Evolutionary genetics of nonself recognition regarding social and sexual interactions of fungi

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Propositions

1. Despite similar components, the Basidiomycete incompatibility system functions in a fundamentally different manner from Ascomycetes.
(this thesis)

2. Manipulation of fungal incompatibility systems will have economic and human health impacts.
(this thesis)

3. Society *under*estimates the importance of science, as novelty rapidly becomes routine.

4. Society *over*estimates the importance of genetics, particularly when applied to humans.

5. Basing scientific careers on the ability to write compelling introduction and discussion sections makes published science unreliable.

6. The requirement for international mobility of scientists is inequitable but essential for science.

7. The *requirement* for PhD propositions outside of Science is fundamentally elitist of the candidate and the university.

Propositions belonging to the thesis, entitled

Evolutionary genetics of nonself recognition regarding social and sexual interaction of fungi

Ben Auxier
Wageningen, March 31, 2023

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This research was conducted under the auspices of the C. T. de Wit Graduate School for Production Ecology and Resource Conservation

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Thesis
submitted in fulfilment of the requirement for the degree of doctor
at Wageningen University,
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 31 March 2023
at 4 p.m. in the Omnia Auditorium.

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The Evolutionary Implications of Fungal Social and Sexual Interactions

254 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)
With references, with summary in English

ISBN: 978-94-6447-593-7
DOI: 10.18174/587592

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Chapter 1

General Introduction

The main problem facing mycologists is to think like a fungus. This paraphrases a statement from my MSc supervisor, Dr. Mary Berbee, that has stuck with me throughout my MSc and now my PhD studies. In some respects, fungi are like other organisms in that they grow throughout their environment in search of food and mates, encountering other organisms, whether friend or foe, along the way. However, in other, more fundamental ways, fungi are distinct from most of life that we know. Inside their filamentous thread-like hyphae reside millions of nuclei that coexist inside a common cytoplasm. This makes a sort of free-floating shared existence that breaks many of the evolutionary assumptions that biologists routinely use. Hopefully, this thesis I shows some examples of how “thinking like a fungus” informs mycological research.

1.1 *Shibboleths and group identity*

"Then said they unto him, Say now ***Shi***bboleth: and he said ***Si***bboleth: for he could not frame to pronounce it right. Then they took him, and slew him at the passages of Jordan: and there fell at that time of the Ephraimites forty and two thousand."

– Judges 12:6 KJV
(Emphasis added)

Artist's depiction of the 'shibboleth incident.' Detail from art by H. de Blois, from The Bible and Its Story Taught by One Thousand Picture Lessons, vol. 3, edited by Charles F. Horne and Julius A. Bewer, 1908

The need to distinguish self from nonself is fundamental. From our own viewpoint, resource sharing within a group comes with benefits, but the question then becomes how group membership itself is defined. To start with an anthropogenic example, distinguishing self from nonself is found in the Hebrew Bible, the book of Judges chapter 12. To set the scene, following a great battle, the tribe of Ephraim attempted to retreat into territory held by the tribe of Gilead. As the Ephraimites tried to cross the border, the resident Gileadites required a method to determine if these strangers at their border were also Gileadites, difficult in the days before passports, or were instead invaders — a method to sort self from nonself. For this, the Gileadites used the pronunciation of the word *shibbólet*, meaning an ear of grain, as a sort of password. The Ephriamites pronounced the first syllable as Shi, while the Gileadites used the Si sound. This difference in pronunciation is learned through culture, and thus reflects their ancestry. So, the "correct" pronunciation becomes a useful proxy for your relationship to this other person. If this syllable is pronounced "incorrectly" (at least to your viewpoint), they are most likely unrelated and may even be invaders trying to steal your resources. Unfortunately for the

Ephraimites, they were unable to “correctly” pronounce *shibbólet*, and suffered the dire consequences. Clearly, there is no actual correct pronunciation, but only the correct pronunciation for a local setting. Instead, these sounds formed a mark to exclude other cultures. In principle, any feature can be used. In the Netherlands during World War II, it was the pronunciation of the Dutch seaside city Scheveningen, which German spies despite great training could not imitate the pronunciations of properly, re-enacted in the 1977 production “The Soldier of Orange”. The pronunciation of these words forms a simple test of one’s ancestry.

There is no reason that language is needed for this purpose, it could have just as easily been the clothes the Ephraimites were wearing if they preferred a color of jacket or such. However, the ease of changing clothing limits the usefulness for distinguishing self from nonself. As seen by the repeated imposition of various regimes for people of the Jewish faith to wear distinguishing clothing, it requires an intensive policing system for clothing to be effective in distinguishing self from nonself. Conversely, the color of one’s skin is largely immutable, and it has become all too obvious how this can be used to distinguish between groups. If your skin is one color, and someone else’s is a different color, then it can be confidently assumed that they are unrelated. The savage effectiveness of this feature, the color of one’s skin, has led to its widespread use as a means of discrimination globally. In a desperate effort to avoid this discrimination, the skin “care” industries in countries like India highlight the lengths to which people will go to avoid such discrimination.

1.2 *Individual distinction in human language*

The above rather distressing examples highlight how groups can discriminate, the negative sense. However, discriminate also has a neutral definition of “recognize a distinction” (Oxford English Dictionary, Twelfth Edition). A much less unpleasant example of how we distinguish ourselves from each other is a naming system. You could imagine a simple naming system, with one letter, names such as “K” or “T”. This would allow groups up to 26 to be unique, and still be reasonably useful up to sizes of 50 people or so. Most people you would encounter would have a different name, “my name is K and your name is E”, and so names would still have some value. However, we exist in a larger society and can encounter many, many people in our lifetimes. Some estimates are that we maintain groups of 1500 people who we can recognize socially (Dunbar, 1992), although differing estimation methods may come to values closer to 200 people (Bernard et al., 1987). To allow us to distinguish within these large social circles, we have developed names that are several characters long, so that the combinations of possible letters easily exceed reasonable population sizes (There are 26^3 or 17,576 three letter names) although many options are not usable due to phonetic limitations from the anatomy of our throat (Dautriche et al., 2017). Based on these physical limitations, we have settled on names that range between 3-7 letters long, generally with two syllables (Meertens

Institute; www.meertens.knaw.nl/). The English language is generally accepted to have 44 different sounds (Hay & Bauer, 2007), and so two syllables produces ±1600 different names. This is seemingly sufficient for the number of people an average person would encounter. The structure of our names shows some principles of how to meaningfully distinguish ourselves from each other. While the consequences of sharing names with other people are not generally life threatening, many people likely have memories of school classes with several of the same first name and the confusion it inevitably brings along.

The example of names naturally brings up the discussion of the cultural forces acting upon them. While at first it may seem odd to consider selective forces on a person's name, parents do not choose a name randomly. Clearly some names are more common than others. While there is some evidence that parents choose a name to represent an idea or concept based on the sound of the phonemes (Sidhu & Pexman, 2015), that seems insufficient to explain the persistence of certain names over centuries/millennia, and the adoption of new names on a regular basis. Previous research has focused on looking for patterns of selection on first names, but results have been inconclusive (Hahn & Bentley, 2003). However, recent work has shown that by integrating the effect of time, a clear pattern of increased preference for rare names emerges (Newberry & Plotkin, 2021). These results show that as a name becomes more common, the chance that parents use this name for their child decreases. This can be thought of as a form of "fitness" for the name, and the fitness in a Darwinian sense is not from the name itself, there is no "best" name, but rather it is context dependent (Heino et al., 1998). When rare, a name is desirable, and when common it becomes less so. The result of this pattern of frequency dependent selection is that names are unlikely to go extinct, as once a name becomes extremely rare, it often encounters a resurgence (Newberry & Plotkin, 2021). As will be expanded upon below, this is rather a general pattern of nonself recognition mechanisms. Once a feature becomes common in a population, be it a name or a variant in protein recognition molecules, the usefulness of such a feature decreases.

2.1 *The need for nonself recognition in multicellular organisms, including fungi*

The abundance of fungi, like other forms of multicellular life, presents a question — Why would a set of cells agree to work together for the "greater good"? This answer seems obvious at first from our anthropogenic viewpoint, that cells *should* work together. However, even simplistic game theory models instead predict cooperation is unlikely because cells benefit directly from competing but only benefit indirectly from co-operating (Guttman, 1996). This predicts that any mutations that increase competition would be favored, while those favoring cooperation would not. Individuals with mutations leading to reduced co-operation are commonly referred to as "cheaters", because they selfishly do not equally contribute to the group.

Although this language is rather anthropogenic, such usage is sometimes surprisingly useful (Ågren & Patten, 2022; Okasha, 2018). This represents a paradox — abundant multicellular life in the face of the predictions against such a state. One resolution of this paradox is that perhaps cooperators are more likely to interact with other cooperators, as early models assumed populations were evenly mixed every generation (Epstein, 1998; Ifti et al., 2004; Nowak & May, 1992). With these preferential interactions, cooperators propagate into small populations of cooperators. However, the resulting communities of cooperators are susceptible to invasion by competitors. To prevent intrusion they can either use proxies of cooperation such as kin recognition (Grafen, 1990), like the shibboleths above, to increase the group stability, or the decision to cooperate can be directly tied to recognition using "greenbeard genes" (Dawkins, 1976). A common way to establish that multicellular organisms begin with single type, whether cooperators or cheaters, and not a mix of cooperators and cheaters, is organismal development from a single-cell bottleneck. This bottleneck means that all the cells of for example a frog or a tree are genetically related, stabilizing the cooperation (Buss, 1987; Maynard Smith & Szathmáry, 1998).

The need for multicellular organisms to protect self from nonself is most viscerally described with the example of vertebrate cancers. While often thought of as a disease, cancer is simply the evolutionary outcome of a cell that stops cooperating with the rest of the body (Aktipis et al., 2015). Mutations causing cancer are often defects in the response to cell cycle regulators, allowing cells to escape the otherwise dictatorial influence of the organism (Malumbres & Barbacid, 2009). These cancers are really just a group of cheating cells that take advantage of the resources available through the blood system of the body. One defense that animals have against invasive cancerous growths is the production of cancer killer cells and other mechanisms to prevent or limit the spread of these non-cooperative cells, using proteins such as P53 and RB (Goodrich et al., 1991; Lane & Crawford, 1979). Arguably, the strongest protections against these overly competitive cells is not found in molecular biology, but rather in the single-cell bottleneck, as well as the early sequestration of the germline in vertebrates (Buss, 1987; Maynard Smith & Szathmáry, 1998). As the sperm and egg cell unite into a single cell, if this cell is not of a cooperating type, it will suffer the consequences. This limits the transmission of cancerous cells to be within a body, although the results can often be lethal for the individual. If instead development occurred by the merging of several cells into an embryo, this would allow non-cooperating cells to persist across generations. Such organismal forms are possible, arising from aggregation instead of clonal outgrowth, but this development mode seems not to be associated with highly specialized life forms. As commonly encountered in laboratory culture of the yeast *Saccharomyces cerevisiae*, aggregating behavior reliably evolves, and can be easily exploited by cheater genotypes (Pentz et al., 2020), this is in contrast to the clonal outgrowth of “snowflake” yeast which is resistant to cheating (Ratcliff et al., 2015). It is interesting

to consider that some vertebrates *do* have cancers transmitted *between* individuals, a facial tumor disease transmitted through fighting between Tasmanian Devils (*Sarcophilus harrisii)* as well as the venereal cancer of domesticated dogs, transmitted by sexual activity. Perhaps unsurprisingly, these tumors have been shown to escape the nonself recognition mechanism, facilitating their spread. In dogs the Canine Transmissible Venereal Tumor seems to escape the watch of the immune system only temporarily, and eventually the host's acquired immunity learns to detect it (Mukaratirwa & Gruys, 2003). This delayed detection may explain the relatively low fatality rate, which may facilitate the spread of this cancer. In Tasmanian Devils, it was thought that while the immune system can recognize the transmitted tumor, the low genetic diversity particularly at immune loci may be allowing the spread of at least some lineages of the transmissible tumor (Caldwell et al., 2018; Pye et al., 2016).

2.2 *The fungal lifecycle and vulnerabilities to cheating*

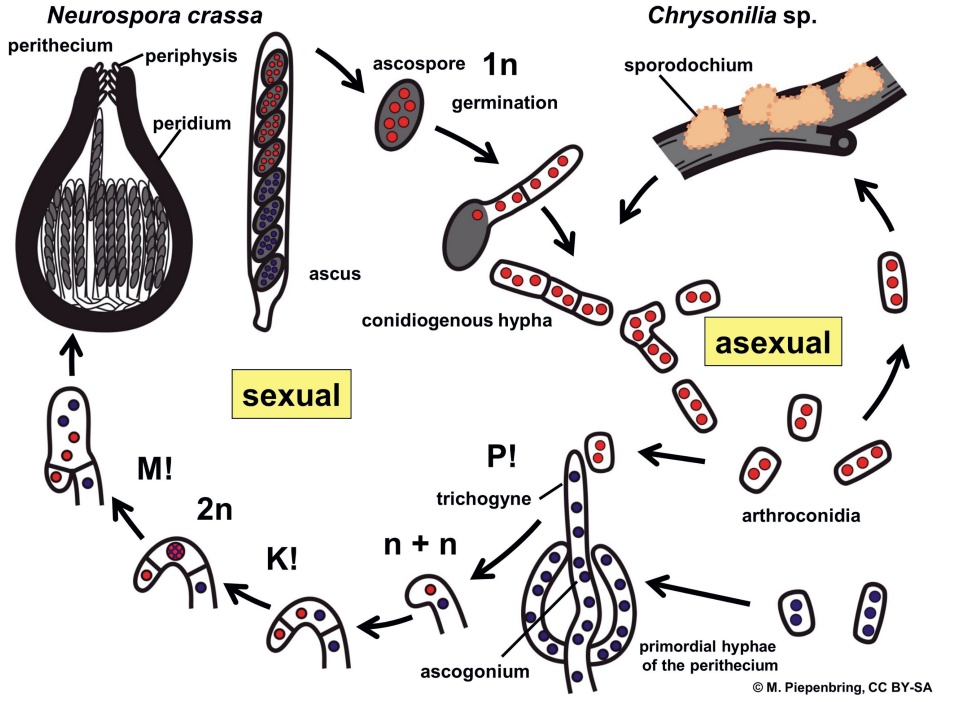

Figure 2: The lifecycle of *Neurospora crassa* is shown above as an example of an ascomycete lifecycle. In this lifecycle the dark melanized ascospores (top middle) germinate into hyphae with a single genotype depicted by the color red. This fungus can propagate asexually, and through fusion with another fungus of the opposite mating type (plasmogamy indicated by P!), a short-lived dikaryotic life stage is entered with two distinct haploid nuclei that divide and eventually fuse into a diploid (larger nucleus with mix of red and blue in 2N stage) nucleus immediately prior to meiosis (M!). The meiotic products are haploid, and genotypes segregate 1:1 in the offspring.

Fungi possess a set of characteristics that set them apart from most other multicellular organisms. For animals and plants, the strict cell separation and single-cell bottleneck means that the individual is the unit of selection (Lewontin, 1970). Importantly, while fungi are commonly referred to as multicellular organisms, it is more accurate to say they are syncytial or coenocytic since they lack true separations between compartments like animal cells (Figure 2). Inside of cylindrical walled tubes, the hypha, fungal “cells” are connected by cross walls, termed septa, which generally have a large central pore. In many species these pores are closed off during most developmental periods, but these blockages are not permanent. The blocking of the septa between compartments allows for exchange of cytoplasmic materials, including vesicles and in some cases mitochondria but generally prevents movement of nuclei depending on life stage. Thus, the cytoplasm of a fungal individual becomes a type of shared commodity, and nuclei can cheat by using more resources (calories or nutrients) than they obtain on their own. This ability for nuclei to migrate means that the nucleus can be considered as the main unit of selection although higher levels of selection still exist (Booth, 2014; Lewontin, 1970). Thus, mutants for processes like nuclear division and hyphal growth can be selected, even if this also reduces the contribution to the sometimes-costly developmental structures. In fungi, they often present with phenotypes of reduced sporulation and flattened growth on agar media (Bastiaans et al., 2016; Fleißner et al., 2005). Recent work on laboratory evolution in fungi has shown that actually such cheating mutations are commonly encountered in many species (Grum-Grzhimaylo et al., 2021; Nandimath, 2022).

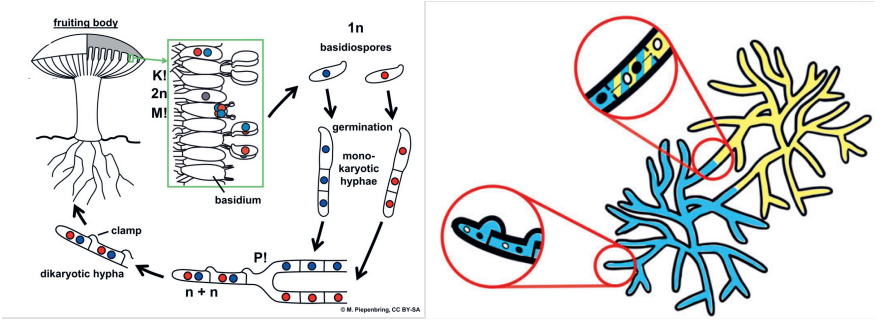

Figure 3: The basidiomycete lifecycle. Left depicts the transition from haploid basidiospore clockwise to monokaryotic hyphae and following mating to dikaryotic hyphae, which subsequently form a fruiting body producing another cycle of haploid basidiospores. Note that the mating step (labelled P!, plasmogamy) only depicts the outgrowth of dikaryotic hyphae and does not highlight reciprocal nuclear migration. Note that meiosis (labelled M!) occurs only in the specialized basidial cells inside the mushroom gills. The right image shows this reciprocal nuclear migration in the top red zoom window, with the white and black nuclei both migrating. The lower zoom window shows the stable dikaryon established following growth, with clamps growing in retrograde, unlike the incorrectly depicted forward growing clamps from the left image. Sources: Left M. Piepenbring, CC BY-SA. Right Vreeburg et al. 2016.

While largely similar to the ascomycete lifecycle, the lifecycle of basidiomycetes, particularly those that produce mushrooms has some notable differences. As members of Dikarya, the phylogenetic group of ascomycetes and basidiomycetes, these fungi also have a dikaryotic stage, the stage that gives the name to the group. In most basidiomycete species this dikaryotic stage is the dominant life stage, often existing for several decades while in ascomycetes it is generally restricted to a few cell divisions. The most charismatic difference found in the basidiomycete lifecycle is the mating reaction. In this, two monokaryotic colonies meet, and reciprocally exchange nuclei. This is shown in the right of Figure 3, with the reciprocal migration of white and black nuclei. This nuclear migration has been shown to occur up to several millimeters per hour, an order of magnitude faster than mycelial growth (Ross, 1976). From an evolutionary perspective, the matings in basidiomycetes are fundamentally different from those in ascomycetes, as both the monokaryons are fertilized in their entirety and so, at least initially, share all resources equally. However, an equal sharing of resources would assume that mating occurs between similar sized colonies, and while there is not much evidence in wild systems, it appears that most mating may occur between extremely asymmetrical individuals (Nieuwenhuis et al., 2013).

This difference in size during mating is relevant, as in the interconnected hyphal network means resources of a large individual has collected are then shared with the genome of the smaller partner, which has fewer resources. This means that while both individuals share equally, the benefit is not equal. While fungi are considered to be isogamous, since the nuclei are the fertilizing agents, the difference in size of mating partners may indicate some similar dynamics may be at play as in egg and sperm producing species. Further complicating the issue of resource management during mating, a monokaryon with a large mycelial network may encounter more than one mating partner simultaneously, and these partners may compete to colonize the mycelium being fertilized. This competition may be one reason why nuclear migration is so rapid in this group — once colonization has started it pays off to colonize the entire mycelium.

Once the two nuclear genotypes reside in a common cytoplasm, a new set of issues arise. Unlike a typical diploid organism, the haploid nuclei remain separate, in a so-called “dikaryotic state”, and thus their evolutionary interests do not necessarily align. In basidiomycetes, this dikaryotic stage retains the ability to mate with monokaryons that are encountered. While a dikaryon can be considered a mated organism, since it can produce sexual offspring via mushroom production, the two haploid genotypes retain the ability to fertilize any additionally encountered monokaryons. This produces a tension, as either of the two resident nuclei are generally capable of fertilizing the monokaryon, but empirical evidence indicates that only one will ultimately be successful (Ellingboe & Raper, 1962a; Nogami et al., 2002). Interestingly, there appears to be variation in fertilization success rates from within a dikaryon, with some genotypes having 100% fertilization rates, compared to

the 50% expectation (Nogami et al., 2002). Whether this is a trait under sexual selection in natural conditions or not remains an unexplored topic, but previous research has shown that in laboratory experimental evolution conditions, there is a response to selection (Nieuwenhuis & Aanen, 2018).

3.1 *Fungal mechanisms of nonself recognition*

The nonself response in fungi has been a productive area of research over the last 30 years. Mainly focused on ascomycete fungi, this research has shown that these fungi do not tolerate fusion between genotypes. Fusion between cells leads to a mixing of cytoplasms produced by nuclei of different genotypes, which triggers a form of programmed cell death. The components of the cytoplasm that trigger this cell death have been characterized both genetically and biochemically. Typically, these involve proteins from the Nod-Like Receptor (NLR) family, the same protein module found in plants. The interaction between the NLR proteins of different genotypes leads to cell death, based on alleles found in the population. The allelic determinants of nonself recognition have been studied in great detail in the species *Neurospora crassa*, *Podospora anserina,* and *Cryphonectria parasitica* (Dementhon et al., 2003; Glass & Kaneko, 2003). Generally, there are two to three alleles at a given locus which produce proteins that when expressed in a common cytoplasm trigger cell death (Daskalov et al., 2019; Heller et al., 2018). In some cases, these interactions have been traced to the individual amino acids that trigger nonself recognition, although in most cases this is difficult due to the high sequence divergence between alleles of a given protein-coding gene.

While the above-mentioned genes limit social interactions within a shared cytoplasm, that is not the only regulator. Recently, it has been shown that at least some of the pre-fusion steps also use similar mechanisms to limit even the act of fusion to within an organism. At least in the model *Neurospora* nonself cell fusion itself is restricted through two mechanisms. The interaction between *doc* alleles seems to involve cell wall receptors or ligands, and differences at the *doc* locus downregulates fusion (Heller et al., 2016). A second pathway seems to involve the *cwr* genes, a chitin lytic polysaccharide monooxygenase, which are also highly polymorphic and where allelic differences also downregulate cell fusion (Detomasi et al., 2022). While these two pathways have similar outcomes, limiting sustained fusion between nonself, the mechanisms are likely very different. The blocking of fusion itself has the additional advantage that it does not result in cell death, and so may be a less energetically costly mechanism. While prefusion restriction also effectively limits social interactions, these largely fall outside the scope of this thesis.

3.2 *More general mechanisms of nonself recognition*

Having discussed the need for nonself recognition, both in general as well as for fungi specifically, we now transition to how this is accomplished at a molecular level. We begin with bacteria, where the system is surprisingly similar. At first glance, one might wonder why bacteria would need to keep track of who is who. As a mother cell divides, it does not fuse with the daughter cells it produces, so such a system seems unnecessary. The answer is found inside the cell, where the genome is stored. If a bacterium is infected with a virus, it is useful to detect this, so that the virus can be killed to protect nearby, often clonally related, bacteria (Koopal et al., 2022). This is of course not accomplished through first names, nor any other verbal *Shibboleth*, but rather by the use of marking their DNA (Murray, 2002; Pleška et al., 2016). A given species of bacteria will epigenetically mark specific sequence motifs of their DNA with a different chemical tag from other bacteria, and so when invading DNA (like from a virus) comes into the cell, it can be noticed as it is missing the particular marks (Pleška et al., 2016). The incoming virus in effect cannot say *shibbólet*. As the DNA missing the marks indicates that the bacterial cell may be being taken advantage of, this triggers the cell to die and stop the virus from replicating (Koopal et al., 2022). This protects the bacteria surrounding it, which are often related clones. The mechanisms that bacteria use for this purpose have been described in exquisite detail, and in fact many are the very restriction enzymes, like EcoRI or BamHI, that formed the basis of molecular biology for several decades (Murray, 2002). Without perhaps realizing it, many thousands of biologists have been using parts of bacterial nonself recognition machinery to make many of the recent fundamental insights into biology and that the great diversity in enzyme specificities came about through selection for nonself recognition.

In plants, nonself recognition has been studied from two directions. The first is how plants recognize other potential pathogenic organisms, using their innate immune systems. This immune system uses a mechanism different from the bacterial example above, but is commonly found across eukaryotes, the NLR system. This class of genes was first found in nematodes with the CED-4 protein (Ellis & Horvitz, 1986; Inohara & Nuñez, 2003). In plants this immune system functions with a set of genes, between dozens to hundreds of genes per plant species (Maekawa et al., 2011). These NLRs either directly detect an invading pathogen, or detect a protein modification that a pathogen would produce (Maekawa et al., 2011). This has produced two conceptual models for how this innate immune system functions, the guard and the decoy model (Dangl & Jones, 2001; van der Hoorn & Kamoun, 2008). Under the guard model, an NLR monitors the biochemical properties of a protein that would be altered during infection by a pathogen (Dangl & Jones, 2001). The decoy model is similar, but the NLR monitors not the actual protein that a pathogen would degrade, but rather a decoy

molecule that only exists to be attractive to a pathogen (van der Hoorn & Kamoun, 2008). In effect, under either model, the NLR is thought to function by consistently asking the target molecule for the *shibboleth*. If at any point the other protein says something other than *shibbólet,* then it is an indication that the cell should be killed to prevent invasion.

A second direction of study of the plant immune systems is to look for cases where self is recognized as nonself, termed hybrid necrosis. First described as early as 1934, when some homozygous genotypes are crossed, the heterozygous F1 offspring develop a lethal reaction (Wiebe, 1934). Despite being recognized for some time, only recently has serious genetic characterization been undertaken (Bomblies et al., 2007; Jeuken et al., 2009). Surprisingly, as arising from mutations in the plant immune system, where the NLR complement of one genotype can "detect" the other genotype, in effect the hybrid offspring believes it is under attack from its own cells (Chae et al., 2014). This triggers a cell death response, and the plants often do not survive past seven to ten days after germination. It has been shown that the genetic variants that cause hybrid necrosis when mixed are under selection for pathogen avoidance, and the evolution of isolated populations to avoid pathogens either not found in other populations, or through incompatible mechanisms seems to be causing this incompatibility (Todesco et al., 2014).

In animals, there is a distinction between the adaptive immune systems, based on exposure to new peptides, and the innate immune system. The adaptive immune system is unique to vertebrates, and not relevant to this thesis. However, the innate immune system shares many homologous elements with plants, largely centered around NLR modules (Maekawa et al., 2011; Uehling et al., 2017). Similar to described above for plants, animals NLRs recognize molecular targets through their various domains, and trigger downstream pathways leading to cell death (Maekawa et al., 2011). Looking across the domains of life, we see a general pattern of nonself recognition. In eukaryotes there is a common theme of the involvement of NLR proteins, although these are not the only players. The example of hybrid necrosis in plants provides an excellent example of how nonself recognition must be regulated to avoid detecting self as nonself. To avoid this, there must be some level of tolerance for differences.

4 *Selective forces*

This above-mentioned high divergence between allelic versions of these nonself recognition genes leads to questions about how this divergence arises and is maintained. Similar to the pattern seen in cultural items (Newberry & Plotkin, 2021), the alleles for genes involved in nonself recognition appear to be under a regime with increased fitness for rare alleles. While the proximate selection forces remain unclear, the pattern seems to be that when an allele for a gene that can

trigger nonself recognition becomes common, it loses its utility as individuals with that allele are more likely to encounter other individuals with the same allele, while when the allele is less common such encounters are also less likely (Grafen, 1990). This rare-allele advantage leads to a form of balancing selection, where alleles are found at intermediate frequency, without fixation of any one allele. Alleles for these types of genes are often found in 1:1 or 1:1:1 ratios, for example in *Cryphonectria* (Ament-Velásquez et al., 2022; Milgroom et al., 2018). This occurs over evolutionary timescales, and even extends across species boundaries when alleles are shared between species. Examples of this have been reported in diverse systems such as mammalian MHC genes, as well as fungal nonself recognition genes (Hedrick & Thomson, 1983; Milgroom et al., 2018; Tian et al., 2002).

An interesting biochemical fact of the fungal nonself recognition system is that the genes are highly redundant, differences at a single gene are sufficient to trigger cell death, although a population may be polymorphic for between 5-15 different genes (Nauta & Hoekstra, 1994). These numbers are surprising as early simulations regarding this found that as few as three loci were sufficient based on what we understand of fungal population dynamics. The source(s) of the selective forces that maintain this high number of such diverse loci are unknown. Potentially, some of this may be explained by the occurrence of repeated interactions. It has recently been shown that by modelling individuals having multiple social interactions, there can be stronger and more dynamic selective forces than there would be if a fungus only had a single lifetime interaction (Scott et al., 2022). Additionally, considering spatial scale in interacting partners also has been found to lead to increased genetic diversity in nonself recognition modules (Czárán et al., 2014).

The fact that fungal networks have been observed in microscopic sections of various field samples has led to theories about cooperation between fungi. Starting with the mycological pioneer Buller, the concept of fungal networks fusing for the greater good was introduced (Buller, 1930). This concept was widely taken up, and persisted in the literature for quite some time (Harley, 1971), although there were mycologists who protested that different networks fusing was not compatible with experimental data (Caten & Jinks, 1966). An important milestone for much of the community was set by Alan Rayner who synthesized experimental evidence on the antagonistic interaction between fungal individuals, with contemporary ideas about levels of selection (Buss, 1987; Maynard Smith & Szathmáry, 1998; Rayner, 1991; Rayner et al., 1984). Rayner suggested rather strongly that fungal individuals, like other organisms, do not cooperate unless highly related. Given the high spore dispersal of most fungi, for example with rust spores travelling across the North American continent (Stakman & Christensen, 1946), it is unlikely that following sexual reproduction, closely related fungi would persist near each other, and structured family level relationships are not expected. It should be noted that other researchers do not find these arguments compelling, and their research supports a more cooperative network paradigm (Chagnon, 2014; Croll et al., 2009; Scott et al.,

2019; Yildirir et al., 2020). Such interactions would perhaps select for cooperative genetic variants, although this would go against many fundamental evolutionary principles.

5 *Sexual reproduction and other genetic exchanges*

Up to this point, an individual's main goal seems to be the preservation of its resources, excluding other through nonself recognition. However, there is a key moment where this barrier must be dismantled. The key step of sexual reproduction is the union of two nuclear genotypes into a common cytoplasm. The contact of these nuclei clearly requires that nonself recognition systems are either bypassed, turned off or at a minimum turned down. In many animals, this is likely accomplished through the difference in size between egg and sperm, termed anisogamy. Effectively, the sperm only donates a nucleus, contributing little cytoplasm that would contain proteins triggering nonself recognition. Fungi do not have egg or sperm cells, but often small spores are involved in fertilization, termed spermatia, at least in ascomycete fertilizations. The size difference between a single spore and a larger hyphal individual acting in a female role, is magnified by the fact that many ascomycetes use a long, thin, trichogyne (Figure 2) through which the nucleus of the spermatia travels down (Brun et al., 2021; Dodge, 1935). This rapid nuclear migration would separate the cytoplasm of the fertilizing strain, perhaps limiting or even preventing nonself recognition responses. As this migration does not include the cytoplasmic mitochondria, this also has the effect of achieving uniparental mitochondrial transmission. The rapid nuclear migration during basidiomycete mating (discussed above) is surprisingly similar to this ascomycete scenario and may have similar functions.

However, this is not the only way that sexual reproduction differs from social interactions. Sexual reproduction between two individuals requires them to merge genomes, and subsequently segregate their genetic differences into diverse progeny. While fungi were for a long time considered to have a significant number of asexual species, these "asexual scandals" (Maynard Smith, 1986) have largely been resolved to be sexual, through population genomics, as well as more complete culturing methods (O'Gorman et al., 2009; Taylor et al., 2015). As many fungi produce asexual propagules, they could theoretically exist without meiotic recombination, however the evidence points otherwise. For many species, where population genomics remains unavailable, it has been common to consider the genes of a species, whether these are consistent with meiotic recombination. As many of the core meiosis genes have been well characterized, it has become routine to assess the complement of the meiotic machinery. Such analyses have shown that species like Arbuscular Mycorrhizal Fungi (AMF), long thought to be asexual, actually contain all conserved meiotic machinery, and it is inferred that they likely regularly engage in meiosis, although this remains unobserved (Halary et al., 2011;

Taylor et al., 2015). For human pathogens, there have been some species that truly appear asexual, such as species of *Trichophyton* or *Candida* although these may simply represent a lack of sufficient study as genomic surveys find largely complete meiotic toolkits (Gabaldón & Fairhead, 2019; Hofstatter & Lahr, 2019). However for other human pathogens, like *Aspergillus fumigatus*, a sexual cycle has been clearly demonstrated (O'Gorman et al., 2009; Paoletti et al., 2005).

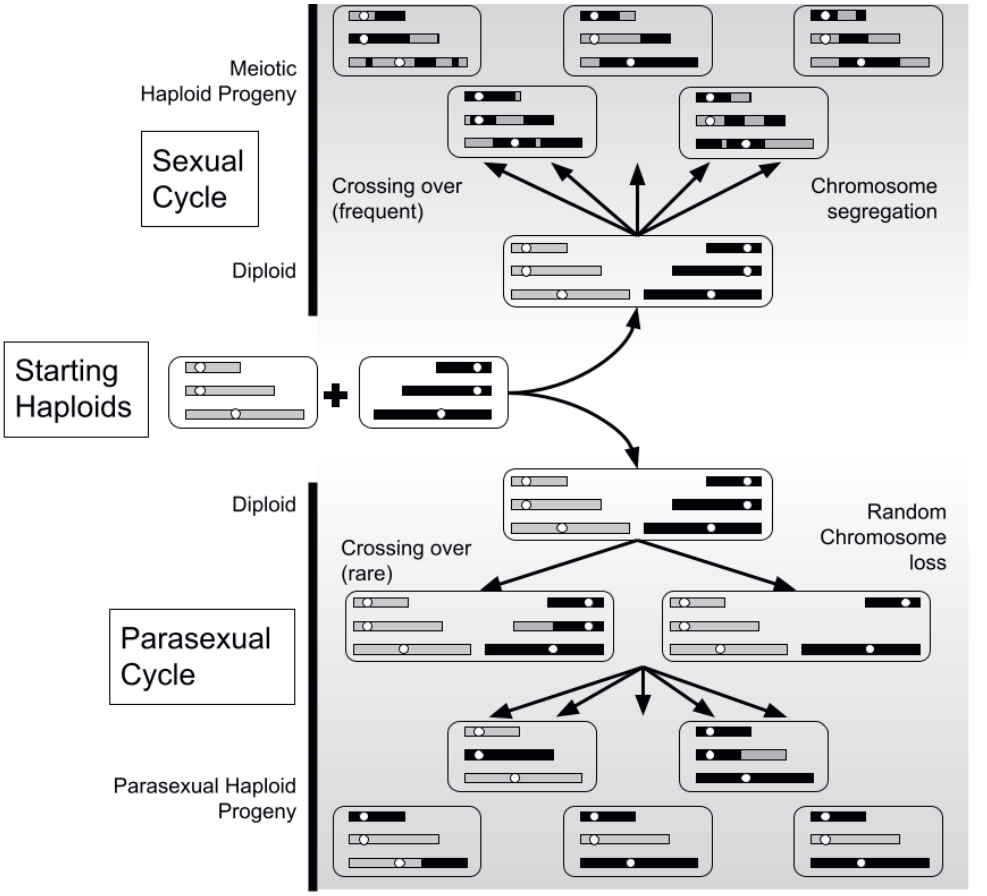

Figure 5: Comparison of the sexual and parasexual cycles in haploid fungi. Starting from two haploid progenitors (centre), a diploid cell is formed in both pathways. In meiosis (top), this diploid cell undergoes frequent crossovers, and resulting haploid gametes are recombinants of both chromosome segregation and crossovers. In the parasexual cycle (bottom), the diploid cell undergoes infrequent crossovers, and eventual haploid formation as the result of random chromosome loss. The haploid progeny of the parasexual cycle are genetically diverse largely due to chromosome segregation, with occasional crossover events. In diploid fungi like some *Candida* sp., or diploid animal cells, the parasexual cycle is similar but proceeds through a tetraploid intermediate.

However, meiosis does not cover all genetic exchanges. As fungi exist in a coenocytic state with multiple nuclei per cell compartment, parasexual interactions also occur. First identified by Baker, and popularized by Pontecorvo, parasexual recombination involves genetic exchange between nuclei in a shared cytoplasm (Baker, 1944; Pontecorvo & Gemmell, 1944). These exchanges occur through the transient production of a nucleus with higher ploidy (two haploid producing a diploid, or two diploids producing a tetraploid). Inside this higher ploidy nucleus, crossing over can occur between homologous chromosomes, as well as segregation of chromosomes during ploidy reduction back to the typical ploidy for a species. In fungi, this has been demonstrated in both ascomycetes and basidiomycetes, and has been suggested in arbuscular mycorrhizal fungi (Baker, 1944; Chen et al., 2018; Ellingboe & Raper, 1962b). It has also been shown to occur in human cells, indicating likely widespread occurrence across animals (Martin & Sprague, 1969; Miroshnychenko et al., 2021). However, an underappreciated factor in these interactions is the nonself recognition response. This means that in nature, parasexual interactions are likely only to be found between clones of a genotype. For animals, parasex can happen within an individual, but not between individuals. In ascomycetes, two individuals meeting each other will likely trigger nonself recognition, as described above. For fungi, this means that to imply that parasexual recombination will be restricted to occur between genomes which are already very similar, and so parasex is unlikely to contribute much genetic variation, although when it occurs it can have significant fitness benefits (Engel et al., 2020; Schoustra et al., 2007). In basidiomycetes, the situation is slightly different, as in a dikaryon two different genomes reside in a common cytoplasm for extended periods of time. However, in this case it is likely beneficial to repress parasex, as the genetic variation within an individual is required to produce diverse offspring. Although reports of parasexual activity have been reported in literature (Clark & Anderson, 2004; Ellingboe & Raper, 1962b), dikaryotic individuals sampled from the field largely maintain heterozygosity over time, and so such parasexual interactions do not appear to be a strong force in nature (Anderson & Catona, 2014).

Outline of this Thesis

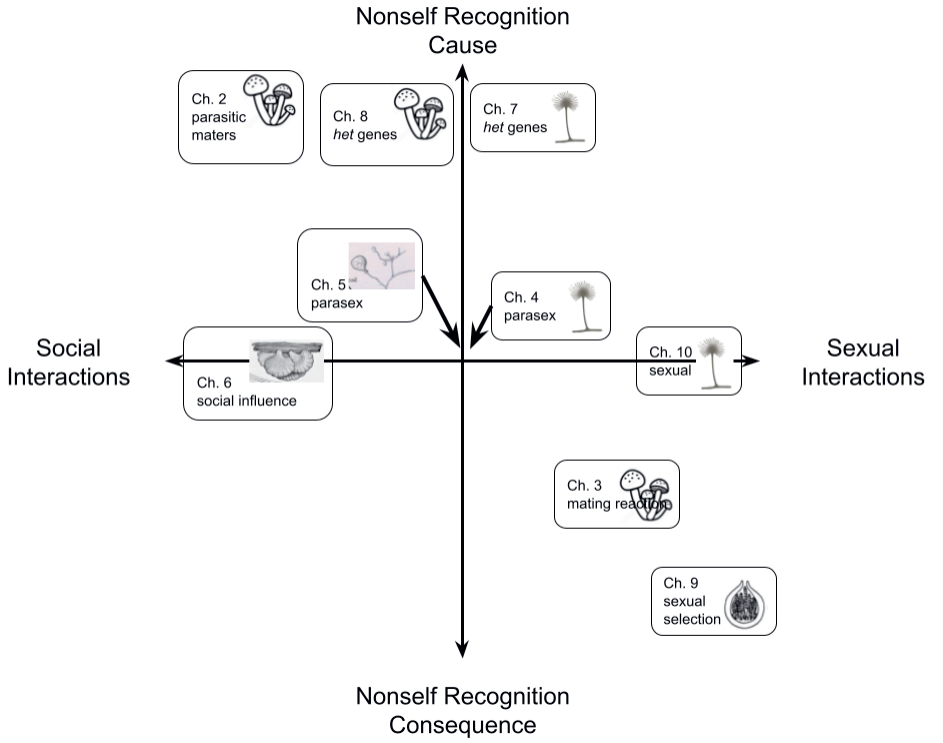

Figure 6: Schematic overview of this thesis.

6 *Conclusion*

The topics covered by thesis span a broad range of organisms and methods, primarily addressing two themes. The title of this thesis includes "evolutionary genetics of nonself recognition", which is visualized in the vertical axis of Figure 6 as the cause and the consequence of nonself recognition. The social and sexual lives of fungi are displayed on the horizontal axis, with the parasexual cycle falling, somewhat artificially, in the middle of this axis. Each chapter is placed on this grid, to approximate where on this scale the topic lands.

The social lives of basidiomycete fungi, particularly those that produce mushrooms, are exceptionally interesting. The extended dikaryotic phase with two distinct nuclear genotypes is similar to a diploid, but unlike a diploid allows for additional mating opportunities. In **Chapter 2** I explore the outcomes these additional mating opportunities have on selection at the nucleus level, and this is then translated back to the mycelial individual. This dikaryotic state also produces some difficulties in how we understand nonself recognition to operate mechanistically. If a dikaryotic basidiomycete fungus wishes to prevent fusion with other individuals, then how can a fertilization between a dikaryon and monokaryon occur? In **Chapter 3** I

speculate on the role of nuclear migration in this process. Based on historical observations, we produce a model where nonself recognition is active at both the monokaryon and dikaryon life stage, but the rapid nuclear migration found in basidiomycetes still permits mating.

As this thesis will hopefully emphasize, the social and sexual lives of fungi can be sometimes difficult to delineate. In **Chapter 4** I show in *Aspergillus niger* and *A. fumigatus* how such social interactions can result in parasexual genetic recombination. The differences between the parasexual and sexual processes unfortunately often lead to confusion, and in **Chapter 5** I attempt to correct the published literature regarding a widely publicized claim of a novel genetic mechanism in the enigmatic Arbuscular Mycorrhizal Fungi. Following on the influence of nuclei during social interaction, it is interesting to consider the interaction between nuclei in naturally occurring basidiomycete dikaryons, unlike the forced heterokaryons of **Chapter 4**. Using three strains of *S. commune*, **Chapter 6** investigates the effect of mating on gene expression, and specifically tests for the effect of the male or female role during such matings.

Moving onto the regulation of such social interactions, this thesis describes the genetic basis of nonself recognition in two species. In **Chapter 7** I identify the first genes controlling nonself recognition in a basidiomycete, using the genetic model mushroom *Coprinopsis cinerea*. Carrying on in this topic of regulation of social interactions, in **Chapter 8** I transition to the human pathogen *Aspergillus fumigatus*, where we characterize four *het* genes, and a fifth *het* locus, the first for any Eurotiomycete.

A common theme of nonself recognition genes is that they fall under balancing selection. **Chapter 9** tests an intriguing hypothesis that nonself recognition loci may fall under additional sexual selection during mating, like that observed in many animal species. If true, this would be an interaction between the sexual and social lives of fungi. Moving to the truly sexual world, in **Chapter 10** I describe the recombination landscape in *A. fumigatus*, showing to have the highest rate of crossover recombination in any Eukaryote characterized to date.

As the preceding paragraphs show, this thesis covers a broad range of topics, species, and methods. In **Chapter 11** I attempt to synthesize what has been learned about the social and sexual lives of fungi, and future research opportunities.

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Chapter 2

Modelling the Consequences of the Dikaryotic Life Cycle of mushroom-Forming Fungi for Genomic Conflict

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Published As :

Auxier et al. 2022, *eLife*
https://doi.org/10.7554/eLife.75917

Abstract:

Generally, sexual organisms contain two haploid genomes, one from each parent, united in a single diploid nucleus of the zygote which links their fate during growth. A fascinating exception to this are basidiomycete fungi, where the two haploid genomes remain separate in a dikaryon, retaining the option to fertilize subsequent monokaryons encountered. How the ensuing nuclear competition influences the balance of selection within and between individuals is largely unexplored. We test the consequences of the dikaryotic lifecycle for mating success and mycelium-level fitness components. We assume a tradeoff between mating fitness at the level of the haploid nucleus and fitness of the fungal mycelium. We show that the maintenance of fertilization potential by dikaryons leads to a higher proportion of fertilized monokaryons, but that the ensuing intra-dikaryon selection for increased nuclear mating fitness leads to reduced mycelium fitness relative to a diploid life cycle. However, this fitness reduction is lower compared to a hypothetical life cycle where dikaryons can also exchange nuclei. Prohibition of fusion between dikaryons therefore reduces the level of nuclear parasitism. The number of loci influencing fitness is an important determinant of the degree to which average mycelium-level fitness is reduced. The results of this study crucially hinge upon a tradeoff between nucleus and mycelium-level fitness. We discuss the evidence for this assumption and the implications of an alternative that there is a positive relationship between nucleus and mycelium-level fitness.

Introduction:

Kin-selection theory shows that altruistic interactions between individuals, or between other replicating entities, can evolve between genetically related vehicles (Hamilton, 1964). Examples are altruistic interactions between individuals in social insects, cooperation between clonally related mitochondria within eukaryotic cells, and between clonal cells of multicellular individuals (Bourke, 2011; Queller, 2000). In sharp contrast to social altruistic interactions, sexual interactions often are avoided among genetically related and promoted among genetically unrelated individuals, either via behavior in animals or via incompatibility systems in plants and fungi (Herron & Freeman, 2014). This difference in preferred relatedness creates a tension between sexual and social interactions. This is illustrated in parental conflict over resource allocation to offspring. In species where females can carry embryos from multiple males, paternal genomes compete to extract nutrients from a common uterus/ovary (Haig, 1993). The competition between genetic elements, in this case paternal alleles from different embryos, inevitably leads to conflict with the mother over resource provisioning. Any paternal competitive trait expressed in the embryo provides the selective ground for modifiers of maternal origin to repress these competitive traits (Rice & Holland, 1997). This parent-of-origin conflict over maternal resource provisioning can be suppressed by mechanisms such as genomic imprinting.

Most sexually reproducing organisms have mechanisms to police competition among the unrelated genetic elements or to re-establish high genetic relatedness upon mating. For example, social insects typically are monogamous, meaning that social interactions occur among full siblings (Boomsma, 2009). Clonal relatedness among organelles is maintained by uniparental transmission and a bottleneck during sexual reproduction (Cosmides & Tooby, 1981). Also, multicellular organisms typically develop by clonal outgrowth of a fertilized egg, and thus the cells are genetically identical barring *de novo* mutations (Buss, 1987). In diploids tension is reduced between the two gametic genomes that form the zygote because cell fusion (plasmogamy) is immediately followed by nuclear fusion (karyogamy), aligning the fates of the two genomes until the next meiosis. Tension is reduced between the unrelated haploid genomes because the benefits of a competitive allele from one genome are shared equally with the other genome. However, basidiomycetes, the fungal clade containing mushrooms, rusts and smuts, provide an exception to this general principle.

In these fungi the fusion of plasma membranes occurs between two multicellular gametes (monokaryons), but nuclear fusion is delayed until immediately prior to meiosis. The mated individual with two separated haploid nuclei, termed a dikaryon, is genetically similar to a diploid by having two copies of the nuclear genome per cell, although gene regulation may differ due to the compartmented genomes (Banuett, 2015; Schuurs et al., 1998). The dikaryotic state differs from diploidy as the separate haploid nuclei retain the ability to fertilize further

monokaryons (reviewed in Raper, 1966). A consequence of this delayed karyogamy in basidiomycetes is an increase in mating opportunities (Anderson & Kohn, 2007). Since the fertilization of a monokaryon is not associated with resource investment in the resulting new dikaryon, the dikaryotic lifecycle corresponds to the retention of the male role (Aanen et al., 2004). This unique form of mating between a dikaryon and monokaryon is known as di-mon mating, often called Buller mating (Buller, 1934; Quintanilha, 1938). Di-mon mating, clearly impossible for a diploid organism, has been thought to be the main benefit of the dikaryotic lifestyle (Raper, 1966 p.264). The exchange of nuclei between dikaryons is thought to be prevented in nature since fusion of two dikaryons triggers nonself recognition followed by apoptosis, quickly killing the fused cell (Auxier et al., 2021; Aylmore & Todd, 1986). The prolonged mating opportunities of the dikaryon allow a nuclear genotype increased access to resources, as it retains its share of resources in the original dikaryon, and gains access to additional resources of the newly fertilized dikaryon. However, the benefits to a nucleus of this delayed karyogamy may carry a cost due to persistent tension in social interactions between the unrelated nuclei.

These di-mon matings present a competitive arena for the paired nuclei. Due to the high number of mating types in basidiomycetes, both nuclei of a dikaryon are generally capable of fertilizing an unrelated monokaryon. This results in competition between the nuclei, the dynamics of which are poorly understood. Initially both nuclei appear to invade hyphae of the monokaryon, but ultimately only one of the two dikaryotic nuclear haplotypes fertilizes the entire monokaryon (Anderson & Kohn, 2007). Competitive success within the dikaryon could be based on variation in rates of nuclear division, migration or percentage of hyphae colonized (Anderson & Kohn, 2007; Raper, 1966; Vreeburg et al., 2016). Evidence of variation for mating success has been found in natural systems, where replicate di-mon matings consistently result in the same fertilizing nucleus (Ellingboe & Raper, 1962, 1962; Nieuwenhuis et al., 2011; Nogami et al., 2002). In one study, the direct interaction between competing nuclei was found to determine success in a Buller mating , with a hierarchy of mating competitiveness (Nogami et al., 2002). However, there is also evidence that the interaction of the different nuclei with the receiving monokaryon determines mating success in a Buller mating (Nieuwenhuis et al., 2011). Intraspecific variation in mating success may be due to variation in the strength of selection for mating success and differences in the historical frequency of di-mon matings. However, since mating success is difficult to quantify, the magnitude of this variation remains unknown. Alternatively, variation in nuclear competitiveness could be maintained due to tradeoffs with mycelium-level fitness components.

Two common measures of fungal fitness are somatic (mycelial) growth, and spore production (Pringle & Taylor, 2002). While these undoubtedly capture only a fraction of actual fitness variation between individuals, fitness components related to aspects like enzymatic variation have not yet been described (Allison et al., 2018).

For these sessile organisms, vegetative growth is required to explore nearby resources, and spore production is needed for dispersal to new sites. It has been thought that fungal vegetative growth and spore production may be under a life history trade-off (Schoustra & Punzalan, 2012; but see Anderson, Nieuwenhuis, & Johannesson, 2019). Such a tradeoff assumes that high vegetative growth rate to explore resources comes at the expense of either rapid or abundant spore production (Gilchrist et al., 2006; Pringle & Taylor, 2002).

Here we explore the consequences of di-mon matings in the dikaryotic life cycle for the proportion of fertilized monokaryons and for mycelium-level fitness components, assuming a three-way tradeoff between growth, spore production, and mating success. We simulated the outcome for three lifecycles: diploid, standard dikaryon (with di-mon matings) with retention of the male role, and a hypothetical open dikaryon with retention of the male and female role (similar to a life cycle without nonself recognition between dikaryons). While this open dikaryon is not known from nature, we explore potential consequences of such a dikaryon for the balance between selection among nuclei within mycelia and among mycelia. The unique lifecycle of the standard dikaryon allows for additional matings through di-mon matings, but may come with the cost of selection at the level of the nucleus (James, 2015). Our results show that the frequency of unmated monokaryons is reduced in the standard dikaryon life cycle, but selection at the level of the nucleus reduces average dikaryon mycelium-level fitness. However, this cost is greatly reduced compared to a hypothetical lifecycle where exchange between dikaryons is also allowed. Our simulations also show that these costs are increased if the loci associated with mating fitness are spread throughout the genome.

Materials and Methods:

The model

The arena: The "substrate" on which the simulated mycelia live is two-dimensional, represented by a square lattice each site supporting a single mycelium, or section thereof. The lattice takes a toroidal topology with the first and the last site of each row/column being neighbours, so that the lattice has no edges. At time 0 the lattice is inoculated with a random initial pattern of spores from a pool of *m* different mating types. Each inoculated site contains the monokaryotic mycelium sprouted from a single spore.

Nuclear fitness: The nuclear fitness of each spore (and the corresponding monokaryon and dikaryon) consists of three heritable (genetically encoded) components:

1) vegetative fitness w_v which determines the rate *G* at which the mycelium expands to neighboring empty sites: $G = g \cdot w_v$, where *g* is the basic rate of mycelial growth.;
2) reproductive fitness w_r which determines the rate *R* at which the mycelium produces spores: $R = r \cdot w_r$, where *r* is the basic rate of spore production; note that only dikaryotic mycelium produces spores, so this fitness has an effective value of zero as a monokaryon.
3) mating fitness w_m that determines the propensity of the nucleus to become part of a dikaryon upon mating.

These three fitness components are traded off so that any one of them can increase only at the expense of the other two in a linear fashion: $w_v + w_r + w_m = 1.0$.

Non-linear fitness: We also explored the effects of non-linear trade-offs on possible combinations of w_v, w_r and w_m such that any actual combination remains below the trade-off surface defined by $1 = (w_v^{\beta} + w_r^{\beta} + w_m^{\beta})$. Results of the non-linear fitness are shown in Supplemental Figure 3. Unless otherwise stated, in our modelling $\beta = 1$.

Dikaryotic fitness: The vegetative and the reproductive fitness components (W_v and W_r) of dikaryons depend on the nuclear fitness components (w_v and w_r) of the two nuclei it harbors and on the dominance interaction, *Θ*. For example, dikaryotic mycelia grow into neighbouring sites at a rate $W_v = g\left[w_{v,max} \cdot Q + w_{v,min} \cdot (1 - Q)\right]$, where *g* is the basic mycelial growth rate, $w_{v,max}$ is the vegetative fitness of the fitter nucleus and *Θ* specifies its phenotypic dominance over the less fit nucleus (of nuclear vegetative fitness $w_{v,min}$). *Θ* = 1.0 is absolute dominance, *Θ* = 0.0 means absolute recessivity. Reproductive fitness (i.e., the spore production rate) of the dikaryon is calculated similarly.

Mating: In all scenarios, monokaryotic mycelia of different mating types coming into spatial contact (i.e., those on neighboring sites) will sexually fuse and produce dikaryons if they are of different mating types. In case of more possible mates, the focal ("female") monokaryon chooses one of the compatible nuclei from the

neighboring mycelia, with the chance that a given “male” nucleus is chosen depending on its mating fitness w_m. The three mating scenarios (“diploid”: mon-mon only; “standard dikaryon”: mon-mon and di-mon; open dikaryon: mon-mon, di-mon and di-di) differ in the number of nuclei competing for becoming one of the two actual nuclei taking over the fused mycelia. In mon-mon matings only the nuclei from monokaryons surrounding a focal receiving monokaryon; in di-mon matings the two nuclei of each dikaryon compete as well as surrounding monokaryons , and in the di-di scenario two of the four nuclei win, with the chances depending on w_m. The winning pair of nuclei spread all over the spatially connected parts of the affected male and female mycelia, transforming them into the same dikaryon.

Mutation: Spore production occurs with the sexual fusion of the two nuclei of the dikaryon, preceded by mutations during the dikaryotic state, and followed by meiosis. Mutations affect the nuclear fitness components (w_v, w_r and w_m), such that mutant fitness (w_v', w_r' and w_m') are drawn from Gaussian ditributions with standard deviation σ centered on the parental values and scaled back to satisfy $w_v + w_r + w_m = 1.0$. The mutation step is shown in Figure 1A as a set of open circles denoting the starting fitness as a position on the ternary diagram, and the arrow showing the mutational step to the new fitness values.

Number of fitness related loci: To partially simulate the genetics underlying the phenotypes of this model, we implemented a parameter, λ, affecting the inheritance of fitness phenotypes , ranging between 0 and 1. $\lambda = 0$ corresponds to multiple loci throughout the genome, where recombination between the parents leads to offspring with the average phenotype of two parental nuclei. Alternatively, $\lambda = 1$ indicates that fitness variance is located inside a single mendelian locus and offspring resemble either one parent or the other.

Updating algorithm: One generation of the simulations consists of N elementary updating steps; N is the number of sites in the lattice. An updating step starts with choosing a random site, which if occupied dies with probability d. If the site is instead empty, the mycelia on the neighboring eight sites compete to occupy it, chances to win depending on their vegetative fitness. If the focal site is occupied and survives, it may engage in mating in the “female” role, provided that it is capable of mating with at least one of its neighbors. Mating type, mating scenario compatibility and mating success w_m of the potential “male” partners determines the actual outcome of mating, resulting in a dikaryon in accordance with the rules detailed in the *Mating* section above. If the focal site contains a dikaryotic mycelium, it may produce spores which are locally dispersed onto sites within the dispersal radius R of the parental dikaryon, which germinate to monokaryotic mycelia in the next generation. One spore per site may survive on each empty site that has received spores; the survivor is chosen at random. Identical nucleotypes instantly fuse somatically upon spatial contact.

The rules and the algorithm of the simulations are summarized in Figure 1. Pseudocode for the simulation can be found in Supplemental Text 1. Animations of sample runs for the Diploid, Standard and Open Dikaryon can be found in Supplemental File 2, 3 and 4, respectively.

Competitions: To compare the competitive advantage of dikaryotic male function (the standard dikaryon scenario), the simulation was modified to represent an allele conferring dikaryotic male function (DMF) linked to the mating locus, having 15 mating types with this allele, and 15 without. Homokaryons of either type were reproductively compatible, and the case of heterozygotes was evalued for the DMF allele being recessive, dominant, or co-dominant where only the nucleus with the DMF allele could participate in di-mon matings. Due to algorithmic complexity, we did not attempt a similar competition for dikaryotic female function (the open dikaryon scenario).

Parameter settings: Due to the number of parameters, we could not assess all possible combinations. Preliminary results showed that simulations with grid sizes of less than 150 squares had stochastic outcomes (data not shown). To combat this, results shown here were performed with a grid size of 300x300, except data from Figure 5 and 6 where due to computational resources, smaller grid sizes of 200x200 were used instead. Except where otherwise specificed the base parameters were: g = 0.1; r = 1; d = 0.3; σ =0.01; grid sizes of 300x300 and simulations were run for 1000 iterations.

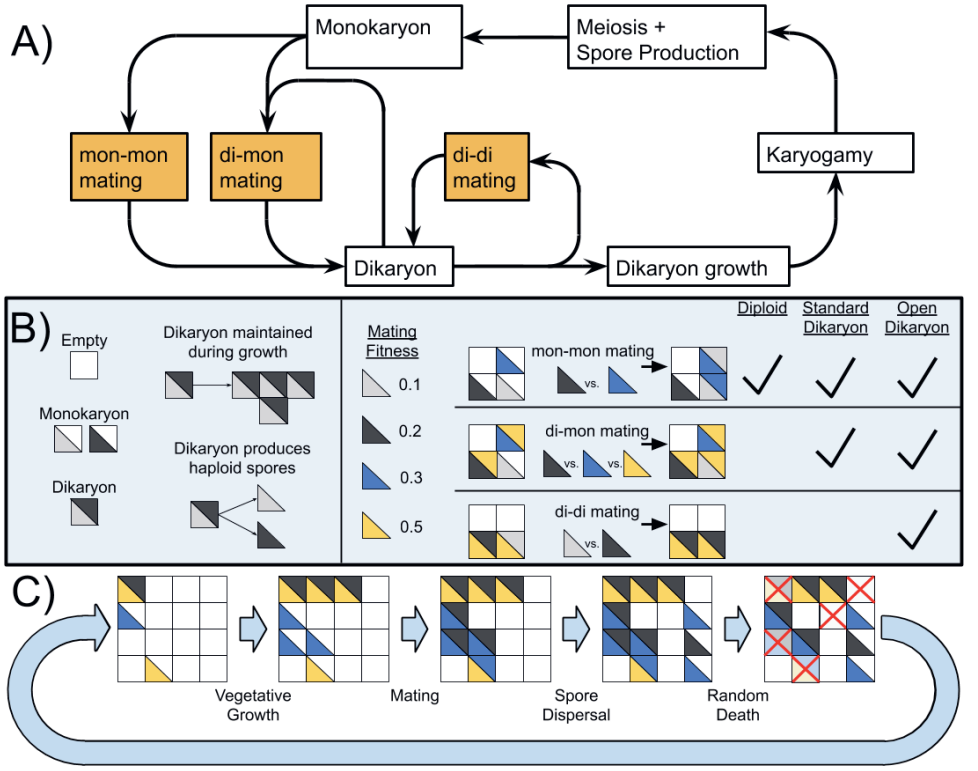


Figure 1: Lifecycle of basidiomycetes and schematic of model. A) Lifecycle of dikaryon showing the three different mating possibilities (mon-mon, di-mon, and di-di), as well as the separation between mating and karyogamy. B) Shows some rules of the algorithm and three types of mating, and which matings are found with each of the three lifecycles. C) Example of the actions taken during one generation of the simulation. Note that in this diagram matings are shown as deterministic based on mating fitness, but in the simulations the outcome of competition is probabilistic.

Results:

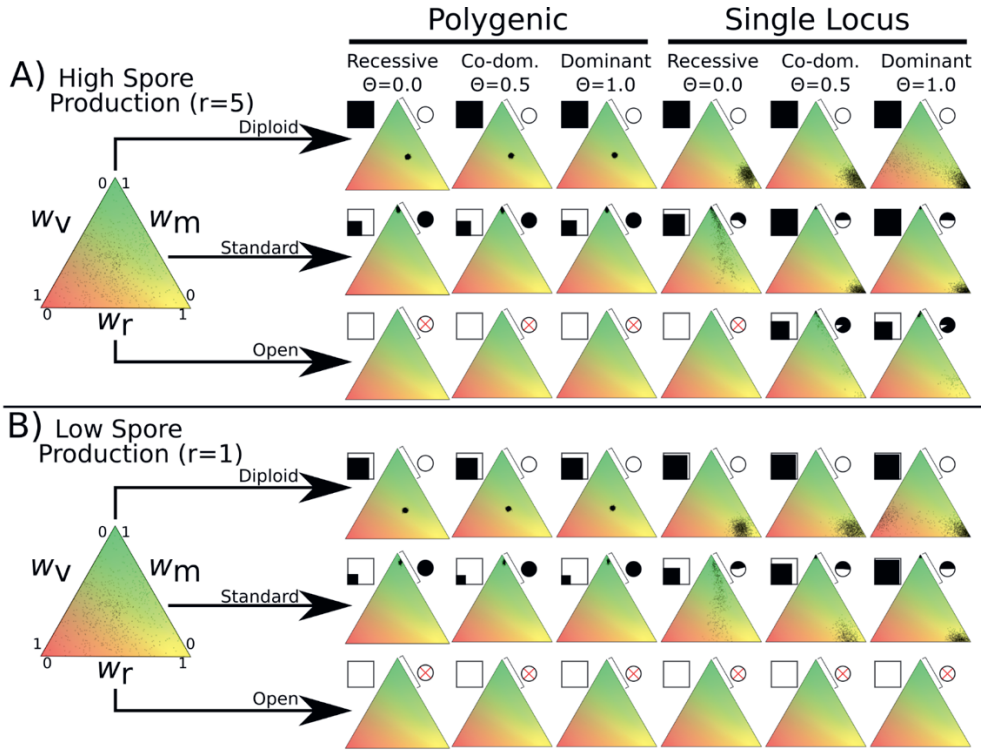


Figure 2: Fitness distributions after 1000 generations under different values of λ and dominance coefficients for the three dikaryon scenarios with A) high spore production (r=10) or B) low spore production (r=1). Simulations were initialized with 60000 homokaryotic individuals with initial fitness values drawn from a normal distribution, as shown in the leftmost triangle. Points in resulting triangles represent individual nuclei remaining on the 300x300 grid at end of simulation. Note that there is significant overlap of points, thus the amount of black is not proportional to population size. Shading of pie charts show percentage of nuclei with >66% mating fitness (w_m; region covered by bar). Shaded square indicates proportion of cells occupied at end of simulation. Simulations with no surviving population have no dots and associated pie charts have a red cross.

Genetic assumptions have a strong influence on equilibrium fitness distributions

Initial simulations run under different scenarios (one locus/many loci, recessive/co-dominant/dominant) showed a strong influence of these parameters on the resulting nuclear fitness (Fig. 2).

Diploid The diploid life cycle, where matings only occur between monokaryons, resulted in stable populations over the entire tested parameter space. When fitness is polygenic, the resulting nuclei cluster near the middle of the fitness space, without maximizing any specific trait. If instead fitness is monogenic the nuclei form a cluster with maximum spore production. When the mycelium-level phenotypes (w_v and w_r) are fully dominant (Θ = 1.0) with monogenic fitness traits (rightmost column) the simulations result in one set of nuclei optimizing spore production and a much

smaller cluster balancing vegetative growth and mating fitness, particularly with high growth rates (Fig Suppl. 2). Although the diploid does not take part in di-mon matings, competition during monokaryon matings continues to make use of some level of mating fitness. There is no obvious difference between the diploid simulations under the high spore (Fig. 2A) or low spore (Fig. 2B) state.

Standard dikaryon When dikaryotic male function is allowed through di-mon matings, the standard dikaryotic state found in nature, there is a strong drive for mating fitness. With polygenic fitness, the nuclei exclusively optimize mating fitness. However, if fitness variance is monogenic a clear polymorphism emerges with one set of nuclei strongly optimizing mating fitness, and the other optimizing spore production. Changing the dominance coefficient of the vegetative growth and spore production phenotypes had quantitative effects on the proportion of the nuclei optimizing mating fitness (Figure 2 pie charts).

Open dikaryon When di-di matings are allowed, the hypothetical open dikaryon situation, the increased selection for mating fitness caused populations to collapse in most scenarios. In scenarios with low spore production (Figure 2B), the open dikaryon population crashes, regardless of dominance or genetics of fitness variance. When spore production is increased (Figure 2A), the populations survive as less spore production fitness is required, but all nuclei optimize mating fitness. When phenotypes are recessive and fitness is monogenic, even this increased level of spore production is not sufficient to maintain the population.

To assess the stability of these results, we performed similar simulations with two further modifications to the global parameters, either increased basal growth rate, g, of 0.5 or increased mutational width, σ of 0.05. Increased basal growth rate allowed the open dikaryon to maintain stable populations (Supplemental Figure 1). Increased mutational width allowed the open dikaryon to persist under a wider set of parameters. For the diploid polygenic scenario, increased mutation width resulted in optimization of fitness around high spore production instead of a balance between the fitness components.

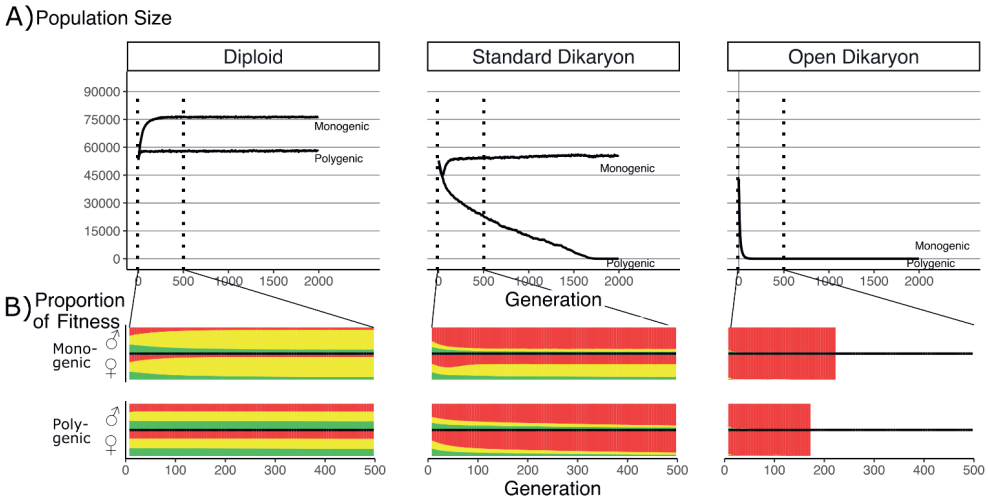


Figure 3: Effect of number of fitness loci on the impact of the fitness tradeoff. A) Population size with □ varying from monogenic (λ =0) to polygenic (λ =1) across the three life strategies, initialized with 60000 squares occupied. Dotted vertical lines indicate region shown in B. B) Average proportion of fitness in nuclei per generation for the first 500 generations. Decreasing λ values are shown on separate rows. The different fitness components are indicated by color: Red (top) is mating fitness, w_m, yellow (middle) is spore production, w_r; and green (bottom) is vegetative growth, w_v. White areas in Open Dikaryon are from dead populations. For every value of λ , the average fitness is calculated separately for nucleus 1 (the nucleus of the original monokaryon) and nucleus 2 (the fertilizing nucleus) Note that increased non-mating fitness in the open dikaryon with λ =0.99 is due to extremely small population sizes.

Limited fitness loci partition mating fitness in the standard dikaryon

As the initial simulation showed clear differences between the monogenic and polygenic states, we more thoroughly investigated this influence. The population size dynamics are shown in Figure 3A, and average population fitness proportions are shown in Figure 3B. As population size results from the balance of dispersal/growth and the random death process, this size is an outcome of average fitness at the level of the mycelium (Gilchrist et al., 2006). Therefore, Figure 3A shows an element of average mycelial fitness, while Figure 3B shows the fitness components of the individual nuclei.

In the diploid situation, the monogenic scenario had an increased population size compared to the polygenic fitness scenario. Figure 3B shows that this increase in population size was co-incident with an increase in the average spore production fitness (yellow), and a roughly symmetrical decrease in vegetative growth and mating fitness. When simulated under similar parameters, the open dikaryon monogenic and polygenic situations resulted in population collapse, with rapid and irreversible increase in mating fitness (red) after only a few generations (Figure 3B).

In the standard dikaryon, the polygenic situation leads to a continuous decline in population, as mating fitness increases in all nuclei (Figure 3A). The increased mating fitness is seen in both the male and female nuclei of the dikaryons in the population (Figure 3B). However, the monogenic situation allows for a polymorphism in the population between the fitness of the nuclei that were the

original monokaryon (the female role) versus those of the fertilizing nucleus (the male role). The male role is then still performed by nuclei specialized for mating fitness, while the female role is performed by nuclei that retain a significant amount of spore production fitness. Interestingly, in the standard dikaryon the monogenic situation shows an initial increase in mating fitness in the female role as well, but this is purged after approximately 75 generations.

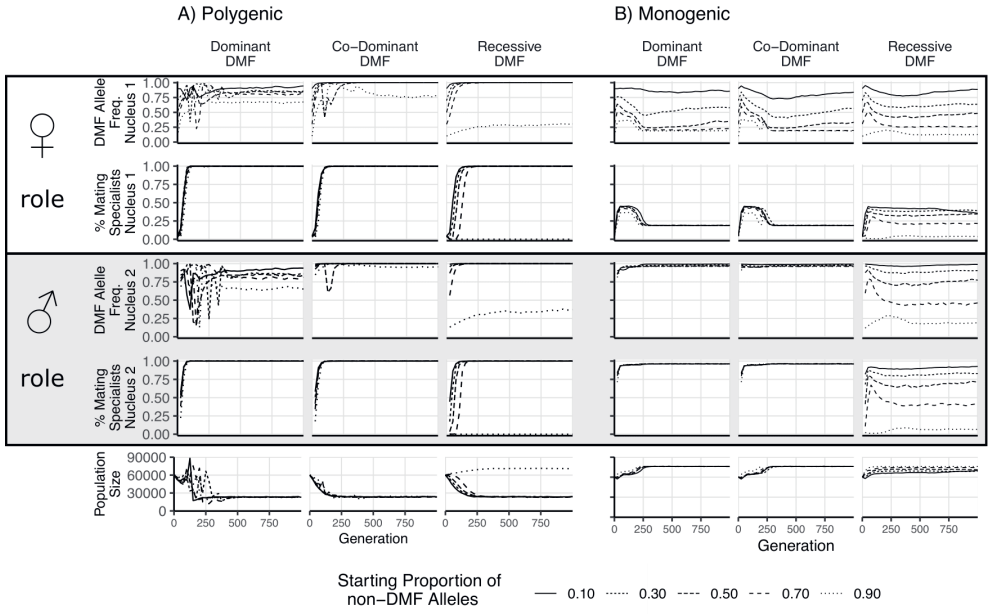


Figure 4: Results of direct competition between nuclei with and without DMF function. Different initial proportions of DMF:non-DMF nuclei are shown with different line styles. Initial DMF and non-DMF nuclei fitness parameters were drawn from the same distribution, with the same tradeoff imposed. Proportion of DMF nuclei and proportion of parasitic nuclei (>66% mating fitness) are shown for the female nucleus (nucleus 1) in the top two rows, while the male nucleus (nucleus 2) is shown below. Note that since the population begins as completely homokaryotic, the Nucleus 2 position is empty for generations 0. The bottom row shows the total population size.

Dikaryotic male function increases in frequency in direct competition

Although our simulations aimed to assess the balance between the levels of selection in the different life cycles, and not to study the transitions between them, it was important to assess whether the dikaryotic state was competitive under the imposed fitness tradeoff. To this end, we competed mixtures with varying starting proportions of nuclei with and without Dikaryotic Male Function (DMF). We simulated DMF as a dominant, co-dominant (each nucleus acting independently), or recessive trait with the fitness tradeoff as either a mono- or polygenic trait. As seen in Figure 4, in most cases when DMF is allowed, it increases in frequency (rows 1 & 3), but with different outcomes between the two nuclear positions. With a polygenic fitness tradeoff, the DMF allele frequency increases, particularly in the male nucleus. When

the fitness tradeoff is monogenic, the DMF allele still increases in the male nucleus, except when DMF is recessive, but the population size remains large. Interestingly, in this monogenic scenario, the initial increase in population size is delayed due to increasing parasitic nuclei in the female role, but this is purged with the first 250 generations regardless of the starting proportion. This purging of high mating fitness genotypes in the female role is less effective in the recessive case, as deleterious alleles are hidden from selection at low frequencies. As the decreased population size with increased mating fitness is based on an assumed fitness trade-off, we tested the effects of a non-linear tradeoff (Fig. S6). Regardless of the shape of tradeoff surface, the DMF allele increased in frequency, and the effect of the non-linearity only affected the rate of increase and the resulting population size.

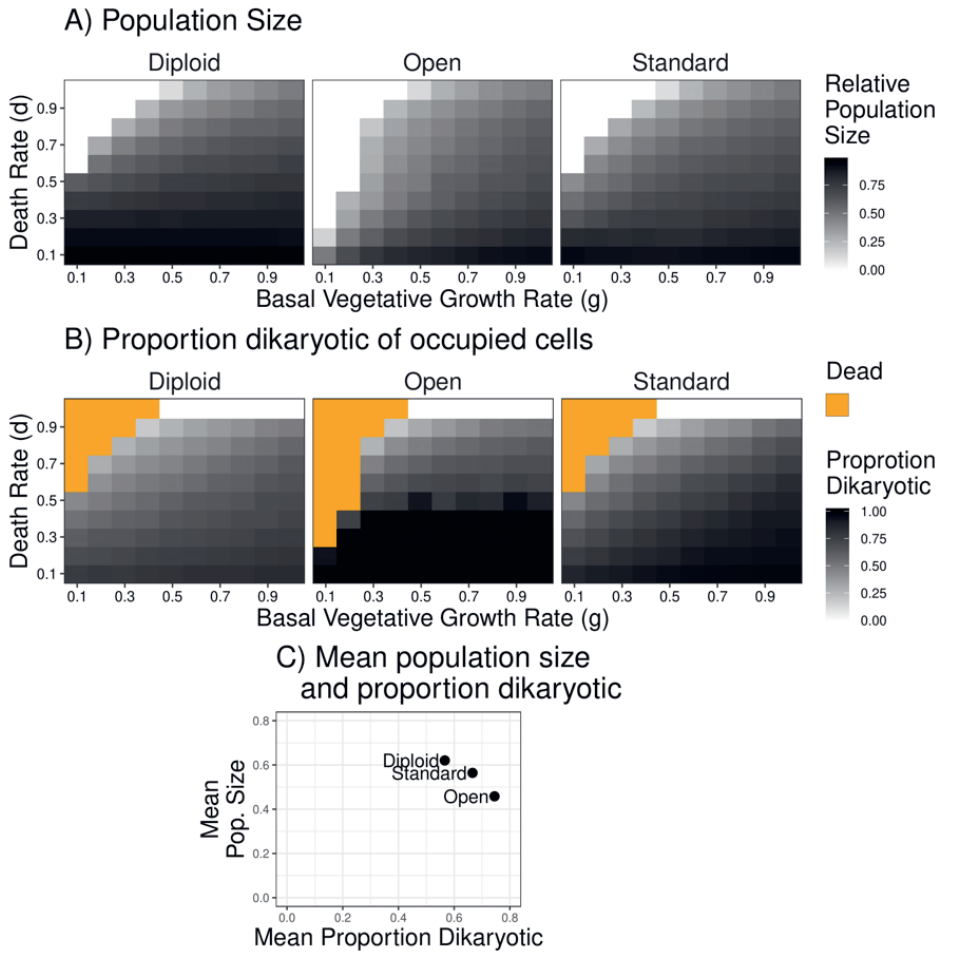


Figure 5: Population size and mating success with differing environmental influences. A) Population size after 500 generations across a range of death and growth rates. Intensity of black is proportional to population size. B) Mating success of simulations from panel A), but intensity of black refers to proportion of occupied cells that are dikaryotic. Orange values indicate simulations had no surviving cells. C) Grand mean of values used in A) and B), grouped by scenario to highlight the relative differences in mating success and population size between the three scenarios.

Effect of environmental pressures

To test the effect of environmental stability on the resulting populations, we varied the parameters of basal growth rate and the death rate, *g* and *d*, respectively. When these two rates are equal a given space has the same probability of dying as it does of being grown into again. When the death rate is higher than growth rate, then a larger proportion of cells will be available to be colonized by spores. As can be seen in Figure 5A, the populations were smaller with increasing death rate (y-axis) in all three types of life cycle, and low growth rate combined with high death

rate led to population collapses (white squares in Figure 5A). The open dikaryon was susceptible to population crashes across a larger range of parameter values. In general, the diploid had the largest population size, the standard dikaryon intermediate, and the open dikaryon the smallest. Looking at the mating success, the proportion dikaryotic, in almost all scenarios the open dikaryon is 100% dikaryotic, while the diploid has a much higher percentage of monokaryons (Figure 5B). As it is difficult to visually compare the three scenarios based on color intensity, the overall averages of surviving populations are plotted in Figure 5C. This shows that indeed the open dikaryon has the highest proportion of dikaryons, but the lowest population size, and the opposite for the diploid. The standard dikaryon is intermediate between the other two scenarios. Notably, there seems to be a negative correlation between population size and proportion dikaryon across the three scenarios.

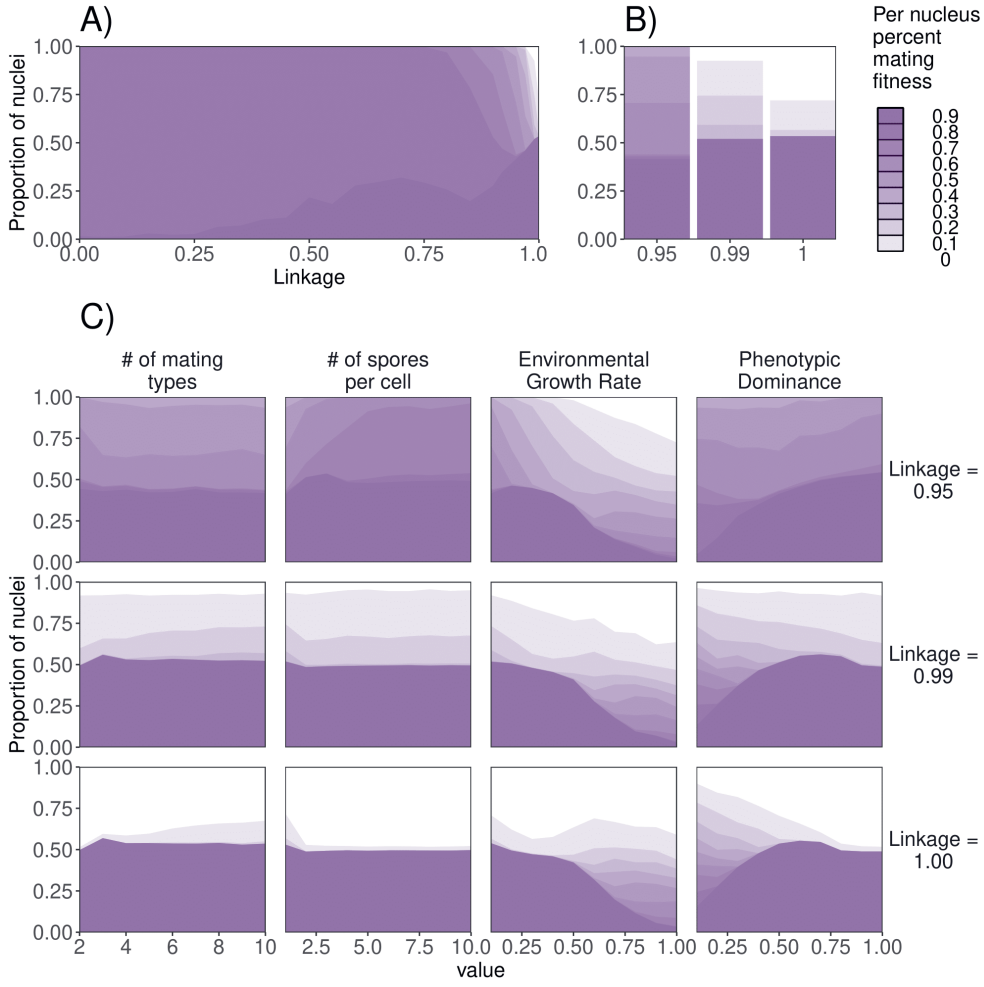


Figure 6: Effect of genetic factors on the prevalence of nuclei with detrimental levels of mating fitness. For all subpanels, mating fitness is binned from all nuclei remaining at the end of 1000 generations. A) Effect of λ on mating fitness in the standard dikaryon. B) Mating fitness distributions for λ values of 0.95, 0.99, and 1.00 which are selected to highlight differences in outcomes. C) Effect of parameters on the proportion of parasitic nuclei in the standard dikaryon scenario. Rows show results under linkage values of 0.95, 0.99, and 1.00. Columns from left to right show results of modifying the number of mating types, spore production, growth rate, and dominance. Shading is proportional to mating fitness, calculated in steps of 10% across 4 replicates. White area represents nuclei with < 10% mating fitness.

Factors that reduce spread of genotypes detrimental to the dikaryon

We investigated potential factors that in the standard dikaryon may influence the proportion of nuclei maximizing their mating fitness, which is detrimental to the dikaryon due to the imposed fitness tradeoff. We varied four factors: the number of mating types, total potential spore production, the basal growth rate, and the phenotypic dominance (Figure 6). As the number of loci affecting this fitness trade-

off is apparently an important factor for the levels of mating fitness, we first calculated the levels of mating fitness across a range of λ values, from λ =1 the monogenic situation, to the polygenic scenario of λ =0. The results showed little difference below λ values of 0.75 (Figure 6A). With increasing λ the phenotypes are more unevenly distributed among nuclei. The results in Figure 6A are resemble those from Figure 3B, except that Figure 6A shows different categories of percentage mating fitness per nucleus, while population averages for the three fitness components are shown in Figure 3B. From these linkage values, we selected λ =0.95 which showed almost all nuclei having >90% mating fitness, λ =0.99 which showed nuclei with a range of mating fitness, and λ =1.00 where a smaller proportion of nuclei optimized mating fitness over 90% (Figure 6B). These scenarios resemble the single locus scenarios, but with offspring phenotypes that include a small amount of mixing between the parental phenotypes.

As individuals with the same mating type cannot mate, reduction of the number of mating types could be expected to reduce selection for mating fitness, as more spaces would be occupied by incompatible mates thus leaving fewer compatible mates for a given focal female. However, we saw little effect of this, and the only difference noticeable was a slight decrease of mating fitness with only two mating types when linkage was 0.99 or 1.00, but a slight increase in mating fitness when linkage was 0.95. Similarly, increasing spore production had a mild affect, increased parasitism when linkage was 0.95.

Increasing the growth rate also decreased parasitism generally, particularly with values of g greater than 0.5. Particularly with the highest linkage values this decrease was more quantitative, with a similar number of nuclei being selected for mating fitness but instead resulting in less specialization. Increasing the dominance, especially above 0.5, resulted in increasing proportion of mating fitness specialists, particularly with higher linkage.

Discussion

The balance of evolutionary forces acting upon this unique dikaryotic life history have been unclear. Here we assess the impact of different levels of selection, nuclear and mycelial, on the dikaryotic lifecycle. The benefits of increased mating opportunities for a nucleus in a dikaryon seem obvious but the potential costs are perhaps less clear. Importantly, the dikaryotic state results in an increased proportion of individuals capable of sexual reproduction, particularly the male role, in the population compared to a diploid, due to the increased mating opportunities. As monokaryons generally have no reproductive output in basidiomycetes, the resources they control (e.g., a section of a tree stump, or ectomycorrhizal tree roots) represent an opportunity for nuclei from dikaryotic mycelial neighbors. Since the nuclei within a dikaryon have low genetic relatedness, competition between them for these additional mating partners provides a tension, potentially resulting in genomic conflict. Our results show that even in the face of an imposed fitness tradeoff at the organismal level, a dikaryotic lifecycle will still select for mating success. Our results further show that the costs and benefits of this dikaryotic lifecycle are influenced by the environment, as the density of individuals influences both the importance of mycelial level fitness, and the chances for repeated matings.

A clear result of our simulations is the strong drive for mating fitness in the standard and open dikaryon. Under all tested scenarios and parameters, the selection for mating fitness resulted in most nuclei specializing in mating fitness. Depending on the parameters, this even resulted in population collapse. The selection benefit of di-mon mating was particularly visible when individual nuclei with and without dikaryotic male function (DMF) are competed directly. The DMF allele spreads through the population, even though it comes with the trade-off, regardless of the shape. This is because the adjacent monokaryons provide a reliable route for gene copy increase of the genotypes with the DMF allele. The marginal decrease in fitness from vegetative growth/spore production, due to the imposed tradeoff, is outweighed by the benefit of additional matings. This drive for mating fitness is even stronger in the open dikaryon, leading to population collapse under a wider range of parameter settings than the standard dikaryon.

Our simulations also indicate that the nature of fitness inheritance has a strong effect on any deleterious effects of nuclear selection. When fitness was determined by a single locus, intermediate, (i.e. non-parental) offspring were restricted to those produced by mutation. The meiotic progeny are differentiated into two types, producing either competitive or non-competitive gametes. However, when fitness was allocated across alleles at multiple loci, matings would result in gametes with competitive alleles at on average half of the loci segregating between the parents, resulting in very low phenotypic variance among offspring, resulting in uniform specialization for mating fitness. While our simulations used a blending inheritance model, this has been shown to have no major effect on resulting social interactions when compared to biologically realistic Mendelian particulate

inheritance (Gardner, 2011). In general, basidiomycete mating type loci are idiomorphic (highly polymorphic alleles), and have suppressed recombination, spanning from a general 50-150Kb up to several Mb (Branco et al., 2017; Brown & Casselton, 2001). Of potential interest, backcrossing of wild isolates has shown that variation in mating success potentially resides near/within the mating-type locus (Nogami et al., 2002). Future studies should investigate if alleles conferring mating success truly reside inside or near the mating locus, and if so, if this is correlated with growth and/or reproduction. Such "parasitic" mating-types alleles could be stable in a population since the tradeoffs between reduced growth and increased mating success would be linked within a single non-recombining locus.

A key assumption in our model is the tradeoff between fitness components. While we do not specify the underlying nature of the tradeoff, our assumption is consistent with antagonistic pleiotropy of the genes involved (i.e., an allele can be optimized for either fitness component but not all). This may seem a strong assumption. However, even without antagonistic pleiotropy, tradeoffs are likely (Garland, 2014; Matthewson & Weisberg, 2009; Stearns, 1989) particularly if selection occurs at multiple levels (Booth, 2014; Lewontin, 1970). Increased selection at the level of the nucleus may also allow accumulation of mutations deleterious for dikaryon fitness, resulting in a tradeoff. An experimental evolution study attempting to select for competitive nuclei failed to find a tradeoff with mycelium-level fitness components (Nieuwenhuis & Aanen, 2018). However, in this experiment simultaneous selection for multiple fitness components could not be excluded. Supporting the presence of a cost for nuclear selection, modifying our trade-off with non-linearity resulted in population decreases proportional to the non-linearity. This shows that the shape of the fitness tradeoff has a quantitative, but not qualitative effect on selection for mating success. Importantly, nuclei capable of dikaryotic male function are selected for, even when the fitness tradeoff is strongly non-linear, leading to a strong cost of mating fitness. In a population of diploid individuals, an allele conferring dikaryotic male function has increased fitness despite the associated tradeoffs.

The relationship between the different fitness components of dikaryons requires further study. Our model crucially assumes that the competitive success of nuclei during di-mon matings is the result of competition between the two nuclei in the dikaryon. Alternatively, the interaction between a nuclear type and the receiving monokaryon may affect the outcome of nuclear competition, effectively a form of female choice by the receiving monokaryon (James, 2015; Kües, 2015; Nieuwenhuis et al., 2011; Nieuwenhuis & Aanen, 2012). The B locus, one of the two loci determining the mating type of tetrapolar species, encodes both pheromones and pheromone receptors. While a single pheromone-receptor interaction is sufficient for sexual compatibility, typically there is high redundancy in the number of compatible pheromone-receptor interactions, with an excess of compatible pheromones (Kües, 2015; Nieuwenhuis & Aanen, 2012). It has been argued that

this redundancy is a consequence of female choice, and that pheromones may function as an honest signal of nuclear quality (James, 2015; Rogers & Greig, 2009). If female choice is important, a tradeoff between nuclear- and mycelial-level fitness components may not be expected, but instead a positive relationship due to the "good genes" model (Zahavi, 1975). Under this model of female choice, nuclear competition could instead have positive effects on mycelial fitness. However, the exact consequences of different assumptions on the relationship between fitness components should be modelled.

While it is often assumed that in fungi the mycelial individual (whether monokaryon or dikaryon) is the unit of selection, in multinucleate fungi this is not the only level (Booth, 2014; Lewontin, 1970). Our model shows an example of how selection at the level of the nucleus, from competition between unrelated nuclei for mating, occurs despite being detrimental to the level of the dikaryon. Nuclear selection has been shown to act rapidly in hyphal fungi, where cheater nuclei can be selected in experimental evolution (Bastiaans et al., 2016), but also in natural isolates of a dikaryotic ascomycete, *Neurospora tetrasperma* (Meunier et al., 2017). An important factor in dikaryotic fitness considerations is the level of phenotypic dominance, which can mask deleterious mutations. The prevalence of dominant phenotypes in basidiomycetes is currently unknown, although recent work has found little support for dominant-recessive relationships (Clergeot et al., 2019; Hiscox et al., 2010; Nobre et al., 2014).

The idea of nuclear selection was not initially connected with fitness tradeoffs, instead predicting ever increasing sexual fitness as a result of internuclear competition (Raper, 1966). A recent reappraisal of internuclear competition phrased it as the "selfish nucleus model", imagining a scenario where mating fitness could select for nuclei detrimental to the dikaryon, similar to our simulation design (James et al., 2009). Our results, with open dikaryons resulting in detrimental levels of nuclear selection, are consistent with the intuition of James et al. (2009), who postulated the occurrence matings between dikaryons "*provided the negative effects of nuclear competition are not also increased"* (James et al., 2009). Our simulations show that if such negative effects of nuclear competition exist, then open dikaryons will suffer from reduced fitness. Therefore, it is crucial to establish the relationship between nuclear competitiveness in di-mon interactions, and the fitness of the resulting dikaryon. As mentioned above, an alternative model is that the interaction between fertilizing nuclei and receiving monokaryon determines nuclear competitive success, which would be a form of female choice based on gamete testing.

Nuclear exchange between dikaryons, di-di matings in our simulations, have been demonstrated in both natural (Hansen et al., 1993; James & Vilgalys, 2001; Johannesson & Stenlid, 2004) and laboratory conditions (James et al., 2009). However, we would argue this is not equivalent to an open dikaryon. These example of nuclear exchange between dikaryons is mainly documented in *Heterobasidion* species, a common basidiomycete study system, but one in which dikaryons develop

abundant monokaryotic hyphal sectors (Johannesson & Stenlid, 2004). Presumably the observed nuclear exchange between dikaryons occurs through monokaryotic hyphal intermediates. This inclusion of monokaryotic hyphal intermediates makes this more similar to either di-mon or mon-mon mating, depending on how the monokaryotic sections are fertilized. Crucially, there is a qualitative difference between di-di and di-mon matings through monokaryotic intermediates, as novel dikaryons from the latter will be somatically incompatible with the two established dikaryotic progenitors, having no spatial access to further resources (Johannesson & Stenlid, 2004). Such segregation into monokaryotic hyphae with subsequent mating would be compatible with the model recently suggested as "somatic hybridization" in rust fungi (Li et al., 2019; Nelson et al., 1955; Wu et al., 2019). These apparent di-di matings may not experience the same evolutionary forces as if true di-di matings were allowed, due to the physical restriction of the novel dikaryons.

Our simulations aimed to understand the potential consequences of nuclear selection in mushroom-forming basidiomycetes but may also have relevance for other fungal groups. The widespread plant pathogenic rust fungi also have a dikaryotic stage. Nuclear exchange between these rust dikaryons is also reported, "somatic hybridization", in the production of novel pathogenic rust races (Burdon et al., 1981; Li et al., 2019; Nelson et al., 1955; Wu et al., 2019). It is difficult to reconcile the occurrence of frequent somatic hybridization with our results showing that open dikaryons are highly susceptible to nuclear selection. One possibility is that somatic hybridization is exceedingly rare, as a single somatic hybridization event could produce a rust race able to grow on otherwise resistant hosts. Another possibility would be monokaryotic intermediate hyphae, similar to that in *Heterobasidion* (Hansen et al., 1993; Johannesson & Stenlid, 2004). Also, recent evidence indicates that the abundant Arbuscular Mycorrhizal Fungi from the phylum Mucoromycota also exist in a dikaryotic state (Ropars et al., 2016). While hyphal fusion occurs in this lineage (Croll et al., 2009), the potential for di-mon, or even di-di matings, remains unknown.

The open dikaryon of our model corresponds to the "unit mycelium" concept formulated to explain observations of cell fusions in cultured fungal material (Buller, 1934). This "unit mycelium" concept was the working paradigm over many decades, with its assumption of cooperation between genetically unrelated individuals. However, accumulating evidence from natural isolates, showing that neighboring dikaryotic mycelia were instead distinct, led to the influential synthesis of the "individualistic mycelium" (Rayner, 1991). This model of distinct individuals separated by allorecognition responses was consistent with the observation of somatic incompatibility between dikaryons, with fusions between nonself individuals leading to cell death, not cooperation (Booth, 2014). The "individualistic mycelium" model is not only consistent with experimental evidence but also compatible with

predictions that cooperation between non-related dikaryons to be exploited almost immediately (Czárán et al., 2014).

This persistent dikaryotic state has been retained for over 400 million years (Chang et al., 2015), yet few extant basidiomycete species are diploid (Anderson & Kohn, 2007). The retention of the dikaryotic state in the vast majority of basidomycetes leaves open the potential for mycelium-level fitness costs of nuclear selection due to di-mon matings. While there is little data on the prevalence of di-mon matings in the field, they may be more common than is generally assumed (Nieuwenhuis et al., 2013). Given the potential for strong selection for mating fitness within a dikaryon, it is crucial to determine the relationship between nuclear competitiveness and mycelium-level fitness.

Conclusion:

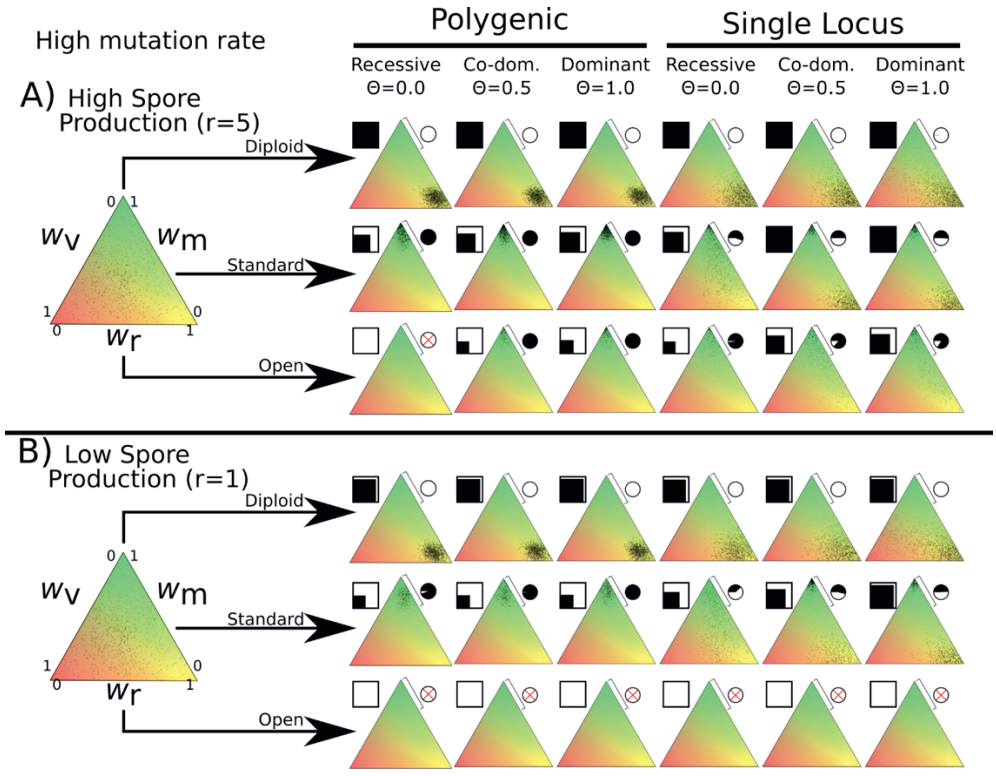
Potential benefits of a dikaryon stage have been a subject of discussion for some time. The presence of the dikaryotic state emphasizes the level of selection on the individual nuclei, something that is often overlooked in evolutionary discussions of fungi. The persistent association between unrelated haploid nuclei will invariably select for mating success, potentially to the detriment of the individual. Our results show the potential costs of selection at the level of nuclei for mating for mycelium-level fitness components, and how those can be reduced. First, restricting fertilization by dikaryons to monokaryons reduces the level of nuclear mating fitness and maintains a higher mycelium-level fitness, compared to completely free exchange between dikaryons. Second, if the variance in mating fitness and its associated trade-off is restricted to a single locus, the costs to mycelial level fitness are reduced. Most importantly, establishing the relationship between the different fitness components is crucial to understand the potential consequences of nuclear competition in the dikaryotic life cycle. We also hypothesize that recent examples of dikaryon nuclear exchange are due to monokaryotic intermediates or result from extremely rare events. Our results show that the consequences of competition between the unrelated haploid nuclei in a dikaryon can be severe, and there must exist mechanisms to police it.

Data Availability: Source to run simulations, as well as scripts to produce figures and analysis are found at https://github.com/BenAuxier/Basid.Sex.Sim

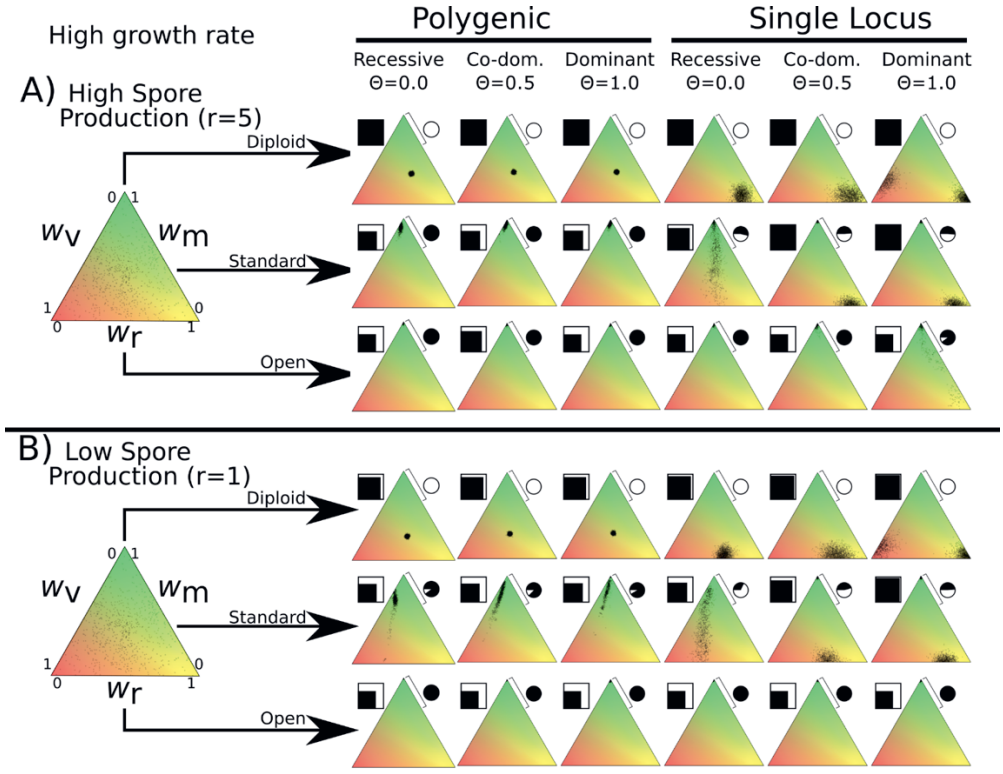
Author Contributions: TC designed Fortran code with input from BA and DKA. BA and TC performed subsequent analysis with input from DKA. BA wrote draft manuscript. All authors edited and approved final manuscript.

Acknowledgments: We gratefully acknowledge the input from anonymous reviewers of a previous version of this manuscript whose critical comments led to substantial improvements in content and clarity.

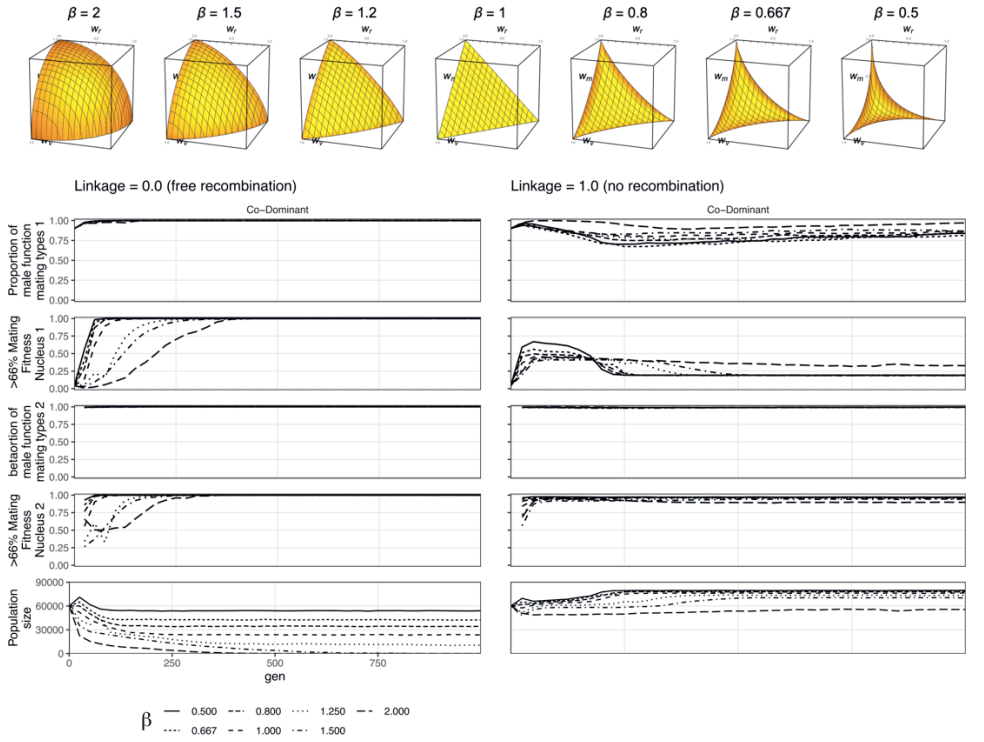
Supplemental Data:



Supplemental Figure 1: Overview of simulation outcomes using higher mutation width of 0.05 instead of 0.01 used in other simulations. Note that in the polygenic diploid scenario the nuclei do not cluster near the middle, but instead optimize spore production similar to the monogenic scenario.



Supplemental Figure 2: Overview of simulation outcomes displayed as in Figure 2 but using higher basal growth rate of 0.5 instead of 0.1 used in other simulations. Note that this increased growth rate allows the open dikaryon to survive and increases the vegetative growth fitness of both diploid and standard dikaryon scenarios.



Supplemental Figure 3: Linearity of the fitness trade-off has limited effect on mating selection**.** It shows that in the co-dominant state, where DMF spreads fastest, the DMF allele spreads through the population regardless of trade-off shape. The beta value has some effect on the strength of selection, so with ß of 2 (the long dash line) in the polygenic scenario the population size is smallest since fitness costs are highest. So, when polygenic selection against the allele slows the spread of parasites, but not of the DMF allele itself, and it takes longest for the parasites to spread in the female role (nucleus 1).

Due to limitations of the print format, the following files available with the online version

Supplemental File 1: Pseudocode for the simulation algorithm.

Supplemental File 2: Animation of the first 100 generations of the diploid life cycle under standard conditions. Dots indicate individual nuclei. Bars indicate population size, and "% maters" which is the proportion of nuclei with >66% of fitness allocated to mating fitness, as in Figure 2.

Supplemental File 3: Animation as found in Supplemental File 2, except of the standard dikaryon life cycle.

Supplemental File 4: Animation as found in Supplemental File 2, except this time with open dikaryon life cycle.

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Chapter 3

Cytoplasmic Mixing, Not Nuclear Coexistence, Can Explain Somatic Incompatibility in Basidiomycetes

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Published As :
Auxier et al., 2022
Microorganisms, 2021, 9(6), 1248;
https://doi.org/10.3390/microorganisms9061248

Abstract:

Nonself recognition leading to somatic incompatibility (SI) is commonly used by mycologists to distinguish fungal individuals. Despite this, the process remains poorly understood in basidiomycetes as all current models of SI are based on genetic and molecular research in ascomycete fungi. Ascomycete fungi are mainly found in a monokaryotic stage, with a single type of haploid nuclei, and only briefly during mating do two genomes coexist in heterokaryotic cells. The sister phylum, Basidiomycota, differs in several relevant aspects. Basidiomycete fungi have an extended heterokaryotic stage, and SI is generally observed between heterokaryons instead of between homokaryons. Additionally, considerable nuclear migration occurs during a basidiomycete mating reaction, introducing a nucleus into a resident homokaryon with cytoplasmic mixing limited to the fused or neighboring cells. To accommodate these differences, we describe a basidiomycete model for nonself recognition using post-translational modification, based on a reader-writer system as found in other organisms. This post-translational modification combined with nuclear migration allows for the coexistence of two genomes in one individual while maintaining nonself recognition during all life stages. Somewhat surprisingly, this model predicts localized cell death during mating, which is consistent with previous observations but differs from the general assumptions of basidiomycete mating. This model will help guide future research into the mechanisms behind basidiomycete nonself recognition.

Introduction

Nonself recognition is a prerequisite for the resilient mycelial network that makes up a fungal individual. This network is built by a set of growing hyphal tips that explore their environment in search of resources. Eventually, this growth results in the meeting of two hyphae, whether originating from the same individual or not. If these tips are from the same individual, then fusion will increase the efficiency of the network [1], while if the hyphae are from different individuals, fusion may permit the spread of parasitic elements [2–5]. In fungi, despite these apparent risks, conspecific hyphal interactions often result in fusion. Identity is then assessed post-fusion and if nonself is recognized the fusion cell is degraded, a process termed somatic incompatibility (SI). In the absence of nonself cellular markers, indicating a self-fusion, a stable fusion cell is formed as part of the interconnected hyphal network. Without such a nonself recognition mechanism, post-fusion continuation would be permitted between different individuals resulting in the risk of spreading elements like viruses and nuclear or mitochondrial parasitic variants.

Nonself recognition followed by cell death reduces these risks but comes with the cost of the hyphal death, as well as reductions in spore production due to a reduced colony size [1]. To minimize the risks of building a hyphal network while allowing the benefits of fusion, a robust and precise system of nonself recognition is essential.

Systems of nonself recognition are not unique to fungi. Other multicellular organisms such as plants and animals also have such systems, often built on similar molecular pathways [6]. This maintenance of cellular identity is a wider phenomenon, also found in bacteria, and is likely shared among all cellular life [7,8]. Thus, even without a multicellular lifestyle, organisms still appear to benefit from preventing the spread of infectious agents, as seen in bacteriophage recognition systems [9]. In these systems, bacteria produce “self” markings on endogenously produced DNA. This allows a bacterium to discriminate against incoming DNA, such as from viruses since they lack these marks. Interestingly, in bacteria, these systems are also somewhat imprecise, and occasionally self-DNA is cleaved [10]. Such self-cleavage in bacteria shows that the costs of nonself recognition systems are common, but the benefits of these systems apparently outweigh the costs.

In basidiomycete fungal species, a SI phenotype is consistently observed between heterokaryons [11]. Thus, a lack of SI between isolates is generally used to identify clonal isolates. Culture-based SI observations are more practical than the use of genetic markers to identify clones and are thus widely used [12–14]. The macroscopic phenotypes of SI in basidiomycetes vary greatly, but SI is generally found between heterokaryons, although occasional reports of macroscopic responses between homokaryons have been mentioned [15]. Despite its repeated use as a tool for experimental mycology, genetics

remains unclear [16–20], and the mechanisms remain completely unknown [11]. Here, we attempt to translate what is known about nonself recognition from ascomycete systems onto basidiomycete biology and explore the consequences of such mechanisms.

Genetics and Principles of Ascomycete SI

Typically, two randomly selected ascomycete individuals from a wild population will show SI following anastomosis. The known molecular determinants of nonself recognition in ascomycete systems are generally based on direct protein interactions [21]. Since ascomycete species are predominantly homokaryotic (i.e., with only one type of nucleus) and have haploid genomes, for any particular gene the products of only a single allele will be found in the cytoplasm. In these organisms, nonself recognition then results from interactions between the products of two alleles of the same gene (allelic; Figure 1 left, or between products of generally tightly linked non-homologous genes (non-allelic; Figure 1 right). Whether allelic or non-allelic, the general principle of ascomycete systems seems to be that for each locus, the cytoplasm of an individual will contain products from a single allele and upon fusion, mixing of two cytoplasms may result in the interaction of these products. The signal from this nonself-recognition is then transmitted to trigger cell death, often based on extremely conserved mechanisms [22]. While there are multiple loci where differences result in nonself recognition, they are not equivalent as the phenotypes can differ greatly both in macroscopic morphology [23] as well as efficacy to prevent the spread of parasitic elements [4]. Regardless of the specifics, the resulting cell death breaks the cytoplasmic connection between the two mycelia preventing, or at least limiting, the spread of parasitic elements. Within a population with multiple loci where differences confer incompatibility, most individuals will differ at a minimum of one locus. Thus, the fusion between two randomly selected individuals will mix these cytoplasms, and incompatibility between any two individuals can be expected [24].

However, cell fusion between genetically different ascomycete homokaryons should not always result in SI, as fusion is necessary during sexual reproduction. In ascomycete species following fertilization both haploid nuclei briefly co-exist in a heterokaryotic state until meiosis. During this stage, clearly cell death should be prevented despite the presence of two genomes. One possible solution is to reduce the concentration of incompatible proteins by dilution of the cytoplasm of one partner. Mating in ascomycete fungi is typically anisogamous since fertilization often involves male spermatia/conidia donating a nucleus with limited cytoplasm, preventing cytoplasmic mixing and subsequent lethal protein-protein interactions. A second solution is the active toleration of otherwise lethal protein combinations. In several *Neurospora* species, the mating locus itself acts as a nonself recognition locus, dependent on the TOL protein. During fruiting body initiation, TOL is downregulated in the sexual structures, allowing both mating types to be co-expressed within specific

tissues [25,26].

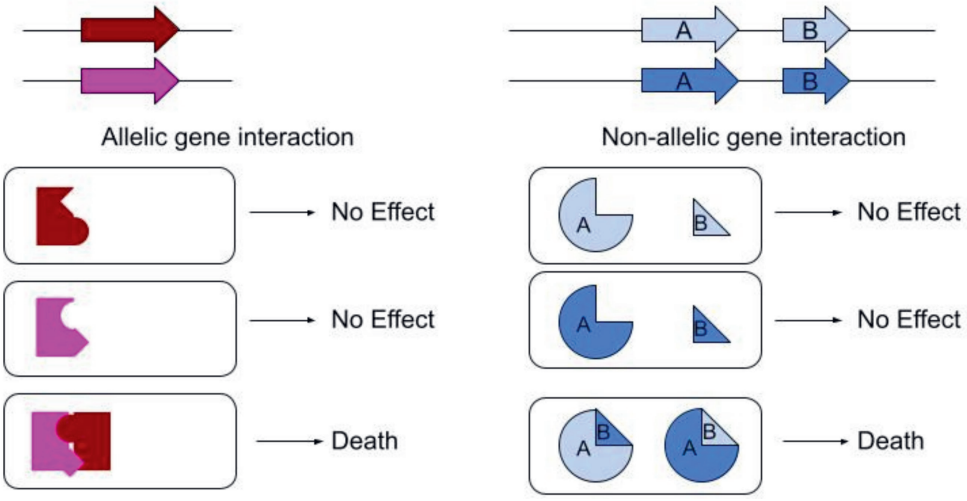

Figure 1. Traditional models for gene interactions leading to cell death from nonself recognition in ascomycete systems. The interactions can be based on direct gene product interactions, whether allelic (alleles of the same gene), or non-allelic (with alternate alleles of genetically linked genes). These systems act similarly upon fusion, but non-allelic systems differ during reproduction, where self-incompatible offspring result from the interaction produced from recombination between genes in a nonallelic locus. Note that non-allelic interactions are often asymmetric (e.g., only the combination of light blue A and dark blue B may be toxic, the interaction of dark blue A and light blue B may have no effect).

Based on the above concepts, a population of an ascomycete species will be polymorphic at a number of loci, either allelic or non-allelic, with each locus being capable oftriggering nonself recognition, resulting in SI. The number of polymorphic loci has been experimentally determined for only a few species, but it appears that there can be between 5 and 11 loci responsible [24]. However, it should be noted that many studies have recognized nonself responses based on macroscopic phenotypes, and recent experiments have identified loci involved in nonself recognition without a macroscopic phenotype [22,27]. The allelic variants of genes conferring nonself recognition specificity are generally found in even allele frequencies and with trans-species polymorphisms, general signs of balancing selection [28–31]. This form of selection occurs when the fitness value of an allele is high at low frequency and decreases as the allele becomes more common in a population as its utility for distinguishing self from nonself decreases [32,33]. The increased fitness of any rare allele leads to stable and even allele frequencies across populations [29]. Whether or not the selection derives from a role for nonself recognition genes in a fungal immunity system [6,34], or other sources of extrinsic selection such as mating loci [35,36], the resulting balancing selection leads to alleles being regularly shared between closely related species [29,30]. However, the polymorphic genes triggering nonself recognition are not regularly shared between higher groups

of taxa. This means that a “core” set of incompatibility genes for all fungi does not exist, although there are domains that are repeatedly found to be involved [37]. This then implies that the mechanisms of nonself recognition are constantly evolving, and the ongoing recruitment of new proteins allows for greater specificity. This lack of a core set of ascomycete nonself recognition genes is consistent with previous research which failed to recover orthologs of ascomycete nonself recognition genes in basidiomycete genomes [38]. While basidiomycete nonself recognition may not involve orthologous genes, we may still be able to apply principles learned from studies in ascomycete species.

Differences between Ascomycete and Basidiomycete Species Regarding Nonself Recognition

Species of basidiomycetes, particularly the mushroom-forming groups, present a challenge for the existing paradigm of fungal nonself recognition. These fungi alternate between a monokaryotic state formed after meiosis and a persistent heterokaryotic state formed from the fusion of two homokaryons, in addition to new outgrowth being heterokaryotic. This outcrossing is facilitated by the extraordinarily high numbers of mating types [39], ensuring almost certain sexual compatibility [40]. It should be noted that while the sexual compatibility system is sometimes referred to as a “nonself recognition system” [41], the sexual system controls nuclear migration (amongst others) and is independent of the cell-death inducing nonself recognition discussed here. While not common, there are examples of homokaryons that have compatible mating loci yet appear to be either sexually incompatible or asymmetrically compatible [15,42]. An important but seemingly underacknowledged observation is that even in sexually compatible matings, cell death in the fusion cell is observed [43]. This cell death between homokaryons has not only been observed at the microscopic level but has been also observed macroscopically, as seen in *Rhizoctonia solani* [44,45].

To achieve near-universal sexual compatibility, any somatic incompatibility between the homokaryons must be overcome [46]. The compatibility of two distinct homokaryons means that nonself recognition must either be turned off when homokaryotic, allowing free fusion, or some other mechanism must allow the migrating nuclei to escape cell death. Following the fusion of two homokaryons, nuclei from each homokaryon reciprocally fertilize the other homokaryon, and rapid nuclear migration leads to both homokaryons becoming heterokaryotic themselves, in addition to new outgrowth being heterokaryotic [47]. Thus, this heterokaryon has two different genomes in a shared cytoplasm (Figure 2b). Ascomycete fungi also form a heterokaryotic stage but this is short-lived and/or occurs in specialized sexual tissues prior to ascospore formation. One solution found in *Neurospora* sp. to tolerate the brief heterokaryotic phase is through suppressing the action of the *tol* gene product, which otherwise triggers nonself recognition due to the mating-type alleles [26]. This suppression of mating-locus-

associated SI is feasible because the ascomycete heterokaryon is short-lived, and reduced *tol* expression can be restricted to a specific time and place in the sexual cycle. This reduced *tol* expression, in theory, permits the spread of parasitic elements, however, the production of the specialized female structures (e.g., perithecial walls and paraphyses) prior to fertilization limits the potential for the spread of these parasitic elements. It has been shown in *Aspergillus nidulans* that the fertilizing nucleus can spread to the Hülle cells, accessory multinucleate cells, but only when strains are somatically compatible, indicating that SI limits this spread [48,49]. As the heterokaryotic stage is the persistent stage in most basidiomycete species, a system analogous to *tol* is not feasible, since prevention of recognition during the heterokaryotic stage is not compatible with the fact that incompatibility occurs between heterokaryons.

In addition to heterokaryon formation during basidiomycete matings, models of non-self recognition must be compatible with matings between heterokaryons and homokaryons, so-called Buller matings [50,51]. These involve the fusion of dikaryotic and monokaryotic hyphae, migration of nuclei from the heterokaryon into the receiving homokaryon, with eventual fertilization of the homokaryon by a single nuclear type from the original dikaryotic partner. These matings are perhaps common in nature [52] and do not seem to be affected by SI. Such a Buller mating produces a fusion cell containing three different nuclei, with nuclear migration from the heterokaryon into the receiving homokaryon (Figure 2c). Interestingly, it is known that SI can result from interactions between heterokaryons constructed to share a “common nucleus”, with one heterokaryon having nuclei A + B and the other A + C, nucleus A being common to both [11,16]. It seems that a fusion cell in a Buller mating, having three different nuclei, has a different outcome than the pairing of two heterokaryons that have a common nucleus, also having three different nuclei. Thus, the difference between a Buller mating and a “common nucleus” heterokaryon pairing cannot be due to genetics, as both involve three nuclear types in the fusion cell. Instead, perhaps the success of Buller matings relates to nuclear migration, which does not occur in pairing between heterokaryons with a common nucleus (Figure 2d). Despite the apparent differences between ascomycete and basidiomycete fungi, we argue that the presence of nuclear migration in basidiomycete species may explain a difference in outcomes but with similar underlying mechanisms.

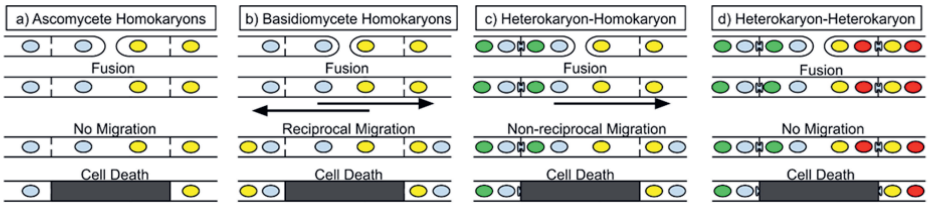

Figure 2. Nuclear migration permits mating in basidiomycetes despite the death of fusion cells. (**a**) The left panel shows the conventional ascomycete scenario, the fusion and subsequent cell death between two genetically different homokaryons; (**b**) shows the basidiomycete scenario when two different homokaryons meet and fuse, it generally leads to mating and reciprocal nuclear migration even though the fusion cell may die; (**c**) shows the outcome of a Buller mating, with unidirectional nuclear migration into the homokaryon individual, producing two different heterokaryons separated by a now-dead fusion cell; (**d**) When two distinct heterokaryons fuse, for example, mated basidiomycetes, it leads to nonself recognition and cell death like the others, but the lack of nuclear migration means that no further matings are produced. The scenario in (**d**) also applies for pseudohomothallic ascomycetes such as *Neurospora tetrasperma* or *Podospora anserina*.

Dikaryotic Ascomycete Fungi May Serve as a Guide to Understand Basidiomycete SI

Somatic incompatibility in ascomycete fungi generally occurs between homokaryons. However, in some pseudohomothallic ascomycete species, incompatibility also functions between heterokaryons. Isolates of *Neurospora tetrasperma* and *Podospora anserina* have nuclei of both mating types, and in these species, SI occurs between the stable heterokaryons, between homokaryons, as well as between heterokaryons and homokaryons. As long as incompatible protein products are expressed, somatic incompatibility results following hyphal fusion. Due to the pseudohomothallic lifecycle (i.e., spores are heterokaryotic for the mating type and the result of selfing), field isolates of these species are, as a rule, heterokaryotic and highly inbred, with little or no het-gene differences within a heterokaryon, and interactions in nature between homokaryons are likely rare.

As *N. crassa* is a primary ascomycete model for fungal nonself recognition, it seems logical to first examine the closely related *N. tetrasperma*, which remains heterokaryotic throughout its entire lifecycle. Members of this species produce heterokaryotic conidia, as well as producing four heterokaryotic ascospores per ascus instead of the standard eight homokaryotic ascospores. Thus, the two genomes reside in the same cell continuously, although homokaryotic ascospores are produced at a low rate. It is then interesting to consider how these organisms handle the complications of incompatibility from the co-habitation of two genomes. *N. tetrasperma* avoids the problem of mating-type associated SI by having inactive tol alleles [53]. Investigation of wild isolates has shown them to be homozygous at all other known het loci [54]. Furthermore, sexual crosses between homokaryons derived from strains that show incompatibility as heterokaryons (thus

homokaryons that differ at het loci) produce heterokaryotic offspring that are self-incompatible, resulting in extremely poor growth [55,56]. This limits outbreeding in this species, as outcrossing produces offspring heterozygous for het loci which restricts growth severely [55]. Interestingly, the severe growth defect of self-incompatibility from heterozygosity at a het locus can be escaped, as mutations in one of the copies of a heterozygous het locus will restore wild-type growth and thus have a strong advantage [54]. Thus, in *N. tetrasperma*, SI between homokaryons is not fundamentally different from SI between heterokaryons.

The other model system for ascomycete SI, *P. anserina*, may also inform a model for basidiomycete nonself recognition. Several incompatibility systems from this species have been characterized, particularly the prion-forming *het-s* [57]. This system has two alleles: *het-S* which contains a HeLO domain that when exposed causes holes in the plasma membrane, and *het-s*, which contains a harmless HeLO domain [57] Figure 1. The HET-s* protein from the *het-s* allele can transition from a harmless non-prion form into the HET-s prion form which can trigger the HET-S protein to cause death [58]. Homozygous *het-s/het-s* heterokaryons producing only HET-s or HET-s* are tolerated since they contain a harmless HeLO domain. Additionally, homozygous *het-S/het-S* heterokaryons are viable since the HET-S is folded to hide the toxic HeLO domain. Heterozygotes *het-s/het-S* producing both HET-s* and HET-S are tolerated, as the rare transitions of HET-s* to HET-s/HET-S result in local cell death preventing prion propagation while the other cells that have remained HET-s*/HET-S remain viable [59]. In effect, this means that the HET-S protein suppresses the action of HET-s as a prion, due to this selection effect [59,60]. However, in homozygous *het-s/het-s* heterokaryons, HET-s* will eventually transform to the prion form and becomes HET-s/HET-s. Such a heterokaryon then triggers cell death if it encounters an individual that produces HET-S, whether that individual is *het-S/het-S* or *het-S/het-s*. Thus, such a system can create incompatibility between heterokaryons as well as between homokaryons, without triggering incompatibility within a heterokaryotic mycelium.

A second model from *P. anserina* may be found in the *het-V/het-V1* incompatibility system [61–63]. In this system, the *het-V* 'allele' is made of two genes: PaMt1, encoding a methyltransferase (writer), and PaMt2, encoding a protein with 2 domains MLKL (an executor, which causes cell death by membrane permeation) and TUDOR (reader: recognizes methylation status of target) [61]. The alternate "allele" *het-V1* is a null allele, with neither PaMt1 nor PaMt2. If the reader (TUDOR domain of PaMT2) senses a lack of methylation from the writer (methyltransferase of PaMT1) in certain target proteins, it directs cell death using the MLKL domain. Timely co-expression of reader and writer is analogous to restriction-modification in bacteria, with no cell death since all target molecules will receive the protective modification [10]. A confrontation

between Podospora *het-V* and *het-V1* homokaryons results in an SI reaction, because the target proteins in *het-V1* cells that are not methylated, will subsequently be recognized by the reader (TUDOR) leading to cell death [61]. In this *het-V/het-V1* system, co-expression neutralizes the antagonistic alleles. Therefore, a heterokaryotic progeny *het-V/het-V1* is stable because the target proteins will be sufficiently methylated, preventing PaMt2 from triggering cell death. This *het-V/het-V1* heterokaryon would have a Het-V phenotype and be compatible with *het-V/het-V* heterokaryons, incompatibility would only result from such individual with Het-V phenotype to a *het-V1/het-V1* individual, highlighting the asymmetry in the *het-V/het-V1* system [62]. So, the *het-V/het-V1* system allows for SI to occur between heterokaryons (as well as between homokaryons), but heterokaryons are tolerated, an important condition of a basidiomycete model. We note that these properties result from the post-translational modification of proteins in the *het-V* system, and similar modifications to other cytoplasmic molecules would likely have similar results.

Outcomes of Traditional Models of Nonself Recognition

We now explore the implications for the different known nonself recognition systems for the typical basidiomycete lifecycle. We consider the lifecycle to include mating between unrelated homokaryons, and reciprocal nuclear migration between homokaryons that are sexually compatible as determined by unlinked mating loci. For the heterokaryotic stage, we assume heterokaryons are incompatible with other heterokaryons, but that nuclear migration from Buller matings is possible. The challenge is how we can explain that SI between two nuclei is avoided in a heterokaryotic cell, which would prevent their stable coexistence during the main stage of the basidiomycete lifecycle.

Sexual Compatibility

Only When Somatic Compatibility Is Excluded (Allelic/Nonallelic Interactions) Direct interactions between two protein products from two alleles, whether or not from homologous genes, are not compatible with the heterokaryotic lifecycle. The fusion of two distinct basidiomycete homokaryons would trigger nonself recognition directly inside the heterokaryon. The observation that homokaryons are generally sexually compatible rules out the possibility that mates are chosen based on compatibility at nonself recognition loci. This makes basidiomycete biology fundamentally different from the heterokaryotic Neurospora tetrasperma [54,55]. While both an allelic and non-allelic system would result in incompatibility between heterokaryons, the subsequent death within the heterokaryon rules out such a mechanism.

Differential Expression

Another possibility is the presence of an allelic or non-allelic system that

is only expressed at the heterokaryon stage, using a mechanism opposite to the *tol* system of
N. crassa. This seems like an attractive option since it allows the fusion of unrelated homokaryons, as they would not express the incompatible gene products, or not express part of the downstream pathway. This would allow for reciprocal nuclear migration. However, once nuclear migration is complete, the now dikaryotic cells would express the incompatible gene products, leading to the death of all dikaryotic cells. It could be possible that only one allele was expressed: allele-specific expression similar to genomic imprinting in plants/animals. Such a system would require precise regulation since a single locus that evades this imprinting would trigger cell death. Additionally, such a mechanism is not consistent with observations of nonself recognition in the fusion cells of sexually compatible homokaryons [43,44].

Post-Translational Modification

Perhaps a more promising model is found in a post-translational modification system. In such a system, heterokaryons are stable since the writers from the two nuclei modify all the target proteins in the cytoplasm, preventing the corresponding readers from triggering cell death. Such a system would be similar to the *het-V/het-V1* system of the *P. anserina* system discussed above in Section 4. The fusion of two heterokaryons would lead to nonself recognition, as long as the set of readers/writers differs between individuals. The fusion of heterokaryons will present a large number of unmodified targets, which will be detected by the corresponding reader molecule and trigger cell death. The fusion of two homokaryons would also present unmodified targets that would be recognized by the reader protein. This would also trigger nonself recognition and cell death. While at first, this seems incompatible with the basidiomycete lifecycle, detailed inspection has shown that cell death is found in sexually compatible homokaryons, although often slower than between heterokaryons [43,44]. It is possible that death of the fusion cell between homokaryons is common, but that occasional nuclear migration can escape prior to the death of the fusion cell. Since two homokaryons growing next to each other will have many hyphal fusions, only a small minority need to be successful for mating to occur due to the subsequent migration and mitosis.

Differential Expression of a Post-Translation Modification System

The above system could be modified by differential expression of the reader, writer, target, or a mix of these three. Limiting the expression of the target or the reader to the heterokaryon would prevent incompatibilities between homokaryons, but as discussed above, nonself recognition between homokaryons may still be compatible with the basidiomycete lifecycle. Furthermore, the targets of non-self-recognition are often essential genes, with other functional roles [27]. As such, the reduced expression would likely come with

deleterious phenotypic side effects.

A Basidiomycete Scenario

Incorporating the previous information, we now consider a model of basidiomycete nonself recognition. We largely draw from the *het-V/het-V1* system, where allelic differences within a heterokaryon are tolerated but incompatibility occurs between heterokaryons based on post-translational modification of cytoplasmic proteins. We consider if such a reader/writer system is consistent with what we know of basidiomycete fungi. We include nuclear migration during mating as a potential explanation for two seemingly conflicting phenomena: Occurrence of cell death, and the formation of a heterokaryon. We first assume a first locus X which encodes the reader and the writer genes, with three alleles; X_A, X_B, and X_C. The genes of X_A both modify a set of proteins (Figure 3, light blue), as well as check their modification (Figure 3, light blue star). Likewise, the protein products of X_B and X_C perform similar functions but with different modifications or different targets.

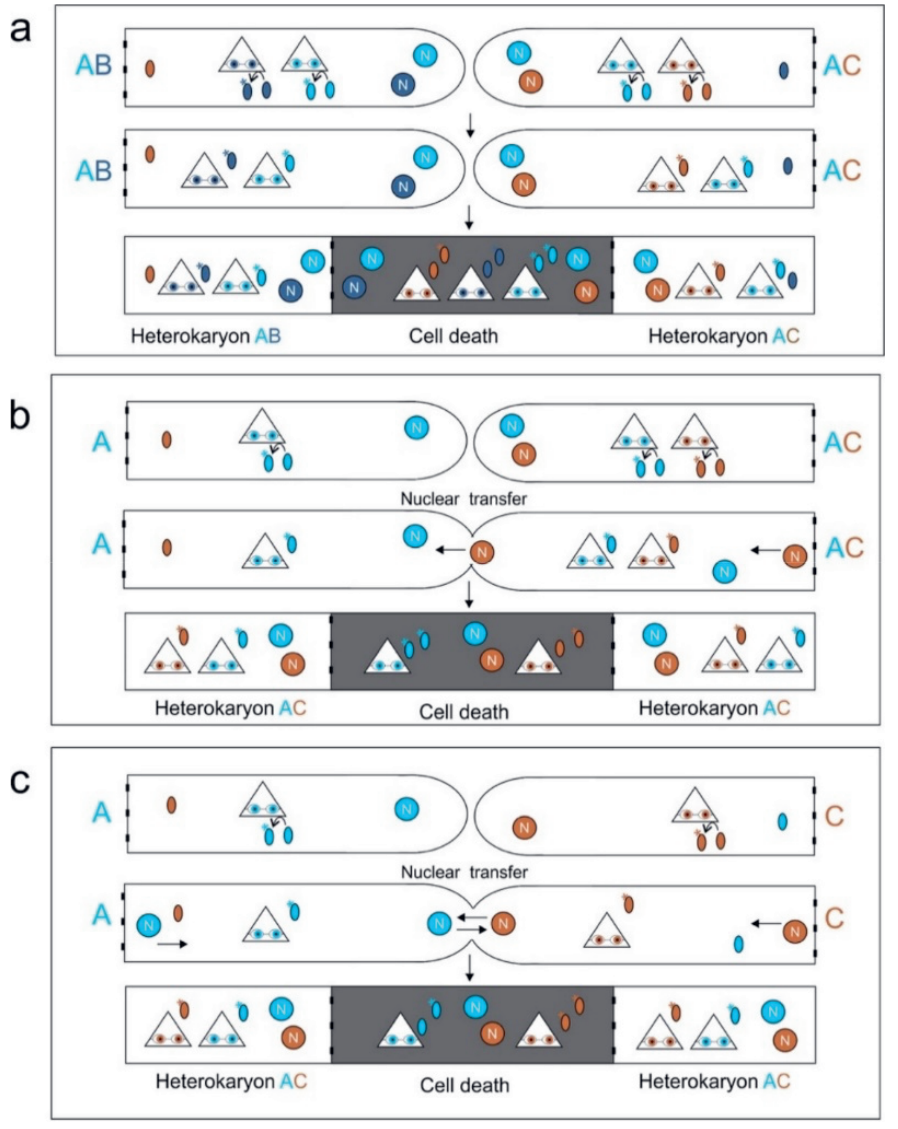

Figure 3. A basidiomycete scenario for a reader/writer involvement in nonself recognition. (**a**) interactions between heterokaryons. Each nucleus (circle with letter N) produces a reader/writer (triangle with glasses) that both modifies (asterisks) its target (oval), as well as monitors for the presence of an unmodified target. Each nucleus of each heterokaryon modifies its own target, but each leaves one target unmodified (e.g., brown targets in the AB heterokaryon). Following fusion of the heterokaryons, these unmodified targets are recognized by the alternate reader/writer leading to cell death. (**b**) Similar to described above, but now nuclear migration in the Buller mating from the AD heterokaryon into the homokaryon allows the escape of cell death by timely expression of the reader/writer in the resultant heterokaryon. (**c**) interactions between homokaryons lead to a mating reaction, as bidirectional nuclear migration allows the escape of the same cell death beyond the fused cell.

Fusion between an AB (X_AX_B) and an AC (X_AX_C) heterokaryon will lead to SI. Inside the AB heterokaryon, for example, (Figure 3a), the X_AX_B genes

will modify targets. Fusion with the AC heterokaryon will then expose X_C to the unmodified targets inside the AB heterokaryon. Similar also for X_B and targets inside the AC heterokaryon. This nonself recognition would then be propagated to the cell death pathways and trigger cell death. A similar situation would occur with Buller matings (Figure 3b) and homokaryon-homokaryon matings (Figure 3c). However, in these cases, the unmodified targets of one individual would be recognized by the proteins of the other individual's X locus. As the cell death process is not instantaneous, and the number of fusion cells is very high, the chance that at least one nucleus can begin migration before cell death is likely quite high.

This model of post-translation modification can explain the survival of heterozygous heterokaryons and the interaction between heterokaryons, but how do we deal with the mating reactions between homokaryons? One potential solution is that the nonself recognition phenotype is only expressed at the heterokaryotic stage, and so the fusion of dissimilar homokaryons would not lead to nonself recognition. When two homokaryons fuse, either the targets of the reader-writer, the reader-writer itself, or some downstream component would not be expressed. However, such differential expression between mono- and heterokaryons is insufficient to explain the success of Buller matings. Under a differential expression scenario, in such a Buller mating the heterokaryotic partner would recognize the homokaryon partner, and cell death would be triggered inside the fusion cell. Since Buller matings are a widely observed phenomenon in basidiomycetes, differential regulation does not seem to fit the observations.

An alternate explanation for a lack of observed incompatibility during homokaryon fusion and Buller matings, is nuclear migration leading to cytoplasmic dilution. When two homokaryons meet, assuming they have compatible mating loci, nuclei reciprocally migrate into each homokaryon. As only the nuclei migrate [47], they bring little to no cytoplasm with them, and both the reader-writer and its targets would remain in the original homokaryon. After migration, transcripts and thus proteins encoded by this new nucleus will begin to accumulate (Figure 4b). Assuming a resident nucleus A and a fertilizing nucleus B, the cytoplasm from A will initially contain the modified targets and the corresponding reader-writer from nucleus A. Transcription and translation of nucleus B will result in the accumulation of the reader-writer of nucleus B. If the accumulation of these proteins is gradual enough, the writer can timely and sufficiently modify the target and the signal produced by the respective readers from nucleus A and B will not reach above the threshold required to trigger cell death (Figure 4a,b, horizontal dotted line). Such a threshold, seen in programmed cell death in general [64,65], combined with nuclear migration, allows for the gradual co-expression of nucleus B inside the previously homokaryotic mycelia of A. Mature heterokaryons will accumulate sufficient target and reader/writer protein, such that fusion between

heterokaryons could produce a signal that exceeds the threshold required for triggering cell death (Figure 4b).

Thus, the fact that two homokaryons are sexually compatible does not necessarily require the absence of nonself recognition. Instead, the rapid nuclear migration could allow for the escape of a small number of nuclei before cell death of the fusion cells. In fact, only a single nucleus needs to escape the nonself reaction for a successful mating reaction. Once a nucleus would make it past the boundaries of the fusion cell, subsequent migration does not bring cytoplasm along, meaning that further triggers of nonself recognition would be avoided during the migration process. Once the migrating nucleus reaches its terminus, *de novo* expression of the reader/writer would then result in gradual modification rather than cell death. We note here again that the death of fusion cells between sexually compatible homokaryons has been described [43,44,66]. The death of a fusion cell between homokaryon hyphae was described as being slower than the death between heterokaryotic hyphae but otherwise appeared similar [43]. In matings of *Agaricus bisporus*, for example, which does not have significant nuclear migration, pairings of homokaryons result in the formation of only a few localized successful heterokaryotic tufts (Figure 4c). This may indicate that although two homokaryons have compatible mating loci, most hyphal fusions do not result in heterokaryon formation. Interestingly, it has been noted that when auxotrophies were introduced into siblings of *Coprinopsis cinerea*, siblings with compatible mating alleles were always successful, but only a fraction of siblings could form heterokaryons if they had identical mating types, which prevents nuclear migration [67]. This lack of heterokaryon formation is a standard indication of SI in ascomycete genetics, and this finding in a model basidiomycete may indicate the similarity of the process if nuclear migration is inhibited. Although the death of fusion cells between homokaryons has been noted for over three decades, the implications of this have not been fully appreciated. What we term somatic incompatibility is a macromorphological phenomenon, and a lack of visible incompatibility does not necessarily mean that nonself recognition is absent, and *vice versa*, a macroscopic manifestation of incompatibility in a certain interaction does not necessarily imply that such an interaction cannot result in a stable heterokaryon.

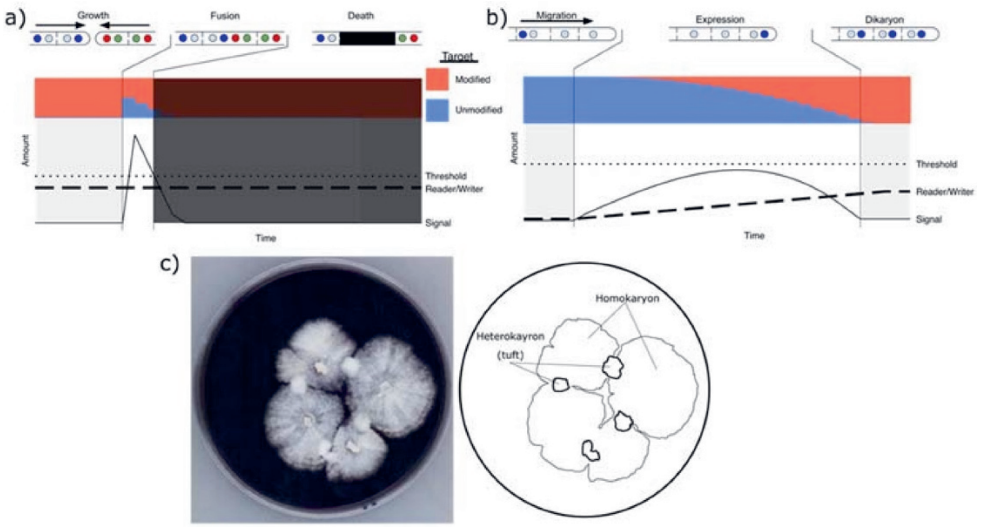

Figure 4. Dynamics of a reader/writer system during hyphal fusion, cytoplasmic mixing, and mating. (**a**) Graph of dynamics of any fusion involving cytoplasmic mixing, in this case between heterokaryotic basidiomycete mycelium. Dotted line shows the threshold above which cell death is triggered. Dark dashed line shows the concentration of the reader/writer of the newly resident blue nucleus. Solid line indicates the signal produced by the activity of the reader, which produces a signal proportional to the amount of unmodified target protein (Blue in the graph above). In this example, the reader has an activity five times higher than the writing activity, and the signal does not rise above the threshold. In this case, the high concentration of reader/writer protein immediately raises the signal above the threshold for cell death, based on the presence of unmodified target protein; (**b**) dynamics of the tip cell of a homokaryon during fertilization; (**c**) Mating reaction in *Agaricus bisporus*, a species with little nuclear migration, showing the relatively limited production of heterokaryons ("tufts") in the interaction zone between homokaryons. (photo credit: Mushroom Group, WUR).

Genetics of Proposed Model

As a rule, pairings of heterokaryotic wild isolates lead to SI [13,19,68,69]. Limited genetic studies have so far indicated that in basidiomycete species, nonself recognition is likely caused by 3–5 loci, some of which may be highly polymorphic in a population [16,17,70]. Although this number is lower than the 5–11 found in ascomycete species, the fact that a heterokaryon contains two genomes means that fewer polymorphic loci may be necessary to ensure a heterokaryon can distinguish itself from others. For example, consider heterokaryons produced from sibling matings from a single mushroom fruiting body. As these spores germinate and mate, they produce numerous heterokaryons from different sibling combinations. Although these siblings all originate from the same mushroom, and the different newly formed heterokaryons are thus closely related, if only a single locus was polymorphic between parents, then already 62.5% of heterokaryons would be incompatible (Table 1). If the parents were polymorphic at two loci, then 86% of offspring heterokaryons would be incompatible (1–0.365*0.365) if the two loci segregated independently. Further, the heterokaryotic state allows for interactions between

multiple loci, since each heterokaryon contains two alleles, there can be up to 4 alleles present. The interaction patterns for polymorphisms at a triallelic locus result in 82% of randomly selected heterokaryons being incompatible due to only a single locus.

Table 1. Compatibility between 37.5% of randomly selected heterokaryons variable at a single biallelic locus. Squares with the letter C indicate compatible heterokaryons.

	A1/A1	**A1/A2**	**A2/A1**	**A2/A2**
A1/A1	C			
A1/A2		C	C	
A2/A1		C	C	
A2/A2				C

Clearly, this system allows for much more specificity than ascomycete systems, where the haploid genetics mean that each allele only confers incompatibility against 50% of other strains. This increased specificity may explain why fewer loci have been recovered in the basidiomycete systems studied to date [16,17,19]. If multiallelic loci are present, then polymorphisms at two triallelic loci would be sufficient to confer incompatibility between 97% of randomly selected heterokaryons (1–0.185*0.185) (Table 2). Assuming higher allelic states could even permit interactions between four alleles at a single locus, further reducing the compatible fraction. If the purpose of nonself recognition is indeed to prevent fusion between conspecific individuals, then very few loci may be necessary.

Table 2. Compatibility between 18.5% of randomly selected heterokaryons variable at a single triallelic locus. Squares with the letter C indicate compatible heterokaryons.

	A1/A1	**A1/A2**	**A1/A3**	**A2/A1**	**A2/A2**	**A2/A3**	**A3/A1**	**A3/A2**	**A3/A3**
A1/A1	C								
A1/A2		C		C					
A1/A3			C				C		
A2/A1		C		C					
A2/A2					C				
A2/A3						C		C	
A3/A1			C				C		
A3/A2						C		C	
A3/A3									C

Conclusions

The particular lifecycle of basidiomycete fungi provides difficulties in understanding how nonself recognition can function, at least at a first glance. The long-term coexistence of two nuclear haplotypes excludes most of the so far known fungal nonself recognition mechanisms. Further, the successful mating between heterokaryons and homokaryons, Buller matings, shows that the expression of nonself recognition is not life-stage dependent. A major difference between ascomycete and basidiomycete fungi is the phenomenon of nuclear migration. Our model suggests that this migration is crucial for understanding how nonself recognition works, possibly through post-translation modification of a reader/writer system in a dose-dependent manner. Such a reader/writer system would allow for the coexistence of two nuclear haplotypes in a single cytoplasm, as well as matings between homokaryons and Buller matings. This limits the ability of parasitic cytoplasmic elements to spread at all life stages. Additionally, such reader/writer systems may act in a dominant fashion, and the use of a "common partner" nucleus (Worrall 1997) in genetic studies may not be as neutral as assumed. Further experiments are necessary to test if this model is compatible with the genetics and mechanism of SI in basidiomycete fungi.

Author Contributions: Conceptualization, B.A., A.J.M.D. and D.K.A.; writing—original draft preparation, B.A.; writing—review and editing, B.A., K.S., A.F.v.P., J.J.P.B., D.K.A. visualization—B.A., K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), grant number ALWGR.2017.010.

Acknowledgments: The authors acknowledge the careful unbiased microscopic observations of previous researchers, without which this paper would not have taken the form it has.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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3

Chapter 4

Most Parasexual Recombination in *Aspergillus* is Likely Due to Chromosomal Shuffling

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Abstract:

The parasexual cycle, the sorting of haploid genetic variation through a mitotic diploid intermediate, is thought to play a potentially important role in shaping genetic variation in many fungi. Particularly in fungi where sexual reproduction is rare or totally absent, parasex may provide a mechanism to escape deleterious mutations and negative epistatic effects. Here, we examine the genetic outcome of parasex in both *Aspergillus fumigatus*, with a regular sexual cycle, and *A. niger*, a species without a known sexual stage. Using two heterokaryon compatible *A. fumigatus* siblings, we obtained a highly heterozygous diploid with 7,822 usable markers. Using isogenic strains of *A. niger*, we obtained a heterozygous diploid with 145 markers spread across the genome. Sequencing of 20 haploids isolated from a starting diploid, as well as three diploids that underwent 10 rounds of single-spore transfer showed that mitotic crossovers are uncommon. When cultivated with only a brief diploid phase, 2% of *A. fumigatus* chromosomes were observed to be involved in a mitotic crossing over, while for *A. niger* 6% of chromosomes had crossovers observed. With extended diploid transfers this increased to 4% for *A. fumigatus* and 29% for *A. niger*. Crossovers were unevenly distributed across chromosomes, but not found to be associated with previously identified hotspots. Calculations with $\bar{r}$ metric show that chromosomal shuffling is responsible for approximately 90% of genetic diversity that occurs during parasex. These results suggest that while short diploid phases will efficiently segregate variation between chromosomes, extended diploid phases are necessary to segregate variation within chromosomes. Further studies on the environmental prevalence of diploids in Ascomycete fungi are needed to understand the biological significance of parasex.

Introduction:

Adaptation to changing environments requires genetic variation which, although ultimately generated by mutation, in most species is increased and segregated by genetic recombination. Sexual reproduction, generating diverse offspring, is known to increase the speed of adaptation and this benefit is further increased in heterogenous environments (Becks & Agrawal, 2010; McDonald et al., 2016). Alternatively, the parasexual cycle, discovered several decades ago in filamentous ascomycetes, can also produce this outcome (Pontecorvo, 1956). During this process, organisms with multiple haploid nuclei per cell or cell compartment can transiently become diploid, and then revert to a haploid. During this reversion, either of the two parental chromosomes can be retained, producing a diverse assortment of genotypes (2^n; n = haploid chromosome set). Additionally, during the diploid stage, mitotic crossing over can produce novel chromosomal genotypes similar to meiotic crossing over. It was later realized that, due to abundant heterokaryon incompatibility, such parasexuality was generally restricted between environmental isolates, (Caten & Jinks, 1966). However, parasex has been successfully used in *A. niger,* where sexual reproduction has not been achieved, to create a classical genetic map (Debets et al., 1993). In this most important filamentous fungus for biotechnology, parasex has been extensively used to localize novel mutations (Jørgensen et al., 2020) and move mutations or alleles between strains, facilitating the cellular factory for which this species is known (Niu et al., 2016; Pel et al., 2007).

While the human pathogenic fungus *A. fumigatus* now has a known sexual cycle (O'Gorman et al., 2009), parasexual recombination still has proven useful in this species. Following the demonstration that parasex operates similar to other *Aspergillus sp.* (Strømnæs & Garber, 1963), parasex has been used to characterize essential genes (Firon et al., 2002). A major human health issue with *A. fumigatus* is azole resistance caused by variation in the *cyp51A* gene, and parasex has been demonstrated to be able to segregate these genetic variants similar to other chromosomal material (Morogovsky et al., 2022). In other fungal species, like the human pathogen *Candida albicans*, parasex is well established in laboratory settings (Bennett & Johnson, 2003; Forche et al., 2008). Although sexual reproduction has not been achieved in the laboratory, many *Candida* species have enough meiosis genes that a sexual stage is likely functional (Butler et al., 2009). Population genetic studies in *C. albicans* have shown abundant evidence for recombination, indicating that (para)sexual reproduction is likely occurring in currently unknown niches (Ropars et al., 2018).

The observation that different genetic individuals generally cannot form stable heterokaryons means that the transition to a heterozygous diploid stage is also prevented (Caten & Jinks, 1966). This precludes exchange between non-isogenic fungal individuals, however a parasexual cycle will still operate within a fungal individual. Within a growing mycelium, different nuclei will accumulate

independent mutations, and by subsequent diploid formation by nuclear fusion (Pontecorvo & Käfer, 1958), these mutations can recombine, potentially produce mycelial sectors with higher fitness (Zhang et al., 2019). This has been experimentally shown in the adaptation of *A. nidulans* to fungicide resistance (Schoustra et al., 2007), and clinically the transition to diploid appears to occur at a low but detectable frequency in *A. fumigatus* in the human lung, possibly enabling adaptation in that environment as well (Engel et al., 2020).

While the diversity produced by chromosomal segregation solely depends on their number, the diversity produced by crossovers is more variable. Previous investigation of parasex in *A. niger* has shown that the rates of mitotic crossing over are higher than in *A. nidulans* (Debets et al., 1993; Lhoas, 1967; Pontecorvo, 1958). This has been postulated as an adaptation to the lack of observed sexual stages in *A. niger* (Pontecorvo, 1958). For many species the lack of a known sexual stage likely reflects limited laboratory investigations, and are not true asexual scandals (Taylor et al., 2015). However, some *Aspergilli* like the *A. niger* complex have been studied for decades without discovery of a sexual cycle (Houbraken & Dyer, 2015). For such fungi, parasex is often suggested as an alternate method to generate genetic diversity (Pontecorvo & Gemmell, 1944). As such, it was hypothesized that asexual species like *A. niger* would have higher parasexual recombination rates, to facilitate genetic diversity (Pontecorvo, 1958). This is in contrast to species like *A. fumigatus* where sex appears common in nature, and meiotic crossovers are frequent (Auxier, Becker, et al., 2022; Zhang et al., 2021). To explore the genetic consequences of parasex in *Aspergilli* we constructed heterozygous diploids from heterokaryon compatible strains of *A. fumigatus* and *A. niger*. Using both short and long diploid stages, we compared the resulting genetic diversity across the entire genome.

Methods

Strains: For *A. fumigatus* studies we used two sibling heterokaryon-compatible strains from a previous cross of AfIR964 and AfIR974 (Auxier, Becker, et al., 2022; Auxier, Zhang, et al., 2022). Sibling 137 (referred to as AfS1) contained the recessive *nia* marker, and sibling 142 (AfS2) contained the recessive *cnx* marker. For *A. niger* we used two strains from the N400 progenitor (Bos et al., 1988). Strain N502 (referred to as AnS1) contained the recessive *bioB2* marker, and strain N522 (AnS2) contained the recessive *metB11* marker. Prior to use, Strain N522 was UV-irradiated for 300 seconds at 0.4 W/cm2 of 298 nm UV-C light to induce additional genetic diversity, of which approximately 1% of spores survived.

Culture: Heterokaryons for *A. fumigatus* and *A. niger* were forced on Aspergillus Minimal Media (MM) (Cove, 1976), which contains NO_3 as a nitrogen source, which no *A. fumigatus* haploid strain could utilize, and insufficient biotin or methionine for the *A. niger* haploids to support growth. Heterokaryons were forced by co-inoculation of two starting strains, followed by five days of growth at 37 °C for *A. fumigatus* or

30 °C for *A. niger.* Heterokaryons were identified based on typical vigorous and irregular growth (Figure 1). The spores from heterokaryons were harvested in saline with 0.05% Tween-80 and heterozygous diploids of *A. fumigatus* and *A. niger* were isolated from heterokaryons by using sandwich agar plates (Bos et al., 1988) after five days of growth (Figure 1).

Diploids for *A. fumigatus* were confirmed based on spore size using a Casy® TT cell counter (OLS OMNI Life Science, Germany), as previously described (Engel et al., 2020). The constructed diploids of *A. niger* could additionally be confirmed based on colour, as N502 produces olive spores due to recessive *olv*A1 and N522 produces fawn spores due to recessive *fwn*A1, thus black spore production provides a further confirmation of heterozygous diploid formation. Haploidisation of *A. fumigatus* was performed on MM + 5 mg/mL urea with benomyl stress at 1 mg/mL, while for *A. niger* MM + 0.8 mg/L biotin and 15 g/L L-methionine with 1 mg/L benomyl was used. Sectors growing on benomyl supplemented media were purified with a 24-hour growth stage prior to being single colony streak purified. The spore size of resulting haploids was measured as for the diploids.

Propagation: For short-term diploid cultures, the initial diploid starting culture was immediately after isolation transferred to benomyl to begin haploidisation. For long-term diploid cultures, a single diploid starting culture for either *A. fumigatus* or *A. niger* was streaked to form three single-spore colonies. The spores from these colonies were streaked again to single-spore colonies. This process was repeated 10 times for each of the three parallel lines, being grown on CM to support any resulting homozygosity at auxotrophic markers. All cultures were subsequently stored as frozen glycerol stocks.

DNA extraction and sequencing: Spores from the starting strains, and the diploid or haploid derivatives were added to 3 mL of Aspergillus Complete Media, and DNA extracted from the mycelial mats after 24 hours of incubation, as described previously (Auxier, Becker, et al., 2022). DNA was sequenced using Illumina HiSeq 6000 with 150 bp pair-end reads. Sequence data for the *A. fumigatus* starting strains was downloaded from NCBI SRA (142:SRR17574060, 137:SRR17574055).

Bioinformatic analysis: For *A. fumigatus*, reads were aligned to the genome of AfIR974, one of the parents of the siblings, which was previously produced as a chromosome-level assembly (Auxier, Becker, et al., 2022). For *A. niger,* reads were aligned to CBS 158.81 (Pel et al., 2007). Reads were aligned using bwa-mem2 (Vasimuddin et al., 2019), sorted and duplicates removed using samtools (Li et al., 2009). Variants were called using freebayes (Garrison & Marth, 2012), and filtered using criteria of “QUAL > 1, SAR > 1, SAF > 1”. These quality filtered variants were then filtered for the criteria of homozygous reference in one starting haploid, homozygous alternate in the other starting haploid, and heterozygous in the starting diploid, using vcfR (Knaus & Grünwald, 2017). Plots were made using ggplot2 (Wickham, 2016).

Results

Heterokaryons of *A. fumigatus* and *A. niger* that were selected on Minimal Media had the expected phenotype of irregular, but vigorous growth (Figure 1A). The subsequent diploids isolated from spores from these heterokaryons showed again regular growth phenotype and larger spore size. Additionally, diploids of *A. niger* produced black conidia as expected due to complementation of the tightly linked recessive color markers *olv*A1 and *fwn*A1. Well-growing segregants isolated from the diploid on benomyl plates showed smaller conidia for both species (Figure 1B). For *A. fumigatus* the presumed haploids had a unimodal peak in spore diameter approximately 2.5 µm while the diploid spores were approximately 3.0 µm. For *A. niger* the presumed haploids fell into two peaks, one around 2.1 µm, and one around 3.7 µm, while the diploid spores were approximately 4.5 µm. The difference in spore size correlated with spore color with olive spores having average diameter of 2.26 µm, while fawn colored colonies produced spores with an average of 3.64 µm (Supplementary Table 1).

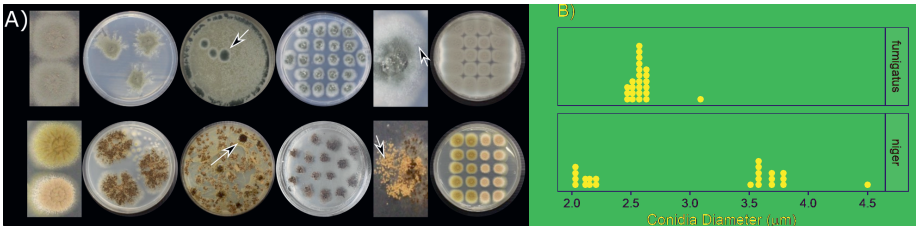


Figure 1: Heterokaryon and diploid formation. Left: Phenotypes of isolates during parasexual procedure. Left to right, images represent starting haploid strains, heterokaryotic stage, diploid stage (arrow), diploids on benomyl media to haploidise, zoom in on sector to show sectoring (arrow) and finally haploidised strains. Right: Spore sizes of *A. fumigatus* and *A. niger* haploids and diploids, singular dot at 3.0 µm for *A. fumigatus* represents diploid, and at 4.5 µm for *A. niger*.

For *A. fumigatus* a total of 7,822 variants were identified that were homotypic in both focal strains, and heterozygous in the diploid. Analysis of the variants across the eight chromosomes shows that most presumed haploids were homotypic across all eight chromosomes, while two strains, AfH18 and AfH23, show signs of heterozygosity across some, but not all, chromosomes (Figure 2). Across the 20 haploids, there were two recombination events that included more than two markers: one in AfH6 on the right side of Chromosome 1, and one in AfH14 on the right side of Chromosome 7. An additional observation was that all haploids appeared to have a crossover on the left end of Chromosome 4, with the same breakpoint. Ignoring this crossover on Chromosome 4, discussed below, this results in two crossovers out of 2/109 haploid chromosomes (1.8%).

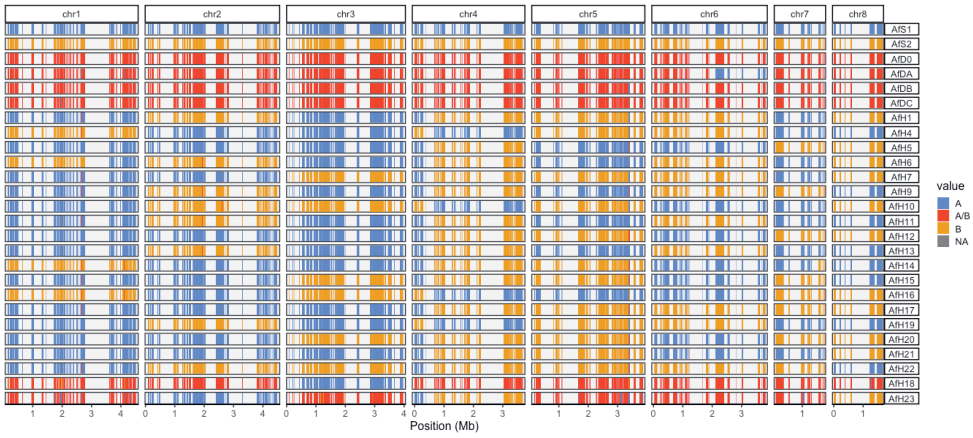


Figure 2 - Parasexual recombination events across eight chromosomes in *A. fumigatus* diploid AfD0 that occurred during ten-times single-spore subculturing (AfDA-C) and the process of haploidisation (AfH1-23) . Alleles of the focal strain AfS1 (strain 137) are shown in blue, and of AfS2 (strain 142) in orange while heterozygous sites are shown in red. White regions indicate non-variant regions, and rare grey indicates alleles not conforming to either AfS1 or AfS2 alleles.

Comparison of allele ratios across chromosomes showed that heterozygosity in samples AfH18 and AfH23, was likely due to aneuploidy. Allele ratios of haploid starting isolate AfS1 show a strongly bimodal distribution at 0 and 1, reflecting the homozygous nature of haploids (Figure 3 top row). In contrast, the diploid strain AfD0 shows unimodal peaks centered at 0.5 resulting from the diploid heterozygosity (Figure 3 second row). Strain AfH18 showed chromosomes 2, 4, 6 and 7 to have unimodal peaks, chromosome 3 to be strongly bimodal, and the remaining chromosomes 1, 5 and 8 to have bimodal peaks intermediate from 0.5 or fixation at 0 or 1. The other aneuploid strain, AfH23 showed a similar pattern, but without any chromosomes with a characteristic unimodal peak.

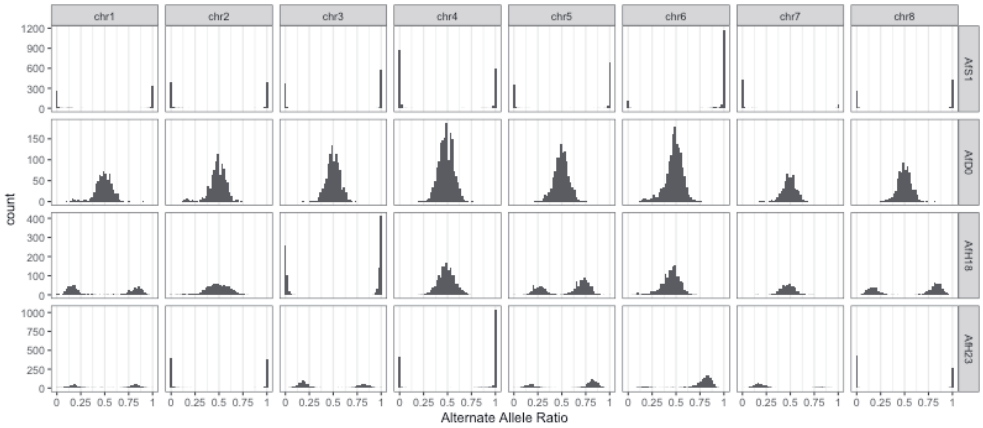


Figure 3: Allele balance indicates chromosome specific ratios. Histograms of allele ratio for all variants across each chromosome. Columns indicate chromosomes, rows indicate strains. Top row represents one starting haploid strain AfS1, AfD0 shows starting diploid, and AfH18 and AfH23 show strains that suggested aneuploidy from previous figure.

After variant filtering, a total of 178 variants were retained for *A. niger*. Analysis of the three ten-times transferred diploids showed loss of heterozygosity events on chromosomes 2, 3, 4 and 6. Diploid AnDC showed near complete homozygosity for chromosome 7, except for two markers. In the haploids one isolate had a crossover on chromosome 2, in the same position as the diploid AnDC. Two haploids had crossovers on chromosome 4, at the same location as in the three diploids. On chromosome 6, three haploids showed a crossover, between the same markers as the crossover seen in diploid AnDA. Notably, AnH17 showed a transition, going from left to right, of AnS2 to AnS1 to AnS2 alleles. Chromosomes 1, 3 and 8 showed no crossovers in the haploid samples.

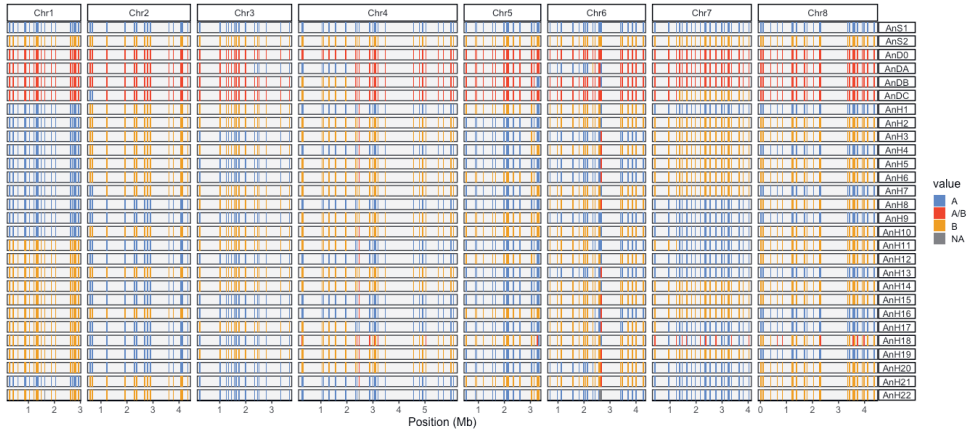


Figure 4: Parasexual recombination in *A. niger*. Genotypes of 178 markers for 22 randomly selected haploidised strains (AnH1-22), and three propagated diploid strains (DA-C). AnS1 (N502) alleles are blue, and AnS2 (N522) are orange. Red is heterozygous. D0 is the initial *A. niger* diploid, DA, DB and DC are three independent diploids after 10 single-spore transfers.

Across all *A. fumigatus* samples, during haploidisation there was a total of two crossovers in the haploids, equal to 2% of all chromosomes (Table 1). In the diploid, a single loss of heterozygosity event was detected, out of the 24 chromosomes. In *A. niger*, the number of crossovers was higher, both in the haploids with 17 crossovers detected, 10% of chromosomes, as well as in the diploids, with eight crossovers detected, 33% of chromosomes (66% if corrected for co-segregation of reciprocal recombinant chromatids).

Table 1: Crossover counts for both *Aspergillus* species after haploidisation (haploids) or 10 times subculturing (diploids).

	A. fumigatus			*A. niger*		
Chromosome	markers	haploid	diploids[2]	markers	haploids	diploids
1	667	1	0	24	0	0
2	855	0	0	20	1	1
3	984	0	0	17	0	1
4	1,576	(1) 0	0	28	2	3
5	1,095	0	0	18	8	1
6	1,423	0	1	22	5	1
7	503	1	0	24	1	1
8	719	0	0	25	0	0
Average[1]		(0.02) 0.01	0.04		0.10	0.33

[1]Average crossovers per total haploid chromosomes, excluding 11 aneuploid chromosomes.

[2]Numbers in parentheses count recombination within diploid as discussed in Main Text.

Discussion:

The generation and segregation of genetic diversity in fungi has long fascinated mycologists. As in most organisms, mutations arise mostly as errors during mitosis, and this accumulated genetic variation is then segregated during sexual reproduction. However, in many fungi, there exists a prolonged stage with multiple nuclei per cell compartment that can also lead to parasexual reproduction. Previous research in *Aspergillus* has been limited to studies using classical markers, requiring sometimes laborious phenotyping of strains. Using an unbiased approach, we explore the segregation of genome-wide variation in two *Aspergillus* species. We show that when such genetic variation has accumulated, the parasexual cycle segregates this variation largely by chromosomal shuffling. While crossovers could be detected in both species, they were relatively rare although more common in *A. niger* than in *A. fumigatus*.

Traditionally, mitotic mapping in fungi has been performed with phenotypic markers, mostly various auxotrophies (Bos et al., 1988; Debets et al., 1993; van de Vondervoort et al., 2007). Here we use a genome-wide sequencing approach. While normally only isogenic lines can form heterokaryons for such analysis, for *A. fumigatus*, we use the fortuitous isolation of two siblings that are isogenic for the five segregating *hetA-E* loci (Auxier, Zhang, et al., 2022). These are the offspring of a cross between two environmental isolates, and they still retain ~8,000 markers, a much higher density of markers than in a typical parasexual analysis. For *A. niger* we used lines from the N400 series, both of which had undergone several independent rounds of UV irradiation to introduce several markers. As a useful side product, these rounds of UV also would be expected to introduce additional genetic background variation, supplemented with our additional UV exposure. Although it may seem unfair to compare the results of a dataset with ~8,000 markers to one with ~150 markers, we do not think this strongly affects our interpretation. First, when we made a random subset of our much denser *A. fumigatus* map to approximately 150 markers, we recovered the same pattern of crossovers as when using the full dataset. Additionally, our finding that *A. fumigatus* has fewer crossovers than *A. niger* despite the 50X denser markers means that high marker density is not necessary to detect mitotic crossovers. As these crossovers involve large chromosomal sections even sparse markers would be sufficient to detect such large events.

A clear result from our work, consistent with the results of others is the relevance of the duration of the diploid stage to crossovers. Unlike in meiosis, mitotic crossovers are sporadic and so a longer diploid stage with multiple mitotic divisions will have a higher chance of crossovers. This is seen in our study, with an approximately five times higher number of crossovers when the diploid was repeatedly transferred, regardless of species. Thus, for parasex to unite or break apart mutations on the same chromosome, an extended diploid stage is essential. In *Aspergillus nidulans*, such extended diploidy of six months of continuous growth

was shown to increase fitness through parasexual recombination compared to haploids (Schoustra et al., 2007). In patients with chronic cystic fibrosis, such long-lasting diploids have been shown in *A. fumigatus* (Engel et al., 2020). Under such situations, crossovers within chromosomes may play a somewhat larger role than shown here, where diploids were grown for ~30 days for *A. fumigatus* and ~50 days for *A. niger.* For species like *Candida albicans*, the observation that mating locus compatible diploids relatively quickly transition to a tetraploid state, without an extended heterokaryotic stage (Bennett & Johnson, 2003; Forche et al., 2008), may indicate that *Candida* species would be expected to have more mitotic crossovers compared to *Aspergillus*. However, the fact that *Candida* species generally have eight chromosomes, similar to *Aspergillus*, means that chromosome shuffling is still likely to generate more genetic variation than crossovers (see below for discussion about $\bar{r}$ metric).

The results presented here highlight some level of predictability to the placement of mitotic crossovers. In the two chromosomes which underwent the most recombination in *A. niger*, chromosomes 4 and 6, all crossovers occurred between the same markers. Notably, these markers are near to, but not spanning, the centromere. This level of specificity has been previously reported to occur on chromosome 3 as well, in the 80 kb region between *lysA* and *hisH* (van de Vondervoort et al., 2007). Interestingly, we did not observe any crossovers at this location in the haploids, but one of the diploids did show loss of heterozygosity near this marker. We note here for clarity that the mutation used, *hisH*8 originating from our laboratory, is not homologous with the *hisHF* identified in *A. nidulans* (Valerius et al., 2001). In *A. fumigatus* the total amount of recombination was lower, preventing the identification of hotspots. Notable however is the recombination on the left arm of chromosome 4, found in all offspring. The region covered by the recombination spans the rRNA genes, which are found in dozens to hundreds of repeated copies, and such highly repetitive DNA is known to undergo mitotic crossing over at a higher rate (Eickbush & Eickbush, 2007; Szostak & Wu, 1980). We therefore interpret the recombination in all derived haploids to have originated from a single event. This crossover occurring during the brief diploid stage may indicate this as a recombination hotspot, as a subsequent 10 transfers of three independent diploids showed one additional crossover event resulting in a LOH. As LOH events only represent ½ of crossovers, since maintenance of heterozygosity is equally likely as loss, we would then estimate a total of two crossover events over the 10 transfers of the three independent lines.

Soon after its discovery, Pontecorvo postulated that mitotic recombination might be more common in asexual species, mitigating the effects of accumulating mutations (Pontecorvo, 1958). Our data is consistent with this hypothesis, with approximately five times more crossovers in *A. niger* than in *A. fumigatus.* However, here again our results indicate that crossing overs are rare enough that chromosomal shuffling is still likely to generate most of the genetic diversity. To

illustrate this, we use the $\bar{r}$ metric of genetic recombination, a calculation of genetic diversity in the production of offspring that incorporates both chromosomal shuffling as well as crossovers within a chromosome (Veller et al., 2019). Both *Aspergillus* species in this study, as well as most others, have eight chromosomes, which produces an $\bar{r}$ of ~0.4 (out of a maximum of 0.5) solely from the random shuffling of chromosomes. Conversely, having less than one crossover per chromosome, as seen in our data, the resulting contribution to $\bar{r}$ is below 0.05. Thus, in species like *A. niger* or *A. fumigatus*, the act of chromosomes being shuffled leads to ~10 times greater genetic diversity than crossovers. It should not be forgotten however that these crossovers, although rare, are necessary to bring together mutations on the same crossover in the case of parasex (Schoustra et al., 2007). While the length of the diploid stage is unknown in natural *Aspergilli*, and a longer diploid phase would increase the contributions from crossovers, the chromosome shuffling generates an order of magnitude more diversity. Although the diploid stages used here were relatively short, only extremely extended diploid stages (in the order of years of continual growth) could produce similar genetic diversity.

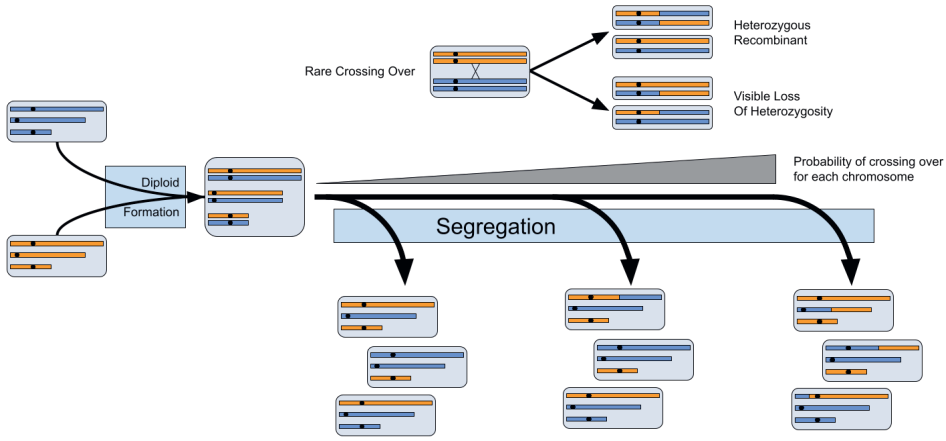


Figure 5: Diagram indicating the relevance of time to the production of crossovers, but not to chromosomal segregation. Starting from two haploids (left), a diploid is formed and reproduces over time (horizontal direction). If segregation to a haploid occurs early, crossovers are rare and diversity is due to chromosomal shuffling. Over time, crossovers become more common and the top figure highlights that observed LOH inside diploid nuclei only shows half of the total crossovers. The subsequent segregation of recombinant diploids will reveal this diversity.

Considering the relevance of the parasexual cycle, it is important to remember that such a process is only possible between heterokaryon compatible individuals. Heterokaryon incompatibility is generally encountered between isolates of *Aspergillus* species (Seekles et al., 2022; van Diepeningen et al., 1997; Zhang et al., 2019) and is likely determined by several genes (Auxier, Zhang, et al., 2022; Dales et al., 1993). Thus, as a fungal individual encounters another in the

environment, they would likely be heterokaryon incompatible, and the possibility to form a heterozygous diploid would be prevented at an early stage. However, we note that some studies have found that nutrient deprivation can permit heterokaryon formation even between incompatible strains (Vangalis et al., 2021). The evolutionary relevance of these diploids will depend on the frequency of their occurrence and the duration of the stage (Figure 5). In this study, and *Aspergillus* parasexual studies in general, the formation of a diploid is selected for by the investigator, in our case through sandwich agar plating. While the natural occurrence in *A. fumigatus* of diploids in samples from patients with long term fungal infections (on the order of months to years) is estimated to be on the order of 1 in a 100 isolates (Engel et al., 2020), screening of hundreds of wild isolates failed to find diploids (Engel et al., 2020). A similar screening of 642 isolates in *A. niger* recovered a single isolate producing spores with two nuclei, but no diploidy was recovered (van Diepeningen et al., 1997). The low frequency at which diploids are recovered from nature suggests that long-lived diploids are uncommon, although when they occur, they can strongly affect the route of adaptation.

The relevance of parasexual recombination to other fungi is unclear. In basidiomycete fungi, the extended dikaryotic stage may make parasexual crossovers more common. While such recombinants can be selected for, often termed somatic recombination in basidiomycetes, using auxotrophies or plant pathogenic ability (Chen et al., 2017; Ellingboe & Raper, 1962). However, extended growth under non-selective conditions of the mushroom *Schizophyllum commune* only results in one detectable loss of heterozygosity event, although only a few markers were tested (Clark & Anderson, 2004). Additionally, genetic analysis of environmental isolates of long-lived mushrooms like *Armillaria gallerica,* which lives as a persistent diploid and would be expected to have higher crossover rates, showed two LOH events in an individual spanning 200 m x 60 m (Anderson & Catona, 2014). Likewise, over more than a decade of growth, the fairy-ring mushroom *Marasmius oreades*, showed no genetic exchange between component nuclei (Hiltunen et al., 2022). The termite associated *Termitomyces cryptogamus*, which persists in a dikaryotic state for several years, also does not show any large regions of homozygosity (Vreeburg, Auxier et al. *in review*). As basidiomycetes can have dikaryotic stages lasting several decades prior to meiosis, it is perhaps not surprising that mitotic crossovers are rare, as if they were common many mushrooms would produce largely homozygous meiocytes and consequently less diverse progeny. The long-term stability of basidiomycetes compared to the ascomycetes studied is likely to be due to a difference in diploid formation. As mitotic crossovers are rare during a diploid phase, they would not be expected to contribute to a difference in stability.

Conclusion:

The parasexual cycle is often considered as a mechanism where fungi without a known sexual stage can generate diversity, including purging of deleterious mutations or bringing together adaptive mutations. However, this is often given in general statements, and has so far lacked a quantitative estimate of the recombination potential. This work shows that most of the recombination occurs through the shuffling of chromosomes, with mitotic crossovers playing a minor role under short diploid phases. However, there appears to be a large variation between species with *A. niger* having much more frequent crossovers. This work informs our understanding of fungal genetic evolution — that due to heterokaryon incompatibility parasex is largely restricted to *de novo* mutations, and that primarily mutations on different chromosomes will be affected. An important factor that requires further study is the prevalence and persistence of diploids, both in natural communities, as well as within mycelia.

Author Contributions

JZ and AJMD designed experiments, JZ performed experiments. BA designed and performed analysis. BA wrote draft manuscript. All authors edited manuscript. All authors approved final version of the manuscript.

Code and Data Availability

Code for analysis is available on https://git.wur.nl/ben.auxier/mitotic.recomb. DNA sequencing data generated for this study for both *A. fumigatus* and *A. niger* will be made publicly available upon publication. Strains generated are available on request to the corresponding author.

Acknowledgements

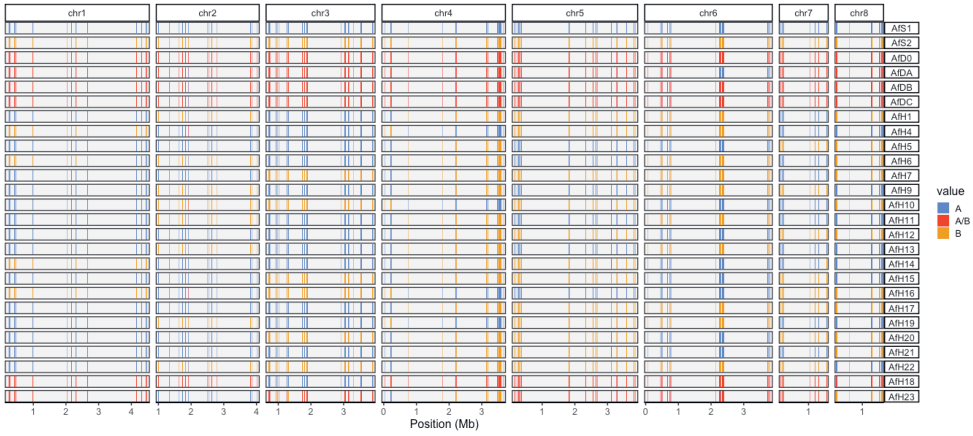
We would like to thank Dr. Adrian Tsang for assistance with the *A. niger* reference genome.

Supporting Material:

Supplemental Table 1: Spore size measurements of recovered haploid strans and diploid progenitor

Species	Strain	Size (µm)	Color	Species	Strain	Size (µm)	Color
fumigatus	AfH1	2.52	green	*niger*	AnH1	2.01	olive
fumigatus	AfH2	2.59	green	*niger*	AnH2	2.05	olive
fumigatus	AfH3	2.62	green	*niger*	AnH3	2.15	olive
fumigatus	AfH4	2.5	green	*niger*	AnH4	2.21	olive
fumigatus	AfH5	2.63	green	*niger*	AnH5	2.2	olive
fumigatus	AfH6	2.62	green	*niger*	AnH6	2.13	olive
fumigatus	AfH7	2.55	green	*niger*	AnH7	2.15	olive
fumigatus	AfH8	2.56	green	*niger*	AnH8	2.01	olive
fumigatus	AfH9	2.62	green	*niger*	AnH9	2.05	olive
fumigatus	AfH10	2.53	green	*niger*	AnH10	2.1	olive
fumigatus	AfH11	2.63	green	*niger*	AnH11	3.51	fawn
fumigatus	AfH12	2.57	green	*niger*	AnH12	3.56	fawn
fumigatus	AfH13	2.58	green	*niger*	AnH13	3.57	fawn
fumigatus	AfH14	2.55	green	*niger*	AnH14	3.56	fawn

fumigatus	AfH15	2.48	green	*niger*	AnH15	3.8	fawn
fumigatus	AfH16	2.64	green	*niger*	AnH16	3.6	fawn
fumigatus	AfH17	2.53	green	*niger*	AnH17	3.58	fawn
fumigatus	AfH18	2.59	green	*niger*	AnH18	3.67	fawn
fumigatus	AfH19	2.49	green	*niger*	AnH19	3.71	fawn
fumigatus	AfH20	2.55	green	*niger*	AnH20	3.8	fawn
fumigatus	AfH21	2.45	green	*niger*	AnH21	3.78	olive
fumigatus	AfH22	2.56	green	*niger*	AnH22	3.69	fawn
fumigatus	AfH23	2.58	green	*niger*	diploid	4.5	black
fumigatus	diploid	3.09	green				



Supplemental Figure 1: A reduced density mitotic map of *A. fumigatus*. Figure shows same data as Figure 2 of the main text, but with only 150 randomly sampled markers. Colors and labels are as shown for Figure 2.

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Chapter 5

Comment on: “Single Nucleus Sequencing Reveals Evidence of Inter-Nucleus Recombination in Arbuscular Mycorrhizal Fungi”

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Published As:
Auxier & Bazzicalupo, 2019
eLife
https://doi.org/10.7554/eLife.47301

Abstract:

We report a reanalysis of the data presented in Chen et al., 2018. After filtering the data by excluding heterozygous sites in haploid nuclei, duplicated regions of the genome, and low-coverage depths base calls, we find the evidence for recombination to be very sparse.

Introduction:

For many years, Arbuscular Mycorrhizal Fungi (AMF) have been presumed to be asexual, as no one has witnessed sexual structures in these fungi. What is most puzzling is that AMF retain core meiosis genes (Halary et al., 2011), indicating that a meiosis-like process most likely occurs in this lineage. Studies finding recombination up to this point have shown to be based on duplicated gene copies (Croll and Sanders, 2009), or ribosomal RNA sequences that turned out to be paralogs (Pawlowska & Taylor, 2004; Maeda et al., 2018). Recently, a novel finding of recombination in these fungi was published, based on comparing single nuclei whole-genome sequences to bulk sequencing data (Chen et al., 2018). The isolates studied are dikaryotic, containing nuclei of two classes defined by their mating type locus, *MAT* (Ropars et al., 2016). Each sequenced nucleus was PCR-amplified to attempt to assign a mating type class, 'MAT-1' up to 'MAT-5'. Recombination was then inferred based on (i) base-pair calls classed as one mating type found in the alternate mating type, (ii) nuclei of the same mating type showing variation in consecutive blocks of single nucleotide polymorphisms (SNPs), (iii) SNPs from nucleus 7 (SL1 strain) being more similar to SNPs of the alternate mating type, consistent with a recombination event spanning the MAT locus.

Here, we asked how strong the signal of within-strain recombination was in the data from Chen et al. (2018) if we excluded heterozygous sites in haploid nuclei, duplicated regions of the genome, and low-coverage depths base calls. By removing data that cannot confidently be distinguished from sequencing errors and repeated regions, we find that the evidence for recombination is very sparse. We also report specific examples of these possible errors to justify our more stringent filtering of the data.

Results

The effect of filtering positions based on reads:

We began with the dataset reported in Supplement 6 of Chen et al. 2018, used for both Figures 2 and 3 of that manuscript. We then filtered out: (1) positions where any single nucleus was heterozygous, (2) any individual site with less than 5 reads coverage, and (3) positions with more than one high-confidence BLAST hit using the settings specified in Chen et al., 2018. We applied the filters individually, or in combination, to see how many of the variable sites inferred as signals of recombination would be removed. Applying each filter individually removed between 19-77% of recombined positions. Filtering of low coverage sites had the strongest effect, a ~75% reduction. Applying all three filters together removed 91% of recombined sites (Figure 1). Notably, these filters had much less effect on the total number of analyzed sites, with the combined application off all three filters reducing the total number of sites by only ~22%.

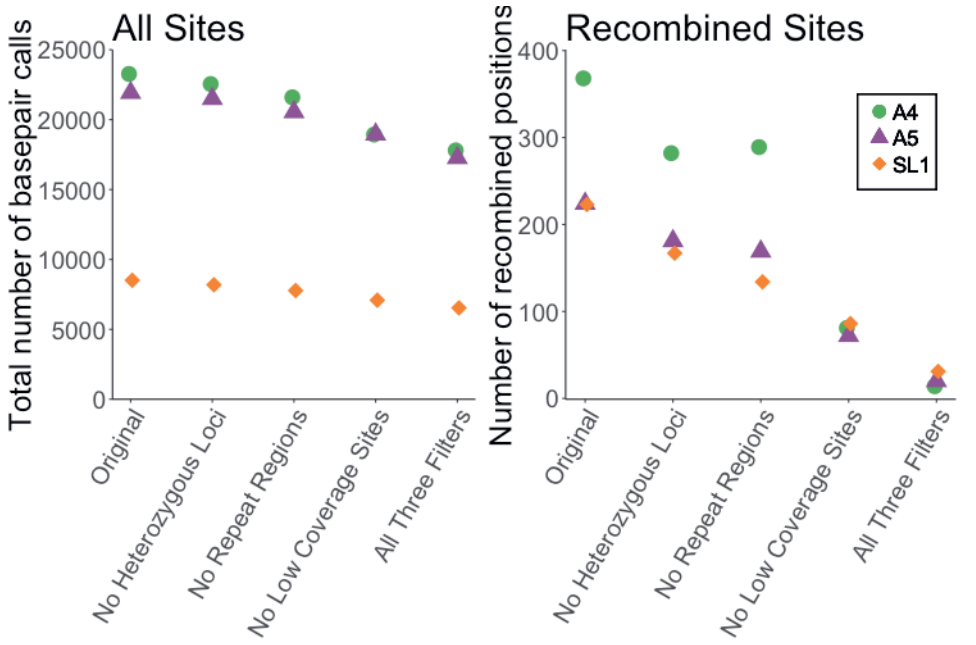


Figure 1: Filtering SNP data shows a decrease of 91% in number of recombined sites, but only ~20% decrease in total sites. Left panel shows the effect of filtering on all sites included in Supplement 6, while right shows the effect on the number of recombined positions. Recombined positions identified based on second criterion in Supplemental Figure 1. Different symbols show the effect on the three different strains (A4, A5, and SL1) used in the study.

In general, recombination exchanges physical blocks between homologous chromosomes, and consecutive allelic differences are a hallmark of this process. Table 1 shows the length of the longest consecutive SNP difference, as well as total number of consecutive SNP blocks, before and after additional filtering. Based on the analysis in Chen et al., all isolates show at least one recombined block spanning over a thousand base pairs. However, applying our three filters significantly reduced these blocks. Strain A4 showed no consecutive SNPs. Strains A5 and SL1 had 2 and 3 recombined blocks remaining, respectively, but the filtering reduced the length of the longest block.

	Original Data			Filtered Data		
Isolate (Mating types)	Number of recombined positions	Number of recombined blocks	Number of SNPs of longest block	Number of recombined positions	Number of recombined blocks	Number of SNPs of longest block
A4 *Mat-1/Mat-2*	54/314	25	5 (1131)	0/14	0	1 (1)
A5 *Mat-3/Mat-6*	41/183	21	22 (2145)	2/18	2	6 (670)
SL1 *Mat-5/Mat-1*	111/112	11	16 (1872)	22/9	3	5 (430)

Table 1: Lengths of recombination events before and after additional filters.

A specific example of repeated regions associated with biallelic sites in haploid nuclei:

Chen et al. (2018) interpreted consecutive SNPs differing between nuclei of the same mating type as a sign of recombination, as highlighted in Figure 3 of their article. The filtering used in Chen et al. 2018 removed multi-copy sites “[i]f BLAST results returned more than two good hits”, but retained regions with two BLAST hits. This could lead to the inclusion of SNPs that are heterozygous due to duplicated regions of the genome.

To show how repeated regions may lead to a false signal of recombination, we focused on an example highlighted in their Figure 3, which was discussed in the main text of Chen et al. (2018). We found that several positions on scaffold 70 from isolate A4 were heterozygous in several nuclei (Figure 2), although they are treated as homozygous in Chen at al. (2018). High sequencing coverage (>30) eliminates rare sequencing errors as the cause. To test if duplicated regions could be the cause of the heterozygosity, we performed a BLAST search against the A4 reference genome with sequences from scaffold 70:100354-100657. This search resulted in two BLAST matches: the self match on scaffold 70, as well as an additional match on scaffold 3570 (Figure 2B). When the short reads of the dikaryon (bulk sequencing of all nuclei) were aligned to the reference genome, both these SNPs on scaffold 70 and their match on scaffold 3570 were heterozygous, and the BLAST hit result for both showed 100% identity match. This repeated sequence of the genome seems to have been assembled as a chimera of the two variants in both scaffolds, thus the short reads from either copy are assigned equally to both.

We feel this example illustrates the need to exclude repetitive regions from analyses of recombination.

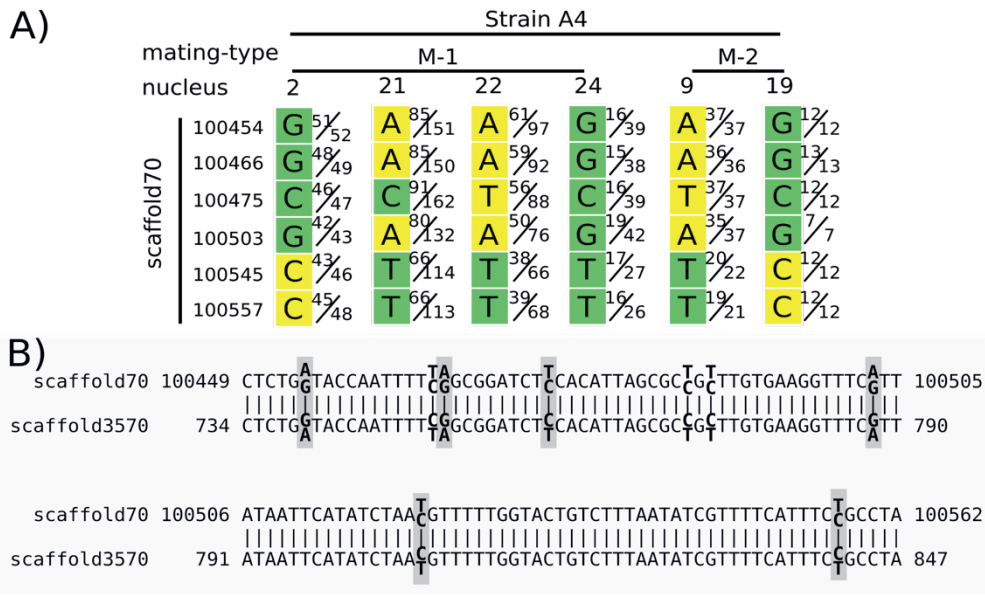


Figure 2: An example of heterozygosity in a duplicated region identified as evidence for a long stretch of recombination. (A) Base calls for positions on scaffold 70 of 6 nuclei (four nuclei of mating type M-1 and two of M-2, as indicated in the top row). Each base call was assigned by a mating type class (green or yellow) in Chen et al. (2018) based on an unspecified criterion. This variation is interpreted as recombination among nuclei. We added after each base pair the fraction representing the number of reads for the base call and the alternative allele for that nucleus that we recovered from the raw read data. For example, in nucleus 2, position 100454, the base was called as a 'G' with a mating type 'green', whereas we found the fraction G/A in the raw reads to be 51/52. For nucleus 21 only 85 of 151 reads were A, and the rest G. (B) Alignment of the region shown in (A) with its best BLAST hit region on scaffold 3570. Heterozygous sites in the dikaryon are indicated in bold, with the two alternate and reference bases shown slightly above/below. Grey boxes surround those sites included in (A). Note that both regions are heterozygous at the same aligned sites, and with the same alternate base for each heterozygous site. Graphic of (A) modified from Figure 3 of Chen et al. (2018), with the addition of nuclei 21 and 24.

Confidence in low coverage sites to infer recombination:

The data presented in Chen et al. 2018 was filtered with a minimum of 2 reads. This is a very low threshold, and insufficient for a consensus in the event of disagreeing reads. To look at the effect of low coverage on the signal of recombination, we compared the distribution of read depths between random and recombined sites. We first needed to identify recombined sites, so we applied a parsimony criterion as detailed in Supplementary Figure S1. This method certainly underestimates recombination, as it cannot identify recombined sites when equal numbers of nuclei within a mating type have alternate genotypes, but identifies 733 positions, sufficient for analysis. Looking at the distribution of read depths, overall

SL1 nuclei had ~95% fewer high coverage sites (average of 97 sites > 10 read depth for nuclei from SL1 versus 2290 for A4 and 2441 for A5) compared to A4 and A5 (Figure 3). We note here, as described in Table 1 of Chen et al. 2018, that SL1 nuclei cover much less of the genome (14%) compared to A4 and A5 (53% and 42%, respectively). Another fact visible from Figure 3 is that, for A4 and A5, recombined sites are overrepresented by sites with a read depth of 2 compared to random sites. We note that for nuclei from isolate SL1, fewer overall recombined sites can be identified since the decreased breadth of coverage reduces the overlap between nuclei, making it difficult to say whether this pattern of excess low-coverage sites is also present.

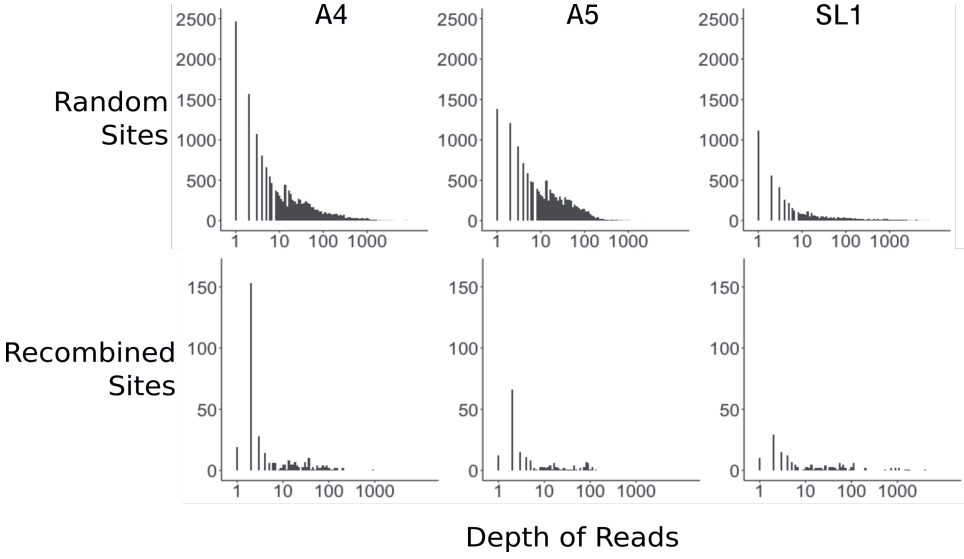
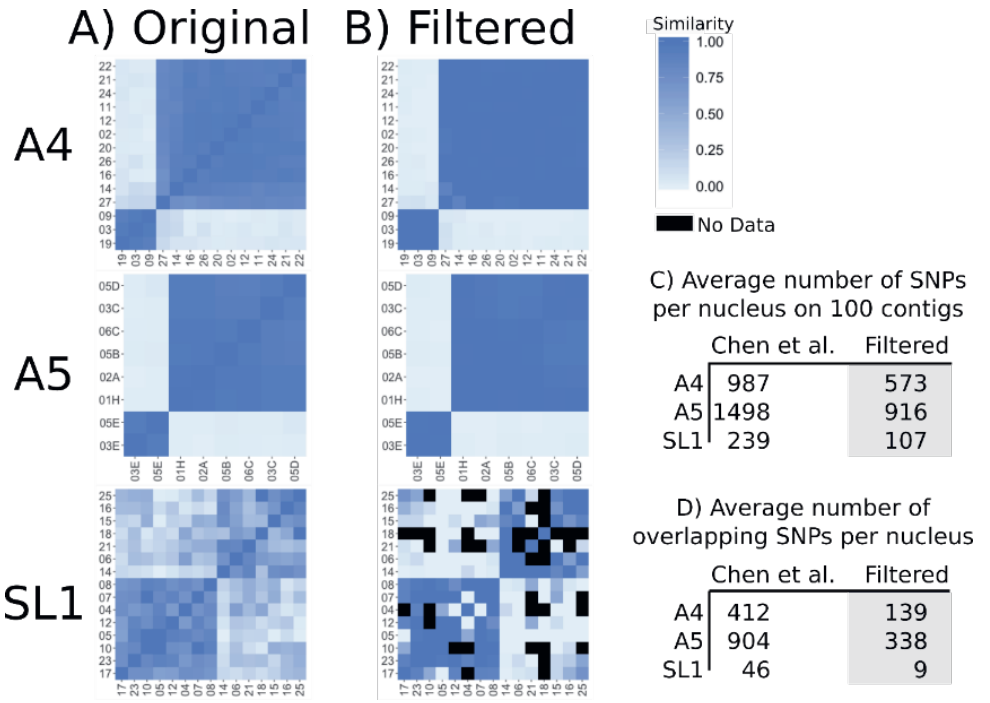


Figure 3: Recombined sites are overrepresented for low coverage sites. Top row: Distribution of read depths for random sites for the three isolates, showing decreased coverage overall for SL1. Bottom row: Distribution of read depths for recombined sites does not mirror the distribution of random sites. Sites identified as recombined based on the parsimony criterion diagrammed in Supplemental Figure 1. Note that read depth is plotted on a log scale.

Genome-wide pairwise SNP differences are reduced after filtering:

We then assessed the evidence for genome-wide recombination based on pair-wise SNP differences. This is the analysis presented in Figure 2 of Chen et al. 2018, showing overall more recombination in SL1 than A4 and A5, indicated by a "mosaic pattern". We should note here that the low sequencing coverage of SL1 nuclei (14%) means that very few sites are shared between nuclei of SL1 (2% on average), reducing the value of this type of analysis. After applying our filters, we find that in A4 and A5 almost all differences between nuclei of one mating type disappear (Figure 4). For nuclei from SL1, the filtering reduced the differences within a mating type, but since these nuclei cover so little of the genome, the dataset is reduced such that on average nuclei only share 9 SNPs that can be compared. Many

nuclei have no overlapping SNPs and no comparison can be made. A few nuclei of opposite mating types, such as nuclei 17 and 25, show high similarity, but for these pairs the similarity is based on only one or two shared SNP positions.



C) Average number of SNPs per nucleus on 100 contigs

	Chen et al.	Filtered
A4	987	573
A5	1498	916
SL1	239	107

D) Average number of overlapping SNPs per nucleus

	Chen et al.	Filtered
A4	412	139
A5	904	338
SL1	46	9

Figure 4: Genetic similarity of SL1 is strongly affected by SNP filtering methods. A) left column panels show heatmaps generated from original data presented in Supplement 6 of Chen et al. B) Right panels show data after filtering. Black squares represent pairs that do not share any SNPs. C) Average number of SNPs in the dataset from Chen et al. 2018 and after filtering. Note that SL1 has the lowest number of SNPs due to low sequencing breadth. D) Average number of pairwise overlapping SNPs per nucleus, note that after filtering nuclei from SL1 have fewer than 10 SNPs overlapping on average, again due to low sequencing breadth.

Confirming the placement of nucleus 07 (SL1) could be strong evidence for recombination:

In the original paper, nucleus 07 of SL1 shows a strong similarity with nuclei of the alternate mating type, seen by the clustering of nucleus 07 with *MAT-5* in Figure 2 of Chen et al., 2018. However, this nucleus was PCR-genotyped to be of mating type *MAT*-1, making the placement of nucleus 07 critically important. The incongruence between PCR-genotyped mating type and the SNP clustering would be strong evidence of recombination spanning the *MAT* locus. To confirm this, we looked in the mapped reads of each nucleus to find reads mapping to either alternate mating type locus (Supplemental Figure S2). We found that there were no Illumina sequencing read of nucleus 07 mapped to either mating type locus. The lack of Illumina data for the mating loci suggests that the MDA step likely did not amplify

that region. As such, we have no publicly available evidence for the mating type of this nucleus. Without corresponding Illumina evidence, we consider the PCR product the sole evidence of this recombination event. This PCR experiment that we cannot confirm with Illumina data represents the only remaining strong evidence for recombination after read filtering. We find cross-contamination to be a more likely scenario in the face of many billions of sequenced bases from an Illumina run.

Conclusion:

Finding a balance between filtering poor data and losing informative data is a critical component of any analysis. For this dataset, we provide evidence for the necessity of reasonable filtering to avoid inferences based on erroneous or misleading data. We do not consider our filters to be particularly strict, as removing low coverage sites, repeated regions, and heterozygous sites from haploid data is commonplace in genomic analyses. In SL1, three blocks of consecutive SNPs remained after our filtering, and two regions in A5. Some of these regions remain because our heterozygosity filter requires a minimum of ten reads, thus low coverage heterozygous sites are not excluded. This analysis used the first 100 contigs, covering approximately 10 Mb. As such, finding only a handful of putative recombined SNPs certainly cannot be confidently separated from amplification/sequencing noise.

In order to map recombination inside repetitive regions one would need long reads from single nuclei, a formidable task but one which has already been accomplished with individual pollen cells (Sun et al., 2018). Notably, the use of a more contiguous reference genome will include additional repetitive regions, making the exclusion of repetitive regions more important. As the apparent recombined blocks are much smaller than the contigs, a more contiguous genome assembly would not change our analysis. To use low coverage sites, a model-based approach could be used which incorporates the associated uncertainty (Hinch et al., 2019; Bloom et al., 2013). Lastly, removing heterozygous sites from haploid single-nuclei seems like a self-evident requirement.

The conserved meiosis genes found in the genomes of Glomeromycotan species strongly suggests a meiosis-like process allowing recombination and re-shuffling of genetic material among genomes. Uncovering the details of this process would be a major scientific breakthrough.

Given this importance, claims made regarding recombination in Glomeromycota require rigorous examination. While we acknowledge that the models presented in Chen et al (2018) are valuable framework to test hypotheses for meiosis-like mechanisms found in these fungi, the data presented are not robust enough to support or reject them. As such, we believe that one of the greatest remaining mysteries in mycology remains unknown.

Acknowledgments

We thank Dr. Duur Aanen, Dr. Anna Rosling, Dr. Marisol Sanchez-Garcia,and Mathijs Nieuwenhuis for critical feedback.

Supplementary Materials:

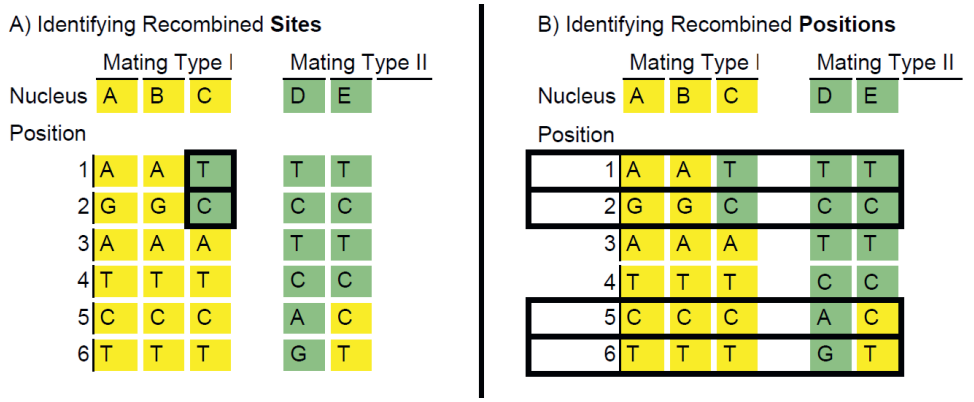


Figure S1: Example of the two criteria used to identify recombination. To identify individual recombined **sites** a parsimony criterion, used for Figure 2B was applied as seen in A), where the recombined nucleus was determined based on the majority genotype for a given mating type. Note that position 5 and 6 for nucleus E are not identified as there is one nucleus of mating type II with each genotype, this criteria cannot identify sites when there are equal number of nuclei of a mating type with each genotype. To more fully identify recombination **positions**, we also used a criteria of a difference in genotype between nuclei of the same mating type. This criteria is capable of identify examples such as position 5 and 6, but does not identify which sites are recombined.

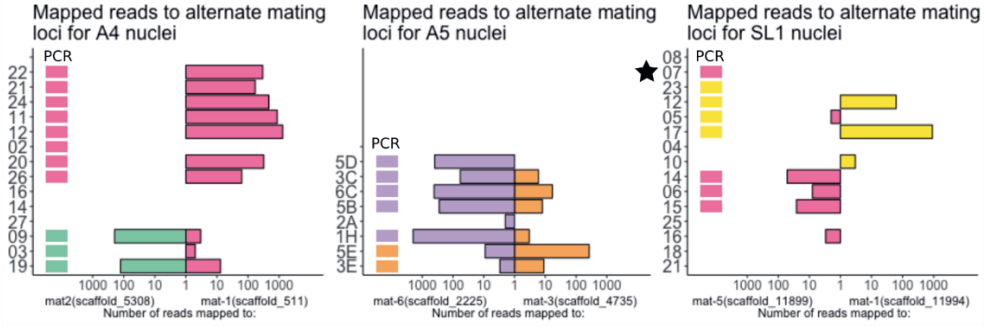


Figure S2: The critical mating type of nucleus 07 (black star) from SL1 is unsupported by short-read data. The two mating type regions for A4, A5, and SL1 were identified *in silico* with BLAST searches using the primer sequences and methodology of Ropars et al., 2016. Two mating type loci were identified for each of the three isolates. Reads mapping to either of the two alternate mating type loci are shown below on a log scale. Colors are according to Figure 3 of Chen et al. 2018, and colored boxes near labels indicate mating type based on PCR amplification as reported in Chen et al., 2018.

This figure shows a general congruence between PCR validated mating types and the number of reads that mapped to either of the two alternate mating loci. For isolate A4, for all nuclei with aligned reads, the PCR results matched the majority of the reads (note the log scale), and 02 and 03 were successful for PCR with no short reads mapped. For isolate A5, all nuclei with a PCR amplified mating type also had a majority of reads mapping to one mating type. Nuclei from SL1 had less success with both short read or PCR, likely due to decreased sequencing breadth. Critically, nuclei 07 from SL1 had no short-reads mapped to either mating locus.

Materials and Methods:

Raw data

We obtained the SPAdes assemblies for the three dikaryotic *R. irregularis* isolates from NCBI, as well as the paired-end read libraries from the dikaryons and the paired-end reads of the single nuclei. Details of the accession numbers used are found in Chen et al., 2018.

Read processing

Short reads were cleaned with FASTP (cite), then aligned using BWA mem with the -M flag as in Chen et al., 2018. Reads per nucleus were analyzed using the python modules pysam, and BLAST searches were scripted using biopython. Scripts used are available on GitHub repository XXXX. The parsimony criterion shown in Supplemental Figure 1A, for identification of recombined sites, was performed manually. Filtering of excel files and calculations of recombined sites was performed with the R statistical language, which was also used to prepare plots with ggplot2 and distance matrices using ape (cite ggplot and ape).

Mating-type loci identification and mapping:

As the location of the mating type loci was not specified in the results of Chen et al., 2018, we identified them based on data from Ropars et al., 2016. Specifically, we used the primers sequences kary001, kary002, and kary003 as query sequences for BLAST searches against the A4, A5, and SL1 genomes. Each primer sequence had strong matches against two different scaffolds, consistent with divergent ideomorphs as found in Ropars et al., 2016. As these primers only target a small region, we extended the locus using the annotations found on NCBI to identify the boundaries of the pair of genes. These locations are reported in Supplemental information. As no annotations are available for SL1, we used the entire sequence of the ideomorph on scaffold511 of A5 as a BLAST query to identify the homologous regions.

With the mating locus identified, we then counted the number of reads that mapped to each sequence using samtools.

To calculate the expected number of overlapping SNPs found in Figure 4D, we used the following formula:

$$\left(\frac{\text{number of bases called for isolate in Supplement 6}}{\text{number of positions}}\right)\left(\frac{1}{\text{number of nuclei}}\right) = \textit{Proportion covered per nucleus}$$

$$(\text{Proportion covered per nucleus})^2 = \textit{Expected pairwise overlap proportion between nuclei}$$

$$(\text{Expected pairwise overlap proportion})(\text{number of positions}) = \textit{Expected number of shared positions}$$

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bioRxiv 484022; doi: https://doi.org/10.1101/484022

Chapter 6

The influence of a mating partner on individual gene expression in *Schizophyllum commune*

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Abstract:

In Eukaryotes, mating generally occurs between two haploids leading to a diploid phase prior to meiosis. Basidiomycete fungi, particularly those that form mushrooms (Agaricomycetes), present an interesting exception to this pattern. In these fungi, mating between two haploid individuals occurs by the reciprocal donation of nuclei by both partners, followed by extremely rapid nuclear migration across both individuals. Instead of forming a diploid, a long lived dikaryotic stage with two haploid nuclei per cell is maintained. As each fertilizing individual gains access to the resources accumulated by the other individual, a tension exists between the resident genome and the incoming, unrelated, fertilizing genome. This tension may be increased by the occurrence of multiple, simultaneous, fertilizing partners. Here we use RNA-seq of reciprocal matings between three wild isolates of the common wood-degrading fungus *Schizophyllum commune* to ask if mechanisms have evolved in response to this tension, by modifying expression of genes from the fertilising, or the fertilised, nucleus. Utilizing the extremely high sequence divergence of *S. commune* we were able to distinguish expression of the alternate alleles of the vast majority (>99%) of genes. Our analyses recover significant numbers of differentially expressed genes both at the monokaryotic stage, as well as at the dikaryotic stage. Depending on the genetic background, there was strong correlation in the ratio of allele expression across life stages. While there is significant evidence of differential expression within the dikaryons, we did not find a strong effect of mating role on subsequent expression. The results of this study indicate that haploid nuclei inside a basidiomycete dikaryon largely retain much of their individuality.

Introduction:

The process of sexual reproduction involves the mixing of two genomes, packaged into genetically diverse offspring. The development of these offspring comes at a metabolic cost to the maternal parent. In oviparous organisms, those with little embryonic development from the maternal parent, these costs are invested early into eggs with only maternal genetic contribution. This is thought to allow for equal resources to be safely apportioned to each egg. In contrast, in organisms with significant embryonic development occurring post fertilization, like mammals or plants, there can be significant genetic conflict between parental genomes in the embryos. In this case, any paternal allele that takes excess resources from siblings will have a selective advantage, particularly when multiple matings are common so that unrelated paternal genomes compete (Moore & Haig, 1991). To resolve this conflict, it is often observed that the two genomes, maternal and paternal, epigenetically regulate each other, termed genomic imprinting. Consistent with theoretical predictions, genomic imprinting is observed in organisms with significant embryonic development, like plants and mammals, but not in egg-laying animals like bird or reptiles (Wilkins & Haig, 2003).

The interaction between genomes during mating can have surprisingly deleterious outcomes. In organisms where nourishment of the embryo occurs by the mother after fertilisation and where multiple simultaneous matings are possible, as in many plants and mammals, there will be genomes from more than two potential fathers, competing for maternal provisioning (Haig & Westoby, 1991; Queller, 1984). While the mother has an evolutionary interest in producing the highest number of viable offspring, favouring prudent and equal investment in each offspring, different fathers may favour more than optimal investment in their own offspring, even if that comes at a cost of total offspring number. The classic example of this is found in mammals with variation in the insulin-like growth factor (IGF-II) alleles conferring a fitness benefit in mice (Haig, 2004). As litters can have pups from different fathers, embryos with alleles with higher IGF-II production will grow faster in the womb, and subsequently consume more of the mother's milk as pups, which is a limited resource. As an evolutionary response, during the production of a zygote, the maternal copy of IGF-II is silenced epigenetically, leaving only the paternal copy to be expressed and the corresponding receptor IGF-IIr, which reduces the effect of IGF-II, has opposite methylation and expression patterns (Barlow et al., 1991; DeChiara et al., 1991). While the details have been best studied in this system, the same evolutionary tension will occur whenever multiple paternal alleles compete for limited maternal resources. While the evidence is still accumulating, it appears that plant genomes also show evidence of this genetic conflict, which is expected as the pollen that fertilize a style can come from multiple sources (Geist et al., 2019; Queller, 1983).

The fertilization process in basidiomycetes, the group of fungi including those that produce mushrooms, poses distinctive areas for conflict during and after

mating. In diploid organisms the gametes from two meioses are united into a single diploid cell, uniting the interests of the two genomes, and reducing the conflict between parental genomes, during somatic growth. In mushroom-forming basidiomycete fungi the haploid nuclei in mycelia produced from sexual spores, called primary mycelia, represent the gamete stage. The fertilization process occurs after two primary mycelia encounter each other, with subsequent fusing of hyphal tips. This mating is often depicted as occurring between two partners of similar size — i.e. similar to isogamy. Since the control of cytoplasmic inheritance in basidiomycetes is not linked to an individual monokaryon, it has been said that fungi do not have sexes (Hurst & Hamilton, 1992). However, the fact that cytoplasmic inheritance occurs in both directions means that fungi can be considered to have sex *roles*, as in a mating between two monokaryons each partner acts both as male and female, in a sense two mating reactions with different cytoplasms (Aanen et al., 2004). Additionally, accumulating evidence shows that perhaps mating between partners of two dramatically different sizes — i.e. similar to anisogamy— is more common than previously thought. This mating may occur through spores that colonize at different timepoints leading to mycelia of different sizes, or through sexual spores landing on a resident monokaryotic mycelium leading to fertilization (James & Vilgalys, 2001; Nieuwenhuis, Nieuwhof, et al., 2013). A further source of these anisogamous matings arises through the process of di-mon mating, where one genotype of a mated dikaryon fertilizes other monokaryotic mycelia encountered (Nieuwenhuis, Nieuwhof, et al., 2013). The prevalence of equally sized monokaryon-monokaryon matings, mating between a germinated spore (a very small mycelium) and a monokayron matings, or between a dikaryon and a monokaryon is an active area of research (Jenssen et al., 2020). In laboratory experiments, it has been shown that if multiple matings occur in parallel, the fertilizing nuclei will race to fertilize the mycelia, and once they meet migration will stop, and two dikaryons will be produced (Todd and Rayner, 1983). This observation indicates that differences in nuclear migration speed may result in differential resources for that genotype.

Aside from the sexual aspect of mating, there is also a social side. Following the fusion of two hyphae with haploid nuclei, the resulting genomes do not merge, but remain isolated in a dikaryotic state. This dikaryotic state means that the genomes may retain a measure of individuality. Early studies in *Schizophyllum commune* indicated that gene expression may be different between the nuclei in the dikaryon perhaps due to the physical spacing (Schuurs et al., 1998). Recent investigations using genome-wide approaches have demonstrated significant allele specific expression for many genes in several species of mushroom (Gehrmann et al., 2018; Merényi et al., 2022), and also in the ascomycete *Neurospora tetrasperma* (Meunier et al., 2022).

The species *Schizophyllum commune* represents an important model species for basidiomycete biology. It was originally used to study, and subsequently identify, the genetic control of mating compatibility (Raper & Raper, 1966; Wendland

et al., 1995). Additionally, this species has been an important fungal cell biology model (Wessels et al., 1991; Wösten et al., 1999). In addition to detailed laboratory-based analyses, the abundance and ease of isolation has made *S. commune* an important species for studying how mushroom-forming fungi disperse in their environment (Baranova et al., 2015; James & Vilgalys, 2001; Nieuwenhuis, Nieuwhof, et al., 2013). This species is globally dispersed, although there appears to be limited gene flow between continents (James et al., 1999). Genetic comparison of populations in this species has recovered nucleotide diversity, p, of 0.1, the highest reported value for any species, potentially due to a combination of a global population and a high mutation rate (Baranova et al., 2015; Bezmenova et al., 2020). While there are other competing model systems for laboratory-based analyses such as *Coprinopsis* (Stajich et al., 2010), *S. commune* is an important bridge linking laboratory studies to fungal ecology (Nieuwenhuis, Nieuwhof, et al., 2013).

Matings in basidiomycetes involve gametes with significant resources, and thus presents a tension. The obtained resources, both in terms of calories as well as physical space, will be shared with the fertilizing nucleus due the nuclear migration. However, if there are multiple matings then the question arises how the resources will be shared between the fertilising partners. Thus, the fertilization process presents a selection area for the incoming nuclei, where rapid migration and nuclear division is presumably favoured, but the resident mycelium may also have an active role in deciding the limits of the activity of this incoming nucleus. Using a trio of wild *S. commune* isolates constructed to have the same mitochondrial background, we tested the effect of mating both on the individual nucleus level as well as the dikaryon level. Further, by analysing expression we looked for an effect of the individual in the female role.

Methods:

Culture conditions:

Strains and culture conditions: three strains of *Schizophyllum commune* were used. The reference strain 4.8A, as well as LoenenD and TattoneD (Marian et al., 2021; Ohm et al., 2010). Strains were cultured at 25ºC on Schizophyllum Complete Media (SCM) except where noted below (Dons et al., 1979).

Mitochondrial swapping: The LoenenD and TattoneD strains were mated with 4.8A by co-culture on SCM, and dikaryons were confirmed visually by the presence of clamp connections. To isolate the original LoenenD or TattoneD with 4.8A mitochondrial genotypes, dikaryons were formed between each strain and 4.8A, and then samples isolated from the 4.8A side of the mating. These dikaryons were then protoplasted as previously described (Nieuwenhuis, Debets, et al., 2013). From the regenerated colonies, genotyping was performed to isolate the LoenenD or TattoneD nuclear genotype. The isolates with swapped cytoplasms were used for all matings in this study, and for simplicity will be referred to simply as Loenen and Tattone.

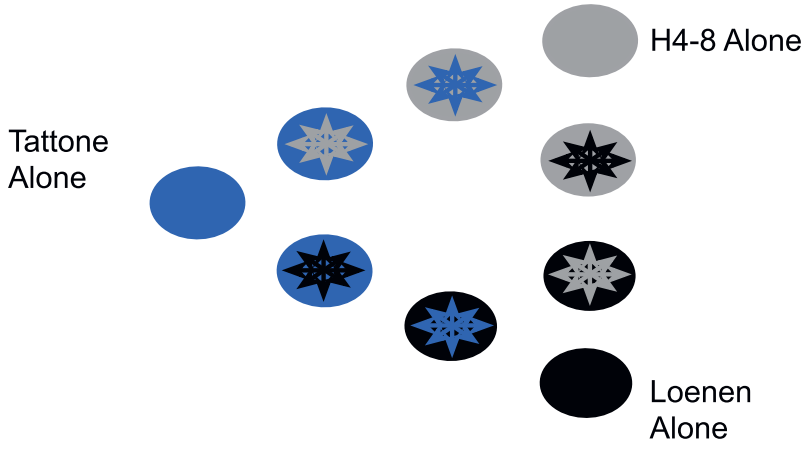

Figure 1: Schematic of samples used for reciprocal matings. Three individuals are indicated with grey, blue or black color. Matings were performed by inoculation of one genotype into the centre of a pre-grown plate of a different genotype (radially expanding arrows), in both directions.

Experimental matings: For matings a plate of SCM media was first overlaid with a sterile cellophane membrane to allow diffusion of nutrients while retaining mycelia. On top of this, a plug of the strain to be used in the female role was added and incubated for 3 days. Following this, the strain to be used in the male role was inoculated by placing a plug of colonized agar in the centre of the colonized plate. Following mating, plates were incubated for either 3 days and mycelia were

harvested (T1) or dikaryotic mycelia was transferred to a second plate and grown for 3 days, where all new growth was harvested (T2).
While all matings were attempted, the matings with Tattone acting as the accepting mycelia were initially unsuccessful. After repeated inoculation of the fertilizing partner, successful dikaryons were established. However, this resulting in missing data for T1 of crosses with Tattone as receiving partner.
RNA extraction: For T1 and T2 samples, RNA was extracted by scraping all mycelia from the cellophane membrane. Total RNA was extracted using the Qiagen RNEasy Micro kit (Cat. No. 74004). Following the extraction, rRNA depletion, cDNA synthesis, library preparation, and sequencing was performed by Novogene Co. Ltd. Using the Illumina platform producing 125 bp paired-end reads.
Othologous gene identification: Predicted transcriptomes for each of the three strains (LoenenD v.1.0, TattoneD v1.0, H4-8 v3.0) were obtained from the Mycocosm webserver. Orthologous genes between the predicted transcriptomes of the three isolates were predicted using a three-way reciprocal best-BLAST hit (RBBH) criterion. The three pairwise RBBH searches (4.8A-LoenenD, 4.8A-TattoneD, LoenenD-TattoneD) were conducted using custom script blast_rbh.py. The results of these three searches were then combined using custom script get_orthogroups.py. Orthologous gene sets were retained where the RBBH match between 4.8A and Loenen was the same 4.8A transcript as the RBBH between 4.8A and Tattone.
RNA-seq read mapping: Hybrid orthologous transcriptomes were constructed by combining the orthologous transcripts of two strains, producing three transcriptomes (4.8A/Loenen, 4.8A/Tattone, Lonene/Tattone). Raw RNA-seq reads were error-corrected with fastp (Chen et al., 2018). For each set of matings, the RNA sequence data of both the mono- and dikaryons were analysed using Kallisto (Bray et al., 2016).

Results:

Ortholog Identification:

Transcriptome	# Transcripts	Pair	# RBBH	Informative Orthologs	Median % DNA Identity
H4.8A	16319	H4.8A/ Loenen	9934	9918	90.9
LoenenD	13827	H4.8A/ Tattone	10312	10296	90.5
TattoneD	15199	Loenen/ Tattone	10991	10262	97.2
			# Orthologs		
		All 3	9057		

Table 1: Statistics of transcriptomes used for identifying orthologs transcripts between the three *S. commune* isolates.

As the transcriptomes were previously predicted *de-novo* for each genome, we used Reciprocal Best Blast Hits (RBBH) to identify orthologous transcripts for further gene expression analysis. The initial transcriptomes had between 13,827 and 15,199 predicted genes, of which approximately 10,000 were shared between any two (Table 1). To allow for gene quantification across all treatments, a final criterion was used of RBBH between all three strains with a minimum similarity of any RBBH of 70% nucleotide similarity. The final dataset, termed All3, of the three-way best BLAST hits represented more than half of the starting transcriptomes of each strain (H4.8A: 55.4%; Loenen: 65.5%, Tattone: 59.5%). This analysis also showed that of the genes that were not included in the All3 dataset, most were unique to a single isolate, as the All3 dataset included ~90% of the pairwise RBBHs between any of the three pairings. Of these orthologs identified, the vast majority were bioinformatically informative, with one or more differences between them. The transcriptomes of Loenen and Tattone were most similar, with approximately 700 orthologs with identical sequence, and this pair also had the highest nucleotide similarity, at 97.2%. The transcriptomes of H4.8A and either Loenen or Tattone were equally similar with average nucleotide identity of ~90% and approximately 20 identical genes.

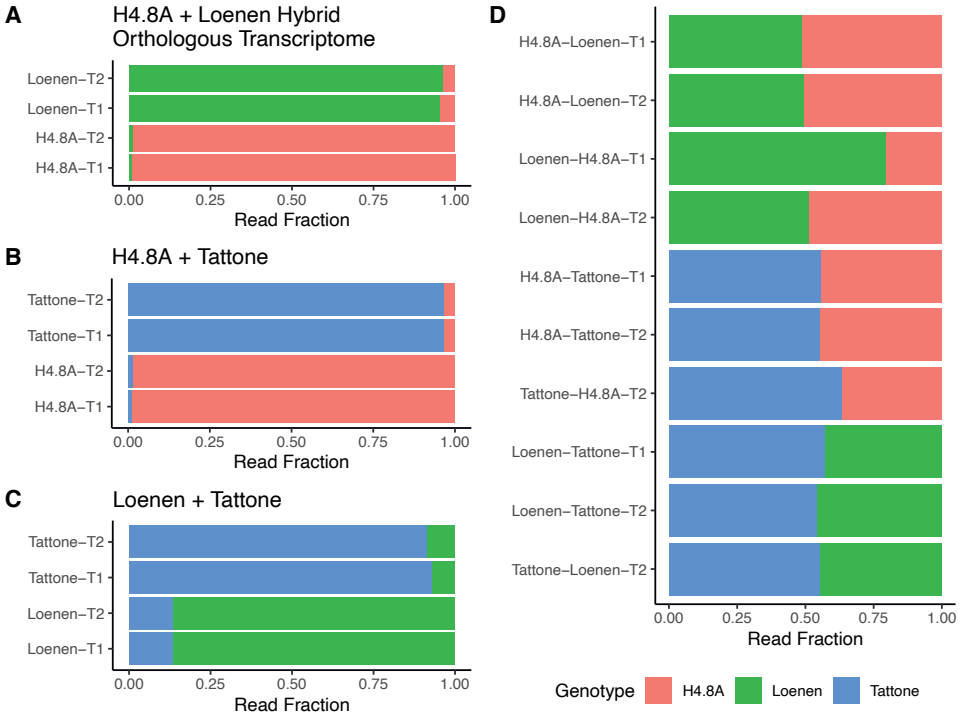

Figure 2: Mapping percentages of reads from differing biological sources against various hybrid transcriptomes. (A-C) Mapping of RNA-seq reads from a monokaryotic strain against hybrid orthologous transcriptomes comprised of two strains, as indicated. Colour indicates the fractions of reads with an assigned identity, with T1 and T2 indicating timepoints. Bars represent mean of three replicates. (D) Read fraction from constructed dikaryons. Names of component strains are listed with female role first, and male role second, with timepoints as above. Note that the second timepoint for Tattone-Loenen and Tattone-H4.8A are not shown (See Discussion).

Mapping specificity of hybrid orthologous transcriptomes:

To evaluate the effectiveness of the identified orthologous groups, we mapped the RNA-seq reads from each homokaryon against a constructed hybrid orthologous transcriptome. This consisted of the transcripts of the 9057 orthologs, from both parents (Figure 2). The pairings of both H4.8A and Loenen as well as H4.8A and Tattone showed high specificity, with >90% of reads being correctly identified (Figure 2A+B). The comparison of Loenen and Tattone showed reduced, but still high, specificity, with slightly less than 90% of reads being correctly assigned (Figure 2C). Within the dikaryons the overall RNA contributions from each parent were approximately 50% in all cases, except for the first timepoint with Loenen in the female role, and H4.8A in the male role (Figure 2D).

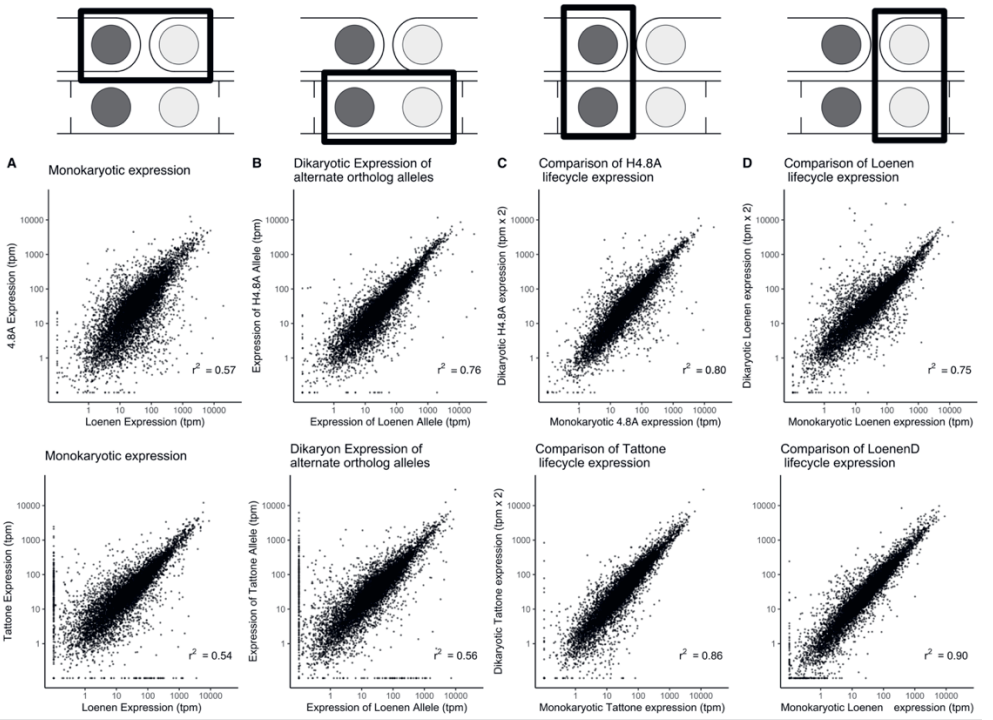

Figure 3: Comparison of gene expression during monokaryotic and dikaryotic lifecycles. Expression of alleles of H4.8A or Loenen in either monokaryotic or dikaryotic state. (A) Expression per gene for monokaryons Loenen and H4.8A (top) or Loenen and Tattone (bottom). (B) Expression of either allele during dikaryotic growth of Loenen and H4.8A (top) or Loenen and Tattone (bottom). (C) Comparison of monokaryotic versus dikaryotic expression of H4.8A allele (top) or Tattone (bottom). D) Comparison of Loenen allele expression across lifestages for Loenen and H4.8A (top) or Loenen and Tattone (bottom) as in C. Note that tpm expression values from alleles within dikaryons are multiplied by two when comparing against monokaryons.

Orthologous gene expression of monokaryons was generally consistent between strains. Comparison of monokaryotic expression between H4.8A and Loenen resulted in a moderate positive correlation (r^2: 0.57), with a similar correlation between Loenen and Tattone monokaryons (r^2: 0.54) (Figure 3A). When growing as a dikaryon, the correlation between expression of the two alternate alleles was higher, with the correlation between H4.8A and Loenen alleles having r^2 of 0.76, and the correlation between Loenen and Tattone alleles having r^2 of 0.56 (Figure 3B). Notably, there were a higher number of alleles expressed in only one of the Loenen and Tattone monokaryons (Fig 3A bottom vertical and horizontal edges) compared to the Loenen and H4.8A monokaryons (Figure 3A top). For each genotype, comparing expression levels during monokaryotic or dikaryotic lifecycles, correlations were high ($r^2 > 0.75$; Fig 3C + D). Notably, the correlation for Loenen alleles between monokaryon and dikaryon was higher when paired with Tattone (r^2=0.90) than when paired with H4.8A (r^2=0.75).

Analysis of differentially expressed genes:

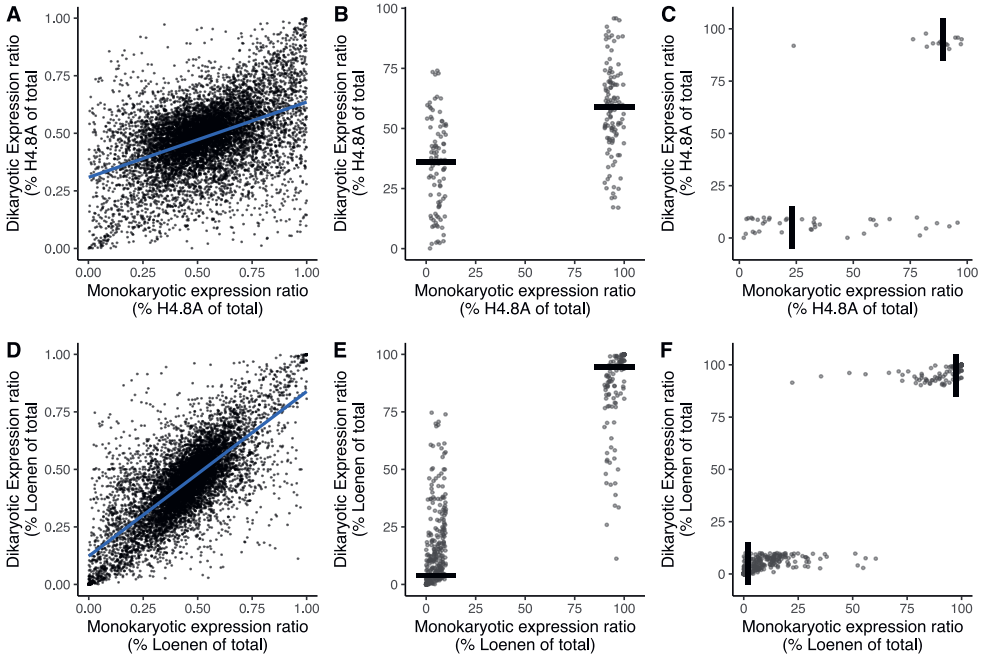

Figure 4: Effect of partner interactions on most divergently expressed genes. (A) Comparison of H4.8A allele expression ratio in dikaryon (y-axis) to expression ratio between monokaryons (x-axis). Dots represent mean of three replicates for each individual gene, and blue line indicates linear best-fit (B-C) Same data as in A but restricted to genes whose alleles are reliably differentially expressed in the monokaryotic stage (B) or differentially expressed between dikaryotic strains (C). Solid lines indicate median expression of the set of differentially expressed genes. (D-F) Similar to A-C but with the matings of Loenen and Tattone.

We then compared the ratio of expression of the two alleles within the dikaryon to the ratio of expression between the two monokaryotic strains (Figure 4). Overall, there was a general positive association between ratio of H4.8A transcripts between monokaryons and within the dikaryon (i.e. if a gene had higher expression in H4.8A than Loenen monokaryons, it was likely to also have a higher fraction of reads within the dikaryon) (Figure 4A). A linear model of dikaryotic allele expression ratio as a function of monokaryotic expression ratio resulted in a significant relationship ($p = 4.5x10^{-8}$), with low explanatory power (r^2: 0.003) for H4.8A and Loenen. For the mating of Loenen and Tattone, the correlation was much higher ($r^2 = 0.55$). To examine the regulatory influence of the dikaryotic interaction, we focused on genes with 10-fold difference in expression and excluded lowly expressed genes (tpm < 5). For genes differentially expressed between monokaryons (Figure 4 B+E) there was also allele specific expression in the dikaryon, with genes with high Loenen expression having higher Loenen allele expression within the dikaryon. This was similarly found for genes with allele specific expression within the dikaryon showed

unequal expression between the monokaryons (Figure 4 C+F). Reflecting the differences in r^2 values, for these strongly differentially expressed genes, the ratios showed more similarity between monokaryon and dikaryon expression in the mating of Loenen and Tattone, than they did for the mating of H4.8A and Loenen.

Analysis of T2 mating data:

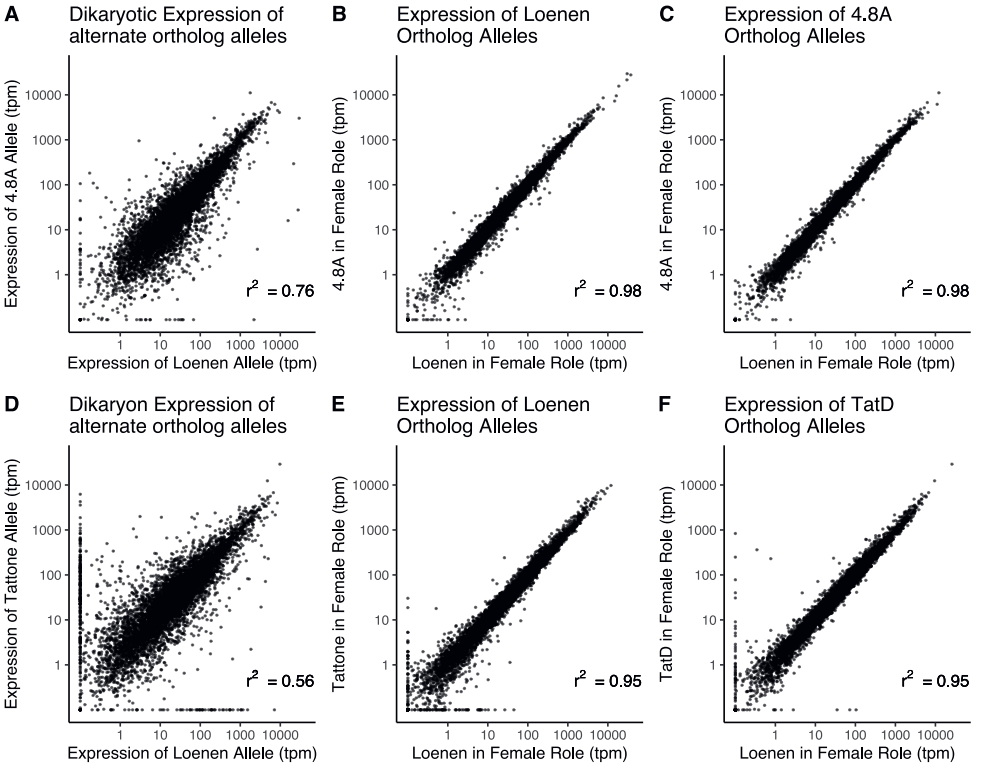

Figure 5: Allelic expression following reciprocal matings. A) Expression of alternate alleles for each orthologous gene at timepoint T2 from mating of H4.8A and Loenen. Dots represent mean expression of 3 replicates. (NOTE: same panel as Figure 3B) B) Expression of Loenen allele in a dikaryon, x axis showing expression with Loenen in a female role, y-axis showing expression for the same gene with Loenen in the male role. E) Expression of H4.8A allele for each gene with x-axis showing expression when H4.8A used as female strain, and y-axis showing expression when used in male role. D-F) similar plots to A-C) but with mating between Loenen and Tattone.

Within the dikaryon, expression of the two alternate alleles was similar (Figure 5A+D), with moderately positive correlations (r^2 0.76 for H4.8A and Loenen, r^2 of 0.56 for Loenen and Tattone). When comparing the expression of alleles of a genotype in either direction, we found the correlation was very strong, with r^2 >0.95 (Figure 5 B+C, E+F). Within the H4.8A and Loenen mating, there were no genes with differential expression based on which parent had the female role. For the mating of Loenen and Tattone, there were several genes with differential expression

of the Tattone allele (Figure 5F), but none reached significance as expression was high when Tattone was in the female role, but below 1 tpm when Loenen was in the female role.

Discussion:

Schizophyllum commune, like other fungi, does not have true sexes, in the sense of separate individuals that do or do not donate cytoplasm to an offspring. However, like other hermaphroditic organisms there is still a distinction to be made between the male and female role during matings. Here, using a reciprocal mating design we set out to test the effect of this role, if any, during mating. Our design shows that there is significant variation in gene expression both between monokaryotic strains, as well as between the alleles inside a dikaryon. Our results indicate that the partner during mating has an influence on overall gene expression, but that the genotypes retain a large fraction of the monokaryotic identity. While we recovered large differences in expression between genotypes, the changes occurring as a result of dikaryotic growth do not show evidence of imprinting based on parental role.

A critical aspect of this experiment was the use of a constant mitochondrial background. One of the largest effects of any reciprocal matings, often referred to as the maternal effect, is the mitochondrial genotype donated. Currently there is little data available for basidiomycetes on the effect of mitochondria on nuclear background. However, the example of differential virulence for *Heterobasidion annosum* depending on mitochondria indicates that mitochondria probably play a role in many traits (Clergeot & Olson, 2021; Giordano et al., 2018). The effect of these mitochondria can be due to epistatic relationships between nuclear genes and mitochondria (Mossman et al., 2019; Parikh et al., 1987), or as a direct effect of the genetic differences in mitochondria themselves (Wolters et al., 2018). Thus, it should not be inferred from our results that the direction of mating in nature does not have an effect, as we purposefully excluded the primary maternal effect of the mitochondrial genotype.

Our results indicate that the differences in expression between monokaryons are somewhat retained when growing together inside a dikaryon. This effect was noticeably larger between the Loenen and Tattone nucleus, and less so between the H4.8A and Loenen nucleus. It is common to deconstruct such differences into those from the chromosome itself, *cis,* from those acting from a distance, *trans* (Bell et al., 2013; Osada et al., 2017). In this study we did not attempt such calculations. While dikaryons seem genetically similar to a diploid, with two copies of each gene, the physical separation of nuclei allows for an additional level of gene regulation (Schuurs et al., 1998). In diploids there are both *cis*- (i.e. those produced by one chromosome acting on itself) and *trans*-effects (i.e. diffusible elements like transcription factors) that can act on both the paternal and maternal components of the genome. However, elements like transcription factors may not act in *trans* as

expected within a dikaryon. If such a factor is transcribed in the cytoplasm near the nucleus of origin, then proportionally more of it will enter that same nucleus. In *Agaricus bisporus* widespread differences in allelic expression were found but some of what would be called *trans* effects in a diploid may appear to act in *cis* within a dikaryon, perhaps leading to the excess of nucleus specific expression seen in *Agaricus* (Gehrmann et al., 2018). As it is relatively easy to produce true diploids in *S. commune* (Frankel & Ellingboe, 1976), the comparison of allele specific expression between a heterozygous diploid and the isogenic dikaryon would be illuminating.

This study was specifically enabled by the genetic diversity within *S. commune*. While for many species only a minority of genes contain enough variation to confidently identify allele-specific expression, in the crosses used here almost all genes had multiple variants. This allowed us to identify differences in allele expression almost comprehensively, whereas for many species much of allele specific expression may be undetected. However, this level of sequence divergence comes with a complicating factor of whether *S. commune* truly represents a single species. This globally dispersed species has been described as containing the highest nucleotide diversity of any known species (Baranova et al., 2015) yet population genetics suggests that little to no gene flow occurs between populations on different continents (Baranova et al., 2015; James et al., 1999). Although isolates from around the world are capable of forming dikaryons, the trait traditionally used as a criterion for delimiting basidiomycete species, the geographical separation may mean there are instead several independent lineages. These independent lineages would suggest that *S. commune*, already does not meet the criteria of an evolutionary species, and can be seen instead as a species complex (Wiley, 1978). For our data, this would indicate that the crosses between Loenen and Tattone likely reflect intraspecific combinations, while Loenen/Tattone and H4.8A may represent sister species. This difference in divergence may explain some of the differences seen in the gene expression patterns.

The physical separation of nuclei within the dikaryon allows for additional evolutionary pressures. The retained ability to mate with additional monokaryons sets up a tension as ultimately only one genotype will successfully fertilize a particular monokaryon (Buller, 1930; Nogami et al., 2002; Quintanilha, 1937). This competition has been suggested to select for “selfish” nuclear types (James, 2015) and simulations have shown that this selfish drive can persist even in the presence of a strong fitness trade-off due to lower-level selection (Auxier et al., 2022). Since differences in mating success between the nuclei in dikaryons have been demonstrated in nature (Nieuwenhuis et al., 2011; Nogami et al., 2002), the source of this variation is important to uncover. The role of gene expression regulation, if any, should be a high priority for future research.

The results here do not indicate any effect of imprinted genes on the outcome of matings in *S. commune.* This would indicate that a monokaryotic mycelium that is fertilized may share resources equally with the fertilizing partner, although not its mitochondria. This finding may be limited by the bias of strongly growing monokaryons. A previous study of the ascomycete truffle forming fungus, *Tuber melanosporum*, indicates that in this species fruiting bodies appear to be fertilized by a lineage that is not recovered from the plant roots with which it is symbiotic (Taschen et al., 2016). In this species, the “male” lineage appears to contribute little to somatic growth. The monokaryotic strains used in our study all grow relatively vigorously. Perhaps using strains or species where there is some indication of more constitutive maternal/paternal role would better reveal a role for imprinting in a fungal species.

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Chapter 7

Genetic Association of Somatic Incompatibility and NLR-like Protein Domains in *Coprinopsis cinerea*

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Abstract:

Fusion between hyphae has potential benefits, but to limit risks of exploitation or infection it should be restricted to be within a genetic individual. To achieve this, sustained fusion is restricted based on the identity of polymorphic allorecognition genes. The genes responsible for this nonself recognition have been unknown in basidiomycetes. Since basidiomycetes experience an extended dikaryotic phase, nonself recognition likely functions differently from known mechanisms in ascomycetes. We present results of genetically mapping the first known basidiomycete nonself recognition locus in the model mushroom *Coprinopsis cinerea*. Using a set of ~600 F1 offspring, combined with independent backcross lines, we identified a region on chromosome 5 whose alleles are strongly associated with nonself recognition. Fine-mapping of this region combined with genomic comparisons of additional *C. cinere*a isolates provides evidence that nonself recognition is driven by ancient polymorphic alleles of an NLR-like system. The polyallelic locus we identified appears to involve a Leucine Rich Repeat, a novel finding for fungal nonself recognition. These results provide a first understanding of how basidiomycetes regulate individuality.

Introduction:

The distinction between individuals is complex in basidiomycete fungi, the group that produces mushrooms as well as rusts and smuts. These fungi exist both as monokaryons, composed of haploid nuclei with a common genotype, as well as dikaryons, with two distinct haploid genotypes in a stable coexistence. Initially, studies by the foundational mycologist Buller suggested that basidiomycetes formed fused networks between individuals, producing chimeric colonies, termed the "Unit Mycelium" (Buller, 1930). However, further work clarified that, at least when dikaryotic, these fungi regularly distinguish self from nonself. Based largely on observations of the common wood decaying fungus *Trametes versicolor*, Rayner produced the influential synthesis of the "Individualistic Mycelium" (Rayner, 1991; Rayner et al., 1984). Using both field and laboratory experiments, it was shown that distinct dikaryotic isolates produced melanized barrage zones during interactions, while clones of an isolate did not. Observations of *Heterobasidion annosum* also showed that genetic markers tracked with patterns of antagonism in confrontation assays, and a lack of antagonism between strains was found only when genetic markers were identical (Stenlid, 1985). Further work showed antagonistic interactions between dikaryotic isolates for many species, and often the antagonistic phenotypes were reduced when isolates were more closely related (Hansen et al., 1993; May, 1988).

The correlation between genetic identity and antagonism naturally led to investigations of its genetic basis in basidiomycetes. Using a set of sibling heterokaryons from a single fruiting body, it was determined that somatic incompatibility (SI) was controlled by 3-4 loci in the tree pathogen *Heterobasidion annosum* (Hansen et al., 1993), which were subsequently mapped to the genome to produce several candidate QTL (Lind et al., 2007). Similar genetic studies in *Amylostereum*, a fungus that is symbiotic with wood wasps, found two loci on two different chromosomes, termed *hetA* and *hetB* (van der Nest et al., 2008, 2009). Experiments performed on siblings of *Collybia fusipes* found evidence for 3-4 loci controlling SI in this species (Marcais et al., 2000). Genetic analysis of the tree pathogen *Phellinus gilvus* provided evidence of a single locus with large effect, but likely a set of smaller effect loci were also involved (Rizzo et al., 1995). An interesting exception to the general pattern of 3-5 loci being involved is found in *Serpula lacrymans* which is an invasive fungus that grows in human housing building materials. This fungus has only two loci, potentially due to the low genetic diversity of the species. Each locus is biallelic, resulting in eight somatic compatibility groups (Kauserud et al., 2004, 2006).

While the pattern of nonself recognition between basidiomycete dikaryons is now widely accepted, its molecular basis remains unknown. In ascomycete fungi, the genes involved are often related to Nod Like Receptors (NLRs), which are well studied in both plant and animal immune systems (Uehling et al., 2017). Primarily studied in the species *Neurospora crassa* and *Podospora anserina*, there is a

common protein domain identified termed the HET domain, of unknown function (Glass & Dementhon, 2006). Testing the assumption that nonself recognition machineries may be shared between fungal phyla, two studies bioinformatically surveyed basidiomycete genomes for candidate genes, pointing to some shared mechanisms between ascomycetes and basidiomycetes, but with an absence of consistent HET domains in basidiomycetes (van der Nest et al., 2014). Further investigations of NLR proteins in fungi found no clear candidates for basidiomycete SI controlling genes (Dyrka et al., 2014).

In mushroom-forming basidiomycetes, there are two primary model genetic systems. Originally used for identification of the mating loci (Raper & Raper, 1966; Wendland et al., 1995), *Schizophyllum commune* has become a widely used organism to understand both the function of hydrophobin proteins as well as fruitbody formation (Ohm et al., 2010; Wessels et al., 1991). However, *S. commune* lacks an obvious nonself recognition phenotype when wild isolates are paired in the laboratory (B. P. S. Nieuwenhuis et al., 2013) so despite the considerable genetic toolbox that is available, this species is not suitable for genetic dissection of this trait. The other main genetic model in mushroom-forming fungi is *Coprinopsis cinerea*, which also has a well-defined sexual cycle, a high-quality annotated reference genome available (Moore & Pukkila, 1985; Stajich et al., 2010) and a reliable transformation system (Binninger et al., 1987). Importantly a collection of wild isolates from South East Asia is available (M. Wu et al., 1983). This species has the benefit of a clear nonself recognition response (May, 1988).

In the last several years, the increased ease of whole genome sequencing has revolutionized the process of genetic mapping. Traditionally, genotyping of markers from a mapping population limited the resolution of mapping experiments. However, high-throughput sequencing has turned this on its head, as the number of markers is now limited by the genetic diversity of the species itself, while phenotyping often becomes a limiting factor. One relatively standard methodology is to use Bulk Segregant Analysis (BSA) which involves grouping offspring into pools based on phenotype (Michelmore et al., 1991). These pools are then sequenced and allelic differences between pools are expected to be highest near the causal genes. BSA is limited by the number of recombination events (Shen & Messer, 2022) and does not allow for finer analyses due to the pooled DNA. A more recent alternative is low-coverage sequencing, where each individual is sequenced at ±0.1-1.0X, much lower than the standard 30X sequencing for most genome analyses. This approach utilizes the fact that the offspring are related, so you do not require high confidence in the genotype calls from each individual since the genotypes can be determined for the population as a whole. Since for most mushroom species there are only a few crossover events on a chromosome, genotypes can be called in blocks which integrates the low coverage sequencing information for a region. This technique has been used increasingly to save costs and allows for much larger mapping populations (Bloom et al., 2019; Huang et al., 2010).

For genes involved in nonself recognition, across organisms and mechanisms, there are similar evolutionary pressures. As the nonself recognition alleles are most useful when rare, fitness of alleles becomes negative frequency dependent (Spurgin & Richardson, 2010). This leads to alleles that are older than would be expected under neutral processes, and often the alleles are older than the species in which they are found, resulting in alleles shared across species boundaries in so called Trans-Species Polymorphisms (Charlesworth, 2006). Across plants, animals and fungi, the effects of balancing selection were detected early on in DNA sequence data at a population level (Hedrick & Thomson, 1983; Tian et al., 2002; J. Wu et al., 1998). The even allele frequencies and trans-species polymorphisms provide clues that balancing selection is acting; however, the causes can be multiple and disentangling them can be difficult to impossible (Lobkovsky et al., 2019; Spurgin & Richardson, 2010).

The extensive research of ascomycete nonself recognition provides a background for understanding basidiomycete SI. However, bioinformatics surveys have failed to identify clear causal genes for this biologically important phenotype. Here we leverage the increased availability of sequencing information, using both genetic and bioinformatic techniques, to map genetic variants of SI in the model species *C. cinerea*.

Materials and Methods:

Strains:

Coprinopsis cinerea strains used are listed in Table 1. For crosses, Okoyama-7 and Java-6 were used as parents. For all experiments in this manuscript, all strains were mated with FSCG #25194 in the "common nucleus" method (Worrall, 1997).

Table 1: Strains used in this study

Strain	Comment	Reference
Okayama-7	Founding parent; reference genome	(Moore et al., 1979)
Java-6	Founding parent	(Binninger et al., 1987)
FGSC 25192	Japanese Wild Homokaryon	(M. Wu et al., 1983)
FGSC 25194	Japanese Wild Homokaryon; used as common partner	(M. Wu et al., 1983)
FGSC 25196	Japanese Wild Homokaryon	(M. Wu et al., 1983)
FGSC 25197	Japanese Wild Homokaryon	(M. Wu et al., 1983)
FGSC 25198	Japanese Wild Homokaryon	(M. Wu et al., 1983)
11-13-8	3 generations backcrossed to Okoyama-7; somatically incompatible to Okoyama-7	This study
8-24-15-7	4 generations backcrossed to Okoyama-7; somatically incompatible to Okoyama-7	This study

Culture Conditions:

Strains were maintained on MYA (20 g/L Malt Extract, 15 g/L Agar) and grown at 37 °C for routine culturing. For sexual crosses, strains were co-inoculated onto test tubes with ~2g of sterilized moistened horse dung (Moore & Pukkila, 1985), and incubated at 25 °C in 12h:12h day:night cycles. Basidiospores were collected by placing several gill pieces into water in microcentrifuge tubes, and heat shocked at 70 °C for 1 hour prior to dilution plating on MYA. Isolated colonies resulting from basidiospores were harvested using a tungsten needle (Moore & Pukkila, 1985). Long-term storage of strains was accomplished by freezing at -80 °C in 30% v/v glycerol.

For incompatibility screening, we phenotyped at the dikaryotic stage. For this, all strains were dikaryotised with FGSC #25194 and dikaryon production was confirmed by visual inspection for clamp connections. For incompatibility testing, dikaryons were inoculated at opposite edges of either 9 cm petri dishes or in 100 mm 5x5 grid plates (Thermo Fisher Scientific Inc. Part #11339273). Incompatibility testing was performed on MYA, as initial comparisons with YPSS media (May, 1988) indicated little difference in the resulting phenotype.

DNA Extraction:

DNA for single isolates was isolated from mycelia scraped from agar plates using a CTAB buffer lysis step, followed by isopropanol precipitation, ethanol washing, and RNAse treatment (M. Nieuwenhuis et al., 2019). For *in-silico* bulk segregant analysis, mycelia were grown in liquid Malt Extract (20 g/L) and transferred to deep well 96-well plates. The mycelia were ground in a bead beater using 2 mm glass beads. DNA was extracted using a SPRI bead extraction, and DNA concentration was normalized using Quant-iT™ PicoGreen™ (ThermoFischer Scientific Inc.) fluorescence. Multiplex low-coverage barcoded libraries of offspring were performed using the hackflex protocol (Gaio et al., 2022). All DNA was sequenced using 150 bp paired-end reads on the Illumina Novaseq 6000 platform (Novogene Inc.).

Single Isolate Genetic Analysis:

DNA reads from single isolates were mapped to the reference Okoyama-7 using bwa-mem (Li, 2013), and variants called with freebayes v1.3.1-19-g54bf409 (Garrison & Marth, 2012). Predicted effects of variants were annotated using snpEff v5.0e (Cingolani et al., 2012). For comparative genomics, genes were predicted using Augustus v2.5.5 (Stanke et al., 2006), and syntenic regions were compared using clinker (Gilchrist & Chooi, 2021).

For genetic analysis of offspring from Okoyama-7 and Java-6, we used variants identified from Java-6 reads mapped to the Okoyama-7 as a starting set. The low-coverage sequence of each offspring was mapped using bwa-mem as above, but only the known variants from Java-6 were used in downstream mapping

analysis. For *in silico* BSA, we combined the low coverage sequences from offspring based on the compatible or incompatible phenotypes and genotyped the pools at the identified variant sites. We then used GATK VariantsToTable to format the input for use with QTLSeqR (Mansfeld & Grumet, 2018; Poplin et al., 2018). Genetic differences between bulks were assessed using the sliding window G' value with a window size of 100 kb (Magwene et al., 2011).

Results:

Interactions between dikaryons of Okoyama-7+25194 and Java-6+25194 produced clear barrage reactions after four weeks while we observed no barrage with controls of interacting clones (not shown). To phenotype the interaction between the offspring and the parents, for all interactions we dikaryotized offspring and parents with FGSC#25194 but for simplicity we further refer only to the members of the cross. Of the 576 F1 offspring of Okoyama-7 x Java-6 tested, we recovered a range of phenotypes (Supplemental Figure X). Of these offspring, we classified 71 offspring (12.3%) as compatible to Okayama-7+25194. The other offspring classified as incompatible had a range of phenotypes ranging from dark pigmented lines to less visible gaps in the hyphal growth front between two interacting colonies. In general, incompatible phenotypes developed following 4-6 weeks of incubation.

To simplify the genetics of the system, we backcrossed several lines of incompatible F1 offspring (when dikaryotized) to Okoyama-7 in parallel, selecting somatically incompatible offspring at each successive round. Following four rounds of successive backcrossing of offspring incompatible to Okoyama-7, we found that offspring segregated in approximately 1:1 compatible:incompatible, when tested against the Okoyama-7 parent, consistent with a single mendelian locus. For two of these backcrossed lines, 11-13-8 (backcrossed three times) and 8-24-15-7 (backcrossed four times), we processed offspring for bulk segregant analysis (BSA).

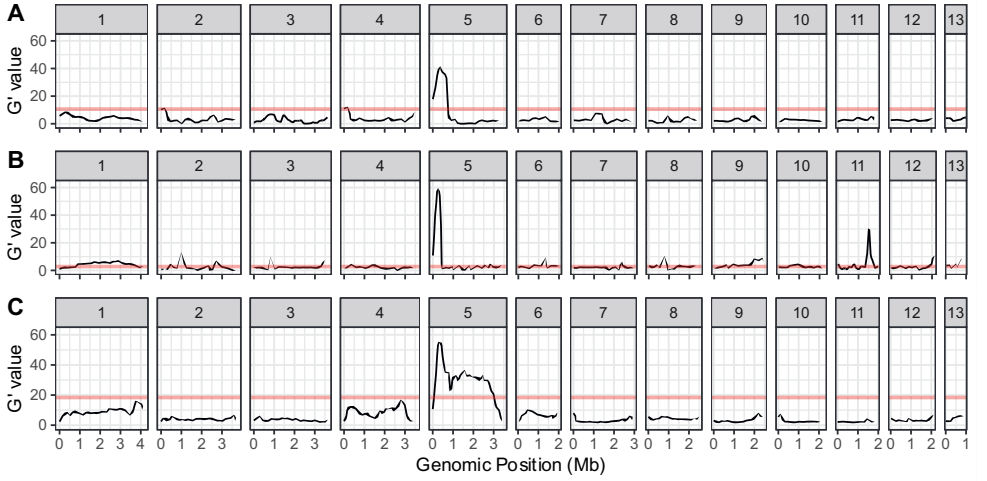

Figure 2: Genetic mapping of Somatic Incompatibility in *C. cinerea*. G' value represents difference in allele frequency between Compatible and Incompatible bulked groups smoothed over 100kb windows across the 13 chromosomes. Top row represents compatible and incompatible bulks from backcrossed line 11-13-8. Middle row represents bulks from backcrossed line 8-24-15-7 and bottom row represents values from bulks of F1 progeny. Horizontal red line shows permuted G' at α=0.05, values above this line can be considered as statistically significant.

Analysis of sequencing data from offspring produced 307,611 high confidence segregating variants, of which 221,414 were single nucleotide variants, 25,266 were insertions or deletions and 60,834 were complex polymorphisms. Analysis of the bulks of 20 compatible and 20 incompatible offspring from the backcrossed line 11-13-8, which had had three rounds of backcrossing, showed a single peak, with variation restricted to a region spanning ~0.5 Mb (Figure 2A). Comparison of the bulks of 19 compatible and 51 incompatible offspring from the backcrossed line 8-24-15-7 showed a peak at a similar location but narrowed compared to the 11-13-8 line (Figure 2B). The 8-24-15-7 backcrossed line also showed a second, smaller, peak on the right side of chromosome 11, albeit with a reduced genetic association (G' approximately 25). Analysis of the compatible and incompatible F1 offspring bulks (65 compatible and 35 incompatible) showed a large portion of chromosome 5 had a high G' value of approximately 55, with a peak near the left-hand side (Figure 2C). In the analysis of F1 progeny, no other locus rose above the significance threshold. The region on the left side of chromosome 5 was selected for further study, and termed *somA*. This nomenclature refers to its association with somatic incompatibility.

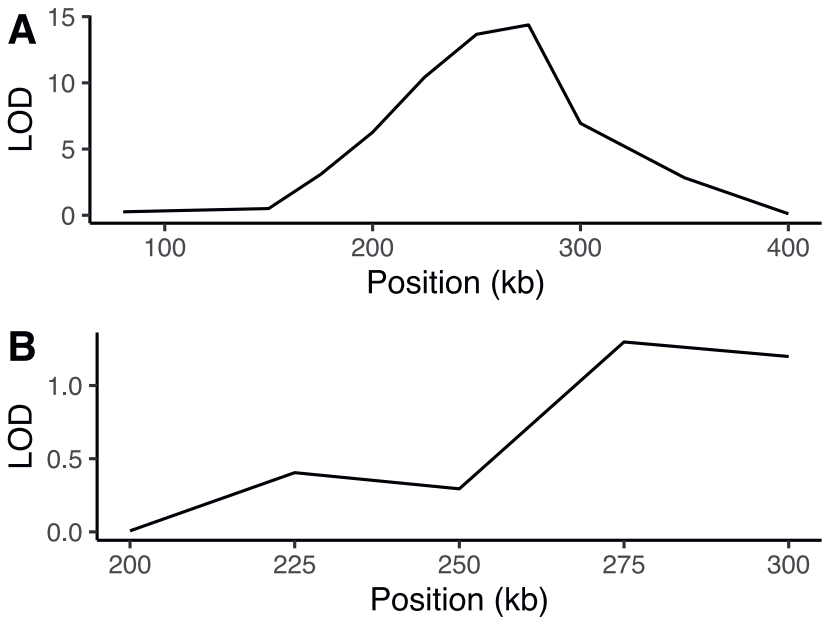

Figure 3: Fine mapping of the *somA* locus. A) LOD scores of QTL mapping using a panel of 47 recombinants across the 80kb – 400kb region of Chromsome 5 from the 8-24-15-7 inbred line.

To fine map the region involved in the 8-24-15-7 backcrossed line, we selected recombinants across the region on Chromosome 5 spanning from 80 kb to 400 kb. From 200 offspring of 8-24-15-7 X Okoyama-7, we recovered 47 recombinant offspring across this window. Phenotyping of these offspring as well as genotyping at six markers results in a further refinement of the QTL peak to be between 200 kb and 300 kb (Figure 3A). Further analysis of an additional 16 recombinants across the 200-300kb window showed less significant association, but with an increased association in the region 250-300kb (Figure 3B). The window spanning 200-300 kb along Chromosome 5 contains 41 genes, with 20 genes in the candidate region of 250-300 kb. To assess which genes could be causal to the trait, variants between Java-6 and Okayama-7 are summarized in Table 1.

Table 1: Predicted effects of Java6 coding variants across the *somA* locus. Genes in greyed section are found within the 200-250kb regions not indicated to be causal from the fine mapping.

Gene	Type	Start	End	Missense	Synonymous
CC1G_06702	N/A	201015	202384	7	3
CC1G_06701	N/A	202728	203944	9	3
CC1G_06700	alpha-1,2-mannosidase	204348	209421	26	35
CC1G_06699	N/A	209691	210513	6	11
CC1G_06698	RTA1-like	212295	213767	5	9
CC1G_06697	N/A	214355	215418	9	8
CC1G_06696	N/A	215706	216076	4	6
CC1G_06695	Histone-like Transcription Factor	219150	220077	1	3
CC1G_06694	rRNA pre-processing	220307	220873	0	4
CC1G_20105	tRNA	221023	221108	1	0
CC1G_06693	N/A	221555	222526	10	5
CC1G_20106	tRNA	222981	223066	-	-
CC1G_06692	N/A	224697	225068	2	0
CC1G_06691	Deaminase	225494	227500	23	18
CC1G_14539	N/A	228209	230050	10	3
CC1G_06688	NUDIX hydrolase	230408	231756	3	2
CC1G_06687	N/A	232545	236308	39	28
CC1G_06686	Zn(2)-C6 Transcription Factor	236786	241814	9	17
CC1G_06685	Pre-Ribosomal Associated Protein 1	242088	249852	50	44
CC1G_06684	LysM	250345	250905	0	1
CC1G_06683	N/A	251993	253062	13	2
CC1G_06682	LysM	253586	254306	0	2
CC1G_06681	Alpha/Beta Hydrolase Fold	255114	256503	4	0
CC1G_14540	N/A	256664	256976	5	1
CC1G_06679	N/A	258308	260468	19	10
CC1G_06678	Chondroitin A/C alginate lyase	260952	263760	1	16
CC1G_06677	N/A	264977	266615	38	39
CC1G_06676	Kinase	267088	268496	16	22
CC1G_06675	P-loop NTP-ase	269273	270675	35	16
CC1G_06674	Kinase	273554	275077	0	0
CC1G_06673	P-loop NTP-ase	275537	277145	0	0
CC1G_06672	Leucine Rich Repeat superfamily	277319	278602	6	20
CC1G_06671	N/A	279163	280764	5	23
CC1G_06670	N/A	281284	282185	10	7
CC1G_06669	N/A	282714	283038	1	0
CC1G_06668	Zn(2)-C6 Transcription Factor	285262	289101	10	61
CC1G_06667	N/A	290350	291659	0	13
CC1G_06663	Transcriptional Repressor TCF25-like	298244	301185	4	11
CC1G_06666	RuvB-like Helicase	291991	293851	1	21
CC1G_06664	N/A	296934	297747	1	6

Within the region of interest, namely 250-300kb, three neighbouring genes, CC1G_06677 to CC1G_06675 had the most coding differences, both missense and synonymous variants. However, the neighbouring two genes CC1G_06674 and CC1G_06673, while similar in size, had no variants annotated, neither missense nor synonymous. There appeared to be a repeated set, with CC1G_06676 and CC1G_06674 both predicted to encode kinases, and CC1G_06675 and CC1G_06673 predicted to encode P-loop NTPases. Closer inspection of the region covering CC1G_06674 and CC1G_06673 showed that the lack of annotated variants was due to a lack of reads mapping to this region, rather than an actual lack of genetic variation (Supplemental Figure 1). As such lack of coverage across a genomic region may indicate structural variation between strains, we then compared *de novo* annotations of a set of environmental strains.

Using BLAST, we searched the Okoyama-7 genome for similar domains to the LRR, P-loop NTPase, and kinase predicted protein sequences. This search resulted in a match to Chromosome 8 from positions 1,959,624-1,977,775, which also contained P-loop NTPase and two kinase encoding genes in a head-to-head orientation, but with a LRR gene flanking on either end. Due to the similarity in protein domains and organization, we refer to this locus on Chromosome 8 as the *somB* locus, although experimental evidence is currently lacking. The P-loop NTPase in this locus was also annotated as a NACHT domain, described in fungal cell death (Koonin & Aravind, 2000). Alignment of the two P-loop NTPase genes from the Okayama-7 *somA* locus with the NTPase annotated as a NACHT domain from this region on chromosome 8 from Okayam-7 showed that the two NTPases in the *somA* locus share the same diagnostic regions as the NACHT domain, primarily the glycine near the Mg^{2+} binding aspartate residue (Supplemental Figure 1). As such, we further refer to all NTPases in both the *somA* and *somB* regions as NACHT domain containing proteins.

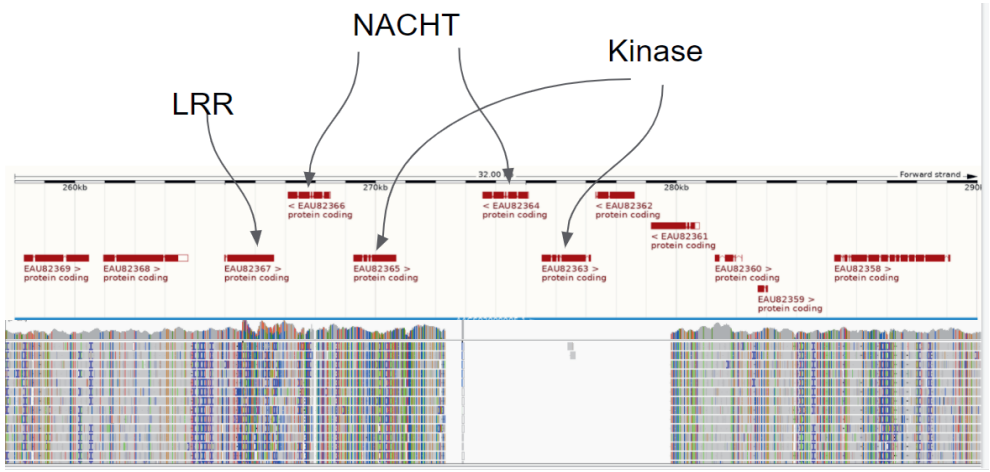

Figure 5: Sequence alignment of reads from Java6 against the reference genome of Okoyama-7. Genes annotated from the Ensembl website. Note large gap spanning one pair of NACHT/Kinase genes to which no reads from Java6 map.

Due to the high sequence diversity in the *somA* locus between Okayama-7 and Java-6, and to further assess the variation at this locus across strains, we produced *de novo* assemblies of 7 additional *C. cinerea* samples from Illumina short read data. These assemblies were significantly less contiguous than the reference Okoyama-7 assembly, although the total genome assembly length was comparable (Supplementary Table 1). The corresponding region of each assembly to the *somA* locus was extracted using CC1G_06671 and CC1G_06677 as flanking genes. Alignment of these regions showed that while the flanking genes were highly similar, the core NACHT and Kinase genes were diverse (Figure 6). Within this locus, each strain had at least one kinase and one NACHT encoding gene, although some strains had two pairs, and some had three pairs. Across all strains, each kinase and NACHT partner was found in head-to-head orientation, regardless of the number of pairs. Between the Java6 and Okoyama-7 parents of the cross, the LRR gene had more synonymous variants (20) compared to missense (6), and this was seen in the multiple alignment as well. (Figure 5). The amino acid sequence similarity was high, compared to the NACHT/kinase genes. The kinase genes across all strains were similar enough to cluster together using BLAST searches (Figure 6, red colour), as well as the NACHT genes (Figure 6 orange colour).

Analysis of syntenic regions for the locus on Chromosome 8 that also contained a LRR/Kinase/NACHT triplet, also each expressed on alternate DNA strands, but of the sampled isolates was only recovered in Okoyama-7 and AmutBmut. In the other isolates, the same flanking genes were identified, but without the central 3 (Figure 7).

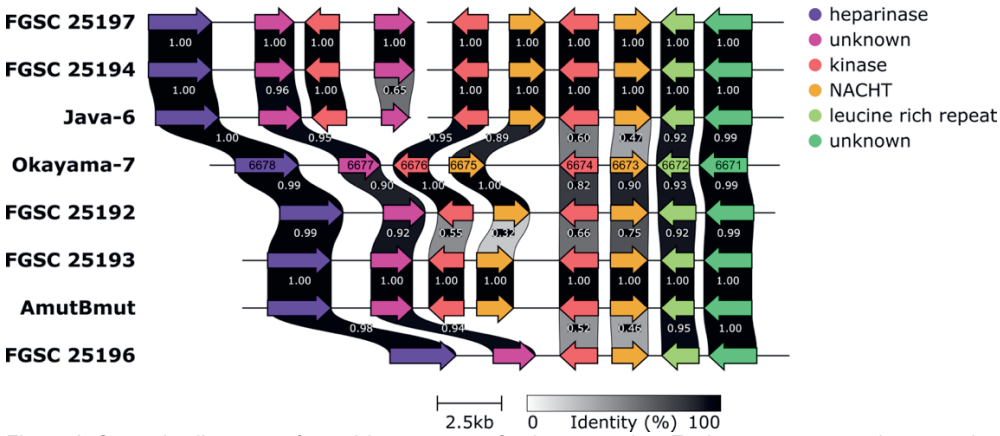

Figure 6: Syntenic alignment of *somA* locus across *C. cinerea* strains. Each row represents the syntenic region of a particular strain, while arrows and direction indicate coding sequence orientation. Genes are colored according to similarity groups. Shaded regions connecting strains indicate amino acid sequence similarity, with amino acid similarity indicated in the text box. For Okoyama-7, the 4 number code for the corresponding locus is given (i.e. 6675 = CC1G_06675). Note that for strains Java6, 25197 and 25194, the region is split across two contigs, indicated by the break in genomic region.

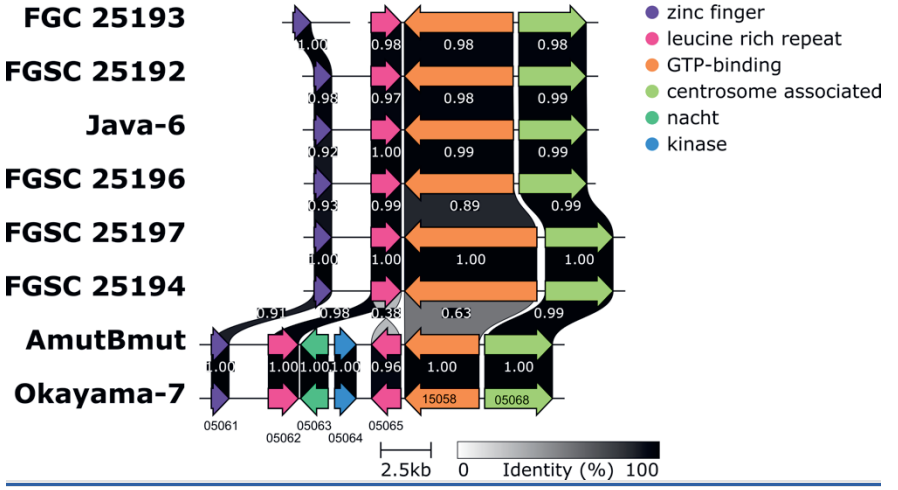

Figure 7: Synteny across *C. cinerea* strains across the *somB* locus on Chromosome 8 with similar protein organization as the *somA* locus. Genes and shading as in Figure 6.

Discussion:

The genetic basis of basidiomycete nonself recognition has eluded identification for several decades, while in this same timespan several ascomycete species have yielded many of their secrets. Presumably at least a partial cause for this is the difficulty of a tetrapolar mating system, which complicates crossing schemes, as well as the added complication of monokaryotic and dikaryotic lifecycles. Our results here show that nonself recognition in basidiomycetes likely shares many biochemical features with SI in other fungal groups, but with some key differences.

This study assumes that SI occurs between dikaryons, and so we dikaryotized all offspring with a common partner. Although the influence of this common nucleus is unclear, fixing this to a single genotype simplifies the genetics of this system, as it reduces the variation segregating in the crosses. Our finding that 12% of the F1 offspring are compatible with a parent is consistent with 3-4 loci segregating between the parents, similar to reports in other mushroom-forming basidiomycetes. In fact, all described species except *Serpula lacrymans* appear to have this range of loci, and the lower number of *S. lacrymans* is likely related to a recent population bottleneck in that species (Kauserud et al., 2004, 2006). This is much lower than the ~10-loci generally found in ascomycete species (Nauta & Hoekstra, 1994). One explanation may be that in basidiomycetes so far only a few biparental populations have been used for the species studied, and so are limited regarding the variation between the parents and perhaps by the interaction in the common nucleus. Conversely, in ascomycetes large crossing schemes have been devised to identify all segregating loci. However, this reduced number of loci could also reflect that allelic diversity for basidiomycete nonself recognition loci may be much higher. For ascomycetes typically there are two, occasionally three, alleles for each locus while here we find four alleles from only eight monokaryotic strains sequenced although we have not tested phenotypic variation. If basidiomycetes do generally have a higher number of alleles, it would reduce the number of loci necessary to maintain a robust nonself recognition system (Nauta & Hoekstra, 1994). Sequencing a wider population would not only provide valuable information regarding the allelic diversity, but potentially identify additional loci that were homozygous between Okayama-7 and Java-6 and so did not segregate in our crosses. An additional explanation for why only 3-4 loci could be sufficient may be related to the dikaryotic stage itself, which has two genomes that contribute allelic diversity to the organism. Currently, it is unclear if these alleles act in a dominant/recessive or a co-dominant fashion. If the effect of the alleles is co-dominant, then the heterozygosity in a dikaryon at these loci may provide additional diversity. We have speculated on this previously (Auxier et al., 2021), but data on this is currently lacking. Further experiments on the interactions between SI alleles within and between dikaryons will be needed to understand the phenomenon.

Although our segregation ratios indicated approximately 3-4 loci involved in SI, we recovered the same single QTL both in the F1s as well as the backcrossed

lines. We did recover a second locus located on chromosome 11, but since this was not found in the F1 compatibility testing, it is unclear if this represents a false positive. A likely reason for consistent recovery of a single locus is that *somA* represents the strongest phenotype, and therefore the bulking of F1 offspring into compatible or incompatible may have largely been effectively sorting based on this locus. This would also affect the backcrosses, as they were performed by selecting on a strong incompatibility phenotype, which would be subject to the same bias as for the F1 experiment. This is also observed in other species, like *Phellinus* (previously *Piptoporus*), where loci have different strengths of phenotypes (Adams et al., 1981; Rizzo et al., 1995). To avoid this, future work could use markers targeting the *somA* locus to isolate incompatible lines with the same alleles for *somA*, thus revealing additional loci. However, an alternative may be that a second incompatibility locus resides in one of the reciprocal translocations found between Okoyama-7 and Java-6. Many offspring would be heterozygous for these regions, and these would complicate QTL mapping. Reciprocal translocations between these two parents have been known previously (Zolan et al., 1994), and future work should avoid such parents. As the parental isolates for this work came from populations likely geographically separated, between Japan and Indonesia, similar crosses using parents from the same interbreeding population may be a useful selection step to avoid such genetic complications.

An important consideration to the results presented here, although it does not affect the genetic mapping, is the use of a common nucleus. In all phenotyping reported here, strains were paired with FGSC#25194 to form dikaryons for SI visualization. Worrall termed this method a “common nucleus”, although this strategy had already been in use (Hansen et al., 1993; Worrall, 1997). This method allows for genetic simplification of SI, since the common nucleus is fixed in all dikaryons, and its influence can be controlled for. However, since we kept the common nucleus constant, it should be inferred that this nucleus is not interacting. In fact, the opposite is likely true. As we have previously speculated on, it is possible that these alleles act in a dominant fashion, and experiments utilizing different common nucleus partners would result in differing sets of compatible offspring. Alternatively, if alleles are co-dominant the role of the common partner would be nullified by this approach and any common nucleus would produce similar results (Auxier et al., 2021). Within the cross used here, Java-6 and the common nucleus FGSC 25194 have the same *somA* locus. Thus, the only difference between dikaryons at *somA* is the allele of Okayama-7. Whether this is by chance, or reflects the nature of the molecular interaction within a dikaryon (i.e. the common nucleus having a third allele would prevent genetic identification) remains unclear. Future research in basidiomycete incompatibility would benefit from the use of additional common nuclei.

Our finding of an NLR-like genetic structure of the *somA* locus is consistent with other nonself recognition systems. The concept of NLRs in fungal nonself recognition has been extensively documented (Choi et al., 2012; Heller et al., 2018;

Uehling et al., 2017). As well, the role of NLR-like genes in relation to immunity in both animals and plants is well established, and in fungi is being rapidly acknowledged as relevant. In the well-established models for the action of NLRs, a protein interaction domain, typically composed of repeated elements, like Ankyrin repeats, WD-40 or Leucine Rich Repeats, is thought to repress the action of the central nucleotide binding domain (Uehling et al., 2017). The locus we identify would be another example of NLR involvement in fungal nonself recognition, but with an important distinction. The *somA* locus has the general structure of an NLR-like structure, with a kinase that could function as a signalling molecule, a NACHT domain, combined with a relatively consistent LRR domain. However, the protein domains of *somA* are found on independent proteins. In other NLR systems, it is thought that intramolecular forces within the multidomain NLR protein allow regulation (Heller et al., 2018). Importantly, the head-to-head orientation of the coding sequence of these genes allows to confidently exclude the possibility that it is solely a genome assembly or annotation artifact. Understanding how this NLR-like protein functions in the absence of intramolecular interactions will be an exciting future study topic. If protein-protein interactions can be confirmed, this would also represent the first LRR type NLR found in fungi, as all other NLRs found in fungi use repeat regions like Ankyrins or WD40 domains (Dyrka et al., 2014; Uehling et al., 2017). Previous reports that LRR type NLRs are not found in fungi would have missed such isolated proteins in NLR searches, as such searches begin with searching for proteins with multiple target domains. Additionally, the NACHT proteins we identify here are quite diverged from ascomycete NACHT proteins, complicating searches with current techniques.

The nonself recognition locus we identify here appears to be under strong balancing selection, with several highly divergent alleles found in a population. Particularly the NACHT and kinase genes from different alleles have little sequence similarity at either the nucleotide or protein level. Balancing selection is a common feature of nonself recognition genes, with varying levels of sequence divergence (J. Wu et al., 1998). The relatively high levels of synonymous variants compared to missense variants at the LRR gene indicate it is under purifying selection. The surrounding regions of a locus under balancing selection also experience this selection, but decreasing with distance and recombination (Charlesworth, 2006). Theory predicts such regions should accumulate not only neutral mutations, but also deleterious mutations, although empirical studies are currently equivocal on this second point (Veve et al., 2022).

Conclusion:

We identify the first genetic locus responsible for incompatibility in a basidiomycete fungus, *somA*. This locus can be narrowed genetically to a region containing several genes that share organization with known NLR mechanisms from other nonself recognition systems. While we currently lack causal proof that differences at this locus lead to incompatibility, the genetic association, and similarity

to known mechanisms provides an exciting first step towards understanding how individuality is determined in basidiomycete fungi.

Acknowledgements:

This work was supported by NWO support to BA. We thank Mayke Schelhaas for assistance with fine mapping of the *somA* locus. This work would not have been possible without the resources provided by the Fungal Genetics Stock Centre.

Code and Data Availability

Illumina short read data for all strains and low coverage sequence data are available on public databases following publication. Recombinants offspring are available by contacting authors, and parental strains are available from the Fungal Genetics Stock Centre. All code used for bioinformatic, statistical, and plotting can be found on at GitHub at https://github.com/BenAuxier/Coprinopsis_Somatic.

Supplementary Data:

```
CC1G_06675      MS-------EAAPILPAISVDIPLAKFKKIR-----N--WDPRKEPMPKREPPGLKEKLF 46
CC1G_05063      MAADSNAQRLSHCNLPIFLIPVPKDKIKGPD----------NLNSSHSFKRPGWFQENFL 50
CC1G_06673      --------------------MPKATSDGVDFPTLPTIIQGYIPEEHHADLPSGFMQTWW 39
                                    :*  . .               .     *  : :.

CC1G_06675      DSYFTEFTAFYLHVANLMEMDHRAT-------VAQGVVYDTEDE---GTLKVLFDRIVYL 96
CC1G_05063      DRYWKNFTSHYLHVADGVRSRHMDN-------VAAGRPFAPGTK---DGIDRSLNTIAEL 100
CC1G_06673      DPYYKKYAAYWLYRADVVGARTVALKTNTGRDSQRSHPFQSPRHVDIRAIEKFLTKIADI 99
                * *:.::::.:*: *: :                 .  :    .     :.  :  *. :

CC1G_06675      EGEWRESVKTKDRLHAQGFSREISETVKAYDSRRRASSRKA-YKLLQTKVALRSPGYRIL 155
CC1G_05063      AMEYQSACADKDTVHARAVAQKIQKKASALRVLECLSNMEC-SKLLENKTAQLSPCYETI 159
CC1G_06673      AEKYILDIQRGDKRAAQSRAETIGTEVRQLEVFERQSNRRSRARAIELSFVTNDETYQSI 159
                  ::       *   *:. :. *   .      .  *. ..  : :: . .  .  *. :

CC1G_06675      LKKRRLEASLFYAFHRIQKFNADFNEDVVADFARWMRNVSRQKPVFWLRGHDRCGKTVIT 215
CC1G_05063      VNKRDNRASLLSPLHTIQMCNPKFRKDILMECTTWIAECTRDNCIFWLNGEPGCGKSVVA 219
CC1G_06673      VKRRRVEASLVHAVTTIHDWNIGFRQRIILDFARWLEEDG-QKRILWVYGEPGCGKSVIT 218
                :::*  .***.  .  *:  *  *.: :: : : *: :   :: ::*: *.  ***:*::

CC1G_06675      YYFAQRCKDNAALAASYRFTDNINFEEFQTDFIASVVGEMSSYLGDSFSESLARLTDKLD 275
CC1G_05063      HWFADGCEKRNSLAASYVFTKPTSTAALLDGFVANLATDFAEYLGDPWRNALVSTATNIG 279
CC1G_06673      YWFADHCSQLKSLAASYKFTASCTQRIVQDTFLANLVTDFETYIGSSFTNALADVVGDFR 278
                ::**: *..  :***** **   .   .   *:*.:. ::  *:*. : ::*.  . .:

CC1G_06675      LANPVVEREFLSRPLKDQLNELVVPAFNALPEDEKRPLLVVL<u>**DGIE**</u>RCGDPECWALASLF 335
CC1G_05063      LE--KYADQFFTRPLRHQMGKLLVPTFFVIDPTQQKPLLVVL<u>**DGLD**</u>KCDD---FALKALF 334
CC1G_06673      PQ---DREDFFQRPPKTQLLDLIVPTFNKLPERDKKPLLVVL<u>**DGID**</u>KCDE---WAAKDVF 332
                       :*: ** : *: .*:**:*  :   :::********:::*.:   :*   :*

CC1G_06675      DFITEAMAKLPICFFITSSTSSPKAGKINRFFTETPIAAQVAGREIAY--DPMKTPKRAT 393
CC1G_05063      ELIEEAILHLPICFFISSTETK----VLTAYLRQGPLSKHVQECHIPGPYLNRPGPNRAS 390
CC1G_06673      HLIKVAIEHLPICFFISCHQND----TIDYFLR-RILADHVERCDVPF--KPNPDSKKNK 385
                .:*  *: :*******:.  ..     :  ::    :: :*   .:          :: .

CC1G_06675      FPSNLY--TDD---SDDSDPEWE--------------------------           411
CC1G_05063      TPTPVNINLPASE-------------------------------------           403
CC1G_06673      TPTAANFTIPEHFDAEDMSPPMTPLPDLYPASDDEGTVARLEPFGTNGGP           435
                 *:
```

Supplemental Figure 1: Multiple sequence alignment of the three NACHT protein sequences encoded by Okoyama-7. Alignment produced using CLUSTAL-Omega. Asterisk below columns indicate complete conservation, while colons and periods indicate similar amino acid types. The defining residues surrounding the Walker B motif, mentioned in the main text, are underlined and bolded.

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Chapter 8

Identification of Heterokaryon Incompatibility Genes in *Aspergillus fumigatus* Highlights a Narrow Footprint of Ancient Balancing Selection

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This work is available on bioRxiv at:
https://www.biorxiv.org/content/10.1101/2022.11.25.517501

Supplementary material described in this chapter can be found online with the bioRxiv submission

Abstract:

In fungi, a phenomenon termed heterokaryon incompatibility restricts hyphal fusion to occur within an individual since fusion between individuals leads to cell death. Generally, the genes involved are found to be under balancing selection from negative frequency dependent fitness. Here, we assess this in *Aspergillus fumigatus,* a human pathogenic fungus with an extremely high crossover rate. Using auxotrophic markers we screened sexual progeny for compatibility to identify genes involved in this process, the so-called *het* genes. In total, 5/148 (3.4%) offspring were compatible with a parent and 166/2142 (7.7%) sibling pairs were compatible, consistent with several segregating incompatibility loci. Genetic mapping resulted in five loci, four of which could be fine mapped to individual genes, of which we tested three through heterologous expression, confirming their causal relationship. Surprisingly, a population-level analysis of two available independent datasets did not show an increase in Tajima's D near these loci, normally a hallmark of balancing selection. However, analysis of closely related species did show trans-species polymorphisms across >10 million years, and equal allele frequencies within *A. fumigatus*. Using available *de novo* assemblies, we show that these balanced polymorphisms are restricted to within several hundred base pairs flanking the coding sequence, potentially due to this species' high crossover rate. In addition to identifying the first *het* genes in an *Aspergillus* species, this work highlights the interaction of long-term balancing selection with a high recombination rate. Future mechanistic work on these *het* genes may provide novel routes for clinical therapies, as well as opportunities for strain improvement in biotechnology.

Introduction:

The ability to differentiate self from nonself is a ubiquitous factor of multicellular life. Perhaps best studied in mammalian MHC loci, such processes prevent fusion between genetically distinct organisms, in effect determining what constitutes an individual (Billingham et al. 1953; Boehm 2006). This ability to distinguish self from nonself is also important for fungi. During the growth of a fungal colony, it benefits from fusions within its own mycelial network (Bastiaans et al. 2015). However, fusion between mycelia of different individuals allows for the propagation of selfish genetic elements such as viruses (Anagnostakis 1983), plasmids and mitochondria (Debets et al. 1994), and nuclei (Bastiaans et al. 2016; Grum-Grzhimaylo et al. 2021). To allow for a fungus to discriminate between self and nonself, fungi possess a robust multigenic system of heterokaryon incompatibility, based on *het* genes (genes which prevent formation of heterokaryons), which trigger cell death when gene products from two alleles are found in the same cytoplasm (Glass and Dementhon 2006). Within a fungal population these *het* genes are highly polymorphic and segregation of the various alleles results in each fungal individual being virtually guaranteed to have a distinct mix of *het* gene alleles compared to a different strain (Nauta and Hoekstra 1994; Czárán et al. 2014; Gonçalves and Glass 2020).

A common observation of such *het* genes is that they appear to be under balancing selection. First shown in *het*-c of *Neurospora crassa*, this balancing selection results in alleles being found in even frequencies in the population of this fungus (i.e. two alleles found at a 1:1 ratio, or three alleles at 1:1:1) (Wu et al. 1998). This pattern of even allele frequencies appears to be quite general, and has subsequently been found in other fungal species (Bastiaans et al. 2014; Milgroom et al. 2018; Ament-Velásquez et al. 2022). While it can be difficult to disentangle the cause of balancing selection (Spurgin and Richardson 2010), the generally accepted cause for nonself recognition genes such as *het* genes is negative frequency dependent selection (Nauta and Hoekstra 1994; Muirhead et al. 2002). This relationship between fitness and allele frequency emerges as a result of *het*-gene action being dependent on the alternate allele. As an allele becomes common in a population, more individuals will share this allele and this allele will be less useful to distinguish other individuals. Conversely, individuals with the alternate allele, which is now rare, will be able to detect other individuals as they will carry the alternate allele. This prevents either allele from reaching fixation, and thus both alleles are maintained longer than would be expected by random processes, with alleles often shared between related species in trans-species polymorphisms (Charlesworth 2006). Such patterns have been found in the genera *Neurospora, Cryphonectria,* and *Podospora* (Wu et al. 1998; Milgroom et al. 2018; Ament-Velásquez et al. 2022).

Through various cell mechanisms, fusion between two fungal individuals, generally differing at multiple *het* loci, leads to the death of the nascent fused cell (Garnjobst and Wilson 1956; Glass and Kaneko 2003). Some known *het* genes

trigger death due to protein-protein interaction encoded by different alleles of the same genes (allelic), while other *het* gene reactions are based on protein products of alleles of separate tightly-linked genes (non-allelic) within a larger haplotype (Kaneko et al. 2006). The biochemical pathway leading to death of this cell fusion differs depending on the *het* gene involved. Some *het* genes utilize systems based on Nod Like Receptors (NLR-like), with strong parallels to the immune system of animals and plants (Uehling et al. 2017; Heller et al. 2018). The action of *het* genes can also be based on prion formation of certain proteins, converting the alternate protein product into a cytotoxic protein that destabilizes the plasma membrane (Debets et al. 2012; Saupe and Daskalov 2012). For many *het* genes, the mode of action remains unknown. In the *Sordariomycete* genera *Neurospora* and *Podospora* many *het* genes contain a protein domain of unknown function, termed the HET domain (Saupe and Glass 1997; Zhao et al. 2015). While this domain is found in other fungi, the relationship between the HET domain and functional *het* genes in other fungal lineages remains uncertain (Fedorova et al. 2008; Dyrka et al. 2014; van der Nest et al. 2014).

The fungus *Aspergillus fumigatus* is globally dispersed and primarily decays dead plant material. Its spores are inhaled daily by birds and mammals, generally presenting no concern to the health of the organism. However, when the immune system is suppressed, such as during chemotherapy, immune suppressive therapy following organ transplantation or co-infection with influenza or SARS-cov2 in intensive care units this fungus can invasively grow into the lung tissue, leading to a condition called invasive aspergillosis (Schwartz et al. 2020; Arastehfar et al. 2021; Arné et al. 2021; Chong and Neu 2021). *A. fumigatus* can also chronically colonize human lungs in Cystic Fibrosis (CF) or Chronic Obstructive Pulmonary Disease (COPD) patients, and the same clonal isolate can be recovered from a patient sequentially for years (Bhargava et al. 1989; de Valk et al. 2009). First-choice treatment for severe *Aspergillus* disease is the azole antifungal class targeting the fungal ergosterol pathways. Increasingly, antifungal resistance is found due to environmental exposure of the isolates prior to infection of a human host (Verweij et al. 2009). As a potential alternative to traditional drug targets, the manipulation of cell death processes has been proposed to manage fungal infections (Weaver 2013; Hardwick 2018; Kulkarni et al. 2019). The first step for such research would be the identification of the genes that trigger heterokaryon incompatibility, the *het* genes, in *A. fumigatus.*

The genetics of *het* genes in other *Aspergilli* has been investigated previously, although not at a molecular level. Experimental studies in *A. nidulans* and *A. heterothallicus* both showed that incompatibility segregated in the progeny of sexual crosses, and was due to the action of several loci, although this was before the genomic era and specific genes were not identified (Kwon and Raper 1967; Anwar et al. 1993; Dales et al. 1993; Coenen et al. 1994). Using bioinformatic tools, sequence divergence combined with gene function information has been used as

criteria to identify putative *het* loci in *A. fumigatus* (Fedorova et al. 2008). These diverged regions were termed “islands” of diversity in the *A. fumigatus* genome, with a highly divergent region flanked by large stretches of almost identical sequence. More recently, a reverse genetics study of genes encoding a HET domain in *A. oryzae* showed that expression of alternate alleles of most genes with a HET domain did not lead to an incompatible reaction, although some led to a modest reduction in growth (Mori et al. 2019). Within *A. fumigatus* it has been shown that environmental strains are not capable of forming stable heterokaryons, indicating abundant heterokaryon incompatibility genes (Weaver 2013; Zhang et al. 2019). Currently, validated *het* genes remain unidentified both in *A. fumigatus* as well as the genus *Aspergillus* in general.

Here, we combine whole-genome sequence data of sexual progeny from two heterokaryon incompatible environmental strains with phenotypic interactions to map the (in)ability to complement two auxotrophic mutations and form stable heterokaryons. Our presumption is that the inability to complement is related to *het* gene allelic differences. We then investigate the putative function and evolutionary history of these loci across related species and finally, we consider how the exceptional recombination rate of *A. fumigatus* may affect the process of balancing selection.

Results:

Heterokaryon compatibility testing between offspring and parental strains

To map the genes causing incompatibility between our parental strains, we used heterokaryon complementation of auxotrophic nitrate pathway mutations. In this method, two haploid strains with different nitrate-deficiency mutations (e.g. one *nir* and the other *cnx*) will show vigorous heterokaryotic growth on nitrate as a sole N-source when compatible, but not when incompatible, due to sustained cell fusion. We produced from each of the two parents (AfIR974 and AfIR964) a *nir*, *cnx* and *nia* mutant, from which we crossed the AfIR974 *cnx* and the AfIR964 *nir* mutants. Among the 193 offspring, the distribution of genotypes (42 *nir/*wt; 51 *cnx/*wt; 55 *nir*/*cnx;* 45 wt/wt) was not significantly different from the expected 1:1:1:1 Mendelian ratio for two segregating loci (χ^2 test, p=0.56)

In total 148 offspring were of a genotypic class that allowed heterokaryon compatibility testing to *nia* mutant versions of the parents (42 *nir/*wt*,* 51 *cnx/*wt *and* 55 *nir/cnx*). From these, we recovered five successful combinations forming a heterokaryon (3.4%) after three days of incubation. Three offspring (#118, 189, 108) could form heterokaryons with the parent AfIR964 *nia*, and two offspring (#166, 142) were compatible with the parental AfIR974 *nia*. Offspring that were compatible with a parent were only compatible with one parent. To increase the number of compatible pairs recovered, we tested each of the 42 *nir* strains against each of the 51 *cnx* strains of the offspring, resulting in testing of 2142 sibling pairings. Of these, 166 pairs (7.2%) formed heterokaryons. Of these 166 pairs, visible heterokaryons developed within three days for 133 pairs, whereas for 33 pairs, heterokaryons were visible only after five days.

Genetic Mapping

From the 166 heterokaryon compatible sibling pairings and the 5 compatible parental-offspring pairs, we could form 15 vegetative compatibility groups (VCGs) (Supplemental File 2). Using the assumption that VCGs will have identical alleles at all *het* genes, we compared the genome sequence of the strains in each VCG at 14,153 variant sites previously identified (Auxier et al. 2022). As genotypes are expected to be similar within, but not between, groups we used the Shannon's Entropy metric to determine similarity (see Methods). Mapping using the 133 isolates forming heterokaryons after three days resulted in peaks on chromosomes 2, 5, 6 and 8, which we termed *hetA, hetB, hetC and hetD,* respectively (Figure 1A). Each of these peaks rose above the significance threshold of 0.567, the highest 5% of the permutations. For each of these peaks, the window of significant markers spanned approximately 40kb with up to 20 predicted protein coding genes (Figure 1B-E). Within the larger VCGs, we observed a polymorphism where some pairings formed heterokaryons within three days, while other pairings formed heterokaryons after only five days (Supp.offspring.genotypes.xlsx; Pairwise Interactions tab). To investigate the genetic basis of this, we split the VCGs into sub-VCGs and performed

a similar Shannon Entropy analysis (Supplemental Figure 1). This analysis recovered a single region on Chromosome 6, which we termed *hetE*.

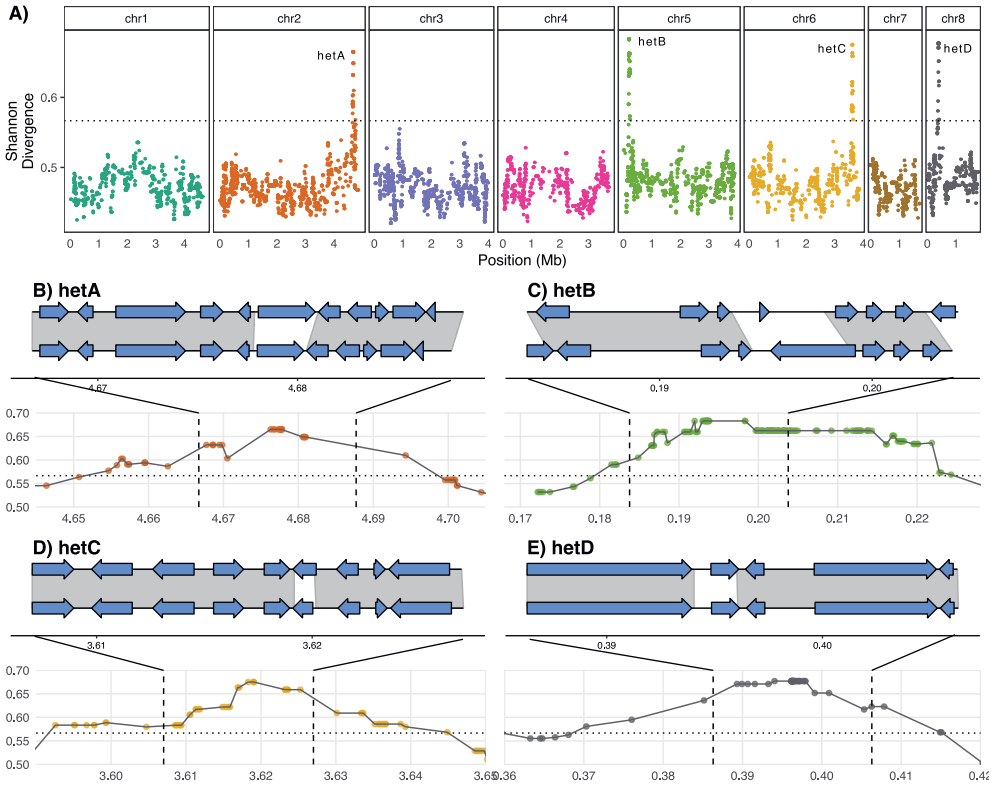


Figure 1: Mapping of *A. fumigatus het* loci based on heterokaryon-forming pairs. A) Genome wide mapping of heterokaryon compatibility after three days of incubation. Dots indicate variant positions, and associated Shannon Entropy phenotypic association. Significance threshold is determined by the top scoring 5% of 1000 random phenotypic permutations. B-E) Fine mapping of *hetA/B/C/D* loci. The lower part of each figure shows a closeup of the locus in A) that rises above the significance threshold. Dashed vertical lines indicate the variable genomic region with gene models shown with AfIR964 above AfIR974. Arrows indicate predicted gene models, and grey shading indicate regions with high sequence similarity between the two parental genomes. In these four loci, there is a region in the middle of the locus, with one associated gene, that is highly differentiated between parents, and lacks grey shading.

For each of the four *hetA/B/C/D* loci, the sequence similarity was high across the locus between the parents except near the centre of the locus with a single coding region with low sequence similarity (grey shading Figure 1B-E). For *hetA/C/D* this central divergent gene had two similarly sized alleles. For *hetB* we instead found that the two alleles had greatly differing coding sequence lengths, and in opposite directions. The *hetE* region was larger than the others, spanning approximately 80kb, with ~40 predicted protein coding genes. The *hetE* locus contained three coding sequences in the AfIR974 allele, and four coding sequences in the AfIR964 allele, all of which had low sequence similarity between the parents (Supplemental Figure 1).

To verify that the five loci identified represented all the *het* loci segregating between these two parents, we backcrossed offspring predicted to differ at only one identified *het* locus from a parent. We then scored 40 offspring from each of nine such crosses, two independent crosses for *hetA/B/C/D* and one for *hetE*, for compatibility to the parental strain. In all crosses compatibility to the parent segregated as expected and did not significantly deviate from a 1:1 ratio (Supplemental Table 1).

***het* Gene Identification and Validation**

The predicted protein products of the *hetA* alleles both contained a nucleotide phosphorylase PNP_UDP, as well as a predicted nucleotide binding NB-ARC domain for the AfIR964 allele (Figure 2A). The *hetB* gene has two idiomorphic alleles, with the AfIR964 allele predicted to encode a 1327 amino acid protein that was predicted to contain a CHAT (Caspase HetF Associated with Tprs) domain, while the alternate *hetB* allele from AfIR974 was 149 amino acids long and had no predicted domains (Figure 2B). The alleles of the *hetC* gene were both predicted to produce a patatin-like protein of similar length (Figure 2C). The *hetD* alleles were also both predicted to encode similar sized proteins, both with a single PNP_UDP phosphorylase domain (Figure 2D). In contrast to the other *het* genes, the *hetE* locus contained multiple candidate genes all of which were polymorphic between parents (Figure 2E). Of these, one gene lacked identifiable domains, while another was the ortholog of a *A. nidulans* characterized gene *rosA* (See Discussion) and one was an uncharacterized C6 transcription factor (Figure 2E). The final candidate gene in the *hetE* locus contained a NACHT domain as well as Ankyrin-repeats and was found in the AfIR964 allele and not present in the AfIR974 allele. Comparison with Af293 showed an alternate allele of the NACHT/Ankyrin gene with low sequence similarity (Figure 2E).

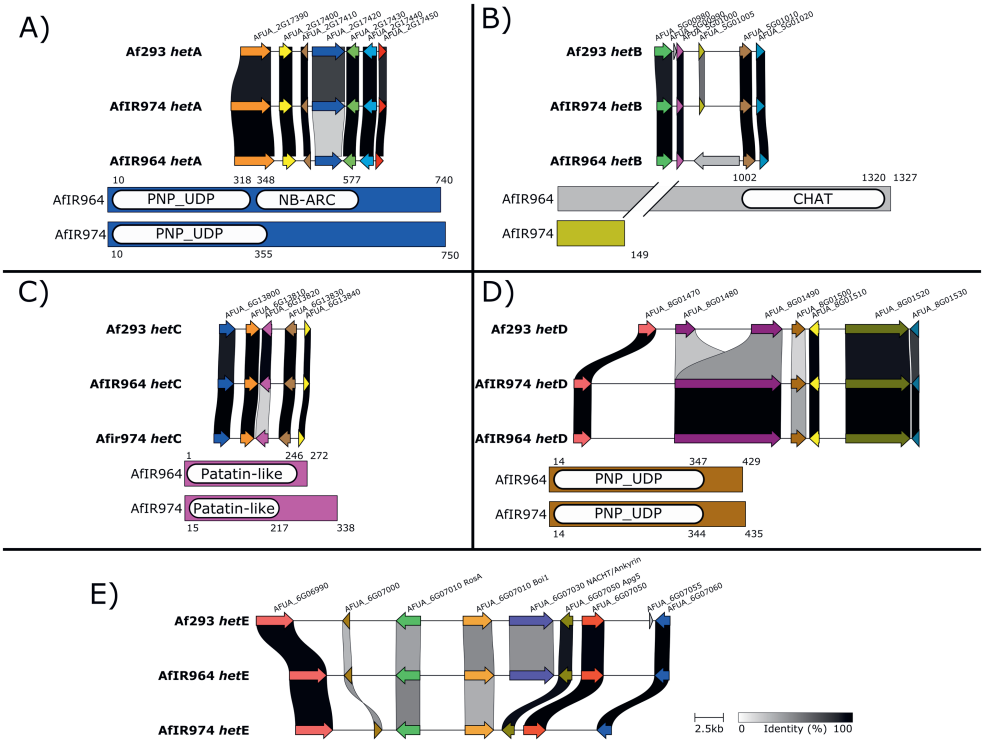
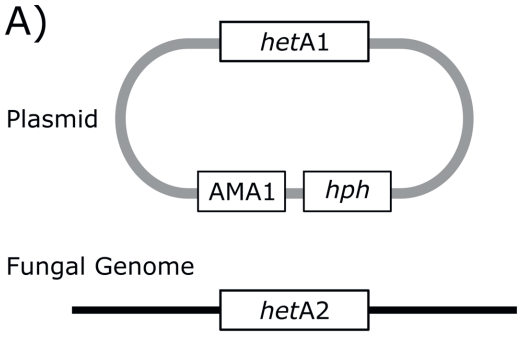
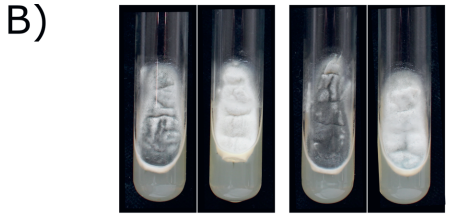


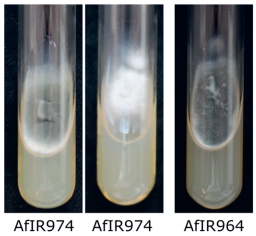
Figure 2: Genomic details of *het* genes. A-E) Top shows syntenic region of *het* gene with adjacent genes for AfIR974, AfIR964 and the reference strain Af293. Colours indicate gene identity, and links between genes indicate amino acid sequence similarity (legend in E). Below shows the protein sequence for each allele, with domains as predicted by InterPro. Colours of proteins reflect synteny analysis above. Note that in panel E) it was noticed at a late stage that Boi1 is incorrectly labelled and should instead be associated with gene ID AFUA_6G07020.

We confirmed that the candidate *het* genes were causal to the phenotype using heterologous expression. Using a nuclear-localized AMA1 plasmid we cloned each allele including ~500bp of flanking sequence for *hetA*, *hetB* and *hetC* (Figure 3A). We did not attempt validation of either *hetD* or any of the genes in the *hetE* locus. For each of the three loci tested, expression of the resident allele on the replicating plasmid led to no change in phenotype. However, introduction of the alternate allele led to an aberrant phenotype of abundant white mycelium and a complete absence of sporulation (Figure 3B-D). For both *hetA* and *hetC* both alleles had equal effect, with the same white mycelial phenotype. For *hetB*, expression of the *hetB2* allele in AfIR974 produced this same white non-sporulating phenotype, but the expression of *hetB1* in AfIR964 resulted in initial non-sporulating colonies that were not viable after transfer on selective media. Transfer of colonies to media without selection pressure allowed rapid sectoring, restoring the wild-type green sporulation, further indicating the phenotype resulted from the presence of the plasmid with the antagonistic *het* allele and not the transformation procedure (data not shown).

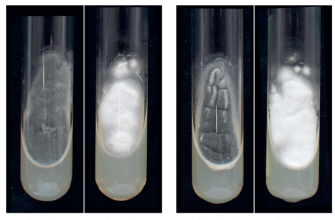




Strain	AfIR974	AfIR974	AfIR964	AfIR964
Endogenous Allele	hetA1	hetA1	hetA2	hetA2
Introduced Allele	hetA1	hetA2	hetA2	hetA1
Sporulation	+	-	+	-



Strain	AfIR974	AfIR974	AfIR964	AfIR964
Endogenous Allele	hetB1	hetB1	hetB2	hetB2
Introduced Allele	hetB1	hetB2	hetB2	hetB1
Sporulation	+	-	+	X



Strain	AfIR974	AfIR974	AfIR964	AfIR964
Endogenous Allele	hetC1	hetC1	hetC2	hetC2
Introduced Allele	hetC1	hetC2	hetC2	hetC1
Sporulation	+	-	+	-

Figure 3: Validation of predicted *A. fumigatus het* genes using heterologous expression. A) Schematic of introducing an alternate *het* allele by transformation with the non-integrative AMA1 plasmid. B) Phenotypes of *hetA* transformants growing on hygromycin selective media, with transformants with endogenous alleles showing wildtype green sporulation, and transformants with the alternate allele with visible white mycelia due to lack of sporulation. C) Phenotypes as in B, but with transformants for *hetB* alleles. Note that AfIR964 with *hetB1* was not viable. D) Similar to B, but with transformants for *hetC* alleles.

Balancing Selection

As heterokaryon incompatibility genes are expected to be under balancing selection, we assessed the strength of this selection across *A. fumigatus* in two datasets. Using genome-wide variants of 213 UK samples, as well as 178 German samples, we calculated Tajima's D values across populations in sliding windows of 10 kb (Supplemental Figure 2). These two independent datasets had similar average Tajima's D values (UK; average D = 0.05, Germany; average D = -0.31). Using window sizes of 10kb, we did not find increased Tajima's D associated with any of our identified *het* genes nor with the mating-type locus (Supplemental Figure 2 A + B). *hetB* was the closest gene to a high Tajima's D value, on chromosome 5, although this high D value is near the start of the chromosome, and careful inspection of the window surrounding *hetB* shows a D value below 2 for both population datasets.

As an alternate measure of balancing selection on these *het* genes, we reconstructed the phylogenetic relationships between amino acid sequences of *A.*

fumigatus as well as the closely related *A. fischeri, A. lentulus* and *A. udagawae* for which multiple genome assemblies were available (Figure 4). For both *hetA* and *hetC*, the topology showed a trans-species polymorphism of two alleles, with each allele being found in all four species. The phylogeny of *hetD* showed a similar pattern of trans-species polymorphism, but instead with three alleles (Figure 4D). Of these three alleles for *hetD,* the D2 allele was present in all four species, but *hetD1* was not recovered in *A. udagawae*, while *hetD3* was not recovered from *A. fischeri*.

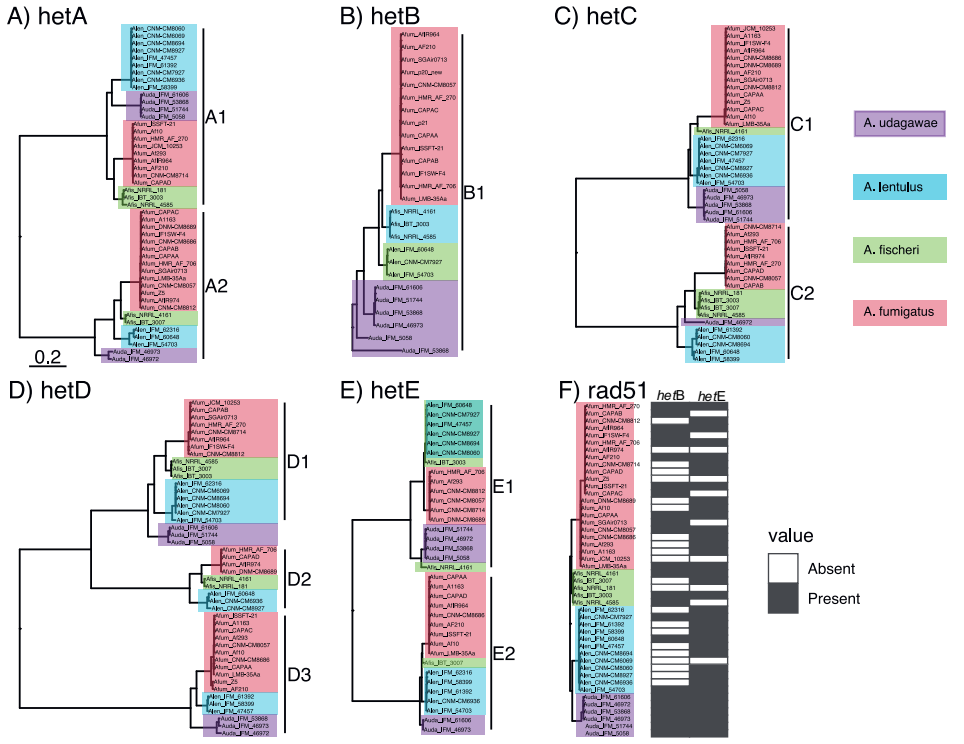


Figure 4: Phylogeny of predicted *het* protein sequences shows trans-species polymorphisms. Maximum likelihood unrooted phylogeny of amino acid sequence of related *Aspergilli*. Branch lengths indicate amino acid change, with tips coloured according to species. Salmon indicates *A. fumigatus*, green *A. fischeri*, light blue *A. lentulus*, and purple *A. udagawae*. Note that B) and E) only include the alleles with a protein present, and the absence alleles are indicated in F).

For *hetB* and *hetE*, which showed a presence/absence polymorphism between our parents (Figure 3), we reconstructed the phylogenetic relationships of the *het* alleles with the predicted coding sequence (Figure 4B and 4E) as well as recorded the status as presence/absence (Figure 4F). The *hetB* protease allele was present in approximately 50% (22/46) of the genome assemblies, and notably in all six *A. udagawae* assemblies. For *hetE*, the NACHT+Ankyrin repeat protein coding sequence was found in approximately 75% of genome (36/46), and the phylogeny showed two distinct protein coding alleles, meaning three alleles including the null allele, with a trans-species polymorphism. The absence allele was found in *A.*

fumigatus, *fischeri* and *lentulus*. As a control analysis, alleles of the DNA repair gene *rad51* were separated between the species, with the alleles from each species being monophyletic (Figure 4F).

Fine-scale population genetics of balancing selection in *A. fumigatus*

To attempt to reconcile the lack of population-level balancing selection with evidence for trans-species polymorphisms, we made use of public *de novo* genome assemblies from 300 previously sequenced individuals of *A. fumigatus*. Extracting the coding sequence and 5000 bp up- and downstream sequence allowed us to compare divergence between alleles of *hetA/B/C/D* as well as the *mat* locus. The Tajima's D value was highest across the coding sequence, reaching values of >6 (Figure 5, top row). However, the drop-off to a neutral D value was abrupt, with values dropping below 2 within 500bp of the start/stop codon. This abrupt and rapid change was mostly mirrored in the nucleotide diversity within and between the allele classes (Figure 5, bottom row). An exception was found in the *mat* locus, where the nucleotide diversity was somewhat lower across the left-hand side of the coding region. Close inspection of the alignments revealed that this was due to a sharing of the terminal ~300bp of the α-box protein.

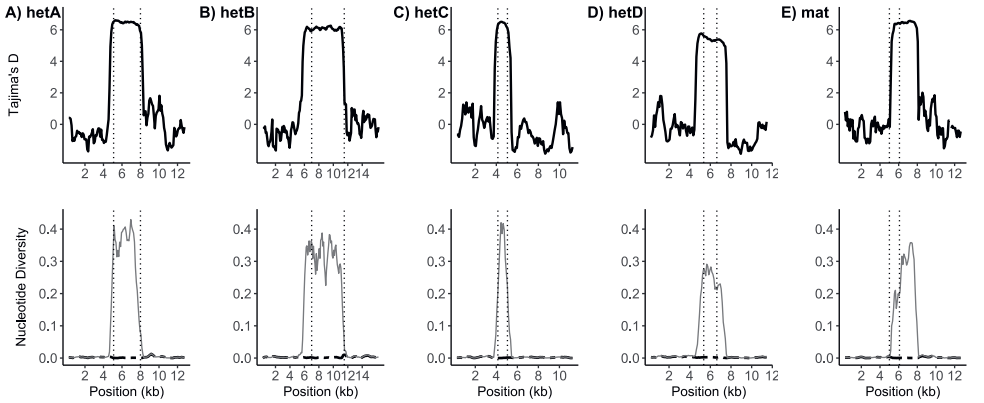


Figure 5: Signatures of balancing selection in *A. fumigatus* are highly restricted. Above is plotted the Tajima's D value for the region of each of the *hetA/B/C/D* as well as the *mat* locus and 5kb of flanking sequence. Dashed vertical lines indicate the start and stop codon of the respective gene, based on the Af293 annotation. The nucleotide diversity is plotted below, with thin grey lines indicating the difference between alleles, and the thick black dashed line indicated nucleotide diversity within an allele.

Discussion:

It is assumed that fusion between conspecific individuals is a risky behaviour, and so it is rigorously policed in fungi. As such, heterokaryon incompatibility is expected to be common between strains of *A. fumigatus*, and has been reported previously (Weaver 2013; Zhang et al. 2019). Our results here reveal the polygenic nature of this heterokaryon incompatibility. For our two parents, both isolated in 2005 in Dublin, Ireland, five loci contribute to incompatibility. Using a single cross and

approximately 150 offspring, we were able to map all responsible loci with high resolution, highlighting the power of genetic mapping in *A. fumigatus* with its high recombination rate (Auxier et al. 2022). These identified *het* genes are to our knowledge the first identified in any *Aspergillus* species, and the first for any member of the class *Eurotiomycetes*.

Two of these *het* genes, *hetC* and *hetD* are consistent with effector domains commonly found in NLR genes, but without the other components (Uehling et al. 2017). The alleles of *hetD* encode similarly sized PNP-UDP domains, while the alleles of *hetC* both encode a patatin-like domain. These patatin domains are also found in the patatin-like protein (PLP-1), which interacts with the SNARE domains of the nearby *sec*-9 gene in *Neurospora crassa* (Heller et al. 2018). However, the action of the gene products of *hetC* and *hetD* seems to occur in isolation, since there are no neighbouring genes with divergent alleles. It is interesting to note that in *N. crassa* there is another *plp* gene, *plp*-2, which also shows evidence of balancing selection, but non-allelic differences between *plp*-2 and *sec*-9 did not trigger cell death (Heller et al. 2018). As differences at a patatin-like protein appear sufficient to trigger heterokaryon incompatibility in *A. fumigatus*, perhaps there are also allelic interactions in *N. crassa plp*-2 that do not require a partner gene to trigger nonself recognition.

Alleles from two of the *het* genes we identify*, hetA and hetE*, have a more traditional NLR-like structure, common to fungal *het* genes as well as immune-system genes in plants and animals (Uehling et al. 2017). Differences at *hetA* completely block heterokaryon formation, while heteroallelism at *hetE* delays, but does not prevent, heterokaryon formation. This is strikingly similar to the “partial” *het* genes previously identified in *A. nidulans* (Coenen et al. 1994). There multiple alleles were identified that caused delayed heterokaryon compatibility, however differences at multiple of these “partial” *het* genes was sufficient to fully block heterokaryon formation (Coenen et al. 1994). It would be interesting to test the interaction between alternate alleles of *hetE* both encoding NLR proteins, and whether this interaction would also only delay or completely block heterokaryon formation. Across *A. fumigatus* and related species, we recovered two different NLR-encoding alleles for *hetE,* and one allele without a coding sequence. This situation, with two alternate protein coding alleles and one null allele, is similar to the ABO blood group system in primates where even the null allele shows trans-species polymorphism (Ségurel et al. 2012). However, it is possible that the likely candidate gene, producing the NACHT+Ankyrin repeat protein, is not acting alone. Adjacent to this gene is AFUA_6G07020, an ortholog of *boi1*. This gene is known from yeast to function in vesicle fusion during exocytosis (Kustermann et al. 2017: 1; Masgrau et al. 2017: 1). Interestingly, a similar pairing has recently been described in the *het* genes of the plant pathogen *Botrytis cinerea*: *Bcvic1*, a NACHT+Ankyrin encoding gene, and *Bcvic2,* which encodes a SNARE syntaxin protein (Arshed et al. 2022). These SNARE domains are also known from the *sec*-9/SNARE system in *N. crassa,* and

hetQ of *Podospora anserina,* which both involve an NLR type protein and a SNARE protein (Heller et al. 2018). It may be that *hetE* of *A. fumigatus* has an interaction with neighbouring *boi1* in an analogous role. Notably, the *sec*-9/*plp*-1 interaction in *N. crassa* has a strong phenotype at the germling stage, but not in developed mycelia (Heller et al. 2018). This may point to a stronger phenotype for *het*E here, if tested during germling fusion. Recently, methods for visualizing germling fusion have been developed which may assist in further studies (Macdonald et al. 2019).

The *hetB* locus in *A. fumigatus* presents two alleles that share no protein similarity, idiomorphs, with one allele encoding a large protease, and the other allele a short protein without annotated domains. This large protease is predicted to contain a CHAT domain, known to function in mammalian apoptosis, and found in other metacaspases in non-mammalian lineages (Hoffman et al. 1997; Aravind and Koonin 2002; Bouchier-Hayes and Martin 2002). This domain has not previously been identified to be involved in heterokaryon incompatibility in a fungal lineage. The involvement of a CHAT domain raises interesting questions about downstream pathways since fungal cell death is not considered homologous to mammalian apoptosis (Hardwick 2018; Kulkarni et al. 2019). The mechanism behind this protease triggering incompatibility may be related to a recent report of proteolytic cleavage leading to cell death described in the *het*-z system in *Podospora anserina* (Clavé et al. 2022). Verification of the biochemical target of the protease of *hetB*, whether it is produced by the small coding sequence of the alternate allele, is an important next step.

A conspicuous absence in our data is an association of the mating-type locus and heterokaryon incompatibility. In an obligately outcrossing species, the mating-type locus experiences balancing selection due to frequency-dependent selection. As an increased number of loci under balancing selection across the genome comes with a cost of reduced genetic diversity at other locations (Wittmann et al. 2022), it may seem logical that such a locus would also be involved in heterokaryon incompatibility, which has been previously suggested (Crozier 1986). This situation is found in many fungal species such as *N. crassa*, where the mating locus also produce heterokaryon incompatibility (Newmeyer et al. 1973). Even within *Aspergilli* the mating locus in outcrossing *A. heterothallicus* was genetically very tightly linked with *het* gene activity, although the molecular genetics are unknown (Kwon and Raper 1967). However, the absence of mating-type associated heterokaryon incompatibility has also been observed, for example in *Cryphonectria parasitica* (McGuire et al. 2004). A by-product of this absence of mating-type associated incompatibility in *A. fumigatus* may be the formation of a sexually fertile diploid heterozygous for the mating type. This has been recently suggested in the diploid formation model of *A. latus,* although this model did not incorporate the difficulties imposed by heterokaryon incompatibility between wild isolates (Steenwyk et al. 2020). While diploids have been previously observed in clinical isolates of *A. fumigatus*, these diploids were almost completely homozygous having arisen by

genome duplication, avoiding incompatibility (Engel et al. 2020). Regarding the mating locus itself, population level analysis showed that the effects of balancing selection was not as sharp as expected. This is due to a partial sequence of the transcription factor of the MAT-2 allele being also found in the MAT-1 allele, as reported previously (Paoletti et al. 2005).

Of the loci described here, only for *hetE* are non-allelic interactions, the interactions between alleles of linked genes instead of homologs, probable. In other species studied in detail, such non-allelic interactions seem common: *het*-R/V and *het*-C/D/E in *Podospora* (Saupe et al. 1995; Saupe 2000), the *hetC*/*pinC* and *het*-6 systems in *Neurospora* (Smith et al. 2000; Kaneko et al. 2006), the *Bcvic1/Bcvic2* system in *Botrytis,* and both the *vic2* and *vic6* loci in *Cryphonectria* (Choi et al. 2012). The extremely high recombination rate in *A. fumigatus* (1% of offspring will be non-parental across a distance of 2.5kb) (Auxier et al. 2022) may mean that such non-allelic interaction present a fitness cost. Any offspring where there was a crossover between the two genes in a non-allelic interaction would then contain an incompatible pair of alleles, and would trigger non-self-recognition within its own cytoplasm (Chevanne et al. 2009). However even for *hetE*, which may have non-allelic interactions with *boi1*, there is a large region of high sequence divergence, which is likely restricting recombination, as the meiotic machinery requires stretches of identical sequences during the initial homology search (Li et al. 2006). Therefore, the fact that *hetE* sits within a large divergent locus may itself prevent crossovers that would otherwise lead to self-incompatible offspring.

A surprising finding of this study was the lack of population level signatures of balancing selection when calculated using window sizes common to such studies (Heller et al. 2018; Ament-Velásquez et al. 2022). It is well established that the mating locus as well as *het* genes are under negative frequency dependent selection, leading to balanced polymorphisms. Our finding of extensive trans-species polymorphism confirmed that balancing selection was acting as expected for these alleles. However, we failed to find a signature with standard population genomics analysis. It appears this discrepancy can be reconciled through a closer analysis of genotypes. Genome-wide studies typically use blocks of 10 kb or higher to average across the genomic stochasticity of individual variants. However, this assumes that the signal from balancing selection, like any other selection, will be carried partially to the neighbouring sequence, the “footprint” of selection, due to linkage with the selected locus. In *A. fumigatus*, we see that variation between allelic classes only extends ~200bp up- or downstream of the coding regions. This means that standard windowed analysis for this species is likely to skip over strong signals of selection, whether from balancing selection or otherwise. Compounding this narrow footprint of balancing selection, the separation between these alleles has existed for so long that the sequences have diverged to a point that standard genomic methods fail. Within such regions, typical short-read DNA data will not be able to be mapped to

the syntenic region between isolates. Downstream analysis will then generally remove such variant sites, due to “missing” data.

The *het* genes we identified appear to have been under balancing selection for several millions of years at minimum. We find shared alleles across a group of four related species. However, we were limited in our sampling to species with multiple available genome assemblies, and thus closely related species like *A. oerlinghausenensis* could not be used (Houbraken et al. 2016), and neither more distant species like *A. clavatus*. We would expect that the alleles for these five genes are shared across an even wider number of species, when additional genome assemblies become available. Across the species studied, we found that the allelic diversity was limited to a region only ~250bp outside the coding region of the gene. This is likely related to the high recombination rate in this species, reducing linkage to nearby regions. This observation has been described before as “genomic islands of divergence”, but the cause was unclear at the time (Fedorova et al. 2008). Although there are few genome-wide empirical analyses of negative frequency dependent selection, it seems that generally the window of balancing selection is wider than observed here. A recent analysis of balancing selection on the S gametic self-incompatibility locus showed that increased nucleotide diversity was seen several tens of kilobases from the selected locus (Veve et al. 2022). Recent analysis of the fungus *Podospora anserina* showed that signatures of balancing selection could be determined from population level analysis looking at 10kb windows (Ament-Velásquez et al. 2022). However, the general relationship between recombination rate and the size of the footprint is unclear, as the divergent region surrounding the *MLO2b* immunity gene in *Capsella sp.* is also tightly restricted, despite a much lower rate of recombination (Sicard et al. 2011; Koenig et al. 2019). The interaction between the age of balancing selection, recombination rate, and the resulting window of sequence divergence requires future study.

Fungal heterokaryon incompatibility involves a sort of paradox. It is understood to be ancestral at least to Dikaryotic fungi, and present in all extant filamentous species. Yet there are no ubiquitous fungal “*het*” genes. Thus, each species, or set of related species, require independent study to understand the repertoire of fungal *het* genes (Paoletti 2016). The five loci we identify here are unlikely to be the only variable *het* genes in the species, and analysis of additional isolates of this species likely will reveal additional *het* genes. However, detailed understanding of additional nonself recognition responses is still only an initial step in understanding heterokaryon incompatibility. The mechanism by which these allelic variants trigger cell death remains largely unknown and requires further study. Deciphering the programmed cell death pathway in this species may present novel targets for treating human aspergillus infections and may open the way to eliminate incompatibility barriers to parasexual recombination for improvement of industrially important asexual *Aspergilli*.

Materials and Methods:

Media: For testing of auxotrophic mutants and heterokaryon compatibility testing, Minimal Medium (MM) was used. MM consists of 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$. $7H_2O$, 0.5 g KCl, 10 mg of $FeSO_4$, $ZnSO_4$, $MnCl_2$, and $CuSO_4$ and 15 g agar per 1000 mL H_2O (pH 5.8) (Zhang et al. 2019). Alternative nitrogen sources (nitrate, nitrite and hypoxanthine) were added at 5 mM as necessary. MM supplemented with urea (0.3g/L) was permissive for all mutant growth and was used for general culturing of auxotrophic mutants and sexual offspring.

Recessive markers introduction in the parental isolates:

To allow for heterokaryon testing, recessive auxotrophic markers (nitrate non-utilizing mutations *nia*, *nir* and *cnx*) were isolated from spontaneous mutations in spore suspensions of parental isolates AfIR974 and AfIR964. To isolate these spontaneous mutations, spore suspensions (approximately $1x10^6$ spores) were spread on MM medium supplemented with 200 mM of chlorate and 5 mM urea. As chlorate is toxic to wild-type *A. fumigatus* this method selects for strains deficient in nitrogen metabolism. After three days of growth at 37 °C chlorate resistant sectors were isolated, purified and classified as *nia*, *nir* or *cnx* mutants based on differential N-source media tests (Cove 1976).

Recessive markers segregation of sexual offspring:

The sexual cross was made from parental isolates AfIR964*cnx* and AfIR974*nir*, which were co-inoculated on Oatmeal Agar followed by incubation at 30 °C. The genotypes of the offspring were determined for a previous study (Auxier et al. 2022). Offspring were phenotyped for *cnx* and *nir* markers as above.

Heterokaryon compatibility testing:

Heterokaryon compatibility of offspring was tested by co-inoculating conidia (approximately 10^5) of isolates with complementing *nir*, *nia* or *cnx* markers on MM with NO_3 as the sole nitrogen source (Zhang et al. 2019). All possible combinations of single mutant offspring with complementing *nir* and *cnx* markers were tested for compatibility. In addition, all *nir*, *cnx* and *nir/cnx* offspring were tested for compatibility with *nia* mutants of parental isolates. Following co-inoculation, plates were incubated at 37 °C, and heterokaryon formation, vigorous growing yet irregularly shaped colonies, was recorded after three and five days of incubation. Pairings that successfully formed heterokaryons were re-tested in triplicate, with additional testing of spore mixtures on MM+NO_3 to ensure heterokaryotic growth was not due to contamination.

Segregating crosses

Segregation of individual *het* loci was tested by backcrossing offspring that differed from one of the parents at only one *het* locus, (i.e. shared alleles for *hetA*,*C*,*D*,*E* but

not *hetB* for example), and with the opposite mating type. Crosses were performed on Oatmeal Agar as above, and sexual offspring were isolated from cleistothecia following a 1 hour 70 °C heatshock treatment to kill asexual spores. Only *cnx* offspring were used, 40 of which were tested for compatibility against the *nia* version of the parental isolate as above.

het loci association mapping

To map the regions associated with incompatibility, the 166 offspring and 2 parents were split into 15 compatibility groups based on heterokaryon testing. For each of the 14,153 bi-allelic markers in this mapping population identified previously (Auxier et al. 2022), the genetic similarity within a group was tested using the combined Shannon Entropy of the allele frequency within a group. As each group should be defined by genetic similarity within a group for a set of loci, Shannon Entropy allows to test whether the genotypes in a group are informative for a phenotype, without influence of the variation in compatibility group size. As the markers were all biallelic, the formula was:

$$\sum_{hcg1}^{hcg15} \left[P(a1) \times \log\big(P(a1)\big)\right] + \left[P(a2) \times \log(P(a2))\right]$$

Where hcg1 to hcg15 are the different heterokaryon compatibility groups, and P(a1) and P(a2) are the allele frequencies of the two alleles within each group. Thus, the association for a marker is the summed Shannon entropy for the 15 recovered compatibility groups. To compute a null distribution, the genotypes of the compatible groupings of the 166 offspring were randomly distributed into 1000 replicate populations with compatibility group sizes the same as our actual results. Threshold cut-off was determined on the 95 % of the distribution. Fine-mapped regions were visualized with gggenes and clinkr (Gilchrist and Chooi 2021; Hackl and Ankenbrand 2022), and domains of candidate genes annotated with InterProScan (Paysan-Lafosse et al. 2022).

het gene confirmation

To validate our predicted *het* genes as causal to the incompatible phenotype, we expressed the alternate alleles from autonomously replicating nuclear AMA1 plasmids. First, we PCR-amplified the predicted gene from gDNA of either parent using BioVeriFi polymerase (PCR Biosystems Inc, Pensylvannia USA; PB-10.42-05), including the upstream and downstream intergenic space (Supplemental Table 2). To include the native regulatory elements, we included approximately 750 bp of flanking regions, in some cases up to ~50 bp of the adjacent gene. The plasmid backbone was also amplified with BioVeriFi polymerase and ligated with the insert using NEBuilder HiFi DNA Assembly Master Mix (New England Biolab Inc. #E2621L) according to manufacturer directions and transformed in to Mach1 competent *E. coli*

cells (ThermoFisher Scientific Inc.). Plasmid was then extracted from these cells, and ~1 µg was used in PEG-mediated protoplast transformation (van Rhijn et al. 2020). Transformed colonies were selected based on growth on Malt Extract Agar (20 g/L) + 300 µg/mL Hygromycin B (H0192; Duchefa Biochemie, Haarlem, the Netherlands).

Cross-species comparison

To assess the extent of trans-species polymorphisms, we made use of publicly available genome assemblies (Supplemental File 1). We used tblastn v2.8.1 to search the genomes of related *Aspergillus* species using predicted protein alleles from AfIR964, AfIR974, and Af293 (Camacho et al. 2009), using an e-value cutoff of 1e-75. To avoid partial matches based on conserved domain, we filtered results to be >300bp long. The corresponding DNA sequences were extracted, and aligned with mafft v7.427 using the --auto command (Katoh and Standley 2013). Phylogenetic relationships were inferred using iqtree v1.6.1using default settings (Nguyen et al. 2015). The resulting most likely tree was midpoint rooted and visualized with ape and ggtree (Paradis et al. 2004; Yu et al. 2017).

***A. fumigatus* population genome scan**

To assess the effects of balancing selection surrounding genes expected to be under balancing selection, we used data from two resequencing studies (Barber et al. 2021; Rhodes et al. 2022). From the UK dataset of Rhodes et al., we downloaded the VCF file from the publication, while for the Barber et al. dataset we called variants as described previously (Auxier et al. 2022). Tajima's D values were calculated in windows sizes of 10,000 bp using vcftools (Danecek et al. 2011).

***het* gene diversity**

To determine the nucleotide diversity across the *A. fumigatus* species, we made use of genome assemblies from a recent large population resequencing study (Barber et al. 2020; Barber et al. 2021). The genome assemblies of the *A. fumigatus* strains used for the trans-species polymorphism analysis (see above) were added to this dataset, making a total of 334 *A. fumigatus* genome assemblies. For each of *hetA/B/C/D*, as well as the mating type as a positive control, we extracted the corresponding gene from Af293, as well as 5 kb of flanking sequence. Due to the high sequence divergence of the *het* alleles, we used the high sequence similarity of flanking regions to isolate homologous sequences. This sequence was used with BLASTN to search the genomes of each assembly, and the region inside the borders of the flanking sequence was retained. The sequence was extracted using samtools, and aligned using mafft v7.427 using the GINSI algorithm as we expected low similarity in the centre of the alignment (Li et al. 2009; Katoh and Standley 2013).

Acknowledgments:

This work was supported by the Netherlands Research Organization (NWO ALGR.2017.010 to BA; ZonMV 451001022 to ES). We thank Drs. Sandra Lorena Ament-Velásquez and Ivain Martinossi-Allibert for valuable discussion regarding fungal balancing selection. We thank Wendy Adriaens for assistance with the *Aspergillus* trans-species polymorphisms. We particularly thank Ke Luan for assistance with phenotyping of the heterokaryon compatibility pairings.

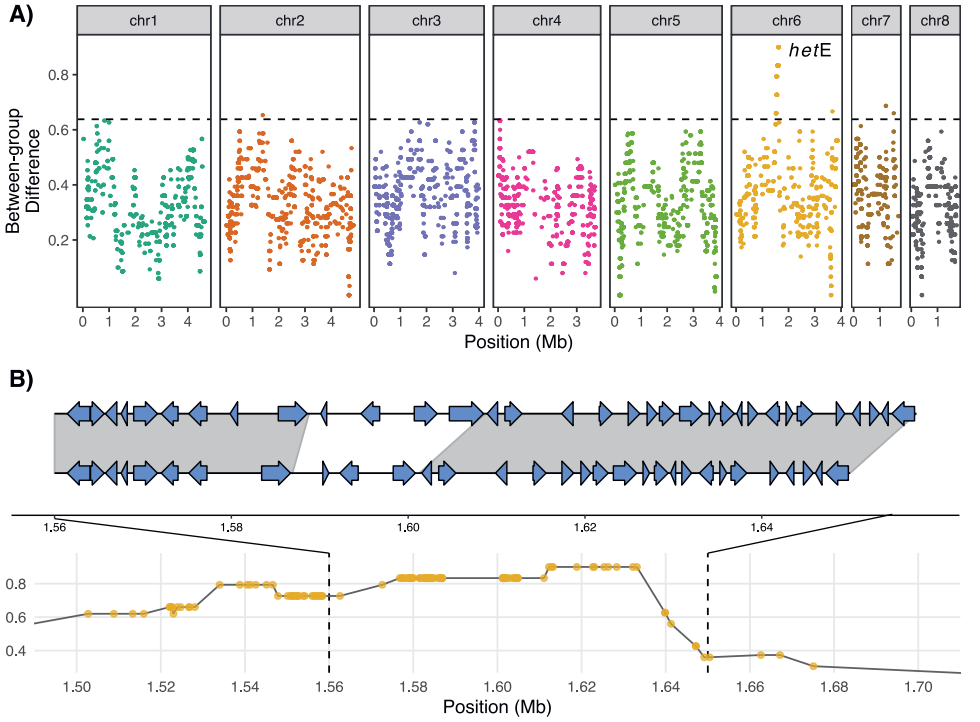
Data and material availability:

Strains and plasmids are available by contacting corresponding authors. Code and data for analyses used in this manuscript is available at: https://github.com/BenAuxier/asp_fum_het

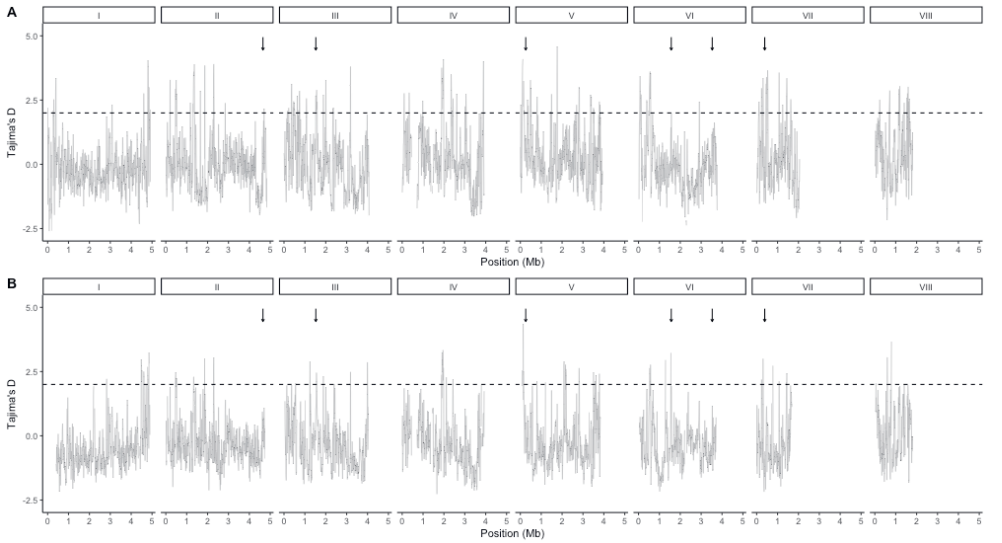
Author Contributions:

Conceptualization: AJMD, BA and ES. Experimental work: JZ, KL, KS, FRM, BA. Bioinformatic analysis: BA, JvdH. Manuscript draft: BA. Manuscript revisions: BA, AJMD, DA, ES, JZ. All authors read and approve publication of the manuscript.

Supplementary Data:



Supplemental Figure 1: Genetic mapping of locus controlling compatibility after five days. Identification of *hetE* locus based on difference between heterokaryon formation at three vs. five days. A) Genome wide plot of allelic differences between 3-day group and 5-day group (See Methods). The peak on Chromosome 6 is indicated as *hetE*. B) close up of *hetE* peak similar to that found in Figure 2A. Note that the divergent window for *hetE* covers several predicted coding genes.



Supplemental Figure 2: Genome-wide balancing selection in *A. fumigatus*. A) shows data from VCF file consisting of 213 UK samples (Rhodes et al. 2022). B) shows data computed from German samples described in (Barber et al. 2021) consisting of 178 German samples. For both populations, Tajima's D is shown in 10kb windows, and an arbitrary threshold of 2 is applied as a dotted horizontal line. Arrows indicate position of *hetA*, *mat*, *hetB*, *hetC*, *hetE*, and *hetD*, in that order.

Supplemental Table 1: Data from 6 segregating crosses to test identified loci.

parent1	**parent1 geno**	**mat**	**parent2**	**parent2 geno**	**segregating**	**Spores tested**	**Compatible with parent**	**χ^2 value**	**P value**
192	11101	MAT-1	AfIR964	11111	hetD	40	20	0	1
215	10111	MAT-1	AfIR964	11111	hetB	34	18	0.11	0.73
71	11110	MAT-1	AfIR964	11111	hetE	40	18	0.4	0.52
66	1000	MAT-2	AfIR974	00000	hetB	40	19	0.1	0.75
171	11011	MAT-1	AfIR964	11111	hetC	24	11	0.16	0.68
169	10000	MAT-2	AfIR974	00000	hetA	40	22	0.4	0.52
154	11110	MAT-1	AfIR964	11111	hetE	no cross	no cross	N/A	N/A

140	10111	MAT-1	AfIR964	11111	hetB	no cross	no cross	N/A	N/A

Supplemental Table 2: Primers used to construct *het* AMA1 plasmids

Primer Name	5' – 3' Sequence	Amplify AfIR974 Allele	Amplify AfIR964 Allele
hetA_F	atatactgggcccgggaagatcTCGTGAGGCGTATCCGCCATCG	X	X
hetA_R	gtcttgaatcgcgcattggatcAGGCTAATTCGGCGCAGTAGGG	X	X
hetB_20F	atatactgggcccgggaagatcCTGAGCGGGTCAAGACATAGGC	X	
hetB_20R	gtcttgaatcgcgcattggatcGAGTCATCACTAGCATTCATCCTG	X	
hetB_21F	atactgggcccgggaagatcGTTGCTGAGCGGGTCAAGGA		X
hetB_21R	cttgaatcgcgcattggatcGAGTCATCACTAGCATTCATCCTGACA		X
hetC_F	atatactgggcccgggaagatcGGGGATATACTCTTCCATGATG	X	X
hetC_R	gtcttgaatcgcgcattggatcAACAGTTTCTTGTCAATTGATTTC	X	X

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Chapter 9

Mate Choice on *het* Gene Diversity in *Neurospora crassa*

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Abstract:

The fungal *het* genes, which restrict fusion between individuals, are characteristically found to be under balancing selection. The source of this balancing selection has been hypothesized to result from deleterious cytoplasmic elements, or perhaps from bacterial pathogens. However, the diversity of *het* genes seems to be in large excess compared to simulations. Here we explore the hypothesis that the increased diversity at these loci may in part be due to mate choice. Using a set of inbred as well as wild strains, we systematically test for the effect of *het* gene diversity on mating fitness. Using spore color of harvested ascospores we found no effect of *het* gene diversity on mating success using inbred lines, but we did find strong effects when competing inbred versus unrelated strains. This increased fitness of outcrossing compared to inbreeding may be partially or wholly due to post-zygotic inbreeding depression, through previously described so-called “bubble asci”. Whether there exists true pre-zygotic mate choice requires further study.

Introduction:

The ability for a fungus to distinguish self from nonself is fundamental. This recognition allows for sustained hyphal fusions to be restricted to within a clone, ensuring that social actions occur within an individual (Gonçalves et al., 2020). By restricting social interactions, nonself recognition prevents the transmission of parasitic elements, such as mycoviruses or selfish nuclei, which is generally thought to be its the main benefit (Aanen et al., 2008; Debets & Griffiths, 1998; Gonçalves & Glass, 2020; Muirhead et al., 2002). An additional, non-exclusionary, benefit may be a general nonself recognition response, where the detection of "other" is achieved, to detect bacteria, fungi, or small animals (Paoletti, 2016; Paoletti & Saupe, 2009). While difficult to study in natural settings, it is likely that the benefit from nonself recognition arises from multiple facets.

The proximate act of fungal nonself recognition, the biochemical steps leading to cell death, results from alternate alleles of so-called *het*erokaryon incompatibility, *het*, genes. The presence of two alternate alleles of a gene activates currently unknown downstream pathways (Saupe et al., 1996). Originally, *het* genes in *Neurospora* were described based on reduced mycelial growth in a heterozygous state, generally using chromosomal translocation strains (Garnjobst & Wilson, 1956; Mylyk, 1976). However, recently more detailed examination has revealed a plethora of new loci, now with 17 loci identified in *N. crassa* (Gonçalves & Glass, 2020). In other species fewer loci have been identified (Choi et al., 2012; Nauta & Hoekstra, 1994), although this may reflect the increased study of *Neurospora* as a model organism.

A common characteristic of *het* genes is the effect of balancing selection. Originally this was identified through sequence analysis of *het*-C alleles in *Neurospora*, with the recovery of two alleles maintained in a population, with the same two alleles being found in related species, termed a trans-species polymorphism (Wu et al., 1998). While the presence of two alleles is also consistent with incomplete lineage sorting, the balancing selection acting on fungal *het* genes means that these alleles are found in roughly even frequencies (Auxier et al., 2022; Milgroom et al., 2018). These features – multiple alleles found in even frequencies with transspecies polymorphisms – have been recognized in *het* genes across all fungal species studied so far (Ament-Velásquez et al., 2022; Auxier et al., 2022; Milgroom et al., 2018; Wu et al., 1998). Not only found in *het* genes, the signature of balancing selection is also a common feature of other immune system genes, like the MHC loci in vertebrates or plant immunity genes (Hedrick & Thomson, 1983; Tian et al., 2002).

The source of balancing selection has been unclear, and the topic of mate choice has been a topic of intense study, at least in vertebrates. Work in various fishes, and particularly mammalian systems has looked at observed genotypes of adult individuals, which are often different from random mating assortment (Chaix et

al., 2008). This non-random assortment could arise simply from increased risk of death of juveniles homozygous for disease resistance genes, or it could instead arise from mate preference for partners with diverse nonself recognition genotypes, falling under the so-called “good genes” hypothesis (Eizaguirre et al., 2009; Milinski, 2006). The evidence for this is somewhat of a mixed bag, with experiments in both salmon and mice supporting this, as well as humans, but with many follow-up experiments failing to recover support (Chaix et al., 2008; Consuegra & Garcia de Leaniz, 2008; Dandine-Roulland et al., 2019; Jordan & Bruford, 1998; Milinski et al., 2005; Promerová et al., 2017). Perhaps the best supported experimental evidence is found in mice, where MHC loci seem to affect the volatiles found in the urine, which provides an olfactory cue that can be used by other mice when choosing a mate (Singh et al., 1987; Yamaguchi et al., 1981).

Assuming that *het* genes are primarily involved in nonself recognition with conspecifics, it is natural to ask what selective forces maintain diversity at these loci. It is important to note that balancing selection can result from different types of underlying selection, common examples are negative-frequency dependant selection (NFDS) where the rare allele has increased fitness, heterozygote advantage, or temporally fluctuating selection due to varying climate or migration (Lobkovsky et al., 2019). As *Neurospora* crassa exists as haploid individuals, heterozygote advantage at least cannot be operating during somatic growth. Somewhat surprisingly, individual level simulations showed that even a few polymorphic loci segregating in a globally mixed population is sufficient for nonself recognition. (Nauta & Hoekstra, 1994). The sufficiency of such a low number of loci can be understood since fungi are sessile, and they likely do not encounter very high numbers of conspecific individuals in their lifetime. However, subsequent modelling showed that incorporating limited local dispersal allowed for the maintenance of more diversity at nonself recognition loci (Czárán et al., 2014).

It is known that the locus that determines the mating type, *mat,* also functions as a nonself recognition gene (Newmeyer et al., 1973). Thus, at least for this locus balancing selection will result both from mate choice as well as the selection that other *het* loci encounter. However, it is unclear if balancing selection for other *het* genes in *N. crassa* may, at least partially, also be explained by mate choice. To test this, we performed mate choice experiments using a set of both isogenic lines, as well as a set of wild strains.

Materials and Methods:

Strains: Strains used are FGSC lines, except for NC201, which is a mat-a derivative of FGSC 2489, a hygromycin insert *alb2* mutant. NC number refers to internal culture collection code.

FGSC Number	NC number	Description	*het*
2489	147	mat-A, *hetCde*	Cde
4200	148	mat-a, *het*Cde	Cde
2657	106	mat-a Pan-1, al-2	Cde
2658	107	mat-A Pan-1, al-2	Cde
1425	004	mat-A Pan-1, al-2	cDE
1429	008	mat-a Pan-1, al-2	cDE
17611	189	mat-A ∆al-2::hph	Cde
N/A	201	mat-a ∆al-2::hph	Cde
1945		Wild isolate; Groveland 1-a	unknown
10639		Wild isolate; JW179	unknown
10646		Wild isolate; JW193	unknown
10647		Wild isolate; JW196	unknown

Media: Strains were propagated on Vogel's Minimal Media (VMM) for asexual conidia production in 15 mm glass tube slants. For sexual crosses, Synthetic Complete (SC) was used with 1% glucose in 60 mm petri plates for mate choice experiments, and 15mm glass tube slants for perithecial observations. For counting plates for both the initial asexual spore harvests and the sexual spore progeny, VMM media was used with supplementation of 20 g/L sorbose to restrict colony size. All VMM media was supplemented with 0.005% pantothenic acid to complement auxotrophies.

Inoculation of strains in female role: 60 mm petri plates of SC media was inoculated with the respective strain as a point inoculation, then plates were sealed in plastic bags and incubated for 5-7 days to allow for full media colonization and visible protoperithecia. Subsequently, these female cultures were fertilised by a 1:1 mixture of asexual spores from two male strains differing in spore colour to measure their relative success.

Inoculation of strains in male role: To fertilize colonized plates with a 1:1 mixture of asexual spores, conidia were harvested from mature (5-7 day old) VMM slants of

appropriate parents by adding H_20+0.05% Tween 80 as a surfactant. The mixture was vortexed to release conidia, and the concentration of the suspension was estimated using a haemocytometer. The spore suspensions were normalized to ~1x10^5 conidia/mL and mixed in a 1:1 ratio. 400 uL of this mixture was added to the colonized SC plates, and gently agitated to ensure all the female mycelia was wetted by spore suspension. In addition, the ratio of white:orange in the fertilizing spore suspension was further estimated by diluting the mixture and plating on counting plates at ~150 spores per plate.

Harvesting of ascospores: After 14 days incubation, shot ascospores were harvested by inverting the lids of the petri plates and pipetting 1 mL of H_20+0.05% tween 80 to resuspend visible ascospores. The spore suspension was heat shocked to kill conidia and hyphae and trigger germination of ascospores by incubation at 70 °C for 30 minutes. The density of the spore suspension was then estimated using a haemocytometer, and ascospores were again plated at ~150 spores per plate on VMM+sorbose counting plates. Plates were incubated for 7 days prior to counting.

Fitness of white spore parents was determined using by dividing the fraction of recovered white colonies on VMM+sorbose, divided by the fraction of white spores inferred from white colonies produced from the inoculation mix on VMM+sorbose medium. The fitness was multiplied by two to account for the contribution of the maternal color gene, which was orange in all crosses. Ratios, both of the output and the input spores, was determined in triplicate counts for each of 4 biological replicates.

$$fitness_{(white\ parent)} = 2 * \frac{fraction\ recovered\ white\ colonies}{fraction\ input\ white\ spores}$$

Perithecial observations: To observe development of perithecia, strains were crossed by stab inoculation of two compatible strains onto a SC slant, followed by incubation. Periodically, perithecia were removed using a dissecting microscope and observed under light microscopy using a Zeiss Axio.1 Microscope.

Results:

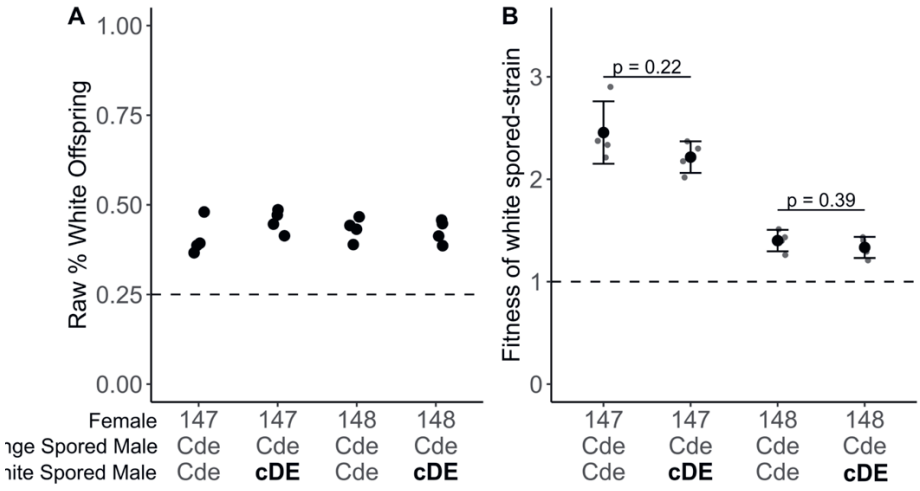

Figure 1: Fraction of white colonies from harvested ascospores of *het*-tester crosses. Names for each cross indicate the female strain NC number above, then the orange-spored fertilizing strain, and the white-spored fertilizing strain at the bottom. A) The % white colonies from each cross, each dot representing an independent replicate. B) Data from A but normalized based on plate counts of the starting conidia mix. The mean and standard deviation is indicated by the whisker plot. Bold names highlights crossings with *het* allelic differences.

The crosses using the Cde and cDE tester strains successfully crossed. When counting the white-spored colonies resulting from crosses, all were consistently higher than the 25% that would be expected from the 1:1 ratio of orange:white conidia used to fertilize, after accounting for the contribution of orange genes from the maternal parent (Figure 1A). When corrected based on colony counts of the fertilizing conidia mix, there was a clear difference based on the parental strain used in the female role. When NC147 was used as the female, the fitness of the white spored parent was consistently higher than when NC148 was used as the female, although the fitness of the white spored strain was higher than 1 in all cases (Figure 1B). Comparing the fraction of white spores where there was no *het* differences (i.e. Cde vs. Cde) to fertilization where there were differences at

the *het* loci (i.e. Cde vs. cDE) showed no significant difference when either NC147 (p=0.22) or NC148 (p=0.39) was used as the female strain.

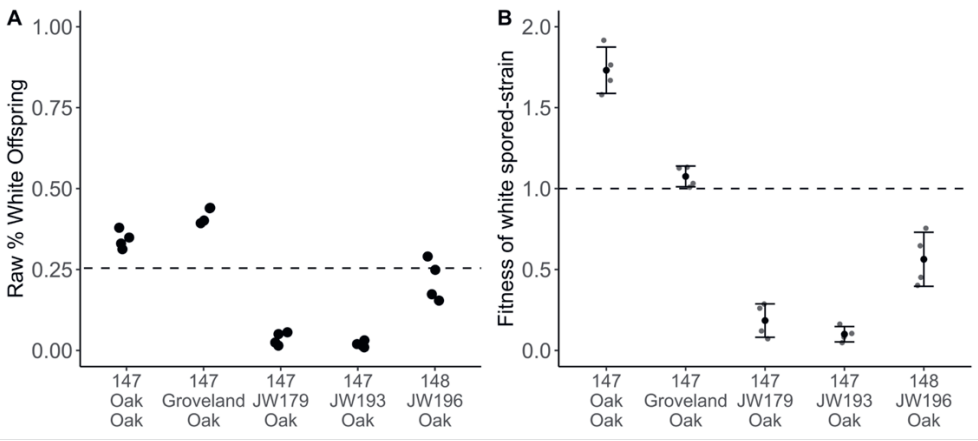

Figure 2: Fraction of white colonies from ascospores of outcrossed strains. Names for each cross indicate the female strain NC number above, then the orange-spored fertilizing strain, and the white-spored fertilizing strain at the bottom. A) Raw % white colonies per paternal mix, each dot representing an independent replicate. B) Fitness of white strain after normalizing for the % white spores in the fertilizing conidia. Whisker plot represents one standard deviation from the mean.

Matings between Oakridge strains and other wild strains showed more variability than the het-tester strains. Two of the crosses, Oakridge with JW179 or JW193 had less than 10% white-spored colonies in the progeny (Figure 2A), and even after correcting for starting spore ratio the fitness of the white-spored strain was greatly reduced (Figure 2B). The control pairing, of White vs. Orange Oakridge strains (left column, Figure 2A+B) showed a fitness greater than one. The competition between Oakridge and Groveland competition showed equal fitness of the white-spored strain. The competiton between Oakridge and JW196 showed reduced fitness of the white-spored Oakridge, but less so than in competition with JW179 or with JW193.
As the above experiments all used Oakridge as a female strain, we attempted to produce isogenic lines of the other strains. Initial attempts to introgress the opposite mating type from Oakridge into Groveland/JW179/JW193/JW196 had limited success. While the F1 cross was generally successful, subsequent backcrosses to make isogenic lines were unsuccessful despite repeated attempts (Data not shown).

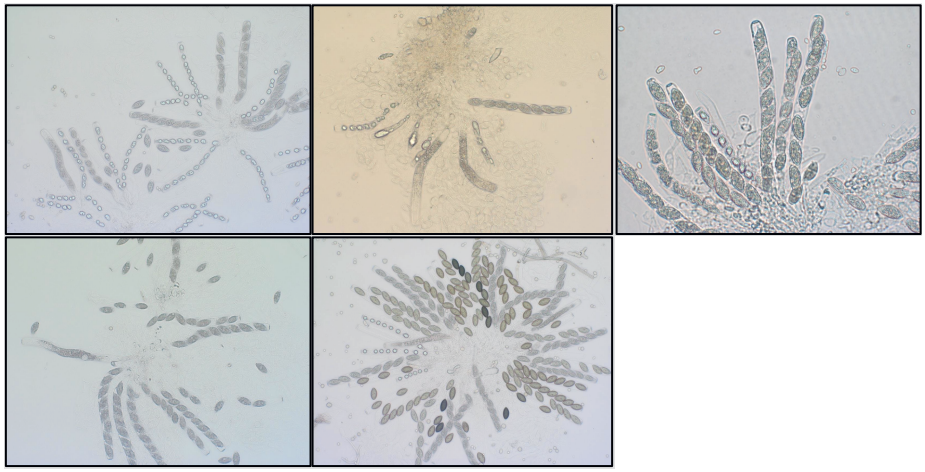

Figure 3: Rosettes of developing ascospores of *N. crassa*. Top-left, inbred cross of Oakridge showing roughly 50% bubble asci. Top-middle, outcross of Oakridge and Groveland 1-a, showing many distorted and aborted asci. Top-right, outcross of Oakridge and JW178 showing limited production of bubble asci. Bottom-left, outcross of Oakridge and JW193 without bubble asci visible. Bottom-middle, outcross of Oakridge and JW196 showing small percentage of bubble asci. Image takes after 8-9 days incubation, and under 400X magnification (except Top-middle at 630X).

As the failure of described backcrossing experiments indicated some genetic incompatibilities, we directly observed perithecia from crosses between our collection. Representative images can be found in Figure 3, while a numerical summary is found in Table 1. Rosette preparations of developing asci showed great variation in the prevalence of inviable bubble asci between crosses. The highest percentage of bubble asci was found in the inbred cross of Oakridge, with ~75% bubble asci (Figure 3 top left). The cross between Oakridge and Groveland produced a lower fraction of bubble asci, although it should be noted that many asci failed to develop even to the stage of ascospore delineation (Figure 3 Top middle). Crosses between Oakridge and JW179,193,196 produced low, but non-zero, frequencies of bubble asci.

Table 2: Bubble asci frequencies in outbred and inbred *N. crassa* crosses

Cross	% Bubble Asci	Total Asci (n)
Oakridge x Oakridge	73	279
Oakridge x Groveland	40	5
Oakridge x JW179	13	49
Oakridge x JW193	0	119
Oakridge x JW196	3	404

Discussion:

Our results here provide a first investigation into mate choice in the model organism *N. crassa*. Using crosses within the *het*-tester strains we found equal reproductive success, indicating similar mating success and fecundity either with the isogenic strain or the strain with differences at three het *loci*. This indicates similar levels of mating success for either strain within a mix. While it is possible that the lack of a statistical difference is due to small effect sizes, or insufficient sampling, at least the latter is unlikely in our case. Our experiments were sensitive enough to detect an association between the mating type of the female mycelium and the color of spores, with the *A* mating type having slight preference for white-spore matings, while the *α* mating type having slight preference for orange-spore matings. Thus, any mating preference for *het*CDE must be smaller than the effect of the mating type.

The reciprocal cross design showed an effect of the mating type on mating preference. This is difficult to explain, as the tester strains all originate from the Wilson-Garnjobst set, and all strains used carried the *pan*-2 mutation, so we can exclude pleiotropic effects of this auxotrophy on mating fitness. As the alternate mating types of Oakridge are highly inbred, it seems unlikely that a gene could be segregating between FGSC2489 and FGSC4200 that would select for/against mates based on spore color. It is perhaps more likely that the *het* tester strains themselves have undescribed mutations causing changes in mating behaviour. Indeed, it has already been noted that many of the *het*-tester strains themselves cause segregation distortion (Jacobsen et al. fgsc.net/fgn42/jacobs1.html).

However, the *het*-tester experiments leave open the possibility of mate choice in wild populations on alleles other than the het*CDE* loci. To explore this, we used a set of wild strains, which are expected to vary at a minimum of eleven loci of varying mechanisms. These wild strains also avoid the complications of the Wilson-Garnjobst *het* testers. These experiments more directly test if mate choice is found between wild strains. These experiments make use of a different white strain, D*al-2::hph*, which likely has less pleiotropic effects than the *pan*-2 mutation in the *het*-testers. At first glance, these experiments show strikingly strong effects of mate choice, with the percentage of white-spored offspring being significantly different from a 1:1 ratio. This is particularly apparent with the cross between Oakridge and JW179 and JW193, where ~10% of resulting colonies were white spored. The direction of this preference is also consistent with mate choice, as fewer white-spored colonies were found indicating increased fertilization with the other wild-types instead of the *al-2::hph* parent. The cross between Oakridge and Groveland was somewhat different, with a higher fitness for the inbred, white-spored parent. This should be interpreted with caution however, as the cross between Oakridge and Groveland produced perithecia with severely reduced spore counts, and the overall spore production was greatly reduced, although not systematically tested in these experiments.

The evidence presented here for mate choice used data from bulk ascospore harvest, and this data is more complicated than it first appears. The production of bubble asci, which do not develop to produce viable ascospores, means that inbreeding carries a direct cost in terms of offspring produced, aside from mate choice. The production of these bubble asci was originally observed several decades ago (Leslie & Raju, 1985). These bubble asci seem to arise from recessive mutations that only have effect during the brief diplophase of meiosis, and appear to be unique to each strain (Raju et al., 1987). The exact effect of the mutations likely varies, but they lead to death of the developing ascospore prior to cell wall deposition (Raju & Leslie, 1992). While the bubble asci themselves do not distort the segregation of markers, as all eight ascospores in an ascus die, they do affect the yield. Thus, the low percentage of white-spored offspring can be at least partially explained by the low ascospore yield when the white inbred parent fertilizes a protoperithecium, versus the higher spore yield when fertilized by the wild strain. It is important to note that assuming inbred Oakridge strains suffer a 75% fitness costs as we observed, with the dilution of the white spores from the maternal parent, that only approximately 10% white-spored colonies are expected. So, while our results are consistent with mate choice against the isogenic mate a significant fraction, perhaps all of it, is due to this inbreeding depression. As we observe even less than 10% white-spored colonies, we cannot at this point say that there is no mate choice occurring.

This reduced spore yield when crossed with a related strain is a form of inbreeding depression, but since it occurs post fertilization it would not be mate choice *per se*. As such, many mate choice experiments in *N. crassa* using bulk ascospore harvesting will inevitably be confounded by inbreeding depression. Further experiments to study mate choice in *N. crassa* should instead study fertilized perithecia directly, prior to ascospore release. The *per*-1 mutations, where the developing outer walls of the perithecia produce varying colors, are one option for this (Howe, 1976). Using a *per*-1 mutant as the female, the fertilizing mix would contain a suitable *per-1* partner, as well as a wild type cross. The experiments described here would also be simplified, as the fertilisation ratio can be determined directly from the mating plate, without need for further harvesting/culturing. An alternative option for future experiments would be to use the bubble asci themselves as a marker. As the production of bubble asci in the Oakridge inbreedings is ~75%, while much reduced for the others, observation of such a large difference is likely to be reliable. By instead making observations of mature perithecia, the fraction of bubble asci could be used as a marker for inbreeding.

These experiments replicate previous findings of bubble asci, which have been described as a form of inbreeding depression. Such recessive deleterious mutations, leading to ascus abortion, indicate that outbreeding is the rule in natural populations as they would otherwise be purged by selection (Raju et al., 1987). The reduction in bubble asci in random wild matings indicates that *N. crassa*

heterozygous matings have strong heterozygote advantage, with inbred matings leading to reduced numbers of offspring. The presence of such strong inbreeding depression may facilitate the emergence of a mate choice scenario, as it provides a direct benefit in terms of viable offspring produced. The active growth of the long thin hyphae of the trichogyne from the developing protoperithecia means that fertilization of *N. crassa* is not a passive process (Brun et al., 2021), and selection could occur in this organ.

The concept of *het*-gene based mate choice is logical in a diploid organism. Offspring produced from two dissimilar parents are more likely to be heterozygous at immune loci, which is thought to confer higher fitness than homozygosity (Lobkovsky et al., 2019). While it is commonly assumed that, by definition, haploid fungi cannot have heterozygote advantage, there is a brief heterozygote stage formed during mating. Indeed, previous research has shown in *Aspergillus nidulans* that outcrossed strains generally have larger fruiting bodies with more sexual spores (Baracho et al., 1970). A recent study in *Podospora anserina* showed no evidence of sexual selection, despite inbreeding depression in the crosses used (Martinossi-Allibert et al., 2022). These results are consistent with our findings here, however in this study the matings were performed across alternate sides of a several centimetres of mycelial growth, and it unclear whether this truly represents a competition between mating partners.

Whether *het* genes function as an anti-bacterial immune system, or to police cytoplasmic fusion, it may be beneficial to have offspring that have a diverse set of these genes. When a mating occurs between two individuals homozygous at all *het* loci, all offspring will then have the same *het* loci as each other as well. Thus, they would all share the same vulnerabilities. However, matings between individuals dissimilar at several *het* loci would produce diverse offspring, which is predicted to be beneficial when faced with co-evolving pathogens (Otto & Michalakis, 1998). Depending on assumptions about dispersal, the observed number of *het* genes seems to be greatly in excess of what is necessary to prevent invasion of parasites or pathogens (Czárán et al., 2014; Nauta & Hoekstra, 1994). It remains to be seen if mate choice is contributing to this excess in diversity and number of loci. If some other overlooked mechanism is contributing to the maintenance of this genetic diversity, this may help us more fully understand the function of *het* genes.

Conclusion:

While often unappreciated, fungi have the capability to execute decisions based on environmental signals. The fertilization leading to ascospore production presents such an opportunity. Using a set of both isogenic as well as wild strains, we tested whether such mate choice could be detected. We found no evidence of mate choice acting directly on *het* genes, but did find evidence of strong inbreeding depression. Our experiments could not rule out mate choice during the outcrossings. Further experiments should focus on the pre-zygotic stage of matings, to avoid the complications introduced by inbreeding depression. Using perithecial markers, or perhaps the production of bubble asci themselves, may present a simple method towards this.

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Chapter 10

Meiosis in *Aspergillus fumigatus* has the highest known number of crossovers

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Available As:
Auxier et al., 2022 *bioRxiv*
https://doi.org/10.1101/2022.01.14.476329

Abstract: Sexual reproduction involving meiosis is essential in most Eukaryotes. This step produces offspring with novel genotypes, both by segregation of parental chromosomes as well as crossovers between homologous chromosomes. A sexual cycle for the human pathogenic fungus *Aspergillus fumigatus* has been described, but the genetic consequences of meiosis remained unknown. Some, but not all, *Aspergillus* species have high crossover rates, yet the rate in *A. fumigatus* was unknown. Here, we show that *A. fumigatus* produces the highest number of crossovers. In contrast to the general production of one to several crossovers per chromosome *A. fumigatus* produces an average of ~29 per chromosome, more than twice as high as any known organism. This crossover rate is high enough to produce a common highly drug-resistant haplotype in the *cyp51A* gene in each sexual event from parents with single mutations. Such a recombination rate likely facilitates the rapid and global spread of epistaticly interacting variants. These results suggest that future genomic studies in this species may need to re-address common assumptions about linkage between genetic regions. This unparalleled crossover rate in *A. fumigatus* provides future opportunities to understand why crossover rates are not higher in other Eukaryotes.

Main Text:

Invasive aspergillosis, caused by the ascomycete fungus *Aspergillus fumigatus* is a serious life-threatening human disease. Clinical and environmental isolates are often resistant to antifungal azole treatments, developing from mutations either during clinical treatment or previous azole exposure in agricultural settings (Chowdhary et al. 2013; Perlin, Rautemaa-Richardson, and Alastruey-Izquierdo 2017; Verweij et al. 2009). Long suspected based on population genetic data, the sexual nature of *A. fumigatus* was confirmed by the discovery of a laboratory sexual cycle (O'Gorman, Fuller, and Dyer 2009; Paoletti et al. 2005). However, the impact of sex on genetic diversity remained unclear. The related genetic model *A. nidulans* has a high recombination rate, producing ~9-10 crossovers per chromosome (Christians et al. 2011; Clutterbuck 1997). With growing interest in population and mendelian genetics of *A. fumigatus* (Barber et al. 2020, 2021; Fan et al. 2021; Lofgren et al. 2021; Rhodes et al. 2021), understanding the outcome of sexual recombination is increasingly important. To investigate this, we constructed the first recombination map of *A. fumigatus*.

We used previously identified fertile strains AfIR964 and AfIR974 as parents in our cross (Sugui et al. 2011). Genome assembly using short- and long-read data recovered all eight contiguous chromosomes for each parent (Table S1). Relative to the reference Af293, our parental genomes are largely syntenic, with little structural variation between them which could interfere with recombination (Fig. S1). After crossing, we randomly isolated 195 offspring from several cleistothecia, and generated ~90X depth of short-read data from each. Mapped against the AfIR974 parent, we identified 14,113 high confidence variants based on quality and segregation criteria (Table S2). These variants were unevenly spaced across the genome with a median inter-marker distance of 206 bp and a mean of 2010 bp.

Using a naïve criterion of recombination as non-parental adjacent markers (i.e.; A-B or B-A instead of A-A or B-B parental), we identified an average of 132.5 recombination events per offspring (16.6 per chromosome), producing a surprisingly long genetic map length of 14,843 cM (Fig. 1A; Table S3). The mean recombinant fraction between adjacent markers was 1% (max 38%), indicating that our markers can provide an accurate genetic map (Fig. S3). This criterion includes both crossovers, the physical exchange of homologous chromosomes, as well as smaller gene-conversion events that occur during resolution of meiotic double-stranded breaks (Mancera et al. 2008). Since gene conversion events appear as two closely spaced crossover events, they can greatly inflate the genetic map (Mancera et al. 2008). Therefore, this distance is an overestimate and requires correction.

To remove gene conversions, we first used a rarefaction of our markers. Since gene conversion tracts are short, typically 0.5-2 kb (Chen et al. 2007), a high density-marker set has increased power to detect them while fewer markers are necessary to detect crossover events, which affect chromosomal segments orders

of magnitude longer (Mancera et al. 2008). Thus, increasing the gaps between markers, reducing the numbers of markers used, reduces the detection of gene conversions but less so for crossovers. Consistent with this, rarefaction of our markers reduced the map length asymptoticly (Fig. 1A). The reduced map lengths are not simply due to a reduced number of markers. For example, the 559 markers at 20 cM spacing resulted in a 12,512 cM map, while the 257 markers at 50 cM spacing produced a map of 11,966 cM — a ~50% reduction in markers only resulted in a 4.5% reduction in map length.

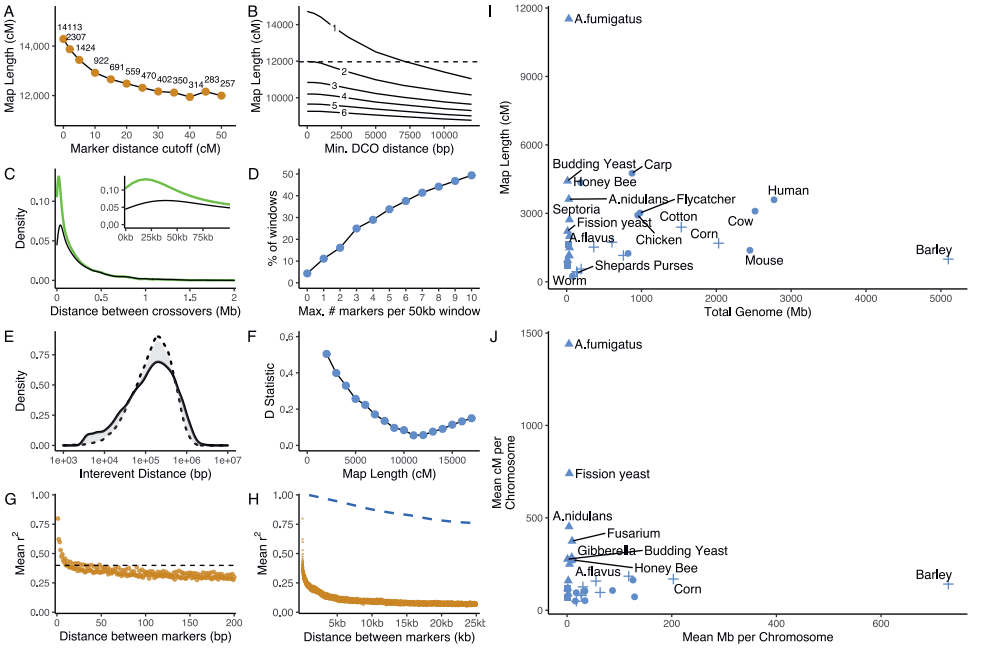

Figure 1: Genetic Map of *A. fumigatus*. **(A)** Total map length after removing markers closer than specified interval (x-axis), lengths represent mean of 5 samplings. Number of markers at specified spacing indicated near points. (**B**) Genetic map length after removing putative gene conversions based on length (x-axis). Criteria of minimum required markers is indicated in-line. **(C)** Distribution of DCO distances in raw data (green line) or after removing putative GC events (black line), inset shows zoom on first 100 kb distance. (**D**) Fraction of the 577 50 kb genomic windows with a minimum number of markers. **(E)** Distribution of DCO distances from empirical data (solid line) versus simulated data (dashed line) of an 11,000 cM genetic map. **(F)** Plot of D statistic (grey shading in E) comparing observed data to simulated genetic map lengths. (**G**) Decay of Linkage Disequilibrium (LD) across the *A. fumigatus* genome. Dots indicate average r^2 between markers across 175 published individuals (Barber et al. 2021), dotted line indicates 50% reduction in LD. **(H)** LD decay across longer distances, orange dots indicate population level decay, while blue dotted line indicates LD of the mapping population. (**I**) Comparison of the genetic map of *A. fumigatus* to other species**. (J)** Similar to G but shown per chromosome. Data for I+J derives from (Fernandes et al. 2018) with the addition of (Christians et al. 2011; Gell, Horn, and Carbone 2020) and our data. Comparison to an alternate dataset is found in Fig. S4.

This rarefied distance is consistent with alternate methods. *Post hoc* criteria for removing gene conversions, such as minimum number of markers or length supporting a double crossover (DCO), had differing effects on the resulting map length (Fig. 1B). Increasing the minimum DCO length up to 12 kb resulted in an 11,037 cM map. However, increasing the minimum number of markers for a DCO had a stronger effect on map length, with a three-marker criterion reducing the map to 10,839 cM (Fig. 1B). This stronger effect of marker number is likely due to portions of the genome with few markers, as 16% of 50 kb genomic windows have less than three markers (Fig. 1D). As a further map length method, we simulated different map lengths of uniform crossovers across the genome. As the related *A. nidulans* lacks crossover interference, it produces uniformly distributed crossovers and the DCO distances form a Gamma distribution (Egel-Mitani, Olson, and Egel 1982; Otto and Payseur 2019). We found that lengths between 11,000 and 13,000 cM best fit the data, minimizing the D statistic between observed and simulated data (Fig. 1E+F; additional map lengths Fig. S2).

We therefore conclude that the map length of *A. fumigatus* is between 11,000-13,000 cM, and we use the 11,966 cM estimate resulting from 50 cM marker rarefaction. To our knowledge this map length (0.422 cM/kb) is the longest estimated for any organism (Fig. 1I, Figure S4A) even after correcting for chromosome length (Fig. 1J, Fig. S4B). Further supporting this recombination rate, a previous study with different parents showed >5% recombination between two spore-color genes, *alb1* and *abr2*, spaced 8.3 Kb apart (>0.6 cM/kb) (Sugui et al. 2011). However, the high recombination rate found in *A. fumigatus* does not appear to be widespread across *Aspergillus* species. High-density genetic maps for *A. nidulans* and *A. flavus* indicate lengths of 3,705 cM and between 1,500 and 2,000 cM, respectively (Christians et al. 2011; Gell, Horn, and Carbone 2020). Using population level variation from a previously published dataset of 175 *A. fumigatus* German clinical and environmental isolates (Barber et al. 2021) the population level effect of sexual recombination is visible. The linkage between genetic variants decays rapidly, within 50 bp using the LD_{50} statistic (Fig. 1G+H). This rapid decay of linkage, with LD_{50} values for other species generally >1kb (Hill and Robertson 1968; Taylor et al. 2015), indicates that sexual recombination in field populations is ongoing, as the single recombination events in our mapping population did appreciably decrease LD (Figure 1H dotted line).

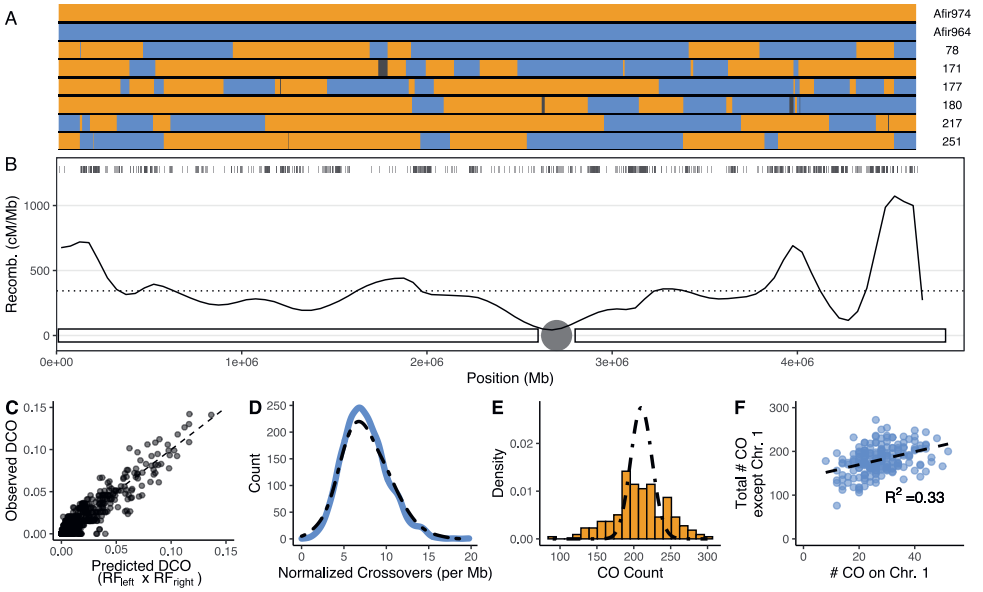

Figure 2: Crossover distribution in *A. fumigatus* shows no sign of interference. (A) Genotype parents and 5 representative offspring for Chromosome 1. Blue indicates Afir964 genotype, orange indicates Afir974. Black regions indicate regions removed from gene conversion criteria. **(B)** Recombination rate across Chromosome 1. Solid line indicates recombination rate across windows of 50kb, dotted line indicates mean recombination across Chromosome 1. Tick marks above indicate markers used for mapping, and chromosome with centromere is indicated below. Plots for all chromosomes are found in Fig. S5. **(C)** Correlation between predicted DCO based on adjacent intervals and observed DCO across the same interval in the 195 offspring. Diagonal dotted line indicates a 1:1 relationship, a coefficient of correlation of 1. **(D)** Histogram of normalized # of crossovers per Mb of chromosome, compared to Poisson distribution of same mean (dotted line) **(E)** Number of crossovers per individual in the dataset (orange bars) compared to the Poisson distribution of the same mean (dotted line) **(F)** Scatterplot of # of crossovers on Chromosome 1 versus the remaining total # of crossovers per genome, dots represent offspring.

As suspected based on the relationship to *A. nidulans*, we find no evidence of crossover interference – the nonrandom distribution of crossovers found in most organisms (Otto and Payseur 2019), although recombination is not uniform across the physical chromosome (Fig. 2B). We base this on three observations. First, as a direct measurement of interference (Capilla-Pérez et al. 2021), we find a coefficient of coincidence of ~1, indicating no deviation between observed and expected double crossover rates (Fig. 2C). Secondly, the distribution of crossovers per chromosome is very similar to the expected Poisson distribution (Fig. 2D). Finally, in the absence of interference the shape parameter of the interevent Gamma distribution, ν, is expected to be 1, with values greater than 1 indicating interference (most species $5<\nu<30$) (Otto and Payseur 2019). The $\nu=0.74$ (Fig 1C) of our cleaned dataset does not indicate interference. The related *A. nidulans* lacks crossover interference, as well as a meiotic synaptonemal complex (SC), similar to *Schizosaccharomyces pombe* (Egel-Mitani, Olson, and Egel 1982; Snow 1979). We expect that *A. fumigatus* also lacks a SC, however sequence similarity of the structural proteins is

only detectable within closely related species (Bogdanov, Grishaeva, and Dadashev 2007). Thus, bioinformatic analyses for presence/absence of SC proteins are not feasible as the nearest species characterized is *Sordaria* (Espagne et al. 2011, 4). Interestingly, the number of crossovers per individual in our data (min. 80; max. 300) was over-dispersed compared to the expected Poisson distribution (Fig. 2E). This is due to correlation between numbers of crossovers between chromosomes, a recently recognized phenomenon (Fig. 2F) (Henderson and Bomblies 2021; Wang et al. 2019).

As an independent line of evidence for this exceptional genetic map length, we tested how this translated to genetic mapping. Fortuitously, the parents had phenotypic variation in resistance to acriflavine (Fig. 3A), an antifungal with a rich history in fungal genetics (Roper and Kāfer 1957). Phenotyping of the offspring on 50µg/mL acriflavine identified a single locus on chromosome 6 between positions 657 kb and 675 kb (Fig. 3B). The resolution of this genetic mapping, an 18 kb window, validates the high recombination rate as well as highlights the power of genetics in this species to identify novel mechanisms. The two variants in this region fall within the coding sequence of AFUA_6G03080 (Fig. 3B). This gene encodes an undescribed ABC efflux transporter in the family containing Multi Drug Resistance 1 (*mdr1*) (Tobin, Peery, and Skatrud 1997). The difference in LOD scores between these two variants, from recombinant offspring across this distance of 410 bp, allows for immediate fine mapping of the presumed causal variant (PheàCys). While genetics has previously been used to identify resistance mechanisms in *A. fumigatus,* this study utilized sequentially evolved isogenic clinical isolates (Camps et al. 2012). Here we show that variants in *A. fumigatus* can be easily mapped with wild-type isolates, even for genes without known mutant phenotypes. This method should facilitate genetic dissection of traits related to host infection and colonization (Kowalski et al. 2016).

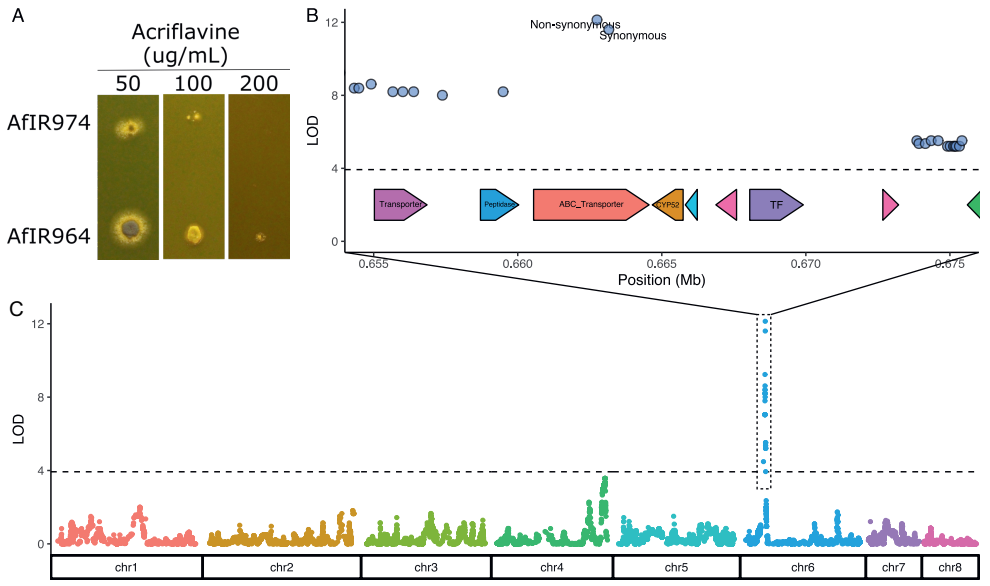

Figure 3: Validation of *A. fumigatus* recombination rate through mapping of acriflavine resistance. **(A)** Phenotypes of both parental strains grown at increasing concentrations of acriflavine after 2 days incubation **(B)** Closeup of the significant QTL window. Gene models shown below the dotted line indicate location of genes in the Afir964 parent. **(C)** Genome-wide significance values for acriflavine resistance in the cross between Afir964 and Afir974. Horizontal dotted line indicates the permutation-based significance threshold with α=0.05.

The high recombination rate of *A. fumigatus* may further explain observed patterns of antifungal resistance, a major concern in the clinical setting (Perlin, Rautemaa-Richardson, and Alastruey-Izquierdo 2017). Azole-resistant environmental isolates commonly contain haplotypes of 2-3 variants in the *cyp*51A gene involved in ergosterol synthesis. These resistant haplotypes are composed of a tandem repeat (often 34 or 46bp) in the promoter element (TR) combined with at least one non-synonymous polymorphism (e.g. TR_{34}/L98H or TR_{46}/Y121F/T289A). There are strong epistatic effects, as the effects are non-additive between specific combinations of non-synonymous coding polymorphisms and promoter mutations causing increased antifungal resistance (Mellado et al. 2007; Snelders et al. 2015). Curiously, it has been noted that in other fungal species resistance to the same azoles is instead generally due to single mutations in *either* the promoter *or* the coding sequence and not by a strong epistatic interaction of both (Verweij et al. 2009). The *cyp*51A haplotypes in *A. fumigatus* have been hypothesized to arise from mutation/tandem duplication at one position, and then an additional mutation/tandem duplication at the second position (Verweij et al. 2009). Our data here suggests an alternative explanation of each mutation arising in independent strains, and then being united through recombination, which is considered a general benefit of sexual recombination (Barton 1995; McDonald, Rice, and Desai 2016).

As our parental strains had no variation within or near the *cyp*51A gene, recombination could not be observed in this region, although it has been noticed at a population level (Barber et al. 2021). However, the 0.422 cM/kb recombination rate indicates that if one parent had the TR_{34} variant, and the other had the L98H variant (365bp apart), then 0.075% of offspring would have both resistance variants, (99.85 with either parental single variant and 0.075% with neither) (Fig. 4A). Recombination in the TR_{46} haplotype is expected to be slightly higher (TR_{46} and Y121F spaced 434bp apart). Since a single fruiting body produces >10,000 spores (Sugui et al. 2011), recombinants within *cyp*51A gene are surprisingly, expected in each sexual event.

To validate this predicted intragenic *cyp*51A recombination, we crossed previously generated single mutants (Paul, Diekema, and Moye-Rowley 2017) with AfIR974, generating sexually fertile offspring with either L98H or TR_{34} variants which each confer intermediate levels of azole resistance (4µg/mL itraconazole). After crossing these, we selected recombinants based on their positive epistatic effect by incubation on 10 µg/mL itraconazole (Fig. 4C+D). These highly tolerant offspring were produced at a rate of 0.096% (mean number resistant colonies 18, mean total ascospores 18,708). Sequence analysis of the itraconazole resistant offspring confirmed them as recombinants, with both the L98H and TR_{34} variants (Fig. 4B).

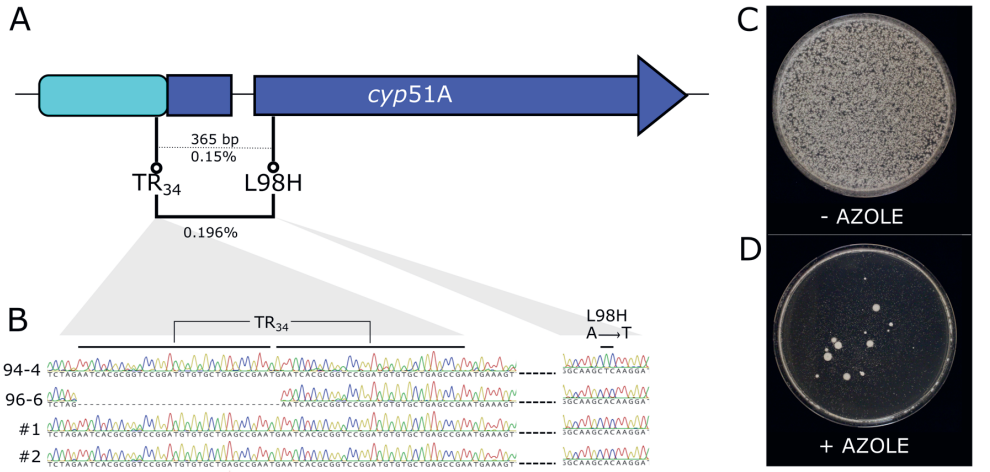

Figure 4: Sexual recombination consistently produces recombinant *cyp*51A haplotypes. **(A)** Diagram indicating distances and inferred recombination rates between positions in the *cyp*51A gene, leading to commonly encountered TR_{34} and TR_{46} azole resistance haplotypes. Base pair distances are indicated above dotted line, predicted recombination rates indicated below. Observed recombination rate from B indicated with solid line connecting TR_{34} and L98H. **(B)** Chromatograms of *cyp*51A sequence showing TR_{34} region and L98H region from parental strains with either variant, as well as two randomly selected azole resistant offspring **(C)** Ascospores of a single sexual fruiting body on non-selective media. **(D)** As above, but with addition of 10µg/mL itraconazole, which is above the tolerance of either single mutant strain. Large colonies indicate resistant offspring. Non-recombinant offspring are visible as scattered small dots. Note that plate for Fig. 4C was incubated 16 hours less than D to prevent overgrowth of abundant colonies. Images of all cleistothecia used are found in Fig. S6

Fungi are known to have higher recombination rates (Stapley et al. 2017), but our finding of up to 64 crossovers per chromosome in *A. fumigatus* brings this to a new level. Across eukaryotes generally only a few crossovers occur per chromosome (Rosu, Libuda, and Villeneuve 2011), and a negative correlation between population size and recombination rate indicates that excess crossovers are detrimental (Buffalo 2021). This detriment can result from the breaking up of beneficial combinations of mutations (Barton 1995) as well as physical meiotic defects, for example contributing to Trisomy 21 in humans (Koehler et al. 1996), however this has been contested by recent experiments (Crismani et al. 2012). This higher crossover number may be explained as the lack of a SC requires an increased number of crossovers to ensure at least one crossover per chromosome, necessary for proper meiotic chromosome reduction (Egel-Mitani, Olson, and Egel 1982; Snow 1979). If this high crossover rate is simply a consequence of lacking a SC, it is unclear why the rate is so much higher than found in *A. nidulans* and *S. pombe*, which also lack a SC.

Our data shows that a physical limitation of crossover number does not explain the general low crossover rate across eukaryotes. Under a narrow range of parameters, a constantly changing environment can select for higher recombination

rates (Otto and Michalakis 1998). What ecological or evolutionary factors differentiate *A. fumigatus* from other *Aspergillus* species with a lower recombination rate is unknown. Whatever the evolutionary cause, this recombination rate likely facilitates azole-resistant haplotypes (Verweij et al. 2009). This can now explain the rapid and global distribution of the azole resistant haplotypes, as sex appears to occur in bulb waste material heaps containing azole residues (Fisher et al. 2018; Zhang et al. 2017, 2021). This high recombination rate also affects the interpretation of population-level genome scans as it practically eliminates linkage between genes/markers (Lofgren et al. 2021). Understanding the adaptive advantage, or lack thereof, of this unparalleled number of crossovers requires further enquiry.

Acknowledgments: We thank Dr. Eugene Gladyshev and Dr. Raphael Mercier for input on a previous version of this manuscript. We gratefully acknowledge Dr. W.M. Moye-Rowley for sharing the L98H and TR_{34} single mutants used in the crosses for Figure 4. We thank Dr. Paul Verweij and Dr. Paul Dyer for sharing the AfIR964 and AfIR974 parental strains. Dr. Erik Wijnker as well as other members of the Laboratory of Genetics provided valuable feedback during the writing process.

Funding:

Nederlands Wetenschappelijk Organisatie ALWGR.2017.010 (BA)

The European Society of Clinical Microbiology and Infectious Diseases 2018 Research Grant #3184200058 (ES)

Data and materials availability: All fungal strains used are freely available from the corresponding authors. Data and code supporting the analysis used are available at https://github.com/BenAuxier/aspergillus_recombination. Genome assemblies of parental strains AfIR964 and AfIR974 are undergoing submission as SUB10510768 and SUB10510426, respectively. Illumina data for all offspring is currently under submission at NCBI SRA SUB10935968.

Supplementary Materials Can be found with the assocated bioRxiv posting

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10

Chapter 11

General Discussion

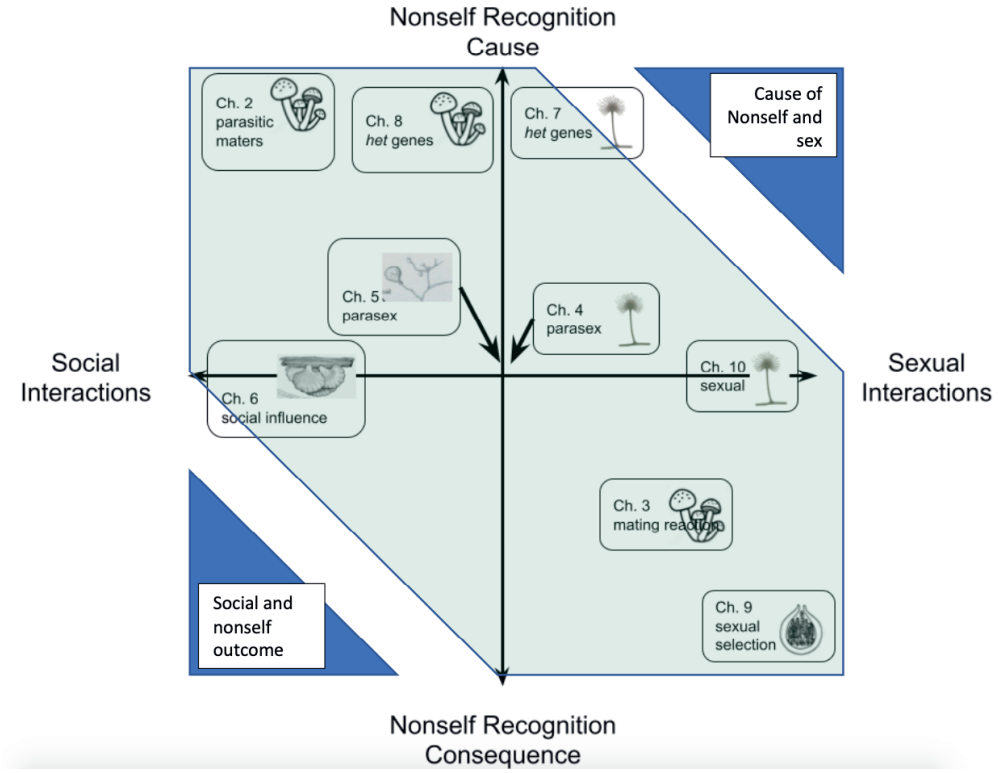

Figure 1: Thematic organization of the chapters of this thesis

Before discussing the topics of this thesis, it is perhaps useful to reflect on Figure 1 from the General Introduction. The chapters span much of this space, but there is a general correlation of the topics studied. Using this admittedly artificial two dimensional layout, when chapters cover social interactions, they generally relate to the cause of nonself recognition. Conversely, when sexual interactions are covered, it is more related to the consequence of nonself recognition. This is perhaps not surprising, since the bottom left space should by definition be empty as nonself recognition limits social interactions. Similarly, the emptiness of the top right is somewhat expected as sexual interactions are not expected to be an evolutionary source of nonself recognition selection.

Whether it is true that there are no social interactions in the face of nonself recognition is unclear. While nonself recognition restricts social interactions that require continuous cytoplasm, it would not restrict extracellular social interactions. This thesis was limited to these cytoplasmic social interactions, but it is important to be aware of these other types. Their study is likely to lead to rather different conclusions. Likewise, it may not be entirely true that sex is not a cause of nonself

recognition. A recent report showed that allelic differences at a *het* locus were split across two sister species, potentially acting to reinforce the species barrier (Ament-Velásquez et al., 2022). Whether this is a more widespread phenomenon — the use of a nonself recognition module to alter the outcome of sex — is unclear.

The regulation of fungal social interactions

As fungal individuals take part in numerous social interactions, the regulation of these interactions is clearly important. I would argue that heterokaryon incompatibility is the *key* regulator of intracellular social interactions, as this forms the basis for the decision with whom to share cytoplasm. It is interesting to consider why such abundant fusion occurs in the first place, as the unrelated, but morphologically similar oomycetes do not have abundant anastomosis (Rayner, 1991), and other fungal lineages, like mucoromycetes similarly lack anastomosis (Ootaki, 1973). In fungi that do fuse, the social interactions between clonally related nuclei seem to be advantageous, as cell fusion mutants generally have reduced reproductive fitness (Bastiaans et al., 2015). However, there seems to be strong selection to limit these social interactions, based on the diversity and ubiquity of nonself recognition loci. The combined action of these loci means that social interactions of ascomycetes will be restricted to occur within clonally related monokaryotic individuals and restricted in basidiomycetes to within clonally related dikaryotic individuals.

The evidence presented in **Chapter 8** increases our knowledge about these genes in *A. fumigatus*. Here we find that the genes involved are unlike those described already in *Neurospora / Podospora / Cryphonectria*, specifically that none of the genes identified are predicted to contain a HET domain. This HET domain has long fascinated researchers, and has been used in multiple bioinformatic genomic surveys (Dyrka et al., 2014; Mori et al., 2019; Seekles et al., 2022; Wu et al., 1998). This result highlights that there is no replacement for experimental work, as bioinformatic searches are limited to the motifs/domains that are already known. Without continued experiments to identify the genes triggering nonself recognition, our understanding will remain biased to what we already know. Additionally, experiments are required to show the actual effect of such allelic differences, as not all *het* loci in *A. fumigatus* produced equivalent phenotypes. While it is generally thought that a difference at any *het* gene is sufficient to trigger cell death, our example of *hetE* showed that some are significantly stronger than others, as described as the "partial" *het* genes previously (Coenen et al., 1994). Whether these genes even trigger cell death, or perhaps act in a different fashion, would be a fascinating area for future research. As genes like *hetE* seem to only delay heterokaryon formation, not prevent it, the effectiveness in then preventing the spread of deleterious elements seems limited.

Further expanding on the idea that experiments are necessary to increase our understanding of biology, **Chapter 7** shows the first genetic details regarding the restriction of social interactions in a basidiomycete fungus. This chapter presents evidence that the genes involved are not so different from those commonly found in ascomycete fungi, being also based on an NLR-like system. While previous bioinformatic searches have attempted to localize the genes triggering somatic incompatibility, this chapter presents the first genetic locus mapped to the resolution of a gene. However, this work also leads to the question of how this is regulated. Interestingly, analysis of unpublished data showed that expression of the genes inside this locus do not noticeably differ between monokaryotic and dikaryotic growth (data from Rob Powers & Tim James). This would suggest that the nonself recognition modules in monokaryons are equally as active at a molecular scale as dikaryons, something that was doubted previously (Dr. Louise Glass, personal communication). The question then arises of how dikaryons can be formed between two genetically different monokaryons, each with their own nonself recognition alleles. Previously the idea that interactions escaped the death of somatic incompatibility has been termed the “sexual override” theory (Rayner et al., 1984; Rayner & Todd, 1980). This theory supposed that the mating factors somehow overrode the somatic incompatibility system. In **Chapter 3**, we provide a possible mechanism facilitating this “override”. It may be that the nuclear migration so common to basidiomycetes during mating is precisely the mechanism whereby fusion between monokaryons evades nonself recognition. Instead of a complicated regulatory mechanism, the rapid migration strips the nucleus of its cytoplasm, meaning the “sexual override” is enacted in a physical manner. Put differently, it is perhaps not that the genes responsible for mating directly regulate the transcription or translation of nonself recognition genes, but rather that the rapid movement they impart provides a literal escape. Importantly, this hypothesis can explain the regular production of nonself recognition proteins in all life stages, while still permitting the unidirectional mating between dikaryons and monokaryons. Previous attempts to explain the “override” theory based on differential regulation had difficulty explaining the observation of mating between dikaryons and monokaryons, since under this theory dikaryons should not tolerate nonself fusions.

An interesting bias to consider in laboratory studies is our obsession with a visual phenotype. While we assume that all multicellular fungi engage in nonself recognition, not all show a visible phenotype on agar media. The other genetic model mushroom, *Schizophyllum commune,* was ruled out as a system for **Chapter 7** based on the inability to produce a nonself recognition phenotype in the laboratory (Nieuwenhuis et al., 2013). For many species the study of this requires media recipes that are optimized for this phenotype, such as the agar used in **Chapter 7**, or the barrage agar used for *Podospora* research (Ament-Velásquez et al., 2022). The applicability of such phenotypes to natural systems remains an open question. Recent

work by others has shown that, at least in some species, genetically distinct individuals *can* form heterokaryons under extremely nutrient depleted conditions (Lu et al., 2021; Vangalis et al., 2021). If this more reduced nutrient context better reflects reality, could it be that nonself recognition is only active when conditions are rich, and that nonself fusions are tolerated in times of trouble? This scenario could represent the evolutionary response to an “opportunity cost” where an individual takes on the risk of a nonself fusion if it appears that local conditions cannot support further growth. While such comparisons to decision making may seem absurd to some, the evolutionary parallels with rational decision making are often useful to understand evolved mechanisms (Okasha, 2018). This could represent a parallel to the common observation of sexual reproduction in poor conditions, where the formation of new genotypes outweighs the cost of recombination (Burt, 2000). Future work is required to determine whether these nonself fusions are truly a common occurrence or are instead unique to the species studied so far.

The balance between social and sexual interactions

It is generally considered that sexual and social interactions are independent, or even opposing. One only needs to look at the names “asexual” and “sexual” that is routinely used in fungi. However, the work presented here adds to the evidence that this is a false dichotomy. Fungi engage in social interactions, both short and long, some of which also lead up to sexual interactions. For many species, the social interactions between individuals are short-lived and result in antagonism, while when sexually receptive they lead to meiotic reproduction. However, the distinction is not always so clear. The most obvious example of this is found in basidiomycete fungi, where two genomes coexist in a single mycelium for decades to centuries. While this partnership is directed towards eventual sexual reproduction, the fact that both nuclei remain isolated means that social interactions continue during somatic growth. In **Chapter 2** we show that these ongoing social interactions between the nuclei inside a dikaryon can lead to deleterious outcomes. To investigate the social interactions that occur during and shortly after the mating reaction, in **Chapter 6** we investigated the effect of female or male role on the resulting nuclear coexistence. This result showed that the social interactions between genomes of basidiomycetes are likely not regulated in a parent-of-origin manner, although there is significant impact of the social partners on each other despite the isolated genomes.

In fungi, the generation of genetic diversity is often partially attributed to a parasexual cycle. This cycle, of the formation of a diploid from haploid (or tetraploid from a diploid as in *Candida albicans*) progenitors, followed by reduction involving random chromosome assortment is seen as separate but parallel to sex, hence the name parasex. However, it is generally not recognized that this is fundamentally a

social interaction. The process of parasex is blocked by nonself recognition (Caten & Jinks, 1966), and so parasex is effectively restricted to be within a clone. This restriction on the generation of diversity casts some doubt on claims made otherwise in arbuscular mycorrhizal fungi (Chen et al., 2018; Yildirir et al., 2020), which **Chapter 5** shows to be bioinformatic artifacts. Indeed, such recombination is not likely to be useful in generating diversity in an individual that already has two genomes, whether diploid or dikaryotic. Recombination between the two genotypes will result in loss of heterozygosity 50% of the time, thus reducing diversity, not increasing it, which we detail in *Aspergillus* in **Chapter 4** As has been suggested previously, this parasexual cycle is only beneficial under strong changes in the environment (Hickman et al., 2015). In basidiomycetes, this process must be restricted to an even greater degree, due to the long dikaryotic stage. While somatic recombination has been demonstrated in *Schizophyllum commune*, the phenotype that was selected for (recombinants for unliked mating loci) was under strong selective pressures, and so even exceedingly rare events could be reliably detected (Ellingboe & Raper, 1962). The mechanisms that reduce the rate of parasexual recombination so drastically in basidiomycetes *versus* ascomycetes are not clear. It may simply be due to the increased spacing of dikaryotic nuclei (Kamada & Tanabe, 1995; Schuurs et al., 1998), but that seems insufficient to explain for polynucleate basidiomycetes like *Agaricus* or *Termitomyces*.

The experiments of **Chapter 9** highlight a stage of the lifecycle that is generally ignored regarding social interactions, the sexual stage. While we did not find a significant impact of mate choice during mating in *Neurospora crassa*, this stage still represents a significant social interaction arena (Brun et al., 2021). Recent live cell imaging has shown that the fusion of fertilizing conidia with the trichogyne can involve several different partners, similar to the dikaryon-monokaryon situation in basidiomycetes. This has been investigated in *Aspergillus* previously (Bruggeman et al., 2004), and also deserves further attention. This is similar to the fertilization process in dikaryon-monokaryon matings in basidiomycetes, where one nucleus from the dikaryon eventually colonizes the entire monokaryon (Anderson & Kohn, 2007). The details of this fertilization process in basidiomycetes are unclear, but it appears that at least initially both nuclei invade some of the monokaryon, with one becoming dominant (Anderson & Kohn, 2007).The anthropomorphic urge to see social interactions as we experience them is surely blinding us to interesting and important biology.

The causes and consequences of balancing selection

The effects of balancing selection on fungal genomic regions have been observed for several decades already. Fungal species have shown an abundance of loci under balancing selection for nonself recognition loci, far in excess of that

predicted to be necessary to limit fusion to within an individual (Nauta & Hoekstra, 1994). It is perhaps surprising then that we still have little firm evidence for the proximate cause of this selection. So far experiments have shown that nonself recognition can limit transfer of both viruses, defective mitochondria, and parasitic nuclei under laboratory conditions (Bastiaans et al., 2016; Debets & Griffiths, 1998), but this seems insufficient to explain the high number of loci involved. Perhaps a more realistic metric for balancing selection would be an increase in fitness for individuals with a rare nonself recognition locus. While this can be inferred from the previous experiments, a formal demonstration is still lacking. Until this is done, it is possible that we are missing the ultimate source of balancing selection. A difficulty with balancing selection is that it can have multiple contributing causes, and so it is not sufficient to solely show that pathogen avoidance results in balancing selection. Instead, the question becomes, does pathogen avoidance increase fitness in natural settings? An alternative explanation could perhaps be pathogenic bacteria, although evidence for this is still lacking (Paoletti & Saupe, 2009). Until the source, or sources, of this selection is identified, we will not truly understand the evolution of these mechanisms.

It may be useful to look towards other biological spheres for strategies of how to understand the causes balancing selection. There are some hopeful signs, with recent work on balancing selection in *Drosophila* showing that environmental variation over multiple generations (winter-adapted alleles vs. summer-adapted) leads to maintenance of intermediate frequencies (Rudman et al., 2022). This suggests that at least some balancing selection can be explained through elegant experimental designs. However, our enthusiasm should be tempered by the fact that the first balanced polymorphisms to be recognized, the A/B/O blood type system in humans, lacks an explanation to this day (Saitou & Yamamoto, 1997). Despite the clear medical implications, we still lack an explanation for the source of the balancing selection over these millions of years. Likewise, the cause of the balancing selection on the alleles at the Major Histocompatibility Loci (MHC Loci) remains unclear. Perhaps it is rare-allele advantage due to parasites/pathogens (Lobkovsky et al., 2019; Spurgin & Richardson, 2010), or perhaps it is sexual selection acting on these loci for a “good genes” hypothesis (Dandine-Roulland et al., 2019; Milinski et al., 2005)? In **Chapter 9** we showed a first examination of whether sexual selection could be contributing to the observed balancing selection. While this chapter showed no strong influence, a wider study of sexual selection in fungi seems warranted.

Since it seems that understanding the ultimate source of the balancing selection may be difficult, it may be productive to instead focus on the consequences. It remains unclear in natural settings what are the problems that can arise in the absence of a nonself recognition system. While previous experiments have shown that nonself recognition mitigates the transfer of cytoplasmic elements, these

elements often have little to no phenotypic effect in natural systems (Choi et al., 2012; Debets et al., 1994; van Diepeningen et al., 1997). This represents an unfortunate bias in mycology, as we typically isolate fungi from environmental samples that match our "idea" of what a particular fungal species should look like. This means we are likely to miss the most deleterious elements, for example a virus with very strong fitness costs is likely to strongly reduce hyphal growth. These situations are of course where the strongest evidence could be found for selection to avoid infection. One way to mitigate this is to find systems where selective isolation conditions are specific to a certain fungal species, and then to investigate *all* samples that result, regardless of morphology. This method has been used successfully using tannic acid to select for *Aspergillus niger* (van Diepeningen et al., 1997). Since this species can tolerate extremely high concentrations of tannic acid, any isolate that grows is likely to be *A. niger* regardless of morphology. Using this isolation method, several transmissible mycoviruses were recovered that had significant effects on growth and sporulation (van Diepeningen et al., 1997). Unfortunately, a sexual stage for *A. niger* remains unknown, reducing its use as a model system. Thus, future work should turn to species with a specific niche, or where a specific additive can be used for isolation. In such a system, isolating samples with reduced mycelial growth may indicate infection with some agent. Such an approach could reduce isolation bias so that strongly deleterious, but not lethal, infections can be recovered. By isolating such detrimental elements, a clearer picture of the need for nonself is likely to emerge.

The basidiomycete lifecycle and social interactions

While this thesis uses both ascomycete and basidiomycete as study systems the emphasis at the outset was towards the basidiomycetes. While it is easy to say your study organism is "interesting", for this group of fungi there are some truly remarkable aspects. The extended dikaryotic lifecycle is unique to this group and has somewhat surprising evolutionary consequences. While this dikaryotic state is genetically identical to a diploid, the co-habitation of two haploid genomes inside a single mycelium leads to unusual social interactions. While the two genomes that contribute to a diploid can be considered together as one organism (Queller & Strassmann, 2009), in a dikaryon the limits to this "organismality" are less clear. With the independent mating opportunities for the nuclei, the chance for conflict is certainly increased. The work presented in this thesis further explored this idea, suggesting in **Chapter 2** that the selective benefit of mating can persist even under a strong fitness trade-off. Fundamentally, this is because unlike diploid organisms such as plants or animals, here the evolutionary fates of the two genomes are not so tightly linked (Boomsma, 2009; Okasha, 2018). The basidiomycete lifecycle highlights the difficulty

of separating social from sexual fungal interactions, while the dikaryotic state is necessary for sexual reproduction, social forces persist within the dikaryon.

These social interactions within a dikaryon are also interesting from a cell biology point of view. The establishment of the dikaryon involves the incredibly rapid movement of nuclei, up to a speed of several millimetres per hour (Ross, 1976). Following fusion, the fertilizing nucleus races across the other mycelium, and subsequently backfills the resident mycelia by mitotic divisions. The mechanism of this rapid migration has not been explored, but a potential connection may be found in the so-called "trunk hyphae" recently described in *Coprinopsis cinerea* (Schmieder et al., 2019). At first glance, such rapid movement does not seem necessary and so it is interesting to consider what adaptive value it may have. It has been thought that this rapid movement is under sexual selection resulting from multiple simultaneous matings (Nieuwenhuis et al., 2013). Sexual selection would arise if a monokaryon fused with two other individuals within a few days of each other, the nuclei from both fertilizing partners will enter the resident monokaryon. In such a case, the speed of nuclear migration would determine the amount of fertilized area for a particular genotype. This seemed like a clear benefit of rapid nuclear migration. However, this thesis predicts a second, somewhat surprising, consequence. In **Chapter 3** we suggest that this rapid migration is necessary for the fertilization itself, independent of any sexual selection. This hypothesis suggests that in the absence of rapid nuclear migration, the nonself recognition response of the monokaryons will kill the fused cell and prevent mating itself, as discussed above.

Interestingly, the speed of nuclear migration is quite variable across basidiomycetes, from several millimetres per hour in model systems like *Schizophyllum* or *Coprinopsis* (Snider & Raper, 1958; Swiezynski & Day, 1960), to practically non-existent in *Termitomyces* or *Agaricus* (Hintz et al., 1988; Nobre et al., 2014). To the best of my knowledge, the variation of migration rates within a species has never been measured. As there is evidence for differential sexual fitness in basidiomycetes (Nieuwenhuis et al., 2013; Nogami, Kamemoto, & Kitamoto, 2002; Nogami, Kamemoto, Ohga, et al., 2002), the potential intraspecific correlation between sexual fitness and nuclear migration should be investigated further. While experimental evolution of nuclear migration rate has been suggested to understand the outcomes of such selection (Aanen, 2008), the results of such an experiment are not available. An interesting experiment by a former PhD in our group used a similar setup, but included a sexual reproduction phase, which likely added additional selective forces on the haploid phase (Nieuwenhuis & Aanen, 2018). Over the course of my thesis an experiment of repeated nuclear passage was attempted both with *S. commune* and *C. cinerea*, but technical limitations led to its abandonment (data not shown). Personal communication with Dr. Jim Anderson has indicated that they have attempted a similar experiment in *S. commune*, also beset with technical challenges.

Whether through experimental evolution, or by sampling wild diversity, this is an area of fungal biology that deserves further study.

Aspergillus fumigatus: meiosis questions and a new model for fungal cell death?

The discovery of a laboratory inducible sexual cycle in *A. fumigatus* opened up the possibility of genetics in this important human pathogenic fungus (Camps et al., 2012; O'Gorman et al., 2009; Sugui et al., 2011). However, the usefulness of genetics in this species has remained underexploited. In our study of meiotic recombination in *A. fumigatus* **(Chapter 10)** we surprisingly found a higher rate of crossovers than in any other known Eukaryotic species. To geneticists, this is extremely valuable as it greatly reduces the work required to dissect genetic traits. We showed the mapping of variation for resistance to acriflavine to an ATP-binding cassette (ABC) gene, and other groups have also recently demonstrated mapping of azole antifungal resistance (Ashton et al., 2022). But this extreme recombination rate is also of fundamental scientific interest. Is this increased crossover rate due to more double-stranded DNA breaks during meiosis, or is it that a higher fraction of such breaks is converted to crossovers instead of gene conversions? Currently we have no evidence for or against either position. Studying mutants for the *A. fumigatus* genes homologous to those known to regulate crossovers in model organisms may provide a useful starting point. A further question is how far this high recombination rate extends into the *Aspergillus* phylogenetic tree. Future work should explore the recombination rate in closely related species such as *A. oerlinhausensis, A. fisheri* and *A. lentulus.* Although some of these species are predominantly selfing, there are many genetic methods that can be used to allow genetic analysis (Bruggeman et al., 2003). The increased use of low coverage sequencing in genetics studies as we used in **Chapter 7** may also facilitate broader sampling.

Moreover, I would propose that *A. fumigatus* is useful as a model to understand fungal cell death. With our identification of the first five *het* genes in this species in **Chapter 8**, we have now identified triggers of cell death in a human pathogen. The process of heterokaryon incompatibility triggering programmed cell death has been suggested to provide insights into biology processes as well as potential therapies for human fungal pathogens (Hardwick, 2018; Kulkarni et al., 2019). The programmed cell death pathway of fungi is largely undescribed and *A. fumigatus* provides strong benefits for the study of this pathway. One is that this species is strictly outcrossing. In the 1990's the cell death pathways from the *het*R/V system in *Podospora* have been studied (Dementhon et al., 2003, 2004; Pinan-Lucarré et al., 2003). However, this work was hampered by the difficulty in isolating homozygous offspring to identify mutant genes. Working with an obligate outcrosser,

with such a high recombination rate simplifies this greatly. Furthermore, the multiple *het* genes we have identified may not utilize the same cell death pathways, increasing the knowledge that can be gained. In addition to cell death pathways, manipulation of the *het* genes in strains of *A. fumigatus* could allow for directed transmission of described hypovirulence causing viruses (Özkan & Coutts, 2015). Such use, the targeted treatment of a patient by introduction of a deleterious element would be similar to phage therapy for bacterial infections. Such knowledge of *het* genes has already proven useful to control *Cryphonectria* blight outbreaks in forest settings (Choi et al., 2012), and there is no fundamental reason that such an approach could also not be used in clinical settings. Clearly, the *het* genes of *A. fumigatus* will be providing answers to us for some time.

The fungal genome as a resource to be protected

In the preceding sections I have discussed how the regulation of social interactions restricts them to be within a genetic individual, or to between two individuals in the case of basidiomycetes. However, at some point a sexual cycle will occur, a true sharing between different genotypes, which is explored in *A. fumigatus* in **Chapter 10**. At this point, while the benefits of sex have been discussed extensively (Becks & Agrawal, 2010; Otto & Michalakis, 1998), I wish to focus on some aspects of the sharing of the genomes. As calculated by the $\bar{r}$ metric (Veller et al., 2019), for most organisms the shuffling of whole chromosome provides most of the diversity during meiosis, rather than genetic recombination through crossovers. As we show in **Chapter 5,** for parasex the reduced crossover rate does not impede the production of genetic diversity. Why then is parasex limited by heterokaryon incompatibility, while meiotic sex is not? There are a few explanations for this, some overlapping. First, it is thought that a vital component of meiosis is the single cell stage, which has important evolutionary implications (Buss, 1987; Maynard Smith & Szathmáry, 1998). As the parasexual cycle lacks this regulation, unrestricted parasex would likely lead to great increases in selfish behaviour, such as cheating. As the parasexual cycle lacks any mechanism to ensure equal segregation, the spread of selfish elements would be unrestricted. Thus, in this scenario, while it may seem that fungi should use parasex to sample increased genotypic space, the lack of a single-cell stage would result in disastrous levels of asocial behaviour. In this way, the genome of a fungal individual can be thought of as a resource, at least in the sense that it should be protected.

This concept, of the fungal genome as a resource, is also consistent with what we see in basidiomycetes. In these organisms, there is often a period of shared cytoplasmic coexistence that can last several decades. This is a social interaction between two individuals, and it is not surprising that recombination between the two

nuclei is so uncommon (Ellingboe & Raper, 1962). The alternative situation would be the merger of the two genomes into a diploid nucleus, such as seen in *Armillaria*, where somatic recombination is also rare (Anderson & Catona, 2014). The mechanisms that limit recombination in basidiomycetes are apparently much stronger than in ascomycetes, where heterokaryons are rare. While in **Chapter 2** we describe the dikaryon as sharing resources, the retention of individual genomes shows the limits of this resource sharing. To speculate on this even further, the expression of a genome can be seen as a resource in basidiomycetes. As we showed in **Chapter 6**, the effect of the partner nucleus in a dikaryon is generally to moderate expression of a monokaryon. However, other research has shown that during different developmental stages, the two nuclei have independent roles (Gehrmann et al., 2018). The two nuclei do maintain some level of independence, perhaps by limiting diffusion of transcription factors by physical nuclear spacing (Schuurs et al., 1998), as has been suggested for Arbuscular Mycorrhizal Fungi (Kokkoris et al., 2021; Limpens et al., 2022). Investigation into this in ascomycetes, where some dikaryotic species are known, is limited by the lifecycle. In *Neurospora tetrasperma*, dikaryons are almost completely homozygous, and so differential expression cannot be tested (Jacobson, 1995). A better example might be found in *Podospora anserina*, where heterozygous dikaryons can be formed, so long as certain *het* gene combinations are avoided (Ament-Velásquez et al., 2022; Chevanne et al., 2009). It would be interesting to see whether such similar nucleus-specific expression patterns could be found in an ascomycete species.

In this thesis, I have studied the social and sexual lives of fungi, and the interaction between. A few words on how this relates, if at all, to the situation in other multicellular lineages like animals and plants. In these lineages, fusion ranges from absent to rare. In the budding polyp, *Botryllus schlosseri*, there can be fusion between multicellular individuals, but this is controlled by allelic differences at a homologous gene to the vertebrate MHC genes (Weissman et al., 1990). Otherwise, in vertebrates there is a strong nonself response, from the MHC loci even in the absence of fusion between individuals (Billingham et al., 1953). The evolutionary causes for this remain unclear. In plants, fusions are more common, at least in some lineages like Chestnuts where these fusions have led to precipitous declines due to fungal pathogens. Not to mention the abundant grafting performed in agricultural settings between different individuals. While fusions are uncommon in natural settings, they are easily performed, and widely tolerated without any noticeable nonself recognition response. Possibly, this is tolerated in plants since the cells are immobile. This has been suggested as a major difference in the multicellular nature of plants *versus* animals and fungi where cells or organelles are much more mobile (Buss, 1987). However, this argument does not seem to account for the cytoplasmic connections between adjacent plant cells, termed plasmodesmata, that allow transmission of viruses and

even some cytoplasmic genetic elements (Gurdon et al., 2016). The reasons for this tolerance of fusion are still unclear, and unfortunately seem to be largely ignored by the botanical scientific community.

The interaction between sexual and social lives of fungi:

I would argue that when compared to other multicellular organisms, fungi are not so different. In general, plants and animals develop from a single diploid nucleus, which then develops mitotically. This has a major implication — a plant or animal individual is made up of clonally related cells. The individuals of plant or animals do not fuse, with some exceptions (Weissman et al., 1990), and so this progenitor cell faithfully represents the final organism. This means that social interactions, such as phloem transport in plants, or blood transport in animals can be performed. The cells that put sugars into these fluids can trust that the cells taking up the sugars are clonally related. However, in most filamentous fungi, the fusion of hyphae introduces a possibility of cheating on similar shared resources. As shown here, to ensure faithful cooperation in the absence of clonal development, a robust nonself recognition mechanism is necessary. The polygenic nature of this nonself recognition means that cooperative social interactions are restricted to clonemates, and thus fungi can be thought of as functioning like any other multicellular organism in terms of social interactions.

Sexual interactions are another often misunderstood fungal facet. For a long time, many fungi had a different Linnean binomial for the sexual (teleomorphic) and the asexual (anamorphic) stage. While this was due to historical uncertainties in the absence of DNA sequences, the asexual set of names has unfortunately persisted with some people, among whom mycologists, believing that many fungi are asexual. However, in almost all cases, detailed inquiry has shown that fungal species are sexual, just like a plant or animal (Taylor et al., 2015). This belief that some fungi may lack a sexual stage has manifested into an odd obsession with the parasexual cycle. While a parasexual stage can be demonstrated easily in a fungus, it should always be remembered that responses of nonself recognition will prevent parasex between wild isolates. As such, it seems that standard sexual reproduction should be expected to occur in all fungi, just as it is the default for all plants and animals. While exceptions do exist, like apomixis in plants, these will confirm the rule as the expectation should still be sexual reproduction.

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Summary:

Background
This thesis was initially aimed to understand the regulation of nonself recognition in Basidiomycetes. However, to understand the regulation of nonself regulation I felt it was important to study both the causes and consequences of this process. The preceding chapters provide a wide ranging survey of the topic. While some of the topics covered consider more general topics, most chapters are focused on the fungal lifecycle. The fungal lifecycle, with coenocytic hyphae which undergo anastomosis, present some unique challenges and opportunities for the definition of an individual.

Over the course of the thesis, it became clear that considering the outcome of nonself recognition in isolation was unsatisfactory. As nonself recognition effectively delimits social interactions, further study of the social interactions was needed. However, in fungi the distinction between social and sexual interactions is often unclear, and so the scope of the thesis again broadened to include the exchange of genetic material, whether through meiotic sex or parasexual interactions.

The social and sexual interactions of fungi remain elusive. Due to small size and their growth being embedded in substrate, observations are more difficult than some other organisms. As such, mycologists often retreat to laboratory observations to make inferences about nature, which undoubtably biases the inferences made. This thesis is no exception to this pattern. The methods used in this thesis only describe a facet of the fungal lifecycle, and undoubtably much interesting biology remains unexplored.

Aims
1. Understand the role of social interactions, particularly how basidiomycetes differ from ascomycetes.

2. Identify the genetic loci underlying nonself recognition in basidiomycetes, and further explore these genes in ascomycetes.

3. Explore some consequences of nonself recognition as it relates to balancing selection.

Results
In Chapter 2, I show that the social interactions in Basidiomycetes can lead to selection at the level of the nucleus, despite negative consequences to the higher-level functioning. Particularly, the unique mating system, of non-reciprocal mating between dikaryons and monokaryons, can select for mating fitness even in the

face of strong trade-offs. This chapter highlights that regulation of the dikaryotic individual is of primary importance to limit lower level selection, providing another selective benefit to nonself recognition.

In Chapter 3, I review results surrounding Basidiomycete nonself recognition, and suggest a mechanism for this phylum. I propose that the rapid nuclear migration is essential for mating, and that nonself recognition occurs even during mating, although restricted to a few cells. This hypothesis can explain how mating between dikaryons and monokaryons could occur, while restricting it to be one-directional.

In Chapter 4, I show an example of parasexual recombination between nuclei in *Aspergillus niger* and *Aspergillus fumigatus*. In contrast to Chapter 5, here we see that recombination occurs largely by sorting of chromosomes from a starting diploid, and that smaller tracts of recombination, whether gene conversion or crossovers, are rare.

In Chapter 5, I comment on a report of genetic recombination within a dikaryon, a genetic outcome of a social interaction. The initial claims were that recombination was abundant and relevant for evolution. I show that the claims made are unreliable, as applying only a few filters to the data removes almost all evidence for recombination. While parasexual recombination in these fungi has been suggested to produce diversity, there is no strong evidence that this occurs at relevant rate.

In Chapter 6, I explore the social interactions during the initial stages of a dikaryon formation. Using three highly diverse isolates of wood-rotting Basidiomycete *Schizophyllum commune*, I explore how the interaction between the two genotypes influences gene expression. While many genes are differentially expressed between the isolates during the monokaryotic stage, many of these differences are strongly reduced or eliminated during dikaryotic growth. This indicates that much, but not all, of the differences in expression are due to *trans* regulatory elements. Further, this chapter shows that the male or female role of mating does not have a strong regulatory effect, meaning that while such mating roles exist, the extension to genomic imprinting is unlikely.

In Chapter 7, I explore how nonself regulation is achieved in Basidiomycetes. Using a cross between described strains of *Coprinopsis cinerea*, I genetically map the first nonself recognition gene in this phylum. Surprisingly, the genes identified are similar to known NLR mechanisms, although with important protein domain differences that perhaps have limited bioinformatic discovery. This discovery opens the field of nonself recognition in Basidiomycetes, which has been largely stagnant for several decades.

In Chapter 8, I then describe the first *het* genes in an *Aspergillus* species. Using a cross between isolates of the human pathogen *A. fumigatus*, I describe four genes, and a fifth locus, that cause incompatibility between individuals. The genes identified encompass a wide range of molecular mechanisms, some of which are novel such as the isolated patatin domain of *het*C. Further, I show that the strong balancing selection at these loci is restricted to small regions up- and downstream of the coding sequence, without large shoulders found in other organisms. This work provides a useful starting point for further studies on fungal cell death, particularly as it targets a human fungal pathogen.

In Chapter 9, I further explore the concept of balancing selection on *het* genes using *Neurospora crassa*. While *het* genes, and nonself recognition genes in general, are known to be under balancing selection, the causes remain unclear. Demonstration of the benefit of nonself recognition diversity under artificial conditions of virus/plasmid/cheater nuclei challenge in the laboratory is routine, but alternative explanations exist. In this chapter I test whether sexual selection for diversity at *het* loci could contribute to the existing diversity. Using a set of isogenic strains, I show that there is no evidence for sexual selection at *het* loci. A limitation of this chapter is that strong inbreeding depression, in the form of bubble asci, limit the accuracy of measurements.

In Chapter 10, I explore what happens when two individuals truly decide to share genomes, in a true meiotic cycle. Using the same two strains of *A. fumigatus* as in Chapter 8, I show that *A. fumigatus* has the highest recombination rate of any described eukaryote. This recombination rate likely explains the narrow footprint of balancing selection from Chapter 8. While the reasons for this high recombination rate are unclear, this fact facilitates future genetic studies in this organism.

Future Prospects

The field of nonself recognition is undergoing a period of revival. Initially studied only phenotypically, several decades of molecular biology built the framework to truly understand this process. However, the recent technological progress in DNA sequencing is rapidly allowing the understanding of this process at a population level. It appears that nonself recognition is increasingly being seen as a social interaction, which helps to anchor it in an evolutionary framework. This evolutionary framework will hopefully assist to anchor future results.

As nonself recognition prevents fusion between fungal individuals, its manipulation will likely prove useful in the future. It has been suggested that this process may provide a druggable target for filamentous pathogens like *A. fumigatus*, which this thesis provides a hopefully useful starting point. Furthermore, this knowledge may provide useful clues for developments in biotechnologically

relevant *Aspergillus niger*, where parasex is currently limited to isogenic strains. The initial goal of the funding for this project, the bypassing of nonself regulation to allow mixing during mushroom cultivation may be more difficult than anticipated but understanding the role of the common nucleus may provide a path forward.

Acknowledgements

This thesis, like all good scientific work, was not performed in isolation. Any progress I have made was only possible with the group I was working in. The contributions of everyone listed below, including those I unfortunately have overlooked, have helped me immensely towards this goal. In his final interview, the astronomer Carl Sagan said “Science is a way of thinking”, and all of your inputs are fundamental to help me “sceptically interrogate the universe”.

First I must mention Duur. In 2017 I emailed you basically asking “can we do a PhD together?”. Luckily, we were able to briefly meet that summer, and then you were able to secure funding for my project. It seemed a bit surreal, moving to Europe to agree to work for 4+ years with someone whom I had only met for a few hours. But I did, and it worked out better than I could have hoped. You showed me how a scientist should question all the assumptions of those around you. Most importantly, you showed me that it is important to be curious about everything around you, the most fundamental insights often come from the most basic observations.

Bas, your role in the beginning was more the formal role of Promoter. However, over time your role as advisor became increasingly important. Your insights into how to navigate the political waters of Science have been invaluable. You have shown how to swim upstream, and how to challenge the system whenever necessary.

Fons, in the beginning of my PhD you seemed to have a smaller role about technical genetic matters. However, over the years I transitioned more and more towards *Aspergillus* and *Neurospora*, for which you provided a wealth of experience. Your creativity for new experiments while still remembering what has been done before is an inspiration. Your passion for teaching genetics of all kinds to students has been infectious, and if I can have even a fraction of that enthusiasm, I will be satisfied.

Eveline, when I started my PhD, you were a post-doc working on *Aspergillus*, but at one point Fons suggested we work together, starting our “heterokaryon meetings”. This developed into quite a few projects and working with you has been very enjoyable. I hope our attempts to dismantle the system continue.

Around half of the work in this thesis is experimental laboratory work. I need to thank Eric particularly for showing me all the different tricks for both *Neurospora* and *Aspergillus*, and how powerful these systems can be. I am eternally indebted

to Frank for showing me the magic of SPRI beads and 96-well plates, which opened up a world of experiments I didn't think possible. And Francisca, your assistance the last few years has been essential for getting the molecular biology to work, as well as providing valuable feedback on matters both scientific and social.

However, the laboratory work is not only detailed technical work but also the general atmosphere, Gabriella and Jordy, it has been very enjoyable discussing all kinds of topics in the labs over the years, something I hope can continue. The energy in the lab would not be the same without Corrie and José keeping control of the chaos (at least usually).

Of the former "Group Duur" members, I should thank Sabine and Margo for showing me how to work with *Termitomyces*. Although this fungus somehow did not end up in this thesis, it did occupy quite a bit of my time. While I never worked with you Alex, you still showed to me the importance of repeating experiments, and rigorously testing the data before you can trust it. And of course, Lennart and Matthijs for making the working atmosphere more fun, particularly those very long lunches discussing the topic of the day. Anouk, while you joined later in my time in the group, it was very enjoyable working with you. While you were not a true "Group Duur" member Krithi, your enthusiasm and love of large experiments really inspired some of the larger project that I did in my PhD. You taught me that a few hundred petri plates is nothing to be afraid of.

I particularly thank Jitpanu, René, Roel and Tom for showing me what can be done with some backcrossed and some inbred lines. Seeing how much you plant people suffer to map genes gives me great inspiration for what is possible with genetics.

I also need to thank the other members of the Laboratory of Genetics. Having people like Arjan, Sijmen, Hilje, Joost and Joost, provided many opportunities to discuss scientific ideas. These valuable conversations contributed greatly to my development during my PhD. Wytske and Marjan, without your help I think I would have been buried under a mountain of paperwork.

Contributing to the social and scientific atmosphere, particularly on Friday Afternoons/Evenings; Alanna, Francesco, Helena, Jitpanu, Louise, Mariska, Mariana, Murambiwa, René, Roel, Suzette, and Tom. The weekly Vrijmibo made this whole process a lot more fun.

The research of this PhD would also not have been possible without the assistance of student thesis projects. In approximate order of appearance: Timon, Anqi, Binghan, Chantal, Julia, Mayke, Serena, Jorrit, Elbert, Sarah, Jan-Pieter, Kira, Wendy, Axel, Niels, Fleur, Bram, Jamie, Leonne, Floor, Ymke, Floris, Astrid and Haarika. Although much of your work does not make it to the final research output, it does not make the work any less valuable. And the additional pleasant working experiences made my entire PhD much more enjoyable.

I must acknowledge the role my supportive wife Jenny has played in all of this. First off, being willing to move to Europe allowed this whole crazy adventure to happen in the first place. While here, you started your own PhD as well, and having you as a sounding board for all my ideas over the years has helped immensely. During corona we were basically two PhD students locked up in a prison cell, and all the discussions about “what is science” or “how to define truth” has made me a better scientist, and a better person.

About the Author:

Ben Auxier was born in Vancouver, Canada on July 19th 1985. Growing up in Vancouver, first worked in several horticultural greenhouses in various positions. He then started university studies with his BSc (Honours) in Plant Biology in 2016 in the laboratory of Dr. Mary Berbee. Staying at UBC he then completed an MSc thesis working on fungal cytoskeleton research in 2018 also with Dr. Berbee. His time at UBC was complemented by a research exchange to the Univeristy of Georgia to work in the laboratory of Dr. Michelle Momany. Following this, and a research internship in the lab of Dr. Eva Stuckenbrock, he moved to the Netherlands in 2018 to start his PhD.

During his time at WUR, Ben was part of various committees including the Wageningen Ecology and Evolution Seminar (WEES) series, and the Netherlands Society for Evolutionary Biology (NLSEB) Phd/Postdoc Organizing Committee.

Following the completion of this thesis, Ben will start as an Assistant Professor at Wageningen University. As the Dutch lifestyle, specifically the cycling culture, has been positive since moving here he expects this next career stage to be both exciting and enjoyable.

Other Publications by the Author:

Leale A, **Auxier B**, Smid E, & S Schoustra. Community diversity affects functionality and species sorting during propagation of a natural microbial community (2023). bioRxiv. 2023.02.24.529839

Vreeburg S, **Auxier B,** Jacobs B, Bourke PM, van den Heuvel J, Zwaan BJ, & DK Aanen. (2023) A genetic linkage map and improved genome assembly of the termite symbiont *Termitomyces cryptogamus*. *BMC Genomics* in press

Van Creij J, van Creij J, **Auxier B,** An J, Wijfjes RY, Bergin C, ... & E Limpens (2023). Stochastic nuclear organization and host-dependent allele contribution in *Rhizophagus irregularis*. *BMC Genomics 24*(1), 53

van de Peppel LJ, de Beer ZW, Aanen D K, & **Auxier, B.** (2022). Termitomyces cryptogamus sp. nov. associated with *Macrotermes natalensis* in Africa. *Mycotaxon, 137*(1), 41-50

Mateus ID, **Auxier B,** Ndiaye MM, Cruz J, Lee SJ, & IR Sanders (2022). Reciprocal recombination genomic signatures in the symbiotic arbuscular mycorrhizal fungi *Rhizophagus irregularis.* PLoS One 17(7), e0270481

van de Peppel LJ, Nieuwenhuis M, **Auxier B,** Grum-Grzhimaylo AA, Cárdenas ME, de Beer ZW, ... & DK Aanen (2021). Ancestral predisposition toward a domesticated lifestyle in the termite-cultivated fungus Termitomyces. *Current Biology, 31*(19), 4413-4421

Auxier B, Dee J, Berbee ML, & Momany M (2019). Diversity of opisthokont septin proteins reveals structural constraints and conserved motifs. BMC evolutionary biology, 19(1), 1-11

Bazzicalupo A, Berch S, … **Auxier B** … & M Berbee (2017). White paper on strategies to reduce risks and expand appreciation of foraged wild mushrooms. McIlvainea: Journal of American Amateur Mycology, 26

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Elmhirst, JF, **Auxier B**, & Wegener LA (2013). First report of box blight caused by *Cylindrocladium pseudonaviculatum* (*C. buxicola*) in British Columbia, Canada. *Plant Disease, 97*(4), 559-559

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review / project proposal (4.5 ECTS)

- Allorecognition of basidiomycete fungi

Post-graduate courses (4 ECTS)

- Evolutionary quantitative genetics workshop; Friday Harbour Labs (2017)
- SLiM Genetic modelling Workshop; Messler Lab (2020)

Invited review of journal manuscripts (4 ECTS)

- Bioessays; ancient fungi (2019)
- Functional Ecology: ant fungi (2019)
- G3: aspergillus genomics (2021)
- Frontiers in Cell. and Inf. Micro.: aspergillus genetics (2022)

Competence strengthening / skills courses (0.6 ECTS)

- Publication of comment on Chen et al. eLife manuscript; Ben Auxier (2019)
- Genetics society of America career development day; GSA (2019)

Scientific integrity / ethics in science activities (0.6 ECTS)

- Research integrity; PE&RC (2021)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.6 ECTS)

- PE&RC Day (2018, 2022)

Discussion groups / local seminars or scientific meetings (7.4 ECTS)

- NLSEB (2018)
- KNMV (2018)
- WEES (2018-2022)
- NLSEB; poster presentation (2019)
- KNMV; oral presentation (2019)
- Evolution Journal Club (2019-2022)
- Canadian Fungal Network (2020)
- Evolution-Ecology (2020-2022)
- KNMV (2021)
- NVMM; oral presentation (2021)

International symposia, workshops and conferences (8.4 ECTS)

- EMPSEB; oral presentation; Spain (2018)

- ESEB; poster presentation Finland (2019)
- Asilomar fungal genetics; poster presentation; USA (2019)
- Asilomar fungal genetics; oral presentation USA (2022)

Committee work (3 ECTS)

- Committee member Wageningen evolution and ecology seminar series (2018-2020)
- Graduate student representative for ESEB board of directors (2019)
- NLSEB PhD/Post-doc meeting organization committee (2019-2020)

Lecturing/Supervision of practicals/tutorials (3.6 ECTS)

- GATC (2018-2022)
- Population and quantitative genetics (2019)
- PE&RC Ecological and evolutionary genomics (2019)

BSc/MSc thesis supervision (15 ECTS)

- Non-self recognition in fungi
- Sexual selection in fungi
- Population genetics of fungi

The research described in this thesis was financially supported by the Dutch Research Council NWO (ALWGR.2017.010)

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

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