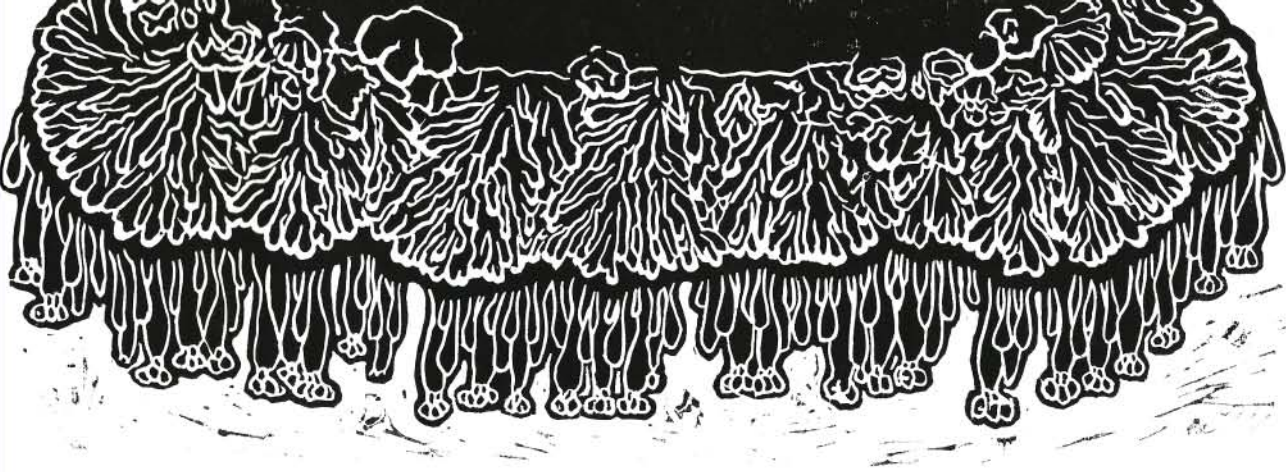
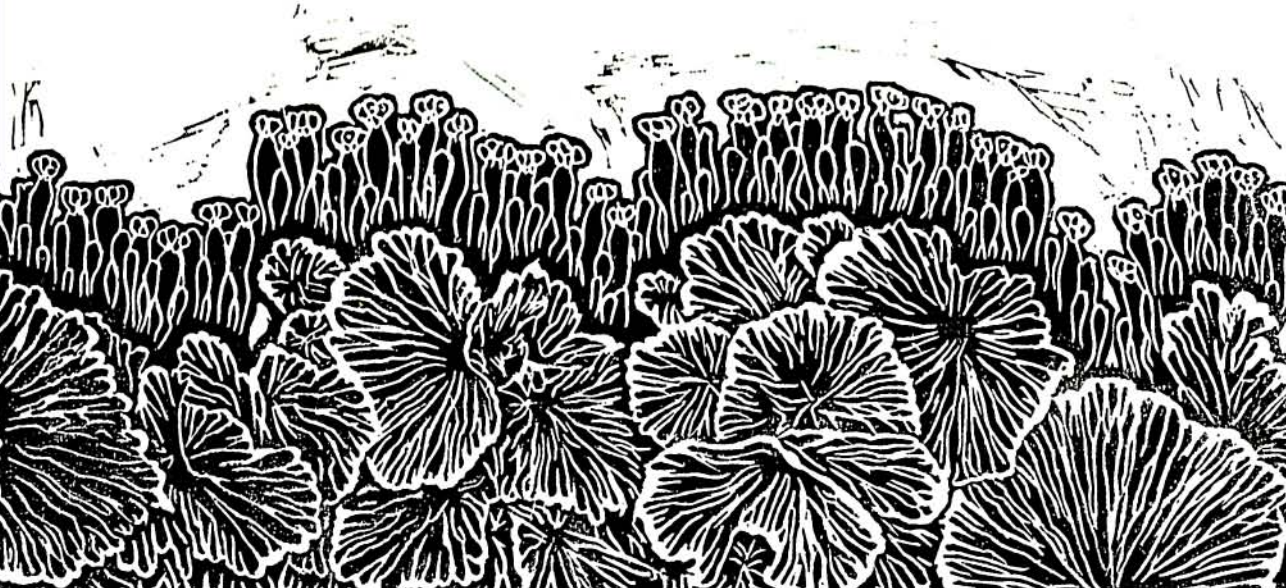


Sexual selection in fungi

Bart P.S. Nieuwenhuis



Sexual selection in Fungi

Bart P. S. Nieuwenhuis

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Sexual selection in Fungi

Bart P. S. Nieuwenhuis

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Contents

Chapter 1

General introduction

Bart P. S. Nieuwenhuis

Sexual selection

The theory of natural selection tells us that the individuals best adapted to their environment will survive best and get most offspring. The genes that code for these characteristics will increase in frequency and as a result extremely well adapted species evolve: perfectly streamlined fish, octopuses with environment dependent camouflage, or plants that catch insects in nutrient poor soils. On the other hand, any trait that is maladaptive is expected to be selected against. Nevertheless, there are many species with traits that appear not well adapted to their environment at all, and these traits are often present in only one of the two sexes. For instance, male Montezuma swordtail fish with large tails swim less well, than males with smaller tails, which reduces their chance to escape predators (Kruesi & Alcaraz, 2007) and male collared lizards with more conspicuous coloration have an increased predation risk (Husak et al., 2006). Even though these traits lead to reduced survivorship, long tails or colourful skins are very common in natural populations of these species.

Darwin was puzzled by these traits that appear to be detrimental for survival but are nevertheless maintained. Already in 'On the Origin of Species' (Darwin, 1859) he documented that such traits might give an advantage for a different component of natural selection than survival, *viz.* the relative mating success. He expanded on this theory in 'The Descent of Man' (Darwin, 1871). Next to staying alive, an individual of an outcrossing sexually reproducing species needs to find a mating partner and successfully mate in order to reproduce. The traits that seem to be a burden might actually contribute to success in mating. This part of natural selection is known as *sexual selection*.

In many species, males have the opportunity to mate with multiple females and produce offspring with all of them. Potentially, one male can have a large share of the total offspring in the population, while other males reproduce much less or not at all. The number of offspring mainly depends on the number of females he mates with and, if a female mates with multiple males, the proportion of offspring per female. Because females can only produce a limited number of offspring, they are usually limiting for reproduction. This results in an operational sex ratio that is effectively skewed towards the males and thus the males will be in competition for the females. The more partners a male can mate with, the larger his share of the next generation will be, and, therefore, the higher his fitness. A female only needs to mate enough to get all her eggs fertilized, and can suffice with a few or even a single mating, and mating more often will not increase her fitness (Bateman, 1948). The most successful male is the one that is most effective in competition for the females. Males actively fight over females, as for instance seen in stags that gather the greatest harem. Another common scenario is when males are in competition to be chosen by the female. Because there are effectively more males that compete for each female, the females can be choosy to take only that mate (or those mates) that are most beneficial for her fitness. Famous examples of female choice are birds in which the males are showing off their quality, by singing, beautiful plumage, or performing complicated dances. Even though in general the males are in competition with each other for the females, competition is not restricted to

males. Females compete with each other for males that can supply them with the best territory, paternal help, or that increase offspring quality (Clutton-Brock, 2009).

Also during and after copulation, competition for fertilization continues between the gametes (Parker, 1970). If a female has mated multiple times, sperm from different males compete to fertilize the eggs. Different traits to increase this post-copulation competitiveness have evolved. For instance, in many species the penis has a dual function; next to transferring sperm, it removes sperm from competing males that mated with that female earlier, thus reducing direct sperm competition (e.g. Waage, 1979). Still, the female can affect the outcome of post-copulatory competition through female cryptic choice (Eberhard, 1996). In response males try to manipulate the female to use their sperm, for instance by producing seminal fluid proteins (Chapman, 2001). In many seed beetles, the male have spines on the penis that brutally harm the female genitalia to discourage her from re-mating at all, thereby monopolizing the female and her gametes (Hotzy & Arnqvist, 2009). Even when manipulations by the male are harmful to the female, and reduce her fitness, selection might favour such adaptations if the male manipulation increases his fitness (Parker, 2006).

Sexual selection is an interesting driver of evolution, because it can select for traits that are harmful for viability and the probability to survive. Since individuals are only in competition over mating with individuals of their own sex, traits that increase successful mating but reduce survival, will most likely only come to expression in the sex where it is beneficial. Sexual selection can thus lead to dimorphism between males and females that are not primarily meant for reproduction.

Sexual selection theory is mostly applied to animals (Andersson, 1994; Carranza, 2009). Recently, sexual selection has also been recognized in plants, where selection has led to impressive flowers for attracting pollinators (Andersson & Simmons, 2006), and male pollen are competing to fertilize the female ovules (Snow & Spira, 1991). In sharp contrast, in fungi, sexual selection has not been generally considered. The traits in fungi that might be under sexual selection are less obvious than those investigated in macro-organisms, and in fungi there is no separation into males and females. Even though sexual selection in fungi is not so easily observed, fungi are not fundamentally different from other organisms: for sexual reproduction mating has to occur, and during mating different individuals can compete to increase their number of matings. In this thesis, I aim to show that sexual selection is also acting in fungi and I argue that it shapes the evolution of traits involved in fungal mating.

Fungi as model organisms

Many fundamental questions on the evolution and maintenance of sex and the mechanisms of sexual reproduction remain to be answered, such as: What are the benefits of sexual reproduction? How do sex chromosomes evolve? How does meiosis work? Many of these questions can be studied using fungi, because the fungal kingdom is very diverse. Many different mating systems (e.g. inbreeding, selfing) and breeding systems (e.g. sexual compatibility) are present

in fungi, which makes them very interesting to study (Billiard et al., 2012). Comparing related species with different systems (e.g. López-Villavicencio et al., 2010), or unrelated species that show convergent evolution (e.g. Billiard et al., 2011) can be used to answer questions on how and when sex occurs.

Fungi have been used for many years to study sexual reproduction. In contrast to most animals and plants, most fungi are haploid for a large part of their life cycle. Whereas in a diploid organism the two different alleles at a locus both can influence a trait of interest, in a haploid organism there is only one copy and its effect can be seen immediately. Another very convenient factor is that fungi produce meiotic products that remain together. This gives the opportunity to investigate how crossing-over occurs during a single meiosis. The four haploid gametes can be grown separately and a trait or gene of interest can be followed through meiosis. Add to this that most fungi can be maintained in the laboratory practically indefinitely, that they can be multiplied clonally and genetically modified rather easily, and it is clear that fungi are great model organisms. It is not surprising that many important discoveries in amongst others molecular and cell biology were achieved using fungi as model systems (Stajich et al., 2009).

Fungi in nature: the great unknown

Even though much studies have been performed on fungi in the laboratory, and from this much has been learned, very little is known about how these mechanisms came about. For instance in *Neurospora crassa* – a fungus used as a model species for many decades – mechanisms such as cell growth, circadian rhythm or fusion have been investigated (Iyer, 2010; Roca et al., 2010; Baker et al., 2012), but very little is known about the role of these mechanisms in natural populations. Only sporadically, studies are performed that investigate evolutionary forces that shaped such traits (e.g. Ellison et al., 2011). Also on the mechanisms of fungal mating and fertilization, much knowledge has been obtained (extensively reviewed in Heitman et al., 2007), but very little is known on their actual function. And when the function is investigated, because most investigation were performed in laboratory settings, the traits observed might function differently or might even not function at all under natural conditions.

To better understand the function of the various sometimes very complex mating mechanisms, it is of importance to understand how selection acts on them. Because most fungi reproduce sexually and most of the sexually reproducing species are obligatorily outcrossing, they need to find a partner to perform sexual reproduction (Whitehouse, 1949; Raper, 1966; Kües et al., 2011). The complex mechanisms observed in laboratory conditions are very well likely to have evolved to increase fitness by assuring mate acquisition, but this has not been studied in great detail. In this thesis I focus on those selective forces that might have shaped traits to regulate and possibly manipulate mating opportunities.

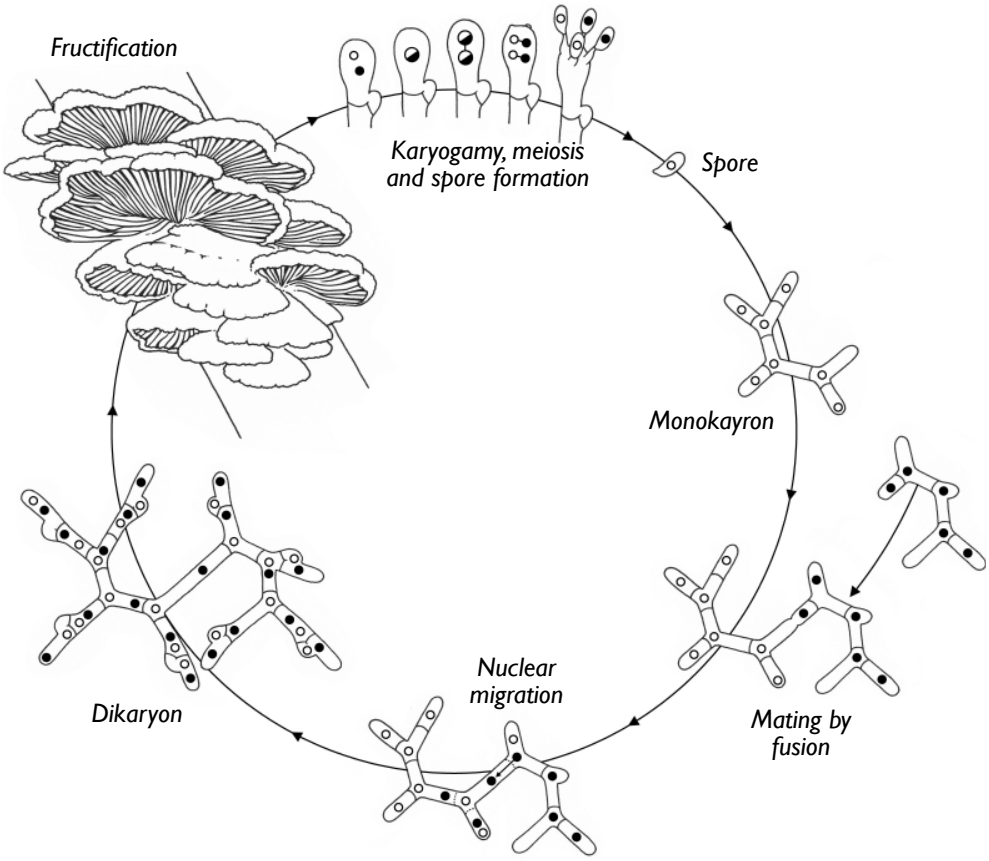

Figure 1.1 Life-cycle of *Schizophyllum commune*. A meiotically produced haploid spore germinates to form a mycelium. The mycelium grows as a monokaryon, in which each hyphal compartment contains a single nucleus. When two compatible monokaryons meet, they fuse and reciprocally exchange nuclei. The nuclei divide and migrate into each other's mycelium. When the entire mycelium is dikaryotized, growth continues vegetatively by cell divisions with clamp connections. A dikaryon is capable of producing fruiting bodies, the mushrooms, on which basidia are located. A basidium is a specialized cell in which the two haploid nuclei fuse and form a diploid nucleus that immediately goes into meiosis. The four meiotic products are wrapped separately into individual spores. These spores disperse via the air. Drawing by Marc Maas.

Schizophyllum commune

For the research described in this thesis I used the mushroom-forming basidiomycete species *Schizophyllum commune*. This species is a saprotrophic fungus and opportunistic tree pathogen with a worldwide distribution. The many spores that are produced disperse over large distances – even far out on sea spores can be collected – which results in a population structure at the continent level, but with occasional gene flow between the subpopulations, thus maintaining species integrity (James et al., 2001; James & Vilgalys, 2001). Probably due to the high spore

density, *S. commune* spores are inhaled regularly what in immuno-deficient humans can lead to infection of respiratory tracts. *S. commune* has recently been classified an emergent fungal pathogen (Chowdhary et al., 2012). The species has been used for many years as a model species in mushroom-fungus research (Raper, 1966), and its full genome was one of the first basidiomycete genomes published (Ohm et al., 2010b).

The mushroom-forming fungi or Agaricomycotina (from here on *mushrooms*) are part of the Basidiomycota, one of the two major clades of fungi (~34% of described fungi; Hibbett et al., 2007; Stajich et al., 2009). Much is known about sexual reproduction in mushrooms and the largest part of this thesis will thus be on sexual selection in this group of fungi. In chapter 7 sexual selection will be discussed more generally, not just in basidiomycetes, but also in the other main fungal clade, the Ascomycota, which is the sister-group of Basidiomycota (~64% of described species; Stajich et al., 2009). Unfortunately, very little is known about mating in fungi of the smaller clades, and only when possible, references to these groups will be made. To better understand how and when mating plays a role in the fungal life cycle, and to understand how sexual reproduction influences fungal fitness, I will first describe the life cycle of *S. commune* (see Fig 1.1). The *S. commune* life cycle is a schoolbook example of the generalized basidiomycete life cycle, but within the basidiomycetes there are many variations to this it, which will be mentioned throughout this thesis, when necessary.

Generalized life cycle of a mushroom fungus

Mushrooms produce haploid spores which function mainly for dispersal. A spore that lands on a suitable substrate can germinate and form a haploid mycelium. The mycelium consists of connected hyphae, which are tube-like cells compartmentalized by septa. In most basidiomycetes, each compartment contains a single haploid nucleus (this mycelium is referred to as a monokaryon, for *single nucleus*), but in many species each compartment contains multiple haploid nuclei (a homokaryon, for *similar nucleus*, because all nuclei are genetically identical). The monokaryon continues to grow asexually and occupies substratum from which it extracts resources, which can later be used to produce offspring – for *S. commune* in the form of spores. The size of a mycelium is therefore highly correlated with its fitness (Pringle & Taylor, 2002). Contrary to most other fungi, most mushrooms cannot produce asexual propagules for dispersal, but can only disperse via sexual spores (although some species do produce asexual 'oidia' on their mycelia; Ramsdale & Rayner, 1994; Polak et al., 1997). Only after fertilization, sexual spores can be produced.

Fertilization of a monokaryon occurs when the mycelium encounters another individual. According to the textbooks, a monokaryon grows until it meets another monokaryotic mycelium and when the two mycelia are compatible, fertilization will occur (but see Chapter 3). Compatibility is controlled by the mating-type system, which will be discussed in Chapter 2. The two monokaryons will fuse and reciprocally exchange nuclei. Each monokaryon donates nuclei to the other mycelium, which incorporates them into its own cytoplasm. These haploid nuclei divide (Kües, 2000) and are actively transported through the entire mycelium (Gladfelter & Berman,

2009), until the mycelium is fertilized completely (Buller, 1930). Unlike other eukaryote kingdoms, in which nuclei fuse immediately after fertilization, in ascomycetes and basidiomycetes the two haploid nuclei remain separate for an extended period. In ascomycetes this stage generally last only a few mitotic divisions, but in basidiomycetes this so-called dikaryon (or heterokaryon for species with multiple nuclei per compartment) can last indefinitely (Anderson & Kohn, 2007). The basidiomycete dikaryon grows in a complex way by producing a short side-branch which immediately fuses back to the main hypha, forming a so-called clamp (see next paragraph 'Nuclear competition and conflict in basidiomycetes' and Chapter 5). A dikaryon continues to grow vegetatively and thereby increases occupied substrate and resources. Often induced by external stimuli, the dikaryon will produce fruiting bodies, the mushrooms, on which basidia are located. Basidia are specialized cells in which the nuclei fuse to form a diploid nucleus, immediately followed by meiosis and formation of four sexual spores. One mushroom can produce billions of spores and each spore can disperse independently to establish a new monokaryon.

Nuclear competition and conflict in basidiomycetes

The uniqueness of cells in which two haploid nuclei remain separate makes the basidiomycete fungi very interesting for the study of sexual selection. The two genomes in the dikaryon work together to increase the fitness of the mycelium, and thereby increase their own and each other's fitness. Because the two remain separate, one nucleus can also increase its own fitness, at a cost of fitness at the dikaryon level, as long as its increase in personal fitness sufficiently compensates for this loss. This is in contrast to diploid organisms in which the two genomes fuse into a single diploid nucleus, and remain together until the moment of meiosis. In diploids only during meiosis one allele can increase its fitness at a cost to the other (Haig, 2010), because at any other moment the two copies remain together and thus their fate is linked.

Buss (1987) saw this potential for conflict between the two levels of selection – the level of the mycelium and of the individual nuclei within the mycelium – and suggested that, in order to resolve the conflict, the two nuclei must keep each other in control so that neither will be cheated by the other. In basidiomycetes, a complex system of nuclear division has evolved, which limits the possibilities for nuclear cheating. During each cell division, the two nuclei divide synchronously, followed by growth with clamp connections, which leads to an equal distribution of exactly two different nuclei over the newly formed cells (Iwasa et al., 1998). In species that do not have highly regulated growth, one of the nuclei can increase in the mycelium and produce hyphae with only one type of nuclei (Fig 1.2a; e.g. Ramsdale, 1999).

In *S. commune*, growth is highly regulated and during vegetative growth, cheating is not possible. However, when a dikaryon meets a monokaryon, generally both nuclear types in the dikaryon can perform fertilization of the monokaryon (Fig 1.2b). The entire domain occupied by the monokaryon becomes available to the nucleus that fertilizes the monokaryon, and therefore the nucleus that performs the fertilization potentially increases its fitness considerably (Pringle & Taylor, 2002; Anderson & Kohn, 2007; Stenlid et al., 2008). Next to direct competition for

fertilization between the nuclei in a dikaryon, other types of competition for mates are expected to occur in nature, for example between multiple spores landing on a compatible monokaryon. In this thesis, I investigated the possibilities for competition during mating in fungi.

Setup and aim of the thesis

This thesis aims to prove that also fungi are under sexual selection – a selective pressure that has not been generally considered for this group of organisms. Furthermore, it aims to describe under which circumstances sexual selection is of importance, using the mushroom fungus *S. commune* as a model system.

One of the major traits likely to be under sexual selection in fungi is the mating type. Mating types are genetically defined mechanisms that describe sexual compatibility between gametes and in most species regulate mating. Sexual selection acts during mating, and therefore has an effect on the genes that define the mating types. In its turn, sexual selection is affected by

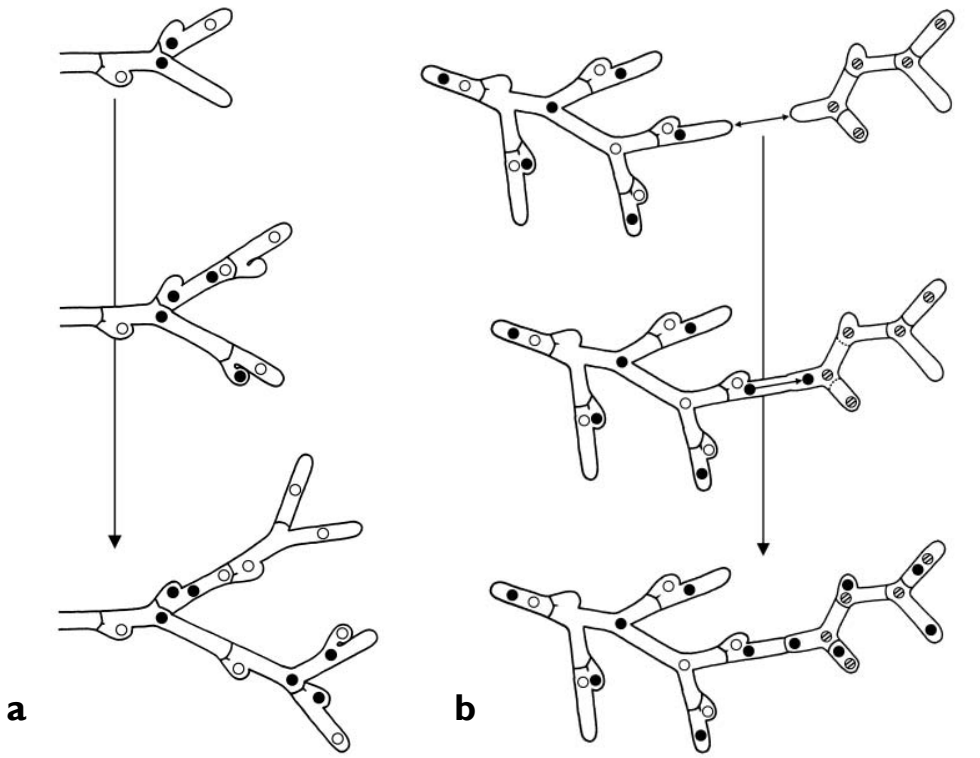

Figure 1.2 Two possibilities in which one of the nuclei in a dikaryon can break free from the other nucleus in the mycelium and increase its fitness individually. **a)** One of the nuclei in a dikaryon escapes from the mycelium and continues to grow as a monokaryon. This is very uncommon in species with clamp connections. **b)** During mating one of the two nuclei can escape by fertilizing a monokaryon. During such a dikaryon-monokaryon mating only one of the two can get established in the original monokaryon, while the other nucleus is restricted to the original dikaryon. Drawing by Marc Maas.

the mating types themselves. Mating can only take place between gametes of different mating types, and competition for mates is therefore different for gametes of different mating types. Due to the central role of mating types during mating, throughout this thesis mating types and their functions will be discussed. In **Chapter 2**, I start with clearly describing what mating types are, and what their function is in mating. I will focus mainly on the difference between mating types and sexes (males and female). Both sexes and mating types are of crucial importance for fungal mating, and even though sexes and mating types both describe if syngamy between gametes is possible, they are very different.

Using the model organism *S. commune*, I will then show in four consecutive chapters, that sexual selection also plays a role in fungi. **Chapter 3** describes how mating occurs in nature and that in natural settings multiple mating occurs. This is an important finding, because for sexual selection to act, multiple individuals must meet for competition and choice to be possible. In **Chapter 4**, we tested if the relative mating success of the two nuclei of a dikaryon, competing to fertilize a monokaryon, is genetically defined, and whether this success depends on the receiving monokaryon. By repeatedly performing dikaryon-monokaryon matings, the relative competitive ability of different nuclei could be assessed as well as the role of the receiving mycelium during mating. In **Chapter 5** we assessed whether the consistently observed difference between nuclei fertilizing a mycelium is due to suppression of mitosis in the other nucleus. In **Chapter 6**, the final empirical chapter, an experimental-evolution approach was taken to investigate if strains of *S. commune* could increase in their competitive ability to fertilize a non-evolving monokaryotic mycelium. The chapter describes the evolution experiment, the adaptations that occurred during evolution, and compares these findings with adaptations in plants and animals.

Sexual selection not only plays a role in mushroom-forming basidiomycetes, but also in other fungi. In **Chapter 7** we give an overview of the general importance of mating in fungal evolution and show that during mating there are many opportunities for sexual selection to affect evolution. In this chapter, we give examples from nature, and show directions for further research on what to focus to investigate sexual selection in fungi. Additionally we show that fungi are good model organisms to experimentally study fundamental aspects of sexual selection.

Chapter 8 integrates the results from previous chapters. I will give an overview of the important findings for sexual selection in *S. commune* and elaborate on the costs and benefits of mating and sexual selection for the receiving mycelium, as well as for the migrating nuclei. Finally I will present additional ideas and give directions for further research.

Chapter 2

Why mating types are not sexes

Bart P.S. Nieuwenhuis, Manuela López-Villavicencio &
Duur K. Aanen

Summary

Mating types are often equated with sexes. However, although both refer to sexual compatibility and asymmetry between gametes, they are not the same. Sexes are defined by the difference in size of their gametes (anisogamy). In contrast, mating type refers to genetic mechanisms allowing discrimination between gametes, independent of size dimorphism, a characteristic that is mainly seen in fungi, algae, flowering plants and protozoa. Because mating types generally are decoupled from sexes, gametes of isogamous species (i.e. species with no sexes), as well as gametes of anisogamous species, can have mating types. In this paper we clearly introduce sexes and mating types, and show the differences between them. We focus on the evolutionary origin, the implications on mating, and indicate the evolutionary consequences of mating types and sexes. Both, genetic mechanisms of disassortative mating (i.e. mating types) and developmental mechanisms that regulate disassortative mating between gametes of different sexes are discussed. We argue that comparing the similarities and differences between mating types and sexes can yield important insights in the evolutionary forces that lead to and maintain asymmetry during mating, but that to make such comparisons and have a clear discussion, we need to be precise and explicit in defining them.

Introduction

Although sexes and mating types are both characteristics of sexually reproducing organisms and refer to sexual compatibility between individuals or gametes, they are not synonyms. Intuitively, people know what sexes are, especially for animals, but the identification of sexes in other organisms is often less trivial. Probably because sexes are more intuitive than mating types, already in the first account of mating types, these were referred to as sexes (Blakeslee, 1904a; Blakeslee, 1904b). But also in recent literature, mating types are often still equated with sexes (e.g. Kothe, 1996; Ni et al., 2011).

This mix-up does not necessarily lead to incorrect conclusions – sexes and mating types can have the same evolutionary consequences – but it can work confusing. Because mating types and sexes are affected differently by natural and sexual selection, it is important to be precise when using these terms. This is of special importance because mating types and sexes can influence each other's evolution.

Here, we will clearly introduce sexes and mating types, and describe the different opinions on the evolutionary origin of sexes and of mating types and their subsequent evolution. As the evolution of mating types and sexes (anisogamy, see below) has recently been reviewed extensively (anisogamy in Togashi & Cox, 2011, mating types in Billiard et al., 2011, and their interplay in Lessells et al., 2009), our focus will be on the distinction between mating types and sexes.

Defining sexes

According to the Oxford English Dictionary, the word sex is derived from the Latin word *sexus* meaning 'either of two divisions of organic nature distinguished as male or female, respectively'. This definition limits the number of sexes to two.

The fundamental biological difference between the sexes is in the type of gametes produced: males produce many small motile gametes, whereas females produce fewer large immotile gametes. These gametes are often referred to as 'male' (e.g. pollen or sperm) and 'female' gametes (e.g. the eggs), respectively. Generally, gametes of one sex cannot fuse with gametes of the same sex. Hermaphrodites do not violate our maximum of two sexes, because they are not a third sex, but have both sex functions – a single individual can produce both male and female gametes.

There are many characteristics by which the gametes can be distinguished and some researchers have argued that any characteristic that leads to bimodality during mating can be used as a criterion to distinguish sexes (e.g. Hoekstra, 1990). The main characteristics of gametes associated with differences between the two sexes are: i) size- and number dimorphism, ii) incompatibility between gametes of the same type, iii) motility difference, and iv) contribution of cytoplasmic genes.

Box 2.1 – Characteristics to define gamete sex

In general, male gametes are motile, small and contribute no resources to the zygote, while female gametes are immotile, large and account for almost all resources for the zygote. As discussed in the main text, gamete size is the most commonly used characteristic for defining the sex of a gamete. Could incompatibility, cytoplasmic gene contribution or motility also be used to define the sexes of gametes?

Incompatibility

Incompatibility is a clear gamete trait by which gametes can be assigned to a different group. A problem here is that no sex can be assigned to a certain group when there are no other gamete characteristics to base this assignment on. In isogamous species, groups are therefore often referred to as + and –, or *a* and α, and not male or female, because both types are phenotypically identical. Sexes can therefore not be assigned to incompatibility of the mating types (see also Box 2.2).

Cytoplasmic contribution

Another difference between gametes is that one gamete type usually contributes most of the cytoplasm and cytoplasmic organelles to the zygote, which is the large gamete class (female), and one does not, which is the small gamete class (male). Also mating types often regulate the transmission of organelles, and it has therefore been proposed to use this criterion as the defining characteristic of the sexes (Hurst and Hamilton, 1992). Again, defining sexes based on this is not universal. There are two clear objections. First, in some gymnosperm species mitochondrial genes are maternally inherited, and chloroplast genes paternally (Neale & Sederoff, 1989). Second, in some species of slime-molds cytoplasmic inheritance depends on the hierarchy of the gamete with which mating occurs. If gamete X mates with Y, X transmits both nuclear and cytoplasmic genes and Y only nuclear genes, but when X mates with Z, it can be Z that transmits cytoplasmic genes (Moriyama & Kawano, 2003). In such a case gamete nuclei could be considered potentially both male and female (in terms of transmission) up to the moment of mating, when they perform one sex role. There are male or female roles during each mating, but there are no size differences or any other pre-determined sexes. Defining sexes based on cytoplasmic inheritance leads away from the natural understanding of male-female distinction and is therefore not preferred.

Motility

In order to be a criterion for male-female distinction, motility needs to be different between gamete types. In general, male gametes are motile and female gametes are less motile. Motility increases the chance of fertilization. This can occur during com petition over mates, which will only be of importance if gametes of different types are present in different numbers which is a result of size dimorphism (Parker et al., 1972). Alternatively, motility can select for diversification of investment in either motility or gamete size (Cox & Sethian, 1985; Iyer & Roughgarden, 2008; Roughgarden & Iyer, 2011). Smaller gametes will be selected because they are more motile, while the other gamete type increases size to compensate for loss of zygote size, which is assumed positively correlated with zygote fitness (Iyer & Roughgarden, 2008). Even though motility is associated with gamete

(box 2.1 continued)
sex, it is not universally so. For example, many broadcast spawners do have anisogamous gametes that fuse disassortatively, but neither type of gamete is motile (Yund, 2000), while in species of the water mould genus *Allomyces*, both male and female gametes are motile (Machlis, 1958). Also, there are isogamous species where two gamete types differ in motility - migrating gametes show male behaviour and sessile gametes female behaviour. Using the terms male and female in such systems is tempting, but one should be aware that other asymmetries generally found between gametes of different sexes are not present here.

Anisogamy (size difference) is the most commonly used characteristic to define male and female gametes (Parker et al., 1972; Bell, 1982; Stearns, 1987b; Charlesworth, 1994; Lehtonen & Kokko, 2011). Using this criterion, sexes only occur when there is anisogamy, i.e. when there is a difference in gamete sizes. Anisogamy is the only characteristic that invariably can distinguish the sexes. For all other differences associated with this size difference between male and female gametes (motility, cytoplasmic inheritance and incompatibility), exceptions exist, which makes these characteristics less useful as a criterion for the definition of males and females (see Box 2.1).

Only two sexes

In anisogamous species, only two sexes exist, which is intriguing because it limits the average number of mating partners per individual to only one half of the individuals in the population. Being of a third sex that is compatible with both others would be advantageous to an individual, because it would increase the number of available partners. One can imagine more than two gamete size-classes, which, based on the anisogamy definition of sexes, would imply that there are more than two sexes. However, evolutionary theory predicts that a third class will not be evolutionarily stable (Parker et al., 1972; Parker, 1978; Hoekstra, 1987). A major argument for this is that, if competition occurs between gametes of different individuals, it can be advantageous to increase the numbers of gametes produced. Assuming that resources are limiting, producing more gametes will result in smaller gametes. If, as is likely, viability of a zygote, produced by fusion between two gametes, increases with its size, gametes of the relatively large type will be selected to become even larger (Parker et al., 1972; Charlesworth, 1978; Parker, 1978).

Also when there is no competition for fertilization, but the supply of gametes limits fertility, anisogamy can evolve (Iyer & Roughgarden, 2008; Lessells et al., 2009). Selection to increase the chance of encounter and fertilization can lead to division of labour between a migrating small and a sessile large gamete (Cox & Sethian, 1984; Cox & Sethian, 1985; Iyer & Roughgarden, 2008; Lessells et al., 2009; Roughgarden & Iyer, 2011). Also in this scenario a third type cannot be evolutionarily maintained. Production of larger microgametes leads to reduced fertilization probability as fewer can be produced, and production of smaller macrogamete reduces zygote survival. Only the two optimum sizes (and therefore only two sexes) can co-exist. Interestingly, some species produce different types of male gametes with different functions

(e.g. micro and macro-conidia in some fungi in which the latter can also function for asexual reproduction; Maheshwari, 1999), but because both are only compatible with female gametes, and not with each other, they are not a third sex.

Compatibility between gametes

Male and female gametes can form a zygote only with gametes of the other sex. No anisogamous species are known, in which fusion between two big or two small gametes is possible (but see Davidovich et al., 2010 in which morphologically different gametes can fuse). No single evolutionary explanation for the prevention of fusion between gametes of same size is generally accepted. If we assume that anisogamy evolved from an isogamous ancestor in which all gametes could fuse with each other (which is not necessarily true, see below), initially, in anisogamous species no mechanisms prevented small-small and large-large fusions. Because the fitness of the zygote is considered to depend on its size, recognition mechanisms could have evolved to prevent small-small gamete fusions, which would produce less viable zygotes. It would, of course, be in the interest of larger gametes to fuse only with large gametes. However, a large gamete mutant that blocked fusion with small ones would not find other large gametes to fuse with, because the other large gametes would already have been fertilized by the much more abundant microgametes (Parker et al., 1972; Parker, 1978). Nevertheless, this does not explain why large-large fusion is impossible (Billiard et al., 2011). More likely, disassortative mating evolved before anisogamy, and mating between different-sized gametes was superimposed on an existing system of disassortative mating based on a different mechanism than size (Charlesworth & Charlesworth, 1978; Hoekstra, 1990). This seems a reasonable assumption, as in most isogamous species where no size differences exist between gametes, mechanisms that prevent the fusion of gametes of the same type occur. Because anisogamy is considered to be derived from isogamy, it has been proposed that disassortative mating mechanisms preceded anisogamy (Hoekstra, 1987). Consistent with this hypothesis, for the green alga Volvox carteri linkage between the mating-type locus, which regulates recognition between the gametes, and a gene or genes controlling gamete size difference has recently been shown (Ferris et al., 2010, Charlesworth & Charlesworth, 2010).

Disassortative mating mechanisms

Disassortative mating is common in both isogamous and anisogamous species. In most anisogamous species, the mating class of the gamete is defined during cell development, while in most isogamous species disassortative mating is genetically defined. We will reserve the term mating type specifically for systems in which compatibility between gametes is genetically defined at the mating-type locus or loci of the gamete itself. We refer to gametes with disassortative mating mechanisms that arise due to developmental differences as different epigenetic mating classes.

Because mating types are genetically defined at the level of the gamete, gametes of different mating types are per definition genetically different. On the other hand, gametes of different epigenetic mating classes can be genetically identical. In species with a hermaphroditic haploid phase, such as the gametophytic phase in some mosses or ferns, individuals can produce both male and female gametes. These gametes will be genetically identical but of a different epigenetic class, which is epigenetically defined during development, when the two kinds of gametes are formed in different organs of the parent.

Genetic mating types

In fungi and most algae, the mating types are genetically defined: compatibility between gametes is regulated by alleles at the mating type locus of the gametes themselves — to be compatible, the gametes must differ at this locus. Gamete recognition is mediated by an interaction between proteins produced by each gamete, either by dimerization of proteins (Casselton, 2002) or agglutinins (Ferris et al., 2005) or by a pheromone-receptor interaction (Kothe, 2008). Most often, there are only two different mating-type alleles, and the genes located on the different alleles are often not homologous (Glass et al., 1990a; Goodenough et al., 1995) and in ascomycete fungi the alternative alleles are therefore called idiomorphs (Metzenberg & Glass, 1990). Due to this bimodality, mating types are often referred to as sexes (e.g. Blakeslee, 1904a; Ni et al., 2011), but, as we mentioned, this is incorrect and confusing. This becomes clear when we consider anisogamous species that also have mating types. For such species, there is no coupling between mating type and sex of the gamete, as each mating type can be associated with both male and female gametes (see below and Box 2.2).

To indicate how mating types affect compatibility in fungi, the terms heterothallism and homothallism are used to specify if a haploid individual can produce gametes that can fuse with each other. In heterothallic fungi, syngamy is only possible between haploids presenting different alleles (or idiomorphs) at the mating type locus. Homothallic species, on the other hand, produce haploids that can fuse with any other haploid in the population (which is called "haploid selfing" Giraud et al., 2008), most often because one haploid presents both mating types in its genome (Coppin et al., 1997).

Some basidiomycete fungi, slime molds and ciliates have more than two mating types (Hurst, 1995 and reference therein), with some mushroom species even more than ten thousand (Raper et al., 1958). Mating types are expected to be under negative frequency-dependent selection, because novel mating types have an increased probability to find a compatible mate when rare. However, with an increasing number of mating types, the frequency of each goes down. This reduces the selective benefit of novel mating types up to the point where a balance is reached between genetic drift and negative frequency dependent selection (Wright, 1939; Stamberg & Koltin, 1973; May et al., 1999). In basidiomycete fungi to which also mushrooms belong, a gamete's mating type is generally defined by two separate loci that both have to be different to be compatible (Raper, 1966).

Box 2.2 Mating types ≠ sexes: the case of the Ascomycota

The here presented example of Ascomycota fungi, illustrates the difference between genetic mating types, phenotypic mating classes and sexes (see Fig 2.1). It gives a simplified account of how these three gamete characteristics are present in these fungi, how they function and how they affect each other.

Mating types and sexes

Many multicellular ascomycetous fungi produce male and female gametes. In heterothallic species one haploid mycelium produces gametes that are all of the same genetic mating type and thus incompatible with each other, but only compatible with gametes of the opposite mating type (e.g. *N. crassa*). Similarly, gametes of the same sex cannot fuse with each other, even when they are of a different mating type. To assure this disassortative mating, phenotypic differences between the male and female gametes are necessary. Most likely the compatibility between the gametes is mediated by receptors and their ligand (often pheromones) that are produced in either the female or the male gametes, respectively. In different heterothallic species male gametes produce pheromones (Turina et al., 2003;

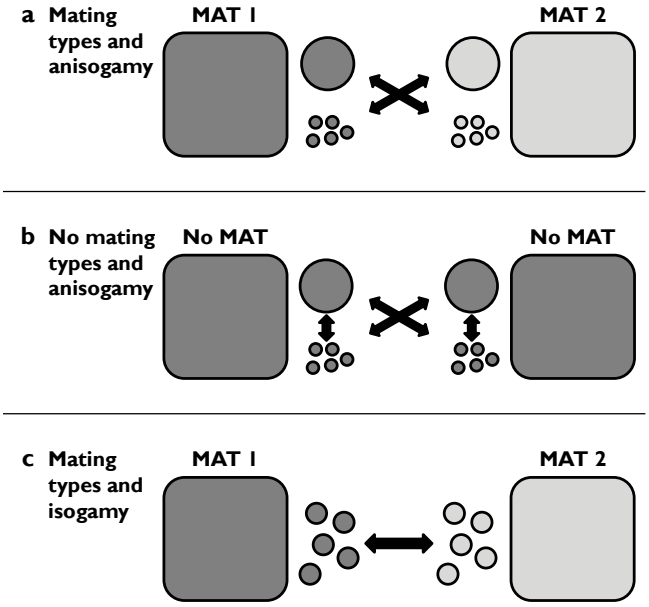

Figure 2.1 Example of the difference between sexes and mating types in three different mating systems. **a)** Two hermaphroditic individuals of different mating types produce each two differently sized gametes that are only compatible with gametes of the other sex and the other mating type. **b)** Two hermaphroditic individuals that do not have mating types produce gametes of different size (anisogamy). Gametes can fuse with all other gametes, but only if they are of the other sex. **c)** Two individuals of different mating types produce one size of gametes (isogamy) that are compatible with gametes of the other mating type.

(box 2.2 continued)
Coppin et al., 2005; Kim & Borkovich, 2006) and the female gametes produce a compatible receptor (Kim & Borkovich, 2004). Loss of pheromone genes results in male sterility and loss of receptors leads to female sterility.

Sexes without mating types
The same mechanism of phenotypic mating classes might also be functional in self-compatible species. In homothallic species one haploid individual produces gametes that are compatible with each other (e.g. *Aspergillus nidulans*, *Sclerotia sclerotinium*). In many homothallic species, the haploid gamete has genes from both mating types located in the genome, so each gamete is effectively of both mating types, and potentially compatible with all gametes (Dyer, 2007). Nevertheless, even though there are no mating types, still male and female gametes are produced that can only fuse with gametes of the opposite sex, due to a mechanism that assigns them to a different phenotypic mating class. It is likely that this phenotypic recognition mechanism in homothallic fungi is similar to that in heterothallic species. Glass et al. (1990b) and Coppin et al. (1997) suggested the possibility that homothallic species produce gametes that are functionally heterothallic. The female gametes transcribe for example only the receptor that will be activated by pheromones that are only produced by the male gamete. To our knowledge this has not been empirically verified.

Mating types without sexes
The pheromone/receptor mechanism for recognizing compatible mates is also functioning in isogamous species (e.g. *S. cerevisiae* and *Schizosaccharomyces pombe*), however, in isogamous species both pheromone and receptor are expressed in the same gamete. Each gamete produces a pheromone that is incompatible with the own receptor, but compatible with that of the other mating type (Tsong et al., 2007). In these species there are no male and female gametes so all gametes that are of different mating types can successfully fuse with each other.

Finally, we want to point out two things about mating types. First, the term 'mating types' as has been used in oomycetes (or water moulds) is incorrect according to our definition. Oomycetes are diploid organisms that have traditionally been classified as fungi, but actually are close relatives of heterokont algae. Due to their historic classification as fungi, frequently fungal terminology is incorrectly used in oomycetes. A diploid individual that can sexually reproduce on its own is referred to as 'homothallic' and an individual that cannot is 'heterothallic' (Judelson, 2007). Oomycetes in 'heterothallic' species come in different types, called 'mating types', which require each other to reproduce sexually. An individual from one group will not initiate gamete production, unless stimulated by pheromones produced by an individual of the other group. However, there are no mechanisms leading to incompatibility at the gamete level: once gametes are produced, they are able to fuse with gametes of either individual, which can result in 1-95% "diploid selfing" (Judelson, 1997). These peculiar details of oomycete sex imply that neither the terms homo- and heterothallism, nor the term mating type are applicable to oomycetes. The

first is reserved for organisms where compatibility is defined at the gametic level (Billiard et al., 2012), the second refers to genetic incompatibility of gametes. Unfortunately, little is known on the mechanisms and genetics of sexual compatibility in oomycetes and what is known is mostly limited to *P. infestans* (Judelson, 2007).

The second point is that mating types should not be confused with genetically controlled sex determination. In many species with separate sexes, the sex of the individual that produces the gametes is genetically controlled (sometimes involving sex chromosomes). For instance, in males with XY sex chromosomes, two types of sperm are produced with either a copy of the X or of the Y chromosome. Both sperm types are male gametes that can only fuse with female gametes, irrespective of the genetic makeup of the sperm cell. The gamete class is thus phenotypically defined by the sex organ producing it. In some species (e.g. some mosses) the haploid gametophyte produces only male or only female gametes, regulated by the sex chromosomes (designated U and V). A gametophyte with the U chromosome will only produce female gametes that all carry the U chromosome, and vice-versa a gametophyte with a V will only produce male gametes that carry the U chromosome. The sex chromosomes in these species thus are associated with the sex of the gamete.

Self-incompatibility in angiosperm plants

Many flowering plants also have genetically controlled 'incompatibility types', which only allow mating between conspecific individuals whose types differ. In most cases, the pollen protein does not interact with the egg cells, but with a protein expressed in the flower's stigma or style tissue, which are both distant from the ovary (Charlesworth et al., 2005). The pollen and pistil proteins are encoded by distinct, but linked genes, ensuring that the individual's pollen is incompatible only with the pistil carrying the same self-incompatibility (SI) allele.

SI has evolved multiple times independently in flowering plants and it is generally accepted that it evolved to avoid selfing and the costs of inbreeding depression (Charlesworth & Charlesworth, 1979). Once SI exists, negative frequency-dependent selection favours new incompatibility alleles, because a new and thus initially rare type will have an increased chance of finding a compatible mate (Wright, 1939). In some species this has led to many different incompatibility types.

Incompatibility types in the SI system in angiosperms do not qualify as mating types according to our strict definition. Even though the SI-type of the pollen for gametophytic SI-systems (GSI) is defined by the genome of the gamete, the female part of the system is defined by the diploid stigma of the receiving flower and in a sporophytic system (SSI) also the male part is defined at the diploid level (Charlesworth et al., 2005). Even though there are many similarities between the two systems, the dynamics of mating types and SI systems will be different. For instance, whereas two mating types suffice to achieve heterothallism, in GSI at least three alleles, and in SSI four alleles are needed for the system to function.

Evolution of genetic mating types

There is no single accepted ultimate explanation for the origin of mating types and the forces maintaining them are expected to differ depending on the system (see Billiard et al. 2011 for an extensive review). In diploid species, it has been proposed that genetic mating types evolved to avoid diploid selfing and inbreeding depression. This mechanism can explain the origin of self-incompatibility systems in plants where recognition is defined by or acts during the diploid phase. In plants, the pollen interacts with the stigma, which expresses both SI-alleles, and recognition leads to incompatibility (see above).

Nevertheless, diploid selfing (and eventually inbreeding depression) is not prevented in diploid species where gamete fusion is only regulated at the level of the gamete, such as in basidiomycete fungi. In these species, diploids are heterozygous at the mating type locus and produce gametes carrying complementary mating type alleles that will be able to fuse, so that inbreeding is not necessarily prevented, although the frequency of inbreeding is reduced (Giraud et al., 2008).

Moreover, in haploid species, inbreeding depression should not be an important force maintaining mating types. Contrary to diploid organisms where recessive deleterious mutations are hidden at the diploid stage, in haploid organisms, recessive deleterious mutations are exposed to selection and should be quickly eliminated from the populations. Inbreeding avoidance thus probably plays a minor role in fungi and algae that spend much of their lives as haploids (although it may play a role in basidiomycetes, where many species spend most of their lives as dikaryons, in many ways equivalent to diploids; Clark & Anderson, 2004). Nevertheless, many such species have mating types. Because in these species mating types will only prevent haploid selfing, it has been suggested that the main force behind their origin and their maintenance is precluding sexual reproduction with identical clones and allowing the benefit of the recombinatorial advantage of sex. Because sexual reproduction is costly (Lehtonen et al., 2011), mating with identical individuals will give some costs of sex, but not the benefits of recombination (Czárán & Hoekstra, 2004).

Recently, Haag (2007) suggested that genetic mating types might have evolved to assess the ploidy level of the cell — with an activated mating type signalling that the cell is diploid, and triggering a different growth mode. Nevertheless, ploidy level is also assessed in species where no mating types exist, such as homothallic fungi, which suggests that ploidy assessment is not the original function of mating types (Billiard et al., 2011; but see Perrin, 2012).

Finally, it has been proposed that mating types evolved to regulate cytoplasmic inheritance (Hoekstra, 1990; Hurst & Hamilton, 1992). Consistent with this hypothesis, cytoplasmic inheritance in isogamous algae and fungi is often regulated by the genetic mating type, such that only one gamete with a particular mating type provides the cytoplasm to the offspring (e.g. Boynton et al., 1987; Yan et al., 2004). However, also species with mating types exist with biparental cytoplasmic inheritance (e.g. S*accharomyces cerevisiae*, Xu, 2005).

Why mating types should not be called sexes

As we have explained, there are essential differences between mating types and sexes. But as mating types and anisogamy show many similarities, it can be tempting to use the more familiar term 'sexes' instead of the abstract term 'mating types'. Often researchers discuss only mating types or sexes, and in these circumstances it seems unproblematic that the two terms are exchanged with each other. However, as this can work confusingly and even lead to wrong insights, we argue that this mix-up should be avoided. Below, we give three examples of such mix-up and describe the confusion this can cause.

Thousands of 'sexes'

It is often claimed that mushroom species have thousands of sexes, when referring to thousands of mating types (e.g. Kothe, 1996). In fact, Hurst and Hamilton (1992), who favoured the hypothesis that binary mating types and anisogamy evolved to regulate uniparental mitochondrial transmission, argued that mushroom forming fungi have zero sexes, as sex in this group essentially occurs without cytoplasmic mixing. These fungi thus do not face the problem of regulating cytoplasmic transmission and can therefore have many more than the regular two mating types!

Fungal 'sex' chromosomes

Another example is the non-recombining regions around mating types. At mating-type loci, multiple genes are located in non-recombining regions on the chromosome, reminiscent of the lack of recombination between the different sex chromosomes (Fraser & Heitman, 2004; Fraser & Heitman, 2005). Especially when these regions of non-recombination around the mating types are big, they are often referred to as sex chromosomes. However, there are likely very different evolutionary reasons for the loss of recombination on sex chromosomes and around mating-type loci. For true sex chromosomes, suppressed recombination is thought to be driven by the advantage of accumulating genes that are beneficial in only one sex and detrimental in the other sex close to the sex determining locus (Rice, 1987). This is only possible if there are sexes. Mating types are generally not associated with asymmetry (even if there is anisogamy, see Box 2.2) and the adaptive significance for expansion of the non-recombining region as happens in sex chromosomes is therefore not obvious. Furthermore, the two different sex chromosomes generally experience different mutation rates, effective population sizes and sexual selection, which are again not likely for mating types for which no inherent asymmetry exists (Bachtrog et al., 2011). However, the mating-type regions can become linked to genes regulating gamete size (Charlesworth & Charlesworth, 2010), after which sex chromosome evolution due to antagonistic selection could start. To understand how selection drives expansion of non-recombining regions around mating type loci, which shows remarkable similarities with non-recombining regions of sex chromosomes and why these regions show increased degeneration (Fraser & Heitman, 2005; Whittle & Johannesson, 2011), the differences between sexes and mating types need to be taken into account.

Evolution of 'hermaphroditism'

Many species of fungi have evolved from a heterothallic system with mating types towards a homothallic system in which all gametes are compatible (Lin & Heitman, 2007). Some researchers have referred to this transition as a change from different sexes to hermaphroditism (e.g. Nauta & Hoekstra, 1992). Even though similarities exist between homothallism and hermaphroditism, such as universal compatibility between individuals, there are some very important differences. One of the major differences is that there is no resource allocation trade-off in production of each mating type, as both are produced in equal numbers during meiosis and there is no reason to assume that one gamete type is more equally expensive than the other. But there is a trade-off in sex allocation. This trade-off is of special importance for the transition between hermaphroditism and separate sexes (Charlesworth & Charlesworth, 1978; Charnov, 1979), but these insights are not applicable to the heterothallism-homothallism transition. Nevertheless, because both homothallic as well as heterothallic fungi generally are hermaphrodites, differential selection on male and female roles is possible (Nieuwenhuis et al., 2011; Chapter 7). By confounding hermaphroditism and homothallism, possible sex allocation trade-offs might be overlooked.

Conclusion

Equating mating types with sexes may obscure interesting or essential properties. Focusing on the characteristics that mating types and sexes have in common can give insights in the importance of asymmetry during mating and the evolutionary forces that drive it. Why are there exactly two sexes and often also only two mating types? Are hermaphroditism and homothallism solutions to similar evolutionary problems (e.g. mate availability in low-density populations)? If differential selection on gametes of different sexes leads to sex specific adaptations (e.g. motility or size; Togashi & Cox, 2011), can mating-type specific selection also lead to divergent mating-type evolution in a similar way? Comparing mating types and sexes on such fundamental questions can lead to important insights, but for this it is essential to keep clear the biologically important differences between them.

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Chapter 3

On the asymmetry of mating in the mushroom fungus *Schizophyllum commune*

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Summary

Before a mycelium of a mushroom-forming basidiomycete develops mushrooms, the monokaryotic mycelium needs to become fertilized. Although the mechanistic details of mating in mushrooms have been studied thoroughly in laboratory research, very little is known on mating patterns in nature. In this study, we investigated three populations of *Schizophyllum commune* from their natural substrate (i.e. dead beech branches). From the three branches, 24, 12 and 24 fruiting bodies were isolated and for each mushroom, the origins of its nuclei and cytoplasm were reconstructed using DNA markers. Nuclear genotypes were determined using sequencing data and mating types, and mitochondrial haplotypes using SNP markers. From these combined data we reconstructed colonisation and mating patterns of the mycelia. On each branch, we found multiple dikaryons (3, 3 and 8, respectively); in two instances one nucleus was shared between two dikaryons and in two other cases a nucleus was shared between three dikaryons. Each dikaryon always had a single mitochondrial haplotype. These findings indicate that mating usually is not symmetrical and that a monokaryon is most likely fertilized by a small monokaryon, a spore or a dikaryon. Sharing of nuclei between different dikaryons resulted either from multiple fertilizations of a single monokaryon, when the dikaryons had identical mitochondrial haplotypes, or, when the dikaryons had different mitochondrial haplotypes, most likely from secondary matings between a monokaryon and a dikaryon (Buller phenomenon). We conclude that mushroom mating between same-sized monokaryons with reciprocal migration, as generally described in textbooks, is rare in nature. We discuss the implications of non-symmetric mating for basidiomycete evolution.

Introduction

Mating is an essential phase of the lifecycle of sexually reproducing species. For an obligatorily outbreeding organism it is essential to find a compatible partner to mate with. Most sexual higher fungi are obligatorily outcrossing (~90%; Whitehouse, 1949; Raper, 1966) and thus need to find a compatible mate to complete their sexual lifecycle. Over the last few decades, the mechanisms of mating have been extensively investigated. The interactions between individuals have been studied at the physiological and molecular level and much of the underlying genetics is well characterized (Heitman et al., 2007). Fungal mating involves complex adaptations, such as highly specialized fertilization organs (e.g. trychogynes), cell recognition mechanisms (Murphy et al., 2006), or nuclear migration with septal breakdown (Stankis and Specht, 2007). Because these mechanisms function during interactions between different conspecific mycelia, to fully understand their ecological function, more knowledge is needed on the natural frequency of different interactions between mycelia during mating.

The extensive knowledge obtained in laboratory research is in sharp contrast to the limited knowledge of mating patterns in nature (Billiard et al., 2012). At the population level, some studies have estimated the frequency of in- and outbreeding in nature (e.g. Giraud et al., 1997; Kauserud and Schumacher, 2001; Knop, 2006) and population structure (e.g. James et al., 2001; Marra and Milgroom, 2001; Kauserud and Schumacher, 2003; Giraud et al., 2010). Some studies show that there is much outbreeding, which indicates that genetically different individuals mate. However, interactions at the level of the individual mycelia in nature have been investigated for only few species (Hiscox et al., 2010; Stenlid et al., 2008).

In mushroom-forming fungi, mating occurs long before mushrooms are formed, often inside the soil or another growth substrate of the fungus. Generally, it is assumed that a monokaryotic spore colonizes a substratum, grows for a limited period until it meets a compatible monokaryon with which it mates (but see e.g. Anderson and Kohn, 2007). During mating, nuclei are exchanged reciprocally (both mycelia fertilize each other), followed by migration of these nuclei throughout both mycelia. This results in a single dikaryotic mycelium, all cells of which have an identical pair of genetically different nuclei, one from each mating partner. Nevertheless, this mycelium is a mosaic for its cytoplasmic genes, because mitochondria do not migrate during mating (Fig 3.1a; May and Taylor, 1988). Next to meeting a monokaryon, fertilization of the monokaryon might occur by a spore or by one of the two nuclei from a dikaryon (Fig 3.1b-c; Buller, 1931; Nieuwenhuis et al., 2011). In those cases migration is unidirectional (Aanen et al., 2004; Anderson and Kohn, 2007). It has not been studied how often a monokaryon is fertilized by a monokaryon, by a dikaryon, or by a spore in natural populations. Important parameters for mating are the longevity of the monokaryon phase and size of the monokaryon at mating, on which little knowledge is available (Crockatt et al., 2008; Hiscox et al., 2010).

To obtain more insight in the presence of monokaryons and the details of basidiomycete mating in nature, there are a number of approaches. One possibility is to sample natural substrates in high resolution both in space and time, as performed by Rayner and co-workers

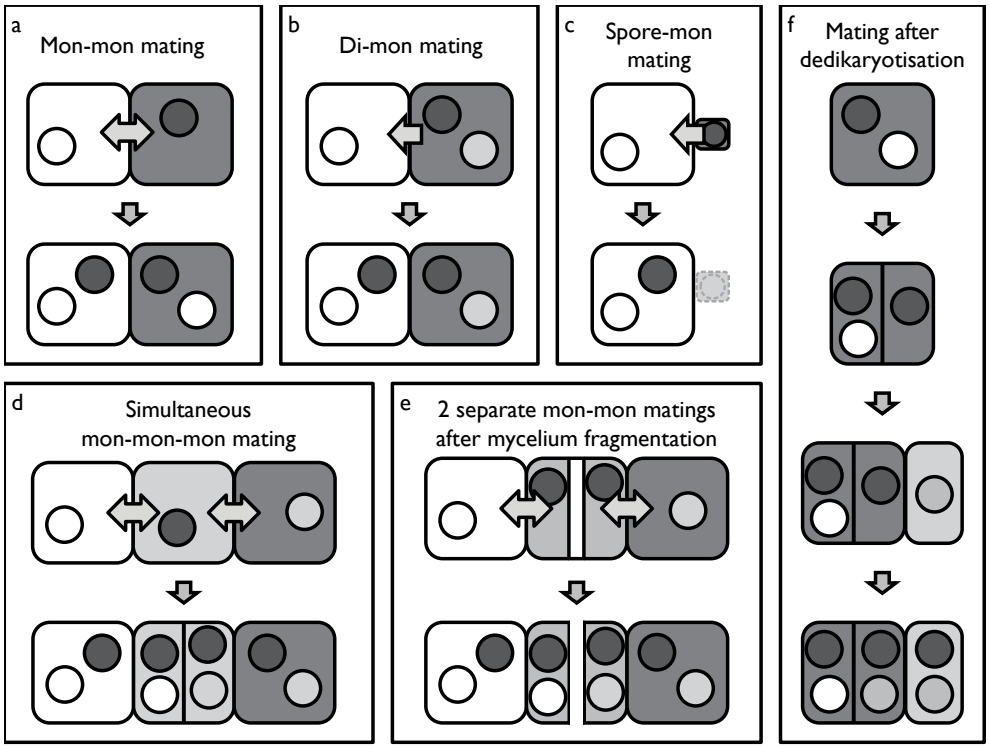

Figure 3.1 Six different possibilities of how mating occurs and the pattern of nuclear and mitochondrial variation it yields. The rounded squares represent a mycelium and the filling represents the cytoplasmic type. The circles in each figure depict the nuclei and nuclei of different types have different filling. **a)** Symmetrical monokaryon-monokaryon mating results in two equally sized dikaryons with identical nuclei, but different mitochondia. **b)** Dikaryon-monokaryon mating results in two dikaryons that share one nucleus but that have different mitochondria. **c)** Spore-monokaryon mating results in one dikaryon. The mitochondria from the spore cannot be retrieved. **d)** Multiple matings when three monokaryons meet each other, which results in four different dikaryons that all share one nucleus. The mitochondrial type from the mycelium that was fertilized twice is found in two different dikaryons with one shared nucleus. **e)** When a monokaryon is fragmented, each monokaryon can be fertilized separately. The outcome is the same as in d, but the mating does not have to be simultaneously. **f)** A dikaryon dedikaryotizes, after which the newly arisen monokaryon can become fertilized again. The re-fertilized monokaryon will share one nucleus and the mitochondria with the original dikaryon.

in elaborate field experiments in the early 1980s (Coates and Rayner, 1985a; Coates and Rayner, 1985b; Coates and Rayner, 1985c; Coates and Rayner, 1985d; Rayner and Todd, 1979; Williams et al., 1981). Although these types of experiments give important insight into colonization dynamics and interactions between individuals at a local scale, they are very labour intensive and not very specific. Furthermore, the species of interest may not be present at all sampling locations, which complicates this kind of investigations.

Alternatively, mating dynamics can be reconstructed from an established population of dikaryons by sampling their sporocarps. To reconstruct mating patterns, first the number of dikaryotic individuals in close proximity should be known, but because mycelia are cryptic, assigning individuals is difficult (Rayner, 1991; Todd and Rayner, 1980). For saprophytic and

ectomycorrhizal fungi, somatic incompatibility (e.g. Johannesson and Stenlid, 2004; Rayner et al., 1984) and genetic markers have been used (e.g. Egger, 1992; Johannesson and Stenlid, 2004; Smith et al., 1990) and these have shown that there is large variation between species in the number and size of dikaryotic individuals at small spatial scales. However, knowledge on the number of dikaryotic individuals only does not tell how mating occurred, as this requires knowledge of the genetic makeup of the different individuals, both at the level of individual nuclei, and of the mitochondria.

In this paper, we investigated the genetic makeup at the cytoplasmic and nuclear level of fruiting bodies from three populations derived from a single substrate. By determining the nuclear identity of each fruiting body, the size and distribution of different dikaryotic individuals could be reconstructed. By combining these findings with the mitochondrial background of each fruiting body we reconstructed the initial number of monokaryons that produced the dikaryons we sampled, and deduced if these were fertilized by a monokaryon, a spore or a dikaryon.

Material and Methods

Strains, media and growth conditions

In the area around Wageningen, The Netherlands, we collected two large branches and one shorter stick of dead European Beech (*Fagus sylvatica*) with multiple fruiting bodies of *Schizophyllum commune* (see Table 3.1 and Fig 3.2 for more information). From these three branches we collected 24, 12 and 24 mushrooms respectively, and isolated dikaryotic mycelium from the non-hymenoid part of the mushroom (Simchen, 1966b) on minimal medium (MM; Dons et al., 1979). The cultures were grown at 15°C, 8h dark - 16h light day cycles to produce mushrooms from which single spore cultures were isolated by inoculating dilutions of the obtained spores in sterile water on MM plates supplemented with 500µl 5% Triton80 which reduces hyphal spreading and keeps colonies small. Sub-culturing was done in 24 well plates with 1ml MM and crosses were performed on CM (Dons et al., 1979) at 15°C in the dark. Higher temperatures resulted in loss of phenotype. Mycelium for DNA extraction was grown for three days on MM covered with cellophane to harvest mycelia (~15g) from the medium. Isolates are named as follows: Sc(branch number).(mushroom number)-(spore isolate) (e.g. Sc31.4-7 for the 7th single spore isolate from the 4th mushroom of branch 31). DNA was extracted using the Chelex method

Table 3.1 Information on the three branches from which mushrooms were isolated.

	Diameter in cm	Length in cm	#fruiting bodies	Location
Branch 30	12	167	24	51° 58' 7.6"N, 5° 42' 8.8"E
Branch 31	3.5	44	12	52° 0' 41.4"N, 5° 40' 23.8"E
Branch 32	7 - 12	187	23	51° 58' 7.6"N, 5° 42' 8.8"E

(Walsh et al., 1991) for PCR of the nuclear genes (see next paragraph) and QIAGEN DNEasy Plant mini kit for mitochondrial markers.

Genotyping of nuclei

To distinguish the different nucleus compositions of each mushroom we used both molecular techniques and crosses. We started by testing if genetic diversity was present and grouped those mushrooms that were likely derived from the same mycelium and therefore clones. Unfortunately, somatic incompatibility to group clones did not yield unambiguous results, because *S. commune* does not produce strong barrages; therefore, we used molecular techniques in combination with mating tests of monokaryons derived from a mushroom to identify the individual nuclei of dikaryons.

We tested six single-copy genes that were expected to be highly variable between individual nuclei (for PCR protocols per primer pair, see the respective references). Of these, elongation factor 1 alpha (EF595F & EF1160R; Kauserud and Schumacher, 2003), the second largest subunit ribosome polymerase (fRPB2-7CF & fRPB2-11aR; Liu et al., 1999), laccase (LCS1F & LCS1R; James and Vilgalys, 2001) and beta-tubulin (B36F & B12R; James and Vilgalys, 2001) were not sufficiently variable to distinguish the nuclei. Intron regions in the second largest subunit ribosome polymerase (bRPB2-6F & bRPB2-7.1R; Matheny, 2005) and super oxide dismutase (SOX1R & SOX1L; James and Vilgalys, 2001) showed high levels of polymorphism and were used for further analysis. PCR products were purified (Gen Elute PCR Clean-Up kit, Sigma) and sequenced by Eurofins MWG Operon Sequencing Department (Martinsried, Germany). For both loci we analysed DNA isolated from the dikaryon of each isolate and for SOX we also analysed a single monokaryon derived from that strain. By comparing the electropherograms of the dikaryotic and the monokaryotic sequence, and subtracting the sequence of the monokaryon from the polymorphic sites at the dikaryon, both alleles for the SOX locus were obtained.

Furthermore, the mating types of the mushrooms were used to distinguish the different nuclei present. The mating type is composed of two unlinked loci (designated A and B) that both must be different for compatible mating. The natural variation of mating types in *S. commune* is very large: 288 A and 81 B factors are predicted, resulting in over 23 thousand possible combinations (Raper et al., 1958; Raper, 1966). Based on this large diversity and on large spore dispersal (James and Vilgalys, 2001), we assume that in our rather small sample no repetitions at both A and B mating type loci occur, except for nuclei that originate from the same monokaryon. For each dikaryon, twelve spores were isolated. One monokaryon was used as reference to cross the other 11 monokaryons with. Using macroscopic and microscopic phenotypic characteristics the mating type of each monokaryon relative to the tester was assessed (Papazian, 1950 ; Miles et al., 1966). For each dikaryon (say with mating type loci A1B1/A2B2) all possible combinations of alleles at the two mating type loci were chosen (i.e. A1B1, A2B1, A1B2 and A2B2), which in sib matings resulted in the right phenotype. This check was performed to assure no recombination

Table 3.2 Different groups per tree based on the nuclear genotypes and mitochondrial haplotypes. For each group the number of individuals in the group and their designation are given, as well as the mitochondial (mt) haplotype, the genotype of the dikaryon for RPB2, and the separate alleles for Sox1. The polymorphic sites for the sequences are given in Tables S3.2-S3.4. The last column indicates the probability of sampling only one cytoplasmic background type when two backgrounds occur in equal numbers, calculated as described in the text.

Reconstructed Dikaryon	Mushrooms per group	Mushrooms in group	Mating type nucleus 1	Mating type nucleus 2	mt haplotype	RPB2	Sox1-A	Sox1-B	Prob 1 mt haplotype
30A	4	30.01, .03, .06, .22	?[a]	?[a]	mtA	R10	S03	S04	0.077
30B	6	30.02, .07, .20, .21, .23, .24	?[a]	?[a]	mtA	R10	S01	S04	0.014
30C	14	30.04, .05, .08, .09, .10, .11, .12, .13, .14, .15, .16, .17, .18, .19	?[a]	?[a]	mtA	R05	S01	S04	<0.001
31A	9	31.02, .03, .04, .05, .06, .07, .08, .09, .10	A1, B1	A2, B2	mtB	R01	S07	S07	-
31B	2	31.01, .11	A3, B3	A4, B4	mtB	R06	S08	S06	-
31C	1	31.12	A5, B5	A4, B4	mtB	R06	S05	S06	-
32A	7	32.01, .02, .03, .04, .05, .06, .24	A11, B11	A12, B12	mtE	R02	S09	S02	0.006
32B	1	32.23	A11, B11	A22, B22	mtE	R02	S13	S02	1.0
32C	2	32.15, .16	A11, B11	A20, B20	mtB	R04	S11	S02	0.429
32D	3	32.18, .19, .21	A15, B15	A16, B16	mtD	R03	S11	S01	0.182
32E	2	32.13, .17	A21, B17	A18, B18	mtB	R09	S11	S01	-
32F	1	32.22	A15, B15	A23, B14	mtB	R07	S12	S01	-
32G	6	32.08, .09, .10, .11, .12, .14	A17, B17	A19, B19	mtC	R07	S11	S11	0.014
32H	1	32.07	A13, B13	A14, B14	mtC	R08	S03	S10	1.0

[a] Mating types for these strains could not be assessed. See 'Results' in the main text.

at the mating type locus had occurred, which could generate a new mating type (Stamberg and Koltin, 1973).

Per mushroom, two compatible isolates, and therefore isolates of opposite mating type, were chosen. Mushrooms expected to be clones based on the molecular markers were mated with each other to confirm clonality and grouped accordingly (see Table 3.2). Per group, two testers were mated with two testers of all other groups to assess mating types.

Mitochondrial genome haplotyping

We genotyped each mushroom isolate at 8 single nucleotide polymorphism (SNP) sites to assess the mitochondrial DNA haplotype. A library was constructed for the mitochondrial genome by performing whole genome Illumina sequencing of pooled whole genome DNA of all collected strains and the sequenced reference strain 4.8A (derived from 4-40 strain; Forget et al., 2002; Ohm et al., 2010b) by Service XS B.V. (Leiden, The Netherlands). A total of 1438 SNPs relative to strain 4.8A were found, of which 1165 SNPs were fixed in the local population. We selected 8 SNPs for which allele frequencies were between 20% and 80% in the population and that had no SNPs in the flanking regions and designed primers for them (see Table S3.1 in supplementary materials). SNPs were identified using KASPar v4.0 SNP Genotyping Systems (KBioscience, Hoddesdon, England) on a CFX96 PCR system (Bio-Rad Laboratories). Due to low GC-content of mtDNA, MgCl2 concentration was increased to 2.2mM and the standard KASPar thermocycling protocol was adjusted such that in step 2 the touchdown ran over 13 cycles dropping 0.6°C/cycle and step 3 was repeated for 35 cycles. The 'allelic discrimination' tool of CFX Manager Software (V2.0, Bio-Rad Laboratories) was used to assess the SNP allele for each strain.

Results

Based on the sequence data for the nuclear markers, we were able to assess the number of different nuclear genotypes on each tree (see Table 3.2, S3.2 and S3.3). These results were tested by performing crosses between monokaryons derived from the mushrooms. From these crosses, also shared nuclei could be deduced (see Table 3.2 and Fig 3.2). For each collected sample the mitochondrial haplotype was determined based on eight SNP markers (see Table 3.2 and S3.4).

On each of the three investigated braches, multiple individuals were found. Based on the nuclear genotypes from the RPB2 and Sox1 loci, 3, 3 and 8 different genotypes could be assigned to branch 30, 31 and 32, respectively. For branches 31 and 32 (for branch 30 see below), the crosses between monokaryons derived from each separate mushroom within each group, showed the presence of two alleles for each mating type locus (see Table 3.2). This confirms that all individuals in each group were most likely composed of the same two nuclei. On branch 31, groups 31B and 31C shared one allele of Sox1 and one of both mating-type loci, which suggests that one nucleus is shared between these groups. The same is true for groups 32D and 32F, and the groups 32A, 32B and 32C.

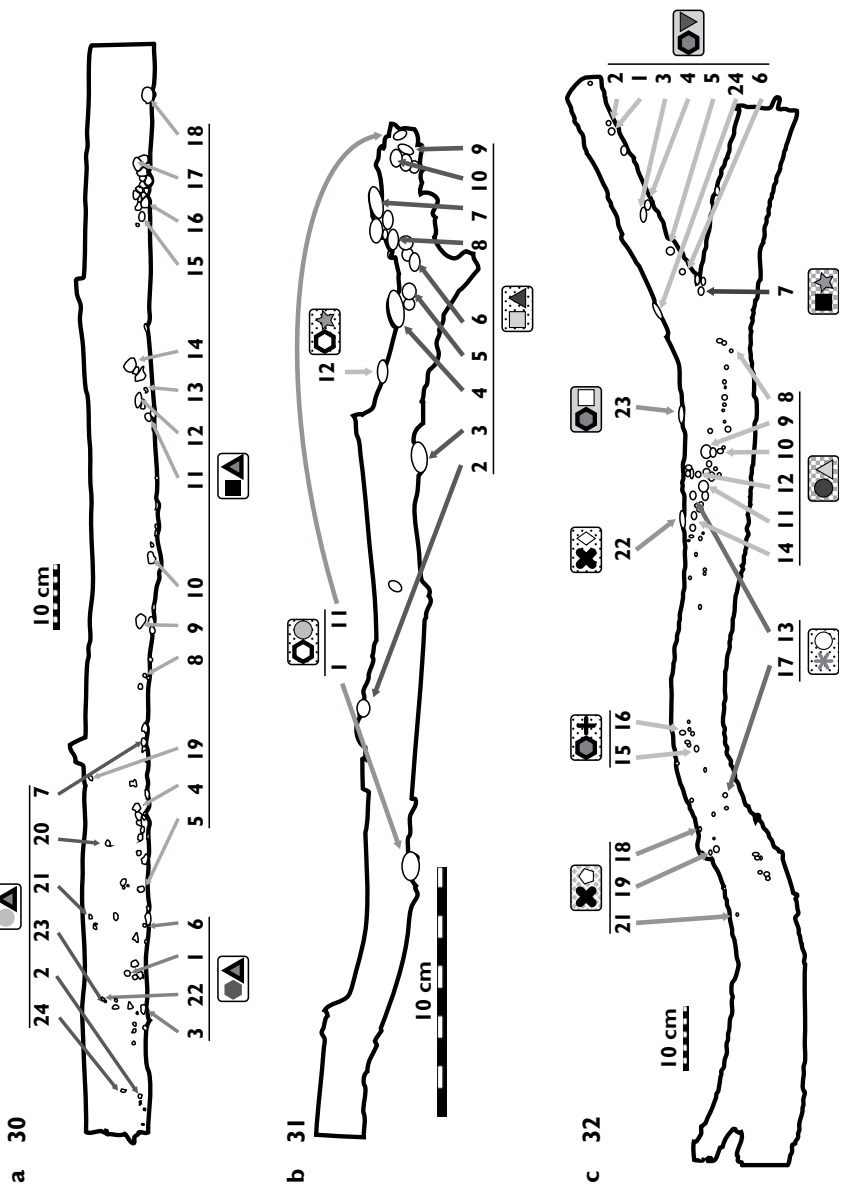

Figure 3.2 Drawing of the branches 30, 31 and 32 (**a**, **b** and **c** respectively) and the location of all mushrooms on each branch. The collected mushrooms are indicated by arrows. Numbers indicate the designation of each mushroom. Per tree, each symbol indicates a nucleus type. Mushrooms with a shared nucleus have one symbol in common (indicated by thick lined symbol). The pattern of the square around the two symbols indicates the mitochondrial genotype of the mushrooms. The designation of the nuclei is based on molecular markers and on crosses, except for branch 30, which is based solely on molecular markers. Reference bars are 10cm.

At the cytoplasmic level, individuals within each group all had the same mitochondrial haplotype, which indicates that each group is derived from a single monokaryotic 'mother' mycelium that incorporated another nucleus. Some mitochondrial haplotypes were shared between the different groups. To establish whether this is caused by low resolution due to the limited number of SNPs, we SNP-genotyped eight more mushrooms from different substrates from the area around Wageningen. Three of these showed unique new haplotypes (mtF, mtG and mtH), and five were of the same already known haplotype mtB. In contrast to the other haplotypes found, haplotype mtB thus is very common.

Branch 30. The genotypes of the strains isolated from branch 30 suggest that all share one nucleus type and that each of the three dikaryon groups has a different second nucleus. All isolates have the same Sox1 allele (S04), which is unique to this branch and the two different dikaryotic Rpb2 genotypes potentially have one shared allele. This is also consistent with the finding that all mushrooms have the same mitochondrial haplotype. This haplotype is unique for this tree and it is thus unlikely that the three different groups by chance have the same cytoplasm (see also section '*Discussion: Dikaryons are not derived from mating between equally-sized monokaryons*'). Even though the molecular characterization gives a clear picture, the crosses performed between the monokaryons derived from dikaryons on branch 30 did not show a clear pattern of compatibility and we were unable to assign mating types to them. It is unclear if the crosses were not performed correctly, or if the stains behave not according to the known mating type paradigm. In three separate experiments, performed by two of the authors individually, inconsistencies between crosses were observed. For instance, in a cross, monokaryons X and Y show a clear common-A reaction (Papazian, 1950) and should thus have the same A mating type allele. In a following cross of each monokaryon to a third monokaryon Z, one cross again showed a common-A phenotype, whereas the other showed a compatible reaction. This indicates that the alleles of X and Y even though different (as indicated by the cross with Z), do not activate each other (as indicated by the first cross). Similar results were observed for the B locus. All monokaryons were able to produce dikaryons and monokaryotic identity was confirmed by checking for clamps, as well as by testing for fruiting ability. We were unable to come up with a satisfactory explanation for these incongruences and could not finish this puzzle. Further investigations into the strains derived from this branch will be conducted to investigate this.

Discussion

In this research we investigated the nuclear and cytoplasmic composition of populations of *S. commune* at the scale of a single branch, to assess how mating occurs in nature. We observed that 1) on each branch multiple genetically different dikaryotic individuals were present, that 2) all individuals similar at the nuclear level had the same mitochondrial haplotype, and that 3) for seven of the 14 dikaryotic individuals a nucleus was shared with a different dikaryon.

Multiple individuals

Finding multiple genetic individuals on each separate substrate indicates that multiple colonisations of the substrate occurred before one individual was able to colonize the entire substrate. Because each investigated mycelium was dikaryotic and colonisation generally occurs by monokaryotic spores, finding multiple individuals per tree implies that multiple matings have taken place at a small spatial scale, i.e. the scale of a single branch.

Dikaryons are not derived from mating between equally-sized monokaryons

The generally assumed agaricomycete lifecycle describes two monokaryotic mycelia growing separately for a short period, and which then meet, fuse, and have reciprocal nuclear donation and incorporation into the mycelium by nuclear migration. The result of this type of mating is a mycelium identical at the nuclear level, *viz.* identical pairs of two nuclei, but mosaic for mitochondrial genotype, because the cytoplasm does not co-migrate (Fig 3.1a; Barroso et al., 1995; Hintz et al., 1988; Specht et al., 1992). Although in a laboratory mating, this will be the observed outcome, we did not find any dikaryotic mycelia, identical at the nuclear level but with two different types of cytoplasm. For four dikaryons we could reject the hypothesis that the dikaryon was the result of a mating between same-sized monokaryons with a significance level of 0.05 (Table 3.2). This was calculated as follows. If we assume that two equally-sized monokaryons of different mitochondrial haplotypes performed the fertilization, both should produce the same number of fruiting bodies. We sampled approximately 25% of the fruiting bodies randomly from the entire branch (see Fig 3.2). Suppose that four mushrooms of the same nuclear composition were collected; the total number of mushrooms of the same nuclear genotype on the branch was then 16, and assuming equally-sized monokaryons at mating, there were eight of each mitochondrial haplotype. The probability that only one mitochondrial type is sampled is 16/16 for the first, 7/15 for the second (seven of the remaining 15 are of the same type), 6/14 for the third etc. The probability that only one type of mitochondrial type was collected can thus be calculated for each dikaryon using

$$Pr = \prod_{i=1}^{n-1} \frac{2n - i}{4n - i}$$

where n is the number of sampled mushrooms. These values are given for each dikaryon in the last column of Table 3.2. Based on these probabilities, it is highly unlikely that for all dikaryons only one mitochondrial background was sampled. More likely, at mating the monokaryons were not of equal size, but one was large and was fertilized by another small mycelium which did not produce mushrooms itself.

Probably it is difficult for a new mycelium to colonize a substrate, but when a monokaryon is present, it can become fertilized readily, most likely by spores (but see section '*Discussion: multiple fertilizations of one monokaryon*'), as was shown for *Coriolus versicolor* (Williams et al., 1981). Spores can be considered as the smallest monokaryon possible (Fig 3.1c; Anderson and Kohn,

2007; James and Vilgalys, 2001). Only the primary mycelium that is able to colonize a substrate will therefore provide cytoplasm to the formed dikaryon. Also for *Armillaria gallica*, the only species for which this has been investigated in natural isolates, no cytoplasmic mosaics were found (Anderson and Kohn, 2007).

It should be noted that our finding might also be caused by low discriminatory power of our method for detecting variation in mitochondrial haplotypes or by low overall standing variation in the population. Our analysis consisted of eight SNP markers, and unequal frequencies of haplotypes might lead to false classification of mitochondria from different monokaryons in the same cytoplasmic group. Especially for individuals of haplotype mtB, which is found on 7 of the 11 substrates tested, this might be the case. Because of its apparent abundance, no conclusions will be drawn based on this haplotype mtB. The other seven haplotypes however, were all unique for each substratum, and of these, only mtC is shared between individuals that do not share at least one nucleus. It is difficult to estimate how many alleles are present in the population and what their frequencies are. However, assuming the observed eight haplotypes are the only haplotypes in the population occurring at the same frequency (1/8), and assuming random mating, the chance that two monokaryons that together form a dikaryon carry the same allele is $(1/8)^2 = 0.0156$. This is a very conservative estimate, because probably many more alleles exist and the frequency of mtB is higher, which both lead to lower frequencies of non-mtB alleles.

Multiple fertilizations of one monokaryon

The dikaryons 32A and 32B share one nucleus, and have the same cytoplasmatic background. This pattern can be caused by two simultaneous matings of the same monokaryotic mycelium which was of mating type A11B11 and mitochondrial haplotype mtE (Fig 3.1d). (The same is likely true for dikaryons 31B and 31C, but because – as described in the previous paragraph – haplotype mtB is very common, we cannot be sure these dikaryons are derived from the same monokaryon.)

Nuclear migration in *S. commune* is fast relative to mycelium growth (Snider and Raper, 1958), and therefore multiple fertilizations must have occurred close after each other on a mycelium that is large enough, to give both fertilizing genotypes the opportunity to get established successfully (Williams et al., 1981). Even though nuclear migration in the field might be different from measurements as performed in laboratory conditions – for instance, because natural mycelia are older when fertilized which reduces migration speed (Ross, 1976; Snider and Raper, 1958), high migration speed reduces the chance for one mycelium to become fertilized twice.

Alternatively, two other scenarios could lead to this pattern. First, a big monokaryon may become fragmented in the substrate, due to interspecific competition or other causes of hyphal death (Fig 3.1e). Each fragment can then become fertilized independently of the other fragment. Second, a dikaryon might have lost one nucleus type and become fertilized by the other nucleus type (Fig 3.1f). For fungi without clamp-connections, monokaryotic outgrowth is known to occur (e.g. Hui et al., 1999). However, for fungi with clamp connections, this is generally believed not to happen (Buss, 1987; see also chapter 5). (Although monokaryotic outgrowth

occurs, when grown on medium containing bile acid, because the two monokaryotic cells in the clamp cannot fuse with each other [Miles and Raper, 1956]. It is unknown if circumstances that prevent clamp fusion occur in nature.) Also in the species *Heterobasidion annosum*, a monokaryon that shared its mitochondria as well as its nucleus with those of a dikaryon has been observed in nature (Garbelotto et al., 1999). It is unknown which of the three described scenarios acted here; monokaryotic outgrowth is a likely option because clamp connections are less regular in this species (Stenlid and Rayner, 1991).

Even though monokaryons in some species can be readily found in nature and be long-lived (e.g. Coates and Rayner, 1985a; Garbelotto et al., 1999; Redfern et al., 2001), more information on the longevity of monokaryons in nature is needed to understand the dynamics of basidiomycete mating and to understand how multiple fertilizations of a monokaryon are possible.

Dikaryon-monokaryon matings

Fertilization of a monokaryon can also occur by donation of one of the nuclei from a dikaryon, known as the Buller phenomenon (Fig 3.1b; Buller, 1930; Quintanilha, 1937). A dikaryon-monokaryon mating results in two dikaryons that share one nucleus, but have different cytoplasms. Dikaryons 32D with 32F, and 32A and 32B both with 32C show this pattern. The same pattern can arise when one monokaryon simultaneously fertilizes two others and we only sampled the latter two, or if one monokaryon fertilizes one mycelium and becomes fertilized by another mycelium, for instance if the initial fertilization was unilateral (Harder and Aanen, 2009). Three monokaryons meeting simultaneously might occur in nature, but, as discussed in '*Discussion: Multiple fertilizations of one monokaryon*', due to the high nuclear migration rate, the time frame for this to happen is very short. Also unilateral migration is not very common for *S. commune* (Koltin et al., 1979). Therefore, the most likely way that identical nuclei end up in different cytoplasmic backgrounds, while paired with a different nucleus is by the Buller phenomenon, which has also been shown to occur readily between compatible strains in laboratory settings (Ellingboe and Raper, 1962; Nieuwenhuis et al., 2011).

Conclusions

Our analysis of 60 isolated *S. commune* mushrooms collected from three branches, shows that Basidiomycete mating in nature occurs differently from what is generally described in the textbooks. Anderson and Kohn (2007) suggested asymmetry between mating individuals, because abundant spores in the air should readily lead to fertilization of a newly established monokaryon (e.g. Hallenberg and Kúffer, 2001; James and Vilgalys, 2001). Our finding that none of the dikaryons sampled had different mitochondrial backgrounds confirms this asymmetry. Even though asymmetry during mating is observed, monokaryons are most likely not just short lived and rapidly fertilized after establishment. The shared nuclei between different dikaryons in the same cytoplasmic background suggest that monokaryons can colonize a substrate and grow to a considerable size before becoming fertilized. Also the observed dikaryon-monokaryon matings

can probably only have resulted in mushrooms when the mycelium was of considerable size before fertilization. We thus conclude that monokaryotic mycelia are generally not fertilized by equally sized monokaryons, but by smaller monokaryons, by spores, or by nuclei derived from a dikaryon.

Even though it seems likely that a monokaryon after establishment quickly becomes fertilized by a spore, apparently this is not what happens in nature. Because the monokaryon grows within the wood and not on the surface, the exposed surface area might be very small, especially when the wood is still covered with bark as often seen in *S. commune* infested wood. This is in contrast to, for example the wood disks or cut logs used by Williams et al. (1981) or spore traps (Hallenberg and Kúffer, 2001; James and Vilgalys, 2001), in which a large monokaryon is completely exposed to spores from the air. Also for *H. annosum*, which lives in protected substrates, a prolonged monokaryotic phase has been observed (Stenlid et al., 2008). It is important to increase our knowledge of the monokaryotic life-phase in nature. To what size can a monokaryon grow before it becomes fertilized? What are its competitive abilities (e.g. Crockatt et al., 2008; Hiscox et al., 2010)? Where in its substratum does the mycelium reside and how easily is it accessible? Answers to these questions are needed to fully understand the intriguing mating biology of mushroom-forming fungi and how it evolved.

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Chapter 3 - Supplementary materials

Table S3.1 Primers designed for SNP detection in *Schizophyllum commune* mitochondria, to be used with the KASPar v4.0 SNP Genotyping System (KBioscience, Hoddesdon, England). SNP position relative to reference sequence NC_003049 (*S. commune* mitochondrion, complete genome).

SNP pos	Allele*	Primer sequence 5' → 3'
474	G (FAM)	GAAGGTGACCAAGTTCATGCTAAAAATTACAAATTATATTCGACTTAAGTAAAG
	C (VIC)	GAAGGTCGGAGTCAACGGATTAAAAATTACAAATTATATTCGACTTAAGTAAAC
	reverse	AAACTTACAGGACTATTTGTTATTACCTTT
6280	T (FAM)	GAAGGTGACCAAGTTCATGCTAACTACCCTTAAACAATATAAATCGACTCA
	C (VIC)	GAAGGTCGGAGTCAACGGATTCTACCCTTAAACAATATAAATCGACTCG
		CCTTAATTAGGAGATAACCAATAAAGGAAT
7274	A (FAM)	GAAGGTGACCAAGTTCATGCTAGAATGTAATGTCTAGTAGATTTACTTAATATT
	G (VIC)	GAAGGTCGGAGTCAACGGATTAGAATGTAATGTCTAGTAGATTTACTTAATATC
		GGCTAGGGTTTCCTAGCTATTTATTTGAT
15534	A (FAM)	GAAGGTGACCAAGTTCATGCTATACAAAGATATGTGGTAATACCCCCA
	C (VIC)	GAAGGTCGGAGTCAACGGATTACAAAGATATGTGGTAATACCCCCC
		CTACACCCACTAATAATTAATCTATAAGAA
21031	A (FAM)	GAAGGTGACCAAGTTCATGCTCTGACAAGTGTTAGCTCTTTTATTTTTAATA
	G (VIC)	GAAGGTCGGAGTCAACGGATTCTGACAAGTGTTAGCTCTTTTATTTTTAATG
		GCTTCTTTCAACTCAGGTTTATCACTATTT
30738	T (FAM)	GAAGGTGACCAAGTTCATGCTATTGCTTTCTTAGGGTATGTTTTACCTTTT
	C (VIC)	GAAGGTCGGAGTCAACGGATTGCTTTCTTAGGGTATGTTTTACCTTTC
		ATTAGTAATAACTGTAGCTCCCCATAATGA
43198	T (FAM)	GAAGGTGACCAAGTTCATGCTCACATGTCATTCACATACATAAATTACATGT
	G (VIC)	GAAGGTCGGAGTCAACGGATTACATGTCATTCACATACATAAATTACATGG
		GAGTATTTCTCAGGAAAATTTTCCGATTTC
43418†	A (FAM)	GAAGGTGACCAAGTTCATGCTCAAATTACAAGTGTAAAGCCATAGTATAAAAA
	C (VIC)	GAAGGTCGGAGTCAACGGATTCAAATTACAAGTGTAAAGCCATAGTATAAAAC
		GGTGAATGTTGTACTAAGTGTGCACTAAA

*VIC and FAM refer to the fluorescence type from the KASPar system associated with the primer for each allele

† In some strains this primer pair yields no result with either SNP. Most likely this is due to a 2bp insertion in the mtDNA at location 43414, which makes the forward primers not compatible. This insertion was present in ~19% of our population.

Table S3.2 Genotypes for the haploid Sox1 alleles. Only polymorphic positions are given. Polymorphic sites are relative to the reference gene sequences for Sox1 (XM_003031349.1).

	370	385	391	418	421	442	457	458	461	475	481	487	503	505	520	547	550	580	587	595	638
S01	g	g	t	t	g	c	c	c	c	c	t	c	t	g	c	t	t	g	c	g	a
S02	g	a	c	c	g	a	c	c	c	t	c	c	c	a	c	c	a	g	c	g	a
S03	a	g	c	c	g	c	t	c	c	c	c	t	c	g	c	t	t	g	c	g	a
S04	g	g	c	c	g	c	t	c	t	c	t	t	c	g	c	c	a	g	t	c	g
S05	g	g	c	c	g	c	t	c	t	c	t	t	c	g	c	c	a	c	t	c	g
S06	g	a	c	c	g	a	c	c	c	t	c	c	c	a	c	c	a	c	c	g	a
S07	g	g	t	c	t	a	c	c	c	t	c	c	c	a	c	c	a	c	c	g	a
S08	g	a	c	c	g	a	c	c	c	t	c	c	c	a	t	c	a	c	c	g	a
S09	g	g	t	c	t	c	t	c	c	c	c	t	c	g	c	t	t	g	c	g	a
S10	g	g	t	t	g	c	c	c	c	c	t	c	t	g	c	t	t	c	c	g	a
S11	g	g	t	c	t	a	c	c	c	t	c	c	c	a	c	c	a	g	c	g	a
S12	g	g	t	c	g	c	c	c	c	c	t	c	t	g	c	t	t	g	c	g	a
S13	g	a	c	c	g	c	t	c	c	c	c	t	c	g	c	t	t	g	c	g	a

Table S3.3 Genotypes for the dikaryotic RPB2 alleles. Only polymorphic positions are given. Polymorphic sites are relative to the reference gene sequences for RPB2 (XM_003038777.1).

	1653	1656	1686	1707	1713	1716	1725	1731	1734	1746	1761	1779	1785	1794	1797	1803	1812	1818	1821	1830	1836	1867	1896	1899	1926
R01	s	s	r	g	r	r	c	c	g	c	y	a	y	c	y	r	r	c	y	r	t	y	r	y	r
R02	g	c	a	g	g	g	c	c	g	c	c	a	c	c	c	a	a	c	t	r	t	t	r	c	g
R03	g	c	a	g	g	g	c	c	g	c	c	a	c	c	c	a	a	c	t	a	t	t	g	c	g
R04	a	c	g	a	t	g	c	c	g	t	c	a	c	c	c	a	a	c	t	g	c	t	a	c	g
R05	r	c	g	r	k	g	c	c	g	t	c	a	c	c	c	a	a	c	t	g	y	y	a	c	g
R06	r	c	g	g	r	g	c	y	r	c	c	a	c	c	c	a	a	y	y	g	w	y	a	c	g
R07	r	c	r	r	k	g	c	c	g	y	c	a	c	c	c	a	a	c	t	g	y	t	a	c	g
R08	g	c	r	g	g	g	y	y	g	y	m	m	t	y	y	r	a	c	y	g	y/c	t	r	c	r
R09	a	c	g	a	g	g	t	t	g	t	a	c	t	t	t	g	a	c	c	g	c	t	g	c	a
R10	a	c	g	a	k	g	y	y	g	t	m	m	y	y	y	r	a	c	y	g	c	y	r	c	r

Table S3.4 Haplotypes for the mitochondrial genome based on 8 SNPs. Position relative to reference genome NC_003049.

	474 (c/g)	6280 (c/t)	7274 (g/a)	15534 (a/c)	21031 (a/g)	30738 (t/c)	43198 (t/g)	43418 (c/a)
H4.8 (NC_003049)	c	c	g	a	a	t	t	c
mtA	c	t	g	c	a	t	g	a
mtB	c	t	g	c	a	c	g	a
mtC	g	c	a	c	a	c	g	a
mtD	g	c	a	c	a	c	g	n†
mtE	g	c	a	c	g	c	g	n†
mtF	c	c	a	c	a	c	g	a
mtG	g	t	g	c	a	c	g	a
mtH	g	c	g	c	a	c	g	a

† In some strains results for neither SNP was retrieved. Most likely this is due to a 2bp insertion in the mtDNA at location 43414, which makes the forward primers not compatible. According to whole genome sequencing of our pooled population this insertion was present in ~19%, which corresponds remarkably well to 'No signal' as observed for 11 from the 59 samples (18.6%).

Chapter 4

Sexual selection in mushroom-forming basidiomycetes

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Summary

We expect that sexual selection may play an important role in the evolution of mushroom-forming basidiomycete fungi. Although these fungi do not have separate *sexes*, they do play female and male *roles*: the acceptance and the donation of a nucleus, respectively. The primary mycelium (monokaryon) of basidiomycete fungi, growing from a germinating sexual spore, is hermaphroditic, but it loses female function upon the acceptance of a second nucleus. The resulting dikaryon with two different nuclei in each cell retains a male potential as both nuclei can fertilize receptive mycelia. We tested the occurrence of sexual selection in the model species of mushroom forming basidiomycetes, *Schizophyllum commune*, by pairing monokaryons with fully compatible dikaryons. In most pairings, we found a strong bias for one of the two nuclei although both were compatible with the monokaryon when paired alone. This shows that sexual selection can occur in mushroom-forming basidiomycetes. Since the winning nucleus of a dikaryon occasionally varied depending on the receiving monokaryon, we infer that sexual selection can operate through choosiness of the receiving individual (analogous to female choice). However, in other cases the same nucleus won, irrespective of the receiving monokaryon, suggesting that competition between the two nuclei of the donating mycelium (analogous to male-male competition) might also play a role.

Introduction

Sexual selection is defined as the component of natural selection associated with variation in reproductive success caused by competition for access to gametes of the opposite sex (see Chapter 7; Darwin, 1871; Andersson, 1994). It is reflected in competition between individuals of the same sex for a mating (usually strongest in males: 'male-male competition') and preference for some individuals as mates (usually strongest in females: 'female choice'). Sexual selection is known to be of importance in the animal and plant kingdom (Andersson & Simmons, 2006; Leonard, 2006; Shuster, 2009), but so far this has not been recognized in fungi (but see Rogers & Greig, 2009). In plants and animals the traits and behaviors associated with sexual selection are often quite elaborate, but in fungi such traits are more difficult to observe. For sexual selection to occur, heritable variation in mating success needs to be present leading to increased fitness (Kokko et al., 2006). In this chapter we show that sexual selection occurs in the basidiomycete fungus *Schizophyllum commune*.

The lifecycle of most basidiomycetes encompasses two distinct phases, that of the monokaryon and of the dikaryon. Initially, a meiotic haploid spore germinates, giving rise to a mycelium with uni-nucleate cells, the monokaryon. This mycelium can grow vegetatively and, when it meets another monokaryon of the same species, hyphal fusions occur between the two mycelia (see Fig 4.1). At that moment fertilization of the mycelium can occur. In most mushroom-forming basidiomycetes, fusion is followed by exchange of nuclei but not cytoplasm (Hintz et al., 1988; May & Taylor, 1988), resulting in a mycelium with bi-nucleate cells, the dikaryon. Nuclei migrate from the contact zone through the whole receiving mycelium (Gladfelter & Berman, 2009). The exact process of dikaryotization is unknown, but it must involve many nucleus duplications (Kües, 2000) because the outcome of dikaryotisation is that all cells of both receiving mycelia contain both nucleus types (see Fig 4.1b). Just like the monokaryon, the dikaryon can grow vegetatively, but it is also able to form sexual fruiting bodies (the mushrooms). In the fruiting bodies the two nuclei fuse, directly after which meiotic spores are produced. A dikaryon can no longer accept other nuclei, but it can still donate nuclei to a monokaryon (Buller, 1930; Snider & Raper, 1958), a phenomenon called the 'Buller phenomenon'.

Even though basidiomycetous fungi are considered to have no sexes (Day, 1978; Hurst & Hamilton, 1992), clear male and female roles can be distinguished in their general life cycle (Aanen et al., 2004; Billiard et al., 2011). Using the common criterion that male and female gametes are defined by small and large size respectively (see Chapter 2; Parker et al., 1972; Bell, 1982; Lehtonen & Kokko, 2011), the acceptance of a nucleus by a large mycelium that contributes all cytoplasm can be seen as a female-like function, and the donation of a nucleus as a male-like function. Previously, people have referred to mating types in basidiomycetous fungi as being different sexes (e.g. Kothe, 1996). Note that we do not. We will treat mating types as sexual compatibility systems, comparable to self-incompatibility systems in plants. We will go into more detail on this topic in the Discussion (see also Chapter 2). The male and female-like functions imply that a monokaryon is hermaphroditic, but that it can function only once as a female during

mating, while after having been fertilized it retains its male potential via the Buller phenomenon. Furthermore, spores that have not germinated can also act as males by fertilizing a monokaryotic mycelium (Adams et al., 1984). According to this view, the nucleus functions as the male gamete and the receiving mycelium as the female gamete. The consequence of this is that in nature the ratio of male and female functions is strongly male biased (Anderson & Kohn, 2007).

Sexual selection is expected to occur during a dikaryon-monokaryon (di-mon) mating because both nuclei (analogous to male gametes) of the dikaryon are able to fertilize the receiving monokaryon (analogous to the female gamete). An important prediction is that the monokaryon should be choosy as a female: after fertilization by a nucleus, it is engaged in a life-long relationship with that nucleus. In other words, the monokaryon can play its female role only once. In contrast, the nuclei of the dikaryon are expected to be promiscuous as the fertilization of a monokaryon is essentially cost free and they can play the male role over and over again. Therefore, the two nuclei compete for fertilization, which potentially selects for traits that increase success in male-male competition. It has been shown that systematic differences in mating success between the two nuclei of a dikaryon can occur in di-mon matings (Ellingboe & Raper, 1962; Crowe, 1963; Ellingboe, 1964). However, this has not been recognized as sexual selection and has not been studied systematically for many strains. Furthermore, it is unknown whether this difference is based on female choice or male-male competition.

Here, we test the occurrence of sexual selection in *S. commune*. To show its occurrence, we investigate if selection during matings occurs based on a genetic characteristic that favors one nucleus type over another in fertilization. Assuming that sexual selection occurs, we expect to observe consistent differences between nuclei in their mating success in a given pairing. Because fungi can be multiplied clonally, we have been able to perform the exact same mating in many replicates. Furthermore, due to the hermaphroditic character of the nuclei, we can use the male and female characteristics of the same genotype to experimentally distinguish between the two main causes of sexual selection, male-male competition and female choice. With male-male competition one of the two nuclei in the dikaryon should have a consistently higher fertilization success, irrespective of the receiving monokaryon. In contrast, with female choice, which nucleus wins will be dependent on the receiving monokaryon.

Materials & Methods

Strains, media and growth conditions

In this research six different monokaryotic strains were used, designated A through F, which were derived as follows. Six dikaryotic mycelia were isolated from fresh fruiting bodies of *S. commune*, collected in the Netherlands (A, B, E & F), Germany (C) and Slovenia (D) and were fruited in the laboratory (Simchen, 1966a). From each fruiting body, we isolated a monokaryon originating from a single spore. To exclude effects of cytoplasmic elements, we placed each nucleus in the same cytoplasmic background. For this, we crossed each of the six above described

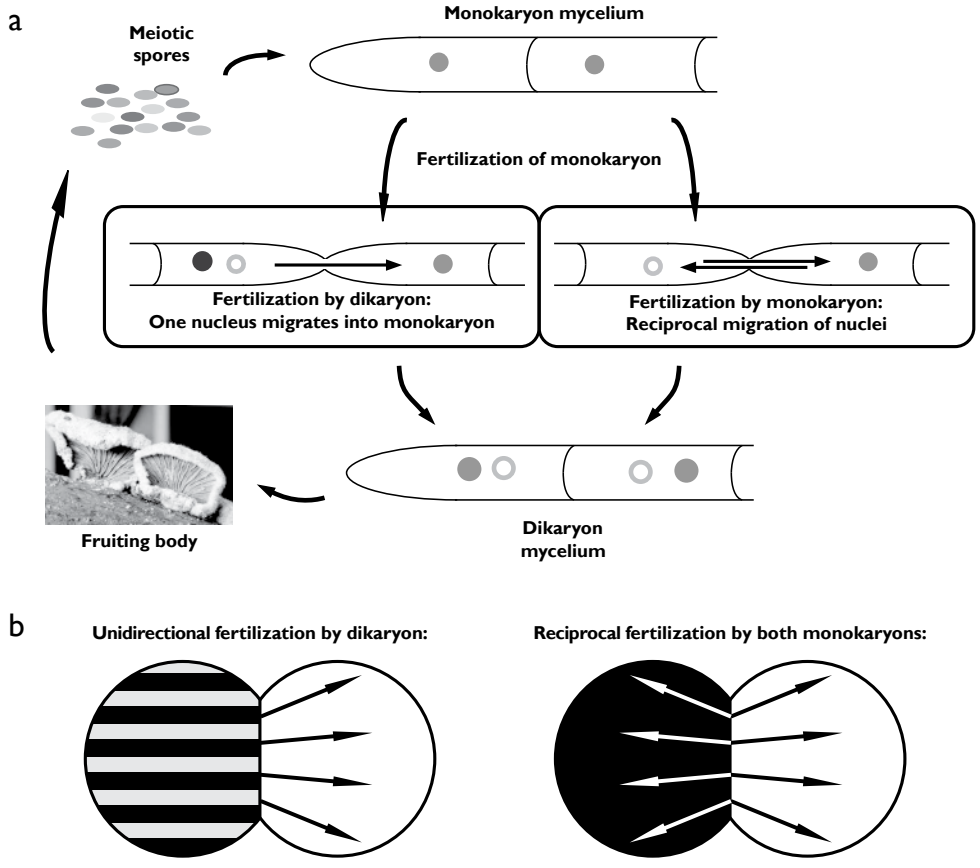

Figure 4.1 Lifecycle and fertilization of *Schizophyllum commune*. Representation of lifecycle of Schizophyllum commune with a monokaryon-monokaryon mating and dikaryon-monokaryon mating at **a)** the hyphal level and **b)** the mycelium level.

monokaryons in their male function with a seventh monokaryon to establish a dikaryon. We de-dikaryotized these dikaryons using protoplast regeneration according to the method of de Vries and Wessels (1972). During this process monokaryotic mycelia can be obtained that only possess one of the nucleus types of the dikaryon. From the retrieved monokaryons we selected the original monokaryons based on the mating types.

Furthermore, for each strain we created a transformant that contains a dominant resistance marker to the antibiotic nourseothricin (construct pGEMNour; kindly provided by Luis Lugones) using protocols described in van Peer et al. (2009). All strains were grown at 27°C in the dark on Minimal Medium (Dons et al., 1979).

Dikaryon-monokaryon matings

We created all 15 possible dikaryons from the six monokaryon combinations (Raper & Hoffman, 1974). To control for marker effects and role in dikaryon formation, per pair of monokaryons four types were created: with either nucleus containing the resistance marker and with either nucleus as receiving mycelium (e.g. A_{res} B, A B_{res}, B A_{res} and B_{res} A; the first letter indicates the receiving mycelium and the second the donating mycelium). All dikaryons were tested against the four monokaryons with which no nucleus was shared (see Table 4.1) with 10 replicates per combination. In total 2400 pairings were performed (15 dikaryons * 4 treatments * 4 receiving monokaryons * 10 replicas). The actual crosses were performed by placing a plug of the dikaryon 5mm from the edge of a three days old monokaryon. After five days incubation, two mycelium plugs from the initially monokaryotic mycelium – that by then had been dikaryotized completely – were taken and tested for nourseothricin resistance. Because the marker is dominant, the dikaryon can directly be tested for growth on plates containing nourseothricin (15µg/ml). For a subset also mating type was used as a marker (Papazian, 1950) to confirm that the marker functioned correctly. No incongruence was found between the resistance marker and mating types.

Table 4.1 Results for all dikaryon-monokaryon matings. The fertilizing dikaryon is given in the rows and the receiving monokaryon in the columns. Each intersection shows the nucleus that performed most of the fertilizations ($p<0.0009$; $n=40$). *ns* indicates there was no significant deviation from 1:1 ratio. Also the ratio of the winning nucleus is given. When there was no significant difference, the ratio of the first nucleus mentioned is given. The intersections indicated with "–" were not tested because one of the nuclei was shared between di- and monokaryon.

	A	B	C	D	E	F
AB	-	-	A 1.00	B 0.85	- [a]	- [a]
AC	-	C 0.90	-	ns 0.70	C 0.83	ns 0.30
AD	-	D 0.90	D 0.90	-	D 0.90	ns 0.55
AE	-	E 0.85	A 1.00	A 1.00	-	ns 0.30
AF	-	F 1.00	F 0.90	A 0.80	A 0.80	-
BC	B 0.80	-	-	ns 0.65	B 0.80	ns 0.25
BD	ns 0.37	-	D 1.00	-	D 0.98	D 1.00
BE	B 1.00	-	B 0.98	E 0.90	-	E 0.80
BF	B 0.80	-	F 0.80	F 1.00	F 1.00	-
CD	D 0.85	D 0.85	-	-	D 0.95	ns 0.60
CE	ns 0.60	C 0.85	-	C 0.93	-	C 0.95
CF	ns 0.70	ns 0.70	-	F 0.93	F 0.88	-
DE	D 0.83	D 0.85	D 1.00	-	-	D 0.88
DF	F 0.75	F 0.93	ns 0.53	-	D 0.90	-
EF	F 0.85	F 0.95	F 1.00	F 0.88	-	-

[a] due to contaminations of the samples no data for these crosses was obtained

Results

We performed all possible dikaryon-monokaryon matings between six monokaryon strains and all their 15 dikaryon combinations (Table 4.1). For each mating we established the frequency of fertilization per nucleus type. We did not find an effect for marker (e.g. $A_{res}B$ or AB_{res}) nor for maternal effects (i.e. whether a nucleus in the fertilizing dikaryon descended from the receiving or from the donating monokaryon [e.g. AB or BA]) upon the fertilizing success of nuclei. Therefore, we treated all four kinds of dikaryon containing the same nuclei as additional replicates. We first give the results of each mating individually and will then subsequently discuss the results from the dikaryon (male) point of view and from the receiving monokaryon (female) point of view.

For 46 out of 58 di-mon matings we found a ratio that significantly differed from 1:1, after Bonferroni correction for multiple (N=58) replicates (binomial test, p<0.0009, n = 40), which indicates that selection of one of the two nuclei occurred. Across all pairings, the mean value of the most successful nucleus was 0.85 (StDev 0.124).

For six of the 15 tested dikaryotic strains, the nucleus fertilizing (male) depended on the receiving mycelium (female). For nine dikaryons always the same nucleus was most successful with all four receiving monokaryons. To test whether this result was caused by an inherent difference between the two nuclei irrespective of receiving monokaryon, or by the low number of tested receiving monokaryons (four), we tested four of these strains (BD, CE, DE and EF) with five additional receiving monokaryons (strains G-K; each originating from a different dikaryon; G collected in Brazil and H-K in the Netherlands). For one dikaryon (CE) in one pairing this time it was the other nucleus that was more successful, whereas for the other dikaryons again the same nucleus always won (data not shown).

From the receiving monokaryon perspective, half of the monokaryons (B, C and D) showed a clear transitive hierarchy in fertilizing nuclei (if nucleus Y was preferred over X, and Z over Y, than Z was also preferred over X). A comparison of the ranking between these three strains showed no clear pattern that would indicate a shared preference (rankings given in Table 4.2). Monokaryon F had too few comparisons to make a complete ranking. For the receiving monokaryons A and D preference was not hierarchical.

Discussion

Sexual selection acts in mushrooms. Our results show that a highly reproducible strong bias for either one of the two potentially fertilizing nuclei in natural isolates of *S. commune* exists – indicating sexual selection – and that this bias depends partly on the receiving mycelium – indicating female choice. Next to female dependent fertilization, for nine dikaryons we found that always the same nucleus performed the fertilization, irrespective of the female. This indicates that some nuclei are more successful males than others, either in being chosen, or in direct competition with other nuclei.

Table 4.2 Fertilization ranking per receiving monokaryon. For each receiving monokaryon, a ranking is indicated of the success of fertilizing nuclei in Buller pairings. For four receiving monokaryons a ranking is found, for A and E no ranking can be made (see also Table 4.1). The ranking for monokaryon F is based on few comparisons due to many non-significant interactions.

A	No ranking								
B	F	>	D	>	C	>	E	>	A
C	F	=	D	>	A	>	B	>	E
D	A	>	F	>	C	>	E	>	B
E	No ranking								
F	D	>	C	>	E	>	B		

The separation of sexual selection in male-male competition and female choice is somewhat artificial and both processes are not mutually exclusive. Only when one of the two sexes is in full control of the fertilization, such a distinction will be applicable. Our results show that in some di-mon matings female choice acts, because the nucleus in the dikaryon chosen depends on the receiving monokaryon. Even though female choice can be shown with our experiment, unfortunately, we cannot be so conclusive about male-male competition. When always the same nucleus in a dikaryon is more successful, irrespective of the receiving monokaryon, this might be caused by a direct interaction between the two nuclei, *i.e.* male-male competition. However, it is still possible that female choice acts, but that all receiving mycelia have the same preference. These two processes cannot be distinguished here.

It is unclear on which criteria the observed selection, be it driven by female choice or by male-male competition, is based. If selection would be based on a single quantitative trait, then we should be able to create a hierarchy; if Y is preferred over X, and Z over Y, than Z should be preferred over X. The same goes for competition. For half of the receiving monokaryons a hierarchy cannot be made (Table 4.2). This either means that competition and preference act at the same time in opposite directions, or that preference depends on a non-linear trait or multiple traits. An example of the latter might be that next to a hierarchical trait also heterozygosity is selected for. A candidate trait might be the mating type. For the Buller phenomenon the mating type locus (or loci) has been suggested as a trait for selection, in which the nucleus in the dikaryon that is more different at this locus in relation to the receiving monokaryon wins (Crowe, 1963; Raper, 1966).

Basidiomycete fungi have a sexual compatibility system, comparable to the self-incompatibility system of angiosperm plants, determined by one or two mating type loci. Only when the mating type factors are different, successful mating will occur and consequently a dikaryon will always be heterozygous at the mating type locus or loci. Because of the high diversity in mating type alleles in *S. commune* (like in many mushroom forming basidiomycetes), about 97% (mon-mon) and 95% (di-mon) of the matings between two individuals in nature will be fully compatible (Raper & Krongelb, 1958; Fraser et al., 2007). Nuclear exchange and maintenance

of the dikaryon phase are mediated by the interaction of the genes of the mating types of the interacting nuclei (reviewed in Heitman et al., 2007) and can partly be used to predict nucleus selection in isogenic lines (Ellingboe & Raper, 1962; Crowe, 1963).

The B-locus, coding for one of the two mating type factors, has also been identified as an important determinant for recovery of monokaryons from dikaryons after artificial de-dikaryotization using protoplast regeneration (see Material & Methods and de Vries & Wessels, 1972; Raper, 1985). Raper (1985) found a transitive hierarchy of recovered nuclei, which was caused by an interaction between the two nuclei in a dikaryon. It was suggested by Nogami et al. (2002) that the recovery success of nuclei after de-dikaryotization is correlated with the relative success of nuclei in Buller pairings; this could be interpreted as an example of male-male competition. Using three strains of *Pholiota microspora (P. nameko),* they observed the same hierarchy for monokaryon recovery as for Buller fertilization, but because of the low number of strains used, each time only two strains could be compared and only for one receiving monokaryon. Even though we did not find a consistent hierarchy in our matings, the described interaction between the nuclei could act during a Buller mating (see Table 4.2). This discrepancy between these studies and ours can be caused by their use of highly inbred strains that were only different for mating types, whereas we used natural isolates. It has been found that other genes than the mating type genes also affect nuclear success in Buller matings (Raper, 1966 pp. 123, BPSN unpublished results), which might be an explanation for the non-hierarchical pattern in the Buller matings reported in this paper. A follow-up study on the comparison between Buller matings and asymmetrical protoplast recovery will be described in Chapter 5.

Sexual selection is considered an important component of natural selection driving evolution in many different groups of sexual organisms, but to our knowledge it has until now not been recognized in filamentous fungi. The strong preferences that we found in natural isolates shows that sexual selection potentially is very significant in the life cycle of mushrooms, in which di-mon matings are likely to be frequent (Chapter 3; Raper, 1966; Fowler & Vaillancourt, 2007), and that it should be considered when studying mushrooms. Recently, Rogers and Greig (2009) showed in a very elegant experiment with the single celled fungus *Saccharomyces cerevisiae* that selection in a very sex biased environment also leads to sexual selection. In this experiment, female preference for high pheromone levels led to selection for high pheromone production. However, in this species such bias in natural situations is not very likely.

It will be interesting to study how sexual selection affects other fitness components of the resulting dikaryon. Because fertilization has direct effects on the receiving mycelium (e.g. changed growth rate [Simchen, 1966a] and protein expression [de Vries & Wessels, 1984]) and indirect effects through offspring fitness, fitness measurements (cf. Pringle & Taylor, 2002) should be performed on the dikaryon itself as well as on monokaryons originating from basidiospores from mushrooms formed by the dikaryon. To understand the evolutionary advantage of female choice and to explore if male-male competition can arise, more needs to be known on the ecology of mushroom species: How long is the monokaryon phase? How many monokaryotic

and dikaryotic individuals will a mycelium meet? What is the cost of inbreeding? In Chapter 8 this will be discussed more elaborately.

Our findings show that sexual selection is more broadly present than was previously thought and that it also acts in fungi. This example confirms that, whenever variation occurs in fertilization success between individuals, no matter how cryptic, a potential for the evolution of sexually selected traits exists. Bateman (1948) suggested that selection between males and related effects may have influenced the evolution of animals and plants in various ways for which much support has been found over the years. Our findings indicate that this might also be true for fungi.

Acknowledgements

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Chapter 5

Fungal fidelity: Nuclear divorce from a dikaryon by mating or monokaryon regeneration

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Summary

Basidiomycete fungi perform fertilizations by incorporation of nuclei into a monokaryotic mycelium to establish a dikaryon. The dikaryon cannot incorporate another type of nucleus, but can still act as a nucleus donor in a dikaryon-monokaryon (di-mon) mating, known as the Buller phenomenon. Previously, it has been observed that: 1) in a particular di-mon mating, one of the nucleus types of the dikaryon generally performs better as a donor than the other, and 2) when nuclei from a dikaryon are separated to form monokaryons again (dedikaryotisation), recovery of monokaryons of the two nucleus types is usually unequal. In this study, we investigated if these two observations of asymmetry are functionally related. We tested this hypothesis by performing both di-mon matings and dedikaryotisation of dikaryons derived from five different monokaryons. When a single mechanism controls both processes, the nucleus better at fertilizing a monokaryon in a Buller pairing should also be recovered upon dedikaryotisation with a higher frequency. The results showed a hierarchical structure for recovery among nuclei in dedikaryotisation, but this hierarchy did not correspond to the fertilization success during di-mon mating. These findings thus show that the mechanism causing asymmetric regeneration of nuclei, is most likely not the same as the mechanism responsible for increased chance of fertilization in di-mon matings. We discuss the complexity of the interactions which occur during di-mon matings with regards to the mating-type loci.

Introduction

Basidiomycetous fungi germinate from a spore to form a primary monokaryotic mycelium in which each cell compartment contains a single haploid nucleus. At fertilization nuclei from a compatible mycelium are incorporated into the own mycelium after which a secondary mycelium is formed: the dikaryon (Raper, 1966; Kües, 2000). In the dikaryon the two nuclei have a very tight connection with each other. Functionally, the dikaryon acts like a diploid (but see Wessels et al., 1999). For example, both nuclei together regulate cell growth, and deficiencies in one nucleus are compensated by the other genome (Clark & Anderson, 2004). However, because the two nuclei remain separate, evolutionary forces can still act on the individual nuclei. If one of the two nuclei can increase its own fitness at the expense of the other's, this can be selected for, even if it is at a cost to the dikaryon (Buss, 1987; Aanen et al., 2004; Aanen et al., 2008). There are two specific moments during which selection at the level of the nuclei can occur: during mating and during asexual propagation (See Fig 5.1a-b; Buss, 1987; Nieuwenhuis et al., 2011). At both moments, mechanisms to take advantage of the other nucleus could arise and be selected. In response counter mechanisms could evolve to keep the other nucleus in track, in order not to be cheated and to enforce fidelity (Buss, 1987).

Competition during mating

After a dikaryon is established, another nucleus cannot be incorporated anymore, but the mycelium is still able to fertilize monokaryons by donating nuclei. These dikaryon-monokaryon (di-mon) matings were first described by Buller (1930) and are therefore referred to as the Buller phenomenon (Quintanilha, 1937). Mating in basidiomycetes is regulated by two different mating type loci (*matA* and *matB*), which both have to be different for compatibility. Because in many species multiple alleles exist at each of the mating type loci, in most cases, both nuclei in a dikaryon are in principle capable to donate nuclei to a monokaryon (Raper, 1966; Anderson & Kohn, 2007). However, per di-mon mating, only one of the two nuclei will successfully perform fertilization of the mycelium (Ellingboe & Raper, 1962). The two nuclei of a dikaryon are thus in competition with each other in fertilizing the monokaryon they meet (Nieuwenhuis et al., 2011). Because the size of a filamentous fungus is highly correlated with its available resources and its growth and fructification potential (Pringle & Taylor, 2002), winning the competition will be highly advantageous.

When repeatedly performing the same fully compatible di-mon mating, based on chance, one would expect each nucleus type to perform the same number of matings. However, often the same nucleus type is more successful during mating (Quintanilha, 1939; Ellingboe & Raper, 1962; Nieuwenhuis et al., 2011). We recently showed that during competition, the receiving mycelium usually determines which nucleus wins the competitions, but that in some combinations the outcome of competition depends on a direct interaction between the two nuclei (Nieuwenhuis et al., 2011). We do not know which mechanism is responsible for this direct interaction, but potentially the competitive ability between the nuclei during mating is associated

with another mechanism that is known from vegetative growth (Nogami et al., 2002; Anderson & Kohn, 2007).

Competition during growth

Because each nucleus remains separate from the other, a potential conflict arises between the nuclei during asexual propagation (Buss, 1987; Ramsdale, 1999). For instance, if one of the two nuclei divides faster than the other within the mycelium, it can increase its relative abundance within the mycelium, even if this decreases the fitness of the mycelium. Alternatively, a nucleus can be opportunistic in positioning itself towards the hyphal tip. Because growth in filamentous fungi occurs at the edge of the colony, those nuclei that position themselves at the hyphal tips take part in growth and can replicate (Xiang & Fischer, 2004). In most ascomycetes and some basidiomycetes, especially those without clamp connections (see below), mitotic growth is not well regulated (Gladfelter & Berman, 2009). These fungi form multinucleate heterokaryotic cells, in which the ratios between nuclei can deviate strongly from fifty-fifty (Davis, 1959; Hui et al., 1999; James et al., 2008). This can lead to escape of monokaryotic hyphae (e.g. *Agaricus bisporus*; (Wang & Wu, 1974) and to the production of monokaryotic asexual spores (oidia) favouring the nucleus that is in the majority (e.g. *Heterobasidion annosum*, *H. parviporum, Pholiota microspora* [*P. nameko*]; Arita, 1979; Ramsdale & Rayner, 1994; Ramsdale & Rayner, 1996; Hui et al., 1999; James et al., 2008).

Buss (1987) suggested that the basidiomycete clamp connections have evolved, to suppress selfish behaviour at the nucleus level, meaning that a nucleus can increase its fitness only at the level of the mycelium. Clamp connections are a well-regulated growth form, which ensures partner fidelity between the nuclei (Buller, 1933). During apical cell growth after the nuclei divide, one copy of each nucleus migrates towards the hyphal tip, while the other copy either migrates into the sub-apical cell, or migrates into the clamp cell, which then fuses back to the sub-apical cell (Fig 5.1c). The order of nuclei changes after each cell division, which further reduces the possibility for selfish behaviour of either nucleus (Iwasa et al., 1998; Badalyan et al., 2004). In species that lack clamp connection or that grow only partially with clamps, one of the two nuclei can increase in relative abundance within the mycelium or in the oidia (Hansen et al., 1993; Ramsdale & Rayner, 1996; Hui et al., 1999).

Synchronized nuclear division in the apical cells (conjugate nuclear division) is regulated by the mating type loci. Synchronous division is initiated by *matA* (Raper & Raper, 1966; Erdmann et al., 2012), but appears to be suppressed by *matB* (Raper, 1985). Raper (1985) suggested that suppression in nuclear division by *matB* is analogous to mating-type hormone mediated cell-cycle arrest in *Saccharomyces cerevisiae*. Activation of the mating type specific receptors to which the pheromones are ligand leads amongst others to cell-cycle arrest in *S. cerevisiae* (Bardwell, 2005). Pheromones produced in basidiomycetes are analogous to the *a*-factor in *S. cerevisiae* and might have similar functions in basidiomycetes (Fowler et al., 1999; Fowler & Vaillancourt, 2007). Suppression becomes apparent when the two nuclei from a dikaryon are separated to form monokaryons (these 'dedikaryotized' monokaryons are referred to as 'neohaplonts' to

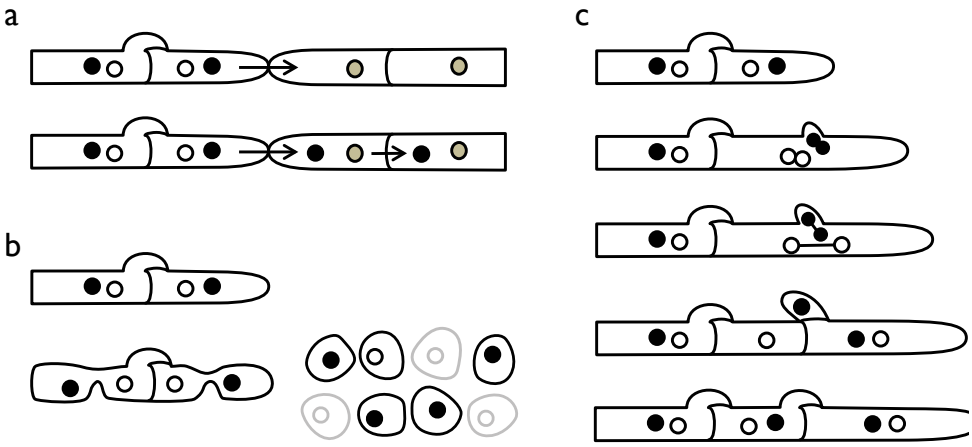

Figure 5.1 a) Di-mon mating in which the black nucleus type preferentially performs fertilization of the monokaryon. **b)** Unequal recovery of monokaryons after protoplasting of a dikaryon, even though equal numbers of protoplasts of each nucleus type were formed. **c)** Different steps during vegetative growth with clamp connections in which the nuclei change position after each cell division. Clamp connections ensure faithful nuclear inheritance of both nuclei in the dikaryon. (c Reproduced from Iwasa et al. 1998)

indicate their dikaryotic origin; Fries & Aschan, 1952). Irrespective of the method – whether it is by protoplasting, macerating or surgical manipulation – in many species, one of the two nuclei is more successful than the other in recovering and continuing growth as a monokaryon even when the nuclei in the mycelium are present in a 1:1 ratio (e.g. Raper, 1985; Kay & Vilgalys, 1992; Ikeda et al., 2003). Raper (1985) observed that among the protoplasts derived from dedikaryotized dikaryons, 25-28% remained uninucleate, whereas only 1-5% of the protoplasts from monokaryons remained uninucleate. Apparently, the interaction between the two different nuclei in the dikaryon reduces nuclear division after separation of the nuclei. This affects one of the two nuclei stronger than the other and results in asymmetry in monokaryon recovery. Another observation is the hierarchy in monokaryons that suppress regeneration of the other nucleus. If nucleus X predominates over nucleus Y, and Y predominates over Z, then X will also predominate over Z.

Research aim

In this study we test the hypothesis that suppression of the competing nucleus in mitotic division is beneficial in competition over fertilization in Buller-matings (Nogami et al., 2002; Anderson & Kohn, 2007). After fusion and initial migration of the fertilizing nuclei into the mycelium, nuclear division has to occur in order to continue fertilization of the mycelium. Even though there is some debate about when and how often this division occurs (Nguyen & Niederpruem, 1984; Kües, 2000), eventually all parts of the mycelium become dikaryotized. Observations that only a few nuclei can accomplish complete dikaryotization confirm that new nuclei are produced by mitosis (Williams & Todd, 1984). After fusion, both nucleus types migrate into the myce-

lium, but only one of the two eventually succeeds in forming the dikaryon (Ellingboe, 1964). A nucleus that can divide more rapidly increases the number of migrating propagules, which can divide, thereby increasing the chance of fertilizing the mycelium. This hypothesis has not been tested systematically yet, but there are indications that nuclei with higher hierarchy in protoplast regeneration also perform better in di-mon fertilizations (Nogami et al., 2002). To investigate the suggested relationship between asymmetry in regeneration and mating, we tested neohaplont recovery of strains for which the competitive ability during mating was previously established (Nieuwenhuis et al., 2011).

Material and Methods

Strains and cultivation

In this research, we used six unrelated fully compatible monokaryotic strains of *Schizophyllum commune* as described in Nieuwenhuis et al. (2011). These strains have been placed in the same cytoplasmic background and are designated A through F. All culturing and crosses were performed in the dark at 27°C on minimal medium (MM; Dons et al., 1979), except when stated differently. Strains A-E were crossed with each other in all combination to form 10 different dikaryons, indicated by a two-letter combination. Each dikaryon can be constructed in two different ways: for example AD and DA (mycelium A which incorporates D nuclei, and vice versa, respectively). In this experiment we only used one of the dikaryons for each combination of monokaryons, because pilot experiments using five reciprocal dikaryotic strains showed no significant difference between the reciprocal dikaryons (data presented in Table 5.1).

Protoplasting

Protoplasting was performed based on the methods described by De Vries & Wessels (1972). The dikaryons described above were grown on solid medium for 5 cm, transferred and grown again to obtain stable dikaryons. Half a plate (5 cm diameter) covered with each dikaryon was macerated with a Waring blender and grown in a 500 ml erlenmeyer containing 100 ml liquid MM, shaken at 120 rpm. After 48h the mycelium was macerated once more, 50 ml fresh MM was added and grown for an additional 24h for production of many fresh hyphal tips. The growth medium was removed from the mycelium by filtering the mycelium on sterile cheesecloth and rinsed with 1M MgSO4. The mycelium (1-3 ml) was re-suspended in a filter-sterilized lysis solution that consisted of 10 ml 1M MgSO4 (adjusted with malate buffer to pH5.8) and 20 mg lysis enzymes (PRI Mushrooms, Wageningen UR, The Netherlands). The mix was incubated for 2.5 hours at 30ºC (not shaken), after which the total volume in the tubes was doubled, by adding sterile MilliQ water. After one more hour incubation, the supernatant was sieved through sterile glass wool twice to remove mycelium fragments, and centrifuged at 2200x*g* for 15 minutes. The pelleted protoplasts were washed once with 0.5 M MgSO4, spinned down once more and re-suspended in 50µl 0,5M MgSO4. A dilution series in 0.5M MgSO4 with penicillin/streptomycin

Table 5.1 Protoplast regeneration ratios for each given dikaryon and, for strains for which this was measured, the reciprocal ratios. For each dikaryon also is given which nucleus won competitions in Buller matings. '—' were not tested because one nucleus is shared between monokaryon and dikaryon, 'ns' indicates no significant result and 'x' was not measured.

	Protoplast regeneration				Winning nucleus per receiving monokaryon in di-mon matings[a]					
Di-karyon	Winner	% Majority	Neohaplont ratios	Reciprocal[b]	A	B	C	D	E	F
AB	A	96.1%	73 : 3	93.9% (n=49)	—	—	A	B	*x*	*x*
AC	A	81.4%	57 : 13	75.9% (n=29)	—	C	—	ns	C	ns
AD	A	70.9%	39 : 16		—	D	D	—	D	ns
AE	A	98.0%	49 : 1		—	E	A	A	—	ns
BC	C	72.2%	15 : 39	78.6% (n=28)	B	—	—	ns	B	ns
BD	D	90.0%	5 : 45	91.1% (n=45)	ns	—	D	—	D	D
BE	ns (B)	52.9%	27 : 24	52.1% (n=48)	B	—	B	E	—	E
CD	D	74.5%	13 : 38		D	D	—	—	D	ns
CE	C	74.5%	41 : 14		ns	C	—	C	—	C
DE	D	96.0%	48 : 2		D	D	D	—	—	D

[a] data from Chapter 4

[b] ratio of dedikaryotisation of the reciprocal dikaryon (see section '2.1 Strains and cultivation')

(1 mg/ml) of the protoplasts was plated out on MM+0.5M MgSO4 plates and left to incubate at 27°C. After 6-8 days the colonies formed were checked for clamp connections and all monokaryons were transferred to fresh MM plates. After 3 days of growth all neohaplonts were crossed with either parental type to test the mating type of the neohaplont and at least 50 neohaplont were tested per dikaryon. Because protoplast regeneration did not always yield 50 neohaplonts in one session, for 7 strains protoplasting was repeated once more.

Results and Discussion

Separation of the nuclei of ten different dikaryons showed significant asymmetric recovery of monokaryons in nine cases (see Table 5.1; binomial test with bonferroni for multiple comparisons $n=14$, $p<0.0037$). Furthermore, the neohaplont recovery data clearly showed a hierarchical structure for the five used monokaryons, which are ordered as follows:

A > D > C > E = B.

Monokaryon A is predominant over all other monokaryon types, D is dominant over all, except over A etc. This result is in agreement with previous findings (Raper et al., 1958; Raper, 1985), which showed a hierarchy for recovered types of neohaplonts. For each strain, the percentage of recovered neohaplont is negatively related with the ranking of the other nucleus of the dikaryon (see Fig 5.2). The higher the other nucleus is ranked, the fewer neohaplonts are formed. This was tested by scoring if with increase in rank, the recovery of a nucleus increased or decreased. If ranking and recovery are not related a 1:1 ratio is expected. The data showed that for 11 of the steps up in rank the ratio of nuclei decreased, and for one step (BA → BD) it decreased, which significantly deviates from 1:1 (binomial test, $p < 0.01$). This is further supported by the results of a chi-squared test on the absolute number of recovered neohaplonts from the dikaryon with the lowest ranked using the recovered neohaplonts with the highest ranked nucleus as expected values, which showed a highly significant difference ($p < 0.001$; $\chi^2 = 1067$; $df = 4$).

No clear correlation is found between the asymmetric recovery of neohaplonts and the success in matings as described in Nieuwenhuis et al. (2011; see Table 5.1). For three of the used dikaryons, dominance depends on the receiving mycelium, and for these strains the success in fertilization can thus not be attributed solely to the interaction between the two nuclei. For the seven dikaryons with one dominating nucleus irrespective of the receiving mycelium (underlined in Table 5.1), we can assume that dominance depends on the interaction between the two nuclei (although this conclusion is conditional on the tested receiving mycelia). For four of these dikaryons the same nucleus that won the fertilization also predominated in neohaplont recovery, while in three cases the opposite occurred. From these findings we can conclude that we have to reject our hypothesis that the mechanism that regulates protoplast regeneration is also responsible for fertilization dominance.

Even though our data show that dominance in mating cannot be explained by the predominance in regeneration, there might still be a link between both phenomena of asymmetry. Nuclear mitotic divisions are regulated by both *matA* (induction) and *matB* (suppression) (Raper & Raper, 1966; Raper, 1985) and also preferential Buller matings have previously been attributed to both the *matA* and the *matB* locus (Crowe, 1963; Raper, 1966). For the latter however, genes unlinked to the mating type loci have been reported that can overrule the effect of the mating types (Raper, 1966). Furthermore, because in Buller matings not only the two nuclei in the dikaryon are of importance, but also the receiving monokaryon (Nieuwenhuis et al., 2011), multiple mechanisms appear to affect selection during mating, of which suppressed nuclear division might be one.

Even if we suppose that dominance in di-mon matings would solely be caused by mating type mediated differential nuclear division, predicting the outcome of a mating would be hard. Most likely dominance is a result of the interplay between the alleles at both mating type loci, of both fertilizing nuclei and of the receiving mycelium. First, interaction in the dikaryon is complex. Nuclear division is induced by *matA* for which multiple gene copies are present (Ohm et al., 2010b), and thus multiple proteins, which will probably affect each other differently, and

react differently to suppression of nuclear division by *matB*. Furthermore, also for *matB* multiple gene copies are present located on two different sub-loci. At each B sub-locus genes for one receptor and many different pheromones are located, the pheromone/receptor system (P/R), for which a high allelic variety exists (Fowler et al., 1999; Kües et al., 2011). An apparent redundancy of pheromones is observed at each *matB* allele, which might be responsible for the quantitative response in nuclear division and protoplast recovery (see Fig 5.1 and Fowler & Vaillancourt, 2007). Secondly, as soon as nuclei migrate into the monokaryon, mating-type genes from the monokaryon can interact with the migrating nuclei. Which interactions will be of importance depends on the lag time of the interaction between the migrating and the resident nucleus, but also on the residual effect of the interaction between the two nuclei in the dikaryon prior to mating. For instance, the receptors of *matB* are membrane bound and unlikely to migrate with the nucleus (Gola et al., 2000; Erdmann et al., 2012), whereas *matA* produces homeodomain transcription factors which after dimerisation are localized to the nucleus and therefore will probably co-migrate (Spit et al., 1998). Add to this the fact that each mating type locus consists of two sub-loci (Raper, 1966; in *Coprinopsis cinerea* even three, Casselton & Kües 2007); predicting

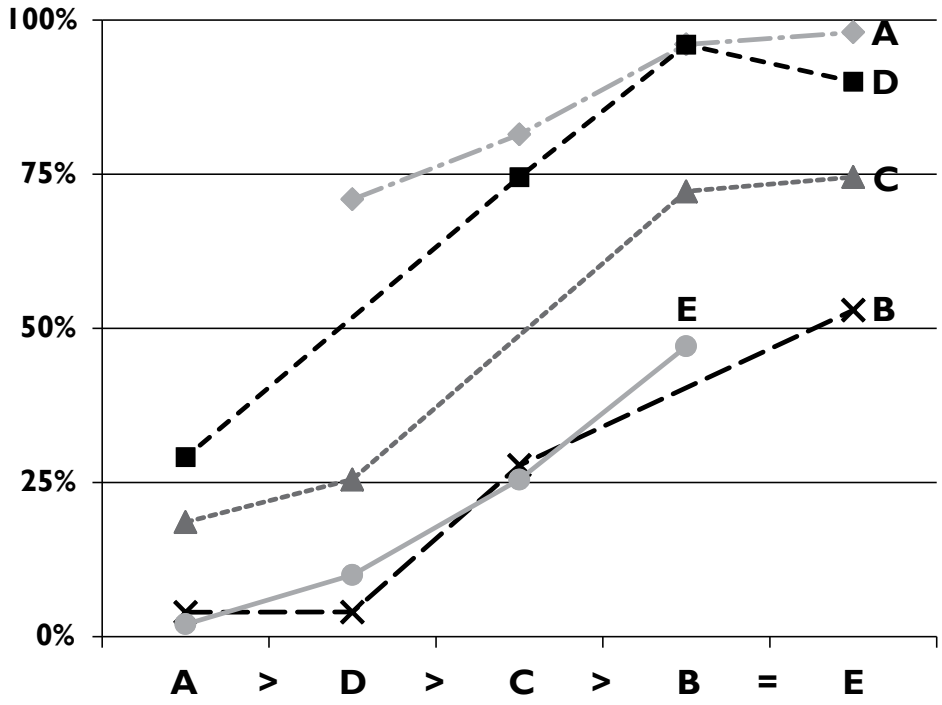

Figure 5.2 Percentage of neohaplonts of each type recovered dependent on the alternate nucleus in dikaryon. Nuclei on the X-axis are ordered by their ranking in qualitative trait 'winning or losing' (A > D > C > B = E). B and E are ranked at the same level. Note that no dikaryons are possible between identical monokaryons and that therefore each line consists of four data points (indicated by markers).

the outcome of each interaction therefore becomes very difficult. Performing this research with a bipolar species (where compatibility is determined by a single mating type locus) might give more insights.

Using the bipolar basidiomycete *P. microspora,* Nogami et al. (2002) showed that for three monokaryons the same hierarchy in di-mon matings as in protoplast regeneration was found – a conclusion that differs from our findings. However, due to the low number of monokaryons used, the association between mating and regeneration dominance found by Nogami et al. could just be based on chance. If their conclusion turns out to be real, the different observation might be caused by the bipolar nature of *P. microspora*. The compatibility system of *P. microspora* lacks the P/R mating type locus (Yi et al., 2008). In *P. microspora*, P/R genes are still present, but all monokaryons have the same allelic variety. The skew in neohaplont mating types observed for *P. microspora* can therefore not be caused by allelic variation of the P/R system, as was observed for *S. commune* (Raper, 1985), but must be caused by another multiallelic locus or multiple loci. The obvious candidate is the *matA*, for which at least six alleles are known in *P. microspora*, and which is involved in nuclear division and clamp formation (Yi et al., 2010). In bipolar species with a non-mating type P/R system, the two nuclei apparently react differently to suppression of nuclear division, which leads to skewed ratios in multinucleate hyphae and even escape of hyphae of a single nucleus type (James et al., 2006; Yi et al., 2010). Repeating this research with more monokaryons of *P. microspora* might give more insights.

Our findings show that selection during mating is not or at least not completely regulated by the same mechanism that leads to asymmetric recovery of protoplasts as was suggested by Anderson & Kohn (2007). Nevertheless, we argue that the latter mechanism most likely, at least partly, affects the competitive ability of nuclei. It will be of interest to compare findings from both bipolar and tetrapolar species to increase our understanding of the many complex functions that mating types regulate, both during mating as well as after. A dikaryon is an interesting growth form in which forced fidelity between the two nuclei assures optimal fitness at the level of the dikaryon. Obtaining more insight in the benefits for the dikaryon of the measures taken to prevent unwanted nuclear divorce, and the costs that arise from nuclear extramarital behaviour, will give more insights in how selection acts at these different levels.

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Chapter 6

Nuclear arms races: sexual selection for masculine mushrooms

Bart P.S. Nieuwenhuis & Duur K. Aanen

Summary

When many gametes compete to fertilize a limited number of compatible gametes, sexual selection will favor those traits that increase competitive advantage during mating. In animals and plants, sperm and pollen competition have yielded many interesting adaptations for improved mating success. In fungi, similar processes have not been directly shown yet. We test the hypothesis that sexual selection can increase competitive fitness during mating, using experimental evolution in the mushroom fungus *Schizophyllum commune*. Mating in mushroom fungi occurs by donation of nuclei to a mycelium. These fertilizing 'male' nuclei migrate through the receiving 'female' mycelium. In our setup, an evolving population of nuclei was serially mated with a non-evolving female mycelium for 20 sexual generations. Four of the twelve tested strains had significantly increased competitive fitness and one had decreased fitness. The main characteristic that explained fitness change was the relative success in colonization of the female mycelium. In most cases, no trade-offs were found with other fitness components. Our results show that sexual selection in mushroom fungi can select for increased competitive ability during mating. We compare these findings with examples of sperm and pollen competition and show that many similarities between these systems and nuclear competition exist. Finally we discuss how these findings can affect mushroom evolution..

Introduction

When two male crickets mate with a female, the male producing most sperm will sire more offspring (Gage & Morrow, 2003). In competition with other males, increasing the number of male gametes increases the chance of fertilization and thus male fitness (Parker et al., 1972; Parker, 1978; Lessells et al., 2009). Not only the *number* of gametes, but also specific *characteristics* of the gametes that increase the ability to perform fertilizations can be selected due to male-male competition. Sperm of animals often have adaptations that give them a competitive advantage in competition with other sperm (e.g. Schärer et al., 2011; Higginson et al., 2012). In plants, pollen are selected for increased growth speed of the pollen tubes to outcompete other pollen on the stigma (e.g. Snow & Spira, 1991; Lankinen et al., 2009).

Competition for mating occurs whenever there is a skew between the gametes to be fertilized and the gametes fertilizing. While members of the sex producing the surplus of gametes are in competition, members of the limiting sex can be choosy. Only recently, it has been realized that these two aspects of sexual selection, *viz.* 'male-male competition' and 'female choice', also apply to fungi (Nieuwenhuis et al., 2011). In many groups of fungi, different sex roles can be distinguished, and there is a skew between gametes fertilizing and gametes to be fertilized (Chapter 7). We have recently demonstrated that there is genetic variation in competitive ability and in choice between fungal individuals during mating (Nieuwenhuis et al., 2011). However, so far, evidence that such traits in fungi are sexually selected is only circumstantial. In this paper, we directly demonstrate sexual selection for increased mating success in fungi, using an experimental evolution approach, and test if the general predictions of sexual selection theory, mostly developed for animals and plants, can also be applied to fungi.

We performed an evolution experiment with the mushroom-forming basidiomycete *Schizophyllum commune*, in which an evolving population of nuclei was repeatedly allowed to mate in the male role with a non-evolving receiving (female) mycelium. In mushroom-forming basidiomycete fungi, a special form of mate competition occurs. In the general life cycle of these fungi, a haploid meiotic spore germinates and forms a mycelium, which grows vegetatively, acquires resources, and occupies substrate (see Fig 1.1). This mycelium can mate in a hermaphroditic fashion by hyphal fusion with a different mycelium. In its male role, it fertilizes other mycelia by donating nuclei. In its female role, fertilizing nuclei are incorporated into the mycelium's own cytoplasm. The incoming fertilizing male nuclei are actively transported through the entire mycelium, in which they divide and which they eventually occupy completely (Raper, 1966; Kües, 2000; Gladfelter & Berman, 2009). The receiving mycelium effectively functions as one single large female gamete. In the fertilized female mycelium, the male nuclei do not fuse with the resident female nuclei, but remain separate. This mycelium, referred to as a dikaryon, is thus entirely composed of cells with two genetically different haploid nuclei. The nuclei only fuse just before new spores are produced via meiosis in the sexual fruiting body, the mushroom. Because of the modular structure of the fungus, fruiting bodies can be formed anywhere on the mycelium (Buss, 1987). Fertilizing nuclei can therefore increase their fitness by occupying as

much of the mycelium as fast as possible, before other nuclei colonize it. In fungi with fertilization by nuclear migration, nuclei migrate through the female mycelium at very high speeds, up to 90 times as fast as the mycelial growth rate (Snider & Raper, 1958; Ross, 1976).

We experimentally test the hypothesis that increased mating success in a mushroom-forming basidiomycete can be sexually selected, using an experimental evolution approach. We find that male competitive fitness has significantly increased after 20 sexual generations. Lines with increased competitive fitness all have increased colonization efficiency into the receiving mycelium, but do not have increased nuclear migration rates. This suggests that the competitive advantage is obtained during the initial stages of entering the mycelium. Finally, we test the hypothesis that increased male fertility trades off with other fitness components, such as mycelium growth rate which can be considered the most important female component of reproduction. Surprisingly, we do not find evidence for such trade-offs.

Material and Methods

Outline of experimental setup

We performed an evolution experiment with the mushroom-forming basidiomycete *Schizophyllum commune* in which an evolving population of nuclei was repeatedly mated in the male role with a non-evolving receiving 'female' mycelium. During each cycle, a fresh non-evolving monokaryon functioning as a receiving mycelium was inoculated with sexual spores. The nuclei could migrate through the mycelium and fertilize it entirely. The most distant part of the fertilized monokaryon, now a dikaryon, was induced to form fruiting bodies and spores, which were collected and used to fertilize a next unfertilized non-evolved mycelium (see Fig 6.1). After twenty cycles of nuclear migration and sexual reproduction, the fitness of the evolved strains was measured, relative to the non-evolved parental strains. For the strains with a change in fitness, we also measured specific fitness components, which might have caused this change.

Strains and culture conditions

In *S. commune* strain H4-8 (matA43, matB41; FGSC no. 9210; Fowler et al., 1999) a resistance marker against the antibiotic nourseothricin (N) or phleomycin (P) was introduced by transformation (plasmids and transformation protocol are described in van Peer et al., 2009). The N marker was crossed into the compatible isogenic strain H4-8b (matA41, matB43; Ohm et al., 2010a). All strains used in this experiment were derived from a single cross between H4-8P and H4-8bN and are expected to be isogenic for all but mating type loci and resistance markers. Monokaryons are referred to as *d* or *e* for mating types A43B41 or with A41B43 respectively with added P or N to indicate resistance (e.g. *dP* for monokaryon with mating type A43B41 and phleomycin resistance marker). Dikaryons during the experiment are named as follows: replicate strain, marker, transfer (e.g. 3N10 for replicate strain 3 with nourseothricin marker after 10 transfers). Parental unevolved dikaryons are referred to as P0 (*dP* x *eP*) and N0 (*dN* x *eN*).

All culturing was performed on defined Minimal Medium (MM; Dons et al., 1979) at 27°C in the dark. Mushrooms were formed in 24h light regime, with Petri-dishes placed upside-down, such that all spores produced would fall on the lid.

Evolution protocol

For the evolution of increased fertilization capabilities, 20 replicate lines (10 of each resistance marker) were started and went through the following setup (see Fig 6.1). Mushrooms were produced from dikaryons P0 and N0, respectively. All spores were harvested in saline, concentrated by spinning them down, and a 40µl droplet containing ~10^6 spores was inoculated on one side of a 9cm Petri dish covered with monokaryon *dP* or *dN*, respectively, grown for three days from macerated mycelium. The spores were allowed to fuse to the mycelium, after which their nuclei migrated through it and completely colonized it. Seven days after inoculation with spores, a strip of agar (20x5 mm) with dikaryotized mycelium was cut from the other side of the Petri dish, inoculated on a fresh plate and grown for seven days in the light. During this time the dikaryotic mycelium grew vegetatively and produced dozens of tiny mush-

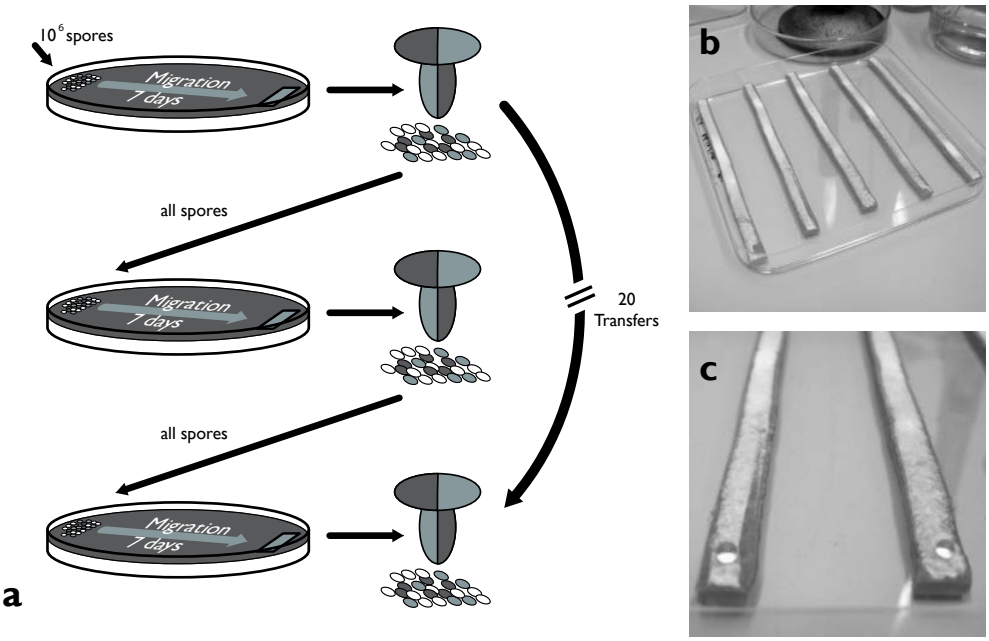

Figure 6.1 Setup evolution experiment and competition measurements. a) Basic setup for the evolution experiment. Spores are inoculated on to a fresh receiving monokaryon (~10^6). Spores fuse to the mycelium and migrate through it for seven days. Then piece of mycelium is removed from the far side of the plate and induced to produce mushrooms and sexual spores for 7 days. All spores are harvested and used for a next round of selection. Each transfer takes 14 days and we performed 20 transfers. Dark grey symbolize strain of mating type *A43B41* and light grey of type *A41B43*. Spores are produced sexually and therefore half is recombined (white). Only 1/4 is compatible with the receiving monokaryon. All mycelia and spores contain the same resistance marker. **b-c)** Racetracks used for the competition experiments. On one side each 9cm long track was inoculated with a droplet containing a mixture of differently marked spores. A piece of mycelium from the far end was taken after 7 days of migration.

rooms, which all produced spores. All spores were harvested in 1ml saline. This dilution was centrifuged for 5 min at 3800xg after which the bottom most 40 µl again was used to inoculate a fresh corresponding monokaryon *dP* or *dN*, respectively. Even though during evolution one of the strains started to produce an increased number of spores, more than fit in 40 µl, always only spores from the bottom 40 µl were used. This procedure was repeated for 20 rounds of experimental evolution.

During each transfer, spores with four different mating types are produced, but only spores of A41B43 are compatible with the receiving monokaryon, and can perform fertilizations. Nevertheless, because the heterokaryon is homozygous for the resistance marker all spores do carry the same marker.

For future reference, 2x2mm blocks of agar with mycelium were cut out of the dikaryon that grows next to the mushrooms and immediately frozen at -80°C. This is a convenient way of storing the mycelium, because a single small block from -80°C can be placed immediately on a fresh MM plate and will continue to grow. Additionally, after every fifth transfer dikaryotic mycelium was grown on cellophane and stored in 15% glycerol at -80°C.

Competitive fitness measurements

The relative fitness of evolved strains at transfer 20, was measured in direct competition with the parental strains. An agar block with either the evolved or parental dikaryon stored at -80°C was placed on fresh MM and grown for 1 week. From this plate a 5x20 mm piece of agar was taken from which mushrooms were grown (see 'Evolution protocol'). To obtain enough spores for all competitions, 20 plates were inoculated for parental lines, 10 of each marker, from which all spores were collected and pooled. All spores were harvested and suspended in 1 ml saline. This suspension was spinned down (5 min at 3800xg) after which 900µl supernatant was removed to concentrate the spores. Next, the spore density of this suspension was determined by counting with a hemocytometer, and the suspension was diluted to 10^7 spores/ml. For each evolved strain, 100µl of this dilution was mixed with an equal volume of spores dilution of the parental strain that carried the alternate resistance marker and vortexed twice, to obtain a homogeneous dilution of 10^7 spores/ml with equal numbers of parental and evolved spores. Of this mixture, 10µl was pipetted on one side of a 'competition track' of unmated mycelium without a resistance marker. A 'competition track' is created by cutting strips of agar (90x8mm) covered with mycelium of monokaryon *d* (i.e. without resistance marker) from a square 12cm Petri dish with 45ml MM. Five tracks were lined next to each other in an empty 12cm square Petri dish (see Fig 6.1b). In every second plate one track was not inoculated to check for cross fertilizations, which were never observed. To assess the exact ratio of evolved and parental strain that was used as inoculation, three dilutions of the mixture were plated on MM+0.5ml 5% Triton-80 to form colonies in 6 replicates. After 3 days the colonies of those dilutions, which yielded between 50 and 500 colonies were counted, after which each plate was covered with 500µl antibiotic of either nourseothricin (0.4 mg/ml) or phleomycin (1.25 mg/ml) and 2 days later the colonies that

continued to grow were counted. Per strain 9 replicate competitions were performed. Additionally, the parental strains were competed against each other for control.

After 7 days, the last 5mm of the track was placed on a fresh MM plate to form mushrooms and spores. To determine the ratio of the evolved vs. parental types we performed quantitative PCR using the resistance genes as targets (NFw: 5'-CACTCTTGACGACACGGCTTAC, NRev: 5-AAGGACCCATCCAGTGCCTC, PFw: 5'-AAGTTGACCAGTGCCGTTCC and PRev: 5'-AAGTCGTCCTCCACGAAGT). The spores produced are the result of a meiosis from a fusion of one nucleus from the unmarked receiving mycelium, and one from either of the two marked fertilizing spores. Therefore, half of the spores will not carry any marker and the other half can be of either marker, following Mendelian segregation. If both nuclei perform equally well during fertilization, the ratio of the markers is 1:1, otherwise there will be a deviation from 1:1.

Using a monokaryotic strain carrying both markers as single copy inserts, the efficiency of the qPCR reaction for each marker could be measured for exactly equal ratio. These efficiencies were used to measure the ratio of each marker within one sample. Spores were collected for DNA isolation using a modified protocol from Sambrook et al. (1989). All spores were harvested in 700µl LETS buffer and spore walls were destroyed by freezing the dilution at -20°C followed by incubation with 20µl Proteinase K (20 mg/ml) for 4h at 56°C. qPCR was performed on a Biorad CFX96. Each reaction consisted of 10µl containing 5µl of undiluted DNA and 5 µl SYBR Green 2X (Biorad) with 200 nM final concentration of each primer. The cycling conditions were 1 cycle at 95°C/10 min, followed by 40 cycles of amplification (95°C/10 sec, 62.5°C/30 sec, followed by a plate read). For each sample both markers were measured in the same run, with one technical replicate. For analysis we used the software package CFX Manager (Biorad) with standard settings for baseline and thresholds. The ratios of marked strains (R = Evolved/Parental) before and after competition were used to estimate the relative competitive fitness ($W = \mathrm{Log}[R_{before}/R_{after}]$).

Fitness component and possible trade-offs measurements

For five evolved strains that showed changed competitive fitness (6N, 7N, 2P, 9P and 10P; see 'Results') and the parental strains (P0 and N0), specific components of fitness were measured.

Maximum nuclear migration speed

Per strain, 20 'competition tracks' of 12 cm length (see above) were inoculated with ~10^6 spores on one side and incubated. Each 24h, 5 random racetracks per strain were sacrificed and cut into 1 cm pieces. Each piece was placed in a well containing 0.5ml MM of a 24 well plate and incubated for two days. After two days outgrowth of each piece was checked for clamp connections to confirm if the piece of mycelium had become dikaryotized, which indicates that at least one fertilizing nucleus was present in that piece of mycelium at the time of isolation (Raper, 1966).

Colonization efficiency

The competitive ability in colonizing and occupying the female mycelium after 7 days was measured by measuring the abundance of fertilizing nuclei relative to the parental strain. For the five strains with fitness change and parental strains against each other, we repeated the measurements as described in 'Competitive fitness measurements', and additionally, we sampled the mycelium in the 'competition track' at two points, 10 and 50 mm after inoculation. At each position 10mm of the track was cut out and DNA was isolated from it using a QIAGEN DNEasy Plant mini kit. qPCR was performed on undiluted DNA derived from spores and 10 times diluted DNA from the mycelium. Relative competitive colonization efficiency was calculated in the same way as relative competitive fitness. This experiment was performed with two different female mycelia: nine replicates with monokaryon *d* as receiver, and nine replicates with monokaryon *e* as receiver.

Spore yield and spore size

The total number of spores produced seven days after inoculation of the dikaryon, were measured on a Z2 Coulter counter (BeckmanCoulter), in threefold. The Z2 also gives size measurements, but because the spores are 'banana-shaped', this method did not give a good size estimate. Alternatively, spore size was measured by taking photographs at 200X magnification with a phase contrast microscope and measuring the length of at least 40 spores using ImageJ V1.44n (http://imagej.nih.gov/ij/). To assure the spores had not started germinating, which starts with swelling of the spores, we used spores that were produced within 2 hours before measurement, which were harvested in saline, immediately stored on ice, and photographed within 10 minutes after harvesting.

Mating type ratio

Because only spores of mating type A41B43 are compatible with mycelium *d* (see 'Results and Discussion'), evolution of meiotic drive by one of the mating type genes or linkage of the mating types loci might be advantageous, because it increases the number of compatible spores. For each strain at least 48 spores were isolated their mating type was determined by performing crosses by the method described in Papazian (1950).

Monokaryotic and dikaryotic mycelium growth rate

Finally, from each dikaryon, 24 single spore colonies were isolated. For each monokaryotic isolate a small mycelium plug was inoculated on a 9cm petridish and radial growth was measured after 3 days of incubation. The same was done in threefold for each dikaryon.

Results

Competitive fitness

Fitness was measured relative to the parental strain. From the twelve evolved lines for which we could measure fitness (see '*Fitness components: Spore yield and mushroom morphology*' for explanation on the other 8 strains), five had significantly changed fitness (Fig 6.2). Four strains (6N, 2P, 9P

and 10P) showed an increase in fitness relative to the parental strain (two-sample T-test, df = 8) and one (7N) a decrease. The difference between the two parental lines showed no marker effect ($W_{\text{N relative to P}}$ = 0.005, S.E. = 0.206).

Competition with strain 2P20 initially showed a reduction in fitness (W_{2P20} = -0.732, S.E. = 0.324). Measurements of fitness for the 'Colonization experiment' however showed an increase in fitness (W_{2P20} = 1.404, S.E. = 0.291). All the other strains showed similar results between both replicates. We therefore performed a third essay in which again 2P20 was tested against N0 which showed results similar to those from the 'Colonization experiment' (W_{2P20} = 1.391, S.E. = 0.227). All controls were consistent over the different essays. We do not know why the results in the first essay differed so much from the second two. Data presented in figure 6.2 is from the last essay.

Fitness components

Maximum nuclear migration speed

All strains were able to fertilize the mycelium and no differences were observed between the parental strains and any of the evolved lines (Fig 6.3, ANOVA, $F_{5,24}$=1.8). After 24 hours migration had occurred in most replicates (average 16.8 mm) which increased to 75.2mm after 48h and 114.4mm after 72h. On day 4, all tracks were completely dikaryotized.

Colonization efficiency

We measured the nuclear ratios after seven days in the female monokaryons *d* and *e* (Figs 6.4a and 6.4b respectively), 10mm and 50mm from the point of inoculation, and measured the overall fitness (*i.e.* the representation of the evolved and parental genomes in the spores formed, as previously described). Measurements of nuclear ratios in the mycelium showed that already after 10mm the ratio between the two nuclei was highly skewed, and that these values could almost completely explain the final fitness increase measured after spore formation (Fig 6.4a). Strain 7N20 shows a decrease, indicating that the loss in fitness might be caused by a change in the same mechanism that gives the other strains an advantage. These findings indicate that the main competitive benefit was obtained already after the first 10mm of migration, which concurs with the findings by Ellingboe (1964) on migration as described in the discussion. Except for strain 10P20, no significant further increase in nucleus ratio was observed between the measurement at 10mm and later.

Spore size

No difference in spore size was observed between the evolved and the parental strains.

Spore yield and mushroom morphology

During the experiment, the lines 5P and 10N stopped producing mushrooms (after 15 and 17 transfers respectively), even though dikaryons with clamp connections were formed. This dikaryon sterility might be caused by mutations at the mating type loci, which are involved in mushroom development (Wosten & Wessels, 2006). Once fixed in an evolved line, mutations

at these loci cannot be purged, because they do not recombine with the female genome. After reviving the evolved strains from the -80°C, six additional strains (5N, 1P, 4P, 6P, 7P, 8P) were incapable of producing mushrooms, even though after the last transfer mushrooms from the same mycelium were present. We were not able to induce fructification in these strains to perform the fitness essays, and additional investigations were therefore not conducted with them.

For two lines, spore yield had increased relative to the parental strain (Fig 6.5). Strain 7N20, which had reduced competitive fitness relative to the parental strain, showed a 4.1 fold increase in spore production. The decrease in fitness for strain 7N20 becomes even more striking when considering that more spores are produced. Strain 6N20 had a stunning 1500 fold increase in spore production relative to the parental strain. Even though 6N20 also had the highest fitness measured, the increased spore production does not explain the advantage observed in the previously described essays. After 10mm the 6N20 strain had completely oppressed the parental type, which cannot be the result from an advantage in numbers, because the inoculum for the competitions was a 1:1 mix of the evolved and parental strain. Increased spore production is not needed to explain increased competitive fitness, nevertheless, it is expected to be selected during the evolution experiment. Additionally, the increase in spores might have assisted evolution of other beneficial traits by increasing the effective population size for this strain, thereby increasing the mutation supply rate. The increase in spore number in strain 6N started already after four transfers (data not shown).

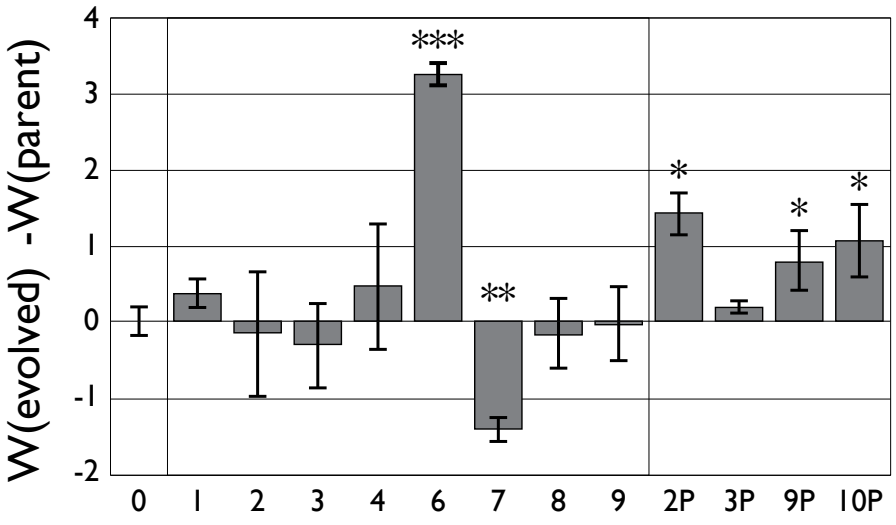

Figure 6.2 Relative competitive fitness of the 12 tested strains. 0 indicates competition between the parental strains P0 & N0. The other values are competitions of lines from transfer 20, relative to the parental strain of the opposite resistance marker. Positive values indicate increased fitness and negative reduced fitness. Measurments of 9 replicates. Error bars indicate standard error. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

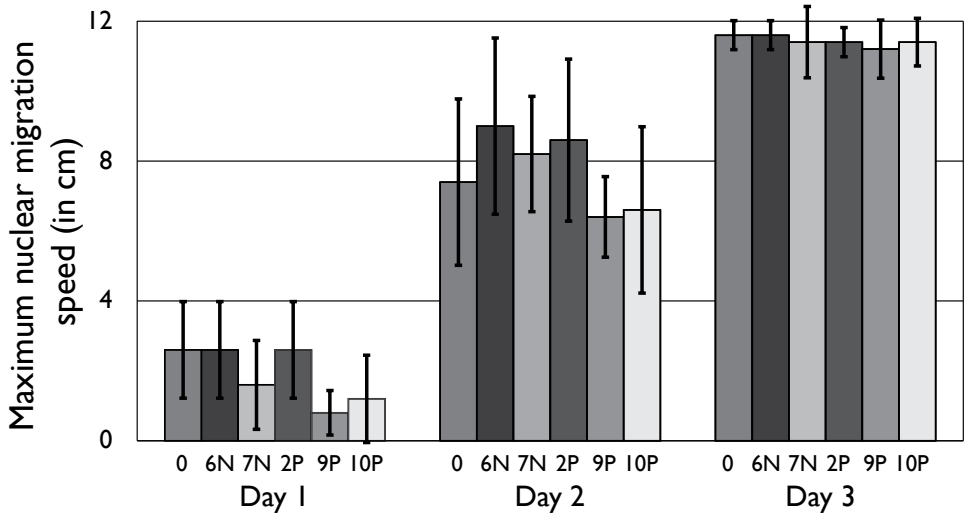

Figure 6.3 Average maximum migration speed of nuclei through a female mycelium after 1, 2 and 3 days for the parental (0) and the five evolved strains at transfer 20. Measurements indicate the furthest 1 cm piece of a 12cm 'race track' that was dikaryotized. Average from 5 race tracks, error bars indicate standard error.

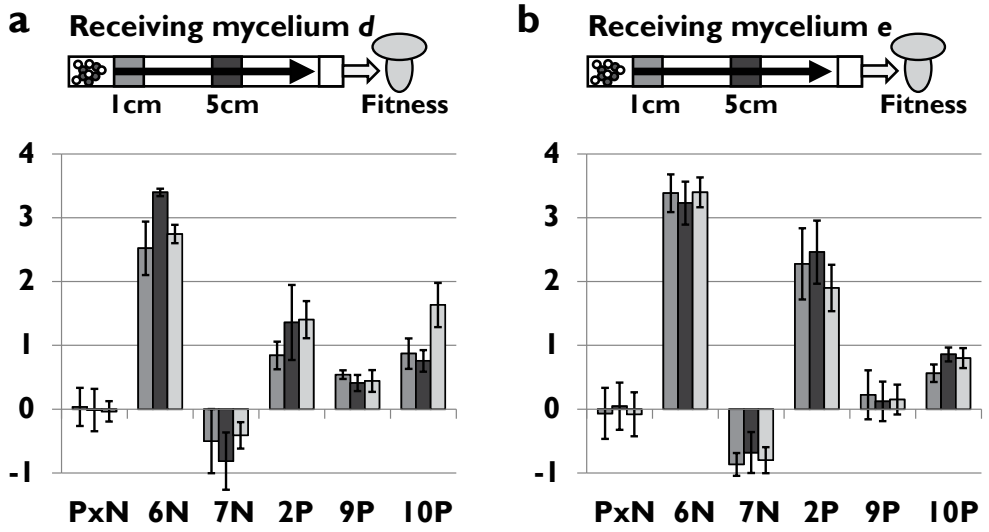

Figure 6.4 Colonization efficiency and relative competitive fitness of the parental line and the five evolved strains. **a)** Bars indicate relative competitive ability in colonization of mycelium *d* (mycelium used as female during evolution) after 1 and 5 cm migration, as indicated in the figure above each graph (first two bars) and relative compatitive fitness (light bar). **b)** as a) but with receiving mycelium *d* was used. Measurments of 9 replicates. Error bars are standard error.

No obvious changes were observed in mushroom morphology, except for strain 6N. At the fourth transfer, part of the mycelium did not produce many small mushrooms as seen in the parental strain, but produced larger mushrooms directly in the growth medium and from transfer 9 (Fig 6.6a), the strain appeared to produce one single large mushroom. This different mushroom phenotype also produced many more spores. High spore production is most likely a wild type characteristic in natural isolates. When performing crosses between monokaryons from the parental or the 6N20 strain and natural isolates, all strains produced spores in quantities comparable to 6N20. The mutation causing increased spore production appeared to be closely, but not completely, linked to the A mating type locus (Fig 6.6b).

Mating-type ratios

During meiosis, four types of spores are produced (A43B41, A43B43, A41B41 and A41B43). Only spores of type *e* (A41B43) are compatible with the receiving mycelium *d* (A43B41). Theoretically, there are several possibilities for sexual to increase the fraction of compatible spores. First, meiotic drive for either the A41 or B43 allele will produce more compatible spores. Second, linkage (or a modifier for repressed recombination) between mating-type alleles A41 and B43 would increase the share of compatible spores from 25% to 50%. To explore these possibilities, single spore monokaryons were isolated from the mycelium, and their mating types were determined (Table 6.1). No deviation from normal Mendelian segregation was detected (χ^2-test; $p>0.5$).

Mycelium growth rate

Growth rate of the evolved strains was measured over three days of growth. No difference was seen between the dikaryons (One-Way ANOVA, $F_{5,12}=3.03$, mean growth = 8.96 mm/day, S.E.=0.273, data not shown). For the monokaryons, growth rate was increased for strain 2P20 and decreased for strain 9P20 (Fig 6.7a, One-way ANOVA, $F_{5,138}=14.548$). Strain 2P20 shows average increased growth rate, but with a similar distribution as seen in the parental strain (Fig 6.7b-c). Strain 9P20 shows a bimodal distribution in which 10 strains fall in the lower and 14 in the higher category (Fig 6.7d). This indicates that this strain has a deleterious mutation for growth, which is complemented in the dikaryon phase. Because the strain is not selected for growth as a monokaryon, no selection against this mutation occurred during evolution, and the deleterious trait might thus be considered neutral for mating in the male role. Due to recombination, a neutral trait that is only present in the male genome will be diluted during each transfer. If the trait is stable over longer periods, the neutral trait has to be linked to a beneficial locus, or to the mating type, which is always selected (linkage to mating type was not observed, data not shown). Another possibility is that the trait itself is beneficial in the male role, which trades off with growth in the female role. If this were true, this indicates conflict between the male and female mating roles (Rice, 1996). This should be tested by performing competitions in the male role with spores derived from fast and slow growing monokaryons.

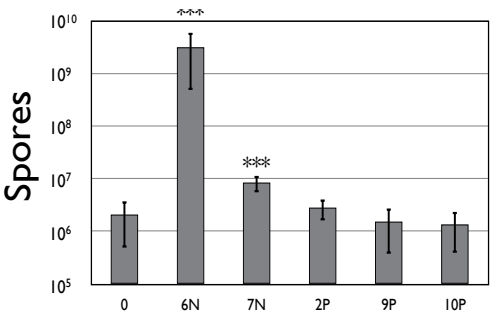

Figure 6.5 Average spore yield. Total number of spores produced after 7 days for parental and evolved strains at transfer 20. Error bars indicate standard error. Y-axis is log scaled. (***: $p<0.001$)

Discussion

We have experimentally shown the evolution of increased mating success in a mushroom-forming basidiomycete. By repeatedly mating a monokaryon with a non-evolving receiving 'female' monokaryon, four of the 12 tested strains significantly increased competitive fitness relative to the parental strain, while one strain decreased in fitness. Increased fitness most likely occurred due to the selection of spontaneous mutations beneficial at some stage between fertilization to sporulation. Measurements of nuclear ratios in the mycelium showed that already after 10mm of nuclear migration, the ratio between the evolved and unevolved nuclei was highly skewed towards the evolved nuclei, indicating that the main competitive benefit must be achieved at an early stage of mating. Furthermore, two evolved strains had increased spore numbers.

The decrease in fitness observed in one strain (7N20) is less easy to explain. In different essays (data not shown), this strain was consistently outcompeted by the parental strain. One possible explanation is genetic drift, due to a small effective population size of selected nuclei. Indeed, on some occasions only few spores had been produced at the moment of transfer. Furthermore, even with a large sample of transferred spores, we do not know the effective population size of spores that contributed to the next generation. For example, we do not know the number of mushrooms from which we sampled spores, and neither from how many nuclei a single mushroom develops. Nevertheless, deleterious mutations are not expected to be maintained. In each round, the genome of the migrating nucleus is recombined with the parental strain, during which the new deleterious mutation can be purged. A deleterious mutation cannot

Table 6.1 Mating type percentage of *n* tested single spore isolates derived from parental and evolved lines. A43B41 was used as the female mycelium (monokaryon *d*) during evolution, A41B43 the compatible male.

Strain	A43B41 ♀	A43B43	A41B41	A41B43 ♂	*n*
0	25.0%	20.8%	33.3%	20.8%	48
6N	18.8%	29.0%	29.0%	23.2%	69
7N	21.6%	21.6%	28.4%	28.4%	116
2P	27.1%	22.9%	29.2%	20.8%	48
9P	29.2%	20.8%	18.8%	31.3%	48
10P	22.9%	27.1%	20.8%	29.2%	70

be purged, only when it is located in or closely linked to one of the two selected mating type alleles – the only parts of the genome that do not recombine.

Strain 7N20 did allow us to test if the same mechanisms that increase fitness in the adapted strains reduce fitness in this maladapted strain. The five strains that had changed in competitive fitness and the parental line were used to measure changes in different fitness components. Below we further discuss these measurements.

The fitness value of strain 6N20 should be considered as a minimal fitness measure. After competition, the parental marker type was below the detection level reached in 40 amplification cycles and more cycles would be prone to give false positives, so no measurements for Rafter could be calculated. Nevertheless, we calculated a minimum relative fitness for this strain, by artificially setting detection value of the parental strain at cycle 40.

Male and female roles during mating

We realize that male and female sex roles in a mushroom fungus cannot unambiguously be assigned to all aspects of the reproductive cycle. In general, male traits are associated with fertilization, and female traits with becoming fertilized. Whereas some traits are exclusively female, such as monokaryotic growth or migration of the nuclei into the own mycelium, others cannot be exclusively given one sex role. Germination for instance might increase fertilization efficiency, but is also essential for monokaryon establishment. The same is true for spore number, which will increase chance of finding a suitable substratum and initiate a mycelium, but also the chance

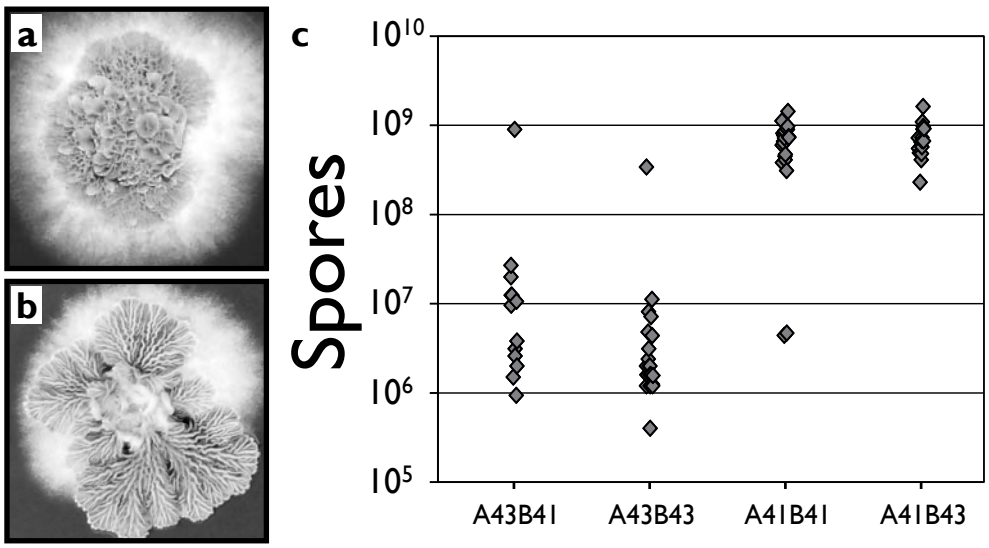

Figure 6.6 Mushroom morphology and spore production. **a)** Mushroom morphology of unevolved strain which creates many small mushrooms. **b)** Mushroom morphology of strain 6N9 which produces fewer large mushrooms that produce many spores. **c)** Spore production defined by mating type of dikaryons composed of a monokaryon derived from 6N20 crossed with a parental monokaryon of opposite mating type. Grouping is based on mating type of the monokaryons from 6N.

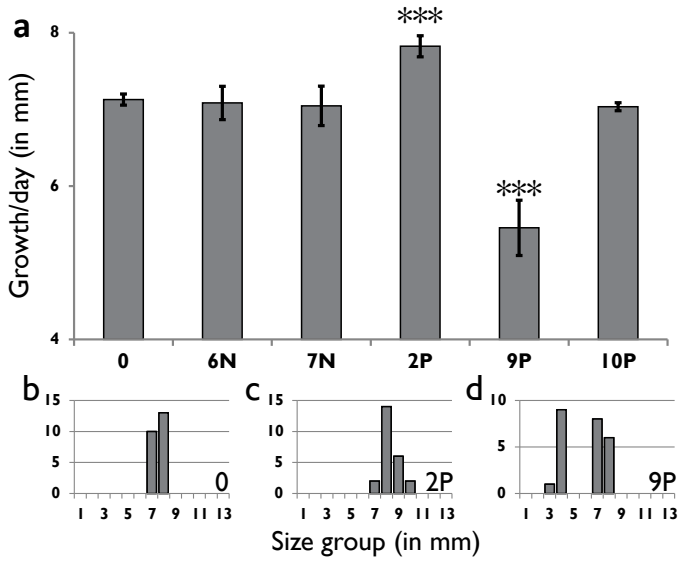

Figure 6.7 Monokaryon mycelium growth rate. a) Average growth rate for 24 monokaryons derived from parental and evolved strains at transfer 20 (errorbars indicate standard error; ***: $p<0.001$). **b-d)** Histogram of data per strain for parental strain and the two evolved strains with changed monokaryon growth rate. Strain 9P20 shows a clear bimodal distribution with a slow and a fast growing group. The mycelia from the fast growing group grew with the same rate as the parental strain.

to find and fertilize another mycelium. Finally there are some traits that are not associated with either a male or female role, such as growth as a dikaryon or mushroom production. Depending on the occurrence and importance of the different sex roles in nature, selection will have shaped traits to optimize fitness.

In the regular life cycle performed in the laboratory, a spore germinates to form a monokaryon, after which it simultaneously fertilizes a different monokaryon and becomes fertilized by that monokaryon in a symmetric mating. In our experimental setup, fertilization was directly possible from a spore. The experiment was designed to increase competitive ability for fertilizing a non-evolving female mycelium and increase the significance of the male role during mating. As selection during the monokaryon growth stage was relaxed in our experiment, we could test if traits beneficial during fertilization, were detrimental for this aspect of female fitness. In one case, we found reduced monokaryotic growth (9P20), but in all other cases, no negative effect on female fitness could be shown. Furthermore, all strains were still able to perform mating in the female role, *viz.* to incorporate nuclei in the mycelium. Our general failure to find a trade-off between male and female roles might be a consequence of the low cost of mating in the male role, which implies donating nuclei is essentially cost-free. Consequently, there may be no antagonism between the two sex roles.

Fertilization by spores may be a common fertilization mechanism in nature (Anderson & Kohn, 2007; Chapter 3), which gives the potential for evolution of traits that increase spore fertilization performance. Because the receiving monokaryon can compensate for traits detrimental during the dikaryon stage, recessive mutations harmful for growth or vegetative competitive ability do not have to result in reduced fitness (Clark & Anderson, 2004; Hiscox et al., 2010). Detailed studies of mating patterns and the competitive ability of nuclei in natural populations, and theoretical modelling will be required to study the equilibrium frequency of mutations with sex-role specific consequences under various population structures.

Next to selection of traits that improve fertilization capabilities, fitness can also be increased after the dikaryon has been established, when no clear sex roles can be distinguished any more. For instance, increased dikaryon growth might increase the number of mushrooms produced and thereby the proportion of spores. Also the functioning of the mushroom can be altered to produce more spores. Except for the mushroom morphology of 6N20, no clear changes were observed in the dikaryon phase. Because the strains used are adapted to a laboratory environment, where fast growth and efficient fructification are probably preferred and therefore might be optimized for these traits, it is not strange that no improvements were found. High spore production is probably not necessary, because only a few spores are enough for most experiments.

Changes in fitness components and parallels to animals and plants

Spore yield and spore size

In animals and plants, a great diversity is seen in sexually selected adaptations that increase mating success. The most obvious adaptation are increased numbers of male gametes and, as a consequence, reduced size (e.g. Gage & Morrow, 2003). In our experiment, we saw for two strain that spore numbers had increased (though these did not trade off with size) which give these strains higher chances in scramble competition. Furthermore, it might have increased opportunity for evolution of other traits by increasing the effective population sizes for these strains.

Another trait that increases competitive ability are chemical or physical alterations to increase receptiveness by the female mating organ (e.g. Hosken et al., 2001; Lankinen & Kiboi, 2007; Schärer et al., 2011). We did not observe alterations in spore size or shape. It is very well possible that chemical alterations have occurred, which might increase the attractiveness of the spore to the female mycelium (Voorhees & Peterson, 1986; Chapter 8). This will be discussed below at '*Colonization efficiency*'.

Nuclear migration speed and spore germination speed

There are parallels between migration speed of nuclei in basidiomycete fungi, and swimming speed of sperm towards the egg in animals and pollen-tube growth towards the ovule in plants (Snow & Spira, 1991; Walsh & Charlesworth, 1992; Tourmente et al., 2011). Increasing migration rate would be very advantageous, because it leads to an increasing difference in travelled distance.

However, in basidiomycetes, nuclear migration speed is most likely determined by the receiving mycelium and not by the male nuclei. Nuclei migrate along microtubules of the receiving mycelium, propelled by dynein from the receiving mycelium which is powered by the receiving mycelium (Gladfelter & Berman, 2009). Only when the nucleus can manipulate the non-evolving female mycelium migration speed might be increased. Indeed, in our selection experiment, migration speed did not change.

It has been shown that interactions between multiple incompatible dikaryons reduce total fitness (Rayner et al., 1984; Aanen et al., 2009). Single mating would thus be beneficial for a receiving nucleus to avoid splitting up the mother mycelium into multiple incompatible domains. (However, for different plants and animals, multiple matings have been shown to be advantageous (Mulcahy & Mulcahy, 1975; Lankinen & Madjidian, 2011). The costs and benefits of multiple matings for mushroom fungi, will be further discussed in Chapter 8.) Therefore, we predict that the receiving monokaryon has an interest in fast nuclear colonization, which will be accomplished by fast nuclear migration, so that migration speed may already be optimized.

In pollen, next to increased migration, faster germination can also increase the chance of fertilization (Lankinen & Kiboi, 2007; Lankinen & Madjidian, 2011). We observed that germination frequency was 100%, but unfortunately we did not obtain data on the rate of germination. It is probable that the spores have adapted in this respect, because increased germination rate will likely increase the probability to become established in the female mycelium. However, this need not be true, because even without germination, fusion to the mycelium is possible (Voorhees & Peterson, 1986).

Colonization efficiency

As observed by Ellingboe (1964) in dikaryon-monokaryon matings (see Chapter 4), both different fertilizing nuclei migrated into the mycelium, and both had approximately the same speed. The nucleus eventually winning the fertilization was the one that established in the mycelium more efficiently, thereby gaining a head start. This might also be the case for our evolved strains.

As we did not find differences in migration speed between evolved and unevolved strains, but did find differences in colonization efficiency, we hypothesise that the spores of evolved strains gain an advantage in entering the mycelium. This might be due to increased germination speed of the spores, but germination is not required as a spore itself can fuse with the mycelium. When a basidiospore of *S. commune* is close enough (>~15μm) to the female mycelium is has the ability to attract hyphae, which grow towards it and fuse (Voorhees & Peterson, 1986). It is unknown how the spore attracts the hypha, but probably the spore excretes a chemical used for chemotaxis by the hypha. If spores produce more of this chemical, they might be more successful in attracting hyphae and increase their head start in migration. It would be interesting to perform the tests described by Voorhees & Peterson (1986) and see if attraction can occur over larger distances for evolved strains, or, when presenting a parental and an evolved spore, whether the evolved strain is preferred. An obvious candidate for such a compound are the pheromones encoded on the *B* mating type. In other basidiomycetes they are used for extracellular com-

munication (Kües & Navarro-González, 2009), and in *S. commune* recent findings indicate that the pheromones, at least over small distances, must be functional extracellular (Erdmann et al., 2012). Changes of the pheromones themselves are not in agreement with the experiments where a receiving mycelium of a different mating type was used, and in which the same results were obtained. Adaptations of other traits that are involved in pheromone signalling, such as for instance increased excretion, and that are located in other parts of the genome might still be functional in interactions with other mating types.

If pheromones indeed have acquired beneficial mutations, the fitness and colonization results of monokaryon *e* are not easily understood. We hypothesize that if the mating types changed during evolution, due to adaptation to the non-evolving female mycelium, this adaptation would be specific, and the evolved strain would lose its advantage in a pairing with a different mycelium. The evolving mating-type allele is not compatible with monokaryon *e*, and therefore, only spores that carry mating type *d* can fertilize this mycelium. The *d* mating-type alleles can only be derived from the non-evolving female, and thus will not carry the beneficial mutation. What we observed was that only strain 9P20 fulfilled this expectation (Fig 6.4b). For the other strains there was no difference in fitness for the evolved strains on mycelium *d*, and on mycelium *e*. There are two possibilities that can explain this result. First, the trait that causes differential fertilization is not located on the mating type, but somewhere else on the genome. However, in that case, the reduced fitness of strain 7N20 cannot be explained, because according to our previous argumentation this characteristic should reside at, or be closely linked to, one of the mating type loci. Alternatively, the fitness change is not defined by the genome of the spore, but epigenetically, during spore development. In the mushroom, the spore develops. This development is most likely defined by the dikaryon, and might affect the spore performance epigenetically. In the basidia, where meiosis and spore formations occur, the B mating type plays an important role in nuclear sorting (Debuchy, 1999; Erdmann et al., 2012), which might explain the reduced fitness of strain 7N20. If this hypothesis is true, the spores derived from a cross between an evolved line and *e* should be as fit as the parental strain. Finally, multiple mutations in both the mating type loci and other parts of the genome might have occurred.

During fertilization of the mycelium migration of nuclei through the receiving monokaryon occurs, but some of the nuclei are left behind to establish a dikaryon (Snider & Raper, 1958). It is not clear if there is a trade-off between the migration efficiency and the ability to colonize a mycelium. On the one hand, a nucleus that settles in the mycelium cannot migrate further. On the other hand, during migration mitotic division of the migrating nuclei is expected to occur which might increase the number of migrating nuclei of a certain type (Kües, 2000). It would be of interest to investigate the rate of nuclear division of evolved strains either after germination, or inside the female mycelium.

Conclusion

We have experimentally shown that sexual selection can increase mating success in a mushroom-forming basidiomycete. Increased mating success was due to a combination of increased spore formation and colonization efficiency of the receiving mycelium. In most cases, no trade-offs were found with other fitness components. Future research is needed to further determine the details of the mating process to identify the targets of sexual selection in mushrooms.

Acknowledgements

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Chapter 7

Sexual selection in fungi

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Summary

Sexual selection is a significant component of natural selection in many sexually reproducing organisms, which is well established for plants and especially animals. We argue that fungi are not fundamentally different from these other kingdoms in this respect and that sexual selection is also acting here. We give background information on differences in the relative importance of sexual reproduction among fungal groups and discuss under which circumstances sexual selection is expected to occur. Even though fungi do not have separate sexes, in many cases distinct sex roles during mating can be distinguished, which can result in sexual selection. Moreover, even when no distinct sex roles can be distinguished, sexual selection can act at the level of the mating type. We present the hypothesis that the high variety of mating types in mushroom-forming basidiomycetes are a consequence of sexual selection. We argue that the realization that sexual selection can occur is highly relevant for mycologists and that fungi are well suited to experimentally study fundamental aspects of sexual selection

Introduction

The conspicuous ornaments of some birds and fish puzzled Darwin as these characteristics seem difficult to explain by natural selection. His solution was to distinguish sexual selection, the component of natural selection associated with variation in mating success (Andersson, 1994, pp. 3), from other components of natural selection (Darwin, 1859; Darwin, 1871). The basic idea is that peacocks with a more conspicuous tail are more attractive to peahens than dull cocks, and obtain more mates, thus compensating for reduced survival. We argue that also in species as inconspicuous as fungi sexual selection can act.

The aim of this paper is twofold. First, we argue that fungi *sensu lato* (including Oomycetes) are not fundamentally different from animals and plants with respect to mate competition, and that sexual selection can explain various traits of fungi that seem difficult to explain by natural selection alone. We will introduce general processes of sexual selection acting in fungi, supported with specific examples from different fungal groups. We argue that sexual selection should specifically be taken into account in studies on fungi to better understand their evolution and ecology of which still surprisingly little is known (Douhan et al., 2011; Billiard et al., 2012). Because until now very little attention has been given to sexual selection in fungi this paper will pose several hypotheses to encourage further research on sexual selection in fungi. The second aim of this paper is to show that fungi are powerful model organisms for experimental studies on fundamental mechanisms of sexual selection, because of the high diversity in fungal life cycles, mating systems and ecology, and because fungi easily can be manipulated experimentally (see Stajich et al., 2009 and Alexopoulos et al., 1996 for an introduction to or in-depth overview of fungal diversity, respectively).

Defining sexual selection

Many different definitions of sexual selection are commonly used, some more restrictive than others (Shuker, 2010). We use the definition given by Jennions and Kokko (2010): "sexual selection [is selection favoring] investment in traits that improve the likelihood of fertilization given limited access to opposite sex gametes due to competition with members of the same sex". This definition is close to the original definition given by Darwin, which has been used by the majority of students of sexual selection (Shuker, 2010) is broadly applicable and has been used to describe selection in many groups of animals (Andersson, 1994; Levitan, 1998) and plants (Queller, 1983; Charlesworth et al., 1987; Skogsmyr & Lankinen, 2002; Bedhomme et al., 2009).

Competition for mating implies that there is a limited number of compatible mates or gametes available. The members of one group compete for access to gametes of the other group. When different sexes (i.e. males and females) can be distinguished, a skew in the number of gametes is very likely, because of the innate asymmetry between the sexes in their investment in gametes. Theory predicts selection for equal investment in the sexes (Fisher, 1958), and because investment is bigger for each large female gamete than for each small male gamete,

many more male than female gametes can be produced (Parker et al., 1972; Parker, 1978). The stronger the skew in the operational sex ratio (OSR), the more opportunity for sexual selection to act. The definition by Jennions and Kokko only refers to species with separate sexes, but also in hermaphroditic species competition for mating can occur (Charnov, 1979; Delph & Ashman, 2006; Leonard, 2006). In hermaphrodites, competition can occur for mating in a specific *sex role*. An individual can increase its fitness in the male role by fertilizing more eggs than another individual. Traits that increase the number of fertilizations in the male role will thus be under sexual selection. On the other hand, in the female role, the hermaphroditic individual can be selective for which males to mate with. The realization that sexual selection can occur in hermaphrodites is of importance, because no fungi with separate sexes are known, but many are hermaphroditic during mating (Nauta & Hoekstra, 1992; Leslie & Klein, 1996; Bruggeman et al., 2003; Nieuwenhuis et al., 2011).

Sexual selection and mating types

Mating types regulate sexual compatibility of the gametes (or haploid structures which function like gametes), and gametes of the same mating type cannot form zygotes (Billiard et al., 2011 and see Box 7.I). Mating types are of essential importance for sexual selection. First of all, mating types in fungi not only define compatibility between gametes, but also regulate many aspects of the actual mating process (Heitman et al., 2007). Species with mating types can still have different sex roles and mate in both male and female roles, but mating types are not associated with either

Box 7.1: Mating types

Mating types are of great importance in fungal mating, as they regulate syngamy between gametes or haploid structures that function as gametes. Only gametes with different mating types can fuse, analogous to self-incompatibility systems in angiosperm plants and corals (Charlesworth, 1994; Idnurm et al., 2008). In some species, individuals produce gametes that are all compatible (homothallism), while in other species gametes are produced that are only compatible with gametes with a different mating type, and not with gametes of the same mating type (heterothallism). Homothallism is most common in the Zygomycota and Ascomycota (Lin & Heitman, 2007). Almost all heterothallic ascomycetes have a system with two different mating types, which usually implies that each individual is compatible with half the population (if both mating types occur in equal frequencies, which is the case whenever sexual reproduction occurs frequently). Many Basidiomycota (especially the mushroom-forming ones; Kües et al., 2011), some Physarum species (Collins, 1975), and at least one ascomycete Gibberella cingulata (Cisar & TeBeest, 1999) have a system with more than two, sometimes up to hundreds, different mating types (see also 'Sexual selection in mushroom fungi' in the main text). In a population with many mating types, gametes are compatible with almost all unrelated gametes in a population.

Fungal mating types are genetically defined. At the mating-type locus one or often multiple tightly linked genes are encoded. In ascomycetes, the genes of the two mating types are not

(box 7.1 continued)

homologous and are therefore referred to as idiomorphs (Metzenberg & Glass, 1990). Depending on the species, the genes at the mating type loci can both regulate functions that operate during mating such as extracellular signaling (Kothe, 2008; Raudaskoski & Kothe, 2010), cell fusion (Glass et al., 2000; Fraser et al., 2007), inheritance of cytoplasmic genes (Yan & Xu, 2003), and establishment of a diploid or heterokaryotic individual (Crowe, 1963; Fraser et al., 2007), and after zygote formation, for instance in regulating cell division (Raper, 1985), sexual reproduction (Van Heeckeren et al., 1998), and virulence (Kwon-Chung et al., 1992) (for an extensive overview of the known molecular mechanisms see Heitman et al., 2007). Basidiomycetes have evolved a unique bifactorial mating type system with two unlinked mating type loci, which both have to be different for successful mating to occur (Raper, 1966; James et al., 2006). Different groups within the basidiomycetes have reversed to a unifactorial system, either by losing one of the mating type loci or by recombination causing linkage between the two loci (Kües et al., 2011).

Inter and intra mating type sexual selection

In sexually reproducing populations the mating types are expected to be in equal frequencies. Using the same reasoning that predicts equal investment in the sexes (Fisher, 1958), we can predict that negative frequency dependent selection will select for equal investment in the different mating types (May et al., 1999). However, in contrast to the different sexes, there are no inherent differences between different mating types in investment in gametes, so that an innate asymmetry as present between the sexes, is unlikely. Therefore, equal investment in mating types also means equal frequencies of mating types. This is the case for species with two mating types, and also for species with multiple mating types. Only when events of sexual reproduction are separated by long stages of vegetative growth or many rounds of asexual reproduction, and one mating type has a higher asexual growth rate, increased virulence, or reduced mortality, systematic skews will be possible in the mating type ratio. This is only known from rare examples, such as *Cryptococcus neoformans* (Kwon-Chung et al., 1992).

In many species of the basidiomycetes, more than two mating type alleles are present (Kües et al., 2011). Due to negative frequency dependent selection all alleles are expected in equal ratios (1/n for n alleles), because more common alleles have a reduced population level compatibility. If mating occurs randomly, each mating type allele will then obtain 1/n of the matings. A mating type allele that is more successful in conquering gametes than other mating types can increase in the population to a frequency above 1/n. Competition in this situation will be between the different mating types. Even though this is a clear example of sexual selection, because fitness is increased solely by increasing the number of mates obtained, this case does not comply to the definition by Kokko & Jennions (2010) as given in the main text. According to this general definition sexual selection occurs within one group (i.e. within one sex or one mating type), but in this special form of sexual selection, it occurs between groups at the level of the whole population. Selection for high pheromone production as discussed in 'Sexual selection in mushroom fungi' in the main text is an example of this specific case.

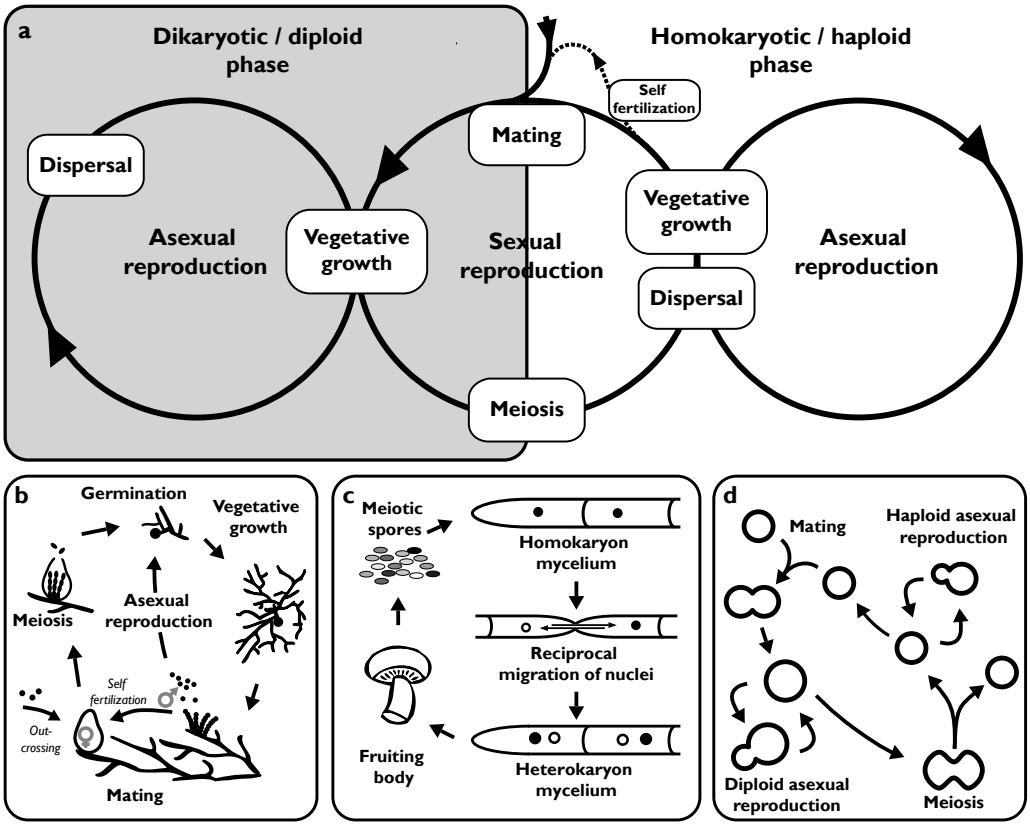

Figure 7.1 The general fungal life cycle (a) and the three distinguished categories of variation on this general theme (b-d). (a) Reproduction can occur sexually and asexually. Asexual reproduction can occur prior to and after mating. In many fungi fusion of nuclei (karyogamy) after mating is postponed to the moment just before meiosis; the nuclei from different gametes can coexist in a single mycelium, called the heterokaryon. The ratio between sexual and asexual reproductive cycles differs greatly between species, and also cycles or parts of cycles can be absent. **(b)** Life cycle typical for filamentous ascomycetes. The spores produced for asexual reproduction also function as male gametes, which fertilize gametes in female structures formed on the mycelium. Often, these species are self-fertile, but they usually can also perform outcrossing. Examples: *Aspergillus nidulans, Neurospora crassa,* and *Puccinia graminis* **(c)** Mushroom-forming basidiomycetes start their life cycle as a haploid mycelium (a homokaryon) from a germinated sexual spore. The mycelium grows vegetatively until it becomes fertilized; either by another homokaryotic mycelium, a heterokaryotic mycelium or by a spore that lands on the mycelium. During fertilization nuclei migrate into the mycelium so that finally all cell compartments contain two different nuclei. This heterokaryon can now continue to grow vegetatively and can also initiate fruiting by producing mushrooms, in which meiotic haploid spores are formed to start the life cycle over. In general, basidiomycetes are heterothallic. Examples: *Coprinopsis cinerea* and *Schizophyllum commune* **(d)** In many single-celled fungi or yeasts, growth occurs by asexual reproduction either in the haploid or the diploid stage. During mating, two isogamous haploid cells fuse, and form a diploid cell or heterokaryon. This phase can be long-lived, or immediately be followed by meiosis, which produces new haploid cells. In many species, the haploid cells fuse directly with close kin. Examples: *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, and *Ustilago maydis*

sex (i.e. male or female; see Chapter 2). Zygote formation is then possible only between gametes of different sexes that are also of different mating types. Some of the traits regulated by the mating types differ between the female and male role during mating, and these traits – and therefore the mating types which regulate them – are likely to be under sexual selection. In homothallic fungi that do not have mating types (see Box 7.1) sexual selection might still act, because sex roles can still be present.

Furthermore, even though 'sexual' in 'sexual selection' originally refers to competition between individuals of one sex for matings with individuals of the other sex (Darwin, 1859; Jennions & Kokko, 2010), one can imagine that competition for mates can also occur between individuals of groups that are not defined by sexes but by the mating types. Most fungi have only two different mating types (Kües et al., 2011) and if one of the mating types is systematically in the majority, which is comparable to a skewed OSR, this will lead to increased competition for access to the other mating type. Because a systematic skew in mating types is generally not expected, as we explain in Box 7.1, we do not think that this type of competition is likely. In Box 7.1 we also describe the special case when more than two mating types are present, which *can* lead to selection between the mating types. Finally, even without a skew in mating type ratio, there is still potential for sexual selection to act when there is difference in quality of the mates. When quality of the mate affects zygote viability or offspring fitness, sexual selection can lead to traits that increase competitive ability relative to individuals of the same mating type.

The various functions that mating types have or regulate on the one side, and the possibilities for sexual selection that arise due to the presence of mating types on the other side, might lead to confusion. To avoid confusion when discussing the specific examples of sexual selection., we will indicate if selection occurs within sexes or mating types, and whether there is evolution of mating types or other traits in response to sexual selection.

When can sexual selection act?

To assess whether sexual selection plays an important role in fungi, i.e. whether fungi respond to sexual selection, a few prerequisites need to be fulfilled. First, obviously, but in fungi not trivially, sexual selection requires sexual reproduction. Although many fungi can reproduce asexually (Dyer, 2007; Billiard et al., 2012), most species go, at least occasionally, through sexual stages (Lee et al., 2010; Ni et al., 2011; see Fig 7.1a for a schematic generalized life cycle). The relative importance of selection during the sexual phase will depend on the frequency of sex compared to vegetative growth and asexual reproduction (Aanen & Hoekstra, 2007; Giraud et al., 2008). Second, we need to show that competition for mates exists, either due to existence of different sexes or sex roles during sexual reproduction, leading to a skewed OSR, or due to limited access to high quality mates. Third, there must be heritable variation in traits influencing the competitive ability of one sex (or sex role), but not of, or opposite to, the other sex (or the other sex role). Fourth, these traits should be costly and thus trade off with other fitness components, such as survival. Fifth, competition should actually occur in nature, which means that multiple

individuals potentially need to meet each other for competition or choosiness to be possible. Also when sexual selection acts not between sexes or sex roles, but between mating types, these prerequisites need to be met.

Male and female roles in fungal mating

As explained above, sexual selection is most likely to act when different sexes or sex roles are present. This usually implies that one of the sexes or sex roles is limiting for reproduction, so that there is increased competition for mating in the other sex role. For single-celled fungi, yeasts, two equally-sized gametes fuse during mating to form a diploid zygote and no sexes or sex roles can thus be distinguished (see Fig 7.1d). In contrast to yeasts, for filamentous fungi different sex roles can be distinguished. Most filamentous ascomycete fungi produce anisogamous gametes (see Fig 7.1b). In these fungi, usually many small, and fewer larger gametes are produced (Nauta & Hoekstra, 1992), which results in a skewed OSR. Also when mating occurs by fusion of hyphae, due to the high investment per zygote by the female, there is a limited number of female gametes available (Bruggeman et al., 2003). In a different group of filamentous fungi, mushroom-forming basidiomycetes, mycelia do not produce female and male gametes, but nevertheless male and female sex roles can be distinguished. Fertilization in these fungi occurs upon fusion between different mycelia, followed by donation and/or acceptance of nuclei (see Fig 1c). The donation of nuclei corresponds to the male role of the mycelium, with the nuclei as the male gametes and the acceptance of a nucleus corresponds to the female role of the mycelium, with the mycelium as a single female gamete. While the donation of nuclei is essentially cost free and can occur repeatedly, the acceptance of a nucleus by a mycelium implies sharing this mycelium with a different nucleus and can occur only once. As there are many more male gametes than female gametes, this results in an OSR biased towards the male role (Nieuwenhuis et al., 2011 and see paragraph 'Sexual selection in basidiomycetes').

Finding a mate and pheromone signaling

For mating to occur, first a mate must be found. When densities are low and fertilization is not guaranteed, any trait that increases the chance to meet a compatible gamete – by increasing the ability to find (e.g. motility) or being found (e.g. gamete size or attractiveness) – will be selected (Levitan, 1998; Lessells et al., 2009). Motility and attractiveness are functional at high densities too, either directly to outcompete rivals, or indirectly, by being more attractive to the mate. Which trait is sexually selected, and whether competition occurs between males or females, depend both on the density (Levitan, 2004; Kokko & Rankin, 2006) and the OSR (Clutton-Brock, 2009; Rosvall, 2011). For instance, when densities are high, females can be selective and choose the best male as happens at a lek where many males are gathered (Kirkpatrick & Ryan, 1991), but when males are rare, females have to attract them, even though this might lead to increased predation, which occurs in some moth species (Svensson, 1996).

Many fungi use extracellular compounds to attract each other (Kothe, 2008; Xue et al., 2008; Kües & Navarro-González, 2009). Often female gametes produce pheromones as a signal for chemotaxis, to attract male gametes or hyphae (the antheridia) to initiate fertilization. For instance, the female gametes of aquatic chytridiomycetes of the genus *Allomyces* produce the pheromone sirenin, which attracts male gametes (Machlis, 1958; Pommerville et al., 1990). When male gametes are limiting, they can choose which female gamete to fertilize, based on female pheromones. The female gamete that signals 'loudest' has the highest chance to become 'heard' and thus fertilized. Theoretically this can lead to Fisherian runaway selection for increasing pheromone production (Fisher, 1958). This aspect of pheromone production has not been investigated for *Allomyces*. In the aquatic oomycete species of the genus *Achlya*, that also uses pheromones to attract a compatible partner, much variation in female pheromone production and male reaction has been described between isolates from different ponds (Raper, 1951). These findings indicate for this trait that potentially there is selectable genetic variation on which sexual selection can act. Because all studies were performed in laboratory setups, it is unclear what the actual function of these pheromones is in nature. To gain more insight in the presence of sexual selection in aquatic species, research is needed on natural gamete density, motility, and pheromone production and function.

Pheromones for quality assessment

Pheromone production can also function in quality assessment of gametes. When production of pheromones is costly, pheromones can be used as an honest signal according to the handicap principle (Zahavi, 1975; Iwasa et al., 1991; Maynard Smith, 1991). According to this handicap principle, low-quality mates cannot pretend to be of high quality, because they cannot afford to pay the costs to produce the signal. In the budding yeast, *Saccharomyces cerevisiae*, pheromones are used during courtship to distinguish between mates, and a preference for higher pheromone production, including rejection of low-level signalers, has been experimentally established (Jackson & Hartwell, 1990a; Jackson & Hartwell, 1990b). In yeast, no sexes or sex roles are present, but competition occurs for high quality mates of the other mating type. Because the costs of pheromone production are high due to post-translational modifications, this signal can be used to assess gamete quality (Nathon et al., 1995; Smith & Greig, 2010). Choosing high-quality mates can occur for indirect benefits because it increases offspring quality, but choosiness could also provide a direct benefit to the diploid zygote (Smith, 2011). This benefit can even lead to evolution of preference when choice only takes place within a meiotic tetrad, when two high quality gametes of opposite mating type choose each other as partner (Pagel, 1993; Tazzyman et al., 2012). The same form of preference might act in basidiomycetes with high intra-tetrad mating such as *Microbotrium violacea* (Hood, 2002; Giraud et al., 2008).

Rogers and Greig (2009) used the intrinsic preference of *S. cerevisiae* for high pheromone-producing gametes to experimentally show sexual selection. In a population artificially skewed for mating-type ratio, choosiness led to selection of high pheromone producer genotypes (Rog-

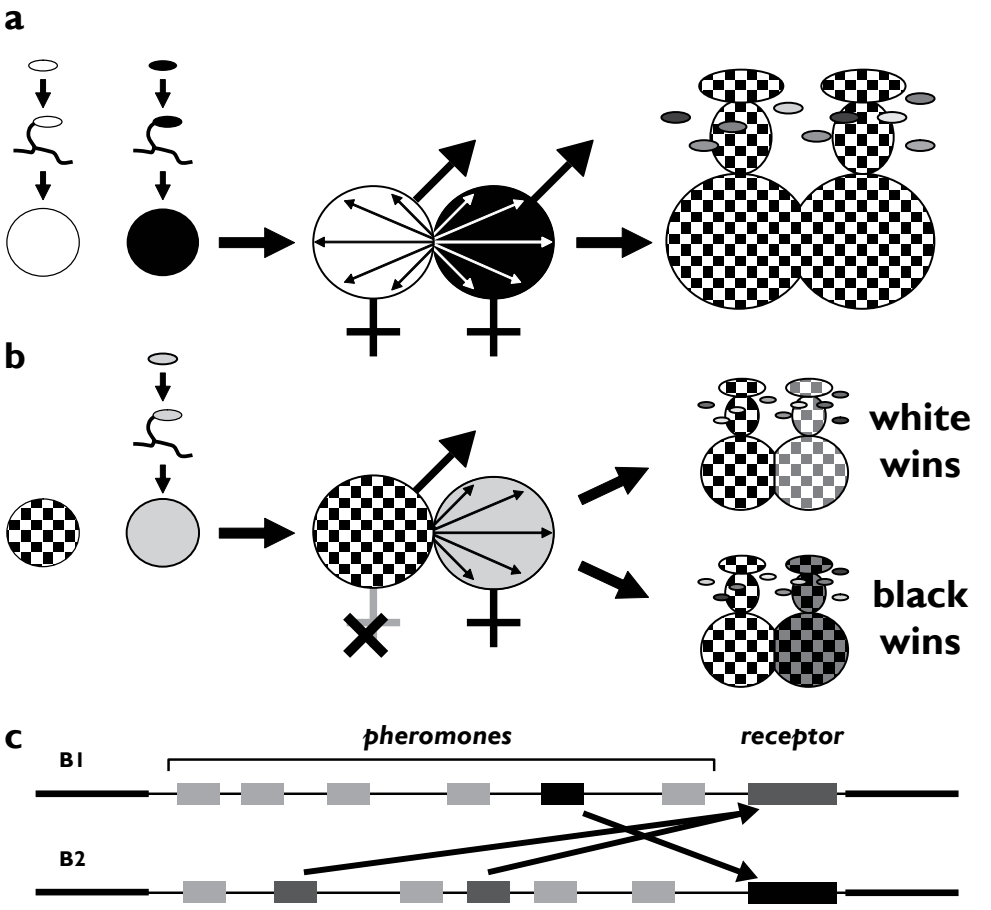

Figure 7.2. Pheromones in a heterokaryon-homokaryon mating. a) In basidiomycetes, mating between two homokaryons occurs in a hermaphroditic fashion: both mycelia simultaneously fertilize and get fertilized by donating a nucleus and receiving a nucleus from their partner, respectively. Nuclei migrate into the receiving homokaryotic mycelium that becomes a heterokaryon (checkered), which can form fruiting bodies in which the nuclei fuse and sexual spores are produced. **b)** The heterokaryon can still donate a nucleus to other homokaryons, but not receive a nucleus, so that the heterokaryon loses female fertility, but retains male fertility. When a heterokaryon with two compatible nucleus types (indicated in black and white) fertilizes a homokaryon (gray), only one of the two types can perform the fertilization. The end result of the heterokaryon-homokaryon mating is either a black-and-gray or white-and-gray heterokaryon. **c)** Schematic organization of two hypothetical alleles (allele B1 and B2) of the *B* locus that each code for a single receptor and multiple pheromones. None of the pheromones activates the receptor located on the same allele, but at least one pheromone on the allele will activates the receptor of *any* other allele. Nucleus B1 produces a pheromone which activates the receptor of its mate (B2) and will migrate into the mate's mycelium, and, *vice versa*, the pheromones produced by the mate activate the receptor on allele B1 induce migration into mycelium B1. Nuclear migration occurs in both ways. There is redundancy in the pheromones produced by B2: two compatible pheromones are produced, while one compatible pheromone is enough to initiate migration and successful mating.

ers & Greig, 2009), even though pheromone production is costly for the gamete during asexual growth (Smith & Greig, 2010). Runaway selection occurs, but contrary to selection as presumed in *Allomyces* (described above), here selection occurs at high mate densities. Such selection requires strong competition between gametes of the same mating type, effectively resulting in a biased OSR. In nature a skew is not expected, due to preferential switching of mating types during asexual growth (Haber, 1998; Haber, 2007) and due to the equalizing effect of sexual reproduction (each zygote produces exactly two haploid offspring of each mating type). These experiments do show, however, that under the right ecological circumstances sexual selection can evolve, using mechanisms present in fungi for mate finding (Kothe, 2008) or mate selection (Jackson & Hartwell, 1990a; Murphy et al., 2006).

Sexual selection in mushroom fungi

In mushroom-forming fungi (Basidiomycota) there are good arguments to believe that there is a male-biased operational sex ratio, and that sexual selection occurs, where pheromones are used as a criterion for selection.

Generalized life cycle of mushroom-forming basidiomycetes

In this group of fungi the generalized life cycle begins with a haploid sexual spore that germinates to form a homokaryotic mycelium (see Fig 7.1c). The homokaryon grows vegetatively until it becomes fertilized by another individual. The homokaryon is hermaphroditic: it can fertilize another homokaryon by donating nuclei and can simultaneously be fertilized by other individuals by receiving nuclei. Generally, fertilization occurs in a special way: the mycelium takes up compatible nuclei (based on mating type) and becomes a heterokaryon with two nucleus types, which remain separate and do not fuse immediately. The receiving mycelium acts as one large female gamete while the fertilizing nucleus acts as a male gamete. The exact process of fertilization is unclear but it is well established that the fertilizing nuclei actively migrate through the mycelium, leaving behind mitotic copies and finally populating the entire mycelium (Fig 2a; Raper, 1966; Gladfelter & Berman, 2009). The heterokaryon can continue to grow vegetatively, but can also form sexual fruiting bodies (mushrooms). In the mushroom the two nuclei will fuse to form a short-lived diploid nucleus, directly followed by meiosis and the formation of haploid sexual spores, which can initiate a new cycle.

The two genomes in a heterokaryon are 'condemned' to each other: after being fertilized, the heterokaryon is not capable to incorporate another nucleus type, and neither to replace one of its nucleus types for a different one. The choice of a partner nucleus by the receiving homokaryon is therefore critical as this determines fitness of the resulting heterokaryon.

Sexual selection in mushroom-forming basidiomycetes

Although a fertilized mycelium cannot be fertilized again, it can still donate a nucleus when it meets another homokaryon, i.e. function as a male (Fig 2b; Buller, 1930), an event expected to occur frequently in nature (Raper, 1966; Williams et al., 1981; Chapter 3). Also spores can directly act as male gametes (Williams & Todd, 1984; James & Vilgalys, 2001; Chapter 6). Consequently, the OSR of basidiomycetes is male biased, as there are more potentially fertilizing mycelia ('males') than potentially receiving mycelia ('females')(Aanen & Hoekstra, 2007). Recently, we investigated whether selection occurs between nuclei from a heterokaryon fertilizing a homokaryon (Nieuwenhuis et al., 2011). While the two nucleus types of the heterokaryon (functioning as male gametes) are in direct competition for fertilization, simultaneously, the receiving homokaryotic mycelium (acting as a female gamete) has the choice between these two male gamete types. In most pairings, a very strong bias for one of the two nuclei was found. In some pairings, the observed bias was determined by preference of the female mycelium, which shows that in the heterokaryon-homokaryon mating the receiving mycelium is choosy, leading to sexual selection on the male function of the nuclei. In Chapter 6 we showed that sexual selection can lead to adaptations that increase fitness in direct competitions. Because male-male competition and female choice are not mutually exclusive, it is also possible that next to female choice there is direct competition between fertilizing nuclei. Consistent with this hypothesis, some of the nuclei were very successful in fertilization, irrespective of the receiving mycelium, suggesting that direct competition between the two nuclei of the heterokaryon can also occur (Nieuwenhuis et al., 2011). There are many different traits that might respond to sexual selection, such as characteristics that increase migration of the migrating nuclei or the speed of mitotic divisions, or mechanisms that suppress similar characteristics in the competitor.

Pheromones for selection

It is not known on which trait (or traits) the receiving mycelium bases its choice. Even though sexual selection might be independent of the mating type, it is likely that selection is based on one of the mating type loci: these are always different between the two nuclei in the heterokaryon and they regulate many aspects of mating (Heitman et al., 2007). Selection might either be balancing, for example for increased heterozygosity – comparable to sexual selection at the major histocompatibility complex (MHC) in animals (Milinski, 2003; Kempenaers, 2007) – or directional in which case exaggerated traits might evolve.

Most mushroom-forming basidiomycetes have a unique mating-type determination by two unlinked mating type loci, the *A* and *B* locus (in some species they are named differently), which both are multi-allelic in many species (Raper, 1966; Casselton & Kües 2007). The *B* locus consists of two or three closely linked subloci, each coding for large families of pheromones and receptors. Each sub-locus typically codes for one receptor and several pheromones (Fig 7.2c). While none of the pheromones will induce activation of the receptor on the same allele, the combination of pheromones on each of the naturally occurring alleles can activate all other

alleles. Most investigated species have hundreds of different *B* alleles, resulting in potential mate compatibility approaching 100% in an outcrossing population (Raper, 1966). Even though a single pheromone activating the receptor of its partner is sufficient to induce nuclear migration into this partner mycelium, a high redundancy in pheromone-receptor compatibility is found (Fowler & Vaillancourt, 2007).

We present the hypothesis that the observed redundancy in pheromone-receptor interactions is a consequence of sexual selection, reinforced by a male-biased OSR. Our hypothesis infers that there is a correlation between the number of compatible receptor-pheromone interactions and the probability to be selected in competition with other nuclei. So, although one compatible pheromone-receptor interaction is sufficient for a compatible mating, a nucleus with multiple compatible interactions causes a stronger compatibility reaction in the receiving mycelium which results in higher chance of fertilization compared to a nucleus with fewer compatible pheromones (see also Chapter 8). Therefore, acquiring an extra copy of a pheromone gene (for example via gene duplication, possibly followed by mutation, or via recombination; Kothe et al., 2003) will be advantageous in competition with other nuclei. Sexual selection may thus favor a *B* allele with additional copies of pheromone genes. Without sexual selection, all alleles are expected in equal frequencies (May et al., 1999; Richman, 2000), but sexual selection can increase the frequency of an allele higher than expected from negative frequency dependent selection. What the effect of sexual selection is on the number of alleles in the population is unclear. Sexual selection might increase mating type numbers due to selection for new alleles with more or more divers pheromones, or reduce their numbers due to increased frequencies of attractive alleles what might lead to loss of less attractive alleles. According to this novel hypothesis, selection for additional incorporated pheromones explains the extraordinary redundancy in pheromone-receptor interactions of *B* alleles found in most species.

Our hypothesis remains to be tested directly, but some circumstantial evidence supports it. First, in all known cases, the number of different pheromone genes per mating type allele is higher than the number of receptors (Fowler & Vaillancourt, 2007; Ohm et al., 2010b), consistent with the hypothesis that there is more competition for male than for female fertility. Second, most mushroom-forming basidiomycetes are obligatorily heterothallic and have efficient dispersal structures, disfavouring inbreeding (Whitehouse, 1949; Giraud et al., 2008; Kües et al., 2011), so that sexual selection is stronger than in most other fungi where asexual reproduction and homothallism are more prevalent. Third, nuclear migration, regulated by pheromone-receptor interactions (Raudaskoski, 1998), can be interpreted as a quality test of the male gamete (comparable with pollen tube growth in plants; e.g. Snow & Spira, 1991).

Direct male-male competition

In the previous examples, selection for increased attractiveness lead to higher chance of mating, but selection can also favor increased direct competitiveness between the gametes. In fungi, it is difficult to imagine fighting between individuals such as occurs in animals, but post-copulatory

competition, similar to sperm or pollen competition, is more easily envisioned. In animals, the speed and numbers of sperm are associated with mating success (Parker et al., 1972; Gage & Morrow, 2003). Both factors can affect fungal mating competitiveness as well. For instance in the heterokaryon-homokaryon matings described above, increased migration speed in the receiving mycelium or suppression of mitotic division of the competing nucleus, could lead to higher mating success (Ellingboe, 1964; Raper, 1985; Aanen, 2008; Chapters 5 and 6). Alternatively, increased numbers of gametes could be produced to increase competitive ability.

Consider the generalized life cycle of a filamentous ascomycete such as *Neurospora crassa* (see Fig 7.1b). Its mycelium has indeterminate vegetative growth, and can reproduce asexually by forming mitotic spores. The mycelium can also reproduce sexually as a hermaphrodite, producing both male and female gametes. The female gametes remain connected to the mycelium, which after fertilization acts as a 'nursing' tissue and supplies the zygote with resources (Leslie & Klein, 1996; Bruggeman et al., 2004). After fertilization by a compatible male gamete, the zygote directly enters meiosis, resulting in recombined haploid sexual spores. Female gametes are thus generally more costly to produce, so that fewer of them are produced per male gamete (Charnov, 1979; Bruggeman et al., 2004). Effectively, the costs of male gametes are even further reduced, because in many species the male gametes can also function as asexual spores, so that the male gametes still have the asexual option if they fail to fertilize a partner.

Even though the double function of gamete and asexual spore seems very advantageous, in some fungal species, two distinct types of male gametes are produced: small and large ones (Maheshwari, 1999). Whereas both are equally capable of fertilizing a female gamete (Bistis, 1983), the smaller gametes (known as microconidia) have lost the asexual function (Maheshwari, 1999; Fukumori et al., 2004). It seems likely that this specialization into two types of male gametes has evolved due to gamete competition at a cost of asexual reproduction. Although the dimorphism here is within the class of male gametes, the factors that influence the evolution of this dimorphism are comparable to those driving the sperm-egg dichotomy in animals (Parker et al., 1972; Lessells et al., 2009). Sexual selection will favor an increase in gamete number and gamete motility to increase the probability to fertilize the relatively rare female gametes – factors that both trade off with gamete size. As the zygote obtains its resources from the female mycelium (Bruggeman et al., 2004), reduced male gamete size is unlikely to have a strong effect on zygote survival. Even though microconidia are observed in many different species (Alexopoulos et al., 1996), their function has been investigated only for a few species.

Meeting multiple individuals

For competition to actually occur in nature, multiple individuals potentially need to meet each other. Although elaborate laboratory research has yielded much knowledge on the mechanisms and genetics of fungal sex, very little is known about the details of sexual reproduction in nature. For instance, there is a lack of knowledge on the numbers of genetically different individuals and their densities in natural habitats, while this is an important factor for the intensity of both sexual

and natural selection (Levitan, 2004; Kokko & Rankin, 2006; Zeyl & Otto, 2007). The few studies that have investigated physical interactions between mycelia of a single species, mostly have shown the presence of multiple genotypes at small spatial scales, resulting in many interaction zones (Burnett & Partington, 1957; Ramsdale & Rayner, 1994; Johannesson et al., 2001; Zhan et al., 2002; Powell et al., 2003; Hamelin, 2006; Chapter 3). However, in some fungi mycelia have been shown to reach extreme sizes (e.g. Smith et al., 1994), resulting in less interactions between different individuals. It has also been shown for several fungal groups that spores can readily be isolated from air samples (Malloch & Blackwell, 1993; James & Vilgalys, 2001; O'Gorman et al., 2009), which indicates constant supply of male gametes. To increase insight in the importance of mating and mate competition, more information on these factors and the frequency of mating and the different types of mating in natural populations is needed.

Sexual conflict in fungi

Distinguishing sexual roles in fungal mating not only implies the potential for sexual selection, but also for sexual conflict. Sexual conflict arises because of the different evolutionary interests of males and females (Parker, 1979; Rice, 1996) or the different sex roles (Charnov, 1979). Especially multiple mating leads to male-male competition, which can lead to male adaptations that are harmful for the female function (Rice, 1996).

It seems likely that such sexual conflicts also occur in fungi. For example, on an ascomycetous homokaryotic mycelium, multiple female fruiting organs are present, which each can be fertilized by a different male gamete (see Fig 7.1b). Potentially, unrelated male gametes are thus in competition and will be selected to extract more resources towards the fruiting body that they fertilized, which may lead to reduced overall fitness for the mycelium. Conversely, the 'mother' mycelium is equally related to the offspring of all female fruiting bodies, irrespective of the number of genetically different male gametes (Haig, 2000), and will thus be selected to divide resources equally over the fruiting bodies, or to the best of her offspring (Stearns, 1987a). The potential for such processes has already been shown. For instance, in *Aspergillus nidulans* differential allocation of female resources to developing fruiting bodies has been experimentally shown (Bruggeman et al., 2004). Furthermore, in *Neurospora crassa*, fruiting bodies can inhibit the development of additional fruiting bodies on the same mycelium (Howe & Prakash, 1969; Metzenberg, 1993). Potentially, this could be a consequence of male-male antagonism. However, because it is unknown which genome regulates these traits and benefits from differential investment, it remains to be shown if these traits result from sexual conflict.

Also in basidiomycetes (see '*Sexual selection in mushrooms*' and Fig 7.1c), multiple mating can occur. For example when multiple homokaryons simultaneously interact, when a heterokaryon fertilizes a homokaryon (Fig 7.2b), or when multiple compatible spores land on a single homokaryon. Multiple mating will lead to a mosaic of heterokaryons, which will interact antagonistically, leading to a reduced total fitness relative to a singly mated mycelium (Williams et al., 1981; Schmit, 2001; Aanen et al., 2009). Therefore, we predict that it is in the interest of the

receiving mycelium to avoid multiple mating. One possible way of achieving this is by female choice of one nucleus type (Nieuwenhuis et al., 2011) or to increase the migration speed of the fertilizing nuclei to assure homogeneous fertilization of the entire mycelium (see also Chapter 8). Furthermore, if the production of pheromones is costly, sexual selection for increased numbers of pheromones due to a male-biased OSR, may decrease other components of heterokaryon or homokaryon fitness than fitness through the male role.

Non-recombining regions around mating types

Regions around the mating-type loci have no or severely reduced levels of recombination. This has many parallels to sex-chromosome evolution in gonochorists (Fraser et al., 2004; Whittle & Johannesson, 2011). In some cases the size of the non-recombining regions has increased dramatically. For instance, in *Ustilago hordei* the region of non-recombination for the two mating types is ~430kb and ~500kb (Bakkeren & Kronstad, 1994) and in *Microbotrium violacea* the region around the two different mating type loci even spans 2.8Mb and 3.5Mb and the chromosomes that harbor them are consequently noticeably different in size (Hood, 2002). Sex-chromosome evolution is mainly driven by selection to reduce recombination between alleles with different effects on males and females (Bachtrog et al., 2011)2011. These are not only sex-determining genes, but also genes involved in sexual selection and genes with sexually antagonistic effects.

Box 7.2 - When is sexual selection expected?

To obtain better insights in the importance of sexual selection in fungi, more research is needed on sexual reproduction in nature. Here, we predict which factors promote sexual selection in nature.

When sexual reproduction is of importance in the lifecycle

The potential of sexual selection depends on how often sex occurs in the life cycle and how important sex is. If there are many asexual cycles per sexual cycle, sexually selected traits that are detrimental for the asexual cycle might not be maintained. Species that are obligatorily sexual have a higher potential for sexual selection (Aanen & Hoekstra, 2007). However, even though some pathogenic species need to be fertilized before host infection takes place, infection is often followed by asexual reproduction, which can affect sexual dynamics strongly (e.g. Giraud et al., 2010). It is of interest to see how much asexual reproduction is allowed for sexual selection to be maintained.

When many individuals meet

Only when multiple individuals or their gametes come in contact, sexual selection can happen. Especially with high densities of individuals, when gametes can migrate easily over longer distances, or when mycelia are long lived, will multiple mating be likely to occur. For instance, mixing of many genetically different individuals is expected in aquatic fungi. Also in long lived mycelia of canker forming ascomycetes one mycelium is likely to have many different mates (Marra et al., 2004).

Because fungi are hermaphrodites, the male and female roles are not associated with the mating-type loci and sexually selected traits will therefore not be linked to these loci. So far, surprisingly few differences in phenotypes between individuals of different mating-types have been described, even for strains with large non-recombining regions, although to our knowledge this has not been studied extensively. However, for some species, differences are known. For instance in the well-studied human pathogen *Cryptococcus neoformans*, inheritance of cytoplasmic genes is regulated by the mating types (Yan & Xu, 2003). Especially cytoplasmic inheritance is of interest with respect to sexual selection, because it might lead to disruptive selection on cytoplasmic investment between the mating types, ultimately leading to mating-type associated anisogamy (Charlesworth & Charlesworth, 2010). More knowledge on phenotypic differences between the mating types can give more insight in the analogies of these non-recombining regions and sex chromosomes. Research should focus on traits that are functional during mating and on the functional genes that are situated on the non-recombining regions around the mating-type genes. Fungi are well suited to experimentally investigate sex-chromosome evolution, because the outcome of antagonistic interactions during sexual reproduction can be investigated in the haploid offspring.

(box 7.2 continued)

When there is high gamete pressure

A high density of gametes creates intense competition for fertilizations. This can lead to selection for more and smaller gametes. Also traits of importance for mating might be selected, such as increased motility (Cox & Sethian, 1985), higher pheromone production (Jackson & Hartwell, 1990a), or nuclear migration speed (Aanen, 2008).

When a skew between groups of compatible gametes exists

For instance, when mating types are present in unequal numbers, competition between gametes of the most common mating type will occur for fertilization of the minority mating type (Rogers & Greig, 2009). If one of the mating types systematically gains an advantage during asexual growth (for instance if there is a coupling of the mating type to a virulence factor), a skew in the mating type ratios can arise, comparable to a skewed OSR. During the sexual phase, gametes of the majority mating type are then in competition for gametes of the other mating type.

When there is variation for selectable traits

Gametes can differ greatly in genetic makeup and compatibility. Selection for the right gamete can have direct effects for the mycelium to be fertilized, especially in species with a long-lasting diploid or heterokaryotic phase (e.g. Simchen & Jinks, 1964; Tazzyman, 2011). If a selectable trait, such as pheromone production, is correlated with 'fitness' (Pagel, 1993), and if quality at the gamete level correlates with fitness at the zygote level (e.g. Simchen & Jinks, 1964), then we expect choice to evolve.

Experimental sexual selection with fungi

Microorganisms in general, and fungi in particular offer excellent opportunities for experimental work on sexual selection, especially because the sexual cycle of many fungi has been studied at the genetic level (reviewed in Heitman et al., 2007). Other advantages are their ease in laboratory experiments, the availability of molecular tools and their small genome sizes, which already has resulted in hundreds of sequenced genomes. Their short generation times facilitate experimental-evolution studies and because they can be multiplied clonally and stored in suspended animation, direct tests between experimentally evolved and ancestral strains can be performed (Elena & Lenski, 2003). Furthermore, parallel evolution can be studied, starting with replicate identical genotypes.

The different factors that are at the basis of sexual selection theory can be manipulated easily in fungi, either by choosing the right species or mutant, genetically modifying a strain or by manipulating the environment. Obvious candidate traits to be studied in detail are the direct and indirect benefits of different mates, the cost of a trait, the strength of display traits, or the initial choosiness for a mate. Also the effects of density or OSR (e.g. Rogers & Greig, 2009) can be tested. This line of research can also give experimental insight in the role of sexual selection on the evolution of anisogamy for which so far only comparative empirical evidence exists (e.g. Bell, 1985; Randerson & Hurst, 2001).

By manipulating the fungal life cycle we can experimentally see how sexual selection influences adaptation. For example, we can force strains to repeatedly mate exclusively in a male or in a female role with strains that are not coevolving. Afterwards, we can test the change in traits, either as a direct consequence of sexual selection, or as an associated trade-off of sexually-selected traits. This can give insight in the traits influencing the evolution of gonochorism vs. hermaphroditism, most likely traits that influence allocation to one or the other sex role during mating. By artificially linking different mating types to either the male or the female role, also the genetic association of antagonistic traits as described in the previous paragraph *'Non-recombining regions around mating types'* can be studied in this way.

Because many fungi are haploid, the underlying genetic mechanisms can be investigated more easily than with diploid organisms. All of this gives ample opportunity to study basic questions of sexual selection and sexual conflict, such as the evolution of sex-role specialization during mating or possible antagonism between sex roles.

Conclusions

We have argues that in fungi sexual selection can act, and that all the prerequisites that we defined in the introduction can occur in fungi. To investigate the importance of sexual selection in fungal biology, some outstanding questions need to be addressed. Especially the sources of variance in reproductive success need to be investigated, such as the presence of skews in gametes acting in different sex roles (e.g. Anderson & Kohn, 2007), resource allocation towards male and female

gametes (e.g. Coppin, 2002), skews in mating-type frequencies resulting in exclusion of part of the population when sex is required (e.g. Kwon-Chung et al., 1992; Rogers & Greig, 2009), or differences in pre- and post-mating investment in offspring (e.g. Bruggeman et al., 2003). Also the consequences and origins of mate choice need to be investigated. Is choice based on genetic compatibility, or on honest signals that indicate mate quality? Research on these questions can be supported by the now well-developed theory for sexual selection and conflict in hermaphrodites (Leonard, 2006; Anthes et al., 2010), which can also be applied to fungi. In Box 7.2, we give some predictions where sexual selection can be expected in fungi.

Sexual-selection theory can provide important insights in the processes that shape the biology of fungi and, *vice versa*, fungi are good model systems to experimentally test basic aspects of sexual selection theory. Fungi can show the generality of sexual selection and show that this type of selection is generally present whenever there is potential for variation in mating success. The mycologists will also benefit; applying sexual selection theory to fungi will possibly shed light on some of the peculiarities of the sex life of fungi, which until now have been difficult to understand. Interaction between students of sexual selection and fungi will, therefore, be reciprocally illuminating.

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Chapter 8

General discussion: male and female fitness

Bart P.S. Nieuwenhuis

Introduction

In this thesis I have described different forms of sexual selection in fungi. I investigated how a monokaryon from the mushroom-forming basidiomycete *Schizophyllum commune* can become fertilized and that during fertilization, different nuclei are in competition to mate with the monokaryon. Even though the presence of selection has been shown, both for competition between the fertilizing nuclei (chapters 4, 5 and 6) and for selection by the receiving mycelium (chapter 4), the evolutionary forces that select for these traits have not been dealt with in detail. To better understand the benefits of sexually selected traits, we need to investigate what the effects of mating are. In most animals and plants, mating only occurs for reproduction and fitness is mainly affected by the number and quality of the offspring produced. In mushroom fungi, mating additionally affects the life and growth of the individual that becomes fertilized itself. To assess the effects of mating, this has to be taken into account as well. In this chapter, I will investigate these direct effects of mating for the female monokaryon, how this monokaryon influences mating, and how this might have shaped adaptation of male nuclei in response.

Mushroom fungus life histories

Before their adulthood in which they reproduce sexually, many organisms go through a sterile juvenile phase. During this phase investment in development takes place to ensure optimal performance when adult. When to make the switch towards adulthood depends on internal, for instance size or age (Kozłowski, 1992), and external cues, such as predation (Abrams & Rowe, 1996) or crowding (Abrahamson, 1975). Timing for this switch is shaped by evolution to optimize total fitness by balancing the advantage of short generation times and benefits of postponed reproduction, accounting for increased risk of mortality over time (Stearns, 1992). The switch to sexual maturity can be after only a few days as in mites (Plaistow et al., 2004), years as in for instance albatrosses (Weimerskirch, 1992) or even decades as in many trees (Verdu, 2002) and is expected to be optimized for the particular life history.

Different from other organisms where the change or metamorphosis occurs by a physiological change of the organism itself, in mushroom fungi maturity occurs due to addition of a second genome to each cell. After the switch to the dikaryotic phase, the organism can potentially reproduce sexually, but only under the right external and probably also internal conditions (Wosten & Wessels, 2006; and see below). If no sexual reproduction is possible yet, for instance due to external circumstances, mating is only beneficial if the formed dikaryon performs better than the monokaryon. Fitness of a clonal long-lived organism is different from individuals composed of a single somatic unit (Harvell & Grosberg, 1988; Pringle & Taylor, 2002). Fitness increases not only by producing sexual offspring, but also by adding mycelium through vegetative growth, which in its turn can reproduce. If mating, and thus switching to the dikaryotic phase, reduces vegetative abilities, it might be advantageous to wait.

Vegetative ability of the monokaryon vs. the dikaryon

The monokaryon colonizes substrate by growing vegetatively, and thereby secures territory, and the resources it represents, for future investment into fruiting and eventually offspring. The fungus competes with conspecific and heterospecific individuals, and has to fend off organisms that might want to parasitize or eat it. How effective a monokaryon is in occupying the substrate and defending it relative to a dikaryon is species specific. Research by Boddy and coworkers showed that in natural substrates, homokaryons of *Trametes versicolor* are as competitive as heterokaryons (Hiscox et al., 2010), and homokaryons of *Hericium coralloides* even outcompete the heterokaryons (Crockatt et al., 2008). In *T. versicolor* mating is thus not beneficial, and in *H. coralloides* mating should be postponed to the moment of fructification, as fertilization results in a competitively weaker mycelium.

In other species, mating can increase the performance of the monokaryon. For *S. commune* no competition studies have been performed, but growth rate, which might be used as a proxy for performance, has been measured for dikaryons and monokaryons. Simchen (1966a) found higher growth rates for dikaryons, but Clark & Anderson (2004) found the opposite. To investigate this further, I performed crosses between six monokaryons to create 30 dikaryons and measured the growth rate of all mycelia (strains and growth conditions as described in Chapter 4). From the 30 dikaryons tested, 10 showed a significant increase in growth rate relative to the unfertilized monokaryon (up to 53% faster than the best monokaryon), one had decreased, and the rest stayed the same (data given in Table 8.1; χ^2-test with $p<0.0017$ for Bonferroni correction for multiple replicates $n=30$). On average, a dikaryon grew 27% better than the unfertilized monokaryon. Only in one case was mating detrimental, and in all other cases mating appears to be beneficial for the performance of the monokaryotic genome.

Of course, growth rate is not the same as competitive ability, and the monokaryon and dikaryon differ from each other in many other characteristics that might have a negative effect on the performance of the mycelium relative to the monokaryotic phase (de Vries & Wessels, 1984; Ohm et al., 2010b). It would be interesting to perform competition assays as well, to better understand the effects of mating on these important life phases for *S. commune*. For now, assuming the dikaryotic mycelium is more vigorous, we can conclude that mating in the female role should increase fitness and mating should not be postponed.

Why not mate?

There are other potential costs of mating to the mycelium. First, there is the chance that during mating the mycelium attracts a sexually transmitted disease. Many fungi are known to contain cytoplasmic viruses (Milgroom, 1999) and in *S. commune* virus like particles (VLP) have been observed, which reduce growth rate of the monokaryon (Koltin et al., 1973). Even though virus transmission is expected to be reduced because there is little mixing of cytoplasm (Hurst,

1996), the VLPs were able to infect other mycelia after anastomosis. This is also known to occur in other species (e.g. Ihrmark et al., 2002). Because the VLPs were also transferred to the offspring (up to 83%), catching a virus can be very costly. How common infectious elements are is unknown.

Secondly, there are nuclei out there, that do not just fertilize the mycelium and move in, but take it over. In a spore trapping experiment by James & Vilgalys (2001), where a monokaryon was used as bait for aerial spores, in one of their traps the nucleus of the bait monokaryon appeared to have been replaced by nuclei from two different spores. After mating, the parasitic nuclei were able to remove the resident nucleus (James & Vilgalys, 2001). Such practices might also occur during di-mon matings (Erika Kothe, personal communications). It would be interesting to see if parasitic nuclei are common in nature. This can be tested by using a strain with a recessive mutation and a dominant resistance marker (A) as a receiving monokaryon, and mating it with a dikaryon that is composed of a nucleus with a different resistance marker (B) with the same recessive mutation and a wild type nucleus. Performing di-mon matings between these strains, and testing mycelial plugs from the receiving mycelium on the far side of the crossing zone on selective plates containing antibiotic B, should show growth only if both migrating nuclei are present. Adding antibiotic A as well can test for trikaryons. In this way many natural isolates can be easily tested.

A third potential danger is that mating will reduce the chance of successful fertilization of another monokaryon in future matings. After fertilization, the entire mycelium becomes dikaryotized and the nucleus thus has to compete with a different nucleus in future fertilizations. Because fertilizing other mycelia can potentially increase fitness tremendously, depending on the size of the to be fertilized monokaryon, losing out on this fertilization can be very costly.

Table 8.1 Growth rate in mm (average±95%CI) per day for 6 monokaryons and 30 dikaryons. Each row indicates the monokaryon that was complemented with the nucleus given in the columns. A crossing of two identical nuclei indicates growth of the monokaryons (bold). Dikaryons with significant increase relative to the monokaryon are shaded dark and with decrease is shaded light.

		Migrant nucleus					
		A	**B**	**C**	**D**	**E**	**F**
Receiving monokaryon	**A**	**6.78 ±0.11**	12.19 ±0.52	12.08 ±0.28	13.39 ±0.68	13.42 ±0.53	11.22 ±0.47
	B	11.14 ±0.80	**9.89 ±0.47**	9.83 ±0.33	11.71 ±0.07	10.08 ±0.25	11.44 ±0.71
	C	11.14 ±0.29	8.31 ±0.33	**7.92 ±0.66**	9.28 ±1.03	8.33 ±1.05	9.67 ±0.16
	D	13.92 ±0.81	11.61 ±0.27	9.61 ±0.27	**10.11 ±0.27**	10.06 ±0.96	10.03 ±1.14
	E	12.44 ±0.76	9.61 ±0.38	8.22 ±0.11	10.58 ±0.41	**9.75 ±0.09**	10.00 ±0.66
	F	11.81 ±0.22	11.42 ±0.09	10.28 ±0.20	10.58 ±0.50	9.78 ±0.72	**7.72 ±0.67**

Finally, mating might be costly, when mating occurs simultaneously multiple times, what might lead to breaking up the own mycelium. A monokaryon shares resources freely within the mycelium because it is a single genetic unit. When the mycelium becomes fertilized multiple times, it will become a mosaic in which each part shares the cytoplasm and one of the nucleus types, but each with another second nucleus (Fig 8.1; Williams et al., 1981). The different dikaryons will not recognise each other as like, and will form an interaction zone in which neither dikaryon grows (Rayner et al., 1984). The combined surface of the balkanized mycelium will be (almost) the size of one big mycelium, but even though fitness of a mycelium is correlated with size (Pringle & Taylor, 2002), this correlation is probably not linear. Due to a minimal threshold size for fructification, increased redistribution of resources through the mycelium, and the non-homogeneous environment, fungal fitness likely benefits from larger mycelium size (Aanen et al., 2008; Aanen et al., 2009; Bastiaans et al., in prep.). Multiple matings will therefore likely lead to reduced fitness.

Mate or wait?

It is difficult to assess whether mating in the female role is net beneficial, without having better knowledge on the actual benefits and costs of mating, but overall there appear to be more benefits than costs. If we suppose that postponing mating is beneficial, the next question is if a monokaryon is capable to delay mating?

The female mycelium is in charge of nuclear migration, because the molecular machinery used for migration of male nuclei is part of the receiving monokaryon (Gladfelter & Berman, 2009). Migration is initiated by activation of a pheromone receptor by pheromones from the male nucleus, and when this receptor is deleted a monokaryon is female sterile (Fowler et al., 1999; Kothe et al., 2003). If a monokaryon can disrupt the signalling cascade that normally occurs after fusion (Olesnicky et al., 1999), migration might also be delayed or aborted. To my knowledge, delayed migration has not been observed, however, this might be due to lab conditions in which migration is generally tested. As discussed above, mating is always beneficial when sexual reproduction can be initiated, which for fungi like *S. commune* is when a mushroom can be created. For this, there has to be enough space and resources, and some light. In laboratory conditions on a Petri-dish, these characteristics are always available, but inside the wood, where fertilizations are likely to occur, no mushrooms can be formed. Only when the mycelium reaches the surface a fertilized mycelium is needed.

If the monokaryon is in charge, it can wait for fertilizations to happen, up to the moment it is needed for fructification to occur and the nuclei can be transferred quickly through the entire mycelium. If multiple nuclei are waiting for migration to start, the monokaryon can select the best nucleus by female choice. Choice is only possible, when multiple mates are present. If the female is in charge, competition can be synchronized, comparable to female induced synchronized germination of pollen on plants' stigmas (Lankinen & Kiboi, 2007; Lankinen & Madjidian, 2011). However, as described above, multiple matings might lead to reduced fitness,

due to breaking up the mycelium. If migration is indeed defined by the mycelium, the benefits of mating as a male might still occur, as donation of nuclei would still be possible (Fowler et al., 1999).

The male nuclei do not benefit from waiting for mating, but will be selected to manipulate the mycelium to initiate migration. The conflict between males and females during mating is common in nature and leads to an arms race between male and female specific adaptations (Rice, 1996; Arnqvist & Rowe, 2002; Chapman et al., 2003). In for instance the plant *Collinsia heterophylla*, the female stigma defines her receptivity to select the best pollen, the male pollen induce early receptivity of the stigma to increase their chance in pollen competition (Lankinen & Madjidian, 2011).

Even if female control is possible, it is very well possible that delayed mating is not beneficial in *S. commune*, but in species where mating results in competition loss, such as *H. coralloides* (Crockatt et al., 2008), it might be a good strategy. Is there evidence that monokaryons postpone mating?

Longevity of the monokaryotic phase

It is generally assumed that the monokaryon is a short-lived phase, which soon after establishment becomes fertilized by another monokaryon to establish a dikaryon (e.g. Kauserud et al., 2006; Anderson & Kohn, 2007). The strongest support for the idea that monokaryons are quickly fertilized comes from spore trapping experiments which suggest that for many species spores are very abundant and readily lead to dikaryotization of the trapping monokaryon (e.g. Williams

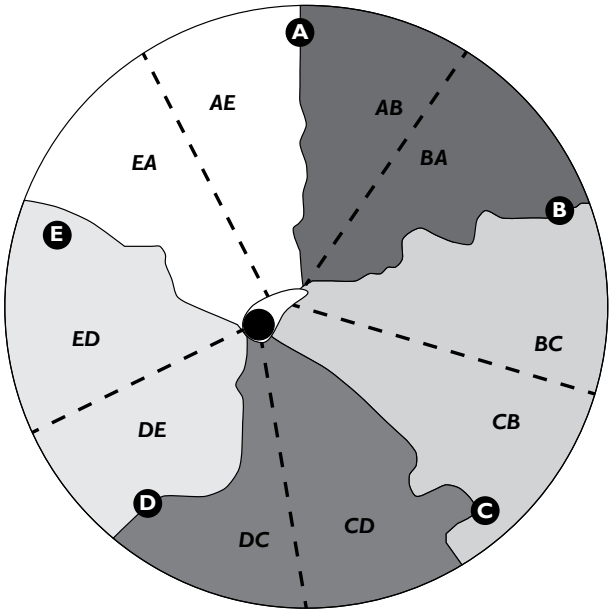

Figure 8.1 A representation of five fully compatible monokaryons of *Coriolus versicolor* that are inoculated simultaneously on a petridish. Each at one of the black dots at the edge of the plate. The dashed lines indicate until where the monokaryon grew. Each monokaryon mated at the zone of contact where reciprocal nuclear exchange occured. The monokaryon was simultaneously fertilized from both contact zones and the nuclei migrating from each side met at the solid line demarkating the mosaic of the receicing monokaryon. The color of the filled areas describe the size of the dikaryon as defined by it nuclear composition. Letter combinations indicate a dikaryon between two monokaryons, where the first letter defines the female monokaryon. *Reproduced from Williams et al. 1981*

& Todd, 1984; Vilgalys & Sun, 1994; James & Vilgalys, 2001; Kauserud & Schumacher, 2001). It is difficult to track longevity of the monokaryotic phase in nature. The mycelium often grows inside a substrate and cannot be observed easily. The few studies that investigated the presence of monokaryons in natural populations showed however, that monokaryotic mycelia can be found in nature and might be long(er) lasting (e.g. Garbelotto et al., 1999; Redfern et al., 2001; Stenlid et al., 2008). Unfortunately, non-destructive sampling of natural substrates is difficult.

Analysis of the nuclear and mitochondrial composition of *S. commune* dikaryons derived from three natural substrates showed that a monokaryon can be fertilized multiple times (Chapter 3). This is only possible when a monokaryon is either large at the moment of fertilization, when a dikaryon dedikaryotizes (Hui et al., 1999a), or when nuclear re-assortment occurs as observed in *Heterobasidion annosum* in which nuclei from two heterokaryons reassemble in a new combination (Johannesson & Stenlid, 2004). For *S. commune* dedikaryotization is not likely due to growth with clamp connections (but see Miles & Raper, 1956). Nuclear reassortment has to my knowledge never been observed, but parasitic nuclei that might oust one of the nuclei in a dikaryon, have been reported (James & Vilgalys, 2001; see above). Assuming that no re-assortment or dedikaryotization occurs, the mycelium must have been large enough for two nuclei to fertilize the mycelium more or less simultaneously so that the first nucleus had not completely dikaryotized the monokaryon, before the second one fertilized it from another point. Alternatively, before initial fertilization, the mycelium became fragmented which also implies some size.

We also observed that monokaryons do not appear to mate often with each other, but are more likely to be fertilized by a spore, or via the Buller phenomenon, by a dikaryotic mycelium (Chapter 3). Dikaryons with the same two nuclear types but with different mitochondrial backgrounds – the expected outcome of a mon-mon mating (Fig 3.2a) – were not observed. The observations that monokaryons are long lived, and that mon-mon matings are uncommon appear contradictory. If a monokaryon can be sustained for a long period, the chance increases that this monokaryon meets another mycelium that also has not been fertilized yet.

Growth in nature might occur in a different way from what we observe in laboratory settings and is likely not radial, but more linear due to the physical structure of the wood colonized by *S. commune*, resulting in more heterogeneity. Consequently, fewer cross connections between different parts of the mycelium arise than on a plate and the zones of growth are reduced. Fewer zones of growth will lead to fewer chances to meet a monokaryon, opposite to what occurs in spore trapping where a large surface of the mycelium is exposed. Furthermore, heterogeneity and fewer cross connections reduce the speed with which migration of nuclei can occur, which increases the time frame for multiple fertilizations.

Finally, larger monokaryons might arise due to postponed acceptance of nuclei.

Benefits of choice

The switch from dikaryon to monokaryon normally is a permanent one. After the dikaryon is formed, it is not expected to break up into a monokaryon again (see Chapter 5). For the

receiving mycelium, it is beneficial to choose the best nucleus possible. If the female mycelium postpones mating until multiple nuclei are present that can be tested, the best one can be chosen. Female plants and animals also increase sperm or pollen competition by reducing 'false starts' and increasing competition, thereby inflating quality differences (e.g. Lankinen & Madjidian, 2011; Higginson et al., 2012).

It is uncertain if choice by the monokaryon is made for increased quality of the fertilizing nucleus or for compatibility with the own genome. We were not able to find a correlation between winning nucleus and the fitness of the mycelium (based on mycelium growth rate) for the crosses we performed (Nieuwenhuis et al., 2011). However, even if the mycelium cannot distinguish between quality of incoming nuclei, the ability of distinguishing between nuclei in itself is already beneficial. As explained above, multiple mating might be detrimental, because it leads to fragmentation of the mycelium. To avoid fragmentation, the mycelium should avoid multiple matings and therefore, when mating with a dikaryon, select for only one nucleus, even if both nuclei are equally good. Whatever the trait is that monokaryons use to base their choice on, to avoid fertilization at other locations, it should be the same over the entire mycelium, which suggests that choice should be genetically defined. Nuclei that express the selected trait strongest will benefit most, and female choice will therefore infer sexual selection on this male trait.

Pheromones for selection

Whether nuclei wait for multiple potential partners and then choose, or whether they only select between nuclei in a dikaryon-monokaryon mating, choice has to be made on a characteristic of the migrating nuclei. In Chapter 4 we showed that the receiving mycelium is capable of influencing which nucleus in a dikaryon performs the fertilizations. We suggested that the trait on which selection is based, might be located at the mating type loci. In Chapter 7, we expanded on this idea and suggested that the pheromone genes, which are located at the B mating type locus are good candidates for sexual selection. The pheromones ligate with the pheromone receptor of the mate, which is also located at the B locus, and when the receptor is activated induce amongst others nuclear migration (Raper, 1966; Fowler et al., 1999). A receptor responds to multiple different pheromones, but has a different affinity to the different pheromones (Gola et al., 2000; Gola & Kothe, 2003). Furthermore, there is a qualitative response of the cell to the pheromones, which suggests that more, or more different pheromones have a stronger effect on the receiving mycelium (Gola & Kothe, 2003). Increasing the number of pheromones might therefore be advantageous for male nuclei that are in competition to be selected by the female mycelium. This competitive advantage might have led to the high diversity and redundancy in pheromones per mating type allele, which can be observed in *S. commune* (Fowler & Vaillancourt, 2007; Ohm et al., 2010b). High pheromone production is known to increase mating chance in *Saccharomyces cerevisiae* (Jackson & Hartwell, 1990a; Rogers & Greig, 2009).

A prerequisite for this hypothesis is that monokaryons react preferentially to nuclei that produce more or more diverse pheromones. Strains that have been used for di-mon matings

contained wild-type alleles and because of the complexity of compatibility between pheromones and receptors, it is difficult to quantify for each strain how many compatible pheromones to the receiving receptor are present. To properly test if female mycelia prefer more pheromones, competitions should be performed between nuclei with defined levels of pheromones.

A strain created by Cardy and John Raper using radiation had a large deletion, which included the entire B mating type locus might offer a solution (Raper & Raper, 1973). This so-called B-null strain has been used successfully to transform strains and test the function of pheromones and receptors (e.g. Fowler et al., 1999; Gola et al., 2000; Fowler et al., 2001). Transforming this strain with the same pheromone with different levels of expression, or with a different number of different pheromone types, and performing di-mon matings as in Chapter 4, will give the possibility to test if pheromones influence preference for nuclei. Because the pheromones and pheromone receptor interactions have been characterized for many different varieties and their sequences are known, different known combinations can be produced.

For a di-mon mating, a dikaryon is needed with two nuclei that are compatible with each other, i.e. they need to be different at the B mating type locus (see Fig 8.2 for a graphical representation of the interactions). Because the pheromones used to initiate the first dikaryon should not affect the di-mon mating, a pheromone should be used that does not interact with

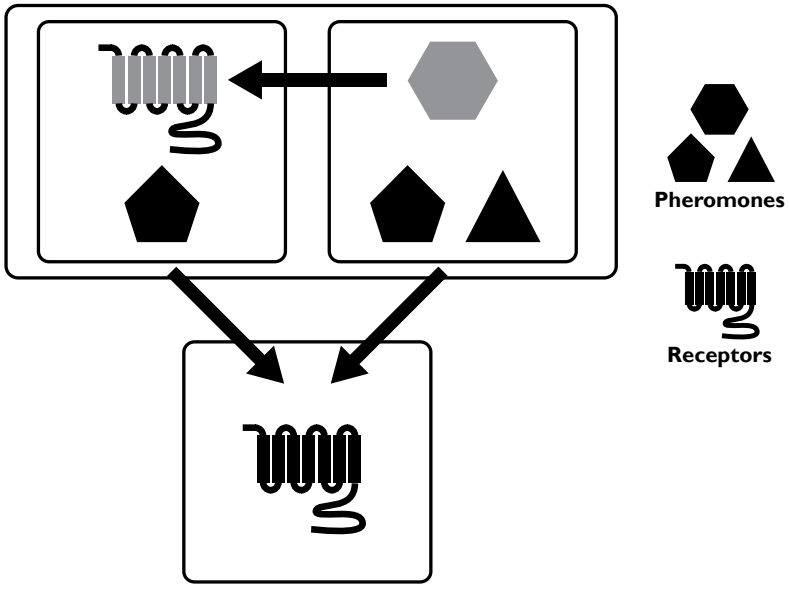

Figure 8.2 Setup to test preference for increased pheromone production in di-mon matings in *S. commune*. The top represents the dikaryon in which two nuclei are present that interact with each other via the grey pheromone and receptor combination. Both nuclei are transformed with pheromones (in black) that interact with the receptor of the monokaryon (square at bottom). The grey pheromone in the right dikaryon nucleus does not interact with the black receptor in the monokaryon. If pheromones are used as a trait for preference, in this example, the right nucleus will be winning most matings.

the receptor of the receiving monokaryon used in the di-mon mating. The B-mating type locus in *S. commune* is composed of two separate subloci, which each have their own set or receptors and pheromones, and between which little cross talk exists (Fowler & Vaillancourt, 2007). To form the initial dikaryon, receptors of one sublocus can be used, and for the di-mon mating, receptors of the other.

Unfortunately, there is no null mutant for the A mating type, so that each nucleus needs to be different for this locus. To control for possible effects of the A locus on the di-mon mating, reciprocal crosses need to be performed with high or diverse pheromones in each A background.

Conclusions

In this thesis I have shown that also in the fungal kingdom sexual selection acts and can lead to a diversity of adaptations. The implications of identifying sexual selection in fungi are that next to adaptations that increase fungal survival and ability to reproduce, attention should be paid to mating. Sexual interactions in fungi have been studied elaborately, but up to now have been based on the paradigm that mechanisms evolve for efficient reproduction and maximal reproductive output. Sexual selection suggests that mechanisms might evolve not for maximal output, but to maximize output relative to other competing individuals in the populations. This can even happen when absolute reproduction goes down, as long as that of competitors is reduced even further.

The potential for sexual selection is largest when sex roles are present in mating, because the intrinsic asymmetry between the sexes leads to a skew in the operational sex ratio. The other consequence of sex roles is that sexual conflict can arise over matings. The male role has different interests than the female role, which can lead to divergent selection on the sex roles. Sexual selection and antagonism between male and female sex roles can lead to evolution of interesting mechanism for mating. In hermaphroditic species, and especially in micro-organisms, such traits might be difficult to observe. Awareness of the importance of mating for the sexual cycle of any organism can yield explanations for phenomena which apparently are in conflict with the adage that the strongest will survive.

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Summary

Sexual selection is an important factor that drives evolution, in which fitness is increased, not by increasing survival or viability, but by acquiring more or better mates. Sexual selection favours traits that increase the ability of an individual to obtain more matings than other individuals that it is in competition with. For many sexually reproducing organisms, obtaining mates is an essential part of the lifecycle, sexual selection can therefore be very strong. A trait that leads to more matings can be selected, even if it strongly reduces other components of fitness, for instance predator escape. Often sexual selection leads to sex specific traits, which can become very extravagant. In animals and plants, it has been well established that this form of selection is an important evolutionary force, but it has not been considered for fungi. This thesis revolves around the idea that in this aspect, fungi are not fundamentally different from animals and plants and that also for species from this kingdom sexual selection influences evolution. Many fungi reproduce sexually and need to find a partner before reproduction can proceed. Furthermore, it is likely that not all individuals that benefit from mating can perform mating, hence a struggle for mate acquisition will occur.

In my research I have investigated how likely it is that in fungi such struggles occur and which mechanisms might act during competitions. For these studies I used the mushroom forming basidiomycete fungus *Schizophyllum commune* as a model organism. I studied the potential for mate competition in natural populations, performed laboratory mating essays to test competition and preference, and experimentally tested if sexual selection can increase competitive ability.

Sexual reproduction in fungi is highly regulated. Many molecular mechanisms are known that modulate each step, from meiosis to gamete production and from mate finding to gamete fusion. In most fungi these characteristics are regulated by genes located on the mating type locus or loci. These genes do not only regulate mating, but also define compatibility between the gametes: gametes with the same alleles at a mating type locus cannot fuse. Because of this double function, fungal mating types are potentially very important for sexual selection. Besides that mating types are a target for sexual selection because they affect traits that might increase competitive ability, since the mating types determine compatibility, they also define who competes with whom.

Sexual selection describes how within one sex competition occurs for individuals of the other sex and is therefore always intra-sexual. Fungi do not have different sexes, but do have sex roles. Sexual selection will therefore act if there is competition for mating in the male or female role. Compatibility between sex roles is different from compatibility between mating types. The first is defined by the size of the mycelium, and the second by a genetic recognition mechanism. This difference is of importance to understand how sexual selection can act in fungi and is explained in Chapter 2.

Mating in mushroom fungi occurs by reciprocal exchange of nuclei. In the female role nuclei from a compatible mate are incorporated into the haploid mycelium. These nuclei migrate though the mycelium until in each cell of the mycelium two haploid nuclei are present, it becomes a so called dikaryon. Only a dikaryon can produce mushrooms that produce spores. In the male role, a mycelium can donate nuclei to a haploid mycelium. A mycelium can thus be considered hermaphroditic. After fertilize, the dikaryon can still act as a nucleus donor, but not incorporate more nuclei – this type of mating is known as the Buller phenomenon. Also spores can act as male, but as they have no mycelium, not as a female. Due to the presence of more individuals that can mate in the male role than there are female mycelia (monokaryons), competition over fertilizations is expected.

Competition can only occur when there are multiple individuals. Fungi are sessile organisms that can only meet other individuals when they are in the same locality. To test whether there is potential for sexual selection in nature, the number of individuals that meet each other needs to be defined. Not much knowledge on numbers of individuals is known, because mushroom fungi generally grow by mycelium expansion inside a substratum and each part of the mycelium can produce mushrooms. Therefore, all mushrooms on a tree can be one genetic individual, but it is also possible that each mushroom is a separate individual. We sampled 24, 12 and 24 mushrooms from the same substrate of three natural populations to analyze how mating occurred (Chapter 3). We determined the identity of the two different nuclei in each mushroom, as well as the mitochondria. Because mitochondria do not migrate during mating, they are specific for each female mycelium. We found that multiple genetic individuals (3, 3 and 8) are present in a small area, and that many matings must have occurred. Even though it is generally assumed that matings occur between two monokaryons, none such matings were found. The data suggest that mating in nature occurs between a monokaryon and a spore, or a monokaryon and a dikaryon.

During a dikaryon-monokaryon (di-mon) mating only one of the two nucleus types from the dikaryon is successful in fertilizing the monokaryon. The nucleus type that is successful will likely increase its fitness considerably, as the entire female mycelium becomes colonized. Sexual selection is expected to select for nuclei that are better in performing this fertilization. Furthermore, during mating the receiving monokaryon meets two different nuclei, and might be able to choose between them. We performed crosses between 15 dikaryons and six different monokaryons to test if selection occurs, and whether selection occurs by male-male competition, or by female choice (Chapter 4). When confronting the same dikaryon with different

monokaryon, in some of the cases the female mycelium decided which of the two nuclei won. In most cases however, the same nucleus always fertilized the monokaryon, irrespective of which monokaryon. This suggests that nuclei are able to either manipulate the monokaryon in incorporating them into the mycelium and not the other type, or that the nuclei of one type can directly suppress mating by the other nuclei in the dikaryon.

Nuclei in a dikaryon have a strict way of cell division in which the different types divide in synchrony. Probably the two nuclei keep each other in check to assure this synchrony. Experiments in which the two nuclei in a dikaryon are separated into monokaryons suggest that the two nuclei suppress each other's mitotic division, and that one of the two nuclei is better in suppression than the other. Consequently, after de-dikaryotization more monokaryons of one type are recovered than of the other. We tested if this mechanism of suppression might be responsible for the dominant nuclei in the di-mon matings (Chapter 5). Separating the two nuclei confirmed earlier findings that always one of the two nuclei is dominant and that a hierarchy in dominance exists. This pecking order did not correspond with the results from the winner in the di-mon matings, which suggests that the mechanism of suppressed mitotic division is not responsible for dominance in di-mon matings. Nevertheless, we argue that the hypothesis that a link between the two mechanisms exists should not be completely written off. Because the interactions that take place during di-mon matings are very complex, the functioning of this mechanism might be obscured during mating.

The observed variance in mating success described above might lead to sexual selection, however, it does not show that sexual selection actually led to traits that improve increased mate acquisition. To show that traits can evolve that increase fitness by higher mating success, we performed an evolution experiment (Chapter 6). An evolving population of nuclei was continuously mated with a non-evolving monokaryon. This setup selected for traits that increase competitive ability over matings. After 20 transfers, four out of twelve evolved lines had increased in competitive fitness and one line had decreased. Different fitness components were measured to investigate which traits had resulted in changed fitness. Fertilization success was mainly determined at the moment of fusion with and in initial migration into the receiving monokaryon. Two strains showed increased spores production, but this did not add to the increased fitness caused by fusion and initial migration. Little fitness change occurred during migration or in the dikaryon phase. We observed no clear trade-offs between the competitive ability of fertilizing in the male role, and female characteristics. This experiment showed that sexual selection can act in mushroom fungi.

Sexual selection can also play a role in other groups of fungi than the mushroom forming fungi. So far this has not been considered, and little research has been done to show how mate competition might influence evolution. We reinterpreted the current knowledge on mating in fungi and assessed whether and when sexual selection might play a role (Chapter 7). Sexual selection is most likely to occur when sex roles can be observed during mating, as this can lead to skewed sex ratios. Also when there is large difference in quality between potential mates

sexual selection might lead to evolution of choice. Directions are given where sexual selection is expected to function in fungal mating. Examples are given of how sexual selection might have led to for instance the evolution of micro-conidia in ascomycetes and pheromone redundancy in basidiomycetes. Furthermore, the existence of different sex roles in fungi, can lead to sexual conflict between the genomes derived from the paternal and the maternal gametes of which examples are given. The realization that sexual selection can also act in fungi gives great opportunity to test how universal general theories of sexual selection are in another important group of organisms. Additionally, because fungi are easy to manipulate, predictions on sexual selection can be tested experimentally using fungi.

Mushroom forming fungi have a life history which differs from animals and from plants. Sexual selection will therefore affect mushroom fungi in a different manner than it would animals and plants. In the general discussion of this thesis (Chapter 8) I will assess how mating influences fungal fitness, teasing apart the benefits and costs of mating in the male and female roles. I give directions for future research and discuss a setup to directly measure the effect of pheromones on female choice in mushroom fungi.

There are still many unanswered fundamental questions about sexual selection. Adding knowledge from a third important kingdom can help increase the understanding of the principles that drive evolution by sexual selection. Furthermore, applying sexual selection theory to fungi might elucidate the functioning of the sometimes very complex mechanism that have evolved for fungal mating.

Nederlandse Samenvatting

Dit is een uitgebreide Nederlandse samenvatting voor allen die, ongeacht biologische voorkennis, geïnteresseerd zijn in het in dit proefschrift beschreven onderzoek.

Seksuele selectie

De theorie van natuurlijke selectie voorspelt dat individuen die het beste zijn aangepast de meeste kans hebben te overleven en ook de meeste nakomelingen zullen krijgen. De kenmerken voor het 'aangepast zijn' liggen vastgelegd in het genetisch materiaal dat wordt doorgegeven aan hun nakomelingen. Op hun beurt zullen de best aangepaste nakomelingen overleven en zelf nakomelingen produceren zodat op den duur zeer goed aangepaste soorten zullen evolueren: perfect gestroomlijnde vissen, octopussen die afhankelijk van hun omgeving zich een camouflage aanmeten, of planten die insecten te vangen als de bodem te schraal is om ze van voedsel te voorzien. Nadelige eigenschappen zullen juist verdwijnen, omdat slechter aangepaste individuen eerder sterven of minder nakomelingen zullen produceren. Toch zijn er vele soorten die helemaal niet goed aangepast lijken. Gekleurde guppies bijvoorbeeld, vallen eerder ten prooi aan predatoren dan onopvallende (Reznick & Endler, 1982) en het is dus te verwachten selectie zal plaatvinden voor onopvallende guppies. Bij vogels zijn grote staarten die het vliegen bemoeilijken niet te verwachten – deze vogels komen moeilijker aan eten en ontsnappen minder makkelijk aan predatoren dan vogels met een kortere staart (Fig S1; Pryke & Andersson, 2005). Toch zijn er vele voorbeelden in de natuur van vogels met lange staarten en van fel gekleurde vissen.

Soorten die op het eerste gezicht niet goed aangepast lijken, maar wel behouden blijven trokken de aandacht van Darwin (1859, 1871). Ruim twee decennia na het verschijnen van 'On the origin of species' verschijnt een tweedelig boekwerk 'The descent of man, and selection in relation to sex' waarin hij uitgebreid uitleg geeft van een andere vorm van selectie, namelijk *seksuele selectie*. Om voort te planten moeten, naast overleven, de meeste soorten namelijk ook een partner vinden en paren. Darwins theorie – die hij onderbouwde met talloze voorbeelden uit het dierenrijk – is dat veel van de eigenschappen in de natuur die nadelig zijn voor overleven een voordeel kunnen geven bij succes in het veroveren van een partner en bij het paren.

Figuur S1. Ondanks dat bij roodkeelwida's een lange staart leidt tot hogere kans op predatie hebben mannetjes extreem lange staarten. Photo courtesy of GR Davis

Een mannetje kan met meerdere vrouwtjes paren en nakomelingen bij meerdere vrouwtjes hebben. De kosten per nakomeling zijn voor een mannetje relatief laag omdat de meeste kosten door het vrouwtje worden gedragen. Doordat het vrouwtje veel investeert per nakomeling, kan zij maar een beperkt aantal nakomelingen produceren. Hierdoor ontstaat een scheve verhouding richting meer mannetjes in de 'operationele seks ratio'. De mannetjes zijn hierdoor in competitie voor het veroveren en bevruchten van de beperkte hoeveelheid vrouwtjes. Eén mannetje kan potentieel een groot deel van de nakomelingen produceren, terwijl andere mannetjes helemaal geen nageslacht zullen hebben. Hoe succesvoller het mannetje is in paren, hoe meer nakomelingen hij zal hebben, en eigenschappen die daartoe bijdragen zullen dus geselecteerd worden. Een sterk mannetje of bijvoorbeeld een mannetje met een groot gewei kan andere mannetjes bevechten om vrouwtjes te veroveren. Aan de andere kant zijn meerdere bevruchtingen voor een vrouwtje niet voordelig, omdat zij aan een paar of zelf één paring genoeg heeft en vaker paren haar fitness niet zal verhogen (Bateman, 1948). De vrouwtjes kunnen hierdoor selectief zijn en alleen met die mannetjes paren die voor haar het voordeligst zijn. Vaak worden deze keuzes gebaseerd of uiterlijke kenmerken. De meest aantrekkelijke mannetjes krijgen zo de meeste nakomelingen, wat kan leiden tot selectie voor extravagante kermerken, zelfs als de eigenschap nadelig is voor overleving (Fisher, 1958). Vrouwtjes van de roodkeelwida (*Euplectes ardens*) hebben een voorkeur voor mannetjes met een langere staart (Andersson, 1982) en vrouwtjes guppies prefereren mannetjes met een felle kleur (Breden & Stoner, 1987). Ondanks de verminderde overleving van mannetjes met extreme kermerken, hebben deze mannetjes toch een verhoogde fitness.

Ondanks dat seksuele selectie meestal wordt geassocieerd met competitie tussen de mannetjes, zijn ook vrouwtjes vaak in competitie met elkaar. Zij beconcurreren elkaar bijvoorbeeld voor mannetjes die hen kunnen voorzien van het beste territorium, broedzorg, of die op een andere manier hun fitness doen toenemen (Clutton-Brock, 2009).

Maar ook tijdens en na de copulatie, is er nog steeds concurrentie voor bevruchting, maar nu tussen de gameten (Parker, 1970). Als een vrouwtje meerdere keren heeft gepaard, zal het sperma van verschillende mannetjes strijden om de eicellen te bevruchten, wat heeft geleidt tot de evolutie van vele verschillende eigenschappen die het concurrentievermogen na paring vergroten. Bijvoorbeeld, in veel diersoorten heeft de penis een dubbele functie; naast de overdracht van sperma, verwijdert het sperma van concurrerende mannetjes die eerder met hetzelfde vrouwtje paarden om spermacompetitie te verminderen (bijv. Waage, 1979). Ook het vrouwtje kan de uitkomst van de uiteindelijke bevruchting nog beinvloeden door middel van cryptic female choice (Eberhard, 1996). Mannetjes proberen vervolgens deze keuze te manipuleren

ten voorkeur van hun sperma, bijvoorbeeld door de productie van eiwitten die samen met het sperma worden overgedragen (Chapman, 2001). Veel zaadkever mannetjes hebben een andere strategie om concurentie te voorkomen. De mannetjes in deze soorten hebben nare stekels op de penis die vrouwelijke genitaliën beschadigen en zo het vrouwtje weerhouden opnieuw te paren, waarmee ze het vrouwtje monopoliseren (Hotzy & Arnqvist, 2009). Zelfs wanneer mannelijke eigenschappen schadelijk zijn voor het vrouwtje en haar fitness verminderen, bijvoorbeeld door ze zo te verwonden, kunnen dergelijke aanpassingen toch voordelig zijn voor het mannetje (Parker, 2006).

Seksuele selectie is voornamelijk onderzocht in dieren (Andersson, 1994; Carranza, 2009), maar komt ook in planten voor, waar selectie onder andere heeft geleid tot evolutie van indrukwekkende bloemen voor het aantrekken van bestuivers (Andersson & Simmons, 2006; Yakimowski et al., 2011) en tot versnelde kieming en groei van pollen in de bloemstijl om als snelste de eicel te bevruchten (Lankinen & Madjidian, 2011). In schimmels is seksuele selectie echter niet algemeen aanvaard. In dit proefschrift stel ik dat ook in schimmels seksuele selectie voorkomt.

Seksuele selectie in schimmels

Schimmels zijn niet fundamenteel anders dan planten en dieren. De meeste schimmels reproduceren regelmatig seksueel. Terwijl een deel van de schimmelsoorten zich kan voortplanten door zelfbevruchting, moeten de meeste soorten een partner vinden om mee te paren en de seksuele fase van de levenscyclus te volbrengen (Whitehouse, 1949; Raper, 1966). Alleen die individuen die er in slagen een partner te vinden en te bemachtigen kunnen zich voortplanten. Ook bij schimmels bestaat dus de mogelijkheid dat seksuele selectie zal plaatsvinden voor kenmerken die paring bevorderen.

De meeste schimmels hebben een levenscyclus die voornamelijk of zelfs volledig cryptisch is – groei en ook bevruchting vinden plaats in het substraat waarin de schimmel groeit (Stajich et al., 2009). De kenmerken die onder invloed van seksuele selectie bij schimmels evolueren zullen daardoor niet duidelijk zichtbaar zijn zoals een hertengewei of het verenkleed van vogels. De te verwachten aanpassingen zullen een voordeel bieden dat specifiek voordeel biedt bij paring zoals voorkomt in de schimmels levenswijze. Ondanks dat er zeer veel bekend is over schimmels doordat in de afgelopen eeuw veel onderzoek is gedaan met schimmels, is seksuele selectie niet herkend – waarschijnlijk door het cryptische karakter van de seksueel geselecteerde eigenschappen.

Algemene levenscyclus van schimmels

Er bestaan vele verschillende soorten schimmels met vele verschillende typen levenscycli (Alexopoulos et al., 1996). Een seksuele schimmel heeft een haploïde en een diploïde fase en om diploïd te worden moet een haploïde individu een andere haploïd vinden om mee te fuseren. Een diploïd

gaat na verloop van tijd door meiotische reductie deling en vormt opnieuw haploïde individuen (zie Fig S2a). Meestal is in iedere fase groei mogelijk, door aseksuele deling of door vegetatieve groei van het mycelium. De duur van iedere fase verschilt per soort en is meer afhankelijk van de levensvorm dan van fylogenetische verwantschap (Fig S3). Bijvoorbeeld de meeste meercellige Ascomycota zijn bijna hun hele leven haploïd, terwijl de gisten van Ascomycota, net als de gistachtige Basidiomycota, een lange diploïde fase hebben.

Het moment waarop seksuele selectie kan plaatsvinden is wanneer een haploïde individu diploïd wordt – het moment van paring (*mating*) wat tegelijkertijd bevruchting is (*fertilization*). In de meeste hogere planten en dieren vindt paring plaats tussen diploïde individuen waarbij door hen geproduceerde (haploïde) gameten van het ene individu (pollen of sperma) de gameten van het andere individu (de eicel) bevruchten. In schimmels, net als in bijvoorbeeld mossen en varens, vindt paring niet tussen diploïden plaats, maar is er een haploïde individu dat bevrucht wordt door een ander haploïde individu, of door losse gameten. In het laboratorium zijn vele, vaak complexe, mechanismen beschreven die tijdens de bevruchting van schimmels plaatsvinden. Waarschijnlijk bevorderen deze mechanismen de kans om een succesvolle paring te laten plaatsvinden, maar dit is niet in detail onderzocht. In dit proefschrift richt ik me op de selectiedruk die kan hebben geleid tot de evolutie van deze mechanismen waarvan ik aanneem dat ze zijn geselecteerd om tijdens de paring een competitief voordeel te hebben over concurrenten.

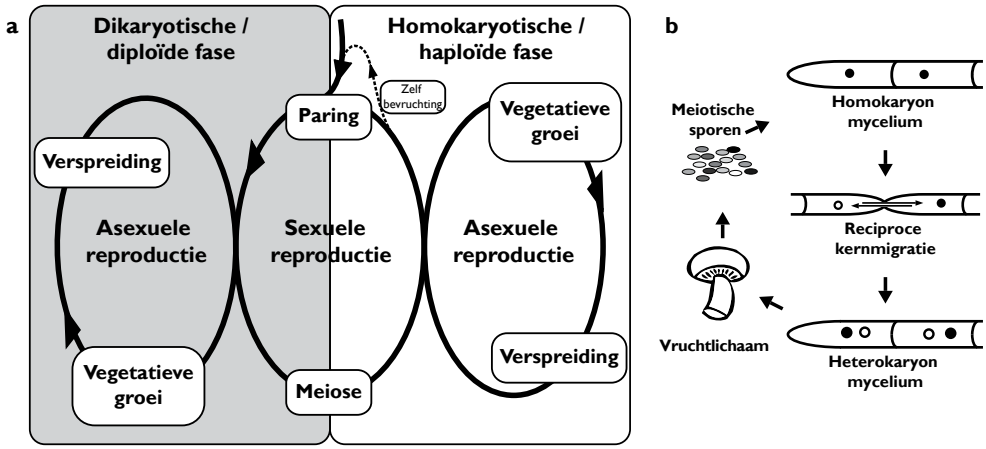
Schizophyllum commune

Voor het onderzoek beschreven in dit proefschrift gebruikte ik de paddenstoelsoort *Schizophyllum commune* (het Waaiertje). Deze soort is een saprofytische schimmel en opportunistisch boompathogeen dat ook luchtwegen van immuno-deficiënte mensen kan infecteren (Chowdhary et al., 2012). *S. commune* heeft een wereldwijde distributie en wordt al vele jaren als model organisme voor paddenstoelen gebruikt (Raper, 1966), en was een van de eerste basidiomyceten waarvan het volledige genoom is gepubliceerd (Ohm et al., 2010b).

Levenscyclus

De vruchtlichamen van *S. commune*, de paddenstoelen, produceren haploïde sporen die via de lucht verspreiden en wanneer zij op een geschikt substraat terechtkomen ontkiemen en een haploïde mycelium vormen (Fig S2b).

Het mycelium bestaat uit een netwerk van hyfen, lange cellen die door septa in compartimenten worden verdeeld. In de meeste basidiomyceten bevat elk compartiment een kern en een mycelium wordt monokaryon genoemd, maar in andere soorten zijn vele kernen per compartiment aanwezig (homokaryon genoemd). Een monokaryon kan vegetatief verder groeien. In tegenstelling tot de meeste dieren, kunnen schimmels zich op ieder deel van hun mycelium voortplanten, doordat er geen voorgeselecteerde kiembaan is (Buss, 1987). Hoe groter het mycelium, des te meer paddenstoelen en ook nakomelingen. Fitness is dus afhankelijk van de grootte van het mycelium (Pringle & Taylor, 2002). Voor een monokaryon paddenstoelen kan maken

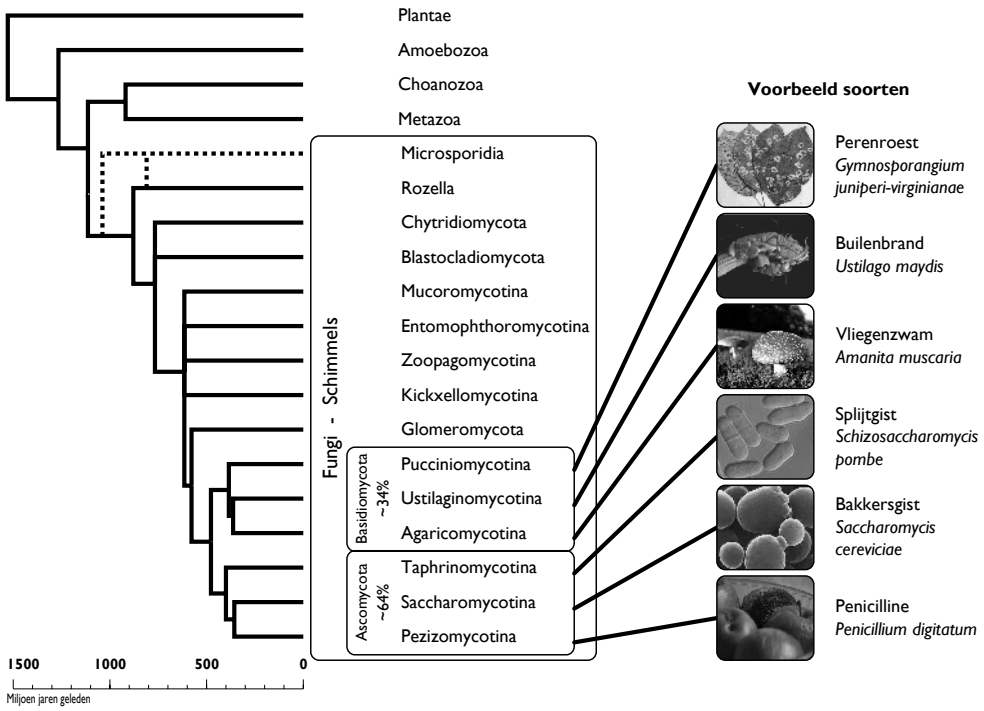


Figuur S2. Algemene schimmel levenscyclus en algemene levenscyclus van een paddenstoel-vormende schimmel. **a)** De meeste schimmels kunnen zich zowel sexueel als asexueel reproduceren, zowel in de haploïde en de diploïde levensfase. **b)** Paddenstoel-vormende schimmels kunnen zich over het algemeen alleen sexueel voortplanten. Asexuele reproductie is meestal beperkt tot groei van het mycelium.

moet het eerst bevrucht worden. In *S. commune* vindt bevruchting van het mycelium plaats wanneer twee mycelia elkaar ontmoeten. De hyfen fuseren, waarna kernen uit het ene mycelium in het andere mycelium migreren. Die kernen, die ook haploïd zijn, bewegen door het hele mycelium en laten kopieën achter zodat na verloop van tijd het hele mycelium bevolkt wordt door de nieuwe kern. De nieuwe inkomende en de reeds aanwezige kernen fuseren echter niet, maar blijven naast elkaar als haploïde kernen in de cel-compartimenten aanwezig. Een mycelium waarin twee verschillende kernen aanwezig zijn per cel heet een dikaryon, maar in sommige soorten zijn meerdere kopieën van de twee verschillende typen kernen aanwezig, wat een heterokaryon wordt genoemd (Raper, 1966). Onder de juiste omstandigheden produceert het mycelium speciale cellen voor seksuele reproductie, vaak gelocaliseerd op vruchtlichamen, de paddenstoelen. In deze cellen, basidia genaamd, fuseren de twee kernen waarna de diploïde kern direct in meiose gaat en vier haploïde kernen oplevert, die ieder een spore vormen. Een paddenstoel kan miljarden sporen produceren die ieder kunnen uitgroeien tot een nieuw monokaryon.

Conflicten en concurrentie tussen kernen in basidiomyceten

De twee kernen in een mycelium moeten samenwerken om zo goed mogelijk te functioneren op het niveau van het mycelium, maar tegelijkertijd zijn de kernen onafhankelijk van elkaar op het kernniveau. Terwijl er bij diploïde organismen in een individu tussen de twee genoom kopieën, die immers samen in één kern zitten, slechts competitie kan optreden tijdens de meiose (Haig, 2010) kan in een dikaryon een kern zijn fitness in het mycelium doen toenemen, ten koste van de andere kern of ten koste van het mycelium (Buss, 1987). Om dit te voorkomen, is in de basid-



Figuur S3. Overzicht van de verschillende groepen schimmels en hun verwantschap en voorbeelden uit de belangrijkste groepen. De meeste schimmels die tot nog toe beschreven zijn behoren tot de Ascomycota of de Basidiomycota. Tot de Ascomycota behoren onder andere industriele producenten als *Aspergillus* en *Penicillium*, economisch belangrijke gisten, maar ook plant- en dier-pathogenen. Tot de Basidiomycota behoren onder andere plant pathogene gisten en alle paddenstoelvormende schimmels (Stamboom gebaseerd op Stajich et al., 2009).

iomyceten een complex systeem van kerndeling geëvolueerd, dat de mogelijkheden voor kernen om 'selfish' gedrag te vertonen beperkt. Tijdens elke celdeling, delen de twee kernen synchroon, waarna twee verschillende kernen in de tip van de cel blijven, en de twee andere kernen achterblijven. Een aftakking met een kern erin wordt gevormd die direct terugbuigt en terugfuseert met de cel met daarin de andere kern (Iwasa et al., 1998). In soorten waar groei niet sterk is gereguleerd kunnen kernen wel *selfish* gedrag vertonen en in het mycelium toenemen waardoor ze ook meer nakomelingen kunnen vormen dan de andere kern (Fig 1.2a ; Ramsdale, 1999).

In *S. commune*, wordt de groei sterk gereguleerd en tijdens de vegetatieve groei is vals spelen dan ook niet mogelijk. Wanneer een dikaryon een monokaryon bevrucht, is er een moment van competitie tussen de kernen in het dikaryon (Fig 1.2b). Omdat maar een van de twee type kernen de bevruchting kan uitvoeren en het bevruchten van een monokaryon veel fitness voordeel kan opleveren, en omdat deze zogenaamde dikaryon-monokaryon (di-mon) paringen waarschijnlijk veel voorkomend zijn in de natuur (Anderson & Kohn, 2007; Pringle & Taylor, 2002; Stenlid et al., 2008) is sterke selectie om beter te zijn dan een andere kern in het uitvoeren van de bevruchting te verwachten.

Bevindingen

In dit proefschrift toon ik aan dat seksuele selectie ook bij schimmels voorkomt en beargumenteer ik dat het een selectiedruk is die niet moet worden vergeten bij schimmels, omdat het eigenschappen kan verklaren die anders moeilijk te begrijpen zijn. Verder heb ik onderzocht onder welke omstandigheden seksuele selectie een rol kan spelen bij de evolutie van schimmels, met name de paddenstoel vormende basidiomyceten.

'Mating types'

Veel van de mechanismen die plaatshebben tijdens de paring bij schimmels zijn gereguleerd door de mating types. Mating types zijn genetisch vastgelegde compatibiliteitskenmerken en alleen twee gameten die van een verschillend mating type zijn kunnen succesvol een zygote vormen. Mating types doen sterk denken aan seksen – alleen gameten van verschillende sekse kunnen immers fuseren – echter mating types zijn niet hetzelfde als seksen. Seksen worden bepaald door het formaat van de gameet, waarin mannetjes veel kleine en vrouwtjes grotere, maar minder gameten produceren. Er is dus altijd een asymmetrie tussen de gameten van verschillende seksen en tussen hun aantallen. Deze asymmetrie zorgt dat altijd een deel van de mannelijke gameten niet kan paren, omdat er maar een beperkt aantal vrouwelijke gameten zijn. Er is dus competitie tussen de gameten van de mannetjes om een vrouwelijke gameet te vinden. Mating types verschillen alleen van elkaar door het genetische kenmerk van de gameet die compatibiliteit bepaalt, maar verder kunnen de gameten volledig gelijk zijn. Tussen gameten van verschillende mating types is dus niet de intrinsieke asymmetrie aanwezig die wel bestaat tussen gameten van verschillende seksen. Verder zijn er maar twee seksen mogelijk, terwijl er meerdere type mating type kunnen bestaan, die allemaal met elkaar compatibel zijn.

Mating types zijn niet alleen belangrijk bij schimmels omdat ze de compatibiliteitsreactie regelen, maar ook omdat ze vele andere aspecten van paring, en ook van de groei als dikaryon en tijdens de reproductie reguleren. In het proefschrift beargumenteer ik dat, omdat mating types compatibiliteit tijdens paring regelen, maar ook betrokken zijn bij andere onderdelen van de bevruchting, ze een waarschijnlijke target zijn voor seksuele selectie.

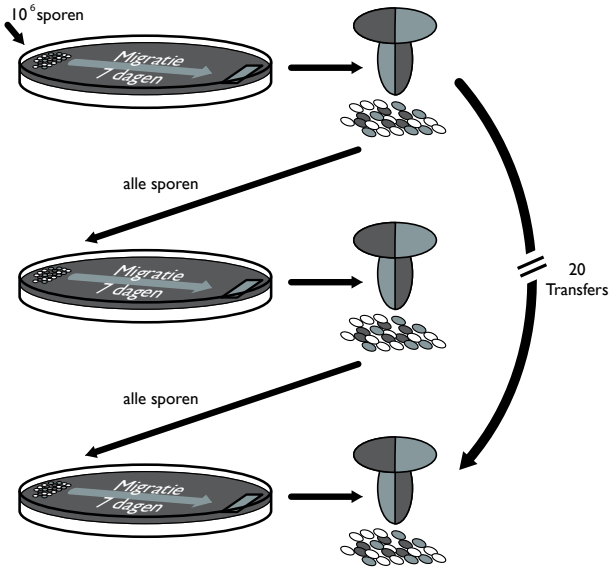
Empirisch onderzoek

Met behulp van het model organisme *S. commune*, heb ik laten zien dat seksuele selectie inderdaad een rol speelt bij schimmels. Ondanks dat veel bekend is over de mechanismen van bevruchting in *S. commune*, zijn er vele vragen over hoe paring in de natuur plaatsvind. Wij hebben voor drie verschillende zeer lokale populaties, ieder afkomstig van een enkele tak, bepaald hoeveel verschillende individuen aanwezig zijn, en welke kernen aanwezig zijn in ieder individu. Omdat een mycelium meerdere paddenstoelen kan maken is het moeilijke te bepalen hoeveel genetische individuen aanwezig zijn op één substraat; iedere paddenstoel kan een ander individu zijn, maar alle paddenstoelen kunnen ook genetisch identiek zijn en aan hetzelfde mycelium ontspruiten. Wij vonden dat altijd meerdere mycelia aanwezig zijn en dat op kernniveau tussen de myce-

lia kernen worden gedeeld. Dit is een belangrijke bevinding, want seksuele selectie kan alleen plaats vinden, als meerdere individuen aanwezig zijn die met elkaar concurreren of om tussen te kiezen. Door informatie van de kernen te combineren met die van de mitochondriën – die tijdens paring niet migreren en dus een kenmerk zijn van het monokaryon (zie Fig 3.1) – konden we reconstrueren hoe paring moest zijn gelopen.

Vervolgens hebben we getest of het succes van een kern in een dikaryon die strijdt met de andere kern in dat dikaryon om een monokaryon te bevruchten genetisch is bepaald, en of dit succes afhangt van het ontvangend monokaryon. Hiervoor construeerden we verschillende dikaryons die herhaaldelijk met monokaryons paringen uit voerden. Voor sommige combinaties van kernen in een dikaryon zagen we dat een van de twee consequent vaker een monokaryon bevruchtte dan de andere kern – een kern is sterker dan de andere, net als sommige herten mannetjes competitie voor vrouwtjes winnen. In andere dikaryons won soms de ene kern en soms de andere, afhankelijk van welk monokaryon bevrucht werd – het monokaryon heeft voorkeur voor een van de twee kernen, zoals in bijvoorbeeld stekelbaarsjes waarin het ene vrouwtje mannetje X en een ander vrouwtje mannetje Y verkiest. Zowel directe competitie tussen de kernen (male-male competitie) als ook indirecte selectie door het ontvangende mycelium (female choice) spelen een rol tijdens de bevruchting in paddenstoelvormende schimmels.

De bevinding dat sommige kernen altijd beter zijn dan andere tijdens de bevruchting is mogelijk als de sterkere kern altijd aantrekkelijker is en dus gekozen wordt door het ontvangende mycelium, of als de kern een andere kern kan onderdrukken tijdens de bevruchting. Een mogelijkheid is dat er een directe interactie in het dikaryon plaatsvindt, die tot onderdrukking leidt. Tijdens mitotische deling is onderdrukking waarschijnlijk, omdat zo wordt voorkomen dat een van de twee kernen zich tijdens groei vermeerdert. Als de kernen in een dikaryon fysiek



Figuur S4. Opzet van selectie experiment voor evolutie van kernen die succesvoller zijn in het bevruchten van een ontvangend mycelium. Sporen die op een monokaryon worden aangebracht zullen met dit mycelium versmelten en door het mycelium migreren en een dikaryon vormen. Een deel van het dikaryon wordt gestimuleerd paddenstoelen te vormen en de hierop geproduceerde sporen worden gebruikt om een nieuw monokaryon te inoculeren. Alle sporen worden overgezet, maar alleen die sporen waarvan de kernen succesvol zijn in migratie en sporulatie bereiken de volgende generatie. Eigenschappen die dit bevorderen en genetisch zijn vastgelegd worden op deze manier geselecteerd. Selectie is 20 transfers herhaald.

worden gescheiden, door middel van protoplastering of door microchirurgische ingrepen, blijkt bijna altijd dat een van de twee kernen de ingreep overleeft en kan regenereren als monokaryon. Waarschijnlijk doordat de andere kern mitose onderdrukt. Wij hebben getest of regeneratie en bevruchting gecorreleerd zijn, en of dit mechanisme verantwoordelijk kan zijn voor bevruchting. We vinden geen aanwijzingen dat overleving na dedikaryotisatie samenhangt met bevruchtingssucces.

Om aan te tonen dat competitie voor bevruchtingen – seksuele selectie dus – ook bij schimmels kan leiden tot nieuwe aanpassingen die de kans op bevruchtingen vergroten hebben we een evolutie experiment uitgevoerd. Sporen van *S. commune* zijn gebruikt als 'mannelijke' gameten die sequentieel een ontvangend 'vrouwelijk' mycelium konden bevruchten. Door consequent de meest succesvolle kernen te nemen en deze een volgende ronde van bevruchting uit te laten voeren werden mutaties die tot dit succes leidden geselecteerd (zie Fig S4). Doordat de oorspronkelijke lijnen die gebruikt waren aan het begin van het experiment (de 'ouder-lijnen') onveranderd in een -80°C vriezer in leven gehouden kunnen worden, kunnen de geëvolueerde en de ouder-lijnen direct met elkaar vergeleken worden. Na twintig keer overzetten (twintig seksuele generaties) waren vijf van de twintig geëvolueerde lijnen veranderd in hun competitieve vermogen ten opzichte van de ouder-lijn. Dit voordeel wordt voornamelijk bereikt, doordat de geëvolueerde kernen sneller in staat zijn om het ontvangend mycelium te bevruchten wat leidt tot kolonisatie van een grotere deel van het vrouwelijk mycelium en tot productie van meer sporen.

In de empirische hoofdstukken hebben we onderzoek gedaan aan seksuele selectie bij paddenstoel vormende schimmels en laten zien dat dit inderdaad voorkomt. Echter, ook in andere schimmel-groepen kan seksuele selectie een rol spelen. De meeste schimmels hebben een bevruchtingssysteem waarin mannelijke en vrouwelijke rollen zijn te onderscheiden, wat in veel gevallen leidt tot een scheve verhouding in de aantallen mannelijke en vrouwelijke gameten. Omdat er meer mannelijke gameten zijn die met elkaar in competitie zijn om de beperkte hoeveelheid vrouwelijke gameten te bevruchten zullen eigenschappen geselecteerd worden die de bevruchting bevorderen. In sommige schimmels zijn er daardoor speciale, kleinere mannelijke gameten ontstaan waarvan er meer geproduceerd kunnen worden, waardoor de kans op bevruchten vergroot. Ook als er geen scheve verhouding in compatibele gameten is kan seksuele selectie optreden. Als er veel verschil is in de kwaliteit van de gameten is het voordelig om onderscheid te maken in kwaliteit. In bijvoorbeeld gisten kunnen gameten juist erg kieskeurig zijn in met wie ze paren, waarbij feromooneiwitten gebruikt worden om de beste partner te kiezen, wat leidt tot selectie voor 'veel feromonen productie'. Ook hier spelen mating types weer een belangrijke rol, omdat de feromonen gereguleerd worden door de mating types genen. Tegelijkertijd bepalen de mating types ook compatibiliteit tussen gameten en zijn er dus twee niveaus waarop seksuele selectie en de mating types interactie hebben.

Wij voorspellen dat seksuele selectie bij schimmels vooral in die soorten is te verwachten waar seksuele reproductie een belangrijke rol in de levenscyclus heeft en waar een groot aantal gameten met elkaar in competitie zijn. Vooral als er veel genetisch verschillende individuen elkaar kunnen tegenkomen, is competitie te verwachten, omdat dan meer variatie voor kermerken aanwezig is. Verder is competitie vooral te verwachten als er verschillen in aantallen compatibele groepen gameten bestaan, wat het meest waarschijnlijk is als er mannelijke en vrouwelijke gameten worden gevormd of als in mannelijke en vrouwelijke rol wordt gepaard.

Conclusies

De bevindingen van mijn proefschrift tonen aan dat seksuele selectie niet alleen in dieren en planten plaatsvindt, maar ook in een ander belangrijke groep organismen: de schimmels. Door dit onderzoek kunnen eigenschappen die in schimmels waren gezien worden verklaard die voorheen moeilijk te verklaren waren. Door de voordelen van werken met schimmels kan experimenteel onderzoek naar fundamentele vragen aangaande seksuele selectie worden gedaan, wat met andere groepen organismen niet mogelijk is. Tevens toont dit onderzoek aan dat de kenmerken van seksuele reproductie die tot seksuele selectie leiden universeel toepasbaar zijn, ook in schimmels.

Dankwoord

In the end these words of thanks have almost become a complete chapter. But then again, how often does one have the chance to publicly thank all those people that helped with all the science stuff, or, and this might be even more important, created all the right circumstances that made it possible for me to finish this project.

Working these last five years in the laboratory of Genetics was a true treat. I can only hope that any group I will work at in the future will be filled with people as creative, friendly, enthusiastic, sweet and clever, as the people I've met in this lab in Wageningen during my thesis research. Being part of this group has made the project a wonderful experience in which the thin line between science, sociability and sheer fun often faded completely, whether it was at seminars or discussion groups, coffee- or lunch breaks, at *de Vlaam*, during breakfast or dinner parties, excursions or lab-onions. Thank you all for creating this great environment.

I want to start by thanking my supervisor and co-promotor Duur: thanks for everything these last 5 years. I remember well the moment you walked into the lab at the Botanical Center and unexpectedly told me I got the job, completely skipping the interview. From that moment on I was a PhD student and you my supervisor. Where we started off with great ideas on genomic conflicts as written in the grant proposal, we soon discovered that the conflicts of Schizophyllum were more inter-sexual than inter-genomic. I had to digress onto what was merely a sidetrack in the proposal, and wander into the unknown territories of sexual selection... in fungi. This great adventure I undertook with you was really gratifying. I've enjoyed your ability to reinterpret established facts and the wideness of your interests that you somehow know to combine. The trust you had in me and the enthusiasm for our experiments made this a successful expedition. Every paper climaxing in an e-mail 'ping-pong' without which none of the chapters in this booklet would ever have been finished. It was great to be part of 'Group Aanen' and have our weekly discussions with Anna, Tânia, Eric and all the students that came and went. I've really liked working together with you and hope we can work together some more in the future to get a few of those fantastic ideas tested.

I'm also very proud to be one of the last PhD students that can say they graduated with Rolf Hoekstra as a promotor. Due to your retirement we had less contact these last few years, but the contact was not less appreciated. Your calm, friendly and insightful comments regularly had me rethink my often (too) strong opinions.

Working in a perfectly run lab made experimenting so easy. Marijke and Bertha, thank you for always having everything working, in stock, ordered when needed, clean, organized, etc. even while moving to and within Radix. Also your technical help and experience is greatly appreciated. Thanks for all the qPCRs you did for me Bertha, and the help with getting any technique running. Marijke, you learned me all the ins and outs of working in a fungus lab when I studied ALEX (I hope ALEX 2.0 will be a great success!), which you continued to do during my PhD work. Thank you for helping me get everything working for yet another (smelly) species in the lab and for all your solutions to so many practical problems. The two of you always create a very pleasant environment for all of us and for the many students to work in freely.

Fons, your creativity, optimism and enthusiasm are endless. It's so much fun discussing ideas and results with you, as it always ends in crazy but great new ideas, often accompanied by simple experiments to test them. I really appreciate our conversations on many of you visits to the lab. Arjan, my life at the Genetics lab actually started when I first met you at ESEB in Krakaw, all those years ago, and ever since I've had a great connection with you. Talking science, but also about personal life, scientific careers, and of course the importance of an academic climate. The latter leading to the initiation of the WEES seminar series.

Sil you were my best (and only) MSc student that I had the privilege of supervising during my studies. It was great working with you. Even though the results we obtained from your hard labor were rather inexplicable – the biological reality is apparently different from what we can explain with our current knowledge – and the techniques regularly failed us, you managed to finish the project and eventually we produced a nice manuscript from it. I hope your PhD work at NIOZ will go smoother. Let me know when you have your next gig, I'd love to see you play again. Manuela, it was good fortune to meet you and Sandra in Edinburgh. You've shown me that proper science discussions via e-mail are indeed possible, which even can lead to a (somewhat forced) co-authorship. I expect that this Mexican *muchacha* will try to make Paris as entertaining for me (given I get there), as Wageningen has always been for you. And do know that at any conference you can hang your poster next to mine. Wytske, thank you so much for taking such good care of everything and more importantly everybody. You are always very helpful with all that has to be arranged, amongst others the WEES finances and website. I had great fun meeting in secret with you and Erik, organizing the many activities as the 'social committee', culminating in a visit of Sinterklaas! Aafke, thank you for taking care and arranging everything when I started the project.

Also thanks to Han, Luis, Jim, Anton and Johan, for helping me with practical advice and techniques to get things started with the Schizo work.

This booklet is the result of 5 years research, but also of 5 years working in the same group. Many moments of lab work, analysis, presentations and discussions, but also of lots of fun with great colleagues and friends. People to celebrate the high, ease the low, and entertain the medium moments.

First of all there are the PhD students in the evolution group with whom I shared my room (or room-like confined open area space) and my opinions (most of the time unasked – sorry for that). Stefan, you were almost finished when I started, I enjoyed our Friday beers and discussions on music. You're a dad of two now! Anna, it was very nice we overlapped so much time during our PhD. Eric, thanks for so many things: help me move twice, go ice-skating, share your house, the regular food and beer nights, and your relativizing comments. I enjoyed discussing the selfish nuclei stuff that is in both our research topics – it's about time we write that paper on it together with Duur. Good luck with the final year.

And then the new group, Alex, Tina, Florien, Jelle and Olga, thanks for all the good and random fun moments, and good luck with the research! Florien, take good care of WEES, with your organizational talent and experience, that will not be a problem. Of course also thanks to the other people in the cubicle: Zeshan, Ana-Carolina, Ya-fen and (local candy and SNP distributor) Frank.

Also the postdocs should not be forgotten: Anne, Tânia, Merijn, Siemen, Martijn and Bart. Anne, with your current position we're bound to meet at mycological meetings. Tânia, it was great to have all those meetings together. Good luck in Portugal! Siemen, thanks for always being so interested in everything and organizing all those borrel-sessions. Martijn, your deadpan humor and your calculations of the number of days to my final deadline have been greatly appreciate. Bart, thanks for your help with the grant proposals. Klaas, you also many thanks, especially your insights in education and university politics taught me much. Claudius van de Vijver, thanks for your help in getting me back on track when that was most needed.

The last three years, I was part of an enthusiastic group of people that organized the Wageningen Evolution and Ecology Seminar series. Every month we invited some outstanding scientists to Wageningen. I really loved the crazy e-mail conversations and great discussions in the pub. Thanks a lot everybody, for making it a great success.

During my thesis two lab-changing events occurred which did not so much change the way how science was performed, but which did affect the social architecture of life at the lab. One event was the move to Radix. From sharing a large building with two other groups we now shared a building with many groups, resulting in less space and more noise, but also much more interaction with the other parts of Genetics and with the Plant Physiologists. Thanks PPH, for all the shared lunches, cakes, bowling nights and drinks. On top of that Bas Zwaan started as a professor, pushing the cyto-, plant and evolutionary genetics groups to more unity. All in all a lot of reasons to get to know many people, broaden knowledge of scientific topics, share practical information and strongly increased chances for birthday pie. Bas, thank you for integrating the genetics-es, as it helped me realize the necessity of the genetic level in evolution, and because it

led to exchange of knowledge that I didn't know was so close at hand. And for your comments, questions, paper review, and introducting the Oxford comma to me.

And then I moved to Wageningen, halfway my phd. There was the shock of actually living in a village again. (Ooh, sorry, 'city'... and please excuse me if once or twice I was a bit too negative about this lovely city, but you have to admit, it is rather small, don't you think?) Luckily there were many great things about Wageningen too, especially great people that I was lucky enough to meet.

First of all, by chance, I ended up in a lovely house downtown. Thank you Marieke, Rio, Rik, Ebel, Alex, Linda and all the other people that lived for some time at N9. The warmth I received (and still when I go there) made my stay in the cold house very enjoyable. Then there was a volleyball team that is great on the field, and at least as good after the game. Thanks *Tercera mano*, I really enjoyed our games and chats over beers.

Most importantly I had many colleagues that also became good friends. Many of the people running around WU-campus showed up regularly at bars, dances, random parties, pub-quizzes, barbeques, canoe trips, intense climbing sessions, Rhine visits, etc. enzovoorts. Alex, Anna U., Anna V., Bart vS., Benjamin, Catarina, Charlito, Cezary, Eric, Filipe, Florien, Jennifer, Manickam, Merijn, Natalia, Neli, Niall, Parisa, Tina, Veronica, and especially Julio, Erik and Pádraic (who somehow seem to be present in almost all memories from the last few years – from proposition pubs, late night discussions, to abductions from my flat) thank you very much for keeping me company, if I was not off to Belgium of course. Great to have so many awesome people around to make life very pleasant.

Merijn, you were my buddy throughout my entire stay in Wageningen, a constant factor from when I started my MSc project to the final submission of this thesis. We played squash and volleyball, shared many a talk in de Vlaam and de Zaaier, discussing love, life, music and the future, and somehow ended up living in the same tower block. Thanks for all of this and that on the day that I formally close the chapter on Wageningen you're here as my paranymph.

Moving to Wageningen meant leaving behind Amsterdam and the people that go with it. Luckily Wageningen was not too far away which meant regular visits back and forth. Allert, Alex, Anne, Eric, Femke, Jessica, Jurgen, Laurens, Marieke, Nellie, Nick, Peter, Roos, Rosanne and Vicencio. Thank you Popjes, for being such a great group of friends. Whenever, wherever, whatever, you're always there when needed. Nellie thank you for all the support those first years. Nickie, even though you were not in Amsterdam enough, you somehow were always present anyways. Thanks for being here now as my paranymph. And thanks of course also the other people in Amsterdam, especially Annemieke, Mirek, Paulien, Steven, and *Extra stout*.

Kathryn, after my stay in Edinburgh, you stayed an amazing friend. Thanks for all those long conversations and the invites to Wales. I hope none of your made-up words you made me believe were genuine English ended up in this thesis. Ellen, great friend on this or the other side

of this globe, great that we could also share the PhD experience. The too infrequent but still regular visits that you and Phil made were always a joyful happening and it was wonderful to visit you in New Zealand!

And then there is my family. Papa, you have always encouraged our curiosity, watching lake water under the microscope, building radios, answering any question we could come up with. And these last few years, asking me questions on anything you could come up with, trying to completely understand any little aspect of what my work was about. I cherish our discussions on science till late in the night, that most often were actually about life. Thank you for your support, love, listening and especially asking. Mama, thank you for all the support and encouragement you always gave us, your caring nature, but also the push to go and 'figure it out yourself'. Especially when times are rough, your '*alles komt goed*' is terrifically missed. My little sister Marieke, a full grown doctor now! I enjoyed our regular dinners out, somewhere halfway, and the warm talks that went with it. Thank you for your unconditional support. Thanks also for introducing Ana who made the marvelous cover of this booklet. Ingrid, Tim and Ybo, my family in the States. I'm always amazed by how you can make the combination of two post-docs with a full and wonderful family life seem so easy. We'll soon come by soon and observe first-hand how you do that. Thank you for everything.

Finally, Catarina. Only three months after we got together you decided to move to Ghent, what meant many weekends back and forth to Belgium again. And finally, when the lab-work was done, I could spend the last few months of writing everything up here in Belgium, at home with you. Thank you for your support, the long nights writing on our theses together, for the love, joy, fun, and friendship. Obrigado por tudo! Now that my thesis is finished and yours is also getting there, it's time to explore what adventures lie ahead of us.

Bart

Curriculum vitae

Bart Pieter Sjef Nieuwenhuis, was born on 19 May 1980 in Heemskerk, the Netherlands. After obtaining his highschool degree from 'Sint-Oelbert Gymnasium' Oosterhout, the Netherlands he moved to Amsterdam to start his studies at the University of Amsterdam. In his first year he enrolled in the *Beta-Gamma Propedeuse* program, an interdisciplinary propaedeutic variant focusing on the relationships between sciences after which he continued studying Biology at the UvA. During his studies he occupied a number of possitions at the *SVBG* study association and was chairman of the student faculty board.

In his first MSc research projects at the University of Amsterdam he experimentally investigated introgression of pathogen resistance cultivated lettuce into its wild relative prickly lettucs (*Lactuca serriola*). For his second project, he went to Edinburgh, Scotland, where he modelled how habitat preference and local adaptation can lead to speciation, followed by a literature study on the application of Adaptive Dynamics. His final MSc research was performed at Wageningen University, where he used experimental evolution to investigate adaptation of a filamentous fungus. After his graduation in 2006, he shortly worked as a research assistant at Wageningen University.

In September 2007, Bart started working as a PhD student at the Laboratory of Genetics, at Wageningen University under supervision of Dr. Duur Aanen of which the results are described in this thesis. The research of the thesis focused on the evolution of sexual strategies in basidiomycete fungi. During this time he initiated and coordinated the Wageningen Evolution and Ecology Seminar series (WEES). Currently he is searching for a postdoc position to continue his work on the effects of sex and sexual selection on evolution and adaptation.

Publications

Nieuwenhuis, B.P.S. & D.K. Aanen. 2012 Sexual selection in fungi. *Accepted by J. Evol. Biol.*

Nieuwenhuis, B.P.S., A.J.M. Debets & D.K. Aanen. 2011 Sexual selection in mushroom-forming basidiomycetes. *Proc. R. Soc. B.* **278**, 152-157.

Hooftman D.A.P., B.P.S. Nieuwehuis, K.I. Posthuma, J.G.B. Oostermeijer & J.C.M. den Nijs. 2007 Introgression potential of downy mildew resistance from lettuce to *Lactuca serriola* and its relevance to plant fitness. *Basic Appl. Ecol.* **8**, 135-146.

Nieuwenhuis, B.P.S., S. Nieuwhof & D.K. Aanen. On the asymmetry of mating in the mushroom fungus *Schizophyllum commune. Submitted to Fungal Genet. Biol.*

Nieuwenhuis, B.P.S., A.J.M. Debets & D.K. Aanen. Fungal fidelity: Nuclear divorce from a dikaryon by mating or monokaryon regeneration. *Submitted to Fung. Biol.*

Nieuwenhuis, B.P.S. & D.K. Aanen. Fungal nuclear arms race: experimental evolution for increased masculinity in a mushroom. *In prep.*

Nieuwenhuis, B.P.S., M. López-Villavicencio & D.K. Aanen. Why mating types are not sexes. *In prep.*

Nieuwenhuis, B.P.S., M.E. Hood, S. Vuilleumier, M. López-Villavicencio, S. Billard, E. Petit & T. Giraud. Evolutionary advantages and drawbacks of uni- versus bi-factoriality in mating type determinism. *In prep.*

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational 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 of literature (6 ECTS)

- Sexual selection in fungi (2010)

Post-graduate courses (3.6 ECTS)

- Summer school 'the evolution of sex chromosomes'; Munich Graduate School for Evolution, Ecology and Systematics (2008)
- Introduction to *R* for statistical analysis; PE&RC and WIMEK/SENSE (2011)

Invited review of (unpublished) journal manuscript (1 ECTS)

- Israel Science Foundation: local adaption in ascomycetous fungi (2010)

Competence strengthening / skills courses (2.8 ECTS)

- Workshop presentation skills; CENTA/WGS (2008)
- Scientific writing; CENTA/WGS (2009)
- PhD Competence assessment; WGS (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.9 ECTS)

- PE&RC Day: 'expect the unexpected' (2008)
- PE&RC Day: 'origin of communication' (2009)
- PE&RC Day: 'innovation for sustainability' (2011)
- Organisation and attendance of the Wageningen Evolution and Ecology Seminars (WEES) (2009-2012)

Discussion groups / local seminars / other scientific meetings (6 ECTS)

- 'Experimental evolution' discussion group (2007-2012)
- 'Biologist's guide to modelling' discussion group (2008-2009)

International symposia, workshops and conferences (18.9 ECTS)

- 11th Congress of the European Society for Evolutionary Biology (ESEB); Uppsala, Sweden (2007)
- Workshop Plant and Animal Sexuality; Neuhausen auf den Fildern, Germany (2008)
- 12th Congress of the European Society for Evolutionary Biology (ESEB); Turino, Italy (2009)
- 15th European Meeting for PhD Students in evolutionary Biology (EMPSEB); Schoorl, the Netherlands (2009)
- 'Friends or Fiends? Consequences of social interactions for artificial breeding programs and evolution in natural populations' (2009)
- 9th International Mycological Congress; Edinburgh, UK (2010)
- 13th Congress of the European Society for Evolutionary Biology (ESEB); Tubingen, Germany (2011)
- NVvM Section Mycology yearly meeting; Utrecht, the Netherlands (2011)
- Netherlands Annual Ecology Meeting; Lunteren, the Netherlands (2012)

Lecturing / supervision of practical's / tutorials (13.5 ECTS)

- Molecular evolutionary ecology; 15 days (2008)
- Molecular evolutionary ecology; 15 days (2009)
- Molecular evolutionary ecology; 15 days (2010)

Supervision of 1 MSc students; 30 days

- The distribution of nuclei in a natural population of the basidimycete *Schizophyllum commune*

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Linocut of *Schizophyllum commune* mushrooms and basidia.

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