

## REVIEW

**Sexual selection in fungi**

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**Abstract**

The significance of sexual selection, the component of natural selection associated with variation in mating success, is well established for the evolution of animals and plants, but not for the evolution of fungi. Even though fungi do not have separate sexes, most filamentous fungi mate in a hermaphroditic fashion, with distinct sex roles, that is, investment in large gametes (female role) and fertilization by other small gametes (male role). Fungi compete to fertilize, analogous to 'male-male' competition, whereas they can be selective when being fertilized, analogous to female choice. Mating types, which determine genetic compatibility among fungal gametes, are important for sexual selection in two respects. First, genes at the mating-type loci regulate different aspects of mating and thus can be subject to sexual selection. Second, for sexual selection, not only the two sexes (or sex roles) but also the mating types can form the classes, the members of which compete for access to members of the other class. This is significant if mating-type gene products are costly, thus signalling genetic quality according to Zahavi's handicap principle. We propose that sexual selection explains various fungal characteristics such as the observed high redundancy of pheromones at the *B* mating-type locus of Agaricomycotina, the occurrence of multiple types of spores in Ascomycotina or the strong pheromone signalling in yeasts. Furthermore, we argue that fungi are good model systems to experimentally study fundamental aspects of sexual selection, due to their fast generation times and high diversity of life cycles and mating systems.

**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 (see Glossary), the component of natural selection associated with variation in mating success (Andersson, 1994, pp. 3), from other components of natural selection (Darwin, 1859, 1871). We argue that also in species as inconspicuous as fungi, sexual selection can act.

The aims of this study are two-fold. 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 study will pose several hypotheses to encourage further research on sexual selection in fungi. The second aim of this study 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].

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### 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 & Kokko (2010): 'sexual selection [is selection favouring] 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 and/or variation in their quality. 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 in a specific *sex role* can occur (Charnov, 1979; Delph & Ashman,

2006; Leonard, 2006). 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. Although no fungi with separate sexes are known, most mate as hermaphrodites, either by producing male and female gametes, or by donation and acceptance of nuclei upon fusion of mycelia (Nauta & Hoekstra, 1992; Alexopoulos *et al.*, 1996; Leslie & Klein, 1996; Bruggeman *et al.*, 2003; Nieuwenhuis *et al.*, 2011). The main exceptions are yeast species or species that mate in the yeast phase of their life cycle (see below 'Male and Female Roles in Fungal Mating') or species in which fusing hyphae equally contribute to the zygote (e.g. the group of the Zygomycota; Alexopoulos *et al.*, 1996).

### Sexual selection and mating types

Mating types regulate sexual compatibility of the gametes (or haploid structures that function like gametes), and gametes of the same mating type cannot form zygotes (Billiard *et al.*, 2011 and see Box 1). Species with mating types can still have different sex roles and mate in both male and female roles, but mating types are not linked to either role (i.e. male or female). Zygote formation is thus possible only between gametes of different sexes (or structures acting as gametes mating in different sex roles) that are also of different mating types. In homothallic fungi, which do not have mating types (see Box 1), different sex roles can still be present.

#### Box 1: Mating types

Mating types regulate fusion (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*), whereas in other species individuals produce gametes that are only compatible with gametes of 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 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 signalling (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

**Box 1** (Continued)

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 at 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.

In many species of basidiomycetes, more than two mating-type alleles are present (Kües *et al.*, 2011). Due to negative frequency-dependent selection, all alleles in a well-mixed population are expected to have 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 higher frequency than  $1/n$ . 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 Jennions & Kokko (2010) as given in the main text. According to the general definition, sexual selection occurs among the members *within* one class (i.e. within one sex or one mating type), but in this special form of sexual selection, it occurs *between* classes at the level of the whole population. An example of this specific case occurs in basidiomycetes as discussed in 'Sexual Selection in Mushroom Fungi' in the main text.

Mating types are of essential importance for sexual selection. First of all, mating types in fungi not only define compatibility between gametes, but genes at the mating-type locus or loci also regulate many aspects of the actual mating process (reviewed in Heitman *et al.*, 2007). As some of these aspects differ between the female and male role during mating, sexual selection is likely to affect the mating types. For instance, in different heterothallic ascomycetes, male gametes produce pheromones (Turina *et al.*, 2003; 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. In some yeast species, cytoplasmic contribution is regulated by the mating type (e.g. Yan & Xu, 2003). In the ascomycete *Neurospora crassa*, mating types regulate the development and abundance of the female fruiting bodies (Debuchy *et al.*, 2010). Throughout the text, the function of mating types in a sex-specific role during mating will be mentioned when known.

Second, even though 'sexual' in 'sexual selection' originally referred to competition between individuals of one sex for mating with individuals of the other sex (Darwin, 1859; Jennions & Kokko, 2010), competition for mates can also occur between individuals of classes 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 systematically is 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 1, this type of competition is unlikely to play an important role. However, even without a skew in the numbers of mating types, there is still potential for sexual selection if gametes differ in quality. When quality of the gamete affects zygote viability or offspring fitness, sexual selection by mate choice can favour traits that signal the gamete's quality relative to gametes of the same mating type. In Box 1, we also describe the special situation when more than two mating types are present, which can lead to selection between the mating types.

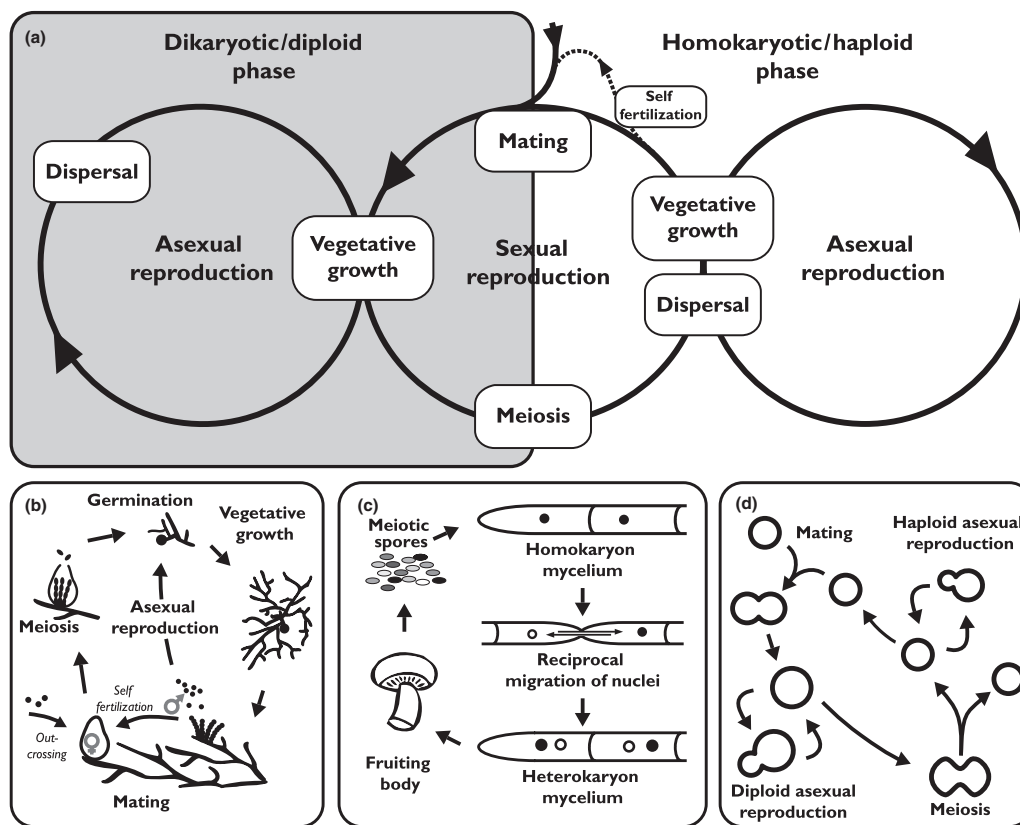
The various functions that mating types have or regulate on the one hand, and the possibilities for sexual selection between gametes of one mating type for access to gametes of the other mating type *per se*, on the other hand, might lead to confusion. To avoid confusion when discussing the specific examples of sexual selection, we will indicate whether selection occurs within sexes (or sex roles) 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, that is, 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. 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, which is expected to be strongest when the OSR is skewed, or when access to high-quality mates is limited. Third, there must be heri-

table variation in traits influencing the competitive ability to access mating partners. 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 does not occur between members of one sex or between individuals in one sex role, but between gametes of one mating type, these prerequisites need to be met.



**Fig. 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 or 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 in 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*

## Male and female roles in fungal mating

As explained above, sexual selection is most likely 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. 1d). In contrast to yeasts, for most sexually reproducing filamentous fungi, different sex roles can be distinguished (Alexopoulos *et al.*, 1996). Sexually reproducing filamentous ascomycetes either produce anisogamous gametes (see Fig. 1b) or mate by fusion of hyphae. In species with distinct gametes, usually many small motile, and fewer larger gametes are produced (Nauta & Hoekstra, 1992), which results in a skewed OSR. Also, if mating occurs by fusion of hyphae, there is a limited number of female gametes available, due to the high investment per zygote by the female (Bruggeman *et al.*, 2003). Fertilization by fusion also occurs in a different group of filamentous fungi, the mushroom-forming basidiomycetes. In these fungi, fusion between different mycelia is followed by donation and/or acceptance of nuclei, whereas the two cytoplasms remain separate (see Fig. 1c; Aanen *et al.*, 2004). 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. Although the donation of nuclei is essentially cost free and can occur repeatedly, the acceptance of a nucleus by a mycelium implies a permanent sharing of this mycelium with a different nucleus, as acceptance 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 mushroom fungi'). As mycelia usually cannot monopolize mating partners, sexual selection usually occurs through gamete competition.

## Finding a mate and pheromone signalling

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 to be 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, both depend on the density (Levitan, 2004; Kokko & Rankin, 2006) and the OSR

(Clutton-Brock, 2009; Rosvall, 2011). For instance, when male 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). In contrast, 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). Female gametes can 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 (Evans *et al.*, 2012). 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*, which 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 that there may be selectable genetic variation for this trait on which sexual selection can act. Because all studies were performed in laboratory set-ups, the actual function of these pheromones in nature is not known. To gain more insight into the occurrence of sexual selection in aquatic species, research is needed on natural gamete density, motility and pheromone production and function.

Pheromones are also involved in attraction in ascomycetes and basidiomycetes (Kothe, 2008), in which growth occurs towards the highest concentration (e.g. Urban *et al.*, 1996; Kim & Borkovich, 2004). Communication in these groups most likely occurs over very small distances. To our knowledge, for these groups, no studies have been performed on variation in pheromone production and its effect on mating success, except for the yeast examples described in the next paragraph.

## Pheromones for quality assessment

Pheromone production can also function in quality assessment of gametes. If pheromone production 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*, mating-type pheromones are used during courtship to distinguish between mates of the opposite mating type, and a preference for higher pheromone production, including rejection of low-level signalers, has been experimentally established (Jackson & Hartwell, 1990a,b). 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 select for preference if choice only takes place within a meiotic tetrad, and 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 occur in some basidiomycetes that have a high frequency of intratetrad mating, such as *Microbotrium violacea* (Hood, 2002; Giraud *et al.*, 2008).

Rogers & 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 (Rogers & Greig, 2009), even though pheromone production is costly for the gamete during asexual growth (Smith & Greig, 2010). Runaway selection occurred, but in contrast to the *Allomyces* example described earlier, here selection occurred at high mate densities. Such selection requires strong competition between gametes of the same mating type, analogous to a biased OSR. In nature, a skew between the mating types is not expected, due to preferential switching of mating types during asexual growth (Haber, 1998, 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 occur, 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

Most mushroom-forming fungi (Agaricomycotina, a subdivision of the Basidiomycota) have obligate sexual reproduction (Raper, 1966) and a male-biased operational sex ratio (Anderson & Kohn, 2007; Nieuwenhuis *et al.*, 2011), providing conditions for sexual selection. Pheromones may be used as a criterion for selection.

### Generalized life cycle of mushroom-forming basidiomycetes

In this group of fungi, the generalized life cycle (see Fig. 1c) begins with a haploid sexual spore that germinates to form a homokaryotic mycelium. 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 receiving nuclei from other individuals. Generally, fertilization occurs in a special way: the mycelium takes up compatible nuclei (based on mating type) and becomes a heterokaryon with two nuclear types, which remain separate and do not fuse immediately. Based on their relative contribution of resources, male and female roles can be attributed to the mating mycelia. The receiving mycelium acts as one large female gamete, whereas 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 of incorporating another nuclear type nor replacing one of its nuclear 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, that is, function as a male (fig 2b; Buller, 1930), an event expected to occur frequently in nature (Raper, 1966; Williams *et al.*, 1981; Nieuwenhuis *et al.*, in prep.). Also, spores can function as male gametes by donating their nucleus to a homokaryon (Williams & Todd, 1984; James & Vilgalys, 2001; Nieuwenhuis *et al.*, in prep.). 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 nuclear types of the heterokaryon (func-

tioning 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. 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 these 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; Kempaers, 2007) – or directional in which case exaggerated traits might evolve.

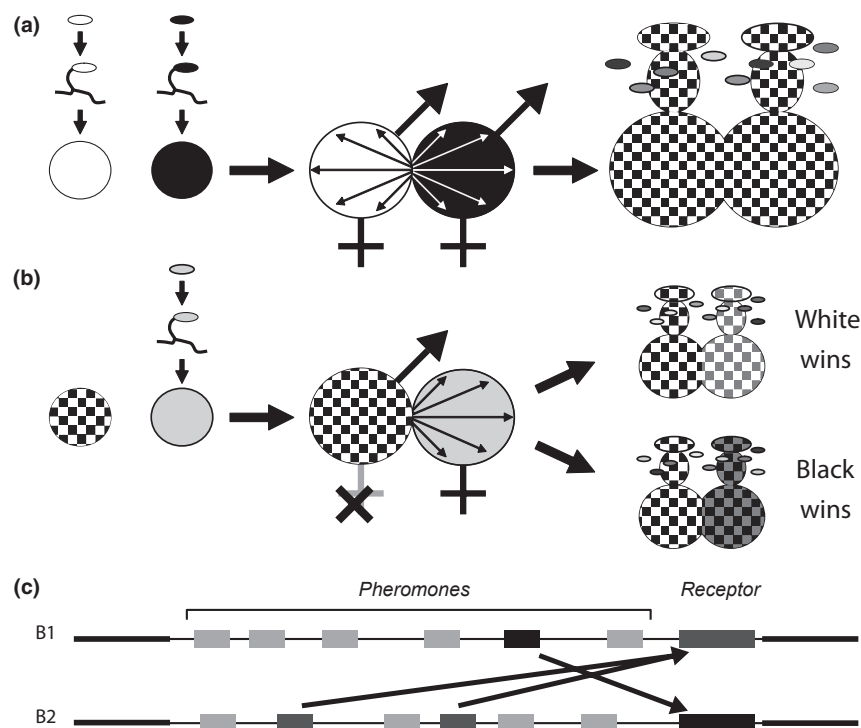
Most mushroom-forming basidiomycetes have a unique mating-type determination by two unlinked mating-type loci, the *A* and *B* locus (in some species, they are named differently), which both are multi-allelic in many species (Raper, 1966; Casselton & Kües, 2007). The *B* locus consists of two or three closely linked sub-loci, each coding for large families of pheromones and receptors. Each sublocus typically codes for one receptor and several pheromones (Fig. 2c). Whereas 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. 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 favour a *B* allele with additional copies of pheromone genes. Without sexual selection, all alleles are expected at equal frequencies (May *et al.*, 1999; Richman, 2000), but sexual selection can increase the frequency of an allele to a value higher than expected from negative frequency-dependent selection. It is unclear what the effect of sexual selection is on the number of alleles in the population. Sexual selection might either increase mating-type numbers due to selection for new alleles with more or more diverse pheromones, or reduce their numbers due to increased frequencies of attractive alleles, inevitably leading to loss of less attractive alleles. According to this novel hypothesis, selection for additional incorporated pheromones explains the extraordinarily 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.*, 2010), 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, disfavoured 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 led to higher chance of mating, but selection could also favour increased direct competitiveness between the gametes. In fungi, it is difficult to imagine



**Fig. 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 nuclear types (indicated in black and white) fertilizes a homokaryon (grey), only one of the two types can perform the fertilization. The end result of the heterokaryon-homokaryon mating is either a black-and-grey or white-and-grey heterokaryon. (c) Typical organization of the *B* locus, schematically illustrated for two hypothetical alleles (allele B1 and B2) 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 activate the receptor of *any* other allele. Nucleus B1 produces a pheromone that 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 sufficient to initiate migration and successful mating. In many species, two or three such loci are present at the *B* mating-type locus, each with pheromones and a receptor. Pheromones and receptors of different subloci do not interact.

fighting between individuals such as that 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 earlier, increased migration speed in the receiving mycelium or suppression of mitotic division of the competing nucleus, which have been observed in *S. commune* (Ellingboe, 1964; Raper, 1985), could lead to higher mating success. 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. 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 are produced in female sexual structures that supply the zygote with cytoplasm. Upon fertilization, these gametes remain connected to the mycelium, which acts as a 'nursing' tissue (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, and fewer of them are produced than male gametes (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 favour an increase in gamete number and gamete motility to increase the probability to fertilize the relatively rare female gametes and both factors 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, we do not know much about 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; Nieuwenhuis *et al.*, in prep.). However, in some fungi, mycelia reach extreme sizes (e.g. Smith *et al.*, 1994), resulting in fewer 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 into 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). At least one type of sexual conflict is likely to occur in fungi. Multiple mating can select for adaptations in the male role even if these decrease the total number of offspring of an individual acting in the female role (Rice, 1996). For example, on an ascomycetous homokaryotic mycelium, multiple female fruiting organs are present, which each can be fertilized by different male gametes (see Fig. 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 her 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 her best offspring (Stearns, 1987). 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 parent regulates these traits and benefits from differential investment, and how this could be regulated, it remains to be shown if these traits result from sexual conflict.

Also in basidiomycetes (see above and Fig. 1c), multiple mating can occur. For example, multiple homokaryons can interact simultaneously, a heterokaryon can fertilize a homokaryon (Fig. 2b), and multiple compatible spores can land on a single homokaryon. Multiple mating can have a direct fitness cost for the mycelium: it will lead to a mosaic of heterokaryons, which, by interacting antagonistically, reduces 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 nuclear type (Nieuwenhuis *et al.*, 2011) or to increase the migration speed of the fertilizing nuclei to assure homogeneous fertilization of the entire mycelium. Sexual conflict may also be a consequence of sexual selection for increased numbers of pheromones due to the male-biased OSR in mushroom-forming basidiomycetes; the increased production of costly pheromones may trade off with other components of dikaryon or monokaryon 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 regions of non-recombination for the two mating types are ~430 and ~500 kb (Bakkeren & Kronstad, 1994), and in *Microbotrium violacea*, the regions around the two different mating-type loci even span 2.8 and 3.5 Mb and the chromosomes that harbour 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). These are not only sex-determining genes, but also genes involved in sexual selection and genes with sexually antagonistic effects.

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). Mating-type-regulated cytoplasmic inheritance is of special 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 into the analogies of these non-recombining regions and sex chromosomes. Research should focus on traits that are of importance during mating and on the genes in 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.

### 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 of fungi 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.

Different basic elements 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. For example, the direct and indirect benefits of mating with particular genotypes, the costs of traits, the strength of display traits or the initial choosiness for a mate can be varied. Also, the effects of density or OSR (e.g. Rogers & Greig, 2009) can be tested. This line of research can also give experimental insight into the role of sexual selection for 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 determine 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 into 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 (Nieuwenhuis and Aanen, in prep).

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 argued that sexual selection is an important driver of fungal evolution. However, 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-

**Box 2: When is sexual selection expected?**

To obtain better insights into 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 life cycle**

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 thus 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).

**When many individuals meet**

The opportunities for sexual selection increase when multiple individuals or their gametes come in contact. Especially with high densities of individuals, when gametes can migrate easily over longer distances, or when mycelia are long-lived and thus big, this is likely. 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).

**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 ratio 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, minority, mating type.

**When there is variation for selectable traits**

Gametes can differ greatly in genetic make-up 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 *et al.*, 2012). 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), we expect choice to evolve.

type frequencies resulting in exclusion of part of the population when sex is required (e.g. 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, indicating 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 2, we give some predictions where sexual selection can be expected in fungi.

Sexual selection theory can provide important insights into 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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- Heterokaryon:** A mycelium with two (or very rarely more than two) genetically different types of nuclei in the same cytoplasm. Each cell or cell compartment contains two or more nuclei that are of different types. In some basidiomycete species, a strict coupling of nuclear division with cell division guarantees precisely two different nuclei per cell, in which case the mycelium is also called dikaryon.
- Heterothallic:** A reproductive system in which sexual reproduction will only be possible between gametes carrying different alleles at the mating-type locus, preventing selfing at the haploid level [intra-haploid mating see ref (Giraud *et al.*, 2008)].
- Homokaryon:** A mycelium with one type of haploid nuclei. In basidiomycetes, each cell often has a single nucleus and the homokaryon is then called monokaryon. In other basidiomycetes and most other fungi, each cell or cell compartment contains more than one nucleus, but all of the same type.
- Homothallic:** A reproductive system in which individuals produce universally compatible gametes. This implies that gametes produced by one haploid individual will be able to fuse.
- Hypha:** A tubular filamentous structure that branches to form a mycelium.
- Idiomorph:** Alternative versions of the mating type that occupy the same locus but that code for genes of nonhomologous origin. Term specifically used in ascomycetes. In other organisms with mating types (e.g. basidiomycetes or algae), the different versions are referred to as ‘alleles’.
- Male gametes:** Those gametes that hardly contribute resources; these gametes are said to fertilize the gamete that contributes most resources.
- Mating type:** A genetically defined sexual compatibility trait. In heterothallic fungi, mating can only take place between gametes with different mating types.
- Mycelium:** A network of undifferentiated hyphae that constitutes the soma of the fungus. In general, all parts of a mycelium can produce asexual and sexual reproductive structures.
- Operational sex ratio (OSR):** The ratio of male vs. female individuals that are available for mating at any given time.
- Sexes:** The property or quality by which organisms are classified as female or male on the basis of their relative size in gametes. Males produce small and females large gametes.
- Sexual selection:** Selection favouring investment in traits that improve the likelihood of fertilization given limited access to compatible gametes due to competition with gametes of the same sex (see also paragraph ‘Defining sexual selection’).

## Glossary

**Female gametes:** Those gametes that contribute most resources to the zygote; these gametes are said to become fertilized by the less contributing gamete.

**Gonochorist:** Sexually reproducing species in which individuals have just one of two distinct sexes. Opposite to hermaphrodite.

**Hermaphrodite:** Sexually reproducing species in which each individual is of both sexes. Opposite of gonochorist.

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