

The cryptic sexual strategies of human fungal pathogens

Iuliana V. Ene and Richard J. Bennett

Abstract | Sexual reproduction is a pervasive attribute of eukaryotic species and is now recognized to occur in many clinically important human fungal pathogens. These fungi use sexual or parasexual strategies for various purposes that can have an impact on pathogenesis, such as the formation of drug-resistant isolates, the generation of strains with increased virulence or the modulation of interactions with host cells. In this Review, we examine the mechanisms regulating fungal sex and the consequences of these programmes for human disease.

Parasexual reproduction

Form of reproduction in which transfer of genetic material and recombination occurs without meiosis or the development of sexual structures.

Despite the prominence of sexual reproduction in eukaryotic species, the benefits of this reproductive strategy continue to be debated. Current models suggest that the ability of sexual reproduction to promote genetic variation is important for lineage survival. Sexual reproduction can promote adaptation to fluctuating environments and also limit the accumulation of deleterious alleles. However, sexual reproduction is associated with increased 'costs', such that asexual reproduction is predicted to be advantageous under many conditions, particularly as a short-term evolutionary strategy^{1–3}. Moreover, sexual reproduction carries the risk of genetic conflicts and the breakdown of well-adapted genetic combinations. There is also the twofold cost of sexual reproduction, in which only 50% of parental alleles are passed on to any single progeny, and two parents are required to make one progeny.

The benefits of sexual versus asexual reproduction are further complicated in pathogenic species. Host–pathogen interactions can lead to cycles of co-adaptation and may result in species that have increased rates of genetic variation. Such an evolutionary 'arms race' between host and pathogen was formulated as the Red Queen hypothesis (BOX 1). In this model, sexual outcrossing in the host promotes adaptation, which enables the host to escape potential destruction by a co-evolving pathogen⁴. Evolution therefore favours faster-evolving lineages, enabling such species to stay ahead in the arms race. Conversely, lack of sexual reproduction results in the host being unable to outrun the pathogen, leading to extinction. Support for the Red Queen hypothesis has come from two experimental models in which sexual populations were found to be more resistant to infection but asexual (that is, self-fertilizing) populations were selected against^{5,6} (BOX 1).

Studies show that co-evolution also drives accelerated evolution rates in pathogens^{7,8}. Although the host persists by keeping one step ahead, the pathogen also adapts by undergoing co-evolutionary fine-tuning⁹. Moreover, in the case of a fungal plant pathogen, sexual reproduction enabled the rapid evolution of the pathogen and the infection of a more resistant host¹⁰. Thus, co-evolutionary interactions drive adaptive events both in the host and in the pathogen, and rapid evolution can be driven by sexual reproduction.

Historically, fungal studies have been used to address key questions concerning the molecular evolution of sexual reproduction and its role in promoting genetic diversity. Several prominent pathogenic species were traditionally thought to be clonal and thus restricted to asexual modes of reproduction. This was often due to the inability to observe sexual reproduction under laboratory conditions or to the long time periods that are required for productive mating. The idea that certain species were asexual was challenged by an increasing body of genomic evidence, which, supported by subsequent experimental approaches, has led to the discovery of extant sexual cycles in many clinically relevant species.

In this Review, we describe the sexual programmes of the most prevalent human fungal pathogens. These species are responsible for a wide range of diseases, from oral thrush and skin infections to fungal meningitis and bloodstream infections. Although largely clonal, most of these species have retained the molecular machinery and the ability to undergo sexual reproduction. Sexual or parasexual reproduction is used by these pathogens to shuffle genetic material within the cell, generating recombinant isolates that have altered drug resistance and pathogenicity. Furthermore, we discuss how sexual programmes exhibit high plasticity and the novel

Department of Microbiology and Immunology, Brown University, Providence, Rhode Island 02912, USA. Correspondence to R.J.B. e-mail: Richard_Bennett@brown.edu doi:10.1038/nrmicro3236

Box 1 | **The Red Queen hypothesis**

The Red Queen model is an allusion to the race with the Red Queen in *Through the Looking-Glass* by Lewis Carroll, in which Alice and the Red Queen are constantly running but remain in the same spot. This model was proposed by Leigh Van Valen in 1973 to indicate that organisms must constantly adapt and evolve to survive a co-evolutionary ‘race’ with other organisms in an ever-changing environment¹. In particular, sexual reproduction might enable organisms to escape and ‘outrun’ pathogens. As the adaptation of a species modifies the environment of neighbouring species and imposes selection onto these, cohabiting species play a ‘null sum game’ within their ecosystem.

The Red Queen hypothesis has received empirical support, which highlights that organisms capable of sexual reproduction have better chances of survival under stressful conditions than asexual species^{5,6}. One study compared sexual and asexual snail lineages in an environment that contained natural parasites. Although initially more resistant to infection, the asexual lineage was soon outcompeted and the initial fitness advantage was lost. By contrast, sexual lineages persisted throughout the study, which supports the hypothesis that sexual reproduction gives animals an advantage in the battle against co-evolving parasites⁵. This was also the case in an interaction between *Caenorhabditis elegans* and a natural bacterial parasite, as all of the obligately selfing populations were driven to extinction, whereas the outcrossing population persisted⁶.

mechanisms that have evolved between species to control this developmental pathway. Overall, studies of sexual reproduction in pathogenic fungi have redefined the paradigms concerning the life cycles of these species, their evolution and their ability to adapt to the mammalian host.

Discovery of extant sexual cycles

Among eukaryotes, sexual reproduction is ubiquitous, and exclusively asexual lineages are often evolutionary ‘dead ends’ owing to the accumulation of deleterious mutations; this is known as Muller’s ratchet. It was therefore surprising that prominent fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* were, for many years, considered to be obligate asexual species. Analysis of genome sequences revealed that these species had retained many genes associated with mating and meiosis. Furthermore, they contained a mating-type (*MAT*) locus, which is a genetic locus encoding the transcription factors that are the master regulators of sexual reproduction^{11–14}. Despite these discoveries, the exclusive use of genome sequences to establish the sexual fecundity of a species has proven to be difficult. Although a core set of conserved genes is associated with sexual reproduction in many species (for example, the ‘meiosis detection toolkit’ (REF. 15)), some genes that are described as markers of sexual activity (for example, *DMC1* and *HOP1*) are absent in species that are now known to be sexual^{13,16}. These exceptions have challenged the idea that a set of conserved genes is shared by all sexual species, suggesting considerable plasticity in sexual strategies between species.

In the case of the three most important human fungal pathogens — *C. albicans*, *A. fumigatus* and *Cryptococcus neoformans* — the existence of sexual or parasexual cycles has now been established. Strikingly, each species seems to restrict access to this mode of reproduction, generating mostly clonal populations in nature. Why these species have retained the machinery for

sexual reproduction despite the associated fitness costs, and the importance of sexual programmes for mediating interactions with the mammalian host, are now beginning to be revealed.

Mating-type loci and mate recognition

Fungi often exhibit bipolar or tetrapolar mating systems that are controlled by transcription factors encoded at the *MAT* locus (Supplementary information S1 (figure)). Bipolar systems have a single bi-allelic locus and include pathogenic ascomycetes such as *C. albicans* and *A. fumigatus* (Supplementary information S2 (figure)) as well as the model yeast *Saccharomyces cerevisiae*. Tetrapolar systems have two unlinked, multiallelic sex loci as exemplified by many basidiomycetes. Bipolar systems enable mating with half of the sibling offspring, thereby enabling both inbreeding (50% of offspring) and outbreeding (50% of offspring). By contrast, tetrapolar systems favour outbreeding by restricting mating to 25% of the sibling offspring (Supplementary information S1 (figure)).

Mate recognition occurs by mating-pheromone signalling. For bipolar systems, the expression of pheromone and pheromone-receptor genes is regulated by *MAT*-encoded transcription factors¹⁷. For tetrapolar systems, one *MAT* locus encodes regulatory homeodomain transcription factors, whereas the other locus encodes pheromones and pheromone receptors; the correct combination of pheromone, pheromone receptor and transcription factors is necessary for successful mating. Following stimulation, pheromone receptors turn on a highly conserved MAPK (mitogen-activated protein kinase) pathway, which results in the induction of a transcriptional mating response¹⁸.

Interestingly, the most prominent human fungal pathogens exhibit bipolar mating-type systems. This is the case for *Cryptococcus gattii* and *C. neoformans*, even though the ancestor to these species had a tetrapolar mating system¹⁹. Studies have suggested that the ancestral pheromone locus and transcription factor locus gradually expanded by sequential rounds of gene acquisition, ultimately fusing into a single *MAT* locus that is >100 kb and encodes >20 genes^{19–21} (Supplementary information S2 (figure)). Recombination at this locus led to the acquisition of several essential genes that restricted further large-scale rearrangements^{19,22}. Recent studies identified recombination hot spots both flanking the *C. neoformans MAT* locus and within this locus^{23,24}. These observations suggest that there is ongoing gene flow between the two mating types and that gene conversion can occur at genomic regions that were previously thought to be cold spots for recombination^{23,24}.

The mating-type-like (*MTL*) locus in *C. albicans* has also undergone expansion since diverging from the related hemiascomycete, *S. cerevisiae*. The *C. albicans MTL* locus includes three genes that are not present in the *S. cerevisiae MAT* locus, two of which are essential for growth and have roles that are unrelated to sexual reproduction^{13,16,25} (Supplementary information S2 (figure)). The *C. albicans a1* and *a2* transcription factors encoded at the *MTL* locus, control sexual mating by

Muller’s ratchet

The accumulation of deleterious mutations in an asexual population, which becomes so great that it leads to the extinction of the population.

Ascomycetes

One of the two large divisions in the fungal kingdom; they are commonly known as sac fungi. Their name stems from their defining sexual feature, ascus (in the form of an ascocarp or cleistothecium), which is where nuclear fusion and meiosis take place, resulting in the formation of ascospores.

Basidiomycetes

One of the two large phyla of Fungi that are typically known as higher fungi. They are most commonly filamentous fungi that reproduce sexually by forming round-shaped cells known as basidia, which bear external basidiospores.

Homeodomain

A 60 amino acid protein domain that folds into a helix–turn–helix compact structure and binds to DNA. Homeodomain folds are commonly found in transcription factors and they are found exclusively in eukaryotes, where they often induce cellular differentiation.

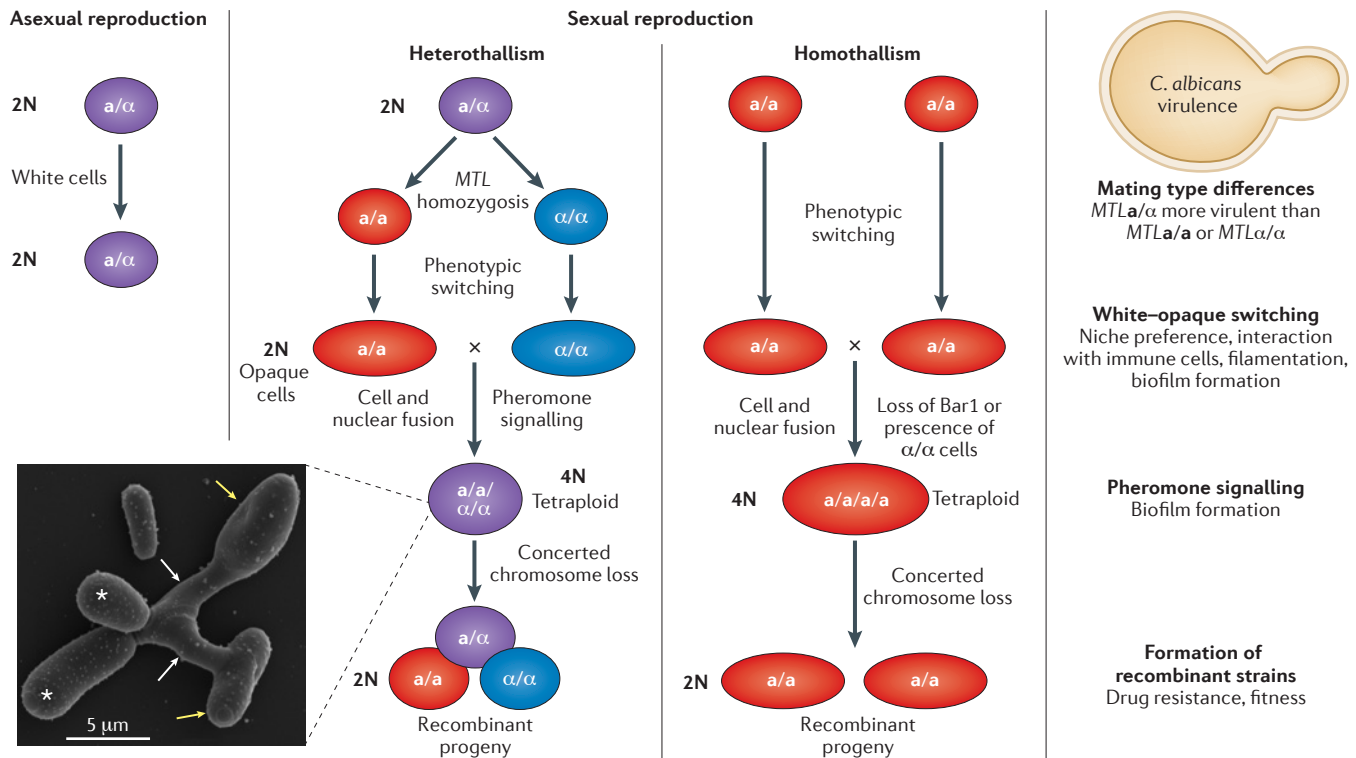
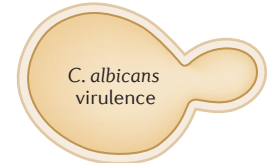


Figure 1 | Asexual and parasexual reproduction in *Candida albicans*. *Candida albicans* cells are diploid (2N) and can divide asexually or can undergo heterothallic or homothallic mating. Mating type-like locus **a** (*MTLa*) and *MTLa* cells must switch from white to opaque to become mating-competent. Opaque cells secrete pheromones that result in the formation of conjugation tubes, and subsequently, cell and nuclear fusion occur to form tetraploid (4N) cells. Homothallic mating also occurs and can be driven by loss of Bar1 protease in *MTLa* cells. Mating products can be induced to undergo concerted chromosome loss to return to the diploid state. The relationship between virulence and parasexual reproduction is indicated on the right-hand side of the figure. Inset, a scanning electron micrograph of a *C. albicans* mating zygote is shown. Parental opaque cells (yellow arrows) form mating projections (white arrows) and subsequently fuse and generate daughter cells (white asterisk). Scanning electron micrograph courtesy of M. P. Hirakawa, Brown University, Rhode Island, USA.



Mating type differences
MTLa/α more virulent than *MTLa*/a or *MTLa*/α.

White–opaque switching
 Niche preference, interaction with immune cells, filamentation, biofilm formation

Pheromone signalling
 Biofilm formation

Formation of recombinant strains
 Drug resistance, fitness

inhibiting a unique phenotypic switch that is necessary for conjugation (discussed below). Thus, both *C. albicans* and *C. neoformans* have evolved to have mechanisms that may restrict efficient outbreeding.

Studies on the evolution of sex-determining regions in fungi also have implications for the development of sex chromosomes in higher eukaryotes. Analogous to the case in *C. neoformans*, sex determinants that are located on one chromosome may have accrued additional sex regulatory genes via genome rearrangements, eventually forming sex chromosomes that are dimorphic between the two sexes^{3,26}.

Homothallism versus heterothallism

Fungi can have heterothallic sexual cycles — in which mating occurs between partners of different sexes and promotes outbreeding — or homothallic sexual cycles — in which organisms are self-fertile, promoting inbreeding. Transitions between heterothallic and homothallic lifestyles are common during fungal evolution; the switch from outcrossing to selfing enables the expansion of a species to niches in which encounters with opposite mating types are rare³.

Limiting outbreeding might be particularly beneficial in human pathogens. Studies in protozoan parasites

first indicated that eukaryotic pathogens generate highly clonal populations that undergo limited recombination²⁷. Homothallic reproduction may help to preserve genomic configurations that are well adapted for growth in the host, while still enabling strains to undergo occasional recombination and reshuffling of their genomes¹².

Studies in the model yeast *S. cerevisiae* have also shown differences among different populations in their tendency to undergo outbreeding. In particular, human-associated isolates (including clinical and vineyard isolates) showed evidence of sexual outbreeding followed by long periods of clonality²⁸. In this case, the limitation of sexual activity was linked to decreased sporulation and increased pseudohyphal growth. Although both responses are stimulated by nutrient limitation, pseudohyphal growth was hypothesized to have a more beneficial response in human-associated environments.

In *C. albicans*, both heterothallic and homothallic mating have been observed. Heterothallic mating occurs between diploid **a** cells and α cells to generate tetraploid **a**/α cells and is mediated by pheromone signalling between the two mating types (FIG. 1). However, in cells that lack the Bar1 protease, same-sex **a**–**a** mating can occur²⁹. Bar1 normally degrades α-pheromone but, in the absence of this protease, **a** cells do not destroy

MAPK
 (Mitogen-activated protein kinase). A serine/threonine-specific protein kinase that signals cellular responses to a wide range of stimuli, including pheromones, mitogens, osmotic or heat stress.

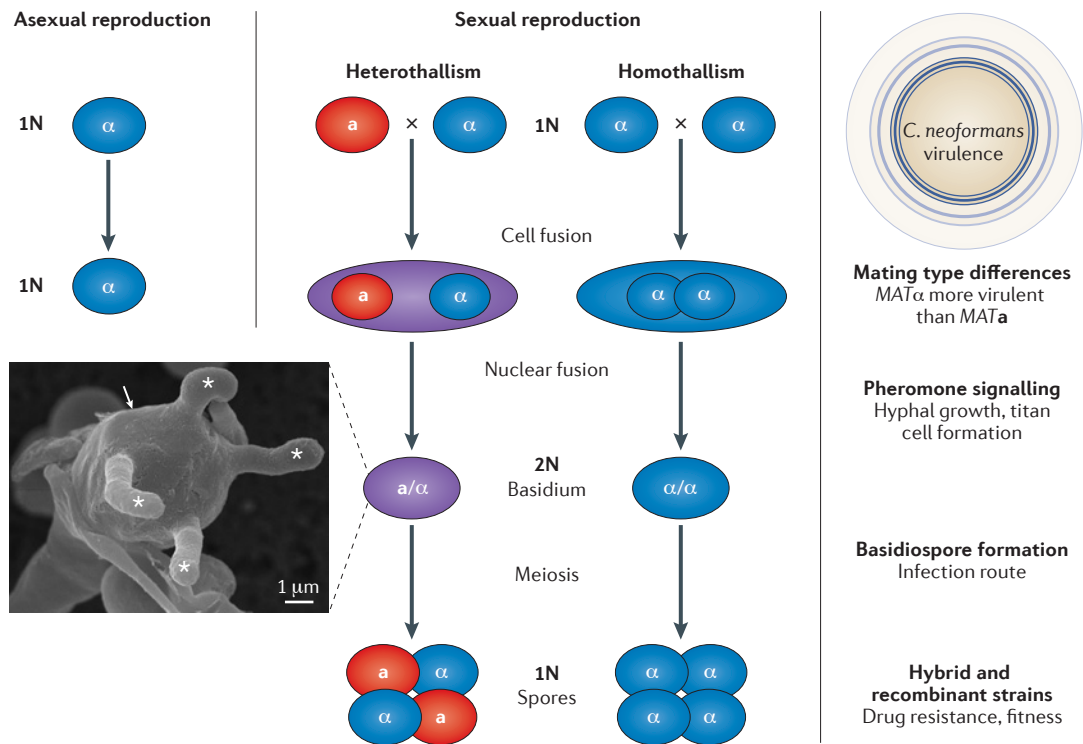


Figure 2 | **Sexual and asexual reproduction in *Cryptococcus neoformans*.** *Cryptococcus neoformans* cells are haploid (1N) and can divide asexually or enter a heterothallic or homothallic mating cycle. In heterothallic mating, pheromone signalling between **a** cells and **α** cells results in cell–cell fusion. Nuclei do not fuse but form a filamentous dikaryon. The tips of the filamentous cells differentiate into basidia, in which nuclear fusion and meiosis occur. Additional rounds of mitotic division produce multiple, haploid basidiospores, which results in the formation of four long chains. The consequences of sexual reproduction for virulence in *C. neoformans* are shown on the right-hand side of the figure. Inset, a scanning electron micrograph shows a *Cryptococcus gattii* basidium (white arrow) with four emerging basidiospores (white asterisk). MAT, mating-type locus. Scanning electron micrograph reproduced from REF. 185.

the α -pheromone that they produce and the resulting autocrine feedback loop drives same-sex mating (FIG. 1). Homothallic mating is also observed in *ménage à trois* matings in which α -pheromone secreted by **a** cells induces productive mating between two **a** cells²⁹. *C. neoformans* has similarly been shown to undergo both heterothallic and homothallic mating. Although conventional mating between *C. neoformans* **a** cells and α cells had been well documented^{30–32}, a surprising unisexual mating cycle between *C. neoformans* α cells has been described^{33,34} (discussed below and shown in FIG. 2). Unisexual α – α mating is thought to have given rise to highly virulent strains of *C. gattii*, which are the cause of an ongoing outbreak in the Pacific Northwest of the United States and Canada^{35,36}.

What are the benefits of unisexual reproduction? The most obvious benefit is the ability to undergo sexual reproduction even in the absence of a partner of the opposite mating type. This could be crucial for *C. neoformans*, as most natural isolates are of a single mating type³⁷. Furthermore, ploidy changes can function as capacitors for evolution even in the absence of outbreeding. This was elegantly shown by a study of *Aspergillus nidulans*, which showed that diploid strains achieved greater fitness than haploid strains during long-term evolution experiments³⁸. The fitter diploid strains

had reverted to haploidy via parasexual recombination, and increased fitness was due to the accumulation of recessive deleterious mutations in the diploid state, which were beneficial when subsequently unmasked in haploid recombinants³⁸.

Same-sex mating cycles in *C. neoformans* and *C. albicans* also promote adaptation owing to the formation of chromosome aneuploidies^{39–41}. Although aneuploidy often negatively affects fitness, certain aneuploid chromosomes increase resistance to antifungal drugs in both species^{40,42,43}. Studies in *S. cerevisiae* have shown that aneuploidies can also drive adaptive evolution^{44,45}, and it is therefore probable that the karyotypic changes that are introduced by self-mating provide a diverse pool of isolates upon which selection can act⁴⁶. Indeed, a recent study by Ni *et al.* showed that unisexual mating in *C. neoformans* resulted in phenotypic variants, most of which were due to chromosome aneuploidies⁴⁰. Same-sex mating can provide an additional selective advantage to *C. neoformans*, as hyphal forms that are induced during mating enable nutrient foraging over an increased area⁴⁷.

We also note that mechanisms similar to fungal homothallism are encountered in protozoan parasites. For example, *Toxoplasma gondii* comprises three highly clonal lineages that have arisen via an ancestral sexual event⁴⁸; however, this parasite can undergo selfing to generate

Aneuploidy

A change in chromosome copy number that does not parallel a change in the entire haploid or diploid genome.

Box 2 | A phenotypic switch regulates the parasexual cycle of *C. albicans*

Candida albicans cells typically exist as white cells but can transition to an alternative ‘opaque’ state. The white–opaque switch was originally described in the clinical isolate WO-1 by Soll and colleagues¹⁶⁵. White cells are round and generate bright, shiny colonies, whereas opaque cells are elongated and form greyer, flatter colonies¹⁶⁵. The white–opaque transition is reversible and heritable. A complex between $\alpha 1$ and $\alpha 2$ proteins inhibits this switch, so that only α cells or α cells efficiently switch to the opaque state. Opaque cells are mating-competent and secrete and respond to sexual pheromones to undergo efficient conjugation^{57,66,166}. Repression of the switch by the mating type-like (*MTL*) locus is relaxed under certain conditions, which enables a subset of natural α/α isolates to also undergo this transition¹⁶⁷.

The transcriptional circuitry that regulates the white–opaque switch has been described, and the master regulator of the opaque state is the white–opaque regulator 1 (*Wor1*) transcription factor. The formation of opaque cells is induced by the expression of *Wor1* and is stabilized by positive feedback of *Wor1* on its own promoter^{168–171}. The $\alpha 1$ – $\alpha 2$ heterodimer inhibits *Wor1* expression, thus limiting the ability of α/α cells to undergo this phenotypic switch¹⁷². *Wor1* forms part of an integrated circuit that contains the transcription factors *Wor2*, *Wor3*, *Czf1* (zinc cluster transcription factor 1), *Efg1* (enhanced filamentous growth protein 1) and *Ahr1* (a zinc finger transcription factor), which act together to regulate the bistable white–opaque switch^{171,173,174}.

In addition to regulating mating, the white–opaque switch influences multiple aspects of *C. albicans* biology, including filamentation, metabolic regulation, biofilm formation, interactions with immune cells and virulence^{59,78,81,175,176}. Furthermore, strains that overexpress *Wor1* hypercolonize the mouse gastrointestinal tract and can undergo a distinct morphological transition to the gastrointestinally induced transition (GUT) phenotype¹⁷⁷. It is therefore apparent that *Wor1* and phenotypic switching have a central role (or roles) in regulating the *C. albicans* lifestyle.

A similar phenotypic switch regulates the mating programme of *Candida tropicalis*, and entry into this programme is also *Wor1*-dependent^{68,178}. However, the transcriptional circuits that control the white–opaque switch have diverged among *Candida* species¹⁷⁸. In addition, phenotypic switching in *C. tropicalis* is independent of *MTL* control, which indicates that the switch may have an even broader role in the biology of this species^{178,179}.

highly virulent isolates as well as infectious spores⁴⁹. Similarly, the protozoan *Giardia lamblia* undergoes a novel type of selfing known as ‘diplomixis’, which involves nuclear fusion in an analogous manner to unisexual mating in pathogenic fungi^{3,50}. Both outcrossing and selfing have also been observed in *Plasmodium* species, whereby a single protozoan can generate both male and female gametes⁵¹. It is therefore apparent that unisexual mating is a prevalent mechanism that is used by eukaryotic microorganisms to adapt to their environment.

Sex in successful human pathogens

More than 100,000 fungal species have been identified so far, but fewer than 200 are associated with humans and only a minority of these cause disease⁵². Members of the *Candida*, *Cryptococcus* and *Aspergillus* genera are the most important in clinical settings. In this section, we compare mating strategies in *C. albicans*, *C. neoformans* and *A. fumigatus*. We subsequently consider sexual cycles in other fungal pathogens that can have an impact on human health.

Sexual reproduction in *C. albicans*. The most clinically relevant *Candida* species are not found in the environment but are commensal microorganisms of humans, suggesting co-adaptation with the host. These species are also frequent opportunistic pathogens: the

most commonly isolated species is *C. albicans*, which is a cause of both mucosal infections (such as thrush and vaginitis) and life-threatening bloodstream infections⁵³. Initially classified as an asexual organism, a novel parasexual programme has now been established for *C. albicans*^{54–58}. Mating is regulated by a unique phenotypic switch: α cells and α cells mate efficiently only if they undergo a heritable and reversible transition from the typical ‘white’ state to the mating-competent ‘opaque’ state (BOX 2; FIG. 1).

Once the sexual state of *C. albicans* had been discovered, additional questions arose. What are the signals that trigger switching to the opaque state, and is this switch regulated by conditions that are encountered in the host? It is now evident that the white–opaque transition is sensitive to many environmental cues that include starvation, haemoglobin, temperature, CO₂, *N*-acetyl glucosamine (GlcNAc) and genotoxic and oxidative stress (reviewed in REFS 59,60). In the laboratory, opaque cells are unstable at 37 °C and rapidly revert to the white state. However, conditions that stabilize the opaque state have been identified, including the presence of CO₂ and GlcNAc, which suggests that opaque cells can persist in some niches in the host^{61,62}. Furthermore, high rates of white–opaque switching have been observed during the passage of a clinical isolate through the mouse intestines, and this may be stimulated by anaerobic conditions in the gastrointestinal tract⁶³. Mating of *C. albicans* cells has also been shown in several *in vivo* models, including systemic infection, as well as during colonization of the skin or intestines^{54,59,64,65}.

The white–opaque switch evolved relatively recently in the *Candida* lineage and is limited to *C. albicans*, *Candida dubliniensis* and *Candida tropicalis*^{66–68}. In each of these species, the opaque forms are the mating-competent state, but the question arises as to why this switch evolved to regulate mating. It is probable that the requirement for cells to switch to the opaque state limits promiscuous sex and restricts mating to specific niches in the mammalian host⁶⁹. In addition, the white–opaque switch may promote mating *in vivo* by regulating the formation of pheromone-induced sexual biofilms (discussed below). Sexual reproduction in non-*albicans* *Candida* species is discussed in BOX 3.

Parasex but no meiosis in *C. albicans*. Despite observations of efficient mating between diploid cells, a conventional meiosis has not been observed in *C. albicans*. Instead, tetraploid mating products undergo a parasexual process of concerted chromosome loss to generate diploid and aneuploid progeny^{39,56}. Genetic recombination is observed in a subset of parasexual progeny and is dependent on *Spo11* — a conserved endodeoxyribonuclease that is required for meiotic recombination in diverse eukaryotes — indicating that there are parallels between the parasexual cycle and a conventional meiosis³⁹.

Could the parasexual cycle have specific benefits for the commensal lifestyle of *C. albicans*? A classical meiosis would be expected to generate spores, which are infectious particles that could be highly immunogenic in the

Biofilms

Complex communities of microorganisms that are commonly found attached to a prosthetic surface in the host. Cells adhere to the surface and to each other and promote the formation of extracellular matrix, which protects the biofilm community from external stress (including antifungal drugs).

Gastrointestinally induced transition

(GUT). A phenotypic transition that enables *C. albicans* cells to hypercolonize the gastrointestinal tracts of mice.

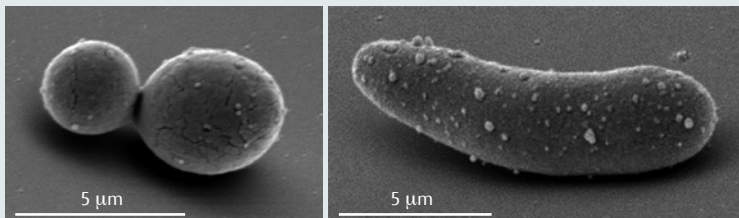
Box 3 | Sexual reproduction in other pathogenic *Candida* species

Genomic and experimental evidence indicate that the regulation of sexual reproduction has been rewired among *Candida* clade species^{13,16}. In contrast to *Candida albicans*, several *Candida* species exhibit complete sexual cycles that culminate in meiosis and sporulation. For example, both *Candida lusitanae* and *Candida guilliermondii* are heterothallic species that can mate and form meiotic ascospores¹⁶, despite lacking the conserved genes that are central to meiosis in *Saccharomyces cerevisiae*^{13,16}. Moreover, in these *Candida* species, meiosis is no longer under control of the a1–a2 complex as it is in *S. cerevisiae*, as *C. lusitanae* lost the gene that encodes a2 and *C. guilliermondii* lost the genes that encode a1 and a2 during evolution^{13,16}. *C. lusitanae* was also recently shown to have coupled mating and meiosis, which enables this pathogen to have only a transitory diploid stage¹⁸⁰. These results exemplify the plasticity in the regulatory mechanisms that control sexual reproduction and meiosis in fungal species.

In addition to *C. albicans*, the white–opaque transition has been observed in *Candida dubliniensis*⁶⁷ and *Candida tropicalis*^{68,179}. As in *C. albicans*, the white–opaque regulator 1 (Wor1) transcription factor controls phenotypic switching and mating in *C. tropicalis* (see the figure) but also regulates other traits that are associated with pathogenesis, including filamentous growth and biofilm formation¹⁷⁸. In more distantly related ascomycetes, such as *S. cerevisiae* and *Histoplasma capsulatum*, orthologues of Wor1 function as transcriptional regulators of filamentation^{181,182}. The ancestral Wor1 protein therefore seems to have been a transcriptional regulator of morphogenesis, with the white–opaque switch having evolved relatively recently in the lineage that leads to *C. albicans*, *C. dubliniensis* and *C. tropicalis*¹⁷⁸.

Candida parapsilosis is also a prevalent pathogen and is a member of the *Candida* clade but does not exhibit sexual activity. Isolates are exclusively *MTLa/a* and the *MTLa1* gene is a pseudogene, which suggests that the *MTL* locus might be degenerating in this species¹⁸³. Two species that are closely related to *C. parapsilosis* are *Candida metapsilosis* and *Candida orthopsilosis*, but only in *C. orthopsilosis* is there a mixture of mating types, suggesting the presence of an extant sexual cycle¹⁸³.

Candida glabrata is more closely related to *S. cerevisiae* than to other *Candida* clade species, but it is often the second most commonly isolated species after *C. albicans*⁵³. Despite its close relationship with *S. cerevisiae*, pheromone genes are not expressed in most *C. glabrata* isolates, and neither a nor a mating types respond to pheromone. Mating and meiosis have not been described for this species, and the population structure is mostly clonal; thus, it remains to be seen if sexual reproduction occurs in this species¹⁸⁴. Scanning electron micrograph of white (left-hand panel) and opaque (right-hand panel) *C. tropicalis* cells courtesy of M. P. Hirakawa, Rhode Island, USA.



Candida clade

A group of related *Candida* species; members of this group share an altered genetic code in which the CUG codon is translated as leucine instead of serine as in the universal genetic code. This group includes most pathogenic *Candida* species, except for *Candida glabrata*, which is more closely related to *Saccharomyces cerevisiae*.

host¹¹. By contrast, generation of progeny via the parasexual cycle allows for a reduction in ploidy and recombination between chromosome homologues but avoids potentially harmful spore formation. In addition, chromosomal aneuploidies are formed at high frequency during parasexual reproduction and can generate additional phenotypic diversity, including the potential formation of drug-resistant isolates^{70–72}. It remains to be seen whether the parasexual pathway is the only mechanism by which a mating cycle can be completed in *C. albicans* or whether a cryptic meiosis remains to be discovered.

In a recent development, Hickman *et al.* reported the surprising isolation of haploid strains of *C. albicans*⁷³. Although *C. albicans* has long been thought to be unable to form a viable haploid state, rare haploid cells were

recovered from both *in vitro* and *in vivo* experiments. Haploid cells were fully competent to undergo the white–opaque switch and could mate to regenerate diploid cells. Haploid cells were the products of parasexual chromosome loss and exhibited fitness defects that were consistent with the unmasking of recessive mutations⁷³. The existence of a haploid state provides a potentially exciting new tool with which to study *C. albicans* biology and further highlights the extensive karyotypic plasticity that is exhibited by this pathogen.

Pheromone signalling, biofilm formation and sex in *C. albicans*. *C. albicans* mating seems to be rare in nature, as the population structure is clonal and there is only limited evidence of recombination^{74,75}. It is possible that stressful conditions (such as oxidative stress or anti-fungal drugs) stimulate the parasexual cycle, as stressors can promote loss of heterozygosity at the *MTL* locus, white–opaque switching and concerted chromosome loss^{70,76}. This is also consistent with theoretical models of sexual reproduction, as organisms are predicted to undergo mating more often when stressed and stress-induced recombination can facilitate adaptation under stress⁷⁰. In the case of *C. albicans*, expression of *MTL* genes could also be regulated by environmental factors in such a way that a/a cells function phenotypically as a cells or α cells; this could enable mating in populations that were previously thought to be unable to undergo sexual reproduction⁷⁷. Pheromone signalling could also affect pathogenesis, as *C. albicans* white cells mount a novel response to mating pheromones that are secreted by opaque cells. Although opaque cells respond to pheromone by forming mating projections and undergoing conjugation (FIG. 1), white cells respond by upregulating genes that are involved in adhesion and biofilm formation^{78–81}. Such ‘sexual’ biofilms enable the stable formation of pheromone gradients between mating partners, which enables opaque cells to locate one another and mate more efficiently^{78,82}. As biofilms are important in establishing *Candida* spp. infections⁸³, the formation of sexual biofilms could directly promote infection in the host⁸⁴.

C. albicans white and opaque cells have also been shown to respond to pheromones from closely related species⁸⁵. Pheromones from non-*albicans* *Candida* species were able to induce biofilm formation in *C. albicans* white cells as well as same-sex mating in *C. albicans* opaque cells⁸⁵. The pheromone–receptor interaction therefore displays surprising plasticity and further broadens the potential conditions under which pheromone signalling, mating and/or biofilm formation might occur in nature.

Sexual reproduction in *Cryptococcus* spp.

C. neoformans is a basidiomycete yeast that is associated with soil, trees and pigeon guano. During infection, *Cryptococcus* spores or desiccated yeast cells are inhaled and lodge in lung alveoli, from where the organism is either cleared or establishes a latent infection⁸⁶. Subsequent immunosuppression leads to reactivation of the latent fungus, followed by dissemination to the central nervous system (CNS), which results in meningoencephalitis^{3,86}.

Three varieties of *Cryptococcus* spp. are responsible for cryptococcosis and these have been classified on the basis of their capsular aggregation reactions and genome sequences. Most infections (95% worldwide) are due to *C. neoformans* var. *grubii* (serotype A)⁸⁷, <5% of infections are caused by *C. neoformans* var. *neoformans* (serotype D), and a hybrid serotype (AD) also exists that may be more pathogenic than serotype D alone⁸⁸. Although these varieties predominantly infect immunocompromised individuals, *C. gattii* strains (serotypes B and C), which are mostly found in tropical regions, can infect immunocompetent, healthy individuals.

C. neoformans strains are heterothallic and both **a** and α mating types are capable of causing disease, although most clinical isolates are α strains³⁷. Unlike most basidiomycetes, in which mating is regulated by a tetrapolar mating locus⁸⁶, the *MAT* locus of *C. neoformans* is bipolar and encodes homeodomain proteins, pheromones and pheromone receptors, all of which contribute to cell identity^{89–91}. Mating in *C. neoformans* and *C. gattii* was reported almost 4 decades ago and involves the fusion of **a** cells and α cells to produce dikaryotic filaments^{30,92} (FIG. 2). Following nuclear fusion and meiosis, long chains of sexual basidiospores are produced, which contain recombinant haploid nuclei^{30,92,93}.

Although heterothallic mating of *C. neoformans* strains was observed in the laboratory, how mating could occur in nature remained an open question, as most (>98%) isolates are of the α mating type. For *C. neoformans* var. *grubii* (serotype A), it became apparent that although α strains are distributed worldwide, the **a** mating type is restricted to sub-Saharan Africa, where there is evidence of recent or ongoing sexual recombination^{94,95}. Studies of mating in this serotype have shown that *C. neoformans* var. *grubii* strains have mating specificity beyond **a** and α mating types, and this may further promote inbreeding of more highly adapted, pathogenic strains⁹⁶.

Hybrid AD serotypes are the result of mating between intervarietal strains and often contain both mating types (for example, **a**AD α or α AD α)^{31,32}. These hybrids germinate poorly to produce diploid or aneuploid products, which suggests that the sexual cycle is inefficient in crosses between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*.

Same-sex mating in *Cryptococcus* spp. *C. neoformans* strains had been known to undergo a programme of monokaryotic fruiting, during which cells formed filaments and underwent sporulation⁹⁷. Although this was originally thought to be an asexual programme, Lin *et al.* showed that fruiting α cells underwent same-sex fusion to form α/α diploids and that these subsequently sporulated to produce recombinant haploid spores³³ (FIG. 2). Progeny formation was dependent on Spo11 and Dmc1, which are two conserved proteins that are necessary for the formation and repair of meiotic DNA double-strand breaks in yeasts and mammals^{33,98}.

Analysis of natural isolates of *C. neoformans* has since confirmed the existence of α/α diploids and α A/D α hybrids, which indicates that same-sex mating occurs in nature^{34,99,100}. This strategy enables inbreeding and

recombination to occur even in geographical regions in which only one mating type is present. Although both **a** strains and α strains of *C. neoformans* undergo monokaryotic fruiting¹⁰¹, the *MATa* locus promotes hyphal growth, and this ability could further contribute to the prevalence of α isolates in nature¹⁰².

Unisexual mating between two closely related α isolates of *C. gattii* is thought to have produced a highly virulent strain that is responsible for an ongoing outbreak that started on Vancouver Island, British Columbia, Canada^{35,103}. The outbreak is associated with high mortality rates (>25%) in both healthy and immunosuppressed individuals as well as in domestic and wild animals¹⁰³. Since the first cases were reported, the outbreak has spread into the Pacific Northwest of the United States and Canada^{36,103,104}. Several genotypes are responsible for these infections, but the major genotype, VGIIa, is the presumed product of a homothallic mating event between closely related isolates of the VGII type³⁵. The VGIIa isolates are highly virulent in animal models of infection^{35,36}, but how this infection emerged from Vancouver Island remains to be determined.

Mating and virulence in *Cryptococcus* spp. Do sex and mating type have an impact on pathogenicity? Several pieces of evidence suggest that there is a close connection between mating type, sexual reproduction and virulence in *Cryptococcus* spp. First, sexual specification has been directly linked to virulence in serotype D isolates, with α cells being more virulent than **a** cells¹⁰⁵ (FIG. 2). Genes from outside the *MAT* locus interact with genes from within the α locus to direct pathogenesis^{96,106}. The two mating types also differ in their interactions with the mammalian host: *C. neoformans* var. *grubii* α isolates are better able to disseminate through the CNS than congenic **a** isolates¹⁰⁷. Moreover, sexual reproduction can generate new variants that are hypervirulent in the host, such as those that are responsible for the Vancouver Island outbreak. Sexual spores of *C. neoformans* can also function as infectious particles, leading to high mortality rates in a mouse inhalation model of infection^{108,109}. Spores might be more successful at causing infection than yeast cells as they are highly resistant to various environmental stresses^{110,111}.

Another mechanism by which mating can affect virulence is via the pheromone-induction of specialized cell types. Pheromone signalling has been shown to promote the formation of titan cells by *C. neoformans*^{112,113}. Titan cells can reach 50–100 μ m in diameter, which is 5–10 times larger than a typical cryptococcal cell. These giant cells are polyploid (4N–64N), are resistant to phagocytosis by macrophages and promote the virulence of *C. neoformans* in a mammalian model of infection^{114,115}. Taken together, it is therefore apparent that mating and virulence are interconnected processes in *C. neoformans* and *C. gattii*.

A sexual revolution in *Aspergillus* spp.

Most *Aspergillus* infections are associated with immunodeficiency and involve pulmonary aspergillosis, which

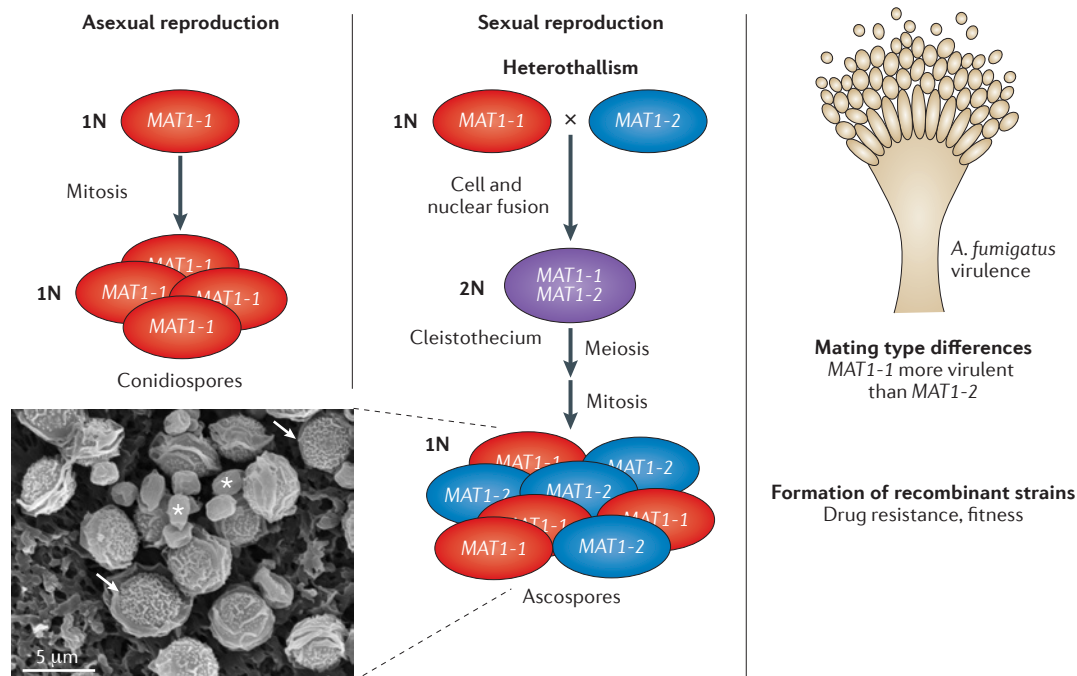


Figure 3 | Sexual and asexual reproduction in *Aspergillus fumigatus*. *Aspergillus fumigatus* undergoes asexual reproduction via the mitotic division of haploid (1N) cells or via the formation of asexual conidiospores. It can also undergo heterothallic mating between mating-type locus 1-1 (*MAT1-1*) and *MAT1-2* mating types. The products of mating are cleistothecia that contain multiple ascospores. Sexual reproduction and virulence are linked in *A. fumigatus*, as specified on the right-hand side of the figure. Inset, a scanning electron micrograph shows *Aspergillus lentulus* cleistothecia (white arrows) with emerging ascospores (white asterisk). Scanning electron micrograph courtesy of C. M. O’Gorman and P. Dyer, University of Nottingham, UK.

can often develop into infection of the CNS^{116,117}. The most common causative agent for these infections is the ascomycete *A. fumigatus*, which is widely found in the soil and in decaying materials¹¹⁸. The primary mode of reproduction is the formation of conidia (that is, asexual spores), which are easily dispersed in the environment and can survive a wide range of conditions.

Although *A. fumigatus* was originally thought to be an asexual fungus, genome sequencing revealed the presence of multiple genes implicated in sexual reproduction^{119–121}. In addition to the *MAT* locus, genes that encode putative pheromones, receptors and pheromone-signalling components were identified^{120–122}. The functionality of *A. fumigatus* *MAT*-encoded proteins was first shown in complementation assays using *Aspergillus nidulans*, while also revealing important differences between the two species^{123,124}. *A. fumigatus* mating types are present in equal ratios in the environment, and there is evidence for recombination in the population, which suggests that mating occurs in nature^{125,126}. However, it was not until 2009 that O’Gorman *et al.* demonstrated the productive mating of *A. fumigatus* in the laboratory. These experiments involved extended incubation periods (>6 months) of strains on oatmeal agar medium in the dark¹²⁷ (FIG. 3). The prohibitive conditions that were required raised the question of where in the environment *A. fumigatus* might undergo sexual development and whether it might be evolving into an asexual organism^{125,128}. Follow-up

studies showed that *A. fumigatus* isolates from patients with invasive aspergillosis could mate in less stringent conditions (co-culture for >6 weeks at 30 °C) and a ‘supermater’ pair was subsequently identified^{129,130}. *MAT1-1* and *MAT1-2* idiomorphs function as master regulators of *A. fumigatus* mating by controlling the expression of pheromone and pheromone-receptor genes¹²⁹.

Does *A. fumigatus* mating have an impact on pathogenesis? Similarly to *C. neoformans*, the mating types of *A. fumigatus* differ in their propensities to cause infection, and the *MAT1-1* mating type is associated with increased invasive growth and increased virulence^{131,132}. Moreover, the discovery of a sexual cycle implies that recombination could yield progeny that have increased virulence and/or altered antifungal resistance (FIG. 3).

Mating in other *Aspergillus* species. Many aspects of the life cycle of *Aspergillus* species have been extensively studied using *A. nidulans*, which is a species that has a homothallic life cycle and is usually non-pathogenic^{133,134}. Similarly to *A. fumigatus*, mating is regulated by two *MAT* genes, *MAT1-1* and *MAT1-2*, which encode an α-box domain protein and a high mobility group (HMG) domain protein, respectively¹³³. Overexpression of these genes induced sexual development in *A. nidulans*, which suggests that the transition from a homothallic to a heterothallic life cycle (or vice versa) can occur by rewiring *MAT* regulation alone^{133,134}.

Idiomorphs

Distinct fungal mating type genes, which, in contrast to alleles, generally lack homology and do not seem to share an obvious ancestry.

Sexual cycles have also been uncovered in *Aspergillus flavus* and *Aspergillus parasiticus*. These species are plant pathogens as well as the primary producers of aflatoxin — a mycotoxin that is a risk factor for hepatocellular carcinoma, particularly in Africa and Asia¹³⁵. *A. flavus* is also an important human pathogen in its own right and causes both invasive and non-invasive aspergillosis¹³⁶. *MAT1-1* and *MAT1-2* idiomorphs are present in both *Aspergillus* species and are found in equal ratios in the environment, which supports the existence of sexual reproduction in nature⁶³. *A. flavus* and *A. parasiticus* undergo heterothallic mating, which results in the development of ascospore-bearing ascocarps^{137,138}.

A heterothallic mating programme has also been uncovered in *Aspergillus lentulus*, which is a close sibling of *A. fumigatus*¹³⁹ (FIG. 3). *A. lentulus* is an emerging human fungal pathogen that causes invasive aspergillosis with high mortality rates in immunocompromised patients and that exhibits decreased susceptibility to antifungal drugs¹⁴⁰. Thus, another supposedly asexual species undergoes a cryptic sexual cycle and hence has the potential to rapidly evolve under selective pressure.

Sex in other human fungal pathogens

Among the ascomycetes, several filamentous species are associated with human disease, including *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii*, *Blastomyces dermatitidis*, *Penicillium marneffeii*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* (Supplementary information S3 (figure)). Although most of these species grow as moulds in the environment, the increase in temperature that is experienced during human infection triggers a switch to the pathogenic yeast state¹⁴¹.

Coccidioidomycosis (also known as Valley Fever) is primarily caused by the inhalation of arthroconidia of the ascomycetes *C. immitis* and *C. posadasii*. These species are commonly found in the soil of arid regions of the United States, Mexico and Central and South America, and they cause progressive pulmonary and disseminated disease in otherwise healthy individuals¹⁴². Genomic analysis has revealed a single mating-type locus (*MAT1-2-1*) that encodes an HMG-box protein, although a complete sexual cycle has not yet been defined for either *C. immitis* or *C. posadasii*¹⁴³. Population genetics suggests that sexual reproduction occurs in both species, but there is no interspecies flow between them¹⁴⁴.

H. capsulatum is closely related to *Coccidioides* and is ubiquitous in the soil, where it generates asexual microconidia that, when inhaled, cause histoplasmosis¹⁴⁵. The sexual cycle of this ascomycete was defined decades ago, and mating types (which are designated + and –) are found at equal ratios in the environment, which is supportive of a sexual cycle^{146,147}. In humans, only the – mating type causes infection, although the two mating types do not differ in their abilities to cause infection in a mouse model¹⁴⁸.

Pneumocystis jirovecii is an airborne ascomycete that can cause a lethal pulmonary infection in immunocompromised individuals¹⁴⁹. *Pneumocystis* species have not been cultivated *in vitro*, and thus, observations regarding

their life cycles have been made on the basis of cells in infected lung tissue. The life cycle is thought to include both a sexual phase and an asexual phase¹⁴⁹. Genome sequencing of this organism revealed a cluster of genes that are potentially involved in pheromone sensing and signalling, which could represent a putative *MAT* locus in this species¹⁵⁰.

Recently, a *MAT* locus was identified in the ascomycete *B. dermatitidis*¹⁵¹. This dimorphic fungal pathogen is the leading cause of blastomycosis, which causes severe respiratory and disseminated disease in immunocompetent people. Distribution of mating types and population genetics studies suggest that *B. dermatitidis* might reproduce sexually, but the *MAT* locus had not been described^{152,153}. The identification of the *B. dermatitidis* *MAT* revealed that, unlike the *MAT* loci of other dimorphic fungi, it contains transposable elements that make it unusually large and may increase sequence diversity between the two *MAT* idiomorphs, while decreasing recombination within this region¹⁵¹. However, it remains unclear whether this fungus undergoes sexual reproduction in nature and, if so, whether the resulting sexual spores are infectious.

Among the *Penicillium* species, *P. marneffeii* stands out as it is thermally dimorphic and can cause lethal systemic infections that are similar to disseminated cryptococcosis. *P. marneffeii* displays a pattern of extreme clonality, being genetically and spatially restricted, and it is endemically associated with AIDS in Southeast Asia^{154,155}. Two mating loci have been identified in *P. marneffeii*, and they seem to be widely distributed among isolates, which suggests that a heterothallic sexual cycle remains to be discovered¹⁵⁶. Although there is considerable evidence to suggest that sexual recombination occurs in this organism, it seems limited to genetically similar and spatially close mating partners, and selfing may occur in this species¹⁵⁷.

Although most fungal species (~95%) belong to the subkingdom Dikarya (consisting of ascomycetes and basidiomycetes), other fungal phyla also include relevant human pathogens. For example, the phylum Zygomycota includes *Mucor circinelloides*, which has defined mating types that are designated as + and –. These mating types encode SexP and SexM, respectively, which are the HMG-domain sex-determining proteins¹⁵⁸. *M. circinelloides* is an emerging opportunistic pathogen in immunocompromised populations. Mating types differ in spore size and virulence, and the – mating type produces larger asexual spores, which are more virulent in mouse infection and more resistant to phagocytosis by macrophages¹⁵⁸.

Finally, we note that the phylum Microspora is also considered to belong to true Fungi (or to be a close sister group to this kingdom). Microsporidia are obligate intracellular parasites that infect both vertebrate and invertebrate hosts and cause gastrointestinal disease in humans as well as infections of other organs¹⁵⁹. In this phylum, evidence for sexual reproduction is also emerging: microsporidia contain a *MAT*-related locus that is similar to that of zygomycetes^{160,161} and some species contain highly heterozygous genomes, which potentially

HMG-box

(High mobility group-box). A protein domain that is involved in DNA binding.

Dimorphic fungi

Fungi that can exist as single cells (yeast) or in a hyphal or filamentous form.

Morphological transitions, such as the yeast–hyphal transition, are driven by a range of environmental conditions (for example, temperature or pH).

Zygomycetes

A phylum of fungi whose name derives from zygospores, which are resistant spherical spores that are formed during mating. Species include *Mucor circinelloides* and *Rhizopus stolonifer*, which is the black bread mould.

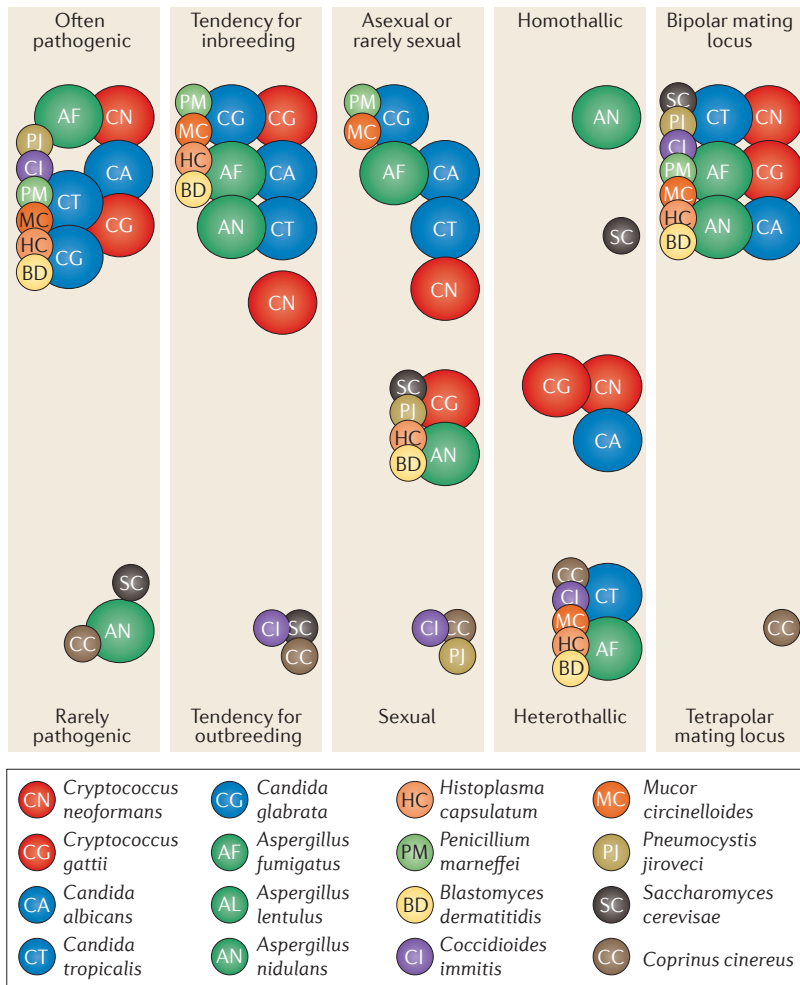


Figure 4 | Comparative analysis of sexual reproduction and virulence leads to emerging trends among human fungal pathogens. Some of the most prevalent pathogenic species tend to promote inbreeding, have restricted and highly specialized sexual cycles, contain bipolar mating loci and often have the potential for homothallic reproduction. *Saccharomyces cerevisiae* and *Coprinus cinereus* have been included as model organisms for ascomycetes and basidiomycetes, respectively. Members of the most commonly isolated genera — *Candida*, *Cryptococcus* and *Aspergillus* — are marked with larger symbols in blue, red and green, respectively. Where possible, we have assigned positions for the less well-studied species.

Commensalism
The close relationship between two organisms, in which one organism (the commensal organism) benefits without affecting its host. The term is derived from the Latin 'commensalis', which means 'sharing a table'.

occur as a direct result of outcrossing¹⁶². One recent study also found homozygous isolates, which is suggestive of selfing in the population¹⁶³. It therefore seems that diverse sexual strategies can be found across the fungal kingdom.

Fungal sex in the context of disease

What have we learned from studying sexual reproduction in fungal pathogens of humans? Many of the assumed advantages of sexual reproduction relate to the ability to increase genetic diversity, despite the

associated costs and the fact that recombination can disrupt well-adapted combinations of alleles¹⁶⁴. In pathogenic species, these costs could prove to be prohibitive. This perhaps explains why prevalent fungal pathogens of humans have preserved the apparatus for sexual reproduction but have restricted the use of these programmes. Thus, these species generate mostly clonal populations but still have the ability to undergo sexual reproduction if dictated by environmental conditions (FIG. 4). Furthermore, the fact that two major pathogens, *C. albicans* and *C. neoformans*, undergo both heterothallic and homothallic mating illustrates a paradigm that has emerged from studies of parasites — microbial pathogens can transition from outbreeding to inbreeding life cycles as they evolve into prevalent pathogens (FIG. 4).

Despite the fact that many of these species seem to limit sexual reproduction, we now recognize that sexual reproduction and sex-associated programmes have important consequences for pathogenesis. Mating types can differ in virulence as well as in their ability to evade the host immune responses; sexual spores can function as infectious particles or can promote persistence by resisting host stresses, recombinant progeny can exhibit increased resistance to antifungal drugs, and pheromone signalling can promote filamentous growth and the formation of biofilms. Moreover, the genetic variation that results from sexual reproduction can mediate the formation of hypervirulent strains. Therefore, even when rare, recombination events can dictate shifts in the lifestyles of these species, and their effects can persist in subsequent clonal lineages. Indeed, as genetic and genomic tools become increasingly available, sexual cycles are likely to be uncovered in many other 'asexual' fungi.

Important questions concerning the sex lives of human fungal pathogens remain. How do these species regulate the balance between inbreeding and outbreeding? How often do fungal pathogens mate in nature, and which niches support their sexual cycles? How are sexual cycles regulated by host cues? For those species that are human commensal microorganisms, does their sexual behaviour modulate the balance between commensalism and virulence? What are the mechanisms that underlie the differences in virulence between cell mating types, and how do altered ploidy states influence virulence?

Given the diversity of mechanisms that have been found to regulate sexual reproduction, it is probable that new paradigms, similar to the white–opaque switch in *C. albicans* and same-sex mating (that is, monokaryotic fruiting) in *C. neoformans*, remain to be discovered. It is therefore apparent that studies of fungal sexual reproduction will continue to improve our understanding of these important pathogens and will uncover novel biological mechanisms that have evolved to regulate entry into, and passage through, the sexual cycle.

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Competing interests statement

The authors declare no competing interests.

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