Sexual reproduction of human fungal pathogens

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Abstract

We review here recent advances in our understanding of sexual reproduction in fungal pathogens that commonly infect humans, including *Candida albicans*, *Cryptococcus neoformans/gattii*, and *Aspergillus fumigatus*. Where appropriate or relevant, we introduce findings on other species associated with human infections. In particular, we focus on rapid advances involving genetic, genomic, and population genetic approaches that have reshaped our view of how fungal pathogens evolve and are evolving. Rather than being asexual, mitotic, and largely clonal, as was thought to be prevalent as recently as a decade ago, we now appreciate that the vast majority of pathogenic fungi have retained extant sexual, or parasexual, cycles. In some examples, sexual and parasexual unions of pathogenic fungi involve closely related individuals, generating diversity in the population but with more restricted recombination than expected from fertile, sexual, outcrossing and recombining populations. In other cases, species and isolates participate in global outcrossing populations with the capacity for considerable levels of gene flow. These findings illustrate general principles of eukaryotic pathogen emergence with relevance for other fungi, parasitic eukaryotic pathogens, and both unicellular and multicellular eukaryotic organisms.

Introduction

A variety of fungal species are now recognised as being major human pathogens, responsible for causing life-threatening infections worldwide (Brown et al. 2012). Some species appear to have evolved to grow commensally, or as pathogens, on animal hosts whilst other species exist primarily in different ecological niches and cause opportunistic infections of humans, notably in those with suppressed immune systems (Tekaia and Latge 2005; Sharpton et al. 2009; Brown et al. 2012). A key aspect of the success of many of these fungal pathogens is their ability to produce infectious propagules by which they can enter and colonise a human host. Most human pathogens have traditionally been considered to reproduce by asexual means, thought to result in largely clonal populations. However, in this chapter we focus on advances involving a combination of experimental, genomic, and population genetic approaches that have reshaped our view of how fungal pathogens reproduce, with consequences for our understanding of how they might be evolving. We consider diverse fungi, focusing on basidiomycete *Cryptococcus* species, hemiascomycete *Candida* species, and euascomycete *Aspergillus* species, casual agents of the diseases cryptococcosis, candidiasis, and aspergillosis, respectively (Brown et al. 2012).

Sexual reproduction of *Cryptococcus neoformans*

The sexual cycles of *Cryptococcus neoformans* and *Cryptococcus gattii* were described to occur under laboratory conditions nearly four decades ago by June Kwon-Chung (Kwon-Chung 1975; Kwon-Chung 1976a; Kwon-Chung 1976b). Her studies revealed a bipolar mating system involving cells of two mating types, α and **a**. However, the observations that the vast

majority of the clinical and environmental isolate population are of mating type α (>95% for serotype D, >99.9% for serotype A, >95% for *C. gattii*) posed a conundrum (Kwon-Chung and Bennett 1978; Lengeler et al. 2000; Yan et al. 2002; Cogliati 2013). This was further compounded by the facts that 1) many isolates were sterile under lab conditions, 2) some lineages (serotype A VNII) are exclusively of one mating type, and 3) in some lineages isolates of **a** mating type are present but geographically restricted (serotype A VNB) (Litvintseva et al. 2003; Nielsen et al. 2003; Litvintseva et al. 2006; Bui et al. 2008; Litvintseva et al. 2011; Litvintseva and Mitchell 2012). Taken together, this led to a prevailing view that the **a**-α sexual cycle might be uncommon, geographically restricted, or absent in nature. Over the past decade robust genomic, genetic, and population genetic studies have provided considerable insight into how, when, and where *Cryptococcus* undergoes sexual reproduction, and have revealed extant heterothallic (**a**-α opposite sex) and homothallic (α-α unisexual) reproduction cycles that contribute to genetic recombination and both the preservation and the generation of genetic diversity (Hull and Heitman 2002; Heitman 2010; Heitman et al. 2013).

a-α opposite sex bisexual reproduction results in a dimorphic transition to hyphal development. The *Cryptococcus* **a**-α bipolar sexual cycle involves fusion of yeast cells of opposite mating type, formation of a dikaryon that undergoes a dimorphic transition to hyphae, and culminates in the formation of basidia where nuclear fusion and meiosis occur (Hull and Heitman 2002; McClelland et al. 2004). The four post-meiotic nuclei undergo repeated rounds of mitosis and budding from the basidium surface to produce four long chains of spores. The initial steps in mating involve pheromone production, secretion, and sensing by the two opposite

mating partners (Davidson et al. 2000). This interaction has features of anisogamy in some lineages, in which the **a** cells form enlarged, round cells whereas the α cells produce long conjugation tubes that fuse with the enlarged **a** partner (Wang et al. 2000; Nielsen et al. 2003). Under some conditions, and in some lineages, conjugation tubes and enlarged cells are less morphologically apparent, or both partners appear to produce conjugation tubes. Cell-cell fusion results in migration of the α nucleus into the **a** partner, and the paired nuclei migrate together and coordinately divide in the hyphae that are produced (McClelland et al. 2004). Concomitantly, mitochondria from the α parent are thought to be destroyed, leading to uniparental mitochondrial inheritance from the **a** parent (Xu et al. 2000). The dikaryotic hyphae produced during sexual reproduction have hallmark features of other basidiomycetes: the generation and fusion of clamp cells to produce fused clamp connections, a process that ensures faithful segregation of both parental nuclei, the formation of blastospores (yeast cells produced by budding from the dikaryotic hyphae), under some conditions formation of haustoria branches, and finally formation of the fruiting structures, the basidia. In contrast to the yeast *S. cerevisiae*, nuclear fusion does not occur immediately following cell-cell fusion, and is instead delayed until basidia form, wherein nuclear fusion and meiosis occur in response to unknown signals (Lee and Heitman 2012).

Monokaryotic fruiting resembles hyphal sexual development. A process that morphologically resembles **a**-α sexual reproduction (formation of hyphae, basidia, spores) but involves the solo culture of only one individual isolate was described by a series of investigators, including Jean Shadomy, Keith Erke, June Kwon-Chung, and Brian Wickes and Jeff Edman, and termed monokaryotic fruiting (Shadomy and Utz 1966; Lurie et al. 1971; Shadomy and Lurie 1971; Erke and Schneidau 1973; Erke 1976; Kwon-Chung 1978; Wickes et al. 1996). Shadomy

and independently Erke first observed clinical isolates that in solo culture produced largely monokaryotic hyphae with unfused clamps. However, terminal or sub-terminal cells were observed to be dikaryotic. This hyphal developmental process was more completely described by Wickes et al as occurring when haploid α cells were cultured on a very low nitrogen synthetic medium (filamentation agar), leading to a dimorphic transition from yeast to monokaryotic hyphae with unfused clamp connections, basidia, and rare spores (Wickes et al. 1996). Because the process occurred when isolates were cultured all on their own, without a partner of opposite mating type, it was hypothesized that this pathway involved an asexual transition to hyphal growth and sporulation that did not involve ploidy changes, meiosis, or recombination (Wickes et al. 1996; Wickes 2002).

While the initial reports focused on α strains that undergo monokaryotic fruiting, it was later appreciated that several isolates of **a** mating type are also competent to undergo this developmental pathway (Hull and Heitman 2002; Tscharke et al. 2003; Lin et al. 2006). Subsequently, detailed genetic studies revealed that this hyphal developmental pathway is a quantitative trait, controlled by as many as ~20 genes in the genome, the most prominent of which was found to be the *MAT* locus for which the α allele made a greater contribution to promote hyphal growth compared to the **a** allele (Lin et al. 2006). In addition to nitrogen limitation, higher temperatures may also promote the formation of hyphae associated with monokaryotic fruiting under some conditions, or with some isolates (Fu et al. 2013).

Unisexual reproduction is novel form of self-fertility. A paradigmatic advance was the discovery that monokaryotic fruiting represents a novel form of selfing sexual reproduction,

which has been termed unisexual reproduction or same sex mating (Lin et al. 2005). Appropriate conditions lead to a transition from yeast to hyphal growth of solo incubated lab strains, and this involves monokaryotic hyphae, unfused clamp connections, basidia and spores. Multiple lines of evidence prove conclusively that this is a true sexual cycle that includes meiosis. First, ploidy changes commonly occur during the process, and diploid blastospores can be recovered from hyphae produced by haploid isolates (Lin et al. 2005; Feretzaki and Heitman 2013a). Second, diploid isolates are enhanced for unisexual reproduction (Lin et al. 2005), and several hyperfilamentous isolates recovered from a genetic screen for altered unisexual reproduction were diploid (Feretzaki and Heitman 2013a). Third, key meiotic genes (*DMC1, SPO11*) are required for sporulation and spore viability, and this includes both Spo11 which inflicts the DNA DSB that provoke meiotic recombination and Dmc1 which is the protein that repairs these double strand breaks (Lin et al. 2005; Feretzaki and Heitman 2013a). Finally, recombination occurs at a high frequency during unisexual reproduction of a genetically marked diploid strain, on par with the frequency of meiotic recombination during **a**-α sexual reproduction (Lin et al. 2005). The karyogamy gene *KAR7* is required for both bisexual and unisexual reproduction, providing compelling evidence that nuclear fusion occurs during both developmental cascades (Lee and Heitman 2012). Laboratory conditions (darkness, room temperature, low CO_2, desiccation, low nitrogen, or presence of inositol) that stimulate bisexual reproduction of *Cryptococcus* similarly support unisexual reproduction of well validated haploid serotype D strains (XL280, JEC21) (Bahn et al. 2005; Idnurm and Heitman 2005; Xue et al. 2007).

Importantly, population genetics studies also provide evidence that unisexual reproduction also occurs in nature for both *C. neoformans* and *C. gattii*. First, the discovery and characterization of both αADα and αAAα isolates from the environment and clinical or

veterinary isolates provides support that unisexual reproduction occurs in nature from fusion of two α cells or endoreplication to generate hybrid diploid or homozygous diploid intermediates (Lin et al. 2007; Bui et al. 2008; Lin et al. 2009). Second, a series of studies provide evidence that recombination occurs in populations that are exclusively of α mating type (Bui et al. 2008; Hiremath et al. 2008; Saul et al. 2008; Chowdhary et al. 2011). Taken together, these laboratory and population genetics studies provide evidence that unisexual reproduction may be a predominant mode of sexual reproduction for both *C. neoformans* and *C. gattii*, and may be a route via which infectious propagules are produced.

Unisexual reproduction can involve cell-cell fusion between genetically divergent isolates, leading to recombination and admixture of genetic information from both parents. Alternatively, unisexual reproduction can also involve two seemingly genetically identical genomes that are brought together into the diploid state either via cell-cell fusion (possibly even between mother and daughter cell) or via endoreplication (involving karyogamy or independent of karyogamy) (Lin et al. 2009; Feretzaki and Heitman 2013a; Feretzaki and Heitman 2013b). In the most extreme examples of selfing, a central question is why undergo sexual reproduction if there is no genetic diversity to reshuffle? The null hypothesis is that there is no evolutionary benefit to this form of selfing, and any cost might be minimal or simply tolerated. On the other hand, the frequent and independent origins of homothallism throughout the fungal kingdom suggest there may be benefits conferred by this novel mode of sexual reproduction. Unisexual reproduction involves a dimorphic transition from yeast to hyphal growth, and recent studies reveal that unisexual strains have an enhanced capacity to explore their environment to forage for nutrients compared to isolates that are asexual (Phadke et al. 2013). Other recent studies reveal that unisexual reproduction can generate phenotypic and genotypic diversity *de novo*, and that

much of this diversity is attributable to aneuploidy (Ni et al. 2013). While a prevailing view is that aneuploidy is uniformly deleterious, in fungi a large number of studies have revealed aneuploidy promotes adaptive evolutionary trajectories and underlies morphogenic switches (Yona et al. 2012; Tan et al. 2013). Similar pathways may operate in animals as well, such as the frequent changes in ploidy and aneuploidy observed in hepatocytes (Duncan et al. 2010). In the specific case of aneuploid progeny produced by unisexual reproduction, these isolates can exhibit azole resistance and virulence equivalent to the euploid parental strain even in the harsh milieu of experimentally infected mice (Ni et al. 2013). It is likely that other adaptive benefits may be conferred by unisexual reproduction that will be brought to light by future studies (Roach et al. 2014).

Insights on sexual reproduction from the vantage point of genetic hybrids. The discovery and analysis of hybrid, diploid *Cryptococcus* isolates reveals that both opposite sex and unisexual reproduction occur in nature, and provides insights into which isolates are undergoing hybridization, possible benefits of hybridization, and species boundaries. These isolates include those between the different serotypes (AD, AB, BD) and within (AA, DD, BB) and provide several key insights. First, the finding of **a**ADα and αAD**a** hybrids with two opposite mating types provides indirect evidence of opposite sex mating that must occur in nature (Lengeler et al. 2001; Xu et al. 2002; Li et al. 2012). Second, the discovery of diploid **a**ADα hybrids found globally that descend from the haploid serotype A VNB lineage that is geographically restricted to South Africa and Botswana (Litvintseva et al. 2003), reveals that hybridization can broaden the environmental niche for otherwise provincial species and sub-types (Litvintseva et al. 2007). This may result from hybrid fitness, as the hybrids are more resistant to UV and more fit at

higher growth temperatures. The ability of these hybrids to survive in distinct environmental niches, such as pigeon guano, compared to the restricted niche of the VNB parental isolates (Mopane trees) may also contribute. Third, the discovery of hybrid serotype A isolates that are αAAα provides evidence that these may represent both an intermediate and a product of sexual reproduction (in addition to haploid recombinant progeny), and also provide evidence of unisexual reproduction in nature (Bui et al. 2008; Lin et al. 2009). This is echoed by the discovery and characterization of αADα hybrids (Lin et al. 2007).

While *C. neoformans* had been thought to be a predominantly haploid organism, diploid isolates represent ~10% of the clinical and environmental isolates examined (Lin et al. 2009). A fundamental question is by which mechanisms are these diploids formed? There are two general routes: (i) endoreplication and (ii) pathways involving karyogamy with or without cell-cell fusion. Endoduplication yields homozygous diploids, and identified αAAα, **a**AA**a**, αDDα, **a**DD**a** homozygous isolates may have been generated by this process (Cogliati et al. 2001; Lin et al. 2007; Li et al. 2012). Other heterozygous diploids (αAA**a**, αDD**a**, αAD**a**, **a**ADα, and αADα) are clearly hybrid isolates that must have been generated by cell-cell and nuclear fusion (Cogliati et al. 2001; Litvintseva et al. 2007; Lin et al. 2009). Diploidization can occur either early or late during both opposite-sex and unisexual reproduction. During opposite-sex reproduction, nuclear fusion typically occurs late, in the basidium, just prior to meiosis. However, nuclear fusion can also occur early during opposite-sex mating, giving rise to diploid isolates that form a hyphal monokaryon with unfused clamp connections, which go on to produce basidia and undergo meiosis. This is a well-established lab method to isolate and study diploids in the lab (Hull et al. 2002; Hull and Heitman 2002). **a**/α diploid strains are thermally dimorphic; the diploid cell grows as yeast at 37°C, but at 24°C, the diploid cell produces

monokaryotic hyphae and completes sexual reproduction (Sia et al. 2000). Nuclear fusion also occurs either early or late during unisexual reproduction, giving rise to either haploid or diploid monokaryotic hyphae. In the case of early nuclear fusion this enhances hyphal growth; in the case of late nuclear fusion, this likely occurs in the basidium just prior to meiosis, similar to opposite-sex sexual reproduction (Feretzaki and Heitman 2013a).

Evidence from population genetics studies on sexual reproduction. Population genetic analysis can be used to detect signatures of sexual recombination as the random association of alleles among individuals within a population. Polymorphic alleles within a genome can be generated via amplification, e.g. DNA fingerprinting and Amplified Fragment Length Polymorphisms (AFLP) or sequencing, e.g. Multi-Locus Sequence Polymorphisms (MLST) and Whole Genome Sequencing (WGS), and there are various approaches to detect statistically significant evidence of clonality or recombination (for examples see (Burt et al. 1996; Carter et al. 2007; Simwami et al. 2011). As fungi can reproduce both sexually and asexually, with either mode favored under different conditions, most fungal species comprise a mosaic of clonal lineages and sexually recombining clusters, with any given population potentially dominated by one or the other type. This can complicate population studies but it can also provide important insights into the expansion and spread of fungi, where migration, propagation, and recombination work together to drive evolution and adaptation. Understanding these can help us to unravel how *Cryptococcus* has evolved to become the important global pathogen that it is today, and may allow us to predict the likelihood of future changes in its virulence, dispersal, and range.

The evolution of a species is driven at the population level, but it can be difficult to define exactly what constitutes a population and at what stage divergent populations are considered different species. *Cryptococcus neoformans* was formerly described as one species, first with two varieties, var. *neoformans* and var. *gattii* (Kwon-Chung et al. 1982), which were subsequently joined by a third, var. *grubii* (Franzot et al. 1999). The recognition of considerable differences in genetic makeup, epidemiology, ecology, host range, and disease manifestation led to the elevation of *C. neoformans* var. *gattii* to full species status as *Cryptococcus gattii* (Kwon-Chung et al. 2002). Serological and molecular analyses have since determined that there are distinctly different serotypes and genotypes within both *C. neoformans* and *C. gattii* (Chen et al. 1996; Meyer et al. 2003; Bovers et al. 2008; Meyer et al. 2009), and it has been argued that some of these may also represent different species (Ngamskulrungroj et al. 2009). Diploid and aneuploid inter-species and intervarietal hybrids have also been reported (Boekhout et al. 2001; Cogliati et al. 2001; Lengeler et al. 2001; Lin et al. 2007), and sub-genotypes have been further defined within some *Cryptococcus* genotypes (Fraser et al. 2005; Chen et al. 2008; Byrnes et al. 2009; Byrnes et al. 2011; Day et al. 2011). It is clear that the population structure of the pathogenic *Cryptococcus* species is complex and is subject to ongoing, dynamic changes. While only two species are currently recognized, as many as six to nine distinct taxa likely populate the pathogenic species complex (VNI, VNII, VNB, VNIV, VGI, VGII, VGIIIa, VGIIIb, and VGIV).

At the global level, *C. neoformans* var. *grubii* (Serotype A; molecular genotypes VNI, VNII, and VNB) causes the vast majority of infections. Within this variety molecular genotype VNI is the most prevalent and is found on every continent except Antarctica, as well as inhabited sub-continents and islands (Cogliati 2013). Weathered pigeon guano is a major environmental reservoir and is thought to be central to its transmission from the environment to humans (Khan

et al. 1978; Gallo et al. 1989; Stenderup et al. 1989; Khosravi 1997; Haag-Wackernagel and Moch 2004). As the common pigeon (*Columba livea*) is also found globally, and *C. neoformans* var. *grubii* can grow and mate on media supplemented with pigeon guano (Nielsen et al. 2007), it is generally assumed that widespread dissemination has co-occurred with pigeons following migratory and trade routes (Littman and Borok 1968). Population analyses of *C. neoformans* var. *grubii* using MLST found a very low level of genetic diversity, with the majority of global strains represented by very few genotypes, and an extreme bias of mating type α strains, consistent with recent, clonal propagation and widespread dispersal to most areas of the world. However, studies conducted at more local levels have found evidence of recombination among isolates sourced from trees in Africa (Litvintseva and Mitchell 2012) and India (Hiremath et al. 2008) and infected animals in Australia (Bui et al. 2008). The presence of ancestral genotypes in African populations originating from native mopane (*Colophospermum mopane*) trees suggests that contemporary global strains associated with pigeon guano originated in Africa (Litvintseva et al. 2011; Simwami et al. 2011; Litvintseva and Mitchell 2012), and the jump in niche to guano enabled a massive amplification and expansion of the species' range.

There is evidence that host shift and range expansion appear to likewise shape modern outbreaks of cryptococcosis caused by *C. gattii*. Phylogenetic analysis indicates molecular genotype VGII is the ancestral *C. gattii* genotype (Bovers et al. 2008; Ngamskulrungroj et al. 2009), and population studies show recombination occurs within this genotype on global (Ngamskulrungroj et al. 2009; Carter et al. 2011) and local (Campbell et al. 2005a; Carriconde et al. 2011) scales. However, this genotype also comprises some highly clonal derived lineages, including sub-genotypes VGIIa, VGIIb, and VGIIc that are responsible for large, ongoing outbreaks of cryptococcosis in Vancouver Island, Canada, and in the Pacific Northwest of the

USA, areas where *C. gattii,* which is generally confined to warmer regions, had not previously been found (Kidd et al. 2004; Byrnes et al. 2009; Byrnes et al. 2010). The ecological niche for VGII is not known; however, recent evidence using coalescent analysis suggests it may have emerged from the Amazon rainforest (Hagen et al. 2013). Isolates from the outbreaks are exclusively mating type α, and amplification in local niches may be via asexual propagation or α-α mating (Fraser et al. 2005).

The other major *C. gattii* genotype that is found globally is VGI (Cogliati 2013). The origin of this genotype is enigmatic, as while it occurs at a relatively high frequency in Australia and can be readily sourced from certain *Eucalyptus* tree species (Ellis and Pfeiffer 1990a; Ellis and Pfeiffer 1990b), the level of genetic diversity across Australia is very limited (Campbell and Carter 2006), which is inconsistent with an ancestral origin in this region. In addition, although both mating types have been found (Halliday et al. 1999), fertility is low (Campbell et al. 2005b), there is no evidence for sexual recombination except on extremely local scales comprising single tree hollows (Campbell et al. 2005a; Saul et al. 2008; Carter et al. 2011), and dispersal even among trees in close proximity appears to be minimal (Halliday and Carter 2003). It is therefore possible that, as with pigeon guano in *C. neoformans, Eucalyptus* trees are not the realized ecological niche of *C. gattii* VGI but are the site of a successful host jump, with subsequent large-scale amplification.

In summary, population studies of *Cryptococcus* indicate a capacity for novel strains to emerge, colonize new hosts, and rapidly amplify in the environment. If this occurs in association with susceptible hosts, as in the case of *C. neoformans* and the HIV/AIDS population, or with an increased propensity to cause disease, as has occurred with *C. gattii* VGIIa in the Pacific northwest, large outbreaks of cryptococcosis can ensue. This has parallels with other fungal

pathogen-host systems, including chytridiomycosis in frogs, white-nose disease in bats, and die-back disease in jarrah forests, where a combination of high virulence, rapid reproduction, long-lived environmental stages and the capacity to infect multiple hosts, can combine to cause massive outbreaks and drive host species to extirpation and extinction (Fisher et al. 2012). *Cryptococcus* manifests all of these attributes, and with ongoing globalization and climate change, capacity for pathogens to spread and evolve may be further enhanced (Garcia-Solache and Casadevall 2010). The biology and population structure of *Cryptococcus* predicts that it will not be a question of whether further outbreaks will occur, but when, and how large they will be.

Sexual reproduction links to virulence. Sexual reproduction is linked to virulence of *Cryptococcus* via the production of infectious spores, in that α isolates can be more virulent than congenic **a** isolates, and possibly also via the production of a novel cell type (Titan or giant cells) observed in the lungs of infected animals or patients (Okagaki et al. 2010; Zaragoza et al. 2010). Infections by *Cryptococcus* are caused by inhalation of infectious propagules, which are thought to be spores, desiccated yeast cells, or both, all of which are small enough to fit down into the alveoli of the lung. Spores have been documented to be bona fide infectious propagules in a series of studies, including via inhalation and direct intracerebral routes of infection (Zimmer et al. 1984; Sukroongreung et al. 1998; Giles et al. 2009; Velagapudi et al. 2009). Moreover, when spores encounter alveolar macrophages, they can be directly phagocytosed, in contrast to yeast cells which require opsonization (Giles et al. 2009; Velagapudi et al. 2009). Mating type has also been associated with virulence in animal model studies, and found to be linked to α mating type in some serotypes and strain backgrounds, but not in others (Kwon-Chung et al. 1992; Nielsen et al. 2003; Barchiesi et al. 2005; Nielsen et al. 2005a; Nielsen et al. 2005b; Zhai et al.

2013; Zhu et al. 2013). Finally, the discovery of dramatically enlarged giant or titan cells in the lungs of infected animals, in which the ploidy has increased from haploid to octoploid or even higher, may produce a novel cell type that evades phagocytosis and plays roles in latency, immune evasion, or dissemination (Okagaki et al. 2010; Zaragoza et al. 2010). How these cells of higher ploidy are produced is not yet understood at a mechanistic level, but may involve some type of unisexual reproduction occurring *in vivo* (Zaragoza and Nielsen 2013).

Parasexual reproduction of *Candida albicans*

Mating by *Candida albicans* and related species is highly similar, but not identical, to that of its preeminent relative *Saccharomyces cerevisiae*, which has served as a general model and reference point for elucidating mating systems in other fungi. Some of the differences are due to the rewiring of regulatory networks, but others are more biological and appear to be related to the pathogenic life style of members of the *Candida* clade.

For *S. cerevisiae* to become mating-competent, diploids (**a**/α), which predominate in nature, undergo meiosis, and the progeny, which are **a** or α at the mating type locus (*MTL*), and thus are released from **a**1-α2 repression, are immediately mating competent. *C. albicans,* the species that will be highlighted in the *Candida* clade, and its closely related species, are also diploid in nature, but must undergo homozygosis at the mating type locus (*MTL*) to mate, remaining diploid with either an **a/a** or α/α *MTL* genotype (Figure 1A). Other members of the *Candida* clade are haploid and some clearly must undergo meiosis (Butler et al. 2009; Reedy et al. 2009). But even though **a/a** and α/α strains of *C. albicans* are relieved of **a**1-α2 repression, they are still not mating competent. Homozygosis is only the first step in attaining mating

competence. Homozygosis occurs *in vitro* primarily by the loss of one chromosome 5 homolog, which harbors the *MTL* locus (Hull and Johnson 1999), followed by duplication of the retained homolog (Wu et al. 2005). In nature, mitotic crossing over may be the major mechanism generating *MTL* homozygous isolates (Wu et al. 2007). In *C. albicans*, release from **a**1-α2 repression by loss of *MTL***a***1* or *MTL*α*2*, derepresses *MTL***a***2* or *MTL*α*1* respectively, which encode an activator of cell type-specific mating genes (Figure 1A). But to achieve mating-competence in *C. albicans* (Miller and Johnson 2002; Lockhart et al. 2003a) and related species (Pujol et al. 2004; Porman et al. 2011), **a**/**a** and α/α cells must then undergo a second complex phenotypic transition, from a "white" traditional yeast-like morphology, to a unique opaque morphology (Figure 1B), a transition discovered more than 25 years ago (Slutsky et al. 1987). This transition, like mating, is also suppressed by the **a**1-α2 complex (Miller and Johnson 2002). Thus homozygosis releases *C. albicans* switching from **a**1-α2 repression. This transition involves changes in virtually every architectural and physiological aspect of the cell and involves the regulation of more than 5% of the genes in the *C. albicans* genome (Lan et al. 2002; Lohse and Johnson 2010). Switching is regulated by a putative master switch gene, *WOR1* (Huang et al. 2006; Srikantha et al. 2006; Zordan et al. 2006), which in turn is regulated by a network of interacting transcription factors (Zordan et al. 2006; Lohse and Johnson 2010; Hernday et al. 2013). Switching is also regulated by histone deacetylases (Klar et al. 2001; Srikantha et al. 2001; Hnisz et al. 2009) (Figure 1B), alternative regulatory pathways, including one that involves the MAP kinase pathway and the target transcription factor Cph1 (Ramirez-Zavala et al. 2013), and the mediator complex (Zhang et al. 2013). The white-opaque transition occurs spontaneously, but is affected by levels of CO_2 and O_2 (Ramirez-Zavala et al. 2008; Huang et al. 2009), environmental insults such as UV irradiation (Morrow et al. 1989), low and high

temperatures (Slutsky et al. 1987), and the carbon source, in particular N-acetyl glucosamine (Huang et al. 2009) (Figure 1B). Recently, it has been shown that **a**/α cells of *C. albicans* can also undergo switching under select conditions, but are mating incompetent (Xie et al. 2013) and that rare haploid strains of *C. albicans* must switch to mate (Hickman et al. 2013).

In the mating process, **a/a** cells release the **a**-pheromone Mf**a**1 (Bennett et al. 2003; Lockhart et al. 2003b; Panwar et al. 2003) and α/α cells produce the α-pheromone Mfα1 (Daniels et al. 2006). As in *S. cerevisiae*, pheromones play two direct roles in the mating process, first as inducers of the opaque cell mating response and second, as a chemotropic attractant (Figure 1C). In the mating response, cells with the *MTL* configuration **a/a** respond to Mfα1 by up-regulating genes involved in the signal transduction pathway, in the processing and release of pheromones, and in the cellular changes involved in the mating process such as polarization and evagination (shmooing) (Bennett et al. 2003; Daniels et al. 2003; Lockhart et al. 2003b; Panwar et al. 2003; Zhao et al. 2005). Interestingly, pheromones also up-regulate a set of genes involved in hyphal formation (Zhao et al. 2005), which may explain why *C. albicans* can form such long conjugation tubes with some features shared with hyphae (Zhao et al. 2005; Chapa et al. 2011). These genes are not up-regulated in the response of *S. cerevisiae* to pheromone, which involves formation of only small mating evaginations, probably because *S. cerevisiae* lacks many of these hypha-related genes (Zhao et al. 2005). Mating projections of opaque **a/a** and α/α cells, on the other hand, may have to travel long distances to find each other. The pheromones of *C. albicans* also act as chemotropic agents over long distances, as demonstrated by orientation studies of seeded opaque cells in white cell biofilms (Figure 1C) (Daniels et al. 2006). In environments where gradients can form and be protected from diffusion and mechanical perturbations, **a/a** and α/α conjugation tubes move up opposing pheromone gradients, fusing at their tips (Figure 1C).

The nuclei move into the tubes and fuse near the position of tip fusion (Daniels et al. 2003; Bennett and Johnson 2005) (Figure 1C). The first daughter cell forms next to the junction on the **a/a** side (Daniels et al. 2003). The tetraploid nucleus divides and one tetraploid **a/a**/α/α daughter nucleus enters the daughter cell (Figure 1C). The pheromone-induced mating response pathway includes the alternative pheromones (Mfα1, Mf**a**1), their respective receptors (Ste2, Ste3), the trimeric G-protein complex (composed of Cag1, Ste18, and Ste4), the MAP kinase cascade (composed of Cst20, Ste11, Hst7, and Cek1/Cek2), the scaffold for the cascade (Cst5), and the target transcription factor Cph1 (Chen et al. 2002; Magee et al. 2002; Yi et al. 2008; Cote et al. 2011; Yi et al. 2011a) (Figure 1D). These are all orthologs of the components in the *S. cerevisiae* pathway (Figure 1D), suggesting conservation of this pheromone response pathway for mating in the hemiascomycetes.

Although there is the suggestion that *C. albicans* has many of the genes necessary for a full sexual cycle, including meiosis (Tzung et al. 2001), the latter has not been observed. Instead, tetraploids undergo stochastic chromosome loss in order to return to the diploid or near diploid state via a parasexual process (Bennett et al. 2003; Forche et al. 2008; Berman and Hadany 2012). Similarly, it is assumed that the very rare occurrence of haploids (Hickman et al. 2013) is a result of a similar mechanism. Genes for mating systems have also been demonstrated in a variety of other species, both haploid and diploid, in the *Candida* clade, with small variations (Butler et al. 2009; Reedy et al. 2009; Sai et al. 2011). For instance, *Candida lusitaniae* lacks α2 in the *MTL*α locus, *Candida guilliermondii* lacks α2 in the *MTL*α locus and **a**1 in the *MTL***a** locus, and *C. parapsilosis* lacks **a**1 in the *MTL***a** locus, yet they contain all of the nonsex genes and the rest of the **a** and α genes found in the *C. albicans MAT* locus (Butler et al. 2009; Reedy et al. 2009; Sai et al. 2011). Even with such deletions, some of these *Candida*

species, such as *C. lusitaniae,* undergo mating and a complete sexual cycle, including meiosis (Francois et al. 2001; Reedy et al. 2009).

But what may prove to be the most unique aspect of mating, in at least the subgroup of the *Candida* clade that includes *C. albicans*, is the role of the white-opaque transition. The pheromone released by *MTL*-homozygous opaque cells also induces mating-incompetent white cells of opposite mating type to form a biofilm architecturally similar to that formed by **a**/α cells (Yi et al. 2008; Yi et al. 2011a; Daniels et al. 2013). This *MTL*-homozygous biofilm facilitates chemotropism (Daniels et al. 2006) and mating (Park et al. 2013) between seeded minority opaque **a/a** and α/α cells (Daniels et al. 2006). It has been hypothesized that the white-opaque transition may have evolved to facilitate mating in a host (Soll 2013). Although the white biofilm is architecturally similar to the resistant **a**/α biofilm, it does not exhibit the pathogenic traits of impermeability to low and high molecular weight molecules, impenetrability by human polymorphonuclear leukocytes, and drug resistance (Yi et al. 2011b). In stark contrast, *MTL*-homozygous white cell biofilms are permeable, penetrable, and drug susceptible, traits consistent with a microenvironment that will allow pheromone gradients to form, but retard diffusion and protect against mechanical perturbations, and permit conjugation tube extension during chemotropism. Both the opaque mating response and the white biofilm response are coordinately induced by the same pheromones, a necessity if the latter is to facilitate the former (Soll 2013). Furthermore, if there is a paucity of opaque cells, white cells will self-induce the formation of white cell biofilms by a paracrine system in which white **a/a** cells release α-pheromone and opaque α/α cells will release **a**-pheromone, for self-induction (Yi et al. 2011b).

Sexual reproduction of *Aspergillus fumigatus*

The majority of *Aspergillus* species are only known to reproduce by asexual means. The opportunistic pathogen *Aspergillus fumigatus* was typical of these in that for most of its documented history it was presumed to be a purely asexual organism (Samson et al. 2009). However, accumulating evidence from population genetic, genomic, and experimental work strongly indicated that *A. fumigatus* might have a cryptic [i.e. covert, hidden and not yet identified (Heitman 2010)] sexual cycle, with the species exhibiting many signs of 'clandestine' sexual activity (Gow 2005). Population genetic analyses had revealed evidence of genetic divergence and possible recombination within worldwide populations (Varga and Toth 2003; Paoletti et al. 2005; Pringle et al. 2005; Bain et al. 2007; O'Gorman et al. 2009). Genomic analysis had shown the presence of over 200 known 'sex-related' genes in the genomes of representative isolates, and importantly these genes appeared functional, with no evidence of point or frameshift mutations (Galagan et al. 2005; Fedorova et al. 2008; Dyer and O'Gorman 2012). Finally, experimental work had revealed the presence of complementary *MAT1-1* and *MAT1-2* isolates within worldwide populations with a nearly even distribution of mating types, consistent with a heterothallic (obligate outcrossing) sexual breeding system encountered in many filamentous ascomycete fungi (Paoletti et al. 2005) The latter study also showed that mating-type and pheromone signaling genes, associated with sex and mating, were expressed under laboratory conditions in both single and mixed mating-type cultures.

Sexual cycle in *Aspergillus fumigatus*. A major breakthrough was then reported in 2009, with the discovery that a complete heterothallic sexual cycle could be induced *in vitro* for *A.*

fumigatus when isolates of opposite *MAT1-1* and *MAT1-2* mating type were crossed in a barrage formation on oatmeal agar at 30ºC in the dark (O'Gorman et al. 2009). The developmental process took 6-12 months to complete and led to the production of cleistothecia (enclosed sexual fruiting bodies) which contained asci within which were formed sexual ascospores (Figure 2). These sexual ascospores exhibited high temperature resistance, for example being able to survive heat shock at 70ºC for 90 minutes [as is characteristic of many *Neosartoya* species (Samson et al. 2007)], which has recently been correlated with the presence of a relatively thick ascospore cell wall (Kwon-Chung and Sugui 2013) Ascospores germinated to form colonies that showed evidence of genetic recombination based on analysis of mating-type and DNA fingerprint markers (O'Gorman et al. 2009). The newly discovered teleomorph (sexual state) was named *Neosartorya fumigata.* This discovery was of great significance as it indicated that *A. fumigatus* should be able to generate increased genetic diversity via the sexual cycle to allow evolution in response to environmental change. In the context of an opportunistic pathogen this might involve evolution of resistance to antifungal agents (see below) and greater virulence. The sexual cycle also offered a valuable genetic tool to study the inheritance and genetic basis of traits of interest.

The discovery of a sexual cycle triggered much subsequent work. Szewczyk and Krappmann (2010) reported that conserved *MAT1-1, MAT1-2*, and *nsdD* regulators of mating in filamentous ascomycete species were also required for sexual development in *A. fumigatus* (Szewczyk and Krappmann 2010). Alvarez-Perez et al. (2010) reported a possible link between the *MAT1-1* genotype and increased virulence in a collection of Spanish isolates. A major advance in the use of the sexual cycle as a genetic tool was then made by Sugui et al. (2011) who identified a 'supermater' pair of isolates which exhibited high sexual fertility relative to other isolates tested, and which could complete the sexual cycle and produce viable ascospores within

4 weeks (although highest ascospore viability was not reached until 20 weeks of incubation). The supermater isolates, AFB62 (*MAT1-1*) isolated from a case of invasive aspergillosis and AFIR928 (*MAT1-2*) from the environment (O'Gorman et al. 2009), were further characterised and shown to be highly virulent in two different murine models and to have a high recombination frequency. Thus, they provide an excellent tool for genetic studies of *A. fumigatus* (Sugui et al. 2011).

Identification of sexual cycles in other 'asexual' pathogenic *Aspergillus* species. Although *A. fumigatus* is the principal causal agent of aspergillosis worldwide, other species of *Aspergillus* such as *A. lentulus, A. flavus*, *A. terreus*, and *A. niger* are also known to cause infections in immunocompromised hosts (Balajee et al. 2005; Dagenais and Keller 2009). As with *A. fumigatus*, all of these species were once presumed asexual organisms. However, by genetic analysis of the presence of mating-type genes and careful manipulation of laboratory conditions, following on from the discovery of sex in *A. fumigatus*, it has since proved possible to induce heterothallic sexual cycles in all of these species except for *A. niger* (Horn et al. 2009; Dyer and O'Gorman 2011; Dyer and O'Gorman 2012; Arabatzis and Velegraki 2013; Swilaiman et al. 2013), indicating that these opportunistic pathogens also have the opportunity to evolve by sexual recombination. However, it is interesting to note that all of these species are 'fastidious' in respect of their demands for environmental conditions to induce sex relative to many other non-pathogenic *Aspergillus* species, possibly linked to an increased ability to reproduce prolifically by asexual means (Kwon-Chung and Sugui 2009; Samson et al. 2009).

Significance of sexual fertility for *Aspergillus* pathogen population biology. Although a sexual cycle has now been demonstrated in *A. fumigatus* and other aspergilli capable of causing opportunistic disease, one outstanding question regards the degree of sexual fertility within natural populations. It has been suggested that many fungal species, particularly well adapted pathogenic species, might be undergoing a 'slow decline' in sexual fertility as a step towards the evolution of asexuality (Dyer and Paoletti 2005). In the case of *A. fumigatus*, O'Gorman et al. (2009) initially demonstrated sexual fertility of isolates only from a population from Dublin, Ireland and the fertility status of worldwide isolates remained unknown (O'Gorman et al. 2009). However, since then sexual fertility (in terms of ability to produce cleistothecia with viable ascospores) has been demonstrated for many clinical isolates from the USA and other parts of Europe (Szewczyk and Krappmann 2010; Sugui et al. 2011; Camps et al. 2012; C O'Gorman and P Dyer, unpubl.) consistent with *A. fumigatus* representing a globally distributed single species capable of sexual reproduction (Pringle et al. 2005). By contrast, many crosses of the emerging agent of aspergillosis *Neosartorya* (*Aspergillus*) *udagawae* either failed to produce cleistothecia or produced ascospores which did not germinate, suggesting a fertility decline in this species (Sugui et al. 2010). Similarly, the majority of attempted crosses between *MAT1-1* and *MAT1-2* isolates of *A. lentulus* failed to produce cleistothecia, despite the selection of highly fertile tester isolates, again consistent with a slow decline in sexual fertility in this species (Swilaiman et al. 2013).

Significance of sex in *Aspergillus fumigatus* for the evolution and spread of resistance to antifungal drugs. There has been a worrying increase in the incidence of resistance to azole and other antifungal drugs in *A. fumigatus* over the past 20 years. This has followed a greatly

increased deployment of antifungal drugs as a result of the increase in cases of aspergillosis in humans, itself linked to an increase in the number of susceptible, immunocompromised patients (Snelders et al. 2008; Snelders et al. 2011). It is possible that the sexual cycle of *A. fumigatus* might be an important factor contributing to the evolution and spread of such antifungal-resistant strains of the fungus due to genetic recombination and dispersal of wind borne ascospores, which might have a greater ability to survive harsh environmental conditions than asexual conidia. Previous work with the fungal plant pathogen *Tapesia yallundae* demonstrated that sexual recombination could give rise to ascospore progeny showing a greater than three-fold increased resistance in azole fungicide resistance than either parent of a cross (Dyer et al. 2000), whilst spread of disease by wind-borne ascospores has also been reported for various plant pathogenic fungi (Camps et al. 2012), and the increased heat resistance of ascospores might allow survival in ecological niches where high temperatures might be encountered, such as composting vegetation (Swilaiman et al. 2013).

The molecular epidemiology of the most frequent form of resistance to azole antifungals by *A. fumigatus* in Europe, involving TR_{34}/L98H mutations in the region of the target *CYP51A* gene, was studied by Camps et al. (2012). Evidence was found that sexual reproduction might have contributed to early genetic diversification of a pool of resistant isolates, and that sexual crossing was possible between different CSP (putative cell surface protein) -type populations of the fungus, meaning that the resistance genotype might spread further in the future. Intriguingly, it was also found that sexual recombination led to the production of a novel microsatellite marker, presumably due to meiotic slippage, which might confound the use of such markers to monitor the spread of isolates (Camps et al. 2012). Sexual reproduction and gene flow has also been shown to be possible, albeit infrequent, between closely related fungal species, for example

in the plant pathogenic *Ophiostoma* species (Paoletti et al. 2006). This is of concern because there is the prospect that antifungal resistance might spread between closely related *Aspergillus* species, and there have been claims of abortive crossing between *A. fumigatus* and other *Neosartorya* species (Takada et al. 1986). However, attempted crosses between highly fertile isolates of *A. fumigatus* and *A. lentulus* failed to produce cleistothecia, demonstrating reproductive isolation between these sibling species. This was of medical significance because most isolates of *A. lentulus* exhibit increased natural resistance to several azole and echinocandin antifungal agents relative to *A. fumigatus*, so fortunately there would appear to be little risk of gene flow between the species (Swilaiman et al. 2013).

Conclusion

Based on the data presented for *Cryptococcus*, *Candida* and *Aspergillus* species it is clear that rather than being purely asexual and mitotic in nature, reproduction in fungal human pathogens instead appears to be much more varied. There have been the discoveries of typical fungal sexual cycles that had previously been overlooked in *Aspergillus* species, or more unusual systems to promote nuclear fusion and gene flow as seen in the unisexual mating of *Cryptococcus* and diploid mating in *Candida* species. The fact that these diverse fungal taxa have maintained methods to promote genetic variation, which might also confer other selective benefits, illustrates the general principles that we should not underestimate the potential for the continued evolution and emergence of fungal pathogens, a finding of relevance for other parasitic unicellular and multicellular eukaryotic organisms.

Figure legends

Figure 1. **Mating in *Candida albicans.***

Mating in *Candida albians* involves both homozygosis from **a**/α to **a**/**a** or α/α, and a switch from the white to opaque phenotype. A. Homozygosis from **a**/α to **a**/**a** or α/α results in the loss of the **a1-2** corepressor complex, which represses both mating and switching. B. Attaining mating competence is a two-step process, involving first homozygosis at the mating type locus, and a second phenotypic switch from the white to opaque phenotype. The latter involves a network of inter-regulatory and auto-regulatory transcription factors and chromatin modifications, affected by a number of environmental conditions. C. The cell biology of mating between opaque **a**/**a** and α/α cells is similar to that of *S. cerevisiae*, but involves long conjugation tubes and the formation of an **a**/**a**/α/α daughter cell. D. The pheromone response pathway for mating activation has been conserved in the hemiascomycetes, so that all components, pheromone to the major targeted transcription factor, are orthologs of those components in the *S. cerevisiae* response pathway.

Figure 2. **Sexual reproduction in *Aspergillus* species.**

Micrograph showing larger, ornamented lenticular ascospores (white arrowed) of *A. lentulus* relative to smaller, globose less heat resistant conidia (black arrowed). Scale bar indicates 5 µm. Image courtesy of C O'Gorman and S Swilaiman.

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