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The evolution of sexual reproduction and mating-type locus: links to pathogenesis of *Cryptococcus* human pathogenic fungi

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Abstract

Sexual reproduction generates genetic diversity and purges genomes of deleterious mutations, allowing organisms to adapt to changing environments and avoid extinction. *Cryptococcus* species utilize a variety of sexual reproduction mechanisms, which contribute to their ability to occupy myriad environmental niches and to exhibit a range of pathogenic potential in humans. Pathogenic *Cryptococcus* species can undergo both bisexual and unisexual reproduction when stimulated by properties associated with their environmental niches, which proceed through well-characterized signaling pathways and corresponding morphological changes. Genes governing mating are encoded within the mating-type (*MAT*) loci, and influence pathogenesis, population dynamics, and lineage divergence within each *Cryptococcus* species. Although the genes encoded by *MAT* remain largely conserved across these species, *MAT* has undergone several important evolutionary changes within the *Cryptococcus* genus. In *Cryptococcus*, *MAT* can exist as either two, genetically and physically unlinked loci in species with tetrapolar mating systems or as a single, large locus in bipolar mating systems; pathogenic *Cryptococcus* species employ bipolar systems for sexual reproduction, while their characterized nonpathogenic counterparts have tetrapolar mating systems. There is evidence that the transition from the ancestral tetrapolar state in nonpathogenic species to a bipolar mating system in pathogenic *Cryptococcus* species was mediated by intercentromeric recombination followed by multiple chromosomal rearrangements. In addition to the transition from tetrapolar to bipolar, the *Cryptococcus MAT* loci have experienced several internal reconfigurations. Due to the variety of established sexual reproduction mechanisms *Cryptococcus* species utilize and the robust characterization of the evolution of mating and *MAT* in this genus, *Cryptococcus* species provide key insights into the evolution of sexual reproduction.

I. Introduction

Sexual reproduction is ubiquitous throughout eukaryotic organisms. It involves mate recognition, zygote formation through gamete fusion, and then gamete or progeny formation through meiosis. In general, sexual reproduction allows reshuffling of genetic material from two parents, which generates recombinant progeny with variable adaptive potential and allows natural selection to act more efficiently to purge deleterious mutations that have accumulated in the parental genomes. While these basic characteristics are shared, there is

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great diversity in sexual reproduction strategies that have been adopted by different organisms (Goodenough and Heitman, 2014).

In fungi, sexual reproduction is governed by the mating-type locus (*MAT*), and modes of sexual reproduction differ across fungal species (Heitman et al., 2013). There are self-fertile, inbreeding species (homothallic), as well as outbreeding species where sexual reproduction only occurs between two individuals that are mating compatible (heterothallic). There are two primary mating-type systems that govern mating compatibility in fungi: bipolar mating systems (e.g. ascomycetes) in which mating compatibility is determined by a single *MAT* locus, and tetrapolar mating systems (e.g. most Basidiomycetous species) where mating compatibility is determined by two genetically and physically unlinked *MAT* loci. In addition to bipolar and tetrapolar mating systems, the number of functional alleles at the *MAT* loci also varies among different species, from bi-allelic to multi-allelic, which adds additional complexity to fungal mating systems. Moreover, different mating systems can often be identified in species that are closely related. Thus, the fungal kingdom provides a unique opportunity to study the underlying molecular mechanisms, as well as the evolutionary origins, dynamics, and consequences of sexual reproduction [reviewed in (Heitman, 2015; Heitman et al., 2017; Heitman et al., 2007; Kües et al., 2011; Lee et al., 2010; Ni et al., 2011)].

The human pathogenic *Cryptococcus* species complex belongs to the phylum Basidiomycota; based on genetic divergence and reproductive isolation identified through genetic crosses, there are currently seven different species defined in this complex (Hagen et al., 2015; Kwon-Chung et al., 2017; Sun and Xu, 2007, 2009). The *Cryptococcus* pathogenic species are major opportunistic human fungal pathogens that cause cryptococcal meningoencephalitis in both immunocompromised and immunocompetent individuals, and it has been estimated that *Cryptococcus* is responsible for 223,100 cases of meningoencephalitis annually, with an associated mortality rate of 20% to 70% (Rajasingham et al., 2017). Pathogenic *Cryptococcus* species have bipolar mating systems, with a single mating-type locus (*MAT*) and two alternative alleles that define the two compatible mating types: *MAT*α and *MAT***a**. The *MAT* locus in *Cryptococcus* is large (~120 kb), contains more than 20 genes, and has been shown to be associated with virulence (Lengeler et al., 2002; Lin et al., 2008). Interestingly, comparative genomic studies of *Cryptococcus* and its closely related non-pathogenic tetrapolar sister species revealed that the *MAT* locus of the bipolar mating system evolved from fusion of the two *MAT* loci in the tetrapolar mating system through ectopic recombination likely mediated via inter-centromeric recombination (Sun et al., 2017).

While *Cryptococcus* species can outcross through bisexual reproduction between isolates of α and **a** mating types, *MAT*α cells can also reproduce unisexually, which increases inbreeding potential and is an extreme form of inbreeding when it occurs through endoreplication of a solo *MAT*α cell (Fu and Heitman, 2017; Kwon-Chung, 1975, 1976b; Lin et al., 2005). Indeed, evidence of both bisexual and unisexual reproduction has been found in population genetic and genomic studies of natural isolates (Chowdhary et al., 2011; Desjardins et al., 2017; Farrer et al., 2015; Hiremath et al., 2008; Lin et al., 2007).

Thus, the pathogenic *Cryptococcus* species have a unique *MAT* locus with diverse mating systems, which provide excellent opportunities for studying the evolution of mating-type loci and mating systems, as well as the evolutionary dynamics and consequences of sexual reproduction. In this review, we focus on: 1) recent advances in studies of bisexual and unisexual reproduction; 2) how sexual reproduction in *Cryptococcus* affects population structure and dynamics, lineage differentiation, and speciation; and 3) evolution of the bipolar mating system from its ancestral tetrapolar mating system in *Cryptococcus* and other basidiomycetous species.

II. Sexual reproduction of the pathogenic *Cryptococcus* species

Bisexual and Unisexual reproduction

Cryptococcus species occupy a number of known environmental niches including Mopane trees native to sub-Saharan Africa, oak trees in North Carolina, eucalyptus trees, and the soil surrounding these trees, but are also known to associate with pigeon guano and animals including many bird and mammal species (Chowdhary et al., 2012; Farrer et al., 2015; Litvintseva et al., 2005; Lugarini et al., 2008; Malik et al., 2003; O'Brien et al., 2004; Singer et al., 2014). Compounds present within several of these niches stimulate mating of *Cryptococcus*. For example, myo-inositol, a sugar produced by most plants, and the plant hormone indole acetic acid, pigeon guano and various copper concentrations all contribute to initiating mating responses in *Cryptococcus* (Xue, 2012; Xue et al., 2007). Media made with pigeon guano and various copper concentrations have also been shown to stimulate robust mating (Figure 1) (Chitty et al., 2019; Gyawali et al., 2017; Kent et al., 2008; Lin et al., 2006; Nielsen et al., 2007; Staib, 1981). A myriad of additional environmental stimuli can also trigger *Cryptococcus* mating. Nutritional cues such as overall nutrient concentration, the absence of glucose, or the presence of glucosamine or galactose are all known to stimulate filamentation (Alspaugh et al., 1997; Xu et al., 2017). Cell-cell interactions among *Cryptococcus* isolates are also of primary importance in mating. Recently the quorum sensing peptide produced by *Cryptococcus* cells, Qsp1, which is involved in density-dependent growth and virulence, has also been shown to be involved in the regulation of sexual development (Homer et al., 2016; Lee et al., 2007; Tian et al., 2018). Many other abiotic factors including humidity, CO_2 concentration, light, and temperature all contribute to the complex and precise environmental state required to initiate mating in *Cryptococcus* (Figure 1) (Casadevall and Perfect, 1998; Heitman et al., 2011; Idnurm and Heitman, 2005a, b; Sia et al., 2000).

In the proper environment, *Cryptococcus* cells begin secreting small mating-associated lipid-modified peptides, known as pheromones (Davidson et al., 2000; McClelland et al., 2002). These pheromones are unique to either one or the other of the two *Cryptococcus* mating types, mating-type α (*MAT*α) and mating-type **a** (*MAT***a**), and are bound by the G-protein coupled receptors (GPCRs) Ste3α and Ste3**a** (Shen et al., 2002). Pheromone binding to this GPCR activates the pheromone response pathway of *Cryptococcus*, promoting expression of a variety of genes involved in mating including those encoded by the mating-type locus (*MAT*) (Figure 1). A constitutively active pheromone receptor-like GPCR, Cpr2, also activates the same signaling pathway (Hsueh et al., 2009). Following activation, the GPCR

and its associated subunits signal through the PAK kinase Ste20 to a mitogen-activated protein (MAP)-kinase cascade, which includes Ste11, Ste7, Cpk1, and a recently identified Ste50 adaptor protein (Jung et al., 2011; Wang and Heitman, 1999). This signaling cascade activates the transcription factor Mat2, which directly and indirectly regulates expression of genes encoded by the *MAT* locus, orchestrating a complex signaling network through several other transcription factors, including Znf2, and the mating-type-specific homeodomain proteins Sxi1α and Sxi2**a** (Hull et al., 2005; Hull et al., 2002; Kruzel et al., 2012; Lin et al., 2010b; Mead and Hull, 2016). Recent identification of additional novel factors involved in regulating mating in *Cryptococcus,* including the PAS domain protein Pas3 and Bre1, a ubiquitin ligase required for histone modification, illustrate the important role of epigenetics in this process and identify an opportunity for further study (Zhao et al., 2018). A signaling pathway independent of Mat2 has also been discovered and is regulated, in part, by Znf3, which helps to defend the genome by inhibiting transposition during meiosis (Feretzaki et al., 2016; Feretzaki and Heitman, 2013). Transposition is further regulated during sexual reproduction by an RNAi-dependent process known as sex-induced silencing (SIS) (Wang et al., 2013; Wang et al., 2010).

Following activation of the pheromone response signaling cascade, *Cryptococcus* cells undergo a number of morphological and cellular changes to complete the sexual cycle, which vary based on the mode of reproduction and species involved (Figure 2). Initially during bisexual reproduction, one of the mating partners (typically the *MAT*α parent) produces a conjugation tube that extends towards its mate, similar to the shmooing process in *Saccharomyces cerevisiae*. The plasma membranes of the mating partners eventually meet and fuse in a process mediated by the plasma membrane fusion protein Prm1 (Fu and Heitman, 2017). This cell-cell fusion generates a diploid zygote and the zygote subsequently produces a single, elongating hyphal filament, which typically initiates from the *MAT***a** cell. The hyphae are divided intermittently by septa and organelles, like mitochondria and nuclei, are appropriately partitioned. Like many eukaryotes, mitochondria are uniparentally inherited in *Cryptococcus*, typically from the *MAT***a** parent. While several genes involved in sexual reproduction, as well as certain environmental factors have been shown to influence the uniparental mitochondrial inheritance in *Cryptococcus*, the precise mechanism behind this process remains to be elucidated (Gyawali and Lin, 2011; Sun and Xu, 2007; Xu et al., 2000a; Yan et al., 2004; Yan et al., 2007a; Yan et al., 2007b; Yan and Xu, 2003). Once the growing hypha reaches an appropriate length, the hyphal tip differentiates into a terminal, globose basidium. Nuclear fusion and meiosis then typically occur within this structure, generating four recombinant products and marking the penultimate stage of sexual reproduction in *Cryptococcus*. Ultimately these meiotic products undergo repeated rounds of mitosis and bud from the basidium as four basipetal basidiospore chains (Kwon-Chung, 1975; Kwon-Chung, 1976a; Kwon-Chung, 1976b). Each of these processes that *Cryptococcus* undergoes during sexual reproduction are mediated by specific genetic regulation. Genes involved in cell-cell fusion (*PRM1* and *RSC9*), filamentation (*ZNF2*, *PUM1*, *FAS1*, *CFL1*, *PAS3*, *BRE1*), sporulation (*DDI1*, *DST1*, *TOP1*, *UBC5*) and germination (*IRR1*, *PRP11*, *PRP31*, *ISP2*, *EMC3*, *GRE202*) have been identified, although the genetic regulation specific for conjugation tube development and basidium formation has

not yet been characterized (Feretzaki and Heitman, 2013; Fu and Heitman, 2017; Huang et al., 2015; Lin et al., 2010a; Wang et al., 2014; Zhao et al., 2018).

During bisexual reproduction in *C. deneoformans*, nuclear fusion, also known as karyogamy, can occur at various stages following cell-cell fusion and is mediated by several karyogamy genes (Fu and Heitman, 2017; Lee and Heitman, 2012). *C. deneoformans* zygotes produce both dikaryotic hyphae, with two separate haploid nuclei, and monokaryotic hyphae, with a single migrating diploid nucleus, during bisexual reproduction. Conversely, a dikaryotic filament is maintained throughout bisexual reproduction in *C. neoformans* until nuclear fusion occurs in the basidium. In addition to bisexual reproduction, *C. deneoformans* can undergo unisexual reproduction, also known as haploid or monokaryotic fruiting (Fu et al., 2014; Lin et al., 2005; Sun and Heitman, 2015). Unlike bisexual reproduction, filaments produced during unisexual reproduction are exclusively monokaryotic and clamp cells along these monokaryotic filaments, which aid in nuclear segregation in dikaryotic hyphae, remain unfused (Lin et al., 2005). Diploidization during unisexual reproduction can occur through either fusion of two cells of the same mating type or through endoreplication (Feretzaki and Heitman, 2013; Fu and Heitman, 2017; Lin et al., 2005). Additionally, it appears that pheromones, the pheromone receptors, and the homeodomain proteins Sxi1**α** and Sxi2**a** are dispensable during unisexual reproduction in hyperfilamentous *Cryptococcus* strains (e.g. XL280) (Gyawali et al., 2017), although they may still be required for haploid fruiting in strains that are less filamentous (e.g. JEC21). Recently, it has been shown that the quorum sensing peptide, Qsp1, is involved in the initiation and coordination of unisexual reproduction in *Cryptococcus*, in a pheromone- and pheromone receptor-independent fashion. Additionally, an atypical zinc finger regulator Cqs2 has been identified as an important component of the Qsp1 signaling cascade (Tian et al., 2018).

Studies conducted on sexual reproduction in non-*neoformans Cryptococcus* species suggest that related species utilize similar mating mechanisms, but require different environmental conditions for success. For instance, mating in the pathogenic species *C. deuterogattii* is more efficient at lower temperatures and takes much longer to produce basidiospores than mating of the *C. neoformans* and *C. deneoformans* species (Fraser et al., 2003; Kwon-Chung, 1975; Kwon-Chung, 1976a; Kwon-Chung, 1976b). On the other hand, the closely related non-pathogenic *C. depauperatus* constitutively undergoes sexual reproduction regardless of environment and may be an example of an obligate sexual fungus (Findley et al., 2009; Kwon-Chung, 2011; Rodriguez-Carres et al., 2010).

Effects of *Cryptococcus* sexual reproduction on pathogenesis

The most widely acknowledged association between *Cryptococcus* sexual reproduction and pathogenesis is the generation of sexual basidiospores, which have been shown to be infectious propagules in both a murine inhalation virulence model and the invertebrate model host *Galleria mellonella* (Giles et al., 2009; Velagapudi et al., 2009). Compared to yeast cells, sexual spores are not only smaller in size, which makes them ideal for alveolar deposition, but also behave differently within the host (Alvarez and Casadevall, 2006; Botts et al., 2009). For example, while yeast cells cannot be phagocytosed by macrophages without opsonization, spores are readily phagocytosed in the absence of opsonins. In

addition, spores within macrophages are able to germinate and grow into yeast cells that can withstand reactive oxygen and nitrogen intermediates produced during the macrophage killing response, enabling them to proliferate and eventually escape from the macrophages and disseminate (Alvarez and Casadevall, 2006; Giles et al., 2009; Velagapudi et al., 2009).

Another hallmark of *Cryptococcus* sexual reproduction is the transition of growth from budding yeast to hyphae, and studies have shown that this morphological change might also be intricately connected with pathogenesis (Lin et al., 2010b; Neilson et al., 1978; Wang et al., 2012; Zimmer et al., 1984). For example, it has been previously shown that one of the key regulators of the yeast-to-hyphal morphological transition is the transcription factor Znf2 (Lin et al., 2010b). Interestingly, cells in which the *ZNF2* gene is deleted are locked in the yeast phase and exhibit elevated virulence in the murine inhalation model; on the other hand, cells in which the wild-type *ZNF2* allele is replaced with an overexpression allele show elevated filamentation, and consequently, attenuated virulence (Lin et al., 2010b; Wang et al., 2012). Remarkably, a recent study showed that mice pre-treated with either live or heat-killed *ZNF2*-overexpression filamentous cells were protected against subsequent infection with *Cryptococcus* isolates that would have otherwise been highly virulent (Zhai et al., 2015). However, it should be noted that hyphae production is not necessarily always associated with attenuated virulence, as some highly self-filamentous isolates (XL280) are more virulent compared closely related isolates that are less filamentous (JEC21) (Feretzaki et al., 2014).

Pathogenic *Cryptococcus* species are opportunistic human pathogens, which means their primary niches are natural environments, and they spend most of their time interacting with non-human organisms in these natural environments. Thus, from an evolutionary point of view, virulence factors that contribute to pathogenesis in humans may be byproducts of long-term survival in harsh, natural environments and defense strategies developed to combat and compete against natural predators. For example, it has been shown that hyphae (as well as pseudohyphae produced under nutrient limitation conditions) could be beneficial for *Cryptococcus* to survive amoeba, which is presumably one of their major natural predators (Casadevall, 2012; Lin et al., 2015; Magditch et al., 2012; Steenbergen et al., 2001). Additionally, the natural habitats of *Cryptococcus* include trees and bird droppings that are normally nutrient limited (promoting mating) and harsh (e.g. temperature fluctuations and extremes, UV and oxidative stress), which could be more challenging to yeast cells than to spores. Thus, the production of spores, an infectious propagule causing human infections, could also be a byproduct of a long-term strategy adopted by *Cryptococcus* to survive in nature (Botts et al., 2009; Steenbergen et al., 2001).

Another unique morphotype of *Cryptococcus,* referred to as the titan cell, was recently identified in the lungs of infected patients and mice, and was later successfully induced *in vitro* (Crabtree et al., 2012; Dambuza et al., 2018; Gerstein et al., 2015; Hommel et al., 2018; Okagaki et al., 2010; Okagaki et al., 2011; Trevijano-Contador et al., 2018). Titan cells are large (>20 um in diameter compared to ~5 um for typical yeast cells), have polyploid genomes, and produce budding cells that are usually haploid, diploid, or aneuploid. Titan cells are resistant to phagocytosis by macrophages, and thus can better sustain attacks from the host immune system than yeast cells. Thus, they may play unique

roles in latency, dissemination, and infection. The fact that titan cells are polyploid and produce haploid budding cells suggests that their cell cycle involves ploidy changes, mimicking what happens during sexual reproduction, undergoing 1N (parental cells) → 2N (zygotes) → 1N (meiotic progeny) transitions in ploidy. Although it is not known whether the formation of titan cells, as well as the subsequent generation of haploid/diploid/aneuploid daughter cells, involves sex or meiosis, these observations may reflect aspects of unisexual reproduction occurring during infection.

Effects of *Cryptococcus* sexual reproduction on population structure and dynamics in nature

Advances in sequencing technology have propelled the analyses of natural *Cryptococcus* isolates from population genetics to population genomics, allowing more comprehensive genomic studies to identify evolutionary signatures of clonal expansion, recombination, and gene flow, as well as signs of positive and/or purifying selection. Several recent studies of large collections of *C. neoformans* and *C. gattii* isolates showed that natural *Cryptococcus* populations are largely clonal, with signs of sexual reproduction and recombination occurring at low frequencies (Billmyre et al., 2014; Desjardins et al., 2017; Engelthaler et al., 2014; Farrer et al., 2015; Rhodes et al., 2017). Additionally, evidence of recombination has been detected in both VNBI/VNBII lineages that have both *MAT***a** and *MAT*α isolates occurring at comparable frequencies, and the VNI lineage in which the vast majority of the isolates are *MAT*α, consistent with models in which both bisexual and unisexual reproduction can promote recombination. This is in overall agreement with previous population genetics studies of *Cryptococcus* that identified clonal structures among natural *Cryptococcus* isolates (Hiremath et al., 2008; Randhawa et al., 2008). Interestingly, a recent population genomics study of different *C. neoformans* lineages revealed the **a** and α *MAT* loci have distinct evolutionary trajectories, and suggested introgression of the *MAT***a** allele from VNBI lineage to the VNI group (Desjardins et al., 2017). Specifically, the authors found that the *MAT*α alleles display a phylogeny that overall matches the whole-genome phylogeny with one exception: VNBI *MAT*α appeared to be paraphyletic with respect to VNBII, and has 2 distinct alleles – one comprised of some VNBI isolates and the other of the remaining VNBI isolate in addition to all of the VNBII isolates. The phylogeny of the *MAT***a** alleles also differed from whole-genome phylogeny in that the VNI and VNBI lineages are sister groups that are separated by a large distance from VNBII, suggesting that the extant *MAT***a** alleles of the VNI lineage originated from an introgression event from the VNBI group. Furthermore, the *MAT***a** allele transgressed into the VNI lineage carries ~5.3 kb of VNBI sequence upstream of the 5′ end of the *MAT* locus, consistent with previous studies showing the presence of recombination hot spots flanking *MAT* in *Cryptococcus* (Hsueh et al., 2006; Sun and Heitman, 2016; Sun et al., 2012). We hypothesize that the most common global *Cryptococcus* lineage (serotype A VNI) lost the *MAT***a** locus and then regained it by introgression from the divergent VNBI lineage endemic to sub-Saharan Africa. Notably, the VNI lineage is almost exclusively α mating-type (99.9%), consistent with a genetic bottleneck while exiting Africa and transition to unisexual reproduction. Introgression of mating-type alleles by inter-specific hybridization has also been reported in other fungi, such as the Dutch elm disease fungus *Ophiostoma novo-ulmi*. It has been shown that when this plant pathogen first invaded Europe as a series of clonal populations, there

was only one mating type (MAT-2) and a single vegetative incompatibility type. The populations then rapidly became diversified with the appearance of the MAT-1 mating type, which *O. novo-ulmi* likely acquired from another species, *Ophiostoma ulmi*, through inter-species hybridization. This inter-species gene transfer of the MAT-1 mating type has been hypothesized to have facilitated the rapid adaptation of *O. novo-ulmi* to a new environment after the initial invasion (Et-Touil et al., 1999; Paoletti et al., 2006).

Natural populations of *Cryptococcus* are dominated by isolates of the *MAT*α mating type (Randhawa et al., 2008), and *MAT***a** strains have been primarily isolated from African countries, such as Botswana (Litvintseva et al., 2003). Indeed, population genomics studies have shown that isolates from Africa display the clearest signals of sexual reproduction and have increased genetic diversity. However, the discovery of unisexual reproduction among *MAT*α cells, via either cell-cell fusion or endoreplication, suggests that the frequency of sexual reproduction in nature might have been underestimated (Fu and Heitman, 2017; Lin et al., 2005). Just as in bisexual reproduction, unisexual reproduction produces robust numbers of basidiospores, which could serve as infectious propagules in places where *MAT***a** cells are mostly absent. Additionally, it has been shown that compared to bisexual reproduction between *MAT*α and *MAT***a** cells, unisexual reproduction between *MAT*α cells has comparable frequencies of meiotic recombination, and consequently, has similar potential in reshuffling genetic materials from the two parental strains and generating progeny with unique genotypes (Desjardins et al., 2017; Farrer et al., 2015; Rhodes et al., 2017; Roth et al., 2018; Sun et al., 2014). Additionally, during unisexual reproduction, the two parental *MAT*α alleles are collinear, and thus allow proper pairing and crossing over to occur within the *MAT* region, which can enable recombinational repair of the *MAT* genes (Sun et al., 2014). Diploid *Cryptococcus* isolates with two *MAT*α alleles (e.g. αAAα, αDDα) have also been identified among natural isolates, providing further evidence that unisexual reproduction occurs in nature (Lin et al., 2007; Lin et al., 2009; Rhodes et al., 2017).

Effects of *Cryptococcus* sexual reproduction on lineage divergence and speciation

Sexual reproduction facilitates gene flow between diverging lineages, and therefore counteracts the establishment of species boundaries. Without sufficient interbreeding, when lineages of the same species diverge, the accumulation of genetic variants will reduce the viability and/or the fertility of progeny produced by inter-lineage sexual reproduction. Within the pathogenic *C. neoformans*/*C. gattii* species complex, there are currently as many as seven recognized species (Hagen et al., 2015). Additionally, within the *C. neoformans* species there are four lineages that are well-separated from each other phylogenetically (Farrer et al., 2015). It is most likely that the reproductive boundaries among the *Cryptococcus* species and lineages are post-zygotic, as inter-lineage and inter-species crosses often produce normal sexual structures, including hyphal growth, clamp cell formation, basidia formation, and basidiospore production. This is also consistent with studies showing that factors involved in pre-zygotic mating interactions, such as pheromones and pheromone receptor genes, are highly conserved and display mating-type specific topologies (Findley et al., 2009; Findley et al., 2012; Fraser et al., 2007).

Post-zygotic reproductive barriers are likely due to accumulated sequence divergence, including both sequence polymorphisms and gross chromosomal rearrangements that collectively compromise meiosis, particularly chromosomal mis-segregation during meiosis I. This typically increases the frequency of progeny with imbalanced genetic makeups that are either inviable or infertile. Indeed, during sexual reproduction between *C. neoformans* and *C. deneoformans*, progeny exhibit highly reduced viability, and are largely either diploid or aneuploid with various levels of heterozygosity throughout the genome, consistent with compromised meiosis with significantly reduced levels of recombination (Lengeler et al., 2001; Sun and Xu, 2007, 2009). Diploid serotype AD hybrid *Cryptococcus* isolates have also been frequently isolated from natural environments, suggesting either ongoing hybridization between *C. neoformans* and *C. deneoformans*, or that diploid hybrid progeny might be advantageous (hybrid vigor), and thus, persistent in natural environments (Lin et al., 2007; Lin et al., 2009; Xu et al., 2002; Xu et al., 2000b). Additionally, studies have shown that diploid and aneuploid *Cryptococcus* progeny produced from sexual reproduction can undergo LOH events that are either regional or across entire chromosomes through mitotic recombination and whole-chromosome loss and regain, respectively, which could provide additional sources of genetic and phenotypic diversity among meiotic progeny (Sun et al., 2014).

As mentioned earlier, post-zygotic reproductive isolation is likely due to genetic divergence accumulated between different lineages/species, including both nucleotide substitutions as well as chromosomal rearrangements such as inversions and translocations. It has been shown that during hybridization between different yeast (as well as bacteria) species, the DNA mismatch repair system functions to prevent recombination between homologous chromosomal regions with elevated nucleotide divergence. The resulting compromised chromosomal pairing and insufficient crossovers leads to chromosomal mis-segregation during meiosis I and results in progeny with imbalanced genetic material that are often inviable (Chambers et al., 1996; Greig et al., 2003; Hunter et al., 1996; Liti et al., 2006; Rayssiguier et al., 1989; Roeder, 1997). Thus, a similar mechanism may operate in *Cryptococcus* and contribute to the observed low spore viability from inter-lineage/inter-species hybridization. Interestingly, natural *Cryptococcus* isolates with null mutations in key mismatch repair genes have been identified in population genomic studies (Billmyre et al., 2017). These isolates have long branches in their associated phylogenies, consistent with accelerated rates of mutation accumulation. They also show elevated rates of mutation in the lab that lead to a high frequency of resistance to certain anti-fungal drugs. Future studies on the impact of these mismatch repair defects on relaxing reproductive isolation boundaries could yield interesting insights into the evolution of this species.

Another source of genetic variation that contributes to reproductive isolation is chromosomal rearrangements, which have been shown to have significant effects on speciation, or at least during the onset of these events (Delneri et al., 2003; Fischer et al., 2000; Hou et al., 2014). For example, it has been shown that chromosomal rearrangements alone could explain the observed hybrid sterility caused by meiosis I chromosome segregation between two closely related yeast species (Rogers et al., 2018). For *Cryptococcus* species, while it has been known that karyotypic variation and chromosomal rearrangements are present among natural isolates (Boekhout and Belkum, 1997; Dromer et al., 1994; Fries et al., 1996; Klepser and

Pfaller, 1998; Perfect et al., 1993; Polacheck and Lebens, 1989; Sun and Xu, 2009; Wickes et al., 1994), most population genomic studies have focused on nucleotide polymorphisms (Desjardins et al., 2017; Farrer et al., 2015). This is partly due to the technical limitations of analyzing chromosomal rearrangements, which require comparison of high quality *de novo* genome assemblies that, until recently, have been difficult to generate. Improvements in long-read sequencing technologies, such as PacBio and Nanopore, as well as accompanying advancements in genome assembly algorithms, have allowed efficient *de novo* assembly of high quality, end-to-end chromosomal-level genome assemblies for *Cryptococcus* and closely related species (Passer et al., 2019; Sun et al., 2017; Yadav et al., 2018). These advances will enable analyses of chromosomal rearrangements events, including their origins, maintenance, and spread in the population, as well as their potential effects on fitness, pathogenesis, and lineage divergence, thus allowing a better understanding of the evolution and speciation of *Cryptococcus*.

Evolution of sexual reproduction in *Cryptococcus* and closely related species

All of the species in the *C. neoformans*/*C. gattii* complex have a bipolar mating system, in which the two mating types, α and **a**, are determined by a single *MAT* locus that is unusually large (~120 kb in size) and encompasses more than 20 genes (Fraser et al., 2004; Lengeler et al., 2002; Loftus et al., 2005). While recombination within the *MAT* locus is repressed during bisexual reproduction, crossover events within the *MAT* locus have been observed during unisexual reproduction between two *MAT*α cells, and recombination and gene conversion hotspots have been identified in regions flanking and within the *MAT* locus, respectively (Hsueh et al., 2006; Sun et al., 2014; Sun and Heitman, 2015, 2016; Sun et al., 2012; Sun and Xu, 2007). Interestingly, the species that are most closely related to the *C. neoformans*/*C. gattii* complex, such as *Cryptococcus amylolentus*, *Cryptococcus floricola*, and *Cryptococcus wingfieldii* have tetrapolar mating systems, which is the ancestral configuration of the basidiomycetes (Findley et al., 2012; Passer et al., 2019; Sun et al., 2017). Genome comparison between *C. neoformans* and *C. amylolentus* showed that almost all of the genes that are present in the *C. neoformans MAT* locus are also present within the *P/R* and *HD MAT* loci in *C. amylolentus*. This comparison also revealed that the transition from the ancestral tetrapolar system to the extant bipolar mating system in *C. neoformans* is the result of a fusion between the ancestral *P/R* and *HD* loci that was likely mediated by inter-centromeric ectopic recombination (Sun et al., 2017). This fusion occurred once in the last shared common ancestor to the pathogenic species complex, concomitant with the emergence of this monophyletic group of highly successful human fungal pathogen.

So what could be the benefits of having a bipolar mating system for the pathogenic *Cryptococcus* species? One possible advantage of bipolar mating systems might be related to the ability of individuals to find suitable mating partners. For species with bipolar mating systems, the chance that two isolates in a random encounter are also mating compatible is 50% when the two mating types are at equilibrium in a population. This is always higher compared to tetrapolar species with low numbers of alleles at the *P/R* and *HD* loci when the two loci are in linkage equilibrium. In actuality, for tetrapolar species to achieve 50% mating compatibility between two random individuals, both *P/R* and *HD* loci must have more than two alleles and the total number of alleles of the two *MAT* loci must be equal to or greater

than eight (e.g. 2/3 * 4/5 = 8/15, i.e. chance of compatibility is about 53%). While there are many examples of tetrapolar fungal species with large numbers of alleles at both *P/R* and *HD* loci that together constitute hundreds or even thousands of different mating types, such as *Coprinus cinereus* and *Schizophyllum commune* (Kothe, 1996; Kües et al., 2011; Raper, 1966), there could be scenarios where the numbers of mating-type alleles present in the population are significantly reduced or the two loci are in significant linkage disequilibrium (e.g. population bottlenecks and clonal expansions) which could then provide selective advantages for bipolar mating systems. Indeed, there are tetrapolar species that are bi-allelic at one of the two *MAT* loci, such as *Ustilago maydis,* which is bi-allelic at the a locus (*P/R* locus) and multi-allelic at the b locus (*HD* locus) (Kronstad and Leong, 1990). Interestingly, the closely related species *Ustilago hordei* has a bipolar mating system in which the *P/R* and *HD* loci show complete genetic linkage even though they are located far from each other on the same chromosome (Bakkeren and Kronstad, 1994). There are also species with pseudobipolar mating systems, in which the *P/R* and *HD* loci are located on the same chromosomes but are not fused, and populations where recombination between the two *MAT* loci has been detected, such as those in the *Malassezia* species complex (Coelho et al., 2013; Gioti et al., 2013). However, it is not yet clear in these cases how many different functional alleles are present at the two *MAT* loci, and how much recombination is occurring between them in natural populations. Nevertheless, conditions favoring the transition from tetrapolar to pseudobipolar or bipolar mating systems might be more prevalent in nature than currently appreciated.

The population of pathogenic *Cryptococcus* species in Africa has been hypothesized to be the origin of the global *Cryptococcus* population and shows a relatively balanced distribution of the two alleles, α and **a**, of the *MAT* locus. Interestingly though, the global *Cryptococcus* population has a highly skewed *MAT* allele distribution, with the vast majority of the isolates belonging to the α mating type. This disproportionate number of *MAT*α isolates may have initially significantly reduced outcrossing potential. It is possible that this scenario provided selective pressure or an advantage for the evolution of unisexual reproduction, which allowed *Cryptococcus* to take advantage of the benefits of sex, even in the face of a population composed of individuals of the same mating type, by increasing the outcrossing potential to almost 100%.

Another possible advantage of transitioning to a bipolar mating system could be provided by selective pressures on chromosomal regions other than *MAT*. *Cryptococcus amylolentus* is the closest known sister species to the pathogenic *Cryptococcus* species complex and it has a tetrapolar mating system (Findley et al., 2012; Sun et al., 2017). While neither the *P/R* nor the *HD MAT* locus in *C. amylolentus* is tightly linked to its centromere physically, both loci show elevated genetic linkage to their respective centromere and repressed recombination within the inter-*MAT*-*CEN* regions. Thus, it is possible that in the intermediate pseudobipolar stage, in which the *P/R* and *HD* loci have moved onto the same chromosome via inter-centromeric ectopic recombination, the two *MAT* loci are located on opposite sides of the centromere and recombination is repressed across the entire region between the two *MAT* loci that encompasses the centromere. This situation may have provided selective pressure for the two *MAT* loci to be located closer together and to eventually fuse with each

other to form the extant bipolar configuration in the pathogenic *Cryptococcus* species, such that the region that was originally trapped between the two *MAT* loci could be released to freely undergo recombination (Sun et al., 2017). If this is the case, one would expect reduced recombination frequencies in regions between the two *MAT* loci in species with pseudobipolar mating systems, like those in the *Malassezia* species complex, such as *M. sympodialis* and *M. globosa* (Gioti et al., 2013; Sankaranarayanan et al., 2019; Zhu et al., 2017).

For species with bipolar mating systems, progeny will be mating compatible with 50% of their siblings, a high fraction relative to progeny of a tetrapolar species regardless of the numbers of alleles at the *P/R* and *HD* loci in the population. Inbreeding among these progeny could be detrimental in the long-term. However, the long-term cost could be offset if sexual structures (e.g. hyphae and spores) provided immediate benefits in the context of nutrient acquisition, predator defense, or stress tolerance. Additionally, if two sibling progeny are sufficiently genetically divergent, consecutive rounds of inter-sibling mating could also break up allele combinations in the parental strains and generate a progeny pool that represents extensive reshuffling of the parental genetic material, and thus, increase the genetic and phenotypic diversity of the progeny. Additionally, both bisexual and unisexual reproduction can generate phenotypic and genotypic diversity *de novo*, in many cases as a result of aneuploidy (Ni et al., 2013).

For microorganisms that can also reproduce mitotically, sexual reproduction is not necessarily always the optimal form of reproduction. Sexual reproduction produces pools of progeny that are genetically and phenotypically diverse, and only some will have higher fitness. Progeny with higher fitness will likely undergo clonal expansion, generating linkage disequilibrium, which could in turn be broken up during subsequent rounds of sexual reproduction when conditions favoring sexual reproduction arise again. Thus, a possible reproductive strategy for microorganisms could involve rare sexual reproduction interrupted by long epochs of asexual clonal expansion (Heitman et al., 2013).

III. Evolution of the mating-type locus (*MAT*) in *Cryptococcus*

MAT locus structure

Mating-type identity in fungi is controlled by genes at the mating-type (*MAT*) locus. This region of the genome is unique in that it differs considerably between mating partners of the same species. The mating type has been hypothesized to be a virulence factor in *C. neoformans* based on disease epidemiology and animal studies (Kwon-Chung et al., 1992; Nielsen et al., 2005; Nielsen et al., 2003), the infectious properties of basidiospores, and the prevalence of the α mating type in clinical and environmental isolates (Kwon-Chung and Bennett, 1978). Given these intriguing characteristics, differences between *MAT***a** and *MAT*α cell types as well as the genes encoded by the *MAT* locus and their targets became a great source of interest. Therefore, extensive work has been conducted focusing on this unique genomic region of *C. neoformans*.

Early studies by Moore and Edman used a difference cloning procedure to isolate sequences of the α-genome that were absent in the **a**-genome, identifying a 35-kb region, which

included the *C. neoformans* pheromone gene (*MF*α*1*) and co-segregated with the α-mating type (Moore and Edman, 1993). Subsequent efforts analyzing cosmid subclones containing α-specific DNA defined the *MAT*α locus boundaries to a ~50-kb region specific to *MAT*α strains (Karos et al., 2000; Moore and Edman, 1993). This region contained multiple copies of *MF*α and one copy of a gene encoding the GPCR Ste3α. Additionally, three homologs of the *S. cerevisiae* pheromone response pathway (*STE11*α, *STE12*α, and *STE20*α) were identified within 24 kb of *MF*α*1* (Karos et al., 2000), already hinting at the possibility that other genes involved in mating or sexual reproduction would likely also be located within the *MAT* locus, indicative of selection favoring linkage of genes with related functions into co-segregating units.

To understand the organization of the *C. neoformans MAT* locus further, Lengeler et al. carried out sequencing analysis of both *MAT*α and *MAT***a** regions in *C. neoformans* (serotype A, strains H99α and 125.91**a**) and *C. deneoformans* (serotype D, strains JEC21α and JEC20**a**) strains of opposite mating types using genomic BAC libraries (Lengeler et al., 2002). Alignment of the nucleotide sequences revealed identical sequences flanking a region of non-identical DNA sequences between mating types that spanned 105 to 125 kb, demonstrating the *MAT* locus was larger than previously hypothesized (Lengeler et al., 2002) (Figure 3). Additional analyses identified the homeodomain gene *SXI1*α (Sex Inducer 1α) of the HD1 class at the left end of the *MAT*α locus (Lengeler et al., 2002). *SXI1*α was later shown to be a key factor for establishing α-cell identity and progression through the sexual cycle, and interestingly, was sufficient to drive sexual development when expressed in **a** cells (Hull et al., 2002). However, it was obvious that a factor from *MAT***a** cells was also required for completion of the sexual cycle. Using a combination of molecular genetics and sequence analysis approaches, a homeodomain gene counterpart (*SXI2***a**) of the HD2 class specific to **a** cells was successfully identified, which likewise forced α cells to adopt an **a**/α cell fate when introduced into α cells, resulting in production of hyphae, basidia, and basidiospores (Hull et al., 2005).

Subsequent comparative analyses showed that the *C. neoformans MAT* locus is a unique genomic region because it differs considerably between mating types and encompasses more than 20 genes (Fraser et al., 2004; Lengeler et al., 2002), including those encoding mating pheromones (*MF*α or *MF***a**) and their cognate receptors (*STE3*α or *STE3***a**), as well as homeodomain transcription factors (*HD1* or *SXI1*α and *HD2* or *SXI2***a**) that together govern cell-type identity. Of the 20 genes, 5 are essential based on gene disruption studies (*MYO2*, *PRT1*, *RPL39*, *RPL22*, and *RPO41*) (Fraser et al., 2004), while others have diverse functions during mating (*STE11*, *STE12*, and *STE20*), sporulation (*SPO14* and *RUM1*), and in virulence (*CAP1*). It is also noteworthy that in contrast to other basidiomycetes, where a divergently transcribed *HD1*/*HD2* gene pair is present in each mating type (Coelho et al., 2017), the *C. neoformans MAT*α locus encodes only Sxi1α (HD1), while the *MAT***a** locus encodes only Sxi2**a** (HD2). This is, however, an exception because for almost every other *MAT* locus gene, there are similar, yet non-identical, alleles in both *MAT*α and *MAT***a** in *C. neoformans* (Fraser et al., 2004).

Although *MAT***a** is clearly allelic to *MAT*α, the organization of *MAT***a** reveals extensive rearrangement of the genes compared to *MAT*α, a feature associated with suppression of

recombination within *MAT* (Figure 3) (Fraser et al., 2004). This represents a scenario where recessive losses of genes from one mating type that do not play an essential role in haploid cells, as shown for *SXI1*α and *SXI2***a** (Hull et al., 2005), could likely occur because each is sheltered in a permanent hemizygous state. These losses may constitute a first sign of mating-type chromosome degeneration as expected for longstanding, non-recombining regions (Bergero and Charlesworth, 2009). However, the presence of essential genes spaced throughout the *MAT* locus (Fraser et al., 2004) may, in contrast, pose an evolutionary constraint ensuring that large regions of the *MAT* locus are not lost through sexual recombination. Additionally, it has been shown that recombination hotspots associated with the presence of GC-rich motifs are located in the regions that flank the *MAT* locus in *Cryptococcus* (Hsueh et al., 2006), which potentially function as repressors of crossover within the *MAT* locus due to interference (Berchowitz and Copenhaver, 2010; Sun et al., 2017).

To gain further insight into the evolutionary trajectory of the *MAT* locus, Lengeler et al. and Fraser et al. (Fraser et al., 2004; Lengeler et al., 2002) cloned and sequenced extant *MAT* alleles in *C. neoformans* and *C. deneoformans*, which diverged ~20 million years ago (mya), and in *C. gattii*, which diverged from the *C. neoformans*/*C. deneoformans* common ancestor approximately ~40 mya (Casadevall et al., 2017; D'Souza et al., 2011; Sharpton et al., 2008; Xu et al., 2000b) (Figure 3a). This study revealed that the *MAT* gene cohort has been dramatically rearranged during evolution of the three lineages (Fraser et al., 2004) (Figure 3c). The subsequent completion of the genome sequences of representatives of three species (i.e. *C. deneoformans* JEC21, *C. neoformans* H99 and *C. gattii* WM276) (D'Souza et al., 2011; Janbon et al., 2014; Loftus et al., 2005) has broadened and accelerated *Cryptococcus* research. For instance, sequencing confirmed that the *MAT* locus occupies ~6% of a 1.8-Mb chromosome in an ~19-Mb genome (excluding the rDNA repeats that constitute ~5% of the genome) (Loftus et al., 2005). It also became clear that rearrangements at the *MAT* locus are highly atypical compared to non-*MAT* regions of the genome, and likely resulted from intra-allelic recombination between transposable elements and their remnants, which are enriched in the *MAT* locus relative to the rest of the genome (excluding the transposon-rich centromeric regions) (Fraser et al., 2004; Janbon et al., 2014; Loftus et al., 2005; Yadav et al., 2018).

Phylogenetic analyses of the alleles of *MAT* genes across these three *Cryptococcus* species demonstrated that the *MAT* locus is composed of four gene strata of distinct evolutionary ages, including: (i) a set of ancestral genes (*STE3*, *MF* and genes of the pheromone-sensing MAPK pathway) with mating-type-specific phylogenies; (ii) two strata of more intermediate evolutionary origin (e.g. *RPL22*, *SPO14*, *RUM1*, *BSP1*) and (iii) a set of recently acquired genes that have species-specific tree topologies, similar to genes outside *MAT* (Fraser et al., 2004) (Figure 3b,c). However, genes in the *Cryptococcus MAT* locus are no longer organized by age, as they been heavily reshuffled during speciation and mating-type divergence (Figure 3c). Furthermore, some genes seem to have been punctuated by more recent gene conversion events, resetting their molecular clock (e.g. *IKS1* in *C. deneoformans*, Figure 3c), and direct experimental evidence of such phenomena has been more recently obtained (Sun et al., 2012). Nevertheless, by plotting the synonymous

divergence level of each protein coding gene in *MAT* it was possible to infer an ancestral state where one gene cluster contained the pheromone and pheromone receptor genes (*P/R* cluster), and the other cluster contained the homeodomain-encoding gene (*HD* cluster). Together, this led to a model where two ancestral unlinked *P/R* and *HD* loci regions were juxtaposed by a chromosomal translocation, giving rise to the extant bipolar *Cryptococcus MAT* locus (Fraser et al., 2004).

To provide experimental evidence to support this model, strains of *C. neoformans* were genetically engineered so that the homeodomain genes (*SXI1*α or *SXI2***a**) were physically unlinked to the pheromone/pheromone receptor genes (Hsueh et al., 2008). In this way, the *HD* and *P/R* loci could segregate independently during meiosis. These strains mimicking a tetrapolar organization were able to mate and complete the sexual cycle, but when they were independently crossed with a strain harboring a contiguous *MAT* locus, only ~50% of the progeny were fertile. This provides evidence that the transitional "tripolar" state imposes disadvantages, which could in turn facilitate the transition from a tripolar to a bipolar state through recombination.

Evolution of the *Cryptococcus MAT* locus

Several studies support the hypothesis that tetrapolarity is the ancestral state of basidiomycetes. However, there have been multiple reports of transitions to bipolarity in the three major basidiomycete lineages ((Branco et al., 2017; Branco et al., 2018), reviewed in (Coelho et al., 2017)), including the human-pathogenic species of the *C. neoformans/C. gattii* complex as described above. Recent phylogenetic analyses resulted in the taxonomic reclassification of the *Cryptococcus* genus (Hagen et al., 2015; Liu et al., 2015). Several yeast species are now known to be closely related to the human-pathogenic clade, including the homothallic, strictly filamentous fungus *Cryptococcus depauperatus*, and a clade composed of three yeast species: *C. amylolentus*, *C. wingfieldii*, and the recently described *Cryptococcus floricola* (Passer et al., 2019) (Figure 3a).

To further understand how this unique bipolar mating system evolved in the pathogenic *Cryptococcus* species, studies have been conducted to investigate the *MAT* locus structures in non-pathogenic *Cryptococcus* species and also in more distantly related species of the sister genus *Kwoniella* (Findley et al., 2012; Guerreiro et al., 2013; Metin et al., 2010; Passer et al., 2019). In these studies, *C. amylolentus*, *C. wingfieldii*, *C. floricola*, *Kwoniella heveanensis*, and *Kwoniella mangrovensis* were shown to be tetrapolar, with *P/R* and *HD* loci located on different chromosomes (Figure 3a,b). The assignment of these species as tetrapolar is in accord with mating studies and analysis of F1 progeny (i.e. isolates need to differ at both the A and B *MAT* loci to be inter-fertile). Therefore, only in the lineage giving rise to the *Cryptococcus* human pathogens has there been a major genomic alteration, with the two *MAT* loci now residing on a single chromosome. Interestingly, in the non-pathogenic *Cryptococcus* species and in *Kwoniella* spp. the *HD* locus is restricted to an ~2 kb region, encodes two linked and divergently oriented homeodomain genes (*HD1* and *HD2*) like most basidiomycetes, and is multiallelic, with allele numbers varying among species (e.g. *K. heveanensis* has at least 6 different *HD* alleles). This differs from the solo *HD* genes (*SXI1*α, *SXI2***a**) of the pathogenic *Cryptococcus* complex and suggests that one

or the other homeodomain gene was recently lost in *C. neoformans/C. gattii* in association with linkage of the two *MAT* loci. Compared to the *HD* locus, the *P/R* locus in the non-pathogenic species is expanded, spanning ~30 kb in *Kwoniella* spp. (Guerreiro et al., 2013; Metin et al., 2010) and up to 96 kb in *C. amylolentus* (Findley et al., 2012; Sun et al., 2017). These large *P/R* loci encode pheromone and pheromone receptor genes along with several other genes that are integrated within the *MAT* locus of the pathogenic *Cryptococcus* species. This indicates that some of these genes were already linked to the *P/R* locus prior to fusion of *P/R* and *HD* loci in the pathogenic *Cryptococcus* complex (Figure 3c). Therefore, the organization of *MAT* in other *Cryptococcus* species and in *Kwoniella* mirrors key aspects of the proposed intermediates in the evolution of *MAT* in the pathogenic *Cryptococcus* species.

Genome sequencing of hundreds of *Cryptococcus* isolates is currently taking place at an increasing scale and speed. While most of the data is still being generated on the Illumina platform, recent developments in long-read Pacific Biosciences and Oxford Nanopore Technologies sequencing technologies are providing more contiguous genome assemblies and are helping to resolve repetitive regions including the centromeres (Sun et al., 2017; Yadav et al., 2018). Indeed, a recent study by Sun et al. (Sun et al., 2017) revealed that, similar to the pathogenic *Cryptococcus* species (Janbon et al., 2014; Yadav et al., 2018), *C. amylolentus* has regional centromeres that are enriched with species-specific transposable elements. It was also shown that the *MAT* loci of *C. amylolentus* displays linkage with their respective centromeres despite overall collinearity of the intervening genomic regions (Sun et al., 2017). Suppression of recombination between each *MAT* locus and the centromere ensures segregation of mating-type in the first meiotic division, and thus, the generation of only two mating types per meiosis. This arrangement in a tetrapolar species therefore results in similar odds of compatibility between products of the same meiosis as that of progeny produced by linked *P/R* and *HD* loci in bipolar species. Similar instances of mating type loci evolution have been reported (e.g. in the anther-smut fungus *Microbotryum lagerheimii*) (Hood et al., 2015), indicating that equally beneficial phenotypes can emerge through different genomic changes in response to natural selection. Suppression of recombination without substantial rearrangements raises the hypothesis that genetic elements involved in epistatic interactions favor co-segregation of alleles from the same parent. Alternatively, recombination could be restricted in these regions due to the presence of epigenetic factors such as DNA methylation or histone modifications, which could potentially affect double-strand break formation and recombination initiation, as several examples have suggested (Maloisel and Rossignol, 1998; Termolino et al., 2016). Additional studies will be required to unravel the causes of recombination suppression in these regions and to define the role of suppression of recombination in the transition between unlinked and linked *MAT* loci.

Finally, because centromeres are enriched with transposable elements and repetitive sequences, and some elements are shared among different chromosomes, Sun et al. proposed that the fusion of the ancestral *P/R* and *HD* loci that gave rise to the bipolar mating system in the pathogenic *Cryptococcus* species was initiated by ectopic intercentromeric recombination (Figure 4). Consistent with this, several lines of evidence now challenge the view that centromeres are typically recombination-free regions (Jaco et al., 2008; Shi et al., 2010; Symington and Petes, 1988). Recombination at these regions may lead to

chromosomal translocations, which in this case would bring the ancestral *P/R* and *HD* loci onto the same chromosome, as the comparison of the extant *MAT* chromosome of *C. neoformans* with the chromosomes harboring the *P/R* and *HD* loci in *C. amylolentus* suggests (Figure 4).

In conclusion, these analyses give rise to an evolutionary model in which the single *MAT* locus of the human-pathogenic *Cryptococcus* lineages evolved from an ancestral tetrapolar mating system via a series of steps, including gene acquisition, chromosomal translocations involving the centromere, and subsequent intrachromosomal rearrangements possibly mediated by transposable elements or repeat-rich regions.

MAT locus function

One distinct feature of the tetrapolar mating-system and consequently of mating in the Basidiomycota is that the genes encoding pheromones and pheromone receptors have acquired a mating-type defining role, in contrast to what occurs in the ascomycetes (Bennett and Turgeon, 2017; Coelho et al., 2017). The archetype of the *P/R* locus encodes pheromone-receptor genes homologous to the *STE3* receptor gene from *S. cerevisiae*, a G-protein-coupled receptor with seven transmembrane domains, and pheromone precursor genes, which are homologous to the lipid-modified *MF***a** pheromone gene of *S. cerevisiae* (Moore and Edman, 1993). Each of the mating types of *Cryptococcus* encodes a *STE3* receptor gene that is activated by binding of its respective pheromone. It is not unusual for the *MAT* locus of basidiomycetes to encode more than one pheromone precursor gene, and in the case of *Cryptococcus* pathogenic species, each mating type has between three and four pheromone precursor genes (Figure 3c) (Coelho et al., 2017; Kües et al., 2011). Similarly to the pheromones of other basidiomycetes, *Cryptococcus* pheromones are first translated into pheromone precursor proteins which subsequently undergo post-translational processing at both the N- and C-terminal regions. A general feature of pheromone precursor proteins is the presence of a “CaaX” motif at the carboxyl terminus of the precursor, where “C” stands for cysteine, “a” for aliphatic amino acids and “X” stands for any residue (Raudaskoski and Kothe, 2010). The multistep maturation process of the pheromone precursors involves farnesylation, C-terminal proteolytic cleavage of the motif “aaX”, followed by carboxymethylation of the cysteine residue and proteolytic cleavage at the N-terminus, producing the mature lipopetide pheromones (Raudaskoski and Kothe, 2010). In addition to pheromones and pheromone receptors, the *Cryptococcus MAT* locus also encodes one homeodomain transcription factor gene in each of the two mating types, as mentioned previously *SXI1*α for *MAT*α strains and *SXI2***a** for *MAT***a** strains, each of which belongs to one of two distinct classes of homeodomain transcription factors based on structural homologies and distinct protein sequences of the DNA-binding motifs (Hull et al., 2005; Kües and Casselton, 1992; Kües et al., 2011). *SXI1*α belongs to the HD1 class of homeodomain transcription factors that are considered to have an atypical homeodomain, with three additional amino acids between helices one and two of the three helices comprising the homeodomain motif. Conversely, *SXI2***a** belongs to the HD2 class of homeodomain transcription factors that have a canonical homeodomain, with 60 residues forming a three helical structure and a highly conserved DNA-binding region (Hull et al., 2005; Kües and Casselton, 1992; Kües et al., 2011). For these transcription factors to be

functional in a heterothallic mating, it is necessary that they heterodimerize with each other after cell fusion in order to complete the mating process (Hull et al., 2005).

In addition to being key molecular determinants for mating, some of the *MAT* genes are also involved in other cellular processes of major importance for the cell. For example, *SXI1* and *SXI2* are not only important for determining mating-type identity and regulating sexual development, but are also indispensable for uniparental mitochondrial DNA inheritance (from the *MAT***a** cell) after cell fusion in heterothallic mating (Gyawali and Lin, 2013; Yan et al., 2007a). In *C. amylolentus* it was shown that mitochondrial inheritance is also uniparental and controlled by *MAT*-encoded genes, however in this case the *P/R* locus controls this process independently from the *HD* locus (Findley et al., 2012).

The *MAT* loci of the pathogenic *Cryptococcus* species also include genes that have been shown to be involved in virulence attributes, like the production of melanin, the formation of a polysaccharide capsule, and growth at 37°C (Kwon-Chung et al., 1992; Lengeler et al., 2002). For example, it has been reported that the *MAT*α allele of *C. deneoformans* is associated with increased virulence given that *MAT*α cells are more virulent than congenic *MAT***a** cells. However, this result was obtained only in certain genetic backgrounds (JEC21α/JEC20**a** and KN433α/KN433**a**) and not in others (KN3501α/KN3501**a**), and was also absent in *C. neoformans* congenic strains (H99α/KN99**a**), indicating that this phenotype might be not only species-specific but also strain-specific (Hsueh et al., 2011; Kwon-Chung et al., 1992; Nielsen et al., 2005; Nielsen et al., 2003). Additionally, the genes or molecular mechanisms responsible for the increase virulence observed in some of the *MAT*α congenic strains of *C. deneoformans* have yet to be clearly elucidated (Wang et al., 2002). One gene hypothesized to be partially responsible for the increased virulence of *C. deneoformans MAT*α is *STE12*, given that deletion or overexpression of this allele was shown to respectively, downregulate or upregulate the *LAC1* gene, which encodes a laccase necessary for the production of the antioxidant pigment melanin, an important virulence factor of *Cryptococcus* (Chang et al., 2001; Chang et al., 2000; Kwon-Chung et al., 1992; Williamson, 1997). Additionally, both *STE12* alleles influence the capsule size of cryptococcal cells in brain smears obtained from mice infected with *C. deneoformans* (Chang et al., 2001). In *C. gattii*, a *ste12*αΔ mutant showed a diminished production of melanin and attenuated virulence in mice models (Ren et al., 2006). However, *C. neoformans ste12*Δ mutants do not show reduced virulence (Yue et al., 1999), indicating that the relationship between *STE12* and virulence may also be species-specific (Nielsen et al., 2003). The PAK kinase homologue *STE20*α encoded by the *C. neoformans MAT* locus is thought to play a role not only in the pheromone signal MAPK cascade, but also in virulence and thermotolerance (Nichols et al., 2004; Wang et al., 2002). Similarly, the *MAT*-encoded *CAP1* gene is thought to encode a capsule-associated protein involved in the addition of xylose to the polysaccharide capsule of *Cryptococcus*, even though its biochemical functions remain unknown (Klutts et al., 2007; O'Meara and Alspaugh, 2012). Finally, other genes encoded at *MAT* do not appear to be directly involved in mating or virulence, including the genes thought to be essential based on genetic disruption assays, like the *MYO2* gene that encodes a myosin homolog, the *PRT1* gene that encodes a translation initiation factor, genes

RPL39 and *RPL22* that encode ribosomal proteins, and the *RPO41* gene that encodes a mitochondrial RNA polymerase (Fraser et al., 2004).

Considering the speed and magnitude of the technical advances in the field of genome sequencing and analysis, we are likely at the cusp of understanding the differences observed between mating types in much more detail. Exploring the expression of all *MAT*-encoded transcripts (including long non-coding RNAs), the possible DNA or histone methylation states of this region at different stages of development, and the 3D interactions between the *MAT* locus and other regions of the genome, we are bound to gain a greater understanding of the factors that control mating and virulence in this important group of human fungal pathogens that are a cause of significant morbidity and mortality globally.

IV. Conclusion and future directions

We have gained great insights on the effects of sexual reproduction on many aspects of *Cryptococcus*, including its pathogenesis, evolution of the mating-type locus, and population structure and dynamics. Questions that remain to be investigated include:

1. What are the underlying mechanisms governing unisexual reproduction vs. bisexual reproduction in the distinct pathogenic *Cryptococcus* species?
2. What are the driving forces for the evolution of the bipolar mating system in *Cryptococcus*, as well as its large *MAT* locus and the genes resident therein? Are there other genes within the *MAT* locus other than the pheromone, pheromone receptor, and *HD* genes that play critical roles in sexual development in *Cryptococcus* species?
3. How much structural variation is present among the genomes of natural isolates, such as chromosomal translocations and inversions? Do these changes in genome structure have effects on fitness, including virulence? How do they affect sexual reproduction, and vice versa?
4. What are the underlying mechanisms of many cellular processes associated with *Cryptococcus* sexual reproduction, such as sex-induced genome defense against transposable elements as well as uniparental inheritance of mitochondria from the *MAT***a** parent?
5. What is the complete suite of genes and their networks that contribute to the pathogenesis of *Cryptococcus*? Taking advantage of sexual reproduction, combined with availability of high throughput genome sequencing and phenotyping, it is possible to perform high-resolution QTL analyses of virulence related factors, such as high-temperature tolerance and drug resistance, using segregants isolated from laboratory crosses. These analyses have the potential to identify new candidate loci/genes associated with *Cryptococcus* pathogenesis.

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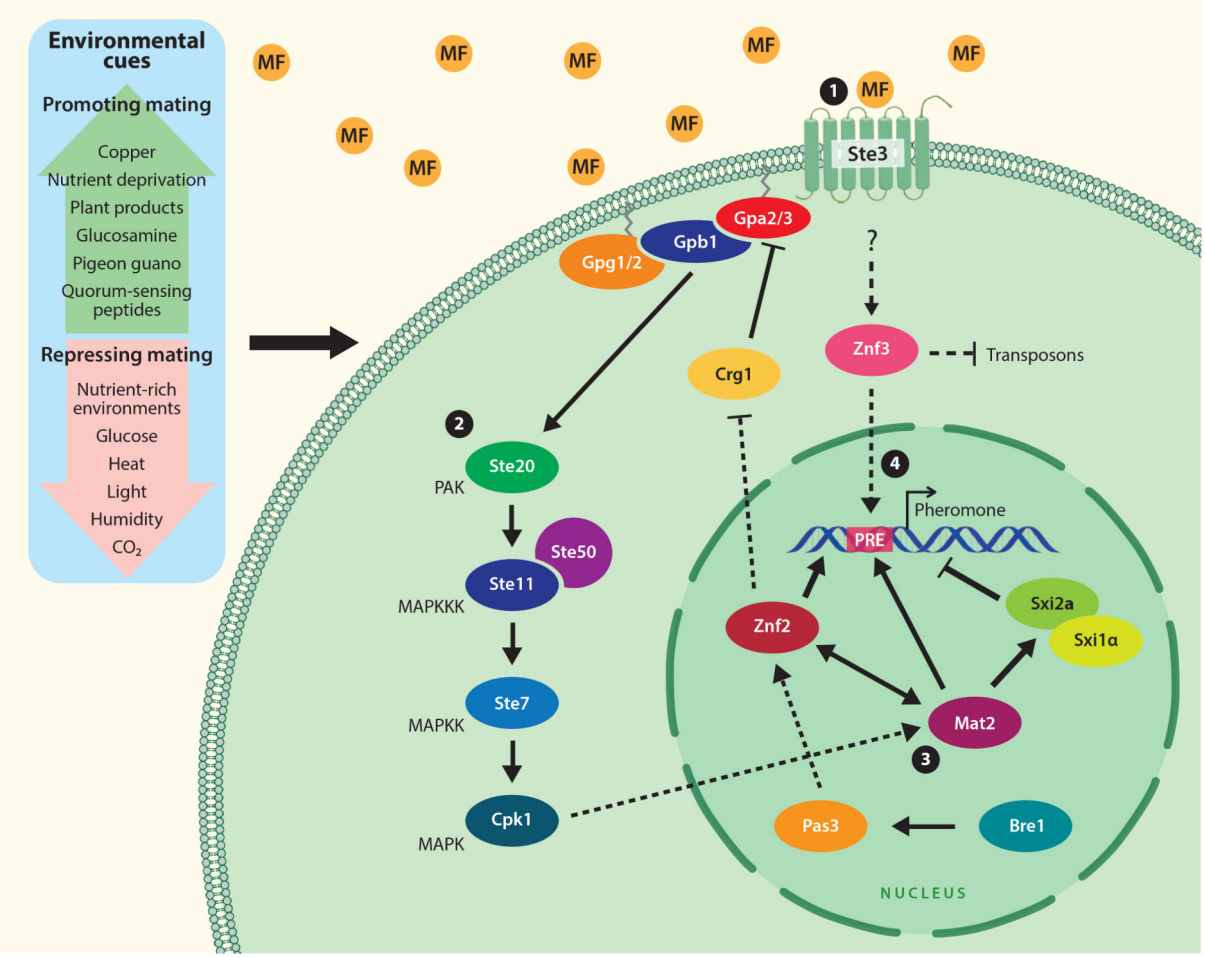

Figure 1. Signal transduction pathways involved in initiation of sexual development in pathogenic *Cryptococcus* species, as well as environmental factors that influence this process.

The various environmental cues that can either stimulate or repress mating are in the blue shaded box to the left. Schematic depicts the signaling pathway that is initiated when pheromone binds to the Ste3 GPCR. An identical signaling pathway is controlled by the constitutively activated pheromone-like receptor Cpr2. Following GPCR activation, signaling proceeds through a mitogen-activated protein kinase (MAPK) cascade, which activates the key high-mobility transcription factor Mat2. Mat2 signals in concert with other transcription factors and also directly binds the pheromone response element (PRE), initiating transcription of the pheromone-encoding gene *MF***a**/α, and also *STE3***a**/α, *SXI1*α, *SXI2***a**, *GPA2*, *GPA3*, as well as many other genes (Kruzel et al., 2012). In addition to signaling mediated by the Mat2 transcription factor, pheromone-response genes are upregulated in response to signaling from the transcription factor Znf3.

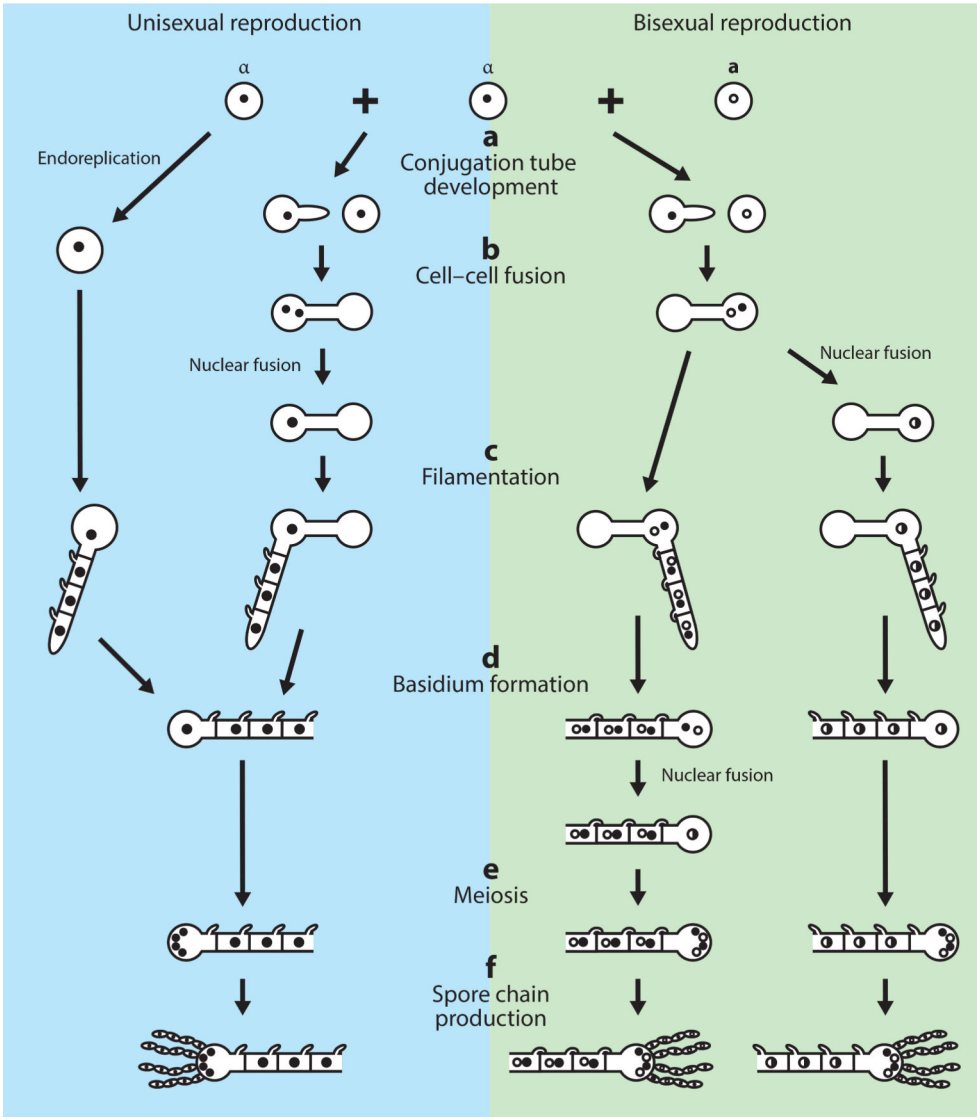

Figure 2. The sexual cycle of pathogenic *Cryptococcus* species.

Cryptococcus pathogenic species can undergo both unisexual (involving only one mating type) and bisexual reproduction (involving two opposite mating types), depicted in the blue and green shaded boxes, respectively. Following mate-recognition in the appropriate environment, one mating partner produces a conjugation tube that grows toward and eventually fuses with the mating partner. Following cell-cell fusion, a filament protrudes and continues to grow, eventually differentiating into a basidium at its terminus. Meiosis then occurs within the basidium and the meiotic products undergo repeated rounds of mitosis, budding from the basidium as four basidiospore chains. In bisexual reproduction, nuclear fusion can occur at various phases: in *C. deneoformans* unisex, nuclear fusion can occur post cell-cell fusion before filamentation; in *C. deneoformans* bisexual reproduction, nuclear fusion can occur post-cell-cell fusion prior to filamentation or within the terminal basidium; in *C. neoformans* bisexual reproduction, nuclear fusion occurs in the basidium. If nuclear fusion occurs post-cell-cell fusion, the subsequent filament is monokaryotic with unfused

clamp cells. If nuclear fusion occurs within the basidium, the prior filament is dikaryotic with fused clamp cells. An alternative mechanism of *C. deneoformans* unisexual reproduction can occur within a single cell in which the nucleus undergoes endoreplication; this pathway is depicted on the far right.

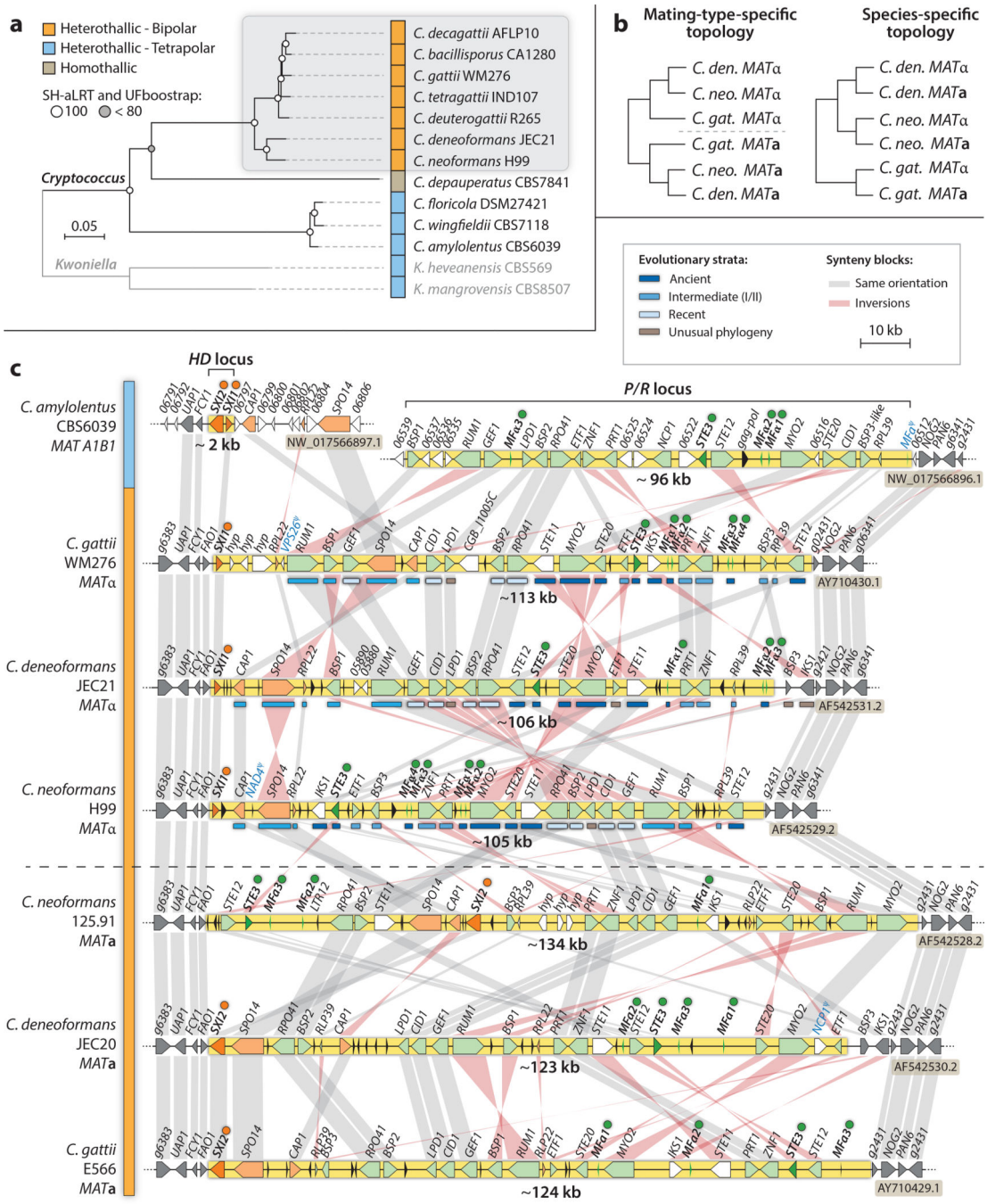

Figure 3. *Cryptococcus* phylogeny and comparative analyses of *MAT* alleles from pathogenic and closely related non-pathogenic *Cryptococcus* species.

(*a*) Seven species are currently recognized among the pathogenic *C. neoformans*/*C. gattii* species complex (shadowed in grey), all of which have linked *P/R* and *HD* mating-type loci (bipolar). Conversely, *C. amylolentus*, *C. floricola*, *C. wingfieldii*, and the two *Kwoniella* species (outgroup) are tetrapolar, with mating-type genes at two genetic loci on different chromosomes representing the ancestral configuration of the *MAT* loci. The tree was inferred from 734 single copy orthologs (149,332 informative amino acid sites), using maximum likelihood and the LG+F+R4 model of amino acid substitution in IQ-TREE.

Branch support was assessed by ultrafast bootstrap (UFboot) and SH-aLRT methods. Scale bar indicates the number of substitutions per site. (*b*) Example of tree topologies observed for genes anciently (left) or recently (right) recruited to *MAT*, representing different evolutionary strata. (*c*) The *MAT* alleles of the pathogenic species are highly rearranged between species and mating types and result from linkage between *P/R* and *HD* gene clusters located on different chromosomes in *C. amylolentus*. The nonrecombining *MAT* region is depicted in yellow (with the number beneath indicating the approximate size), and is bordered by ~10 kb of common flanking regions depicted on each side (4 genes in dark grey), except in *C. deneoformans* where the *IKS1* and *BSP3* genes were evicted from *MAT* and fixed in the flanking region by inversion and recombination. Common genes found in the *HD* and *P/R* regions of *C. amylolentus* are colored in orange and green, respectively, in the extant *MAT* locus of the pathogenic species. Genes encoding homeodomain transcription factors (*SXI1* and *SXI2*), pheromones (*MF*), and pheromone receptors (*STE3*) are bulleted. Blue bars below the genes indicate the evolutionary strata class they belong to as shown in the key. Genes in both the “Ancient” and the “Intermediate” strata classes have mating-type specific phylogenies (they entered *MAT* prior to speciation), while genes in the “Recent” strata class display species-specific phylogenies (they began diverging only after speciation). Genes with unusual phylogenies represent cases where the gene underwent gene conversion in one or the other lineage, thereby fixing one of the two alleles in both mating types and with concomitant loss of the alternative allele. Grey and pink bars connect orthologs with the same or inverted orientation, respectively. Genes depicted in white are not conserved in all species. Pseudogenes are labeled in blue, and black arrows represent transposable elements or their remnants.

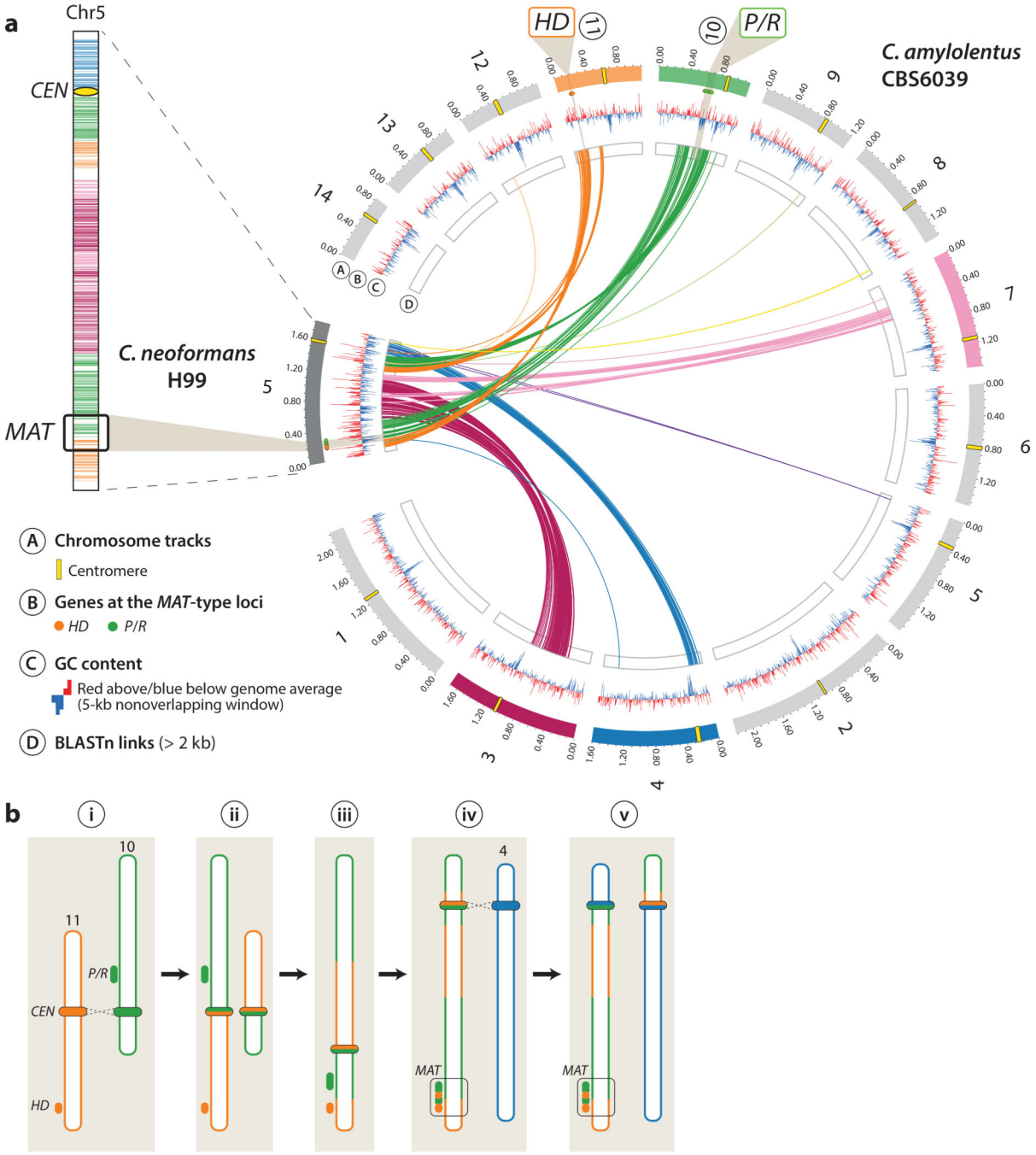

Figure 4. Evolution of a bipolar mating system from a tetrapolar mating system via inter-centromeric ectopic recombination mediated by transposable elements shared between centromeres.

(*a*) Genome comparison between chromosome 5 of *C. neoformans* H99 and *C. amylolentus* CBS6039 chromosomes. Distribution of BLASTN hits showing that chromosome 5 of *C. neoformans* is made up of several *C. amylolentus* chromosome regions, implying multiple rearrangements along the evolutionary history of the two lineages. (*b*) Proposed model for the transition from tetrapolar to bipolar *MAT* organization. This model proposes intercentromeric recombination as a key mechanism bringing the two ancestrally unlinked

P/R and *HD* loci into a single *MAT* locus, thus linking the two sets of genes determining mating type.