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Orchestration of sexual reproduction and virulence by the fungal mating-type locus

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Introduction

Sexual reproduction is ubiquitous across kingdoms and important both to the origins and fitness of species. Fungi, in particular, serve as excellent models to study sexual reproduction and explore how sex is orchestrated. Sex is genetically determined in fungi, governed by a sex-specific region in the genome known as the mating-type locus (*MAT*). The molecular structure of *MAT* was first characterized in *Saccharomyces cerevisiae*, in which homeodomain or α domain transcription factors are encoded by the **a** or α idiomorph and are critical to establish cell-type identity [1]. With the wealth of fungal genome sequences now available, the structure, function, and evolution of *MAT* has been revealed in three major fungal lineages (Ascomycetes, Basidiomycetes, and Zygomycetes) [2-4]. Generally, *MAT* encodes transcription factors that have conserved homeodomain, α domain, or HMG domain DNA binding regions; these transcription factors then control the expression of cell-type specific genes and genes that are required for mating. These advances provide insight into how the sexual cycle is orchestrated in fungi and how this sex-determining region evolves [4,5]. In many fungal pathogens of both plants and animals, the sexual cycle has been associated with virulence. In this review, we discuss how sex is orchestrated and how it influences virulence with a focus on species in the Basidiomycete phylum that infect plants and animals.

Mating systems in Basidiomycetes

Fungi exhibit either of two mating patterns: self-fertile (homothallic) or self-sterile (heterothallic). In heterothallic fungi, mating occurs when cells of different mating-types encounter each other. In homothallic fungi, sexual reproduction can occur in monoculture without the need for a mating partner but most homothallic fungi can also undergo outcrossing. Importantly, several species, including *S. cerevisiae*, encompass both heterothallic and homothallic members in their population. Mating-types are dictated by the *MAT* locus, and fungi can be either bipolar with a single *MAT* locus, or tetrapolar with two unlinked *MAT* loci. A principle advantage of sexual reproduction is genetic exchange, which occurs via two different routes: mating between progeny derived from a cross (inbreeding) and between progeny of a cross with isolates from the broader general population (outcrossing). For successful mating to occur, two parents must be compatible at *MAT*. Because mating is controlled by two unlinked loci in a tetrapolar species, only a quarter of the progeny (25%)

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from a cross are interfertile. In contrast, this amount increases to half (50%) in a bipolar cross. Therefore, from the viewpoint of population genetics, a tetrapolar mating system restricts inbreeding when compared to a bipolar system.

On the other hand, the frequency of outcrossing is not dependent on the pattern of the mating system (bipolar vs. tetrapolar), but rather, is dependent upon the number of *MAT* alleles that exist in the population (Table 1). For example, in the tetrapolar model mushroom *Coprinopsis cinerea*, >200 different alleles are estimated to be present for both *MAT* loci [6], and as a result, the chance of outcrossing is as high as 99.5%. In contrast, in the corn smut fungus *Ustilago maydis*, which has a biallelic pheromone/pheromone receptor locus, the chance of outcrossing in the population is still 50% regardless of the fact that it is also tetrapolar. In the bipolar, biallelic fungi, the relative frequency of inbreeding and outcrossing are both 50%; however, if one of the mating-types is skewed in the population, the relative level of outcrossing is decreased. The human pathogen *Cryptococcus neoformans* is one such example; although bipolar-biallelic, the majority of isolates identified from both the environment and the clinic are α mating-type [7]. Thus, the frequency of α - α outcrossing in nature is likely to be significantly lower compared to other biallelic species in which the distribution of the two different sexes is relatively equal.

The genome of the dandruff-associated basidiomycetous fungus *Malassezia globosa* has recently been sequenced. Although the sexual cycle has not yet been observed in nature or under laboratory conditions, the genome sequence revealed conserved genes involved in mating and meiosis in other fungi, and may be a bipolar *MAT* locus, suggesting that this pathogen may be capable of sex [8]. Mating of *Candida albicans* has been observed on the hairless skin of newborn mice inoculated with suitable mating partners [9], and it has been proposed that mating of *M. globosa* may similarly occur on skin of the human host [8].

Structure and evolution of the *MAT* locus

As mentioned above, the molecular structure of *MAT* has been defined in many fungal species from three major lineages [2-4,10]. Despite that conserved sex-determining transcription factors are ubiquitously present, *MAT* loci vary considerably in size and gene content. For example, *MAT* in the model yeast *S. cerevisiae* is less than 1 kb, and encodes only one or two genes. However, in another hemiascomycete yeast, *C. albicans*, several additional genes (*PAP*, *PIK*, and *OBP*) have been incorporated into *MAT*, extending the length of *MAT* to ~ 9 kb [11].

Even more diversified *MAT* structures have evolved in the Basidiomycota (Fig. 1). There are three major lineages within this phylum: mushrooms, smuts, and rusts. The structures of *MAT* are best known in mushrooms and smuts, while only a few species of the rust lineage have been characterized [12-15]. *U. maydis*, *Ustilago hordei*, and *Sporisorium reilianum* are three closely related smut species but they differ substantially in the structure of *MAT* [15]. *U. maydis* and *S. reilianum* are both tetrapolar, with two unlinked *a* and *b* loci while *U. hordei* is bipolar, in which the *a* and *b* loci are genetically and physically linked. In these species, the *a* locus encodes pheromone/pheromone receptors that function to establish cell-cell signaling during mating. The *b* locus encodes two divergently transcribed homeodomain protein subunits that are important to govern the expression of genes involved in completing sexual reproduction after cell-cell fusion. Both *U. maydis* and *S. reilianum* are multiallelic at the *b* locus but have only two or three alleles at the *a* locus, respectively. The *a* and *b* loci of these two species are also similar in size, spanning a few kb in the genome. In contrast, the *MAT* locus of *U. hordei* is surprisingly large: the distance between the *a* and *b* loci was estimated at 500 and 430 kb for the *MAT-1* and *MAT-2* alleles, respectively [16,17]. Recombination between the two loci is suppressed as a result of genetic divergence and rearrangements in the intervening

genomic region between the *a* and *b* loci. Sequencing of the entire *MAT-I* region revealed that there are 47 protein-coding genes, including the pheromone/receptor and homeodomain genes and a striking accumulation of repetitive sequences and retrotransposons that accounts for ~50% of *MAT* [18]. These features highly resemble the characteristics of sex chromosomes in which repetitive sequences also accumulate in these regions of the genome that are sheltered from recombination. Comparing *MAT-I* with the *U. maydis* *a* and *b* loci showed that this repetitive sequence accumulation is unique in *U. hordei*, and although an overall conservation of synteny is observed in blocks of genes, local rearrangements in gene order are also apparent. These lines of evidence suggest that the tetrapolar mating system harbored by *U. maydis* and *S. reilianum* is likely more ancestral and that the bipolar arrangement in *U. hordei* is derived by a more recent translocation that linked the *a* and *b* loci [18].

The *MAT* locus of the bipolar human fungal pathogen *C. neoformans* is also much larger than most other fungal *MAT* loci. Here *MAT* spans over 100 kb, and consists of a cohort of ~20 genes, including the pheromone/receptor and homeodomain transcription factor genes that function to establish cell-type identity [19]. Interestingly, several genes involved in the pheromone signaling cascade have also been recruited into the *C. neoformans* *MAT* locus. These include *Ste20a/α*, a signal transducing kinase of the PAK (p21-activated kinase) family, *Ste11a/α*, a mitogen activated protein kinase kinase kinase (MAPKKK) and *Ste12a/α*, a downstream transcription factor activated by the MAPK signaling cascade. There are also genes that may be involved in later steps in the mating process including meiosis and sporulation, and others with no apparent functions related to sexual development [19,20]. *MAT* is also rich in repetitive elements, which represent ~25% of the entire structure. How did this unusually large *MAT* gene cluster evolve? Sequencing of *MAT* from the sibling species *C. gattii* that diverged ~40 million years ago enabled a detailed comparison of the architecture, gene order, and phylogenetic histories of the genes, providing insights to advance a model on the evolution of the *MAT* gene cluster [3,20].

Fraser and colleagues proposed that the current extant structures of these *MAT* loci are derived from an ancestral, simpler tetrapolar *MAT* via a series of genomic rearrangement events that occurred during evolution. First, the ancestral tetrapolar pheromone/receptor and homeodomain protein loci acquired additional sex-related genes to form two unlinked gene clusters. Next, the two clusters of one mating-type fused via a chromosomal translocation, resulting in a contiguous *MAT* locus allele containing both pheromone/receptor and homeodomain protein genes while the two loci remained unfused in the other mating-type, forming an intermediate “tripolar” state. Lastly, recombination then occurred between the two alleles, forming two equivalent contiguous *MAT* structures that were then subject to more recent gene inversions and conversions, resulting in suppression of recombination between the two alleles and giving rise to the extant *MAT* locus alleles (Fig. 2) [3,20].

Recently, direct experimental evidence has been provided to support this model involving recapitulation of the ancestral tetrapolar state of *C. neoformans* [21]. By deleting the homeodomain genes in *MAT* and relocating them to a different chromosome, strains were engineered that now have two unlinked *MAT* loci, mimicking the ancestral tetrapolar mating system. These strains are able to complete the sexual cycle, and produce viable and fertile progeny, indicating that a tetrapolar mating system is functional in *C. neoformans*. These studies also further demonstrated that the intermediate tripolar state is likely under strong selection pressure due to a significant reduction of fertility. The chance of inbreeding from a tripolar genetic cross is restricted to 12.5% of the progeny. Furthermore, 50% of the progeny are not only sterile when mated with their siblings, but also are sterile when mated with cells of any other mating-type. This disadvantage might have contributed to the transition of a tripolar intermediate system to the extant bipolar one [21].

Another factor influencing the evolutionary trajectory of the *MAT* locus involves recombination hotspots linked to *MAT* (Fig. 2). Two recombination hotspots flanking the *MAT* locus were identified and found to elevate recombination ~10- to 50-fold during meiosis. These γ -type hotspots are correlated with a higher G + C content compared to other regions of the genome, and it was suggested that they might have facilitated the gene acquisition and translocation in the early steps of the *MAT* gene cluster evolution [22]. In addition, a G + C rich peak within *MAT* is present in both the *a* and *α* alleles of *C. neoformans* and *C. gattii*, between the *RPO41* and *BSP2* genes, which might also function as a recombination hotspot that has driven gene conversion between the two alleles, and further experiments to test this hypothesis are in progress[22].

M. globosa is another basidiomycete that resides on human and animal skin. For over a century, an association between *Malassezia spp.* and dandruff has been known, and some *Malassezia* species secrete antigenic proteins that exacerbate inflammatory skin disorders, but little is known about the biology and life cycle of these fungi. The genome of *M. globosa* has recently been sequenced and the 9 Mb genome is among the smallest genomes of free-living fungi [8]. Although *M. globosa* is phylogenetically closer to the plant pathogen *U. maydis*, many gene families encoded in the genome are similar to those of *C. albicans*, which is a phylogenetically distant species that can infect or reside in similar niches, such as human skin. In addition, an ~ 170 kb region was identified as the putative *MAT* locus of *M. globosa* because it shares homology and a related organization with the linked *a* and *b* loci of *U. hordei* [8]. Interestingly, although the putative *MAT* locus of *M. globosa* is structurally similar to that of *U. hordei* and *C. neoformans* in that the pheromone/receptor locus and the homeodomain protein gene loci are physically linked, a striking difference is a lack of repetitive sequences. Unlike the *MAT* locus of *U. hordei* and *C. neoformans*, in which 50% and 25% of the sequence are repetitive elements and transposons, these sequences are absent in the *M. globosa MAT* locus, indicating that repetitive sequences are not universal in an unusually large bipolar *MAT* locus. Moreover, this region is gene-rich, predicted to encode ~82 genes, and no significant similarities in gene content other than the key sex determinants were observed between the *MAT* loci of *M. globosa*, *U. hordei* and *C. neoformans* [8]. Thus, the translocation events that gave rise to these three bipolar species occurred independently in each lineage, indicating that the transition from tetrapolar to bipolar mating-type determination may be common. Although the sexual cycle of *M. globosa* is still cryptic, the discovery of this *MAT*-like structure implies that this pathogen may be capable of sex and the *MAT* structure implies that a bipolar mating system may be extant in this species.

Besides pathogens, basidiomycete ectomycorrhizal fungi are ecologically important for their roles in symbiosis with trees. Symbiosis depends on the sexual, dikaryon state of the fungi; however, little was known about their sexual cycle or the molecular structure of *MAT*. Recently, the first genome sequence of an ectomycorrhizal fungus, *Laccaria bicolor*, was reported [23]. Sequence analyses demonstrated that the homeodomain gene locus and the pheromone/receptor locus of this tetrapolar fungus are located in two unlinked genomic regions. Furthermore, the overall structures of these genes are conserved with that encoded by *MAT* of Agaricomycotina such as *Coprinopsis cinerea* [24]. Importantly, the gene organization revealed that the homeodomain locus is under strong selective pressure to maintain synteny while the selection pressure for the pheromone/receptor locus appears to be relaxed in that frequent gene duplications, translocations and insertions of transposons are observed in several members in the Agaricales, and thus, the evolutionary trajectory of the two unlinked *MAT* loci of tetrapolar species differs [24].

Tetrapolar mating systems first evolved within the phylum of Basidiomycetes; isolates of other fungal phyla described so far are exclusively bipolar. Therefore, the bipolar system is considered to be the ancestral state. Interestingly, although the tetrapolar mating system is more

commonly observed in basidiomycetes, a significant proportion of species are bipolar. For example, in mushroom fungi, it is estimated that ~65% of the species are tetrapolar, ~35% are bipolar and ~10% homothallic [25]. Both tetrapolar and bipolar mating systems are observed in closely related species, suggesting that conversions between the two mating systems have commonly occurred during evolution. What could cause these transitions during evolution? As first proposed by Raper, simple genetic changes may have such a consequence [25]. For example, a chromosomal translocation event can link two sex-determining regions, which converts a tetrapolar mating system to a bipolar one. The structures of the *MAT* locus in *C. neoformans*, *U. hordei* and *M. globosa* all support this model. Meanwhile, they also illustrate that the transition from a tetrapolar to bipolar mating system occurred multiple times independently during evolution. In addition to chromosomal translocation, mutations in the pheromone/receptor or homeodomain protein locus that render cells self-activated may also trigger the transition from tetrapolar to bipolar mating, and such mutants have been described in the mushroom fungi [13,26,27].

The model smut fungus *U. maydis* does not possess an extensive *MAT* gene cluster, and most of the genes clustered in the *Cryptococcus MAT* locus are dispersed throughout the genome of *U. maydis*. But interestingly, the *U. maydis* genome sequence revealed 12 gene clusters of small secreted proteins [28]. The expression of most of the genes in these clusters is regulated coordinately and induced during the process of plant infection. Furthermore, it was demonstrated that deletion of several such clusters significantly reduced virulence whereas in other cases, deletion caused either no effect or enhanced virulence [28]. A transcription factor that regulates the expression of a subset of the *MIG2* gene cluster was recently identified, named Mzr1, providing a further understanding of the molecular basis for the regulated expression of these virulence clusters in response to the plant host [29]. It is not clear how these functionally-related genes were recruited into clusters during evolution, and we propose that parallel studies on the evolution of *MAT* gene clusters from other basidiomycetes may provide further insights into the origin of gene clusters that are important for virulence.

Sex and virulence

It is clear that sex has been favored by evolution since most organisms employ sexual reproduction as the mode to generate offspring. However, the roles that sex plays in the evolution of pathogenic species are debated. One can imagine that virulence of a pathogen is a polygenic trait, and random chromosomal assortment and recombination during meiosis is likely to disrupt favorable gene combinations necessary for virulence. Nonetheless, one can also advance the counter argument that recombination may generate offspring with enhanced virulence and better fitness, and thus sex might also prove beneficial to the population.

In rust fungi such as *U. maydis* and *U. hordei*, it is clear that sex is necessary for virulence (Fig. 3). The infectious structure, the filamentous dikaryon, is only produced after mating. Cells unable to mate are avirulent because they fail to generate the filamentous structures that grow invasively *in planta* [30]. In addition, the ability of these fungi to complete their sexual cycle in the lab is limited and cues from the plant are necessary to support efficient mating. Similarly, in some phytopathogenic ascomycetes, ascospores are important as inocula to initiate diseases, especially in species that do not produce mitotic spores such as *Sclerotinia sclerotiorum* [31]. Interestingly, it has been recently shown that the debilitating mycovirus in this species cannot be transmitted via ascospores, demonstrating that sex serves as a defending mechanism for virus infection [32]. In contrast, in many human fungal pathogens such as *Aspergillus fumigatus*, *Coccidioides immitis*, *Candida glabrata*, and *M. globosa*, sex has never been observed, despite the fact that they all possess *MAT* loci, machinery for mating and meiosis, and population genetic evidence in support of sexually recombining populations has been reported (Fig. 3) [8,33-37].

The issues linking sex and virulence are especially intriguing in *C. neoformans*. In this human fungal pathogen, the sexual cycle readily occurs in the laboratory but mating has never been directly observed in nature or during infection [38,39]. One major factor that accounts for this discrepancy is the highly skewed mating-type distribution: the vast majority isolates are α , and thus, chances for mating to occur between cells of opposite mating-type are limited. One exception is a unique population found in Botswana, in which 10% of the isolates are **a** mating-type [40]. Recently it was further demonstrated that mating not only occurs on the mating-inducing medium such as V8 juice agar, but also occurs during co-culture with plants, implying that specific conditions in nature may be required for mating to occur (Fig. 3)[41]. Unlike the smut fungi, in which the sexual state is required for infection, it is the yeast form of *C. neoformans* that causes disease development and studies have shown that in α cells are more virulent than **a** cells in certain genetic backgrounds [42,43]. Nonetheless, the sexual cycle is vital to generate spores, which are the suspected infectious propagules. Monokaryotic fruiting, also known as same-sex mating, is an unusual, alternative sexual reproduction mode that typically involves cells of the α mating-type [44]. Several studies have found evidence of recombination in populations with few or no mating-type **a** isolates, suggesting that sexual reproduction is likely to be maintained in these populations via same-sex mating [40,45-48]. Another line of evidence supporting the impact of same-sex mating derives from the *C. neoformans* sibling species *C. gattii*, which has caused an outbreak on Vancouver Island since 1999 [49]. Studies on the origin of the outbreak isolates led to the hypothesis that the majority of these clones descend from two α parents [50]. The resulting majority α isolate is hypervirulent in a murine inhalation model, demonstrating that same-sex mating might have facilitated the emergence of new strains that have evolved to be more potent in infection, and same-sex mating might also contribute to the ongoing production of infectious propagules driving the outbreak [50].

In summary, most fungal pathogens retain the machinery to engage in sex although their ability to undergo sexual reproduction appears in many cases to be restricted, possibly to ensure their virulence traits remain linked but at the same time, retain the potential to diversify their population when faced with strong selection pressures or new environments. Thus, a mixture of clonal and recombining populations is often observed. Finally, what we have learned from the evolution of fungal pathogens is likely to be applicable to other pathogenic microbes, such as bacteria and parasites [39]. Now the challenge for the future is to further understand the underlying bases of sex, sex determination, and the structure of *MAT* in a wider spectrum of species to gain further insights into their evolution and their impact on both sexual reproduction and virulence.

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Figure 1.

MAT structures of representative basidiomycete pathogens. The homeodomain genes (red and blue) and the pheromone/ receptor genes (green and yellow) are highlighted.

Figure 2. Evolution of the *MAT* locus in *C. neoformans*. We hypothesize that the current bipolar *MAT* structure evolved from an ancestral tetrapolar state via additional gene acquisition, translocation and recombination, and that similar but independent translocation events occurred during the evolution of other bipolar fungi (*M. globosa* and *U. hordei*).

Ascomycete	Basidiomycetes		
<i>Candida albicans</i>	<i>Malassezia globosa</i>	<i>Cryptococcus neoformans</i>	<i>Ustilago maydis</i>
Human pathogen			Plant pathogen
<ul style="list-style-type: none"> • Diploid commensal resides in mammalian GI tract and on mucosal surfaces. • Has the ability to mate. Opaque cells can mate on skin or in the anaerobic conditions of the GI tract.	<ul style="list-style-type: none"> • Haploid yeast resides on human and animal skin. • No known sexual cycle but the discovery of <i>MAT</i> and conserved mating machinery has led to the proposal that mating may occur on human skin.	<ul style="list-style-type: none"> • Haploid yeast found ubiquitously in trees, soil, and pigeon guano. • Mating is stimulated by plants or on pigeon guano, but no evidence for mating in the host. • Sexual cycle leads to the production of basidiospores, the presumptive infectious propagules.	<ul style="list-style-type: none"> • Haploid yeast that infects the plant host (maize) as a filamentous dikaryon. • Mating and sexual development occurs <i>in planta</i> and is essential for infection.

Figure 3.

Sex and virulence in fungal pathogens. A comparison of the link between virulence and sex in the human fungal pathogens *C. albicans*, *M. globosa*, *C. neoformans* and the plant pathogen *U. maydis*.

Table 1

Mating systems in Basidiomycetes

Species	Mating system	Host	# of alleles in the population	Chance of inbreeding	Chance of outcrossing
<i>Ustilago maydis</i>	Tetrapolar-bi/ multiallelic	Corn	2 (<i>a</i> locus) >25 (<i>b</i> locus)	25%	50%
<i>Coprinopsis cinerea</i>	Tetrapolar-multi/ multiallelic	Saprotrophic	>90 for both <i>A</i> and <i>B</i> loci	25%	99.5%
<i>Malassezia globosa</i>	Bipolar	Human	Unknown	~*	~*
<i>Cryptococcus neoformans</i>	Bipolar-biallelic	Human	2	50%	<50%
<i>Ustilago hordei</i>	Bipolar-biallelic	Corn	2	50%	50%

~* sexual cycle remains to be identified. Pictures of meiotic spores are shown in the table except for *M. globosa*.