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Review

Seasons of change: Mechanisms of genome evolution in human fungal pathogens

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ABSTRACT

Fungi are a diverse kingdom of organisms capable of thriving in various niches across the world including those in close association with multicellular eukaryotes. Fungal pathogens that contribute to human disease reside both within the host as commensal organisms of the microbiota and the environment. Their niche of origin dictates how infection initiates but also places specific selective pressures on the fungal pathogen that contributes to its genome organization and genetic repertoire. Recent efforts to catalogue genomic variation among major human fungal pathogens have unveiled evolutionary themes that shape the fungal genome. Mechanisms ranging from large scale changes such as aneuploidy and ploidy cycling as well as more targeted mutations like base substitutions and gene copy number variations contribute to the evolution of these species, which are often under multiple competing selective pressures with their host, environment, and other microbes. Here, we provide an overview of the major selective pressures and mechanisms acting to evolve the genome of clinically important fungal pathogens of humans.

Fungi are a remarkably diverse group of 1.5 million species that have been evolving as a distinct kingdom for an estimated billion years (Blackwell, 2009). Over that time, fungi have evolved mutualistic, commensal, and parasitic relationships with multicellular eukaryotes such as plants and animals (Rodriguez and Redman, 2008). Research into plant-fungal interaction laid the groundwork for investigating molecular mechanisms of human fungal pathogenesis (Flor, 1956). This is due, in part, to the long-standing focus on fungi in crop plant diseases that was reinforced by the relative lack of discernable disease burden historically inflicted upon humans by fungal organisms. However, over the last few decades, interest in the impact of fungal interactions on human health has grown (Seed, 2014). Much of this interest stems from Western clinical practices such as immune-suppression and chemotherapy that have sensitized patients to infections by microbes typically restricted by the immune system or the patient's own microbiota, including many fungal species. As a consequence, species that were often considered allergens or benign fungal commensals have risen to prominence as important clinical agents of infection.

1. Environmental and host-associated fungal pathogens

Fungi occupy a plethora of niches including environmental and host-associated spaces. In the environment, fungal organisms have a profound impact on ecological cycles where they are estimated to outnumber metazoan species by a factor of 50:1 (Blackwell, 2011; Hawksworth and Lucking, 2017; Tovey and Green, 2005). These fungi obtain nutrients from dead and decaying organic matter that requires various catabolic pathways to utilize the millions of different compounds present while also resisting the ubiquitous presence of heavy metals, temperature fluctuations, and radiation. Competition with a highly diverse repertoire of microorganisms that includes both predators and prey adds to the complexity of these niches. Thus, fungi face a complex and dynamic environment with a continuously changing set of biotic and abiotic selective forces.

A select few fungi that propagate through defined environmental reservoirs are able to cause disease within healthy individuals. Primary fungal pathogens that can cause disease in otherwise healthy individuals are typically endemic to certain geographical regions that tend to favor warmer climates and include dimorphic fungi among the *Histoplasma*, *Blastomyces*, *Coccidioides*, and *Paracoccidioides* genera (Sifuentes-Osornio et al., 2012). Dimorphic fungi grow as mycelia within temperate soils and produce spores that are dispersed into the environment where they can be inhaled by mammalian hosts. The spores germinate in response to the elevated body temperatures following intake by mammals and begin to proliferate as yeast, which is typically the disease-causing form (Medoff et al., 1987). The transition

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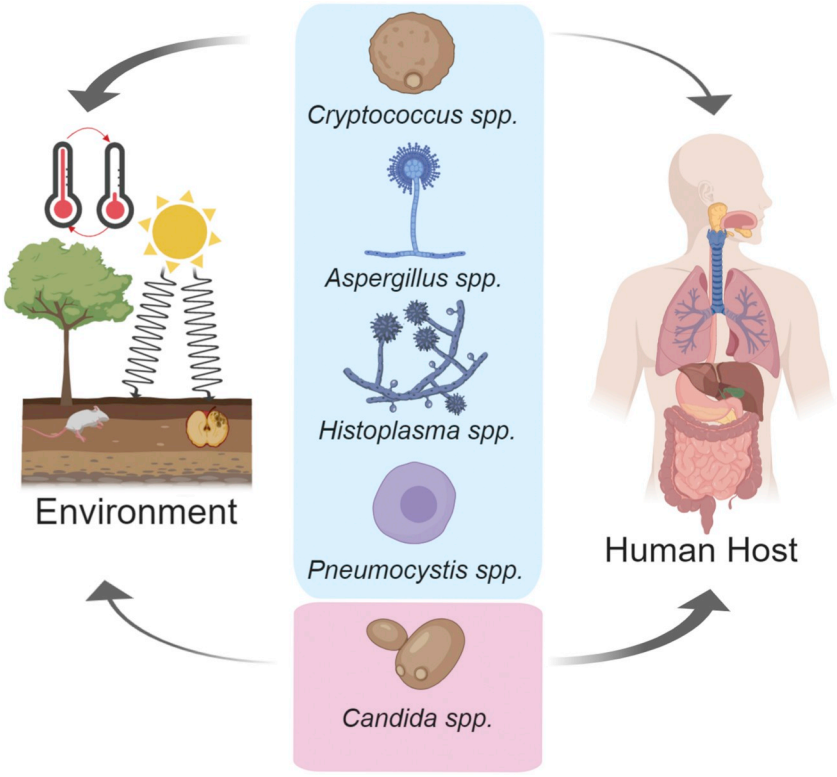

Fig. 1. Differences between fungal pathogen reservoirs. Human fungal pathogens existing primarily within terrestrial environmental reservoirs (blue) must encode genes to utilize energy from diverse carbon sources, modulate large temperature shifts, resist radiation, and compete with other soil residents as well as the human host during infection. In contrast, primarily host-associated fungi (magenta) encode genes for host-specific carbon utilization, grow at relatively stable temperatures, and must contest for space and nutrients with microbes specific to the host in addition to the host. Arrow thickness denotes the prevalence of the fungi within each niche.

to yeast phase growth correlates with production of disease-causing factors including changes to the cell wall structure, presentation of surface antigens, and secreted effectors (Isaac et al., 2015; Marion et al., 2006; Rappleye and Goldman, 2006; Sterkel et al., 2016). If the invasive yeast are engulfed by phagocytic cells, effector molecules restructure the phagosome into a permissive environment suitable for yeast growth and continued proliferation (Beck et al., 2009; Brandhorst et al., 2013).

In contrast to primary fungal pathogens, most environmental fungi are only able to cause disease in immunocompromised hosts that lack innate or adaptive immunity. These fungi represent species among the Ascomycota, Basidiomycota, Zygomycota, and Chitridiomycota fungal phyla (Ecker et al., 2005) (Fig. 1). Infection by these species can spike following environmental disturbances such as natural disasters that lead to rapid and transitory foci of disease from organisms typically restricted by environmental barriers to their dispersal (Benedict and Park, 2014; Schneider et al., 1997). Fungi gain access to the host via a diversity of routes including inhalation, ingestion, and access through the skin. Once inside the host, survival strategies that are critical to microbe-microbe interactions in the environment can directly impact the progression of human disease. For example, the dimorphic fungal pathogen *Cryptococcus neoformans* has evolved a morphologically-distinct polyploid cell state termed the Titan cell (Zaragoza and Nielsen, 2013). Titan cells are resistant to phagocytosis by soil-born parasitic *Acanthamoeba* species that may be encountered by *Cryptococcus* in the environment, while also conferring resistance to uptake by professional phagocytes in the host following inhalation (Derengowski Lda et al., 2013; Okagaki and Nielsen, 2012). Thus, the selective pressures fungi can encounter in a free-living environment may also have direct consequences on pathogenic processes in the human host.

In contrast to environmental fungi, the number of resident fungal species associated with humans are estimated to be in the hundreds (Huffnagle and Noverr, 2013). Host-associated fungi persistently colonize the skin, oral cavity, gastrointestinal tract, and urogenital tract of their human host where mutualistic and commensal relationships dominate their interactions (Li et al., 2018). Niche specificity of commensal fungi varies considerably with species such as *Candida albicans* capable of colonizing all of these body sites, whereas *Malassezia* spp. are found almost exclusively on the skin. Outgrowth of resident fungi is prevented by physical barriers of the host, immune surveillance, and competition with other resident bacterial and fungal species (Parfrey et al., 2011). Loss of these antagonistic forces can lead to parasitic overgrowth of commensal fungi resulting in debilitating superficial and mucosal infections as well as life-threatening systemic infections (Ascioglu et al., 2002; De Pauw et al., 2008). Importantly, the distinctions between host-associated and environmental fungi may be less well defined than previously thought. A recent study reported the identification of *Candida albicans* isolated from oak trees, suggesting that an environmental reservoir for *C. albicans* may exist (Bensasson et al., 2019). Thus, host-associated fungi may more intimately associate with the host as opposed to being completely excluded from the environment.

Work prior to large-scale sequencing efforts predicted co-evolutionary trajectories of commensal eukaryotes matched their hominid hosts during the last 6-26 million years (Berger et al., 2002; Lott et al., 2005). More recent work evaluating the evolution of bacterial species within the microbiomes of hominid species supported this hypothesis. Phylogenies of some bacterial constituents within the gut microbiome correlated tightly with their hominid host (Ochman et al., 2010). No work has yet been performed to test for similar evolutionary histories in commensal fungi but it is reasonable to speculate that similar phylogenetic trends will exist between fungal residents of humans and other hominid relatives. Unfortunately, little research describing the fungal components of the microbiome, the 'mycobiome', exists for apes and other closely-related hominids. The sole study of fungal diversity within the eukaryome of western lowland gorillas in their natural habitat demonstrated the presence of several fungal species occupying the GI tracts that are common to humans including *Candida* and *Pichia* species (Hamad et al., 2014). This similarity in the mycobiome between humans and gorillas could be due to establishment of fungal species within the GI in a common ancestor, interactions between humans and other hominids leading to cross cultivation, or that similarities in diet

Table 1
Genome characteristics of pathogenic fungi

	Genome Length (Mb)	Chromosomes	Genes	Proteins	GC Content (%)	Assembly	Habitat
Aspergillus niger	35.74	8	11601	11190	49.8	GCA_002211485.1	Soil
Aspergillus fumigatus	29.39	8	19832	19260	49.81	GCA_000002655.1	Soil
Aspergillus flavus	37.03		13196	13196	48.3	GCA_000952835.1	Plants/Soil
Coccidioides immitis	29.02		9905	9910	46	GCA_000149335.2	Soil, southwest US
Coccidioides posadasii	28.21		10016	9964	46.3	GCA_000150055.1	Soil, southwest US
Histoplasma capsulatum	38.96		9792	9547	42.1	GCA_000151035.1	Soil, Ohio and Mississippi River valleys
Cryptococcus neoformans	18.91	15		7826	48.2	GCA_000149245.3	Soil, plants, animals
Cryptococcus deneoformans	18.52	14	6609	6578	48.54	GCA_000149385.1	Soil, plants, animals
Fusarium solani	45.81				49.3	GCA_002215905.1	Soil
Fusarium oxysporum	61.38	15	21326	27347	48.43	GCA_000149955.2	Soil
Blastomyces dermatitidis	66.6		10081	11539	37.1	GCA_000003525.2	Soil
Candida albicans	14.28	8	6263	6030	33.48	GCA_000182965.3	Commensal
Candida dubliniensis	14.61	8	6093	5859	33.25	GCA_000026945.1	Commensal
Candida auris	12.11		5541	5382	45.2	GCA_003013715.1	Commensal
Candida glabrada	12.72	13	5543	5293	38.92	GCA_002219185.1	Commensal
Saccharomyces cerevisiae	12.17	16			38.31	GCA_003086655.1	Commensal
Candida tropicalis	14.63		6441	6254	33.2	GCA_000006335.3	Commensal
Candida parapsilosis	13.03		5907	5810	38.7	GCA_000182765.2	Commensal
Candida orthopsilosis	12.65	8	5782	5678	37.62	GCA_000315875.1	Commensal
Cyberlindnera jadinii	13.02	13	6184	6032	44.6	GCA_001661405.1	Commensal
Epicoccum nigrum	34.6		12190	12025	52	GCA_002116315.1	Soil

and/or lifestyle have led to similar microbial profiles. Importantly, populations of macaques, gorillas, and humans have distinct microbiomes when separated geographically, supporting a significant role for diet and geography in likely shaping the eukaryotic residents of each hominid species. (Gomez et al., 2015; Smits et al., 2017; Wilcox and Hollocher, 2018). Thus, changes to commensal fungi within the host likely reflect a mixture of evolutionary and environmental selection in shaping microbial communities.

2. Divergent genome organization of fungal pathogens

Key differences in the genome sequences between fungi that are either predominantly commensal or environmental highlight their unique lifestyles. Genomes of fungal pathogens with known environmental reservoirs are commonly significantly larger than that of commensal fungi (Table 1), often exceeding twice the DNA content (Butler et al., 2009; Desjardins et al., 2011; Farrer et al., 2015; Hirakawa et al., 2015). This variation in genome size generally correlates with the presence or absence of intronic gene structures; dimorphic fungi encode genes with a high frequency of introns whereas genes in commensal yeasts contain relatively few introns (Edwards et al., 2013; Janbon, 2018; Mitrovich et al., 2007; Spingola et al., 1999). Additionally, many opportunistic and primary fungi from the environment contain significantly more genes than their commensal counterparts, often exceeding 10,000 genes compared to 5,000 to 6,000 common in most commensals. Interestingly, the guanine-cytosine (GC) bias of environmentally-derived and host-associated fungi differ considerably as well. Fungal organisms originating from environmental reservoirs harbor larger GC content within the genome that may be the result of GC-biased gene conversion following meiotic recombination (Duret and Galtier, 2009). Most environmental opportunistic pathogens have either defined or putative sexual programs based on population composition and conservation of meiosis-specific genes; whereas multiple host-associated *Candida* clade species lack known meiotic cycles (Ene and Bennett, 2014; Heitman et al., 2014). An absence or reduction in meiosis and, thus, GC-biased gene conversion may explain the reduced GC content of commensal fungi despite errors in DNA replication and mitotic recombination during vegetative growth introducing increased GC content as well (Marsolier-Kergoat, 2013). Extreme examples of reduced genome size and gene content are observed among intracellular Microsporidia eukaryotes (Cuomo et al., 2012; Katinka et al., 2001; Pombert et al., 2015) and bacteria (Bobay and Ochman, 2017; Ochman and Davalos, 2006) during the transition from free-living environmental lifestyles to obligate host-associated forms. Thus, reliance on commensal relationships with the host and its associated microbial communities by fungi may remove the requirement for genes necessary in the environment and spur a reduction in genome size.

The genetic repertoire of commensal and environmental fungi capable of causing human disease reflects their surrounding habitats. Genes required for temperature-induced morphogenic switches, control of transcriptional regulation, and breakdown of an expansive array of carbohydrate and amino acids are present at high numbers in environmental isolates compared to commensals (Desjardins et al., 2011; Munoz et al., 2018). Interestingly, these same environmentally-acquired pathogens have lost many of the genes essential for colonization and metabolism of plant-derived compounds while increasing the genetic repertoires of enzymes used to breakdown amino acids and fatty acids compared to other closely-related species that are incapable of establishing mammalian infections (Munoz et al., 2018).

In the smaller genomes of commensal species, increased pathogenesis correlates with expansion of genes whose products interface with the host cell surface and secreted proteins (Butler et al., 2009). Expansion of cell surface protein repertoires linked to adhesion, invasion of host cell barriers, and iron acquisition are indicators of increased potential for pathogenesis (Almeida et al., 2008; de Groot et al., 2013; Yeater et al., 2007). Secreted lipases, amino acid permeases, and superoxide dismutases are similarly associated with increased virulence due to their ability to destroy tissues and take up nutrients while protecting the cell from host-mediated immunity (Butler et al., 2009; Kraidlova et al., 2011). Indeed, gene family diversity among pathogenic fungi is a reliable way to distinguish between phyla and genus (Fitzpatrick et al., 2006), suggesting variation in gene families leads to niche specification and success in commensal relationships.

3. Evolutionary mechanisms in fungal pathogens

Here we will focus on evolution of the four major human fungal pathogen genera: *Candida*, *Cryptococcus*, *Aspergillus*, and *Pneumocystis*. These genera are clustered primarily within the Ascomycota (*Candida*, *Aspergillus*, and *Pneumocystis*) and include a single Basidiomycota (*Cryptococcus*). Systemic infections of these species are particularly lethal with rates of mortality between 20% and 95% for systemic infections, significantly greater than common bacterial pathogens (Brown et al., 2012; Wisplinghoff et al., 2004).

Iterative mutation is often the common evolutionary mechanism ascribed to fungal species because all major human fungal pathogens can replicate by vegetative growth through repeated mitotic cell division to produce a clonal population of cells. During mitosis, DNA damage or failures during replication and cell division give rise to heritable changes in the DNA of the cell. These changes accumulate over generations to produce genetically distinct but related strains whose evolutionary progression can be traced through 'identity by descent', a phylogenetic method of inferring relatedness by genetic similarity (Butler et al., 2009; Engelthaler et al., 2016; Findley et al., 2009; Odds et al., 2007; Taylor and Fisher, 2003). Most fungal pathogens originating from environmental sources and some commensal organisms can also evolve through meiotic processes of recombination and segregation (Ene and Bennett, 2014; Heitman et al., 2014; Sherwood et al., 2014). Furthermore, many of these species including *C. neoformans*, *A. fumigatus*, and some commensal *Candida* species lacking a meiotic cycle can evolve through parasexual processes, which involves a random process of ploidy reduction and recombination termed 'concerted chromosome loss' (CCL) (Ene and Bennett, 2014; Forche et al., 2008; Li et al., 2012; Seervai et al., 2013). Sexual and parasexual processes facilitate greater genetic diversity and the joining of potentially advantageous allelic combinations.

3.1. Single nucleotide mutations

Base substitutions are a major driver of genetic diversity within these fungal species. Recent sequencing efforts of *Pneumocystis* clonal genetic diversity estimated variation between strains up to 0.1% (Cisse et al., 2018), whereas *C. albicans* strain diversity breaches 1% (Wang et al., 2018). Nucleotide variation among the *C. neoformans* species complex can extend up to ~5% but likely reflects added meiotic contributions that are estimated to have occurred over the last ~10 million years (Farrer et al., 2015; Firacative et al., 2016; Ngamskulrungroj et al., 2009). Relatively little *de novo* genetic variation is often found within *A. fumigatus* and instead gene flow of existing alleles contributes more substantially to the global genetic diversity (Ashu et al., 2017; Rydholm et al., 2006). Surprisingly, the high level of genetic variation present within fungal species does not inhibit formation of viable hybrid genotypes that give rise to novel phenotypes with implications for virulence (Mitchell and Perfect, 1995; Ropars et al., 2018; Wang et al., 2018).

Genome-wide analysis within these species found bias in the base substitutions that accumulated over time. Mutations tend to accrue in centromeric, subtelomeric, and repetitive regions of the genome (Cisse et al., 2018; Ene et al., 2018). Minimal functional consequences result from single nucleotide polymorphisms (SNPs) and other sequence variants emerging in centromeres and repetitive DNA, likely a result of being relatively gene-poor or lacking highly restricted functional sequences altogether (Lephart et al., 2005; Yadav et al., 2018). In contrast, mutations in subtelomeric sequences often reside within gene families members whose products encode cell surface antigens, transcription regulators, and secondary metabolism gene clusters (Anderson et al., 2012; de Groot et al., 2008; McDonagh et al., 2008; Zhang et al., 2012). These mutations drive sequence divergence of already highly plastic regions of the genome (Brown et al., 2010; Carreto et al., 2008). Mutations within the *C. albicans* genome also preferentially accumulated in heterozygous regions of the genome compared to those that have experienced homozygosis via loss of heterozygosity (LOH) over time similar to biases present in multicellular eukaryotes during meiosis (Wang et al., 2018; Yang et al., 2015).

Selection acting on new mutations can provide clues as to what processes and functions are critical to the organism's lifestyle. Among fungal pathogens, signatures of positive selection are overrepresented in multidrug transporters, cell-surface proteins such as antigens, and secreted virulence factors (Butler et al., 2009; Delaye et al., 2018; Farrer et al., 2015; Keely et al., 2005). *C. neoformans* strains also underwent strong selection for sugar transporters, which may reflect the interplay between environmental persistence and mammalian infection (Desjardins et al., 2011). Importantly, genes under positive selection also often reside within subtelomeres consistent with these genomic regions being important sites for adaptive innovation (Dunn et al., 2018; Keely et al., 2005; Mason and McEachern, 2018).

While certain regions of the genome may be more prone to mutation, retention of non-synonymous SNPs is the principle driver of selection across the genome. In *C. albicans* the rate of synonymous substitutions across the genomes of 21 sequenced isolates was fairly constant; however, the frequency of non-synonymous polymorphisms fluctuated greatly (Hirakawa et al., 2015). Consequently, the selection coefficient generally mirrored the distribution of non-synonymous mutations and underscored regions of positive and purifying selection across the genome.

3.2. Insertions/deletions and copy number variation

Small gains and loss of DNA resulting in formation of insertions/deletions (indels) are less well described than nucleotide variation but also provide important contributions to genome evolution in human fungal pathogens. Indels often follow the same phylogenetic relationships as nucleotide polymorphisms in *Candida* species due to their primarily clonal population structure (Pryszcz et al., 2015; Ropars et al., 2018; Wang et al., 2018). Indel frequency is tightly correlated with base substitutions, suggesting that mutational synergy or underlying genomic features in *C. albicans* may enrich for both mutation types (Wang et al., 2018). Often found within repetitive domains, these expansions and contractions can profoundly alter the size of the repetitive domain including the major repeat sequences (MRS) found on all *C. albicans* chromosomes (Chr) with the exception of Chr3 (Chibana et al., 2000; Ene et al., 2018).

Longer indel tracts that cover full functional sequences resulting in gene duplication and/or loss are typically more common than base substitutions (Keith et al., 2016). For example, small intrachromosomal events led to over 4% of the *C. gatti* pan-genome being present as lineage-specific or multilineage-specific genes (Farrer et al., 2015). These genes cover an array of biological phenotypes with enrichment for oxidative stress responses, iron binding, membrane trafficking, genome stability, and RNAi machinery (D'Souza et al., 2011; Steenwyk et al., 2016). Similarly, genes involved in stress resistance and membrane transport vary in copy number among *C. neoformans* sequenced isolates, potentially reflecting selective pressures held in common between the species (Day et al., 2017). An even larger fraction, approximating 10% of the *A. fumigatus* genome experienced copy number variation (CNV) among 71 sequenced isolates (Zhao and Gibbons, 2018). These CNVs followed the strain set's general phylogenetic relationship, suggesting mitotic mechanisms of gain and loss that persisted through evolutionary time are a common feature in *A. fumigatus*.

Analysis of CNVs over longer evolutionary histories between *Candida* species found 21 gene families that increased in size among more pathogenic species (Butler et al., 2009). As with the other fungal pathogens, these gene families encoded cell surface antigens (Hoyer and Cota, 2016), genes involved in iron acquisition (Fourie et al., 2018), and host cell invasion (Yang et al., 2014). Interestingly, a family of transcriptional regulators called the telomere-associated (*TLO*) genes have undergone the greatest expansion in the most clinically relevant *Candida* species, *C. albicans*, although their precise role in virulence is still unclear (van het Hoog et al., 2007). Similarly, mechanisms of gene loss can provide massive advantages to fungal survival in host systems. The obligate biotroph *Pneumocystis jirovecii* lost over 2000 genes compared to related *Pneumocystis* that are thought to reduce its virulence and immunogenicity to facilitate persistence in the human host (Cisse et al., 2014). Thus, organismal lifestyle can profoundly influence retention of indels and specifically those covering functional sequences associated with virulence and persistence in the host.

3.3. Karyotypic changes

Changes in copy number can extend beyond repetitive sequences and single genes to encompass whole chromosomes or chromosome complements. Ploidy changes can occur through mating and defects in cell division whereas aneuploidy arises primarily in the latter. While whole ploidy changes are generally well tolerated or required steps within various mating processes, aneuploidy induces proteotoxic stress and poses a fitness disadvantage under most conditions (Dephoure et al., 2014; Sheltzer and Amon, 2011). Thus, tolerance of chromosomal imbalances due to aneuploidy were canonically thought of as an unusual feature of some highly plastic fungal genomes such as that of *C. albicans*. Yet, more recent work has demonstrated that persistent aneuploidy is widespread across the eukaryotic lineage including *Leishmania* spp. (Mannaert et al., 2012), *Saccharomyces cerevisiae* (Gasch et al., 2016) and crop plants (Wu et al., 2018; Zhang et al., 2013).

Whole ploidy shifts are generally associated with sexual and parasexual processes within these fungal pathogens. For example, *C. neoformans* and *C. gatti* species are typically found as haploid cells but undergo both homothallic (Feretzaki and Heitman, 2013; Lin et al., 2005; Phadke et al., 2014) and heterothallic mating (Campbell et al., 2005; Fraser et al., 2003; Nielsen et al., 2007) to form transient diploid intermediates before meiotic reductions in DNA to haploid sexual products. Haploid *A. nidulans* and *A. fumigatus* species undergo similar processes of ploidy cycling during meiosis beginning and ending in haploid states (Egel-Mitani et al., 1982; O'Gorman et al., 2009). Conversely, no conclusive evidence exists for meiosis in *Pneumocystis* despite ploidy cycling between the haploid trophic form and polyploid precyst form being a defining feature of its life cycle and its genome containing homologs of meiotic proteins (Gigliotti et al., 2014). Changes in DNA content through the life cycle without major shifts in allelic frequencies between the trophic and precyst forms suggest that either conjugation between haploid trophic forms, autodiploidization, or homothallic mating produces the diploid form (Almeida et al., 2015). Among *Candida*, only two species, *C. lusitaniae* and *C. guilliermondii*, have defined meiotic cycles (Ene and Bennett, 2014; Reedy et al., 2009). Furthermore, *C. lusitaniae* has a fused sexual cycle in which mating is followed by meiosis, reducing cells immediately to a haploid state (Reedy et al., 2009; Sherwood et al., 2014).

Fusing of genomic contents via hybridization also contributes to altered ploidy states among fungal pathogens. Mating between haploid *C. neoformans* and *C. deneoformans* has led to hybrid strains that retained a diploid state or degenerated to become aneuploid (Cogliati et al., 2001; Lengeler et al., 2001; Samarasinghe and Xu, 2018). Similarly, hybridization among haploid progenitor *Candida* species gave rise to members of the *Candida orthopsilosis* cluster. Although *C. metapsilosis* emerged from a single ancestral hybridization (Pryszcz et al., 2015), these events are likely fairly common as the *C. orthopsilosis sensu stricto* species arose from multiple independent matings of genetically distinct haploid progenitors (Schroder et al., 2016). Interestingly, all fusant species retained primarily diploid genomes and high levels of heterozygosity. Retention of the fused diploid genome may have due to increased resistance to mutagenic forces (Mable and Otto, 2001), the ability to mask deleterious recessive alleles (Gerstein et al., 2011), or requirements to retain both genomes to maintain differential regulatory processes between the previously haploid genomes.

In contrast to the ordered ploidy reduction characteristic of meiosis, some human fungal pathogens employ parasexual programs of mating and ploidy reduction. Dissection of parasexual programs center on *Candida* species including *C. albicans*, *C. dubliniensis*, and *C. tropicalis* who are all capable of heterothallic mating (Bennett and Johnson, 2003; Pujol et al., 2004; Seervai et al., 2013). Homothallic mating has also been described for *C. albicans* (Alby et al., 2009). Isolates of opposing mating types (a or α) initially fuse by mating and karyogamy to generate a/α mating products containing the full genomes of both parental cells (Hull et al., 2000; Magee and Magee, 2000). These stable mating products can then be induced to reduce their ploidy via CCL (Forche et al., 2008; Hickman et al., 2015). Progeny cells from CCL span ploidy states extending from haploid to highly aneuploid karyotypes. As in meiosis, recombination prior to chromosome loss leads to the production of novel recombinant genotypes and involves the meiosis-specific factor Spo11 (Forche et al., 2008). Until recently, little data supported the existence of *C. albicans* mating and parasex outside the laboratory (Jacobsen et al., 2008; Odds et al., 2007; Tavanti et al., 2005), but two recent studies provided strong evidence of both ancient and more recent genetic exchange within *C. albicans* natural isolates (Ropars et al., 2018; Wang et al., 2018). Therefore, parasex likely contributes to the *C. albicans* population structure and may play similar roles in evolution among other parasexual *Candida* species.

Recovery of aneuploid isolates from clinical infections is a common occurrence among human fungal pathogens. Strains recovered from cryptococcal infections harbored extra copies of various chromosomes (Desnos-Ollivier et al., 2010; Fries et al., 1996; Hu et al., 2011). Furthermore, karyotypically-distinct but genetically-related *C. neoformans* cells can be isolated from a single site of infection (Desnos-Ollivier et al., 2010). The advantages to mixed population persisting throughout the course of infection are unclear but could include balancing selection for different genotypes within the virulent population and/or induction of chromosomal abnormalities in previously isogenic cells. Once isolates are established in the lab, these chromosomal imbalances can persist and, in some cases, arise *in vitro* (Chibana et al., 2000; Rustchenko, 2007; Selmecki et al., 2005). For example, exposure of *C. albicans* to sorbose induced loss of one Chr5 homolog to relieve repression on the Sorbose Utilization (*SOU1)* gene and promote survival (Kabir et al., 2005). Additionally, induction of aneuploidy with mitotic spindle destabilization agents in *A. nidulans* was a common experimental system to dissect the process of generating aneuploidy and the implications of chromosomal imbalance prior to the genome revolution although naturally occurring aneuploidy appears to be quite rare (Kafer et al., 1986).

The frequency of aneuploidy among natural isolates of *C. albicans* continues to be a topic of significant debate. Aneuploidy is a common outcome of laboratory manipulation of *C. albicans* (Arbour et al., 2009), and supernumerary chromosomes are found in a number of historically important laboratory strains such as WO-1 and CAI-4 (Abbey et al., 2011; Chibana et al., 2000; Selmecki et al., 2005). In fact, some aneuploidy states are quite stable during *in vitro* passaging of *C. albicans* cells in rich media (Hickman et al., 2015). Importantly, passage of *C. albicans* through a mouse models of systemic infection is also capable of producing aneuploid progeny and demonstrates the general tendency towards aneuploid formation across environments (Forche et al., 2009b). A large sequencing effort to capture the genetic diversity among 21 *C. albicans* strains found almost 1/3 of sequenced isolates contained aneuploid chromosomes (Hirakawa et al., 2015). Furthermore, these aneuploid chromosomes were readily lost during *in vitro* passaging consistent with *in vitro* derived aneuploid cells (Anderson et al., 2017). However, a subsequent effort constituting 182 genomes observed whole chromosome aneuploidy in only ten isolates, suggesting that exposure to antifungal drugs may be responsible for high levels of aneuploidy observed previously (Ropars et al., 2018). Accordingly, karyotypic changes including the gain and loss of chromosome copy number did occur during sequential isolation of *C. albicans* from the infection site during the course of patient treatment with azole class antifungals (Ford et al., 2015). Indeed, formation of aneuploid cells is a common feature following exposure to azoles in both *C. albicans* and *C. neoformans* (Ngamskulrungroj et al., 2012; Selmecki et al., 2006; Selmecki et al., 2009; Sionov et al., 2010). Aneuploidy functions here to increase expression or efficiency of ergosterol biosynthesis, the pathway targeted by azoles, and drug efflux pumps (Selmecki et al., 2008). Thus, it remains to be determined how frequent aneuploidy arises *in vivo* in the absence of antifungal drug exposure but may be relatively uncommon in the absence of azole drug treatment.

3.4. Loss of heterozygosity

Loss of heterozygosity (LOH) refers to the transition of a genetically heterozygous region of DNA to homozygosity. Recombination often contributes to LOH but can also be produced by whole chromosome or ploidy loss and reduplication of alleles retained by the cell. The importance of LOH to genetic diversity and phenotypic changes has been largely ignored outside the context of cancer biology and human fungal pathogens (Ryland et al., 2015; Wertheimer et al., 2016). Detection of LOH requires at least diploidy and can be found in four basic forms: micro LOH, involving small tracts spanning a few to hundreds of nucleotides; short range LOH, those covering one or a small number of genes; chromosome arm LOH, homozygosis due to crossover or break-induced replication that extends to the telomere; and whole chromosome LOH that implicates chromosome loss and reduplication. Although LOH often does not produce new genetic sequences, it allows phenotypes to emerge from recessive alleles within LOH regions that have important contributions to genome structure and organismal success.

LOH within fungal pathogens is frequent and widespread. Sequencing of *C. neoformans* isolates uncovered LOH tracts in all diploid strains that were clustered towards chromosome ends (Rhodes et al., 2017). Interestingly, hybrid *C. neoformans/C. deneoformans* strains displayed elevated rates of LOH and aneuploidy compared to all other diploids with frequent homozygosis of full chromosomes from one parent or the other (Cogliati et al., 2001; Ni et al., 2013; Rhodes et al., 2017). LOH of the mating-type (*MAT*) locus is required for these hybrid strain to return to a mating-competent *MAT* homozygous state and often occurs via gene conversion (Sun et al., 2012). Similar mechanisms of increased LOH within the mating type-like (*MTL*) locus and towards chromosome ends are found in *C. albicans* (Hirakawa et al., 2015; Wang et al., 2018; Wu et al., 2005). LOH events in the *C. albicans* genome reference strain have important consequences for phenotypic traits relating to biotic stress resistance (Ciudad et al., 2016; Forche et al., 2011), antifungal drug resistance (Ford et al., 2015; Selmecki et al., 2006), and virulence (Lockhart et al., 2005; Wu et al., 2007). Additionally, increasing LOH in response to stress establishes a cyclical pattern in which LOH and stress feedback to promote homozygosis of certain regions that confer a fitness advantage under those same conditions (Forche et al., 2011).

As with aneuploidy, *Aspergillus* species were key systems for developing concepts of heterozygosity in the early era of genetic research (Pontecorvo et al., 1954). These studies dissected the recombinational processes that often underlie transitions from heterozygosity to homozygosity (Kafer, 1976; Kappas, 1978). More recent efforts to assess evolutionary trajectories of *A. nidulans* demonstrated that homozygosis of particular genomic segments can replicate phenotypes seen primarily in haploid strains (Schoustra et al., 2007). In this case, LOH facilitated expression of recessive alleles leading to faster growth rates comparable to haploids and distinct from other diploid cells that retained a heterozygous genome. Relatively little is known about the role of LOH in response to antifungal resistance among *Aspergillus* species although a recent report suggests that genomic instability, which can include aneuploidy and LOH, is likely involved (Dos Reis et al., 2018).

4. Genomic variation by experimental evolution

Unicellular growth of many fungal pathogens provides an ideal system to assess genome variation using experimental evolution approaches. Much of this work using fungal pathogens is still in its infancy, but previous studies using simple passaging of cell populations has begun to provide insight into what future work may hold. For instance, isolation of antifungal drug-resistant *C. albicans* and *Cryptococcus* species through exposure to sub-lethal drug concentrations demonstrated important roles for aneuploidy (Selmecki et al., 2008; Selmecki et al., 2009; Sionov et al., 2010), loss of heterozygosity (Coste et al., 2006; Schubert et al., 2011), and individual mutations in drug targets and DNA repair proteins (Billmyre et al., 2017; Sanglard et al., 2003). Yet, the *C. albicans* genome is capable of significant rearrangement even in the absence of intentional selective pressures. Changes in DNA content (Hickman et al., 2015), gene family composition (Anderson et al., 2015), and centromere organization (Burrack et al., 2016) have all been observed during standard passaging in nutrient-rich conditions. Continual passaging of *C. albicans* strains from diverse backgrounds over 600 generations found a high rate of SNP emergence when compared to indels although both mutation types were enriched in intergenic regions indicating purifying selection worked to remove genic mutations in the absence of a clear selective force (Ene et al., 2018). Importantly, repetitive regions of the genome including expanded gene families, centromeric regions, and repetitive DNA sequences mutated more frequently compared to the rest of the genome. These *in vitro* passaged isolates also experienced frequent and widespread micro-LOH tracts that may have been overlooked in previous large-scale genotyping assays that did not rely upon whole genome sequencing approaches. In *C. neoformans*, continual passaging of the H99 isolate led to reduced virulence (Janbon et al., 2014), a trait that has been associated with laboratory strains across many prominent pathogens (Dunster et al., 1990; Somerville et al., 2002).

Greater effort has been placed on interrogating the processes of *in vivo* evolution of fungal pathogens within their respective host niche. In contrast to the observed decreased virulence of *C. neoformans* H99 following *in vitro* evolution, *in vivo* evolution led to an increase in overall virulence following passage through a rabbit model host (Janbon et al., 2014). Such phenotypic differences may be rapidly selected for within the host, including cells with reduced metabolic states that do not resuscitate immediately after recovery, a common characteristic of dormancy (Alanio et al., 2015). Serial passaging of multiple *C. albicans* strains *in vivo* by repeated isolation from the kidneys, however, did not alter virulence in any strain background, suggesting they are already fit within the host niche, experience relatively little selection during infection and accumulation in the kidney, or did not have a sufficient number of generations for selection to act (Ene et al., 2018; Luttich et al., 2013). In contrast, passage though gut colonization of these same isolates did increase fitness within the GI tract when compared directly to parental genotypes (Ene et al., 2018). Elevated fitness was associated with trisomy of Chr7 across multiple strain backgrounds. More recently, a study detailed fitness advantages within the GI directly linked to inactivating mutations in the *FLO8* transcription factor that also reduces filamentation and virulence within systemic models of infection (Tso et al., 2018). Fitness tradeoffs between gut colonization and systemic disease for *FLO8* mutants mirror results for *efg1*$^{-/}$ strains that also lack a robust filamentation response (Hirakawa et al., 2015; Pande et al., 2013; Pierce and Kumamoto, 2012). Importantly, a greater than 10-fold increase in mutation frequency occurred during *in vivo* passaging experiments compared to *in vitro* passaging, suggesting that host systems facilitate rapid evolution through selection acting on populations and/or induction of mutations directly through stress. An increased rate of LOH, aneuploidy, and whole ploidy shifts also accompanied *in vivo* growth and translated to greater phenotypic diversity when compared to *in vitro* passaging (Forche et al., 2018; Forche et al., 2009a). Thus, both *in vitro* and *in vivo* evolution of human fungal pathogens operate through similar mechanisms but the rates of *in vivo* evolution dwarf their *in vitro* counterparts.

5. Concluding remarks

Major advances in our understanding of the selective pressures acting on organisms followed improvements in genome sequencing and comparative analysis across species. Differences in genome organization between environmental and host-associated fungal pathogens reflect the requirements for survival within their niches and correlate well with trends in genome composition found within bacterial and archeal

species. Mutations that drive this variation in fungi are similar to those in other eukaryotes, but these fungal species have been particularly amenable to the study of how that variation arises. Looking forward, focus on those organisms still lacking extensive genomic investigations and additional studies linking similarities and differences between *in vitro* and *in vivo* evolutionary trajectories will help decode the universal and species-specific requirements for infection while also informing researchers whether or not laboratory conditions are appropriate to understand how host and external environments steer fungal evolution.

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