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Fantastic yeasts and where to find them: the hidden diversity of dimorphic fungal pathogens

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Dimorphic fungal pathogens are a significant cause of human disease worldwide. Notably, the dimorphic fungal pathogens within the order Onygenales are considered primary pathogens, causing disease in healthy hosts. Current changes in taxonomy are underway due to advances in molecular phylogenetics, population genetics, and new emerging dimorphic fungal pathogens causing human disease. In this review, we highlight evolutionary relationships of dimorphic fungal pathogens that cause human disease within the order Onygenales and provide rationale to support increased investment in studies understanding the evolutionary relationships of these pathogens to improve rapid diagnostics, help identify mechanisms of antifungal resistance, understand adaptation to human host, and factors associated with virulence.

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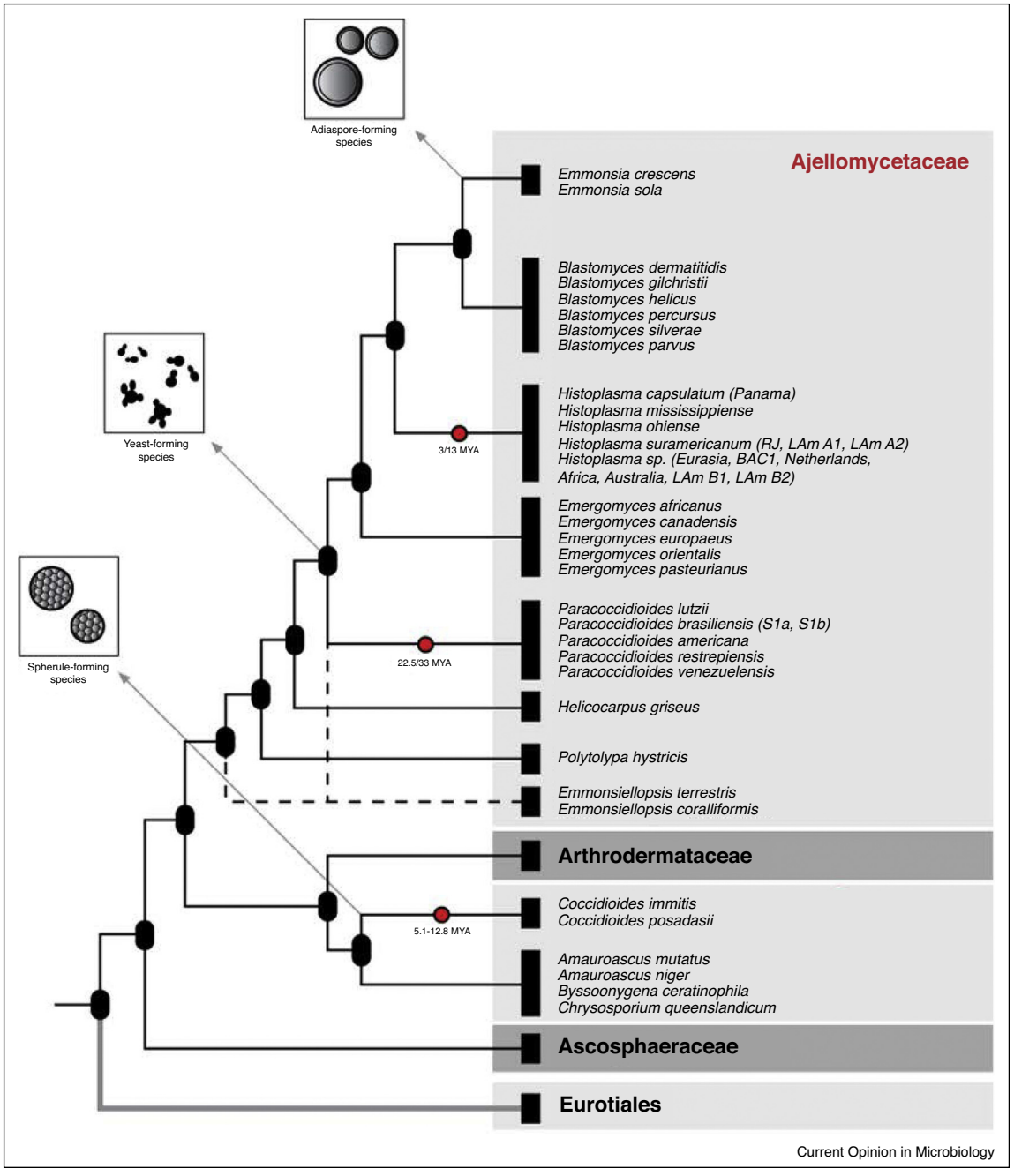
Introduction

The fungal kingdom contains millions of ubiquitous fungal species, and most pose no or trivial direct threat to human health. However, a handful of species can cause devastating disease in both immunocompetent and immunocompromised individuals. Although plants can also be affected by fungal pathogens, this review highlights a distinct group of dimorphic fungal pathogens that cause disease in humans. This specific group of fungi that cause systemic infections is nested within the order Onygenales (Eurotiomycetes, Ascomycota) which include *Coccidioides*, *Histoplasma*, *Blastomyces*, *Paracoccidioides*, *Emmonsia,* and *Emergomyces* (see current phylogenetic representation of these species in Figure 1). These fungal organisms are known to be dimorphic fungal pathogens, which emerged around 150 MYA [1], and are capable of growth in the environment on at a wide range of temperatures and in the human host at 37°C [2]. Dimorphic fungal pathogens in the order Onygenales are known as primary pulmonary pathogens that cause disease in immunocompetent individuals with over 650 000 new infections occurring each year in the United States [3]. These fungi generally live as saprobes producing filamentous mycelium and under certain environmental circumstances produce asexual conidia (e.g. arthroconidia, blastoconidia, etc.). Upon the inhalation of conidia by a susceptible host, these fungi switch into their parasitic form (e.g. yeasts, spherules, adiaspores, etc.) (Figure 2) and live as endozoans, maintaining an active or inactive stage within host granulomas. These fungi may return to the environment upon host death transforming into conidia-producing mycelia [4].

Defining pathogenic fungal species based on morphological characteristics poses many challenges because not all fungi are cultivable in media, not all fungal pathogens easily produce sexual fruiting bodies, and some fungi are homothallic (self-fertile) and therefore will not exhibit a detectable inheritance pattern. Before the genomic era, Genealogical Concordance for Phylogenetic Species Recognition (GCPSR) was the method of choice for defining species boundaries within Onygenales and other fungi [5]. This approach is based on Multi Locus Sequencing Type (MLST) to assess sequence variation of conserved housekeeping genes or other neutral loci. The genetic background of centennial species (i.e. *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*) were resolved leading to new phylogenetically isolated species and new dimorphic fungal pathogens were described (e.g. *Emergomyces*) [5].

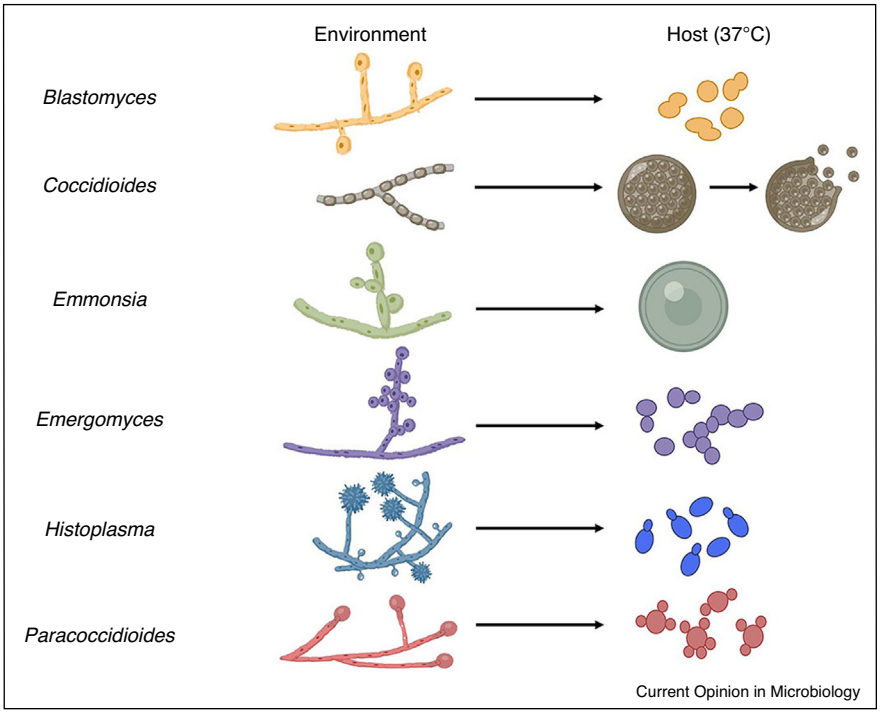
Advances in molecular phylogenetics and population genetics based on genomic science have significantly improved our ability to accurately define fungal species limits and hybridization events that shaped the Onygenalean clade [6]. Sequencing the genomes of Onygenalean fungal pathogens also provides information to better understand shared and unique adaptive traits between species complexes, such as metabolism, mating patterns, gene gain or loss, and chromosomal variations that may be associated with infection and disease progression [1].

Figure 1



Phylogenetic representation of the evolutionary trajectory of the order onygenales.
The branch distribution is based on MLST tree (rPB2, TUB2, TEF3, ITS, and LSU), whole genome phylogentic trees, and node calibrations previously published (4200 core genes) [6,61•,71]. Solid black branches are concordant between both phylogenetic studies and dotted branches are due to uncertainty regarding the true phylogenomic position of *Emmonsiellopsis*. The solid grey branch represents the outgroup formed by Eurotiales species. The solid red square represents the most common ancestor of Onygenales that emerged around 150 MYA and black ellipsis are nodes distinguishing each lineage. Red nodes represent the age of divergence of each species complex previously analyzed [6,12,37,64]. *Blastomyces parvus* (*) and *Emergomyces pasteurianus* (#) were previously known as *Emmonsia crescens* and *E. pasteuriana*, respectively. The forms of pathogenic structures among the Onygenales are displayed along the tree.

Figure 2



Morphological shifts of Onygenalean fungi.
The simple cartoons for each dimorphic Onygenales are shown. In the environment, each of these species is in its hyphal/mycelial form with oval or circular shapes representing the vegetative conidia. In the case of *Blastomyces*, *Emergomyces*, *Histoplasma*, and *Paracoccidioides* in the host, these switch to yeast-phase with some characteristic differences. *Blastomyces* yeast cells display a broad bud neck; *Emergomyces* have yeast cells with unipolar or bipolar budding at narrow base; *Paracoccidioides* have yeast that can be multibudded. In the host, *Coccidioides* switches to endosporulating spherules which then ruptures to release endospores. *Emmonsia* switches to an adiaspore (non-endosporulating spherule). (Illustration created with BioRender).

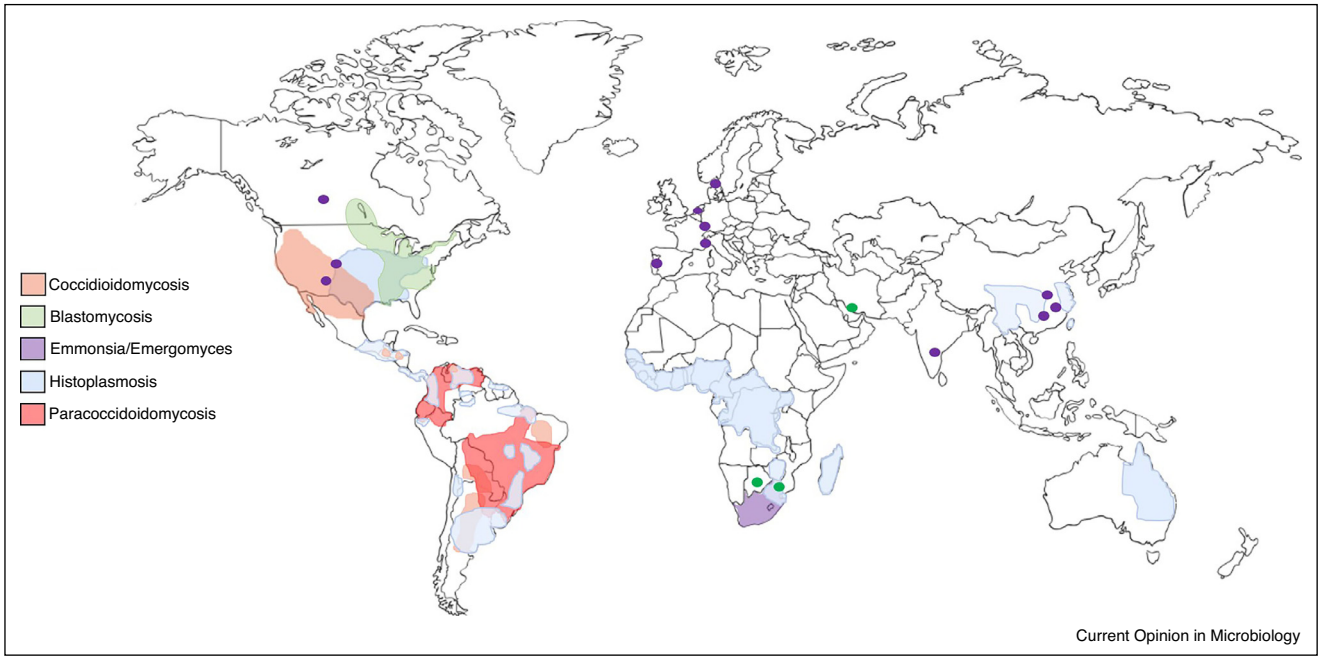
Additionally, comparative genomic analyses between pathogenic and non-pathogenic closely related fungal species provide evidence of recently diverged species and adaptation to mammalian hosts. This review highlights studies on the phylogenetics and evolution of dimorphic fungal pathogens, and provides insight into current taxonomy, pathogenicity potential, recombination patterns, and gene gain/loss within species complexes and across different populations and species.

Coccidioidomycosis

Coccidioidomycosis or San Joaquin 'Valley Fever' is a fungal infection caused by two Onygenaceae species, *C. immitis* and *Coccidioides posadasii* (Figure 1). Coccidioidomycosis occurs most notably in California and Arizona, which together account for a majority of reported cases in the United States (Figure 3) [7]. Outside the United States, there have been cases in the Northern region of Mexico [8], Central America, and arid regions of South America such as Argentina, Brazil, Paraguay, and Venezuela [9]. Although most cases are asymptomatic (60%), the literature states there are about 150 000 new infections each year in the United States [10]; however, new estimates suggest 350 000 new infections are occurring each year in the United States [T Chiller, abstract in Vaccine Strategies for Endemic Fungal Pathogens, NIAID, Rockville, MD, March 2019].

Recently, studies have combined phylogenetic meta-analysis and population genetics to identify at least six distinct populations within the two genetically diverse species, *C. immitis* and *C. posadasii* [11,12]. *C. immitis* is restricted to Northwestern Mexico, California, and Washington. In contrast, *C. posadasii* is widely distributed in the Americas ranging from the Sonora and Chihuahua deserts (including Arizona, Texas, New Mexico, and Northeast Mexico) to Central and South America. The current understanding of population structure within both species complexes has been constantly revisited over the past 20 years. On the basis of multiple markers, these studies reveal three populations within *C. immitis* (San Joaquin/Central Valley California, Southern California/Mexico, and Washington) and three populations within *C. posadasii* (Arizona, Texas/Mexico/South America and Guatemala) (Figure 3) [12–14]. Although these populations are genetically different, limited gene flow

Figure 3



Estimated global distribution of Onygenales that cause human disease.
These geographical locations for endemic sites of Onygenales are an estimate. Histoplasmosis has a worldwide distribution and the map depicts known endemic regions. This map has been adapted from Ref. [32, CDC; https://www.cdc.gov/features/fungalinfections/] with distribution of infections caused by *Emergomyces* (●) and *Blastomyces* (●) adapted from Ref. [53]. (Illustration created with BioRender).

does occur and few phenotypical differences have been identified between species [14].

Both species of *Coccidioides* share the same asexual life cycle switching between saprobic and parasitic life cycles. Despite the divergence of these two species, about 5.1 MYA [14], gene exchange between *C. immitis* and *C. posadasii* is thought to occur. Recent studies utilized a large sample of genomes from each species to quantify the magnitude of gene exchange whereby identifying genomic regions that have crossed from one species to another [15•]. These studies indicated that introgressions have occurred within species at low frequencies establishing that admixture is either rare or deleterious.

Importantly, studies conducted on dimorphic fungi to understand the underlying mechanisms of morphological transition between environmental and pathogenic forms aim to uncover virulence-defining genes and find potential new drug targets. Studies conducted by Hung *et al.* determined a gene that encodes for a glycoprotein present on the cell surface, spherule outer wall glycoprotein (*SOWgp*), that contributes to virulence by functioning as both an adhesin and a host immune response modulator [16,17]. Additional studies determined the *CPS1* gene, homologous to an identified virulence factor in a maize pathogen, to be important for virulence in *Coccidioides* [18]. The deletion of the *CPS1* gene in *C. posadasii* resulted in complete attenuation of disease in a murine model [19]. Studies utilizing microarray gene expression analysis show 2208 *C. immitis* genes were differently expressed in spherules compared to mycelia form [20]. Additional studies by Whiston *et al.* using RNA-Seq demonstrated an upregulation of 1880 genes in the spherule stage of *Coccidioides* [21]. Collectively, these studies demonstrated similar gene expression profiles with differentially expressed genes associated with defense against oxidative stress, cell wall remodeling, lipid metabolism, and genes associated with virulence factors. Previous reports focusing on dermatophytes demonstrated the expansion of proteins related to the peptidoglycan binding domain LysM which has been shown to be involved in fungal immunity [22]. However, recent studies compared *Coccidioides* genomes to other nonpathogenic Onygenaceae species and demonstrated a contraction in LysM gene families in *Coccidioides* species [23]. The authors of this study express their opinion that LysM gene families may be more immunoreactive demonstrating the disadvantage of the presence of these gene families in pathogenic fungi, which could explain the gene family contraction of LysM-containing genes in *Coccidioides* [23].

Histoplasmosis

H. capsulatum sensu lato is a pathogenic fungus that causes histoplasmosis, a life-threatening systemic mycosis [24]. Histoplasmosis is known to be one of the most common pulmonary fungal diseases in the United States [25]; however, cases of histoplasmosis have a worldwide distribution occurring in both temperate and tropical regions (Figure 3) [26–30]. The fungus is usually found in sites enriched with bird or bat guano that allows the development of the aerial mycelial containing infectious airborne microconidia (Figure 2).

Historically, three distinct varieties of *Histoplasma* were determined based on morphology and clinical aspects: New World human pathogen (*H. capsulatum* var. *capsulatum*), African human pathogen (var. *duboisii*), and Old World horse pathogen (var. *farciminosum*) [31,32]. GCPSR studies conducted by Kasuga *et al.* demonstrated that *H. capsulatum* contains at least eight phylogenetic species: LAm A and LAm B (primarily from Latin America), NAm 1 and NAm 2 (restricted to North America), Eurasian (Egypt, India, China, Thailand, and England), Netherlands, Africa, and Australia [27]. The three historical *Histoplasma* varieties were found in multiple monophyletic lineages and were therefore invalidated. Additionally, eleven species-level clades within *H. capsultum*, were identified. The former LAm A and LAm B species were further split into five different genetic clusters as follows: RJ, LAm A1, LAm A2, LAm B1, and LAm B2 [29]. Additionally, a new phylogenetic species, BAC-1 (Mexico), and four different monophyletic lineages from Brazil (BR1–4) were identified (Figure 1) [29]. Recently, whole genome sequencing was used to compare 30 *Histoplasma* isolates demonstrating *H. capsulatum* is composed of at least five groups [33] and four different species were proposed as follows: *Histoplasma sensu stricto* Darling (Panama), *Histoplasma mississippiense* (NAm 1), *Histoplasma ohiense* (NAm 2), and *Histoplasma suramericanum* (LAm A) (Figure 1). The authors also suggest hybridization and gene flow have occurred between these cryptic species, but very infrequently, thus species boundaries are robust even in sympatry [33]. *H. ohiense* and *H. mississippiense* were further examined to determine the possibility of gene exchange [34•]. This study demonstrated recent hybridization between two strains of each species, *H. mississippiense* and *H. ohiense* genomic reference strains, WU24 and G217B. With shared overlapping geographic boundaries of *Histoplasma* species, this study demonstrates the potential for new hybrids of *Histoplasma* in the future.

Studies utilizing *H. capsulatum* demonstrated a group of important genes conserved for the dimorphism of other pathogenic Onygenales: Required for Yeast Phase genes or RYP 1–4 [35,36]. A yeast phase-specific gene, YPS3, was determined to encode a protein that is secreted and localized on the cell wall of certain strains of *Histoplasma* [37]. Additionally, studies demonstrated the role of YPS3 on mammalian virulence *in vivo* using the RNA-mediated interference (RNAi) strategy whereby silencing led to decreased fungal burden compared to wild type infection [38]. Another important virulence factor in *Histoplasma* pathogenicity includes Calcium-binding protein, CBP, where studies demonstrated deletion of CBP1 allowed for rapid clearance of yeast cells from the lungs of infected mice [39]. Furthermore, studies determined the production of siderophores as a virulence factor in *Histoplasma* [40,41]. Disruption of the *SID1* gene, an enzyme important for siderophore production, resulted in a growth defect *in vitro* and attenuation of infection in the murine model of histoplasmosis.

Other Onygenales

Additional human fungal pathogens from the Onygenales order that belong to the family Ajellomycetaceae include *Blastomyces*, *Paracoccidioides*, *Emmonsia*, and *Emergomyces* (Figure 1). The infections caused by these fungi range from asymptomatic to mild pneumonia, acute respiratory distress, and dissemination to multiple organ systems which is often fatal, especially in immunocompromised individuals [42–44].

Blastomyces

Blastomycosis is a lung infection caused by the etiological agent *Blastomyces* spp., endemic to areas of North America including Ohio and Mississippi river valley, the Great Lakes, and the St. Lawrence River (Figure 3) [45,46]. Until recently, *B. dermatitidis* was believed to be the only species; however, studies utilizing population genetic analysis defined two different species: *B. dermatitidis* and *Blastomyces gilchristii* (Figure 1) [47–49]. *B. dermatitidis* and *B. gilchristii* are the most common agents of blastomycosis; these species diverged about 1.9 MYA and studies suggest that additional cryptic populations/species exist [47]. Studies using deep comparative genomic analysis revealed a large genome size for both species, *B. dermatitidis* at 66.6 Mb for the ER-3 stain to 75.4 Mb for the *B. gilchristii* SLH14081 strain, which is nearly double the size of other Onygenales [50]. Furthermore, these studies show evidence that these genomes comprise large isochore-like regions with low GC content. Recent studies have revealed two new distinct species: *Blastomyces helicus* (formerly *Emmonsia helica*) and *Blastomyces percursus* (formerly *Emmonsia*) (Figure 1) [51•,52]. Therefore, infections due to *Blastomyces* are occurring outside the United States demonstrating the underappreciation of the geographical distribution of this organism (Figure 3) [53].

Previous studies have demonstrated BAD1 to be a virulence factor in *Blastomyces* where a knockout strain was shown to be avirulent in mice [54]. Furthermore, studies demonstrated that expression of BAD1 was yeast-phase-specific in both

B. dermatitidis and *H. capsulatum* demonstrating a common mechanism between dimorphic fungal pathogens and the expression of yeast-phase-specific genes [55]. Additional studies found dimorphism-regulating kinase, DRK1, to be required for phase transition from mold to yeast in *H. capsulatum* and *B. dermatitidis*; thereby, functioning as a global regulator of both dimorphism and virulence [56].

Emmonsia and *Emergomyces*

Emmonsia is a genus that until recently was thought to cause adiapiromycosis, a pulmonary infection common in small mammals and rarely associated with human infections and caused by two species: *Emmonsia crescens* and *Emmonsia parva* [51•,57–59]. The pathogenic phase of *E. crescens* is characterized by adiaspore (non-endosporulating spherule) production, whereas yeast-like forms are recognized in infection due to *E. parva*. A recent emergence of infections to *Emmonsia* in HIV-positive patients in South Africa suggests that *Emmonsia* is one of the most common causes of endemic mycosis in that region (Figure 3) [60,61•]. These infections prompted phylogenetic studies which proposed a new species: *Emmonsia pasteuriana* [60]. Additionally, recent MLST analysis of 5 loci showed *E. crescens* to be grouped on a single branch while *E. parva* is closely related to *Blastomyces* species and was therefore re-classified as *B. parvus* because this species produces yeast in its pathogenic form (Figure 1) [51•]. Dukik *et al.* proposed a new genus, *Emergomyces (Es.),* which contains *Es. pasteurianus*, *Es. africanus* [51•], and other novel species: *Es. orientalis* [62] and *Es. canadensis* (Figure 1) [63•]. Recent studies demonstrate that *E. pasteurianus* did not cluster with *E. parva* or *E. crescens* and with the discovery of *Es. africanus*, *Emmonsia pasteurianus* was re-named *Emergomyces pasteurianus* [51•]. Infections due to *Emergomyces* occur endemically in South Africa but have recently been reported in Canada, New Mexico, and Colorado (Figure 3) [53,63]. Recent studies by Jiang *et al.* demonstrate the morphological features of *Emmonsia* and *Emergomyces* in both the environment and the host. *Emmonsia* spp. are morphologically characterized by adiaspores or adiaspore-like cells in the host while *Emergomyces* spp. are characterized by yeast cells in the host environment (Figure 2) [53]. Little is known about the virulence factors in this group, and additional work is needed to better understand how and why these organisms are emerging as significant pathogens.

Paracoccidioides

Paracoccidioides spp. are the causative agents of paracoccidioidomycosis, a pulmonary fungal infection endemic to Latin America, ranging from southern Mexico to northern Argentina (Figure 3) [64]. Recent phylogenetic studies have helped to uncover novel species of *Paracoccidioides* in addition to the original species *P. brasiliensis* (Figure 1). *Paracoccidioides lutzii* is found in mid-western Brazil and morphologically is distinct producing elongated conidial cells compared to other barrel-shaped conidia produced by *Paracoccidioides* species [65]. *P. brasiliensis* is the most dispersed species and comprises two populations, S1a and S1b, which have been found to overlap geographically in Southeast Brazil, Argentina and Paraguay [66,67•]. *Paracoccidioides americana*, formerly PS2, is the rarest species associated with cases reported in states of Brazil, São Paulo, Minas Gerais, and Rio de Janeiro (Southeast Brazil), as well as in Venezuela [68,69]. Two other species, *Paracoccidioides venezuelensis* (former PS4) and *Paracoccidioides restrepiensis* (former PS3), are geographically restricted to Venezuela and Colombia, respectively [67•,70]. Sequencing the genomes of both *P. brasiliensis* and *P. lutzii* was completed and comparison to other dimorphic fungal pathogens helped elucidate the divergence both within the *Paracoccidioides* lineage and among other dimorphic fungal species [71]. These studies found expansions of the fungal-specific kinases family FunK1 and loss of genes involved in carbohydrate metabolism while retaining Onygenales-specific proteases which highlights how *Paracoccidioides* may have adapted to the human host.

Comparative strategies across Onygenales

Dimorphic Onygenales demonstrate mostly mycelia to yeast-phase transitions when introduced to a host or temperature change (37°C); however, *Emmonsia* and *Coccidioides* demonstrate unique structures when introduced to a host (Figures 1 and 2). As discussed above, the pathogenic phase of *Emmonsia* is characterized by adiaspores, spherule-like structures, while *Coccidioides* has endosporulating spherules unique among fungal pathogens. The processes regulating these morphological switches is largely unknown.

As discussed above, studies have found genes with similar function associated with dimorphism and virulence (BAD1, DRK1, CBP, and YPS3). Some factors are associated with multiple fungal pathogens (DRK1 and BAD1; *H. capsulatum* and *B. dermatitidis*) while others (YPS3 and CBP) have been shown to be specific to *Histoplasma* such as YPS3, which has no sequence homology to any other gene in fungal pathogens [37,39,55,56]. Studies have demonstrated that α(1-3)glucan, a major polysaccharide in the fungal cell wall, is a virulence factor conserved across multiple dimorphic fungal pathogens. Focusing on the Onygenales, studies utilized RNAi to deplete α(1-3)glucan from *H. capsulatum* and the organism was no longer able to kill macrophages or cause disease in mice [72]. Additionally, studies observed decreased α(1–3) glucan content in the cell walls of attenuated strains of *Blastomyces* and *Paracoccidioides* [73,74]. To better understand the evolutionary histories of dimorphic fungal pathogens, recent studies have sequenced genomes of non-pathogenic (*Helicocarpus griseus* and *Polytolypa hystricis*) and pathogenic species (*Blastomyces*, *Emmonsia*, *Emergomyces*, *Paracoccidioides*, and *Coccidioides*) and performed comparative genomic analysis across systemic, opportunistic, and

non-pathogenic fungal species [75•]. These studies found conserved proteins related to dimorphic switch across pathogenic (*E. parva*, now named *B. parvus*, and *E. crescens*) and non-pathogenic (*H. griseus* and *P. hystricis*) species suggesting that the presence of these factors does not directly correlate to pathogenicity. Additionally, these studies show retention of genes important for plant degradation in the non-pathogenic species compared to loss of those genes among pathogenic species revealing how Onygenales may have transitioned to use animals as a primary nutrition source [75•].

In conclusion

The studies highlighted above reveal the importance of precisely identifying fungal species causing human disease. Advances in genomic tools have significantly increased our understanding of these pathogens highlighted above and has become a powerful tool in determining phylogenetic relationships among species and populations. As a direct result of gaining this sequence data, there have been taxonomic rearrangements in multiple fungal groups and some species, such as *Histoplasma*, need to be further revised [29]. However, the advances in genomic tools need to be used with parsimony especially in taxonomical studies. Clinical applications can be counfounded by renaming schemes which imply geographical designations (e.g. *H. mississippiense* and *H. ohiense*) when in fact these species occur in sympatry in both Ohio and Mississippi River valleys. Given the increasing rate of infections due to dimorphic fungal pathogens and emerging species and genera, there is a need for increased investment in studies to understand the significance of these evolutionary relationships to improve rapid diagnostics, help identify mechanisms of antifungal resistance, understand adaptation to the human host, and pathogenesis. Despite great advances in comparative phylogenetic analysis, there remains much work to do to better understand the functional significance of the evolutionary transitions that enabled infection of mammals and humans, understand hybridization, and the role of introgression between species.

Conflict of interest statement

Nothing declared.

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