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ADVANCES IN DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS (S CHEN, SECTION EDITOR)

The Black Yeasts: an Update on Species Identification and Diagnosis

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Abstract

Purpose of review Black yeast-like fungi are capable of causing a wide range of infections, including invasive disease. The diagnosis of infections caused by these species can be problematic. We review the changes in the nomenclature and taxonomy of these fungi, and methods used for detection and species identification that aid in diagnosis.

Recent findings Molecular assays, including DNA barcode analysis and rolling circle amplification, have improved our ability to correctly identify these species. A proteomic approach using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has also shown promising results. While progress has been made with molecular techniques using direct specimens, data are currently limited.

Summary Molecular and proteomic assays have improved the identification of black yeast-like fungi. However, improved molecular and proteomic databases and better assays for the detection and identification in direct specimens are needed to improve the diagnosis of disease caused by black yeast-like fungi.

Keywords Black yeasts · Diagnosis · Species identification · *Cladophialophora* · *Exophiala* · *Fonsecaea* *Phialophora* · *Rhinocladiella*

Introduction

Black yeast-like fungi and their filamentous relatives are capable of causing a wide range of often difficult to treat infections. Infections caused by members of the order *Chaetothyriales*, under which these fungi fall, include mycetoma, chromoblastomycosis, and phaeohyphomycoses. Hematogenously disseminated infections can also occur and are primarily caused by *Exophiala* species that are able to produce yeast-like cells [1–3, 4•]. Although rare, case reports of endocarditis due to *Exophiala* species have been reported in the literature [5, 6], and a recent epidemiologic study also reported one case caused by *Fonsecaea pedrosoi* [7••]. Other species are capable of causing central nervous system infections, which are associated with significant morbidity [7••, 8–12]. Significant differences in pathogenicity and virulence have also been reported between closely related species, and this may be a function of the individual species' ability to grow at certain temperatures. Those species that are able to grow at human body temperature (i.e., 37 °C) or higher are capable of causing systemic or disseminated disease. These species include but are not limited to *E. dermatitidis*, *E. jeanselmei*, *E. oligospora*, *Cladophialophora bantiana*, and *Fonsecaea* species [4•]. In addition to causing infections in humans, black yeast-like fungi and their filamentous relatives are also capable of causing disease in cold-blooded animals [13]. Species that grow at temperatures between 35 and 37 °C often are associated with superficial and cutaneous infections in humans but may cause systemic disease in cold-blooded animals. In contrast, mesophilic species, which only grow at temperatures between 27 and 33 °C, are limited to cold-blooded animals, although infections in invertebrates have also been reported [14].

The diagnosis of invasive fungal infections remains challenging. In many patients with probable or presumed invasive mycoses, fungal cultures are rarely positive, thus hampering the diagnosis of these infections. However, it is known that delays in diagnosis and initiation of appropriate therapy are associated with worse outcomes in patients with invasive

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fungal infections [15–17]. Although these studies have primarily involved patients with invasive aspergillosis and candidiasis, it is presumed that delays in appropriate therapy would also lead to worse outcomes in patients with infections caused by other fungi. This review discusses the species of black yeast-like fungi and their filamentous relatives, changes in their nomenclature, and methods for diagnosis and species identification.

Species of Black Yeast-like Fungi, Their Filamentous Relatives and Characteristics

Black yeast-like fungi and their filamentous relatives are dematiaceous, non-lichenized fungi characterized by the presence of a dark yeast-like phase that later becomes a mycelial stage [18]. These fungi exhibit slow to moderate growth and as previously noted are classified under the ascomycete order *Chaetothyriales* (subphylum *Pezizomycotina*). Five families are recognized within this order: Chaetothyriaceae, *Cyphellophoraceae*, *Epibryaceae*, *Herpotrichiellaceae*, and *Trichomeriaceae* [19••, 20]. Most clinically important species are included in the Family *Herpotrichiellaceae* and *Cyphellophoraceae*, mainly in the genera *Cladophialophora*, *Cyphellophora*, *Exophiala*, *Fonsecaea*, *Phialophora*, and *Rhinocladiella* (Table 1) [19••].

Cladophialophora species produce one-celled ellipsoidal to fusiform dry conidia arising through blastic, acropetal conidiogenesis and arranged in branched chains [25]. The genus *Cyphellophora* is characterized by having melanized thalli with phialidic condiogenesis, phialidic openings inserted directly on hyphae or occasionally on flask-shaped conidiogenous cells, and producing small clusters of olivaceous, septate or non-septate, mostly curved conidia [26]. The genus *Exophiala* is comprised of species that exhibit smooth or velvety, initially mucoid, black, olivaceous green or dark brown colonies [27]. Nearly all species within this genus are characterized and recognizable by their production of budding cells, and the yeast-to-hyphae transition mostly proceeds via torulose hyphae. These species are highly pleomorphic, and morphologic characteristics are often determined by factors such as culture media, incubation temperature, and the age of the culture. The genus *Fonsecaea* includes species that are defined by the presence of indistinct melanized conidiophores with blunt, dispersed denticles that bear single or short chains of conidia that eventually become unbranched [28]. The genus *Phialophora* is characterized by melanized hyphae and by the production of slimy, one-celled conidia in heads from phialides with distinct collarettes [29]. Another genus of black yeast-like fungi *Rhinocladiella* is comprised of species with sympodial conidiogenesis. The conidiophores are dark brown, straight, upright, unbranched, thick walled, and produce conidia that are sub-hyaline, smooth- and thin-walled, one or occasionally two-celled, ellipsoidal to clavate in shape [21, 30].

Table 1 List of medically important species in the Family *Herpotrichiellaceae* and *Cyphellophoraceae* [21–24]

Genus	Species
Cladophialophora	*Cladophialophora arxii*
	Cladophialophora bantiana
	Cladophialophora boppii
	Cladophialophora carrionii (formerly *Cladophialophora ajelloi*)
	Cladophialophora devriesii
	Cladophialophora emmonsii
	Cladophialophora immunda
	Cladophialophora modesta
	Cladophialophora mycetomatis
	Cladophialophora samoensis
	Cladophialophora saturnica
	Cladophialophora subtilits
Cyphellophora	*Cyphellophora laciniata*
	Cyphellophora pluriseptata
	Cyphellophora suttonii
Exophiala	*Exophiala asiatica*
	Exophiala attenuata
	Exophiala bergeri
	Exophiala castellanii
	Exophiala dermatitidis (formerly *Wangiella dermatitidis*)
	Exophiala equina
	Exophiala halophila
	Exophiala jeanselmei
	Exophiala lecanii-corni
	Exophiala mesophila
	Exophiala oligosperma
	Exophiala spinifera
	Exophiala "*siphonis*"
	Exophiala xenobiotica
Fonsecaea	*Fonsecaea pedrosoi* (formerly *Fonsecaea compacta*, a dysgonic mutant or variety of *Fonsecaea pedrosoi*)
	Fonsecaea multimorphosa
	Fonsecaea nubica
	Fonsecaea pedrosoi
Phialophora	*Phialophora Americana*
	Phialophora europaea
	Phialophora verrucosa
Rhinocladiella	*Rhinocladiella aquaspera* (formerly *Acrotheca aquaspersa*)
	Rhinocladiella basitona (formerly *Ramichloridium basitonum*)
	Rhinocladiella mackenziei (formerly *Ramichloridium mackenziei*)
	Rhinocladiella similis

Changes in Nomenclature

Until 2013, polymorphic higher fungi (Dikarya) were allowed to carry multiple names to describe their teleomorphic and anamorphic stages. These stages occur independently, and their relationship was not always established. The wide use of molecular methods has facilitated the establishment of

relationships between these various stages in the life cycles of fungi, and has led to the abolition of the dual nomenclature system of fungi and the adoption of the One Fungus One Name system, as provided in the new "International Code of Nomenclature for Algae, Fungi, and Plants" [31, 32]. The classification of fungi has now moved from phenotypic characteristics to phylogenetic relationships. Nucleic acid variation has proved a means of ensuring that genera are monophyletic, and that species that differ at the ordinal and family level are no longer accepted as members of a single genus. The advantage of the phylogenetic approach is that close relatives come together even if they are morphologically quite different. This approach may be useful for predictions of pathogenicity or susceptibility to antifungals. Conversely, distant relationships are expected to predict large differences in clinically relevant parameters [22].

Several black yeast-like fungi have been known by different names, and this has caused confusion within the medical mycology community. For example, *Cladosporium carrionii* and *Cladophialophora ajelloi* were described separately. Due to noticeable similarities in their basic morphology, Honbo et al. conducted a study of their relationship and found that the *Cladosporium* and *Phialophora* synanamorphs produced by *Cladosporium carrionii* were identical to those produced by isolates of *Cladophialophora ajelloi* [23]. *Cladosporium carrionii* was then emended to include the *Phialophora* anamorph, and *Cladophialophora ajelloi* was considered to be a later synonym. *Cladosporium carrionii* was formally redescribed and designated in an appropriate combination as *Cladophialophora carrionii* [33].

Another example of changing nomenclature is that of *Exophiala dermatitidis*. In 1937, Kano described the species *Hormiscium dermatitidis* [34]. Due to its polymorphic nature, confusion was caused regarding its correct placement, and the species has undergone several taxonomic changes (i.e., *Fonsecaea dermatitidis*, *Hormodendrum dermatitidis*, *Phialophora dermatitidis*). The genus *Wangiella* was erected to accommodate *Hormiscium dermatitidis*, as it was found to be invalidly published because of the lack of a Latin diagnosis. Thus, the species was renamed as *Wangiella dermatitidis* [34]. Morphologically, *Wangiella dermatitidis* is intermediate between *Phialophora* and *Exophiala* [34], and it was formally transferred to the genus *Exophiala* as *Exophiala dermatitidis* based on the absence of phialides with collarettes [27].

Fonsecaea compacta was first described as *Hormodendrum compactum* by Carrion in 1935 from a clinical case [35], who observed that although it resembled *Hormodendrum pedrosoi* (now known as *Fonsecaea pedrosoi*), *H. compactum* was morphologically distinct, and was given the species epithet *compactum* due to the compact arrangement of the conidia. Subsequent studies found that the morphologic separation of *F. compacta* does not coincide with molecular data, and that this is no more than a dysgonic mutant or a morphological variety occurring in *F. pedrosoi* [24, 36, 37].

The genus *Ramichloridium* was erected in 1937 by Stahel but was invalid due to the lack of a Latin diagnosis. This genus was resurrected by De Hoog in 1977 to accommodate species with erect, dark, more or less differentiated, branched or unbranched conidiophores and predominantly aseptate conidia produced on a sympodially proliferating rachis [38], and included *Ramichloridium basitonum* and *Ramichloridium mackenziei*. However, a re-evaluation of the genus *Ramichloridium* using morphologic and DNA sequence data resulted in the proper accommodation of these two species in the genus *Rhinocladiella* as *Rhinocladiella mackenziei* and *Rhinocladiella basitona* [30].

Molecular Methods of Species Identification

Traditionally, phenotypic characteristics have been used as the primary method of fungal species identification. For black yeast-like fungi, identification based on morphology alone remains difficult. This is especially true for *Exophiala* species, which are pleomorphic and produce synanamorphs during their complex life cycles [39, 40]. The similarity of these structures, even in distantly related species based on phylogenetic analysis, and the lack of comparable physiological data, make it difficult to use morphologic criteria for reliable species identification [39].

DNA sequence analysis has proven to be a powerful tool for fungal species identification, including the identification of opportunistic and pathogenic organisms. The internal transcribed spacer (ITS) region of nuclear ribosomal RNA is a routinely used target for fungal species identification [41]. ITS is commonly used for routine identification by doing similarity searches in publicly available genetic databases, such as GenBank, and phylogenetic analysis. BLAST similarity search results should be considered carefully, as sequences in publicly available databases may be assigned the wrong taxon due to sample contamination, chimerism, poor taxonomic identification, and other errors [42]. Often, the mislabeled sequences may be corrected in the published manuscript but the correction may not occur within the database.

In black yeast-like fungi, the ITS region exhibits low variability among species and is not useful for discriminating between them [18, 43–45]. Other DNA targets, such as the nuclear ribosomal gene (small and large subunits), elongation factor α-1, and actin have been used in conjunction with ITS to improve sensitivity [13, 19••, 39, 45]. Heinrich et al. observed that the ITS2 region of species in the Family *Herpotrichiellacaea* contains homopolymeric regions mostly of poly (T) that may account for approximately 8% of artificial variances among species [39]. To avoid this problem, barcode identifiers within domain 2 of ITS2 were developed. Domain

2 of ITS2 provides highly conserved flanking sequences with a highly variable section in between, which varies markedly in both nucleotide composition and length between closely related taxonomic entities. These barcode identifiers have been found to be reliable, easy to use, and highly practicable for the rapid identification of clinically important black yeast-like fungi and closely related species [39].

Species identification of black yeast-like fungi has been improved with the introduction of barcode identifiers using the ITS2 region. Although barcode identifiers are useful, this method still involves sequencing of the ITS2 region, which is relatively expensive and does not have a rapid turn-around-time. Rolling circle amplification is an isothermal amplification method that uses padlock probes that hybridize to target DNA or RNA [46]. Two ends of the probe become juxtaposed and can be joined by a DNA ligase when both ends of the probes show perfect complementarity. This allows for the detection of single nucleotide mismatches and prevents non-specific amplification [46, 47]. Najafzadeh et al. reported that this technique could discriminate between *Exophiala* species with correct identification achieved unambiguously [46]. Given that this method is more rapid than DNA sequence analysis, it has potential to be used as an assay in clinical laboratories for fungal species identification.

Other molecular approaches using PCR-based assays have been used in the identification of black yeast-like fungi. One approach utilized a novel species-specific primer pair (i.e., Exdf, Exdr) that targeted the conserved regions of the partial ITS1-complete 5.8S-partial ITS2 rRNA region of *E. dermatitidis* [48]. When tested on presumptive black yeast isolates cultured from sputum samples in a cohort of cystic fibrosis patients, the primers were found to be specific, as cultures were confirmed to be *E. dermatitidis* by DNA sequence analysis. Another method specific for the detection of *E. jeanselmei*, termed *Ejeanselmei*_ITS qPCR, which utilizes SYBR green chemistry and real-time PCR, was shown to be 100% sensitive and specific for this particular species [49]. Although tested on water samples, this method has the potential to be applied routinely for the detection and identification of *Exophiala* species and other black yeast-like fungi in clinical specimens.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Another method that has demonstrated great potential for the species identification of various fungi, including black yeast-like species, is that of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which has proven to be a timely, accurate, and reproducible method for the identification of different microbes [50–53, 54•]. Species identification using this method is based on the detection of mass-to-charge (m/z) ratios of protonated ions from ribosomal proteins. The spectra that are obtained represent a unique fingerprint, known as a peptide mass fingerprint, and are compared to a library of spectra of known species in order to make the identification [55, 56]. MALDI-TOF MS has been found to be a rapid and sensitive method for species identification of bacteria, yeast, and filamentous fungi [54•, 57–61] and may be advantageous to DNA sequence analysis due to rapid turn-around-time and the relatively low cost of consumables. Independent studies have demonstrated that this method is capable of identifying and discriminating between different *Exophiala* species, as well as other related species [54•, 61, 62]. In a recent multicenter study, 31 of 31 *E. dermatitidis* isolates were correctly identified to the species level [54•]. Good results were also observed with *Cladophialophora bantiana* where 28 of 29 isolates were correctly identified. Interestingly, one *C. bantiana* isolate was misidentified as a *Candida collicculosa*. Of note, 7 of the 32 *E. xenobiotica* isolates evaluated were not identified by this particular assay despite confirmation of all species identifications by DNA sequence analysis. Similar success in the identification of *E. dermatitidis* and other *Exophiala* species has been reported by others with the use of MALDI-TOF MS technology [61, 63]. In one study that included 89 isolates, 83 corresponded to 16 known species of *Exophiala* and *Rhinocladiella*, and the remaining 6 represented two novel *Exophiala* species based on phylogenetic analysis [63]. A larger study that included 110 *Exophiala* isolates reported identification rates to the genus or species level between 80.6 and 100% for most species [62]. Thus, MALDI-TOF MS appears to be a robust assay for the identification of *Exophiala* species, although more work is needed for other species.

Detection and Identification in Direct Specimens

A major limitation of DNA sequence analysis and MALDI-TOF MS is the need for isolates growing in culture, as many patients with invasive fungal infections, including those caused by black yeast-like fungi, may be culture negative. This has clinical implications as clinicians may be forced to use empiric therapy for a presumptive fungal infection, and the drugs that are used may be suboptimal against the actual infecting organism. Surrogate marker assays are available and are often used to aid the diagnosis of certain invasive fungal infections. These include the 1,3-β-D-glucan assay and the *Aspergillus* galactomannan assay, which are capable of being performed on biological fluids, such as serum and bronchoalveolar lavage fluid. The 1,3-β-D-glucan assay detects the presence of 1,3-β-D-glucan polymers, a major component of the cell wall of numerous pathogenic fungi, including black yeast-like organisms [64]. However, this pan-fungal assay is incapable of determining which fungal species may be causing the infection, and false-positive reactions have been reported with its use [65]. In contrast, the galactomannan assay

is specific for *Aspergillus* species and is unable to detect black yeast-like fungi and their filamentous relatives.

Another approach for the detection and identification of fungi in direct specimens is the use of PCR-based assays on tissue, including fresh tissue and paraffin-embedded specimens. This approach may be especially useful where cultures are negative, but histopathology is consistent with a fungal infection. These assays have primarily targeted one or more regions within the rRNA cluster, including the 18S, 28S, and ITS regions, as these areas contain both highly conserved and variable regions, and are present in multiple copies, thus improving the sensitivity of these assays [66••]. Studies have reported some success with this approach in the identification of black yeast-like fungi, although the data are quite limited. In one study, *Exophiala* species were detected in three culture-positive specimens, including synovial fluid from one patient and skin from two others, with the use of a pan-fungal PCR assay that targeted the ITS1 region [43, 66••]. Interestingly, one *E. jeanselmei* identified by morphologic characteristics in a skin specimen was found to be a *E. spinifera* based on the results of the molecular assay, while the other *E. jeanselmei* identified by morphology was reported only to the genus level (*Exophiala*) based on the DNA sequence results. A recent study using a similar approach but targeting the ITS2 and D2 region of 28S rRNA in sterile specimens where histopathology was consistent with fungal infection also reported good success [67]. However, only one patient with an infection due to *Exophiala* species was included.

Several molecular-based platforms are now clinically available for the detection and identification of yeasts within the bloodstream. These include the Yeast Traffic Light and QuickFISH assays (AdvanDx) [68, 69], the FilmArray blood culture identification assay (bioMerieux) [70], and the T2 *Candida* magnetic resonance assay (T2Biosystems) [71, 72]. However, none of these assays is capable of detecting and identifying *Exophiala* species, even though these fungi have yeast-like structures and can disseminate via the hematogenous route. One assay, the PLEX-ID system, showed promise in the detection and identification of a large number of different fungi, including *Exophiala* and *Fonsecaea* species by combining multiplex PCR with electrospray ionization-mass spectrometry in order to amplify and identify organisms based on the mass-to-charge ratio of the PCR amplicons [73–75]. This assay was under clinical development for the detection of up to 75 different fungal species and had shown promising results with direct specimens [75]. Unfortunately, its development system has been discontinued by the manufacturer [76].

Conclusions

Black yeast-like fungi and their filamentous relatives are capable of causing a wide range of mycoses, and can cause disease in humans and other warm-blooded animals, as well as cold-blooded animals. Marked changes in the nomenclature and taxonomy of these species has also occurred. The diagnosis of infections caused by these species can be problematic, and species identification based solely on morphologic features may be fraught with difficulty due to similarities in characteristics among different species. Discrimination between closely related species can be accomplished by DNA sequencing, including DNA barcode analysis based on the ITS2 rRNA region. MALDI-TOF MS has also proven to be useful in the species identification of *Exophiala* species, although more work is needed to determine its utility for other species. Work was also done to assess the utility of molecular approaches for the detection of fungal species within direct specimens. However, available data for *Exophiala* species and other related fungi are limited.

Compliance with Ethical Standards

Conflict of Interest Connie F. Cañete-Gibas declares no conflict of interest.

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- •• Of major importance

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