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Analysis of Genes Coding for Small-Subunit rRNA Sequences in Studying Phylogenetics of Dematiaceous Fungal Pathogens

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Because of their ability to display yeast-like growth forms in various environmental conditions, dematiaceous (melanized) hyphomycetes of the form-genera *Exophiala*, *Rhinocladiella*, and *Wangiella* have been informally termed "black yeasts." Cladistic analysis of 1,050 bp of the genes coding for small-subunit rRNA (SSU rDNA) supported a close relationship among species of these black yeasts with other dematiaceous hyphomycetes in the form-genera *Fonsecaea*, *Phialophora*, and *Ramichloridium*. The conventional categories of these fungi based on asexual states are not supported by phylogenetic analysis of SSU rDNA sequences. Isolates exhibiting annellidic modes of blastic conidiogenesis (e.g., *Exophiala* spp.) were not monophyletic and were placed as sister taxa to isolates that produce phialides or sympodulae. The results indicated very close relationships between isolates of *Wangiella dermatitidis* and *Exophiala mansonii* and between *Rhinocladiella aquaspersa* and *Exophiala jeanselmei*. This clade of dematiaceous hyphomycetes was a sister group to a clade comprising members of two orders of cleistothecial ascomycetes, Eurotiales and Onygenales. The etiological agents of chromoblastomycosis were found to be a closely related group (clade), while the agents of phaeohyphomycosis displayed a broader distribution on the SSU rDNA tree.

Dematiaceous hyphomycetes are a large and diverse group of known or suspected ascomycetes that are usually or exclusively observed as asexual forms (14). They are characterized by the production of darkly pigmented hyphae and/or mitotic spores (conidia). A large group of medically important fungi are included among the species of these pigmented hyphomycetes. It is proposed that melanin production is linked to the pathogenicity of many of these fungi (10, 11, 13, 27).

The major infections caused by dematiaceous hyphomycetes are grouped into three broad classes of diseases: chromoblastomycosis, eumycotic mycetoma, and phaeohyphomycosis. Chromoblastomycoses are chronic cutaneous and subcutaneous infections characterized by the production of verrucose lesions, pseudoepitheliomatous hyperplasia, and the presence in tissue of muriform fungal cells that are termed sclerotic bodies (23). Of the fungi known to cause chromoblastomycosis (see Table 1), *Fonsecaea pedrosoi* (Brumpt) Negroni is the most common worldwide, while *Phialophora verrucosa* Medlar is the most common in North America (23). Eumycotic mycetomas are characterized by tumefaction, draining sinus tracts, and granules consisting of mycelial aggregates and tissue elements. The third major type of disease is termed phaeohyphomycosis (2, 23), which represents a broad range of primary and opportunistic mycoses. Phaeohyphomycosis is characterized by the presence in tissue of brownish yeast-like cells, pseudohypha-like cells, true hyphae, or any combination of these forms (23). Unlike chromoblastomycotic lesions, phaeohyphomycotic lesions lack sclerotic cells, are not limited to the skin, and elicit a greater variety of inflammatory responses.

Everyone routinely encounters dematiaceous fungi, which are in the air, water, and soil and are found in association with plants. They have been isolated as saprobes of decaying plant matter (24), members of the microbial flora of bathwater (26), and a major component of the airborne fungal spore flora (24).

In a study of 39 woody plant and soil samples, 43 isolates of dematiaceous hyphomycetes were recovered (12), including pathogenic isolates of *Cladosporium* Link ex Fries, *Phialophora* Medlar, *Exophiala* Carmichael, and *Wangiella* McGinnis as well as others. Similarly, the average airborne-spore count for *Cladosporium* species is several thousand per cubic meter, approximately 10 times the average pollen count (7).

Both the incidence of phaeohyphomycosis and the number of dematiaceous hyphomycetes that have been documented as etiologic agents are increasing. While only five or six agents of chromoblastomycosis are known, more than 70 species have been identified as causing phaeohyphomycosis (1). With increasing numbers of immunocompromised individuals, such as those with organ transplantation, Cushing's syndrome, collagen vascular disease, hematological malignancies, and AIDS, the etiological agents of phaeohyphomycosis are becoming recognized as a global health problem (1, 11).

The majority of dematiaceous hyphomycetes were placed in the form-family Dematiaceae on the basis of the presence of melanin and the lack of a known sexual state (14). Form-families are admittedly unnatural taxonomic groupings, and their use has been criticized recently by mycologists as producing artificial classifications that do not reflect relatedness (28). The result has been confusion due to classification systems that may convey erroneous information to clinical laboratorians, physicians, plant pathologists, and others. Many dematiaceous hyphomycetes whose sexual states are known, for example, are members of the ascomycete orders Chaetothyriales, Dothideales, and Pleosporales of the class "Loculoascomycetes" (3). At this time, supraordinal rankings among ascomycetes are debated and controversial; therefore, the supraordinal rankings contained in this paper are informal and are designated by quotation marks. These include "Euascomycotina," "Hymenoascomycetes," "Loculoascomycetes," "Plectomycetes," and "Pyrenomycetes." The last two categories have been included in the "Hymenoascomycetes" in several classifications at various rankings (3, 5, 20).

The focus of this investigation is a group of dematiaceous hyphomycetes from the form-genera *Exophiala*, *Rhinocladiella*,

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and *Wangiella* that have been informally termed "black yeasts" (9). Depending on the environmental conditions, these fungi may exhibit filamentous as well as yeast-like growth forms. Black yeasts are associated with chromoblastomycosis, phaeohyphomycosis, and eumycotic mycetomas and are the source of considerable taxonomic debate with respect to their identification, species concepts, and higher taxonomic placement. Examples include the most common etiological agents of phaeohyphomycosis, *Exophiala jeanselmei* (Langeron) McGinnis et Padhye and *Wangiella dermatitidis* (Keno) McGinnis (23). The recognition of *W. dermatitidis* has been questioned, as other classifications have treated it as conspecific with isolates of *Exophiala* or other form-genera (18). In this study, we have used cladistic analysis of nucleus-encoded genes coding for small-subunit rRNA (SSU rDNA) sequences to hypothesize phylogenetic relationships of black yeasts with other ascomycetes. This approach will assist in the development of future studies concerning the integration of sexual and asexual forms, species concepts, and population level questions.

MATERIALS AND METHODS

Fungal cultures. Dematiaceous hyphomycetes with known and unknown sexual cycles were sampled to elucidate better their placement among ascomycetes. Emphasis in taxon sampling was placed on the form-genera *Exophiala, Fonsecaea* Negroni, *Phialophora, Ramichloridium* Stahel ex de Hoog, *Rhinocladiella* Nannfeldt, and *Wangiella*. Of these, isolates of *Exophiala* and *Rhinocladiella* are linked to the order Chaetothyriales (3). Dematiaceous hyphomycetes were also sampled from the form-genera *Curvularia* and *Scolecobasidium*, which are treated in the order Pleosporales, and *Cladosporium* and *Aureobasidium*, which are treated in the order Dothideales (3). Additional taxa were included from the ascomycete orders Endomycetales, Eurotiales, Hypocreales, Microascales, Onygenales, Sordariales, Taphrinales, and Xylariales (see Table 1). Three basidiomycetes were used as the out-group. Cultures were grown in potato dextrose liquid media for 1 to 2 weeks, depending on growth rates. Isolates were confirmed by microscopic examination. Mycelium was collected, frozen in liquid nitrogen, and stored at −70°C prior to DNA extractions.

DNA sequences. DNA isolations were performed by the technique of Lee and Taylor (19). The 5′-most 1,150 bp of the SSU rDNA was amplified in symmetric PCRs (25, 29) using primers NS1 and NS4 (36). Reaction conditions were one cycle of 94°C for 3 min; 40 cycles of 94°C for 1 min, 53°C for 30 s, and 72°C for 1 min; and one cycle of 72°C for 5 min. Double-stranded PCR products were purified by using microcentrifuge ultrafiltration cartridges (UFC3 THK 00; Millipore) and concentrated to a final volume of 25 μl. Purified products were sequenced directly by a cycle sequencing technique (U.S. Biochemicals) following the manufacturer's protocol. The sequencing primers used were NS1, NS2, and NS4 (36) and SR11R (5′GGAGCCTGAGAAACGGCTAC3′) and SR7R (35). Sequencing reaction mixtures were electrophoresed on 8% denaturing polyacrylamide gels and then subjected to standard autoradiography.

Sequence analysis. Sequencing gels were read manually and aligned by using the University of Wisconsin Genetics Computer Group software package, and then the alignments were refined by direct examination. Sequence alignments were analyzed cladistically with the software package Phylogenetic Analysis Using Parsimony (PAUP) (34). Informative characters were analyzed by using the tree bisection-reconnection with random sequence addition option; 25 replications were performed. Support for inferred groups was estimated by the bootstrapping technique (15). Five hundred bootstrap replications were performed for the informative characters alone, using the tree bisection-reconnection with random sequence addition option.

Nucleotide sequence accession numbers. SSU rDNA sequences determined for the following species have been submitted to GenBank under the indicated accession numbers: *Cladosporidium cladosporioides* (Fresenius) de Vries, U20381; *Curvularia brachyspora* Boedijn, L36995; *E. jeanselmei* (Langeron) McGinnis et Padhye, L36996; *Exophiala mansonii* (Castellani) de Hoog, U20382 and U20383; *F. pedrosoi* (Brumpt) Negroni, L36997; *P. verrucosa* Medlar, L36999; *Ramichloridium anceps* (Sacc. et Ellis) de Hoog, U20380; *Rhinocladiella aquaspersa* (Borelli) Schell, McGinnis et Borelli, U20512; *Rhytidhysteron rufulum* (Spreng.: Fr.) Petrak, U20506; *Sculecobasidium* sp., U20513; and *W. dermatitidis* (Keno) McGinnis, L37002.

RESULTS

For 33 taxa, an average 1,050 bp of the 1,150-bp fragment was either sequenced or retrieved from GenBank (Table 1). Of the 1,050 bp, 246 were potentially phylogenetically informative (putative synapomorphies). Cladistic analysis of these charac-

TABLE 1. Isolates, GenBank accession numbers of SSU rDNA sequences included in the study, and disease categories of dematiaceous hyphomycetes

Isolate	GenBank accession no.[a]	Mycosis of dematiaceous hyphomycete[b]
Ambrosiozyma platypodis (J. M. Baker & Kreger-van Rij) Walt	L36984	
Aspergillus fumigatus Fresenius	M55626	
Athelia bombacina Pers.	M55638	
Aureobasidium pullulans (de Bary) G. Arnaud	M55639	Ph
Blastomyces dermatitidis Gilchrist et Stokes	M55624	
Candida albicans (C. P. Robin) Berkhout	X53497	
Candida tropicalis (Castellani) Berkhout	M55527	
Cephaloascus fragrans Hanawa	U20355	
Chaetomium globosum Kunze: Fr.	U20379	
Cladosporium cladosporioides (Fresenius) de Vries	U20381*	Ph
Coccidioides immitis Rixford et Gilchrist	M55627	
Cryptococcus neoformans (Sanfelice) Vuillemin	M55625	
Curvularia brachyspora Boedijn	L36995*	Ph
Diaporthe phaseolarum (Cooke & Ellis) Sacc.	L36985	
Exophiala jeanselmei (Langeron) McGinnis et Padhye	L36996*	Ph, M
Exophiala mansonii (Castellani) de Hoog	U20382*, U20383*	Ph
Fonsecaea pedrosoi (Brumpt) Negroni	L36997*	Ch
Hypocrea schweinitzii (Fr.) Sacc.	L36986	
Microascus trigonosporus C. W. Emmons & B. O. Dodge	L36987	
Neurospora crassa Shear & B. O. Dodge	X04971	
Ophiostoma piliferum H. Sydow & Sydow	U20377	
Penicillium notatum Westling	M55628	
Phialophora verrucosa Medlar	L36999*	Ch
Ramichloridium anceps (Sacc. et Ellis) de Hoog	U20380*	?
Rhinocladiella aquaspersa (Borelli) Schell, McGinnis et Borelli	U20512*	Ch
Rhytidhysteron rufulum (Spreng.:Fr.) Petrak	U20506*	
Saccharomyces cerevisiae E. Hans.	M277607	
Sculecobasidium sp.	U20513*	Ph
Spongipellis unicolor (Schw.) Murr.	M59760	
Talaromyces flavus (Klocker) Stolk et Samson	M83262	
Taphrina deformans (Berk.) Tul.	U20376	
Wangiella dermatitidis (Keno) McGinnis	L37002*	Ph
Xylaria hypoxylon (L.:Fr.) Grev.	U20378	

[a] Asterisks indicate sequences determined for this study.
[b] Ph, phaeohyphomycosis; Ch, chromoblastomycosis; M, mycetoma.

ters resulted in four equally most-parsimonious trees (MPTs) of 702 steps with a consistency index and a retention index of 0.506 and 0.707, respectively. A strict consensus of these four MPTs (Fig. 1) was 708 steps with a consistency index and a retention index of 0.501 and 0.702, respectively.

Alignment of sequences was relatively facile. A total of 17 insertions or deletions (indels) were introduced into the align-
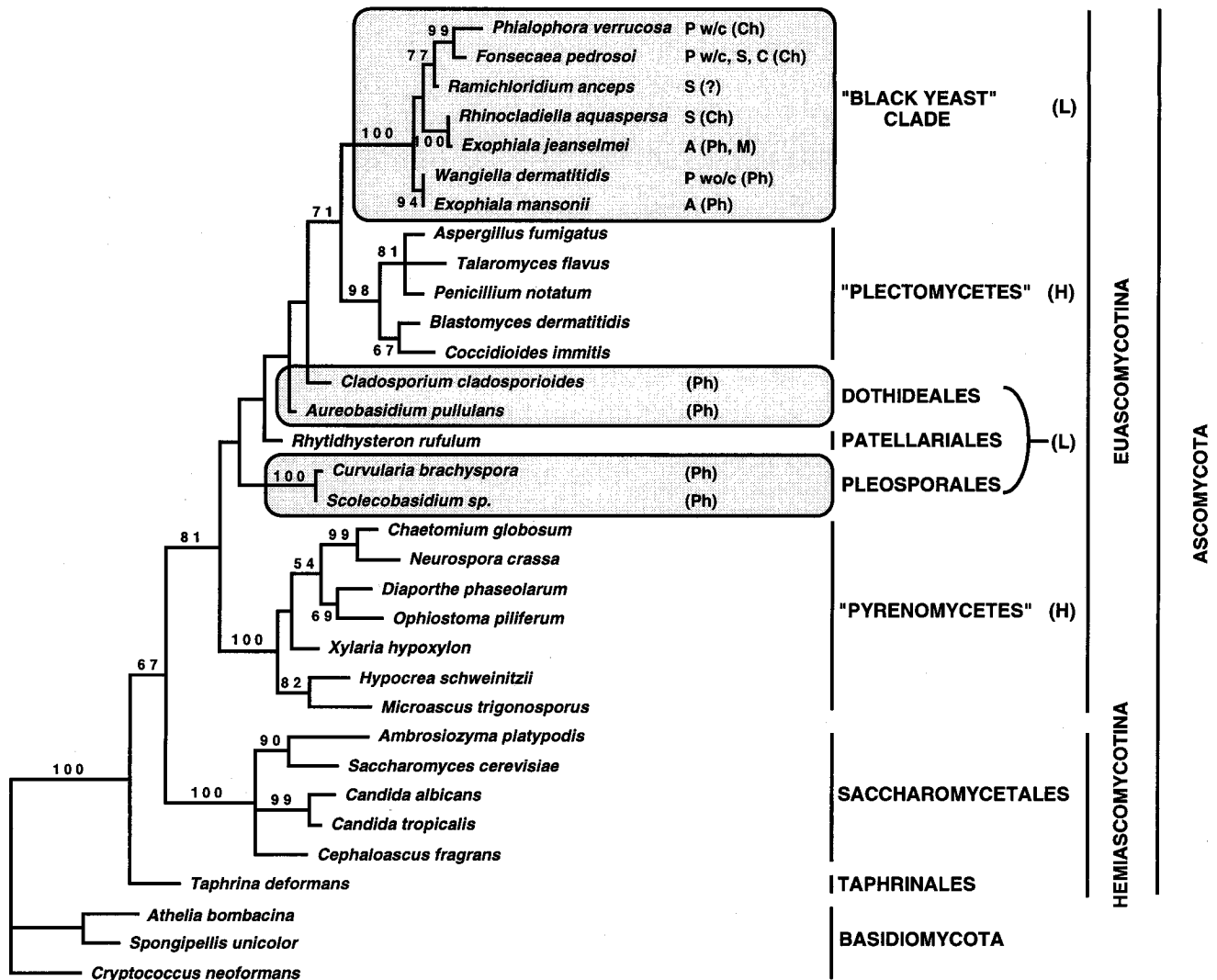
FIG. 1. Strict consensus tree of four equally MPTs. Nodes that received bootstrap values of >50% are shown. Major groupings of organisms are listed on the right; (L), "Loculoascomycetes"; (H), "Hymenoascomycetes." Dematiaceous hyphomycetes (grey boxes) are indicated. Mitotic states of black yeasts and related dematiaceous hyphomycetes are designated as follows: A, annellides; C, *Cladosporium*-like; P w/c, phialides with collarettes; P wo/c, phialides without collarettes; and S, sympodulae. Diseases caused by dematiaceous hyphomycetes are designated as follows: (Ch), chromoblastomycosis; (Ph), phaeohyphomycosis; and (M), mycetoma.

ment. Analyses included indels coded as single events or as multiple events where appropriate or omitted. All analyses produced the same four MPTs. Support for the major clades, however, did vary with respect to variations in coding of indels (data not shown). The tree lengths and indices presented here are those from the analysis with indels omitted. A 400-bp insertion was detected in the sequences of *W. dermatitidis* and *E. mansonii* at position 507. No nucleotide substitutions between the partial sequences of the two inserts were detected.

The isolates sampled from the form-genera *Exophiala*, *Rhinocladiella*, *Fonsecaea*, *Phialophora*, *Ramichloridium*, and *Wangiella* (Fig. 1) formed a monophyletic group (clade). This clade was supported by 19 characters and a bootstrap value of 100%. The form-genus *Exophiala* was not monophyletic. *E. mansonii* (Castellani) de Hoog was a sister taxon to *W. dermatitidis*, and *E. jeanselmei* was a sister taxon to *R. aquaspersa* (Borelli) Schell, McGinnis et Borelli; these groups were supported by bootstrap values of 94 and 100%, respectively. This clade was a sister group to the "Plectomycete" clade and not to

clades containing the other dematiaceous hyphomycetes or "Loculoascomycetes" sampled (Fig. 1). The sister group relationship of the black yeast and the "Plectomycete" clades was supported by 12 characters and a bootstrap value of 71%. The remaining dematiaceous hyphomycetes were either unresolved or not strongly supported with respect to their placement among the Ascomycota.

DISCUSSION

Higher taxonomic placement of black yeasts. Ascomycete systematics is currently in a state of flux. Over the past decade, the tendency has been to de-emphasize supraordinal rankings (17). This has stemmed primarily from phylogenetic conflict among suites of morphological characters. Recently, molecular systematics has had a substantial impact on the field by providing phylogenetic data that are independent of the morphology in question (5, 32). At the present, supraordinal rankings among ascomycetes are controversial.

Some recent classifications (3, 4) propose two major groups within the ascoma-producing ascomycetes ("Euascomycotina"), the "Hymenoascomycetes" and the "Loculoascomycetes." "Hymenoascomycetes" possess true ascomata that are formed from ascogenous hyphae. "Loculoascomycetes" possess pseudothecia, or false ascomata, that are derived from somatic hyphae. Additionally, a high level of correlation exists in "Hymenoascomycetes" possessing asci with a single functional wall layer (unitunicate asci) and "Loculoascomycetes" possessing asci with two functional wall layers (bitunicate asci) (20).

The grouping of the black yeast clade as a sister group to the "Plectomycetes" is inconsistent with morphological classifications. On the basis of morphology, these fungi more closely resemble some of the other dematiaceous hyphomycetes or "Loculoascomycetes" sampled, i.e., *Aureobasidium pullulans* (de Bary) Arnaud and *C. cladosporioides* (Fresenius). These results, however, are in agreement with two complementary phylogenetic studies. In an SSU rDNA analysis of the major groups of higher fungi (37), isolates of *Exophiala dermatitidis* grouped with taxa sampled from the "Plectomycetes"; however, no other closely related dematiaceous hyphomycetes were sampled. Consistent results were also obtained in independent analyses that sampled similar taxa but compared data from chitin synthase genes (6, 8). In those analyses, *E. jeanselmei*, *Phaeococcomyces exophialae* (de Hoog) de Hoog, *Rhinocladiella atrovirens* Nannfeldt, and *W. dermatitidis* were placed as the closest group to the plectomycetous taxa *Aspergillus niger* van Tieghem, *Emericella nidulans* (Eidam) Vuillemin, *Blastomyces dermatitidis* Gilchrist et Stokes, and *Histoplasma capsulatum* Darling. The agreement among these three studies suggests that a closer taxonomic affinity exists between these two groups than predicted or previously appreciated. These data suggest that the characters (as they are currently defined) true ascomata and unitunicate asci have evolved independently and are therefore not homologous. That is, they result from convergent or parallel evolution and do not reflect the natural groupings within the "Euascomycotina."

Conidiogenesis among black yeasts. Ascomycetes usually have two states to their life cycle, a sexual (perfect, meiotic, or telomorphic) and an asexual (imperfect, mitotic, or anamorphic) state. In some species, only one of the two states is known, or the states occur under different environmental conditions. Historically, the sexual and the asexual states of an organism each receive a Latin binomial. There are several examples among the ascomycetes of a particular asexual genus (form-genus) occurring in multiple sexual genera. Similarly, several asexual species (form-species) may be associated with a single sexual species. Fungi that are known to have several modes of conidiogenesis are described as having a polymorphic life cycle or as being pleomorphic (33). This situation reflects the artificial groupings that occur frequently in the grouping of asexual ascomycetes and the taxonomic difficulties presented by these fungi.

As noted, the majority of dematiaceous hyphomycetes are known only from their asexual states. The primary criteria by which these fungi are identified and categorized are the different structural and developmental attributes of asexual reproduction (conidiogenesis). The difficulty arises in that these gross morphologies are often quite reduced, and a single species may be pleomorphic. For example, members of the form-genus *Fonsecaea*, such as *F. pedrosoi*, the most common cause of chromoblastomycosis, may exhibit *Cladosporium*-like (chains of blastoconidia), *Rhinocladiella*-like (sympodulae), and *Phialophora*-like (phialides with collarettes) forms of conidiogenesis (31). An isolate of *F. pedrosoi* may produce each of these states unaccompanied by the other states, as well as different

combinations. Similar controversies exist with the pleomorphic species of other form-genera, such as *Wangiella*, *Exophiala*, and *Rhinocladiella* (9, 21).

The form-genera *Exophiala*, *Wangiella*, and *Rhinocladiella* are currently delimited on the basis of annellidic, phialidic (without collarettes), and sympodial modes of blastic conidiogenesis, respectively (13, 24, 31). In the SSU rDNA analysis, the isolates sampled from these form-genera were interspersed among one another (Fig. 1). These data do not support the grouping of these form-genera on the basis of conidiogenesis as they are currently circumscribed. Additional taxon sampling is necessary to resolve convincingly the discrepancies with morphology. Furthermore, some form-species of *Exophiala* and *Rhinocladiella* have been linked with sexually reproducing species in the genera *Capronia* Sacc. and *Dictyotrichiella* Munk (Herpotrichiellaceae, Chaetothyriales). A close phylogenetic relationship between form-species of *Exophiala* and the genus *Capronia* is consistent with the results of an independent phylogenetic study of SSU rDNA (16) and warrants the inclusion of additional chaetothyrialean taxa in future analyses.

The polyphyly of *Exophiala* demonstrates the inconsistency between conidiogenesis, as currently defined, and the SSU rDNA analysis. *E. mansonii* and *W. dermatitidis* have been the source of considerable taxonomic debate. At various times, *W. dermatitidis* has been placed in the form-genera *Hormiscium* Kunze, *Fonsecaea*, *Hormodendrum* Bonorden, and *Phialophora*, and it has been synonymized in *Aureobasidium mansonii* (Castellani) Cooke ex Borelli, *Phialophora gougerotii* (Matruchot) Borelli ex Borelli, and *Rhinocladiella mansonii* (Castellani) Schol-Schwarz ex Schol-Schwarz (*E. mansonii*) (22). Schol-Schwarz (31) amended *R. mansonii* (*E. mansonii*) to include *Hormiscium dermatitidis* Kano (*W. dermatitidis*). de Hoog (9) later transferred *R. mansonii* to *Exophiala*. McGinnis (22) disagreed with both of these proposals and reserved *Exophiala* for isolates that produced annellides and constructed the form-genus *Wangiella* for the isolates that produce phialides without collarettes. Kwon-Chung and Bennett (18) synonymized *W. dermatitidis* and *E. mansonii* in *E. dermatitidis*. These data support the synonymy of *W. dermatitidis* and *E. mansonii*, although additional taxon sampling of both species and the use of a more variable region of the genome are necessary.

The other form-species of *Exophiala* sampled, *E. jeanselmei*, is a sister taxon to *R. aquaspersa* (30). *R. aquaspersa* is a polymorphic form-species producing predominantly a sympodial anamorph. However, it also produces annellides and phialides similar to those described for the form-genera *Exophiala* and *Wangiella*, respectively. Similarly, *E. jeanselmei* is highly variable; three varieties have been described (9).

The other members of the black yeast clade are *Ramichloridium anceps* (Sacc. et Ellis) de Hoog, *F. pedrosoi*, and *P. verrucosa*. All of these taxa have been proposed to be closely related to the form-genera *Exophiala* and *Rhinocladiella*. This hypothesis is supported by the analysis of the SSU rDNA data. Specifically, these analyses support a close relationship between *Ramichloridium* and some component within the *Exophiala-Rhinocladiella* complex and *Fonsecaea* and *Phialophora* sensu lato. However, additional taxa must be sampled to resolve these questions in the context of the morphological variation that exists within each of these form-genera.

These data do not support the exclusive use of modes of conidiogenesis, as currently defined, to categorize the taxa sampled into the form-genera in question. Sister taxa, as defined by the SSU rDNA analysis, may produce different asexual states. Asexual states are polymorphic within isolates and are hypervariable among form-genera within the black yeast clade. These results underscore the taxonomic challenge of these

fungi and the evidence that phylogenetic analysis of molecular data can provide towards a better understanding of the organisms.

Distribution of diseases associated with dematiaceous hyphomycetes on the SSU rDNA tree. The etiological agents of chromoblastomycosis and phaeohyphomycosis display different distributions on the SSU rDNA tree (Fig. 1). The isolates sampled that cause chromoblastomycosis are all relatively closely related, whereas those that cause phaeohyphomycosis display a broader distribution on the SSU rDNA tree. This observation should not be surprising if one considers that the numbers of causal agents for chromoblastomycosis and phaeohyphomycosis are 5 or 6 and 70, respectively. The agents of chromoblastomycosis are united by a specialized in vivo morphogenesis to produce sclerotic cells (11), whereas the agents of phaeohyphomycosis lack this feature. There are often many ways not to possess a particular trait. More importantly, the production of sclerotic bodies appears to be restricted to a defined group of organisms.

Not all members of the black yeast clade are reported to produce sclerotic bodies. This observation may have several explanations. Some species of the clade may have lost the ability to produce sclerotic bodies, may rarely exhibit this type of growth form, or may be polymorphic for the trait. For example, *W. dermatitidis* has been reported as producing muriform cells (sclerotic bodies) in vitro but not in vivo (10). Alternatively, all members of this clade may be capable of producing sclerotic bodies but erroneous species concepts have led to misidentification. That is, an investigator may automatically exclude certain taxa as the causal agent of the infection if a tissue biopsy is positive for sclerotic bodies. This scenario is complicated further by the observation that mitotic states are variable within the black yeast clade. Molecular systematics and sampling of sclerotic bodies could determine whether sclerotic bodies may occur in mitotic species previously thought not capable of producing such structures.

The application of phylogenetics to the taxonomy of pathogenic fungi will lead to more-accurate identification and provide testable means of assessing relationships of pathogenic and nonpathogenic forms. This knowledge will facilitate a more accurate diagnosis and potentially assist in identifying nonpathogenic model systems and designing more-specific and more-effective treatments. Moreover, such studies provide us with a better understanding of the biology and evolution of pathogenic fungi.

ACKNOWLEDGMENTS

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