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Phylogeny and Phenotypic Characterization of Pathogenic *Cryptococcus* Species and Closely Related Saprobic Taxa in the Tremellales[▽]†

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The basidiomycetous yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* are closely related sibling species that cause respiratory and neurological disease in humans and animals. Within these two recognized species, phylogenetic analysis reveals at least six cryptic species defined as molecular types (VNI/II/B, VNIV, VGI, VGII, VGIII, and VGIV) that comprise the pathogenic *Cryptococcus* species complex. These pathogenic species are clustered in the *Filobasidiella* clade within the order Tremellales. Previous studies have shown that the *Filobasidiella* clade also includes several saprobic fungi isolated from insect frass, but information evaluating the relatedness of the saprobes and pathogens within this cluster is limited. Here, the phylogeny encompassing a subset of species in the Tremellales lineage that clusters closely with the pathogenic *Cryptococcus* species complex was resolved by employing a multilocus sequencing approach for phylogenetic analysis. Six highly conserved genomic loci from 15 related basidiomycete species were sequenced, and the alignments from the concatenated gene sequences were evaluated with different tree-building criteria. Furthermore, these 15 species were subjected to virulence and phenotype assays to evaluate their pathogenic potential. These studies revealed that *Cryptococcus amylolentus* and *Tsuchiyaea wingfieldii*, two nonpathogenic sibling species, are the taxa most closely related to the pathogens *C. neoformans* and *C. gattii* and together with *Filobasidiella depauperata* form a *Cryptococcus* sensu stricto group. Five other saprobic yeast species form the *Kwoniella* clade, which appears to be a part of a more distantly related sensu lato group. This study establishes a foundation for future comparative genomic approaches that will provide insight into the structure, function, and evolution of the mating type locus, the transitions in modes of sexual reproduction, and the emergence of human pathogenic species from related or ancestral saprobic species.

Recent phylogenetic and genomic studies of the fungal kingdom have illustrated that analysis of both distantly and closely related species provides insight into the evolutionary trajectories of fungal species (16, 53). The Fungal Tree of Life (AFTOL) project applied a high-resolution multilocus sequencing (MLS) approach to 170 species, elucidating broad and specific evolutionary relationships among species (16). Yet, the entire fungal kingdom encompasses an estimated 1.5 million species (14), many more than can be analyzed by this approach. More than 100 fungi have been subjected to whole-genome analysis, including distantly related but also, in some cases, closely aligned species (53). Taken together, these approaches illustrate that comparisons of closely related species, first by MLS and then by whole-genome studies, can impact our understanding of how closely related pathogenic and saprobic fungi have evolved to occupy specialized niches in nature.

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The human fungal pathogen and basidiomycete *Cryptococcus neoformans* and its closest relative *Cryptococcus gattii* cause both respiratory and neurological diseases in immunocompromised and immunocompetent patients (7). The sexual states of those species, which form on artificial culture media, were named *Filobasidiella neoformans* and *Filobasidiella bacillispora*, respectively (21, 22). *C. neoformans* and *C. gattii* are members of the pathogenic *Cryptococcus* species cluster, which contains two currently recognized varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, and the sibling species *C. gattii* (7, 23). Recent studies based on MLS typing analysis provide evidence that *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are distinct species and that a few subpopulations are found in *C. neoformans* var. *grubii* (VNI, VNII, and VNB) (6, 28, 29). Furthermore, *C. gattii* can be further subdivided into at least four cryptic species (VGI, VGII, VGIII, and VGIV) (6, 10, 34). Hence, as many as six species define the pathogenic *Cryptococcus* species complex. All six of these pathogenic *Cryptococcus* species cluster together in the *Filobasidiella* clade, which appears to have emerged from the *Tremella* lineage (8, 48). Nonetheless, the genus *Cryptococcus* is polyphyletic, and species cluster within the Tremellales, the Trichosporonales, the Filobasidiales, and the Cystofilobasidiales clades (8, 45, 48), including the less common human pathogens *Cryptococcus albidus*, *Cryptococcus laurentii*, and *Crypto-*

coccus adeliensis (43, 49) and the saprobe *Cryptotrichosporon anacardii* (39), which shares phenotypic traits (melanin production and capsule formation) similar to *C. neoformans*.

Previous taxonomic studies based on ultrastructural features and basidial morphology separated the hymenomycetous yeasts (now classified in the order Tremellomycetes [15]) into two orders: Tremellales, with cruciately septate basidia, and Filobasidiales, with aseptate basidia (19). Because *Filobasidiella* species have aseptate holobasidia, they were included in the Filobasidiales together with the *Filobasidium* and *Cystofilobasidium* species. However, molecular data later showed that *Filobasidiella* species are more closely related to those in Tremellales (48). Most of the species with defined sexual cycles in the Tremellales have septate two- to four-celled basidia (5, 18, 19, 27, 45, 47, 54). The holobasidia of *Filobasidiella* species are thus unique in the Tremellales.

Mating systems also differ among the Tremellales. All known heterothallic *Tremella* species have been reported to have a tetrapolar (bifactorial) mating system with a multiallelic A locus and a biallelic B locus involved in production and sensing of pheromones (13, 65). *Fibulobasidium inconspicuum* has also been shown to have the same type of mating system (3). However, other teleomorphic species in the Tremellales, such as *Auriculibuller fuscus* and *Kwoniella mangroviensis*, are reported to have bipolar mating type systems (5, 46, 54).

Tremellales generally have haustorial branches, which are short branches of hyphae with a basal clamp connection (33). Haustorial filaments of the mycoparasitic *Tremella* species were observed attached to host cells, enabling penetration and parasitic interactions (22, 54, 66). Haustorial branches have also been described in *C. neoformans* (19, 20), suggesting an ancestral mycoparasitism (2).

Previous studies examining the genetic relatedness of the species in the *Filobasidiella* clade, namely, the homothallic filamentous fungus *Filobasidiella depauperata* and the heterothallic yeasts *C. neoformans* and *C. gattii*, are based on internal transcribed spacer (ITS) analysis and/or ribosomal DNA sequence divergence and basidial morphology (10, 20, 24, 25, 57). Results from these studies strongly support the possibility that these three species form a monophyletic clade. Additional studies have shown that these fungi also align closely with *Cryptococcus amylolentus* and *Tsuchiyaea wingfieldii* (8, 12, 23, 36). Because of their proximity to the pathogenic *Cryptococcus* species cluster, these species all constituted the focus of our phylogenetic study. Several additional saprobic anamorphic yeasts, *Cryptococcus bestiolae* (59), *Cryptococcus dejecticola* (59), *Cryptococcus heveanensis*, and *Bullera dendrophila* (61, 63), also appear to be closely related to the *Filobasidiella* clade and to form a monophyletic lineage related to the dimorphic, heterothallic basidiomycetous yeast *Kwoniella mangroviensis* (formerly *Cryptococcus* sp. strain CBS 8507), recently discovered to have an extant sexual cycle (54). Therefore, for a more robust comparison, several members of the *Kwoniella* lineage were also included in our analyses.

Many of the species nested in the Tremellales can be found as mycoparasites and saprobes associated with wood, plants, soil, and arthropod frass (66). Similar habitats and environmental associations have been found for *C. neoformans* and *C. gattii.* These two species have been isolated from a variety of tree species, pigeon guano, and insects. For example, *C. neoformans* has been isolated from beetles (55a), and *C. gattii* has been detected in honeybee hives and insect frass (7a, 17a, 26). This accumulating circumstantial evidence might imply a possible arthropod-associated habitat for the pathogenic *Cryptococcus* species. Previous studies have used several invertebrate model hosts such as *Acanthamoeba* spp., *Caenorhabditis elegans*, *Dictyostelium discoideum*, *Drosophila melanogaster*, and *Galleria mellonella* to model bacterial or fungal infections in mammals (37). These host systems are in some cases genetically tractable, relatively simple to manipulate, and inexpensive (37). Interestingly, the virulence potential of the pathogenic *Cryptococcus* species correlates with the ability of the fungus to cause lethal infections in the heterologous insect host *G. mellonella* (wax moth) (38). Moreover, *C. neoformans* pathogenicity has been studied extensively in *G. mellonella*, and genes required for infection of wax moth larvae are necessary during *C. neoformans* and *C. gattii* infection of mammalian hosts (38). Results from these studies reveal insights into the ecological niche and the evolution of pathogenesis of *C. neoformans* and *C. gattii.*

The fungal species in the Tremellales are numerous, representing over 120 species, and many of their phylogenetic relationships are weakly supported due to the lack of multilocus phylogenetic and phenotypic analyses. Here, we use molecular and phenotypic methods to examine the species surrounding the monophyletic pathogenic *Cryptococcus* complex clustered within the Tremellales. We employed an MLS approach encompassing six highly conserved genomic loci present in the fungal kingdom. The *RPB1*, *RPB2*, *EF1*α, and mitochondrial small subunit (mitSSU) rRNA genes, the nuclear large subunit (nucLSU) rRNA (D1/D2 domains), and the ITS regions were amplified and sequenced to determine the divergence among the 15 fungal isolates tested. The virulence potential of all 15 isolates was also evaluated in *G. mellonella*. These studies provide insight into the genotypic and phenotypic trajectory of a highly successful pathogenic clade that likely emerged from saprobic fungi associated with insects in the environment.

MATERIALS AND METHODS

Fungal isolates. The isolates used in this study are listed in Table 1. All were grown and maintained on yeast-peptone-dextrose (YPD) medium at 24°C.

DNA isolation, PCR, and sequencing. To isolate fungal DNA, cells were harvested after shaking at 24°C in YPD liquid medium overnight, followed by lyophilization. DNA was isolated using the CTAB (cetyltrimethylammonium bromide) method (64). PCR amplification was performed with the following six fungal-specific, highly conserved genes encoding the largest subunit of RNA polymerase II (*RPB1*), the second largest subunit of RNA polymerase II (*RPB2*), elongation factor 1 alpha (*EF1*α), the mitochondrial small-subunit (mitSSU) rRNA, the D1/D2 domains of the nucLSU rRNA (nucLSU [D1/D2]), and the ITS region of the ribosomal DNA unit, which includes the ITS1 and ITS4 regions and the 5.8S rRNA gene. Individual PCRs were performed for each of the six genes. Primer information can be found at http://www.aftol.org/primers.php (the primers used in this study were *RPB1*Af and *RPB1*Cr, *RPB2*-5f and *RPB1*-11bR, *EF1*α1F and *EF1*α1R, mitSSU1F and mitSSU3R, nucLSU LrDNA and nucLSU LR3, and ITS1 and ITS4). PCR products were separated on an agarose gel and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequencing reactions were performed using BigDye chemistry version 3.1 (Applied Biosystems, Foster City, CA) and analyzed with an Applied Biosystems 3730xl capillary sequencer. Sequence reads were trimmed and assembled with a DNA sequence assembly software program, Sequencher (version 4.8; Gene Codes Corporation, Ann Arbor, MI). Individual contigs were generated with Sequencher, and a BLAST (1) analysis was performed to confirm the identity of the

TABLE 1. Description of species studied

Species[a]	Strain	Original substrate and location	Clade
Bullera dendrophila	CBS6074	Frass of buprestid larvae in South Africa	*Kwoniella*
Cryptococcus amylolentus	CBS6039	Frass of beetles in South Africa	*Filobasidiella*
Cryptococcus bestiolae	CBS10118	Frass of the litchi fruit borer in Vietnam	*Kwoniella*
Cryptococcus dejecticola	CBS10117	Frass of the litchi fruit borer in Vietnam	*Kwoniella*
Cryptococcus heveanensis	CBS569	Sheet rubber in Indonesia	*Kwoniella*
Cryptococcus humicola	CBS571	Soil	*Trichosporon*
Cryptococcus gattii VGI*	CBS10510	From debris of *Eucalyptus tereticornis* (strain WM276)	*Filobasidiella*
Cryptococcus gattii VGII*	CBS10514	Bronchial wash fluid of male patient in Canada (strain R265)	*Filobasidiella*
Cryptococcus neoformans var. *grubii* VNI*	CBS8710	Patient with Hodgkin's lymphoma in North Carolina (strain H99)	*Filobasidiella*
Cryptococcus neoformans var. *neoformans* VNIV*	ATCC MYA-565	Serotype D alpha laboratory congenic strain (JEC21)	*Filobasidiella*
Filobasidiella depauperata	CBS7841	Dead spider in Canada	*Filobasidiella*
Kwoniella mangroviensis	CBS8507	Mangrove Cay in Bahamas	*Kwoniella*
Tremella globispora	CBS6972	*Diaporthe* sp. on *Cornus nuttallii* in Canada	*Tremella*
Tremella mesenterica	CBS6973	Fallen branch of *Alnus rubra* in Canada	*Tremella*
*Tremella mesenterica**	ATCC 24925	Dead branch of *Alnus rubra* in Canada	*Tremella*
Tsuchiyaea wingfieldii	CBS7118	Frass of scolytid beetles in South Africa	*Filobasidiella*

[a] *, whole-genome sequences were available for these strains.

sequenced products (see Table S1 in the supplemental material for GenBank accession numbers.).

Phylogenetic analysis. A total of 92 out of 96 DNA sequences (4% of the molecular data was missing) were aligned using ClustalW version 1.81 (60). The FASTA alignment files for each of the six loci were imported into MacClade version 4.08 (32) for manual editing and to identify and correct ambiguously aligned regions (732 characters). Edited files were concatenated, resulting in the inclusion of 3,422 characters for phylogenetic analyses. Heuristic searches for maximum parsimony (MP) (Fig. 1A) and maximum likelihood (ML) (Fig. 1B) criteria were conducted using PAUP version 4.0 (56). MP searches were also conducted with the concatenated data set, with a user-defined step matrix generated in STEP3 matrix for conserved regions (31), and using a matrix generated by INAASE for ambiguously aligned regions (35). Model parameter estimates for ML analysis were obtained using MODELTEST (42). Statistical support was calculated using 1,000 bootstrap replicates from MP and ML criteria, and all trees were rooted using *Cryptococcus humicola* as the outgroup (a member of the order Trichosporonales [8, 48]) (Table 1). MacClade software was also used to trace the habitat preferences (Fig. 2) of the fungi in the current study (32).

Phenotypic analysis of isolates. The phenotypic properties of each isolate were examined using several assays. First the ability of each strain to grow on YPD and yeast nitrogen base (YNB) media at 24°C, 30°C, and 37°C was tested. Each isolate was analyzed for melanin production on Niger Seed, Rose Bengal, and low-glucose (0.1%) media supplemented with the diphenolic molecule L-DOPA (dihydroxyphenylalanine) (100 mg/ml). To assay isolates for capsule production, isolates were grown in capsule induction medium (5 g glucose, 5 g asparagine, 400 mg K_2HPO_4, 80 mg $MgSO_4 \cdot 7H_2O$, and 250 mg $CaCl_2 \cdot 2H_2O$, and 1,000× vitamin-mineral mixture [0.4 g thiamine, 0.057 g $B(OH)_3$, 0.004 g $CuSO_4 \cdot 5H_2O$, 0.01 g $MnCl_2 \cdot 4H_2O$, 2 g $ZnSO_4 \cdot 6H_2O$, and 0.46 g sodium molybdate in 1 liter of water]) with low iron (20 mg/liter of the iron chelator, EDDHA [ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid)]) at 24°C for 2 days, followed by India ink staining. Isolates were also tested at 24°C for urease activity, using Christensen's agar (Beckton Dickinson, Cockeysville, MD). For these assays, all isolates were grown to exponential phase in liquid YPD medium, serially diluted in spots onto YPD, YNB, Niger Seed, Rose Bengal, L-DOPA, and Christensen's agar, and incubated for 4 to 5 days. The phenotypic assays were performed in triplicate. We note that some images of growth patches on the same medium were cropped to produce the composite image shown in Fig. 3.

***Galleria mellonella* survival assay.** To analyze virulence in the heterologous host *G. mellonella*, a previously described protocol (38) was followed with minor modifications. Wax moth larvae (~12) were injected in the last pseudopod with 5×10^5 cells of each isolate, and wax moths were incubated at 24°C. The number of surviving wax moths was monitored and recorded daily. Survival curves were plotted using Prism software (version 4.0a; Prism Computational Sciences, Incorporated, Madison, WI). A P value of <0.05 was considered significant. P values were calculated and compared to those of the reference phosphate-buffered saline (PBS) (or mock inoculation) control and to those of the pathogenic control *C. neoformans* var. *grubii* strain H99 (Fig. 4 and 5). The experiment was performed three times.

RESULTS

***Cryptococcus amylolentus* and *Tsuchiyaea wingfieldii* are the species most closely related to *C. neoformans*.** To establish evolutionary relationships among the pathogenic *Cryptococcus* species cluster and the related saprobic yeasts in the Tremellales, we performed a multilocus phylogenetic analysis of the 15 species listed in Table 1. Six highly conserved, fungal-specific genomic loci were sequenced and analyzed, as follows: *RPB1*, *RPB2*, *EF1*α, mitSSU, nucLSU (D1/D2), and ITS. Phylogenetic trees from concatenated sequences were generated with PAUP (56), and the parsimony heuristic searches in PAUP (56) generated only one tree topology (Fig. 1A). The same topology for the *Filobasidiella* clade was obtained under ML criteria searches (Fig. 1B) when the substitution parameters estimated by MODELTEST were used (42).

This MLS analysis resolved the phylogeny of the pathogenic *Cryptococcus* species complex and its closest relatives. In agreement with previous studies (57), two closely related monophyletic sister clades, the *Filobasidiella* and *Kwoniella* lineages, were identified and showed over 96% bootstrap support (Fig. 1). The *Filobasidiella* clade represents a sensu stricto clade of *Cryptococcus* species (it contains the type species of the genus, *C. neoformans*), while the *Kwoniella* clade contains the majority of the more distantly related insect-associated yeasts, named the sensu lato group (Fig. 1 and 2). These two clades are reminiscent of the sensu stricto and the sensu lato groups used to describe the phylogeny of *Saccharomyces cerevisiae* and related species (62). Our results show that the two closest relatives of *C. neoformans* are the sibling species *C. amylolentus* and *T. wingfieldii.* These findings do not support previous phylogenetic studies (based on a single gene) that placed *F. depauperata* as the closest relative of the pathogenic *Cryptococcus* species (25, 39, 51). However, the current and previous results agree that *F. depauperata* is a member of the

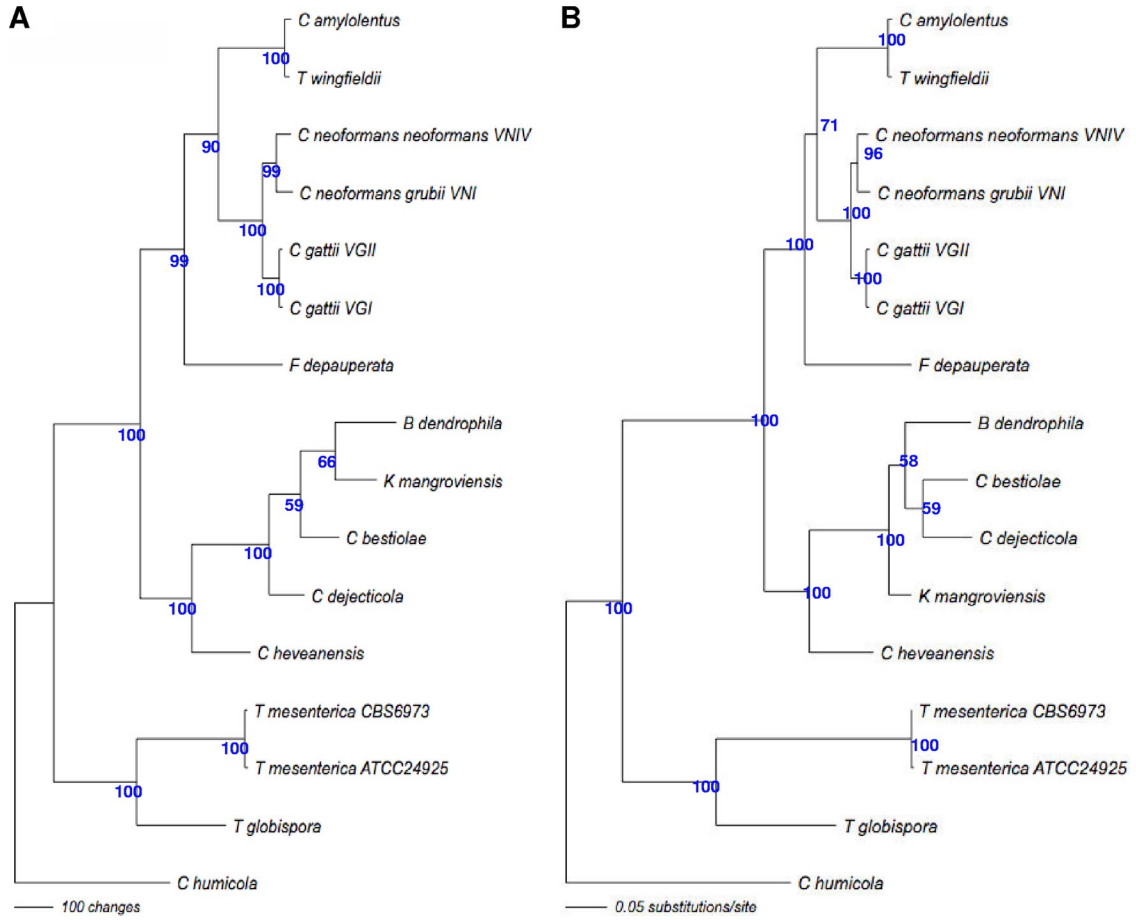

FIG. 1. Phylogenetic relationships among members of the Tremellales. A combined data set of concatenated gene sequences in a parsimony bootstrap phylogenetic tree representing six fungal-specific genomic loci (*RPB1*, *RPB2*, *EF1*α, mitSSU, nucLSU (D1/D2), and ITS) reveals the phylogeny of the *Cryptococcus* pathogenic species complex with the insect-associated species. Single topology for the MP tree is shown in panel A, and an ML tree is shown in panel B. Nucleotide sequences for each gene were aligned and analyzed using Mesquite and PAUP. Numbers on branches are bootstrap values from 1,000 replicates. The outgroup in the analysis is *C. humicola.*

monophyletic *Filobasidiella* clade, which also includes *C. amylolentus*, *T. wingfieldii*, and the pathogenic species *C. neoformans* and *C. gattii.*

The *Kwoniella* lineage includes several arthropod-associated species, *Bullera dendrophila*, *Cryptococcus dejecticola*, *Cryptococcus bestiolae*, and *C. heveanensis* (the *C. heveanensis* strain used in this study was isolated from sheet rubber), and one species isolated from mangrove areas, *Kwoniella mangroviensis* (Table 1 and Fig. 2). Our findings confirm previous studies that suggested the *Kwoniella* clade is split into two monophyletic clusters, one lineage represented by *C. heveanensis* and the other including *B. dendrophila*, *C. dejecticola*, *C. bestiolae*, and *K. mangroviensis* (Fig. 1 and 2).

The three *Tremella* strains used in the analysis represent more distantly related taxa within the Tremellales. Interestingly, the two strains of *Tremella mesenterica* appear to be as divergent from each other as the two *C. neoformans* varieties are and also as diverged as the two *C. gattii* VG groups, suggesting the existence of subpopulations, varieties, or cryptic species within *T. mesenterica* (Fig. 1 and 2). The monophyletic clades that are defined based on this phylogenetic analysis were rooted with *C. humicola*, a member of the Trichosporonales (8, 48, 58).

Phenotypic and morphological differences in close and distant relatives of *C. neoformans*. The virulence potential of the isolates studied was assessed by examining three well-established virulence attributes: growth at 37°C, capsule formation, and melanin production. To assay growth at different temperatures, serial dilutions of individual species were spotted at 24°C, 30°C, and 37°C on both nutrient-rich and minimal media. The three species belonging to the pathogenic *Cryptococcus* species complex, *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii*, all grew at the highest temperature, 37°C (Fig. 3). Additional species clustering in the sensu stricto *Filobasidiella* lineage, *C. amylolentus*, *T. wingfieldii*, and *F. depauperata*, all failed to grow at 37°C. Five of the six species in the sensu stricto *Filobasidiella* lineage were able to grow at 30°C (Fig. 3). The only strain in this group that did not grow at 30°C was *T. wingfieldii*. We speculate that because *T. wingfieldii* is a cryptic or sibling species of *C. amylolentus* (Fig. 1 and 2), the lower thermotolerance of the former might be a distinct trait between the two species; alternatively, this property could

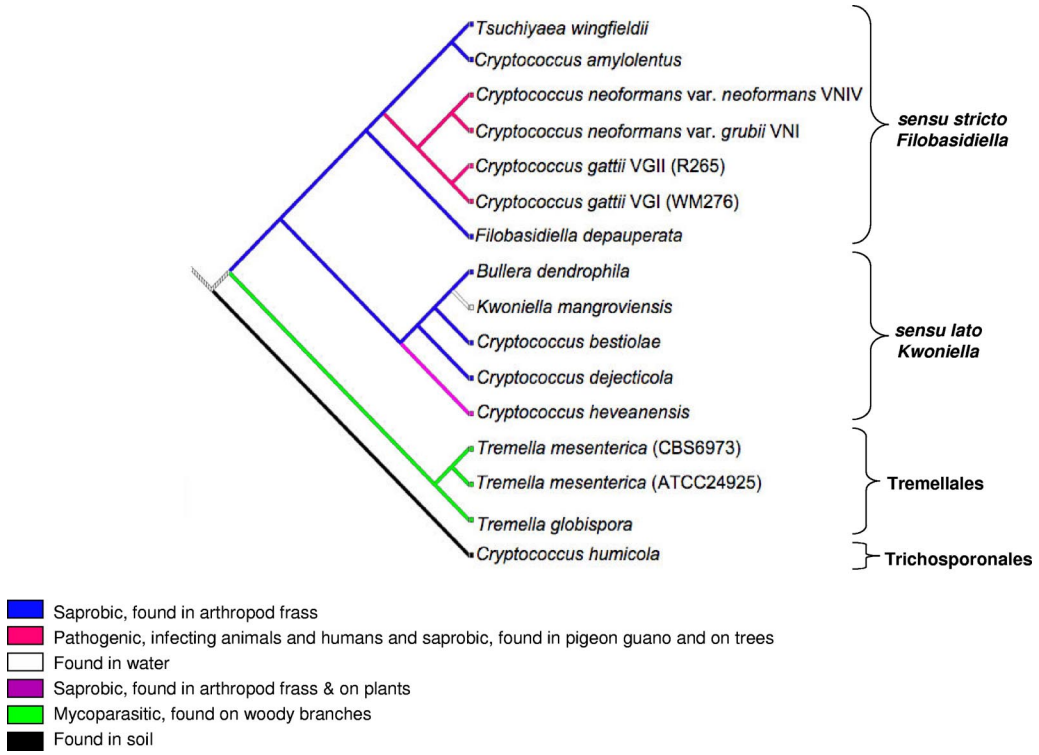

FIG. 2. Phylogram showing the preferred habitats of the fungal species used in this study. The tree diagram is based on data from Fig. 1 and indicates evolutionary lineages and natural habitats of the isolates examined in the study. These species are possibly derived from a common insect-associated ancestor.

vary between isolates of the same species. At present, no other *T. wingfieldii* or *C. amylolentus* isolates are available to explore this point further.

None of the species in the sensu lato *Kwoniella* clade, including *K. mangroviensis*, *B. dendrophila*, *C. bestiolae*, *C. dejecticola*, and *C. heveanensis*, or the species representing the paraphyletic lineages, *Tremella* spp. and *C. humicola*, grew at 37°C (Fig. 3). Only two isolates, *B. dendrophila* and *Tremella globispora*, could not grow at 30°C. A previous report showed that *B. dendrophila* displays little to no growth at 30°C or at 35°C (4). In this case, lack of growth at 30°C seems to be a phenotypic difference that could be of assistance in distinguishing *B. dendrophila* from the rest of the species in the *Kwoniella* lineage (Fig. 3), since the majority of their close relatives are able to grow at 30°C (for a current study, see reference 41). In summary, the saprobic yeasts are distinguished from closely aligned pathogenic yeasts by their inability to grow at 37°C.

To examine capsule production, all strains were grown under conditions (low-iron media) known to induce capsule formation in *C. neoformans* and *C. gattii.* Under the conditions tested, none of the saprobic yeasts produced capsules that could be visualized by India ink exclusion, in contrast to those observed for both *C. neoformans* and *C. gattii* (data not shown). Melanin production was tested using three different melanin-inducing media (L-DOPA, Rose Bengal, and Niger Seed). The species in the pathogenic *Cryptococcus* clade all produced melanin (Fig. 3). Of the remaining species in the study, *C. heveanensis* and *C. humicola* also showed faint coloration that might have been attributable to melanin production (Fig. 3). The ability to hydrolyze urea was also tested with Christensen's agar (40). All 15 species tested hydrolyzed urea, confirming that they are all basidiomycetes. Based on these findings, we hypothesize that the environmental cues triggering capsule production might differ for *C. neoformans* and *C. gattii* compared to that of their saprobic relatives. Furthermore, both melanin production and growth at 37°C are characteristic of human pathogenic species, whereas, other traits, such as the ability to hydrolyze urea, are plesiomorphic.

Virulence potential of *Cryptococcus* and neighboring taxa using *G. mellonella*. To further assess the virulence potential of each isolate in the heterologous host *G. mellonella*, larvae were inoculated with 5×10^5 yeasts and incubated at 24°C for the duration of the experiment. Of the species in the *Filobasidiella* lineage, the two *C. neoformans* varieties and *C. gattii* exhibited the highest virulence potential ($P < 0.0001$, compared to PBS) during infection assays in *G. mellonella* (a sample survival curve is shown in Fig. 3). Of the remaining species in the *Filobasidiella* sensu stricto lineage, *T. wingfieldii* ($P = 0.285$), *F. depauperata* ($P = 0.3458$), and *C. amylolentus* ($P = 1.0$) (Fig. 3) were avirulent in *G. mellonella* compared to the negative control, PBS. Three of the five species clustering in the *Kwoniella* clade, *C. bestiolae* ($P = 0.0128$, PBS; and $P = 0.0160$, compared to strain H99), *B. dendrophila* ($P = 0.0382$, PBS; and $P = 0.0128$, H99), and *C. heveanensis* ($P = 0.0181$, PBS; and $P = 0.0160$, H99) displayed intermediate virulence. The other two species in the *Kwoniella* clade, *C. dejecticola* ($P = 0.1473$, PBS; and $P < 0.0001$, H99) and *K. mangroviensis* ($P = 0.023$, PBS; and $P < 0.0001$, H99) were consistently severely attenuated or

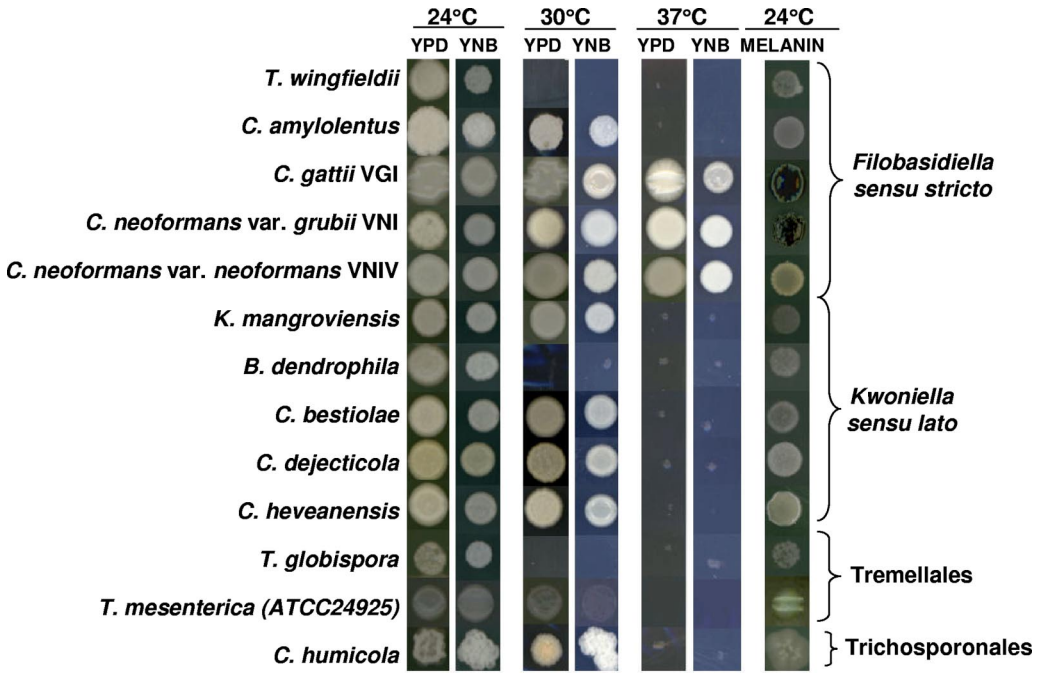

FIG. 3. Phenotype and morphology of species grown under different conditions. Spot assays of individual isolates were grown for 4 to 5 days on different media at 24°C, 30°C, and 37°C. Each isolate was individually tested for the ability to grow on the nutrient-rich medium, YPD, and the minimal medium, YNB. For the melanin assay, strains were grown for 4 to 5 days at 24°C on L-DOPA medium. *F. depauperata* grows more slowly than any of the other isolates tested (approximately 10 days were required to achieve the same colony size shown in Fig. 3) and is therefore not included here.

avirulent compared to the pathogenic H99 control. Furthermore, the *Tremella* outgroup species *T. mesenterica* and *T. globispora* ($P = 1$, PBS; and $P < 0.0001$, H99) were clearly avirulent in this assay. However, the *Trichosporon* outgroup species *C. humicola* displayed an intermediate level of virulence ($P = 0.030$, PBS; and $P = 0.02$, H99). In summary, the pathogenic *Cryptococcus* species displayed the highest virulence in the greater wax moth, whereas the majority of their close relatives were attenuated or avirulent.

DISCUSSION

Previous phylogenetic studies evaluating the phylogeny of the *Filobasidiella* clade used single genomic loci, such as 5.8S, SSU, nucLSU (D1/D2), or the ITS region (8, 12, 45, 48). Results from these analyses were conflicting and failed to accurately resolve the phylogeny of the pathogenic *Cryptococcus* species within the Tremellales. To generate a robust data set to resolve the phylogenetic relationships surrounding this clade, 15 species representing the *Filobasidiella*, *Kwoniella*, and *Tremella* lineages were examined by using a multilocus approach.

The MP and ML trees concordantly defined two monophyletic groups: the sensu stricto *Filobasidiella* and the sensu lato *Kwoniella* lineage, as rooted with *C. humicola* (Fig. 1 and 2). The monophyletic clades defined based on this phylogenetic analysis are analogous to the sensu stricto and the sensu lato groups in Saccharomycetaceae. The sensu stricto species in-

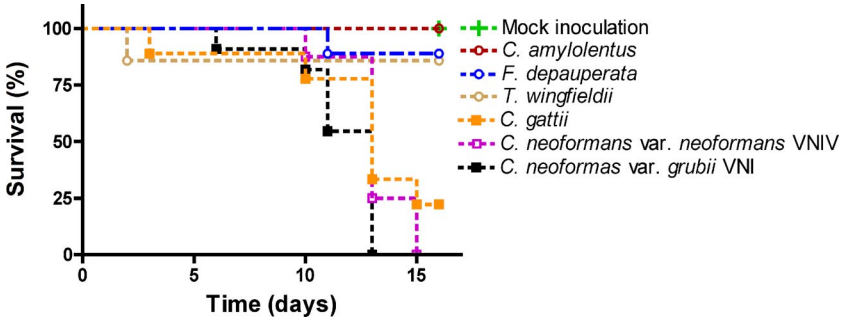

FIG. 4. Infection of the heterologous host *G. mellonella* with species closely related to *C. neoformans.* Survival of *G. mellonella* after inoculation with species in the *Filobasidiella* clade was assayed. At least 12 larvae were injected with 5×10^5 cells for each isolate. After inoculation, larvae were incubated at 24°C and survival was monitored for 17 days postinoculation. The experiment was repeated three times, and results from one representative experiment are presented here. In one of three replicates, *C. amylolentus* exhibited an intermediate virulence level (data not shown). The mock inoculation is injection of a PBS control.

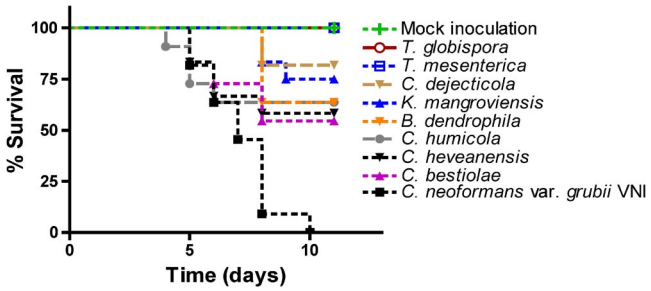

FIG. 5. Infection of the heterologous host *G. mellonella* with species more distantly related to *C. neoformans.* Survival of *G. mellonella* larvae after inoculation with species in the *Kwoniella* clade and outgroup species representing *Trichosporon* and *Tremella* was assayed. At least 12 larvae were injected with 5×10^5 cells of each isolate. After inoculation, larvae were incubated at 24°C, survival was monitored daily, and the experiment was terminated 12 days postinoculation. The experiment was repeated three times, and one representative result is presented here. The mock inoculation is injection of a PBS control.

clude *T. wingfieldii*, *C. amylolentus*, and *F. depauperata*, which form a monophyletic cluster with the pathogenic *Cryptococcus* complex. The previously accepted *Filobasidiella* clade is composed of members with known sexual cycles. Therefore, only *Filobasidiella neoformans* (anamorph *C. neoformans*), *Filobasidiella bacillispora (*anamorph *C. gattii*), and *Filobasidiella depauperata* were included (24, 51). Considerably less attention has focused on *T. wingfieldii* and *C. amylolentus*, although previous molecular evidence suggested they were closely related to the *Filobasidiella* clade (8, 45, 48). The holobasidium of the sexual taxa in the *Filobasidiella* clade (sensu stricto species) is clearly a synapomorphy (shared derived character) for the clade. The phragmobasidium of the remaining taxa in the Tremellales seems to be a simplesiomorphy (ancestral character).

In the sister *Kwoniella* clade, the sensu lato species include *B. dendrophila*, *C. bestiolae*, *C. dejecticola*, *C. heveanensis*, and *K. mangroviensis*. The basidia of the only sexual species in the clade *K. mangroviensis* are similar to those of the remaining members of the Tremellales (54), and we could not identify a synapomorphy for the *Kwoniella* clade. Single-gene phylogenies depicting the relationships between *C. bestiolae*, *C. dejecticola*, and neighboring species, using nucLSU (D1/D2), revealed conflicting views, especially compared to the neighbor-joining analyses of the nucLSU (D1/D2) rRNA gene in *Kwoniella* and closely related species (54, 59). Our multilocus approach confirmed the relatedness among members of the *Filobasidiella* and *Kwoniella* clades as two distinct monophyletic clades, which had been suggested previously based on SSU (57) and ITS (48) sequence analyses. The six genomic loci employed in this study did not completely resolve the species within the *Kwoniella* clade, given the lower bootstrap values (Fig. 1). We attempted to add additional resolution to this clade by including ambiguously aligned regions in the analyses and, although MP (Fig. 1) and neighbor-joining trees (not shown) had identical topologies, results from bootstrapping and the ML topology strongly indicated that these relationships were not well supported. Future studies including additional species, such as recently discovered yeasts that cluster near these fungi or other species in the Tremellales, should increase the phylogenetic resolution of the *Kwoniella* clade. For example, *Cryptococcus cuniculi* (not included in this study), a previously described yeast isolated from rabbit feces in Korea, is phylogenetically related to *C. heveanensis* (50). Additionally, a novel *Cryptococcus* species, *Cryptococcus pinus*, was recently described (11). *C. pinus* was isolated from dead needles of *Pinus sylvestris*, and ribosomal DNA sequence data reveal that *C. pinus* is also a member of the *Kwoniella* clade related to *C. dejecticola* (11). In summary, the robust data set generated in our study provides a platform for future studies exploring the biology, genetics, and genomics of defined species in the Tremellales and those that remain to be discovered.

Unlike many ascomycetous yeasts, which are commonly found in insect communities, basidiomycetes are less frequently isolated from living or dead insects (48). Moreover, these ascomycetes are not limited solely to insect communities but can also be found in association with other habitats like plants and aquatic environments. Although basidiomycetes are rarely associated with insects, most of the sensu stricto and sensu lato species in this study are saprobic yeasts frequently associated with decaying insects and arthropod frass. The data presented here suggest that the fungi in the *Filobasidiella* and *Kwoniella* lineages likely represent a group of phylogenetically related fungi that inhabit similar ecological niches (Fig. 2). Moreover, another possibility is that the successful human pathogenic fungi emerged from an insect-frass-associated ancestor.

Phenotypic assays were performed to identify features shared with or distinct from the extensively studied and well-characterized human pathogen *C. neoformans.* Melanin production and growth at 37°C were observed only in the pathogenic *Cryptococcus* species, whereas growth at 30°C appears to be fairly common in all lineages (Fig. 3). Under the conditions of our tests (low-iron media to induce capsule formation in the pathogenic *Cryptococcus*), visible capsule production was not observed in any of the close or more distant relatives. Previous results have provided evidence that *C. humicola* produces laccase, the enzyme required for melanin production, and a capsule composed of polysaccharides similar to those found in *Cryptococcus* (41). Additional studies have indicated the presence of a capsule in many of the *Tremella* species (9, 52). Like *C. neoformans*, the Trichosporonales species *C. anacardii* (not included in this study) also produces both capsule and melanin. Biochemical studies have reported that *T. mesenterica* produces extracellular polysaccharides, such as those often found in the capsules of other Tremellales (9), as does the pathogenic *Cryptococcus* (41), in which growth on YPD medium was used to assay capsule production. We speculate that these contradictory findings might be the results of different environmental cues triggering capsule production among the different lineages or the sensitivity of the assays used to detect capsule production. Taken together, the results may imply that shared ancestral traits (capsule formation, melanin production, or growth at high temperatures) and also novel traits (or more recently evolved phenotypes) play a role in the pathogenic life styles of the *Cryptococcus* species.

C. neoformans and *C. gattii* displayed the highest virulence in *G. mellonella* (Fig. 4). The species in the *Kwoniella* clade were also able to infect *G. mellonella* larvae, and several exhibited intermediate virulence compared to that of the pathogenic *C. neoformans* and *C. gattii* species (Fig. 5). The outgroup in this

study, *C. humicola*, has been previously isolated from immunocompromised patients (44) and insect frass or the gastrointestinal tracts of insects (55). Although the specific strain used in this study was isolated from soil (Table 1), it also displayed intermediate virulence in *G. mellonella*. All of the *Tremella* isolates (*T. globispora* and the two *T. mesenterica* strains) were found to be avirulent in *G. mellonella* (Fig. 4). Thus, we hypothesize that ancestral characteristics found in both the *Kwoniella* and the *Filobasidiella* lineages may play a role in the pathogenic potential (Fig. 3) of the pathogenic *Cryptococcus* species.

Moreover, several traits associated with *C. neoformans* pathogenic potential, for example, virulence in *G. mellonella* and melanin production, appear to be present in other closely and distantly related species. Therefore, these shared traits might be examples of plesiomorphic, i.e., ancestral, traits retained in these species due to their selection by the environment. Alternatively, some of these characteristics and their distribution, such as the faint melanin-like pigment produced by *C. humicola*, could be the product of convergent evolution. Current advances, such as the *T. mesenterica* genome recently sequenced by the Joint Genome Institute at the U.S. Department of Energy (isolate ATCC 24925), in addition to the sequenced genomes available for several strains of *C. neoformans* and *C. gattii* (30), set the stage for future comparative genomic analyses to differentiate between these divergent evolutionary trajectories.

Within the Saccharomycotina, the human-associated and sometimes pathogenic *Candida* species are clustered within lineages of endosymbiotic and commensal *Candida* species found in the gastrointestinal tracts of insects (55). In a complementary study, four novel anamorphic yeast species were isolated from the gastrointestinal tracts of flower-visiting beetles in China (17). These novel insect-associated *Candida* species closely cluster with the human fungal pathogen *Candida albicans*/*Lodderomyces elongisporus* clade (17). The emergence of human pathogenic *Cryptococcus* species from an ancestral lineage of mycoparasitic and insect frass-associated fungi might be similar to these previously illustrated examples in the ascomycetes. Consequently, strong evolutionary selection might drive the emergence of successful human pathogens from ancestral yeasts associated with insects and/or other insect habitats or selection for isolates that survive at higher temperatures.

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