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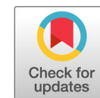

Rapid Identification of Clinically Relevant Members of the Genus *Exophiala* by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry and Description of Two Novel Species, *Exophiala campbellii* and *Exophiala lavatrina*

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ABSTRACT *Exophiala* is a ubiquitous pleomorphic genus comprising at least 40 species, many of which have been associated with superficial, visceral, or systemic infections in humans, other mammals, or cold-blooded animals. In this study, we investigated the potential of matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) for the identification of *Exophiala* species. A total of 89 isolates (including 50 human and 4 animal clinical isolates) stored in the National Collection of Pathogenic Fungi were identified by PCR amplification and sequencing of internal transcribed spacer region 1. Eighty-three of the isolates corresponded to 16 known species within *Exophiala/Rhinocladiella*. The remaining six isolates are shown by phylogenetic analyses based on four loci to represent two novel *Exophiala* species. Four isolates from domestic bathrooms which form a sister species with *Exophiala lecanii-corni* are described here as *Exophiala lavatrina* sp. nov. The remaining two isolates, both from subcutaneous infections, are distantly related to *Exophiala oligosperma* and are described here as *Exophiala campbellii* sp. nov. The triazoles and terbinafine exhibited low MICs against all *Exophiala* isolates *in vitro*. MALDI-TOF MS successfully distinguished all 18 species and identified all isolates after appropriate reference spectra were created and added to commercial databases. Intraspecific mean log scores ranged from 1.786 to 2.584 and were consistently significantly higher than interspecific scores (1.193 to 1.624), with the exception of *E. lecanii-corni* and *E. lavatrina*, for which there was considerable log score overlap. In summary, MALDI-TOF MS allows the rapid and accurate identification of a wide range of clinically relevant *Exophiala* species.

KEYWORDS *Exophiala* species, MALDI-TOF, mass spectrometry, multigene phylogeny, DNA sequencing, antifungal susceptibility, *Exophiala campbellii*, *Exophiala lavatrina*

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Although principally saprobic with worldwide distribution, members of the genus *Exophiala* (Herpotrichiellaceae) are also frequently found in association with petrochemical-polluted materials (1) and hot humid man-made environments (2). Moreover, *Exophiala* contains numerous potential pathogens of warm-blooded (3) and cold-blooded animals (4), with the ability to cause infections in humans being dependent at least in part on the ability to grow at 37 to 40°C. Disseminated fatal human

infections with *Exophiala dermatitidis*, *Exophiala spinifera*, and *Exophiala asiatica* have been reported in immunocompetent and immunocompromised hosts (5–8), and *E. dermatitidis*, *Exophiala lecanii-corni*, *Exophiala oligosperma*, *Exophiala phaeomuriformis*, and *Exophiala xenobiotica* have been identified as the species most commonly associated with systemic infections in general (9, 10). Moreover, *E. dermatitidis* is a recognized agent of neurotropic infections in humans (3, 7). Numerous additional species have also been implicated in cutaneous and subcutaneous infections ranging from solitary subdermal cysts to widespread chromoblastomycosis and eumycetoma (10, 11). Additionally, *E. dermatitidis* has been shown to be one of the principal fungal colonizers of the airways of patients with cystic fibrosis (12–14). An equally wide variety of thermointolerant species, principally encountered in aquatic environments, have been associated with disseminated, visceral, and cutaneous infections in cold-blooded animals, including fish and amphibians (summarized in reference 4).

All *Exophiala* species share common anamorphic features in culture, including melanized, often toruloid hyphae, and annellidic conidiophores that produce conidia in wet masses, as well as in budding (yeast) cells. However, accurate phenotypic identification is hindered by the limited degree of morphological differentiation between different species and their pleomorphic nature. A multitude of recent molecular studies, principally based upon rRNA gene and internal transcribed spacer region 1 (ITS1) sequencing, have begun to elucidate the phylogenetic complexity of the genus and to catalogue the range of species included (4, 8–11, 15–17), with in excess of 40 species described to date. Molecular approaches have also better delineated the spectrum of clinically relevant *Exophiala* species (9, 10), with *E. dermatitidis*, *E. xenobiotica*, *E. lecanii-corni*, *E. oligosperma*, *E. phaeomuriformis*, and to a lesser extent, *E. bergeri* and *E. jeanselmei*, predominating in clinical specimens. The accurate and rapid identification of *Exophiala* species is increasingly important, given the observation that certain species are consistently associated with different infection types (8, 9) and the modest species-specific differences in antifungal susceptibility profiles reported in several studies of well-characterized *Exophiala* isolates (9, 17, 18).

Matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) has shown considerable promise as a rapid and robust method of identifying pathogenic yeast isolates (19, 20) and many species of pathogenic filamentous fungi (21–24), although identification success is highly dependent on robust and expansive databases. Indeed, MALDI-TOF MS has been successfully applied to the identification of *Exophiala* species in several pilot studies. However, both previous studies only employed very restricted numbers of different *Exophiala* species, with only one and four species included in the respective test panels (25, 26), and achieved relatively modest identification success, as judged by mean log scores of $>$2.0 (26). Here, we have performed molecular characterization of 89 isolates of *Exophiala* species stored in the UK National Collection of Pathogenic Fungi (NCPF) and successfully applied them to MALDI-TOF MS for identification. The isolates comprised 15 accepted *Exophiala* species and two novel taxa, which we describe here as *Exophiala campbellii* sp. nov. and *Exophiala lavatrina* sp. nov.

RESULTS

Phylogenetic analyses. Initial identification of the 89 isolates included in the current study was performed by PCR amplification and sequencing of a portion of ITS1, combined with BLASTN searches of the resulting sequences against sequences present in GenBank to identify nearest neighbor reference sequences (Table 1 and Fig. 1A). Definitive identity was confirmed by phylogenetic analyses of the ITS1 locus performed using the maximum likelihood (ML) algorithm, as described in Materials and Methods; an isolate was considered to be the same species as its nearest neighbor if the ITS sequence similarity exceeded 99% and isolates colocated on the same branch of the phylogenetic tree. The resulting phylogenetic tree was broadly consistent with similar recent studies of a variety of *Exophiala* species (see, for example, de Hoog et al. [4]). Using this approach, 83 of 89 isolates were unambiguously identified and comprised

TABLE 1 Isolates employed in the current study

Culture no. by organism	Isolation information[a]			GenBank accession no.			
	Source	Origin	Yr	ITS1	RPB2	TEF1	28S
Exophiala aquamarina							
NCPF 7889	Leafy sea dragon	UK	2015	LT594705			
NCPF 7908	Clown fish	UK	2016				
Exophiala begeri							
NCPF 2236	Diseased eye	UK	1979	LT594666			
NCPF 2353	Subepidermal cyst	USA	1976	LT594667			
NCPF 2395	Unknown	USA	1965	LT594668			
NCPF 2438	Chromoblastomycosis	Canada	1976	LT594669			
NCPF 2513	Unknown	Japan	Unknown	LT594670			
Exophiala campbellii sp. nov.							
NCPF 2274	Foot ganglion	Germany	1981	LT594703	LT594740	LT594739	LT594760
NCPF 2593	Human chest nodule	UK	1984	LT594704	LT594742	LT594741	LT594761
Exophiala cancerae							
NCPF 2404	Unknown	Canada	1980	LT594701			
NCPF 2558	Human, sputum	USA	1981	LT594702			
NCPF 7907	Human, finger	UK	2016				
Exophiala capensis							
NCPF 2517	Cedar tower	Canada	1973	LT594700			
Exophiala dermatitidis							
NCPF 2343	Chromoblastomycosis	Japan	1977	LT594638			
NCPF 2349	Wood	Unknown	1976	LT594639			
NCPF 2354	Knee fluid	USA	1977	LT594640			
NCPF 2355	Unknown	Japan	1966	LT594641	LT594752	LT594751	LT594766
NCPF 2356	Chromoblastomycosis	Taiwan	Unknown	LT594642			
NCPF 2357	Lung	Japan	1977	LT594643			
NCPF 2362	Unknown	Unknown	1979	LT594644			
NCPF 2369	Human	USA	1981	LT594645			
NCPF 2370	Human	USA	1981	LT594646			
NCPF 2384	Knee fluid	USA	1977	LT594647			
NCPF 2396	CNS fluid	Japan	1963	LT594648			
NCPF 2400	Human	Japan	1976	LT594649			
NCPF 2402	Internal organs, bat	USA	1979	LT594650			
NCPF 2403	Unknown	USA	1979	LT594651			
NCPF 2405	Soil	Brazil	1980	LT594652			
NCPF 2414	Unknown	USA	1979	LT594653			
NCPF 2415	Unknown	USA	1979	LT594654			
NCPF 2416	Unknown	USA	1973	LT594655			
NCPF 2418	Chromoblastomycosis	Japan	1972	LT594656			
NCPF 2420	Unknown	USA	1977	LT594657			
NCPF 2422[T]	Chromomycosis, face	Japan	1935	LT594658			
NCPF 2448	Subcutaneous, finger	USA	1981	LT594659			
NCPF 2450	Unknown	Unknown	1979	LT594660			
NCPF 2462	Unknown	Japan	1966	LT594661			
NCPF 2464	Unknown	Venezuela	1964	LT594662			
NCPF 2518	Human	USA	Unknown	LT594663			
NCPF 2519	Human	USA	Unknown	LT594664			
NCPF 2611	Human	Hong Kong	1985	LT594665			
NCPF 7906	Human, BAL	UK	2015				
Exophiala equina							
NCPF 2376	Sputum	USA	Unknown	LT594671			
NCPF 2489C	Paper mill	USA	1959	LT594672	LT594738	LT594737	LT594759
NCPF 2493	Skin scraping	USA	Unknown	LT594673			
NCPF 7890	Human eye	UK	2016	LT594674			
Exophiala heteromorpha							
NCPF 2380	Human	USA	1981	LT594675			
Exophiala jeanselmei							
NCPF 2516	Unknown	USA	1973	LT594689			

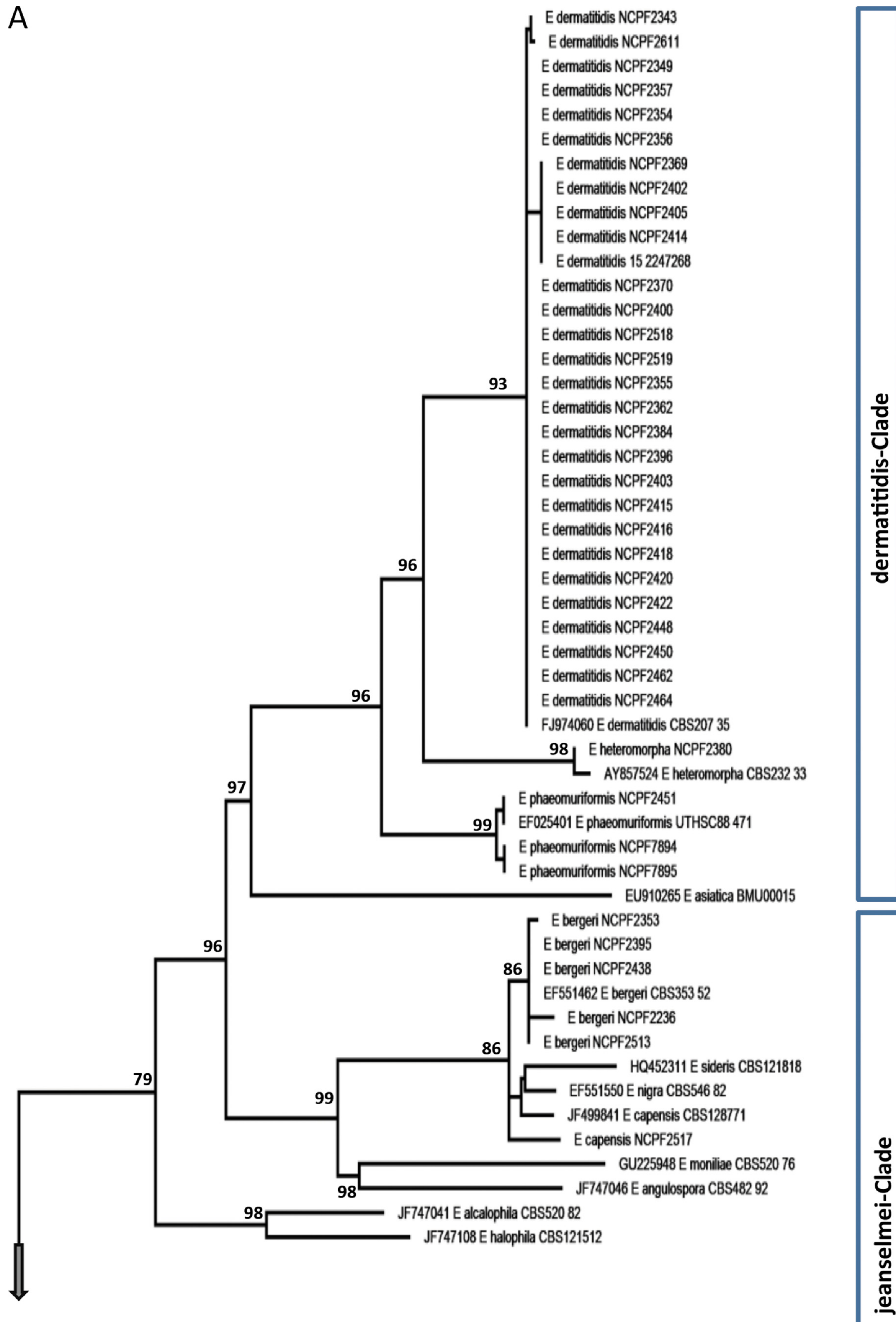
(Continued on next page)

TABLE 1 (Continued)

Culture no. by organism	Isolation information[a]			GenBank accession no.			
	Source	Origin	Yr	ITS1	RPB2	TEF1	28S
Exophiala lavatrina sp. nov.							
NCPF 7893	Domestic bathroom	UK	2016	LT594696	LT594730	LT594729	LT594755
NCPF 7898	Domestic bathroom	UK	2016	LT594697	LT594732	LT594731	LT594756
NCPF 7899	Domestic bathroom	UK	2016	LT594698	LT594734	LT594733	LT594757
NCPF 7900	Domestic bathroom	UK	2016	LT594699	LT594736	LT594735	LT594758
Exophiala lecanii-corni							
NCPF 7891	Human, nail	UK	2015	LT594692	LT594726	LT594725	LT594753
NCPF 7901	Domestic bathroom	UK	2016	LT594693			
NCPF 7902	Domestic bathroom	UK	2016	LT594694			
NCPF 7903	Domestic bathroom	UK	2016	LT594695	LT594728	LT594727	LT594754
Exophiala mesophila							
NCPF 7892	Contact lens	UK	2015	LT594690			
Exophiala oligosperma							
NCPF 2352	Polluted water	USA	1957	LT594676	LT594748	LT594747	LT594764
NCPF 2378	Ear swab	Canada	1980	LT594677			
NCPF 2379	Unknown	Brazil	1978	LT594678			
NCPF 2382	Unknown	USA	1977	LT594679			
NCPF 2383	Thigh abscess	USA	1977	LT594680	LT594750	LT594749	LT594765
NCPF 2436	Wood	UK	Unknown	LT594681			
NCPF 2466	Unknown	Venezuela	Unknown	LT594682			
NCPF 2469	Sputum	USA	1983	LT594683			
NCPF 2490	Human	USA	1977	LT594684			
NCPF 2496	Human	USA	1980	LT594685			
NCPF 2559	Human lung	USA	1981	LT594686			
NCPF 2725	Human	UK	1989	LT594687			
NCPF 7896	Human skin	UK	2015	LT594688			
Exophiala phaeomuriformis							
NCPF 2451	Unknown	USA	1977	LT594706			
NCPF 7894	Blood culture, endocarditis	UK	2015	LT594707			
NCPF 7895[b]	Blood culture, endocarditis	UK	2016	LT594708			
Exophiala pisciphila							
NCPF 2645	Liver, crocodile	Israel	1986	LT594691			
Exophiala spinifera							
NCPF 2358	Nose lesion	USA	1966	LT594709			
NCPF 2454	Palm tree	Uruguay	1978	LT594710			
NCPF 2494	Human	USA	Unknown	LT594711			
Exophiala xenobiotica							
NCPF 2372	Elbow pus	USA	1981	LT594712			
NCPF 2375	Arm ulcer	USA	1979	LT594713	LT594744	LT594743	LT594762
NCPF 2377	Phaeomycotic cyst	USA	1980	LT594714			
NCPF 2455	Bronchial wash, human	USA	1981	LT594715	LT594746	LT594745	LT594763
NCPF 2471	Lesions, abdomen and thigh	USA	1981	LT594716			
NCPF 2472	Wood	Sweden	1977	LT594717			
NCPF 2487	Chromoblastomycosis, back	Japan	1976	LT594718			
NCPF 2555	Human	USA	1977	LT594719			
NCPF 2556	Human hand	New Zealand	1982	LT594720			
Rhinocladiella similis							
NCPF 7897	Human abdomen	UK	2015	LT594721			
NCPF 7904	Human	UK	2015	LT594722			
NCPF 7905	Gastroscope	UK	2016				

[a]CNS, central nervous system; BAL, bronchoalveolar lavage.
[b]This isolate was from the same patient as NCPF 7894.

E. dermatitidis (n = 29), *E. oligosperma* (n = 13), *E. xenobiotica* (n = 9), *E. bergeri* (n = 5), *Exophiala equina* (n = 4), *E. lecanii-corni* (n = 4), *E. phaeomuriformis* (n = 3), *E. spinifera* (n = 3), *Rhinocladiella similis* (n = 3), *Exophiala cancerae* (n = 3), *Exophiala aquamarina* (n = 2), *Exophiala capensis*, *Exophiala heteromorpha*, *E. jeanselmei*, *Exo-*



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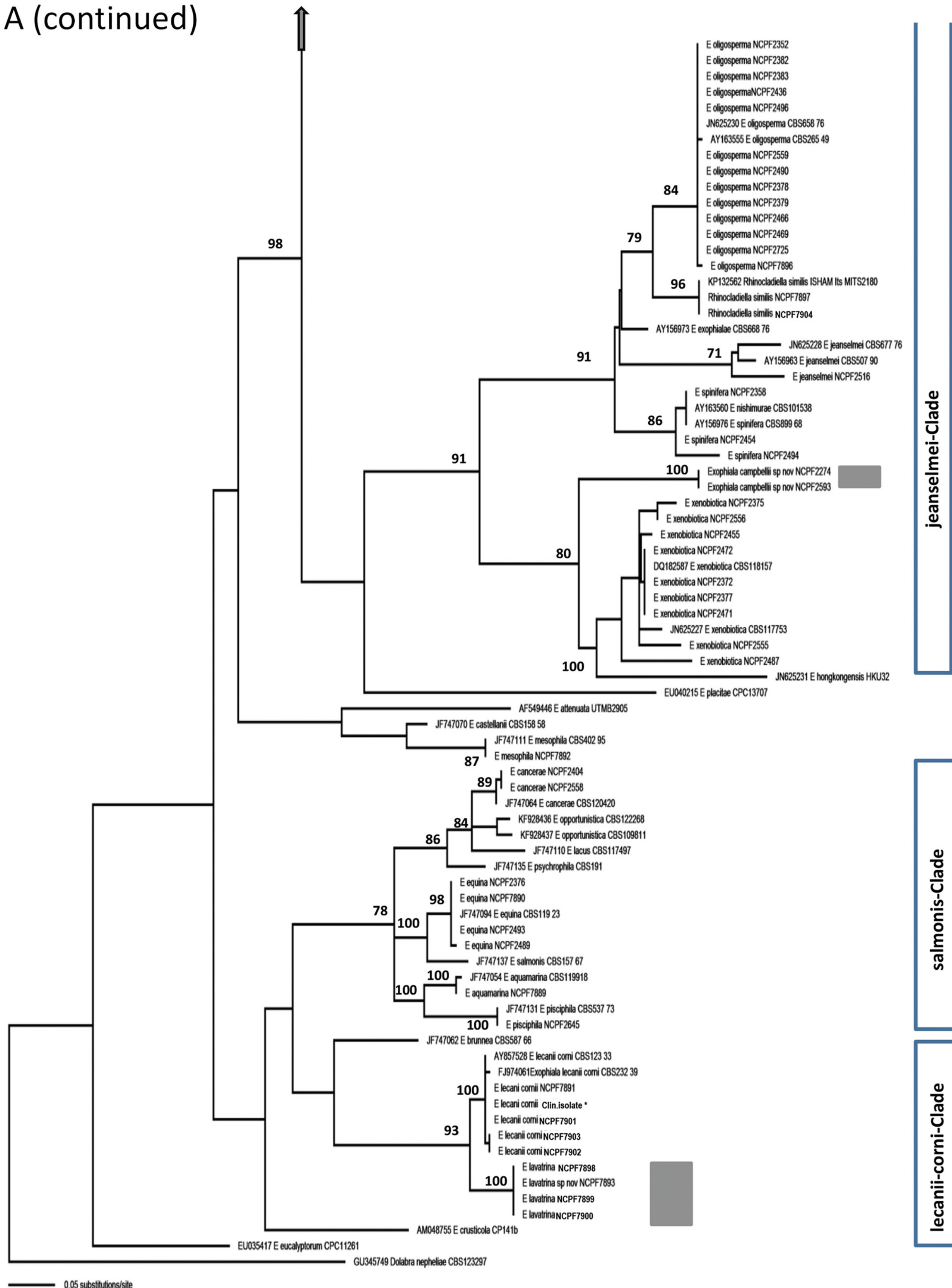

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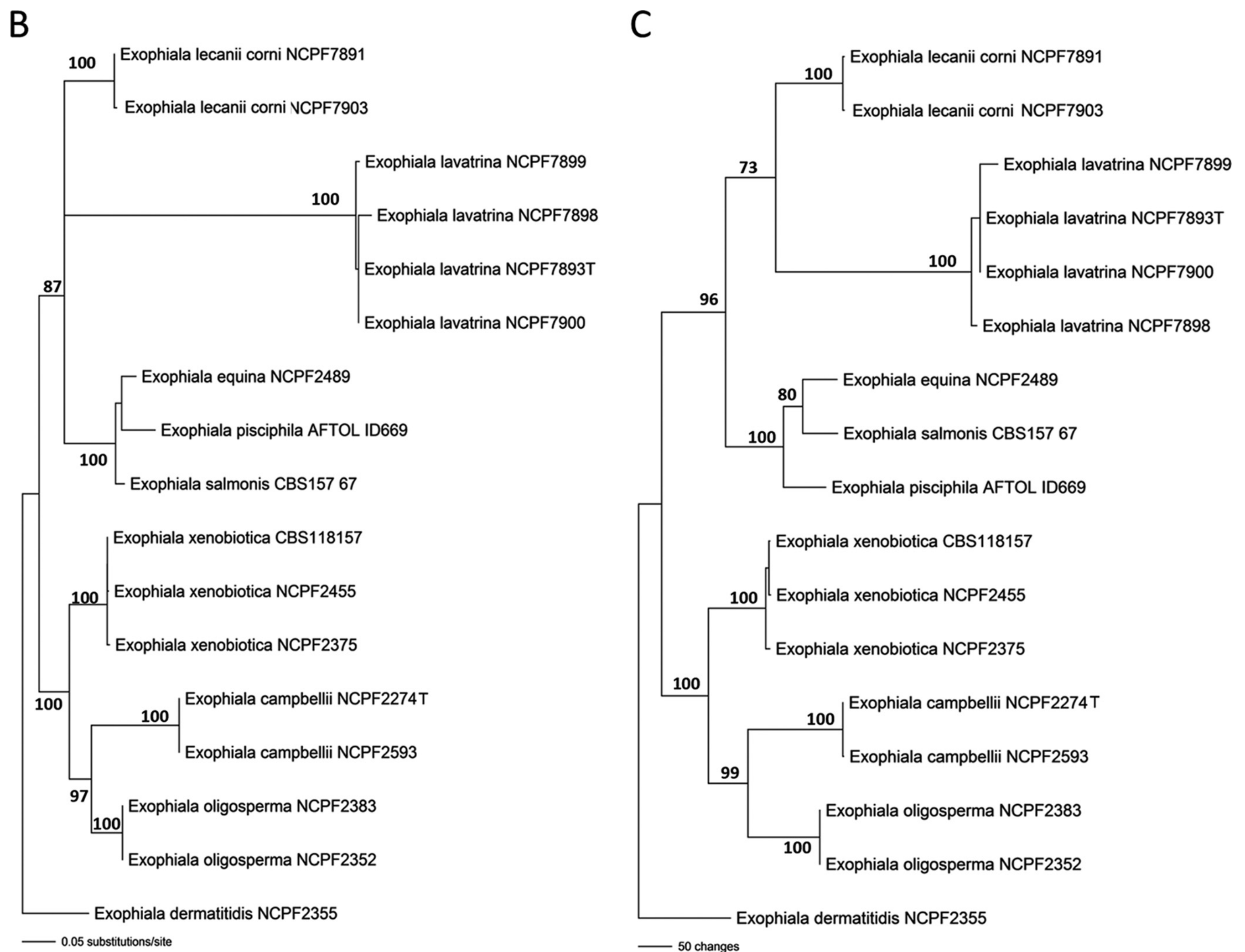

FIG 1 Phylogenetic trees showing the relationships of the 86 isolates included in the current study. (A) The 50% majority rule consensus tree inferred from ITS1 sequence data using the maximum likelihood method and K80+G model. Numbers at nodes represent levels of bootstrap support calculated from 200 replicates. The tree was rooted with *Dolabra nepheliae*. The scale bar represents 0.05 substitutions per site. Accession numbers of comparator sequences retrieved from GenBank are given before non-NCPF isolates. Clin. isolate* represents a clinical isolate of *E. lecanii-corni* that is no longer available for subsequent analyses. (B) Maximum likelihood tree constructed with concatenated partial sequences from the 28S rRNA, ITS1, *TEF1*, and *RPB2* genes of *E. lavatrina* sp. nov., *E. campbellii* sp. nov., and nearest relatives (2,228 nucleotides in total). Branch lengths are proportional to phylogenetic distance. Bootstrap values (using nearest neighbor interchange) above 70% (1,000 replicates) are shown above branches. Scale bar = 0.05 substitutions/site. (C) Most parsimonious tree of the concatenated sequences analyzed in panel B (2,228 characters; tree length [TL] = 1,570, consistency index [CI] = 0.698, retention index [RI] = 0.838, rescaled consistency [RC] = 0.585, homoplasy index [HI] = 0.302). Branch lengths are proportional to phylogenetic distance. Bootstrap values (using tree-bisection-reconnection) above 70% (1,000 replicates) are shown above branches. The scale bar shows 50 changes. (B and C) Trees were rooted with *Exophiala dermatitidis*.

phiala mesophila, and *Exophiala pisciphila* (all $n = 1$). The remaining 6 isolates formed two well-supported clades in the ITS tree, as sister taxa to *E. lecanii-corni* (*E. lavatrina* sp. nov.; $n = 4$) and *E. oligosperma* (*E. campbellii* sp. nov.; $n = 2$).

To further characterize these 6 novel isolates, PCR amplification and sequencing were extended to include portions of the RNA polymerase second largest subunit (*RPB2*) and translation elongation factor 1 alpha (*TEF1*) genes, and together with the long subunit (LSU) and ITS1, these additional loci were used for phylogenetic comparisons with reference NCPF *Exophiala* species that represented their nearest neighbors. A total of 2,217 nucleotides were included, of which 352 nucleotides were derived from LSU, 247 nucleotides were derived from ITS1, 730 nucleotides were derived from *RPB2*, and 888 nucleotides were derived from *TEF1*. Maximum likelihood phylogenetic trees were established for each of the 4 individual loci and inspected for congruence using a 70% bootstrap cutoff. As individual trees were broadly concordant (data not shown),

TABLE 2 MALDI-TOF MS log scores for *Exophiala* species determined in the current study

Species (no. tested)	No. of isolates with Bruker log score of: >1.7	>1.8	>1.9	>2.0	MALDI-TOF log score[a] Mean	Range	Next highest match ID	Maximum log score[b]
Exophiala dermatitidis (17)	17	17	17	15	2.111	1.991–2.363	No unrelated match with score >1.200	
Exophiala oligosperma (13)	13	13	12	10	2.266	1.865–2.584	*R. similis*	1.624
Exophiala xenobiotica (5)	5	5	5	3	2.050	1.929–2.130	*Lactobacillus* sp.	1.485
Exophiala begeri (5)	5	5	5	5	2.216	2.062–2.317	*E. oligosperma*	1.442
Exophiala equina (4)	4	3	3	2	2.002	1.719–2.246	*Pichia fermentans*	1.370
Exophiala lavatrina sp. nov. (4)	4	4	4	3	2.170	1.976–2.379	*E. lecanii-corni*	1.527
Exophiala lecanii-corni (4)	4	4	4	3	2.121	1.964–2.265	*E. lavatrina*	1.917
Exophiala phaeomuriformis (3)	3	3	2	2	2.031	1.880–2.214	*Ralstonia* sp.	1.327
Rhinocladiella similis (3)	3	2	2	2	2.119	1.789–2.313	*E. spinifera*	1.412
Exophiala cancerae (3)	3	3	3	3	2.345	2.002–2.654	*Arthrobacter* sp.	1.519
Exophiala aquamarina (2)	2	2	2	2	2.281	ND	*Ralstonia* sp.	1.333
Exophiala spinifera (2)	2	2	2	2	1.977	1.830–2.124	*Actinomyces* sp.	1.435
Exophiala campbellii sp. nov. (2)	2	2	2	1	2.103	1.908–2.299	*Bacillus* sp.	1.354
Total (%)	67 (100)	67 (100)	65 (97.0)	62 (92.5)	51 (76.1)			
Exophiala capensis (1)	NA	NA	NA	NA	NA	NA	*Jonesia* sp.	1.450
Exophiala heteromorpha (1)	NA	NA	NA	NA	NA	NA	*Pichia anomalus*	1.193
Exophiala jeanselmei (1)	NA	NA	NA	NA	NA	NA	*E. oligosperma*	1.514
Exophiala mesophila (1)	NA	NA	NA	NA	NA	NA	*Arthrobacter* sp.	1.332
Exophiala pisciphila (1)	NA	NA	NA	NA	NA	NA	*E. aquamarina*	1.713

[a]Excludes scores obtained against MSPs generated with the same isolate.
[b]Maximum log score of the nearest incorrect ID by MALDI-TOF MS.

the combined 4-locus data set was used for subsequent analyses using both ML and maximum parsimony algorithms (see Materials and Methods; Fig. 1B and C). The resulting phylogenetic trees had very similar topologies, and both strongly supported, with high bootstrap values, the status of both *E. campbellii* and *E. lavatrina* as novel *Exophiala* species that are related to *E. oligosperma* and *E. lecanii-corni*, respectively. For example, *E. lavatrina* and *E. lecanii-corni* shared only 81% nucleotide identity over the ~2,100-bp regions examined, and pairwise nucleotide identity for the same regions between *E. oligosperma*, *E. xenobiotica*, and *E. campbellii* was 87% (compared to 91% identity between *E. equina* and *E. pisciphila*, and 93% sequence conservation between *E. dermatitidis* and *E. heteromorpha*; data not shown).

MS analyses of *Exophiala* isolates. Since it is well established that the performance of MALDI-TOF MS is highly dependent on spectral database inclusivity (20, 22), representative NCPF isolates corresponding to the 18 species identified by ITS1 sequencing were used for the creation of reference mass spectra (MSPs), exactly as described in Materials and Methods. This in-house database was then used in conjunction with the existing Bruker database for the identification of the remaining isolates, which were processed in an operator-blinded manner using the same extraction methodologies. All species with more than 1 representative isolate gave species-specific spectra which allowed their identification to the species level, and concordance of species-level identification was 100% by MALDI-TOF MS compared to sequencing. With the exception of one isolate each of *E. equina* and *R. similis* (log scores, 1.719 and 1.789, respectively), 65/67 isolates gave log scores above 1.8, with 92.5% (62/67) of the isolates giving scores in excess of 1.9 and 76.1% (51/67) of isolates achieving scores greater than 2.0 (Table 2). For the five species represented by only a single isolate (*E. aquamarina*, *E. capensis*, *E. jeanselmei*, *E. mesophila*, and *E. pisciphila*), the lack of a second independent test isolate precluded evaluation of the robustness of the reference MSPs generated in the current study. However, the log scores of the nearest matches in the existing databases were consistently very low (range, 1.193 to 1.713) and were achieved against unrelated or only distantly related organisms, suggesting that these MSPs might also be reliable and unique (Table 2). Indeed, MSPs were robust for all 16/18 species, as judged by the differences between intraspecific and interspe-

TABLE 3 Geometric mean MIC/MEC values and ranges for *Exophiala* species and antimicrobials

Organism (no. of isolates)	MIC/MEC geometric mean (range) (mg/liter) for[a]:						
	AMB	ITC	VRC	PSC	ANID	TRB	NAT
All (50)	0.60 (0.06 to 4)	0.11 (<0.03 to 0.5)	0.18 (<0.03 to 1)	0.06 (<0.03 to 0.25)	6.28 (0.25 to >8)	0.19 (0.06 to 1)	2.69 (1 to 16)
E. dermatitidis (9)	0.54 (0.125 to 1)	0.23 (0.06 to 0.5)	0.13 (0.06 to 0.25)	0.15 (0.06 to 0.25)	>8 (8 to >8)	0.15 (0.06 to 0.25)	2.94 (2 to 4)
E. oligosperma (11)	0.88 (0.25 to 4)	0.09 (0.03 to 0.25)	0.22 (0.06 to 0.5)	0.06 (0.03 to 0.125)	6.62 (0.5 to >8)	0.22 (0.125 to 0.5)	3.52 (2 to 16)
E. xenobiotica (9)	0.79 (0.5 to 1)	0.13 (0.06 to 0.5)	0.21 (<0.03 to 1)	0.07 (0.06 to 0.125)	4.32 (0.25 to >8)	0.10 (0.06 to 0.25)	3.42 (1 to 8)
E. begeri (3)	0.25 (0.25)	0.05 (<0.03 to 0.125)	0.20 (0.125 to 0.25)	0.02 (<0.03 to 0.06)	1.26 (0.5 to 4)	0.25 (0.25)	2 (2)
E. lecanii-corni (2)	0.5 (0.5)	0.125 (0.125)	0.125 (0.125)	0.125 (0.125)	0.5 (0.5)	ND	1 (1)
E. phaeomuriformis (3)	0.16 (0.06 to 0.25)	0.02 (<0.03 to 0.06)	<0.03 (<0.03)	0.02 (<0.03 to 0.06)	2 (2)	1 (1)	1 (1)
E. spinifera (3)	0.39 (0.25 to 1)	0.12 (0.06 to 0.25)	0.25 (0.125 to 0.5)	0.04 (<0.03 to 0.06)	>8 (>8)	0.5 (0.25 to 1)	2 (2)
E. campbellii (2)	1 (1)	0.06 (0.06)	<0.03 (<0.03)	<0.03 (<0.03)	>8 (8 to >8)	0.06 (0.06)	1 (1)
E. equina (2)	0.17 (0.06 to 0.5)	0.18 (0.125 to 0.25)	0.71 (0.5 to 1)	0.12 (0.06 to 0.25)	ND	ND	ND
Rhinocladiella similis (1)	4 (NA)	0.5 (NA)	1 (NA)	0.25 (NA)	>8 (NA)	0.5 (NA)	4 (NA)
E. aquamarina (1)	4 (NA)	0.25 (NA)	0.125 (NA)	ND	ND	0.125 (NA)	ND
E. capensis (1)	0.125 (NA)	<0.03 (NA)	ND	ND	ND	0.25 (NA)	1 (NA)
E. heteromorpha (1)	0.25 (NA)	0.25 (NA)	0.125 (NA)	<0.03 (NA)	4 (NA)	0.5 (NA)	2 (NA)
E. jeanselmei (1)	1 (NA)	0.125 (NA)	0.25 (NA)	0.06 (NA)	>8 (NA)	ND (NA)	2 (NA)
E. mesophila (1)	1 (NA)	0.125 (NA)	0.5 (NA)	ND	ND	0.5 (NA)	1 (NA)

[a]AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; PSC, posaconazole; ANID, anidulafungin; TRB, terbinafine; NAT, natamycin; ND, not determined; NA, not applicable.

cific log score values (Table 2) and the fact that the nearest incorrect matches belonged to different genera for 11/18 species, and even different kingdoms for 8/18 species. An exception to this concerned *E. lecanii-corni* and its related novel sister species *E. lavatrina*, where interspecific log scores nearly approached intraspecific scores for this particular pair of organisms (Table 2).

Antifungal susceptibility. Limited previous studies have suggested that *Exophiala* spp. might exhibit certain species-specific antifungal susceptibility variations, in particular with respect to amphotericin B (17, 18, 27). Thus, a selection of 50 isolates in the current study were subjected to antifungal susceptibility testing against a panel of antifungal agents covering most drug classes. Data for *E. lavatrina* are omitted, since all four isolates of this organism grew extremely poorly in the RPMI medium employed for antifungal susceptibility testing. Since a proportion of strains had been isolated from superficial sites, including skin and eyes, natamycin and terbinafine were also included in the antifungal panel. The vast majority of isolates exhibited low MICs when tested with amphotericin B, the three triazoles tested, and terbinafine, with natamycin and especially anidulafungin yielding higher MICs. Species-specific variation in antifungal susceptibility was minimal among the 15 species included (Table 3), although the single isolates of *E. aquamarina* and *R. similis* exhibited significantly higher MICs to amphotericin, as did one of nine isolates of *E. oligosperma*.

TAXONOMY

Exophiala campbellii sp. nov. Andrew M. Borman, Mark Fraser, Adrien Szekely, Elizabeth M. Johnson. MycoBank accession no. MB 817129. Etymology: named after the prominent British mycologist Colin K. Campbell, previous long-time curator of the National Collection of Pathogenic Fungi, Bristol, UK. Diagnosis: the species resembles *E. oligosperma*, although colonies on most media are mainly mycelial, with a limited yeast phase, and toruloid mycelial are seen rarely (Fig. 2). Colonies on Sabouraud agar at 25°C restricted, attaining 15 to 18 mm diameter in 14 days, peripherally flat with slightly raised and radially folded center, velvety olivaceous-gray to brown with colony reverse dark greenish-gray. No diffusible pigment produced. Budding cells scant in young cultures, when present oval to ellipsoidal, light olivaceous-green, 2.5 by 3.5 μm, often with a short annellated zone. Torulose mycelium is scant. Hyphae are regular, pale olivaceous to brown, 1.5 to 3.0 μm wide, with dense septation. Conidiogenous cells arising either terminally, at acute angles in simple penicillia as part of a slightly differentiated conidial apparatus, or more commonly at right angles from undifferentiated mycelium, in all cases bearing conidia in clusters. Terminal conidiogenous cells are cylindrical and tapering toward a short apical annellated zone, occasionally with inconspicuous collarettes; lateral conidiogenous cells are flared, vasiform, tapering

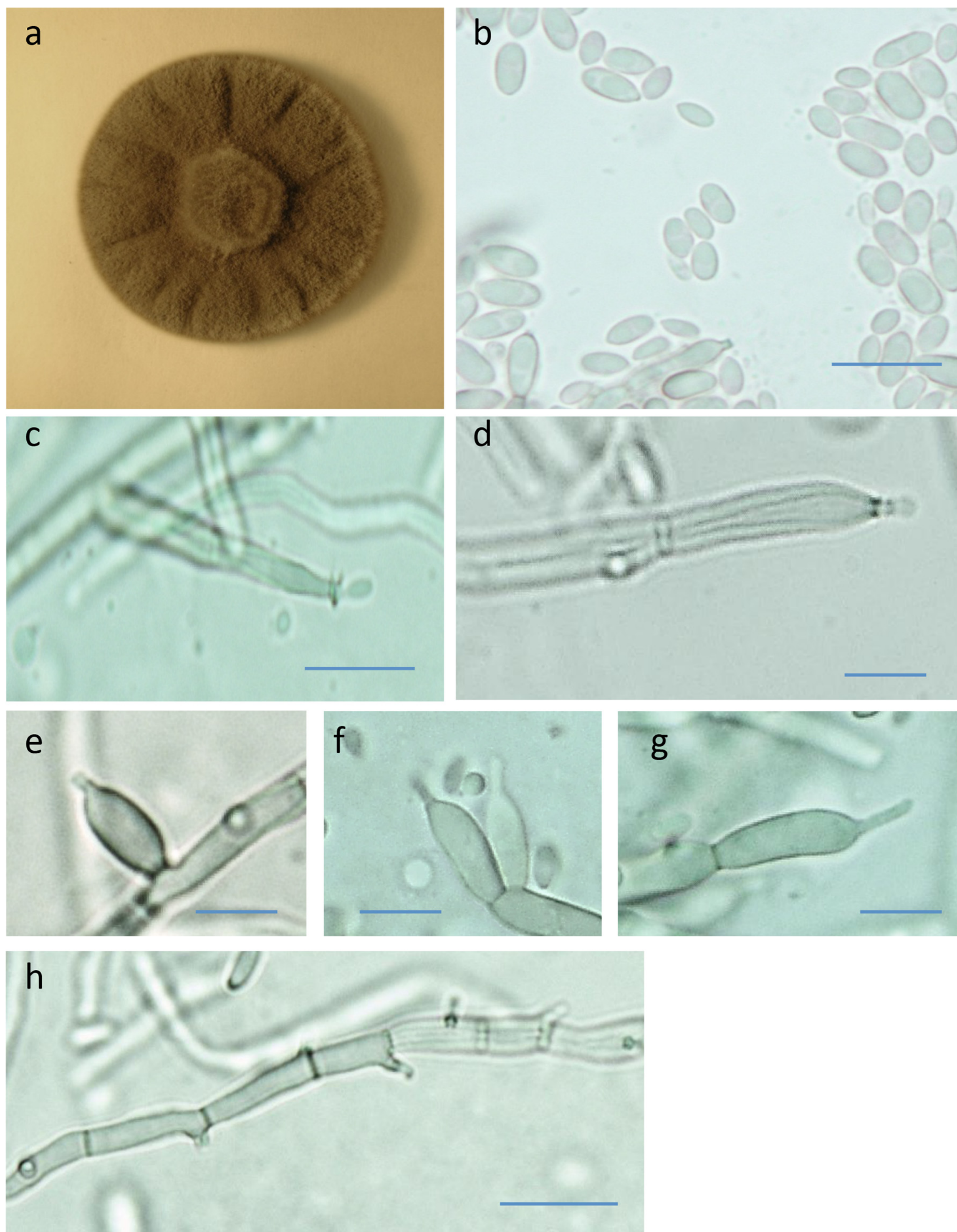

FIG 2 Colonial and microscopic morphological appearance of *Exophiala campbellii* sp. nov. (NCPF 2274) (a) Colony on Sabouraud dextrose agar after 14 days at 30°C. (b to h) Conidiophores, conidiogenous cells, and conidia. (c) *Phialophora*-like phialidic state with collarettes and conidia. Bars = 10 μm.

rapidly toward annellated zones of irregular length. Short annellated conidiogenous pegs are often formed next to septa. Conidia are unicellular, light green, thin-walled, oval to ellipsoidal, and 3.5 to 5.0 by 2.0 to 3.0 μm. Growth observed from 15 to 37°C, optimum 30°C. The teleomorph is unknown. Holotype: (living and dried) NCPF 2274 isolated from a human subcutaneous lesion (foot ganglion), in an otherwise healthy female, 1981, Germany.

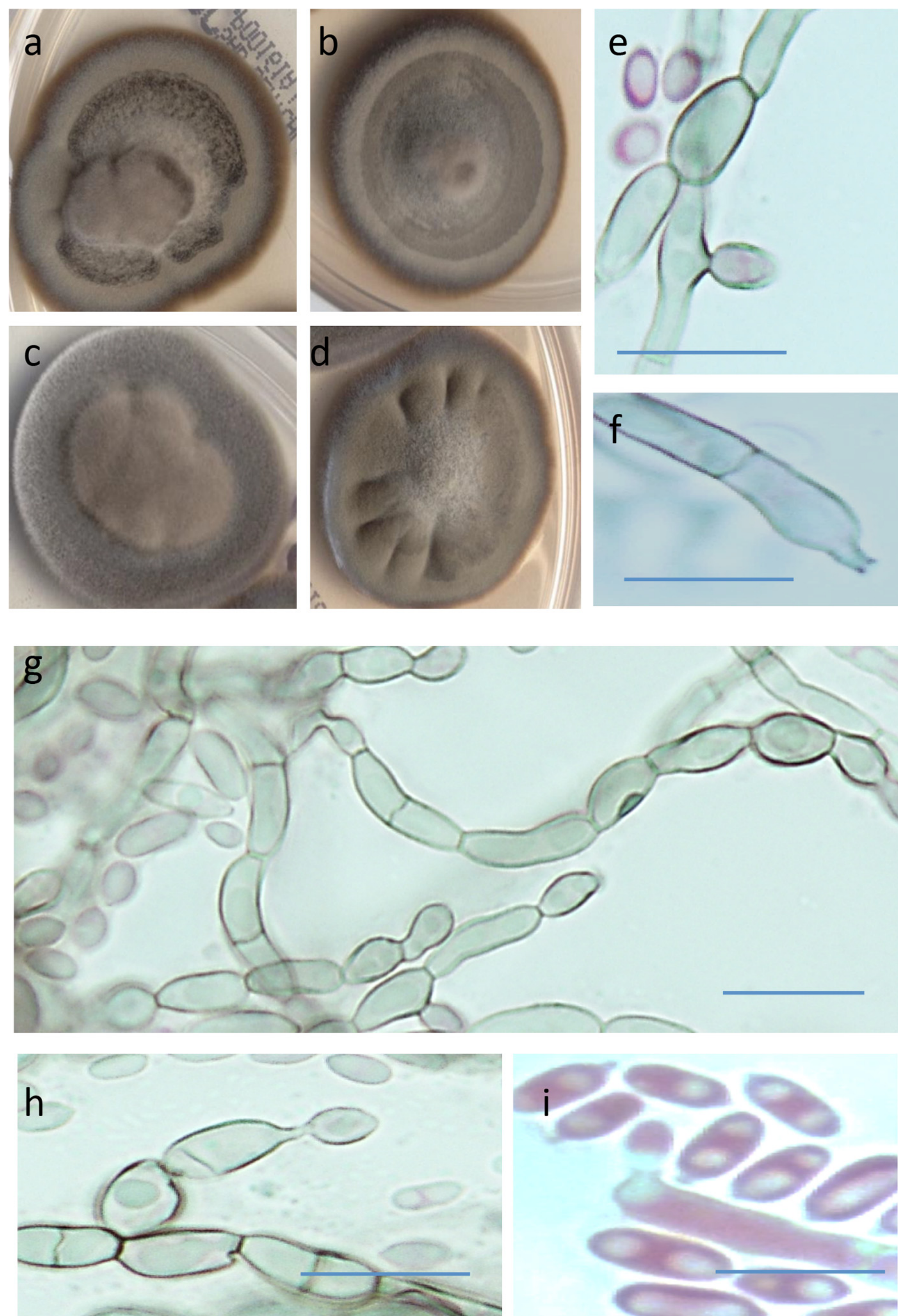

FIG 3 Colonial and microscopic morphological appearance of *Exophiala lavatrina* sp. nov. (a to d) Colony appearance of NCPF 7893 (a), NCPF 7898 (b), NCPF 7899 (c), and NCPF 7900 (d) on Sabouraud dextrose agar after 14 days at 30°C. (e to i) Conidiophores, conidiogenous cells, and conidia of NCPF 7893. Bars = 10 μm.

Exophiala lavatrina sp. nov. Andrew M. Borman, Mark Fraser, Adrien Szekely, Elizabeth M. Johnson. MycoBank accession no. MB 817130. Etymology: from the Latin, domestic bathroom. Colonies on Sabouraud agar at 25°C restricted, attaining 22 to 24 mm diameter in 14 days, peripherally flat with entire margins and with raised, tufted, or radially folded center, velvety to locally powdery, olivaceous-gray to gray-brown with colony reverse dark greenish-gray (Fig. 3). No diffusible pigment produced. Yeast cells mostly scant in young cultures, broadly oval to ellipsoidal, light olivaceous-green, 2.0 by 3.5 μm, often with a short terminal annellated zone. Torulose mycelium is abundant. Hyphae are irregular, pale olivaceous to brown, 1.5 to 4.5 μm wide, with dense septation. Conidiogenous cells arising either terminally as broadly cylindrical phialides

with short conspicuous annelated zones, or more commonly as inconspicuous lateral annellated conidiogenous pegs and in chains of inflated barrel-shaped cells interconnected by narrow septa. Conidia produced in clusters, pale green, broadly ellipsoidal, 4.5 to 7.0 by 2.5 to 4.0 μm. Chlamydospores rarely produced. Growth observed from 10 to 37°C, optimum 30°C. The teleomorph is unknown. Holotype: (living and dried) NCPF 7893 isolated from a domestic bathroom, 2016, United Kingdom.

DISCUSSION

The current study has employed 89 historical clinical and environmental isolates of *Exophiala* spp. to evaluate the robustness of identification by MALDI-TOF MS, using rRNA gene sequence analysis as the gold standard. Formal identification of the isolates by ITS1 sequence analyses revealed that the 89 isolates comprised 18 different species (17 *Exophiala* spp. plus *Rhinocladiella similis*). The spectrum of *Exophiala* species associated with human infections in this UK culture collection appears very similar to that previously described from the United States (9), with a preponderance of *E. dermatitidis*, *E. oligosperma*, and *E. xenobiotica* among clinical isolates in general, and in particular associated with systemic and deep infections. Interestingly, six of the panel of 89 isolates represented two novel taxa, as delineated by extended multilocus sequencing approaches. Four isolates from domestic bathrooms formed a sister species with *E. lecanii-corni* and are described here as *E. lavatrina*. The other two isolates, described here as *E. campbellii*, were both from subcutaneous infections and represent a sister species to *E. oligosperma*. It is likely that *E. lavatrina*, like *E. lecanii-corni*, is an environmental saprobe that is encountered only infrequently in the clinical setting. However, the description here of *E. campbellii* based on two independent isolates from subcutaneous infections in the UK and Germany adds an additional taxon to the list of potentially human-pathogenic *Exophiala* species associated with subcutaneous infections. Further studies will be required to fully ascertain the importance of *E. campbellii* as a human pathogen.

In agreement with previous studies using limited numbers of different *Exophiala* species (25, 26), here, we have shown that MALDI-TOF MS analyses permit the robust and rapid identification of clinical *Exophiala* isolates. For species represented by more than one independent isolate, all isolates could be identified correctly by MALDI-TOF MS, with more than 90% of strains having log scores of $>$1.9 and in excess of 75% of strains having log score values of $>$2.0. With the exception of isolates of *E. lavatrina* and *E. lecanii-corni*, where interspecific log scores were nearly as high as intraspecific scores, the highest log score for an incorrect identification was significantly lower than that for a correct identification and never approached 1.8. Five additional species were represented by only a single isolate, preventing an analysis of the robustness of the specific MSP profile for that species. However, the next best log score for these five species against a different species/genus was considerably below the threshold of 2.0, once again suggesting that these additional MSPs may be useful for identification. Given the rapidity and robustness of MALDI-TOF MS-based identification of *Exophiala* species as described here, and the low cost per isolate after initial MS machine acquisition costs, MALDI-TOF MS appears to be an excellent alternative to ITS sequencing for the accurate identification of *Exophiala* species in the clinical laboratory.

Finally, we have established antifungal susceptibility profiles for a representative selection of 50 of the test panel isolates. In agreement with several previous reports of human-pathogenic *Exophiala* species (17, 27), the amphotericin B and triazole MICs with our *Exophiala* isolates were generally low (17, 27) and lower than those for the echinocandins (18). In the current study, only a limited number of isolates exhibited *in vitro* resistance to amphotericin B, in contrast to previous analyses employing waterborne isolates and those from cutaneous infections in predominantly cold-blooded animals (18).Unfortunately, very few of the waterborne *Exophiala* species and those associated with infections in cold-blooded animals were represented in our collection, precluding a direct comparison in the current study. Further studies will be required to address these questions and to evaluate whether MALDI-TOF MS analysis will be

equally useful for the identification of those *Exophiala* species pathogenic to other animals.

MATERIALS AND METHODS

Fungal isolates. The isolates included in the current study were obtained from the National Collection of Pathogenic Fungi (NCPF), Mycology Reference Laboratory (MRL), Bristol, UK, where they had been stored in sterile water at room temperature in the dark (isolates detailed in Table 1). All isolates were subcultured twice on plates of Oxoid Sabouraud dextrose agar containing 0.5% (wt/vol) chloramphenicol with incubation at 30°C (Unipath Limited, Basingstoke, England).

Morphological examination. Isolates were all subjected to morphological examination at the MRL. Isolates were cultured on slopes of Oxoid Sabouraud dextrose agar containing 0.5% (wt/vol) chloramphenicol, potato dextrose agar (PDA; 20 g of glucose, 4 g of potato extract, 15 g of agar per liter) or potato sucrose agar (PSA; 20 g of sucrose, 4 g of potato extract, 15 g of agar per liter). Cultures were examined for conidiogenesis under a Kyowa Optical SDZ-PL dissecting microscope, and using a Nikon Eclipse E400 light microscope to examine microscopic mounts prepared in lactophenol mounting fluid. The growth rates of novel *Exophiala* species were determined on Sabouraud dextrose agar incubated for 14 days in the dark at 4, 20, 30, 35, 37, 40, and 42°C.

Molecular methods. Genomic DNA was prepared from cultures after 2 weeks of incubation on Sabouraud agar, exactly as described previously (28). Amplification of a region of the large subunit (LSU) gene and the internal transcribed spacer 1 (ITS1) region was performed using the primers described in reference 29. Amplification of a fragment of the actin (*ACT*), RNA polymerase second largest subunit (*RPB2*), and translation elongation factor 1-alpha (*TEF1*) genes used the primer pairs ACT-512F/ACT-738R (30), fRPB2-5F/fRPB2-7cR (31), and EF-3983F/EF-2218R (http://www.aftol.org/pdfs/EF1primer.pdf), respectively. PCR composition, cycling conditions, postamplification analyses, and amplicon sequencing were performed exactly as described previously (32).

Sequence alignment and phylogenetic analyses. Preliminary organism identification was achieved using BLASTN searches against fungal sequences in existing GenBank DNA databases (33). For phylogenetic analyses, sequence alignments included reference sequences from the type strains of key species of *Exophiala* obtained from GenBank (Table 1 and Fig. 1). Initial alignments of each gene were performed separately in MAFFT version 7 (34) and manually adjusted where necessary. Deletions were treated as missing data. The optimal nucleotide substitution models, calculated in PAUP version 4.0a149 (35) using the automated model selection function, were TrNef+G (28S), K80+G (ITS1), TrN+I+G (*TEF1*), and SYM+G (*RPB2*). The concordance of the individual loci was evaluated by comparing individual phylogenetic analyses of each locus performed using the maximum likelihood (ML) algorithm in PAUP, with a 70% bootstrap support cutoff. Since individual trees were broadly concordant, further ML analyses were performed using the GTR+I+G model and the concatenated sequences corresponding to all 4 genes, with nearest neighbor interchange as the heuristic method of tree inference. Topological support was evaluated by a search of 1,000 bootstrapped data sets, with bootstrap values of >70% considered significant. The concatenated sequences corresponding to all 4 genes were also subjected to analyses using maximum parsimony algorithms in PAUP. The combined data set included 2,228 characters, of which 1,456 characters were constant, 50 characters were variable but parsimony uninformative, and 772 positions were parsimony informative. Tree-bisection-reconnection was employed as the heuristic method of tree inference. Topological support was evaluated by a search of 1,000 bootstrapped data sets, with bootstrap values of >70% considered significant.

MALDI-TOF MS sample preparation. Full protein extraction employing sequential ethanol, 70% formic acid, and acetonitrile precipitative steps was performed exactly as described by the manufacturer (Bruker Daltonics, Bremen, Germany), with fungal biomass (approximately 20 to 40 mg) in 300 μl of water harvested by scraping from 48- to 72-h colonies grown at 30°C on Sabouraud agar. Extracts (1-μl volumes) were applied to MSP 96 target ground steel plates (Bruker Daltonics) and left to air dry at room temperature. As soon as spots were dry, they were overlaid with 1 μl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics). Fully dried plates were then directly subjected to MALDI-TOF MS analysis.

MALDI-TOF MS analysis and construction of in-house *Exophiala* database. Dried loaded plates were introduced into a Bruker microflex LT Biotyper, according to the manufacturer's instructions, and analyzed using the MALDI Biotyper RTC software (version 3.0). The linear positive-ion acquisition mode was used, with the standard 2,000- to 20,000-Da mass range and 240 laser shots per sample. For species that were not present in the latest Bruker MSP library database (MBT-BDAL-5989 MSP Library plus Filamentous Fungi version 1.0), new MSP entries were created using selected NCPF isolates representing the 18 species identified by molecular approaches, exactly as described previously (20). The spectra for the remaining 70 test isolates that had not been used to make reference MSPs were analyzed using the FlexControl software (version 3.3) and compared against the Bruker MSP libraries supplemented with the in-house *Exophiala* database. All isolates were analyzed at least twice.

Antifungal agents and CLSI broth microdilution determination of MICs. Antifungal drugs were obtained from their respective manufacturers as standard powders. Amphotericin B and natamycin (both Sigma Chemical Co., St. Louis, NO), anidulafungin and voriconazole (both Pfizer Central Research, Sandwich, UK), and terbinafine (Novartis Pharmaceuticals, Frimley, UK) were dissolved in dimethyl sulfoxide. Itraconazole (Janssen Research Foundation, Beerse, Belgium) and posaconazole (Merck, Sharp, and Dohme, Hoddesdon, UK) were prepared in polyethylene glycol 400 (PEG 400) with heating to 70°C. Serial 2-fold dilutions of the various drugs were prepared in RPMI 1640 medium (with L-glutamine, without bicarbonate; Sigma Chemical Co.), and buffered to pH 7.0 using a 0.165 M solution of

morpholinepropanesulfonic acid (MOPS; Sigma Chemical Co.). MICs were determined according to CLSI methodologies (36) in round-bottomed 96-well plates, as described previously (32), except that conidial suspensions rather than hyphal fragments were employed.

Accession number(s). Sequences from this study were deposited in GenBank under accession numbers LT594638 to LT594722 and LT594725 to LT594766 (Table 1).

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