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Spectrum of Clinically Relevant *Exophiala* Species in the United States▽

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Numerous members of the genus *Exophiala* are potential agents of human and animal mycoses. The majority of these infections are cutaneous and superficial, but also fatal systemic infections are known. We re-identified 188 clinical isolates from the United States, which had a preliminary morphological identification of *Exophiala* species, by sequencing internal transcribed spacer (ITS) region of the rRNA. Molecular identifications of the strains were as follows, in order of frequency: 55 *E. dermatitidis* (29.3%), 37 *E. xenobiotica* (19.7%), 35 *E. oligosperma* (18.6%), 13 *E. lecanii-corni* (6.9%), 12 *E. phaeomuriformis* (6.4%), 7 *E. jeanselmei* (3.7%), 7 *E. bergeri* (3.7%), 6 *E. mesophila* (3.2%), 5 *E. spinifera* (2.7%), 3 *Exophiala* sp. 1 (1.6%), 3 *E. attenuata* (1.6%), 3 *Phialophora europaea* (1.3%), 1 *E. heteromorpha* (0.5%), and 1 *Exophiala* sp. 2 (0.5%) strains. *Exophiala* strains were repeatedly isolated from deep infections (39.9%) involving lung, pleural fluid, sputum, digestive organs (stomach, intestines, bile), heart, brain, spleen, bone marrow, blood, dialysis fluid, lymph node, joint, breast, middle ear, throat, and intraocular tissues. About 38.3% of the *Exophiala* spp. strains were agents of cutaneous infections including skin, mucous membranes, nail, and corneal epithelium lesions. The other strains caused superficial infections (0.5%, including hair) or subcutaneous infection (12.0%, including paranasal sinusitis, mycetoma, and subcutaneous cyst). The systemic infections were preponderantly caused by *E. dermatitidis*, *E. oligosperma*, *E. phaeomuriformis*, *E. xenobiotica*, and *E. lecanii-corni*. Strains of *E. bergeri*, *E. spinifera*, *E. jeanselmei*, *E. mesophila*, and *E. attenuata* mainly induced cutaneous and subcutaneous infections. Since relatively few unknown ITS motifs were encountered, we suppose that the list of opportunistic *Exophiala* species in temperate climates is nearing completion, but a number of species still have to be described.

Black yeasts of the genus *Exophiala* are notoriously difficult to classify and identify. In the past, diagnostic schemes were morphological, and physiological parameters were soon added (10, 11). Several species indeed have marked phenetic characteristics, such as the large conidiophores of *E. spinifera*, or the thermotolerance and absence of nitrite assimilation in *E. dermatitidis*. The majority of species, however, are morphologically variable, due to their passage through complicated life cycles where diagnostic features are variably expressed (14) and, conversely, very similar microscopic structures can be expressed in phylogenetically remote species. In recent years diagnostic approaches have been supplemented by molecular tools, particularly sequence data of the rRNA internal transcribed spacer (ITS) regions (13, 15, 37).

A significant proportion of the known species are regularly encountered as causative agents of human mycoses (see, for example, references 4, 23, 28, 30, 32, 34, and 37). In harboring a wide array of clinically relevant species, the black yeasts and relatives are unique in the fungal kingdom. Because of the lack of tools for species distinction, *Exophiala* species have long been viewed as coincidental opportunists, having their prime occurrence as saprobes on plant material. However, when circumscribed according to modern criteria, some species have turned out to be consistent in their ecology and preferred sites of infection (15). This places the possibility of species-specific virulence and antifungal susceptibility in another light.

We analyzed retrospectively a large number of clinical strains preserved at the Fungus Testing Laboratory in the Department of Pathology at the University of Texas Health Science Center at San Antonio and determined their antifungal susceptibility profiles. Given the difficulty of morphological identification, final identifications were reached after sequencing. An overview of identification results showing discrepancies between morphological and molecular identifications can be sent upon request.

MATERIALS AND METHODS

Fungal strains. A total of 188 clinical strains, previously submitted to the Fungus Testing Laboratory for identification, antifungal susceptibility testing, or both and added to the University of Texas Health Science Center at San Antonio collection were analyzed (Table 1). All isolates were stored at −80°C prior to study and had preliminary morphological identifications as *Exophiala* spp. Sequences were compared to the Centraalbureau voor Schimmelcultures database, which contains thousands of comparable sequences of environmental and clinical *Exophiala* species and related black yeast-like fungi (orders *Chaetothyriales* and *Dothideales*).

DNA extraction. About 1 cm^2 of fungal material was transferred to a 2-ml Eppendorf tube containing a 2:1 (wt/wt) mixture of silica gel and Celite (silica gel H, Merck 7736/Kieselguhr Celite 545; Machery) and 300 μl of TES buffer [2 g Tris (hydroxymethyl)-aminomethane (Merck catalog no. 8382), 0.38 g Na-EDTA

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TABLE 1. Source and identification of strains examined

Identification	No. of strains																																						
	Deep																			Subcutaneous				Cutaneous																
	Lung or pleural fluid	Sputum	Blood	Heart	Stomach, intestine or bile	Stool	Dialysis fluid	Spleen	Bone marrow	Brain biopsy	Lymph node	Breast	Abdominal wound	Throat	Middle ear	Intraocular lesion	Joint	Total		Paranasal sinus	Mycetoma	Subcutaneous cyst	Total	Nail	Skin lesion	Corneal epithelium	Mucous membrane	Total	Superficial infection	Unknown (human)		Animal	Total	%							
E. dermatitidis	16	8	2		1	1				1		1	3		1	2		36		3	1		4		9	2	1	12		3			55	29.3						
E. xenobiotica			1				2									2		5			1	2	3		21		1	22		6		1	37	19.7						
E. oligosperma	10			1	1			1			2				1			16		3	2		5	1	10			11		2		1	35	18.6						
E. lecanii-corni	3				1													4		2	1		3	1	3			4		2			13	6.9						
E. phaeomuriformis	4		1	1					1			1				1		9		1			1	1				1		1			12	6.4						
E. jeanselmei																		0				1	1		6			6					7	3.7						
E. bergeri																		0				1	1		5		1	6					7	3.7						
E. mesophila																	1	1		2			2		1			2	1				6	3.2						
E. spinifera																		0			1	1	2		1			1		2			5	2.7						
Exophiala sp. 1						1												1					0			1	1	2					3	1.6						
E. attenuata																		0					0		3			3					3	1.6						
Exophiala sp. 2																		0					0					0		1			1	0.5						
E. heteromorpha						1												1					0					0					1	0.5						
P. europaea																		0					0	1	2			3					3	1.6						
Total																		73					22					73	1	17	2	188	100						

(Titriplex III; BioRad catalog no. 161-0729), and 2 g sodium dodecyl sulfate in 80 ml of ultrapure water (pH 8)]. The fungal material was ground with a micropestle for 1 to 2 min. The volume was adjusted by adding 200 μl of TES buffer. After vigorous shaking and the addition of 10 μl of a 10-mg/ml concentration of proteinase K to the tube, the mixture was incubated at 65°C for 10 min. The salt concentration was raised by adding 140 μl of 5 M NaCl solution. The mixture was combined with 1/10 volume (~65 μl) of CTAB (cetyltrimethylammonium bromide) buffer 10%, followed by incubation for another 30 min at 65°C. One volume (~700 μl) of chloroform-isoamyl alcohol (vol/vol = 24/l) was added and mixed carefully by hand. After incubation for 30 min at 0°C (on ice water) and centrifugation at 14,000 rpm at 4°C for 10 min, the top layer was transferred to a clean Eppendorf tube. The sample mixed with 225 μl of 5 M NH_4-acetate was incubated for at least 30 min (on ice water) and spun again. The supernatant was transferred to a clean sterile Eppendorf tube and mixed with a 0.55 volume (~510 μl) of ice-cold isopropanol. After being spun for 7 min at 14,000 rpm and 4°C (or room temperature), the supernatant was decanted. The pellet was washed with ice-cold ethanol 70% two times and dried by using a vacuum dryer. The powder was resuspended in 48.5 μl of Tris-EDTA buffer with 1.5 μl of 10 mg of RNase/ml, incubated at 37°C for 15 to 30 min, and stored at −20°C until used.

DNA amplification and sequencing. PCR was performed in 50 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.01% gelatin, 200 mM concentrations of each deoxynucleotide triphosphate, 25 pmol of each primer, 10 to 100 ng of rRNA, and 0.5 U of *Taq* DNA polymerase (Sigma). ITS amplicons were generated for all strains by using the primers V9G (5′-TTA CGT CCC TGC CCT TTG TA-3′) and LS266 (5′-GCAT TCC CAA ACA ACT CGA CTC-3′) (20). Amplification was performed in a GenAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: 95°C for 4 min, followed by 35 cycles consisting of 94°C for 45 s, 52°C for 30 s, and 72°C for 2 min, with a delay at 72°C for 7 min. Amplicons were cleaned with GFX columns (GE Healthcare UK, Ltd., Buckinghamshire, England). For each of the two primers separately, sequencing PCR using 1 μl of template DNA (1 to 10 ng), 3 μl of dilution buffer, 1 μl of BigDye v3.1, and 1 μl of 4 pmol primer filled with 4 μl of MilliQ water to a final volume of 10 μl was performed as follows: 95°C for 1 min, followed by 30 cycles consisting of 95°C for 10 s, 50°C for 5 s, and 60°C for 2 min. Reaction products were purified with Sephadex G-50 Fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and analyzed by using an ABI Prism 3730xl DNA analyzer (Applied Biosystems).

Molecular identification. The sequences were adjusted by using the Lasergene software program SeqMan II (DNASTAR, Inc.) and aligned iteratively using Ward's averaging in the Bionumerics package v. 4.0 (Applied Maths, Kortrijk, Belgium). Nearest neighbors were found by local BLAST searches. The distance trees were based on a realigned file using the DCSE program (17) and calculated by the neighbor-joining method of the Treecon package (38) with Kimura-2 correction; only unambiguously aligned positions were taken into account. A total of 100 bootstrap replicates were used for analysis. Bootstrap values of >90 from 100 resampled datasets are shown. If the similarity of sequences of the ITS region was more than 99% between a studied strain and its nearest neighbor and they were distributed in same branch of the phylogenetic tree, the strain is regarded as belonging to the same species as its nearest neighbor.

Antifungal susceptibility. Antifungal susceptibility testing of four currently available antifungal agents (amphotericin B, itraconazole, voriconazole, and posaconazole) was performed with all strains according to Clinical and Laboratory Standards Institute guidelines (M38-A) (8).

RESULTS

A total of 185 strains from the United States were determined to belong to the genus *Exophiala* as circumscribed by annellidic conidium production and phylogenetic affinity to the order *Chaetothyriales*. Budding cells are mostly present in any stage of the life cycle but are absent from some psychrophilic species. Three strains morphologically attributed to *Exophiala* appeared to be *Phialophora europaea*.

Figure 1 shows a distance tree of partial ITS rRNA of a selection of the strains identified, supplemented with some reference strains. In this tree each *Exophiala* species is clearly individualized in an independent branch supported by a high bootstrap value. Molecular identifications of all of the strains are shown in Table 1; a total of 14 species were identified, which included 2 undescribed novel *Exophiala* species. A comparison between morphological and genetic identifications can be found in Table 2. Only *E. dermatitidis* had a relatively high degree of congruent identifications with morphological and molecular approaches.

In order of frequency, the prevalent agents of *Exophiala* species were *E. dermatitidis* (29.7%), *E. xenobiotica* (20.0%), and *E. oligosperma* (18.9%), comprising more than two-thirds of isolates treated in the present study, followed by *E. lecanii-*

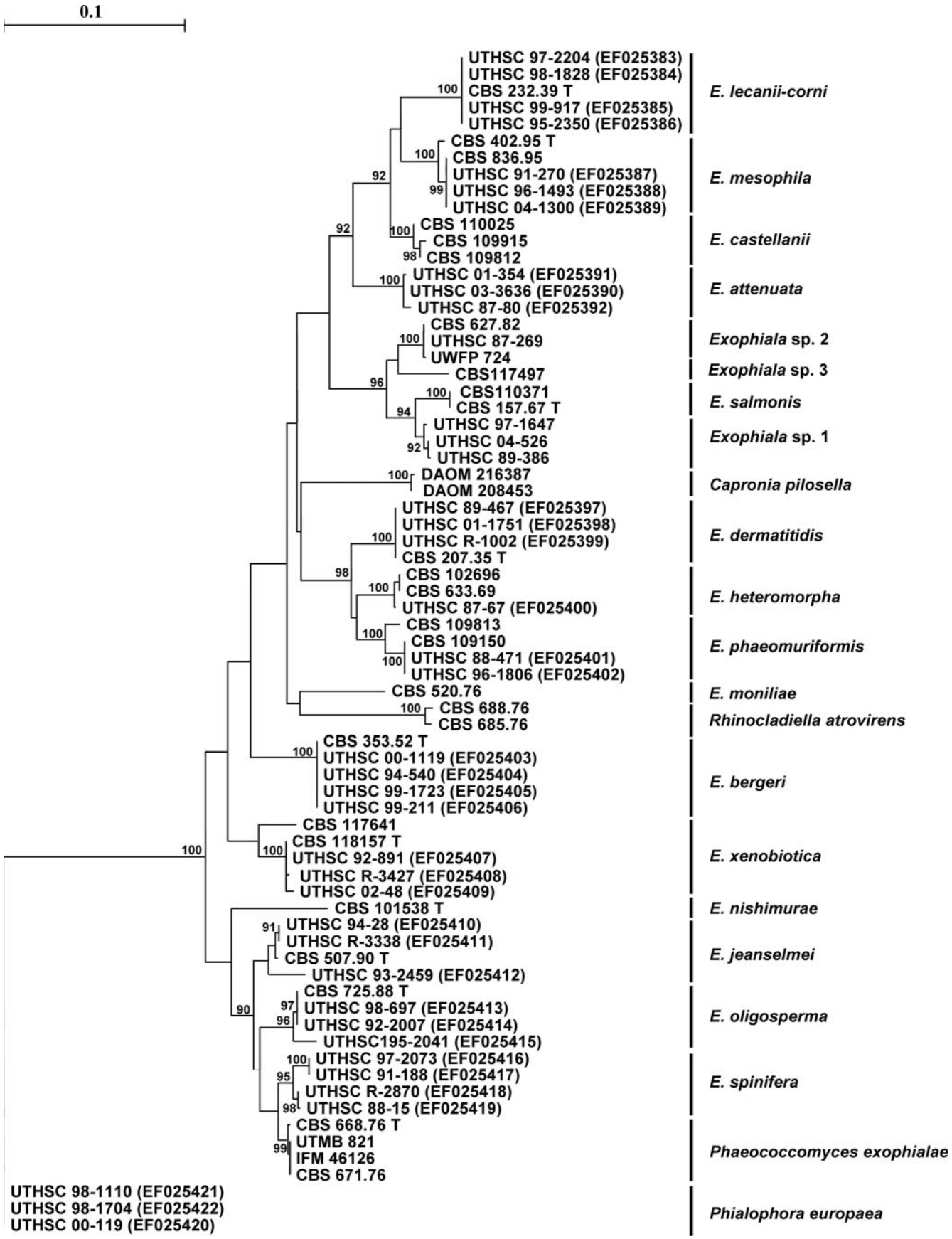

FIG. 1. Consensus tree of ITS rRNA gene of 15 described clinical *Exophiala* and neighboring species, constructed by using the neighbor-joining algorithm in the Treecon package with Kimura-2 correction and 100 bootstrap replications (values of >90 are shown with the branches). *P. europaea* is selected as an outgroup. The numbers in parentheses are GenBank accession numbers for ITS sequences deposited in the GenBank database.

TABLE 2. Comparison of morphological and genetic identifications of strains examined

Genetic identification	Morphological identification (no. of isolates)									Total
	E. castellanii	*E. dermatitidis*	*E. jeanselmei*	*E. jeanselmei* var. *lecanii-corni*	*E. jeanselmei* var. *jeanselmei*	*E. moniliae*	*E. spinifera*	*E. salmonis*	*Exophiala* sp.	
E. dermatitidis		53	1			1				55
E. xenobiotica		3	12	16					6	37
E. oligosperma		1	21	1	3	2	1		6	35
E. lecanii-corni			6	3		2		1	1	13
E. phaeomuriformis	3	2	1	2		2			2	12
E. jeanselmei			4			1			2	7
E. bergeri	1		2	4						7
E. mesophila			2	1					3	6
E. spinifera			1				4			5
Exophiala sp. 1						1			2	3
E. attenuata			1				2			3
Exophiala sp. 2	1									1
E. heteromorpha		1								1
P. europaea			2			1				3
Total	5	60	53	27	3	10	7	1	22	188

corni (7.0%), *E. phaeomuriformis* (6.5%), *E. jeanselmei* (3.8%), *E. bergeri* (3.8%), *E. mesophila* (3.2%), and *E. spinifera* (2.7%) (Fig. 2). The total frequency of the second series of species was more than 25%. *E. attenuata*, *E. heteromorpha*, and two hitherto-undescribed species were seldom isolated.

Exophiala strains were repeatedly isolated from human systemic, single-organ infections (39.9%), particularly those involving the lungs (Fig. 3 and 4). More than 50% of the systemic strains were isolated from the lungs, pleural fluid, or sputum (Fig. 4), whereas isolation from the digestive system and feces was uncommon. Cerebral infections were very rare. Strains from human cutaneous infections, including skin, mucous membranes, nail, and corneal epithelium, were equally common as agents from deep localizations (Fig. 3). Subcutaneous infections in humans were less common (12.0%, involving sinusitis, mycetoma, and subcutaneous cysts), whereas strains were exceptional as commensals (0.5%, involving hair). Two strains were isolated from animals. For a small number of strains no isolation data were available.

The deep infections in human were preponderantly caused by *E. dermatitidis* (36 of 73 [49.3%]), *E. oligosperma* (16 of 73 [21.9%]), *E. phaeomuriformis* (9 of 73 [12.3%]), *E. xenobiotica* (5 of 73 [6.8%]), and *E. lecanii-corni* (4 of 73 [5.5%]) (Table 1). The three most common *Exophiala* agents of cutaneous and subcutaneous infection were *E. xenobiotica* (25 of 95 [27.2%]), *E. dermatitidis* (16 of 92 [17.4%]), and *E. oligosperma* (16 of 92 [17.4%]). *E. jeanselmei*, which in the literature has been regarded as a major agent of cutaneous and subcutaneous mycoses, was rarely observed (7 of 92 [7.6%]). Although *E. dermatitidis*, *E. oligosperma*, and *E. phaeomuriformis* caused different mycoses, they were more frequently isolated from deep infections than from cutaneous and subcutaneous lesions. Strains of the uncommon species *E. jeanselmei*, *E. bergeri*, *E. spinifera*, and *E. attenuata* were rarely systemic.

The results of antifungal susceptibility testing for nine species strains are shown in Table 3. Although there are no defined breakpoints for any species in the genus, MIC data correlated with safely achievable drug concentrations suggests

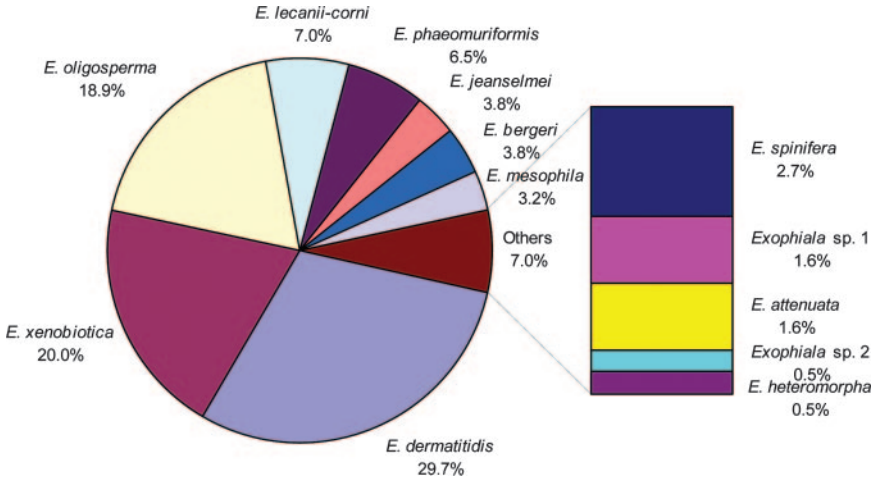

FIG. 2. Spectrum of clinical *Exophiala* species from the United States.

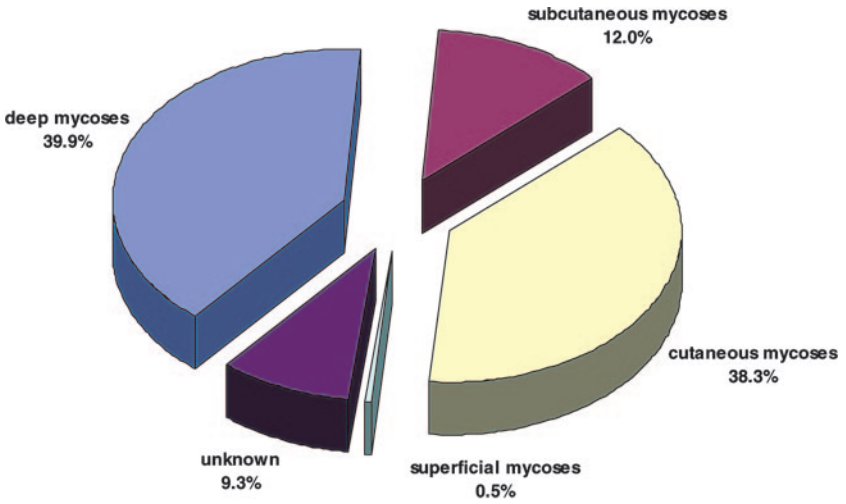

FIG. 3. Localization of infections caused by *Exophiala* species in the United States.

clinical efficacy with each of the antifungal agents evaluated. Since the number of strains of less-common species is low, susceptibility data are not shown. Of note, however, *E. attenuata* (three strains) was found to be resistant to amphotericin B.

DISCUSSION

Although some *Exophiala* species can be identified morphologically and with the help of physiological parameters, most taxa can only be recognized with sufficient certainty by using molecular methods. The sequence diversity of the ITS rRNA region has proven to be reliable for routine species distinction in the genus *Exophiala* (13, 37). For some of the main clinically relevant species (16), the conclusions in the present study have been confirmed by partial sequencing of the elongation factor 1-α and β-tubulin genes. Large distances were observed between nearly all species analyzed, even when these were highly similar morphologically and physiologically. Hence, the use of umbrella names such as "*E. jeanselmei* group," which may be acceptable for daily routine, cannot be applied in the scientific literature. Publication of case reports should be accompanied by sequence data of at least the ITS rRNA. Since relatively few ITS motifs were encountered in the present study that were unknown to us, we suppose that the list of human-associated *Exophiala* species in temperate climates is nearing completion, although a few new species are still on the list to be described.

Species concepts in *Exophiala* have changed considerably after the large-scale application of molecular methods over the last 5 years. In particular, the common clinical species *E. jeanselmei* (9) appeared to comprise a number of cryptic species, such as *E. heteromorpha* (15), *E. lecanii-corni* (22), *E. oligosperma* (15), and *E. xenobiotica* (16), in addition to *E. jeanselmei* in a restricted sense (15). Most of these species have been reported from proven clinical cases, whereby slight, species-specific differences in preferred sites of infection were noted (15). Unfortunately, case reports continue to be published under obsolete concepts (33, 34) and thus create confusion. The present study utilizes species circumscriptions pub-

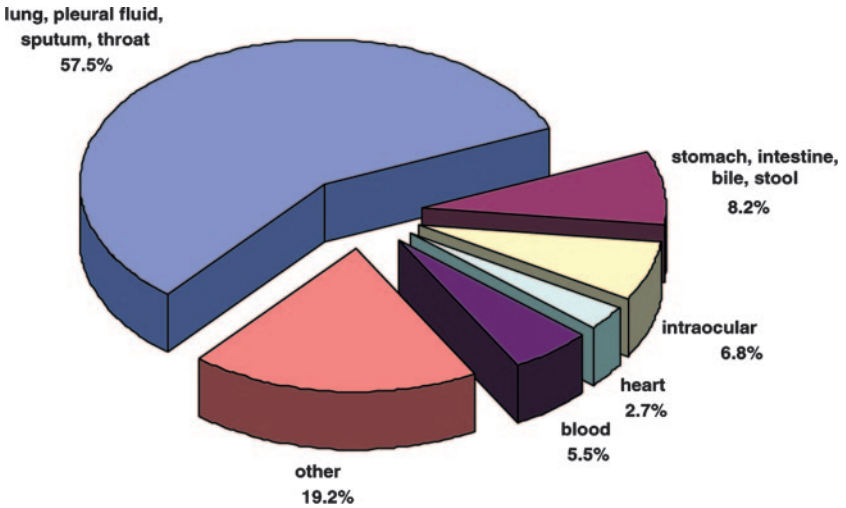

FIG. 4. Distribution of deep mycoses caused by *Exophiala* species in the United States.

TABLE 3. Results of in vitro antifungal susceptibility testing[a]

Organism or type	MIC (mg/liter)							
	Amphotericin B		Itraconazole		Voriconazole		Posaconazole	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
E. phaeomuriformis	0.5	1	≤0.015	0.03	0.03	0.06	≤0.015	≤0.015
E. xenobiotica	0.25	0.5	0.03	0.125	0.125	0.5	≤0.015	0.03
E. bergeri	0.25	0.25	0.125	0.25	1.0	1.0	0.03	0.03
E. lecanii-corni	0.25	0.5	0.06	0.125	0.06	0.5	≤0.015	0.03
E. spinifera	0.5	0.5	0.03	0.06	0.125	0.5	≤0.015	0.03
E. jeanselmei	0.5	1	0.03	0.06	0.125	0.25	≤0.015	0.03
E. mesophila	0.25	0.5	0.125	0.25	0.5	0.25	0.06	0.06
E. dermatitidis	0.5	1.0	0.125	0.25	0.125	0.25	0.03	0.06
E. oligosperma	0.25	0.5	0.06	0.125	0.25	1.0	0.03	0.03
Susceptible[b]	≤1.0		≤0.5		≤2.0		?	
Resistant[c]	>2.0		≥1.0		>2.0		?	

[a] No defined breakpoints are available for the genus *Exophiala*, and the clinical correlates are primarily anecdotal. AMB, amphotericin B, ITRA.
[b] Suggested MIC for clinical efficacy based upon in vitro data.
[c] Suggested MIC for less than favorable clinical outcome based upon in vitro data.

lished after the latest overview of clinically relevant *Exophiala* species, which dates back to the year 2000 (9).

Thus far, 18 of 29 known *Exophiala* species have been proven or were suggested to cause infections in or colonization of humans and animals. The list includes the recently described taxa *E. xenobiotica* (16), *E. oligosperma* (1), and several mesophilic species (M. J. Harrak et al., unpublished data). No reliable data are available on the incidence of diseases, since the strains studied were those that were sent to reference laboratories for identification and thus may not be representative for their actual prevalence; the current data can only be taken as indicative of the frequencies of the main species. Unfortunately, only limited clinical data were available for the present set of strains (not shown). Proof of the clinical significance of a number of species thus has to be judged from future case reports.

Epidemics caused by these species have not been observed, but repeated reports of pseudoepidemics due to contaminated fluids have been published (2, 33, 34). In the large set of strains analyzed for the present study, 12 of the 17 known invasive species were encountered, with the exception of *E. castellanii*, *E. moniliae*, *E. nishimurae*, *E. salmonis*, and *E. pisciphila*. *E. dermatitidis*, and two segregants of *E. jeanselmei*, viz. *E. xenobiotica* and *E. oligosperma*, are the three major agents of mycoses caused by *Exophiala* species.

In the present overview, the frequency of deep mycoses in the studied set of isolates is almost two-fifths (39.9%) and thus significantly higher than that of the categories of subcutaneous and superficial mycoses and also slightly higher than that of cutaneous mycoses. Predisposing diseases or metabolic factors listed by clinicians at the submission of strains include solid organ or bone marrow transplant, hematologic or nonhematologic malignancy, diabetes mellitus, and, exceptionally, human immunodeficiency virus infection. Microbial contamination at injury also occurs repeatedly in immunocompetent individuals. The most frequent deep infections are those of the respiratory system caused by *E. dermatitidis*, *E. oligosperma*, *E. phaeomuriformis*, and *E. lecanii-corni*. Pulmonary infections are mostly not invasive, but probably subclinical colonization is involved, as observed in patients with cystic fibrosis (18, 24). In Europe, this is one of the preponderant clinical pictures by *Exophiala* species, particularly *E. dermatitidis* (18, 24). This fungus occurs with a frequency of 2 to 8% in the susceptible patient population (21); similar screening has thus far not been done in the United States.

Cerebral infections caused by *Exophiala* species are very rare in the United States. Until now only a single fatal case has been reported, caused by strain CDC B-6450. It concerned a contaminated steroid injection, the fungus being directly inoculated into the circulation (19). Strain R-1002 was listed as originating from human brain, but the clinical data and origin were not specified, neither whether it was a primary cerebral infection nor whether there were any predisposing conditions. In Asia, cerebral infection in healthy adolescents is a remarkable clinical syndrome. At least 11 fatal cases were reported (6, 23, 26). Primary cerebral infection caused by *E. dermatitidis* appears to occur nearly only in Asian patients, the possibility of race-dependent virulence has been suggested (24). Strains of *E. spinifera* were mainly involved in cutaneous and subcutaneous mycoses. Outside the United States several cases of disseminated infection caused by this species have been reported (5, 31, 36, 40) in individuals without known immune disorder. The reason for the absence of systemic disease in the United States is unknown.

In cutaneous sources, the recently described species *E. xenobiotica* appeared to be the most frequently detected black yeast. This species is a recent segregant of *E. jeanselmei*, differing at the molecular level and having a different predilected site of infection (16). Also, *E. dermatitidis* and *E. oligosperma* occur in cutaneous infections. The underdiagnosis of *E. xenobiotica* and *E. oligosperma* is certainly due to recent developments in the taxonomy of black yeasts, which led to the description of these taxa, after they had been deposited in reference collections, mostly either as "*E. jeanselmei*" or "*Exophiala* sp."

In conclusion, we suggest that infections with black yeasts of the genus *Exophiala* are severely underdiagnosed in the United States. In the case of the occurrence of *E. dermatitidis* in cystic fibrosis and in stool, frequencies have been published in Europe (12, 21); data from the United States are unlikely to be

different. Underdiagnosis of *Exophiala* infection in superficial and cutaneous disorders is a worldwide problem, and the clinical significance of individual species is therefore hard to establish. Systemic and disseminated cases may be severe, particularly because they can take a fatal course in young and otherwise-healthy individuals. The reason why the severe syndromes seem to be relatively rare in the United States is currently not understood.

Most strains of *Exophiala* species tested appeared to be susceptible in vitro to the four widely used antifungal agents evaluated in the present study, except that *Exophiala attenuata* was resistant to amphotericin B; no significant differences were noted with the different phylogenetic positions of the species concerned. Particularly low MICs were noted for posaconazole. Similar results have been shown previously (3, 25, 27, 33, 39). Infections by *Exophiala* species may require a combination of surgical and medical treatment. Although amphotericin B and itraconazole, with or without additional flucytosine, are currently regarded to be efficacious against cutaneous and subcutaneous lesions, the newer triazole agents, voriconazole and posaconazole, expand the therapeutic options for these mycoses. The clinical outcome in deep infection, however, is dismal (6, 7, 29, 23, 26, 33, 35). Improvement may be expected, since posaconazole has shown striking effects in treating a case of disseminated infection (31).

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