Journal of Clinical Microbiology

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J. Clin. Microbiol. **2012, 50(1):66. DOI: 10.1128/JCM.06133-11.**

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Antifungal Susceptibility and Phylogeny of Opportunistic Members of the Order *Mucorales*

Roxana G. Vitale,[a,b,c] G. Sybren de Hoog,[c,d,e] Patrick Schwarz,[f] Eric Dannaoui,[f] Shuwen Deng,[c] Marie Machouart,[g] Kerstin Voigt,[h,i] Wendy W. J. van de Sande,[j] Somayeh Dolatabadi,[c,d] Jacques F. Meis,[k] and Grit Walther[c,h]

CONICET[a] and Ramos Mejia Hospital, Parasitology Unit, Mycology Section,[b] Buenos Aires, Argentina; CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands[c]; Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands[d]; Peking University Health Science Center, Research Center for Medical Mycology, Beijing, and Sun Yat-sen Hospital, Sun Yat-sen University, Guangzhou, China[e]; Institut Pasteur, Unité de Mycologie Moléculaire, Centre National de Référence Mycologie et Antifongiques, Paris, France[f]; Service de Parasitologie-Mycologie, CHU Brabois, Nancy, France[g]; Institute of Microbiology, Department of Microbiology and Molecular Biology, University of Jena, Jena, Germany[h]; Leibniz-Institute for Natural Product Research and Infection Biology – Hans-Knöll-Institute, Jena Microbial Resource Collection, Jena, Germany[i]; Erasmus Medical Centre, Department of Medical Microbiology and Infectious Diseases, Rotterdam, The Netherlands[j]; and Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands[k]

The *in vitro* susceptibilities of 66 molecularly identified strains of the *Mucorales* to eight antifungals (amphotericin B, terbinafine, itraconazole, posaconazole, voriconazole, caspofungin, micafungin, and 5-fluorocytosine) were tested. Molecular phylogeny was reconstructed based on the nuclear ribosomal large subunit to reveal taxon-specific susceptibility profiles. The impressive phylogenetic diversity of the *Mucorales* was reflected in susceptibilities differing at family, genus, and species levels. Amphotericin B was the most active drug, though somewhat less against *Rhizopus* and *Cunninghamella* species. Posaconazole was the second most effective antifungal agent but showed reduced activity in *Mucor* and *Cunninghamella* strains, while voriconazole lacked *in vitro* activity for most strains. Genera attributed to the *Mucoraceae* exhibited a wide range of MICs for posaconazole, itraconazole, and terbinafine and included resistant strains. *Cunninghamella* also comprised strains resistant to all azoles tested but was fully susceptible to terbinafine. In contrast, the *Lichtheimiaceae* completely lacked strains with reduced susceptibility for these antifungals. *Syncephalastrum* species exhibited susceptibility profiles similar to those of the *Lichtheimiaceae*. *Mucor* species were more resistant to azoles than *Rhizopus* species. Species-specific responses were obtained for terbinafine where only *Rhizopus arrhizus* and *Mucor circinelloides* were resistant. Complete or vast resistance was observed for 5-fluorocytosine, caspofungin, and micafungin. Intraspecific variability of *in vitro* susceptibility was found in all genera tested but was especially high in *Mucor* and *Rhizopus* for azoles and terbinafine. Accurate molecular identification of etiologic agents is compulsory to predict therapy outcome. For species of critical genera such as *Mucor* and *Rhizopus*, exhibiting high intraspecific variation, susceptibility testing before the onset of therapy is recommended.

The fungal order *Mucorales*, belonging to a section of lower fungi that until recently was referred to as zygomycetes, constitutes a phylogenetically ancient group of organisms. In the fungal tree of life the group encompasses a number of widely spaced, ancestral lineages. Over time, mutations are hypothesized to have accumulated, which is reflected, e.g., in an immense degree of sequence diversity of evolutionary markers such as the ribosomal operon. By assessment of identical genes, mucoralean species are separated from each other at branches much longer than those of species of more recent fungi, such as *Aspergillus* or the dermatophytes. As a result, ancestry is difficult to reconstruct, leading to phylogenetic trees with poorly resolved backbones. For similar reasons, the phylum *Zygomycota* has been abandoned: phylogenetic distances are so large that no taxonomic hierarchy can be constructed and no umbrella group defined that would unite all fungi attributed to the *Zygomycota* in the classical sense (23).

The opportunistic members of the *Mucorales* are classified in the families *Cunninghamellaceae*, *Lichtheimiaceae*, *Mucoraceae*, *Saksenaceae*, and *Syncephalastraceae*, with the great majority of human infections being caused by members of *Mucoraceae* and *Lichtheimiaceae*. In molecular phylogenetic analyses (31, 43), the genus *Rhizomucor* was positioned outside the *Mucoraceae*, and in the present article the Index Fungorum (http://www.indexfungorum.org) is followed, classifying the genus in the *Lichtheimiaceae*.

Infections generally occur in severely debilitated patients and are acute, destructive, and with a rapid course and fatal outcome (14, 37). In general, different types of underlying conditions predispose for different types of infection. Major skin abrasion and burn wounds may lead to erosive subcutaneous infection. Rhinocerebral and pulmonary infection are linked to ketoacidotic diabetes and severe neutropenia, respectively, while immunosuppression and prolonged deferoxamine therapy predispose for disseminated infection (11). Chronic disorders observed in individuals without severe immune or metabolic dysfunction are exceptional cases (25). Also, renal mucormycosis tends to occur in immunocompetent individuals (21, 27).

Given the enormous phylogenetic diversity of the *Mucorales* (31, 43), it is remarkable that frequent case reports appear referring to the etiologic agent without proper species identification (e.g., references 19 and 26). Practical reasons for this are the difficulties of cultivating these fungi from biopsy samples (22, 34) and of differentiating zygomycete species by classical mycological techniques in clinical microbiology laboratories. Previous com-

Received 27 April 2011 Returned for modification 23 June 2011
Accepted 18 October 2011
Published ahead of print 9 November 2011
Address correspondence to Grit Walther, grit_walther44@yahoo.de.

doi:10.1128/JCM.06133-11

prehensive studies on susceptibility profiles against antifungal drugs in the *Mucorales* revealed considerable variation among and within genera and species, as defined either by applying classical parameters (5, 16, 35, 39, 40) or more recently by using molecular taxonomic methods (1, 12). A recent review of *in vitro* activity of antifungals against zygomycetes was provided by Alastruey-Izquierdo et al. (2).

Application of molecular methods for species identification in *Mucorales* frequently leads to the unexpected detection of novel sibling species (3, 6, 9). For this reason, and because of the difficulty of morphological species identification, studies on the taxon specificity of susceptibility profiles in this group of fungi need to be preceded by molecular identification of the strains under study (1). Otherwise, in clinical practice, when the only intention is to determine the most appropriate antifungal as quickly as possible, susceptibility tests could also be performed directly. We chose the internal transcribed spacer (ITS) region for taxonomy at the species level because it earlier has been shown to be the species marker of choice in *Mucorales* (36).

Classification of *Mucorales* above the species level is in a state of flux, since molecular phylogenetic analyses found polyphyly of the majority of morphology-based families and genera (31, 41). The D1/D2 region of the nuclear ribosomal large subunit (LSU) was chosen to reconstruct phylogeny because it could be sequenced directly, while all protein-encoding genes tested revealed paralogs in numerous species (3). Furthermore, the LSU is alignable over the entire order. A robust molecular phylogenetic hypothesis is necessary to address the main question of this study: do phylogenetic taxa (species, genera, and families) of the *Mucorales* possess more or less characteristic susceptibility profiles?

MATERIALS AND METHODS

Strains. All isolates used in this study were taken from the reference collection of the CBS-KNAW Fungal Biodiversity Centre (CBS, Utrecht, The Netherlands), from the Institut Pasteur (CNRMA/IP, Paris, France), or from the American Type Culture Collection (ATCC, Manassas, VA). Strains selected for phylogenetic reconstruction represented all relevant taxa of the *Mucorales* including the clinically relevant species.

Extraction of genomic DNA, amplification, cloning, and sequencing. Genomic DNA was extracted from 2-day-old malt extract agar (MEA) cultures according to the procedure reported by Möller et al. (28) with several modifications described in detail by Alastruey-Izquierdo et al. (3). DNA segments comprising the complete ITS region and the D1/D2 region of the LSU were amplified using the primer pair V9G (18) and LR3 (41). The PCR mixture (25 μl) contained 0.4 μM each primer, 0.185 mM each deoxynucleoside triphosphate (GC Biotech, Alphen a/d Rijn, The Netherlands), 10× NH_4 BioTaq reaction buffer (GC Biotech), a final concentration of 1.5 mM $MgCl_2$, 0.8 U BioTaq DNA polymerase (GC Biotech), and about 20 ng DNA. The cycling conditions included one initial cycle at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C, with one final cycle of 7 min at 72°C. PCRs were performed on a thermal cycler 2720 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reaction products were analyzed in 1% agarose gels. Both strands of the PCR products were directly cycle sequenced using the BigDye sequencing kit (Applied Biosystems), and the primer set ITS1 and ITS4 (44) for the complete ITS region and NL1 and LR3 for the D1/D2 region of the LSU (30) were used. Cycle-sequencing products were analyzed on an ABI 3730XL automatic sequencer (Applied Biosystems). In a few cases, direct sequencing failed and the PCR products were cloned in the competent *Escherichia coli* cell line JM109 by using the pGEM-T Easy vector (Promega, Leiden, The Netherlands) as instructed by the manufacturer. Colony PCRs were performed using the primer pair M13f (5′-GTAAAACGACGGCCAGT-3′) and M13r (5′-GGAAACAGCTATG ACCATG-3′). Products of colony PCRs were sequenced as described above.

Species identification and phylogenetic analysis. Consensus sequences were constructed by means of the SeqMan program v.7.2.2. (DNASTAR, Lasergene). The D1/D2 region of the LSU was used to reconstruct the phylogeny of the *Mucorales*, while the ITS region served as a marker for molecular species recognition. For both markers, sequences were aligned using the server version of the MAFFT program (http://www.ebi.ac.uk/Tools/mafft) and manually corrected in the program Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) (33). Sequences of the ITS spacers were not alignable over the entire order. Therefore, ITS alignments for each studied genus were prepared including sequences of all relevant ex-type strains to ensure an accurate identification. They were rooted by the nearest neighbors according to the work of O'Donnell et al. (31). Phylogenetic relationships were estimated using the maximum likelihood method with the server version of RAxML-VI-HPC v.7.0.0 (38), as implemented on the Cipres portal. The robustness of the trees was estimated by a bootstrap analysis with 1,000 replicates.

***In vitro* susceptibility testing.** A set of 66 strains was tested (Table 1), including species of *Cunninghamella* ($n = 8$), *Lichtheimia* (syn. *Absidia pro parte*, *Mycocladus*) ($n = 13$), *Mucor* ($n = 12$), *Rhizomucor* ($n = 8$), *Rhizopus* ($n = 19$), and *Syncephalastrum* ($n = 6$). *In vitro* susceptibilities were determined by a broth microdilution technique following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M38A document (29) with few modifications. RPMI 1640 medium with L-glutamine but without sodium bicarbonate (Sigma-Aldrich, Saint Quentin Fallavier, France) buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich) was used as the test medium. Isolates were grown on Sabouraud dextrose agar for 7 days at 30°C, and stock spore suspensions were prepared by washing the surface of the slants with sterile saline containing 0.05% Tween 80. Spore suspensions were counted with a hemacytometer and then diluted into RPMI medium to a concentration of 2×10^4 spores/ml (2× final concentration). Pure powders of known potency of amphotericin B (AMB; Sigma-Aldrich, Saint Quentin Fallavier, France), voriconazole (VCZ; Pfizer Central Research, Sandwich, United Kingdom), itraconazole (ITZ; Janssen-Cilag, Issy-les-Moulineaux, France), posaconazole (PCZ; Schering-Plough, Kenilworth, NJ), 5-fluorocytosine (5-FC; Sigma-Aldrich), terbinafine (TBF; Novartis Pharma, Basel, Switzerland), caspofungin (CAS; Merck, Rahway, NJ), and micafungin (MCF; Astellas Pharma, Inc., Ibaraki, Japan) were used.

Briefly, microplates containing the antifungal drugs were prepared by batch and stored frozen at −20°C for less than 1 month. MIC endpoints were determined by an automated microplate reader spectrophotometer (Multiscan RC-351; Labsystems Oy, Helsinki, Finland) after 24 h of incubation (an optical density [OD] of >0.15 was required for the drug-free control wells) at 35°C (28°C for *Mucor* and *Rhizomucor*). MIC endpoints were defined as ≥50% reduction in growth compared to the drug-free wells, except for AMB, for which a 90% reduction endpoint was used. For echinocandins, minimum effective concentrations (MECs) were determined by reading the microplates with the aid of an inverted microscope. Two reference strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, were included in each set of determinations to ensure quality control.

Statistics. For calculation purposes, high off-scale MICs/MECs were raised to the next higher concentration, while low off-scale MICs were left unchanged. Associations between the phylogenetic order and MICs of the various antifungal agents were tested with the two-tailed Mann-Whitney test using GraphPad InStat version 3.00 (GraphPad Instat Software, Inc., San Diego, CA).

Nucleotide sequence accession numbers. Sixty LSU sequences (GenBank accession numbers HM849659 to HM849674, HM849676 to HM849707, HM849709, HM849710, and HM849715 to HM849724) and 38 ITS sequences (GenBank accession numbers HM999950 to HM999986 and HQ186304) were newly generated for this study.

TABLE 1 Strains analyzed with present molecular identification and *in vitro* susceptibility data[a]

Name	Strain	Source	Country	MIC/MEC (μg/ml) for:							
				5-FC	MCF	CAS	TBF	VCZ	ITZ	PCZ	AMB
Mucoraceae											
Mucor											
M. circinelloides (syn. *Rhizomucor regularior*)	CBS 384.95 T	Human, face	China	>64	>4	>4	16	>8	4	1	0.25
M. circinelloides	CBS 416.77	Fermenting rice	Unknown	>64	>4	>4	4	>8	2	1	0.5
M. circinelloides	CNRMA 03.154	Human, skin	France	>64	>4	>4	32	>8	1	0.5	0.25
M. circinelloides	CNRMA 03.371	Human, muscle	France	>64	>4	>4	2	>8	2	1	0.5
M. circinelloides f. *circinelloides*	CBS 195.68 NT	Air	Netherlands	>64	>4	>4	32	>8	1	1	0.25
M. circinelloides	IP 1873.89	Human, feces	France	>64	>4	>4	32	>8	2	1	0.25
M. circinelloides	CNRMA 04.805	Human, muscle	France	>64	>4	>4	2	>8	2	2	0.5
M. ramosissimus	CBS 135.65 NT	Human, nasal lesion	Uruguay	>64	>4	>4	2	>8	1	1	0.25
M. racemosus f. *racemosus*	CBS 260.68 T	Unknown	Switzerland	>64	>4	>4	0.125	2	0.25	0.125	0.5
M. hiemalis f. *hiemalis*	CBS 201.65 NT	Unknown	USA	>64	>4	>4	0.25	1	0.5	0.5	0.5
M. irregularis (syn. *Rhizomucor variabilis*)	CBS 103.93 T	Human, hand	China	>64	>4	>4	0.5	>8	1	1	0.5
M. indicus	CNRMA 03.894	Human, muscle	France	>64	>4	>4	0.5	>8	1	1	0.5
Rhizopus											
R. arrhizus	IP 4.77	Human, brain	France	>64	>4	>4	32	8	0.5	0.5	1
R. arrhizus	CNRMA 03.413	Unknown	France	>64	>4	>4	32	4	0.5	0.25	0.5
R. arrhizus	CNRMA 03.410	Human, sputum	France	>64	>4	>4	32	8	1	0.5	1
R. arrhizus	CNRMA 03.412	Human, sputum	France	>64	>4	>4	1	4	0.5	0.125	1
R. arrhizus	CBS 112.07 T	Unknown	Netherlands	>64	>4	>4	32	8	0.5	0.5	1
R. arrhizus	CNRMA 03.411	Human, sputum	France	>64	>4	>4	4	8	0.5	0.25	1
R. arrhizus	CNRMA 04.48	Human, skin	France	>64	>4	>4	32	8	0.5	0.25	2
R. arrhizus	CNRMA 04.160	Human, sputum	France	>64	>4	>4	16	8	0.5	0.25	1
R. arrhizus	CNRMA 03.253	Human, lung	France	>64	>4	>4	4	4	0.25	0.03	1
R. arrhizus	CNRMA 03.395	Human, skin	France	>64	>4	>4	32	4	0.5	0.25	1
R. arrhizus	CNRMA 03.375	Human, sinus	France	>64	>4	>4	32	>8	1	0.5	2
R. arrhizus	CNRMA 03.909	Human, sinus	France	>64	>4	>4	8	4	0.5	0.25	0.5
R. arrhizus	IP 1443.83	Unknown	Unknown	>64	>4	>4	2	4	0.125	0.5	0.5
R. arrhizus	CNRMA 03.918	Human, lung	France	>64	>4	>4	32	8	1	0.5	1
R. microsporus var. *azygosporus*	CBS 357.93 T	Tempeh	Indonesia	>64	>4	>4	0.125	4	0.5	0.5	1
R. microsporus var. *rhizopodiformis*	CNRMA 04.1469	Human	France	>64	>4	>4	0.125	8	4	2	2
R. microsporus var. *chinensis*	CBS 631.82 T	Bread	China	>64	>4	>4	0.25	4	1	0.5	1
R. microsporus var. *rhizopodiformis*	IP 1123.75	Unknown	Unknown	>64	>4	>4	0.06	4	0.5	0.5	1
R. microsporus var. *rhizopodiformis*	IP 676.72	Human, skin	France	>64	>4	>4	0.125	4	0.5	0.25	1
Syncephalastraceae											
Syncephalastrum											
S. "*racemosum*" (species II)	CBS 199.81	Soil	Kuwait	64	>4	>4	0.25	>8	0.5	0.25	0.06
S. "*racemosum*" (species II)	CBS 421.63	Soil	Zaire	>64	>4	>4	0.25	>8	0.5	0.5	0.03
S. "*racemosum*" (species I)	CBS 302.65	Soil	Brazil	>64	>4	>4	0.25	>8	0.5	1	0.25

Continued on following page

TABLE 1 (Continued)

Name	Strain	Source	Country	MIC/MEC (μg/ml) for:							
				5-FC	MCF	CAS	TBF	VCZ	ITZ	PCZ	AMB
S. "*racemosum*" (species I)	CBS 441.59	Dung of coyote	USA	64	>4	>4	0.25	8	0.5	0.25	0.06
S. "*racemosum*" (species I)	CNRMA 03.414	Human, skin	France	>64	>4	>4	0.06	1	0.03	0.06	0.25
S. "*racemosum*" (species I)	CBS 370.49	Air	Indonesia	>64	>4	>4	0.125	8	0.25	0.5	0.06
Lichtheimiaceae											
Rhizomucor											
R. pusillus	CBS 354.68 NT	Corn meal	Netherlands	>64	>4	>4	0.125	2	0.25	0.25	0.5
R. pusillus	CNRMA 04.210	Human, bone	France	>64	>4	>4	0.125	>8	0.25	0.25	0.5
R. pusillus	CNRMA 04.503	Human, sputum	France	>64	>4	>4	0.125	2	0.25	0.5	0.5
R. pusillus	IP 1956.90	Human, bronchia	France	>64	>4	>4	0.125	2	0.25	0.25	0.5
R. pusillus	ATCC 36606	Cat, brain	France	>64	>4	>4	0.06	4	0.25	0.125	0.5
R. pusillus	CNRMA 03.1205	Human, lung	France	>64	>4	>4	0.125	2	0.25	0.25	0.5
R. pusillus	IP 1127.75	Unknown	Unknown	>64	>4	>4	0.06	4	0.25	0.125	0.5
R. miehei	CBS 182.67 T	Retting plant	USA	>64	>4	>4	0.25	4	0.06	0.125	0.5
Lichtheimia	CNRMA 03.611	Human, bronchia	France	>64	>4	>4	0.06	8	0.125	0.25	0.5
L. corymbifera	IP 1129.75	Air	Morocco	>64	>4	>4	0.125	8	0.25	0.25	0.25
L. corymbifera	CNRMA 03.697	Human, bone	France	>64	>4	>4	0.125	8	0.25	0.25	0.25
L. corymbifera	IP 1279.81	Unknown	Unknown	>64	>4	>4	0.125	8	0.25	0.06	0.25
L. corymbifera	CNRMA 04.732	Human, lung	France	>64	>4	>4	0.25	>8	0.25	0.25	0.5
L. corymbifera	IP 1280.81	Unknown	Unknown	>64	>4	>4	0.125	>8	0.25	0.25	0.25
L. ramosa	CBS 100.55	Unknown	Unknown	>64	>4	>4	0.12	8	0.5	0.06	0.125
L. ramosa	CBS 100.49	Dung of cow	Indonesia	>64	>4	>4	0.25	2	0.03	0.06	0.06
L. ramosa	CBS 124198	Culture contaminant	Netherlands	>64	>4	>4	0.25	2	0.12	0.06	0.25
L. ramosa	CBS 582.65 NT	Cacao seeds	Ghana	>64	>4	>4	0.5	>8	0.5	0.25	0.125
L. ramosa	CBS 223.78	Soil	Unknown	>64	>4	>4	0.25	>8	0.25	0.25	0.125
L. ornata	CBS 958.68	Unknown	Unknown	>64	>4	>4	0.25	2	0.06	0.06	0.25
L. ornata	CBS 291.66 T	Dung of bird	India	>64	>4	>4	0.25	>8	0.12	0.06	0.25
Cunninghamellaceae											
Cunninghamella											
C. bertholletiae	CBS 186.84	Human, lung	USA	>64	>4	>4	0.06	>8	16	1	2
C. bertholletiae	CBS 693.68	Soil	Yugoslavia	>64	>4	>4	0.25	>8	>64	1	1
C. bertholletiae	CBS 372.95	Soil	China	>64	>4	>4	0.06	>8	16	0.5	1
C. bertholletiae	CBS 190.84	Human, heart	USA	>64	>4	>4	0.06	>8	16	0.5	2
C. bertholletiae	CBS 191.84	Human, tibia	USA	32	1	4	0.06	>8	>64	0.5	2
C. echinulata var. *antarctica*	CBS 545.75	Soil	Chile	>64	>4	>4	0.125	>8	1	0.5	2
C. echinulata	CBS 766.68	Unknown	Unknown	>64	>4	>4	0.06	>8	>64	1	1
C. echinulata	CBS 156.28	Unknown	Unknown	>64	0.5	4	0.25	>8	16	1	2

[a] Susceptibility data are in μg/ml for 5-fluorocytosine (5-FC), micafungin (MCF), caspofungin (CAS), terbinafine (TBF), voriconazole (VCZ), itraconazole (ITZ), posaconazole (PCZ), and amphotericin B (AMB). Genera are placed in line with the phylogenetic tree of Fig. 1 except for *Rhizomucor*. Within the genera, strains are arranged according to their position in the trees of Fig. 2. Species boundaries are marked by vertical space. NT, neotype; T, ex-type.

RESULTS

The LSU tree (Fig. 1) comprises members of the clinically significant genera *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia*, *Mucor*, *Rhizomucor*, *Rhizopus*, *Saksenaea*, and *Syncephalastrum*, all belonging to the order *Mucorales*. Sequences could be aligned with relative confidence but showed considerable diversity. In contrast, morphological identification of species appeared to be nonpredictive, with extreme differences occurring between neighboring molecular taxa. Ribosomal DNA (rDNA) distances between species belonging to a single genus were considerable. ITS diversity was up to 20% in *Lichtheimia* and 35% in *Mucor*, while *Rhizopus arrhizus* and *Rhizopus microsporus* deviated 29.8% (data not shown). ITS sequences showed very limited similarity over the entire data set and could not be aligned. Separate trees were therefore constructed for molecular identification (Fig. 2).

With a single exception, all strains included in susceptibility

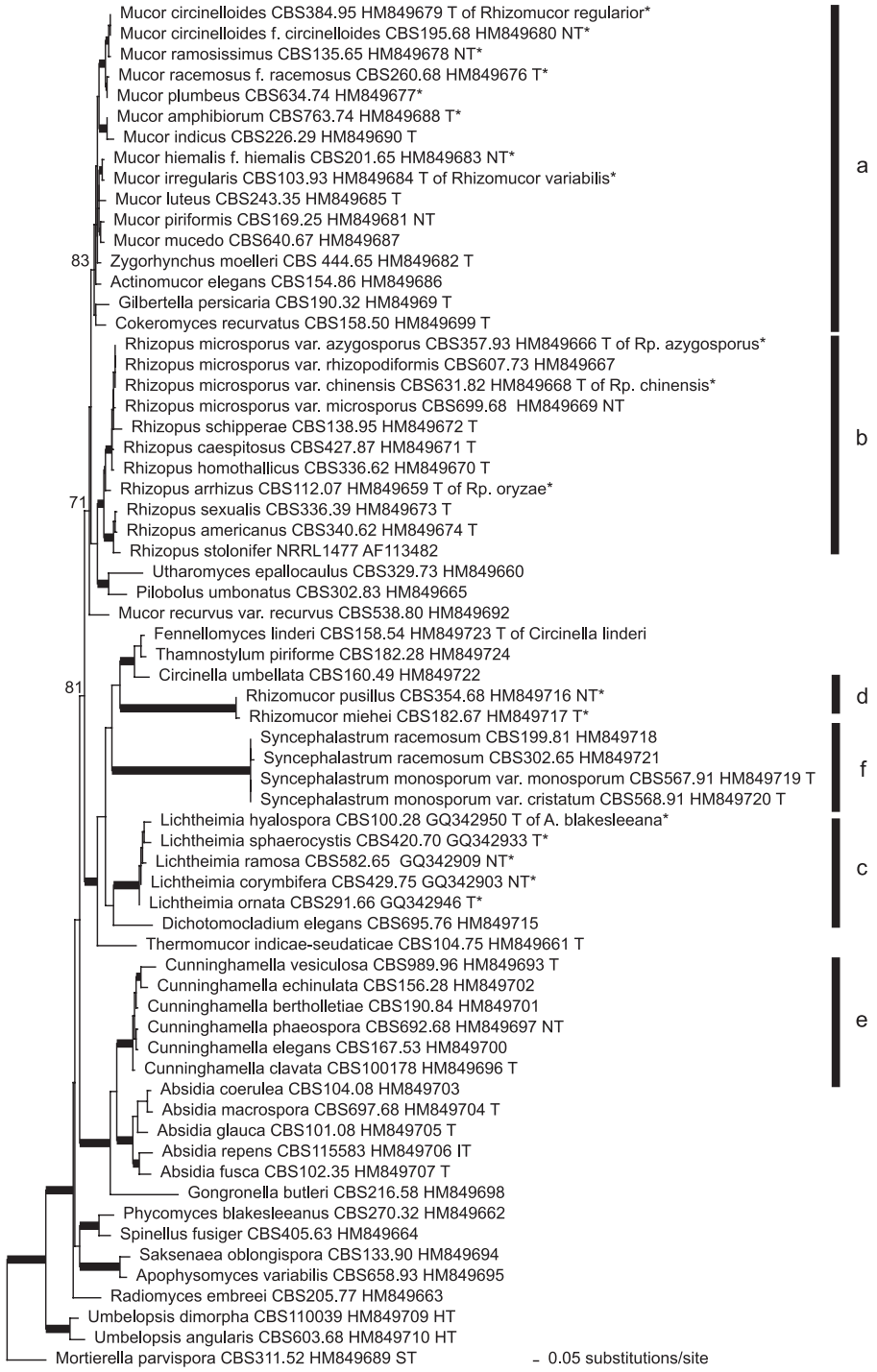

FIG 1 RAxML phylogram based on the D1/D2 region of the LSU. Branches with bootstrap values of 85 or higher are printed in bold. Important branch support values lower than 85 are indicated by numbers near the branches. Ex-type strains are indicated by T (type strain), NT (neotype strain), IT (isotype strain), and HT (holotype strain) following the strain numbers. Asterisks indicate strains included in the susceptibility study. Bars (a to f) mark the genera represented by an ITS dendrogram in Fig. 2.

testing could be identified reliably to the species level by using the ITS region, because of their position in shared clades with ex-type strains (Fig. 2). The correct identification of *Syncephalastrum racemosum* turned out to be problematic because this species has not been typified yet, and strains morphologically identified as *Syncephalastrum racemosum* formed two clearly separate clades in the ITS tree (Fig. 2f): some *S. racemosum* strains were part of a well-supported clade with *Syncephalastrum monosporum*, while others (including the clinical strain CNRMA03.414), represented by two types of ITS sequences (note different clone numbers of the same

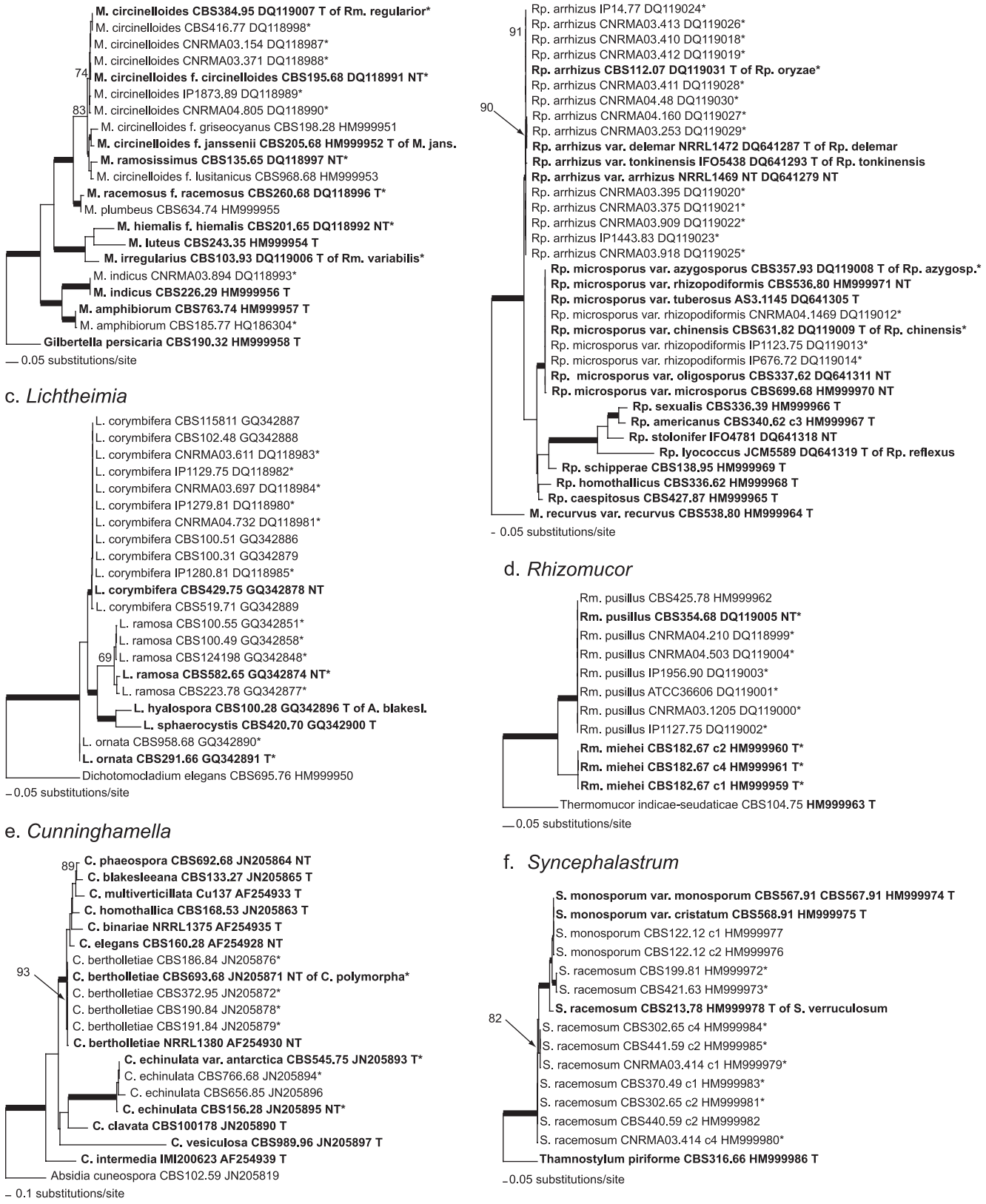

FIG 2 RAxML phylograms based on the complete ITS region. Branches with bootstrap values of 85 or higher are printed in bold. In cases of less space or important values lower than 85, branch support values are indicated by numbers near the branches. Ex-type strains are printed in bold and indicated by T (type strain), NT (neotype strain), IT (isotype strain), and HT (holotype strain) following the strain numbers. Asterisks indicate strains included in the susceptibility study. Clones are specified by a "c" followed by the clone number.

TABLE 2 MIC ranges and geometric means (GMs) for genera and species inferred from the phylograms[a]

Name (no. of strains)	MIC range (GM) (μg/ml) for:							
	5-FC	MCF	CAS	TBF	VCZ	ITZ	PCZ	AMB
Mucor (12)	>64 (>64)	>4 (>4)	>4 (>4)	0.125–32 (2.69)	1–>8 (>6.0)	0.25–4 (1.19)	0.125–2 (0.79)	0.25–0.5 (0.37)
Mucor circinelloides sensu lato (8)	>64 (>64)	>4 (>4)	>4 (>4)	2–32 (8)	>8 (>8)	1–4 (1.68)	0.5–2 (1)	0.25–0.5 (0.32)
Mucor racemosus (1)	>64	>4	>4	0.125	2	0.25	0.125	0.5
Mucor hiemalis (1)	>64	>4	>4	0.25	1	0.5	0.5	0.5
Mucor irregularis (1)	>64	>4	>4	0.5	>8	1	1	0.5
Mucor indicus (1)	>64	>4	>4	0.5	>8	1	1	0.5
Rhizopus (19)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–32 (3.84)	4–>8 (>5.5)	0.125–4 (0.58)	0.03–2 (0.33)	0.5–2 (1)
Rhizopus arrhizus (14)	>64 (>64)	>4 (>4)	>4 (>4)	1–32 (21.11)	4–>8 (>5.9)	0.125–1 (0.5)	0.03–0.5 (0.28)	0.5–2 (0.95)
Rhizopus microsporus (5)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–0.25 (0.12)	4–8 (4.6)	0.5–4 (0.87)	0.25–2 (0.57)	1–2 (1.15)
Syncephalastrum (6)	≥64 (>64)	>4 (>4)	>4 (>4)	0.06–0.25 (0.18)	1–>8 (>5.6)	0.03–0.5 (0.28)	0.06–1 (0.31)	0.03–0.25 (0.09)
Syncephalastrum "*racemosum*" species II (2)	≥64	>4	>4	0.25	≥8	0.5	0.25–5	0.03–0.06
Syncephalastrum "*racemosum*" species I (4)	≥64 (>64)	>4 (>4)	>4 (>4)	0.06–0.25 (0.15)	1–>8 (>4.7)	0.03–0.5 (0.21)	0.06–1 (0.29)	0.06–0.25 (0.12)
Rhizomucor (8)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–0.25 (0.11)	2–>8 (>3.1)	0.06–0.25 (0.21)	0.125–0.5 (0.21)	0.5 (0.5)
Rhizomucor pusillus (7)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–0.125 (0.1)	2–>8 (>3.0)	0.25 (0.25)	0.125–0.5 (0.23)	0.5 (0.5)
Rhizomucor miehei (1)	>64	>4	>4	0.25	4	0.06	0.125	0.5
Lichtheimia (13)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–0.5 (0.18)	2–>8 (>5.8)	0.03–0.5 (0.18)	0.06–0.25 (0.13)	0.06–0.5 (0.24)
Lichtheimia corymbifera (6)	>64 (>64)	>4 (>4)	>4 (>4)	0.06–0.25 (0.12)	≥8 (>8)	0.06–0.5 (0.22)	0.06–0.25 (0.20)	0.25–0.5 (0.31)
Lichtheimia ramosa (5)	>64 (>64)	>4 (>4)	>4 (>4)	0.125–0.5 (0.25)	2–>8 (>4.6)	0.03–0.5 (0.19)	0.06–0.25 (0.11)	0.06–0.25 (0.12)
Lichtheimia ornata (2)	>64	>4	>4	0.25	2–>8	0.06–0.125	0.06	0.25
Cunninghamella (8)	32–>64 (>58.7)	0.5–>4 (>2.59)	≥4 (>4)	0.06–0.25 (0.09)	>8 (>8)	1–>64 (>19.0)	0.5–1 (0.71)	1–2 (1.54)
Cunninghamella bertholletiae (5)	32–>64 (>55.7)	1–>4 (>3.03)	≥4 (>4)	0.06–0.25 (0.08)	>8 (>8)	16–>64 (>27.9)	0.5–1 (0.66)	1–2 (1.52)
Cunninghamella echinulata (3)	>64	0.5–>4	≥4	0.06–0.3	>8	1–>64	0.5–1	1–2
All isolates (66)	32–>64 (>63.3)	0.5–>4 (>3.8)	≥4 (>4)	0.06–32 (0.62)	1–>8 (>5.5)	0.03–>64 (>0.66)	0.03–2 (0.33)	0.03–2 (0.48)

[a] MIC ranges (GMs) for 5-fluorocytosine (5-FC), micafungin (MCF), caspofungin (CAS), terbinafine (TBF), voriconazole (VCZ), itraconazole (ITZ), posaconazole (PCZ), and amphotericin B (AMB). Genera are placed in line with the phylogenetic tree of Fig. 1 except for *Rhizomucor*. Within the genera, species are arranged according to their position in the trees of Fig. 2.

strains), formed a second group. *Syncephalastrum racemosum* has been described by Schroeter (original reference cited in reference 13) as "inter *Aspergillus oryzae* in *Oryza* et pane," i.e., on moldy rice and bread, in Wroclaw, Poland, and no type material is known to be preserved. None of the isolates studied morphologically completely matched the description given by Schroeter, and more-detailed taxonomic studies are needed to select a neotype for this species. Therefore, we designate the putative species in the following as *Syncephalastrum* "*racemosum*" species I and *S.* "*racemosum*" species II.

Judging from LSU and ITS phylograms (Fig. 1, 2) *Rhizomucor regularior* and *Rhizomucor variabilis* were found to be related to *Mucor* but belonged to the species complexes of *M. circinelloides* and *M. hiemalis*, respectively; *R. variabilis* has recently been renamed as *M. irregularis*, and *R. regularior* has been synonymized with *M. circinelloides* (9). The genus *Rhizomucor* is restricted to thermophilic species forming spherical spores, such as *R. pusillus* and *R. miehei*. *Mucor ramosissimus* belongs to *M. circinelloides*. The varieties *azygosporus*, *chinensis*, *rhizopodiformis*, and *tuberosus* of *Rhizopus microsporus* possessed identical ITS sequences and could not be discriminated molecularly (Fig. 2b). Therefore, the morphology-based assignment to the varieties was retained. In *Rhizopus arrhizus* (syn. *R. oryzae*), small molecular differences between varieties were observed, but because of the limited number of reference strains available representing each of the varieties, identifications were done at the species level only.

A total of eight antifungal compounds were tested on six mucoralean genera: *Mucor* and *Rhizopus* (*Mucoraceae*), *Lichtheimia* and *Rhizomucor* (*Lichtheimiaceae*), *Syncephalastrum* (*Syncephalastraceae*), and *Cunninghamella* (*Cunninghamellaceae*). The *Cunninghamellaceae* and the *Syncephalastraceae* are unigeneric, and conclusions drawn on the genus are also valid for the family. The positions of the genera in Tables 1 and 2 refer to their positions in the LSU phylogram of Fig. 1, except for *Rhizomucor*. *Rhizomucor* as a member of the *Lichtheimiaceae* is placed with *Lichtheimia* because its position closer to *Circinella* in the LSU phylogram is not supported. Within the genera, strains are arranged according to their position in the phylogenetic trees of Fig. 2. Because clinical breakpoints for filamentous fungi have not been assigned to the majority of antifungals, we refer to those given by Almyroudis et al. (5) and de Hoog et al. (17), *viz.* AMB $\leq$ 1; CAS $\leq$ 2; 5-FC $\leq$ 16; ITC $\leq$ 0.5; PCZ $\leq$ 0.5; and VRC $\leq$ 2.

For 5-FC, MCF, and CAS, little variation in susceptibility was noted: for 5-FC and CAS, all strains showed high MIC/MEC values of 32 to >64 and ≥4 μg/ml, respectively. Except for two strains of *Cunninghamella*, high MEC values (≥4 μg/ml) were also obtained for MCF. All strains included in this study were resistant or less susceptible to VCZ.

For the remaining compounds, considerable variation was found at the family and generic levels inferred from the LSU phylogram. Overall, AMB *in vitro* was the most effective antifungal agent against *Mucorales*, but its efficacy proved to be ambiguous in *Rhizopus* and *Cunninghamella*, where relatively high MICs were obtained. Three out of 19 *Rhizopus* strains and 5 out of 8 *Cunninghamella* strains tested had MICs of 2 μg/ml, exceeding the assumed breakpoint for this drug. MICs of these two genera were significantly elevated when compared to the other *Mucorales*, for which the amphotericin B MICs ranged between 0.03 and 0.5 μg/ml (Mann-Whitney, $P < 0.0001$ for both genera). Together with those for posaconazole, amphotericin B MICs showed the

smallest range of variation over the entire set of *Mucorales* tested (Table 2). Among the azoles, PCZ was the most effective antifungal drug, with all strains being inhibited by concentrations of 2 μg/ml. The genera *Mucor* (defined in a phylogenetic sense) and *Cunninghamella* showed significantly lower degrees of susceptibility to PCZ (Mann-Whitney, $P < 0.0001$ for both genera). In *Rhizopus* and *Syncephalastrum*, resistant strains occurred only sporadically. The *Lichtheimiaceae*, represented by *Lichtheimia* and *Rhizomucor*, completely lacked strains with reduced susceptibility for PCZ.

Itraconazole was significantly less active against the *Mucoraceae* (defined in a phylogenetic sense) and *Cunninghamella*, in which especially high MIC values were obtained (Mann-Whitney; $P < 0.0001$ for the *Mucoraceae* as well as for *Cunninghamella*). In contrast, the *Lichtheimiaceae* and *Syncephalastrum* (*Syncephalastraceae*), united in a well-supported clade in the LSU phylogram, did not include strains with reduced susceptibility for ITZ.

For TBF, elevated MIC values were found only in the *Mucoraceae*, while the remaining families, namely, the *Cunninghamellaceae*, the *Lichtheimiaceae*, and the *Syncephalastraceae*, were fully susceptible. The susceptibility of *Cunninghamella* to TBF is of special importance because TBF was the only thoroughly active antifungal against this genus in our study. The susceptibility to TBF in the *Mucoraceae* appeared to be species dependent. Terbinafine MICs were strikingly different between *R. microsporus* (geometric mean [GM] MIC of 0.12 μg/ml) and *R. arrhizus* (GM MIC of 21.1 μg/ml) (Mann-Whitney, $P = 0.0020$) but not between *M. circinelloides* sensu lato (GM MIC of 5.66 μg/ml) and the remaining *Mucor* species (MICs of ≤0.5 μg/ml), although a trend was noted (Mann-Whitney, $P = 0.0746$). Susceptibility values of PCZ and TBF were more heterogeneous in the *Mucoraceae* than in the remaining families.

Maximum susceptibilities were reached with PCZ (MIC = 0.03 μg/ml) for *Rhizopus arrhizus*, ITZ (MIC = 0.03 μg/ml) for *Lichtheimia ramosa* and *Syncephalastrum* "*racemosum*" species I, and AMB (MIC = 0.03 μg/ml) for *Syncephalastrum* "*racemosum*" species II, while maximum resistance was observed with 5-FC (MIC > 64 μg/ml) for nearly all strains tested and with ITZ (MIC > 64 μg/ml) for 3 *Cunninghamella* strains.

Intrageneric differences of *in vitro* susceptibility in *Mucor* and *Rhizopus* ranged from 4 to 9 $\log_2$ dilution steps. With respect to PCZ and ITZ, the genus *Mucor* seems to contain a higher proportion of strains that are resistant to or show reduced susceptibility than *Rhizopus* (Mann-Whitney, $P = 0.0042$ and $P = 0.0369$, respectively). In contrast MICs for VCZ were lower in *Mucor* than in *Rhizopus* (Mann-Whitney, $P < 0.0001$). Some intraspecific variability of *in vitro* susceptibility was found in all genera tested (Table 1). Strain CNRMA 04.1469 of *R. microsporus* deviated from remaining strains of this species in its response to azoles.

DISCUSSION

A robust taxonomy and phylogeny of the *Mucorales* require species validation by their ex-type materials. These reference materials serve to calibrate additional strains examined, such that a taxonomic scaffold is provided, allowing meaningful comparison of susceptibility data at the levels of species, genera, and families.

The genera *Mucor* and *Rhizomucor* have been misapplied in some medical publications. Judging from ITS sequence data, *Rhizomucor regularior* (as *R. variabilis* var. *regularior*) was declared to be a synonym of *Mucor circinelloides* (9) as suggested before (6, 36). The typical variety of *Rhizomucor variabilis* var. *variabilis* fits elsewhere in the genus *Mucor* (1, 10, 42), and recently Álvarez et al. (9) proposed a name change to *Mucor irregularis*.

Our study supports AMB as the antifungal of choice for most genera of *Mucorales*. High MICs for AMB have been reported for some genera not tested in the present study, such as *Apophysomyces* and *Saksenaea* (7, 8, 12, 15, 35, 39). Results for *Rhizopus* and *Cunninghamella*, however, are ambiguous, with MICs of 2 μg/ml for AMB. Similar results were obtained by other authors (1, 35, 39, 40). Alastruey-Izquierdo et al. (1) listed *R. arrhizus* and *Cunninghamella bertholletiae* with MIC ranges as wide as 0.03 to 32 μg/ml and 2 to 32 μg/ml, respectively. In concordance with previous studies (1, 5, 16), PCZ was the second most active drug, all strains being inhibited by 2 μg/ml or less (Table 2). The success rate of therapy was reported to be 79% (20).

5-FC, MCF, and CAS were ineffective in all or nearly all *Mucorales*, showing high MIC/MEC values of ≥32, ≥4 (for 64 out of 66 strains), and ≥4 μg/ml, respectively. This resistance is in accordance with literature data (1, 5, 16, 40). With MICs consistently ≥1 μg/ml in all *Mucorales* analyzed, VCZ was less active than other azoles. The poor activity of this antifungal has been highlighted previously (e.g., references 1, 5, and 16).

Phylogenetic distances within the *Mucorales* are reflected in *in vitro* susceptibility profiles against antifungal drugs on all levels of family, genus, species, and strain (Tables 1 and 2). Members of the *Lichtheimiaceae* were consistently susceptible to PCZ, ITZ, TBF, and AMB and exhibited smaller MIC ranges than the *Mucoraceae*. This matches with MIC values published (5, 16, 24, 32, 35, 39), although Torres-Narbona et al. (40) and Alastruey-Izquierdo et al. (1, 4) reported on individual *Lichtheimia* strains that were resistant to PCZ, ITZ, and TBF and to ITZ, respectively.

The consistent *in vitro* activity of TBF against *Cunninghamella* is of clinical relevance because the proportion of strains that are resistant or have reduced susceptibility to PCZ and AMB is relatively high in this genus, as shown in this study and by other authors (1, 5, 39). Alastruey-Izquierdo et al. (1) tested the susceptibility of *Cunninghamella* against TBF with a similar result. However, the authors found an individual strain resistant to TBF.

The species-specific differences in the susceptibility to TBF found (*Rhizopus arrhizus* [resistant] versus *R. microsporus* [susceptible] and *Mucor circinelloides* sensu lato [resistant] versus remaining *Mucor* species [susceptible]) were also in agreement with earlier studies (1, 16). Significantly deviating resistance to azoles was found in a single strain of *R. microsporus*; Alastruey-Izquierdo et al. (1) reported that TBF data were variable in that species.

In conclusion, AMB and PCZ were the most effective antifungal agents against *Mucorales*. Susceptibility profiles (restricted to the drugs that were at least partly active) differed significantly at the familial, generic, and specific levels and reflected relationships as referred from the phylogram; the *Lichtheimiaceae* were fully susceptible to PCZ, ITZ, TBF, and AMB. *Syncephalastrum* (*Syncephalastraceae*) members, positioned at the shortest distance to the *Lichtheimiaceae* in the LSU phylogram, showed similar susceptibility profiles. The only difference was a single strain resistant to PCZ. In contrast, the *Mucoraceae* were characterized by a reduced susceptibility to PCZ, ITZ, and AMB and a lack of activity of TBF in some species. While more *Mucor* strains were resistant to the azoles, only *Rhizopus* strains exhibited a reduced susceptibility to AMB. Susceptibility profiles of *Cunninghamella* (*Cunninghamellaceae*) resembled those of the *Mucoraceae* in terms of the high

proportion of strains resistant to PCZ, ITZ, and AMB but differed in terms of the low MIC values for TBF. Judging from MIC values recorded by other authors, the *Saksenaeaceae* behave similarly to *Cunninghamella*, their closest studied neighbor in the LSU phylogram, in susceptibility tests. Along with *Cunninghamella*, they exhibit a lack of or reduced susceptibility against PCZ, ITZ, and AMB (5, 7, 8, 12, 35) and low MIC values for TBF (7) (tested only for *Saksenaea*).

Obviously, the *Mucorales* cannot be considered as a single entity from an antifungal perspective, since large differences in susceptibility exist between families, genera, species, and strains. The reduced activity of AMB in *Rhizopus* and *Cunninghamella* and the high proportion of *Mucor* and *Cunninghamella* strains that were less susceptible to PSZ are of practical importance because they concern deviations for compounds that are otherwise recommended for antifungal therapy of infections due to *Mucorales*. Molecular identification of the etiologic agent is therefore required unless the susceptibility profile of the strain is known. In genera with high intraspecific variation in their antifungal profiles, such as *Mucor* and *Rhizopus*, immediate susceptibility testing is recommended to confirm the most effective and appropriate compound.

ACKNOWLEDGMENTS

We are indebted to Ana Alastruey-Izquierdo and Xuelian Lu for useful comments on the manuscript.

J.F.M. has been a consultant to Astellas, Basilea, Merck, and Schering-Plough and received speaker's fees from Gilead, Janssen Pharmaceutica, Merck, Pfizer, and Schering-Plough.

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