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Two new species of *Mucor* from clinical *samples*

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Two new species in the order Mucorales, *Mucor velutinosus* and *Mucor ellipsoideus*, isolated from human clinical specimens in the USA, are described and illustrated. The former species is similar to *Mucor ramosissimus*, from which it can be differentiated by its ability to grow at 37°C and produce verrucose sporangiospores. *Mucor ellipsoideus* is also able to grow and sporulate at 37°C like *M. indicus*, the nearest phylogenetic species in this study, however, the former has narrow ellipsoidal sporangiospores in contrast to the subglobose to ellipsoidal sporangiospores of *M. indicus*. Analysis of the sequences of the ITS and the D1–D2 regions of the rRNA genes confirmed the novelty of these species. The *in vitro* antifungal susceptibility of the new species showed that amphotericin B was active against all isolates and posaconazole and itraconazole showed low activity.

Keywords *Mucor,* Mucorales, mucormycosis, zygomycetes

Introduction

The phylum Zygomycota is an artificial assemblage of fungi of uncertain taxonomic position. Most of the genera of this phylum are currently included in the subphylum Mucoromycotina [1], *Mucor* being the genus with the highest number of species. Molecular studies have demonstrated that *Mucor* is a polyphyletic genus [2]. Members of *Mucor* are characterized by fast-growing colonies, simple or branched sporangiophores without basal rhizoids, non-apophysate sporangia, and zygospores having more or less equal, opposed, non-appendaged suspensors [3–5]. Several species of *Mucor* have important biotechnological applications, and are used in the elaboration of different kinds of Asian food [6–8]. *Mucor* is, after *Rhizopus*, the most clinically relevant genus of the Mucorales [9,10]. The species most frequently involved in human infections are *Mucor circinelloides*, *Mucor indicus*, *Mucor racemosus* and *Mucor ramosissimus* [3,9].

In a recent phylogenetic study of mucoralean species from the US, where a wide panel of clinical isolates were included, some isolates of *Mucor* could not be properly identified at the species level. These isolates showed important distinctive morphological characters and were resolved into two well-supported phylogenetic clades (*Mucor* sp. 1 and *Mucor* sp. 2) [9]. In the present study we have demonstrated that these two clades represent two new species of *Mucor* which are described and illustrated here.

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Materials and methods

Fungal isolates

A total of seven clinical isolates of *Mucor*, which could not be identified previously [9], are included in the present study. Three of these isolates were designed as *Mucor* sp. 1, a fourth isolate as *Mucor* sp. 2 and the other three were unnamed. The type or reference strains of the most clinically relevant species of the genus and some strains of species morphologically related to some of these isolates, such as *Mucor fragilis* and *Mucor fuscus*, were also included in this study (Table 1). All isolates were subcultured on potato dextrose agar (PDA, Pronadisa, Madrid, Spain) at room temperature (25°C) for 2–5 days.

DNA extraction, amplification and sequencing

DNA was extracted and purified directly from fungal colonies following the Fast DNA kit protocol (Bio101, Vista, Calif., USA), with a minor modification, consisting of a

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Table 1 Isolates included in the study and their origins.

Isolate	Species	Source	GenBank accession nº	
			ITS	28S rDNA
UTHSC 03-1044	*Mucor* sp.	Blood, Utah, USA	FN650648	FN650661
UTHSC 03-1823	*Mucor* sp.	Bronchial, Indiana, USA	FN650649	FN650662
UTHSC 04-2492	*Mucor* sp.	Right thigh, Minnesota, USA	FN650651	FN650663
UTHSC 02-1981	*Mucor* sp. 1	Central venous line, Connecticut, USA	FN650646	FN650672
UTHSC 06-1667	*Mucor* sp. 1	Blood, Minnesota, USA	FN650652	FN650656
UTHSC 04-1961	*Mucor* sp. 1	Blood, New York, USA	FN650650	FN650657
UTHSC 02-2090	*Mucor* sp. 2	Peritoneal dialysis fluid, Florida, USA	FN650647	FN650660
UTHSC 03-2919	*Mucor circinelloides* f. *circinelloides*	Forearm, Minnesota, USA	FN663959	n.d
UTHSC 05-3221	*Mucor circinelloides* f. *circinelloides*	Colorado, USA	FN663960	n.d
UTHSC 06-3784	*Mucor circinelloides* f. *circinelloides*	Utah, USA	FN663961	n.d
CBS 195.68^{NT}	*Mucor circinelloides* f. *circinelloides*	Environment, air, The Netherlands	FN650639	FN650667
CBS 384.95	*Mucor circinelloides* f. *circinelloides*	Face, China	FN663957	FN663953
CBS 108.17	*Mucor circinelloides* f. *lusitanicus*	Unknown	FN650644	FN650664
CBS 108.19	*Mucor circinelloides* f. *lusitanicus*	Unknown	FN650645	FN650665
CBS 236.35	*Mucor fragilis*	Tremella, Hann.-Münden, Germany	FN650655	FN650671
CBS 132.22^{T}	*Mucor fuscus*	Unknown	FN650653	FN650658
CBS 230.29	*Mucor fuscus*	France	FN650654	FN650659
CBS 201.65^{NT}	*Mucor hiemalis* f. *hiemalis*	Michigan, USA	FN650640	FN650668
CBS 226.29^{T}	*Mucor indicus*	Switzerland	FN650641	FN650669
UTHSC 01-667	*Mucor indicus*	Stoma tissue, Pennsylvania, USA	FN663955	n.d
UTHSC 02-2453	*Mucor indicus*	Hand wound, Rhode Island, USA	FN663956	n.d
CBS 260.68^{T}	*Mucor racemosus*	Switzerland	FN650642	FN650670
CBS 135.65^{NT}	*Mucor ramosissimus*	Nasal lesion, Uruguay	FN650643	FN650666
CBS 103.93^{T}	*Rhizomucor variabilis* var. *variabilis*	Man, China	FN663958	FN663954

CBS-KNAW, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA; ^{NT}, neotype strain; ^{T}, type strain; n.d, not determined.

homogenization step repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY, USA). DNA was quantified by the GeneQuant pro (Amersham Pharmacia Biotech, Cambridge, England). The internal transcribed spacer (ITS) region of the nuclear rRNA gene was amplified with the primer pair ITS5 and ITS4 [11] and the D1-D2 domains of 28S rRNA gene were amplified with the primer pair NL1-NL4 [12].

The PCR mix (25 µl) included 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 2.5 mM $MgCl_2$ (10X Perkin-Elmer buffer II plus $MgCl_2$ solution Roche Molecular Systems, Branchburg, NJ, USA), 100 µM each dNTP (Promega, Madison, Wis, USA), 1 µM of each primer and 1.5 U of Ampli*Taq* DNA polymerase (Roche). The amplification program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 1 min at 55°C, and extension for 1 min at 72°C. After PCR, the products were purified with an Illustra GFX™ PCR DNA and Gel Band Purification Kit (General Electric Healthcare, Buckinghamshire, UK) and stored at –20°C until they were used in sequencing. PCR products were sequenced by using the same primers used for amplification and following the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit protocol (Applied Biosystems, Gouda, The Netherlands). Reactions were run on a 310 DNA sequencer (Applied Biosystems). Consensus sequences were obtained using the Autoassembler program (PerkinElmer-Applied Biosystems) and Seqman software (Lasergene, Madison, Wis.). Some sequences, corresponding to several species of *Mucor*, which could be related to our clinical isolates**,** or other genera of the Mucorales which were phylogenetically related to *Mucor* in previous studies [2], were retrieved from GenBank and included in the phylogenetic study.

Phylogenetic analyses

Clustal X 1.8 was used to align the sequences, followed by manual adjustments with a text editor. For the individual analysis of the genes we used the software program MEGA 4.0. The maximum composite likelihood algorithm was used for the determination of evolutionary distances between sequences. Trees were generated using the neighbor-joining (NJ) method. Gaps were treated by the pairwise deletion option of MEGA. Support for internal branches was assessed by a search of 1000 bootstrapped sets of data.

Phenotypic studies

Isolates were cultured on PDA, Czapek agar (CZA; Difco, Becton Dickinson, France) and malt extract agar (MEA; 10 g of malt extract, 20 g of agar, and 1000 ml of distilled water), incubated at 25°C in darkness, and examined daily. Color notations in parentheses are from Kornerup and Wanscher [13]. Microscopic features were determined in

mounts on lactic acid. Photomicrographs were taken using a Zeiss Axio Imager M1 light microscope. Scanning electron microscope (SEM) micrographs were obtained with a Jeol JSM- 6400 scanning electron microscope using techniques described previously by Figueras and Guarro [14]. All isolates were characterized morphologically following traditional criteria [3–5,15–17]. Growth rates of the isolates at different temperatures (4, 15, 25, 30, 37, 42, 45 and 50°C) were determined on 90 mm diameter PDA Petri dishes that had been inoculated at the center, and the colony diameters (in mm) were measured daily for up to 10 days.

Antifungal susceptibility

The *in vitro* activity of amphotericin B (USP, Rockville, MD, USA), posaconazole (Schering-Plough Europe, Brussels, Belgium) and itraconazole (Janssen Pharmaceutica, Beerse, Belgium) against the seven clinical isolates was evaluated following a microdilution reference method (M38-A2) [18].

Nucleotide sequence accession numbers

Sequences of the ITS region and D1-D2 domains of the 28S rRNA gene of the isolates listed in Table 1 were deposited in GenBank. Accession numbers are shown in the same table.

Results

Phylogenetic analyses

With the primers used, the lengths of the amplicons of the ITS regions and D1–D2 domains of the 28S rRNA gene were 530–572 bp and 576–658 bp, respectively. The phylogenetic tree inferred from NJ analysis of the nucleotide sequences of the ITS (Fig. 1) revealed that *Mucor* sp. 1 and *Mucor* sp. 2 were located in two well supported clades. The three isolates of *Mucor* sp. 1 constituted a highly supported branch (100% bootstrap support (bs)) within the biggest clade (96% bs), where also were placed *Mucor circinelloides* f. *circinelloides*, *M. circinelloides* f. *lusitanicus*, *M. fragilis*, *M. plumbeus*, *M. racemosus* and *M. ramosissimus*, and the clinical isolates UTHSC 03-1823, UTHSC 03-1044 and 04-2492. Isolates UTHSC 03-1823 and UTHSC 03-1044 were genetically very close to reference strains of *M. circinelloides* f. *lusitanicus* and to the sequence EU484227 deposited in GenBank. Isolate UTHSC 04-2492 was nested with a reference strain (CBS 236.35) of *M. fragilis* but two sequences retrieved from GenBank (EU862184 and EU484238), deposited as *M. fragilis*, were clearly separated from them. The isolate of *Mucor* sp. 2 was nested in a very distant clade (94% bs) together with *M. amphibiorum* and *M. indicus*, but clearly separated from these species. With this marker, the species of *Mucor* phylogenetically more distant were *Mucor flavus* and *M. hiemalis*, the latter forming a clade with the type of *Rhizomucor variabilis* var. *variabilis*. Species of several genera which other authors [2] reported previously as closely related to *Mucor*, such as *Actinomucor elegans* and *Parasitella parasitica* were more separated.

The phylogenetic tree inferred from analyses of the D1–D2 domains of the 28S rRNA gene (Fig. 2) possessed a topology similar to that of the ITS tree. The seven clinical isolates investigated in this study showed similar genetic relationships to that in Fig. 1.

Phenotypic analyses

The minimum, optimal and maximum growth temperatures of the isolates of *Mucor* sp. 1 were 7°C, 25°C and 37°C, respectively. These isolates grew well on all media tested, producing greyish velvety colonies. Microscopically, their sporangiophores were mostly sympodially branched, and the sporangiospores were globose to subglobose, thick-walled and verrucose. The isolate of *Mucor* sp. 2 also had a minimum, optimal and maximum growth temperatures of 7°C, 25°C and 37°C, respectively, but formed white to pale yellow colonies in all media tested. Microscopically, the sporangiophores were simple or sympodially branched, and the sporangiospores were narrowly ellipsoidal, and thick- and smooth-walled. The minimum, optimal and maximum growth temperatures of the clinical isolates UTHSC 03-1823, UTHSC 03-1044 and UTHSC 04-2492 of *Mucor* sp. were 15°C, 25°C and 37°C, respectively. Microscopic features of these three isolates were similar, i.e. their sporangiophores were sympodially branched, and the sporangiospores were very variable in shape (ovoid, ellipsoidal, subspherical or irregular), and thin- and smooth-walled.

Antifungal susceptibility

The minimal inhibitory concentrations of the antifungal drugs tested are shown in Table 2. Amphotericin B was active against all isolates. Posaconazole and itraconazole demonstrated low activity.

Based on the molecular and phenotypic results, we propose *Mucor* sp. 1 and *Mucor* sp. 2 as the following new species.

Mucor velutinosus **E. Álvarez, Stchigel, Cano, D. A. Sutton & Guarro, sp. nov.** (Fig. 3A–D; Fig. 4; Fig. 5)

= *Mucor* sp. 1 sensu Álvarez *et al*. *J Clin Microbiol* 2009; **47**: 1650–1656.

Ad 25°C in agaro cum decocto malturorum (MEA) coloniae velutinosae, 0.5–2 mm altae, Petri-patellas in die quarto, primum albae, cito cinerea (M.1E1), in adversum albidum. Sporangiophora erecta, simplicia et simpodialiter

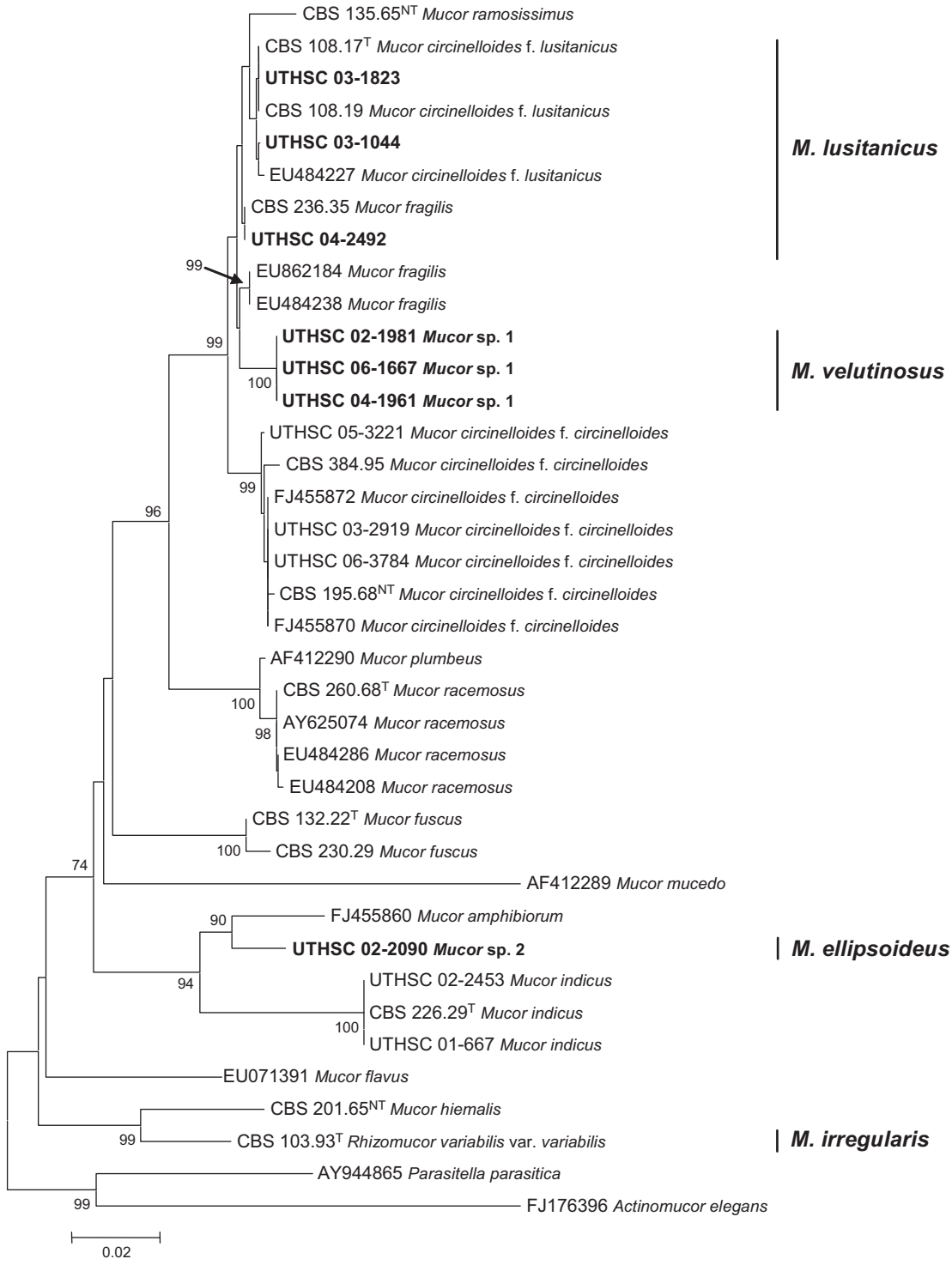

Fig. 1 Neighbor-joining tree based on maximum composite likelihood corrected nucleotide distances among the ribosomal internal transcribed spacer (ITS) regions and 5.8S rRNA gene sequences of the strains of *Mucor* and related taxa. Bootstrap support values above 70% are indicated at the nodes. The bar indicates genetic distance. Sequences of *Parasitella* and *Actinomucor* were used to root the phylogram.

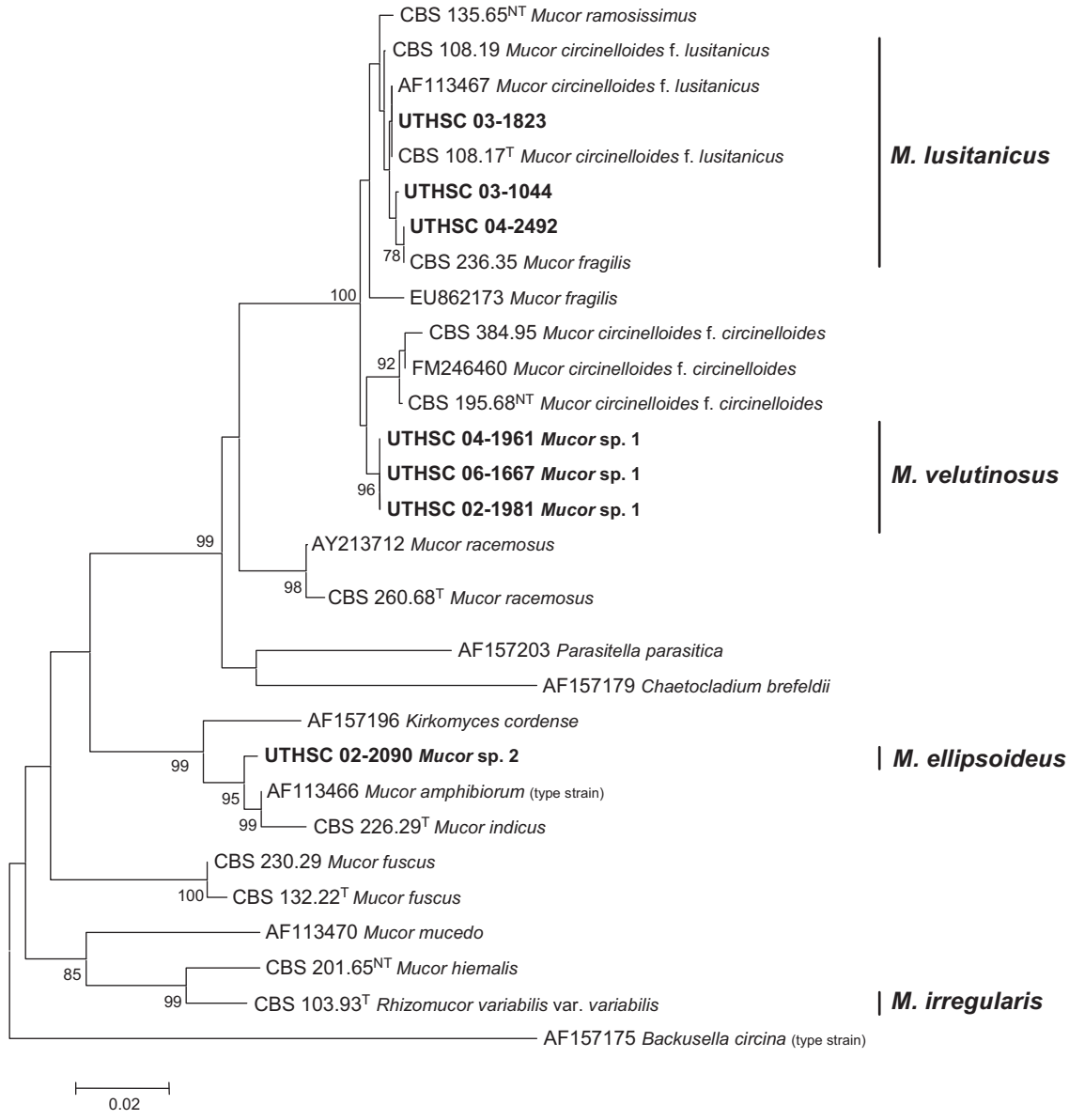

Fig. 2 Neighbor-joining tree based on maximum composite likelihood corrected nucleotide distances among the D1-D2 domains 28S rRNA gene sequences of the strains of *Mucor* and related taxa. Bootstrap support values above 70% are indicated at the nodes. The bar indicates genetic distance. A sequence of *Backusella* was used as the outgroup to root phylogram.

ramosa, 200–1000 µm longa, 4–15 µm lata; rami sporangiophoris 10–200 µm longi, 5–12 µm lati, eseptati vel cum septum 1-pluribus, hyalini vel brunnei, cum sporangio terminatibus nonapophysati. Sporangia globosa vel subglobosa, 15–60 µm in diam, griseola vel griseola-brunnea, parietibus subpersistentibus, lente tabidis vel disruptis. Columellae globosae vel turbinatae, 10–50 µm in diam, hyalinae vel brunneae; collaria distincta. Sporangiosporae globosae, subglobosae, ovoideae vel irregulares, 4–15 µm in diam, griseo-brunneae, eminenter verrucosae. Zygosporae ignotae.

Colonies velvety, 0.5–2 mm high, filling the Petri dish after 4 days of incubation at 25°C on MEA, at first white, soon becoming mid gray (M. 1E1), reverse whitish. Rhizoids abundant. Sporangiophores erect, mostly sympodially branched (5–15 times), 200–1000 µm long, 4–15 µm wide; branches 10–200 µm long, 5–12 µm wide, nonseptate or with one septum, colourless or brownish, simple, terminating in a non-apophysate sporangium. Sporangia globose or subglobose, 15–60 µm diam, grey to greyish-brown, wall slowly dissolving, sometimes broken. Columellae globose to conical, 10–50 µm diam, hyaline to

Table 2 Results of *in vitro* antifungal susceptibility testing.

	MIC (μg/ml)					
	AMB		PSC		ITC	
Strain	24 h	48 h	24 h	48 h	24 h	48 h
Mucor sp.1 UTHSC 02-1981	0.125	0.25	1	4	1	4
Mucor sp.1 UTHSC 04-1961	0.06	0.25	2	2	4	16
Mucor sp.1 UTHSC 06-1667	0.06	0.125	0.5	2	1	2
Mucor sp.2 UTHSC 02-2090	0.06	0.25	2	>16	4	>16

brown; collar evident. Sporangiospores globose, subglobose, ovoid or irregular, thick-walled, greyish-brown, 4–15 μm diam, coarsely verrucose. Chlamydospores terminal or intercalary, single or in short-chains (up to 4), hyaline to subhyaline, globose, ellipsoidal, barrel-shaped, cylindrical or irregular, 9–25 × 6–15 μm, very thick-walled, formed on hyphae, sporangiophores. Zygospores absent.

Colonies on PDA similar to those on MEA. On CZA the colonies were hyaline, and the reverse concolorous, with scarce aerial mycelium up to 2 mm high. The optimum growth temperature was 25°C and the minimum 7°C. At 37°C growth occurred but sporulation was not observed. The fungus failed to grow at 42°C.

Holotype. CBS H-20399, from central venous line site, Connecticut, USA. Living cultures ex-type FMR 10020, UTHSC 02-1981 and CBS 126272

Etymology. The epithet *velutinosus* refers to the velvety texture of the colonies.

***Mucor ellipsoideus* E. Álvarez, Cano, Stchigel, D. A. Sutton & Guarro, sp. nov.** (Fig. 4B; Fig. 6A–D)

= *Mucor* sp. 2 sensu Álvarez *et al*. *J Clin Microbiol* 2009; **47**: 1650–1656.

Ad 25°C in agaro cum decocto malturorum (MEA) coloniae velutinosae, 2–5 mm altae, Petri-patellas in die nono, primum albae, cito flavidae (M. 4A3), in adversum flavidum (M. 4A4). Sporangiophora erecta, simplicia et simpodialiter ramosis ad basim et bifurcata vel trifurcata ad apicem, 1000–3000 μm longa, 4–10 μm lata; rami sporangiophoris 20–1000 μm longi, 3–6 μm lati, eseptati vel cum septum 1-pluribus, hyalini, cum sporangio terminatibus tenuiapophysati. Sporangia subglobosa, (11–) 16–48 × (12–) 18–50 μm, pallide aurantiobrunnea, cum parietibus spinulosus, lente tabidis vel disruptis. Columellae globosae vel subglobosae, 10–45 μm in diam, hyalinae; collaria indistincta. Sporangiosporae 4–8 × 2–3 μm, hyalinae, levitunicatae. Zygosporae ignotae.

Colonies velvety, 2–5 mm high, filling the Petri dish after 9 days of incubation at 25°C on MEA, at first white, soon becoming pale yellow (M. 4A3), reverse pale yellow (M. 4A4). Rhizoids very abundant, arising from various parts of the hyphae, simple or branched, non-septate, straight or curved, soon evanescent. Sporangiophores erect, simple and sympodially branched (5–10 times) at the base, and bi- to trifurcate at the apex, arising directly from superficial and aerial hyphae, 1000–3000 μm long, 4–10 μm wide; branches 20–1000 μm long, 3–6 μm wide, non-septate or with one septum, colourless, simple. Sporangia subglobose, (11–) 16–48 × (12–) 18–50 μm, pale yellowish-brown, with a spinulose wall, slowly dissolving, sometimes broken. Columellae globose to conical, 10–45 μm diam, hyaline, persistent, collapsing at the base; collar not evident. Sporangiospores narrowly ellipsoidal (sometimes flattened at one side), 4–8 × 2–3 μm, hyaline, smooth-walled. Chlamydospores abundant, oidia-like, thick-walled, terminal or intercalary, single or in long chains, hyaline to subhyaline, globose, ellipsoidal, barrel-shaped, cylindrical or irregular, 4–30 × 7–20 μm, very thick-walled, formed on hyphae, sporangiophores, sporangia, and rhizoids. Zygospores absent.

Colony features on PDA were similar to those seen on MEA. On CZA the colonies were hyaline with a concolorous reverse. The optimum growth temperature was 25°C and the minimum 7°C. At 37°C growth and sporulation was observed. The fungus did not grow at 42°C.

Holotype. CBS H-20398, from peritoneal dialysis fluid of a human patient with chronic renal failure, Florida, USA. Living cultures ex-type FMR 10021, UTHSC 02-2090 and CBS 126271.

Etymology. The epithet *ellipsoideus* refers to the shape of the sporangiospores.

Discussion

Variability of nucleotide sequences of the ITS region has been reported by several authors as a useful character to discriminate taxa included in the order Mucorales [19,20]. The ISHAM working group on fungal molecular identification also proposed the use of ITS sequences as a reliable method for that purpose [21]. In a previous study, by using this marker, we were able to identify numerous species of Mucorales from clinical specimens in the US [9]. However, several *Mucor* isolates could not be identified in that study. Here they could be unambiguously identified after morphologically and genetically comparing these isolates with reference strains of related *Mucor* species. The isolates UTHSC 04-1961, UTHSC 02-1981 and UTHSC 06-1667 are described herein as the new species *M. velutinosus*.

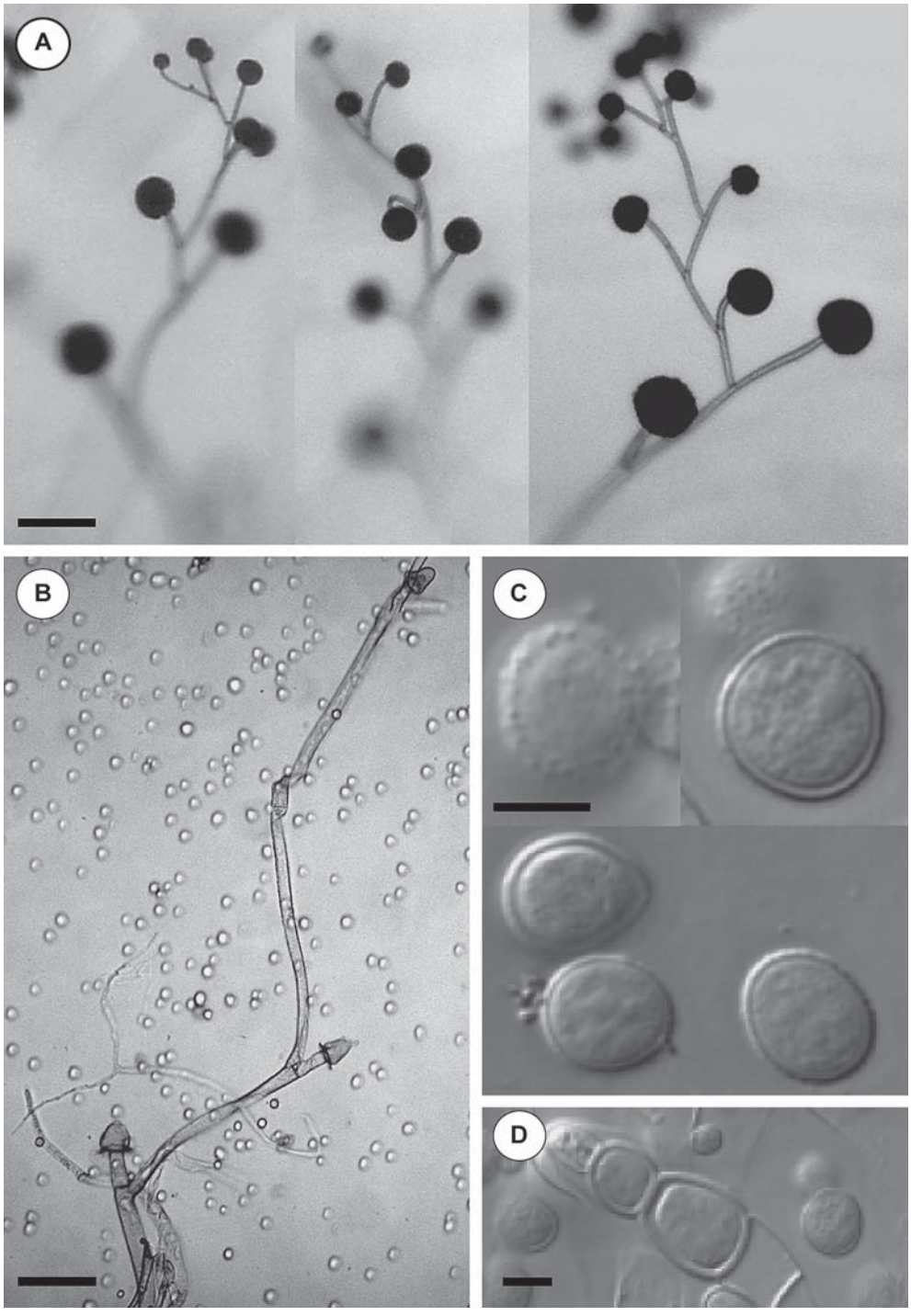

Fig. 3 *Mucor velutinosus* UTHSC 02-1981. (A) Branching pattern of sporangiophores. (B) Columellae. (C) Sporangiospores. (D) Chlamydospores. Bars: A–B = 50 μm; C–D = 5 μm.

This species showed a close molecular relationship with *M. circinelloides* and *M. ramosissimus*, but it can be differentiated from them by the production of larger and ornamented sporangiospores. *Mucor velutinosus* also shared some similarities with other species of *Mucor* with sympodially branched sporangiophores and globose sporangiospores, such as *M. amphibiorum*, *M. fuscus* and *M. plumbeus.* However, *Mucor amphibiorum* does not grow at 37°C, forms taller (up to 25 mm) colonies, broader (up to 20 μm) sporangiophores and smooth-walled and

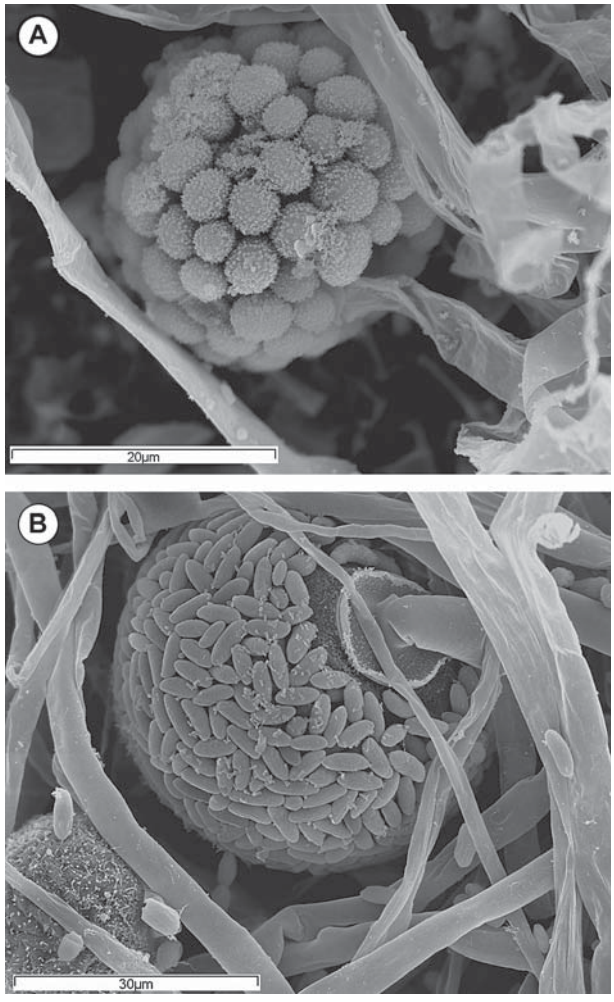

Fig. 4 *Mucor velutinosus* UTHSC 02-1981. (A) Sporangium and sporangiospores (SEM). *Mucor ellipsoideus* UTHSC 02-2090. (B) Sporangium and sporangiospores (SEM). Scale is indicated by bars.

smaller (3.4–5.4 µm diam) sporangiospores; *Mucor fuscus* is the most similar species to *M. velutinosus* since both produce ornamented sporangiospores, but this ornamentation consists of minute wall protrusions in *M. fuscus*, being coarsely verrucose in *M. velutinosus*. Moreover, *M. fuscus* differs from *M. velutinosus* by its lack of growth at 37°C, formation of pyriform and bigger (up to 80 × 52 µm) columellae and sporangia (up to 140 µm diam), and slightly smaller sporangiospores (up to 11 µm diam). *Mucor plumbeus*, also produces globose and verrucose sporangiospores, but its sporangiospores are less coarsely ornamented than those of *M. velutinosus*, it produces columellae with one to several projections, and fails to grow at 37°C.

Isolate UTHSC 02-2090 is described herein as the new species *Mucor ellipsoideus*. This species is genetically related to *M. amphibiorum* and *M. indicus*. However, *M. ellipsoideus* is easily differentiated from these, because

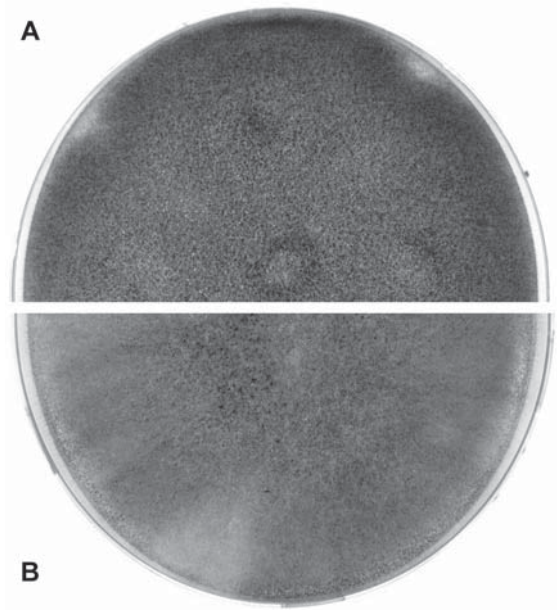

Fig. 5 Morphology of colonies on PDA. (A) *Mucor velutinosus* UTHSC 02-1981. (B) *Mucor racemosus* CBS 260.68.

M. amphibiorum does not grow at 37°C, and produces smaller and spherical sporangiospores, and *M. indicus* grows at 42°C, and produces smaller sporangia and smaller ellipsoidal sporangiospores [3,17].

The other three clinical isolates, UTHSC 03-1823, UTHSC 03-1044 and UTHSC 04-2492, were identified as *M. lusitanicus*. Schipper [17] had considered *M. circinelloides* f. *lusitanicus* as different from *M. circinelloides* f. *circinelloides* based on the shape and size of the sporangiospores. We examined these fungi morphologically and agree with Schipper that they are phenotypically different. These differences were confirmed in our phylogentic study; however, we conclude as previously reported, that these two fungi must be considered as two different species, i.e., *M. lusitanicus* and *M. circinelloides* [22,23], instead of two *formae* of the same species. Consequently, the two clinical isolates were identified as *M. lusitanicus*.

Isolate UTHSC 04-2492 grouped, in our phylogenetic analyses, with a strain of *M. fragilis* (CBS 236.35), and two sequences deposited in the GenBank as *M. fragilis* (EU862184 and EU484238). Unfortunately, the type of this species does not exist and strain CBS 236.35, identified by Zycha in 1935 [24], is the only reference strain available of that species. We agree with Schipper [15] that this strain differs from the original description of the species [25] in important characters, e.g., it does not produce zygospores, and its sporangiospores are larger (4–10 × 3–7 µm vs. 4.2 × 2.1 µm in *M. fragilis* original description) and they are not bluish. By contrast, this isolate is morphologically similar to the strains of *M. lusitanicus*

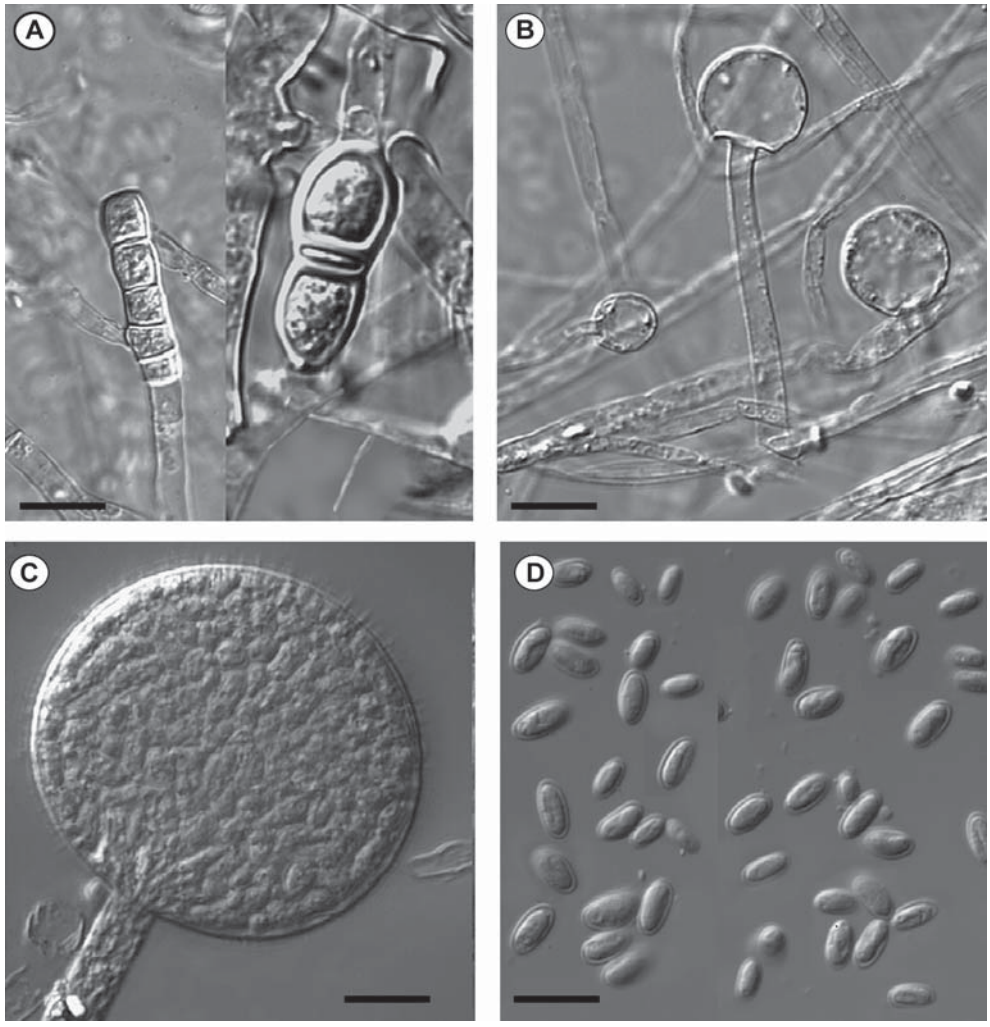

Fig. 6 *Mucor ellipsoideus* UTHSC 02-2090. (A) Chlamydospores. (B) Columellae. (C) Sporangium. (D) Sporangiospores. All bars 10 µm.

included in our study, showing a percentage of similarity with the type strain of this species (CBS 108.17) of 98% (ITS region) and 99.6% (D1-D2 domains).

Taxonomy of the genus *Rhizomucor*, a genus morphologically close to *Mucor*, is unresolved. Previously, we demonstrated that *Rhizomucor variabilis* var. *regularior* and *R. variabilis* var. *variabilis* were two different species genetically related to *Mucor* spp. and clearly separate from *Rhizomucor pusillus*, the type species of the genus [9]. *Rhizomucor variabilis* var. *regularior* is morphologically and genetically indistinguishable from *M. circinelloides* [9] and *R. variabilis* var. *variabilis,* in the present study, is genetically related to *Mucor hiemalis* and *M. mucedo*. Production of rhizoids and thermophilic capabilities have been traditionally considered as the main features that differentiate *Rhizomucor* from *Mucor*; however, these characters should be considered as homoplasious, since several species of *Mucor*, such as *M. circinelloides* and *M. velutinosus* produce rhizoids too, and also some species of *Mucor*, such as *M. indicus,* are thermotolerant. A detailed examination of the most important morphological characteristics of *R. variabilis* var. *variabilis* and *R. variabilis* var. *regularior* revealed that there are not enough features to separate them from *Mucor* and hence we propose the following taxonomic changes:

Mucor circinelloides Tieghem – Annls Sci. nat. 1: 94. 1875

Mucor ambiguus Vuill. – Bull. Soc. Sci. Nancy 8: 92. 1886

Mucor alternans Tieghem in Gayon & Dubourg – Annls Inst. Pasteur, Paris 1: 532. 1887

Mucor javanicus Wehmer – Zentbl. Bakt. ParasitKde, Abt. 2, 6: 619. 1900

Mucor dubius Wehmer – Zentbl. Bakt. ParasitKde, Abt. 2 , 7: 318. 1901 (*nomen provisorium*).

Mucor prainii Chodat & Nechitch in Nechitch – Sur les ferments de deux levains de I'Inde. Le Mucor Prainii et le Dematium chodati, Geneve, p. 38. 1904

Mucor griseo-roseus Linnemann – Beitrag zu einer Flora der Mucorineae Marburgs, p. 189. 1936

Mucor ramificus B. S. Mehrotra & Nand – Sydowia 20: 69. 1966

Mucor mandshuricus Saito – Rep. cent. Lab. S. Mandsch. Railw. Co. 1: 16. 1914 = *Mucor circinelloides* Tieghem var. *mandshuricus* (Saito) Milko – Atlas mucoral'nykh gribov, p. 67. 1971

Circinomucor circinelloides (Tiegh.) Arx – Sydowia **35**: 18. 1982

Rhizomucor variabilis var. *regularior* R. Y. Zheng & G. Q. Chen - Mycosystema **6**: 2. 1993

M***ucor lusitanicus*** Bruderl. – Bull. Soc. Bot. Genève **8**: 276. 1916

Mucor griseo-lilacinus Povah – Bull. Torrey bot. Club **44**: 301. 1917.

Mucor jauchae Lendner – Bull. Soc. bot. Geneve **10**: 374. 1918

Mucor zeicolus Graff – CBS List of Cultures, p. 71. 1939 (*nomen nudum*)

Mucor racemosus Fresen var. *lusitanicus* (Bruderlein) Naumov – Clés des Mucorinées (Mucorales), p. 47. 1939

Mucor circinelloides Tiegh. f. *lusitanicus* (Bruderl.) Schipper – Stud. Mycol. **12**: 9. 1976

Mucor irregularis Stchigel, Cano, Guarro & Álvarez, **nom. nov.**

= *Rhizomucor variabilis* var. *variabilis* R. Y. Zheng & G. Q. Chen, Mycosystema **4**: 47. 1991

Non *M. variabilis* A.K. Sarbhoy, Trans. Br. mycol. Soc. **48**: 559. 1965.

In the D1–D2 rDNA phylogeny, *Chaetocladium brefeldii*, *Kirkomyces cordense*, and *Parasitella parasitica* were genetically related to some species of *Mucor*. Such a relationship was also reported by O'Donnell *et al*. [2] based on phylogenetic analyses of the 18S rDNA and translation elongation factor genes. On the basis of the clear polyphyly of *Mucor*, further molecular phylogenetic analyses are needed to delimit natural taxa and relationships. The genus *Parasitella* was erected by Bainier [25] (1903) to accommodate *Mucor parasiticus*, which is morphologically related to other species of the genus *Mucor*, but differs from them by its ability to parasite other Mucorales and by the presence of digitiform projections on the suspensors of

Key to distinguish the most relevant clinical species of *Mucor*

(1) Sporangiophores unbranched or weakly branched 2
Sporangiophores repeatedly branched 3
(2) Sporangiospores spherical, 3.4–5.4 µm in diam ..... *M. amphibiorum*
Sporangiospores ellipsoidal, 5.7–8.7 × 2.7–5.4 µm ..... *M. hiemalis*
(3) Growth at 40°C ..... *M. indicus*
No growth at 40°C 4
(4) Sporangiospores globose or nearly so 5
Sporangiospores otherwise 8
(5) Sporangiospores verrucose 6
Sporangiospores smooth-walled 7
(6) Growing at 37°C; sporangiospores coarsely ornamented ..... *M. velutinosus*
No growth at 37°C; sporangiospores finely ornamented ..... *M. plumbeus*
(7) Growing at 35°C; sporangiophores with several swellings; sporangia on short lateral branches; sporangiospores 5.0–8.0 × 4.5–6.0 µm ..... *M. ramosissimus*
No growth at 35°C; sporangiophores lack swellings; sporangia on long lateral branches; sporangiospores 5.5–10.0 × 4.0–7.0 µm ..... *M. racemosus*
(8) Sporangiospores ellipsoidal, 4.4–6.8 × 3.7–4.7 µm ..... *M. circinelloides*
Sporangiospores very variable in shape 9
(9) Columellae irregular in shape; sporangiospores 2.5–16.5 × 2.0–7.0 µm ..... *M. irregularis*
Columellae globose; sporangiospores 5.5–17.6 × 3.4–12.8 µm ..... *M. lusitanicus*

the zygospores [26]. Later, other authors [5,24,27] also differentiated *Parasitella* from *Mucor* by the production of nodose vegetative structures derived from the fusion of the parasite and the host hyphae. However, these morphologic and ecologic differences do not appear to be sufficient to consider *Parasitella* and *Mucor* as separated genera. The placement of *Chaetocladium* and *Kirkomyces* intermixed with different *Mucor* species in the phylogenetic trees reported by O'Donnell *et al*. [2] demonstrated the polyphyly of *Mucor*.

Antifungal susceptibility results showed that amphotericin B is the most active antifungal agent against the new species (Table 2), a fact reported by many other authors for other species of *Mucor* of clinical importance [28]. Posaconazole and itraconazole showed limited activity.

A dichotomous key to distinguish the most relevant clinical species of *Mucor* is provided (*M. ellipsoideus* is not included since it was only isolated once from peritoneal dialysis fluid and its true clinical role is problematic).

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References

1 Hibbett DS, Binder M, Bischoff JF, *et al*. A higher-level phylogenetic classification of the fungi. *Mycol Res* 2007; **111**: 509–547.
2 O'Donnell K, Lutzoni FM, Ward TJ, Benny GL. Evolutionary relationships among mucoralean fungi (Zygomycota): Evidence for family polyphyly on a large scale. *Mycologia* 2001; **93**: 286–296.
3 Hoog GS de, Guarro J, Gené J, Figueras MJ. *Atlas of Clinical Fungi*. 2nd ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, University Rovira i Virgili, Reus, Spain; 2000.
4 Schipper MAA. On certain species of *Mucor* with a key to all accepted species. *Stud Mycol* 1978; **17**: 1–52.
5 Zycha H, Siepmann R, Linnemann G. *Mucorales: Eine Beschreibungaller Gattungen und Arten Dieser Pilzgruppe*. Cramer, Lehre (Germany), 1969.
6 Abe A, Sujaya IN, Sone T, Asano K, Oda Y. Microflora and selected metabolites of potato pulp fermented with an Indonesian starter *Ragi Tapé*. *Food Technol Biotechnol* 2004; **42**: 169–173.
7 Millati R, Edebo L, Taherzadeh MJ. Performance of *Rhizopus*, *Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and woodhydrolyzates. *Enzyme Microb Technol* 2005; **36**: 294–300.
8 Shetty PK, Mitra J, Murthy NBK, *et al*. Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermohyalospora* MTCC 1384. *Curr Sci* 2000; **79**: 1381–1383.
9 Álvarez E, Sutton DA, Cano J, *et al*. Spectrum of zygomycete species identified from clinically significant specimens in the United States. *J Clin Microbiol* 2009; **47**: 1650–1656.
10 Ribes JA, Vanover-Sams CL, Baker DJ. Zygomycetes in human disease. *Clin Microbiol Rev* 2000; **13**: 236–301.
11 White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (ed.), *PCR Protocols: A Guide to the Methods and Applications*. New York: Academic Press, 1990: 315–322.
12 O'Donnell K. *Fusarium* and its near relatives. In: D. R. Reynolds & J. W. Taylor (eds), *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*. Wallingford: CAB International, 1993: 225–233.
13 Kornerup A, Wanscher JH. *Methuen Handbook of Color*. 3rd ed. London, UK: Methuen, 1978.
14 Figueras MJ, Guarro J. A scanning electron microscopic study of ascoma development in *Chaetomium malaysiense*. *Mycologia* 1988; **80**: 298–306.
15 Schipper MAA. A study on variability in *Mucor hiemalis* and related species. *Stud Mycol* 1973; **4**: 1–40.
16 Schipper MAA. *Mucor mucedo*, *Mucor flavus* and related species. *Stud Mycol* 1975; **10**: 1–33.
17 Schipper MAA. On *Mucor circinelloides*, *Mucor racemosus* and related species. *Stud Mycol* 1976; **12**: 1–40.
18 National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard-second edition. Document M38-A2. National Committee for Clinical Laboratory Standards, Wayne, PA, 2008.
19 Schwarz P, Bretagne S, Gantier JC, *et al*. Molecular identification of Zygomycetes from culture and experimentally infected tissues. *J Clin Microbiol* 2006; **44**: 340–349.
20 White MM, James TY, O'Donnell K, *et al*. Phylogeny of the Zygomycota based on nuclear ribosomal sequence data. *Mycologia* 2006; **98**: 872–884.
21 Balajee SA, Borman AM, Brandt ME, *et al*. Sequence-based identification of *Aspergillus, Fusarium* and *Mucorales* in the clinical mycology laboratory: where are we and where should we go from here? *J Clin Microbiol* 2009; **47**: 877–884.
22 van Tieghem P, Nouvelles recherches sur les Mucorinées. *Annl Sci Nat* 1875; **1**: 5–175.
23 Bruderlein J, *Mucor lusitanicus* n.sp. *Bull Soc Bot Genève* 1916; **8**: 273–276.
24 Zycha H, Pilze II. *Mucorineae*. Kryptogamenflora der Mark Brandenburg 6a. Leipzig, 1935.
25 Bainier G, Sur quelques espèces de Mucorinées nouvelles ou peu connues. *Bull Soc Mycol Fr* 1903; **19**: 153–172.
26 Burgeff H. Untersuchungen über Sexualität und Parasitismus bei Mucorineen I. *Bot Abh* 1924; **4**: 1–135.
27 Naumov NA, *Clés des Mucorinées (Mucorales)*. Paris: P. Lechevalier, 1939.
28 Almyroudis N, Sutton DA, Fothergill AW, Rinaldi MG, Kusne S, *In vitro* susceptibilities of 217 clinical isolates of Zygomycetes to conventional and new antifungal agents. *Antimicrob Agents Chemother* 2007; **51**: 2587–2590.

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