TAXONOMY AND PHYLOGENY OF CLINICALLY IMPORTANT MUCORALEAN FUNGI

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Dipòsit Legal: T-1721-2011

UNIVERSITAT ROVIRA I VIRGILI

DEPARTAMENT DE CIÈNCIES MÈDIQUES BÀSIQUES

Taxonomy and phylogeny of clinically important mucoralean fungi

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Doctoral thesis

2011

UNIVERSITAT ROVIRA I VIRGILI

Departament de Ciències Mèdiques Bàsiques
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STATE THAT:

The present work entitled “Taxonomy and Phylogeny of clinically important mucoralean fungi” presented by Eduardo Alvarez Duarte to obtain the degree of doctor by the University Rovira i Virgili, has been carried out under my supervision at the Unit of Microbiology of the Department of Basic Health Sciences, and that it fulfills the requirements to obtain the European Doctorate mention.

Reus, July 10, 2011

Dr. Josep Guarro Dr. José F. Cano Lira Dr. Alberto M. Stchigel

Acknowledgments

I would like to end this thesis by acknowledgments the people who one way or another contributed to the success of this work. However it is difficult to recall every person that has assisted, contributed or more importantly encouraged me to pursue these studies.

I would like to show my gratitude to Dr. Josep Guarro for his support, encouragement and guides during these years; and for giving me the opportunity to work in his lab.

I would like to thanks to Dr. José Cano and Dr. Alberto Stchigel for giving me advices and for the guidance in these years.

Thanks to Dr. Josepa Gené and all others professors of the Unit of microbiology of URV, for the assistance among these years.

I would like to sincerely thanks to Dr. Luis Zaror and Dr. Eduardo Valenzuela, two Chilean mycologists from who I learned so much about the fungi.

I am very grateful to Dr. Cony Decock (MUCL; Mycotheque de l'Université catholique de Louvain, Belgique) for accepting me in his research group for 3 months, and for his encouragement and sense of humor. Thanks for sharing your time with me. I also want to express my gratitude to other people who helped me during my stay at the MUCL: Prudence and the little Noora, Mario, Céline, Stéphanie, Olivier, Oscar, Mateus, Paul, Madu…..thanks to all the staff at MUCL.

I really appreciate the friendship, help and encouragement of my labmates at URV: Luis, Hugo, Valentina, Fabiola, Anabel, Haybrig, Mónica, Carolina, Enrique, Mar, Margarita, Keith, Mabel, Carme……many names!!!!, in general, thanks to all people in the lab.

Thanks to my friends Shiva and Max; especially for your good sense of humor and advices.

Thanks to Cati Nuñez and Núria Pilas for your help during these years.

Thanks to Montse and Inma, for your help and good will.

I owe my deepest gratitude to my dear parents, Eduardo and Olga, thanks for giving me solid roots from which I can grow, for being a constant and active presence in my life, for show me the way with your example. In the same way, thanks to my brother and sister, for your love and respect.

Finally, I wish to thank to all people with whom I have shared experiences, to those which with the gift of their company and friendship made my days more enjoyable and worth living.

CONTENTS

ANI	anidulafungin
ABLC	amphotericin B lipid complex
AFLP	Amplified Fragment Length Polymorphism
AMB	amphotericin B
ATCC	American Type Culture Collection
BAL	bronchoalveolar lavage
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
ca.	*circa*, Latin word meaning “around” or “approximately”
CAS	caspofungin
CBS	Centraalbureau voor Schimmelcultures
CFU	colony-forming unit
CI	consistency index
CLSI	Clinical Laboratory Standards Institute
CZA	Czapek`s agar
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP´s	deoxyribonucleotides triphosphate
EF	elongation factor
EMBL	European Molecular Biology Laboratory
et al.	*et alii*, Latin expression meaning “and others”
FMR	Facultad Medicina Reus
g	grams
GMCH	Government Medical College and Hospital
H3	histone 3

ILD	incongruence length difference
IMI	International Mycological Institute
ISHAM	International Society for Human and Animal Mycology
ITR	itraconazole
ITS	internal transcribed spacer
KCl	potassium chloride
Kg	kilogram
L	litre
M	molar
MEA	malt extract agar
MFG	micafungin
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimal inhibitory concentration
ML	maximum likelihood
MLI	maximal level of identity
MP	maximum parsimony
MPT	most parsimonious tree
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NCCLS	National Comitee for Clinical Laboratory Standards
NJ	neighbor-joining
NRCMA	National Reference Center for Mycoses and Antifungals
NRRL	Agricultural Research Service Culture Collection
PCA	potato carrot agar

PCR	polymerase chain reaction
PDA	potato dextrose agar
PFGE	Pulse Field Gel Electrophoresis
PHT	partition homogeneity test
PSC	posaconazole
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragments Length Polymorphism
RI	retention index
RNA	ribonucleic acid
RVC	ravuconazole
TBR	tree bisection reconnection
TER	terbinafine
µL	microlitre
USA	United States of America
UTHSC	Fungus Testing Laboratory, University of Texas Health Science Center
VRC	voriconazole

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1. INTRODUCTION

1.1 General features of zygomycetes

The zygomycetes represent approximately 1% of the species of fungi described so far (Kirk *et al.,* 2001). The number of fungal species that exist is estimated to be around 1.5 million (Hawksworth, 1991, 2001; Kirk *et al*., 2001), so the number of zygomycetes not yet found could be around 10.000 (Hawksworth, 1991, 2001; Hawksworth and Rossman, 1997; Kirk *et al*., 2001). It has been postulated that the zygomycetes were the first species of fungi to colonize the land, even before the plants (about 600 million years ago) (Berbee and Taylor, 2001; Heckman *et al*., 2001). Their ancestors developed several strategies for interacting with plants, animals and other organisms, ranging from parasitic to mutualistic symbiotic associations. Like other fungi, zygomycetes are heterotrophic and grow inside the substrate, dissolving it with extracellular enzymes and taking up nutrients by absorption. They act as decomposers in soil and dung, thereby playing a significant role in the carbon cycle. Some zygomycetes are important in industry as biocatalysts in the production of steroids, due to the production of organic acids (citric and gluconic) and carotenoid compounds, and because they are involved in various fermented food processes (Certik and Shimizu, 1999; Hesseltine and Ellis, 1973). Some species are used in several Asian foods, for example *Rhizopus oligosporus* in *tempeh*, and *Actinomucor elegans* in the fermentation of *sufu* (Hesseltine, 1991). By contrast, several species, for example *Rhizopus stolonifer* and *Choanephora cucurbitarum* have a negative impact on the economy, causing rot during the storage of plants and meat. Other species like *Apophysomyces elegans*, *Mucor circinelloides*, *Rhizopus oryzae*, and *Saksenaea vasiformis*, are able to infect both immunocompromised and immunocompetent people (de Hoog *et al*., 2000; Ribes *et al*., 2000; Roden *et al*., 2005). Some of these species are commonly isolated from animals and soil in tropical and subtropical areas.

The zygomycetes are characterized by a fast growth on high sugar content substrates, such as berries and grapes. These fungi are common in both terrestrial and aquatic environments, having a worldwide distribution. Macroscopically, they show an expanding mycelium with a woolly appearance (Fig. 1a), mainly white-gray (rarely orange or brown), which can reach several centimeters in height, as in the *Blakeslea* and *Phycomyces* species. Microscopically, they are characterized by thin-walled hyphae (Fig 1b, c), composed mainly of the polysaccharides chitin and glucans, and branched in right angles. Unlike the ascomycetes and the basidiomycetes, whose hyphae are regularly septate, most of the zygomycetes usually (with the exception of the members of the classes Kickxellales and Harpellales) present coenocytic (not divided by septa) hyphae (Fig 1b, c). A unique character that defines the zygomycetes is the production of sexual spores called zygospores (Fig. 1d), which are usually pigmented structures with a smooth or rough-walled surface, formed by the fusion of specialized hyphae (gametangia; Fig. 1e) during the sexual phase (Benjamin, 1979; Benny *et al*., 2001). They have the ability to remain dormant for a long period of time, and in favorable environmental conditions they can germinate, producing a new cycle of life.

Asexual reproduction in zygomycetes is based on the production of numerous propagules called sporangiospores (Fig. 1f, g), which are distinguished from other types of asexual spores, such as the conidia in Ascomycota and Basidiomycota, by its internal production through a split in the cytoplasm of the sporangium (Fig. 1h), a vesicle located in the extremity of the fertile hyphae (= sporangiophore; Fig. 1i). The hyphae walls then dissolve and the spores are released, being spread by wind, water or animals (e.g. arthropods) (Richardson, 2009). The zygomycetes can also produce globose and uni- to few-spored sporangia (= sporangiola; Fig. 1j), and cylindrical sporangia (= merosporangia; Fig. 1k), in which the sporangiospores are disposed in a chain-like

series. A sterile central axis within the mature sporangia (= columella; Fig. 1l) is usually present in many individuals.

Under adverse environmental conditions, the zygomycetes can form arthrospores and chlamydospores (both, a sort of thallic propagule developed from remodeling of the cell wall of a pre-existent hyphae), or blastospores (propagules of neo-formation) (Benny, 1995). Certain vegetative structures, such as rhizoids (root-like structures acting as resting and feeding organs; Fig. 1m) and stolons ("running" hyphae; Fig. 1n), are typically presents in several genera (Kirk *et al*., 2008).

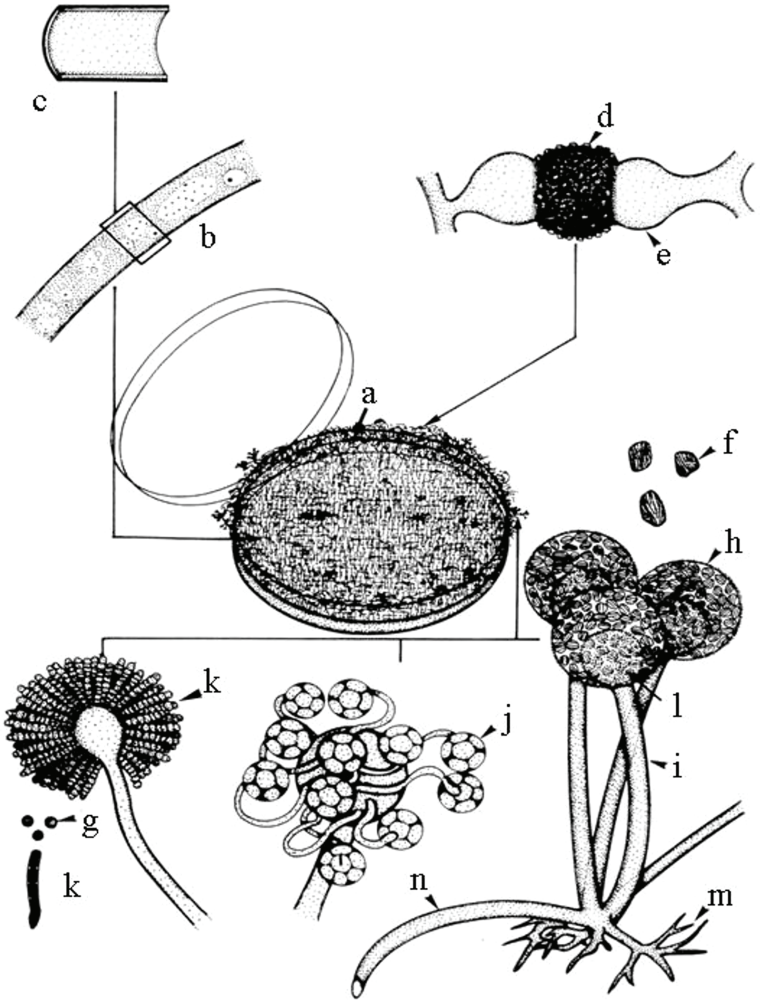

Figure 1. Typical features of the zygomycetes. a, colony; b, coenocytic hypha; c, section of a hypha; d, zygospore; e, gametangium; f, sporangiospores; g, merospores; h, sporangium; i, sporangiophore; j, sporangiole; k, merosporangia; l, columella; m, rhizoids; n, stolon (taken from Guarro *et al*., 1999).

1.2 Taxonomy and phylogeny

Traditionally, the classification of the zygomycetes has been based on phenotypic characters, mainly from the morphological features of the sexual and asexual reproductive structures, but also in the presence and features of certain differentiated vegetative structures, such as stolons and rhizoids (Benny, 1995; Hoffmann *et al*., 2009). Several authors have reported the taxonomic importance of the zygospore in the identification/classification of this group of fungi (Benjamin, 1979; Weitzman *et al*., 1995). Sexual mating (heterothallic or homothallic nature of the taxa), the ornamentation of the zypospores and the suspensors (hyphae that support the gametangia) (Fig. 2) have been used to solve some taxonomical controversies (Hoffmann *et al*., 2007), such as in *Absidia* / *Mycocladus* / *Lichtheimia* (Hoffmann *et al*., 2007, 2009). Nevertheless, some studies have demonstrated the usefulness of the size and shape of sporangiospores in their taxonomical classification (Garcia-Hermoso *et al*., 2009; Hoffmann *et al*., 2007).

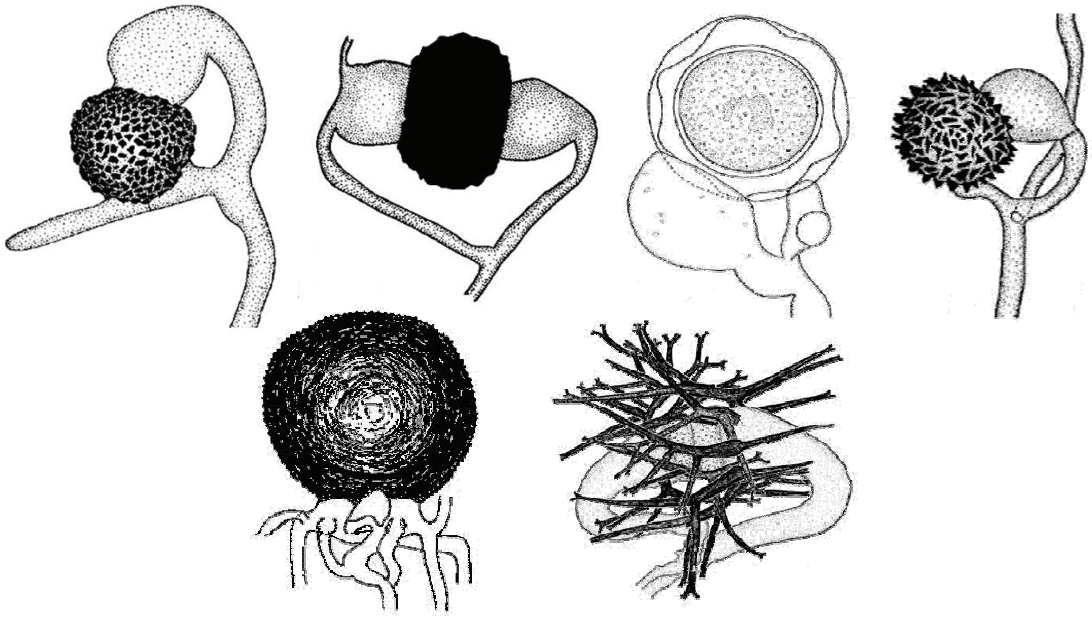

Figure 2: Some types of zygospores, zygospore appendages, and suspensors (from Webster and Weber, 2007).

On the other hand, the use of physiological tests as a taxonomical tool, such as the assimilation of different carbon and nitrogen sources, are less frequently employed, but they are useful for discriminating between some species of clinical importance (Schwarz *et al*., 2007; Vastag *et al*., 1998).

The zygomycetes have suffered a series of changes to their nomenclature. A summary of their successive taxonomic placements is summarized in Table 1. These changes in their taxonomic denomination could explain why the zygomycetes have been named in three different ways: Zygomycetes, Zygomycotina and Zygomycota.

Table 1. Classification of the zygomycetes along the time according different taxonomists.

Year of publication	1931	1971	1973	1983	1995	2007	2007
Author/s	Clements and Shear	Ainsworth *et al*.	Ainsworth *et al*.	Hawksworth *et al*.	Hawksworth *et al*.	Webster and Weber	Hibbett *et al*.
Higher rank		Subdiv. Zygomycotina	Subdiv. Zygomycotina	Subdiv. Zygomycotina	Div. Zygomycota	zygomycota	**Subphylum Mucoromycotina**
2nd higher rank	**Class Phycomycetes**	**Class Zygomycetes**	**Class Zygomycetes**	**Class Zygomycetes**	**Class Zygomycetes**	**Class Zygomycetes**	
	Order Spirogyrales (Zygomycetes)	Order Mucorales	Order Mucorales	Order Mucorales	Order Mucorales	Order Mucorales	Order Mucorales
	Order Protococcales			Order Endogonales	Order Endogonales	Order Endogonales	Order Endogonales
	Order Vaucheriales			-	-	Order Mortierellales	Order Mortierellales
	Order Confervales			-			
				-	-		**Subphylum Entomophthoromycotina**
		Order Entomophthorales	Order Entomophthorales	Order Entomophthorales	Order Entomophthorales	Order Entomophthorales	Order Entomophthorales
							Subphylum Zoopagomycotina
			Order Zoopagales	Order Zoopagales	Order Zoopagales	Order Zoopagales	Order Zoopagales
				Order Kickxellales*	Order Kickxellales*		
				Order Dimargaritales*	Order Dimargaritales*		
					Order Glomales**		

* included in the Class Trichomycetes (Webster and Weber 2007) and in the Subphylum Kickxellomycotina (Hibbett *et al*., 2007)

** included in the Phylum Glomeromycota (Hibbett *et al*., 2007)

Benny (1995) pointed out the limitations of the morphological features in the species delimitation within certain zygomycetes, suggesting the use of molecular tools for solving existing taxonomic controversies. O´Donnell *et al*. (1994) also suggested that the traditional classification scheme for the zygomycetes does not reflect the phylogenetic relationships between their taxa. A later molecular study, which included a large set of species belonging to the zygomycetes and based on the analysis of the sequences of three different genes (18S rDNA, 28S rDNA and elongation factor 1-α [EF1-α]) concluded that several of the recognized families, including the Mucoraceae, were polyphyletic (O'Donnell *et al*. 2001). In recent years, several studies based on multilocus analysis (18S, 28S, EF1-α, RNA polymerase II subunits 1 and 2 and the mitochondrial complete genome) have confirmed the polyphyletic nature of the zygomycetes (Hibbet *et al*., 2007; James *et al*., 2006; Tanabe *et al*., 2004, 2005; Voigt *et al*., 1999; White *et al*., 2006). Based on these reports, a new scheme was proposed, based on the monophyletic relationships comprising the subphyla (= subdivisions) Entomophthoromycotina, Kickxellomycotina, Mucoromycotina and Zoopagomycotina; the orders Zoopagales and Entomophthorales were assigned to the subphylum Zoopagomycotina and Entomophthoromycotina, respectively; the Kickxellales, Dimargaritales, Harpellales and Asellariales, to the Kickxellomycotina; and the Mucorales, Endogonales and Mortierellales to the subphylum Mucoromycotina, which includes 61 genera and around 325 species (Hibbet *et al*., 2007; Kirk *et al*., 2008) (Figs. 3 and 4). Currently, therefore, none of the terms Zygomycetes, Zygomycotina or Zygomycota are valid for taxonomic denomination of the zygomycetes, because they do not reflect their polyphyletic origin.

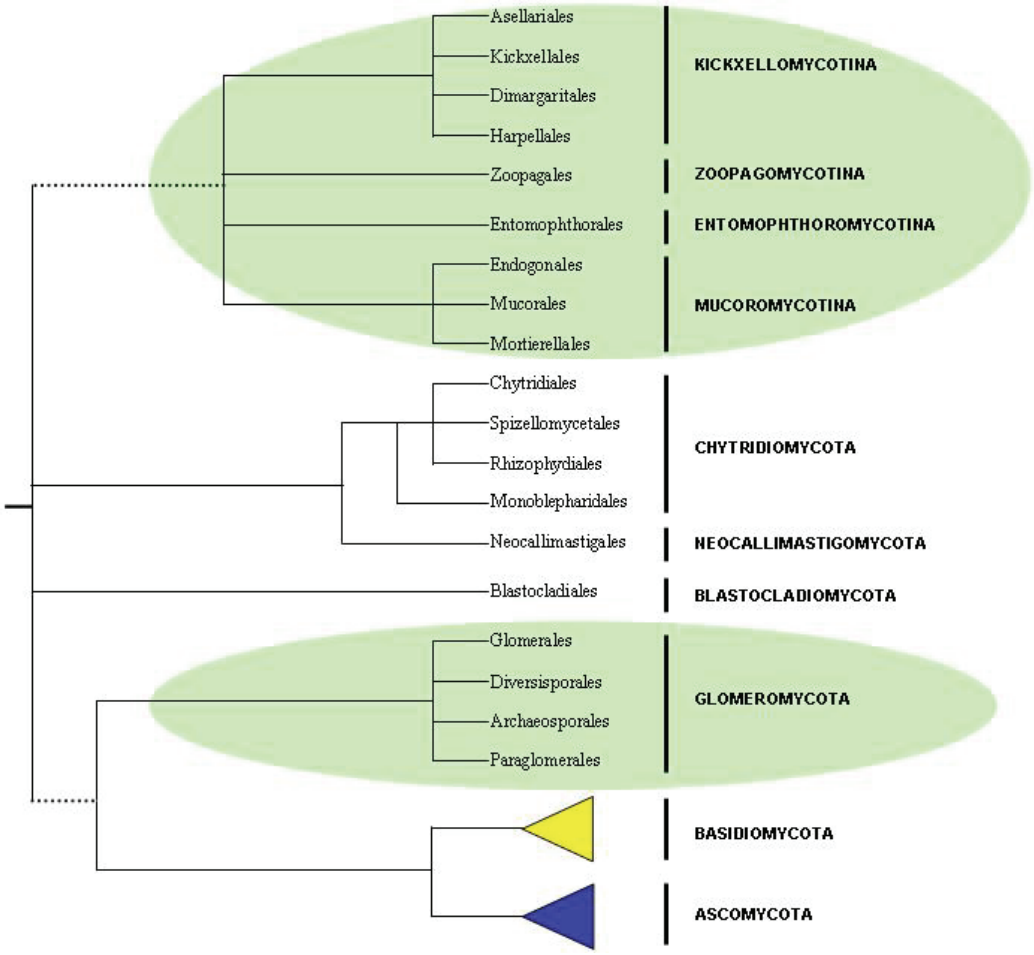

Figure 3. Phylogeny and classification of the Fungi. Highlighted in green: the taxa pertaining to the obsolete phylum Zygomycota. Dashed lines: taxa with uncertain placement (incertae sedis). Adapted from Hibbet *et al*. (2007).

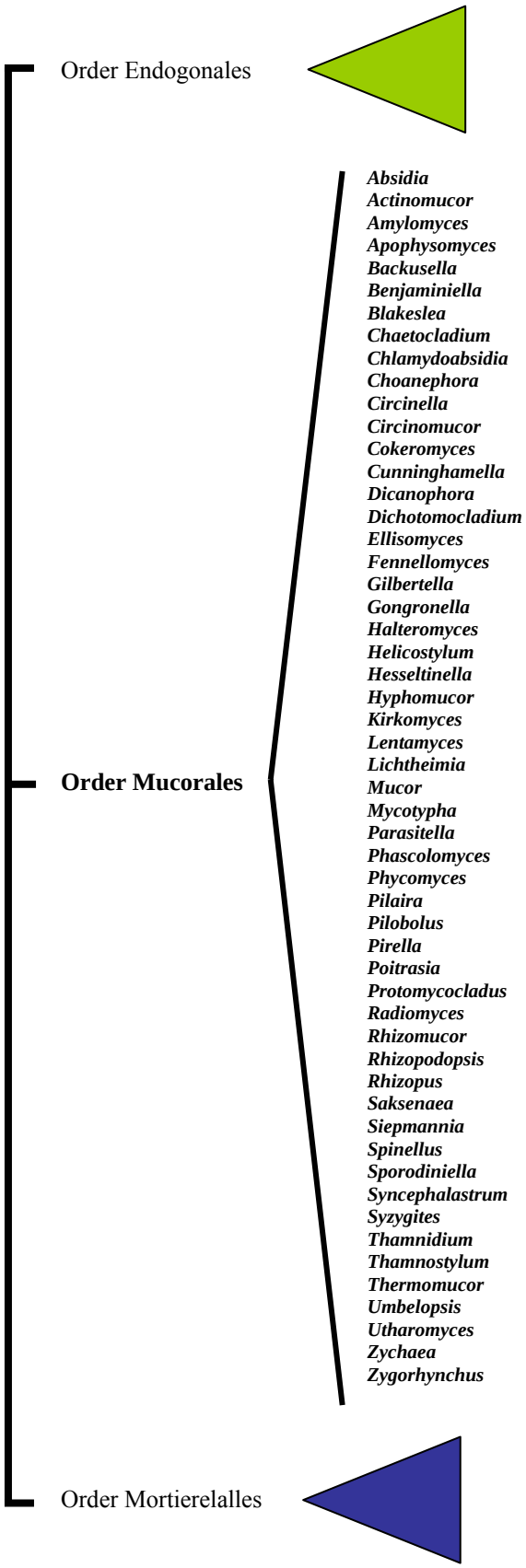

Figure 4. Genera belonging to the order Mucorales.

Four orders of zygomycetes, Basidiobolales, Mortierellales, Mucorales and Entomophtorales, include the most important taxa implicated in animal infections. However, most of the animal (and human) pathogenic species are within the Mucorales, such as species of the genera *Mucor*, *Rhizomucor* and *Rhizopus* (family Mucoraceae), and of *Absidia* (formally *Lichtheimia*) (family Absidiaceae). A fungal infection due to members of the Mucorales is commonly named mucormycosis, but also as zygomycosis, an alternative term which has become less accurate based on the recent taxonomic reclassification of the zygomycetes (Spellberg *et al.*, 2009b; Kontoyiannis *et al.*, 2011).

1.3 Epidemiology of the mucormycosis

In recent years the clinical range of the infections caused by zygomycetes has changed dramatically, because of the increase in the number of the immunocompromised patients (Meis and Chakrabarti, 2009). Roden *et al.* (2005), in the broadest retrospective study on human mucormycosis carried out in the USA (929 cases), found that there was a 70% increase in the number of cases reported between 1940 and 2000. A comparable result (a 40% increase) was found in another retrospective study carried out in France (531 cases) between 1997 and 2006 (Bitar *et al*., 2009). Saegeman *et al.* (2010), in a recent study at a university hospital in Leuven, Belgium, also reported a significant increase in the annual incidence of mucormycosis, from 0.019 to 0.148 cases/10.000 patients/day between 2000 and 2009.

All of those authors remarked that the infections caused by zygomycetes were frequently associated to neutropenic patients, transplant recipients, patients with hematological disorders or diabetes mellitus, patients receiving deferoxamine therapy, and intravenous drug users.

Roden *et al.* (2005) reported that the frequency of infections by organ type involved was: rhino-orbito-cerebral (66% of diabetic patients), pulmonary (around 25% of all cases), followed by cutaneous, disseminated, and gastrointestinal. Despite no information about locations being available for 30.3% of patients, in a study developed in France (Bitar *et al*., 2009) the presentations included digestive tract (23.2%), pulmonary (18.5%), rhino-cerebral (11.8%), and cutaneous localizations (9.4%), and disseminated in 6.8% of the cases. However, the locations found by Saegeman *et al.* (2010) differed from the other authors: 51.5% of infections were pulmonary, 23% disseminated, 13% cutaneous, 6.5% sinus, 3% cerebral, and 3% mycetoma. Moreover, these authors mentioned a high percentage (45%) of co-infections with *Aspergillus* spp.

Most of the clinically important zygomycetes are in the order Mucorales, and comprise approximately 60 genera. Roden *et al*. (2005) established that the most prevalent species were *Rhizopus oryzae* (nearly half of all isolates), followed by *R. microsporus*, *Mucor circinelloides*, *Absidia* (= *Lichtheimia*) *corymbifera*, *Rhizomucor pusillus*, *Cunninghamella bertholletiae*, *Mucor indicus*, *Cunninghamella echinulata*, and *Apophysomyces elegans*. Three recent retrospective studies carried out in the USA (Kontoyiannis *et al.*, 2000, 2005; Sims *et al.*, 2007) included a significant number of cases (24, 27 and 26, respectively). However, one of these (Sims *et al.*, 2007) distinguished only between *Mucor* spp. and *Rhizopus* spp., and in the others two (Kontoyiannis *et al.*, 2000, 2005), only the genera were mentioned (*Cunninghamella*, *Mucor*, *Rhizopus*, and *Syncephalastrum*). Despite the authors mentioning the mycological methods used to identify those fungi (culture-based [Kontoyiannis *et al.*, 2000, Roden *et al.*, 2005; Sims *et al.*, 2007] and culture- and sequencing-based [Kontoyiannis *et al.*, 2005]) in a high number of the cases the identification methods were not described, and most of the diagnoses should be considered doubtful or of

insufficient accuracy. Unfortunately, there have been no similar studies in other countries for comparison purposes.

The main route of transmission for mucormycosis is by the inhalation of spores from environmental sources. It has been demonstrated experimentally that after nasal instillation of sporangiospores of *Absidia* (=*Lichtheimia*) *corymbifera*, *Cunninghamella bertholletiae*, *Rhizopus oryzae*, *R. microsporus* var. *rhizopodiformis*, or *Rhizomucor pusillus* an upper and/or lower respiratory infection develops which can disseminate to the central nervous system (Kamei, 2001; Reinhardt *et al*., 1981; Waldorf and Diamond, 1984). Outbreaks of rhino-cerebral and pulmonary mucormycosis have been described in workers by exposure to aerosols generated in excavation sites, during building construction or renovation, and by inadequate manipulation of contaminated air-conditioner filters (England *et al*., 1981; Krasinski *et al*., 1985; Richardson, 2009).

The agents of mucormycosis are unable to penetrate through unbroken skin to produce cutaneous infection; however, burns, traumatic disruptions or macerations of the skin (Ledgard *et al*., 2008; Stewardson *et al*., 2009; Vega *et al*., 2006), insect bites or stings, (Lechevalier *et al*., 2008; Saravia-Flores *et al*., 2010), birds pecks (Wilson, 2008), traumatic inoculations (Andresen *et al*., 2005; Richardson, 2009; Ribes *et al*., 2000; Snell and Tavakoli, 2007), and any process of skin disruption can facilitate the implantation and colonization of tissues by the spores. Cutaneous infections can be very invasive, also affecting subcutaneous tissues and the adjacent fat, fascia, muscle, and finally bone. In addition, the fungus can affect deep solid organs by hematogenous dissemination.

Gastrointestinal mucormycosis is very uncommon. The ingestion of food or fermented drinks contaminated with spores may play a role in promoting gastric infections and, although it is rare in humans, it can significantly affect animals, i.e.

amphibians, bovines and rodents (Creeper *et al*., 1998; Jensen *et al*., 1994; Ohbayashi, 1971; Oliver *et al*., 1996; Richardson, 2009; Sandford *et al*., 1985; Speare *et al*., 1997).

In the past decade, there has been a reported increase in the incidence of several types of mucormycosis in immunocompetent people, principally due to the species *Apophysomyces elegans* and *Saksenaea vasiformis* (Baradkar and Kumar, 2009; Meis and Chakrabarti, 2009; Stewardson *et al*., 2009). Infections by *A. elegans* have been reported commonly from tropical and subtropical countries, especially India, where around of the 50% of the total number of cases in the world annually occur (Chakrabarti *et al*., 2003, 2006, 2009; Devi *et al*., 2008; Jain *et al*., 2006; Meis and Chakrabarti, 2009; Ribes *et al*., 2000).

An additional factor in the increasing of the mucormycosis has been the use of voriconazole for prophylaxis or empirical therapy. Several studies have reported cases of breakthrough mucormycosis in patients receiving that antifungal drug (Lionakis and Kontoyiannis, 2005; Marty *et al*., 2004; Pongas *et al*., 2009; Singh *et al*., 2009; Trifilio *et al*., 2007). In this way, voriconazole treatment might be associated with the expression of virulence factors such as proteases or siderophores by the fungus. Nevertheless, until now the role of voriconazole in the development of mucormycosis has remained unclear (Kontoyiannis *et al*., 2011; Lamaris *et al*., 2009).

1.4 Clinical presentations of mucormycosis

Based on the clinical presentation and the part of the body affected, mucormycosis are divided in five clinical categories: rhino-cerebral, pulmonary, cutaneous, gastrointestinal, and disseminated. In the largest study ever carried out on mucormycosis, Roden *et al.* (2005) observed that sinus infections were the most common (39%), followed by pulmonary (25%), cutaneous (19%), and gastrointestinal

(7%) infections. Dissemination of the infection was observed in approximately 23% of cases.

1.4.1 Rhino-cerebral mucormycosis

The clinical manifestation of this infection is characterized by the involvement of the paranasal sinuses, although the maxillary sinus, the orbit, cavernous sinuses, and the brain can also be affected (Schutz *et al*., 2006; Sörensen *et al*., 2006; Sundaram *et al*., 2005). Rhino-cerebral mucormycosis is commonly observed in patients with uncontrolled diabetes mellitus, usually with a remarkable keto-acidosis. In these patients, the immune system failed to suppress the germination of spores and to kill the proliferating hyphal elements (Ribes *et al*., 2000) increasing the risk of developing mucormycosis. Symptoms can be nasal obstruction, sinus pain, drainage, headache, odontalgia, maxillary pain, hyposmia or anosmia. The disease may become rapidly progressive, involving the neighbouring tissues. If it extends to the orbital region, edema or proptosis can develop and there can be a blurring or loss of vision when the optic nerve is reached. The infection can readily progress to the brain, causing an altered state of consciousness. The majority of patients with cerebral infections die, though mainly due to a delay in diagnosis and treatment (Mantadakis and Samonis, 2009; Ribes *et al*., 2000; Spellberg *et al*., 2005a).

1.4.2 Pulmonary infection

Pulmonary mucormycosis is more common in neutropenic patients, and in those who undergo haematopoietic stem cell transplantation. Some cases have also been described in patients with diabetic keto-acidosis; although the infections in these patients are less common and do not cause sudden death; these can follow a sub-acute course (Rothstein and Simon, 1986). One of the problems associated to this clinical

presentation is the fact that clinical and radiological features are indistinguishable from those of other pulmonary mycoses, such as aspergillosis. Symptoms of pulmonary mucormycosis include fever, non-productive cough, chest pain, dyspnoea and haemoptysis. Unfortunately, the sputum culture for diagnosis is highly unreliable. Several reports have shown more than 50% negative cultures in real infections (Lee *et al.*, 1999; Reid *et al.,* 2004; Tedder *et al.,* 1994). Without treatment, hematogenous dissemination from lungs to other organs frequently occurs, increasing mortality rates considerably (Mantadakis and Samonis, 2009; Ribes *et al.,* 2000; Spellberg *et al.,* 2005a).

1.4.3 Cutaneous infection

Cutaneous mucormycosis is usually associated to traumatic inoculation, and less frequently to disseminated mucormycosis (Roden *et al.,* 2005). The patients are mainly immunocompromised, following extensive burn injury or with severe soft tissue trauma (Cantatore-Francis *et al.,* 2007; Kobayashi *et al.,* 2001; Mueller and Pabst, 2006; Page *et al.,* 2008). In other cases, the infection has followed car accidents, insect bites, and traumatic inoculation after natural disasters, like tsunamis and earthquakes (Andresen *et al.,* 2005; Devi *et al.,* 2008; Ruiz *et al.,* 2004; Saravia-Flores *et al.,* 2010; Snell and Tavakoli, 2007). Symptoms such as erythema and induration are non-specific; necrotic scars being the diagnostic hallmark. A prompt biopsy of the involved skin is mandatory, due the fast spread to adjacent tissues, especially in neutropenic patients. An often-fatal secondary necrotizing fasciitis may occur in these type of infections. Approximately 44% of cutaneous infections are complicated by extending deeply or by dissemination (Roden *et al.,* 2005). Generally, the cutaneous infections have a favourable prognosis and a low mortality if aggressive surgical debridement is carried out promptly.

1.4.4 Gastrointestinal infection

Gastrointestinal mucormycosis is uncommon, and has been described mainly in infants or in extremely malnourished people (Amin *et al.,* 1998; Sarin, 2010). Apart from malnutrition, immunosuppression and ingestion of contaminated food are the most common predisposing factors. The infection is characterized by fungal invasion into the gut mucosa, submucosa and blood vessels, developing necrotic gastric or intestinal ulcers, which may rupture, causing peritonitis (Shiva Prasad *et al.,* 2008).

1.4.5 Disseminated infection

Disseminated mucormycosis is commonly the result of the spread of a pulmonary form, although it may originate from any other primary site of infection (Rüping *et al.,* 2010; Tomita *et al.,* 2005). In general, pathogenic Mucorales are angioinvasive, able to infect many organs by the hematogenous route (Ribes *et al.,* 2000). The most common sites of dissemination are the brain, spleen, heart, and skin. Cerebral infection following dissemination is distinct from the rhinocerebral mucormycosis and results in abscess formation and brain infarction (Spellberg *et al.,* 2005a). Recently, several cases of mucormycosis have been reported in transplant patients under voriconazole prophylaxis (Imhof *et al.,* 2004; Marty *et al.,* 2004; Pongas *et al.,* 2009). The diagnosis of disseminated infection is difficult, because patients are often severely ill due to other underlying diseases so blood cultures generally remain negative. Misidentifications with aspergillosis are common (Spellberg *et al.,* 2005a).

Disseminated mucormycosis have, in general, a bad prognosis, showing a mortality rate near to 90% (Mantadakis and Samonis, 2009; Roden *et al.,* 2005).

1.4.6 Atypical presentations

The Mucorales may cause infection in virtually any anatomical site, although some of them are atypical. There have been occasional reports of rare manifestations involving the kidneys, usually confirmed at autopsy (Yu and Yu Li, 2006; Singh *et al.,* 2004; Welk *et al.,* 2004), the trachea (Wolf *et al.,* 2004) and heart and cardiac valves (Gubarev *et al.,* 2007; Mehta *et al.,* 2004; Van de Glind *et al.,* 2010).

1.5 Treatment

Without proper treatment, the mucormycoses are rapidly progressive and lethal. In general, the response of the different species of Mucorales to antifungals is quite similar, being susceptible to amphotericin B, itraconazole and posaconazole, and more or less resistant to the rest of the drugs (Alastruey-Izquierdo *et al.*, 2009; Almyroudis *et al*., 2007; Dannaoui *et al.,* 2002c, 2003c; Dannaoui and Garcia-Hermoso, 2007; Ibrahim *et al*., 2009; Pagano *et al*., 2009; Rodriguez *et al*., 2008; Saoulidis *et al*., 2010; Sun *et al.,* 2002a; Spellberg *et al*., 2009b; Spellberg and Ibrahim, 2010; Takemoto *et al*., 2010; Zhao *et al*., 2009).

Lipid preparations of amphotericin B are the only ones active both *in vitro* (Dannaoui *et al.,* 2002b, 2003b; Sun *et al.,* 2002a, Takemoto *et al*., 2010) and *in vivo* (Dannaoui *et al.,* 2002c; Odds *et al.,* 1998; Sun *et al.,* 2002b, Takemoto *et al*., 2010) against the Mucorales, still today being the treatment of choice against these infections (Rüping *et al.*, 2010; Spellberg and Ibrahim, 2010; Takemoto *et al*., 2010). Lipid formulations of amphotericin B, which cause less nephrotoxicity, allows high dosages to be administered (Pearson, 2003; Spellberg *et al.,* 2005a; Sun and Singh, 2011). Based on experimental models, liposomal amphotericin B at 10 mg/kg/day, in combination with surgery, has been proposed as the first line therapy against mucormycosis

(Spellberg *et al.,* 2005a, 2009b). In the treatment of cutaneous infections, the use of amphotericin B and surgical debridement have also had successful results (Dromer and McGinnis, 2002; Ibrahim *et al*., 2005; Singh *et al*., 2009).

Azoles (triazoles) are widely used to treat mycoses other than mucormycosis. Posaconazole, a broad-spectrum antifungal drug, showed a good *in vitro* activity against a range of species of Mucorales, especially for *Absidia* spp., *Lichtheimia corymbifera* and *Rhizomucor* spp. (Alastruey-Izquierdo *et al*., 2009; Almyroudis *et al*., 2007; Dannaoui *et al.,* 2003c; Sabatelli *et al.,* 2006; Spreghini *et al.,* 2010; Sun *et al.,* 2002a). *In vivo*, posaconazole significantly prolonged survival in neutropenic mice infected with *Mucor* spp., and reduced fungal burden in kidneys compared to untreated control (Sun *et al.,* 2002b). However, in another study on immunocompetent mice with disseminated infection, posaconazole was not effective against a *Rhizopus oryzae* infection (Ibrahim *et al*., 2009), but showed a clear dose-response effect for *Rhizopus microsporus* (Rodriguez *et al*., 2009) and was partially active against *Lichtheimia corymbifera* (Dannaoui *et al.,* 2003a). Despite the latest *in vivo* results, and some less frequent resistant clinical isolates (Khan *et al.*, 2009), posaconazole has shown good therapeutic results, and could be an alternative in the treatment of mucormycosis (Greenberg *et al.,* 2006; Peel *et al*., 2008; Spellberg *et al.*, 2009b; Tobon *et al.,* 2003; van Burik *et al.,* 2006). Recent studies have shown that voriconazole had limited activity against the Mucorales (Cornely *et al*., 2007; Dannaoui *et al.,* 2003b; Espinel-Ingroff, 1998, Sun *et al.,* 2002a), and their use in fungal infection prophylaxis was identified as one of the factors that favoured the development of mucormycosis (Cornely *et al.*, 2008; Imhof *et al*., 2004; Kontoyiannis *et al.*, 2005; Marty *et al*., 2004; Singh *et al*., 2009; Siwek *et al*., 2004; Trifilio *et al*., 2007; Ullmann *et al*., 2007). In contrast, itraconazole showed *in vitro* species-dependant activity, showing a low MIC (minimal inhibitory concentration)

for *Lichtheimia* and *Rhizomucor* spp., and a higher MIC, with some intraspecific variability, against *Rhizopus* spp. and *Mucor* spp. (Almyroudis *et al*., 2007; Dannaoui *et al.,* 2003c; Sabatelli *et al.,* 2006; Sun *et al.,* 2002a). *In vivo* studies have shown activity of itraconazole against *Lichtheimia* spp. (Dannaoui *et al.,* 2002c, 2003a; Mosquera *et al.,* 2001), although being ineffective for *Mucor* spp. (Sun *et al.,* 2002b), *Rhizopus microsporus* (Dannaoui *et al.,* 2002c, 2003a), and *Rhizopus oryzae* (Dannaoui *et al.,* 2002c, 2003a; Odds *et al.,* 1998).

Due to the limited activity of the majority of the azoles against the mucormycosis, a combination of two or more drugs could be of interest (Dannaoui *et al.,* 2002a; Dannaoui *et al.,* 2009; Ibrahim *et al*., 2008; Ogawa *et al*., 2011; Reed *et al*., 2008; Spellberg *et al*., 2005b). An evaluation of several combinations against 35 isolates of different species of Mucorales showed that amphotericin B interacted synergistically with rifampicin, terbinafine, and voriconazole. Antagonism was not observed (Dannaoui *et al.,* 2002a). In another study, the combination of amphotericin B with cyclosporine, rapamycin, and tacrolimus were synergic (Dannaoui *et al.,* 2009). Chamilos *et al.* (2006) evaluated the *in vitro* activity of lovastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor against seven clinical isolates of different species of Mucorales: *Cunninghamella bertholletiae*, *Mucor circinelloides*, *Rhizopus homothallicus* and *R. oryzae*, alone and in association with voriconazole, and also carried out an *in vivo* study using a *Drosophila melanogaster* model of mucormycosis. In this study, lovastatin showed significant strain-independent fungicidal *in vitro* activity, showing synergy with voriconazole in all cases. Lovastatin was also synergic with voriconazole in the *Drosophila* model of mucormycosis, itself being active in the infection by *Mucor circinelloides*. More recently, Reed *et al.* (2008) reported that, in patients with cerebral mucormycosis, the response to treatment with

lipid complex amphothericin B (ABLC) plus caspofungin was better than with those treated only with ABLC therapy.

Iron is essential for the growth and virulence of Mucorales. A high plasma level of free iron produces an increase in susceptibility to mucormycosis (Pagano *et al.,* 2009). In this context, the use of an iron chelator like deferasirox, alone or in combination with other antifungal agents, appears a rational option in the therapy of the mucormycosis and is under active investigation (Ibrahim *et al.*, 2006, 2007, 2008; Reed *et al.,* 2006; Spellberg, 2005a; 2009a, 2009b; Lewis *et al.*, 2011).

Nevertheless, due to the lack of a significant number of studies, both *in vitro* and *in vivo*, as well as a reliable identification at species level of many strains isolated from clinical cases, the best antifungal treatment, especially against to uncommon species, remains unclear. In fact, despite efforts in the development of new therapeutic approaches, mortality rate in mucormycosis patients remains high.

1.6 Molecular phylogeny of the Mucorales

As was discussed in section 1.2, molecular techniques have proven to be useful for solving taxonomical problems of the mucoralean fungi, a group traditionally considered homogeneous. In the first comprehensive phylogenetic study carried out by O´Donnell *et al*. (2001), a large panel of genera of Mucorales (63 species belonging to 54 genera, and 13 families) was analysed, based on the partial nucleotide sequences of two nuclear ribosomal genes (18S and 28S) and the translation elongation factor-l-α gene (EF 1-α) exons. The analysis showed that traditional family-level classification schemes for the Mucorales appear to be highly artificial, due to the polyphyletic origin of four families within them (Thamnidiaceae, Mucoraceae, Chaetocladiaceae, and Radiomycetaceae). Moreover, the resultant phylogram suggested the absence of a common ancestor in the evolution of the mucoralean fungi. Recent molecular studies,

based on the nucleotide sequence analysis of several *loci*, including ribosomal DNA (Abe *et al*., 2006, 2007, 2010; Alastruey-Izquierdo *et al*., 2010; Dannaoui *et al*., 2010; Hoffmann *et al*., 2007; Garcia-Hermoso *et al*., 2009; O´Donnell *et al*., 2001), actin (Abe *et al*., 2007; Hoffmann *et al*., 2007), EF 1-α (Alastruey-Izquierdo *et al*., 2010; Abe *et al*., 2007, 2010; Garcia-Hermoso *et al*., 2009; O´Donnell *et al*., 2001), and lactate dehydrogenase (Saito *et al*., 2004) genes, have been carried out for phylogenetic species recognition and for taxonomical reorganization, all of them confirming the conclusions suggested by O´Donnell *et al* (2001). In this way, the results of polyphasic studies strongly support the existence of several monophyletic clades among the Mucorales. Thus, *Lichtheimia* was proposed as a new genus; and their species *L. corymbifera*, *L. ramosa, L. sphaerocystis*, and *L. hongkongensis* were considered different species from *Absidia*, as well as the etiological agents of mucormycosis, because of their ability to grow at 37ºC (Alastruey-Izquierdo *et al*., 2010; Hoffmann *et al*., 2007, 2009; Garcia-Hermoso *et al*., 2009; Woo *et al*., 2010). The new genus *Lentamyces* (Hoffmann and Voigt, 2009) was described to relocate the micoparasitic species *L. parricida* and *L. zychae*. Finally consider three different genera within the genus *Absidia*: *Absidia*, *Lentamyces* and *Lichtheimia*.

Other molecular techniques, such restriction fragment length polymorphism (RFLP) (Chakrabarti *et al*., 2003; Larche *et al*., 2005; Lemmer *et al*., 2002; Machouart *et al*., 2006; Seif *et al*., 2005), amplified fragment length polymorphism (AFLP) (Chakrabarti *et al*., 2010; Kito *et al*., 2009); random amplified polymorphic DNA (RAPD) (Papp *et al*., 2001; Vágvölgyi *et al*., 2001), and pulsed field gel electrophoresis (PFGE) (Diaz-Minguez *et al*., 1999; Nagy *et al*., 2000, 2004) have been used relatively rarely in the identification/taxonomy of the mucoralean fungi. The banding patterns obtained have proven to be useful for the identification of clinical agents of

mucormycosis, but have shown a limited species-level discrimination when they are compared with the sequencing-based techniques (Chakrabarti *et al*., 2003, 2010).

Based on the results of the molecular-based studies, the guidelines for the fungal identification by DNA sequencing were published by the CLSI (CLSI, 2007). For the Mucorales, the ITS region was proposed as the gold standard for generic and species identification. The Working Group on Fungal Molecular Identification of the International Society of Human and Animal Mycology (ISHAM) supported this proposal (Balajee *et al*., 2009).

DNA barcoding, a technique for identifying organisms based on a short and standardized fragment of genomic DNA (because it is like a barcode tag for each taxon), has a promising future. Applying the barcoding makes it possible to achieve a more precise delimitation of species and subspecies and to discriminate between morphologically similar species. DNA barcoding can be a powerful tool when used in conjunction with the traditional methods for identifying specimens from different sources and for discovering new species. Moreover, this strategy can also be used by scientists with less technical expertise in taxonomy, and can be run from specimens that are hard or impossible to identify using the classical phenotypical methods.

2. INTEREST AND OBJECTIVES

2.1 Interest of this work and objectives

Recently, several studies have reported the increasing incidence of mucormycosis in both immunocompromised and immunocompetent patients (Bitar *et al*., 2009; Roden *et al*., 2005; Saegeman *et al*., 2010). Some species belonging to the order Mucorales (subphylum Mucoromycotina) are considered opportunistic pathogens, causing infections with a high mortality rate. Interesting is the epidemiological change observed in both *Apophysomyces elegans* and *Saksenaea vasiformis*, two species considered emerging pathogens, with a high clinical relevance in recent years, due to the infection in immunocompetent people. Additionally, several retrospective studies have been carried out, the largest and most comprehensive review to date being on 929 cases of mucormycosis published by Roden and colleagues (2005) in which critical points like predisposing factors, clinical presentations, treatments, outcomes and etiological agents were analyzed. However, a high percentage of those 929 cases lacked identification to species level, and for most of them the identification was doubtful. In this way, the identification of the Mucorales remains a difficult, time-consuming task and demands trained personnel.

Another relevant aspect is the fact that the Mucorales are resistant to most of the currently available antifungal drugs. Amphotericin B is the only antifungal drug to show potent *in vitro* activity and *in vivo* efficacy. Nevertheless, the clinical efficacy remains limited, with high mortality rate, especially when the infections are caused by either *Saksenaea vasiformis* or *Apophysomyces elegans*.

Over the last few years, our laboratory has been working with the Fungus Testing Laboratory at the University of Texas, which is the reference center for the identification and antifungal susceptibility in the USA. We have been able to identify a

significant number of clinical isolates belonging to the Mucorales from several American hospitals. Also, since 1990, our laboratory has been using several molecular techniques for the study of pathogenic fungi, and having personnel trained in the identification of fungi has made the present study possible.

This thesis aims to increase the knowledge of the taxonomy of some clinically important species of mucoralean fungi by studying those so far poorly explored aspects. The objectives of this thesis are the following:

1. To assess the spectrum of mucoralean species and their frequency in clinical samples from the United States of America, and to try to establish the degree of correlation between morphological and molecular identification.

2. To identify putative cryptic species using a polyphasic approach (i.e. DNA sequence data, morphology and physiology) in several genera so far poorly studied.

3. Having identified and characterized the species involved in human infections, to establish their antifungal susceptibility against antifungal drugs.

3. MATERIALS AND METHODS

3.1 Origin of the isolates and storage

A total of 217 isolates of species of Mucorales, from clinical and environmental samples, including types and reference strains obtained from different international culture collections and reference centers, were studied in the present work. Their geographic origin, substrates, or anatomical sites of isolation are detailed in sections 4.1, 4.2, 4.3 and 4.4.

All strains were conserved as submerged cultures in liquid paraffin or/and water. In the first procedure, fungi were cultured on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) and potato carrot agar (PCA; 20 g potatoes, 20 g of carrot, 15 g of agar Pronadisa ref. 1800, 0.05 g de L-chloramphenicol Acofarma and 1 L distilled water) in slant tubes and the growing part of the agar covered by the liquid paraffin. The tubes were stored at room temperature. In the second procedure, small pieces of sporulated cultures in Czapek agar (CZA; Difco, Becton Dickinson, France) and PDA were transferred to small dark vials (10 mL) containing 5 mL of distilled water and stored at room temperature.

3.2 Phenotypic characterization

The phenotypic characterization of the species included in the present thesis consisted in the study of their most relevant morphological features, both at macro- and microscopic level, the evaluation of their ability to assimilate carbon and nitrogen sources, and their growth response to different temperatures. In order to achieve the optimal growth and sporulation we tested different culture media. The morphological criteria used in the identification of the different taxa had been previously tested by other authors (de Hoog *et al*., 2000; Misra and Srivastava, 1979; Saksena, 1953; Schipper, 1973, 1975, 1976, 1978; Zycha *et al*., 1969).

3.2.1 Morphology

3.2.1.1 Colonies

For morphological studies, a small inoculum of fungal colony was placed in the center of a petri dish (9 cm diameter), with each of the following culture media: Potato dextrose agar (PDA; Pronadisa, Madrid, Spain); Malt extract agar (MEA; 20 g malt extract, 15 g agar, 1 L of distilled water), and Czapek agar (CZA; Difco, Becton Dickinson, France).

Cultures were incubated at 25ºC in darkness. Cultural characteristics were determined at 4-10 days after inoculation. Colony diameters, texture, colour (Kornerup and Wanscher, 1978), and production of exudates and pigments were evaluated.

3.2.1.2 Microscopic features

The micromorphology of the isolates was determined performing slide cultures mounted in lactic acid or water, and examined using a light microscope Zeiss Axio Imager M1. The size, shape, ornamentation, and color of the following structures, when present, were studied and measured:

- hyphae
- apophyses
- columella
- chlamydospores
- sporangiophores
- sporangia
- sporangioles

- sporangiospores
- giants cells
- septa
- rhizoids

3.2.1.3 Electron microscopy

Scanning electron microcopy techniques were used mainly for a detailed study of the ornamentation of several fungal structures but particularly for the surface of the sporangiospores which constitutes a relevant taxonomic character in *Mucor*. The methods used were based on Figueras and Guarro (1988).

- **Fixation**. Small pieces of 5 x 5 mm were selected and transferred to vials containing to 2% glutaraldehyde solution in 0.1 M phosphate buffer (Sabatini *et al*., 1963). To prevent the samples floating, several drops of Tween 80 were added (Carroll and Carroll, 1973). The vials were vacuum-dried for around 1 hour (Campbell, 1972; Hanlin, 1982; Rosing, 1982). Later, the samples were washed for 15 minutes in 0.1 M phosphate buffer. Next, a second fixation was performed with a 1% p/v osmium tetroxide solution (O_4Os) in 0.1M phosphate buffer (Palade, 1952).

- **Dehydration**. The samples were dehydrated through a graded series of aqueous ethanol solutions (30% v/v - 100% v/v). Later, the aqueous ethanol solutions were replaced by amyl acetate-ehtanol mixtures (amyl acetate-ethanol 1:3 v/v - amyl acetate).

- **Dry**. The phase of critical point dry replaces the water present in the samples by CO_2 (Anderson, 1953; Bartlet and Burstyn, 1975).

- **Mount and coat**. Once samples are dried, they were mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium in a Polaron Sputter Coater.

- **Observation**. The samples were examined with a scanning electron microscope Jeol JSM-6400.

3.2.2 Physiological tests

For the identification of the isolates belonging to *Apophysomyces*, *Mucor* and *Saksenaea* the growth of such fungi on 2% MEA at 4, 15, 24, 30, 35, 37, 42, 45 and 50º C were determined in dark. Colony diameters were measured every 2 days to establish the growth rates.

Carbon sources assimilation patterns were determined with the commercial kit API 50CH (bioMérieux, Marcy-l'Etoile, France) according to the protocols described by Schwarz *et al* (2007). To obtain sufficient sporulation, all strains were cultured for 6 days on CZA at 37ºC and 42ºC for the *Saksenaea* and *Apophysomyces* species respectively. A final concentration of 5 x 10^5 CFU/mL was prepared in 20 mL of yeast nitrogen base (7.7g/L; Difco), containing 0.5g/L-chloramphenicol (Sigma-Aldrich) and 0.1% Bactoagar (Difco), and each well of the strips was inoculated with 300 µL of medium. The viability of the conidia was verified by plating 100 µL of serial dilutions of each inoculum onto PDA and incubating at 37/42 ºC for 6 days. The inoculated API 50CH strips were incubated for 48–72 h at 37/42 ºC in dark. After incubation, the strips were read visually and growth or lack of growth was noted. Weak growth was considered as a positive result.

Nitrogen source assimilations were determined using the same inoculum described above, but the yeast nitrogen base broth was replaced by carbon nitrogen base

broth (Difco), and testing was performed in sterile, disposable, multiwell microplates. The medium with the nitrogen sources was dispensed into the wells in 150 µL volumes with a multichannel pipette and each well was inoculated with 50 µL of the conidial suspension. The microplates were incubated at 37 ºC in dark for 48 and 72h. We also determined growth of the strains on NaCl (2%, 5%, 7%, 10%), $MgCl_2$ 2%, and cycloheximide 0.1% (de Hoog *et al*., 1994; Yarrow, 1998). All tests were performed in duplicate.

The production of urease was determined after incubation on Christensen's urea agar slants at 37 ºC for 8 days (de Hoog *et al*., 1994; Ghosh *et al.*, 2002; Yarrow, 1998). In both studies, the final columns of the multiwell microplates were used as positive and negative controls respectively. The positive control consisted in 150 µL of *Bacto Yeast Nitrogen Base* (Difco) with glucose. The negative control consisted in 150 µL of base medium without carbon and nitrogen sources.

3.3 Molecular studies

DNA extraction was performed directly from fungal colonies on PDA after 5-7 days of incubation at 25º C in dark. We followed the Fast DNA kit protocol (Bio 101, Inc., Joshua Way, Vista, California, USA) as described by the manufacturer.

Amplification and sequencing of the 5.8S ribosomal RNA gene and the ITS region (internal transcribed spacers 1 and 2) (Fig. 6) were performed with the primer pair ITS5/ITS4 (Table 1) as described by White *et al* (1990). The D1/D2 domains of the 28S rRNA gene (Fig. 6) were amplified and sequenced using primers NL1/NL4 (Table 1) following the protocol of O`Donnell (1993). The Histone 3 (H3) (Fig. 7) and elongation factor (EF-1α) genes (Fig. 8) were amplified with primer pairs H3-1A/H3-1B (Glass and Donaldson, 1995) and MEF-11/MEF-41 (Table 1) (O`Donnell *et al*.,

2001), respectively, following the Ready-To-Go bead protocol (Amersham Bioscience, Freiburg, Germany) and using the amplification programs described by Gilgado *et al* (2007). PCR products were purified using a GFXTM PCR DNA kit (Pharmacia Biotech, Cerdanyola del Vallès, Spain) and stored at -20 C until sequencing. PCR products were sequenced by using the same primers employed for amplification and following the Taq DyeDeoxy Terminator cycle sequencing kit protocol (Applied Biosystems, Gouda, The Netherlands). DNA sequencing reaction mixtures were analyzed on a 310 DNA sequencer (Applied Biosystems).

The software Autoassember version 1.40 (Applied Biosystems, Perkin Elmer Corp., Norwalk, Connecticutt, USA) and SeqMan (Lasergene, Madison, Wisconsin) were used to obtain consensus sequences from the complementary sequences of each isolate. BLAST searches (Altschul *et al*., 1990) were performed to compare data of the isolates studied with those of other fungi deposited in the GenBank database.

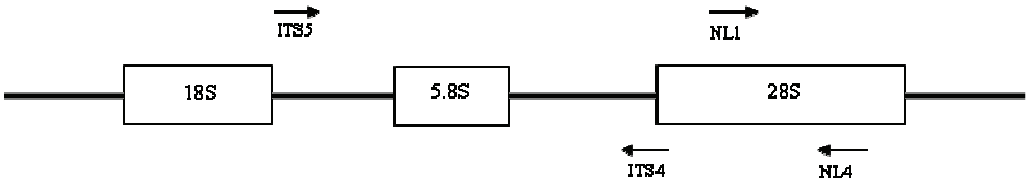

Fig. 6: Structure of the ribosomal RNA gene cluster. Arrows indicate the primers direction.

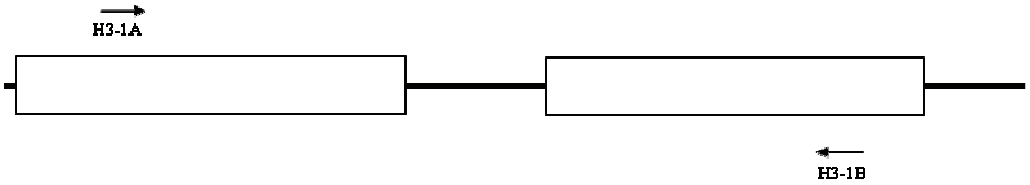

Fig. 7: Map of H3 gene showing exons and introns. Arrows indicate the primers direction.

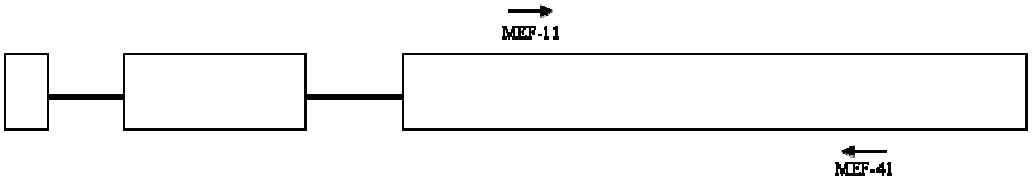

Fig. 8: map of the EF-1α gene showing exons and introns. The arrows indicate the primers direction.

Table 1: Primers used in the present study.

Gene / region	**primer**	**Sequence (5`- 3`)**	**Product size**
ITS	ITS 5	GGAAGTAAAAGTCGTAACAAGG	650-750 bp
	ITS 4	TCCTCCGCTTATTGATATGC	
D1/D2 domains 28S rDNA	NL1	GCATATCAATAAGCGGAGGAAAAG	600-650 bp
	NL4	GGTCCGTGTTTCAAGACGG	
Histone 3	H3-1A	ACTAAGCAGACCGCCCGCAGG	350-400 bp
	H3-1B	GCGGGCGAGCTGGATGTCCTT	
Elongation factor 1α	MEF-11	AAGAAGATTGGTTTCAACCC	400-450 bp
	MEF-41	GCACCGATTTGACCAGGRTGG	

3.3.1 Phylogenetic studies

Nucleotide sequence alignments were performed with ClustalX version 1.81 (Thompson *et al*., 1997), followed by manual adjustments with a text editor (Microsoft Word 2002). In some cases, before the visual correction, the alignments were checked with Gblocks version 0.91b (Castresana, 2000), to remove hypervariable regions.

For the phylogenetic inference, the following methods were used:

- **Neighbor-joining** (NJ): NJ analyses were performed in the MEGA 4.1 computer program (Tamura *et al*., 2007), using the model *Maximum Composite Likelihood* (Tamura *et al*., 2004) with bootstrap analysis of 1000 replicates (Felsenstein, 1985).

- **Maximum parsimony** (MP): phylogenetic inference to find the most parsimonious tree was performed in the PAUP (version 4.0b10) (Swofford, 2001). Heuristic searches was performed with 100 replicates, with random sequence addition and tree bisection-reconnection branch swapping algorithms, collapsing zero-length branches and saving all minimal length trees. Gaps were treated as missing data.

- **Maximum likelihood** (ML): trees were inferred by the PhyML (version 3.0) (Guindon and Gascuel, 2003), with a heuristic search based on 100 replicates, with random sequence addition and nearing neighbor interchange branch-swapping algorithm. The bootstrap analysis was performed with 1000 replicates.

- **Bayesian inference** (BI): The bayesian analyses were performed in the MrBayes (version 3.1) computer program (Huelsenbeck and Ronquist, 2001), running 1.000.000 generations in four chains, saving the trees every 100 generations. The 25% of the all trees obtained were used to construct a 50% majority-rule consensus tree.

  For the analyses of ML and BI, the best-fit models were selected by MrModeltest (Posada and Crandall, 1998).

The sequences generated during our research were deposited in EMBL (http://www.ebi.ac.uk/embl/Submission/).

3.4 Mating tests

To evaluate the formation of sexual structures (zygospores) the isolates were grown on CZA plates at 37 ±1°C in the dark, after which they were paired in all combinations, including selfcrosses, on CZA. Each strain was streaked onto one-half of a CZA plate opposite the streak of another strain, allowing for a central zone of contact when the strains grew. Plates were incubated at 37 ±1°C and were examined macroscopically each week for as long as 4-6 months for the presence of zygospores. All tests were performed in duplicate.

3.5 Antifungal susceptibility testing

The antifungal susceptibility tests were performed for the species belonging to *Apophysomyces*, *Mucor* and *Saksenaea*, using pure powders of known potency.

A total of 9 antifungal drugs were tested. They were: amphotericin B (AMB; Sigma-Aldrich, Saint Quentin Fallavier, France), anidulafungin (ANI; Pfizer Inc., New Cork, NY, U.S.A), voriconazole (VRC; Pfizer Central Research, Sandwich, United

Kingdom), itraconazole (ITC; Janssen-Cilag, Issy-les-Moulineaux, France), ravuconazole (RVC; Bristol-Myers Squibb Co., New Brunswick, NJ, U.S.A), caspofungin (CAS; Merck&Co., Inc., Rahway, NJ, U.S.A), posaconazole (PSC; Schering-Plough Ltda., Hertfordshire, United Kingdom, Schering-Plough Research Institute, Kenilworth, NJ, U.S.A, and Schering-Plough Europa, Brussels, Belgium), micafungin (MFG; Astellas Pharma, Osaka, Japan), and terbinafine (TER; Novartis Pharma AG, Basel, Switzerland).

The isolates were subcultured on CZA for 7 to 20 days at 30°C or 37°C. Sporangiospores were then collected in water, and the suspension was adjusted to 2 x 10^4 UFC^5/mL per well. Antifungal susceptibility testing was performed by a broth microdilution technique according to the guidelines of the National Committee for Clinical Laboratory Standards M38-A (NCCLS, 2002), and M38A-2 (CLSI, 2008).

The antifungal drugs were dissolved in dimethyl sulfoxide (DMSO, Panreac Química S.A., Barcelona, Spain), or in distilled water (MFG). Subsequently, the stocks solutions of antifungal drugs were serially diluted in DMSO, and then each drug concentration was diluted 1:50 in RPMI. Wells of 96-well microtiter plates were filled with 100 µL of each drug concentration. A drug-free well containing RPMI + 2% DMSO in the medium served as the growth control. Each well was inoculated with 100 µL of conidial suspension diluted 1:50 in RPMI. With the aim to evaluate the viability, 100 µL of the dilutions were transferred to PDA plates. The microtiter plates were incubated for 24-48 h at 35ºC in dark. The fungal growth was assessed visually at 48 h, and confirmed at 72 h with the aid of a concave mirror and graded according to NCCLS guidelines as follows: 4, no reduction in growth; 3, slight reduction in growth; 2, prominent reduction in growth; 1, slight growth; and 0, absence of visual growth compared with the growth in the drug-free well.

4. RESULTS AND DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND PHYLOGENY OF CLINICALLY IMPORTANT MUCORALEAN FUNGI
Eduardo Álvarez Duarte
DL: T-1721-2011

4.1 Spectrum of Zygomycete Species Identified in Clinically Significant Specimens in the United States

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Journal of Clinical Microbiology (2009) 47, 1650–1656

JOURNAL OF CLINICAL MICROBIOLOGY, June 2009, p. 1650–1656 Vol. 47, No. 6
0095-1137/09/$08.00+0 doi:10.1128/JCM.00036-09

Spectrum of Zygomycete Species Identified in Clinically Significant Specimens in the United States▽†

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Received 7 January 2009/Returned for modification 5 March 2009/Accepted 9 April 2009

Several members of the order *Mucorales* (subphylum *Mucoromycotina*) are important agents of severe human infections. The identification of these fungi by using standard mycologic methods is often difficult and time consuming. Frequently, the etiological agent in clinical cases is reported either as a *Mucor* sp., which is not the most frequent genus of zygomycetes, or only as a member of the *Mucorales*. For this reason, the actual spectrum of species of zygomycetes and their incidences in the clinical setting is not well known. The goals of this study were to compare the results of the molecular identification of an important set of clinical isolates, received in a mycological reference center from different regions of the United States, with those obtained by using the traditional morphological methods and to determine the spectrum of species involved. We tested 190 isolates morphologically identified as zygomycetes by using sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA. Molecular identification revealed that *Rhizopus oryzae* represented approximately half (44.7%) of these isolates. The remainder was identified as *Rhizopus microsporus* (22.1%), *Mucor circinelloides* (9.5%), *Mycocladus corymbifer* (formerly *Absidia corymbifera*) (5.3%), *Rhizomucor pusillus* (3.7%), *Cunninghamella bertholletiae* (3.2%), *Mucor indicus* (2.6%), *Cunninghamella echinulata* (1%), and *Apophysomyces elegans* (0.5%). The most common anatomic sites for clinically significant zygomycetes, as determined by isolates sent to the Fungus Testing Laboratory for identification and/or susceptibility testing and included in this study, were the sinuses, lungs, and various cutaneous locations, at 25.8%, 26.8%, and 28%, respectively. These sites represented approximately 80% of the isolates evaluated. A high level of correlation (92.6%) between morphological and molecular identifications was found.

Members of the subphylum *Mucoromycotina* (formerly *Zygomycota*) (10) are characterized by the production of a coenocitic mycelium and the formation of asexual spores (sporangiospores) in a variety of fungal structures. A few are homothallic, forming zygospores in culture. They are distributed worldwide and are ubiquitous in soil and organic substrates. Roden et al. (23) reported a 70% increase in the number of cases of zygomycosis between 1940 and 2000. These infections were more frequently seen in neutropenic patients, transplant recipients, patients with hematological disease or diabetes mellitus, patients receiving deferoxamine therapy (9, 18, 21, 32, 38), and intravenous drug users (17). The most common clinical infections in order were rhino-orbito-cerebral, cutaneous, pulmonary, disseminated, and gastrointestinal manifestations (23). The most clinically important zygomycetes are in the order *Mucorales*, comprising approximately 60 genera, some of which are important etiologic agents of human disease, especially in immunocompromised patients (6). *Rhizopus* is the most common genus causing human infection, although other genera such as *Mucor*, *Rhizomucor*, *Cunninghamella*, *Apophysomyces*, and *Mycocladus* (formerly *Absidia*) have also been reported, although less frequently (6, 7, 21).

As has been demonstrated, pathogenic species of the zygomycetes show important differences in their responses to antifungal drugs (1, 31), and their correct identification in human infection is of prime importance (4). However, the etiologic agents of zygomycoses in numerous clinical cases are not identified to the species level or are more commonly being improperly named *Mucor* spp. Routine laboratory tests commonly identify isolates only as a zygomycete or to the genus level at best. In the most comprehensive review of zygomycoses published to date, a high percentage of the 929 cases reviewed lacked identification to the species level, and for most, the identification is doubtful (23). In recent years, it has been demonstrated that the analysis of DNA sequences, especially that of ribosomal DNA (rDNA), is very useful for the identification of zygomycetes (29, 36, 37).

We have retrospectively analyzed a large number of human clinical isolates of zygomycetes preserved at the Fungus Testing Laboratory in the Department of Pathology at the University of Texas Health Science Center at San Antonio. Given the difficulty of morphological identification, final identifications were reached after sequencing the internal transcribed spacer (ITS) region of the rDNA.

MATERIALS AND METHODS

Fungal isolates. A total of 190 clinical isolates submitted to the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio from the period of January 2001 to February 2007 were analyzed (see the supplemental material). In addition, the type or reference strains of *Apophysomyces elegans*, *Cunninghamella bertholletiae*, *Cunninghamella echinulata*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor indicus*, *Mucor racemosus*, *Mucor ramosissimus*, *Mycocladus corymbifer*, *Rhizomucor pusillus*, *Rhizomucor variabilis*, *Rhizo-*

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† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 22 April 2009.

TABLE 1. Comparison of morphological and molecular identification of isolates examined

Morphological identification of species	No. of isolates identified by molecular methods															% Correlation (morphological/ molecular)
	R. oryzae	*R. microsporus*	*M. corymbifer*	*M. circinelloides*	*R. pusillus*	*C. bertholletiae*	*M. indicus*	*Apophysomyces* spp.	*Mucor* sp. strain 1[a]	*Mucor* spp.	*Cunninghamella* spp.	*C. echinulata*	*Mucor* sp. strain 2[b]	*A. elegans*	Total	
Rhizopus oryzae	85														85	100
Rhizopus microsporus		42													42	100
Mucor circinelloides				18											18	100
Mycocladus corymbifer			10												10	100
Cunninghamella bertholletiae						6					3				9	66
Rhizomucor pusillus					7										7	100
Mucor indicus							5						1		6	83.3
Apophysomyces elegans								4						1	5	20
Mucor racemosus										3					3	0
Mucor ramosissimus									3						3	0
Cunninghamella echinulata												2			2	100
Total	85	42	10	18	7	6	5	4	3	3	3	2	1	1	190	92.6

[a] The results of the BLAST search showed 94 to 95% MLI with the *Mucor circinelloides* type strain CBS 195.68 and 97 to 99% MLI with a *Mucor racemosus* non-type strain (ATCC 1216B; AJ271061.1). In the phylogenetic tree (Fig. 2), the sequences of this species and of the *Mucor racemosus* type strain were rather distant.
[b] The results of the BLAST search showed 95% MLI with the *Mucor indicus* type strain CBS 226.29.

mucor variabilis var. *regularior*, *Rhizopus microsporus*, and *Rhizopus oryzae* were also included in the analysis. All isolates, including the reference strains, were subcultured onto plates containing potato dextrose agar (Pronadisa, Madrid, Spain) at room temperature (24°C ± 2°C) for 2 to 5 days to ensure purity. Isolates were subsequently grown on potato dextrose agar slants for 7 days and later covered with mineral oil for long-term room temperature storage.

Morphological identification. The isolates were identified using schemes based on morphological and physiological characters (6, 25–28, 40). Microscopic observation was made from slide preparations mounted in water and lactophenol, using a Leitz Dialux 20EB microscope.

DNA extraction, amplification, and sequencing. DNA was extracted and purified directly from fungal colonies following the FastDNA kit protocol (Bio101, Vista, CA), with a minor modification consisting of a homogenization step repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY). The DNA was quantified by GeneQuant *pro* (Amersham Pharmacia Biotech, Cambridge, England). The ITS region of the nuclear rDNA was amplified with the primer pair ITS5 and ITS4, following the protocol described by Gilgado et al. (8).

The PCR mix (25 μl) included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ (10× PerkinElmer buffer II plus $MgCl_2$ solution; Roche Molecular Systems, Branchburg, NJ), 100 μM of each deoxynucleoside triphosphate (Promega, Madison, WI), 1 μM of each primer, and 1.5 U of AmpliTaq 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 52°C, and extension for 1 min at 72°C. After PCR analysis, the products were purified with an illustra GFX PCR DNA and gel band purification kit (General Electric Healthcare, Buckinghamshire, United Kingdom) and stored at −20°C until they were used in sequencing. PCR products were sequenced by using the same primer 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, WI) Multiple-sequence alignments were performed with the ClustalX (version 1.8) computer program (35), followed by manual adjustments with a text editor. These products were analyzed on an ABI Prism 310 automated DNA analyzer (Applied Biosystems).

Phylogenetic analyses. The phylogenetic analyses were performed with the help of the software program MEGA 4.0 (33). The maximum composite likelihood algorithm was used for the determination of evolutionary distances between sequences. Trees were built using the neighbor-joining (NJ) method. Gaps were treated as pairwise deletions. Support for internal branches was assessed by a search of 1,000 bootstrapped sets of data. The final identification of the isolates was performed using Basic Local Alignment Search Tool (BLAST) searches (2). Only the nucleotide sequences of type or reference strains, deposited in the GenBank/EMBL database, were considered for identification purposes. When the BLAST algorithm aligned and compared the sequences of our isolates with those considered confident, a maximal level of identity (MLI) equal to or higher than 98% was considered for specific identification. MLI values lower than 98% provided identification only to the genus level.

Nucleotide sequence accession numbers. Sequences from one isolate identified as *Mycocladus corymbifer* (UTHSC 06-1655) and another identified as *Rhizopus microsporus* var. *oligosporus* (UTHSC 03-3512) have been deposited in GenBank under accession numbers FN293105 and FN293106, respectively.

RESULTS

The lengths of the amplicons of the ITS1-5.8S-ITS2 region in *Rhizopus oryzae* isolates varied from 530 to 550 bp; in *R. microsporus* isolates, from 601 to 616 bp; in *Mucor circinelloides*, from 524 to 572 bp; in *Mycocladus corymbifer*, from 681 to 700 bp; in *Cunninghamella bertholletiae* and a *Cunninghamella* sp., from 602 to 631 bp; in *Mucor indicus*, from 520 to 537 bp; in *Rhizomucor pusillus*, from 526 to 529 bp; in *Apophysomyces elegans* and an *Apophysomyces* sp., 672 to 818 bp; in the other *Mucor* sp., from 524 to 572 bp; and in *Cunninghamella echinulata*, from 770 to 810 bp.

Table 1 shows the results of the molecular identification. A total of nine known species could be identified. In addition, two undescribed species of *Mucor* (called *Mucor* sp. strain 1 and *Mucor* sp. strain 2), which were morphologically identified as *Mucor ramosissimus* and *Mucor indicus*, respectively, were also detected. The molecular study also revealed that not all the *Apophysomyces* isolates belong to a single species, but unfortunately the ITS sequence of the type strain of *A. elegans* is not in GenBank and was not available for study. Lastly, molecular characterization also supports the possibility of other species of *Cunninghamella* in addition to *C. echinulata*

and *C. bertholletiae*. In order of frequency, the most prevalent agent of zygomycosis was *Rhizopus oryzae*, comprising nearly half of the isolates tested (44.7%), and this was followed by *R. microsporus* (22.1%), *Mucor circinelloides* (9.5%), *Mycocladus corymbifer* (5.3%), *Rhizomucor pusillus* (3.7%), *Cunninghamella bertholletiae* (3.2%), *Mucor indicus* (2.6%), *Cunninghamella echinulata* (1%), and *Apophysomyces elegans* (0.5%). Since only a total of 7.4% of the isolates could not be identified to the species level, the correlation between morphological and genetic methods at the species level was 92.6% and at the genus level it was 100%. The only discrepancies corresponded with those isolates that represented undescribed species. A listing of the anatomic sites for the isolates based upon the information available and cross-referenced by species is provided in Table 2. The majority of the isolates, approximately 80%, were represented by isolates from the sinuses (25.8%), lungs (26.8%), and various cutaneous presentations (28%). The remaining 20% of the isolates consisted of a subset collected from deep sites, such as the brain, bones, liver, bladder, blood, bowel, and heart as well as from a few miscellaneous sites and one isolate from a marine mammal.

Figure 1 shows the NJ tree of the 5.8S rRNA genes of a representative number of the isolates treated in this study, including the type and reference strains of the species mentioned above. Due to the high level of variability in the sequences of the ITS regions, it was not possible to align them all with confidence. Therefore, in this analysis we used only sequences of the 5.8S rRNA gene. Six main clades, each supported by a high bootstrap value and representing a different genus (*Mucor*, *Rhizopus*, *Mycocladus*, *Apophysomyces*, *Rhizomucor*, and *Cunninghamella*), were observed in the phylogenetic tree (Fig. 1). The different species of each genus were also well separated, with the exception of those belonging to the genus *Mucor*. To better determine the phylogenetic relationship among the species of the genus *Mucor*, a new analysis using the sequences of the ITS regions of the isolates of this genus was performed. The sequences of the type strains of *Rhizomucor variabilis* var. *variabilis* and *Rhizomucor variabilis* var. *regularior* were also included to build an NJ tree (Fig. 2). In the ITS tree, the *Mucor* species were well separated in different clades, with each receiving a high level of statistical support. All the isolates of *M. circinelloides* and the type strain of *Rhizomucor variabilis* var. *regularior* were nested in a single clade. A total of 14 isolates could not be assigned to any known species.

TABLE 2. Source and identification of isolates examined

Species	No. (%) of isolates obtained from indicated source(s)																			
	Most common				Systemic										Miscellaneous					Grand total (%)
	Sinuses and related sites	Lungs and related sites	Various cutaneous sites	Total	Brain	Bone	Liver	Urine/bladder	Blood	Peritoneal fluid	Bowel	Heart	Unknown tissue	Nail	Genitals	Unknown tissue	Dolphin	
Rhizopus oryzae	32	14	26	72		1		2			1		6	1	1	1		85 (44.7)
Rhizopus microsporus	5	22	10	34		1		1					1			2		42 (22.1)
Mucor circinelloides	4	2	7	13		1			1				1			2		18 (9.5)
Mycocladus corymbifer	3	3		7	1				1			1						10 (5.3)
Rhizomucor pusillus	2	3	1	6					3		1					1		7 (3.7)
Other *Mucor* spp.	1	1	2	4						1								7 (3.7)
Cunninghamella bertholletiae	1	3	1	5	1													6 (3.2)
Mucor indicus			1	1			2			1			1					5 (2.6)
Other *Apophysomyces* spp.	1		3	4														4 (2.1)
Other *Cunninghamella* spp.	1	2		3														3 (1.6)
Cunninghamella echinulata	1	1	1	3														2 (1)
Apophysomyces elegans																	1	1 (0.5)
Total	49 (25.8)	51 (26.8)	53 (28)	153 (80.6)	2 (1.1)	3 (1.6)	2 (1.1)	3 (1.6)	5 (2.6)	2 (1.1)	1 (0.5)	1 (0.5)	9 (4.7)	1 (0.5)	1 (0.5)	6 (3.1)	1 (0.5)	190 (100)

DISCUSSION

This study contains the largest number of clinical isolates of zygomycetes identified to the species level by molecular characterization. Unfortunately, similar studies performed in other countries for comparison purposes do not exist. In the review of Roden et al. (23), the zygomycetes causing approximately half of the reported cases were identified by culture. However, that study was only a compilation of unrelated cases, with identifications performed in different institutions. In numerous cases, the mycological methods used were not described. Therefore, most of those identifications are considered doubtful or of insufficient accuracy. Three recent retrospective studies performed in the United States (13, 14, 30) included a

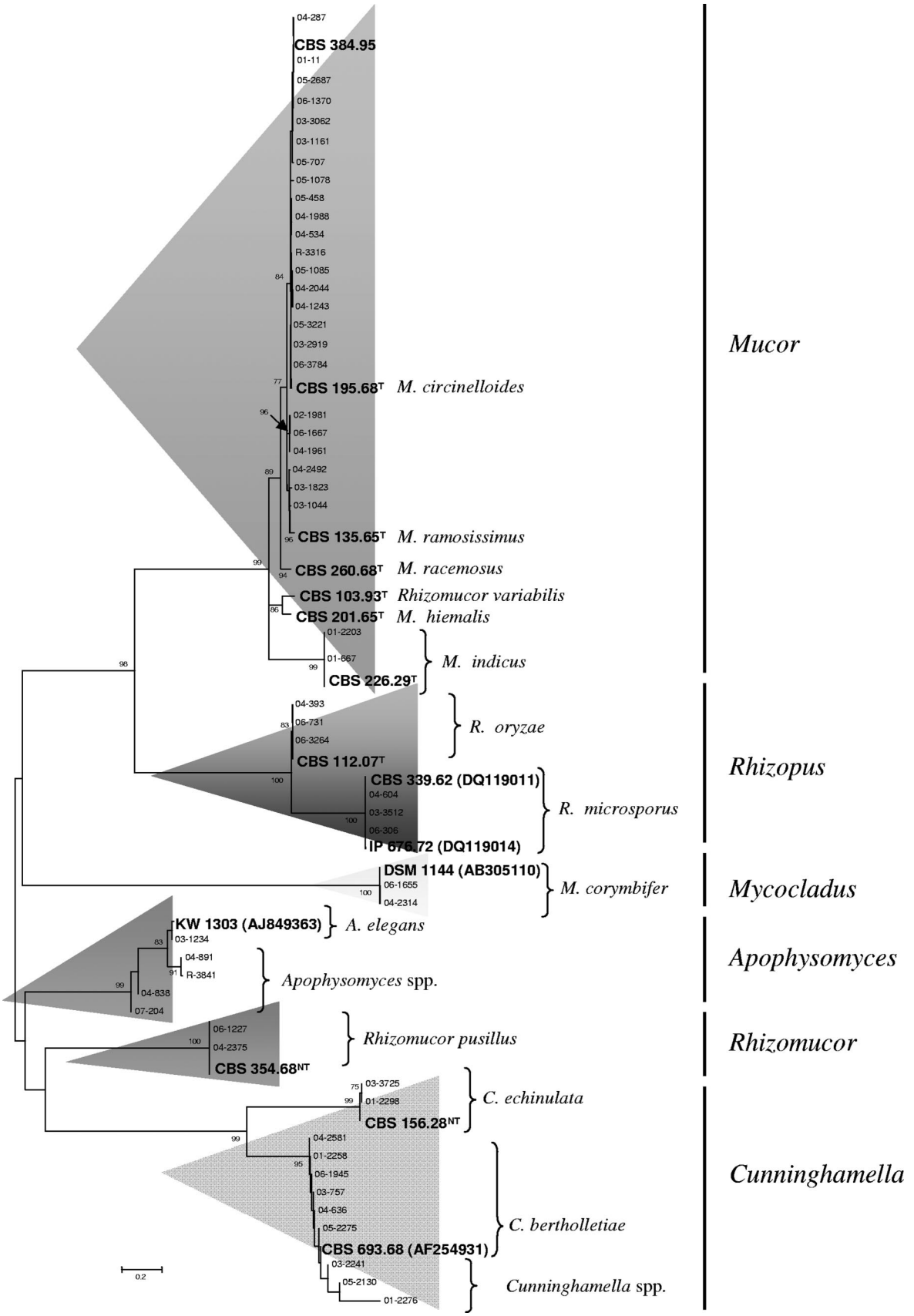
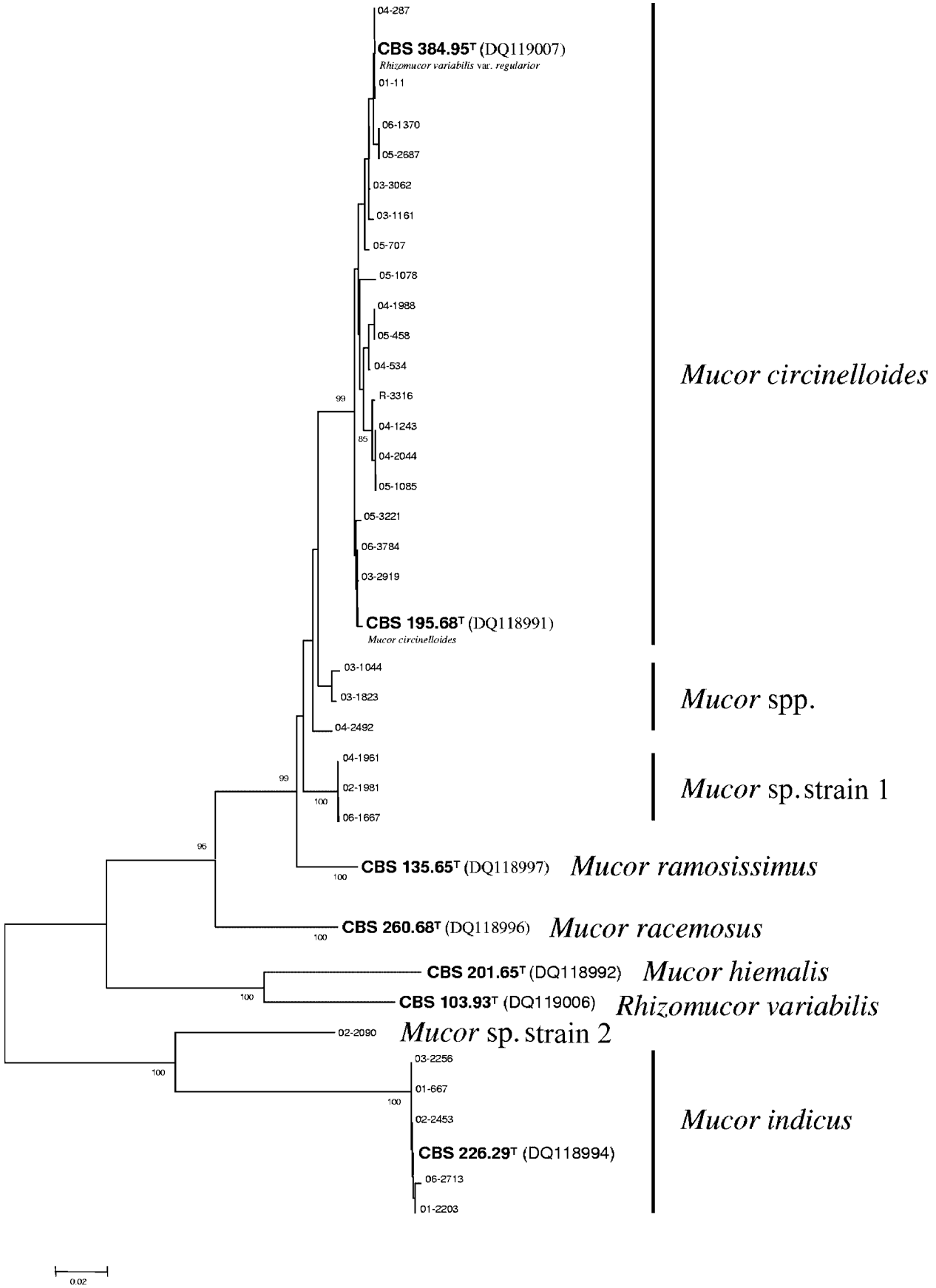


FIG. 1. NJ tree based on maximum composite likelihood-corrected nucleotide distances among 5.8S rRNA gene sequences of representative isolates of the species listed in Table 1. In the tree, branch lengths are proportional to distance. Bootstrap iteration frequencies (1,000 iterations) above 70% are indicated on the nodes. Type or reference strains are indicated in boldface. ^{T}, type strain; ^{NT}, neotype strain.



significant number of cases. However, one of these (30) distinguished only between *Mucor* spp. and *Rhizopus* spp., and in the others (13, 14) only the genus names were mentioned (*Cunninghamella*, *Mucor*, *Rhizopus*, and *Syncephalastrum*). The election of a cutoff score of ≥98% for the molecular identification of the isolates was based in the sequence variability observed within the species represented by well-supported clades (bootstrap values, ≥80%) in the phylogenetic tree (Fig. 1).

In this study, isolates from the sinuses and related areas (sino-orbital, sino-nasal, and hard palate) represented 25.8% of the isolates evaluated. Roden et al. (23) reported that sinus involvement, consisting of rhinocerebral, sinus, and sino-orbital infections, constituted the majority (66%) of infections in diabetic patients.

The lungs and related sites were similarly represented at 26.8%. Among all forms of zygomycosis, cutaneous infection is a less frequent presentation (3, 21), and it is associated with penetrating trauma, burns, motor vehicle accidents, and falls (23). In the present study, however, cutaneous sites accounted for 28% of the isolates, and the most prevalent agent was *Rhizopus oryzae*.

The molecular identification of clinical zygomycetes using the ITS region has been successfully used in recent years (11, 12, 19, 29, 36, 37, 39). However, a BLAST search can constitute an important limitation of such procedures when comparisons are made with inaccurate sequence data (5). In our case for the sequences of *Mucor circinelloides*, the BLAST search gave a similarity of 99 to 100% with the type strain of *Rhizomucor variabilis* var. *regularior*. When we compared the sequences of the type strains of both species, we noticed that they were identical. Considering that in our phylogenetic tree the type species of *Rhizomucor*, *Rhizomucor pusillus*, was placed very far from the *Mucor* clade (Fig. 2), it seems logical to consider *R. variabilis* var. *regularior* a synonym of *Mucor circinelloides*. Schwarz et al. (29) also previously reported a high level of similarity between the sequences of these two species. *Rhizomucor variabilis* var. *variabilis* was also included in our study in the *Mucor* clade, in this case close to *M. hiemalis*. Voigt et al. (37), analyzing the 28S and 18S rDNA loci, also reported that *R. variabilis* var. *variabilis* was phylogenetically closely related to *Mucor hiemalis* and *Mucor mucedo*. The most important morphological feature reported in the literature to differentiate *Rhizomucor* spp. from *Mucor* spp. is the presence of rudimentary rhizoids in the former. However, this does not seem to be a very consistent taxonomic feature, since this study demonstrated that the two varieties of *R. variabilis*, which have such rhizoids, belong to the genus *Mucor*. In addition, *R. variabilis* shows several morphological features typical of *Mucor* spp., such as the size and type of sporangiospores, the presence of chlamydospores, the maximum temperature for growth, and other cultural characteristics.

Infections by *Rhizomucor* spp. are rare in humans and are caused mostly by *R. pusillus* (11, 21). The sequences of the *R. pusillus* isolates analyzed here were very similar, and they can be easily distinguished from those of other genera. These results agree with previous studies reported by different authors (11, 19, 29).

In the present study, the ITS sequences of six isolates included in the *Mucor* clade (Fig. 2) presented a very low level of similarity with the sequences of the species of *Mucor* deposited in GenBank. Three of these isolates showed identical sequences (*Mucor* sp. strain 1) and were distributed into one well-supported subclade. Initially they were morphologically identified as *Mucor ramosissimus*. However, the BLAST search showed a low percentage of similarity (94 to 95%) with the type strain of *M. ramosissimus* (CBS 135.65). The other three isolates, which had different sequences between them, were initially morphologically identified as *Mucor racemosus*. In this case, the BLAST search for these isolates also showed a low similarity (90 to 91%) with the type strain of *M. racemosus* (CBS 260.68). Another isolate of *Mucor* that we could not identify to the species level was UTHSC 02-2090 (*Mucor* sp. strain 2). This isolate was phylogenetically and morphologically related to *M. indicus* isolates. It is of interest that two of the *M. indicus* isolates were recovered from the liver, corroborating previous reports of this organism's ability to disseminate, particularly in immunocompromised and/or neutropenic individuals (34). One report of a bone marrow transplant recipient suggests that the organism may have been acquired following ingestion of naturopathic medicine containing the organism (20).

In general, we found a high genetic variability in the 5.8S rRNA gene sequences of species from *Cunninghamella* and *Apophysomyces*. In the former genus, our analysis was able to clearly differentiate *C. echinulata* and *C. bertholletiae*. However, we were not able to identify to the species level the other three isolates with high percentages of similarity which were morphologically identified as *C. bertholletiae*. The most common and practically the sole species of the genus *Cunninghamella* that is traditionally considered the etiologic agent of human infections is *C. bertholletiae* (21, 24). However, Lemmer et al. (15) reported in Germany a case of human infection by *C. echinulata* which was identified by sequencing the ITS region. In that study, the isolates of *C. echinulata* from environmental and clinical origins showed identical digestion patterns in the ITS restriction fragment length polymorphism by using TaqI and HinfI. Our study confirms the identification of *C. echinulata* as a clinically significant isolate. In the *Apophysomyces* clade, although all the isolates included were morphologically similar to *A. elegans*, the only species of the genus involved in human infections so far, a high level of molecular intraspecific variability was observed. Five of these isolates showed a low level of similarity (87 to 91%) with the sequence of a reference strain of *A. elegans* (AJ849363). Most of the *Apophysomyces elegans* infections reported have occurred in immunocompetent patients (16). In this study, the one isolate with 99% similarity to the reference strain was recovered from a dolphin. This organism, along with *Saksenaea vasiformis*, is a

FIG. 2. NJ tree based on maximum composite likelihood-corrected nucleotide distances among the ribosomal ITS regions and 5.8S rRNA gene sequences of the isolates included in the *Mucor* clade shown in Fig. 1. In the tree, branch lengths are proportional to distance. Bootstrap iteration frequencies (1,000 iterations) above 70% are indicated on the nodes. Type strains are indicated in boldface. ^{T}, type strain.

known aggressive and commonly systemic pathogen in killer whales (*Orcinus orca*), Pacific white-sided dolphins (*Lagenorhynchus obliquidens*), and bottlenose dolphins (*Tursiops truncates*) (22). The other four morphologically similar human isolates occurred in one case of sino-orbital involvement and three cutaneous presentations (Table 2).

In conclusion, although the identification of zygomycetes remains a difficult and time-consuming task, this study has demonstrated that morphological features alone, when assessed by individuals with expertise in fungal identification, can provide a high level of accuracy and that ITS sequencing can be a useful tool in the identification of the most common clinically significant species of zygomycetes and the delineation of undescribed species. The most common species in this set of clinical isolates were *Rhizopus oryzae* and *Rhizopus microsporus*.

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Supplementary material. Table listing the source and molecular identification of the isolates studied

#	Source	Isolates # (UTHSC)	Molecular identification
1	Right hand	01-11	*Mucor circinelloides**
2	Right cribiform	01-149	*Rhizopus microsporus*
3	Face tissue	01-316	*Rhizopus oryzae*
4	Stoma tissue	01-667	*Mucor indicus*
5	Lung cavity	01-983	*Rhizopus microsporus*
6	Slant-not provided	01-1164	*Rhizopus microsporus*
7	Fine needle aspirate left upper lobe	01-1325	*Rhizopus microsporus*
8	Arm tissue	01-1543	*Rhizopus oryzae*
9	Sinus	01-1730	*Rhizopus oryzae*
10	Tissue nasal tip	01-1944	*Rhizomucor pusillus*
11	Lung tissue	01-1972	*Rhizopus microsporus*
12	Hard palate	01-1985	*Rhizopus microsporus*
13	Right maxillary sinus	01-2135	*Rhizopus oryzae*
14	Sinus tissue	01-2181	*Rhizopus oryzae*
15	Liver abscess	01-2203	*Mucor indicus*
16	Ethmoid sinus	01-2258	*Cunninghamella bertholletiae*
17	BAL	01-2276	*Cunninghamella* sp.
18	Sputum	01-2298	*Cunninghamella echinulata*
19	Not provided	02-439	*Rhizopus oryzae*
20	Right lower lobe tissue	02-651	*Rhizopus oryzae*
21	Bronch wash	02-740	*Rhizopus microsporus*
22	BAL	02-991	*Rhizopus microsporus*
23	Central venous line site	02-1981	*Mucor* sp.1*
24	Peritoneal dialysis fluid	02-2090	*Mucor* sp.2*
25	Forearm	02-2126	*Rhizopus microsporus*
26	Sputum	02-2365	*Rhizopus oryzae*
27	Abdomen	02-2425	*Rhizopus microsporus*
28	Hand wound	02-2453	*Mucor indicus*
29	Left leg	02-2517	*Rhizopus microsporus*
30	Lt. maxillary sinus	02-2882	*Rhizopus oryzae*
31	Deep leg	03-112	*Rhizopus oryzae*
32	Sinus	03-201	*Mycocladus corymbifer*
33	Sputum	03-228	*Rhizopus oryzae*
34	Hand tissue	03-285	*Rhizopus oryzae*
35	Lower anterior leg tissue	03-299	*Rhizopus oryzae*
36	Brain abscess	03-328	*Mycocladus corymbifer*
37	Sinus	03-511	*Rhizopus oryzae*
38	Tissue left middle meatus	03-547	*Rhizopus oryzae*
39	Right leg tissue	03-757	*Cunninghamella bertholletiae*
40	Blood	03-1044	*Mucor* spp.*
41	Vascular catheter site	03-1161	*Mucor circinelloides**
42	Sputum	03-1544	*Rhizopus microsporus*
43	Right lower lobe & left lower lobe	03-1802	*Rhizopus microsporus*

44	Bronchial	03-1823	*Mucor* spp.*
45	BAL	03-1826	*Rhizopus oryzae*
46	Bronchial	03-2032	*Rhizopus oryzae*
47	Sputum	03-2241	*Cunninghamella* sp.*
48	Liver abscess	03-2256	*Mucor indicus*
49	Left ethmoid	03-2399	*Rhizopus microsporus*
50	Not provided	03-2426	*Rhizomucor pusillus*
51	Left forearm	03-2548	*Rhizopus oryzae*
52	Tissue right upper lobe	03-2702	*Rhizopus oryzae*
53	Sinus	03-2771	*Rhizopus oryzae*
54	Arm skin	03-2869	*Rhizopus microsporus*
55	Forearm	03-2919	*Mucor circinelloides**
56	Forearm	03-2970	*Rhizopus oryzae*
57	Skin biopsy	03-3062	*Mucor circinelloides**
58	Nasal tissue	03-3110	*Rhizopus oryzae*
59	Forearm	03-3165	*Rhizopus oryzae*
60	Sputum	03-3298	*Rhizopus oryzae*
61	Sinus	03-3348	*Rhizopus oryzae*
62	Pleural fluid	03-3512	*Rhizopus microsporus*
63	Tissue-dolphin	03-1234	*Apophysomyces elegans*
64	Flank lesion	03-3725	*Cunninghamella echinulata*
65	Finger	04-161	*Rhizopus oryzae*
66	Tissue right orbit	04-278	*Rhizopus oryzae*
67	Sinus	04-287	*Mucor circinelloides**
68	Nasal	04-374	*Rhizopus oryzae*
69	Right nasal contents	04-393	*Rhizopus oryzae*
70	Left ethmoid sinus	04-443	*Rhizopus oryzae*
71	Ethmoid sinus	04-534	*Mucor circinelloides**
72	Pleural fluid	04-604	*Rhizopus microsporus*
73	Tissue occipital lobe	04-636	*Cunninghamella bertholletiae*
74	Left leg	04-838	*Apophysomyces* sp.*
75	Right sinus	04-891	*Apophysomyces* sp.*
76	Urine	04-1075	*Rhizopus microsporus*
77	Bronchial	04-1199	*Rhizopus microsporus*
78	Tissue	04-1220	*Rhizopus oryzae*
79	Bronchial	04-1241	*Rhizopus oryzae*
80	Sternal wound	04-1243	*Mucor circinelloides**
81	Cheek tissue	04-1567	*Rhizopus oryzae*
82	Tissue right hand	04-1613	*Rhizopus oryzae*
83	Right leg tissue	04-1848	*Rhizopus oryzae*
84	Blood	04-1961	*Mucor* sp.1*
85	Blood	04-1988	*Mucor circinelloides**
86	Sputum	04-2044	*Mucor circinelloides**
87	Nasal mass	04-2159	*Rhizopus oryzae*
88	Tracheal	04-2314	*Mycocladus corymbifer*
89	Nasal	04-2328	*Rhizopus oryzae*
90	Tissue right upper lobe	04-2375	*Rhizomucor pusillus*
91	Not provided	04-2459	*Rhizopus oryzae*

92	Rt. thigh	04-2492	*Mucor* spp.*
93	Bronchial	04-2581	*Cunninghamella bertholletiae*.
94	Hard palate	04-2602	*Rhizopus oryzae*
95	Sinus tissue	04-2829	*Rhizopus oryzae*
96	Nasal	04-3109	*Rhizopus oryzae*
97	Pleural fluid	04-3292	*Mycocladus corymbifer*
98	BAL	04-3294	*Rhizopus microsporus*
99	Back	04-3360	*Rhizopus microsporus*
100	Maxillary sinus	05-114	*Rhizopus oryzae*
101	Tissue	05-146	*Rhizopus oryzae*
102	Foot	05-197	*Rhizopus oryzae*
103	BAL & skin	05-458	*Mucor circinelloides**
104	Sputum	05-596	*Rhizopus microsporus*
105	Face biopsy	05-641	*Mycocladus corymbifer*
106	Abdominal	05-707	*Mucor circinelloides**
107	Right sphenoid	05-791	*Rhizopus microsporus*
108	Bladder	05-812	*Rhizopus oryzae*
109	Rt. elbow	05-937	*Rhizopus microsporus*
110	Soft tissue lt. axilla	05-1052	*Rhizopus microsporus*
111	Tibial bone	05-1078	*Mucor circinelloides**
112	Skin Bx.	05-1085	*Mucor circinelloides**
113	Lung abscess	05-1147	*Rhizopus microsporus*
114	Left knee	05-1312	*Rhizopus microsporus*
115	Tissue left orbit	05-1502	*Rhizopus oryzae*
116	Rt. lung	05-1582	*Mycocladus corymbifer*
117	Abscess	05-1679	*Rhizopus oryzae*
118	Not provided	05-1817	*Rhizopus oryzae*
119	Left upper lobe lung tissue	05-1850	*Rhizopus microsporus*
120	Rt. maxillary sinus	05-2130	*Cunninghamella* sp.*
121	Not provided	05-2275	*Cunninghamella bertholletiae*
122	Sinus	05-2447	*Rhizopus oryzae*
123	BAL	05-2501	*Rhizopus microsporus*
124	Tissue left hand	05-2658	*Rhizopus oryzae*
125	Tissue left sinus	05-2687	*Mucor circinelloides**
126	Tissue left forearm	05-2827	*Rhizopus oryzae*
127	Sputum	05-2975	*Rhizopus oryzae*
128	Forearm	05-2985	*Rhizomucor pusillus*
129	Leg tissue	05-3032	*Rhizopus oryzae*
130	Right knee	05-3111	*Rhizopus oryzae*
131	Nasal	05-3132	*Rhizopus oryzae*
132	Not provided	05-3221	*Mucor circinelloides**
133	Mid small bowel	05-3226	*Rhizopus oryzae*
134	Right hip	05-3474	*Rhizopus oryzae*
135	Wound	05-3479	*Rhizopus oryzae*
136	Middle turbinate tissue	05-3580	*Rhizopus oryzae*
137	Gingiva	06-26	*Mycocladus corymbifer*
138	Palate tissue	06-89	*Rhizopus oryzae*
139	Bronchial	06-195	*Rhizopus microsporus*

140	Lt. lung	06-229	*Rhizopus microsporus*
141	Wound right lower leg	06-306	*Rhizopus microsporus*
142	Sinus	06-329	*Rhizopus oryzae*
143	Sinus wash	06-731	*Rhizopus oryzae*
144	Bx. tissue	06-1080	*Rhizopus oryzae*
145	Tissue	06-1143	*Rhizopus oryzae*
146	Induced sputum	06-1227	*Rhizomucor pusillus*
147	Sinus	06-1301	*Rhizopus oryzae*
148	Penis tissue	06-1351	*Rhizopus oryzae*
149	Nasal	06-1370	*Mucor circinelloides**
150	Blood	06-1655	*Mycocladus corymbifer*
151	Blood	06-1667	*Mucor* sp.1*
152	Rt. Heart ventricle	06-1670	*Mycocladus corymbifer*
153	Sputum	06-1844	*Rhizopus microsporus*
154	Lung tissue	06-1945	*Cunninghamella bertholletiae*
155	Cheek tissue	06-2013	*Rhizopus oryzae*
156	Forearm	06-2251	*Rhizopus oryzae*
157	Arm	06-2374	*Rhizopus oryzae*
158	Tissue right upper lobe	06-2413	*Rhizomucor pusillus*
159	Right elbow	06-2457	*Rhizopus oryzae*
160	Sputum	06-2588	*Rhizopus microsporus*
161	Peritoneal	06-2713	*Mucor indicus*
162	Right parietal	06-2714	*Rhizomucor pusillus*
163	Sinus tissue	06-2868	*Rhizopus oryzae*
164	Skin biopsy	06-2926	*Rhizopus oryzae*
165	Abdomen	06-3264	*Rhizopus oryzae*
166	Lung aspirate	06- 3126	*Rhizopus microsporus*
167	Tissue	06-3684	*Rhizopus microsporus*
168	Not provided	06-3784	*Mucor circinelloides**
169	Left ethmoid sinus	06-3827	*Rhizopus oryzae*
170	Not provided	06-3848	*Rhizopus microsporus*
171	Sinus	06-3849	*Rhizopus oryzae*
172	BAL lingual	06-3913	*Rhizopus oryzae*
173	Tissue chest	06-4012	*Rhizopus microsporus*
174	Nasal	06-4035	*Rhizopus oryzae*
175	Lt. lung	06-4133	*Rhizopus oryzae*
176	Lung tissue	06-4152	*Rhizopus oryzae*
177	Lt maxillary sinus	06-4282	*Mycocladus corymbifer*
178	Wound	06-4336	*Rhizopus oryzae*
179	Pleural fluid	06-4399	*Rhizopus oryzae*
180	Sputum	06-4430	*Rhizopus microsporus*
181	Right cheek	06-4434	*Rhizopus oryzae*
182	Facial	07-204	*Apophysomyces* sp.*
183	Nephrostomy urine	07-238	*Rhizopus oryzae*
184	Maxillary sinus	07-365	*Rhizopus oryzae*
185	Vertebral	07-371	*Rhizopus microsporus*
186	Tracheal	07-389	*Rhizopus microsporus*
187	Not provided	R-3316	*Mucor circinelloides**

188	Not provided	R-3466	*Rhizopus microsporus*
189	Left maxilla	R-3556	*Rhizopus microsporus*
190	Necrotic face tissue	R-3841	*Apophysomyces* sp.*

- *Mucor* sp.1* The results of the BLAST search showed 94-95 % of maximum level of identity with the strain CBS 195.68 (type strain) of *Mucor circinelloides* and 97-99 % with a non–type strain of *Mucor racemosus* (ATCC1216B, AJ271061.1). In the phylogenetic tree, the sequence of this species and that of the type strain of *Mucor racemosus* were rather distant.

- *Mucor* sp.2* The results of the BLAST search showed 95 % of maximum level of identity with the strain CBS 226.29 (type strain) of *Mucor indicus*

- *Mucor* spp* The results of the BLAST search showed 94-95 % of maximum level of identity with the strain CBS 195.68 (type strain) of *Mucor circinelloides* and 98-99 % with a non–type strain of *Mucor ramosissimus* (UWFP 969, AY213664.1). In the phylogenetic tree, the sequence of this species and that of the type strain of *Mucor ramosissimus* were rather distant.

- *Mucor circinelloides** The percentages of homology in the BLAST search of these strains with the type strain of *Mucor circinelloides* were 98 – 100 %. This seems to demonstrate that this latter specie is a synonym of *Mucor circinelloides*.

- *Apophysomyces* sp.* The results of the BLAST search showed 87-91 % of maximum level of identity with the Kw1303 (GenBank # AJ849363) of *Apophysomyces elegans.*

- *Cunninghamella* sp.* The results of the BLAST search showed 87-96 % of maximum level of identity with the CBS 693.68 (GenBank # AF254931) of *Cunninghamella bertholletiae.*

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4.2 Two new species of *Mucor* from clinical samples

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Medical Mycology (2011) 49, 62–71

Medical Mycology January 2011, **49**, 62–72

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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.

Received 11 March 2010; Received in final revised form 24 May 2010; Accepted 5 June 2010

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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

DOI: 10.3109/13693786.2010.499521

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

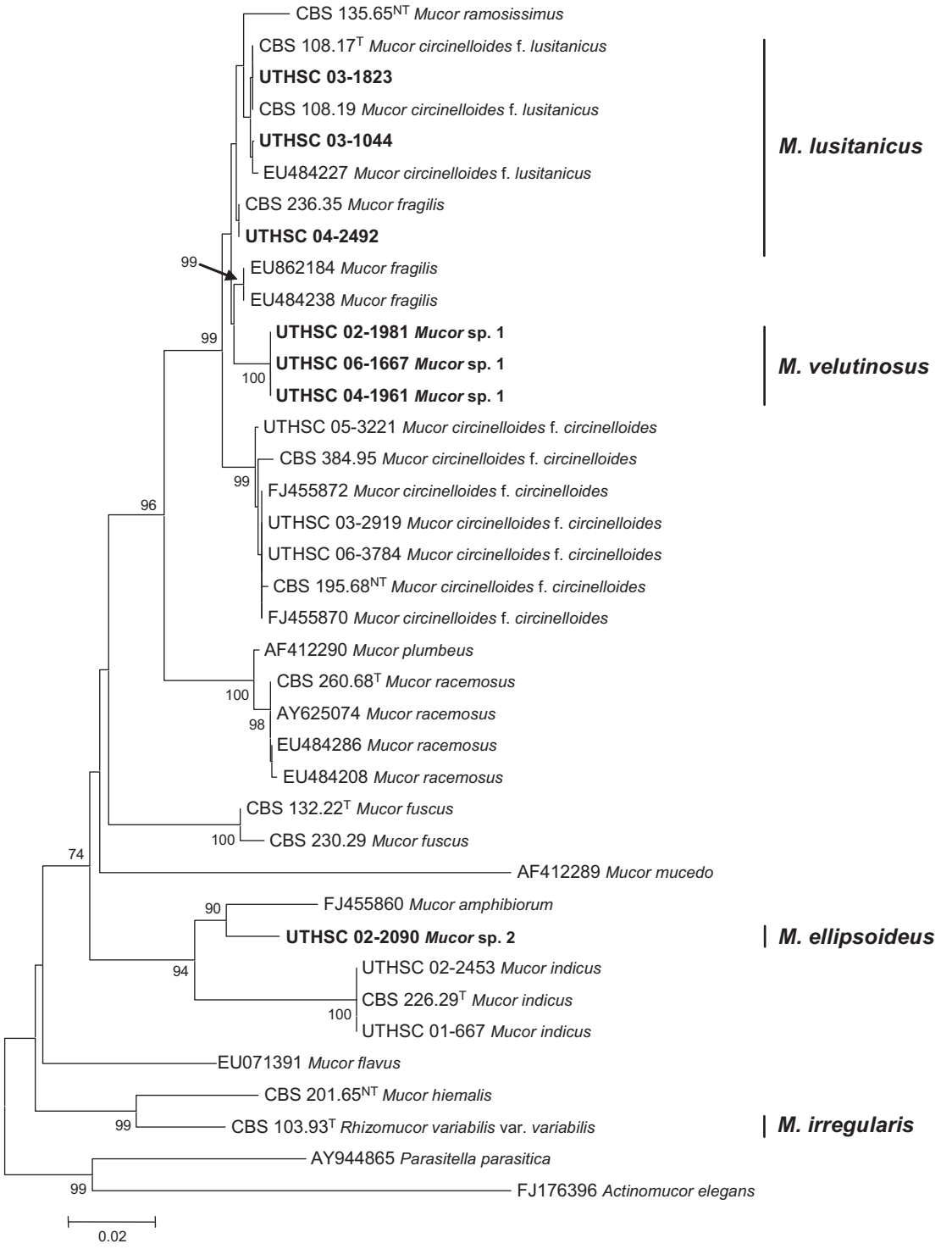

Fig. 1 Neighbor-joining tree based on maximum composite likelihood corrected nucleotide distances among the ribosomal internal transcribed spacer (ITS) regions and 5.8S rRNA gene sequences of the strains of *Mucor* and related taxa. Bootstrap support values above 70% are indicated at the nodes. The bar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

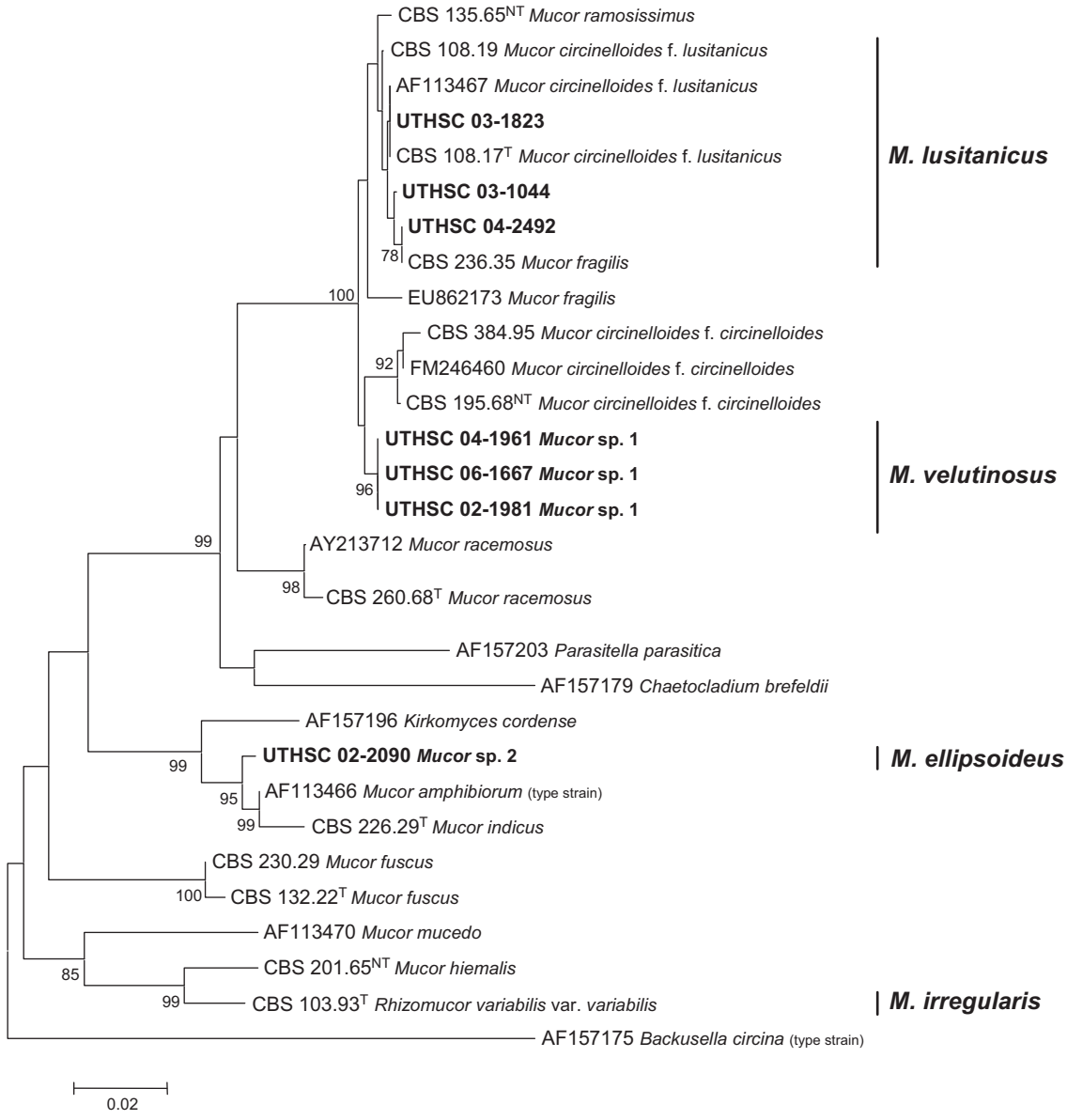

Fig. 2 Neighbor-joining tree based on maximum composite likelihood corrected nucleotide distances among the D1-D2 domains 28S rRNA gene sequences of the strains of *Mucor* and related taxa. Bootstrap support values above 70% are indicated at the nodes. The bar indicates genetic distance. A sequence of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

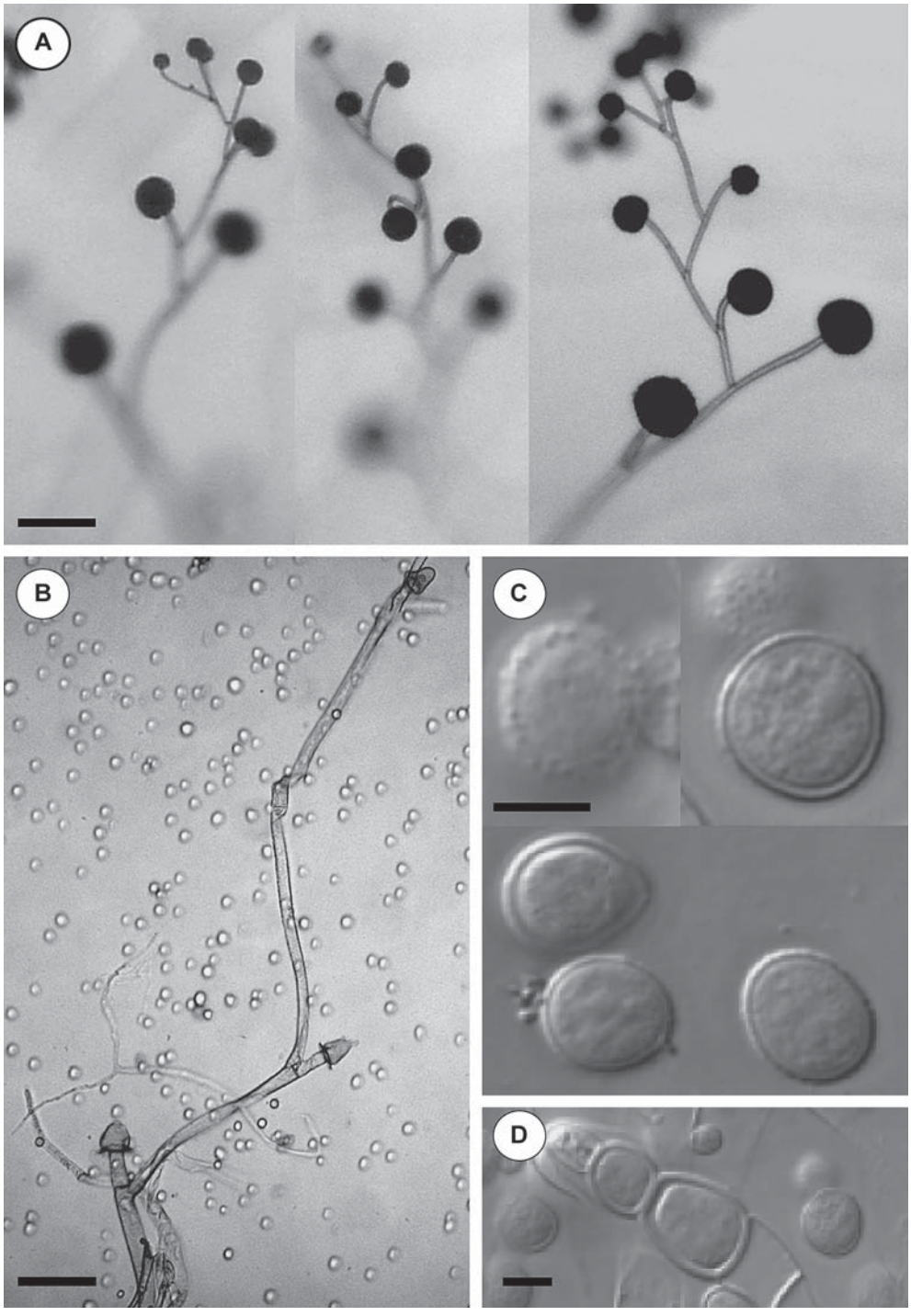

Fig. 3 *Mucor velutinosus* UTHSC 02-1981. (A) Branching pattern of sporangiophores. (B) Columellae. (C) Sporangiospores. (D) Chlamydospores. Bars: A–B = 50 μm; C–D = 5 μm.

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

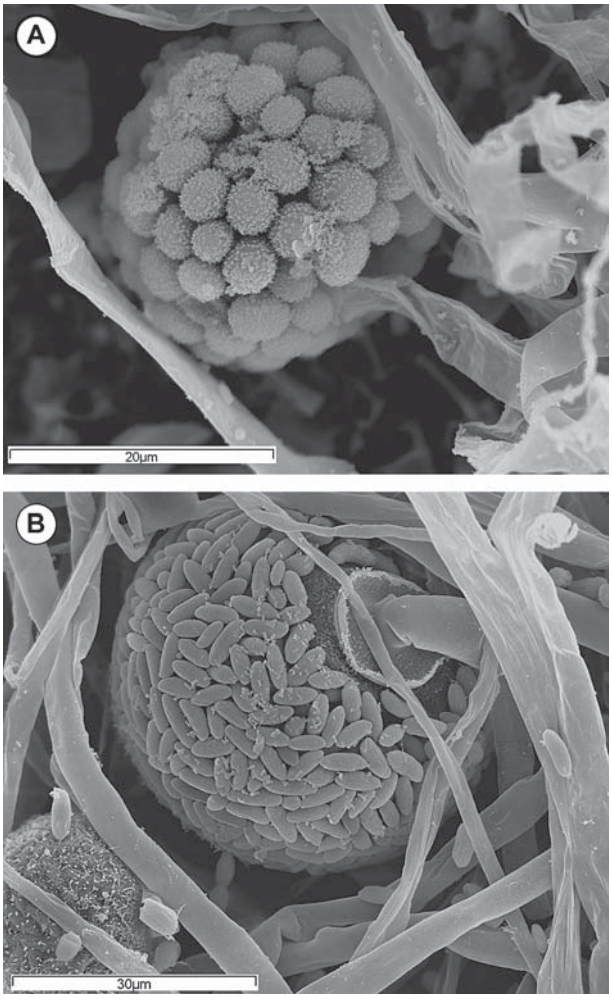

Fig. 4 *Mucor velutinosus* UTHSC 02-1981. (A) Sporangium and sporangiospores (SEM). *Mucor ellipsoideus* UTHSC 02-2090. (B) Sporangium and sporangiospores (SEM). Scale is indicated by bars.

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

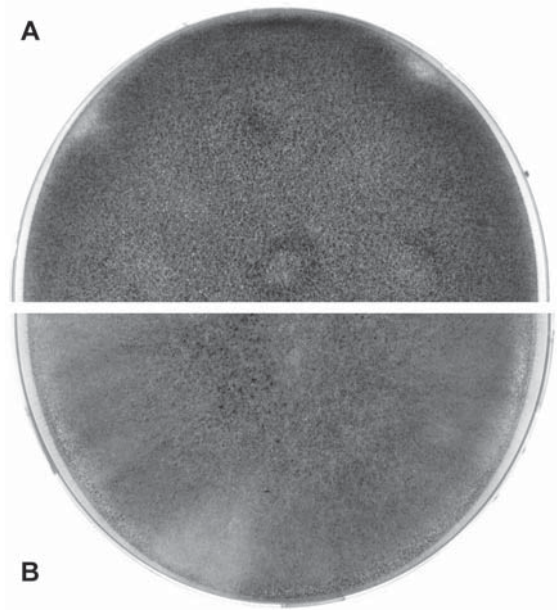

Fig. 5 Morphology of colonies on PDA. (A) *Mucor velutinosus* UTHSC 02-1981. (B) *Mucor racemosus* CBS 260.68.

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*

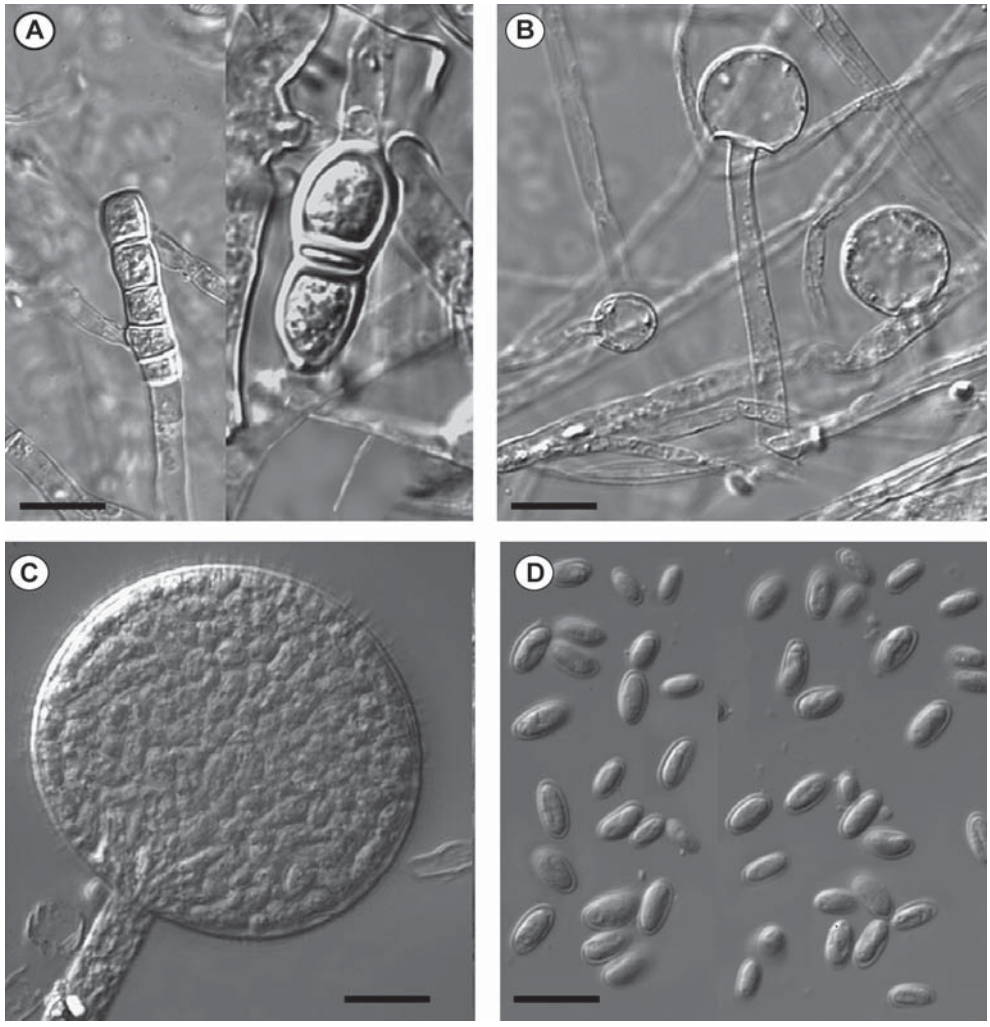

Fig. 6 *Mucor ellipsoideus* UTHSC 02-2090. (A) Chlamydospores. (B) Columellae. (C) Sporangium. (D) Sporangiospores. All bars 10 µm.

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).

Acknowledgements

This work was supported by the Spanish Ministerio de Ciencia y Tecnología grants CGL 2007-65669/BOS and CGL 2009- 08698/BOS. We thank Catalina Nuñez for technical assistance.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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This paper was first published online on Early Online on 21 July 2010.

4.3 Molecular phylogenetic diversity of the emerging mucoralean fungus *Apophysomyces*: Proposal of three new species

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Revista Iberoamericana de Micología (2010) 27, 80–89

Rev Iberoam Micol. 2010;**27(2)**:80–89

Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol

Original article

Molecular phylogenetic diversity of the emerging mucoralean fungus *Apophysomyces*: Proposal of three new species

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ARTICLE INFO

Article history:
Received 13 November 2009
Accepted 12 January 2010
Available online 4 de marzo de 2010

Keywords:
Apophysomyces elegans
Apophysomyces ossiformis
Apophysomyces trapeziformis
Apophysomyces variabilis
Phylogeny
Taxonomy
Zygomycetes

ABSTRACT

Background: Apophysomyces is a monotypic genus belonging to the order Mucorales. The species *Apophysomyces elegans* has been reported to cause severe infections in immunocompromised and immunocompetent people. In a previous study of Álvarez et al.[3] [J Clin Microbiol 2009;47:1650–6], we demonstrated a high variability among the 5.8S rRNA gene sequences of clinical strains of *A. elegans*.

Material and methods: We performed a polyphasic study based on the analysis of the sequences of the histone 3 gene, the internal transcribed spacer region of the rDNA gene, and domains D1 and D2 of the 28S rRNA gene, as well as by evaluation of some relevant morphological and physiological characteristics of a set of clinical and environmental strains of *A. elegans*.

Results and conclusions: We have demonstrated that *A. elegans* is a complex of species. We propose as new species *Apophysomyces ossiformis*, characterised by bone-shaped sporangiospores, *Apophysomyces trapeziformis*, with trapezoid-shaped sporangiospores, and *Apophysomyces variabilis*, with variable-shaped sporangiospores. These species failed to assimilate esculin, whereas *A. elegans* was able to assimilate that glycoside. Amphotericin B and posaconazole are the most active *in vitro* drugs against *Apophysomyces*.

Diversidad filogenética del hongo mucoral emergente *Apophysomyces*: propuesta de tres nuevas especies

Palabras clave:
Apophysomyces elegans
Apophysomyces ossiformis
Apophysomyces trapeziformis
Apophysomyces variabilis
Filogenia
Taxonomía
Zigomicetos

RESUMEN

Antecedentes: Apophysomyces es un género monoespécifico perteneciente al orden Mucorales. La especie *Apophysomyces elegans*, ha sido reportada como causante de infecciones severas en pacientes inmunocomprometidos e inmunocompetentes. En un estudio previo (Álvarez *et al.*, J Clin Microbiol. 2009;47:1650–6), demostramos la elevada variabilidad dentro de las secuencias del gen 5.8S del ARNr en un grupo de cepas clínicas de *A. elegans*.

Material y métodos: Hemos realizado un estudio polifásico basado en el análisis de las secuencias del gen de la histona 3, la región de los espaciadores internos del ADNr y los dominios D1 y D2 del gen 28S del ARNr, así como la evaluación de caracteres morfológicos y fisiológicos relevantes de un grupo de cepas clínicas y ambientales de *A. elegans*.

Resultados y conclusiones: Hemos demostrado que *A. elegans* es un complejo de especies. Proponemos como nuevas especies para la ciencia *Apophysomyces ossiformis*, caracterizada por sus esporangiosporas con forma de hueso; *Apophysomyces trapeziformis*, con esporangiosporas trapezoidales; y *Apophysomyces variabilis*, con esporangiosporas de formas variables. Las nuevas especies no asimilan la esculina, en tanto que *A. elegans* fue capaz de asimilar dicho glicósido. La anfotericina B y el posaconazol fueron los antifúngicos más activos frente a *Apophysomyces*.

The genus *Apophysomyces*, belonging to the subphylum Mucoromycotina (oldest phylum Zygomycota),[11] was erected by Misra et al.[18] in 1979 to accommodate the only species of the genus, *Apophysomyces elegans*, which was isolated from soil samples in northern India. This fungus was characterized by pyriform sporangia, conspicuous funnel- and/or bell-shaped apophyses, and subhyaline, thin-, and smooth-walled sporangiospores that are mostly oblong with rounded ends. It is a thermotolerant fungus that grows rapidly between 26 and 42 °C.[6,18] *A. elegans* is not only isolated from soil, decaying vegetation, and as an environmental

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doi:10.1016/j.riam.2010.01.006

contaminant,[17,18,24] but it is also able to cause severe human infections.[8] Unlike other members of Mucorales, this fungus primarily infects immunocompetent hosts.[17] The infection typically follows traumatic implantation of the agent, but may also result from inhalation of spores into the sinus.[5,6,12,15,17,21] This disease is more common in tropical and subtropical climates. Cases have been reported in Australia,[6,20] India,[5,13,14,26] the United States,[3,15] Sri Lanka,[4] Thailand,[24] and in Central and South America.[21,22]

The genetic population structure of *A. elegans* remains largely unknown and may be due in part to the lack of preservation of strains for study. In a recent survey on the spectrum of species of Mucorales from clinical sources in the United States, we demonstrated a high intraspecific 5.8S rRNA gene sequence diversity in *A. elegans*.[3] Additionally, in a typing study of *A. elegans* using microsatellites markers, it was demonstrated that, in a set of clinical strains, mainly from India, different banding patterns exist.[5] These data suggest that more than one phylogenetic species may be present within the morphospecies *A. elegans*.

To determine possible cryptic species in *A. elegans*, we performed a polyphasic study on a diverse panel of strains, based on a multilocus sequence analysis of three loci (the histone 3 gene (H3), internal transcribed spacer region of the rDNA (ITS), and domains D1 and D2 of the 28S rRNA gene) and the evaluation of different morphological and physiological characters.

Materials and methods

Fungal strains

A total of 16 strains from different origins were included in the study (Table 1). The fungi were cultured on potato dextrose agar (PDA, Pronadisa, Madrid, Spain) and incubated at 35 ± 1 °C for 2–5 days.

DNA extraction, amplification, and sequencing

DNA was extracted and purified directly from fungal colonies following a slightly modified Fast DNA kit protocol (Bio101, Vista, CA, USA), consisting of a 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 rDNA was amplified with the primer pair ITS5 and ITS4, the D1–D2 domains of the 28S rRNA gene were amplified with the primer pair NL1–NL4, and histone 3 (H3) gene was amplified with the primer pair H3-1a–H3-1b.[10]

The PCR mix (25 µl) included 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$ (10 × Perkin-Elmer buffer II plus $MgCl_2$ solution Roche Molecular Systems, Branchburg, NJ, USA), 100 µM of each dNTP (Promega, Madison, WI, USA), 1 µM of each primer, and 1.5 U of Ampli*Taq* DNA polymerase (Roche). The amplification program for the three DNA fragments 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 54 °C, and extension for 1 min at 72 °C. 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 using the same primers employed for amplification and following the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit protocol (Applied Biosystems, Gouda, Netherlands). Reactions were run on a 310 DNA sequencer (Applied Biosystems). Consensus sequences were obtained using the Autoassembler program (Perkin-Elmer-Applied Biosystems) and Seqman software (Lasergene, Madison, WI).

Phylogenetic analyses

The sequences were aligned using Clustal X (version 1.8) computer program, followed by manual adjustments with a text editor. Most-parsimonious tree (MPT) analyses were performed using PAUP* version 4.0b10. One hundred heuristic searches were conducted with random sequence addition and tree bisection–reconnection branch-swapping algorithms, collapsing zero-length branches, and saving all minimal-length trees (MulTrees). *Saksenaea vasiformis* (FMR 10131) was chosen as the outgroup. Support for internal branches was assessed using a heuristic parsimony search of 1000 bootstrapped data sets. The combined data set of the ITS, D1–D2, and H3 was tested for incongruence with the

Table 1
Origin of *Apophysomyces* strains included in the study

Isolate	Source	GenBank accession no.		
		ITS1-5.8S–ITS2	D1/D2 domains of 28S rDNA	H3
CBS[a] 476.78	Soil, Deoria, India	FN556440	FN554249	FN555155
CBS 477.78	Soil, Gorakhpur, India	FN556437	FN554250	FN555154
CBS 658.93	Osteomyelitis, Netherlands Antilles	FN556436	FN554258	FN555161
GMCH[b] 480/07	Cutaneous infection, India	FN556442	FN554253	FN555163
GMCH 211/09	Cutaneous infection, India	FN556443	FN554254	FN555164
IMI[c] 338332	Ankle aspirate, Australia	FN556438	FN554257	FN555159
IMI 338333	Daly river, Australia	FN556439	FN554256	FN555160
UTHSC[d] 03-3644	Dolphin, Florida, USA	FN556431	FN554259	FN555158
UTHSC 04-838	Cellulitis wound leg, Minnesota, USA	FN556432	FN554252	FN555157
UTHSC 04-891	Sinus, Minnesota, USA	FN556433	FN554264	FN555165
UTHSC 06-2356	Dolphin, Texas, USA	FN556427	FN554262	FN555167
UTHSC 06-4222	Dolphin, Bahamas	FN556428	FN554255	FN555162
UTHSC 07-204	Facial cellulitis, Arizona, USA	FN556435	FN554251	FN555156
UTHSC 08-1425	Abdominal tissue, Phoenix, USA	FN556429	FN554261	FN555168
UTHSC 08-2146	Skin biopsy, Colorado, USA	FN556430	FN554260	FN555169
UTHSC R-3841	Necrotic face tissue, Georgia, USA	FN556434	FN554263	FN555166

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[b] GMCH, Government Medical College and Hospital, Chandigarh, India.
[c] IMI, International Mycological Institute, CABI-Bioscience, Egham, UK.
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partition homogeneity test (PHT) as implemented in PAUP*. The Kishino–Hasegawa test was performed to determine whether trees differed significantly. Gaps were treated as missing data.

Morphological studies

The strains were subcultured on PDA, Czapek agar (CZA; Difco, Becton Dickinson, France), malt extract agar (MEA; 10 g of malt extract, 20 g of agar, and 1000 ml of distilled water), and starch agar (SA; 5 g of soluble starch, 15 g of agar, and 1000 ml of distilled water), and incubated at 37 and 42 °C. The microscopic features were determined on the sixth day in wet mounts on water and on lactic acid, which were examined under a light microscope. The strains were identified using schemes based on morphological characters.[8,18]

Physiological studies

Growth rates at 4, 15, 24, 30, 35, 37, 42, and 50 °C were determined on PDA, MEA, CZA, and SA for each of the strains included in the study. The Petri dishes were inoculated in the center, incubated in darkness, and the colony diameters (in millimeters) were measured daily.

Carbon source assimilation profiles were determined with the commercial kit API 50 CH (bioMérieux, Marcy, l'Etoile, France), following protocols described previously but with minor modifications.[23] To obtain sufficient sporulation, all strains were cultured for 6 days on CZA at 42 °C. A final concentration of 5×10^5 CFU/ml was prepared in 20 ml of yeast nitrogen base (7.7 g/l; Difco), containing 0.5 g/l L-chloramphenicol (Sigma-Aldrich) and 0.1% Bacto agar (Difco), and each well of the strips was inoculated with 300 µl of medium. The viability of the conidia was verified by plating 100 µl of serial dilutions of each inoculum onto PDA and incubating at 42 °C for 6 days. The inoculated API 50 CH strips were incubated for 48–72 h at 37 °C in darkness. After incubation, the strips were read visually and growth or lack of growth was noted. Weak growth was considered as a positive result. For nitrogen source assimilations, we used the same inoculum described above, but the yeast nitrogen base broth was replaced by carbon nitrogen base broth (Difco), and testing was performed in sterile, disposable, multiwell microplates. The medium with the nitrogen sources was dispensed into the wells in 150 µl volumes with a multichannel pipette and each well was inoculated with 50 µl of the conidial suspension. The microplates were incubated at 37 °C in darkness for 48 and 72 h. We also determined growth of the strains on NaCl (2%, 5%, 7%, 10%), $MgCl_2$ 2%, and cycloheximide 0.1%.[9,27] All tests were performed in duplicate.

The production of urease was determined after incubation on Christensen's urea agar slants at 37 °C for 8 days.[16]

Mating tests

Sixteen *Apophysomyces* strains were grown on CZA plates at 37 °C in the dark, and then paired in all combinations, including self-crosses, on CZA. Each strain was streaked onto one half of a CZA plate opposite to the streak of another strain, allowing for a central zone of contact as the strains grew. Plates were incubated at 37 °C and examined macroscopically each week for up to 6 months for the presence of zygospores. All tests were performed in duplicate.

Antifungal susceptibility testing

The *in vitro* activity of seven antifungal agents against the 16 strains of *Apophysomyces* was evaluated according to Clinical and Laboratory Standards Institute guidelines (M38-A2).[19] The drugs tested were amphotericin B (USP, Rockville, MD, USA), anidulafungin (Pfizer Inc., New York, NY, USA), caspofungin (Merck & Co., Inc., Rahway, NJ, USA), itraconazole (Jansen Pharmaceutica, Beerse, Belgium), posaconazole (Schering-Plough Ltd., Hertfordshire UK), ravuconazole (Bristol-Myers Squibb Company, New Brunswick, NJ, USA), and voriconazole (Pfizer Inc., New York, NY, USA).

Nucleotide sequence accession numbers

All the sequences obtained in this study were deposited in GenBank database and assigned the accession numbers listed in Table 1.

Results

Phylogeny

With the primers used, we were able to amplify and sequence 684–820, 582–683, and 345–382 bp of the ITS, D1–D2, and H3 loci, respectively. Of the 1630 nucleotides sequenced, 48 characters were parsimony informative in the different strains, the lowest number was eight for the H3 gene, and the highest was 27 for the ITS region. Sequences of the three region genes were analyzed phylogenetically as separate (data not shown) and combined datasets. The partition homogeneity test demonstrated that the three loci sequence data sets were congruent (P=0.05) and could therefore be combined. A total of 36 MPT was produced from a heuristic search, using the combined dataset from the three loci (Fig. 1). The trees had a consistency index of 0.969, a retention index of 0.956, and a homoplasy index of 0.031. Clustering was similar to that observed in the particular trees of the different genes analyzed. Most nodes in the combined analysis showed increased clade support as measured by bootstrapping (six nodes with $\geq$70%). Analyses of the combined partitions support the recognition of four well supported clades (Fig. 1), each of which could be considered separate phylogenetic species. Clade 1 (bootstrap support (bs) 73%) was composed of two strains from India, two from Australia, and one strain each from the United States, Netherlands Antilles, and Bahamas. Within clade 2 (bs 100%) were included the type strain of *A. elegans* and one strain from Indian soil. In clade 3 five strains (bs 96%) were located, four of them of clinical origin from the United States, and one from a dolphin. Finally, clade 4 (bs 98%) consisted of two clinical strains, both from the United States.

Physiology

Carbon assimilation profiles of the different strains on API 50CH strips are shown in Table 2. Assimilation patterns of all the strains were positive for 20 tests. Twenty-seven carbon sources were not assimilated by any strain. The profiles of assimilation of two carbon sources, esculin and D-lyxose, were species- and strain-dependent, respectively. Esculin was weakly assimilated only by the strains nested in clade 2. The assimilation of D-lyxose was highly variable among the strains of the different clades. By contrast, the variability in the assimilations of nitrogen sources, and tolerance to NaCl, $MgCl_2$, and cycloheximide was nule among the species (Table 3). All the strains were positive for 11 nitrogen sources. Nitrite was not assimilated by any of the strains. All strains were able to grow at 2% NaCl and at 2% $MgCl_2$, but failed to grow at 5% NaCl and at 0.1% cycloheximide.

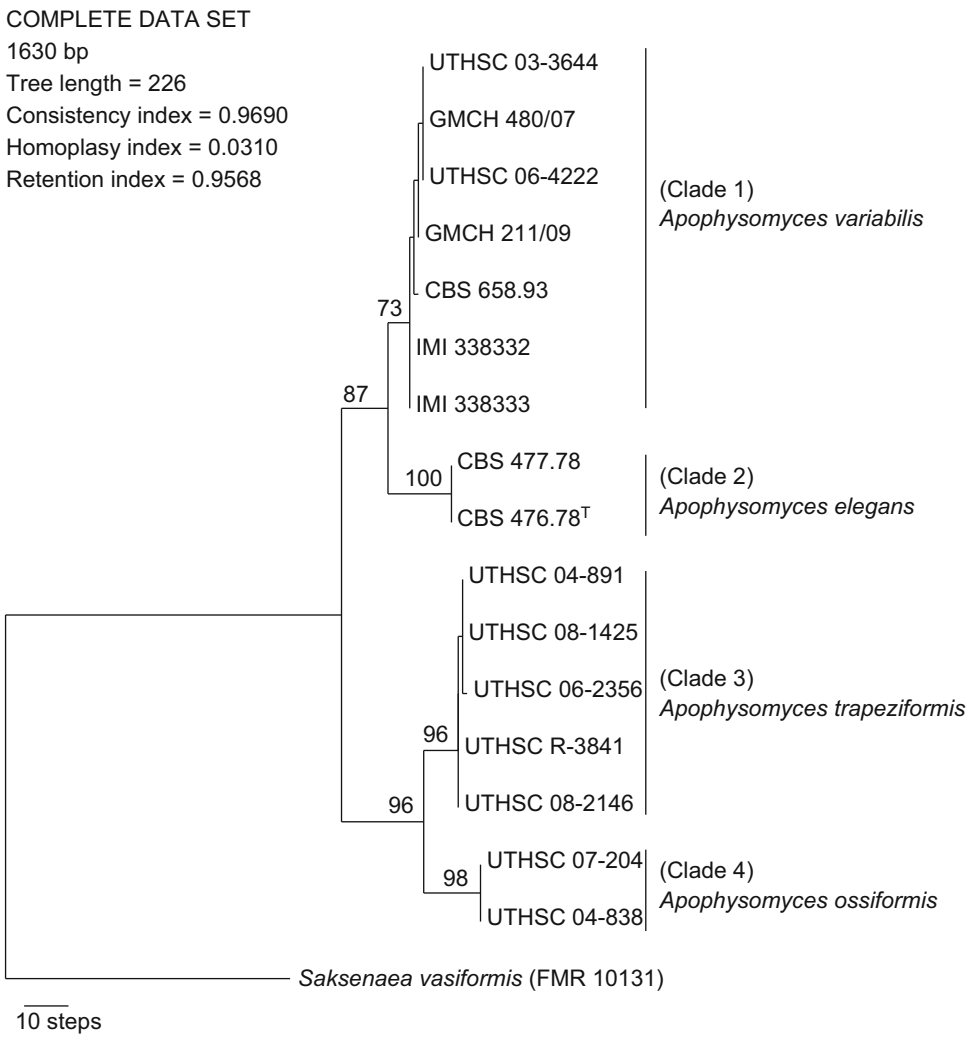

Fig. 1. One of the 36 most parsimonious trees obtained from heuristic searches based on analysis produced from the combined data set. Sequence of *Saksenaea vasiformis* was chosen as outgroup. Bootstrap support values above 70% are indicated at the nodes. T: Type strain.

Morphology

In general, all the strains examined displayed the typical features of the genus *Apophysomyces* described by Misra et al.[18] However, a more detailed microscopic study of these fungi showed important and consistent differences, mainly in the morphology of sporangiophores and sporangiospores, which correlated with the different phylogenetic species. The strains included in clade 1 showed some morphological diversity. The sporangiospores ranged from broadly clavate to ellipsoidal, were flattened on one side, and measured 5–14 × 3–6 μm. The strains included in clade 2, which comprises the type strain of *A. elegans*, showed ovoid, subspherical, broadly ellipsoidal to barrel shaped sporangiospores, although more irregularly shaped spores were also present, measured 6–12 × 5–8 μm, and were the largest for the different species in the complex. The sporangiospores of the strains included in clade 3 were trapezoidal and smaller (5–8.5 × 3–5 μm) while those of strains in clade 4 were thick-walled and clearly biconcave (bone-shaped) in side view, measuring 6–8 × 3–5.5 μm. In addition to differences in spore morphology, the strains in clade 2 also showed two types of sporangiophores: (i) large (up to 540 μm), bearing vase- or bell-shaped apophyses and (ii) shorter (up to 400 μm), bearing funnel-shaped apophyses. The sporangiophores in strains of clades 1, 3, and 4 are similar to the short ones of clade 2.

Mating test

Zygospore formation was not observed after 6 months of incubation in all the mating tests assayed.

Based on the described morphological and physiological differences, which correlated with the molecular data, we concluded that clades 1, 3, and 4 represent three species of *Apophysomyces*, different from *A. elegans* (clade 2), which are proposed here as new species.

Apophysomyces variabilis Alvarez, Stchigel, Cano, D.A. Sutton et Guarro, sp. nov. (Figs. 2A, B; 3H, I).

Coloniae in CZA ad 37 °C rapide crescentes, albae, sed sparsis, inmersis pro parte maxima compositae. Sporangiophora erecta, plerumque simplicia, 100–400 μm longa, 2–3.5 μm lata, brunnea, cum sporangio apohysati. Apophyses plerumque infundibuliformes, 15–20 × 15–20 μm^2. Sporangiosporae variabiles in forma et magnitudine, trapezoides, ellipsoideae, subtriangulares vel claviformis, 5–14 × 3–6 μm^2. Holotypus, CBS H-658.93, ex osteomielitis (cultura viva FMR 10381, CBS 658.93).

Etymology: the epithet refers to the variable morphology of the sporangiospores.

Table 2
Carbon assimilation profiles for *Apophysomyces* species obtained with API 50 CH strips

Carbon source	Species															
	A. variabilis							*A.elegans*		*A. trapeziformis*					*A. ossiformis*	
	CBS 658.93	UTHSC 06-4222	GMCH 211/09	GMCH 480/07	IMI 338332	IMI 338333	UTHSC 03-3644	CBS 476.78	CBS 477.78	UTHSC 04-891	UTHSC 06-2356	UTHSC 08-1425	UTHSC 08-2146	UTHSC R-3841	UTHSC 04-838	UTHSC 07-204
GLY (glycerol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ERY (erythritol)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DARA (D-arabinose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LARA (L-arabinose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RIB (D-ribose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DXYL (D-xylose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LXYL (L-xylose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ADO (D-adonitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MDX (*methyl*-ß-D-xylopyranoside)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GAL (D-galactose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GLU (D-glucose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FRU (D-fructose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MNE (D-mannose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SBE (L-sorbose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RHA (L-rhamnose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DUL (dulcitol)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
INO (inositol)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MAN (D-mannitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SOR (D-sorbitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MDM (*methyl*-D-mannopyranoside)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MDG (*methyl*-D-glucopyranoside)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NAG (*N*-acetyl-glucosamine)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AMY (amygdalin)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ARB (arbutin)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ESC (esculin)	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
SAL (salicin)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CEL (D-cellobiose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAL (D-maltose)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LAC (D-lactose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MEL (D-melibiose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAC (D-saccharose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TRE (D-trehalose)	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
INU (inulin)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MLZ (D-melezitose)	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
RAF (D-raffinose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

AMD (amidon)	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
GLYG (glycogen)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XLT (xylitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GEN (gentiobiose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TUR (D-turanose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LYX (D-lyxose)	–	–	–	–	+	+	–	–	–	–	+	–	–	–	+	+
TAG (D-tagatose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DFUC (D-fucose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
LFUC (L-fucose)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DARL (D-arabitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LARL (L-arabitol)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GNT (potassium gluconate)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2KG (potassium 2-keto-gluconate)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5KG (potassium 5-keto-gluconate)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 3
Nitrogen assimilation and tolerance to chemical compounds for the *Apophysomyces* species included in this study

	Species															
	A. variabilis							*A. elegans*		*A. trapeziformis*					*A. ossiformis*	
Nitrogen source and other tests	CBS 658.93	UTHSC 06-4222	GMCH 211/09	GMCH 480/07	IMI 338332	IMI 338333	UTHSC 03-3644	CBS 476.78	CBS 477.78	UTHSC 04-891	UTHSC 06-2356	UTHSC 08-1425	UTHSC 08-2146	UTHSC R-3841	UTHSC 04-838	UTHSC 07-204
Cadaverine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Creatine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Creatinine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-lysine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrite	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-tryptophan	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-leucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-ornithine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-cysteine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10% NaCl	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2% $MgCl_2$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cycloheximide 0.1%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

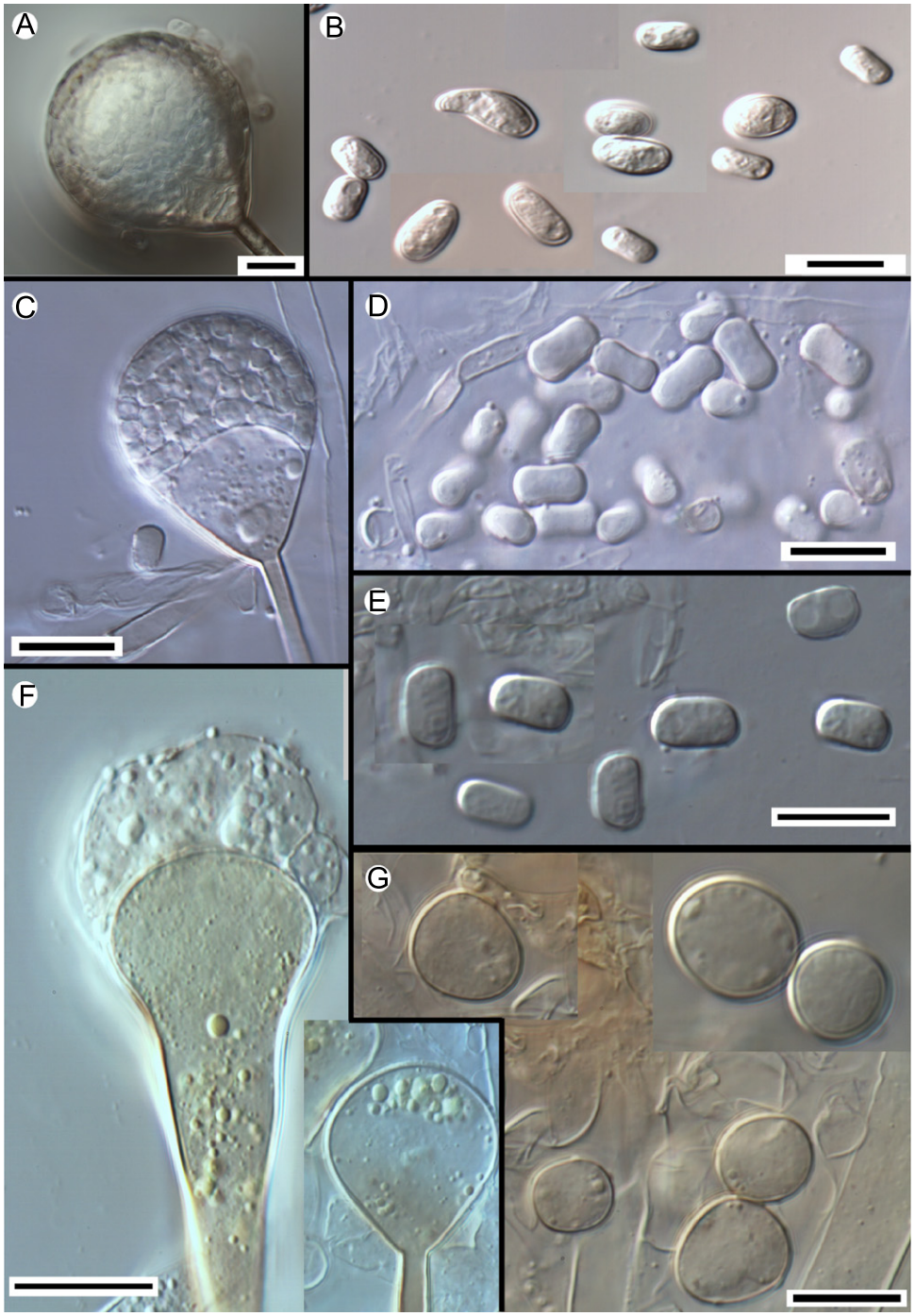

Fig. 2. Morphology of the apophyses and sporangiospores of *Apophysomyces*: (A, B) *A. variabilis* CBS 658.93 (A, apophyses funnel-shaped; B, sporangiospores). (C, D) *A. ossiformis* UTHSC 04-838 (C, apophyses funnel-shaped; D, bone-like shaped sporangiospores). (E) *A. trapeziformis* UTHSC 08-1425 (E, trapezoid-shaped sporangiospores). (F, G) *A. elegans* CBS 476.78 (F, apophyses bell- and funnel-shaped; (G) subspherical to broadly ellipsoidal sporangiospores). All bars = 10 μm.

Colonies attaining a diameter of 90 mm after 4 days of incubation at 37 °C on CZA, whitish, with scarce aerial mycelium; hyphae branched, hyaline, smooth-walled, 3–5.5 μm in diameter; reverse concolorous. Sporangiophores erect, generally arising singly, at first hyaline, soon becoming light greyish brown, generally straight, slightly tapered towards the apex, unbranched, 100–400 μm long, 2–3.5 μm wide, and smooth-walled. Sporangia apophysate, terminal, pyriform, multispored, white at first, becoming light greyish brown when mature, and 15–50 μm in diameter. Apophyses short, funnel-shaped, and 15–20 × 15–20 μm. Sporangiospores variable in shape, trapezoid, ellipsoid, subtriangular or claviform, hyaline to light brown in mass, smooth- and thin-walled, and 5–14 × 3–6 μm. Not able to assimilate esculin.

Colonies on SA, PDA, and MEA showed similar features than on CZA, but they were more floccose, white, and with less sporulation. The optimum growth temperature was 35–42 °C and the minimum temperature of growth was 15 °C. The fungus did not grow at 50 °C.

Apophysomyces ossiformis Alvarez, Stchigel, Cano, D.A. Sutton et Guarro sp. nov. (Figs. 2C, D; 3D, E).

Coloniae in CZA ad 37 °C rapide crescentes, albae, sed sparsis, inmersis pro parte maxima compositae. Sporangiophora erecta, plerumque simplicia, 100–400 μm longa, 2–3.5 μm lata, brunnea, cum sporangio apohysati. Apophyses plerumque infundibuliformes, 15–20 × 15–20 μm. Sporangiosporae ossiformis, 6–8 × 3–5.5 μm. Holotypus, CBS H-20328, ex cellulitis cruris vulnus hominis (cultura viva FMR 9913, UTHSC 04-838).

Etymology: the epithet refers to the bone-like shape of the sporangiospores.

Colonies attaining a diameter of 90 mm after 4 days of incubation at 37 °C on CZA, whitish, with scarce aerial mycelium, branched, hyaline, smooth-walled, and 3–5.5 μm in diameter; reverse concolorous. Sporangiophores erect, generally arising singly, at first hyaline soon becoming light greyish brown, generally straight, slightly tapered towards the apex, unbranched, 100–400 μm long, 2–3.5 μm wide, and smooth-walled. Sporangia apophysate, terminal, pyriform, multispored, white at first,

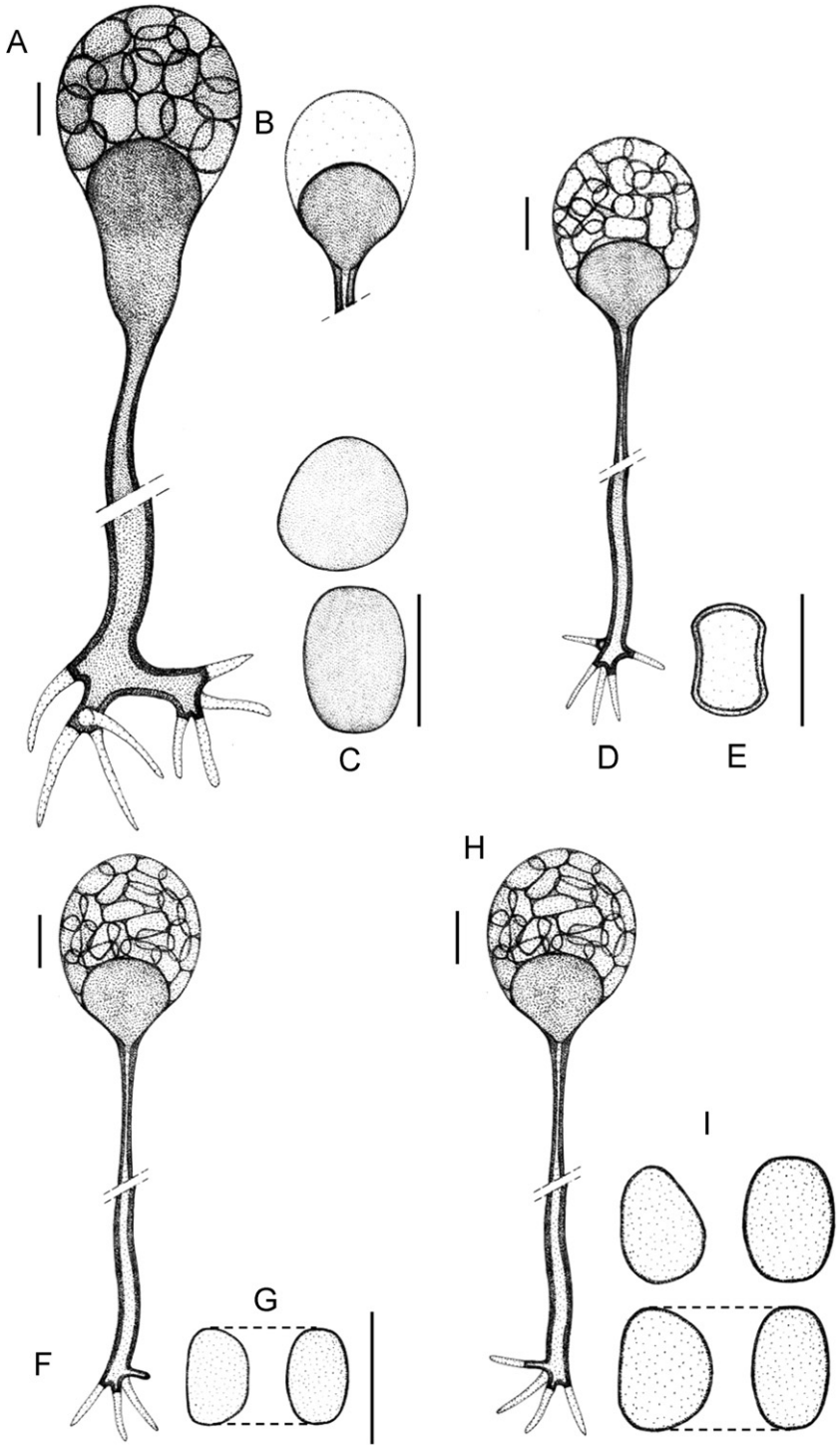

Fig. 3. (A)–(C) *Apophysomyces elegans* (A, vessel-shaped sporangiophore; B, funnel-shaped sporangiophore; C, sporangiospores). (D, E) *Apophysomyces ossiformis* (D, sporangiophore; E, sporangiospore, frontal and side views). (F, G) *Apophysomyces trapeziformis* (F, sporangiophore; G, sporangiospore). (H, I) *Apophysomyces variabilis* (H, sporangiophore; I, sporangiospores). Bars: 10 μm.

becoming light greyish brown when mature, 15–50 μm in diameter. Apophyses short, funnel-shaped, 15–20 × 15–20 μm. Sporangiospores mostly bone-like shaped, hyaline to light brown in mass, smooth- and thick-walled, and 6–8 × 3–5.5 μm. Not able to assimilate esculin.

Colonies on SA, PDA, and MEA showed similar features than on CZA, but they were more floccose, white, and with less sporulation. The optimum growth temperature was 35–42 °C and the minimum 15 °C. The fungus did not grow at 50 °C.

Apophysomyces trapeziformis Alvarez, Cano, Stchigel, D.A. Sutton et Guarro sp. nov. (Figs. 2E; 3F, G).

Coloniae in CZA ad 37 °C rapide crescentes, albae, sed sparsis, inmersis pro parte maxima compositae. Sporangiophora erecta, plerumque simplicia, usque ad 400 μm longa, 2–3.5 μm lata, brunnei, cum sporangio apohysati. Apophyses plerumque infundibuliformes, 15–20 × 15–20 μm. Sporangiosporae trapezoids vel ellipsoideae, 5–8.5 × 3–5 μm.

Holotypus, CBS H-20329, ex abscessus abdominis humanus (cultura viva FMR 10456, UTHSC 08-1425).

Etymology: the epithet refers to the trapezoid shape of the sporangiospores in side view.

Colonies attaining a diameter of 90 mm after 4 days of incubation at 37 °C on CZA, whitish, with scarce aerial mycelium, branched, hyaline, smooth-walled, 3–5.5 μm in diameter; reverse concolorous. Sporangiophores erect, generally arising singly, at first hyaline soon becoming light greyish brown, generally straight, slightly tapered towards the apex, unbranched, up to 400 μm long, 2–3.5 μm wide, and smooth-walled. Sporangia apophysate, terminal, pyriform, multispored, white at first, becoming light greyish brown when mature, and 15–50 μm in diameter. Apophyses short, funnel-shaped, and 15–20 × 15–20 μm. Sporangiospores mostly trapezoid-shaped in side view, more or less cylindrical in front view, flattened at one side and broadly convex on opposite side, hyaline to light brown in mass, smooth- and thin-walled, and 5–8.5 × 3–5 μm. Not able to assimilate esculin.

Colonies on SA, PDA, and MEA showed similar features than on CZA, but they were more floccose, white, and with less sporulation. The optimum growth temperature was 35–42 °C and the minimum 15 °C. The fungus did not grow at 50 °C.

Based on our morphologic and physiologic studies, the type species of *Apophysomyces* is redefined as follows:

A. elegans Misra, Srivastava, and Lata (Figs. 2F,G; 3A–C).

Colonies attaining a diameter of 90 mm after 4 days at 37 °C on CZA, whitish at first, becoming brownish grey, with scarce aerial mycelium; reverse concolorous. Sporangiophores generally arising singly, emerging from aerial hyphae, straight or curved, mainly unbranched or some times branched at the apex, light greyish brown, with two types of morphology, i.e. (i) those that were large (up to 540 μm), bearing vase- or bell-shaped apophyses (15–46 × 11–40 μm) and (ii) those that were shorter (up to 400 μm) and bore funnel-shaped apophyses (15–20 × 15–20 μm), of 4–7.5 μm wide, and smooth-walled. Sporangia produced terminally, pyriform, with distinct apophyses, and 20–60 μm in diameter. Sporangiospores ovoid, subspherical, broadly ellipsoidal to barrel-shaped, frequently irregularly shaped, subhyaline, smooth- and thin-walled, and 6–12 × 5–8 μm. The strains analyzed were able to assimilate esculin.

Similar colonies features as described on CZA were observed on AS, PDA, and MEA, with the exception of lesser production of mycelium in CZA. The optimum growth temperature was 35–42 °C and the minimum 15 °C. The fungus did not grow at 50 °C.

Table 4
In vitro antifungal susceptibility data for *Apophysomyces* species

Species (♯ of strains tested)	Antifungal agent	MIC or MEC (μg/mL) 24 h	
		GM	Range
A. variabilis (7)	Amphotericin B	1.0	1
	Posaconazole	1.1	1–2
	Voriconazole	29.0	16– > 16
	Itraconazole	1.0	0.5–2
	Ravuconazole	1.0	0.5–2
	Anidulafungin	7.2	4–8
	Caspofungin	19.5	4– > 16
A. elegans (2)	Amphotericin B	0.5	0.5
	Posaconazole	0.5	0.5
	Voriconazole	8.0	8
	Itraconazole	1.0	0.5–2
	Ravuconazole	1.0	0.5–2
	Anidulafungin	8.0	8
	Caspofungin	32.0	> 16
A. trapeziformis (5)	Amphotericin B	0.8	0.5–1
	Posaconazole	0.8	0.5–1
	Voriconazole	16.0	8– > 16
	Itraconazole	0.9	0.5–2
	Ravuconazole	0.9	0.5–2
	Anidulafungin	6.1	4–8
	Caspofungin	27.9	16– > 16
A. ossiformis (2)	Amphotericin B	1.4	1–2
	Posaconazole	0.7	0.5–1
	Voriconazole	5.7	4–8
	Itraconazole	1.4	1–2
	Ravuconazole	2.8	2–4
	Anidulafungin	5.7	4–8
	Caspofungin	32.0	< 16

Antifungal susceptibility tests

The results of antifungal susceptibility testing for *Apophysomyces* strains are shown in Table 4. Amphotericin B and posaconazole were the most active antifungal agents. Itraconazole and ravuconazole were more active than voriconazole, and caspofungin and anidulafungin were inactive against all strains.

Discussion

Apophysomyces has been traditionally considered a monotypic genus. However, on the basis of genetic, physiological, and morphological data, we have demonstrated here that the genus constitutes a complex of species. DNA sequences from three different loci were analyzed to infer phylogenetic relationships and species boundaries within strains morphologically identified as *A. elegans*. The informations provided by the three loci evaluated were similar, and proved to be useful markers for species level differentiation in *Apophysomyces*. Although our study included strains from very diverse origins, the number of isolates we could obtain was small, and we anticipate even greater diversity as more strains become available. Given this limitation we were, however, able to recognize at least four phylogenetically, morphologically, and physiologically different species. The shape and size of the sporangiospores, the type of the sporangiophore, and the shape of the apophyses were the most useful characters for this purpose.

As carbon assimilation profiles can be useful for differentiation of human pathogenic mucoralean genera,[23] we tested the assimilation of numerous carbon sources (Table 2). Low interspecific variability within *Apophysomyces* was noted, with the only exception of esculin assimilation, which was positive for *A. elegans*, and negative for the

other species in the complex. In this study, *Apophysomyces* strains also showed negative results for D-galactose, amygdalyn, arbutin, salicin, and gentiobiose assimilation, while in the study of Schwarz et al.[23] these same tests were positive for the members of six other pathogenic genera, i.e. *Cunninghamella*, *Lichtheimia* (*Absidia*), *Mucor*, *Rhizopus*, *Rhizomucor*, and *Syncephalastrum*. In contrast, carbon sources such as L-sorbose, L-rhamnose, dulcitol, inositol, erythritol, D-arabinose, methyl-ß-D-xylopiranoside, methyl-D-mannopyranoside, methyl-D-glucopyranoside, D-tagatose, D-fucose, L-fucose, and inulin were all negative for both the *Apophysomyces* strains and the other six mentioned genera.[23] Other carbon sources such as glycerol were assimilated by *Apophysomyces*, *Rhizopus*, and *Cunninghamella* but not by *Lichtheimia*, *Rhizomucor*, *Mucor*, and *Syncephalastrum;* D-ribose was assimilated by *Apophysomyces*, *Rhizopus*, and *Mucor* and not by *Cunninghamella*, *Lichtheimia*, *Rhizomucor*, and *Syncephalastrum*; L-xylose was assimilated only by *Mucor* and some strains of *Rhizopus*, and D-lactose was assimilated only by *Lichtheimia*, *Rhizomucor*, and *Syncephalastrum*, although it was species dependent in *Rhizomucor*. The nitrogen assimilation profiles and tolerance to various chemical agents for the *Apophysomyces* strains tested in this study were non-discriminatory (Table 3).

From a clinical point of view it is also worth mentioning that none of the clinical strains included in this study belonged to clade 2, which contains the type strain, *A. elegans,* and which has previously been considered a pathogenic species. Clade 2 included only two environmental strains isolated from Indian soils.

The *in vitro* activity of the antifungal drugs tested appeared to corroborate data from previous studies.[1,2,7,25]

Acknowledgments

This work was supported by the Spanish Ministerio de Ciencia y Tecnología Grants CGL 2007-65669/BOS and CGL 2009-08698/BOS.

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UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND PHYLOGENY OF CLINICALLY IMPORTANT MUCORALEAN FUNGI
Eduardo Álvarez Duarte
DL: T-1721-2011

4.4 Molecular Phylogeny and Proposal of Two New Species of the Emerging Pathogenic Fungus *Saksenaea*

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Journal of Clinical Microbiology (2010) 48, 4410–4416

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2010, p. 4410–4416 Vol. 48, No. 12
0095-1137/10/$12.00 doi:10.1128/JCM.01646-10

Molecular Phylogeny and Proposal of Two New Species of the Emerging Pathogenic Fungus *Saksenaea*▽

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Received 16 August 2010/Returned for modification 2 September 2010/Accepted 24 September 2010

Saksenaea **is a monotypic genus belonging to the order *Mucorales* and capable of producing severe human infections. Through a polyphasic study based on analysis of the sequences of the internal transcribed spacer (ITS) region, domains D1 and D2 of the 28S rRNA gene, and the elongation factor 1α (EF-1α) gene, as well as by evaluation of relevant morphological and physiological characteristics of a set of clinical and environmental strains, we have demonstrated that *Saksenaea vasiformis* is a complex of species. We propose as new species *Saksenaea oblongispora*, characterized by oblong sporangiospores and unable to grow at 42°C, and *Saksenaea erythrospora*, characterized by large sporangiophores and sporangia and by ellipsoid sporangiospores, biconcave in the lateral view. Itraconazole, posaconazole, and terbinafine were active against all isolates included in the study, while amphotericin B, voriconazole, and the echinocandins showed low activity.**

The genus *Saksenaea* S. B. Saksena, belonging to the subphylum *Mucoromycotina*, was first isolated from a forest soil in India (38). *Saksenaea vasiformis* S. B. Saksena, the only species of the genus, is a filamentous fungus reported in soil, driftwood, and grains (1, 12, 34), characterized by flask-shaped sporangia, short sporangiophores, oval sporangiospores, and dark rhizoids. It is a thermotolerant fungus that grows between 25°C and 44°C (13, 24, 44) and is able to cause severe human infections in both immunocompromised and immunocompetent hosts. Mucormycosis caused by *S. vasiformis* most often occurs after traumatic implantation of the fungus but can also be due to inhalation of spores (18), spider bites, insect stings, and the use of indwelling catheters (11, 22, 28, 31). Clinical cases seems to be more common in tropical and subtropical climates than elsewhere and have been reported from Australia (16, 19, 22, 40, 48), India (6, 7, 11, 33), the United States (1, 8, 31, 35, 45), Thailand (44), Tunisia (28), the Middle East (2, 25), and Central and South America (9, 47).

Recent molecular studies, based mostly on internal transcribed spacer (ITS) sequences, which have proven to be a good phylogenetic marker in the *Mucorales* (5), have demonstrated unexpectedly high genetic diversity within the most relevant clinical species of this order (3, 4, 21). In the case of *Saksenaea vasiformis*, a few studies have also demonstrated relatively high intraspecific genetic diversity (9, 28), suggesting that more than one phylogenetic species may be present within this morphospecies.

To identify possible cryptic species in *S. vasiformis*, we performed a polyphasic study, based on sequence analysis of three loci, and evaluated different morphological and physiological characteristics for a diverse panel of strains.

MATERIALS AND METHODS

Fungal strains. A total of 11 strains from different reference culture collections were included in the study (Table 1). They were provided by the American Type Culture Collection (ATCC) Manassas, VA; the Centraalbureau voor Schimmelcultures (CBS) Utrecht, Netherlands; the National Reference Center for Mycoses and Antifungal Agents (NRCMA), Institut Pasteur, Paris, France; the Facultad de Medicina de Reus (FMR), Reus, Spain; the Fungus Testing Laboratory at the University of Texas Health Science Center (UTHSC), San Antonio, TX; and the ARS (NRRL) Culture Collection, Peoria, IL. The strains were cultured on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) and were incubated at 37°C ± 1°C for 2 to 5 days.

DNA extraction, amplification, and sequencing. For sequencing of the ITS region, DNA was extracted and purified directly from fungal colonies by following a slightly modified Fast DNA kit protocol (Bio 101, Vista, CA) consisting of a homogenization step repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, NY). DNA was quantified with GeneQuant pro (Amersham Pharmacia Biotech, Cambridge, England). The ITS was amplified with the primer pair ITS5 and ITS4. The PCR mixture (25 μl) included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$ (10× Perkin-Elmer buffer II plus $MgCl_2$ solution [Roche Molecular Systems, Branchburg, NJ]), 100 μM each deoxynucleoside triphosphate (dNTP) (Promega, Madison, WI), 1 μM each primer, and 1.5 U of Ampli*Taq* DNA polymerase (Roche). The amplification program for the three DNA fragments 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 56°C, and extension for 1 min at 72°C. A final extension step at 72°C for 7 min was included at the end of the amplification. The products were purified with an Illustra GFX PCR DNA and gel band purification kit (General Electric Healthcare, Buckinghamshire, United Kingdom) and were stored at −20°C until they were used in sequencing. PCR products were sequenced by using the same primers employed for amplification and following the *Taq* Dye Deoxy Terminator cycle sequencing kit protocol (Applied Biosystems, Gouda, Netherlands). Reactions were run on a 310 DNA sequencer (Applied Biosystems). Consensus sequences were obtained using the Autoassembler program (Perkin-Elmer Applied Biosystems) and Seqman software (Lasergene, Madison, WI).

The D1 and D2 domains of the 28S rRNA gene (D1/D2) and the elongation factor 1α (EF-1α) locus were sequenced at the NRCMA. The mycelium was grown as described previously (17). Genomic DNA was extracted for approximately 100 mg of mycelium. The mycelium was homogenized using green ceramic MagNA lyser beads (diameter, 1.4 mm; Roche, Mannheim, Germany) for

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▽ Published ahead of print on 6 October 2010.

TABLE 1. List of *Saksenaea* isolates included in the study

Isolate[a]	Source	GenBank accession no.		
		ITS	D1/D2 domains of 28S rRNA gene	EF-1α
ATCC 28740	Craniofacial tissue and brain, Mississippi	FR687322	HM776674	HM776685
ATCC 60625	Human wrist lesion at arterial catheter site, Louisiana	FR687323	HM776675	HM776686
CBS 133.90	Forest soil, Brazil	FR687324	HM776676	HM776687
CNRMAF/9-83	Skin lesion, France	FR687325	HM776677	HM776688
FMR 10131	Cutaneous lesion, Tarragona, Spain	FR687326	HM776678	HM776689
NRRL 2443^T	Soil, India	FR687327	HM776679	HM776690
UTHSC 08-3606	Bovine fetus, Texas	FR687328	HM776680	HM776691
UTHSC 09-528	Human tissue, United States	FR687329	HM776681	HM776692
UTHSC 08-379	Eye, Utah	FR687330	HM776682	HM776693
UTHSC 06-576	Blood, Middle East	FR687331	HM776683	HM776694
UTHSC R-2974	Human tissue, Texas	FR687332	HM776684	HM776695

[a] ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CNRMA, Centre National de Référence Mycologie et Antifongiques, Paris, France; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; NRRL, ARS Culture Collection (also known as the NRRL Collection), Peoria, IL; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, TX; ^{T}, type strain.

three cycles of 30 s at 5,000 rpm. The tubes were then centrifuged, and 450 μl of supernatant was recovered. Three microliters of RNase A (100 mg/ml) was added, and the tubes were incubated for 10 min at 65°C. DNA was then extracted by using a DNeasy plant minikit (Qiagen, Germany) according to the manufacturer's instructions.

D1/D2 was amplified with the primer pair NL-1 and NL-4 (26), and a small region of EF-1α was amplified with primers MEF-11 and MEF-41 (32). PCR amplification of D1/D2 and EF-1α and the corresponding sequence analysis were carried out as described previously (17).

Phylogenetic analyses. The sequences were aligned using the Clustal X (version 1.8) computer program, and alignments were corrected using the Gblocks tool (version 0.91b) (10), followed by manual adjustments with a text editor. Neighbor-joining (NJ) phylogenetic trees were constructed using the MEGA package, version 4.1 (43). The distance matrix was based on the maximum composite likelihood method with the pairwise deletion option.

For the phylogenetic inferences using maximum-likelihood (ML) analysis, trees were inferred with PhyML (version 3.0) (20), and we used heuristic searches, with starting trees obtained by random addition with 100 replicates and nearest-neighbor interchange (NNI) branch swapping. For bootstrap ML analysis, we performed 1,000 replicates (NJ starting tree with NNI branch swapping). Bayesian analyses were carried out using MrBayes, version 3.1 (23). Bayesian analyses were performed by running 1,000,000 generations in four chains, saving the current tree every 100 generations. The last 18,000 trees were used to construct a 50% majority-rule consensus tree. The best-fit model of nucleotide substitution for the combined data set selected by Modeltest (36) was the general time-reversible (GTR) model (27, 37). *Apophysomyces elegans* (CBS 476.78) was chosen as the outgroup.

Morphological study. The strains were subcultured on PDA, Czapek agar (CZA; Difco, Becton Dickinson, France), and malt extract agar (MEA; 10 g of malt extract, 20 g of agar, and 1,000 ml of distilled water) and were incubated at 37°C. The microscopic features were determined in wet mounts on water and on lactic acid, which were examined under a light microscope. The strains were identified using the traditional schemes based on morphological characteristics (13).

Physiological study. Growth rates at 4, 15, 24, 30, 35, 37, 42, and 50 ± 1°C were determined on PDA, MEA, and CZA for all strains. The petri dishes were inoculated in the center and were incubated in darkness, and the colony diameters (in millimeters) were measured daily.

Carbon source assimilation profiles were determined with the commercial API 50 CH kit (bioMérieux, Marcy-l'Etoile, France) by following protocols described previously (39). The strains were cultured for 6 days on CZA at 37°C. A final spore concentration of 5×10^5 CFU/ml was prepared in 20 ml of yeast nitrogen base (7.7 g/liter; Difco, Becton Dickinson, France) containing 0.5 g/liter chloramphenicol (Sigma-Aldrich Corp., St. Louis, MO) and 0.1% Bacto agar (Difco, Becton Dickinson, France). Each well of the strips was inoculated with 300 μl of medium. The inoculated API 50 CH strips were incubated for 48 to 72 h at 37 ± 1°C in darkness. After incubation, the strips were read visually, and growth or lack of growth was noted. Weak growth was considered a positive result. To determine nitrogen source assimilation, we used the same inoculum described above, except that the yeast nitrogen base broth was replaced with a carbon nitrogen base broth (Difco, Becton Dickinson, France), and testing was performed in sterile, disposable, multiwell microplates. The medium with the nitrogen sources was dispensed into the wells in 150-μl volumes with a multichannel pipette, and each well was inoculated with 50 μl of the conidial suspension. The microplates were incubated at 37°C in darkness for 48 to 72 h. We also evaluated the growth of the strains on NaCl (at 2%, 5%, 7%, and 10%), 2% $MgCl_2$, and 0.1% cycloheximide (14, 49). All tests were performed in duplicate. The production of urease was determined after incubation on Christensen's urea agar slants at 37 ± 1°C for 8 days (30).

Mating tests. The 11 *Saksenaea* strains were grown on CZA plates at 37 ± 1°C in the dark, after which they were paired in all combinations, including self-crosses, on CZA. Each strain was streaked onto one-half of a CZA plate opposite the streak of another strain, allowing for a central zone of contact when the strains grew. Plates were incubated at 37 ± 1°C and were examined macroscopically each week for as long as 4 months for the presence of zygospores. All tests were performed in duplicate.

Antifungal susceptibility testing. All strains were subcultured on CZA for 7 to 20 days at 30°C or 37°C. Sporangiospores were then collected in water, and the suspension was adjusted to 2×10^4 CFU[5]/ml per well. Pure active powders, of known potency, of amphotericin B (Sigma-Aldrich, Saint Quentin Fallavier, France), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), itraconazole (Janssen-Cilag, Issy-les-Moulineaux, France), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ), terbinafine (Novartis Pharma AG, Basel, Switzerland), caspofungin (Merck & Co., Inc., Rahway, NJ), micafungin (Astellas Pharma, Osaka, Japan), and anidulafungin (Pfizer) were used. Antifungal susceptibility testing was performed by a broth microdilution technique according to the guidelines of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing for the testing of conidium-forming molds (41), with some modifications (17).

Nucleotide sequence accession numbers. All the sequences obtained in this study have been deposited in the GenBank database and assigned the accession numbers listed in Table 1.

MycoBank accession numbers. The descriptions and illustrations of *Saksenaea oblongispora* and *Saksenaea erythrospora* are available online (http://www.MycoBank.org/) under MycoBank accession numbers MB 518626 and MB 518627, respectively.

RESULTS

Phylogeny. With the primers used, we were able to amplify and sequence 641 to 800 bp, 648 to 738 bp, and 486 to 532 bp of the ITS, D1/D2, and EF-1α loci, respectively. The sequences of the three loci were analyzed phylogenetically as separate (data not shown) and combined data sets.

The topologies of the combined data sets by the three methods (NJ, ML, Bayesian inference) were very similar to those observed in the particular trees of the different genes analyzed. Most nodes in the combined analysis showed increased clade

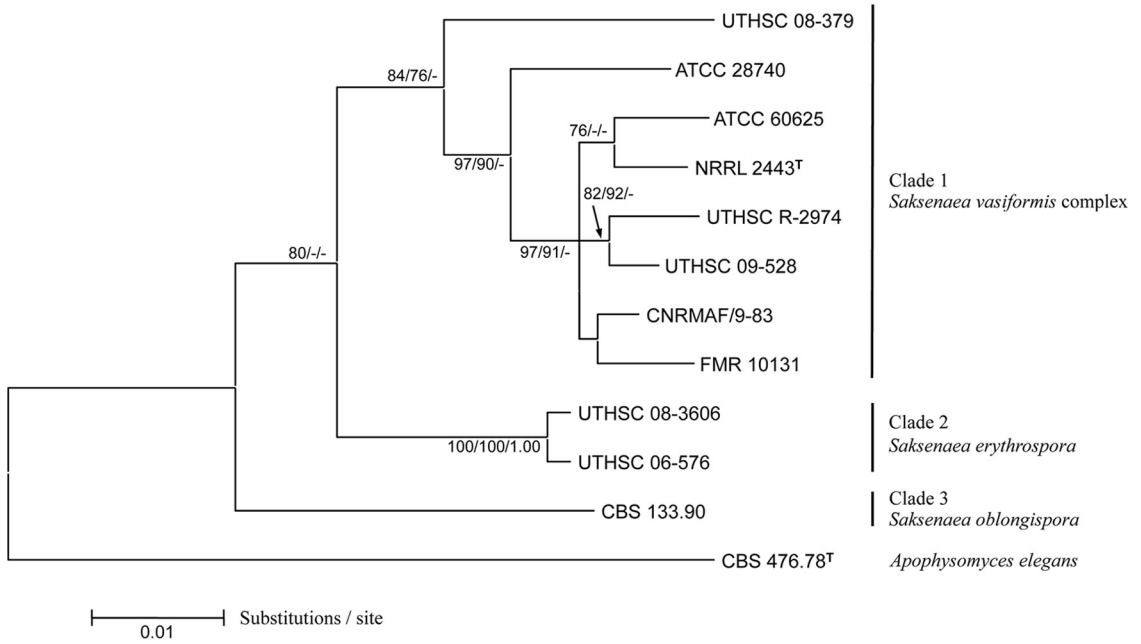

FIG. 1. Maximum-likelihood (ML) tree obtained from the combined DNA sequence data from three loci (ITS, D1/D2, EF-1α). Numbers on the branches are bootstrap neighbor-joining (NJ)/ML values above 75%, followed by Bayesian posterior probabilities above 0.95. Support values of <75% (NJ/ML) and <0.95 (Bayesian posterior probabilities) are indicated by minus signs. T, type strain.

support (≥70% bootstrap support for the NJ and ML methods; ≥0.95 for Bayesian inference). Analysis of the combined partitions supports the recognition of three well-supported clades (Fig. 1), each of which represents a different phylogenetic species. Clade 1 comprises eight strains from different countries and sources, mainly from human and animal clinical samples, including the type strain of *S. vasiformis* (NRRL 2443). This clade shows high intraspecific variability, with three well-supported subclades, two of which contain only one strain each (UTHSC 08-379 and ATCC 28740, respectively). The third subclade contains six strains, including the type strain. The genetic relationships of the members of the clade with the type strain range between 84% and 94% identity, suggesting that this clade represents a complex of species. Clade 2 includes two clinical strains from the United States, and clade 3 consists of only one strain from Brazil.

Of note, two additional clinical isolates, not shown in Fig. 1, were included in the study. Phylogenetic analysis showed that they belonged to clade 1 (CNRMAF/6-50, recovered from muscle lesions of a French patient) and clade 2 (CNRMAF/4-81, from a patient from French Guiana) (9) (data not shown). These isolates were not viable, however, preventing any further study.

Physiology. The carbon assimilation patterns of all the strains were positive in 12 tests (glycerol, D-adonitol, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, *N*-acetylglucosamine, D-maltose, D-trehalose, amidon, and glycogen). Thirty-two carbon sources were not assimilated by any strain (erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, methyl-β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-D-mannopyranoside, methyl-D-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-lactose, D-melibiose, D-saccharose, inulin, D-melezitose, D-raffinose, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, and potassium 5-keto-gluconate). The assimilation profiles of four carbon sources (xylitol, D-arabitol, L-arabitol, and potassium gluconate) were strain dependent. In contrast, variability in the assimilation of nitrogen sources and the tolerance of NaCl, $MgCl_2$, and cycloheximide was null among the species. All the strains were positive for 10 nitrogen sources (creatine, creatinine, L-lysine, nitrate, L-tryptophan, L-proline, L-leucine, L-ornithine, L-cysteine, and arginine). Cadaverine and nitrite were not assimilated by any of the strains. All strains were able to grow in 2% NaCl, 5% NaCl, and 2% $MgCl_2$ but failed to grow in 7% NaCl, 10% NaCl, and 0.1% cycloheximide.

None of the clades obtained in the phylogenetic analysis showed distinctive physiological profiles.

Morphology. In general, all the strains examined displayed the typical features of the genus *Saksenaea*. However, a more detailed microscopic observation of these fungi showed important and consistent differences among them, mainly in the morphology of the sporangiophores and sporangiospores, which correlated with the different clades observed in the phylogenetic analysis. The strains that made up clade 1, including the type strain of *S. vasiformis*, showed the longest sporangiospores (5 to 7 by 2 to 3 μm), which were mainly cylindrical, with rounded ends. The sporangiospores of the strains in clade 2 were mostly ellipsoid, biconcave in a lateral view, and smaller (5 to 5.5 by 2.5 to 3 μm), while the sporangiospores of the clade 3 strain were mainly oblong, measuring 5 to 6.5 by 3 to 4.5 μm (Fig. 2). In addition, the strains of clade 2 showed clearly longer sporangiophores and sporangia than the members of the other clades (Fig. 3).

Mating test. After 4 months of incubation, no zygospore formation was observed for any of the mating combinations performed.

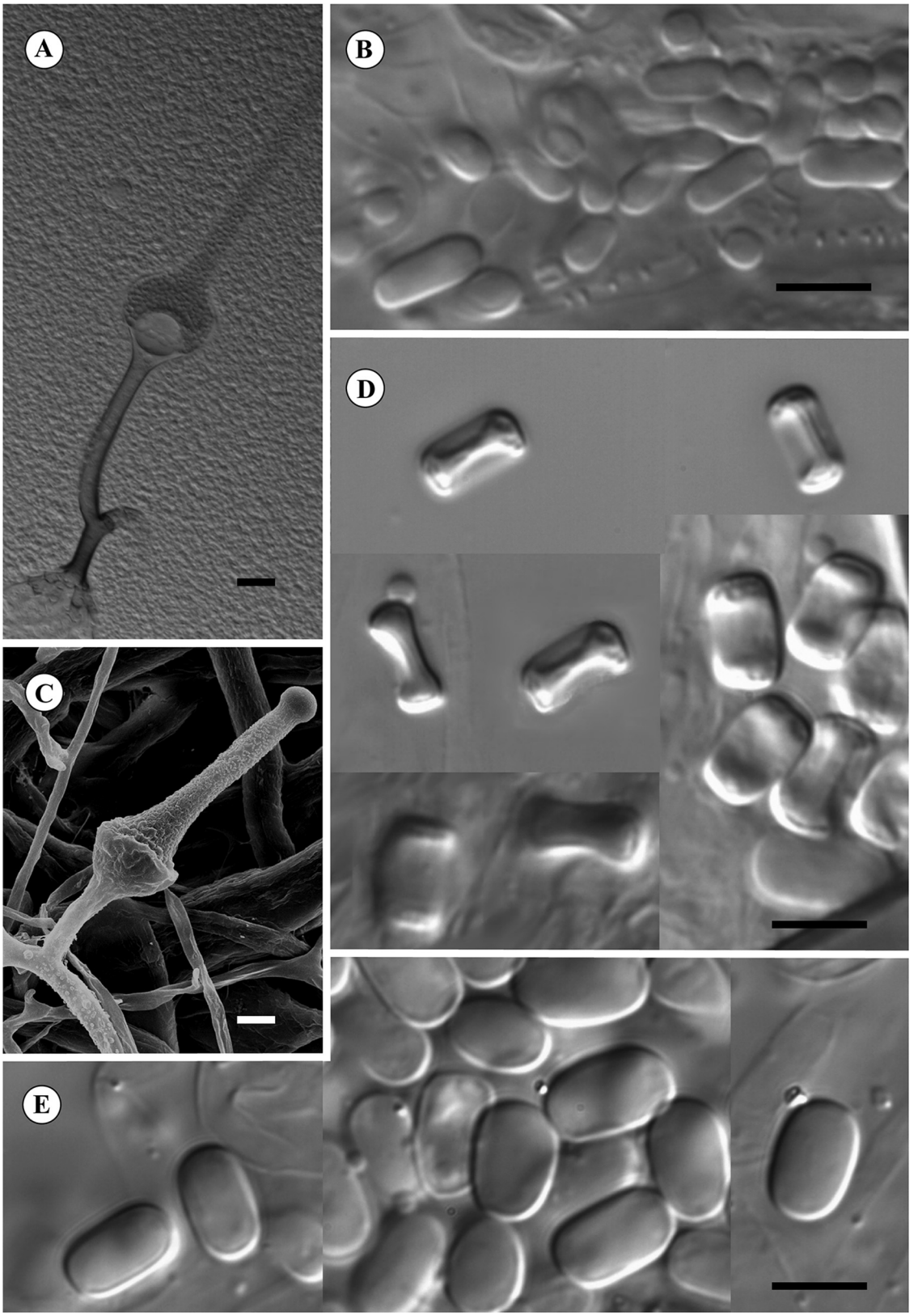

FIG. 2. Morphology of the sporangiophores and sporangiospores of *Saksenaea* spp. (A, B, and C) *S. vasiformis* NRRL 2443. (A) Sporangiophore; (B) sporangiospores; (C) detail of the upper part of the sporangium. (D and E) Sporangiospores of *S. erythrospora* UTHSC 08-3606 (D) and *S. oblongispora* CBS 133.90 (E). Bars, 10 μm in panels A and B and 5 μm in panels C, D, and E.

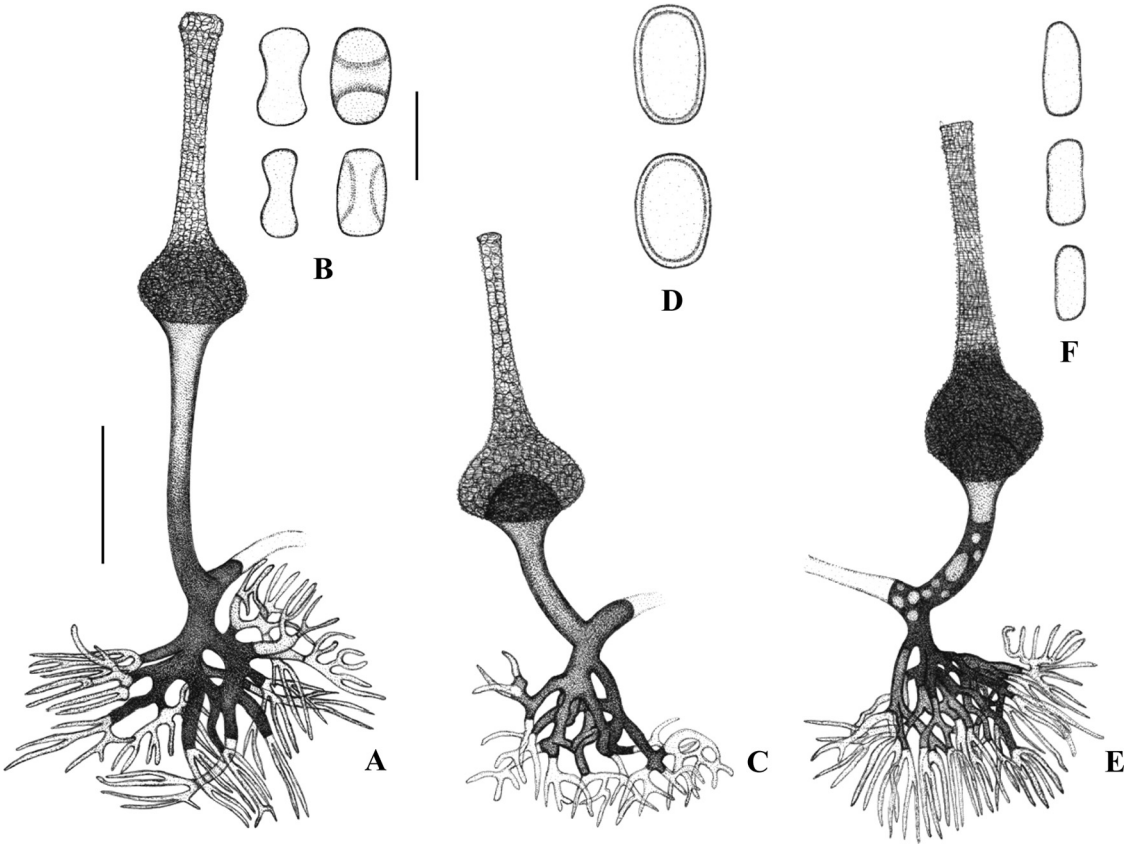

FIG. 3. (A and B) *Saksenaea erythrospora*. (A) Sporangiophore; (B) sporangiospores, frontal and side views. (C and D) *Saksenaea oblongispora*. (C) Sporangiophore; (D) sporangiospores, frontal and side views. (E and F) *Saksenaea vasiformis*. (E) Sporangiophore; (F) sporangiospores. Bars, 50 μm for drawings A, C, and E; 5 μm for drawings B, D, F.

Antifungal susceptibility testing. MICs were determined for the nine strains that produced enough sporangiospores (all except strains ATCC 60625 and ATCC 28740 from clade 1) and are given below as geometric means (ranges), in micrograms per milliliter. High MICs of amphotericin B (4.1 [1 to 8]) and voriconazole (4.7 [2 to 8]) and high minimum effective concentrations (MECs) of echinocandins (4.0 [4 to 8]) were observed for all the strains tested. Itraconazole B (0.2 [0.06 to 1]), posaconazole (0.1 [0.06 to 0.25]), and terbinafine (0.1 [0.03 to 1]) had lower MICs. There was no apparent difference between the species studied, but the small sample size prevents statistical analysis.

TAXONOMY

Based on the morphological differences described, which correlated with the molecular data, we conclude that clades 2 and 3 represent two species of *Saksenaea* different from *S. vasiformis*, which we propose here as new species.

***Saksenaea oblongispora* Alvarez, Stchigel, Cano, Garcia-Hermoso, et Guarro sp. nov.** MycoBank MB 518626. See Fig. 2E and 3C and D.

Coloniae in CZA rapide crescentes, albae, ex mycelium aerium sparsum compositum. Sporangiophora erecta, plerumque simplicia, 80 ad 100 μm longa, 6 ad 10 μm lata, supra massam rhizoidalem saepe bifurcatam positis. Sporangia ampulliformes, 70 ad 110 μm longa. Sporangiosporae plerumque oblongae, 5 ad 6.5 per 3 ad 4.5 μm. Zygospora ignota.

Holotypus, CBS H-20471. Typus lectus in terra, Pará, Capitâo Poço, Brazil, 1990 (cultura viva FMR 6500, CBS 133.90).

Etymology: the epithet refers to the oblong shape of the sporangiospores.

Colonies filling the petri dish (diameter, 9 cm) after 4 days of incubation at 37°C on CZA, whitish, with scarce aerial mycelium; hyphae branched, at first hyaline soon becoming light brown, smooth-walled, 3 to 6 μm in diameter; reverse concolorous. Sporangiophores erect, generally arising singly, hyaline, unbranched, 80 to 100 μm long, 6 to 10 μm wide, with a profusely dichotomously branched rhizoidal complex. Sporangia terminal, multispored, hyaline, flask-shaped, asperulate at low magnification, ornamented with many irregular, bacilliform protuberances under scanning electron microscopy (SEM), 70 to 110 μm long; with a long (60- to 90-μm) neck; apex of the neck closed with a mucilaginous plug, which is gradually dissolved when mature. Sporangiospores mainly oblong, 5 to 6.5 by 3 to 4.5 μm. Zygospores not observed.

Colonies on PDA and MEA showed features similar to those on CZA, but they were more floccose and white, with less sporulation. The optimum growth temperature was 25°C, and the minimum temperature of growth was 15°C. The fungus did not grow at 42°C.

***Saksenaea erythrospora* Alvarez, Cano, Stchigel, Garcia-Hermoso, et Guarro sp. nov.** MycoBank MB 518627. See Fig. 2D and 3A and B.

Coloniae in CZA rapide crescentes, albae, ex mycelium aerium sparsum compositum. Sporangiophora erecta, plerumque simplicia, 100 ad 150 μm longa, 7 ad 11 μm lata, supra massam rhizoidalem saepe bifurcatam positis. Sporan-gia ampulliformes, 100 ad 220 μm longa. Sporangiosporae plerumque ellipsoidae, maturitate membrana circa sporangiospore collapsa, 5 ad 5.5 per 2.5 ad 3 μm. Zygospora ignota.

Holotypus, CBS H-20472, ex fetu bovis, Texas, 2009 (cultura viva FMR 10672, UTHSC 08-3606).

Etymology: the epithet refers the shape of the sporangiospores, resembling the biconcave form of erythrocytes.

Colonies filling the petri dish after 4 days of incubation at 37°C on CZA, whitish, with scarce aerial mycelium, branched, hyaline, smooth-walled, 3 to 6 μm in diameter; reverse concolorous. Sporangiophores erect, generally arising singly, at first hyaline soon becoming light brown, generally straight, unbranched, 100 to 150 μm long, 7 to 11 μm wide. Sporangia terminal, multispored, flask-shaped, asperulate, ornamented with many irregular, small bacilliform protuberances under SEM, 100 to 220 μm long; with a long (80- to 200-μm) neck; apex of the neck closed with a mucilaginous plug. Sporangiospores mostly ellipsoid, biconcave in a lateral view, smooth-walled, their membranes collapsing at maturity, 5 to 5.5 by 2.5 to 3 μm. Zygospores not observed.

Colonies on PDA and MEA showed features similar to those on CZA, but they were more floccose and white, with less sporulation. The optimum growth temperature was 25°C, and the minimum was 15°C. The fungus grew at 42°C and did not grow at 50°C.

Based on our morphological and physiological studies, the type species of *Saksenaea* is redefined as follows.

***Saksenaea vasiformis* S. B. Saksena (Saksena, 1953).** See Fig. 2A to C and 3E and F.

Colonies filling the petri dish after 4 days at 37°C on CZA, whitish, with scarce aerial mycelium; reverse concolorous. Sporangiophores generally arising singly, emerging from aerial hyphae, straight, mainly unbranched, at first hyaline soon becoming light brown, 65 to 100 μm long, 6 to 10 μm wide. Sporangia produced terminally, flask-shaped and at low magnification asperulate, ornamented with small and irregular bacilliform protuberances under SEM, up to 110 μm long; with a long (60 to 90 μm) neck; apex of the neck closed with a mucilaginous plug, which gradually becomes dissolved when mature. Sporangiospores mainly cylindrical, with rounded ends, hyaline, 5 to 7 × 2 to 3 μm.

Colonial features similar to those described on CZA were observed on PDA and MEA, except for reduced mycelium production in the former. The optimum growth temperature was 25°C to 37°C, and the minimum was 15°C. The fungus grew at 42°C and did not grow at 50°C.

DISCUSSION

Despite its reproducibility in severe human infections, *Saksenaea* remains a poorly studied mucoralean genus, mainly due to the lack of sporulation on the mycological culture media routinely used in clinical laboratories and the scarce number of strains preserved in culture collections. Here we demonstrated high genetic and phenotypic diversity among the 11 strains studied based on a polyphasic approach. The information provided by the three loci evaluated was similar, and they proved to be useful markers for species-level delimitation in *Saksenaea*. The genus was determined to be genetically heterogeneous, comprising several species. Apart from the type species, at least other two species were characterized phenotypically. The shape and size of the sporangiospores, the length of the sporangiophore and sporangia, and the maximum temperature of growth were the most useful characteristics for their recognition.

Carbon assimilation profiles can be useful for the differentiation of human pathogenic mucoralean fungi at the genus level. In the case of *Saksenaea*, the test for assimilation of D-cellobiose was negative, while this test was positive for the other genera tested in previous studies (4, 39). However, none of the physiological tests evaluated in this study were discriminatory at the species level.

Antifungal susceptibility profiles also were not discriminatory. However, they matched previously published results (42) for the order *Mucorales*, except for amphotericin B, which has higher MICs for *Saksenaea* than *Mucor* spp.

Up to now, approximately 40 cases of zygomycete infections, mostly cutaneous infections, have been attributed to *Saksenaea* (6, 40, 46, 47), although for the reasons indicated above, it is likely that the actual number of clinical cases has been underestimated. To avoid difficulties in the detection and identification of *Saksenaea* in clinical samples, several authors have emphasized the need for special culture techniques, such as the use of floating agar blocks on water, or the use of Borelli′s lactrimel agar (15, 29) to induce sporulation. However, our use of these techniques yielded poor results (data not shown). In contrast, the use of Czapek agar, a culture medium traditionally used for the phenotypic characterization of *Aspergillus* and *Penicillium* species, produced good *in vitro* sporulation of the *Saksenaea* strains tested in this study. This medium also worked well for the sporulation of another mucoralean genus, *Apophysomyces*, which also sporulates poorly in routinely used mycological media, such as Sabouraud agar or PDA (4).

In addition to the two new species described here, the phylogenetic analysis also demonstrated the existence of more cryptic species within the first clade. However, since these strains could not be distinguished by using phenotypic characteristics, we prefer to refer to clade 1 as *Saksenaea vasiformis* complex. Although our study included strains of diverse origins, the number we could obtain was small, due to the fact that most of the clinical isolates previously recovered were not kept in culture collections and/or were no longer viable. It is likely that when more strains become available, additional new species within the genus *Saksenaea* will be identified.

The study performed here and the DNA sequences generated should be useful for further characterization of clinical isolates and/or identification of causative species from tissue biopsy specimens. They may also allow the identification of other species within the genus or the *S. vasiformis* complex.

ACKNOWLEDGMENTS

This work was supported by the Spanish Ministerio de Ciencia y Tecnología grant CGL 2009-08698/BOS, with cofunding from Fondo Europeo de Desarrollo Regional (FEDER).

We thank Catalina Nuñez for technical assistance. The NRCMA gratefully acknowledges the technical help of Laure Diancourt and Coralie Tran at the sequencing facility (PF-8, Genotyping of Pathogens and Public Health, Institut Pasteur).

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UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND PHYLOGENY OF CLINICALLY IMPORTANT MUCORALEAN FUNGI
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DL: T-1721-2011

5. SUMMARIZING DISCUSSION

The Mucorales have been known as etiological agents of human infections for more than 100 years (Dannaoui and Garcia-Hermoso, 2007; Platauf, 1885; Ribes, 2000). However, the biology of these fungi and the epidemiology of the diseases that they cause are still poorly known. This is due to the relatively low frequency of these infections, despite the continuous increase in the number of immunocompromised people (diabetics, immunodeficiency, transplants, haematological malignancies, AIDS), who are especially susceptible hosts; but it is also due to the difficulty of their isolation and identification at generic and species level, because of their (presumed) poor morphological differentiation. Moreover, two emergent mucoralean pathogens that affect people who are not immunocompromised, *Apophysomyces elegans* and *Saksenaea vasiformis*, fail to produce fertile structures on the culture media that is usually employed in the clinical microbiology lab.

Roden *et al*. (2005) reviewed an impressive number of clinical cases of mucormycosis (ca. 1000), with a detailed relation of the species involved in such infections. That study revealed that the etiological agent was identified at species level in only 50% of cases, and even in those cases identification was doubtful since the mycological methods were not indicated and in numerous cases were carried out by non expert laboratories. Consequently, the value of those data is relative and must be taken with caution. Regarding that, we carried out a preliminary study (Alvarez *et al*., 2009), in order to investigate the spectrum of species involved in mucormycosis in the United States of America, a large number of isolates having been made available by University of Texas Health Science Center at San Antonio (UTHSC), an institution with whom we have frequently collaborated in different investigations. In that work we studied 190 mucoralean isolates, which is the largest set of clinical isolates ever examined in a single study up to now. One of the most relevant findings of our study was to

demonstrate that practically all the species involved in mucormycosis (a total of 9) could be reliably identified using accurate phenotypic methods. When we compared the identification using morphological and physiological criteria against that using the Basic Local Alignment Search Tool (BLAST) search employing ITS nucleotide sequences, we found 100% concordance of the identifications at generic level, and 92% at species level. Only 7.2% of the isolates were not identified using the sequencing/Blast search. Among them, seven of the isolates of *Mucor* spp. presented little similarity with the sequences of the species of this genus deposited in GenBank. For three of these isolates, phenotypically identified as *M. ramosissimus* the BLAST search showed a low similarity (94-95%) with the type strain of the species. The three remaining isolates, which were initially morphologically identified as *M. racemosus*, also showed a low similarity (90-91%) with the sequence of the type strain of this species when the BLAST search was made. A seventh isolate, belonging to the genus *Mucor*, was not possible to identify using both phenotypic and molecular tools although it showed certain morphological features related to *M. indicus*. As a result, the phylogram inferred using the 5.8S rRNA nucleotidic sequences from our isolates and reference/ex-type strains, all those isolates of *Mucor* were placed in separated clusters, in reference to the most morphological and molecularly related species. On the other hand, the placement of *Rhizomucor pusillus* (the type species of the genus) distant from the *Mucor* clade, and the inclusion of *Rhizomucor variabilis* var. *regularior* in the *Mucor circinelloides* clade meant that the former must be considered a synonym of *M. circinelloides*. Something similar happened with *Rhizomucor variabilis* var. *variabilis*, which clustered with *M. hiemalis*, but the morphological differences and the genetic distance supports leaving them as two separated species. *Rhizopus oryzae*, as in other studies (Ribes *et al*., 2000; Roden *et al.*, 2005), was the most frequently isolated in the

studied cases (44.7%), followed by *R. microsporus* (22.1%), *Mucor circinelloides* (9.5%), *Lichtheimia corymbifera* (5.3%), *Rhizomucor pusillus* (3.7%), *Cunninghamella bertholletiae* (3.2%), *Mucor indicus* (2.6%), *Cunninghamella echinulata* (1%), and *Apophysomyces elegans* (0.5%). In reference of the origin of the isolates, the majority of them were from cutaneous infections (28%), lungs (26.8%), and sinuses (25.8%). The remaining isolates comprised a subset collected from different deep sites. These results differ from those obtained by Roden *et al.* (2005), who found that the most commonly involved anatomical sites were the sinuses (39%), followed by lungs (24%) and cutaneous presentations (19%), among others.

Despite the proposal of Balajee *et al.* (2009) to establish a 99% of identity in the nucleotide sequence of ITS region as the cut-off to consider two isolates as pertaining to the same mucoralean species, the higher intraspecific variability that we found in our isolates, and because the unreliability of some sequences available in the nucleotide databases (ie., GenBank), we proposed decrease the cut-off to 98% of identity. In this sense, it still needs to be clarified whether *Cunninghamella bertholletiae*, which presented a high genetic variability in the 5.8S rRNA gene sequences, contain several cryptic species or if the cut-off should be reviewed again.

Apophysomyces was a monotypic genus erected by Misra (Misra *et al*., 1979) to accommodate a single species, *A. elegans*. However, the results obtained in our first study (Alvarez *et al*., 2009), showed a high intraspecific variability in the ITS nucleotide sequences between our isolates and with respect to those of the type strain. In addition, some previously reported findings suggested that more than one phylogenetic species might be present within the morphospecies *A. elegans* (Chakrabarti *et al*., 2003). Based on these previous observations, and due to the availability of 16 strains from

different geographic areas and origins (mostly clinical, but also environmental), we performed a polyphasic study consisting of a phenotypic (morphological and physiological) and a genotypic characterization. We carried out a multilocus study based on the analysis of the nucleotide sequences of the ITS region, D1-D2 domains, and a fragment of the H3 gene. Our results suggested the existence of three new phylogenetic species plus *A. elegans*, which could be confirmed morphologically. Because the origin of the isolates included in the type species clades, including the type strain, is exclusively environmental (soil samples), we concluded that *A. elegans*, traditionally considered an etiological agent of mucormycosis, has not been involved in clinical so far. However, the three new species, *A. ossiformis* (morphologically characterized by its bone-shaped spores), *A. trapeziformis* (producing trapezoid-shaped spores) and *A. variabilis* (which form sporangiospores variable in shape and size), would be the real etiological agents of mucormycosis, particularly the latter, because they are mostly from clinical (human and animal) origin. Recent studies confirmed the clinical importance of *A. variabilis* isolates (Guarro *et al*., 2011; Chakrabarti *et al*., 2010).

In addition to these taxonomical and epidemiological findings, we demonstrated the usefulness of Czapek agar to stimulates the production of fertile structures (sporangiophores and sporangiospores), which are critical in routine (morphological) identification. Other tools, such as the use carbon assimilation profiles, has proven useful for the identification of human pathogenic mucoralean fungi (Schwarz *et al*., 2007), but for the case of the four species of *Apophysomyces* a low interspecific variability was noted, with the exception of the hydrolysis and assimilation of the esculin, which were positive only for both isolates of *A. elegans*.

Mucor is a genus that has been the subject of several taxonomical changes in the past years, being divided into several sections based on growth temperatures, length of the sporangiophores, size of the sporangium, size and shape of sporangiospores, etc. (Schipper 1973, 1975, 1976, 1978; Zycha *et al*., 1969). In our first study (Alvarez *et al.*, 2009) seven of the 30 isolates belonging to the genus *Mucor* were not identified at species level because of the differences in their nucleotide sequences (ITS region) with respect to the morphological closest related species. Previously, only five species had been reported in clinical infections: *Mucor indicus*, *M. circinelloides*, *M, racemosus*, *M. ramosissimus*, and *M. hiemalis* (Prabhu and Patel, 2004; de Hoog *et al.*, 2000). In order to determine the possible existence of cryptic species in this set of unclassified isolates, we performed a polyphasic study (Alvarez *et al*., 2011), which included the phenotypic analysis of the morphologically closest related species, and a phylogenetic study using the nucleotide sequences the ITS region and the D1 and D2 domains of the 28S rDNA. This polyphasic study revealed the existence of two new species: *Mucor ellipsoideus* and *Mucor velutinosus*. The first one, constituted only by a single isolate, clustered together *M amphibiorum* and *M. indicus*. However, *M. ellipsoideus* produces narrowly ellipsoidal (and flattened in one side) spores, whereas they are smaller and globose and regularly ellipsoidal in *M. amphibiorum* and in *M. indicus*, respectively. *Mucor velutinosus* included three of the previously unidentified isolates, and formed a well supported cluster closely related to *M. circinelloides* (f. *circinelloides* and f. *lusitanicus*) and *M. ramosissimus*. However, *M. velutinosus* could be clearly differentiated from these species because of the production of larger and ornamented spores in the former. The most morphologically related species to *M. velutinosus*, i.e. *M. amphibiorum*, *M. fuscus* and *M. plumbeus*, were placed phylogenetically distant, and could easily be differentiated from the former because they failed to growth at 37ºC. Recently, this new

species has been involved in a disseminated skin infection in a female patient with acute myeloid leukemia (Sugui *et al.*, 2011). The remaining three previously unidentified isolates were placed in both phylograms together with reference strains of *Mucor circinelloides* f. *lusitanicus*. Due to the genetic and morphological differences between the reference strains and our clinical isolates of *M. circinelloides* f. *lusitanicus* with the reference strains of *M. circinelloides* f. *circinelloides*, we proposed that both *formae* belonged to different species: *Mucor circinelloides* and *M. lusitanicus*. On the other hand, the *M. fragilis* strains included in this study were placed in the same clade as the other *M. lusitanicus* strains, based on the fact that we did not find any morphological or physiological differences with the *M. lusitanicus* strains. Furthermore in the absence of a type strain of the former, they could be considered as pertaining to *M. lusitanicus*. Another interesting finding was the confirmation of *Rhizomucor variabilis* var. *variabilis* as a species belonging to the genus *Mucor*. *Rhizomucor variabilis* var. *variabilis* was genetically closely related to *Mucor hiemalis* and *Mucor mucedo* and very distant from *Rhizomucor pusillus*, the type species of the genus. For this reason, and despite their ability to produce rhizoids, we suggested the reclassification of *Rhizomucor variabilis* var. *variabilis* as *Mucor irregularis*. Our proposal has recently been supported by Schell *et al.* (2011), who also reported new cases of mucormycosis due to *M. irregularis* from the U.S.A. Our study therefore confirmed the polyphyly of the genus *Mucor*, shown by other authors (O`Donnell *et al*., 2001; Schell *et al*., 2011), sharing common ancestors with species not related morphologically to other genera like *Chaetocladium*, *Backusella*, *Ellisomyces* and *Kirkomyces.*

Saksenaea is a genus considered an emerging pathogen, mainly in India (Baradkar and Kumar, 2009). Several authors have reported an unexpected high genetic diversity among sequences from isolates morphologically identified as *Saksenaea*

vasiformis, suggesting that more than one phylogenetic species may be present within this morphospecies (Blanchet *et al*., 2008; Lechevalier *et al*., 2008). Based on these results, we carried out a polyphasic study, analyzing three molecular markers (the ITS region, the D1-D2 domains, and a fragment of the EF-1α gene) to establish the phylogenetic relationships for a diverse panel of 11 clinical and environmental isolates of *Saksenaea vasiformis*, including the type strain. In the tree of the combined dataset, we observed three well supported clades. Within the main clade, which includes the type strain of the genus, we can found some well supported sister clades, that could be considered as potential phylogenetic cryptic species, especially if we consider the high genetic variability between the isolates (with less than 94 % of the nucleotide sequence identity among them). However, these isolates could not be distinguished phenotypically (they all produced short sporangiophores and more or less cylindrical spores; and grew at 42ºC), so we preferred to refer to this clade as *Saksenaea vasiformis* complex. A future study, that includes more isolates and analyses a bigger panel of physiological characters (i.e. the identification of extrolytes and structural polysaccharides) and many more molecular markers, perhaps could to separate these cryptic species. The other isolates formed another two well supported clades, also showing morphological and physiological features that allowed us to describe them as two new species: *Saksenaea erythrospora* (forming long sporangiophores, and ellipsoid sporangiospores, but biconcave in lateral view; and growing at 42ºC) and *S. oblongispora* (with short sporangiophores, and oblong-shaped sporangiospores; and not growing at 42ºC). As for the *Apophysomyces* species, the physiological tests were not useful for discriminating between the *Saksenaea* species.

Up to now, the prognosis in mucormycosis infections remains poor, with high mortality rates of approximately 50% (Roden *et al*., 2005; Skiada *et al*., 2011). This

could be explained by the high variability in the susceptibility patters to antifungal drugs (Almyroudis *et al*., 2007; Dannaoui *et al*., 2002a, 2002b, 2002c, 2003a, 2003b; Sun *et al*., 2002a). Based on our results (Alvarez *et al*., 2010a, 2010b), amphotericin B is the most active antifungal drug *in vitro* against the species studied. This is the case of our new species of *Mucor*, *M. ellipsoideus* and *M. velutinosus*, which showed that amphotericin B was active against all isolates, and posaconazole and itraconazole showed a low activity (Alvarez *et al.*, 2011). In the case of *A. elegans* and the new species *A. ossiformis*, *A. trapeziformis* and *A. variabilis* (Alvarez *et al.*, 2010b), amphotericin B again and posaconazole were the most active antifungal agents, whereas itraconazole and ravuconazole were more active than voriconazole, and caspofungin and anidulafungin were inactive against all strains. However, the isolates of *Saksenaea* that we studied showed a significant difference with respect to the other Mucorales assayed. We found that itraconazole, posaconazole and terbinafine were active against all *Saksenaea* isolates (Alvarez *et al.*, 2010a), while amphotericin B, voriconazole, and the echinocandins showed low activity. These results are in concordance with previous reports (Almyroudis *et al*., 2007; Sun *et al*., 2002a). Based on our results, and in agreement with other authors (Dannaoui *et al*., 2003b; Sabatelli *et al*., 2006; Sun *et al*., 2002a) we propose the use of pozaconazole as the second line of antifungal drugs (the first line in cases of *Saksenaea* spp. infections), because it has a lower toxicity than other antifungals (Stewardson *et al*., 2009).

Overall, given the high variability of the susceptibility patterns in the Mucorales, we have demonstrated that a correct identification of the etiological agent, through the use of classical and molecular tools will lead to a correct therapy.

UNIVERSITAT ROVIRA I VIRGILI
TAXONOMY AND PHYLOGENY OF CLINICALLY IMPORTANT MUCORALEAN FUNGI
Eduardo Álvarez Duarte
DL: T-1721-2011

6. CONCLUSIONS

1. Phenotypical and molecular methods were used to identify 190 isolates of species of the order Mucorales that had been isolated from clinical samples, mainly in United States of America. The correlation between the two methods was 92%.

2. *Rhizopus oryzae* was the most common species isolated from clinical samples (44.7%), followed by *Rhizopus microsporus* (22.1%) and *Mucor circinelloides* (9.5%).

3. We demonstrated that by the use of the internal transcribed spacer region it was possible to discriminate between closely related species in all genera studied.

4. Three new species of *Apophysomyces*: *Apophysomyces ossiformis*, *A. trapeziformis* and *A. variabilis* have been proposed, based on a polyphasic study that included the sequencing of the histone 3 gene, the internal transcribed spacer region and domains D1 and D2 of the 28S rRNA gene, as well a detailed morphological and physiological study.

5. Our results suggest that *Apophysomyces elegans*, was not the etiological agent of mucormycosis in humans, as previously reported being mainly isolated from soil. The other 3 new species, especially *A. variabilis*, were related to clinical cases.

6. Two new species of *Mucor*, *Mucor ellipsoideus* and *M. velutinosus* were proposed, based on morphology, physiology and DNA sequence data.

7. We demonstrated that *Rhizomucor variabilis* var. *variabilis* and *Rhizomucor variabilis* var. *regularior*, were morphologically and genetically related to *Mucor,* being relocated in this genus.

8. Two new species of *Saksenaea*: *Saksenaea erythrospora* and *S. oblongispora* were proposed, based on morphology, physiology and a multilocus study.

9. A multilocus analysis revealed that *Saksenaea vasiformis* was a complex species with poor morphological differentiation.

10. We corroborated that amphotericin B remains the most active antifungal drug against the majority of the isolates of Mucorales tested in this study. Posaconazole and itraconazole showed high antifungal activity, particularly against *Saksenaea* species.

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