



Taxonomy and epidemiology of *Mucor irregularis*, agent of chronic cutaneous mucormycosis

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Key words

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Abstract Mucormycosis usually presents as a progressive infection with significant angio-invasion. Mucormycosis due to *Mucor irregularis* (formerly *Rhizomucor variabilis* var. *variabilis*), however, is exceptional in causing chronic cutaneous infection in immunocompetent humans, ultimately leading to severe morbidity if left untreated. More than 90 % of the cases known to date were reported from Asia, mainly from China. The nearest neighbour of *M. irregularis* is the saprobic species *M. hiemalis*. The aim of this study was to evaluate the taxonomic position, epidemiology, and intra- and inter-species diversity of *M. irregularis* based on 21 strains (clinical n = 17) by multilocus analysis using ITS, LSU, *RPB1* and *RPB2* genes, compared to results of cluster analysis with amplified fragment length polymorphism (AFLP) data. By combining MLST and AFLP analyses, *M. irregularis* was found to be monophyletic with high bootstrap support, and consisted of five subgroups, which were not concordant in all partitions. It was thus confirmed that *M. irregularis* is a single species at 96.1–100 % ITS similarity and low recombination rates between populations. Some geographic structuring was noted with some localised populations, which may be explained by limited air-dispersal. The natural habitat of the species is likely to be in soil and decomposing plant material.

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INTRODUCTION

Members of *Mucoromycotina* are notorious for their acute, disfiguring, often fatal infections in severely compromised human hosts. The infection usually takes a progressive, invasive course with marked angiotropism and occlusion of blood vessels leading to rapid necrosis of tissue. The mortality rate of affected patients often exceeds 50 % (Roden et al. 2005, Skiada 2011). The most frequently occurring opportunists include *Rhizopus arrhizus* (*R. oryzae*), *R. microsporus* and *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) (Ribes et al. 2000, Alvarez et al. 2009, Dolatabadi et al. In press). All these species have a long history in industrial mycology. Many species

had already been described in the 19th century (Walther et al. 2013) and several are among the classic agents of opportunistic infections in humans (de Hoog et al. 2009). *Mucorales* are among the prevalent pioneer invaders of virgin substrates, such as foodstuffs, which they degrade by the production of lipolytic and a wide array of other enzymes (Hiol et al. 2000, Ghorbel et al. 2005). In many countries in Asia and Africa this property is used for fermentation of foodstuffs based on soy and other lipid-rich agricultural products. *Mucorales* are also primary causes of food spoilage of soy cakes used in traditional foods in Asia (Yao et al. 2010, Kim et al. 2011).

A recently described emerging opportunistic fungus especially being reported from Asia, *Mucor irregularis* (formerly *Rhizomucor variabilis* var. *variabilis*) is unique in many respects. Its clinical infection pattern deviates considerably from that of other *Mucorales* by being chronic and by occurring in immunocompetent patients without apparent underlying disorder (Lu et al. 2009, Hemashettar et al. 2011). *Mucor irregularis* infection usually persists with a history of several years on exposed sites such as the face and the extremities. The infection is cutaneous and subcutaneous without angio-invasion especially at the early stage (Lu et al. 2009, Zhao et al. 2009), and finally leads to severe mutilation (Lu et al. 2009, Li & Lun 2012). Its environmental habitat has as yet not been revealed with certainty.

Mucor irregularis was first isolated from lesions of a Chinese patient with a primary cutaneous infection in 1991 and was named *Rhizomucor variabilis* var. *variabilis* (Zheng & Chen 1991). From that moment onwards, more cases were reported in China during the past 20 yr (Lu et al. 2009, Zhao et al. 2009, Li & Lun 2012). In 2010, a case of secondary and complex infection including *R. variabilis* was reported from Australia (Ribeiro et al. 2010). Subsequently, three cases of primary

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Table 1 Data and GenBank numbers of *Mucor irregularis* strains used in this study.

Strain ¹	Original identification	Source (location, duration)	Geography		GenBank accession No. ²			Reference
			LSU	ITS	RPB1	RPB2		
CBS 103.93 (T)	<i>Rhizomucor variabilis</i> (T)	Human, skin (wrist, 18-year)	China, Jiangsu	JX976212	JX976257	JX976234	JX976279	Zheng & Chen 1991
CBS 100164	<i>Mucor hiemalis</i> f. <i>luteus</i>	Human, skin (face, 2-year)	China, Jiangxi	JX976213	JX976258	JX976235	JX976280	Wang et al. 1998
CBS 609.78	<i>Mucor hiemalis</i> f. <i>hiemalis</i>	Garden soil	Nigeria	JX976215	JX976260	JX976237	JX976282	
CBS 654.78	<i>Mucor</i> aff. <i>varisporus</i>	Owl pellet	India	JX976216	JX976261	JX976238	JX976283	
CBS 700.71	<i>Mucor hiemalis</i> f. <i>luteus</i>	Soil	India	JX976199	JX976247	JX976225	JX976270	
CBS 977.68	<i>Mucor hiemalis</i> f. <i>hiemalis</i>	Unknown	India	JX976214	JX976259	JX976236	JX976281	
CMFCCC B 50e	<i>Rhizomucor variabilis</i>	Human, skin (face, 3-year)	China, Shanghai	JX976217	JX976262	JX976239	JX976284	Zhao et al. 2009
CMFCCC B 50f	<i>Rhizomucor variabilis</i>	Human, skin	China, Shandong	JX976201	JX976245	JX976224	JX976269	
CMFCCC B 50g	<i>Rhizomucor variabilis</i>	Human, skin (face, 3-year)	China, Hubei	JX976218	JX976263	JX976240	JX976285	Hu et al. 2010
CMFCCC B 50h	<i>Rhizomucor variabilis</i>	Human, skin (face, 2-year)	China, Anhui	JX976203	JX976248	JX976226	JX976271	Zhang et al. 2010
CMFCCC B 50i	<i>Rhizomucor variabilis</i>	Human, skin (face, 10-year)	China, Jiangsu	JX976200	JX976244	JX976223	JX976268	Lu et al. 2009
CMFCCC B 50j	<i>Rhizomucor variabilis</i>	Human, skin (neck, right upper extremity, 16-year)	China, Jiangsu	JX976220	JX976265	JX976242	JX976287	Qi et al. 2012
CMFCCC B 50k	<i>Rhizomucor variabilis</i>	Human, skin	China, Jiangsu	JX976205	JX976250	JX976228	JX976273	
CMFCCC B 50l	<i>Rhizomucor variabilis</i>	Human, skin	China, Hubei	JX976219	JX976264	JX976241	JX976286	
CMFCCC B 50m	<i>Rhizomucor variabilis</i>	Human, skin (face, 2-year)	China, Jiangxi	JX976207	JX976252	JX976230	JX976275	Qin et al. 2010
CMFCCC B 50n	<i>Mucor</i> sp.	Human, skin (face, 1-year)	China, Sichuan	JX976208	JX976253	JX976231	JX976276	Jiang et al. 2003
CMFCCC B 50o	<i>Mucor</i> sp.	Human, skin (face, 2-year)	China, Sichuan	JX976221	JX976266	JX976243	JX976288	Ran et al. 2006
CMFCCC B 50p	<i>Rhizomucor variabilis</i>	Human, skin (face, 7-month)	China, Chongqing	JX976206	JX976251	JX976229	JX976274	Zhou et al. 2011
CMFCCC B 50q	<i>Rhizomucor variabilis</i>	Human, skin (face, neck, 22-year)	China, Jiangxi	JX976204	JX976249	JX976227	JX976272	
CMFCCC B 50r	<i>Rhizomucor variabilis</i>	Human, skin (right upper extremity, 7-year)	China, Shandong	JX976210	JX976255	JX976232	JX976277	Li et al. 2004
CMFCCC B 50s	<i>Rhizomucor variabilis</i> var. <i>regularior</i>	Human, skin (face, 9-year)	China, Shandong	JX976211	JX976256	JX976233	JX976278	Li et al. 2006

¹ CBS = Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, The Netherlands; CMFCCC = Center of Medical Fungi, China Committee for Culture Collection of Microorganisms, Nanjing, China; T = Type strain.

² ITS = Internal Transcribed Spacer region; LSU = partial 28S rDNA; RPB1 = partial RNA polymerase II large subunit; RPB2 = partial RNA polymerase II second largest subunit.

infection outside of China were reported, from Japan (Tomita et al. 2011), India (Hemashettar et al. 2011), and the USA (Schell et al. 2011), respectively. Interestingly, all except one case of primary infection in humans were reported from Asia, particularly China, and the disease despite its disfiguring appearance had not been reported before 1990. Today, 17 cases have been reported, suggesting that this is one of the few truly emerging opportunistic fungi. *Rhizomucor variabilis* was renamed as *M. irregularis* because results of rDNA ITS sequencing showed closer phylogenetic distance to *Mucor* species, especially *M. hiemalis* (Voigt et al. 1999, Alvarez et al. 2011). The present paper focuses on the unique mucoralean species *M. irregularis*, its geographic distribution and possible routes of transmission, as well as its taxonomy.

In a large phylogenetic overview of *Mucorales* including more than 75 % of all species known to date, Walther et al. (2013) confirmed that the nearest neighbour of *M. irregularis* is indeed *M. hiemalis*. This saprobic species was first isolated by Behrens in 1902 (Schipper 1973) and has only rarely been reported as an agent of human disease (de Hoog et al. 2009). It was mentioned by Neame & Rayner (1960) as one of the agents of mucormycosis, but no material is presently available to verify this claim. The cases of Costa et al. (1990) and Prevoo et al. (1991) may have been misidentifications (see below). Thus, the origin of the exceptional pathology of *M. irregularis* within the *Mucoromycotina* seems to have emerged *de novo*. A detailed study of the biology of *M. irregularis* therefore seems overdue. In this study, we explored the intra- and inter-species diversity of *M. irregularis* using phylogenetic analysis based on gene sequencing of four loci, compared with cluster analysis based on amplified fragment length polymorphism (AFLP).

MATERIAL AND METHODS

Strains

Sixteen clinical isolates of *M. irregularis*, derived from Chinese cutaneous mucormycosis patients, including the ex-type strain and maintained at the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands (Table 1) were studied. Another five strains from clinical samples (n = 1) and from the environment (n = 4) were also included. The latter strains had been deposited as other *Mucor* species in the CBS collection and identification as *M. irregularis* was verified by rDNA internal transcribed spacers (ITS) sequencing followed by BLAST analysis in GenBank and phylogenetic tree construction. The ex-type stain of *M. luteus* (CBS 243.35) and the ex-neotype strain of *M. hiemalis* (CBS 201.65) were included as outgroups. All strains used in this study are deposited either in the CBS culture collection or at the Centre of Medical Fungi, China Committee for Culture Collection of Microorganisms (CMFCCC), Nanjing, China. Stock cultures were maintained on slants of malt extract agar (MEA 5 %, Oxoid, Badhoevedorp, The Netherlands) and potato dextrose agar (PDA), dextrose (Merck), agar (Oxoid) at 24 °C.

Morphology

Strains were cultured on 5 % MEA (Oxoid) at 30 °C for 5 d. The abundantly sporulating strain CBS 103.93 was taken as model for descriptions and illustrations. Observations were done using both a light microscope (Nikon ECLIPSE 80i, Japan) and a stereo microscope (Nikon SMZ1500, Japan). Microscopic slide preparation was done using water as mounting fluid. Photos were made using a Nikon digital sight DS-5M114780 camera (Nikon, Japan). Magnification was 400–1000× for light microscopy and 100× for stereo microscopy.

Table 2 List of primers used for amplification and sequencing.

Locus	Name of primer	Primer sequence (5'-3')	Reference
LSU	LR0R	ACC CGC TGA ACT TAA GC	Vilgalys & Hester 1990
	LR5	ATC CTG AGG GAAACT TC	Vilgalys & Hester 1990
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	White et al. 1990
	ITS4	TCC TCC GCT TAT TGA TAT GC	White et al. 1990
<i>RPB1</i>	RPB1-Af	GAR TGY CCD GGD CAY TTY GG	Stiller & Hall 1997
	RPB1-Cr	CCN GCD ATN TCR TTR TCC ATR TA	Stiller & Hall 1997
<i>RPB2</i>	fRPB2-5F	GAY GAY MGW GAT CAY TTY GG	Liu et al. 1999
	fRPB2-7cR	CCC ATR GCT TGY TTR CCC AT	Liu et al. 1999

Extraction of genomic DNA

Approximately 1 cm² of 4-d-old cultures on MEA at 24 °C were transferred to a tube containing glass beads (Sigma G9143) and 400 µL TE_x buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands), pH 9.0 (100 mM Tris [hydroxymethyl]-aminomethane, 40 mM Na-EDTA). The samples were homogenized for 3 min using a Genie[®]2 Vortex (MoBio, Carlsbad, CA, USA). Subsequently 120 µL of a 10 % sodium dodecyl sulfate (SDS) solution and 5 U of Proteinase K (10 mg/mL, Sigma-Aldrich) were added followed by 30 min of incubation in a waterbath at 55 °C. In total 120 µL of 5 M sodium chloride and 1/10 volume of 10 % CTAB (cetyl trimethylammonium bromide) were added to the material followed by 60 min incubation at 55 °C. The samples were vortexed again for 3 min. One volume of chloroform : isoamylalcohol 24 : 1 (v/v) was added and the samples were centrifuged for 5 min at 4 °C at 14 000 rpm (20 400 rcf). The supernatant was transferred to a new Eppendorf tube with 225 µL 5 M NH₄-acetate (Sigma-Aldrich), mixed carefully by inverting, incubated for 30 min on ice water, and centrifuged again for 5 min at 4 °C at 14 000 rpm. The supernatant was transferred to another Eppendorf tube with 0.55× volumes of isopropanol. After incubation at -20 °C for at least 30 min DNA was pelleted at 14 000 rpm (20 400 rcf) for 5 min at 4 °C. The supernatant was decanted and the DNA pellet was washed with 1 000 µL ice cold 70 % ethanol. After drying at room temperature, it was re-suspended in 50 µL of TE buffer (Sigma-Aldrich) (Tris 0.12 % w/v, Na-EDTA 0.04 % w/v, pH 8.0). Quality of genomic DNA was verified on agarose gel. Genomic DNA was stored at -20 °C.

DNA amplification and sequencing

Four gene regions were amplified for multilocus sequence typing, i.e. the D1/D2 region of the nuclear large subunit ribosomal RNA gene (nuLSU), rDNA internal transcribed spacers (ITS), partial largest subunit of the RNA polymerase II (*RPB1*), and partial second largest subunit of the RNA polymerase II (*RPB2*) genes. Primers used for amplification and sequencing are listed in Table 2. PCR reactions were performed in 25 µL volumes reaction mixture with 7 µL Go Taq master mix (Promega, Leiden, The Netherlands) containing reaction buffer, MgCl₂, dNTPs, 1 µL of each primer (10 pmol), and 1 µL template DNA. Amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The cycling conditions included 5 min at 94 °C for initial denaturation, followed by 35 cycles consisting of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 2 min, and final elongation for 7 min at 72 °C. The annealing temperature was changed to 51 °C for *RPB1* and *RPB2*. Amplicons were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Buckinghamshire, UK). Sequencing was performed as follows: 95 °C for 1 min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems) with ABI PRISM BigDye[™] terminator cycle sequencing kit (Applied Biosystems).

Alignment and phylogenetic reconstruction

Consensus sequences were assembled using the SeqMan package of Lasergene software (DNASTar, Madison, Wisconsin, USA). Phylogenetic analyses were focused on multilocus sequencing typing (MLST) which included four markers: partial LSU, ITS, partial *RPB1* and partial *RPB2*, which were analysed separately and combined. Sequences were aligned automatically with manual adjustment using BioNumerics v. 4.61 (Applied Maths, St-Martens-Latem, Belgium). Phylogenetic relationships were estimated for all alignments by maximum parsimony analysis done in MEGA v. 5.05 (Tamura et al. 2011) and by use of a Bayesian approach with Monte Carlo Markov chains performed with the MrBayes (v. 3.1) computer program (Ronquist & Huelsenbeck 2003). 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. Phylogenetic reconstructions and bootstrap values were obtained for each locus separately by Neighbour-Joining method and Maximum likelihood method with 500 bootstrap replications using MEGA v. 5.05 (as described above). Bootstrap values ≥ 70 % for the NJ and ML methods, and ≥ 0.95 for Bayesian inference were considered significant (Hillis & Bull 1993). The congruence between loci was assessed using on-line calculation of the congruency index I_{cong} (de Vienne et al. 2007). After performing the partition-homogeneity test (PHT) to verify congruency among the four genes, the loci were combined and aligned using BioNumerics v. 4.61 (Applied Maths). The multilocus phylogenetic tree was constructed by Neighbour-Joining and Maximum likelihood with 500 bootstrap replications using MEGA v. 5.05 (as described above). Moreover, the ITS dataset was used in order to investigate the phylogenetic placement of *M. irregularis* and relationship between *M. irregularis* and *M. hiemalis*. This dataset included ITS sequence data from the present study supplemented with sequences from GenBank (JQ885450, JQ776538, GU355647, JN827387, JN107738, HQ891659, HM639969, HM639970, HM639968, EU196747 and FJ875119). The ITS sequences were compared in a large database maintained at CBS for research purposes and the sequence data of BMU 02530, BMU 2257, dH 18763, dH 19950, KN 97-9 and KN 97-1 were included as well. *Mucor luteus*, *M. hiemalis* and *M. silvaticus* were used as outgroup. Sequences were aligned automatically with manual adjustment using BioNumerics v. 4.61 (Applied Maths). Phylogenetic trees were constructed by Neighbour-Joining and Maximum Likelihood methods with 500 bootstrap replications using MEGA v. 5.05 (as described above).

AFLP analysis

Approximately 50 ng of genomic DNA was subjected to a combined restriction ligation procedure containing 50 pmol of HpyCH4 IV adapter, 50 pmol *Mse*I adapter, 2 U of HpyCH4 IV (New England Biolabs, Beverly, MA, USA), 2 U of *Mse*I (New England Biolabs), and 1 U of T4 DNA ligase (Promega, Leiden, The Netherlands) in a total volume of 20 µL of 1× reaction

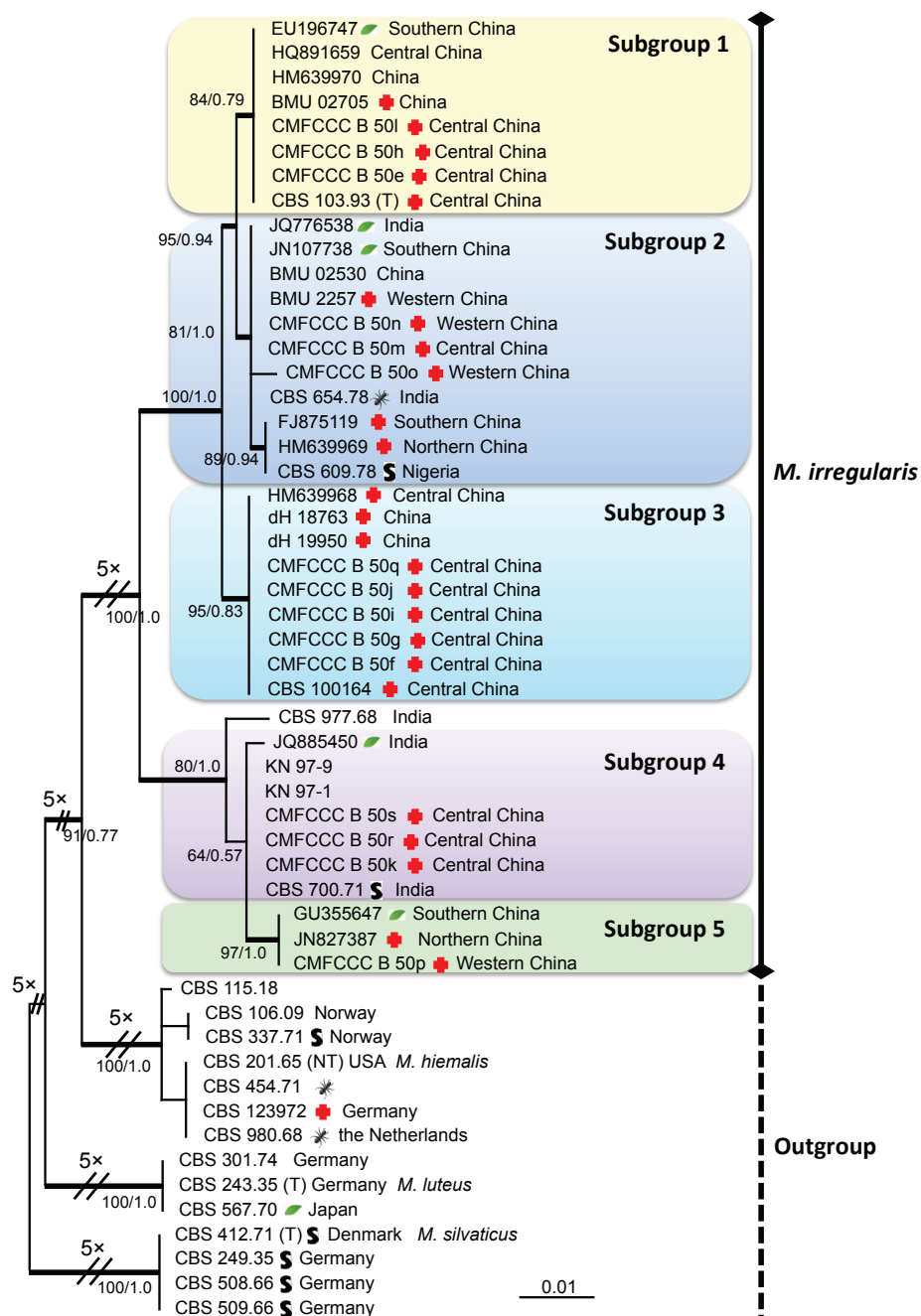


Fig. 1 Phylogeny of *Mucor irregularis* obtained from a Neighbour-Joining analysis of Internal Transcribed Spacer (ITS) using Mega 5 software. Bootstrap support values were estimated based on 500 replicates, and are shown by the numbers near the branches (Neighbour-Joining bootstrap values / Bayesian posterior probabilities, boldface branch for values ≥ 80). Strains forming well-supported subgroups are marked in colour. Three *Mucor* species were taken as outgroup. Species names are given only for ex-type strains. Geographic origins of isolates are given with strain numbers. T = ex-type strain; NT = ex-neotype strain; \blacksquare = strains from clinical samples; \blacksquare = isolates from soil; \blacksquare = isolates from plants; \ast = isolates from animals or animal excretion.

buffer for 1 h at 20 °C. Subsequently, the mixture was diluted five times with 10 mM Tris-HCl (pH 8.3) buffer. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5'-CTCGTAGACTGCGTACC-3' and 5'-CGGG-TACGCAGTC-3' for HpyCH4 IV; 5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3' for MseI) and heating to 95 °C, subsequently cooling slowly to ambient temperature. One microliter of the diluted restriction-ligation mixture was used for amplification in a volume of 25 μ L under the following conditions: 1 μ M HpyCH4 IV primer with one selective residue (5'-FluGTAGACT GCGTACCCGTT-3'), 1 μ M MseI primer with four selective residues (5'-GATGAGTCCTGACTAATGAG-3'), 0.2 mM of each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase (Roche Diagnostics, Almere, The Netherlands) in 1 \times reaction buffer containing 1.5 mM MgCl₂. Amplification was done as follows. After an initial denaturation step for 4 min at

94 °C in the first 20 cycles, a touchdown procedure was applied: 15 s of denaturation at 94 °C, 15 s of annealing at 66 °C, with the temperature for each successive cycle lowered by 0.5 °C, and 1 min of extension at 72 °C. Cycling was then continued for a further 30 cycles with an annealing temperature of 56 °C. After completion of the cycles, incubation at 72 °C for 10 min was performed before the reaction mixtures were cooled to room temperature. The amplicons were then combined with the ET400-R size standard (GE Healthcare, Diegem, Belgium) and analysed on a Mega BACE 500 automated DNA platform (GE Healthcare) according to the manufacturer's instructions. Data were inspected visually and were also imported in BioNumerics v. 6.0 software (Applied Maths) and analysed by UPGMA clustering using the Pearson correlation coefficient. The analysis was restricted to DNA fragments in the range from 80–250 bp.

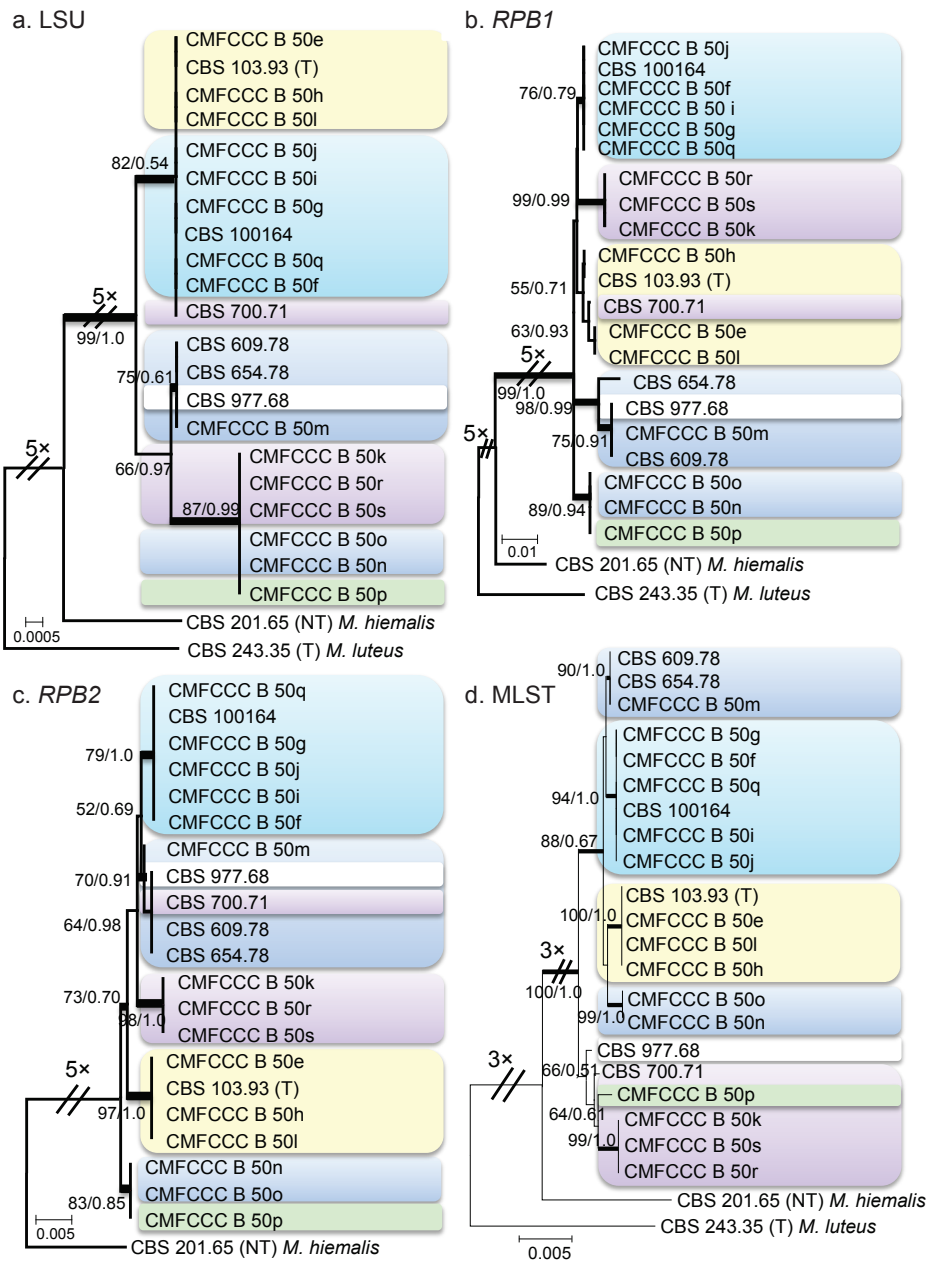


Fig. 2 Neighbour-Joining phylogenies of 21 isolates of *Mucor irregularis* based on four different markers: a. LSU; b. *RPB1*; c. *RPB2*; d. multilocus, combining sequences of LSU, ITS, *RPB1* and *RPB2* by Mega 5 software. Branches with bootstrap values, estimated based on 500 replicates in the Neighbour-Joining analysis, are printed in **bold** when 70 or higher. Branch support values are indicated by the numbers near the branches (Neighbour-Joining bootstrap values / Bayesian posterior probabilities). Group colours used in four phylogenies per strain are the same as in the ITS phylogram. CBS 243.35 and CBS 201.65 were taken as outgroup. T = ex-type strain; NT = ex-neotype strain.

RESULTS

Phylogeny analysis

The four genes could be sequenced directly for all strains except CBS 243.35 for which no editable trace file of *RPB2* could be obtained. Newly generated sequences have been deposited in GenBank (Table 1).

Fig. 1 presents the rDNA phylogeny based on the ITS region representing four species of *Mucor*. Phylogenetic analyses were conducted using Neighbour-Joining, Maximum likelihood (data not shown) and Bayesian approaches. The resulting three phylogenies yield almost identical topologies. Four well-supported groups became apparent that consisted of *M. irregularis*, *M. hiemalis*, *M. luteus* and *M. silvaticus* (all bootstrap values and Bayesian posterior probabilities are 100/1.0, respectively). *Mucor irregularis* showed similarities below 90 % with the outgroups (87.2–88.8 % with *M. hiemalis*, 83.7–84.7 % with

M. luteus and 79.4–79.8 % with *M. silvaticus*, respectively). The intraspecies ITS variability of *M. irregularis* is 96.1–100 %. Clinical isolates are shown with red crosses in the ITS tree (Fig. 1). Almost all clinical isolates are grouped in *M. irregularis* except CBS 123972, which originated from a sputum sample in Germany. But there was no predominant subgroup distribution with respect to clinical and environmental isolates in *M. irregularis*. Among the three *M. hiemalis* species, only a single isolate (CBS 567.70) originated from Asia, while, conversely, only a single isolate (CBS 609.78) of *M. irregularis* originated from outside Asia.

For multilocus sequence analysis, the LSU sequence alignment (a total of 930 characteristics, of which 2 were parsimony-informative intraspecies) resulted in 350 most parsimonious trees (TL = 59 steps), the *RPB1* sequence alignment (a total of 805 characteristics, of which 24 were parsimony informative intraspecies) resulted in 76 most parsimonious trees (TL = 165 steps), and the *RPB2* sequence alignment (a total

of 949 characteristics, of which 13 were parsimony informative intraspecies) resulted in 107 most parsimonious trees (TL = 126 steps). A separate clade corresponding to *M. irregularis* showed high bootstrap-support in all the trees. Similarities between *M. hiemalis* and *M. irregularis* of ITS, LSU, *RPB1* and *RPB2* were 87.2–88.8 %, 96.9–97.0 %, 73.2–74.0 %, and 87.8–88.1 %, respectively. In the ITS tree, five subgroups (subgroups 1–5) appear among the *M. irregularis* group except CBS 977.68 (bootstraps for NJ/Bayesian posterior probabilities = 84/0.79, 81/1.0, 95/0.83, 80/1.0 and 97/1.0, respectively). The strains in subgroup 2 and 4 were isolated from different countries including China, Nigeria and India, while the isolates of remaining subgroups were isolated from China. The strain designations are attributed the same colour if the strains belong to same subgroup in the ITS genealogy (Fig. 1). Because the same colour coding is used in the LSU, *RPB1* and *RPB2* trees, conflicts in gene genealogies of different loci are visualized by the intermixing of colours (Fig. 2a–c). Partition homogeneity test showed congruence of all four genes (LSU, ITS, *RPB1* and *RPB2*), which were therefore combined. The combined sequence alignment (a total of 2 861 characteristics, of which 64 were parsimony-informative intraspecies) resulted in 105 most parsimonious trees (TL = 272 steps). The tree topologies obtained by Neighbour-Joining, Maximum-likelihood and Bayesian Markov chain Monte Carlo analyses were largely similar. Therefore, the Maximum-likelihood and the Bayesian trees are not shown (Fig. 2d). In the combined tree, *M. irregularis* clade showed 100 % bootstrap support, and three well-supported subgroups (bootstrap of subgroup 1, 3, 4 = 100 %, 94 % and 99 %, respectively) were consistent with the ITS tree. Subgroup 2 in ITS tree was divided into two well-supported clade (bootstraps 90 and 99 %, respectively), while subgroup 5 was merged into subgroup 4.

AFLP

Profiles of 20 strains of *Mucor irregularis* were generated including CBS 243.35 and CBS 201.65 as outgroup controls. Fingerprints contained bands in a 50–500 bp range. Dendrograms derived from the AFLP banding patterns of *M. irregularis* were generated by using UPGMA cluster analysis (Fig. 3). At > 73 % similarity, five main clusters were found. Clusters 1, 2, 4 and 5 approximately matched with coloured AFLP subgroups, but particularly subgroup 4 (red AFLP) contained strains with widely different AFLP profiles. Deviating strain CBS 700.71 was

also shown to be different in remaining partitions (Fig. 2), but CMFCCC B 50k, 50r and 50s were together in all genes while the AFLP pattern was deviant. Strains originating from garden soil in Nigeria in 1966, from an owl pellet in India in 1978, and recent strains from Chinese patients with mucormycosis were all found in a single ITS subgroup (2) and clustered together with AFLP.

Morphological characteristics

Colonies grew rapidly at 30 °C on MEA, were expanding, hairy and whitish, and pale-yellow in reverse (Fig. 4a, b). Sporangiophores were hyaline, up to 2 mm high, 10–20 µm wide, sympodially and monopodially branched arising from abundant branched hyphae, branches all ending in a sporangium. Sporangia (sub)spherical, up to 100 µm diam; membrane diffluent; columella spherical, ellipsoidal to cylindrical, about 40 µm wide, with apophysis. Sporangiospores were hyaline, smooth-walled, very variable, mostly subspherical to ellipsoidal, 3–11 × 2–7 µm. Chlamydospores abundant and occur in hyphae and sporangiophores.

DISCUSSION

Recent molecular studies have shown that, being re-classified in the genus *Mucor*, *M. irregularis* had *M. hiemalis* as its closest neighbour (Alvarez et al. 2009, Walther et al. 2013). The *M. hiemalis* group comprises a number of species that are phylogenetically widely different, which were included as outgroup in our study. *Mucor luteus*, *M. hiemalis* and *M. silvaticus* proved to be significantly distinct from one another. When these three species were taken as outgroup in an ITS phylogeny, *M. irregularis* composed a monophyletic clade with high bootstrap support, remote from *M. hiemalis* at similarities below 90 %. With the remaining genes studied comparably high relative distances to *M. hiemalis* were found in all four markers, similarities of ITS, LSU, *RPB1* and *RPB2* being 87.2–88.8 %, 96.9–97.0 %, 73.2–74.0 % and 87.8–88.1 %, respectively.

All clinical isolates studied in the present revision clustered in *M. irregularis*, with the exception of CBS 123972, which proved to belong to the saprobic taxon *M. hiemalis* (Fig. 1). This strain originated from sputum of a human patient in Germany; the remaining strains of the same cluster, as far as is known, were cultured from soil or from faeces. Clinical data of CBS 123972 were too scant to establish whether or not

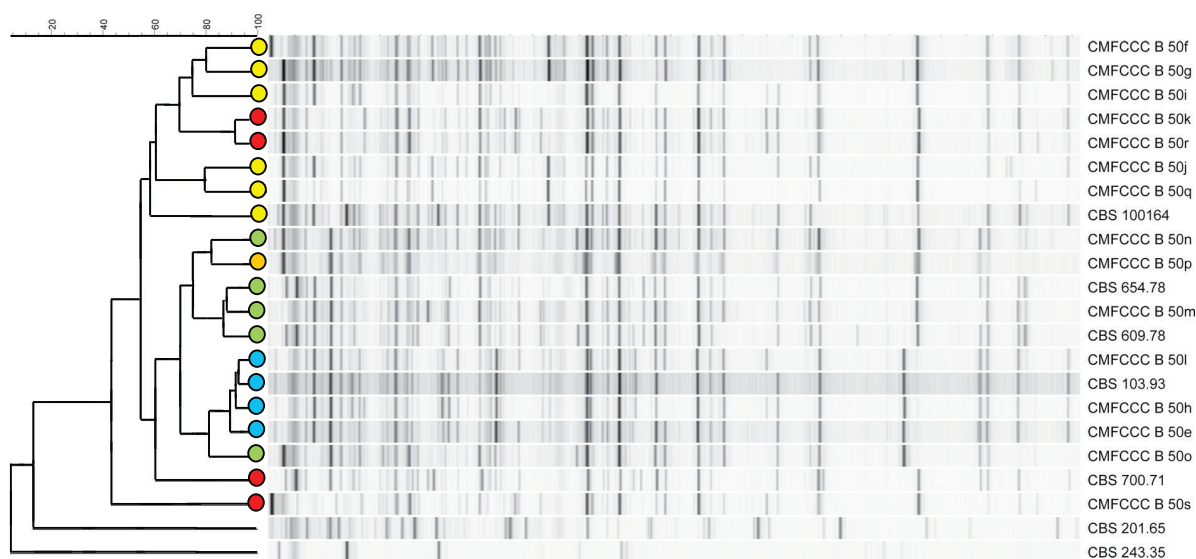


Fig. 3 Dendrogram of amplified fragment length polymorphism (AFLP) profiles of 21 *Mucor irregularis* isolates. *Mucor hiemalis* (CBS 201.65) and *Mucor luteus* (CBS 243.35) are taken as outgroup. The scale bar on the top indicates the percentage similarity. Strain colours: blue = ITS subgroup 1; green = ITS subgroup 2; yellow = ITS subgroup 3; red = ITS subgroup 4; ochre = ITS subgroup 5.

the isolate was involved in a disease process or was just a colonizer. In contrast, strain CBS 100164, originally deposited in the CBS collection as a clinical isolate of *M. luteus* from the skin lesion of a Chinese patient with chronic mucormycosis, was re-identified in our study as *M. irregularis*. In the literature, four clinical cases have been ascribed to *M. hiemalis*. Three of these were cutaneous mucormycosis (Costa et al. 1990, Prevo et al. 1991, de Oliveira-Neto et al. 2006), while the remaining one was a rhinocerebral mucormycosis (Larkin et al. 1986). All four cases were diagnosed based on morphology prior to the description of *M. irregularis* (*Rhizomucor variabilis*), and the samples have not been preserved so that DNA confirmation is not possible. Carefully judging the case reports, we have the impression that *M. irregularis* was concerned rather than *M. hiemalis*, at least in a number of cases. The case reported by Prevo et al. (1991) from The Netherlands presented a slowly extending, indurated, erythematous, and scaling eruption on the right cheek in a healthy young girl after an insect bite. The case described by de Oliveira-Neto et al. (2006) from Brazil presented a similar, 5-year history of a slowly enlarging, erythematous plaque with slight elevated, scaling, circinate borders on the right thigh in a healthy immunocompetent young girl. Comparing these cases with the general clinical course of *M. irregularis* infections, the features of these two cases are clearly consistent with *M. irregularis* infection (Lu et al. 2009), which is unique among the *Mucorales* by being chronic cutaneous in healthy individuals. According to Schipper (1973), who provided physiological data of *M. hiemalis* and *M. luteus*, this species was unable to grow at temperatures higher than 33 °C, which practically excluded human pathogenicity. With a maximum at 37 °C, *M. irregularis* is less thermotolerant than most other clinically relevant members of *Mucorales*, which often grow at temperatures well above 42 °C (Dolatabadi et al. In press). Given the resemblance of '*M. hiemalis*' clinical features to those of *M. irregularis*, the morphological similarities between the two species, and weak temperature tolerance of *M. hiemalis*, we conclude that these cases ascribed to *M. hiemalis* were actually caused by *M. irregularis*. If we include these two cases in the list of cases caused by *M. irregularis*, the pathogen has a world-wide distribution, mainly occurring in Asia with a focus in China, but also occurring in Australia, the USA, South America and Europe. *Mucor hiemalis* is an environmental species without any pathogenic potential.

Mucormycosis due to *M. irregularis* presents with unique clinical features. All cases of primary infection reported in the literature demonstrated a chronic process with a history of at least three months (Table 1; Hemashettar et al. 2011, Tomita et al. 2011). Nearly all patients were immunocompetent, non-diabetic and in good general health. None of the cases showed signs of angio-invasiveness especially in the early-stage, and none of the clinical signs typical of classical mucormycosis was observed. Mucormycosis caused by all other members of the order, such as *Lichtheimia corymbifera* or *Rhizopus microsporus*, nearly exclusively present with an acute, invasive, rapid process with obvious angio-invasion and leading to massive necrosis within days; the disease is only seen in patients with severe underlying disease such as uncontrolled diabetes, leukemia, or with other types of immuno-incompetence, or in wound infections.

In its pathogenicity, *M. irregularis* differs significantly from all remaining *Mucorales*. One of the possible factors may be the difference in temperature tolerance between species. The widely known opportunists in the *Mucorales* are highly thermotolerant or thermophilic, with maximum growth temperatures up to 37–45 °C, such as *Apophysomyces* spp. (Alvarez et al. 2010b, Chakrabarti et al. 2010), *Lichtheimia* spp. (Alastruey-Izquierdo et al. 2010), *Cunninghamella* spp. (de Hoog et al. 2009), *Rhizomucor* spp. (de Hoog et al. 2009), *Rhizopus* spp. except *R. stolonifer* (de Hoog et al. 2009),

Saksenaee spp. (Alvarez et al. 2010a), *Syncephalastrum racemosum* (de Hoog et al. 2009) and *Actinomucor elegans* (Tully et al. 2009, Mahmud et al. 2012). In the genus *Mucor*, the potent human and veterinary opportunists *M. circinelloides*, *M. ellipsoideus*, *M. indicus*, *M. ramosissimus* and *M. velutinosus* (Alvarez et al. 2011) grow well at 37 °C, while *M. amphibiorum*, *M. irregularis* and *M. racemosus* grow at 35 °C but not at 37 °C (de Hoog et al. 2009), and hence are questionable opportunists or amphibian pathogens at most. *Mucor hiemalis* and *Rhizopus stolonifer* show the weakest temperature tolerance (33 °C; Schipper 1973, 1976) and cases reported as caused by these species are doubtful. Cases of mucormycosis due to *R. stolonifer* were reported decennia ago (Sandler et al. 1971, Ferry & Abedi 1983, del Palacio et al. 1999), without molecular identification. Under the name *M. racemosus* only a single case has been reported from China (Wang et al. 2002), which was identified by phenotypic methods as well. This case presented similar features as *M. irregularis* infections, i.e. showing 7-year-history of a cutaneous plaque at the right upper extremity. Judged according to current data, this strain was likely to have been *M. irregularis* as well.

Among the 21 isolates of *M. irregularis* studied, five subgroups were found in phylogenetic trees of four loci (Fig. 1, 2). The position of most isolates was consistent in most subgroups, but some strains changed position in different loci, such as CBS 977.68, CBS 700.71, CMFCCC B 50n and CMFCCC B 50o, which was also observed in AFLP data, indicated possible recombination events. *In vitro* mating tests have classically been performed within the closest relative, *M. hiemalis* and its varieties (Schipper 1973). A recent study in *Rhizopus microsporus* showed that 83.33 % of the strains belonged to either one mating type and produced well-developed zygospores upon crossing (Dolatabadi et al. In press). Schell et al. (2011), however, noted limited formation of zygospores in *M. irregularis*. Two sexually active strains were found with high breeding competence, while most strains could not be mated with either mating type. Similar phenomena are known elsewhere in the fungal Kingdom, e.g. in dermatophytes, where Kawasaki (2011) produced apparent mating between phylogenetically unrelated species by the use of sexually very active strains. In *Mucorales*, interspecific mating mostly leads to the formation of azygospores (Alastruey-Izquierdo et al. 2010, Schell et al. 2011). When presence or absence of mating products do not reflect actual interbreeding communities, the biological species concept is difficult to apply.

Three isolates clustered together in all separate markers and in the AFLP dendrogram as subgroup 2. The group included CBS 609.78 from garden soil in Nigeria in 1966, CBS 654.78 from owl pellets of India in 1978 and CMFCCC B 50m from the lesion of a Chinese patient in 2010. Further strains that clustered with this group in the ITS tree (Fig. 1) originated from China and India, while subgroup 4 contained strains from different origins including China and India as well. Some of the subgroups of *M. irregularis*, however, showed approximate differences in geographic distribution. Strains of subgroups 1 and 3 clustered together in all partitions and comprised strains originating from central China. These subgroups also were partially supported in AFLP dendrogram. The mild local geographic structuring in *M. irregularis* seems to be in conflict with the intuitive knowledge that members of *Mucorales* are easily dispersed by air, but the distance that the airborne spores are able to travel is limited. The localised geographic distribution pattern may partly be explained by some of the morphological characters of *M. irregularis*: the sporangiospores measure 3–11 × 2–7 µm, and are often irregular in shape. In comparison, conidia of the global airborne fungus *Aspergillus fumigatus* are spherical and measure 2.5–3.0 µm diam. Many filamentous fungi such as *Aspergillus* species express water-repellent hydrophobins on

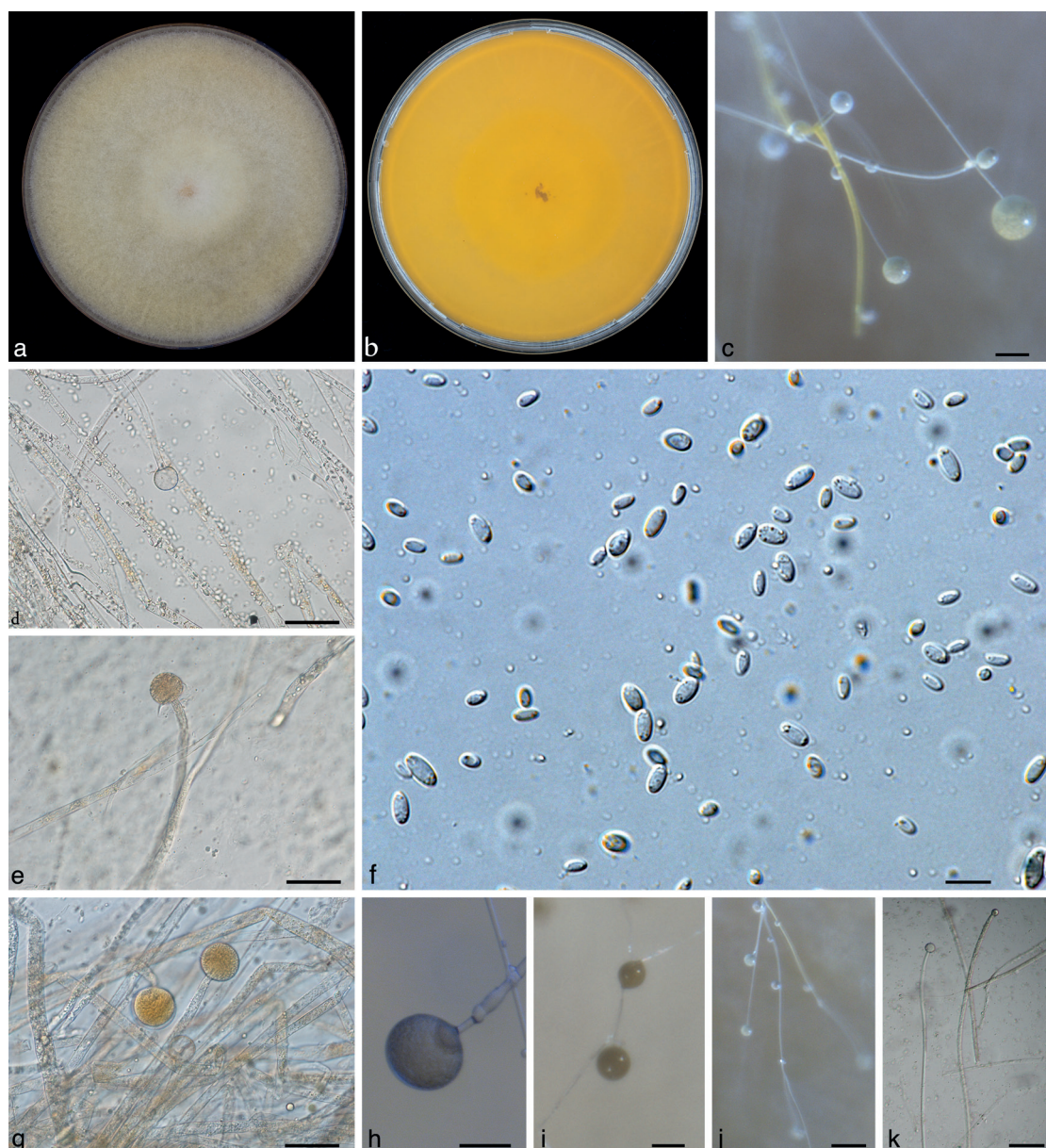


Fig. 4 Macroscopic and microscopic morphology of *Mucor irregularis*. a. Colony on MEA after 4 days incubation at 30 °C; b. colony from backside; c, d, f–k. columella and sporangiospores; e. sporangia. — Scale bar = 10 µm.

the conidial surface, which enhance airborne dispersal (Linder et al. 2005). Sporangiospores of *M. irregularis* are much less hydrophobic and are likely to have a higher sedimentation rate in the atmosphere and hence populations are likely to have a more limited distribution. *Mucor irregularis* thus far has not been encountered as an airborne contaminant, environmental isolates having been isolated from soil, plants and animal faeces. In conclusion, the comparison of gene genealogies based on four markers (LSU, ITS, *RPB1* and *RPB2*) and AFLP results showed evidence of recombination, and hence *M. irregularis* is recognised as a single species despite geographic structuring in some of the subgroups. The species is prevalent in East Asia but has a world-wide distribution. Morphological characters of sporangiospores preventing efficient airborne dispersal may be partially responsible for the observed geographic structuring.

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