



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

X.-L. Lu^{1,2,3}, M.J. Najafzadeh⁴, S. Dolatabadi¹, Y.-P. Ran⁵, A.H.G. Gerrits van den Ende¹, Y.-N. Shen³, C.-Y. Li⁶, L.-Y. Xi⁷, F. Hao⁸, Q.-Q. Zhang⁹, R.-Y. Li¹⁰, Z.-M. Hu¹¹, G.-X. Lu³, J.-J. Wang⁷, M. Drogari-Apiranthitou¹², C. Klaassen¹³, J.F. Meis¹³, F. Hagen¹³, W.-D. Liu^{2,3}, G.S. de Hoog^{1,7,10,14}

Key words

biodiversity
chronic cutaneous infection
epidemiology
Mucor hiemalis
Mucor irregularis
Mucormycosis
taxonomy

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.

Article info Received: 16 November 2012; Accepted: 4 February 2013; Published: 6 March 2013.

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

¹ CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

² Department of Mycology, Dermatology Hospital of Dalian, Dalian Medical University, Dalian, China; corresponding author e-mail: liumyc@hotmail.com.

³ Department of Mycology, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China.

⁴ Department of Parasitology and Mycology, and Cancer Molecular Pathology Research Center, Ghaem Hospital, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

⁵ Department of Dermatology, West China Hospital, Chengdu, China.

⁶ Qilu Hospital of Shandong University, Jinan, China.

⁷ Sun-Yat Sen Memorial Hospital, Sun-Yat Sen University, Guangzhou, China.

⁸ Southwest Hospital, Third Military Medical University, Chongqing, China.

⁹ Huashan Hospital, Fudan University, Shanghai, China.

¹⁰ Peking University Health Science Center, Research Center for Medical Mycology, Beijing, China.

¹¹ The First Hospital of Wuhan, Wuhan, China.

¹² Attikon University Hospital, Athens, Greece.

¹³ Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands.

¹⁴ Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands.

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

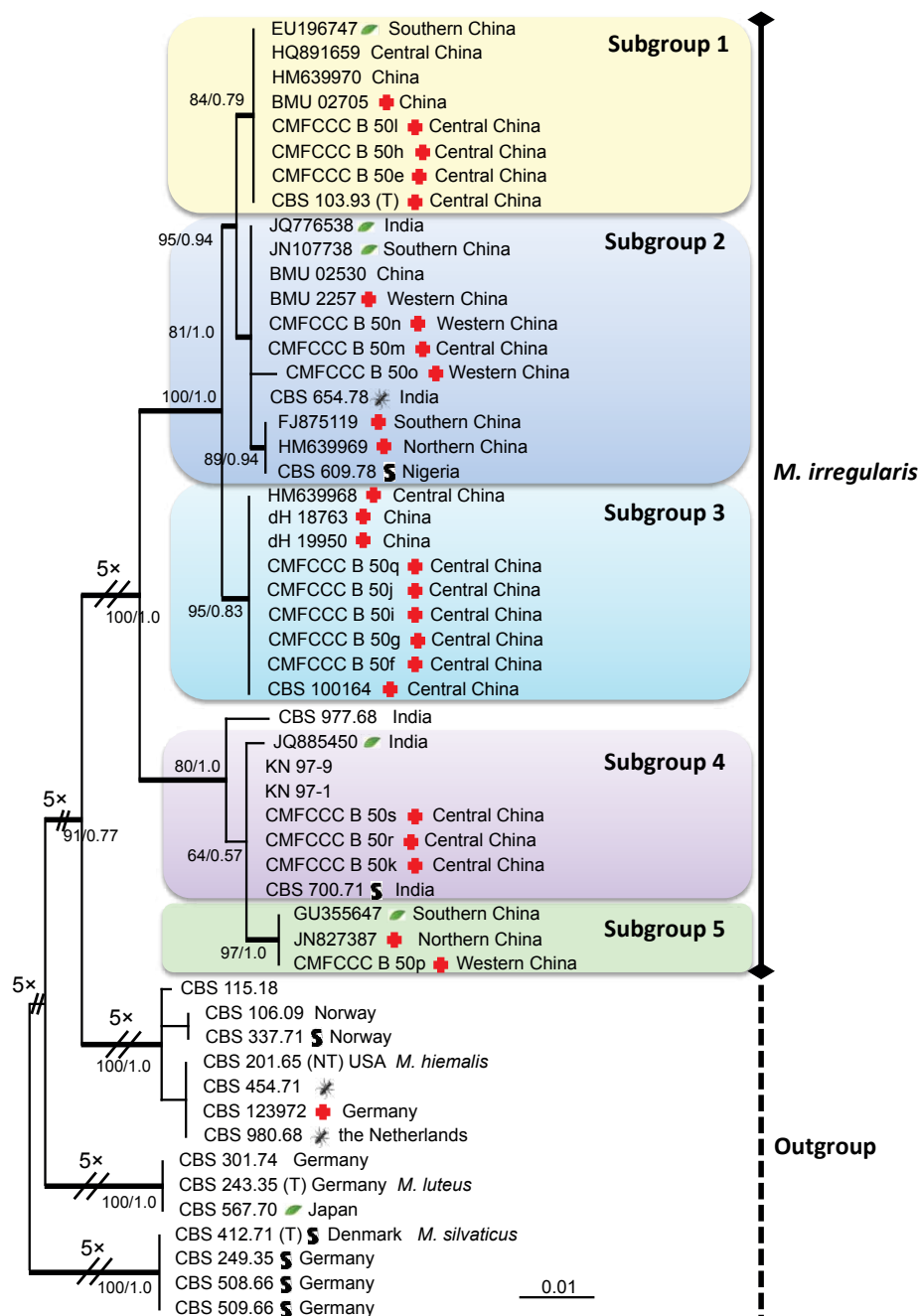


Fig. 1 Phylogeny of *Mucor irregularis* obtained from a Neighbour-Joining analysis of Internal Transcribed Spacer (ITS) using Mega 5 software. Bootstrap support values were estimated based on 500 replicates, and are shown by the numbers near the branches (Neighbour-Joining bootstrap values / Bayesian 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; \blacklozenge = strains from clinical samples; \mathbf{S} = isolates from soil; \blacklozenge = 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'-FluGTAGACTGCGTACCCGTT-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.

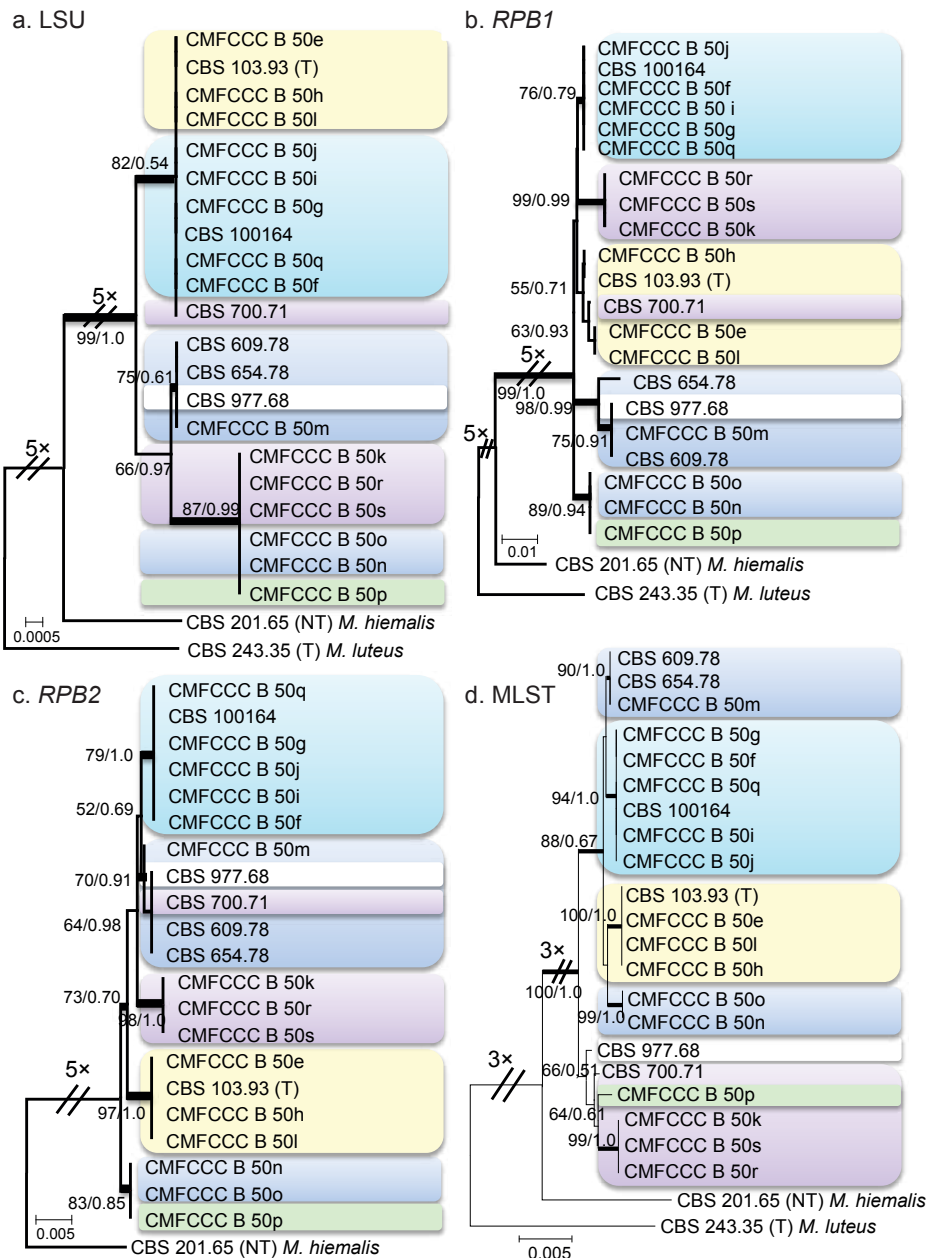


Fig. 2 Neighbour-Joining phylogenies of 21 isolates of *Mucor irregularis* based on four different markers: a. LSU; b. *RPB1*; c. *RPB2*; d. multilocus, combining sequences of LSU, ITS, *RPB1* and *RPB2* by Mega 5 software. Branches with bootstrap values, estimated based on 500 replicates in the Neighbour-Joining 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

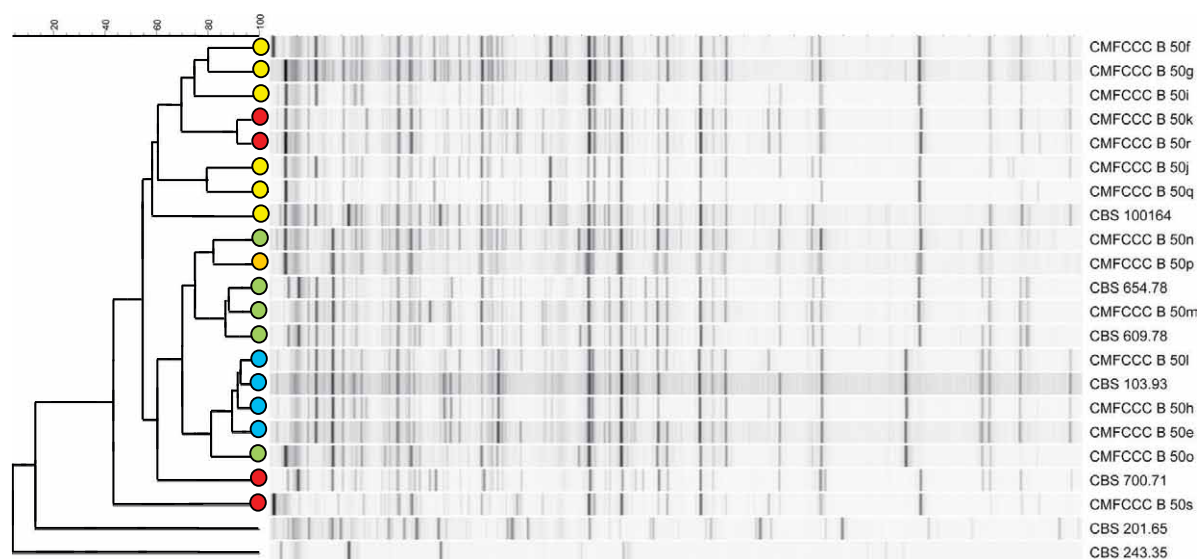


Fig. 3 Dendrogram of amplified fragment length polymorphism (AFLP) profiles of 21 *Mucor irregularis* isolates. *Mucor hiemalis* (CBS 201.65) and *Mucor luteus* (CBS 243.35) are taken as outgroup. The scale bar on the top indicates the percentage similarity. Strain colours: blue = ITS subgroup 1; green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

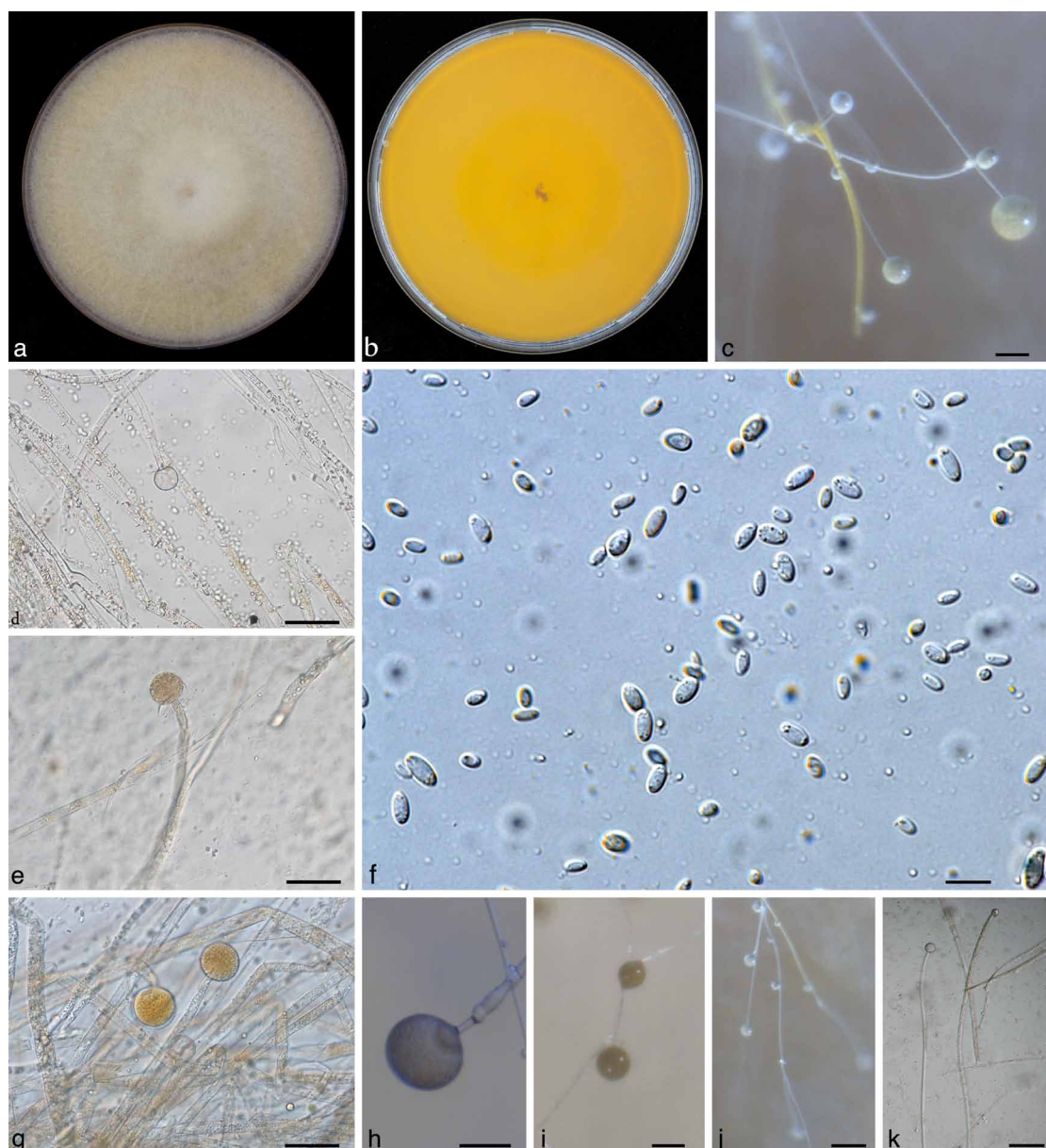


Fig. 4 Macroscopic and microscopic morphology of *Mucor irregularis*. a. Colony on MEA after 4 days incubation at 30 °C; b. colony from backside; c, d, f–k. columella and sporangiospores; e. sporangia. — Scale bar = 10 µm.

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.

Acknowledgements This work was supported by project 11CDP009 of the China Desk of KNAW project, and by the National Natural Sciences Foundation of China (grant number No. 81101232). Grit Walther is acknowledged for useful comments on the text. We are grateful to Erik Geertsen for assistance with AFLP.

REFERENCES

- Alastruey-Izquierdo A, Hoffmann K, Hoog GS de, Rodriguez-Tudela JL, Voigt K, et al. 2010. Species recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Absidia* pro parte, *Mycoclados*). *Journal of Clinical Microbiology* 48: 2154–2170.
- Alvarez E, Cano J, Stchigel AM, Sutton DA, Fothergill AW, et al. 2011. Two new species of *Mucor* from clinical samples. *Medical Mycology* 49: 62–72.
- Alvarez E, Garcia-Hermoso D, Sutton DA, Cano JF, Stchigel AM, et al. 2010a. Molecular phylogeny and proposal of two new species of the emerging pathogenic fungus *Saksenaia*. *Journal of Clinical Microbiology* 48: 4410–4416.
- Alvarez E, Stchigel AM, Cano J, Sutton DA, Fothergill AW, et al. 2010b. Molecular phylogenetic diversity of the emerging mucoralean fungus *Apoophysomyces*: proposal of three new species. *Revista Iberoamericana de Micología* 27: 80–89.
- Alvarez E, Sutton DA, Cano J, Fothergill AW, Stchigel A, et al. 2009. Spectrum of zygomycete species identified in clinically significant specimens in the United States. *Journal of Clinical Microbiology* 47: 1650–1656.
- Chakrabarti A, Shivaprakash MR, Curfs-Breuker I, Baghela A, Klaassen CH, Meis JF. 2010. *Apoophysomyces elegans*: epidemiology, amplified fragment length polymorphism typing, and in vitro antifungal susceptibility pattern. *Journal of Clinical Microbiology* 48: 4580–4585.
- Costa AR, Porto E, Tayah M, Valente NY, Lacaz Cda S, et al. 1990. Subcutaneous mucormycosis caused by *Mucor hiemalis* Wehmer f. *luteus* (Linnemann) Schipper 1973. *Mycoses* 33: 241–246.

- Dolatbadi S, Walther G, Gerrits van den Ende AHG, Hoog GS de. In press. Diversity and delimitation of *Rhizopus microsporus*. *Fungal Diversity*.
- Ferry AP, Abedi S. 1983. Diagnosis and management of rhino-orbitocerebral mucormycosis (phycomycosis). A report of 16 personally observed cases. *Ophthalmology* 90: 1096–1104.
- Fu MH, Chen H, Song LY, Chen H, Chen W, et al. 2006. Facial mucormycosis due to *Rhizomucor variabilis*: a case report. *International Journal of Dermatology and Venereology* 32: 67–69. [In Chinese.]
- Ghorbel S, Souissi N, Triki-Ellouz Y, Dufosse L, Guerard F, Nasri M. 2005. Preparation and testing of *Sardiella* protein hydrolysates as nitrogen source for extracellular lipase production by *Rhizopus oryzae*. *World Journal of Microbiology and Biotechnology* 21: 33–38.
- Hemashettar BM, Patil RN, O'Donnell K, Chaturvedi V, Ren P, Padhye AA. 2011. Chronic rhinofacial mucormycosis caused by *Mucor irregularis* (*Rhizomucor variabilis*) in India. *Journal of Clinical Microbiology* 49: 2372–2375.
- Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systems Biology* 42: 182–192.
- Hiol A, Jonzo M, Rugani N, Druet D, Sarda L, Comeau L. 2000. Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme and Microbial Technology* 26: 421–430.
- Hoog GS de, Guarro J, Figueras MJ, Gené J. 2009. *Atlas of Clinical Fungi*. 3rd CD-ROM ed. Centraalbureau voor Schimmelcultures, Utrecht / University Rovira I Virgili, Reus, Spain.
- Hu ZM, Wang Y, Zhou FH, Chen LQ, Tong ZS, et al. 2010. Primary cutaneous mucormycosis due to *Rhizomucor variabilis*: a case report. *Chinese Journal of Dermatology* 43: 259–262. [In Chinese.]
- Jiang X, Wang S, Zhao TJ, Ran YP. 2003. A case of cutaneous mucormycosis. *Journal of Clinical Dermatology* 32: 271. [In Chinese.]
- Kawasaki M. 2011. Verification of a taxonomy of dermatophytes based on mating results and phylogenetic analyses. *Japanese Journal of Medical Mycology* 52: 291–295.
- Kim JY, Yeo SH, Baek SY, Choi HS. 2011. Molecular and morphological identification of fungal species isolated from bealmijang meju. *Journal of Microbiology and Biotechnology* 21: 1270–1279.
- Larkin JG, Butcher IG, Frier BM, Brebner H. 1986. Fatal rhinocerebral mucormycosis in newly diagnosed diabetic. *Diabetic Medicine* 3: 266–268.
- Li CL, Jia TH. 1993. Primary cutaneous mucormycosis due to new species named *Rhizomucor variabilis*. *Chinese Journal of Dermatology* 26: 352–353. [In Chinese.]
- Li CY, Li Y, Hu ZM. 2004. A case report of primary cutaneous mucormycosis caused by *Rhizomucor variabilis*. *Journal of Clinical Dermatology* 33: 158–159. [In Chinese.]
- Li CY, Xu YH, Hu QF. 2006. Facial cutaneous *Rhizomucor variabilis* caused by *Rhizomucor variabilis*: a case report. *Chinese Journal of Mycology* 1: 284–285. [In Chinese.]
- Li DM, Lun LD. 2012. *Mucor irregularis* infection and lethal midline granuloma: a case report and review of published literature. *Mycopathologia* 174: 429–439.
- Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME. 2005. Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiology Reviews* 29: 877–896.
- Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* 16: 1799–1808.
- Lu XL, Liu ZH, Shen YN, She XD, Lu GX, et al. 2009. Primary cutaneous zygomycosis caused by *Rhizomucor variabilis*: a new endemic zygomycosis? A case report and review of 6 cases reported from China. *Clinical Infectious Diseases* 49: e39–e43.
- Mahmud A, Lee R, Munfus-McCray D, Kwiatkowski N, Subramanian A, et al. 2012. *Actinomucor elegans* as an emerging cause of mucormycosis. *Journal of Clinical Microbiology* 50: 1092–1095.
- Mason-Gamer RJ, Kellogg EA. 1996. Testing for phylogenetic conflict among molecular datasets in the tribe Triticeae (Gramineae). *Systems Biology* 45: 524–545.
- Neame P, Rayner D. 1960. Mucormycosis. A report on twenty-two cases. *Archives of Pathology and Laboratory Medicine* 70: 261–268.
- Oliveira-Neto MP de, Silva M da, Fialho Monteiro PC, Lazera M, Almeida Paes de R, et al. 2006. Cutaneous mucormycosis in a young, immunocompetent girl. *Medical Mycology* 44: 567–570.
- Palacio A del, Ramos MJ, Pérez A, Arribi A, Amondarain I, et al. 1999. Five cases of zygomycosis. *Revista Iberoamericana de Micología* 16: 50–56. [In Spanish.]
- Prevoo RL, Starink TM, Haan P de. 1991. Primary cutaneous mucormycosis in a healthy young girl. Report of a case caused by *Mucor hiemalis* Wehmer. *Journal of the American Academy of Dermatology* 24: 882–885.
- Qi B, Chen X, Ren FL, Xu HB, Shen YN, Gu H. 2012. Primary cutaneous zygomycosis caused by *Rhizomucor variabilis*: a case report and literature review. *Journal of Clinical Dermatology* 41: 329–332. [In Chinese.]
- Qin W, Lu S, Que DM, Xi LY, Li XQ. 2010. A case report of primary cutaneous mucormycosis caused by *Rhizomucor variabilis*. *Diagnosis and Therapy Journal of Dermatovenereology* 17: 14–17. [In Chinese.]
- Ran YP, Xiong L, Hu L, Zhang YZ, Dai YL, et al. 2006. Rhinocerebral mucormycosis after the operation of turbineotomy: a case report. *Chinese Journal of Mycology* 1: 28–30. [In Chinese.]
- Ribeiro NF, Heath CH, Kierath J, Rea S, Duncan-Smith M, Wood FM. 2010. Burn wounds infected by contaminated water: case reports, review of the literature and recommendations for treatment. *Burns* 36: 9–22.
- Ribes JA, Vanover-Sams CL, Baker DJ. 2000. Zygomycetes in human disease. *Clinical Microbiology Reviews* 13: 236–301.
- Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, et al. 2005. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clinical Infectious Diseases* 41: 634–653.
- Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.
- Sandler R, Tallman CB, Keamy DG, Irving WR. 1971. Successfully treated rhinocerebral phycomycosis in well controlled diabetes. *The New England Journal of Medicine* 285: 1180–1182.
- Schell WA, O'Donnell K, Alspaugh JA. 2011. Heterothallic mating in *Mucor irregularis* and first isolate of the species outside of Asia. *Medical Mycology* 49: 714–723.
- Schipper MAA. 1973. A study on variability in *Mucor hiemalis* and related species. *Studies in Mycology* 4: 1–40.
- Schipper MAA. 1976. On *Mucor circinelloides*, *Mucor racemosus* and related species. *Studies in Mycology* 12: 1–40.
- Skiada A, Pagano L, Groll A, Zimmerli S, Dupont B, et al. 2011. Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. *Clinical Microbiology and Infection* 17: 1859–1867.
- Stiller JW, Hall BD. 1997. The origin of red algae: implications for plastid evolution. *Proceedings of the National Academy of Sciences, USA* 94: 4520–4525.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731–2739.
- Tomita H, Muroi E, Takenaka M, Nishimoto K, Kakeya H, et al. 2011. *Rhizomucor variabilis* infection in human cutaneous mucormycosis. *Clinical and Experimental Dermatology* 36: 312–314.
- Tully CC, Romanelli AM, Sutton DA, Wickes BL, Hospenthal DR. 2009. Fatal *Actinomucor elegans* var. *kuwaitiensis* infection following combat trauma. *Journal of Clinical Microbiology* 47: 3394–3399.
- Vienne DM de, Giraud T, Martin OC. 2007. A congruence index for testing topological similarity between trees. *Bioinformatics* 23: 3119–3124.
- Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172: 4239–4246.
- Voigt K, Cigelnik E, O'Donnell K. 1999. Phylogeny and PCR identification of clinically important Zygomycetes based on nuclear ribosomal-DNA sequence data. *Journal of Clinical Microbiology* 37: 3957–3964.
- Walther G, Pawłowska J, Alastruey-Izquierdo A, Wrzosek M, Rodriguez-Tudela JL, et al. 2013. DNA barcoding in Mucorales: an inventory of biodiversity. *Persoonia* 30: 11–47.
- Wang JJ, Liao YX, Xu DX. 1998. Primary cutaneous mucormycosis caused by *Mucor hiemalis* f. *luteus*. *Chinese Journal of Dermatology* 31: 348–350. [In Chinese.]
- Wang JJ, Zhang QQ, Li L. 2002. A case of primary cutaneous mucormycosis due to *Mucor racemosus*. *Chinese Journal of Dermatology* 35: 413. [In Chinese.]
- White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR protocols: a guide to methods and applications*: 315–322. Academic Press Inc, New York, NY.
- Yao Y, Pan S, Wang K, Xu X. 2010. Fermentation process improvement of a Chinese traditional food: soybean residue cake. *Journal of Food Science* 75: M417–421.
- Zhang SP, Hu B, Zhou HL, Jiang FX, Wu AL, et al. 2010. A case of facial cutaneous mucormycosis due to *Rhizomucor variabilis*. *Chinese Journal of Dermatology* 43: 134. [In Chinese.]
- Zhao Y, Zhang QQ, Li L, Zhu J, Kang K, Chen L. 2009. Primary cutaneous mucormycosis caused by *Rhizomucor variabilis* in an immunocompetent patient. *Mycopathologia* 168: 243–247.
- Zheng RY, Chen CQ. 1991. A non-thermophilic *Rhizomucor* causing human primary cutaneous mucormycosis. *Mycosystema* 4: 45–57.
- Zhou CJ, Wang L, Xu Y, Zhong BY, Hao F. 2011. A case of primary cutaneous mucormycosis caused by *Rhizomucor variabilis*. *Chinese Journal of Mycology* 6: 361–362. [In Chinese.]