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MOLECULAR PHYLOGENETIC AND SCANNING ELECTRON MICROSCOPICAL ANALYSES PLACES THE CHOANEPHORACEAE AND THE GILBERTELLACEAE IN A MONOPHYLETIC GROUP WITHIN THE MUCORALES (ZYGOMYCETES, FUNGI)

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A multi-gene genealogy based on maximum parsimony and distance analyses of the exonic genes for actin (*act*) and translation elongation factor 1 alpha (*tef*), the nuclear genes for the small (18S) and large (28S) subunit ribosomal RNA (comprising 807, 1092, 1863, 389 characters, respectively) of all 50 genera of the Mucorales (Zygomycetes) suggests that the Choanephoraceae is a monophyletic group. The monotypic Gilbertellaceae appears in close phylogenetic relatedness to the Choanephoraceae. The monophyly of the Choanephoraceae has moderate to strong support (bootstrap proportions 67% and 96% in distance and maximum parsimony analyses, respectively), whereas the monophyly of the Choanephoraceae-Gilbertellaceae clade is supported by high bootstrap values (100% and 98%). This suggests that the two families can be joined into one family, which leads to the elimination of the Gilbertellaceae as a separate family. In order to test this hypothesis single-locus neighbor-joining analyses were performed on nuclear genes of the 18S, 5.8S, 28S and internal transcribed spacer (ITS) 1 ribosomal RNA and the translation elongation factor 1 alpha (*tef*) and beta tubulin (*βtub*) nucleotide sequences. The common monophyletic origin of the Choanephoraceae-Gilbertellaceae clade could be confirmed in all gene trees and by investigation of their ultrastructure. Sporangia with persistent, sutured walls splitting in half at maturity and ellipsoidal sporangiospores with striated ornamentations and polar ciliate appendages arising from spores in persistent sporangia and dehiscent sporangiola represent synapomorphic characters of this group. We discuss our data in the context of the historical development of their taxonomy and physiology and propose a reduction of the two families to one family, the Choanephoraceae *sensu lato* comprising species which are facultative plant pathogens and parasites, especially in subtropical to tropical regions.

Keywords: Multigene genealogy – ultrastructure – ITS – nrDNA – protein-coding genes

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INTRODUCTION

The Choanephoraceae J. Schröter 1894 consists of fungi parasitic on higher plants causing blossom blights and fruit rots [13, 33, 35]. They are geographically widespread, especially in subtropical and tropical regions. Systematically, the Choanephoraceae is one of thirteen families of the Mucorales (Zygomycetes) recognized in the current classification scheme [17, 18]. The Choanephoraceae comprises three genera, *Choanephora* Thaxter 1903 (originally: *Cunninghamia* Currey 1873), *Blakeslea* Thaxter 1914 and *Poitrasia* P. M. Kirk 1984 with a total of five species, which are characterized by formation of large, pyriform collumellate, *Mucor*-like, multi-spored sporangia and smaller, non-collumellate, few- and uni-spored, dehiscent or non-dehiscent sporangiola borne on separate and distinct sporophores or sporangiophores [17]. The sporangiospores from sporangia and sporangiola are morphologically similar, namely ellipsoidal in shape, pigmented, longitudinally striated and possessing clusters of radiating hyaline appendages at both of their polar ends in sporangia and dehiscent sporangiola [1, 13, 23, 28, 43]. Zygospore morphology appears to be unique for the Choanephoraceae and is proposed to be significant as a family-specific criterion [1, 15–17]. The zygospores are at maturity smooth-walled, golden to dark brown, delicately striated and arise from the fusion of nearly equal-sized gametangia, which are developed by apposed or tong-like suspensors between the tips of basally entwined hyphal branches.

During the past century the genus delimitation of the Choanephoraceae was subject to constant changes. The genera are distinguished primarily by a combination of different sporulating structures, which forced different authors to include different genera into the family. Naumov [22] included only *Choanephora* and *Blakeslea* in the Choanephoraceae. Shanor et al. [32] described a new genus *Cokeromyces* Shanor 1950 and included it in the Choanephoraceae because of the way in which it reproduces asexually (by formation of sporangiola on a capitate vesicle on erect sporangiophores), which is similar to *Blakeslea* and *Choanephora*. The presence of few- and uni-spored sporangiola developing on spherical or longitudinal vesicles of the erect sporangiophore apex was considered to be important. This resulted in the proposal of a polyphyletic concept for the family Choanephoraceae uniting *Blakeslea*, *Choanephora*, *Cunninghamella* Matruchot 1903, *Rhopalomyces* Corda 1839, *Radiomyces* Embree 1959, *Mycotypha* Fenner 1932, *Thamnocephalis* Blakeslee 1905 and *Sigmoideomyces* Thaxter 1891 [43]. The genus *Gilbertella* Hesseltine 1960, which is based on a species isolated from a peach (*Prunus persica*) exhibiting storage rot, was originally included in the genus *Choanephora* as *C. persicaria* Eddy 1925 and was therefore part of the Choanephoraceae [11]. With the introduction of *Gilbertella* for *Choanephora persicaria* and *Gilbertella*'s separation from the genus *Choanephora*, which is based on the formation of non-choanephoraceous *Mucor*-type rough-walled, appendaged zygospores formed between two opposed suspensors, Hesseltine [12] placed *Gilbertella* in the Mucoraceae, a view shared by Zycha et al. [43], von Arx [1] and Kirk [16]. But Hesseltine and Ellis [14] allied *Gilbertella* again with *Choanephora* and *Blakeslea* in the Choanephoraceae. Moreover, other

authors treated *Gilbertella* and *Blakeslea* as synonyms of *Choanephora* [19, 20]. Large-scale phylogenetic studies confirmed the close phylogenetic relatedness between *Gilbertella* and the Choanephoraceae foreshadowing the findings of the present study [25, 40].

These contradictory views and interpretations show the importance of characters that are easy to define, restricted to a few taxa, and taxon-specific. The aim of the present study is to investigate members of the Choanephoraceae using molecular phylogenetics and scanning electron microscopy, and to prove the merging of the families Choanephoraceae and Gilbertellaceae Benny 1991.

MATERIAL AND METHODS

Fungal strains, media and growth conditions

Eight fungal strains of the Choanephoraceae and the Gilbertellaceae were investigated in this study: *Blakeslea trispora* FSU 331 and FSU 332, *Choanephora infundibulifera* f. *cucurbitarum*-FSU 314 and FSU 772, *Gilbertella persicaria* FSU 807 and FSU 808, *Poitrasia circinans* FSU 888 and FSU 889 (Table 1). *Mucor hiemalis* f. *corticola* FSU 3008, which was kindly provided by Prof. George Newcombe (University of Idaho, Moscow, Id, USA), was used as the outgroup taxon for sporangial ultrastructure comparison. All strains are deposited and maintained in the Fungal Reference Centre Jena and available upon request. The fungi were cultivated on MEX medium containing 30 g L^{-1} malt extract (Serva, Heidelberg, Germany) or on pea glucose medium consisting of 500 mL L^{-1} pea extract and 5 g L^{-1} glucose (Merck, Darmstadt, Germany). For pea extract preparation 150 g frozen peas were boiled in 500 mL distilled water for 15 min. For solidification the media were supplemented with 20 g L^{-1} agar agar (Roth, Karlsruhe, Germany). Petri dishes were incubated at 25 °C until sporulation for about 3–6 days. The fungal spores served as inoculum for the mycelial growth for DNA extraction procedures. Liquid cultures appropriate for each of the fungal strains were inoculated with spore suspensions (approximately 10^6 spores ml^{-1}) in 500 ml round flasks. The liquid cultures were incubated for two to four days at 25 °C under constant shaking (120 rpm).

Purification of genomic DNA and PCR amplification

Genomic DNA was purified and amplified according to Einax and Voigt [7]. In typical PCR experiments one assay contained 50–100 ng genomic DNA, 10 pmol forward primer F-βtub1 (5′-CARGCYGGTCARTGYGGTAACCA-3′), 10 pmol reverse primer F-βtub4r (5′-GCCTCAGTRAAYTCCATYTCRTCCAT-3′), 16.0 mM $(NH_4)_2SO_4$, 50.0 mM Tris-HCl pH 8.8., 0.01% (v/v) Tween 20, 2.0 mM magnesium chloride, 0.2 mM of each dNTP (Roth, Karlsruhe, Germany) and 1 unit *Taq* polymerase (InViTek, Berlin, Germany) in a total reaction volume of 50 μl. After an

Table 1
Strains analysed by scanning electron microscopy

Species and strain	Mating type	Equivalent strain designations
Blakeslea trispora FSU 331	+	CBS 130.59, ATCC 14271, NRRL 2456
Blakeslea trispora FSU 332	–	CBS 131.59, ATCC 14272, IMI 195169, NRRL 2457
Choanephora infundibulifera f. *cucurbitarum* FSU 314	+	CBS 150.51
Choanephora infundibulifera f. *cucurbitarum* FSU 772	Nd	DSM 960
Gilbertella persicaria var. *indica* FSU 807	+	CBS 349.64
Gilbertella persicaria var. *indica* FSU 808	–	CBS 442.64
Poitrasia circinans FSU 888 (Type strain of Blakeslea circinans)	+	CBS 153.58, ATCC 13016, IMI 078522ii, NRRL 2546
Poitrasia circinans FSU 889	–	CBS 154.58, NRRL 2548, IMI 078524
Mucor hiemalis f. *corticola* FSU 3008	Nd	None

ATCC: American Type Culture Collection, USA. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. DSM: Deutsche Sammlung Mikroorganismen und Zellkulturen, Braunschweig, Germany. FSU: Friedrich-Schiller-Universität, Jena, Germany. IMI: CABI Bioscience (formerly: International Mycological Institute), London, U.K. NRRL: Agricultural Research Service Culture Collection at the National Center of Agricultural Utilization Research (formerly: Northern Regional Research Laboratories), Peoria, Illinois, USA. Nd: not determined.

initial heat treatment of 5 min at 95 °C all reactions were subjected to polymerase chain reaction in a programmable thermocycler Primus (MWG-Biotech, Ebersberg, Germany) or in a programmable water bath AUTOGENE II (Grant, CLF, U.K.) using 30 cycles of the following temperature profile: 0.5 min at 95 °C for denaturation, 1 min at 52 °C for primer annealing and 1 min at 72 °C for primer extension. The PCR products were electrophoretically separated on 1.2% agarose gels (SeaKem LE; BMA, Rockland, Maine, U.S.A.) and visualised on a TL-312A transilluminator (Spectroline) after staining in 0.5 mg mL^{-} ethidium bromide.

Cloning and sequencing of PCR products

Amplified PCR products were purified by adsorption of DNA to glass particles (GeneClean II, BIO 101, Vista, CA) based on a procedure developed by Vogelstein and Gillespie [38]. The purified PCR products were ligated into the pCR4®-TOPO® vector (Invitrogen, Karlsruhe, Germany) and, after heat shock, cloned in chemically competent Top10 one shot® cells of *Escherichia coli* (Invitrogen, Karlsruhe, Germany). Plasmids were purified after a modified protocol of Birnboim and Doly [4] using additional purification steps (additional removal of proteins with potassium

acetate to a final concentration of 1.0 M and chloroform extraction in a second step). Cycle sequencing with the universal primers T3 (5′-ATTAACCCTCAC-TAAAGGGA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) was conducted in a programmable water bath with the Bigdye™ fluorescent-labelled terminator dye deoxy protocol applying AmpliTaq polymerase (PE Applied Biosystems, Warrington, U.K.) by using the following temperature profile: 20 sec at 96 °C, 30 sec at 55 °C, 240 sec at 60 °C, for 30 cycles. The initial denaturation step was prolonged to 3 min. Each sequencing reaction mixture was precipitated with isopropanol and resuspended in 20 μl formamide (Template Suppression Reagent; PE Applied Biosystems, Warrington, U.K.) before running on an ABI Prism™ 310 (PE Applied Biosystems, Foster City, CA, U.S.A.) automated sequencer. Sequence fragments were assembled with TSE, a DOS text software program (SemWare; Marietta, Ga.). Six new β-tubulin sequences for *Blakeslea trispora*, *Choanephora infundibulifera*, *Poitrasia circinans*, *Gilbertella persicaria*, *Mucor hiemalis* and *Mucor racemosus* were generated in this study, and were deposited in GenBank under accession numbers AY937397-AY937402. All other nucleotide sequences were obtained from GenBank at http://ncbi.nlm.nih.gov. The gene sequences used for phylogenetic analysis shown in Fig. 1 were generated in previous studies [25, 39, 40] and correspond to the following accession numbers: 18S rDNA: AF113405, AF113407, AF113410, AF113412, AF113415-16, AF113421, AF113424, AF113426-33, AF113435-36, AF113438-42, AF157117-70, X89434-37; 28S rDNA: AF113443, AF113445, AF113448, AF113450, AF113453-54, AF113459, AF113462-64, AF113466-74, AF113476-77, AF113479-84, AF157171-224; *tef*: AF157225-304, X54730 and *act*: AJ287132-70, AJ287172-213. The gene sequences used for phylogenetic analysis shown in Fig. 2 were selected from the same data pool, except for ITS 1 and 5.8.S rDNA, where six additional accession numbers were requested from GenBank: AJ278358, AJ278364, AJ278366, AJ278369, AY243949, AY625074.

Sequence alignments and phylogenetic analysis

The nucleic acid sequences of the ribosomal DNA and the coding sequence of the protein-coding genes were aligned using CLUSTAL-W version 1.81 [37]. All alignments were visualised in TSE, a DOS text software program (SemWare; Marietta, Ga.) and manually controlled for ambiguously aligned regions.

Unweighted distance and weighted parsimony analyses were carried out on a total of 336 nucleic acid sequences using PAUP*4.0b10 [34]. The neighbor-joining trees [29] shown in Figs 1A and 2 were inferred from Jukes-Cantor distances. To search for islands of the shortest trees in the maximum parsimony analysis shown in Fig. 1B, heuristic searches were conducted with 1,000 replicates, each with single addition sequence order entry, and the ′MulTrees′ option in effect. Gaps were treated as missing data. Of the 4,151 total nucleic acid characters, 545 ambiguously aligned characteres were excluded, 2,002 were constant, 324 variable characters were parsimony-uninformative and 1,280 variable characters were parsimony-informative. The

ribosomal DNA and the third codon-positions of the protein-coding genes were equally weighted (weight 1). The first and second codon-positions of the protein-coding genes received weights 3 and 2, respectively. Two equally parsimonious trees (tree length = 11,929 steps; consistency index = 0.27; homoplasy index = 0.73; retention index = 0.62) were found and subjected to strict consensus tree computation. The phylograms were displayed and printed in TreeView version 1.6.6. [26]. Bootstrap analyses [8] (50% majority rule) with 1,000 replicates, each with 10 random addition sequence entry order replicates, tree-bisection-reconnection branch swapping algorithm were used to calculate clade stability. 'MulTrees option' was disabled. For all maximum-parsimony analyses MaxTrees were set to 100 and auto-increased by 100. The phylogenetic trees and the aligned data matrices including all accession numbers of the nucleotide sequences used in this phylogenetic analysis are available from TreeBase at http://www.treebase.org/treebase (study accession no. S1847, matrix accession nos. M3395–M3402). These informations are also available from KV upon request.

Scanning electron microscopy

Sporangial specimens were fixed overnight in 3% unbuffered glutaraldehyde. After rinsing in 0.1 M phosphate buffer, they were dehydrated in an ethanol/acetone series (50%, 70%, 90%, 95% and absolute ethanol; 25%, 50%, 75% and 100% acetone) and transferred into liquid CO_2 in a critical point dryer. The dried specimens were mounted, sputter-coated with gold/palladium, and examined in a Philips CM10 scanning electron microscope. Digital pictures were obtained as tagged image file format (TIFF) files.

RESULTS

Molecular phylogenetic analyses

A multi-gene genealogy based on distance and maximum parsimony analyses of a combined four-locus data set of nuclear genes encoding 18S and the D1/D2 domain of the 28S ribosomal RNA, actin (*act*) and elongation factor 1 alpha (*tef*) with complementary bootstrap assessment of phylogenetic confidence is shown in Fig. 1.

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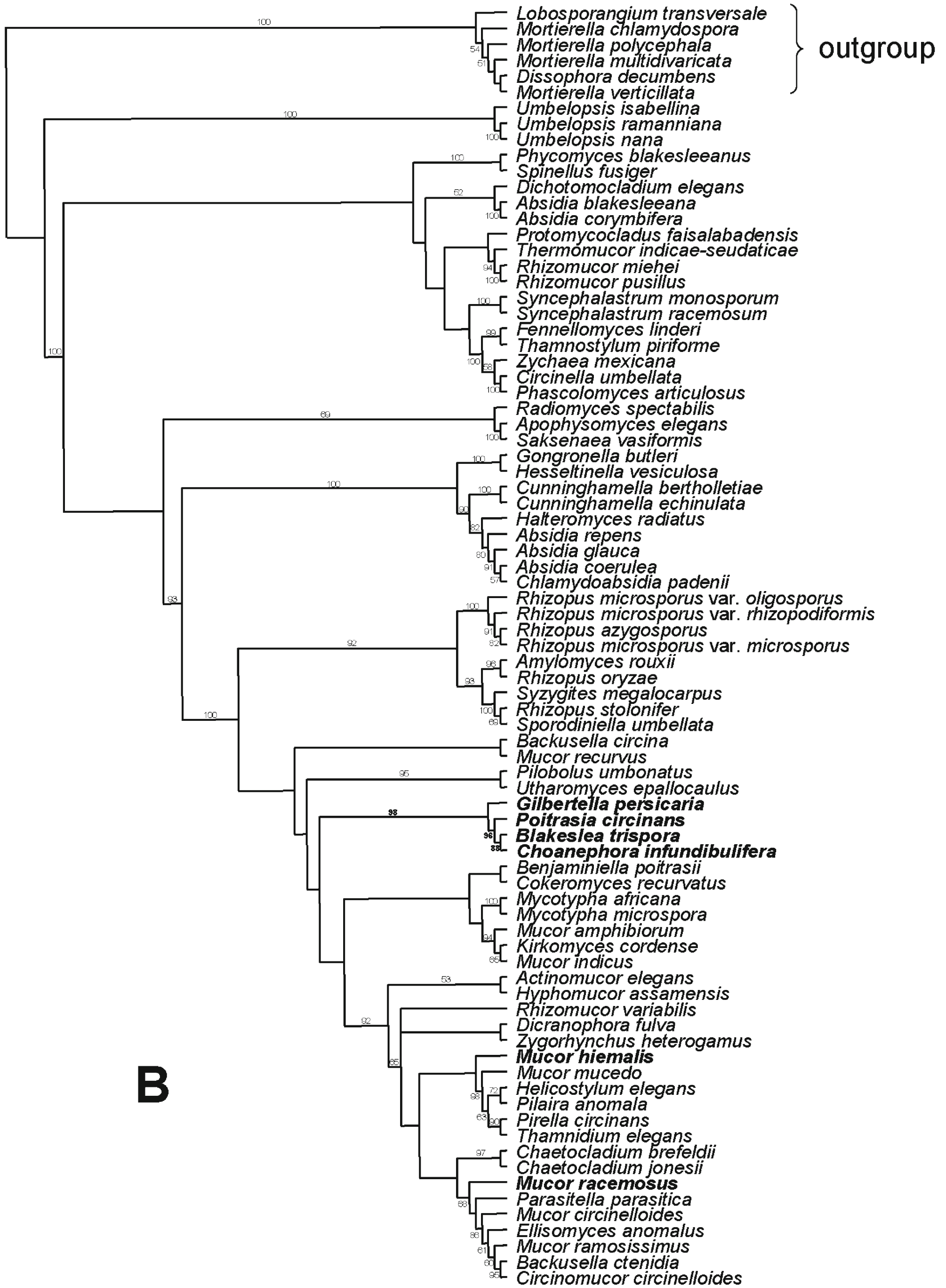
Fig. 1. Phylogenetic analyses of 4,151 aligned nucleotide characters of four genes encoding SSU (18S) rRNA (1,863 characters), the D1/D2 domain of the LSU (28S) rRNA (389 characters), actin (807 characters) and translation elongation factor 1 alpha (1,092 characters) from 75 taxa of the Mucorales and 6 taxa of the Mortierellales, which were used as outgroup taxa. Taxa in bold were subjected to phylogenetic single-gene analyses as shown in Fig. 2. **A:** Unweighted neighbor-joining analysis of Jukes-Cantor distances. Numbers above branches indicate bootstrap proportions > 50% as calculated by bootstrapping 1,000 replicates of neighbor-joining trees using Jukes-Cantor distances. **B:** Strict consensus tree of a weighted maximum-parsimony analysis. Numbers above branches indicate bootstrap proportions > 50% as calculated by bootstrapping 1,000 maximum parsimony replicates, each with 10 random order entry

Lobosporangium transversale
Mortierella polycephala
Mortierella chlamydospora
Mortierella multidivaricata
Dissophora decumbens
Mortierella verticillata
outgroup
Umbelopsis isabellina
Umbelopsis ramanniana
Umbelopsis nana
Phycomyces blakesleeanus
Spinellus fusiger
Radiomyces spectabilis
Apophysomyces elegans
Saksenaea vasiformis
Thermomucor indicae-seudaticae
Absidia blakesleeana
Absidia corymbifera
Circinella umbellata
Phascolomyces articulosus
Zychaea mexicana
Fennellomyces linderi
Thamnostylum piriforme
Rhizomucor miehei
Rhizomucor pusillus
Protomycocladus faisalabadensis
Dichotomocladium elegans
Syncephalastrum monosporum
Syncephalastrum racemosum
Gongronella butleri
Hesseltinella vesiculosa
Cunninghamella bertholletiae
Cunninghamella echinulata
Halteromyces radiatus
Absidia repens
Absidia coerulea
Absidia glauca
Chlamydoabsidia padenii
Backusella circina
Mucor recurvus
Pilobolus umbonatus
Utharomyces epallocaulus
Sporodiniella umbellata
Rhizopus stolonifer
Syzygites megalocarpus
Amylomyces rouxii
Rhizopus oryzae
Rhizopus microsporus var. oligosporus
Rhizopus microsporus var. microsporus
Rhizopus azygosporus
Rhizopus microsporus var. rhizopodiformis
Gilbertella persicaria
Poitrasia circinans
Blakeslea trispora
Choanephora infundibulifera
Mucor amphibiorum
Kirkomyces cordense
Mucor indicus
Benjaminiella poitrasii
Cokeromyces recurvatus
Mycotypha africana
Mycotypha microspora
Actinomucor elegans
Hyphomucor assamensis
Mucor racemosus
Parasitella parasitica
Mucor circinelloides
Ellisomyces anomalus
Mucor ramosissimus
Backusella ctenidia
Circinomucor circinelloides
Dicranophora fulva
Zygorhynchus heterogamus
Mucor hiemalis
Rhizomucor variabilis
Chaetocladium brefeldii
Chaetocladium jonesii
Mucor mucedo
Helicostylum elegans
Pilaira anomala
Pirella circinans
Thamnidium elegans
A
0.1 substitutions per site


Lobosporangium transversale
Mortierella chlamydospora
Mortierella polycephala
Mortierella multidivaricata
Dissophora decumbens
Mortierella verticillata
outgroup
Umbelopsis isabellina
Umbelopsis ramanniana
Umbelopsis nana
Phycomyces blakesleeanus
Spinellus fusiger
Dichotomocladium elegans
Absidia blakesleeana
Absidia corymbifera
Protomycocladus faisalabadensis
Thermomucor indicae-seudaticae
Rhizomucor miehei
Rhizomucor pusillus
Syncephalastrum monosporum
Syncephalastrum racemosum
Fennellomyces linderi
Thamnostylum piriforme
Zychaea mexicana
Circinella umbellata
Phascolomyces articulosus
Radiomyces spectabilis
Apophysomyces elegans
Saksenaea vasiformis
Gongronella butleri
Hesseltinella vesiculosa
Cunninghamella bertholletiae
Cunninghamella echinulata
Halteromyces radiatus
Absidia repens
Absidia glauca
Absidia coerulea
Chlamydoabsidia padenii
Rhizopus microsporus var. oligosporus
Rhizopus microsporus var. rhizopodiformis
Rhizopus azygosporus
Rhizopus microsporus var. microsporus
Amylomyces rouxii
Rhizopus oryzae
Syzygites megalocarpus
Rhizopus stolonifer
Sporodiniella umbellata
Backusella circina
Mucor recurvus
Pilobolus umbonatus
Utharomyces epallocaulus
Gilbertella persicaria
Poitrasia circinans
Blakeslea trispora
Choanephora infundibulifera
Benjaminiella poitrasii
Cokeromyces recurvatus
Mycotypha africana
Mycotypha microspora
Mucor amphibiorum
Kirkomyces cordense
Mucor indicus
Actinomucor elegans
Hyphomucor assamensis
Rhizomucor variabilis
Dicranophora fulva
Zygorhynchus heterogamus
Mucor hiemalis
Mucor mucedo
Helicostylum elegans
Pilaira anomala
Pirella circinans
Thamnidium elegans
Chaetocladium brefeldii
Chaetocladium jonesii
Mucor racemosus
Parasitella parasitica
Mucor circinelloides
Ellisomyces anomalus
Mucor ramosissimus
Backusella ctenidia
Circinomucor circinelloides
B

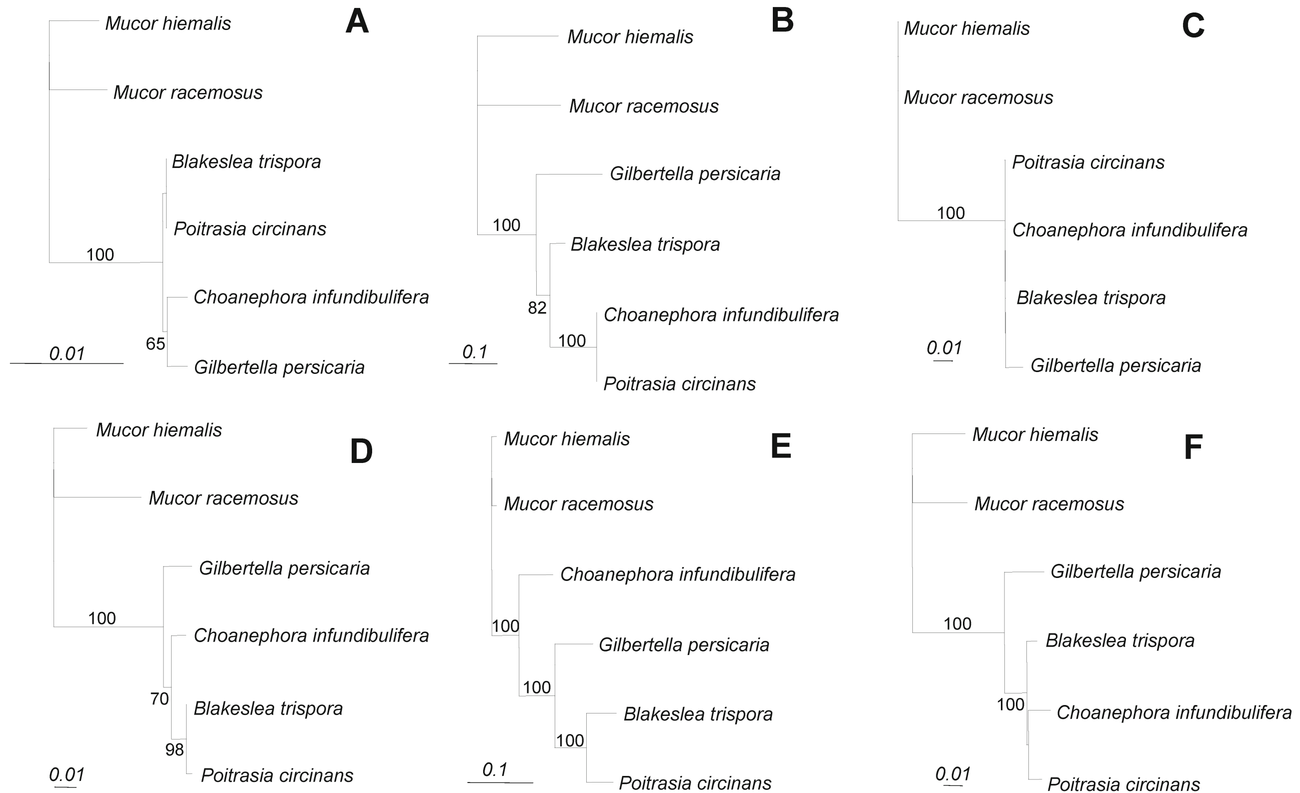


Fig. 2. Molecular phylogenies of the Choanephoraceae based on neighbor-joining analyses of single nuclear genes. **A:** 18S rDNA (1,863 characters), **B:** ITS 1 (232 characters), **C:** 5.8S rDNA (112 characters), **D:** D1/D2 domain of the 28S rDNA (374 characters), **E:** beta tubulin gene (*βtub*: 1,161 characters), **F:** translation elongation factor 1 alpha (*tef*: 1,095 characters). *Mucor hiemalis* and *M. racemosus* were defined as outgroup. Values above branches are bootstrap proportions >50% as calculated by bootstrapping 1,000 replicates of neighbor-joining trees using Jukes-Cantor distances. Scale bars indicate substitutions per site

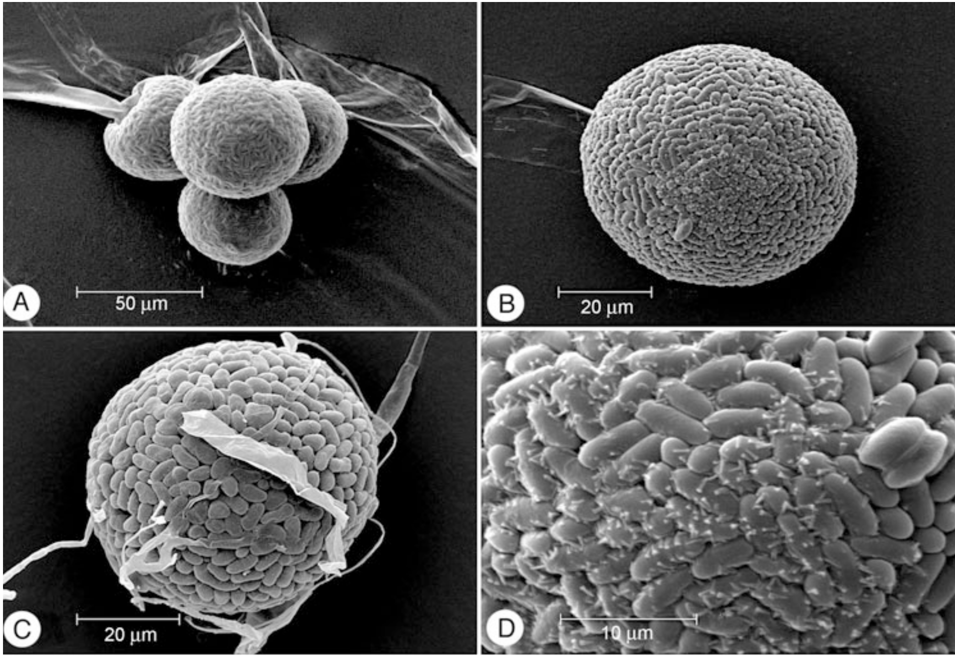


Fig. 3. Scanning electron microphotographs of sporangia (A–C) and sporangiospores (D) of *Mucor hiemalis*. Values on scale bars indicate magnification

Seventy-five species representing all 50 traditionally recognized genera of the Mucorales [17] are included as ingroup taxa. Six species of the genera *Mortierella*, *Lobosporangium* and *Dissophora*, representatives of the Mortierellales Cavalier-Smith 1998 were used as outgroup taxa. Equally weighted distance (Fig. 1A) and unequally weighted maximum parsimony phylogenetic analyses (Fig. 1B) both resulted in a clear dichotomy within the Mucorales clade with the Umbelopsidaceae Gams & Meyer 2003 grouping apart from the core Mucorales group, a phylogenetic relationship supported by a bootstrap proportion (BP) of 100%. Within the core Mucorales group another strongly supported intraordinal sub-clade (BP = 100%) can be distinguished consisting of 44 taxa including the genera *Backusella, Mucor, Pilobolus, Utharomyces, Sporodiniella, Rhizopus, Syzygites, Amylomyces, Gilber-*

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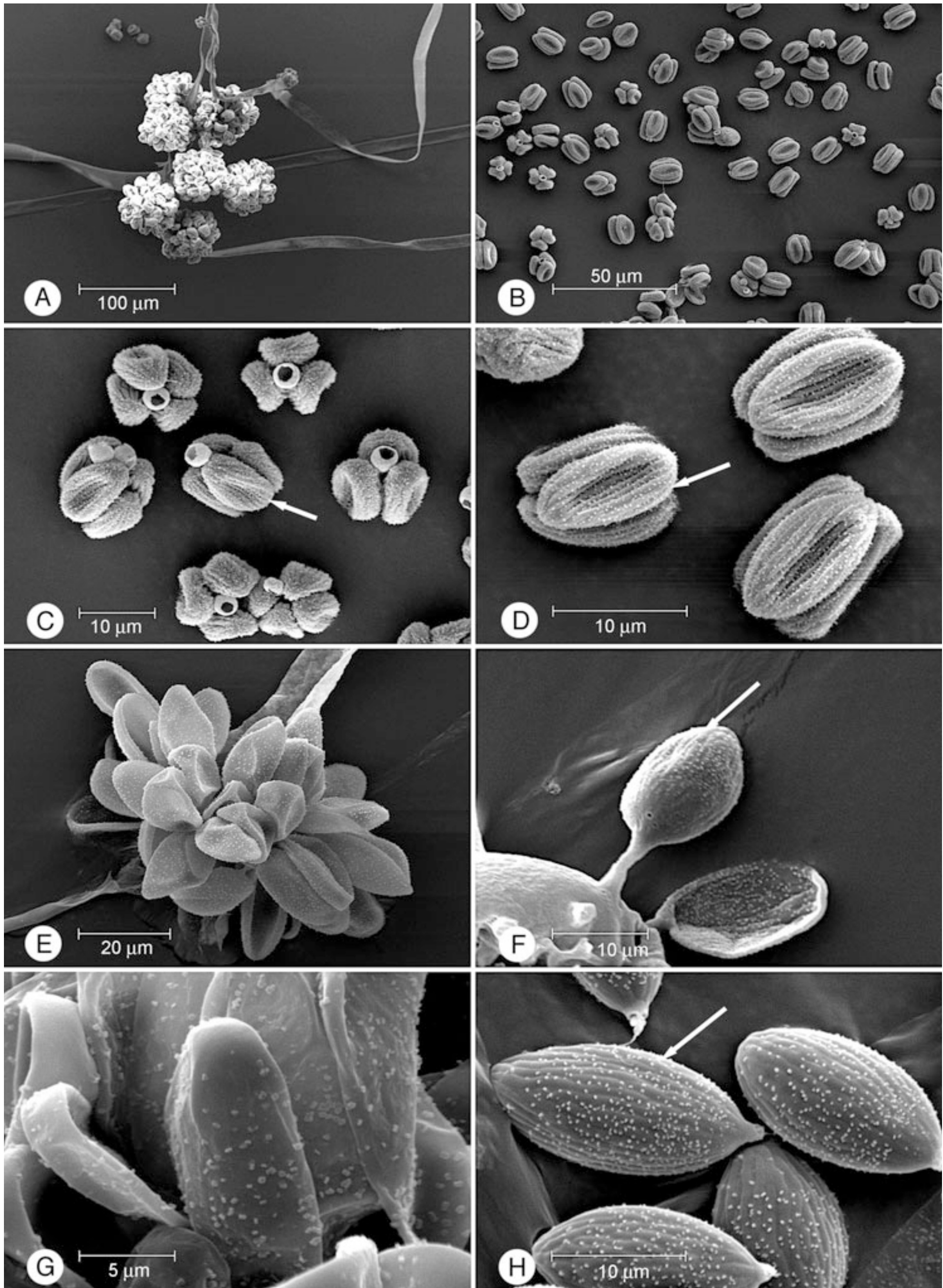
Fig. 4. Scanning electron microphotographs of sporangiola and spores of *Blakeslea trispora* (A–D) and *Choanephora infundibulifera* (E–H). Values on scale bars indicate magnification. Arrows indicate striated ornamentation on the spores. Sporangiola and sporangiospores of both species are covered with calcium oxalate crystals. **A–D:** sporangiola of *Blakeslea trispora*; **B–D:** triplicated and quadruplicated sporangiospore assemblages forming the 3 to 4-spored sporangiola typical for *Blakeslea trispora*; **E–F:** monosporous sporangiola of *Choanephora infundibulifera* on capitate vesicles at the apex of an erect sporophore; **G–H:** uni-spored sporangiola typical for *Choanephora infundibulifera*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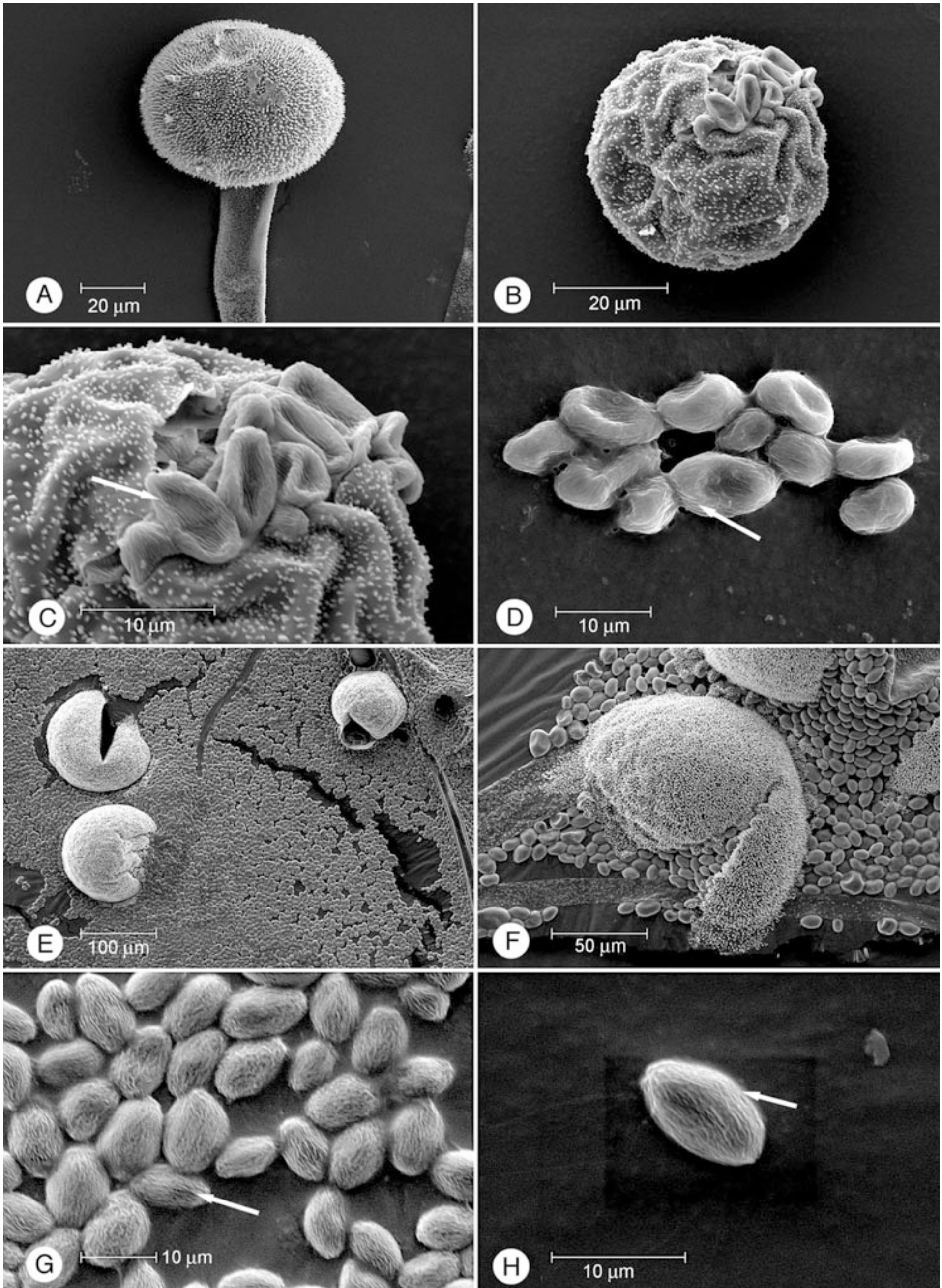
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H
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tella, Poitrasia, Blakeslea, Choanephora, Kirkomyces, Benjaminiella, Cokeromyces, Mycotypha, Actinomucor, Hyphomucor, Parasitella, Ellisomyces, Circinomucor, Dicranophora, Zygorhynchus, Rhizomucor, Chaetocladium, Helicostylum, Pilaira, Pirella and *Thamnidium*. With the exception of one species of *Rhizomucor*, *R. variabilis*, whose generic classification possibly requires revision, all genera appear exclusively within this clade. The Choanephoraceae represented by *Poitrasia circinans* (Naganishi & Kawakami 1955) P. M. Kirk 1984 (formerly: *Choanephora circinans* (Naganishi & Kawakami 1955) Hesseltine & Benjamin 1957), *Blakeslea trispora* Thaxter 1914 (formerly: *Choanephora trispora* (Thaxter 1914) Sinha 1940) and *Choanephora infundibulifera* (Currey 1873) Saccardo 1891 is the only multigeneric family that forms a monophyletic group. Its monophyly is supported by aberrant bootstrap proportions in unweighted distance (BP = 67% representing a low support; Fig. 1A) and in weighted maximum-parsimony analyses (BP = 96% representing a higher support; Fig. 1B). *Blakeslea trispora* is most closely related to *Choanephora infundibulifera* (BP = 100% in Fig. 1A and BP = 98% in Fig. 1B). *Gilbertella persicaria* (Eddy 1925) Hesseltine 1960, representing the monotypic and monogeneric family Gilbertellaceae [2], forms a well-supported monophyletic group together with the Choanephoraceae in both phylogenetic analyses (BP = 100% in Fig. 1A and BP = 98% in Fig. 1B).

Six single-locus data sets were subjected to distance analyses followed by complementary determination of bootstrap proportions (Fig. 2: A – 18S rDNA; B – ITS 1; C – 5.8 S rDNA; D – D1/D2 domain of the 28S rDNA; E – *βtub*; F – *tef*). Attempts to use the actin gene for single-locus phylogenetic analyses failed because of the lack of variable nucleotides that could serve as suitable apomorphies.

The Gilbertellaceae-Choanephoraceae clade is monophyletic with 100% bootstrap proportions in all six single-locus trees (Fig. 2A–F). Interestingly, *Gilbertella persicaria* forms a lineage basal to the Choanephoraceae *sensu stricto* only in the single-gene genealogies of ITS 1, D1/D2 of the 28S rDNA and *tef* (Fig. 2B, D, F). While *Poitrasia circinans* is most closely related to *Choanephora infundibulifera* (BP = 100%) in the ITS 1 phylogeny (Fig. 2B), it appears in a monophyletic group with *Blakeslea trispora* in the 28S rDNA tree (Fig. 2D) supporting a close phylogenetic relationship between *Choanephora*, *Blakeslea* and *Poitrasia*.

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Fig. 5. Scanning electron microphotographs of sporangia and sporangiospores from *Poitrasia circinans* (A–D) and *Gilbertella persicaria* (E–H). Values on scale bars indicate magnification. Arrows indicate striated ornamentation on the sporangiospores. Sporangia of both species are covered with calcium oxalate crystals, an apparent feature which has been frequently observed among *Gilbertella persicaria*, and during this study among the Choanephoraceae and thus, can be considered to pronounce the choanephoraceous nature of the sporangia. **A:** Sporangium typical for *Poitrasia circinans*. Sporangium and sporangiophore are covered with calcium oxalate deposits producing a hydrophobic coating, strenghtening the sporangiophore and keeping the sporangium upright; **B–C:** Setached multi-spored sporangia of *Poitrasia circinans* with longitudinal suture splitting the two sporangial halves apart to ease spore release; D: released sporangiospores of *Poitrasia circinans*; **E–F:** Squeezed multi-spored sporangia of *Gilbertella persicaria* bearing calcium oxalate crystals on their sporangial walls; G–H: released sporangiospores of *Gilbertella persicaria* with longitudinally striated ornamentation typical for the Choanephoraceae

The single-gene analysis of the 18S ribosomal DNA places *Choanephora infundibulifera* in a monophyletic group with *Gilbertella persicaria*, a relationship which is weakly supported (BP = 65%; Fig. 2A). The best resolution with maximum phylogenetic confidence is obtained using the beta tubulin gene in a single-locus phylogenetic analysis (Fig. 2E). *Choanephora infundibulifera* appears to be basal to *Gilbertella persicaria* (BP = 100%). These two taxa form a paraphyletic relationship to the monophyletic group containing *Blakeslea trispora* and *Poitrasia circinans* (BP = 100%).

Scanning electron microscopic analyses

In view of the apparent universality of spore striation and its possible significance as a diagnostic characteristic of the Choanephoraceae [1, 28, 43], the occurrence and extent of striations on the walls of sporangiospores produced by these fungi was investigated. In comparison with *Mucor*, the most prominent genus of the Mucoraceae, where typically multi-spored sporangia with smooth-walled, non-striated sporangiospores are observed (Fig. 3), the presence of striations on the walls of spores produced in sporangiola of *Blakeslea* (Fig. 4A–D) and *Choanephora* (Fig. 4E–F) and in sporangia of *Poitrasia* (Fig. 5A–D) unites the Choanephoraceae and should therefore be considered as a family characteristic of diagnostic importance. While *Blakeslea* and *Choanephora* produce both types, sporangiola and sporangia [9, 43], *Poitrasia* only forms sporangia, never sporangiola [16, 21]. The different spore types of sporangia and sporangiola in *Blakeslea* and *Choanephora* are morphologically similar [28]. Thaxter [36] separated *Choanephora* and *Blakeslea* on the basis of single-spored sporangiola in *Choanephora* and few-spored (3–6) sporangiola in *Blakeslea*. In *Blakeslea trispora* and *Choanephora infundibulifera* sporangiola are produced abundantly on stalks over the surface of terminal capitate swellings or vesicles of the sporangiophores (Fig. 4A, E). The sporangiolar type of *Choanephora infundibulifera* is truly monosporous (Fig. 4F) and similar, except for the number of spores developed in each, to the sporangiola of *Blakeslea trispora* (Fig. 4C). The sporangiola of *Blakeslea trispora* and *Choanephora infundibulifera* are rough-walled with longitudinal striations, uniform in size and not variable in shape, and usually ellipsoidal (Fig. 4D, H). The striations on sporangiola originate in the polar region adjacent to the connective apophysis towards the vesicles and spread over the sporangiola as longitudinal, rarely anastomising lines. On sporangiola of *Choanephora infundibulifera* the striations comprise regular, external ridges, some of which appear to taper slightly and end abruptly. *Poitrasia circinans* forms exclusively multi-spored sporangia with a persistent wall (Fig. 5A), which is characterized by at least one longitudinal suture apparently caused by internal pressure arising from the hydrophilic internal spore mass at sporangium maturity (Fig. 5B–C). Like *Poitrasia circinans*, *Gilbertella persicaria* also never forms sporangiola but rather produces multi-spored and pyriform collumellate sporangia with a single longitudinal suture, causing both sporangial halves to separate at maturity, which gives the sporangium the appearance

of an open clam shell [2, 6, 12]. Deposition of calcium oxalate crystals on the surface of the sporangial wall and the sporangiophore of *Gilbertella persicaria* (Fig. 5E–F) are believed to be the result of detoxifying the organism of calcium and oxalate ions and may function in spore dispersal by producing a hydrophobic coating [41]. Apart from *Gilbertella persicaria*, we also observed calcium oxalate crystal coating on the surface of the sporangial wall and sporangiophores of *Poitrasia circinans* (Fig. 5A–C) and on the surface of the sporangiola of *Blakeslea trispora* (Fig. 4C–D) and *Choanephora infundibulifera* (Fig. 4E–H). Calcium oxalate crystal deposits are found on the surface of a wide range of other mucoralean fungi [5, 37]. Comparison of the exclusively multi-spored sporangia-producing species *Poitrasia circinans* and *Gilbertella persicaria* reveals similarities in the ultrastructure of both the persistent sporangial wall and the sporangiospores (compare Figs 5A–D and 5E–H). The sporangiospores of *Poitrasia circinans* (Fig. 5C–D) and *Gilbertella persicaria* (Fig. 5G–H) possess clear striations and are truly choanephoraceous. Thus, striations were observed on the walls of all spore types produced by all of the species of the Choanephoraceae and the Gilbertellaceae investigated in this study and independent of mating type designations. Therefore, spore striation appears to be of diagnostic importance for both families and separates them ultra-micromorphologically from other mucoralean fungi.

DISCUSSION

The Mucorales comprises ubiquitous, terrestrial fungi that are taxonomically defined by development of a coenocytic mycelium, non-motile mitotic spores which are produced in single- to multi-spored sporangia. The sporangia are always associated with a more or less well-developed collumella, a bulbous vesicle of the sporangiophore apex, and zygospores, where observed, are formed following fusion of sexually compatible hyphae (zygophores). The traditional and current family-level classification schemes for the Mucorales appear to be artificial as evidenced by polyphyly of most of the families containing at least two genera, and require revision [25, 40].

The purpose of this study was the investigation of members of the Choanephoraceae against the background of a comprehensive selection of species comprising all genera of the Mucorales using molecular systematics and scanning electron microscopy to determine their closest phylogenetic relatives. Multi-locus phylogenetic analyses with complementary bootstrap assessment of phylogenetic confidence suggest that the Choanephoraceae, consisting of the genera *Blakeslea*, *Choanephora* and *Poitrasia*, forms a monophyletic group. All other families, including those thought to be well characterized and monophyletic – Chaetocladiaceae Benny&R. K. Benjamin 1993 (*Chaetocladium*, *Dichotomocladium*), Mycotyphaceae Benny& R. K. Benjamin 1985 (*Mycotypha*, *Benjaminiella*) and also the coprophilous Pilobolaceae Corda 1842 (genera *Pilobolus*, *Pilaira*, *Utharomyces*), which are characterized by a specialized active spore liberation mechanism, were resolved as polyphyletic.

During the last century the genus delimitation within the family Choanephoraceae was subject to constant changes. Apart from a generally recognized core group consisting of the genera *Choanephora*, *Blakeslea* and *Poitrasia* other genera were also classified in the Choanephoraceae due to an excessive overemphasis on the presence of sporangiola as representing asexual structures [32, 43]. None of these additional genera could be confirmed to be closely related to the Choanephoraceae in our phylogenetic analyses based on multi-gene genealogies. Chemotaxonomic considerations, mainly stressing the similarity of cell wall composition and the ratio between oleic and linoleic fatty acids in the lipid bilayer, led Feofilova et al. [10] to suggest that fungi of the genera *Blakeslea* and *Cunninghamella* are phylogenetically related and therefore *Cunninghamella* should also be included in the Choanephoraceae. Additionally, *Cokeromyces*, *Cunninghamella*, *Radiomyces* and *Mycotypha* differ strikingly from *Blakeslea* and *Choanephora* in how they propagate sexually. Zygosporogenesis occurs between short, relatively straight copulating branches as in *Mucor* leading to *Mucor*-type opposed suspensors and not between the tips of basally entwining branches of fertile hyphae as in *Blakeslea* or *Choanephora*, which was earlier believed to be the characteristic method of zygospore development in the Choanephoraceae [1, 15, 16, 28]. Genera forming *Mucor*-type zygospores, which are subspherical or short cylindrical, brown, thick-walled, with conical protuberances and swollen, equal-sized and opposed suspensors like *Gilbertella* were excluded from the Choanephoraceae [1, 16]. *Gilbertella* was believed to be an intermediate form between Choanephoraceae and Mucoraceae due to formation of sporangiospores typical for the Choanephoraceae and zygospores typical for the Mucoraceae [12], and was therefore placed in a separate family, the Gilbertellaceae Benny, 1991 [2]. However, results of the phylogeny reconstructed by O'Donnell et al. [25] indicate that the transformation from opposed to apposed zygospore suspensors is homoplasious, having occurred independently at least three times within the Mucorales. Thus, zygospore architecture seems not relevant for segregating *Gilbertella persicaria* from the other Choanephoraceae into a distinct family.

In contradiction to the generally accepted opinion we conclude that the type of zygosporogenesis [28, 32] and details of zygospore morphology [15, 16] are important at generic but not at family level. This argument can be substantiated by the mating and sexual compatibility experiments of Hesseltine and Benjamin [13], who obtained nearly perfect sexual reactions with smooth-walled zygospores on apposed suspensors between *Poitrasia* and *Choanephora* and between *Blakeslea* and *Choanephora*. *Gilbertella persicaria* produces rough-walled zygospores with appendages between opposed suspensors after perfect sexual hybridization with *Blakeslea trispora* but no zygospores with members of the Mucoraceae like *Rhizopus* and *Mucor* [12, 24]. According to Schipper [30, 31] successful mating is a parameter for the synonymous taxonomic classification of the mating partners, substantiating our finding that the Gilbertellaceae are more closely related to the Choanephoraceae *sensu stricto* than to the Mucoraeceae. Our molecular phylogenetic analyses using multi-gene approaches combining four nuclear genes with complementary assessment of high bootstrap proportion support our hypothesis that the

monogeneric Gilbertellaceae with its monotypic species *Gilbertella persicaria* should be included in the Choanephoraceae. This interpretation differs from that of Papp et al. [27] who did phylogenetic reconstructions based on nuclear 5.8.S ribosomal DNA sequences. Therefore, we conducted a variety of single-locus phylogenetic analyses with complementary bootstrap assessments. The results give strong support for uniting the Gilbertellaceae and the Choanephoraceae. We performed scanning electron microscopical investigations to look for ultrastructural criteria, which could serve as distinctive synapomorphies. Evidence was found for the choanephoraceous nature of the sporangiospores of *Gilbertella persicaria*. Our scanning electron microscopic observations demonstrate that the sporangiospores of *Blakeslea*, *Choanephora*, *Poitrasia* and *Gilbertella* all possess an ellipsoidal shape and a clear longitudinal striation. Kirk [16] considered the presence of striations on *Choanephora* spores as too variable to be of any taxonomic value, but it is likely that the ornaments were below the limit of resolution and therefore the spores appear to be smooth. In our opinion, the nature of asexual structures and spores including their ultrastructural morphology are important criteria for the classification at the family level. Once the technical presumptions were established, the presence of striations on the walls of spores of the Choanephoraceae is easy to determine, represents a stable morphological criterion and should be considered as a family characteristic of diagnostic importance, a view shared by Poitras [28]. Numerous authors reported the formation of apical clusters of hyaline cilium-like setae on the polar ends of the sporangiospores for all four genera [1, 2, 12, 23, 43]. Many of these fungi are plant parasites and the spore appendages may aid insects in dispersing spores from flower to flower [3]. These hair-like appendages were described as a typical and unique synapomorphy for the Choanephoraceae *sensu stricto* and the Gilbertellaceae within the Mucorales, providing a further argument for the reduction to one family, the Choanephoraceae *sensu lato*.

The morphology of the multi-spored sporangia also appears to unite the Choanephoraceae with the Gilbertellaceae. The multi-spored sporangia of *Blakeslea*, *Choanephora*, *Poitrasia* and *Gilbertella*, are associated with well-developed pyriform collumellae [2, 13, 43]. The sporangial walls were frequently observed to be covered with calcium oxalate crystals [2, 3]. The distribution and type of calcium oxalate deposits on sporangia and sporangiola may aid in propagule dispersal of the plant parasites [3]. The calcium oxalate deposits produce a hydrophobic coating, which strengthens the sporangiophore, keeps the sporangia upright and eases spore release [41, 42]. This hydrophobic coat may guarantee the internal pressure of the hydrophilic spore mass, which is necessary to split each sporangia into two nearly equal halves along a longitudinal suture in the sporangial wall. While *Gilbertella persicaria* forms a single suture [2], *Poitrasia circinans* can produce more than one circumscisile suture at maturity of the persistent sporangia [16]. Von Arx [1] considered the criterion of the sporangial wall splitting into halves as specific for the family Choanephoraceae, a criterion which can be extended to the Choanephoraceae *sensu lato* including all species designated to the genera *Blakeslea*, *Choanephora*, *Poitrasia* and *Gilbertella*.

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