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The family structure of the Mucorales: a synoptic revision based on comprehensive multigene-genealogies

K. Hoffmann1,2, J. Pawlowska3, G. Walther1,2,4, M. Wrzosek3, G.S. de Hoog4, G.L. Benny5*, P.M. Kirk6*, K. Voigt1,2*

Key words
Mucorales families phylogeny

Abstract
The Mucorales (Mucoromycotina) are one of the most ancient groups of fungi comprising ubiquitous, mostly saprotrophic organisms. The first comprehensive molecular studies 11 yr ago revealed the traditional classification scheme, mainly based on morphology, as highly artificial. Since then only single clades have been investigated in detail but a robust classification of the higher levels based on DNA data has not been published yet. Therefore we provide a classification based on a phylogenetic analysis of four molecular markers including the large and the small subunit of the ribosomal DNA, the partial actin gene and the partial gene for the translation elongation factor 1-alpha. The dataset comprises 201 isolates in 103 species and represents about one half of the currently accepted species in this order. Previous family concepts are reviewed and the family structure inferred from the multilocus phylogeny is introduced and discussed. Main differences between the current classification and preceding concepts affects the existing families Lichtheimiaceae and Cunninghamellaceae, as well as the genera Backusella and Lentamyces which recently obtained the status of families along with the Rhizopodaceae comprising Rhizopus, Sporodinia and Syzygites. Compensatory base change analyses in the Lichtheimiaceae confirmed the lower level classification of Lichtheimia and Rhizomucor while genera such as Circinella or Syncephalastrum completely lacked compensatory base changes.

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INTRODUCTION
The fungal order Mucorales – evolutionary position and characterisation

As a member of the Mucoromycotina, the Mucorales belong to the early diverging, ancient fungi along with the Kickxellomycotina, Zoopagomycotina, Entomophthoromycotina, Mortieliomycotina, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, and Cryptomycota and Microsporidia with the latter two still highly discussed (Schüßler et al. 2001, James 2001a, b, Benny 2012). The Entomophthoromycotina were later elevated to the phylum Entomophthoromycota (Humber 2012). Mucorales are characterised by a usually abundant, rapidly growing mycelium as well as anamorph structures usually formed in large quantities. The mycelium is typically unseptate or irregularly septate. Anamorphic sporangiospores are produced in multi-spored sporangia, few-spored sporangiola or merosporangia. Chlamydospores, arthrospores and yeast cells are, in most species, rarely formed. Sporangia are characterised by the inclusion of a variously shaped columella. This well-developed columella counts as a synapomorphic character for the Mucorales. Conjugation in homothallic species or between compatible mating types of heterothallic species results in the formation of zygospores. Zygospores often display a specific exospore ornamentation (smooth, rough, warty) and protecting appendages (finger-like, antler-like) born on the supporting cells (suspensors) (Zycha et al. 1969). Some species of the Mucorales exhibit dimorphism, possessing the ability to switch between a filamentous, multi-cellular state to a yeast-like state (Bartnicki-Garcia & Nickerson 1962).

Life styles and applications — Fig. 1
Mucoralean fungi are ubiquitous, predominantly saprobioc soil organisms on decaying organic material but parasites of plants, fungi and animals also are known. As one of the largest orders in the basal Fungi, the Mucorales is also one of the most studied groups in the early diverging fungi. These studies on mucoralean fungi encompass physiology and biochemistry, as well as taxonomy and systematics, and potential applications in industry. In general, mucoralean fungi reproduce anamorphically via non-motile sporangiospores released from variously shaped sporangia. If not homothallic, a compatible mating partner is needed for the formation of the zygospore, where meiosis occurs. The different sexual modality of either homo- or heterothallic in the Mucorales was discovered more than 100 yr ago, with most species found to be heterothallic (Blakeslee 1904). Volatiles are responsible for the formation of sexual reproductive structures (Burgeff 1924). These volatiles were identified as trisporoids, derivatives of beta-carotene (van den Ende 1967, Gooday 1968). The trisporic acid precursors are mutually processed by the compatible mating partners, resulting in the formation of a mature zygospore (Werkman 1976). Although the composition of the compounds is species specific to allow only intra-species matings (Sutter et al. 1989), inter-species zygospores are also described with some impact on systematics (Blakeslee & Cartledge 1927).
Stalpers & Schipper 1980). Combining an order-wide trisporoid profiling with the current knowledge on phylogenetic relationships would most likely reveal the 'languages' of the different clades and their potentials for interspecific mutual recognition. But currently, only profiles for few species are known: e.g., *Phycomyces blakesleeanus* (Miller & Sutter 1984) and *Blakeslea trispora* (Caglioti et al. 1966).

Although a general trisporic acid biosynthesis pathway (Schachtenschabel et al. 2005) is widely accepted, the genetic background is resolved only in parts. The synthesis and degradation of beta-carotene is well studied and understood (Almeida & Cerdá-Olmedo 2008, Polaino et al. 2010, Tagua et al. 2012) but most enzymes responsible for trisporic acid production remain undiscovered. So far, only 4-dihydro-methyltrisporate...
dehydrogenase and 4-dihydrotrisporin dehydrogenase are verified (Czempinski et al. 1996, Wetzel et al. 2009). Since an interaction of compatible mating types is essential for mature zygospores to be produced, the information for the mating type is probably genetically coded. The appropriate regions were identified first in Phycocymes blakesleeanus (Idnurm et al. 2008) and subsequently discovered in Rhizopus delemar, R. oryzae (Gryganskyi et al. 2010), Mucor cirenceloides (Lee et al. 2010) and even in a homothallic species, Syzygites megalocaropus (Idnurm 2011). Although heterothallic strains possess only one gene coding for either plus or minus mating type, the phenomenon of rare switches between mating types (Schipper & Stalpers 1980) is not yet explained.

The importance of zygospores for reproduction and distribution compared to the asexual sporangiospores is still unknown, since germination in the natural habitat could not be observed and germination under laboratory conditions has only been described and illustrated for few species (Michailides & Spotts 1988, Yu & Ko 1997). Nevertheless, zygomyces are reported from the fossil records. The earliest zygomycotan fossil known, exclusive of the Glomeromycota, may be Jhimwitea circumtecta, possible Endogonaceae, from the middle Triassic (Krings et al. 2012). Many fossil zygomyces have been found in the Carboniferous and later, including Protoascon missouriensis and others (Taylor et al. 2005, Kar et al. 2010). Calculations of the diverging time of zygomyces using molecular data suggest an origin of around 600 mya (Berbee & Taylor 2001).

The zygomyces are known to be useful for a variety of different applications, including food and food additive production and food preservation. Zygomyces are used as starter cultures for the fermentation of soybean- or rice-based products in Asia, Africa and South America, e.g. beverages, or the well-known tempeh (Henkel 2004, 2005, Hesseltine 1983, 1991, Nout & Kiers 2005, Tamang & Thapa 2006).

Mucorales also are used for diverse biological transformations (Gladkowski et al. 2004, 2011) as well as the production of additives for food, feed, pharmaceuticals (like lycopene) or various applications of chitosan (reviewed by Shahidi et al. 1999), a cell wall component only known to be produced by Mucorales. Yet, Mucorales also are reported as spoilage agents in stored cereals and other food, especially fruits and vegetables (Martin 1964, Wade & Morris 1982, Ray & Ravi 2005). In addition, some organisms also infect living plants, especially the fruits (e.g., strawberry, yellow summer squash or green beans; Fig. 2c, d) (Dennis 1983). Thus, these fungi play an important role as plant pathogens as well (Shlienber 1997). Furthermore, some species of the Mucorales are facultative parasites of other fungi. They can be biotrophic or necrotrophic parasites with a few species (Syzygites megalocaropus, Dicranophora fulva, Spinellus fusiger) able to infect the fruit body of agarics (Fig. 2a; Zycha et al. 1969), a feature that is thus far not well studied. However, well studied is the biotrophic fusion parasitism (Fig. 2b) between Absidia glauca and Parasitella parasitica, a model system for studies of horizontal gene transfer and the link between sexual and parasitic interactions (Burgeff 1924, Kellner et al. 1993, Schultz et al. 2005). Trisporic acid and its precursors are also believed to be responsible for recognition of potential hosts for Chaetocladium (another parasite) and Parasitella, which was assumed from an observed mating-type dependent infection (Burgeff 1924, Schultz et al. 2005). Yet, a strict mating-type dependency was rejected as early as 1926 by a mere tendency which, in addition, seems to be restricted to only few species (Satina & Blakeslee 1926). Currently, an order-wide comprehensive survey of host-ranges for all known biotrophic fusion parasites is lacking. A recent investigation revealed an unstudied mycoparasite, Lentamyes parricida, as the most basal with the highest mycoparasitic potential to infect other mucoralean hosts (Hoffmann & Voigt 2009).

Mucoralean fungi are also known as human and animal pathogens. Mucor corymbifer (currently Lichtheimia corymbifera) was first reported as causative agent of mycosis in a rabbit (Platauf 1885). In the last decades, the reported number of infections caused by members of the Mucorales (mucormycoses) has constantly increased. This is probably due to a rising awareness, an improved identification by the use of molecular methods, as well as a permanent worldwide increase of risk factors such as immunosuppression, malignancies and diabetes (Rodan et al. 2005, Skiada et al. 2011).

The symptoms of infections by Mucorales remain unspecific for a long time, making a diagnosis extremely difficult. A fast, proper and effective therapy is required, since these infections can result in death within hours to a few days. Survival rates for mucormycosis are highly dependent on the location of the infection, but they are very low overall at 53 % (Skiada et al. 2011). The large and still increasing numbers of studies pertaining to the susceptibility of Mucorales to known and new fungicides indicate a pressing need for an effective therapy. And with the discovery of species-specific susceptibility profiles, it became obvious, that the causative agents should be identified.


<table>
<thead>
<tr>
<th>Family</th>
<th>Main characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaetocladiaceae</td>
<td>Unisporid sporangia formed on fertile vesicles, discoid columella, dichotomous branched fertile hyphae, sterile spines, chlamydospores absent, zygospores rough-walled, suspensors opposed</td>
</tr>
<tr>
<td>Choanephoraceae</td>
<td>Sporangia and sporangiolia, on different sporangioles, zygospores stiate, suspensors opposed or tongs-like</td>
</tr>
<tr>
<td>Cunninghamellaceae</td>
<td>(Fig. 3a), unisporid sporangia, sporangia absent, zygospores warty, suspensors opposed</td>
</tr>
<tr>
<td>Gilbertelaceae</td>
<td>Sporangiospores appendaged; zygospores rough-walled, suspensors opposed</td>
</tr>
<tr>
<td>Mucoraceae</td>
<td>Sporangia present, specialized sporangia absent, zygospores smooth to warty, variously shaped suspensors: opposed, naked, appendaged, polyphlytic</td>
</tr>
<tr>
<td>Mycotyphaceae</td>
<td>Sporangia on pedicels (Fig. 4k)</td>
</tr>
<tr>
<td>Phycocymeceaceae</td>
<td>Sporangioles large and unbranched, zygospores with coiled tongs-like suspensors and branched appendages</td>
</tr>
<tr>
<td>Pilobolaceae</td>
<td>Spores are actively liberated, zygospores smooth, suspensors tongs-like or appendaged (Fig. 5)</td>
</tr>
<tr>
<td>Radiomycesaceae</td>
<td>Sporangia absent, sporophores with a primary vesicle bearing secondary ampullae, sporangiolia originating from ampullae, zygospores smooth, suspensors opposed, appendaged</td>
</tr>
<tr>
<td>Saksenaceae</td>
<td>Provisionally classified together with Lobosporangium (currently Mortierellomycofrina) because of the unusual-shaped sporangia</td>
</tr>
<tr>
<td>Syncephalastraceae</td>
<td>Merosporangia, zygospores warty, suspensors opposed (Fig. 4g)</td>
</tr>
<tr>
<td>Tomanidaceae</td>
<td>Sporangia present, sporangia absent or apically on the sporangioles, zygospores warty, suspensors opposed (Fig. 4n, o)</td>
</tr>
</tbody>
</table>
correctly to species level (e.g., Vitale et al. 2012). To investigate and to understand mucormycoses, their susceptibility and their evolutionary relationships need to be comparatively investigated. Understanding evolutionary relationships will elucidate approaches to improve existing or to invent new applications in industry, agriculture or medicine.

**Morphology-based families**

Traditionally, Mucorales were classified using their observable characters, for example physiology, biochemistry and, especially, morphology (Table 1). Unfortunately, Mucorales display only a small number of distinguishable morphological characters and only a few of them have proven to be useful for distinction between species, genera and families. Nevertheless, in early mucoralean systematics, clustering of morphologically similar species resulted in well-defined genera and families accepted before the implementation of molecular data in phylogenetic reconstruction (Table 2).

**Delimitations of morphology**

Traditional approaches used to classify fungi – fossil records, biochemistry and, especially, morphology (e.g., Paterson & Bridge 1994, Benny 1995, Hawksworth et al. 1995) became less important following the emergence of molecular systematics (White et al. 1990). Applying molecular data to phylogenetic analyses has led to the breakdown of the former phylum Zygomycota, combined by the morphological feature ‘zygospore’ into the subphyla Mucoromycotina, Kickxellomycotina, Zoopagomycotina and Entomothrophomycotina (James et al. 2006, Hibbett et al. 2007).

The family structure of the Mucorales is still rather unstable, but with the discovery of new, potentially phylogenetic informative characters (molecular data) and with the availability of higher resolution microscopy (e.g., fluorescence, SEM, TEM) it becomes feasible to reveal smaller, presumably monophyletic clades.

The most significant changes have affected the Thamnidiales, Mucoraceae, Chaetocladiaceae and Absidiales. The first molecular studies addressing the entire order (O’Donnell et al. 2001, Voigt & Wöstemeyer 2001) showed that species traditionally assigned to Thamnidiales and Mucoraceae were scattered over the entire order. A widely accepted classification predominantly based on morphological traits was published by Benny et al. (2001) and is summarised with the molecular studies in Table 3.

Over the following years, several species and genera were studied in more detail, re-evaluated and revised (for a complete list see Walther et al. 2013 in this issue of Persoonia). In the following only studies that influenced family concepts by the dissection of the genus, the exclusion of a genus from a family or the fusion of families are addressed.

**Absidia, Lichtheimia and Lentamycyes**

The genus Absidia was originally defined by its pyriform, apophysate sporangia (Fig. 3b, c, 4h–j). The first phylogenetic analyses (O’Donnell et al. 2001, Voigt & Wöstemeyer 2001) revealed a paraphyletic origin of this genus, a separation was accomplished later. Mesophilic species were retained in the genus Absidia (Cunninghamellaceae), whereas thermotolerant species form a separate phylogenetic clade as genus Lichtheimia (Hoffmann et al. 2007, 2009). In addition to the thermotolerant species separated from Absidia, potential mycoparasitic species were also distinguished in a new genus, Lentamycyes (Hoffmann & Voigt 2009). This genus harbours two species, L. parricida and L. zycheae. At the same time, two new species were isolated from nature and described as Siepmannia lariceti and S. pineti (Kwaśna & Nirenberg 2008a, b). This genus also was supposed to include both species of Lentamycyes. Since molecular data for Siepmannia includes only ITS sequences, with no living material accessible, the relationship between the two genera remains unclear.

**Choanephora and Gilbertella**

Although there are morphological differences in zygosporogenesis in the Gilbertellaceae and the Choanephoraceae, a molecular study combined with ultrastructure supported merging these two families, under the older name, Choanephoraceae (Voigt & Olsson 2008).

**Pilaira**

Due to morphological similarities, this genus was placed traditionally within the Pilobolaceae together with Pilobolus and Uthamycyes. But molecular data (O’Donnell et al. 2001, Voigt & Wöstemeyer 2001) revealed a non-relationship of Pilaira to both other genera, followed by an assignment to the Mucoraceae as published in Index Fungorum. This classification was also suggested on the base of a comprehensive molecular study of the Pilobolaceae (Foos et al. 2011).

### Table 3  Summary of the family structure of the Mucorales based predominantly on morphology (Benny et al. 2001) as well as on combination with molecular data (O’Donnell et al. 2001, Voigt & Wöstemeyer 2001).

<table>
<thead>
<tr>
<th>Family</th>
<th>Genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaetocladiaceae</td>
<td>Chaetocladium, Dichotomocladium</td>
</tr>
<tr>
<td>Choanephoraceae</td>
<td>Blakelea, Choanephora, Potrasia</td>
</tr>
<tr>
<td>Cunninghamellaceae</td>
<td>Cunninghamella</td>
</tr>
<tr>
<td>Gilbertaceae</td>
<td>Gilbertella</td>
</tr>
<tr>
<td>Mortierellaceae</td>
<td>Aquamortierella, Dissophora, Echinusporangium, Modicella, Mortierella, Umbelopsis</td>
</tr>
<tr>
<td>Mucoraceae</td>
<td>Absidia, Actinomucor, Apophysomyces, Chlamydoabsidia, Cincinella, Cincinomucor, Dicranophora, Gongronella, Halteromyces, Hyphomucor, Micromucor, Mucor, Mycoclados, Parasitella, Protoxycomoclados, Rhizomucor, Rhizopodopalis, Rhizopus, Spinellus, Sporodiniella, Syzygites, Thermomucor, Zygorhynchus</td>
</tr>
<tr>
<td>Mycotyphaceae</td>
<td>Benjaminiella, Mycotypha</td>
</tr>
<tr>
<td>Phycomycetaceae</td>
<td>Phycomyces</td>
</tr>
<tr>
<td>Pilobolaceae</td>
<td>Pilaira, Pilobolus, Uthamycyes</td>
</tr>
<tr>
<td>Radiomyctaceae</td>
<td>Hesselinella, Radiomyces</td>
</tr>
<tr>
<td>Saksenaceae</td>
<td>Saksenaea</td>
</tr>
<tr>
<td>Syncyphalaceae</td>
<td>Syncephylstrom</td>
</tr>
<tr>
<td>Thamnidiaceae</td>
<td>Backusella, Cokeromyces, Ellisomyces, Fennellomyces, Helicostylum, Kirkomyces, Phascolomyces, Pirella, Thamnidium, Thannostylum, Zychaeae</td>
</tr>
</tbody>
</table>
Molecular systematics and implications on Mucorales

Molecular systematics is rapidly developing. Taxon samplings, possibilities to combine data and the number of applicable analytical tools are constantly increasing. In addition, with the ability to sequence whole genomes at relatively moderately cost combined with appropriate annotation software, computing capability and open access, genome-wide phylogeny comes within reach (Fitzpatrick et al. 2006, Kuramae et al. 2006, Huerta-Cepas et al. 2008). However, as only a few mucoralean fungi are fully sequenced, elucidating the phylogenetic relationships within this order is usually based on single genes or the combination of a few genes. Currently (April 2012), 24 genome/trancriptome projects for Mucorales are listed in the JGI Genome Online Database (GOLD; Fig. 6), but this includes only four different taxa (Mucor circinelloides, Rhizopus oryzae, Rhizopus stolonifer (each one project), and Phycomyces blakesleeanus (21 projects).

There are currently more than 6 000 sequences of zygomycota deposited in GenBank, approximately one-third of these are protein coding sequences. This is the third largest fraction for basal fungi, but still far behind the derived fungi, the Dikarya (Ascomycota and Basidiomycota; Fig. 6). Molecular data for the Mucorales have been submitted to GenBank since 1993, with a constantly increasing number, reaching more than 1 000 sequences in 2010 and more than 1 400 last year (Fig. 7). Nevertheless, the submitted sequences are restricted to only a few genera and species, with half of the sequences from the two genera Mucor and Rhizopus (Table 4). Around 50 species for Mucor and nine species for Rhizopus are listed in the 10th edition of the Dictionary of the Fungi (Kirk et al. 2008) which is 24 % and 4 %, respectively, of all species accepted in the Mucorales.

Studies predominately concerned with molecular phylogenetic aspects of zygomycetes, especially Mucorales, are still relatively rare. Searching NCBI and the ISI Web of Science with ‘zygomycetes or Mucorales AND phylogeny’ resulted only in between 40 and 50 analyses including 15 studies where at least 2 loci were applied (April 2012, Fig. 8).

Commonly applied markers for phylogeny are sequences coding for rDNA (especially 18S rDNA for relationship levels of families, orders and above-order as well as ITS1 & 2 for relationships of species and genera). Therefore, the majority of studies are using rDNA sequences for phylogenetic approaches although ITS sequences represent the largest fraction of sequences in GenBank (Fig. 9). Protein coding genes predominantly applied so far are actin and translation elongation factor 1-alpha. Establishment of alternative protein coding markers for the whole order remains difficult. Whereas largest and second-largest subunit of RNA polymerase II (RPB1 & 2), ATPase subunit 6 (ATP6), a DNA replication licensing factor (MCM7), a gene required for rRNA accumulation (TSR1) or cytochrome c oxidase I (COX1) proved to be suitable for other fungal groups mostly belonging to the Basidiomycota and Ascomycota (Matheny et al. 2002, Reeb et al. 2004, Seifert et al. 2007, Schmitt et al. 2009), these genes have not be successfully amplified for a broad range of Mucorales and are still under represented in GenBank (Schoch et al. 2012).
The present study focuses on the family structure of the *Mucorales*. Family boundaries are inferred from a molecular phylogeny based on four markers and including 201 isolates and all currently accepted genera. Historical approaches and changes in recent years are revised, the support of the families by the current data is discussed and the families are characterised morphologically and ecologically. The resulting changes on the higher level nomenclature of the *Mucorales* were already briefly introduced by Voigt (2012). In order to ensure that these changes were based on a stable lower level taxonomy the
internal transcribed spacer 2 region (ITS2) was analysed for compensatory base changes (CBCs) as indicators for species boundaries (Müller et al. 2007).

MATERIAL AND METHODS

Strains, DNA isolation, PCR
Strains used for the generation of additional sequences (bold accession numbers in Table 5) were cultivated on 3 % malt extract medium at room temperature. Genomic DNA was extracted as described in Hoffmann et al. (2007). For phylogenetic analyses, sequences of large (LSU) and small (SSU) subunit of ribosomal DNA, ITS (internal transcribed spacer 1 & 2, incl. 5.8 SrDNA), actin (act) and translation elongation factor 1-alpha (tef) were either generated in this study or retrieved from GenBank (www.ncbi.nlm.nih.gov/; Table 5). Primers used for the amplification of LSU were NL1 and NL4 (O’Donnell 1993), NS1 and NS4 for SSU (White et al. 1990), ITS1 and ITS4 for...
ITS (White et al. 1990), Act1/Act1b and Act4R/Act4Ra for actin (Voigt & Wöstemeier 2000) and MEF1 and MEF4/UEF4 for Tef (O’Donnell et al. 2001). PCR fragments were purified using the protocol of Vogelstein & Gillespie (1979) and sequenced on an Applied Biosystems 3730xL DNA Analyzer (ABI, Carlsbad) according to the manufacturer’s instructions.

**Sequence alignment, phylogeny, distance matrices, CBC**

Multiple sequence alignments were generated using MAFFT v. 6.901b (server: mafft.cbrc.jp/alignment/server/) or v. 6.822 as implemented at the CIPRES portal ([//www.phylo.org/; Miller et al. 2010]). Alignments comprised 20T taxa and 1 586 characters for 18S rDNA, 358 characters for 28S rDNA, 807 characters for 100000 and 1 092 characters for translation elongation factor 1-alpha. Phylogenetic trees were calculated using RAxML v. 7.3.0 and MrBayes v. 3.1.2 from the CIPRES portal under the default settings with the following adjustments: RAxML was run choosing rapid bootstrapping (GTRCAT) and GTRGAMMA for final tree inference with 1 000 bootstrap iterations. Bayesian inference was run setting the number of substitution types to 6 (GTR), with among-site rate variation set to invgamma. Analysis was run with four chains each in two runs for 5 million generations. 5 001 trees were sampled, and 2 501 trees were analysed discarding the first 50 % of the samples as burnin.

Distances are expressed as substitutions per 100 bases or amino acids. CBC analyses were done as described previously (Pawłowska et al. In press).

Distances are given (data retrieved April 2012). Also the total number of available sequences for all basal fungal lineages are indicated by different colours (data retrieved April 2012).

**Fig. 7** Chronology of sequences submitted to GenBank since 1993 for the Mucorales (data retrieved April 2012).

**Fig. 8** Number of publications predominantly focused on mucoralean phylogeny retrieved from NCBI and ISI Web of Science by searching ‘Zygomycota/ Mucorales AND phylogeny’. Publications are separated by the molecular marker applied for phylogeny. Nearly half of all published studies included more than one molecular marker. Published combinations of molecular markers are indicated by different colours (data retrieved April 2012).

**Fig. 9** Distribution of available sequences in GenBank for the Mucoromycota. Also the total number of available sequences for all basal fungal lineages are given (data retrieved April 2012).

**Table 4** Sequences available at GenBank (April 2012) for mucoralean genera.

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. of seq.</th>
<th>Genus</th>
<th>No. of seq.</th>
<th>Genus</th>
<th>No. of seq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absidia</td>
<td>184</td>
<td>Gongronella</td>
<td>31</td>
<td>Rhizomucor</td>
<td>200</td>
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<tr>
<td>Actinomucor</td>
<td>66</td>
<td>Halteromyces</td>
<td>5</td>
<td>Rhiizopus</td>
<td>1928</td>
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<tr>
<td>Ambomucor</td>
<td>3</td>
<td>Helicostylum</td>
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<td>Siepmannia</td>
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<td>Apophysomyces</td>
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<td>Hyphomucor</td>
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<td>Backusella</td>
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<td>Sporodiniella</td>
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<td>Blakeslea</td>
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<td>Lichtheimia</td>
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<td>Thammiunium</td>
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<td>Clamydobaissia</td>
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<td>Mycothryza</td>
<td>11</td>
<td>Thermomucor</td>
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<tr>
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Taxa and sequences used for the phylogenetic analyses. GenBank accession numbers in bold are generated within this study.

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P428 Saccharomycyes bayanus CBS380 X97777 AF113892 na

**Basidiomycota**  
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**Blastocladiomycota**  
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**Chytridiomycota**  
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**Eccrinales**  
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KV3 Taeniellopsis sp. MAS517 AY336704 AY336897 na

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P027 Eryniopsis caroliniana ARSEF640 EF392552 EF392387 na
P030 Massospora cicadina ARSEF374 EF392548 EF392377 na
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RESULTS AND DISCUSSION

Species recognition is an essential step to higher level classification. Yet, morphology and/or mating behaviour played a major role in traditional fungal species concepts. Depending on the experience of the mycologist and on experimental conditions, morphology and mating behaviour could profoundly vary, and today, both methods were shown to be unsuitable to define mucoralean species if they are not combined with DNA data. Additional concepts have been surveyed and evaluated for fungi (Mayden 1997) with the genealogical concordance phylogenetic species recognition (GCPSR, Taylor et al. 2000) being the most likely one to recognize natural species. Phylogenetic species recognition (PSR) already revealed more species within originally identified species using morphological or biological species recognition (e.g., Hibbett et al. 1995, Taylor et al. 1999).

The underlying problems of interbreeding and geographic/allopatic speciation were extensively discussed by Taylor et al. (2000). Following the discovery of phylogenetic species, additional biological and morphological characters were revealed that supported those species (reviewed by Taylor et al. 2000). Following the discovery of phylogenetic species, additional biological and morphological characters were revealed that supported those species (reviewed by Taylor et al. 2000). In Mucorales, the application of GCPSR resulted in the detection of several new species (Álvarez et al. 2010a, Walther et al. 2013). In contrast to the naturally existing species there are no concepts for the recognition of higher or lower taxonomic levels. Traditionally, certain morphological features (Table 2) that were regarded as synapomorphies were used to define families.
(Zycha et al. 1969, Hesseltine & Ellis 1973, Benjamin 1979, Benny 1982, von Arx 1982). Later they were adapted based on results of molecular phylogeny. Undoubtedly higher taxa should represent monophyletic groups but the taxonomic rank that a group deserves remains a subjective decision. Genetic distances are helpful in this decision but they cannot be translated directly into higher level taxonomy because of dramatic difference in the phylogenetic age in fungal groups.

Even though studies implementing molecular data are still very rare for Mucorales compared to other fungal groups, the number of sequences submitted to GenBank is constantly increasing (Fig. 7). Yet, sequences deposited are predominantly sequences of the rDNA cluster, (Fig. 9). Protein coding sequences are still under represented. This may be due to the lack of appropriate primers which are able to work over a broad range of isolates and an often encountered problem of direct sequencing of the amplificates (Schoch et al. 2012) and the frequent presence of paralogs (Alastruey-Izquierdo et al. 2010). Studies which do apply this kind of molecular data and which are predominantly focused on mucoralean phylogeny count far below 100 if searching ISI Web of Science and NCBI. Furthermore, most of these studies using only one marker for the analyses (Fig. 8). If
Fig. 10 (cont.)

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0.4 substitutions/site
Fig. 11 Distance matrices for all applied loci based on nucleic acid and amino acid sequences. The range of distances is given for each locus. Families are coded according to Fig. 10.
different sequences are combined in an analysis, it is often rDNA and the genomically linked ITS region, but also rDNA combined with protein coding genes (Fig. 8).

The phylogenetic analysis in Fig. 10 consists of combined sequences coding for LSU, SSU, actin and translation elongation factor. At least one member of all accepted genera is included with a total of 201 isolates, 151 belonging to the Mucorales, and 103 unique species representing around half of all described species in the order. Species were included if at least two loci were present in the alignment.

A distance matrix was calculated for each locus. The order-wide distance analyses were based solely on the isolates in the illustrated tree (Fig. 10). Species-specific variations for each locus were not considered. The inclusion and analyses of all available sequences from GenBank would constitute a separate research project that goes beyond the scope of this study.

As expected, distance matrices derived from protein coding genes vary less if based on amino acids instead of nucleic acids. Based on the underlying data, amino acid sequences of actin are more conserved within the Mucorales with relatively similar distances over the whole order versus the situation for the translation elongation factor. When comparing all distance matrices, three major groups can be distinguished (Fig. 11):

i) Low to moderate distances for the most derived clade of the ‘Mucorineae’ including the Mucoraceae, Mycotyphaceae, Choanephoraceae, Pilobolaceae, Rhizopodaceae and Backusellaceae. All matrices show the lowest distances for Mucoraceae (incl. Mycotyphaceae).

All other groups and clades in the tree show no low distance values to any other group. Shortest distances exist only within each group whereas distances to all other groups are more or less similar. Clades included here are:

ii) the Cunninghamellaceae. Within this family, the shortest distances are between the genera Absidia, Halteromyces, Chlamydooabsidia and Cunninghamella (except for translation elongation factor, where distances between Gongronella/ Hessettinella and Absidia/Halteromyces/Chlamydoabsidia are shorter than to the embedded Cunninghamella.

iii) Lichtheimiaceae/Syncephalastraceae/Lentamycetaceae/ Umbelopsidiaceae/Radiomycetaceae/Phycomycetaceae. High distance values for the more ancient clades of the phylogenetic tree result from the different evolutionary times of origin which gives the more basal groups more time to evolve separately.

In the following, clades of the phylogenetic tree will be discussed including proposed/necessary changes in nomenclature or family delimitation.

A) Well-established and supported clades:

Aa) Umbelopsidiaceae W. Gams & W. Mey.

Species of this family were thought to belong most likely to the Mortierellales, rather than to the Mucorales, mainly because of the highly reduced columella (nearly ‘acolumellata’, Fig. 4a) and non-mucoralean colony morphology. The colony mycelium is very dense and velvety as opposed to floccose. And unlike the colonies formed by species of Mortierella, those of Umbelopsis are reddish, brownish or ochraceous and lack a typical garlic-like odour. This distinction and a relationship to Mucorales are surveyed in detail by Meyer & Gams (2003, including a detailed description of the family). With those slight morphological differences compared to all other mucoralean fungi, this group is currently regarded as the most basal in this order. The family is a monogenic group with a clade support (CS, bootstrap support from the Likelihood analysis and Posterior Probabilities from the Bayesian analysis) of at least 99 %, and a clear distinction from the core Mucorales (CS ≥ 99 %) (Fig. 10).

Ab) Phycomycetaceae Arx

This clade (CS ≥ 99 %) includes only two genera with different life styles. Species of Phycomycetes are saprobic, whereas those of Spinelius are facultatively parasitic on the basidioma of Agaricomycotina (Fig. 2a). Species of Phycomycetes are model organisms for studies of phototropism and geotropism as well as carotenoid synthesis, carotenoid degradation and zygosporogenesis.

Ac) Pilobolaceae Corda

The Pilobolaceae is one of the few families recognized from the pre-genomics era with one taxonomic change. The genus Pilaira (Fig. 5e, f), thought to be a member of the family due to morphological characteristics, was placed in the Mucoraceae and is related most closely to Helicostylum, Thamnidiium, Pirella and Mucor mucedo.

Main characteristics of this family are the formation of tropho- cysts (Fig. 5a), the mode of spore release and the growth on dung of herbivores and rodents (Fig. 5c, d). Both included genera possess a vesicle/swelling below the sporangium, which functions in Pilobolus during active discharge of the sporangium (Page 1964, Zycha et al. 1969). In Utharomycetes (Fig. 5b) spores are passively released. Pilobolus is especially difficult to cultivate on artificial media over several generations, resulting in changes in morphology and eventually in death of the culture. Based on analyses of molecular data, only the size and shape of the sporangiospores is retained as of relevance in species delimitation since this feature is the only one that correlates with molecular phylogenies (Foos et al. 2011).

Ad) Choanephoraceae J. Schröt.

This clade (CS ≥ 99 %) includes species producing only sporan gia (Poitrasia, Gilberella; Fig. 3d, e), or also sporangiola (Choanephora, Blakeslea; Fig. 3f). Sporangia and sporangiolas are produced on separate sporangioshores. The wall of the sporangium is persistent. At maturity the wall ruptures at preformed sutures to release sporangiospores with hyaline, hair-like polar appendages representing a synapomorphy of this family. The species are saprobes or fruit and vegetable inhabiting parasites, sometimes occurring as major post-harvest pathogens in tropical and subtropical regions (Fig. 2c). The newly introduced subfamilies Gilberellioideae (MycoBank IF550022) and Choanephoroideae (MycoBank IF550021) are distinguished by the characters of the zygospore, e.g. suspensors opposed or apposed, zygosporangium ornamented or smooth (Voigt 2012).


Although this clade is highly supported (CS ≥ 99 %), it is one family that should be studied in more detail. While two recent studies dealt with the genus Cunninghamella and incorporated the largest number of isolates studied so far, the sister genera lack such a profound study. The authors evaluated all available information ranging from morphology to growth temperatures, mating experiments and molecular data (Liu et al. 2001, Zheng & Chen 2001). Currently, only Absidia and Cunninghamella are well sampled; Gongronella, and especially Hessettinella, Halteromyces and Chlamydooabsidia, definitely need more isolates to study. Since Chlamydooabsidia is always nested within Absidia, its status as a distinct genus should be evaluated; this might also be extended to Halteromyces. The distances between sequences are very high in this family representing one of the highest variabilities when compared to other clades (Fig. 11).
Af) Lentamycetaceae K. Voigt & P.M. Kirk — MycoBank IF550009
Since the first analyses including species of the genus Lentamycetes (formerly Absidia) it was obvious, that these species should be separated. And since there are no other species of the Mucorales closely related to this genus, a separate family is introduced (Voigt 2012). Species of the Lentamycetaceae (Fig. 4b, c) are homothallic and mycoparasitic, although the mycoparasitic potential of L. zychae was lost during cultivation (Zycha et al. 1969). Kwasina & Nirenberg (2008a, b) introduced the genus Siepmannia that included the two Lentamycetes species besides the new species S. pineti and S. lariceti. A correct classification of these taxa is still unclear because only ITS sequences and no living material are available from S. pineti and S. lariceti. A resampling of strains of Siepmannia is necessary to perform multilocus studies and to determine their mycoparasitic potential.

Ag) Backusellaceae K. Voigt & P.M. Kirk — MycoBank IF550011
Species included here originally were placed in the Mucoraceae or Thamnidiaceae. Like other described families once included in the Mucoraceae (e.g. Pilobolaceae, Chaenephoraceae), this clade should also be distinguished from the Mucoraceae. The monogeneric Backusellaceae are characterised by transitorily recurved sporangiphores and the tendency to produce sporangiola in addition to the sporangia. Several Mucor species owning gigas in addition to the sporangia. Several Mucor species were transferred to Backusella. Clade support for the Backusellaceae is ≥ 99 % (Fig. 10) and it contains three species: Backusella lamprospora, B. circina, B. recurva. The members of the Backusellaceae seem to be saprotrophs found e.g. in soil, on wood and fallen leaves (Walther et al. 2013).

Ah) Rhizopodaceae K. Voigt & P.M. Kirk — MycoBank IF550010
Like the Backusellaceae, the Rhizopodaceae forms a well-supported clade, distinct from the Mucoraceae (CS ≥ 99 %). Within this clade, a trifurcation is observed (each with a CS ≥ 99 %), with one Rhizopus microsporus-clade containing predominantly thermotolerant fungi (growth up to 45 °C), a sub-thermotolerant R. arthriticus-group (37–40 °C) and a mesophilic group containing R. stolonifer, Sporodiniella, and Syzygites. This was already observed applying morphology and growth temperatures (Schipper & Stalpers 1984), establishing a classification accepted as standard for many decades. The application of molecular data and biochemical features (e.g. production of iastic acids) supported those three major clades, but revealed also newcryptic species (Abe et al. 2006, 2007). The implementation of GCPSR, including different genetic markers, resulted in the publication of a new, reliable Rhizopus classification (Abe et al. 2010). Yet, the final clustering in the Rhizopodaceae (Fig. 10) remains unresolved, because some species (R. caesipitous, R. homothallicus, R. lyococcus, R. schipperae, R. sexualis) were not included because of missing data. But the thermotolerant species R. caesipitous, R. homothallicus and R. schipperae, seem to be closely related to the R. microsporus clade (rDNA analysis, Abe et al. 2006). In this study, R. sexualis (mesophilic) is related to R. stolonifer and R. lyococcus (mesophilic) appears as a very basal species (Abe et al. 2006). All species of the Rhizopodaceae are reported to be pathogenic to other organisms. Whereas Syzygites is a parasite of Dikarya (Kovacs & Sundberg 1999), Sporodiniella is a parasite of insect larvae (Evans & Samson 1977, Chien & Huang 1997), and species of Rhizopus are pathogens of plants and opportunists of animals, including humans.

Ai) Radiomycetaceae Hesselt. & J.J. Ellis & Saksenaceae Hesselt. & J.J. Ellis
The Radiomycetaceae contains only one genus with three species (Benny & Benjamin 1991). Radiomycetes is coprophilous and pathogenic to mice (experimental infections, Kitz et al. 1980). The unispored or multispored sporangia are produced on pedicels, which originate from a vesicle. The Saksenaceae contain two genera, Saksenia and Apophysomyces are saprobic in soil and compost. Some species are also known to infect animals and humans (Álvarez et al. 2010a, b).

B) Moderately supported clades:
The Mycotyphaceae currently contains only one genus (Benny & Benjamin 1976). Although the inclusion of adjacent species is proposed (Voigt 2012), the results of molecular phylogenetics are still controversial (Fig. 10). Furthermore, CS is ≥ 99 % for Mycotyphaceae, but strong support for the separation from Mucoraceae is only given for Bayesian analysis (CS ≥ 90 %). Although molecular distances (Fig. 11) of Mycotypha are similar to those of the Mucoraceae, the Mycotyphaceae is maintained as the sister family to Mucoraceae also because of the exceptional sporangiophores bearing terminal, elongate, cylindrical vesicles (Fig. 4k). The unispored sporangiola are of two types, an inner layer that consists of globose spores and an outer layer of spores that are either obovoid or more or less cylindrical.

Species of the genus Absidia growing well at elevated temperatures were transferred to the genus Lichtheimia based on both molecular and physiological data (Hoffmann et al. 2007, 2009). Lichtheimia has appeared as a well-supported sister taxon to Dichotomocladium in many phylogenetic analysis (e.g. O’Donnell et al. 2001, White et al. 2006) requiring an emendation of the Lichtheimia. Dichotomocladium has been included in the Chaetocladiaceae (Benny & Benjamin 1993) based on morphological structures such as sterile spines, unispored sporangiola and branched, tree-like sporangiophores (Fig. 4d–f, l, m). Molecular data, however, revealed that these morphological features are of no phylogenetic significance. A shared feature of Lichtheimia and Dichotomocladium is their tolerance of higher temperatures. Species of Lichtheimia are consistently able to grow at and above 37 °C (Hoffmann et al. 2007), the species of Dichotomocladium tolerate 35 °C and some species, namely D. hesseltinei, D. floridanum and D. robustum are even able to grow at 37 °C (unpubl. data). The subfamilies Lichtheimioideae (MycoBank IF550086) and Dichotomocladioidae (MycoBank IF550087) are proposed for the Lichtheimiaeae (Voigt 2012). Based on a smaller set of sequences a third subclade within the Lichtheimiaeae was suggested: namely the Rhizomucoroidae (MycoBank IF550085) (Voigt 2012) but this classification could not be verified in this study.

Syncephalastrum (Syncephalastraceae) is the only genus in the Mucorales producing sporangiola with the spores arranged in a linear series (merosporangia, Fig. 4g). Whether other genera (e.g. Protomycocladus) should be included in this family needs to be studied in more detail because of the low phylogenetic branch support (Fig. 10), leaving Syncephalastrum the only genus in this family. The final position of Protomycocladus could not be resolved unquestionable due to low branch support in this study as well as other publications (e.g. O’Donnell et al. 2001, Voigt & Wüstemeyer 2001, White et al. 2006, Walther et al. 2013).
Closely related to the families, Lichtheimiaceae and the Syncephalastraceae, are three additional clades: i) Protomycocladus faisalbadensis; ii) Rhizomucor/Thermomucor; iii) Fennelomyces/Circinella/Thamnostylum/Zychaea/Phascolomyces. Clades i) and ii) include thermotolerant species with growth temperature maxima at 45 °C for Protomycocladus (Schipper & Samson 1994), and thermophilic species with growth temperature maxima at 55–57 °C for Rhizomucor (de Hoog et al. 2000) or above 60 °C for Thermomucor (Subrahmanyan et al. 1977). Clade iii) contains species that are predominantly mesophilic, not growing at elevated temperatures. Furthermore, this clade is characterised by cinclata (strong or less pronounced) elements in the sporangiophores (Fig. 4p, q).

For a reliable placement of clades i–iii, in relation to the Lichtheimiaceae and Syncephalastraceae, additional data are needed, since the relationships of the former clades are not significantly supported in any published analyses. Therefore, these clades gain the status incertae sedis till their relationships could be solved unambiguously.

In order to test the taxonomic stability in the newly delimited Lichtheimiaceae, ITS2 sequences of all isolates were searched for compensatory base changes (CBC) as indicators for species boundaries. A comprehensive study on CBC suggests that with a reliability of 93.11 % one CBC is present in two specimens belonging to two different species. But the lack of CBCs does not indicate that two specimens do belong to the same species (Müller et al. 2007). Applying CBC analyses to several clades within the Lichtheimiaceae/Syncephalastraceae, CBC is widely concordant with species concepts in Rhizomucor (Fig. 12), Lichtheimia (Fig. 13, except L. corymbifera and L. ornata), Dichotomocladium (Fig. 14), Zychaea and Thamnostymo (Fig. 15). There are few species in the analyses which could not be clearly separated from others, which is due to the lack of CBCs (e.g. Dichotomocladium hesseltinei and D. floridanum, Fennelomyces heterothallicus, Thamnostymo repens). However, no CBCs at all were detected in the genera Syncephalastrum and Circinella showing that CBC analyses cannot be used generally as a tool for species recognition in Mucorales. CBC analyses between different genera remains difficult if not impossible (especially in the ancient clades of the Mucorales) due to highly diverse ITS2 sequences and thus secondary structure. If differing too much, no comparison of the secondary structure is possible, which results in no detectable CBCs. CBC analyses are in parts suitable for distinguishing species that are highly similar in their morphology (e.g. Lichtheimia ramosa and L. corymbifera) and could assist in supporting molecular phylogenies.

**Bc) Mucoraceae** Dumort.

The Mucoraceae is undoubtedly the largest family and presumably the most derived in the Mucorales (Fig. 10). Traditionally all species lacking features for classification within any other family where assigned to the Mucoraceae making the family polyphyletic. This study has circumscribed a monophyletic Mucoraceae with highly diverse features that characterise different species and genera. All species are saprobes except Dictaphora, Parasitella and Chaetocladium which are facultative mycoparasites (Dictaphora on Agaricomycetes, Parasitella and Chaetocladium on Mucorales). A few species are also described as opportunistic pathogens causing deep and systemic mycoses. Species are either homothallic or heterothallic, the zygospores form a warty to smooth zygosporangial wall with naked (without appendages) opposed suspensors. Sporangia are borne on branched or unbranched, sometimes phototrophic sporangiophores, sporangiola are rare and the sporangia are ± lageniform, ± apophysate and columellate.
SUMMARY AND CONCLUDING REMARKS

Traditional classification in Mucorales was done, as in all Eu-
mycetes, mainly by using morphological characters. Already
eleven years ago large deficiencies in the morphology-based
system were revealed by molecular data. The distinctly extended
dataset of the current study gives now a clearer picture of the
family structure in the Mucorales. Our phylogeny based on four
markers and contains 14 clades that we interpret as families:
(1) Umbelopsidaceae; (2) the newly erected monogeneric Lenta-
mycetaceae; (3) Syncephalastraceae presumably including Proto-
mycetaceae; (4) Lichtheimiae containing Lichtheimia and
Dioctomociadium; (5) Phycymycetaceae; (6) Saksenaceae;
(7) Radiomycectaceae; (8) Cunninghameallaceae inclusively Ab-
sidia s.str.; (9) the newly erected monogenic Backusellaceae;
(10) Pilobolaceae; (11) the newly erected Rhizopodaceae
including the genera Rhizopus, Sporocniella and Syzygites;
(12) Choanephoraceae; (13) Mycotyphaceae; and (14) Mucora-
ceae. Most of these family clades were well supported. Only
the delimitation between the Mucoraceae and the Mycotyphaceae
as well as the Lichtheimiae and the Syncephalastraceae
could not be defined doubtlessly, few subclades are classified
as incerta sedis. The Mucoraceae, Mycotyphaceae and Cun-
ninghameallaceae involve several taxonomic deficiencies and
a detailed study of the phylogenetic relationships in these families
is needed.

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REFERENCES

Abe A, Asano K, Sone T. 2010. A molecular phylogeny-based taxonomy
of the genus Rhizopus. Bioscience, Biotechnology and Biochemistry 74:
1325–1331.

Abe A, Oda Y, Asano K, Sone T. 2006. The molecular phylogeny of the genus
Rhizopus based on rDNA sequences. Bioscience, Biotechnology and Bio-
chemistry 70: 2387–2393.

Abe A, Oda Y, Asano K, Sone T. 2007. Rhizopus delmar is the proper name

Alastreuy-Izquierdo A, Hoffmann K, Hoog GS de, Rodriquez-Tudela JL, Voigt
K, et al. 2010. Species recognition and clinical relevance of the zygomy-
cetous genus Lichtheimia syn. Absidia pro parte, Mycoclaus. Journal
of Clinical Microbiology 48: 2154–2170.

Almeida ER, Cerdá-Olmedo E. 2008. Gene expression in the regulation
of carotene biosynthesis in Phycymycetaceae. Current Genetics 55:
129–137.

2010a. Molecular phylogeny and proposal of two new species of the
emerging pathogenic fungus Saksenaea. Journal of Clinical Microbiology
48: 4410–4416.

Molecular phylogenetic diversity of the emerging mucoralean fungus Apo-
physomyces: Proposal of three new species. Revista Iberoamericana de
Mycologia 27: 80–89.

Arx JA. 1982 ‘1984. On Mucoraceae s.str. and other families of the

Bartrinch-García S, Nickerson WJ. 1962. Induction of yeast-like develop-

Museums of Canada, Ottawa, Canada.

Benny GL. 1982. Zygomyetes. In: Parker SP (ed), Synopsis and classifi-


Benny GL. 2012. Current systematics of the zygomycotan fungi with a brief
discussion of their biology. In: Misra JK, Tewari JP, Deshmukh SK (eds),
Systematics and evolution of fungi. Progress in mycological research:


Note:
New taxa in Voigt 2012 were validated in Kirk 2012 and Kirk & Voigt 2012.