Species recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*)

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Abstract
The zygomycete genus *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*) consists of saprotrophic fungi inhabiting soil or dead plant material. *Lichtheimia corymbifera* (syn. *Absidia corymbifera*, *Mycocladus corymbifer*) and *Lichtheimia ramosa* (syn. *Absidia ramosa*, *Mycocladus ramosus*) may cause fulminant infections in patients with impaired immunity. This study investigates species boundaries in *Lichtheimia* using Genealogical Concordance Phylogenetic Species Recognition (comparing genealogies of ITS, D1/D2 region of the LSU and actin), Biological Species Recognition by mating tests, as well as morphological and physiological characters. The three molecular markers used were selected by evaluating polymorphism and paralogy in several loci including β-tubulin, translation elongation factor EF-1α, the largest subunits of the RNA polymerase II (RPB1 and PRB2), the mitochondrial cytochrome c oxidase subunit I (COI), and the mitochondrial ribosomal small subunit (mtSSU) rDNA among four strains belonging to different putative species. Comparing ITS-, LSU- and actin-gene genealogies, we recognized seven phylogenetic species. However, mating tests did not show intrinsic reproductive barriers for two pairs of the phylogenetic species. Therefore we regard five species in *Lichtheimia* as confirmed: *Lichtheimia corymbifera*, *L. ornata* comb. nov., *L. ramosa*, *L. hyalospora*, and *L. sphaerocystis* sp. nov. Only the first three species seem to have clinical relevance. *Lichtheimia blakesleeana* is reduced to synonymy of *Lichtheimia hyalospora*. We provide a detailed description of *Lichtheimia sphaerocystis* sp. nov. and a key for the identification of all herein accepted species based on morphological traits and growth at different temperatures.

**Introduction**

Mucormycoses, i.e. infections caused by members of the Mucoromycotina are uncommon but often dramatic, requiring immediate action on the basis of accurate diagnosis. The recently observed increase in case reports on mucormycosis (32) can be ascribed to the growing number of patients
with risk factors such as diabetes, neutropenia, bone marrow transplantation, or long-term use of steroids. Although this trend was already observed prior to the availability of voriconazole in medical applications (17, 21), several studies related the increasing incidence of mucormycoses to voriconazole prophylaxis and treatment against aspergillosis infection in immunocompromised patients (18, 37, 43). According to Roden et al. (32) approximately 5% of mucormycoses are caused by species of *Lichtheimia* (syn. *Absidia* p.p., *Mycocladus*). These authors reviewed 25 well-documented cases of *Lichtheimia* (as *Absidia*) infections since 1940. However, the actual incidence of the species may have been underestimated given the fact that no less than 53 clinical *Lichtheimia* strains were sent to the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands) and the Instituto de Salud Carlos III National Centre of Microbiology (CNM-CM, Madrid, Spain) during the past 10 years alone.

Originally the genus *Absidia* was characterized by the formation of pyriform sporangia with a distinct apophysis and branched sporangiophores. Subsequent phylogenetic and physiological studies showed that *Absidia*-like fungi represent three separate lineages (15): 1 – *Absidia* s.str. that shows decreased growth rates above 30°C and no growth above 40°C (mesophilic) and has zygospores, which are protected by long appendages of the suspensors, 2 – *Lentamyces*, which does not grow above 30°C and consists of potential parasites of other fungi (13), and 3 – *Lichtheimia*, consisting of thermotolerant species that show good growth at human body temperature and produce zygospores with equatorial rings and suspensors without appendages. Only the latter group has clinical relevance. It was first named *Mycocladus* typified by *Mycocladus verticillatus* (15).

However, the type of that species turned out to represent a mixed culture of an *Absidia* s.str. and possibly a *Lentamyces* species, thus not congeneric with any of the thermotolerant species. Therefore this group had to be renamed with the oldest available genus name, *Lichtheimia* (14), typified by *L. corymbifera*. 

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The genus *Lichtheimia* consists of basically saprotrophic species inhabiting soil and decaying plant material. According to Hoffmann et al. (14) the genus *Lichtheimia* contained four species: *L. corymbifera* (syn. *Absidia corymbifera*, *Mycocladus corymbifer*), *L. ramosa* (syn. *A. ramosa*, *M. ramosus*), *L. blakesleeanana* (syn. *A. blakesleeanana*, *M. blakesleeanus*), and *L. hyalospora* (syn. *A. hyalospora*, *M. hyalosporus*). Of these, only *L. corymbifera* and *L. ramosa* have been reported from human infections. Whether or not *L. ramosa* and *L. corymbifera* are separate species has been a controversial issue in the past, when studies addressing this question applied phenetic criteria. For example, Ellis & Hesseltine (4) treated *L. corymbifera* and *L. ramosa* as being distinct, whereas Nottebrock et al. (28) and Schipper (36) reduced *L. ramosa* to synonymy of *L. corymbifera*. Recently Garcia-Hermoso et al. (8) re-established *L. ramosa* as a separate species based on sequence-analyses of ITS, D1/D2 region of LSU and partial EF1-α gene, supplemented with phenotypic characters. However, evidence that the clade beside the *L. corymbifera* clade belongs to *L. ramosa* remained pending because their study did not include the neotype strain of *L. ramosa*. They assigned the clade to *L. ramosa* because two isolates included (CBS 269.65 and CBS 270.65) were originally identified as such. The authors found considerable sequence divergence especially in *L. ramosa*. One of the strains that was morphologically similar to *L. corymbifera* could not be assigned to a species by sequence data. Our sequence analysis of strains from all species known in the genus *Lichtheimia* also revealed well-supported subgroups suggesting the existence of additional taxa.

The present study aims to explore species boundaries in the genus *Lichtheimia*, to evaluate the clinical importance of each species, and to provide discriminating characters of clinical as well as environmental species in order to allow a reliable identification. Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (40), based on gene genealogies of the ITS region, the D1/D2 region of LSU, and the partial actin gene was used to define phylogenetic species. Mating tests were performed to recognize biological species (Biological Species Recognition), and
morphology and growth characteristics were used to develop taxonomic concepts and practical
diagnostic features for genus and species. After evaluation of the combined data, we propose to
accept five species in Lichtheimia, namely *L. corymbifera*, *L. ramosa*, *L. ornata* comb. nov., *L.*
hyalospora, and *L. sphaerocystis* sp. nov., of which only the first three are clinically relevant.

MATERIAL AND METHODS

Strains

A total number of fifty-three isolates of *Lichtheimia* comprising 19 environmental, 23 clinical and
11 strains of unknown sources and including all ex-type strains of *Lichtheimia* species available at
the reference collection of the CBS were studied (Table 1). All strains used in this study are either
deposited at the CBS, at the CNM-CM, or at the Fungal Reference Centre (PRZ, Jena, Germany).

Molecular studies

Extraction of genomic DNA

Cultures were grown for 2 days on malt extract agar (MEA, 5%, malt extract agar produced by
Oxoid, Badhoevedorp, The Netherlands) at 24°C. Genomic DNA was extracted according to the
procedure described by Möller et al. (25) with several modifications. Briefly, fungal material was
transferred to a tube containing 2 glass beads and 500 ml TES buffer (124 mM
Tris(hydroxymethyl)-aminomethane, 12.8 mM Na-EDTA, 87 mM sodiumdodecylsulphate (SDS),
pH = 8). The samples were homogenized for 3 min at 30 Hz using the TissueLyser (Quiagen,
Venlo, The Netherlands) and spined for 2 min at 14,000 rpm (20,400 rcf). Afterwards 5.1 U of
Proteinase K (in 10 µl) (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added followed by 30
min of incubation in a waterbath at 55°C. 120 µl of 5 M sodium chloride and 1/10 volume of 10%
CTAB (Cetyl trimethylammonium bromide) were added to the material followed by 60 min
incubation at 65°C. The samples were again homogenized for 3 min (30 Hz) using the TissueLyser.
One volume of SEVAG (chloroform: isoamylalcohol 24:1 v/v) was added and the samples were
spined for 5 min at 4°C at 14,000 rpm (20,400 rcf). The upper phase (aqueous phase) was
transferred to a new tube and 0.55x volumes of isopropanol were added to precipitate the DNA.
After incubation at -20°C for at least 30 min DNA was pelleted at 14,000 rpm (20,400 rcf) for 10
min at 4°C. The supernatant was decanted and the DNA pellet was washed twice with 700 ml 70%
etanol, dried, and resuspended in 50 µl of TE buffer (12.4 mM Tris, 1.34 mM Na-EDTA (pH =
8.0). Genomic DNA was stored at -20°C.

Marker selection

We searched for genomic regions with a polymorphism comparable to that of the ITS region in
order to apply GCPSR (40). Using DNA extracts of four strains belonging to different putative
species (*Lichtheimia blakesleeana*, later reduced to a synonym of *L. hyalospora*, CBS 100.28, *L.
corymbifera* CBS 100.51, *L. ornata* CBS 958.68, *L. ramosa* CBS 582.65) we amplified and
sequenced parts of the following genes: actin, β-tubulin, translation elongation factor EF-1α, ITS,
D1/D2 region of the nuclear ribosomal large subunit (nuclLSU), largest subunit of the RNA
polymerase II (RPB1), second largest subunit of the RNA polymerase II (RPB2), mitochondrial
cytochrome c oxidase subunit I (COI), and mitochondrial ribosomal small subunit (mtSSU). The
primers used are given in Table 2. Direct sequencing was possible only for PCR products
comprising the COI, LSU, mtSSU, and the ITS region except for CBS 100.36 and CNM-CM 4849.
PCR products of the remaining loci had to be cloned before sequencing in the competent cell line
JM109 of *E. coli* using the pGEM-T Easy Vector (Promega, Leiden, The Netherlands) according to
instructions of the manufacturer. Similarity values based on uncorrected distances for the 6 possible
pairings between the 4 strains were calculated for all genomic regions tested with BioNumerics
v.4.61 (Applied Maths NV, Sint-Martens-Latem, Belgium).

For several loci different sequences for the same strains were obtained. In order to ascertain
the origin of this diversity, single-spore cultures of 3 strains of \textit{L. ramosa} (CBS 223.78, CBS 124197, CNM-CM 5111) were achieved by suspending sporangiospores in sterile distilled water and plating several dilutions of these suspensions on MEA in Petri dishes. The outgrowing mycelia were isolated. DNA was extracted and used for PCR amplification, cloning and sequencing as described.

Amplification, cloning and sequencing of the selected marker

DNA segments comprising either the complete ITS region and the D1/D2 region of the LSU, or a large part of the actin gene were amplified using the primer sets listed in Table 2. For both PCR types the reaction mixture (25 µl) contained 0.4 µM of each primer, 0.185 mM of each deoxynucleoside triphosphate (GC Biotech, Alphen a/d Rijn, The Netherlands), 10x NH$_4$ BioTaq Reaction buffer (GC Biotech), a final concentration of 1.5 mM MgCl$_2$, 0.8 U BioTaq DNA polymerase (GC Biotech), and about 20 ng of DNA. The cycling conditions for the ribosomal fragment included one initial cycle at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C, with one final cycle of 7 min at 72°C. For the actin gene fragment the following PCR conditions were applied: predenaturation for 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C, and one final cycle of 7 min at 72°C.

PCR reactions were performed on a Thermal cycler 2720 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reactions products were analysed in 1% agarose gels.

PCR products comprising the actin fragment or the ITS region (in case of CBS 100.36 and CNM-CM 4849) were ligated into pGEM-T Easy Vector (Promega, Leiden, The Netherlands) and cloned in \textit{E. coli} JM109 competent cells (Promega) following the manufacturer’s instructions. Colony-PCRs were performed using the primer pair M13f (5’-GTAAAACGACGGCCAGT-3’) and M13r (5’-GGAAACAGCTATGACCATG-3’). In a first step 4 clones were sequenced for each strain. In case that these clones did not include the required outparalog (see results), we sequenced additional
clones until all strains were represented by at least one sequence of outparalog I in the alignment.

Both strands of the products of the primary PCR (ITS, LSU) or the colony PCR (actin) were
directly cycle-sequenced using the Big dye sequencing kit (BigDye Terminator cycle sequencing
ready reaction kit, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and the primer
sets given in Table 2. Cycle-sequencing products were analysed on an ABI 3730XL automatic
sequencer (Applied Biosystems).

Sequence analysis

Consensus sequences were constructed by means of the SeqMan program v.7.2.2 (DNASTAR,
Lasergene) and deposited in GenBank under the accession numbers GQ342712-GQ342955 (Table
1). Sequences were aligned using the server version of the MAFFT program
(www.ebi.ac.uk/Tools/mafft) and manually corrected in the program Se-Al v2.0a11 (A. Rambaut,
2002; http://tree.bio.ed.ac.uk/software/seal/). In cases where actin gene sequences belonging to the
same outparalog of a single strain were different (inparalogs), the most deviating actin gene
sequences per strain were included in the alignment. Phylogenetic relationships were estimated for
all alignments with a maximum parsimony analysis done in PAUP (Phylogenetic Analysis Using
Parsimony) v.4.0b10 (D. L. Swofford, 2002, Sinauer Associates, Sunderland, MA) and with a
Bayesian approach using Markov chain Monte Carlo performed by the computer program MrBayes,
v. 3.1.2 (33). In maximum parsimony analyses, heuristic search was performed with 1,000
replicates and tree-bisection-reconnection (TBR) as branch-swapping algorithm. Gaps were treated
as missing data. Robustness of the trees was estimated by bootstrap analysis with 1,000 replicates.
The Jukes-Cantor-69-model of DNA substitution was selected by the program MrAIC v.1.4.3 (J. A.
A. Nylander, 2004, Evolutionary Biology Centre, Uppsala University) for all three alignments and
used in the Bayesian analyses. Four simultaneous Monte Carlo Markov chains were run over 2 M
generations using random starting trees, and default starting parameters of the DNA substitution
model. Trees were sampled every 100 generations. After discarding the first 8 K trees sampled (burn-in), a posteriori probabilities were estimated by computing majority rule consensus trees.

*Dichotomocladium elegans* was used as outgroup in analyses of the LSU because it was shown to be closely related to *Lichtheimia* by O'Donnell (29, as *Absidia* p.p.). The analyses of actin and ITS were performed without an outgroup because inclusion of *Dichotomocladium* decreased the quality of the alignment due to large sequence differences.

### Morphological study

**Study of culture characteristic and sporangiophore morphology**

Strains were cultivated on MEA at 24°C and on Synthetic Mucor Agar (SMA; dextrose, Merck, Amsterdam, The Netherlands; asparagine, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands, thiamine chloride, Sigma-Aldrich Chemie BV; agar, Oxoid, Badhoevedorp, The Netherlands) described by Chen & Zheng (1) at 28°C in the dark. After 7 days and 3 weeks, respectively, texture and colour of the colonies were described and in case of the newly described species rated using the charts of Rayner (31). Mounts for microscopic examinations of sporangiophore morphology were made from 7-day-old MEA cultures in two different ways for each species, (i) by pressing a piece of adhesive tape in the colony, and (ii) by squashing a small portion of the colony including the submersed mycelium. The fungal material was mounted in lactic acid with cotton blue (2 mg cotton blue ml\(^{-1}\) lactic acid) and in lactic acid only and examined using a microscope of type Nikon eclipse 80i (Nikon, Amstelveen, The Netherlands). Measurements were performed using the software NIS-Elements D 3.0 (Nikon).

**Study of the giant cells**

In search of additional distinguishing characters we tested development and structure of giant cells on 3 media at different temperatures: on MEA at 24°C, on potato dextrose agar (PDA; dextrose,
Merck, Amsterdam, The Netherlands; agar, Oxoid, Badhoevedorp, The Netherlands) at 24°C, 33°C and 37°C, and on yeast extract agar according to Haynes et al. (10) (YEA; yeast extract, Difco, Alphen a/d Rijn, The Netherlands; malt extract, Difco; dextrose, Merck, Amsterdam, The Netherlands; agar, Oxoid, Badhoevedorp, The Netherlands) at 33°C and 37°C. In this first screening five strains of *Lichtheimia corymbifera* (CBS 101040, CBS 120580, CBS 120581, CBS 429.75, CBS 519.71), five strains of *L. ramosa* (CBS 100.55, CBS 103.35, CBS 223.78, CBS 123197, CBS 124198), and all eleven strains of the remaining species (see Table 1) were included. After the first results became available we extended the study on YEA at 37°C to all strains of *L. corymbifera*. Every week the cultures were screened for giant cells after removal of the aerial mycelium with a sterile needle using a stereomicroscope of type Nikon SMZ 1500 (Nikon, Amstelveen, The Netherlands). Giant cells were taken from the culture and mounted in lactic acid for microscopic examination.

**Mating experiments**

Sporangiospore suspensions were prepared from 5-day-old cultures grown on MEA in Petri dishes at 24°C by adding roughly 2 ml of sterile distilled water and by sucking the water several times into the pipette. One or two drops of the suspension were placed in a distances of approximately 1-2 cm from the drop(s) of the second strain on YEA as described by Haynes et al. (10) and incubated at 31°C in the dark for 7 weeks. A total number of 168 contrasts were tested including 73 intraspecific and 95 interspecific mating tests.

**Growth kinetics**

MEA plates were inoculated with small blocks taken from the edge of 3-day-old colonies. The plates were incubated at the following temperatures: 24°C, 33°C, 37°C, 40°C, 43°C, 46°C, 49°C, and 52°C. The diameter was measured twice a day for 3 days. The diameter of the colony taken 8 hours...
after inoculation was subtracted from all following measurements. Growth rate, measured in millimeters per hour, was calculated for each strain and temperature. Descriptive and comparative statistical analyses were performed. Comparisons were done by variance analysis (ANOVA) including the Bonferroni post hoc test. A $P$ value $\leq 0.05$ was taken as statically significant.

Statistical analysis was done with the help of the software package SPSS 16.0 (SPSS S.L., Madrid, Spain). Growth rates of each species at different temperatures were plotted in a graph performed with SigmaPlot 11.0 (SPSS S.L., Madrid, Spain).

RESULTS

Molecular markers for species recognition in *Lichtheimia*

Table 3 lists the similarity values for the analysed genomic regions of six pairs of strains. In cases where more than a single sequence was found per strain for a certain locus (paralogs), the highest possible similarity value for the respective pair of strains is given in Table 3.

ITS was by far the most variable among all nine regions tested. The second highest degree of polymorphism was found in the nucleotide sequences of $\beta$-tubulin, followed by RPB2, actin, RPB1, LSU, EF-1$\alpha$, and COI. The mitochondrial SSU was strictly monomorphic. For some of the studied genes we detected paralogs originating from different duplication events. In order to avoid confusion between these different paralog types, we introduced the terms ‘outparalogs’, which evolved via an ancient duplication process preceding speciation, and ‘inparalogs’, which, judging from tree topology, evolved subsequent to the speciation event (19). Based on gene genealogies and species concepts maintained in this paper, gene sequences were considered as outparalogous if they formed two or more clades, each clade containing sequences of more than a single species (see small scale Fig. 1b). Clades consisting purely of paralogous sequences of a single species indicate an inparalogous relationship. Inparalogs of a certain species are considered to be co-orthologous to
inparalogs of other species if they form a monophyletic group. This evaluation is required to ascertain the use of the correct inparalogs for phylogenetic analyses.

Our main criteria for the selection of molecular markers for species recognition were variability and, in terms of paralog identities, absent or easy-to-discriminate outparalogs enabling unambiguous assignment of co-orthologous paralogs, and small sequence differences among inparalogs (Table 3). On this basis we chose ITS, LSU, and actin. The absence of outparalogs as well as small sequence differences among inparalogs would have made the RPB2 gene also a promising candidate for species recognition. However, we could not obtain editable trace-files for strain CBS 100.28.

The LSU could be sequenced directly for all strains. Direct sequencing of the ITS region was hampered by polymorphism among ITS copies in two strains. Most species of Lichtheimia possess two outparalogs of actin (I and II), which differed in their intronic and exonic sequences and were easily distinguished. Strains listed as Lichtheimia blakesleeana and L. hyalospora, and their sibling species L. sphaerocystis, a novel species that is described below, deviated in having only outparalog I. For nearly all studied strains we found more than one inparalog originating from the duplication of outparalog I. In all studied species with the exception of L. ramosa, inparalogs of the same strain differed by 1 to 6 basepairs. In L. ramosa inparalogs of the same strain varied by 1 up to 21 basepairs. Each of the three single-spore isolates of the L. ramosa strains CBS 223.78, CBS 124197, and CNM-CM 5111 possessed three different inparalogs originating from outparalog I, suggesting the existence of a gene family. Some of the inparalogs (e.g. clone 2 of CNM-CM4978, clone 1 of CBS 112528) contained single-base deletions in their exonic sequences, which indicates loss of function (pseudogenes). The inparalogs of all Lichtheimia species belonging to outparalog I of the actin gene formed a well-supported clade in the phylogenetic tree matching with the co-orthologous relation of the inparalogs (Fig. 1b).
Molecular phylogenetic analyses

Maximum parsimony analyses based on the ITS alignment (a total of 899 characters, of which 383 were parsimony-informative) resulted in 906 most parsimonious trees (tree length TL = 726 steps), the LSU alignment (a total of 657 characters, of which 78 were parsimony-informative) resulted in 185 most parsimonious trees (TL = 230 steps), and the actin alignment (a total of 885 characters, of which 280 were parsimony-informative) in 228 most parsimonious trees (TL = 653). Tree topologies obtained by maximum parsimony and Bayesian MCMC analyses were largely similar for all three markers and did not contain conflicting well-supported groups. Therefore, the Bayesian trees are not shown but Bayesian posterior probabilities are included in the maximum parsimony trees in Fig. 1.

In Fig. 1, strains are attributed the same colour if they belong to a single, well-supported group in the ITS genealogy. Because the same colour coding is used in LSU and actin trees, conflicts in gene genealogies of different loci are visualized by intermixing of colours in supported groups of LSU and actin trees.

In Fig. 1b the actin tree including outparalog I (blue frame) and II (red frame) is pictured on a small scale. The large scale tree represents only the outparalog I part of the tree (blue frame). We detected only outparalog I in *L. blakesleeana, L. hyalospora,* and *L. sphaerocystis,* described below, although we sequenced at least eight clones for each strain. Similarity of the inparalogous sequences resulted in a lack of distinct subclades in nearly all groups rendering a decision on valid co-orthologs unnecessary. In the *L. ramosa* clade, strains CBS 271.65 and CBS 582.65 possessed different inparalogous sequences that were included in two distinct and well-supported subclades, one (CBS 271.65, CBS 582.65, and CBS 100.24) single-colored (pink) and another multi-colored (pink, black, lilac) in Fig. 1b. In this case we selected the inparalogous sequences of the multi-colored clade as valid co-orthologs because of their higher similarity to the inparalogs of the remaining strains of this species. This selection did not influence the boundaries of the phylogenetic
species.

Tree topologies of ITS, LSU, and actin trees were concordant except for groups of *L. ramosa*. Three groups of *L. ramosa* supported in the ITS genealogy (indicated as lilac, vinaceous, and light blue in Fig. 1a) were not detected in the LSU and actin genealogies. The group of *L. ramosa* marked in pink in the ITS tree was also supported in the LSU tree, but was not detected in the actin tree because the inparalogous sequences constituting a well-supported pink group in the actin genealogy were treated as invalid co-orthologs, based on criteria outlined above.

Considering that phylogenetic species do not necessarily have to be monophyletic in all loci due to different rates of lineage sorting (16), the following criteria (3) were applied to recognize clades that represent independent evolutionary lineages: 1 – genealogical concordance, i.e. presence in all single-locus genealogies regardless of the level of support, 2 – genealogical nondiscordance, i.e. high support of the clade in at least one single-locus genealogy without supported contradiction in another single-locus genealogy. Only relatively distinct lineages were accepted as phylogenetic species in order to avoid that minor tip clades would have to be recognized as phylogenetic species. Boundaries of phylogenetic species were determined in such a way that no strain was left to be unclassified (3). Applying these criteria, seven phylogenetic species in *Lichtheimia* were recognized (Fig. 1a), as follows:

1 – ‘*L. ramosa* subgroup I’ containing the ex-type strains of *Absidia gracilis* (CBS 103.35) and *Absidia idahoensis* var. *thermophila* (AS3.4808);

2 – ‘*L. ramosa* subgroup II’ including the neotype strain of *L. ramosa* (CBS 582.65);

3 – ‘*L. corymbifera*’ including the neotype strain of *L. corymbifera* (CBS 429.75) and the ex-type strain of *Absidia griseola* (CBS 519.71);

4 – ‘*L. ornata*’ with the ex-type strain of *Absidia ornata* (CBS 291.66);

5 – ‘*L. blakesleeana* subgroup I’ containing the ex-type strains of *L. hyalospora* (CBS 173.67), *A. blakesleeana* var. *atrospora* (CBS 518.71), and *A. cristata* (CBS 102.36);
6 – ‘L. blakesleeana subgroup II’ and containing the ex-type strain of *L. blakesleeana* (CBS 100.36);

7 – ‘*L. sphaerocystis*’ containing 3 strains with a morphology of the sporangiophores similar to that of *L. blakesleeana*.

Strain CBS 269.65 that was used as reference strain by Garcia-Hermoso et al. (8) belongs to subgroup I of *L. ramosa*. Strain CNRMA/F05-100 that could not be assigned to a species by Garcia-Hermoso et al. (8) was identified as *L. ornata* based on ITS sequence (data not shown).

**Biological species recognition by successful mating**

Zygospores were found in 17 out of 168 mating experiments including 73 intraspecific matings (Fig. 2). Azygospores were observed in a single case (*L. ramosa* CBS 582.65 x *L. ramosa* CNM-CM3590). Twelve out of the 17 mating pairs producing zygospores were intraspecific matings and five were interspecific according to taxonomic concepts of *Lichtheimia* maintained in this paper.

Mature zygospores developing after intraspecific matings (Fig. 3a-c) were usually dark red brown, (46-) 58-77 (-91) x (38-) 48-67 (-82) µm and possessed 1-3 (-5) equatorial rings. The suspensors were rough and frequently unequal in size. High numbers of zygospores were usually achieved in intraspecific matings. Zygospores resulting from interspecific matings (Fig. 3d-f) were often smaller, less intensively colored (orange brown), they often contained large oil droplets, and the equatorial rings were frequently less pronounced or absent. Only a small number (< 10) of zygospores was found in three out of five interspecific matings. Consequently, we consider large, darkly pigmented zygospores with distinct equatorial rings as indication of successful mating within the same species. Such zygospores were present in high numbers in five matings between the two *Lichtheimia ramosa* subgroups and in a single mating between the two *L. blakesleeana* subgroups (Fig. 2). Therefore, we regard these subgroups as belonging to the same biological species. In contrast, we consider the phylogenetic species ‘*L. ornata*’ and ‘*L.
sphaerocystis', as separate species because we did not find conflicting mating results and because both possess morphological traits that distinguish them from their phylogenetic sibling species, L. corymbifera and L. blakesleena, respectively (see below).

Morphology

Culture characteristic and sporangiospore morphology

Pigmentation of colony reverse on SMA was used by Hesseltine et al. (11) and Chen and Zheng (1) to distinguish two varieties of Absidia idahoensis. Because the ex-type strain of A. idahoensis var. thermophilia was reidentified as L. ramosa, we tested the suitability of this character for species identification. However, intraspecific variability in pigmentation of mycelia and colony reverse did not exceed those between species (data not shown). Therefore this trait is regarded unsuitable for species recognition in Lichtheimia.

The morphology of sporangiophores was very similar in Lichtheimia corymbifera, L. ornata, and L. ramosa. Differences in spore shape between L. corymbifera and L. ramosa were not consistent. Strains with intermediate spore shapes existed in both species (Fig. 4 c,d) although the majority of L. corymbifera strains formed subglobose to broadly ellipsoidal sporangiospores (Fig. 4 a) and many of the L. ramosa strains produced ellipsoidal to broadly cylindrical spores (Fig. 4 b). In Fig. 1a the preponderant spore shape in each strain is mapped on the ITS tree demonstrating the inapplicability of this character for species identification.

In L. blakesleena, all strains predominantly formed subglobose or more rarely broadly ellipsoidal sporangiospores that differed in size and colour depending on the strains. The two strains constituting subgroup II (CBS 100.28, CBS 100.36) formed small (3.8–5.2 µm diam.), hyaline to brownish, smooth to rough sporangiospores. Subgroup I of L. blakesleena includes the ex-type strain of A. cristata (CBS 102.36) producing spores of the same size as subgroup II and two strains with distinctly larger sporangiospores, namely CBS 173.67, ex-type strain of L. hyalospora,
forming large (4.6–8.3 µm diam.), hyaline to subhyaline, smooth to slightly rough sporangiospores, and CBS 518.71, ex-type strain of A. blakesleeana var. atrospora, producing large (6.0–11 µm diam.), hyaline to brownish, smooth to rough sporangiospores. Both large-spored strains originate from fermented food and have probably been cultivated and subcultured for a long time. This fact and their grouping with a small-spored strain suggests that L. hyalospora and A. blakesleeana var. atrospora are morphological mutants rather than separate lineages.

**Giant cells**

Giant cells are strongly swollen, branched or unbranched, often droplet-filled hyphae with thick, refractive walls. Often they are septate especially when branched and in some strains they possess projections. Giant cells are common in all species of Lichtheimia, but their size and complexity depends on medium and growth temperature. On MEA at 24°C numerous strains of all species developed giant cells. They were also common on PDA, where the highest degrees of differentiation were noted at 24°C, and less at 33°C and 37°C. Cultivation on YEA at 37°C was especially appropriate for the discrimination between L. corymbifera and L. ornata. In L. blakesleeana, L. corymbifera and L. ramosa size and shape of giant cells varied strongly with strain, temperature and medium. In most strains they were slightly or distinctly branched. Two strains of L. ramosa (CBS 223.78 and CBS 124198) had globose, thick-walled giant cells with projections. This type of giant cells is characteristic for L. sphaerocystis (see below). L. ornata differed from its sister species L. corymbifera by forming large [380-760 (-900) x 320-660 (-770) µm], compact, densely branched giant cells (Fig. 9) in two-week-old YEA cultures. They were predominantly formed in the mycelial mat attached to the substrate and in the submerged mycelium, but also occurred, though smaller-sized and less developed, in the aerial mycelium. The agar surface of L. ornata isolates appeared characteristically granular on YEA, due to an abundance of
large submerged giant cells. In contrast, we could not find any giant cell in *L. corymbifera* in YEA cultures at 37°C.

**Growth kinetics**

Fig. 5 shows mean growth rate (mm/h) and standard deviations for each species at each temperature analysed. Significant *P* values (*p* < 0.05) were found in all ANOVA analyses performed for each temperature. The largest distinctions between species were found at 40°C and 43°C. At 43°C *L. ramosa* could clearly be distinguished by its growth rate from *L. corymbifera* and *L. ornata* while *L. hyalospora* (including *L. blakesleeana*, which is reduced to synonymy below), and *L. sphaerocystis* did not grow at this temperature. *L. ramosa* was the species with the highest growth velocity at all temperatures. Twenty six out of 30 (86.7%) strains of *L. ramosa* grew at 49°C, but only two strains out of 12 (16.7%) of *L. corymbifera*. According to our experimental design, the maximum growth temperature was 46°C for *L. ornata*, 40°C for *L. blakesleeana*, and 37°C for *L. sphaerocystis* except for a single strain that grew at 40°C.

**Synopsis of the results and taxonomic conclusions**

On the basis of five successful matings suggesting the absence of an intrinsic reproductive barrier and lack of diagnostic morphological and physiological characters, we judge that the two subgroups of *Lichtheimia ramosa* represent a single species. We also favour to retain the two phylogenetic entities, subgroup I and subgroup II of *L. blakesleeana* within the same species, because of the formation of a high number of large, dark coloured zygospores found in a mating between the subgroups, and because of the absence of phenotypically distinctive traits. The basionym of *L. hyalospora* is older than that of *L. blakesleeana* and hence the correct name for the resulting species is *L. hyalospora*. The large-spored strains within this species are considered as mutants because
they form a subgroup with the small-spored environmental strain CBS 102.36 in all three
genealogies.

The two phylogenetic entities, ‘L. corymbifera’ and ‘L. ornata’ were distinct and well-differentiated
in all three gene-genealogies. In addition, the groups formed different types of giant cells.
Therefore, we suggest to maintain L. ornata as a separate species. ‘L. sphaerocystis’ was also
distinct and well-differentiated in all three gene-genealogies and differed morphologically from its
sibling species L. hyalospora by the formation of consistently globose giant cells. For this reasons
we describe it as a new species below.

The following taxonomy is proposed (for more synonyms see refs 4 and 12):

MycoBank MB416447.
Synonyms:
Mycocladus corymbifera (Cohn) J. H. Mirza, in Mirza et al., Mucor. Pakistan (Faisalabad): 95
(1979), comb. inval., Art. 36.1.
Absidia griseola H. Naganishi & Hirahara, in Naganishi & Hirahara, Bull. Hiroshima Jogakuin

Mycobank MB416448.
Replaced synonym: Mucor ramosus Lindt, Arch. Exp. Path. Pharmacol. 21: 269 (1886); nom.
illegit., Art. 53.1.
Synonyms:


8. Synonyms:


15. *Lichtheimia ornata* (A. K. Sarbhoy) A. Alastruey-Izquierdo & G. Walther, **comb. nov.**


*Lichtheimia sphaerocystis* A. Alastruey-Izquierdo & G. Walther, *sp. nov.*

MycoBank MB516505, Fig. 6a-i, Fig. 7a-w.

Etymology: Refers to the globose shape of the giant cells.

Latin diagnosis:

Coloniae extensae, cotoneae vel coactae, albae vel cellulis giganteis copiosis cremeae vel sporangiophoris griseae, reversum ochraceum. Temperatura crescentiae optima 33°C, maxima 40°C. Sporangiophora simplicia vel ramosa, singula vel bina orientia, recta vel incurva vel circinata; septum subsporangiale plerumque absens. Sporangia globosa vel pyriformia, multispora, deliquescentia, atrobrunnea vel atra; sporangia maxima terminalia apophysi conica conspicua praedita, 16–43 µm diam; columellae ellipsoidae vel sursum angustatae vel raro subglobosae, rarissime una vel duabus projectionibus praeditae, 8.5–33 × 6.8–29 mm, collari praesente vel absentе. Sporangiosporae leves vel asperulatae, hyalinae vel dilute brunneae, acervatae fuscae, subglobosae vel late ellipsoidae vel modice irregulares, 3.5–7 µm diam vel 4.2–6.8 × 3.3–5.5 µm. Cellulae giganteae intercalares, globosae, 60–150 µm diam (strato mucido excluso), saepe

Colonies expanding on MEA at 24°C, cottony to felty, at first white, later, depending on the proportion of sporangia and giant cells: grey to dark grey (lavender grey to leaden grey according to Rayner 1970) in colonies with predominant sporangiophores, consistently white to cream-coloured in colonies with predominant giant cells, reverse ochreous. On MEA optimal growth temperature 33°C, maximal growth temperature 40°C, no growth at 43°C. Sporangiophores simple or branched, arising solitarily or in pairs but not in whorls, either directly from the substrate or from aerial hyphae, hyaline, often with light brown apophysis and columella, smooth or slightly rough, straight, bent or circinate. Subsporangial septa mostly absent. Sporangia spherical to pyriform (including apophysis), multi-spored, deliquescent, blackish brown to black, largest sporangia terminal, with conspicuous conical apophysis, 16-43 μm diam. Columella ellipsoidal, ellipsoidal-tapering or more rarely subglobose, occasionally with one or more projections, 8.5-33 × 6.8-29 μm, with or without collar. Sporangiospores smooth- to rough-walled, hyaline to light brown, dark brown in mass, subglobose to broadly ellipsoidal or slightly irregular, 3.6-7.0 μm in diameter or 4.2-6.8 × 3.3-5.5 μm. Large intercalary hyphal swellings (giant cells) in aerial hyphae and in the mycelium attached to the medium, but not in the mycelium permeating the medium (substrate mycelium), spherical, 60-150 μm diam. (excluding the mucous layers), septate, often droplet-filled, thick-walled, often with simple or branched projections. Cell wall of the giant cells consisting of 2 refractive layers with a total thickness of 3-14 μm, enclosed by 2 to several mucous layers up to 31 μm thick. Projections 9-24 (-40) μm long. CBS 648.78 ceased to form giant cells after several transfers. Stolons and rhizoids present. Heterothallic. Zygospores not observed.


Key to the species

Based on our morphological and physiological results we developed the following key for phenotypic identification of all accepted Lichtheimia species. Some characters for the discrimination of Absidia s.str. and Lentamyces were adopted from Hesseltine & Ellis (12) and Hoffmann et al. (13, 15):

1a. Subsporangial septa present; growth of aerial hyphae indeterminate; most of the sporangiophores in whorls; not thermotolerant; no or reduced growth at 37°C; zygospores with appendaged suspensors..........................Absidia s.str.

1b. Subsporangial septa present; aerial hyphae generally ending in a sporangium; whorls of sporangiophores absent; not thermotolerant; no growth above 30°C; homothallic; zygospores warty, without appendaged suspensors..........................Lentamyces

1c. Subsporangial septa absent or rare; aerial hyphae generally ending in a sporangium; whorls of sporangiophores present in some species but not obvious; thermotolerant; typically good growth at 37°C; heterothallic; zygospores with equatorial rings, without appendaged suspensors......................

.................................................................................................................Lichtheimia (2)
2a. Sporangia dark brown or dark grey to black; no continued growth at 43°C (initial growth occurring in rare cases stops after less than a day); mature sporangiospores rough and/or > 6.5 µm in their longest extension……………………………………………………………………………3

2b. Sporangia light brownish grey; continued growth at 43°C; mature sporangiospores smooth and < 6.5 µm in their longest extension………………………………………………………………………………………………………………………………………………………………5

3a. Giant cells consistently globose, 60-150 µm diam. (Fig. 6g-i, Fig. 7s-w) …………………….…

……………………………………………………………………………………………………………………………………………………………………………..Lichtheimia sphaerocystis

3b. Giant cells (if present) more hypha-like, irregularly swollen, simple to strongly branched, never consistently globose (Fig. 8) ………………………………...…………..

Lichtheimia hyalospora (4)

4a. Mature sporangiospores small (< 5.5 µm), rough, and brownish …………………………small-spored variants of Lichtheimia hyalospora

4b. Mature sporangiospores larger (on the majority > 5.5 µm), smooth or rough, hyaline or brownish…………large-spored variants of Lichtheimia hyalospora

5a. Colony diameter at 43°C after 72 h > 40 mm (average growth rate of 1.3 mm/h, growth rate range 0.5 – 3.2 mm/h); spores ellipsoidal to cylindrical or subglobose to broadly ellipsoidal……

…………………………………………………………………………………Lichtheimia ramosa

5b. Colony diameter at 43°C after 72 h < 27 mm (average growth rate of 0.4 mm/h, growth rate range 0.1 – 1.0 mm/h); spores never consistently ellipsoidal to cylindrical…………………….6

6a. Giant cells densely branched (Fig. 9), 380-760 (-900) × 320-660 (-770) µm, present in 2-week-old YEA cultures……………………………………………………………………………………………………………………………………………………………………………………………………...Lichtheimia ornata

6b. Giant cells absent from 2-week-old YEA cultures……………………Lichtheimia corymbifera
Clinical relevance and distribution

Clinical stains are underlined in red in the ITS tree (Fig. 1a). Judging from these data only species with more pronounced thermotolerance, namely *L. corymbifera*, *L. ornata*, and *L. ramosa*, are clinically relevant. *Lichtheimia corymbifera* and *L. ramosa* seem to be relatively common etiological agents, while *L. ornata* has been isolated only twice from clinical sources: once from skin of a 51-year-old female undergoing an amputation in France (8) and a second time from a wound of a 50-year-old male in Spain. The data suggest similar distribution areas for all clinically relevant species although only strains originating from Asia and Europe were available for study and the number of strains was too low to infer geographic distribution.

Discussion

**Phylogenetic species recognition and molecular markers**

As far as we are aware this is the first study applying GCPSR to an entire genus of the Mucorales. We found multilocus analyses in *Lichtheimia* to be hampered by the common presence and the high number of paralogs necessitating labour- and cost-intensive cloning steps. Another problem for applying GCPSR is the limited variability of protein-coding genes compared to the ITS region. Hence, with three molecular markers representing only two loci the number of markers used in our study is relatively low for GCPSR. Nevertheless, we consider the resulting species concepts to be reliable because the molecular results are not conflicting among one another, and are furthermore not in conflict with the recognized biological species that are supported by morphological and physiological findings.

The calculated error rate for the polymerase used in this study reaches from 0.02 to 0.11 errors per thousand basepairs according to the literature cited by the manufacturer (http://www.bioline.com/faq/h_biotaqfaq.asp). However, in our study the polymorphism between
the inparalogs was significantly higher than 1.0 per thousand in most cases. The low error rate of the polymerase used is further supported by the fact that ITS and COI sequences that were generated directly and via cloning were all identical. For these reasons we are convinced that the detected sequence differences are true polymorphisms. Abundance of paralogous sequences is also reported for *Rhizopus arrhizus* (as *R. oryzae*) another member of the Mucorales (20). The analysis of the genome sequence of *R. arrhizus* revealed an ancient whole-genome duplication event together with recent gene duplications leading to expanded gene families with a high number of paralogs.

While most eukaryotes such as animals (26), plants (23), and slime molds (44) are known to have a small actin gene family, fungi were supposed to possess only a single copy for actin, based on results from yeasts like *Saccharomyces cerevisiae* (6) and *Schizosaccharomyces pombe* (24) and filamentous fungi such as *Aspergillus nidulans* (5), *Trichoderma reesei* (22), and *Neurospora crassa* (42). However, recently the zygomycetous species *Abidia glauca* and the basidiomycetous fungi *Schizophyllum commune* and *Suillus bovinus* have been reported to carry two actin-encoding genes (39). Our data suggest the existence of a gene family for actin in the studied species of *Lichtheimia*. The two outparalogs of actin show a high degree of nucleotide polymorphisms including exonic sequences, which suggests a duplication event before the diversification process of the Mucorales. This fact should be taken into account when using actin for phylogenetic analysis, especially at lower taxonomic levels, because the choice of the outparalog may influence the position of the species in the tree topology.

Sequence differences among actin inparalogs originating from the same outparalog were small, and several actin inparalogs were found in all species studied. Cases in which gene copies (repeat copies, ‘inparalogs’) in a genome are more similar to each other than to their respective ‘orthologous’ counterparts (repeats; ‘co-orthologous inparalogs’) in closely related species could be caused: 1 - by duplications following speciation in all species, 2 - by duplications preceding
speciation and the presence of mechanisms such as concerted evolution retaining similarity among ancient duplicates, or 3 - both, duplications before and after speciation as in birth-and-death-evolution combined with permanent losses of gene copies (7, 27). We can only speculate on the origin of the paralogs (‘inparalogs’) in the actin gene family. The most likely process is birth-and-death-evolution because it is assumed for many protein-coding gene families (27), and our finding of pseudogenes supports this assumption. In this case the term ‘inparalog’ used here would be misapplied to some extent, but we kept this terminology for a better understanding. Although the presence of several inparalogs is an obvious disadvantage for the use in phylogenetic studies, we think that the use of the actin gene was legitimate because of the comparatively low sequence variations between actin inparalogs, and the lack of an alternative locus.

**Biological species recognition by mating test**

The relatively low number of successful intraspecific matings (16.4%) corresponds with the results of Ellis and Hesseltine (4), who found only 4.7% of successful matings in *L. corymbifera* (as *Absidia c*.). Successful interspecific matings were previously described for *L. corymbifera* and *L. ramosa* (5) and for *L. ornata* (CBS 958.68, as *Absidia hesseltinei*) and *L. ramosa* (CBS 270.65, as *L. corymbifera*) (36), and also for other genera such as *Mucor* (35). As in the present study, Schipper (35) found zygospores resulting from interspecific matings in the *Mucor racemosus* group to be less coloured and reduced in number and size. The existence of interspecific zygospores reduces the significance of successful mating for species recognition, even though there is doubt about their occurrence under natural conditions, and necessitates a detailed analysis of the zygospores. However, mating test results are useful to detect the lack of an intrinsic reproductive barrier when number, appearance, and size of the zygospores are taken into account. Obviously, mating barrages between species in the Mucorales are not as developed as in other fungal groups.
allowing the formation of zygospores between species that share only 79.8% similarity in their ITS sequences, as in the case of *L. hyalospora* CBS 100.36 x *L. ramosa* CBS 100.49.

**Diagnostic characters**

Our results show that sporangiophore morphology and spore shape are insufficient to differentiate *L. corymbifera*, *L. ornata*, and *L. ramosa* and that additional characters are needed. In contrast, Garcia-Hermoso et al. (8) found significant differences in length, width and length/width ratio of the sporangiospores of *L. corymbifera* and *L. ramosa*. They described further discriminative characters such as different texture of growth or slightly colored sporangiospores in *L. ramosa* that we did not detect in our study. However, their morphological findings were based on 5 strains of each species only, which possibly did not cover variability of these species.

The presence of intermediate spore shapes in *L. corymbifera* and *L. ramosa* found in this study may explain why earlier mycologists tended to treat them as synonyms (28, 36). These intermediate spore shapes might also explain why studies that used the spore shape for discriminating both species encountered numerous intraspecific matings (28). Growth rates at certain temperatures as well as appearances of giant cells provided additional valuable characters for species recognition.

The ITS region is the marker of choice for molecular identification because of its high variability and the possibility of direct sequencing in most cases.

**Taxonomy**

This study shows that *L. corymbifera* and *L. ramosa* are distinct species on the basis of molecular data, as already supposed by Ellis and Hesseltine (4) using morphology and mating tests and by Garcia-Hermoso (8) based on sequence analyses. *Lichtheimia ornata* was distinguished by the formation of ‘peculiar thick-walled globose to elongated bodies with very prominent finger-shaped appendages’ (34, p. 999, as *Absidia ornata*). We consider these structures as early stages of the
giant cells that are characteristic for this species although their size described as 44-70 µm is relatively small.

Schipper (36) already recognized *L. sphaerocystis* and *L. hyalospora* (as *L. blakesleeana*) as distinct taxa based on morphological features, referring to strains of *L. sphaerocystis* as ‘*Absidia aff. blakesleeana*’. The degradation of *L. blakesleeana* to a synonym of *L. hyalospora* is supported by the presence of zygospores between strain NRRL 2983, formerly named *A. hyalospora* and strain NRRL 1306 formerly named *A. blakesleeana* (12, p. 779). However, with respect to *A. blakesleeana* the authors stated on page 782 of the same publication ‘the only closely related species is *A. hyalospora* which has much larger spores; it does not seem to fruit on Czapek’s solution agar, and it does not mate with *A. blakesleeana* tester strains’. All three isolates of *L. hyalospora* studied by Hesseltine and Ellis (12) were isolated from fermented food (‘taosi’ or soybeans). Continued subcultivation of single strains for fermentation might have enhanced the occurrence of mutants whose phenotypes can be characterized by an increase in spore size.

In conclusion, by comparing gene genealogies based on three molecular marker (ITS, actin, and LSU) and by considering mating results and differences in morphology and growth rates at different temperatures, we recognize five species in *Lichtheimia*: *L. corymbifera*, *L. ornata*, *L. ramosa*, *L. hyalospora*, and *L. sphaerocystis*. Only the first three species seem to have clinical significance.

**Acknowledgements**

We are grateful to Walter Gams for providing the Latin diagnosis, for important advices in nomenclatural approaches and experimental design, and for critical reading the manuscript. Grit Walther thanks Ursula Eberhardt for helpful discussions and advices. Kerstin Voigt thanks Ru-yong Zheng (Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology,
Chinese Academy of Sciences, Beijing, China) for providing the ex-type strain of *Absidia idahoensis* var. *thermophila* (strain AS 3.4808).

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Figure legends

Fig. 1. Maximum parsimony phylograms of three different markers: a. ITS, b. actin [small-scale tree includes outparalog I (blue frame) and outparalog II (red frame), large-scale tree represents only outparalog I (blue frame in the small-scale tree)], c. D1/D2 region of the LSU. Branches with bootstrap values in the MP analysis of 85 or higher are printed in bold. Branch support values are indicated by numbers near the branches (MP bootstrap proportions/Bayesian posterior probabilities) but given only for decisive branches. Strains forming a well-supported group in the ITS phylogram are marked in a certain colour. The same colour is used for each strain also in the LSU and the actin phylogram. In the ITS phylogram, clinical strains are underlined in red, names are given at the type strains. For each strain of *L. ramosa*, *L. corymbifera*, and *L. ornata* the predominant spore shape is mapped on the ITS phylogram: white/spherical – subspherical to broadly ellipsoidal, subspherical predominant; light grey/broadly ellipsoidal – subspherical to broadly ellipsoidal; medium grey/broadly ellipsoidal - subspherical to broadly ellipsoidal, broadly ellipsoidal predominant; dark grey/ellipsoidal – broadly ellipsoidal to ellipsoidal; black/ellipsoidal – ellipsoidal to broadly cylindrical. Respective numbers of clones (‘c’) are given after the strain numbers. Actin sequences belonging to single-spore cultures obtained in this study are indicated by ‘ss’.

Fig. 2. Results of 73 intraspecific and 95 interspecific matings in *Lichtheimia*. X- no mating test performed, white field – no zygospore formation, large grey field – formation of large dark brown zygospores, number of zygospores lower than 10, small grey field – formation of smaller orange brown zygospores, number of zygospores lower than 10, large black field - formation of large dark red brown zygospores, number of zygospores higher than 10, small black field - formation of smaller orange brown zygospores, number of zygospores higher than 10.

Fig 3. Zygospores of *Lichtheimia*. A-c. intraspecific matings: a. *L. blakesleeana* CBS 100.28 x CBS
102.36, b. *L. ramosa* CBS 124198 x CBS 100.24, c. *L. ramosa* CBS 271.65 x CBS 223.78. D-f. interspecific matings: d. *L. blakesleeana* CBS 100.28 x *L. ornata* CBS 958.68, e. *L. ramosa* CBS 124198 x *L. ornata* CBS 958.68, f. *L. blakesleeana* CBS 100.36 x *L. ramosa* CBS 100.49. Scale bar = 50 µm.

Fig. 4. Spore shape in *Lichtheimia corymbifera* (a. and c.) and *L. ramosa* (b. and d.): a. subglobe to broadly ellipsoidal shape of *L. corymbifera* (CBS 429.75 NT), b. ellipsoidal to cylindrical shape of *L. ramosa* (CBS 582.65 NT), c. predominantly broadly ellipsoidal shape in *L. corymbifera* (CBS 100.31), d. predominantly broadly ellipsoidal shape in *L. ramosa* (CBS 112528).

Fig. 5. Mean growth rate (mm/h) and standard deviations of *Lichtheimia* species at eight different temperatures.

Fig. 6. *Lichtheimia sphaerocystis*, CBS 420.70 (T): a. aerial hypha bearing sporangiophores, scale bar = 25 µm, b-e. sporangiophores with columella, scale bar = 40 µm, f. sporangiospores, scale bar = 20 µm. g. mature giant cell, h. young giant cell; CBS 648.78: i. mature giant cell, scale bar 40 µm.

Fig. 7. *Lichtheimia sphaerocystis*: a. colony surface of CBS 420.70, b. colony reverse of CBS 420.70, c. colony surface of CBS 648.78 (predominant giant cell formation), d. colony surface of CBS 647.78 (predominant sporangiospore formation), e-f. CBS 647.78, sporangiophores, scale bar 50 µm, h-j. CBS 647.78, sporangiophores, k-m. CBS 420.70, part of the sporangiophore with mature sporangium, n-q. CBS 420.70, columella, r. CBS 420.70, sporangiospores, s-v. CBS 420.70, young (s and t) and mature (u and v) giant cells, w. CBS 648.78, mature giant cell, scale bars (h-w) = 20 µm.
Fig. 8. Giant cells of *Lichtheimia hyalospora* formed in PDA cultures: a. CBS 102.36, b. CBS 100.36, scale bars = 100 µm.

Fig. 9. Giant cells of *Lichtheimia ornata* formed in two weeks old YEA cultures: a. younger giant cell formed by CBS 291.66, b. mature giant cell formed by CNM-CM4978, scale bar = 100 µm.
Table 1. Strains used in this study

<table>
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<th>Strain</th>
<th>Species</th>
<th>Country</th>
<th>Source</th>
<th>GenBank accession no.</th>
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<td>L. corymbifera</td>
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<td>Kurone</td>
<td>GQ342889</td>
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<td></td>
<td>(T of A. griseola)</td>
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<td>Manihot esculenta; stem</td>
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<td>fermented food taosi</td>
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<td>Kurone</td>
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<td>291.66</td>
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<td>n.a.</td>
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<td>human, bronchoaspirate</td>
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<td>Strain</td>
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<td>CBS 647.78</td>
<td>L. sphaerocystis</td>
<td>India</td>
<td>dung of mouse</td>
<td>GQ342899, GQ342911, GQ342757, GQ342759</td>
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<td>CBS 648.78</td>
<td>L. sphaerocystis</td>
<td>India</td>
<td>soil</td>
<td>GQ342901, GQ342916, GQ342758, GQ342762</td>
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Table 2. Primer used for amplification and sequencing of nine different genomic regions in order to select marker for GCPSR.

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<thead>
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<th>Region</th>
<th>PCR primer</th>
<th>Sequencing primer</th>
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<tr>
<td>actin</td>
<td>Act-1 5'-TGGGACGATATGGAIAAIATCTGGCA-3' (46)</td>
<td>Act-1 5'-TGGGACGATATGGAIAAIATCTGGCA-3' (46)</td>
</tr>
<tr>
<td></td>
<td>Act-4ra 5'-</td>
<td>Act-4ra 5'-</td>
</tr>
<tr>
<td></td>
<td>TCITCGTATTCTTGCTTIGAIATCCACAT-3' (46)</td>
<td>TCITCGTATTCTTGCTTIGAIATCCACAT-3' (46)</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>B36f modified 5'-CACCCACTCMCTYGGTGGTG-3' (41)</td>
<td>B36f modified 5'-CACCCACTCMCTYGGTGGTG-3' (41)</td>
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<td>B12r modified 5'-</td>
<td>B12r modified 5'-</td>
</tr>
<tr>
<td></td>
<td>CATGAAGAARTGRAGACGVGGGAA-3' (41)</td>
<td>CATGAAGAARTGRAGACGVGGGAA-3' (41)</td>
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<td>M-cox1-fa 5'-GATATGGGCATTTCCTCGAT-3''</td>
<td>M-cox1-fa 5'-GATATGGGCATTTCCTCGAT-3''</td>
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<td></td>
<td>M-cox1-rb 5'-</td>
<td>M-cox1-rb 5'-</td>
</tr>
<tr>
<td></td>
<td>GGWACTGCAATAATCATTTGTAGC-3''</td>
<td>GGWACTGCAATAATCATTTGTAGC-3''</td>
</tr>
<tr>
<td>EF-1α</td>
<td>MEF-1 5'-ATGGGTAAGARAAGACTCACG-3' (30)</td>
<td>MEF-1 5'-ATGGGTAAGARAAGACTCACG-3' (30)</td>
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<td>MEF-4 5'-ATGACACCCRAACACGCAGGTGTG-3' (30)</td>
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<td>MEF-11 5'-AAGAAGATTTGGTTTCAAACC-3' (30)</td>
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<td>ITS</td>
<td>V9G 5'-TTACGTCCCTGCCCTTTGTGA-3'(2)</td>
<td>V9G 5'-TTACGTCCCTGCCCTTTGTGA-3'(2)</td>
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<td>LR3 5'-GGTCCGTGTGTCTGTTAAGAC-3'(45)</td>
<td>LR3 5'-GGTCCGTGTGTCTGTTAAGAC-3'(45)</td>
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<td>ITS1 5'-TCGCAGTGGAACCTGCAGG-3'(47)</td>
<td>ITS1 5'-TCGCAGTGGAACCTGCAGG-3'(47)</td>
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<td>ITS4 5'-TCCTCCTCCTATGGATATGC-3'(47)</td>
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<td>LS266 5'-GCATTCACAAACACTGACTC-3'(9)</td>
<td>LS266 5'-GCATTCACAAACACTGACTC-3'(9)</td>
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mtSSU  mrSSU1 5'AGCAGTGAGGAATATTGGTC-3' (48)  mrSSU1 5'AGCAGTGAGGAATATTGGTC-3' (48)
mrSSU3r 5'ATGTGGCAGCCTATAGCCC-3' (48)  mrSSU3r 5'ATGTGGCAGCCTATAGCCC-3' (48)
nucLSU  V9G 5'TTACGTCCCTGCCCTTTGTA-3' (2)  NL1 5'GCATATCAATAAGCGGAGGAAAAG-3'(29)
LR3 5'GGTCCGTGGTTTCAAGAC-3'(45)  LR3 5'GGTCCGTGGTTTCAAGAC-3'(45)

RPB1  RPB1-Df modified 5'-
TAYAACCGNGATTTCGATGG-3' (38)  RPB1-Df modified 5'-
RPB1-Fr modified 5'-
CCTTCAGACCACCATAGC-3' (38)  RPB1-Fr modified 5'-

RPB2  RPB2-6f modified 5'-
CCYGCWGAACKCCMGAAGG-3b  RPB2-6f modified 5'-
bRPB2-7r1 5'-
CCCATRGCGTGYTTMCCATDGC-3b  bRPB2-7r1 5'-

1
2  a designed for this study.
3  b B. Matheny, 2006, PCR primers to amplify and sequence rpb2 (RNA polymerase II second largest
4  subunit) in the Basidiomycota (Fungi)
5  (http://www.clarku.edu/faculty/dhibbett/rpb2%20primers.htm).
6
Table 3. Maximal similarity values between six pairs of strains for nine different genomic regions (ITS, partial β-tubulin, partial RPB2, partial actin, partial RPB1, D1/D2 region of the LSU, partial EF-1α, partial COI, partial mtSSU).

<table>
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<tr>
<th>pairs of strains</th>
<th>similarity values in % between the pairs of strains</th>
<th>paralogous conditions</th>
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</thead>
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<tr>
<td>ITS</td>
<td>86.0 82.0 79.2 81.3 92.4 81.1</td>
<td>polymorphism among copies (paralogs) detected in two strains only, remaining strains directly sequenced</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>91.2 90.0 91.2 88.4 92.4 88.1</td>
<td>several paralogs with large sequence differences, selection of co-orthologous paralogs not possible</td>
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<tr>
<td>RPB2</td>
<td>n.a. n.a. n.a. 90.0 97.5 89.7</td>
<td>no outparalogs detected, several inparalogs with small sequence differences</td>
</tr>
<tr>
<td>actin</td>
<td>91.3 91.0 94.2 91.6 96.2 92.1</td>
<td>two outparalogs in 3 species, several inparalogs with small sequence differences</td>
</tr>
<tr>
<td>RPB1</td>
<td>92.4 92.6 91.1 91.8 96.7 92.1</td>
<td>two outparalogs, several inparalogs with small sequence differences</td>
</tr>
<tr>
<td>LSU</td>
<td>93.9 93.7 93.4 95.5 98.1 95.3</td>
<td>probably no polymorphism among copies (paralogs) (directly sequenced)</td>
</tr>
<tr>
<td>EF-1α</td>
<td>94.4 94.1 94.9 94.0 97.7 93.0</td>
<td>no outparalogs detected, numerous inparalogs with large sequence differences forming subclades (at least 2 duplications after speciation)</td>
</tr>
<tr>
<td>COI</td>
<td>97.8 98.2 98.4 97.9 98.2 98.1</td>
<td>no paralogs detected</td>
</tr>
<tr>
<td>mtSSU</td>
<td>100 100 100 100 100 100</td>
<td>probably no paralogs (directly sequenced)</td>
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<td>L. blakesleeanus (T of A. cristata) (-)</td>
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<td>L. blakesleeanus var. atmospera (T)</td>
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<td>L. blakesleeanus (-)</td>
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<td>L. cereus (-)</td>
<td>CBS 190.36</td>
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<td>L. cereus (-)</td>
<td>CBS 207.70</td>
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