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Mortierellomycotina subphyl. nov., based on multi-gene genealogies

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Abstract — The *Mucoromycotina* unifies two heterogenous orders of the sporangiferous, soil-inhabiting fungi. The *Mucorales* comprise saprobic, occasionally facultatively mycoparasitic, taxa bearing a columella, whereas the *Mortierellales* encompass mainly saprobic fungi lacking a columella. Multi-locus phylogenetic analyses based on eight nuclear genes encoding 18S and 28S rRNA, actin, alpha and beta tubulin, translation elongation factor 1alpha, and RNA polymerase II subunits 1 and 2 provide strong support for separation of the *Mortierellales* from the *Mucoromycotina.* The existence of a columella is shown to serve as a synapomorphic morphological trait unique to *Mucorales,* supporting the taxonomic separation of the acolumellate *Mortierellales* from the columellate *Mucoromycotina.* Furthermore, irregular hyphal septation and development of subbasally vesiculate sporangiophores bearing single terminal sporangia strongly correlate with the phylogenetic delimitation of *Mortierellales,* supporting a new subphylum, *Mortierellomycotina.*

Key words — *Zygomycetes,* SSU rDNA, LSU rDNA, protein-coding genes, monophyly

Introduction

The type species of *Mortierella* Coem. 1863, *M. polycephala* Coem. 1863, was originally isolated from a parasitic interaction with a mushroom and named in honour of M. Du Mortier, the president of the Société de Botanique de Belgique (Coemans 1863). However, the common habit of mortierellalean species is as soil saprobes, enabling the fungi to grow on excrements, decaying plants, or (not infrequently) on decaying mushrooms and mucoralean fungi (Fischer 1892). Members of the *Mortierellales* do occasionally occur as opportunistic pathogens in animals and humans as well (de Hoog et al. 2009: 79). Because of the morphological differences to other mucoralean-like fungi, the genus was classified in a separate family, *Mortierellaceae* A. Fisch. 1892 (Fischer 1892), which is the only family within the *Mortierellales* Caval.-Sm. 1998 (Cavalier-

Smith 1998). In addition to *Mortierella,* the *Mortierellaceae* includes a further five genera, *Aquamortierella* Embree & Indoh 1967, *Dissophora* Thaxt. 1914, *Gamsiella* (R.K. Benj.) Benny & M. Blackw. 2004, *Lobosporangium* M. Blackw. & Benny 2004 (≡ *Echinosporangium* Malloch 1967, nom. illegit.), and *Modicella* Kanouse 1936. Currently, the *Mortierellales* are classified within the subphylum *Mucoromycotina* Benny 2007 with the *Mucorales* Fr. 1832 and *Endogonales* Moreau ex R.K. Benj. 1979 (Hibbett et al. 2007). This classification, based upon informal supertrees, abandons the phylum *Zygomycota* and recognizes four subphyla of uncertain phylogenetic position: *Zoopagomycotina, Entomophthoromycotina, Kickxellomycotina,* and *Mucoromycotina.* Supermatrix-based phylogenetic approaches using multiple genes in concatenated analyses place the *Mortierellales* basal to the *Mucorales* (Tanabe et al. 2004, James et al. 2006). In contradiction to this, the *Mortierellales* has also been shown to be more closely related to the *Dikarya* and *Glomeromycota* (Benny et al. 2001, Liu et al. 2009, Voigt et al. 2009).

This study provides evidence for the missing sister relationship between both orders. All attempts to constrain the monophyly were statistically insignificant and rejected under the null hypothesis. Therefore, the possibility of a common ancestor for both orders is unlikely. Combined Bayesian inference, maximum likelihood, maximum parsimony and distance phylogenetic analyses imply clear separation of the *Mortierellales* and the *Mucorales.* Both orders appear as well-separated, monophyletic groups in single and combined analyses. Two synapomorphic morphological features are identified that reliably characterize, strongly support, and justify the introduction of a new subphylum, *Mortierellomycotina.*

Materials & methods

Sequence and Phylogenetic Analyses. Nucleotide sequences encoding the nuclear small (18S) and large (28S) ribosomal RNA (rDNA), actin (act), translation elongation factor 1 alpha (tef), RNA polymerase II largest subunit (rpb1), RNA polymerase II second largest subunit (rpb2), alpha- (atub) and beta-tubulin (btub) from twenty-seven fungal species were retrieved from GenBank (www.ncbi.nlm.nih.gov), aligned and subjected to single and combined phylogenetic analyses using Bayesian inference, maximum likelihood, maximum parsimony and distance algorithms. In addition, amino acid sequences were also obtained from the protein-coding genes and used for validation of the tree topologies. Furthermore, nucleic acid sequences available from genome projects were screened for genomic regions, which are orthologous to the phylogenetic marker genes selected in this study. Accession numbers of the sequences retrieved from GenBank as well as the loci from the genome projects (http://genome.jgi-psf.org/ and www.broadinstitute.org/) are indicated by the taxon labels in Fig. 1. Individual alignments of the ribosomal DNA sequences and the protein-coding sequences were performed using ClustalW implemented in BioEdit version 7.0.9.0 (Hall 1999). Considering the triplet-based coding of the protein sequences, these sequences

were re-translated into their nucleic acid sequences using RevTrans (Wernersson & Pedersen 2003, www.cbs.dtu.dk/services/RevTrans/). Ambiguously and divergently aligned blocks were removed from the alignments prior to phylogenetic analysis, which was finally conducted in individual and all possible gene combinations of concatenated analyses. Two phylogenetic trees, which are representative for these analyses, are shown in Fig. 1. The phylogram presented in Fig. 1A bases on a combined alignment of the nuclear ribosomal DNA consisting of a total of 2103 characters (1518 and 585 characters for 18S and 28S rDNA, respectively). The phylogram presented in Fig. 1B was computed based on an aligned supermatrix concatenating a total of 5382 characters of the protein-coding genes (807; 1515; 1890 and 1170 characters for act, rpb1, rpb2 and btub, respectively). For the trees shown in Fig. 1A and 1B, respectively, the combined nucleotide data sets of the ribosomal DNA and the protein-genes were subjected to MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The Bayesian inference was initiated from a random starting tree. Two independent runs with four chains each were conducted for 2,500,000 generations with samples collected every 2500th generation. After discarding the first 25% of the generated trees (burn-in) the consensus tree was calculated using the halfcompat option. Posterior probabilities (in percent) are indicated at the nodes and represent node confidence values. Additional bootstrap analyses (Felsenstein 1985; 50% majority rule) with 100 and 1000 replicates of maximum likelihood (RaxML; Stamatakis 2006; Stamatakis et al. 2008) and neighbor-joining (Saitou & Nei 1987) searches, respectively, were used to calculate clade stability. The amoeboid protist genus *Nuclearia* was chosen as outgroup taxon because it appears to be the closest extant relative to the fungi (Liu et al. 2009). The probability of alternative phylogenetic tree topologies constraining the phylogenetic relationship of the *Mucorales* and *Mortierellales* unified within a common monophyletic clade was evaluated by several independent statistical tests, such as (i) the Kishino-Hasegawa test (Kishino & Hasegawa 1989), (ii) the Templeton test (Templeton 1983) and (iii) the winning-sites test (Prager & Wilson 1988), based on the evaluation of the number of steps necessary to find the most parsimonious tree as implemented within PAUP*v4.0b10 (Swofford 1998). Additionally, the number of trees found after reaching a stationary phase (stationarity) from the constraint topology in the Bayesian analyses were compared to the number of trees without particular topological constraints using PAUP*v4.0b10.

Identification of Sequence Signatures. Exonic nucleotide and protein sequences of the genes encoding act, rpb1, and tef were identified as suitable marker genes for short unique sequence regions that differentiate the *Mortierellales* from the *Mucorales*. The sequence fragments were subjected to BLAST (Altschul et al. 1990, 1997) at NCBI in order to exclude identical sequence regions within the order *Mucorales*. These discriminatory sequences were defined as signature sequences and are displayed in table 1.

Results & discussion

Phylogenetic tree reconstructions

In 2007, Hibbett et al. proposed a re-classification of the *Fungi,* which is now widely accepted, abandoning the phylum *Zygomycota* which was reported as

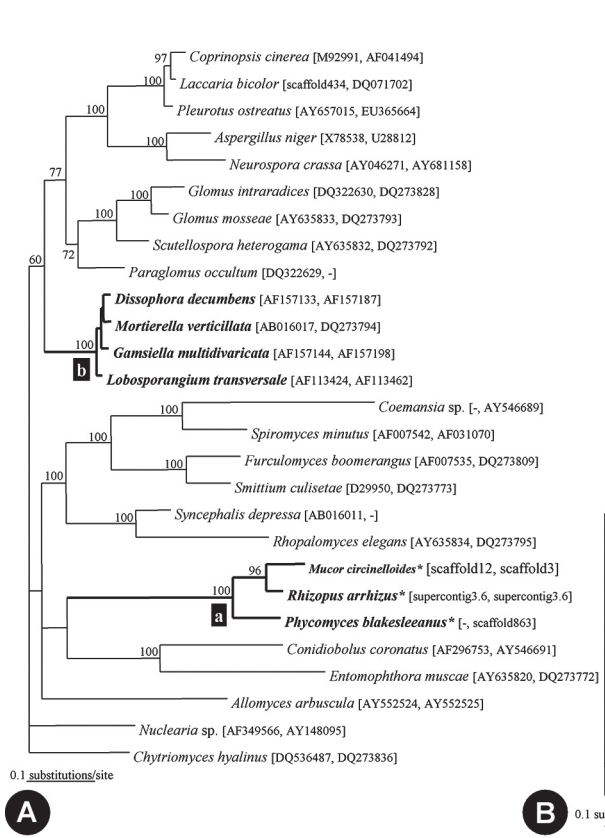
Table 1. Signature sequences derived from sequences in GenBank of all available genera of the subphylum *Mortierellomycotina* (as of 1 March 2010). Signature sequences were identified based on aligned amino and nucleic acid data from genes encoding actin, TEF, and RPB1. As verified by BLAST searches at NCBI, the signature sequences unequivocally characterize the *Mortierellales* and are not present in any member of the order *Mucorales*.

	AMINO ACID SEQUENCE [5`- 3`]	NUCLEIC ACID SEQUENCE [5`- 3`]
ACTIN (ACT), CONSENSUS	YLM(K/R)IL(M/L)ER GYSF(N/S/Q)T(S/T)A	TACCTSATGARGATYCTSMTGGAGCGHGGYTACTCYTTCHMBACCWCBGCY
Dissophora decumbens AJ287155	YLMKILMERGYSFNTSA	TACCTCATGAAGATCCTCATGGAGCGTGGTTACTCCTTCAACACCTCCGCC
Gamsiella multidivaricata AJ287168	.....S...	.....A.....T...TCG.....T...
Lobosporangium transversale AJ287156	.....S...	.....T.....T...TC...T..T..T
Mortierella alpina AM411535	.....S...	.....T.....C.....TC......G...
M. chlamydospora AJ287167	.....S...	.....TCT.....
M. indohii EU736237	.....S.T.	.....C.....TC....A.....
M. minutissima EU736238	...R.....S.T.	.....G...T.....TC....A.....
M. minutissima EU736239	.....S.T.	.....T.....TC....A.....
M. polycephala AJ287169	.....S.T.	.....C.....T...TC....A....T
M. verticillata AJ287170	.....S.T.	.....TC....A.....
M. wolfii AJ287171	......L......Q...	.....G.....T..GC.....C.....T...C.G.....G...
TRANSLATION ELONGATION FACTOR 1ALPHA (TEF), CONSENSUS	VKKVGYNPK(S/T/A)V (P/A)FVPIS	GTCAAGAAGGTCGGTTACAACCCCAAGDCYGTYSCYTTCGTCCCCATYTCY
D. decumbens AF157247	VKKVGYNPKSVPFVPIS	GTCAAGAAGGTCGGTTACAACCCCAAGTCCGTTCCCTTCGTCCCCATCTCC
G. multidivaricata AF157260	.....T.A.....	.....A.T...G.....T
L. transversale AF157248	.....A.A.....	.....G.T...G.....T..T
M. alpina EU736263	.....A.....	.....G.T.....
M. chlamydospora AF157259	.....A.A.....	.....G.T..CG.T.....
M. indohii EU736264	.....A.....	.....G.T.....T
M. minutissima EU736265	.....A.....	.....G.T.....T...
M. polycephala AF157261	.....A.....	.....T...G.T.....
M. verticillata AF157262	.....A.....	.....G.....T...
RNA POLYMERASE II LARGEST SUBUNIT (RPB1), CONSENSUS	GWEGFLPTPAILKPKPL	GGTTGGGARGGWTTCCTRCCWACYCCTGCWATYCTMAAGCCCAAGCCYCTS
M. cf. elongata AB097422	GWEGFLPTPAILKPKPL	GGTTGGGAGGGATTCCTGCCAACCCCTGCAATCCTCAAGCCCAAGCCCCTG
M. verticillata DQ294595	.....	.....A..T.....A..T..T.....T..T..A.....T..C

paraphyletic in several previous phylogenetic studies (e.g. Gehrig et al. 1996, Tanabe et al. 2000). The hypothesis of a close relationship between *Mortierellales* and *Mucorales* (classified as *Mucoromycotina*) was recently rejected by Liu et al. (2009) and, moreover, the justification for a classification of the *Mortierellales* into a separate supra-ordinal taxon distinct from the *Mucoromycotina* was proposed by Voigt et al. (2009). In our single and combined phylogenetic analyses both orders appear as distinct clades (Fig. 1a–b). Although, the actual relationship between both clades is not resolved, none of the sequence analyses supported a sister-clade relationship between *Mucorales* (clade 'a') and *Mortierellales* (clade 'b'). Based on ribosomal DNA data, the *Mortierellales* are more closely related to the *Glomeromycota* and the *Dikarya* (*Ascomycota* + *Basidiomycota*), but less related to the *Mucorales* (Fig. 1a). This relationship was also shown by Liu et al. (2009), but here the *Glomeromycota* were not included, using instead a phylogeny inferred from mitochondrially encoded sequences. Analyses of the protein-coding sequences show a minimal sister group relationship between *Mucorales* and *Glomeromycota* but fairly unresolved relationships between all other groups, probably due to a lack of informative characters and the phylogenetic signal of the marker genes for clade 'b' (Fig. 1b). Both phylogenies support the monophyly and also, therefore, the phylogenetic distinctiveness, of both clades 'a' and 'b' by bootstrap proportions (BP) of 100%. Eight-locus phylogenies using RaxML analyses resulted in the coherence of the *Mortierellales* supported by a BP of 77% grouping apart from the *Mucorales* (BP 90%; data not shown).

Rejecting the coherence of a *Mortierellales–Mucorales* clade

In single-locus analyses none of the eight loci supported the phylogenetic coherence of the *Mortierellales* with the *Mucorales* (Table 2). Using 18S, 28S rDNA and btub the individual clades of *Mortierellales* and *Mucorales* are supported by BP values of 100% each. But a coherent node for a unified *Mortierellales–Mucorales* clades collapses due to a BP below 50%. Also, no support of a monophyletic *Mortierellales–Mucorales* clade occurs if rpb1 and rpb2 are used, with 92% and 100% BP for a monophyletic *Mucorales* clade separated from the *Mortierellales*, respectively, for each individual gene. Since there is currently only one mortierellalean nucleotide sequence available for each of the phylogenetic markers rpb1 and rpb2, this order appears without statistical support. For act and tef only the *Mortierellales* are well supported (100% BP for tef and 81% BP for act), there is also no statistical support for a monophyletic origin of both orders. With the exception of atub, from which no sequences were available for the *Mortierellales,* single-gene based bootstrapping of all other loci suggests the incoherence of the *Mucoromycotina* as a whole in its current circumscription, supporting the separation of the *Mortierellales* from the *Mucorales* (Table 2).


Coprinopsis cinerea [M92991, AF041494]
Laccaria bicolor [scaffold434, DQ071702]
Pleurotus ostreatus [AY657015, EU365664]
Aspergillus niger [X78538, U28812]
Neurospora crassa [AY046271, AY681158]
Glomus intraradices [DQ322630, DQ273828]
Glomus mosseae [AY635833, DQ273793]
Scutellospora heterogama [AY635832, DQ273792]
Paraglomus occultum [DQ322629, -]
Dissophora decumbens [AF157133, AF157187]
Mortierella verticillata [AB016017, DQ273794]
Gamsiella multidivaricata [AF157144, AF157198]
Lobosporangium transversale [AF113424, AF113462]
Coemansia sp. [-, AY546689]
Spiromyces minutus [AF007542, AF031070]
Furculomyces boomerangus [AF007535, DQ273809]
Smittium culisetae [D29950, DQ273773]
Syncephalis depressa [AB016011, -]
Rhopalomyces elegans [AY635834, DQ273795]
Mucor circinelloides* [scaffold12, scaffold3]
Rhizopus arrhizus* [supercontig3.6, supercontig3.6]
Phycomyces blakesleeanus* [-, scaffold863]
Conidiobolus coronatus [AF296753, AY546691]
Entomophthora muscae [AY635820, DQ273772]
Allomyces arbuscula [AY552524, AY552525]
Nuclearia sp. [AF349566, AY148095]
Chytriomyces hyalinus [DQ536487, DQ273836]
0.1 substitutions/site
A

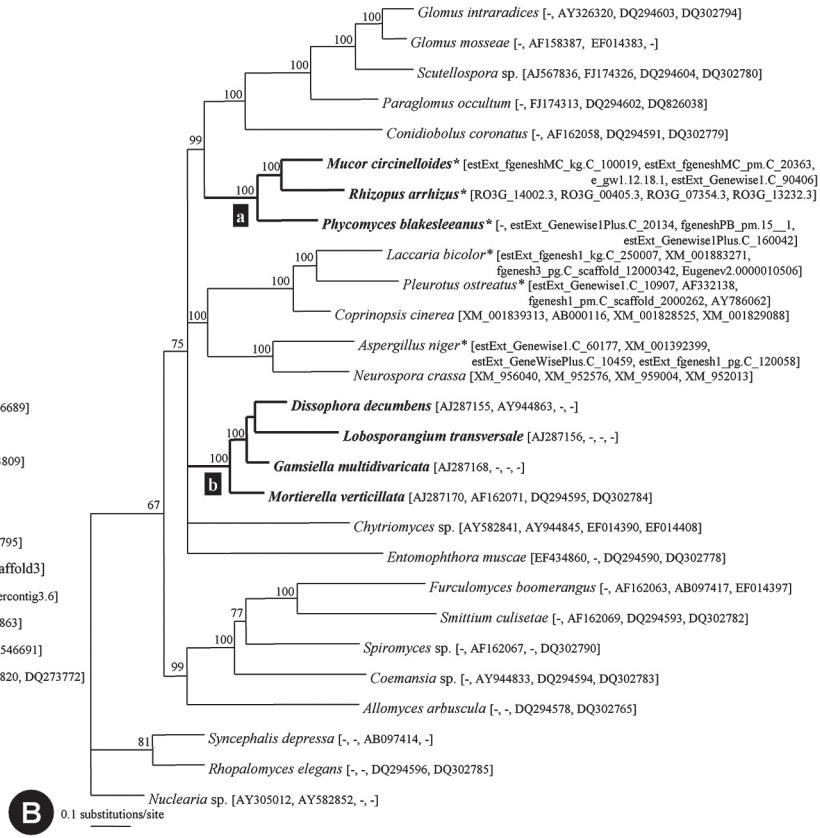

Glomus intraradices [-, AY326320, DQ294603, DQ302794]
Glomus mosseae [-, AF158387, EF014383, -]
Scutellospora sp. [AJ567836, FJ174326, DQ294604, DQ302780]
Paraglomus occultum [-, FJ174313, DQ294602, DQ826038]
Conidiobolus coronatus [-, AF162058, DQ294591, DQ302779]
Mucor circinelloides* [estExt_fgeneshMC_kg.C_100019, estExt_fgeneshMC_pm.C_20363, e_gw1.12.18.1, estExt_Genewise1.C_90406]
Rhizopus arrhizus* [RO3G_14002.3, RO3G_00405.3, RO3G_07354.3, RO3G_13232.3]
Phycomyces blakesleeanus* [-, estExt_Genewise1Plus.C_20134, fgeneshPB_pm.15__1, estExt_Genewise1Plus.C_160042]
Laccaria bicolor* [estExt_fgenesh1_kg.C_250007, XM_001883271, fgenesh3_pg.C_scaffold_12000342, Eugenev2.0000010506]
Pleurotus ostreatus* [estExt_Genewise1.C_10907, AF332138, fgenesh1_pm.C_scaffold_2000262, AY786062]
Coprinopsis cinerea [XM_001839313, AB000116, XM_001828525, XM_001829088]
Aspergillus niger* [estExt_Genewise1.C_60177, XM_001392399, estExt_GeneWisePlus.C_10459, estExt_fgenesh1_pg.C_120058]
Neurospora crassa [XM_956040, XM_952576, XM_959004, XM_952013]
Dissophora decumbens [AJ287155, AY944863, -, -]
Lobosporangium transversale [AJ287156, -, -, -]
Gamsiella multidivaricata [AJ287168, -, -, -]
Mortierella verticillata [AJ287170, AF162071, DQ294595, DQ302784]
Chytriomyces sp. [AY582841, AY944845, EF014390, EF014408]
Entomophthora muscae [EF434860, -, DQ294590, DQ302778]
Furculomyces boomerangus [-, AF162063, AB097417, EF014397]
Smittium culisetae [-, AF162069, DQ294593, DQ302782]
Spiromyces sp. [-, AF162067, -, DQ302790]
Coemansia sp. [-, AY944833, DQ294594, DQ302783]
Allomyces arbuscula [-, -, DQ294578, DQ302765]
Syncephalis depressa [-, -, AB097414, -]
Rhopalomyces elegans [-, -, DQ294596, DQ302785]
Nuclearia sp. [AY305012, AY582852, -, -]
0.1 substitutions/site
B

Table 2. Bootstrap proportions of the *Mucorales* and *Mortierellales* nodes and the combined *Mucorales–Mortierellales* clade in single-locus analyses.

NA-sequences	18S	28S	atub	btub	rpb1	rpb2	tef	act
Mucorales (Mu.)	100	100	78	100	92	100	51	54
Mortierellales (Mo.)	100	100	ni	100	nd	nd	100	81
Mu. + Mo.	0	0	-	0	0	0	0	0

*NA = nucleic acid sequences coding for: 18S/28S: small/large subunit nuclear ribosomal DNA; atub/btub: alpha-/beta-tubulin; rpb1/2: RNA polymerase II subunit 1 or 2; tef: translation elongation factor 1alpha; act: actin. ni: not included; nd: not determined because there is only one member of the *Mortierellales* included in the analysis.

Statistical tests for a monophyletic relationship between *Mortierellales* and *Mucorales*

The *Ascomycota, Basidiomycota* and *Kickxellomycotina* are well resolved and show high clade stability support in both analyses presented in fig. 1. However, the relationships among those groups and the other groups are less clear-cut, showing no significant support values. Statistical tests for an alternative topology with a monophyletic *Mucorales–Mortierellales* clade allowed the exclusion of that hypothesis of a biordinal monophyletic relationship, which justifies their classification into a common subphylum. All tests rejected the phylogenetic alliance between *Mucorales* and *Mortierellales* with $P<0.0001$ under the assumption of the null hypothesis. In Bayesian inference analyses only 23 trees out of 2,002 were found after stationarity showing the topological constraint unifying both orders using the ribosomal DNA data set and no tree was found based on the combined protein data set. Therefore, it can be concluded that the probability of a monophyly for the *Mucorales–Mortierellales* clade is 1.15% for the ribosomal DNA data set and zero for the protein data set.

Identification of signature sequences

Based on act, tef and rpb1, consensus amino and nucleic acid sequences were identified which represent diagnostic signatures unique for the *Mortierellales* (Table 1). These are the protein sequence ylm[k/r]il[m/l]ergysf[[n/s/q]t[s/t]a with its corresponding nucleic acid 5`-tac cts atg arg aty cts mtg gag cgh ggy tac tcy ttc hmb acc wcb gcy-3` for act, vkkvgynpk[s/t/a]v[p/a]fv pis with 5`-gtc aag aag gtc ggt tac aac ccc aag dcy gty scy

Figure 1. Bayesian inference analysis displaying the relationship between the orders *Mucorales* and *Mortierellales*. The phylogenetic reconstruction is based on A: a total of 2103 aligned nucleotide characters from the nuclear small and large subunit ribosomal DNA (1518 and 585 characters of the 18S and 28S rDNA, respectively) and B: a total of 5382 nucleotide characters from aligned protein-coding genes (807; 1515; 1890 and 1170 characters of act, rpb1, rpb2 and btub, respectively), each from 27 taxa. The protistan genus *Nuclearia* was chosen as outgroup taxon. Posterior probabilities are indicated as clade stability support values above the branches. Clade 'a' represents the subphylum *Mucoromycotina* and clade 'b' is represented by the *Mortierellomycotina*.

TTC GTC CCC ATY TCY-3` for TEF and GWEGFLPTPAILKPKPL with 5`-GGT TGG GAR GGW TTC CTR CCW ACY CCT GCW ATY CTM AAG CCC AAG CCY CTS-3` for RPB1 (Table 1). These signatures based upon sequences currently available in GenBank (as of 1st of March, 2010).

Identification of micromorphological traits

Typical micromorphological structures are shown in Fig. 2. The *Mucorales* develop a columella, a sterile swelling of the sporangiophore apex protruding into the multi-spored sporangium. Conversely, the *Mortierellales* lack this structure, suggesting that the columella is a synapomorphy of the *Mucoromycotina*. The vegetative hyphae of the *Mucorales* are coenocytic, with septation only rarely occurring below the columella within the sporangiophore. In contrast, the *Mortierellales* develop irregularly septate vegetative hyphae and, in addition, septa also appear among the hyphae lacking generative structures and sporangiophores. Also, the sporangiophores are typically subulate, being broad at the base and tapering towards the apex, whereas the sporangiophore diameters in the *Mucorales* remain more-or-less constant (Benny et al. 2001). Rhizoids are more pronounced and occur more frequently in the *Mucorales* than among the *Mortierellales;* giant cells are absent in the *Mortierellales*.

Taxonomic description

Mortierellomycotina Kerst. Hoffm., K. Voigt & P.M. Kirk, **subphylum nov.**

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Mycelium hypharum bifurcate ramosarum anastomosantium compositum. Sporangiophora basin inflata, apicem attenuata, erecta, non ramosa vel ramosa, ab initio non-septata, sed irregulariter septata ubi plene creta. Sporangia sphaerica, unispora vel multispora; columella carens. Sporangiosporae globosae usque ad ellipsoideas, vel irregulares, muris laevibus ornamentatisve. Stylosporae, rhizoides et chlamydosporae interdum praesentes. Zygosporae muris crassis; zygosporangium laeve; suspensores sine appendicibus.

Typus: *Mortierella* Coem. 1863

Zygomycota, incertae sedis. Mycelium with anastomosing hyphae, dichotomously branching, bearing stylospores. Hyphae sporangiferous, sporangiophores basally inflated and elongating towards the sporangiophore apex, erect, coenocytic initially, but irregularily septated at maturity. Asexual reproduction via sporangia and sporangiola. Sporangia sphaerical, multi-spored; columella absent. Ramifications gracilous, primarily horizontally expanding, erecting hyphae sometimes terminate with sporangiola. Spores globose to ellipsoid or irregular, smooth or ornamented. Rhizoids only occasional. Giant cells absent. Zygospores naked.

Exemplar genera: *Mortierella*, *Dissophora*, *Modicella*, *Lobosporangium, Gamsiella, Aquamortierella*.

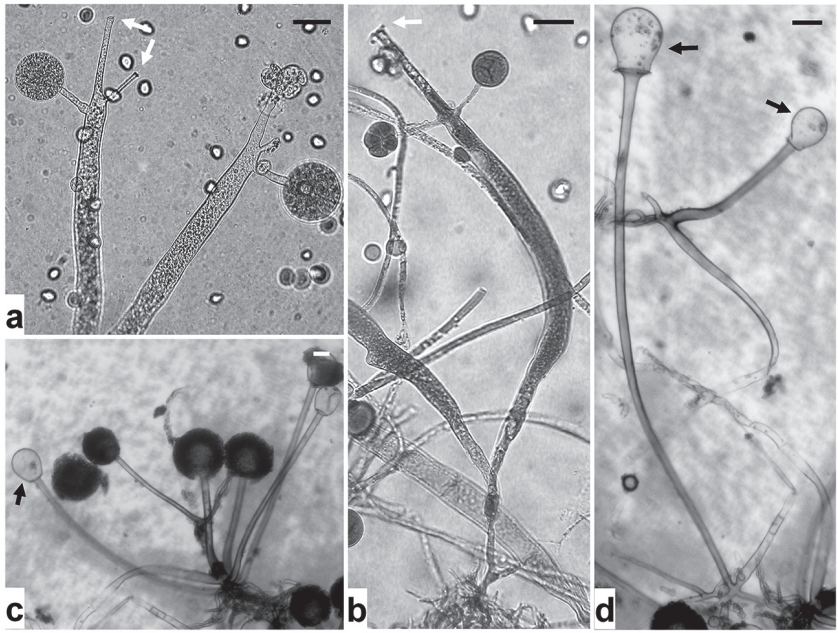

Figure 2. Micromorphological characteristics of prominent members of the subphyla *Mucoromycotina* and *Mortierellomycotina*. a) Sporangiophore of *Mortierella* sp. lacking a columella (white arrows). b) Tapering sporangiophore with rhizoids of *Mortierella* sp. Rejuvenating towards the sporangiophore apex is characteristic for the *Mortierellomycotina*. c+d) Non-tapering sporangiophores with rhizoids of *Rhizopus/Rhizomucor* sp. with sporangia. After spore release a well-developed columella remains (black arrows).

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