A MULTI-GENE MOLECULAR SYSTEMATIC STUDY OF THE KICKXELLOMYCOTINA, INCLUDING THE EXAMINATION OF TWO NEW GENES (MCM7 AND TSR1) FOR PHYLOGENETIC INFERENCE

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DEDICATION

I would like to dedicate this thesis to my parents, Ellen and Dennis Tretter, for helping to create and nurture in me a love of science that has continued to the present day.

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ABSTRACT

Kickxellomycotina is a recently described subphylum of Fungi defined by the presence of a unique disciform septal pore with a lenticular plug. The relationship between members of the group has proven difficult to resolve with traditional methods due to the degree of morphological and ecological variation among taxa within the clade. Furthermore, existing phylogenetic studies have lacked either the taxonomic coverage or had insufficient phylogenetic resolution to reveal the evolutionary history of the group.

In chapter one, I investigate the phylogenetic utility of two single-copy protein-coding genes, MCM7 and TSR1, to improve phylogenetic resolution within the clade. Suitable primers were developed and tested for both genes within the Kickxellomycotina and other early-diverging fungal clades. Trees produced with MCM7 and TSR1 were compared to those produced with rDNA, the gene region used in most previously published studies. MCM7 proved to have considerable phylogenetic utility within the group compared to the rDNA results, while TSR1 was found to be less useful, although still potentially valuable for resolving relationships among closely related taxa.

In chapter two, I utilize eight genes (18S rDNA, 28S rDNA, 5.8S rDNA, MCM7, TSR1, RPB1, RPB2, and β-tubulin) to produce a phylogenetic tree of the Kickxellomycotina within the greater context of Fungi. The Kickxellomycotina are found to be monophyletic, with the Zoopagomycotina suggested to be their closest relatives. Eight clades were identified within the tree, including the four orders previously defined

within the subphylum (Asellariales, Dimargaritales, Harpellales, and Kickxellales). Four genera (*Barbatospora*, *Orphella*, *Ramicandelabler*, and *Spiromyces*) do not cluster within the order in which they are currently placed, representing novel clades.

Dimargaritales and *Ramicandelaber* are the first groups of Kickxellomycotina to diverge, although the relationship between the two remains unclear. The remaining six clades form a monophyletic grouping, from which *Barbatospora* diverges first, followed by a split that divides the group into a clade composed of Asellariales and Harpellales, and a clade composed of Kickxellales, *Orphella*, and *Spiromyces*. Ancestral character state reconstruction revealed that some characters previously thought to be isolated to single taxonomic clades, such as the endosymbiotic life history of the Asellariales and Harpellales, are instead distributed throughout the tree. This suggests that the evolutionary picture within the Kickxellomycotina is likely more complex than previously thought.

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LIST OF ABBREVIATIONS

MCM	Mini Chromosome Maintenance
TSR	20S rRNA Accumulation
PCR	Polymerase Chain Reaction
rRNA	Ribosomal RNA
rDNA	Ribosomal DNA
SSU	Small Subunit
LSU	Large Subunit
DNA	Deoxyribonucleic Acid
SH	Shimodaira-Hasegawa
AU	Approximately Unbiased
RPB1	RNA Polymerase II, Largest Subunit
RPB1	RNA Polymerase II, Second Largest Subunit
BSA	Bovine Serum Albumin
GTG	Genetic Technology Grade
EDTA	Ethylenediaminetetraacetic Acid
TAE	Tris-Acetate-EDTA

AICc	Akaike Information Criterion (Corrected for small sample size)
BI	Bayesian Inference
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
GTR	General Time Reversible
LG	Le and Gascuel
ML	Maximum-Likelihood
AWTY	Are We There Yet
BPP	Bayesian Posterior Probability
MLBP	Maximum-Likelihood Bootstrap Proportion
bp	Base pairs
aa	Amino Acids
NRRL	Northern Regional Research Laboratory
ARSEF	ARS Collection of Entomopathogenic Fungal Cultures
AFTOL	Assembling the Fungal Tree of Life
ITS	Internal Transcribed Spacer
EF-1A	Elongation Factor 1 Alpha
YGCH	Yeast Extract, Glycerol, Casein Hydrolysate
DMSO	Dimethyl Sulfoxide
PAUP*	Phylogenetic Analysis Using Parsimony (* and Other Methods)

MCMC	Markov Chain Monte Carlo
RAxML	Randomized Axelerated Maximum Likelihood
TNT	Tree Analysis Using New Technology
PSR	Parsimony Symmetrical Resampling
MMW	Dr. Merlin M. White
BTUB	Beta Tubulin

EXAMINING NEW PHYLOGENETIC MARKERS TO UNCOVER THE EVOLUTIONARY HISTORY OF EARLY-DIVERGING FUNGI: COMPARING MCM7, TSR1, AND RRNA GENES FOR SINGLE- AND MULTI-GENE ANALYSES OF THE KICKXELLOMYCOTINA

Abstract

The protein-coding genes MCM7 and TSR1 have shown significant promise for phylogenetic resolution within the Ascomycota and Basidiomycota, but have remained unexamined within other fungal groups (except for Mucorales). We designed and tested primers to amplify these genes across early-diverging fungal clades, with emphasis on the Kickxellomycotina, zygomycetous fungi with characteristic flared septal walls forming pores with lenticular plugs. Phylogenetic tree resolution and congruence with MCM7 and TSR1 were compared against those inferred with nuclear small (SSU) and large subunit (LSU) rRNA genes. We also combined MCM7 and TSR1 data with the rDNA data to create 3- and 4-gene trees of the Kickxellomycotina that help to resolve evolutionary relationships among and within the core clades of this subphylum. Phylogenies suggest that *Orphella*, *Barbatospora*, *Spiromyces*, and *Ramicandelaber* may represent unique lineages. These two new protein-coding genes may be more broadly useful for phylogenetic studies among other groups of early-diverging fungi.

Introduction

The molecular revolution has transformed our understanding of the evolutionary relationships between groups of fungi—with examples of both artificial and natural clades being refuted or recognized, respectively. However, in the early-diverging fungi, the process has been only partially successful. Some monophyletic groups have been broken up. For example, Chytridiomycota was shown not to be monophyletic, resulting in two new phyla, Blastocladiomycota and Neocallimastigomycota (James et al. 2006b, Hibbett et al. 2007). The Zygomycota has been split into numerous subphyla (Hibbett et al. 2007). However, the relationships between and sometimes within these groups have resisted efforts with existing phylogenetic techniques for genes in broad usage. James et al. (2006a) were unable to define well-supported relationships between most of the basal groups, leading them to be regarded as *incertae sedis* within the most recent reclassification of Fungi (Hibbett et al. 2007). Additional genes might provide better support for phylogenetic analyses and understanding of these evolutionary relationships, especially when combined with increased taxon sampling. Ultimately, well-supported phylogenies (depicted as trees) allow one to (re-)evaluate and hopefully improve classification systems, as well as understand the ancient environmental pressures that have guided and shaped fungal diversity.

While improving molecular tools and phylogenomics undoubtedly will provide the best evidence to address these questions, the results may not be evident for some time. Firstly, only a limited number of early-diverging taxa have been genome sequenced. Efforts with additional taxa are in progress, but currently only three

species of Chytridiomycota, one each of Blastocladiomycota and Kickxellomycotina, as well as four species of Mucoromycotina have their genomes available (based on available online searches and the list at http://www.fungalgenomes.org). Furthermore, many early-diverging fungi will prove difficult to genome-sequence as they have not yet been cultured axenically and offer genomic DNA samples that are low in concentration and potentially contaminated with host DNA. This is particularly true of the symbiotic members of the Entomophthoromycotina, Zoopagomycotina, and Kickxellomycotina, each of which has at least one major clade with no member species yet successfully cultured. For this reason, finding powerful single-copy genes that can be amplified and sequenced using current techniques (for available samples) remains a reasonable phylogenetic option in pursuit of answers to critical evolutionary questions.

Fortunately, the wealth of information emerging from genomic sequencing projects can be utilized concurrently to discover candidate single-copy genes for such purposes. Using a bioinformatics approach, Aguileta et al. (2008) mined genomic sequences among *Fungi* to identify clusters of orthologous single-copy genes. Individual phylogenetic trees, inferred from the predicted protein sequences, were compared to a phylogenetic tree based on a concatenated alignment of protein sequences. Two genes, MS456 and MS277, demonstrated high topological congruence with the overall consensus tree using all of the genes in the study (Aguileta et al. 2008). MS456 corresponds to the MCM7 gene, a DNA replication licensing factor that forms part of a hexameric protein complex required for DNA

replication (Moir et al. 1982, Kearsey & Labib 1998). MS277 corresponds to the TSR1 gene, a ribosome biogenesis protein (Gelperin et al. 2001).

Although Aguileta et al. (2008) demonstrated the utility and power of these two genes for phylogenetic analysis, neither primer sequences nor PCR protocols were provided. Schmitt et al. (2009) aligned amino acid sequences (from GenBank) to design new degenerate primers to amplify regions of both MCM7 and TSR1. With these primers, they were able to sequence MCM7 and TSR1 for 42 species of lichenized ascomycetes. The resulting phylogeny was well-resolved and demonstrated the potential use of these genes for other taxa. Raja et al. (2011) performed additional testing of MCM7 among the Ascomycota and found that it resolved relationships more strongly than the ribosomal large subunit (LSU), one of the most commonly used genes within the ascomycetes. Morgenstern et al. (2012) generated a phylogeny using MCM7 sequences from genome-sequenced fungi, which included some early-diverging taxa. Hermet et al. (2012) utilized both MCM7 and TSR1 in a study of *Mucor*, demonstrating the potential utility of the MCM7 and TSR1 genes outside of the Dikarya. Despite the apparent phylogenetic potential beyond the Mucorales (Hermet et al. 2012), these genes have not yet been investigated for their power to resolve relationships among the early-diverging fungi.

To address this and potentially improve our understanding of evolution within this section of the fungal tree of life, we attempted to amplify and sequence the MCM7 and TSR1 genes for putative species within the Kickxellomycotina. This subphylum is a diverse group, among which members may be saprotrophic, mycoparasitic, or obligate symbionts of arthropods. Natural affinities among its members have long

been suspected on morphological grounds, such as the unique septal pore and plug structure or the ‘coemansoid pattern’ of growth (Moss & Young 1978). Some molecular-based studies (James et al. 2006a, Sekimoto et al. 2011) have suggested the subphylum is monophyletic, whereas others (White et al. 2006c) have suggested the relationship between the Kickxellomycotina and closely related taxa may be more complex. Some studies have been inconclusive on the matter, with different genes disagreeing on the monophyly of the clade (Tanabe et al. 2004). Furthermore, the relationships between the four orders (Asellariales, Dimargaritales, Harpellales, and Kickxellales) that comprise the subphylum are not fully substantiated.

Our primary goal was to assess the phylogenetic utility of MCM7 and TSR1 for these early-diverging fungal taxa. In so doing, we compared these genes against a combined nuclear 18S and 28S rDNA phylogeny, with attention to tree resolution and congruence. Additionally, 3-gene (18S+28S+MCM7) and 4-gene (18S+28S+MCM7+TSR1) phylogenies were examined to assess their use in combination. These data provide an opportunity to assess the inferred evolutionary relationships and history among members of the Kickxellomycotina, one of the first multi-gene phylogenies with such a focus (but also see Wang 2012).

Materials and Methods

DNA samples used for this study were extracted according to White (2006). Some samples were prepared from axenic cultures, whereas others were prepared from the dissection of host arthropods (Table 1.1).

PCR Amplification

MCM7

Initial attempts to amplify MCM7 were conducted using the primers MCM7-709for and MCM7-1348rev of Schmitt et al. (2009). The PCR products from three taxa (*Coemansia braziliensis, Dipsacomyces acuminosporus*, and *Smittium culisetae*) were amplified successfully and sequenced using the same primers. However, further attempts using these primers with other taxa were unsuccessful. We subsequently designed new primers, assuming that the taxa of interest had primer sites that were not well-matched to the originals of Schmitt et al. (2009).

Specifically, using those three sequences (above) with others from GenBank and several from genome-sequencing projects published online (see Table 1.1), a reference alignment of MCM7 protein sequences was compiled, spanning the Dikarya and several groups of early-diverging fungi, that was used to design six new degenerate primers (Table 1.2). Two sets of primers were used for the majority of our data collection. One set uses the Schmitt et al. (2009) primer MCM7-709for but with our reverse primer (MCM7-16r). The latter appeared to be more conserved amongst a greater diversity of taxa and worked well on the majority of early-diverging fungi tested, except for members of the *Harpellales* where a second set, MCM7-8bf and MCM7-16r, was compatible with the majority of the taxa tested from that order. Both primer combinations amplified a region of approximately 850 base pairs.

The PCR reagents used for the MCM7-709for and MCM7-1348rev primer combination included 11 µL of Promega Go-Taq Green Hot Master Mix, 2.20 µL of each primer at 10 µM concentration, 0.44 µL of 25 mM $MgCl_2$ (to a total concentration of 2.5 mM), 4.16 µL dH_2O, and 2 µL of genomic DNA. Cycling

conditions used an initial denaturation step of 95 °C for 2 min, 45 cycles of 95 °C for 30 sec, annealing at 56 °C for 45 sec, and extension at 72 °C for 1 min 15 sec, a final extension at 72 °C for 10 min, followed with a final hold step at 4 °C. Reagents for the MCM7-8bf and MCM7-16r primer were identical except that 0.35 µL of 50 µg/µL BSA was added (while reducing the water by an equal amount). Cycling conditions included an initial denaturation step of 95 °C for 2 min, with 45 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 45 sec, and extension at 72 °C for 1.5 min, followed by a final extension step at 72 °C for 10 min, before a final hold at 4 °C.

TSR1

As with the MCM7, except with greater sequence variation of the TSR1 gene, a reference alignment was prepared but used first to conduct *in silico* testing before attempting amplifications of it. Specifically, the translated protein sequences of both primers were compared visually to the translated protein sequences in the alignment to assess their conservation and putative compatibility. Again, sequences from GenBank and various genome sequencing projects (see Table 1.1) were used to make this initial assessment. When published primers (Schmitt et al. 2009) did not appear compatible with the early-diverging fungi, based on estimated compatibility with the Chytridiomycota, Blastocladiomycota, and Mucoromycotina, the closest relatives to the Kickxellomycotina for which we had data, we considered the development of new primers. Ultimately, three new primers were developed and tested (Table 1.2). One set (TSR1-1018f with TSR1-2356r) successfully amplified products 1250-1300 bp for most non-harpellid Kickxellomycotina. The other set (TSR1-1492f to TSR1-2356r)

generated fragments from 700-800 bp but was more broadly compatible within the *Kickxellomycotina*. Because the latter products were generated entirely from within the range of the gene region amplified by the other set, only this shorter region was used within the analysis (longer sequences were truncated accordingly).

rRNA Genes

Ribosomal RNA gene sequences were amplified and sequenced as well as obtained from GenBank (Table 1.1). Wang et al. (2013) developed primers for both the small rDNA subunit (18S), specifically primers NS1AA and NS8AA, and the large subunit (28S), with primers NL1AA and LR7AA. Those primers were specifically designed to avoid amplification of host DNA from mixed genomic DNA samples, a situation that is not uncommon when fungi are prepared as micro-dissections from arthropod digestive tracts. PCR reagents used for the NS1AA and NS8AA primer combination included 11 µL of Promega Go-Taq Green Master Mix, 0.66 µL of each primer at 10 µM concentration, 0.88 µL of 25 mM $MgCl_2$ (to a final concentration of 2.5 mM), 0.35 µL of 50 µg/µL BSA, 6.45 µL dH_2O, and 2 µL of genomic DNA. Cycling conditions included an initial denaturation step of 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 30 sec, annealing at 62 °C for 45 sec, and extension at 72 °C for 3 min, with a final extension step at 72 °C for 10 min and a final hold at 4 °C. The PCR cocktail used for the NL1AA and LR7AA primer combination included 11 µL of Promega Go-Taq Green Hot Master Mix, 0.66 µL of each primer at 10 µM concentration, 0.44 µL of 25 mM $MgCl_2$ (to a total concentration of 2.5 mM), 2.20 µL of 5M Betaine, 0.35 µL of 50 µg/µL BSA, 4.69 µL dH_2O, and 2 µL of genomic DNA. Cycling conditions included an initial

denaturation step of 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 45 sec, and extension at 72 °C for 3 min, with a final extension at 72 °C for 10 min followed by a final hold at 4 °C.

Electrophoresis and Sequencing

For all amplified sequences, the PCR product was electrophoresed in 1% Lonza Seaplaque GTG agarose (low EDTA 1X TAE buffer), stained with Gelstar nucleic acid stain (Cambrex), and visualized on a Clare Chemical DR46B transilluminator. Bands of the appropriate size were excised with medium sized pipet tips (pre-cut by a few mm to increase the bore and suitability for bands being extracted) and DNA was extracted using a 'freeze and squeeze' method. Briefly, pipet tips with excised gel cores were placed in a 1.5mL microcentrifuge tube, frozen at -20 °C, spun at 14500xG for 10 minutes, frozen again at -20 °C again, and similarly centrifuged once more. Cycle sequencing reactions were set up using the Applied Biosystems BigDye v. 3.1 kit for bidirectional sequencing. The resulting products were sent to the University of Wisconsin Madison Biotechnology Centre for capillary electrophoresis.

Phylogenetic Analyses

DNA sequences were first aligned using the MUSCLE algorithm (Edgar 2004) and then imported into Mesquite (Maddison & Maddison 2011) for final manual adjustment. Introns were removed from the MCM7 sequences via visual inspection for translation into hypothetical proteins. For the MCM7 protein alignment, the reading frame was determined, and the nucleotide sequences translated into proteins.

This protein alignment was then re-aligned with MUSCLE (Edgar 2004). Regions of poor or ambiguous alignment were manually removed.

Each of the alignments was tested using an appropriate model selection program. The 18S and 28S nucleotide sequences, as well as each of the three individual codon positions of the MCM7 nucleotide alignment, were tested with jModelTest (Guindon & Gascuel 2003, Posada 2008). Model selection was based on the corrected AIC (AICc) score. For all sequences tested, except for the 2nd codon position of the MCM7 nucleotide alignment, the GTR+ Γ+I method had the highest AICc score. For the 2nd codon position, the GTR+ Γ model was slightly higher; however, for simplicity of analysis, the GTR+ Γ+I model was used in all cases. The ProtTest programme (Drummond & Strimmer 2001, Guindon & Gascuel 2003, Abascal et al. 2005) was used on preliminary MCM7 and TSR1 datasets to determine the best model of amino acid evolution for these genes. The LG+ Γ+I model, described by Le and Gascuel (2008), consistently received the highest AICc score and was used.

Phylogenetic inference was conducted through both the Maximum-Likelihood (ML) method and Bayesian inference (BI). The Bayesian tree was used as the primary tree for all analyses, but because of possible Bayesian overestimation of branch supports (Suzuki et al. 2002), Maximum-Likelihood results are also provided. MrBayes v. 3.1.2 was used for Bayesian inference (Huelsenbeck et al. 2001, Ronquist & Huelsenbeck 2003, Altekar et al. 2004). The LG+ Γ+I model of protein evolution, mentioned above, is not implemented natively in MrBayes v.3.1.2 and was done by setting a fixed GTR model and using the LG exchange matrix and equilibrium frequencies as a dirichlet prior. The online version of AWTY was used to assess tree

convergence (Wilgenbusch et al. 2004). GARLI v. 2.0 was used for maximum-likelihood calculations (Zwickl 2006).

Nine analyses were performed – MCM7 nucleotide, MCM7 protein, TSR1 protein, 18S, 28S, nuclear 18S+28S, 3-gene (18S + 28S + MCM7 protein), 4 gene (18S + 28S + MCM7 protein + TSR1 protein), and 18S + 28S for the taxa in the TSR1 protein alignment only. For the MCM7 nucleotide tree, each codon position was treated as an independent partition. For all trees, different genes were always treated as unlinked partitions. For all trees, 10 million generations (BI) and 100 bootstrap replicates (ML) were performed, with half of the BI generations treated as burn-in.

A total of nine phylogenetic trees were produced by our analyses (Fig. 1.1-1.5, 1.7-1.10). Six of the analyses used a large number of taxa (76–81) and were primarily intended to investigate the use of MCM7, whereas three of the analyses used a smaller number (38–39) and were intended to evaluate the use of TSR1 and the combined four-gene analysis (see Table 1.5). For all of the analyses, branches were considered well-supported (and shown in figures with heavy bold lines) if they had a Bayesian posterior probability (BPP) of > 0.95 and a maximum-likelihood bootstrap proportion (MLBP) of > 0.70.

Results

We report 68 new MCM7 sequences, 26 new TSR1 sequences, and 46 new rDNA sequences (Table 1.1) for a variety of taxa within the early-diverging fungal lineages. We amplified most of the lineages tested with at least one primer combination for

each gene. Recommended primer combinations for MCM7 (Table 1.3) and TSR1 (Table 1.4) are provided for each primer and clade tested.

For MCM7, primer combination MCM7-709f and MCM7-16r was effective for most taxa, with the exception of the Harpellales. MCM7-16r appeared to be more conserved than MCM7-1348rev, amplifies a larger region, and is highly conserved. Fewer spurious bands were noted in PCR attempts with MCM7-16r. We were unable to develop a primer closer to the beginning (on the 5' end) of the MCM7 gene. Several clades within the Kickxellomycotina appear to be variable at the MCM7-709f priming site. For these orders, the MCM7-8bf forward primer, which is further downstream, appears to have a higher success rate but is also specific to the Kickxellomycotina and is not recommended for use with other clades. Both primer combinations appear to work well for genomic samples derived from axenic cultures, but sometimes amplify bacterial genes in vouchers prepared from dissected insect guts. Additionally they occasionally amplify host MCM genes, although not MCM7. For example, the MCM2 gene for the host arthropod was amplified in a few of our attempts from mixed genomic samples. These issues are similar to those we observe for primers used to amplify other genes used for phylogenetic studies, such as RNA polymerase II largest subunit and second largest subunity (RPB1 and RPB2), when used under similar conditions. Schmitt et al. (2009) did not report any size variation or introns within their MCM7 data set. However, we observed spliceosomal introns for some species within the Blastocladiomycota, Chytridiomycota, Entomophthoromycotina, Kickxellomycotina, and Zoopagomycotina. No pattern was observed regarding intron position or presence. The largest fragment sequenced in

this study was 1139 bp, about 300 bp larger than the observed average size, within our study, of 850 bp (for primers MCM7-709 and MCM7-16r). We also experienced minor size variation (~10 aa) within the translated amino acid sequences. Our alignment also had a small ambiguously aligned region (approx. 15 aa), which was excluded from analysis.

For TSR1, primer combination TSR1-1018f – TSR1-2356r worked best for members of the Blastocladiomycota, Entomophthoromycotina, and Zoopagomycotina. In our view, it is preferable over TSR1-1492f –TSR1-2356r because it amplifies a larger region. This region appears to be more conserved and is recommended for the Chytridiomycota, which we found did not have the correct primer site for TSR1-1018f. Within the Kickxellomycotina, TSR1-1492f and TSR1-2356r appears to work for most clades. TSR1-1018f and TSR1-2356r works for many groups, except for the Harpellales.

Schmitt et al. (2009) reported the presence of both introns and hypervariable regions within TSR1, and both of these phenomena were observed within our sample set as well. Introns often occurred within highly variable sections of the gene that were not well aligned, making them difficult to precisely locate. At this scale of phylogenetic comparison, the introns could not be reliably aligned between various taxa and were thus excluded from further consideration. Introns are listed and positions given within Fig. 1.6. Several hypervariable regions could not be aligned and also needed to be excluded within the dataset. Of the 9253 characters in the complete alignment, 1226 of them were excluded in the final analysis. The overall

rate of success for amplifications seemed to be lower with TSR1 than with MCM7, but no host insect sequences were observed for TSR1 during the course of this study.

To assess the congruence of the MCM7 gene to the phylogeny of the trichomycete fungi produced by the most well-accepted gene used previously, the topology of the MCM7 nucleotide and protein trees was compared (Figs. 1.1 and 1.6) to a tree based on 18S and 28S rDNA (Figs. 1.3, 1.8, and 1.9) as well as to existing analyses. The MCM7 nucleotide tree (Fig. 1.6) had one significant and well-supported incongruity with the rDNA tree (Fig. 1.3) as well as the accepted phylogeny: the basidiomycete *Ustilago maydis* was placed in a well-supported group including the Kickxellomycotina, the Zoopagomycotina, and the Blastocladiomycota, instead of with the other basidiomycetes. In general, the MCM7 nucleotide tree failed to recover a higher-level classification of the fungi that was congruent with the accepted phylogeny (James et al. 2006a, White et al. 2006c, Hibbett et al. 2007). We suspect that the third codon base is saturated at this level of taxon selection and is introducing noise into the analysis. It is recommended that future studies utilizing MCM7 to study the entire tree of Fungi or large clades either use the amino acid translation, or at least consider excluding the third codon base from analysis, if it is not otherwise down-weighted.

The MCM7 protein analysis (Fig. 1.1) was, in general, more congruent with both the rDNA (Fig. 1.3) and the accepted phylogeny. No well-supported incongruities between the MCM7 protein analysis and the accepted phylogeny were apparent. The MCM7 protein analysis did have one incongruity involving *Coemansia reversa*, *Coemansia braziliensis*, and *Spirodactylon aureum* that was also shown by the

MCM7 nucleotide analysis. This is discussed more in-depth in the section on *Kickxellales* below.

To assess the congruence of TSR1, we compared its topology to a smaller rDNA analysis containing only the taxa for which we had data on TSR1. A few well-supported incongruities were noted. *Dimargaris bacillospora* placed within the *Mucoromycotina*; this species is an obligate mycoparasite of *Mucorales* that is often cultured with *Cokeromyces recurvatus* (Benny 2005), and it is likely that our DNA isolate was derived from such a mixed culture. As this may indicate that our sequence is derived from the host, not from *Dimargaris*, we removed this taxon from the four-gene analysis. This analysis also placed *Coprinopsis cinerea* with *Ustilago maydis*, instead of with *Cryptococcus neoformans*, placed *C. reversa* with *C. braziliensis* similar to the MCM7 analysis, and was incongruent in several places within the order *Harpellales*. This unusual placement may be due to significant sequence length variation and the difficulty in accurately identifying and removing introns within this group.

Discussion

Overall Assessment of MCM7 and TSR1

We developed and tested, along with those from Schmitt et al. (2009), primers for MCM7 and TSR1 that amplify regions of these genes suitable for phylogenetic reconstruction among the early-diverging fungi. Within the Kickxellomycotina, we were able to sequence three of the four orders for MCM7, as well as four other genera that may represent new orders. For TSR1, we were able to sequence two of these

orders and three of these other genera. Finally, we amplified and sequenced other groups of early-diverging fungi, including members of the Blastocladiomycota, Chytridiomycota, Entomophthoromycotina, and Zoopagomycotina for comparative purposes. The Glomeromycota, Mucoromycotina, or the Neocallimastigomycota were not tested. Our assessment of each primer (and combinations of them) for both MCM7 and TSR1 among the clades tested are detailed in Tables 1.3 and 1.4.

Phylogenetic resolving power for the genes varied. The translated MCM7 protein sequences appear to be similar to the SSU rDNA (see Table 1.5), potentially making it a valuable single copy protein-coding gene contribution to multi-gene studies. Congruity with earlier multi-gene trees (Aguileta et al. 2008) suggests that it is resistant to environmental selection and long-branch attraction. Whereas MCM7 analyses were generally congruent to those from rDNA, without having phylogenies based on whole-genomes for comparison, it is difficult to estimate whether the MCM7 protein or the rDNA tree better reflects the evolutionary history in the few cases where they disagree.

While it was possible to reconstruct a phylogeny of fungi with TSR1 that was congruent on the large scale with previous analyses (Liu et al. 2009, White et al. 2006c, James et al. 2006a) and with the combined rDNA analysis here, it did present more hindrances in this regard than MCM7. Furthermore, introns needed to be removed while preparing the alignment file. Nonetheless, at this time, these issues do not seem severe enough to suggest eliminating TSR1 from future consideration. These issues should, however, be taken into account for those considering its potential utility. In our view, TSR1 was more challenging (and perhaps less useful)

when compared to MCM7, at least at the taxonomic scale of this study. TSR1 is likely to be more useful in studies on clades of more closely related species, where its greater variability will be beneficial.

Combined analyses using both the three- and four-gene datasets had greater resolving power than any single-gene analysis. The three-gene analysis utilizing the rDNA (SSU and LSU) along with MCM7 protein sequences yielded high resolving power across the greatest number of taxa, whereas the four-gene analysis utilizing these genes along with the TSR1 protein sequences had the highest proportion of fully-supported branches of any analysis (noting also the differences in taxon number between them). Because this, along with Wang (2012), represent the first multi-gene studies concentrating primarily on the Kickxellomycotina that include sequences from both rDNA and protein-coding genes, we also present a phylogenetic perspective on the various clades presented.

Phylogenetic Analyses

Kingdom Fungi

Except for the Kickxellomycotina, taxon sampling limits our commentary about the other fungal groups. However, by comparing our trees to those presented by others, we offer our assessment of the power of MCM7 and TSR1 for large-scale phylogenetic reconstruction. The relationships within the early-diverging fungal lineages are still in need of refinement. Hibbett et al. (2007) could not distinguish between early-diverging fungal clades at higher taxonomic levels due to limited molecular phylogenetic support (and to some extent taxon sampling). However, existing analyses do offer hints. James et al. (2006a) used a combination of three

rDNA genes and three protein-coding genes to place the Entomophthoromycotina, Kickxellomycotina, and Zoopagomycotina on an unsupported branch along with the Dikarya, the Glomeromycota, and the Mucoromycotina. However, our MCM7 protein tree (Fig. 1.1) placed the Entomophthoromycotina, the Kickxellomycotina, and the Zoopagomycotina in a group together with Blastocladiomycota, and separate from the Dikarya and the Mucoromycotina. That branch was supported by the Bayesian but not by the maximum-likelihood analysis (BPP: 98.3%, MLBP: 37/100). The four-gene tree (Fig. 1.5) placed representatives of the Entomophthoromycotina together with Blastocladiomycota in a well-supported group (BPP: 100.0%, MLBP: 80/100); the Dikarya, Kickxellomycotina, Mucoromycotina, and Zoopagomycotina were placed in another well-supported group (BPP: 99.9%, MLBP: 78/100).

With regard to the later-diverging fungi, the TSR1 protein tree (Fig. 1.2) placed the Dikarya on a well-supported branch (BPP: 100.0%, MLBP: 70/100). The MCM7 protein tree (Fig. 1.1) placed the Ascomycota together with the Mucoromycotina, but was not well supported (BPP: 69.1%, MLBP: 40/100). Multi-gene analyses (Figs. 1.4 and 1.5) recovered a well-supported Dikarya (3-gene: BPP: 98.7%, MLBP: 72/100. 4-gene: BPP: 99.9%, MLBP: 94/100) as well as a well-supported Dikarya+Mucoromycotina clade (Three-gene: BPP: 100.0%, MLBP: 90/100; Four-gene: BPP: 100.0%, MLBP: 98/100).

Kickxellomycotina

The Kickxellomycotina, the primary focus of this study, are a subphylum of fungi previously placed within the Zygomycota. The Kickxellomycotina are differentiated from other fungi by the production of septal walls with a lenticular pore, containing a

plug of material (Hibbett et al. 2007). This characteristic septal pore has been confirmed from all four orders within the Kickxellomycotina. Members of the Kickxellomycotina produce branched or unbranched septate thalli, sometimes with aseptate regions, such as in the main axis of *Pteromaktron*. They include arthropod symbionts (Harpellales and Asellariales), haustorial mycoparasites (Dimargaritales), and saprobes (Kickxellales except for *Martensella*, which is a non-haustorial mycoparasite). Asexual one- or two-spored merosporangia are produced (in Harpellales, these merosporangia are referred to as trichospores) as well as zygospores. The sexual spores can vary in shape, being spherical in the *Dimargaritales*, Asellariales, and Kickxellales, biconical (or rarely uniconical) within the Harpellales, and coiled within *Orphella* (Moss & Young 1978, Valle & Santamaria 2005, Valle & Cafaro 2008.)

The MCM7 protein tree (Fig.1.1) recovered a monophyletic Kickxellomycotina with seven major subclades (BPP: 100.0%, MLBP: 86/100). These included three of the four known orders, Dimargaritales, Harpellales, and Kickxellales, and four genera, *Barbatospora*, *Orphella*, *Ramicandelaber*, and *Spiromyces* (likely to represent new orders in a subsequent publication). The TSR1 protein tree (Fig. 1.2) also recovered a monophyletic Kickxellomycotina, with five of the main subclades represented (the Asellariales, Dimargaritales, and *Orphella* have yet to yield sequences), although it was only strongly supported below *Ramicandelaber* (BPP: 100.0%, MLBP: 74/100). The four-gene analysis (Fig. 1.5) was also able to recover a monophyletic Kickxellomycotina (BPP: 99.8%, MLBP: 72/100), but the three-gene analysis (Fig. 1.4) was not (BPP: 99.9%, MLBP: 42/100). It placed *Rhopalomyces*

elegans (Zoopagomycotina) in a clade with *Dimargaris* and *Ramicandelaber*. This may be due to long-branch attraction between *Dimargaris* and *Rhopalomyces*, as both have highly divergent rDNA sequences.

All trees presented a branch that contained all members of the orders Harpellales and Kickxellales except for the genus *Ramicandelaber*. This branch was well supported in both the MCM7 (Fig. 1.1; BPP: 99.9%, MLBP: 74/100) and TSR1 (Fig. 1.2; BPP: 100.0%, MLBP: 74/100) single-gene analyses, and in both the three-gene (Fig. 1.4; BPP: 100.0%, MLBP: 97/100) and four-gene (Fig. 1.5; BPP: 100.0%, MLBP: 100/100) multi-gene analyses. This strongly suggests that the Harpellales and Kickxellales (except for *Ramicandelaber*) form a monophyletic group, and *Ramicandelaber* may not be closely related to the Kickxellales. Within this group, no tree (in which they are present) places the genera *Barbatospora* or *Orphella* in a monophyletic clade together with only the Harpellales, and no tree places the genus *Spiromyces* together with only the Kickxellales. Thus, our suggestion is that these genera may represent distinct evolutionary clades.

Harpellales

Harpellales is a diverse order of symbiotic fungi that live within the guts of aquatic insect larvae or rarely isopods (White 1999). Along with the Asellariales, they are often referred to as the ‘gut fungi,’ and can shift between parasitic, commensalistic, and mutualistic roles (Lichtwardt et al. 2007). The Harpellales have a unique zygospore, whether biconical or uniconical, that distinguishes them from other orders within the Kickxellomycotina. Most species of Harpellales also produce unispored merosporangia (Moss & Young 1978) for asexual reproduction, referred to

as trichospores (noting that *Carouxella* and *Klastostachys* spores remain attached to the generative cell, which is dehiscent, similar to the arthrospores of the Asellariales). These spores are specialized for the aquatic environment, with many species having mucilaginous non-motile appendages. Moreover, trichospores are sensitive to the precise condition of the insect gut in which they germinate, and rapidly extrude a sporangiospore when appropriate conditions are detected within the correct host gut. During this extrusion process, a mucilaginous holdfast is excreted that secures the thallus to the gut lining of the host. Some genera of Harpellales (*Genistellospora*, *Harpella*, and *Pennella*) are also known to occasionally infest the ovaries of developing black flies, replacing the eggs with ovarian cysts containing spores in the adult black flies (White et al. 2006a). The flying adult then oviposits these cysts among egg masses, allowing for effective dispersal and upstream transmission.

The existing classification of the Harpellales includes two families – the Legeriomycetaceae, which are members that have branched thalli and are usually found in the hindgut, whereas the Harpellaceae are all unbranched and typically found in the midgut of their host (Lichtwardt et al. 2007). However, molecular-based phylogenetic analyses have typically not supported this separation. The most complete phylogenetic analyses of the Harpellales to date were provided by White (2006), White et al. (2006c), and Wang et al. (2013). White (2006) designated a '*Smittium*' clade consisting of *Smittium* and a few related genera, and a 'non-*Smittium*' clade for of *Smittium culisetae* and most of the other genera of the *Harpellales*. Wang et al. (2013) have moved *Smittium culisetae* to a new genus (we use *S. culisetae* here, ahead of print). That 2-gene study again found evidence of a

Smittium /Non-*Smittium* phylogenetic split and further defined the '*Smittium* allies' to include *Austrosmittium, Coleopteromyces, Furculomyces, Pseudoharpella, Stachylina,* and *Trichozygospora.*

Our three-gene analysis (Fig. 1.4) provides further evidence of this split, with *Coleopteromyces, Furculomyces, Smittium, Stachylina,* and *Trichozygospora* all placed together and well supported (BPP: 99.8%, MLBP: 70/100). Another well-supported clade includes *Bojamyces, Capniomyces, Genistelloides, Graminella, Harpella, Lancisporomyces, Legerioides, Legeriomyces, Pennella, Pteromaktron,* and *Smittium culisetae* (BPP: 100.0%, MLBP: 98/100). *Harpellomyces* and *Caudomyces* were placed as just outside this group, although only strongly supported by the Bayesian analysis (*Harpellomyces*: BPP: 100.0%, MLBP: 58/100). The MCM7 protein analysis alone is not as well-resolved, but still contains all of the same clades, supporting the conclusion that the both of these analyses are underlying the correct species tree. This result is another indication that family structure will need to be reconsidered, pending improved taxon sampling, to more naturally represent the actual relationships.

The TSR1 analysis of the Harpellales (Fig. 1.2) does not fully agree with the phylogeny provided by the MCM7 protein or rDNA tree (Figs. 1.1 and 1.3). Although a monophyletic Harpellales was obtained, the topology within the group is not completely congruent with the other analyses, or analyses using RPB1 and RPB2 (not shown, Dr. Merlin White personal communication). TSR1 presented difficulties from aligning the nucleotide sequences to identifying and removing introns, and finally in aligning the proteins and removing ambiguously aligned regions. Sampled members

of the *Harpellales* seemed to have more introns as well as greater size variation within the protein, compared to related groups. Additional taxon sampling within the *Harpellales* might help to resolve these issues. The four-gene tree incorporating the TSR1 protein (Fig. 1.5) did have the same topology as the one from the three-gene analysis (Fig. 1.4).

The 'non-*Smittium*' clade represents a diverse assemblage with variable characteristics, whereas the *Smittium* clade has much greater morphological similarity. Nearly all members of the *Smittium* clade have a single appendage as well as a collar left where the trichospore dehisces from the fertile thallus. *Trichozygospora* is the exception, with its large number of thin appendages on both the sexual and asexual spores, but is otherwise similar in spore shape and collar presence. Many members of the non-*Smittium* clade have more than one appendage, and most of them have no collar on the trichospore. A collar is present in *Smittium culisetae* and *Bojamyces*, but for both it is flared, unlike most examined species of *Smittium.* Additionally, phylogenetically related genera *Graminella* and *Pteromaktron* have a ball-like or knob-like structure on the appendage near its attachment to the spore. Whether or not this knotted portion of the appendage might represent some remnant of an earlier dehiscent collar or collar-like structures, homologous to the collar of *Smittium*, is unknown. The *Smittium* clade is also almost completely restricted to Diptera hosts (except for *Coleopteromyces,* with one species from aquatic *Coleoptera*), whereas the non-*Smittium* clade has members that utilize a diverse group of hosts including not only *Diptera*, but also *Ephemeroptera, Isopoda*, *Plecoptera*, and *Trichoptera*.

Asellariales

The Asellariales represent a much smaller grouping of endosymbiotic fungi, consisting of *Asellaria* and *Baltomyces* (within Isopoda) as well as *Orchesellaria* (in Collembola). The Asellariales produce branched, septate thalli within the hindgut of their host, extending from a specialized holdfast cell with a secreted mucilaginous holdfast (Lichtwardt & Manier 1978). This order is distinguished by the fragmenting of the thallus at maturity to produce arthrospores. The general similarity in growth form and life history, along with the similarity between the arthrospores of *Asellaria* to the asexual reproductive propagules of *Carouxella* (Harpellales) have been used to suggest that the two orders may be sister taxa (Moss & Young 1978). On the other hand, spherical zygospores have been observed for *Asellaria* (Valle & Cafaro 2008), unlike the biconical zygospores of the Harpellales. Septal structure has been observed for both *Asellaria* and *Orchesellaria*, and is characteristic of the Kickxellomycotina (septa with a lenticular pore and an electron-dense plug), but without the spherical occluding bodies of the Dimargaritales (Moss 1975).

Despite significant effort with all primer combinations listed in this paper (along with some other attempted but unsuccessful primers not provided), we have been unable to amplify and sequence MCM7 or TSR1 for any member of *Asellaria*. Unpublished RPB1 and RPB2 sequences for *Asellaria* have been known for some time (Hibbett et al. 2007), and we have successfully amplified additional sequences for these genes as well as the SSU and LSU rDNA (for another manuscript), but even with working genomic samples we were unable to amplify or sequence MCM7. Some bands were visible in the gels, but either would not sequence directly or were deemed

incorrect products. Similarly, all attempts to amplify and sequence TSR1 with *Asellaria* failed to even produce bands. We also attempted to amplify and sequence both of these single-copy genes for *Orchesellaria* and *Baltomyces*, but with no success to date.

Kickxellales

The Kickxellales are primarily saprobic (except one genus, *Martensella*, which is mycoparasitic) fungi in the Kickxellomycotina. Saprobic members of this group have been found associated with soil, dung, and insect carcasses (Benny 2005). Members of this order reproduce asexually by means of sporocladia that produce multiple, unispored merosporangia supported upon small basal cells, the pseudophialides, and also sexually through spherical zygospores (Benny 2005). The sporocladia may be either single- or multi-celled (Benny 2005). Most Kickxellales genera release their spores in a droplet of liquid at maturity, referred to by Moss and Young (1978) as 'slime spores,' with only the genera *Spiromyces* and *Spirodactylon* being dry-spored (Benny 2005). Moss and Young (1978) described this slime as possibly being related to a special intracellular structure found in the pseudophialide, referred to as the 'labyrinthiform organelle' and possibly homologous to the trichospores appendage produced by the Harpellales. They also compared the morphology of the reproductive structure of the two groups, describing the structures as having a shared 'coemansoid' morphology and suggesting the two groups may be closely related. This relationship has since been supported by several molecular-based phylogenetic studies (O'Donnell et al. 1998, James et al. 2006a, White et al. 2006c). The Kickxellales have a septal

structure similar to the Harpellales and Asellariales, with a lenticular septal pore with an electron-dense plug (O'Donnell et al. 1998).

All trees inferred in this study revealed a strongly-supported and monophyletic *Kickxellales* clade that includes *Coemansia*, *Dipsacomyces*, *Kickxella*, *Linderina*, *Martensiomyces*, and *Spirodactylon* (three-gene: BPP: 100.0%, MLBP: 100/100; four-gene: BPP: 100.0%, MLBP: 100/100). This clade never included *Ramicandelaber*. *Spiromyces* is included as a strongly-supported sister clade to this group in the four-gene analysis (BPP: 100.0%, MLBP: 100/100), but for that analysis *Orphella* was not available. Within the three-gene analysis (Fig. 1.4; BPP: 100.0%, MLBP: 100/100) and the rDNA-based analysis (Fig. 1.3; BPP: 100.0%, MLBP: 100/100), *Orphella* seems to be more closely related to the monophyletic Kickxellales group than *Spiromyces*. As such, it appears that *Ramicandelaber* is not part of the *Kickxellales*, and *Spiromyces* may not be, unless *Orphella* (currently, still a member of the *Harpellales*) is considered to be a member of the Kickxellales as well (see more on this in the *Spiromyces* and *Ramicandelaber* sections).

Within the monophyletic *Kickxellales*, relationships seem to be difficult to resolve. The group consisting of *Coemansia reversa*, *C. braziliensis*, and *Spirodactylon aureum* was first shown by O'Donnell et al. (1998) and again by White et al. (2006c). This relationship is further demonstrated by both the MCM7 (Fig. 1.1; BPP: 98.2%, MLBP: 75/100) and TSR1 phylogenies (Fig. 1.2; BPP: 100.0%, MLBP: 100/100), providing multi-gene support. However, in both the MCM7 (Fig. 1.1; BPP: 100.0%, MLBP: 100/100) and TSR1 (Fig. 1.2; BPP: 100.0%, MLBP: 100/100) analyses, *C. reversa* and *C. braziliensis* are placed together, while in the rDNA

analysis (as for the previous published analyses, which also utilised rDNA) *C. reversa* is placed together with *S. aureum*, which renders *Coemansia* polyphyletic (Fig; 1.3; BPP: 99.9%, MLBP: 86/100). This may represent a true instance of incomplete lineage sorting within the Kickxellales. Alternatively, it may be due to long-branch attraction related to *Spirodactylon*, which appears to be more diverged from the other Kickxellales with regard to rDNA than MCM7 or TSR1.

Other relationships between the members of the Kickxellales are more difficult to resolve. Both the MCM7 and TSR1 individual gene trees (Fig. 1.1 and 1.2) are not well resolved within the Kickxellales clade. The three-gene tree (Fig. 1.4) is well resolved with regard to internal members of the group; a poorly-supported group consisting of *Dipsacomyces* and *Martensiomyces* is the most early-diverging member (BPP: 86.5%, MLBP: not present), followed by well-supported individual branches containing *Linderina* (BPP: 100.0%, MLBP: 78/100) and *Kickxella* (BPP: 100.0%, MLBP: 81/100). The four-gene analysis (Fig. 1.5), however, does not strongly support these internal branches (possibly due to contrasting signal from TSR1), but does strongly support the relationship between *Dipsacomyces* and *Martensiomyces* (BPP: 100.0%, MLBP: 83/100). This relationship is present, although not as strongly supported, in all four individual-gene analyses (Fig. 1.1, 1.2, 1.8, and 1.9) as well as previously by O'Donnell et al. (1998).

Dimargaritales

Dimargaritales is an unusual group of Kickxellomycotina. Mycoparasites of Mucorales and Ascomycota, they have several morphological and life history features that differentiate them from other Kickxellomycotina. While they retain the

diagnostic lenticular septal cavity with an electron-dense plug (Jeffries & Young 1979, Brain et al. 1982), the plug has globose bodies to either side of the septum in the Dimargaritales (Benjamin 1959), which can be dissolved in 2-3% KOH, unlike the septal plugs of the Kickxellales. Other unique features include bispored merosporangia (all other Kickxellomycotina are unispored) and the presence of haustoria.

We attempted to amplify and sequence MCM7 and TSR1 for our single representative of this order, *Dimargaris bacillosporus* (see Table 1.1). We were able to successfully sequence MCM7. Unfortunately, for TSR1, our sequence appears to be that of a mucoralean contaminant. *Dimargaris bacillosporus* is often grown in co-culture with its host, *Cokeromyces recurvatus*. The phylogenetic position of the *Dimargaris* within the TSR1 tree suggests strongly that our sequence is that of the host fungus. Our MCM7 sequence does not appear to show any affinity to the Mucorales, and thus it appears to be genuine.

The MCM7 analysis reveals a monophyletic Kickxellomycotina that includes *Dimargaris* (BPP: 100.0%, MLBP: 86/100) as part of an early-diverging group that also contains *Ramicandelaber*, although the connection between *Ramicandelaber* and Dimargaritales was only supported by the Bayesian analysis (BPP: 97.5%, MLBP: 59/100). Multi-gene analysis was less clear; the three-gene analysis placed *Dimargaris* in an unsupported group with *Rhopalomyces* (BPP: 66.7%, MLBP: not present). The possibility that Dimargaritales is one of the most early-diverging members of the Kickxellomycotina is evocative. Several features of Dimargaritales bear close resemblance to members of the Zoopagomycotina, particularly

Piptocephalis and *Syncephalis* that are mucoralean mycoparasites (Benny 2005). Beyond the lifestyle, these genera also have multispored merosporangia and appear to have a similar growth form. It may be that the Kickxellomycotina either descend from within the Zoopagomycotina or form a sister clade to it. Molecular analyses thus far, including this one, have been unable to fully resolve the phylogenetic position of the Zoopagomycotina, and point to the need for further study.

The MCM7 gene will be particularly useful for Dimargaritales due to the consistent sequence length and reliable alignment. Dimargaritales have demonstrated extremely diverged and variable rDNA sequences that make them difficult to align and result in long-branch attraction artefacts (White et al. 2006c). MCM7 does not suffer from this problem and trees have relatively consistent branch-lengths, at least as demonstrated by our *Dimargaris* representative.

Distinct Lineages: Barbatospora, Orphella, Ramicandelaber, and Spiromyces

Several genera of Harpellales and Kickxellales have consistently not clustered with their respective orders. These unique genera (lineages) are examined here.

Barbatospora has not been reported since the type *B. ambicaudata* was described from blackflies in the Great Smoky Mountains National Park, USA (White et al. 2006b). Although the general growth form of *Barbatospora* resembles the Harpellales, with a branched, septate thallus and a secreted holdfast, it also presents unique morphological features. These include a ‘cap-like’ structure at the terminal end of the trichospores, which typically falls away at maturity, to reveal a set of appendages or appendage-like structures on either end of the asexual spore. However, much about the morphology of this species is not known, including the presence and

form of zygospores, the septal wall structure, and the method of spore extrusion and germination. *Barbatospora* was placed, on morphological grounds, in Harpellales within the family Legeriomycetaceae.

Phylogenetically, *Barbatospora* consistently places within a branch that includes the Harpellales, the Kickxellales, *Orphella*, and *Spiromyces*. This placement is well-supported in the MCM7 (Fig. 1.1; BPP: 99.9%, MLBP: 74/100), TSR1 (Fig. 1.2; BPP: 100.0%, MLBP: 74/100), 3-gene (Fig. 1.4; BPP: 100.0%, MLBP: 97/100), and 4-gene (Fig. 1.5; BPP: 100.0%, MLBP: 100/100) analyses, and is present (but not completely supported) in the rDNA (Fig. 1.3; BPP: 100.0%, MLBP: 68/100) analysis as well. Within this group, the Harpellales, Kickxellales, *Orphella*, and *Spiromyces* are together on a strongly-supported branch within the TSR1 (Fig. 2; BPP: 100.0%, MLBP: 75/100), rDNA (Fig. 3; BPP: 100.0%, MLBP: 90/100), three-gene (Fig. 4; BPP: 99.9%, MLBP: 91/100), and four-gene analyses (Fig. 1.5; BPP: 100.0%, MLBP: 95/100), and a branch that is not strongly supported within the MCM7 (Fig. 1.1; BPP: 86.4%, MLBP: 48/100) analysis. The position of *Barbatospora*, which is one of the most consistent and well-supported evolutionary hypotheses provided by this study, may suggest that the species is an ‘offshoot’ from an ancestral clade that split to form the Kickxellales and Harpellales. Thus, *Barbatospora* might offer valuable insights into the early evolution of this group.

Orphella, also currently a member of the Harpellales, has unusual morphological features for that order (see review by Valle & Santamaria (2005)). *Orphella* is unique among gut fungi in releasing both trichospores and zygospores as multi-celled dissemination units, and in having allantoid to coiled asexual spores and (to some

extent) coiled zygospores (Valle & Santamaria 2005). At maturity, both spore forms extend, attached to the thallus, beyond the anus of the host. Valle and Santamaria (2005) reported that *Orphella* has a characteristic 'coemansoid' growth form, which pointed to a relationship with the Kickxellales. This relationship was also suggested by molecular-based studies (James et al. 2006a, White et al. 2006c), where *Orphella* is clustered with the Kickxellales. Aside from the unusual spore features, its morphology resembles the Harpellales, with an extruded mucilaginous holdfast, a specialized holdfast cell, and a branched, septate thallus.

Again, for *Orphella*, we were able to sequence MCM7 but not TSR1. The MCM7 analysis does not offer any additional insight into the relationship between *Orphella* and the other taxa within the Kickxellomycotina, beyond suggesting that *Orphella* is apart from the other Harpellales. The three-gene analysis (Fig. 1.4; BPP: 100.0%, MLBP: 100/100 both above and below the branch containing *Orphella*) supports the phylogeny demonstrated by previous studies (James et al. 2006a, White et al. 2006c), noting the possible disproportionate phylogenetic signal from rDNA.

Spiromyces is currently a member of the Kickxellales, though previous phylogenetic analyses have placed it apart from that order. It is separated from the Kickxellales by *Orphella* (White et al. 2006c), but sometimes it appears ancestral to both the Kickxellales and Harpellales (James et al. 2006a). Morphologically, *Spiromyces* is an unusual member of the Kickxellales because rather than pseudophialides, it produces merosporangia from enlarged sections of the sporangiophore, similar to the collar regions of the generative cells of Harpellales (Moss & Young 1978). It is also one of the few Kickxellales that is dry-spored at

maturity. *Spiromyces* species are saprobic and usually associated with dung. We were able to amplify and sequence both MCM7 and TSR1 for *Spiromyces*, but neither single-gene tree is able to place it reliably (Figs. 1.1 and 1.2). Within the 3-gene tree (Fig. 1.4), *Spiromyces* is placed as a sister clade to a group consisting of the Kickxellales (except *Ramicandelaber*) and *Orphella* (BPP: 100.0%, MLBP: 88/100). Within the 4-gene tree (Fig. 1.5), *Spiromyces* is with the Kickxellales (except *Ramicandelaber*) as the earliest-diverging member (but recall *Orphella* is not available for this tree) (BPP: 100.0%, MLBP: 100/100).

Ramicandelaber is another genus within the Kickxellales that may not belong with the order. This genus has an unusual growth form for the Kickxellales. It forms both stolons and rhizoids and in *R. brevisporus*, and may form supporting branches (Benny 2005). It is also unusual how, in age, *Ramicandelaber* sporocladia broaden and become covered with more pseudophialides, which become subspherical (Ogawa et al. 2001). Previous molecular studies have placed *Ramicandelaber* apart from the Harpellales and Kickxellales (Ogawa et al. 2005, White et al. 2006c). On the MCM7 tree (Fig. 1.1), *Ramicandelaber* is placed within the Kickxellomycotina on an early-diverging branch along with *Dimargaris* (note however that this branch was only supported by the Bayesian analysis; BPP: 97.5%, MLBP: 59/100). Within the TSR1 analysis (Fig. 1.2), it was placed as an unsupported branch as the earliest-diverging member of the Kickxellomycotina (recall that the Dimargaritales sample was not placed correctly on this tree due to amplification of the fungal host of *Dimargaris*; BPP: 85%, MLBP: 40/100). Within all five analyses (Figs. 1.1-1.5), *Ramicandelaber* is placed outside of a well-supported clade that contains all other members of the

Kickxellales and the Harpellales (except, in the case of the rDNA analysis, *Barbatospora*). These results suggest that the rDNA-based placement of *Ramicandelaber* outside of the Kickxellales is likely an accurate one, and that this genus may well represent a unique, early-diverging lineage of the Kickxellomycotina.

Conclusion

The comparison between the rDNA-based and MCM7-based phylogenies suggest that MCM7 is a valuable gene for phylogenetic inference within the Kickxellomycotina, although it does not seem to have the same degree of resolving power that it does within Ascomycota (Schmitt et al. 2009, Raja et al. 2011). While it is unlikely to be sufficient to resolve complex relationships on its own, the relative ease of amplification and sequencing (for a single-copy, protein-coding gene) and the high degree of resolving power make it a valuable addition to rDNA-based or multi-gene studies. In addition, MCM7 seems to not be plagued with the long-branch attraction problems demonstrated by the rDNA of the Dimargaritales, and to a lesser extent, *Ramicandelaber*, making it an excellent alternative to consider for accurate phylogenetic inference.

As we have pursued the use of TSR1 over a shorter time frame, its potential utility is more difficult to ascertain. While the large-scale phylogenetic resolution of TSR1 appears to be quite good, difficulties in identifying and removing introns, which may have resulted in incongruities between the TSR1 tree and the rDNA tree, make it uncertain at those levels, and specifically how trustworthy it may be within the Harpellales. Additional studies with it for more representatives of the Harpellales for TSR1 should make it easier to reliably remove introns and align amino acid

sequences. To date, TSR1 appears to be more difficult to amplify and sequence compared to MCM7, although in our laboratory we have found it to be far easier to work with than RPB1 or RPB2 (Dr. Merlin White personal communication). When TSR1 amplifies, it is specific to the correct gene and to *Fungi*, and the variability could be an asset within groups of closely related species.

In addition to their utility within the Kickxellomycotina, general congruence with accepted phylogenetic studies across the broader fungal tree and successful amplification within several early-diverging lineages suggests that MCM7 and TSR1 can potentially be used by those studying other groups of early-diverging fungi. In particular, they are also likely to be useful within the Entomophthoromycotina and Zoopagomycotina, groups that traditionally have proven difficult to culture (for some taxa at least) and place. While the gene regions require some manual adjustment (intron removal, translation, and removal of poorly aligned regions) to be useful, this is true of the majority of phylogenetically-informative genes, including well-accepted ones (such as RPB1 and RPB2), when used over wide taxonomic ranges. We are poised to consider them for our revisionary efforts on the gut fungi, within the Kickxellomycotina, and hope they will be considered by others exploring the earliest branches of the fungal tree of life.

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Table 1.1 Fungal species, isolate number, and source, amplified with specific primer combinations.

Species	Isolate #	Source / Host	Culture	Country	Collector	Primer Combination				GenBank Accesssion or Genome Identifier			
						18S	28S	MCM7	TSR1	18S	28S	MCM7	TSR1
Batrachochytrium dendrobatidis[8]	JEL423	Broad Institute sequencing project[3]	-		-	-	-	-	-	Supercontig 14 188674-190464	NG_027619	BDEG_04439.1	BDEG_02071.1
Spizellomyces punctatus[8]	DAOM BR117	Broad Institute sequencing project[3]	-		-	-	-	-	-	Supercontig 32 115-2731	NG_027618.1	SPPG_04788.3	SPPG_02880.2
Rhizophlyctis rosea	JEL318	AFTOL DNA sample	-		-	-	-	7f-16r	-	AY635829.1	DQ273787.1	**KC297581**	-
Allomyces macrogynus[8]	ATCC 38327	Broad Institute sequencing project[3]	-		-	-	-	-	-	Supercontig 63 186963-188875	Supercontig 63 164434-171606	AMAG_00422.2	AMAG_17353.1
Allomyces arbuscula	Brazil 2	AFTOL DNA sample	-		-	-	-	7f-16r	1018f-2356r	NG_017166.1	DQ273806.1	**KC297582**	**KC297629**
Coelomomyces stegomyiae	DUH0008925	AFTOL DNA sample	-		-	-	-	7f-16r	1018f-2356r	NG_017164	NG_027644.1	**KC297583**	**KC297630**
Mucor circinelloides[8]	CBS277.49	JGI sequencing project[5]	-		-	-	-	-	-	Scaffold 11 800000-801950	Scaffold 11 804000-809666	Scaffold 14 106477-109288	Scaffold 1 4335584-4338189
Phycomyces blakesleeanus[8]	NRRL 1555	JGI sequencing project[5]	-		-	-	-	-	-	NG_017190.1	NG_027559.1	Scaffold 5 753037-756512	Scaffold 24 361829-364983
Rhizopus oryzae[8]	99-880	Broad Institute sequencing project[4]	-		-	-	-	-	-	Supercontig 6 2079534-2081357	Supercontig 6 2074749-2079337	RO3G_11608	RO3G_12091.3
Coprinopsis cinerea[8]	okayama7#130[6]	GenBank	-		-	-	-	-	-	M92991	AF041494	AACS02000002.1	AACS02000003.1
Cryptococcus neoformans[8]	B-3501A	GenBank	-		-	-	-	-	-	BR000310.1	BR000310.1	AAEY01000032.1	AAEY01000024.1
Ustilago maydis[8]	521[7]	GenBank	-		-	-	-	-	-	X62396	AF453938	AACP01000247.1	AACP01000184.1
Aspergillus nidulans[8]	FGSC A4	GenBank	-		-	-	-	-	-	U77377.1	EU840227.1	AACD01000102.1	AACD01000107.1
Saccharomyces cerevisiae[8]	S288c	GenBank	-		-	-	-	-	-	NC_001144.5	NC_001144.5	BK006936.2	BK006938.2
Schizosaccharomyces pombe[8]	972h-	GenBank	-		-	-	-	-	-	CU329672.1	NC_003421.2	CU329671.1	CU329670.1
Rhopalomyces elegans	NRRL A-10835	AFTOL DNA sample	-		-	-	-	7f-16r	1018f-2356r	NG_017191.1	NG_027654.1	**KC297584**	**KC297631**
Conidiobolus coronatus	NRRL 28638	AFTOL DNA sample	-		-	-	-	7f-16r	-	NG_017182.1	NG_027617.1	**KC297585**	-

Entomaphaga conglomerata	ARS-2273	Adult Chironomidae	yes		-	-	-	7f-16r	-	AF368509.1	-	**KC297586**	-
Entomophthora muscae	ARSEF3074	AFTOL DNA sample	-		-	-	-	7f-16r	1018f-2356r	NG_017183.1	NG_027647.1	**KC297587**	**KC297632**
Barbatospora ambicaudata	TN-49-W4a	*Simulium vandalicum*	yes	United States	MMW	SR1R-NS8	ITS1F-NL4	8bf-16r	1492f-2356r	**KC297614**	**KC297566**	**KC297588**	**KC297633**
Coemansia reversa	NRRL 1564	JGI sequencing project[5]	yes		-	-	-	-	-	Scaffold 121 3595-5518	Scaffold 121 7595-11423	Scaffold 81 3398-5725	Scaffold 24 40229-42782
Coemansia braziliensis	NRRL-1566	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007532.1	AF031069.1	**KC297589**	**KC297634**
Kickxella alabastrina	NRRL-2693	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007537.1	AF031064.1	**JX155485**	**KC297635**
Dipsacomyces acuminosporus	NRRL-2925	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007534.1	AF031065.1	**KC297590**	**KC297636**
Martensiomyces pterosporus	NRRL-2642	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007539.1	AF031066.1	**KC297591**	**KC297637**
Linderina pennispora	NRRL-3781	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007538.1	FJ517544.1	**JX155486**	**KC297638**
Spirodactylon aureum	NRRL-2810	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007541.1	AF031068.1	**KC297592**	**KC297639**
Spiromyces minutus	NRRL-3067	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007542.1	DQ273810.1	**KC297593**	**KC297640**
Spiromyces aspiralis	NRRL-22631	ARS Culture Collection	yes		-	-	-	8bf-16r	1492f-2356r	AF007543.1	NG_027560.1	**KC297594**	**KC297641**
Orphella catalaunica	NOR-40-W10 + W12	Leuctrid	no	Norway	MMW	NS1AA-NS8AA	ITS1F-LR5	8bf-16r	-	**KC297617**	**KC297569**	**KC297598**	-
Orphella dalhousiensis	NS-34-W16	*Paracapnia* sp.	no	Canada	GenBank / MMW	-	-	8bf-16r	-	DQ322626.1	DQ273830.1	**KC297599**	-
Ramicandelaber longisporus	ARSEF 6175	Eggs/cysts, *Heterodera glycines*	yes	China	-	SR1R-NS8	NL1-LR11	7f-16r	1492f-2356r	**KC297615**	**KC297567**	**KC297595**	**KC297642**
Ramicandelaber longisporus	ARSEF 6176	Eggs/cysts, *Heterodera glycines*	yes	China	-	SR1R-NS8	-	7f-16r	-	**KC297616**	-	**KC297596**	-
Dimargaris bacillispora	NRRL 2808	AFTOL DNA sample	yes		-	-	-	7f-16r	1018f-2356r	NG_017180.1	NG_027650.1	**KC297597**	**KC456420**[9]
Harpellomyces montanus	TN-22-W5B	Thaumaleidae	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297618**	**KC297570**	**KC297600**	-
Harpellomyces eccentricus	NOR-56-W1	Thaumaleidae	no	Norway	MMW	-	-	8bf-16r	-	-	-	**KC297601**	-

Bojamyces sp.	CA-18-W17	Ephemeroptera	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	JX155619	JX155645	**JX155471**	-
Capniomyces sasquatchoides	ID-130-N5	Plecoptera	no	United States	NKR	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297619**	**KC297571**	**KC297602**	-
Capniomyces stellatus	MIS-21-127	*Allocapnia* sp.	yes	United States	GenBank / RWL	-	-	8bf-16r	1492f-2356r	DQ367451.1	EF396194.1	**JX155472**	**KC297643**
Caudomyces sp.	OR-8-W10	Tipulidae	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	1492f-2356r	**KC297620**	**KC297572**	**KC297603**	**KC297649**
Caudomyces sp.	UT-1-W16a	*Antocha* sp.	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	1492f-2356r	JX155620	JX155646	**JX155473**	**KC297650**
Furculomyces boomerangus	AUS-77-4	*Tanytarsus* nr. *inextentus*	no	Australia	RWL	-	-	8bf-16r	-	AF277013.1	AF031074.1	**JX155466**	-
Genistelloides hibernus	KS-19-M23	Capniidae	no	United States	GenBank / JKM	-	-	8bf-16r	1492f-2356r	DQ367456.1	JQ302921	**JX155474**	**KC297651**
Graminella microspora	NOR-35-1	*Baetis rhodani*	no	Norway	RWL	-	-	8af-16r	-	JQ302867	JQ302945	**KC297604**	-
Harpella or *Pennella* sp.	NF-MC-15	Adult *Prosimulium mixtum* ovarian cysts	no	Canada	MCB	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297621**	**KC297573**	**KC297605**	-
Harpella melusinae (cysts)	NF-15-5A	Adult *Prosimulium mixtum* ovarian cysts	no	Canada	RWL	SR1R-NS8	NL1AA-LR7AA	8bf-16r	1492f-2356r	JX155621	JX155647	**JX155475**	**KC297652**
Harpella sp.	NF-MC-18	Adult *Prosimulium mixtum* ovarian cysts	no	Canada	MCB	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297622**	**KC297574**	**KC297606**	-
Lancisporomyces falcatus	NS-X-2	*Paracapnia angulata*	no	Canada	DBS	-	-	8bf-16r	-	JQ302865	JQ302943	**JX155477**	-
Lancisporomyces vernalis	SPA-X-40	Nemoura	no	Spain	LGV	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297623**	**KC297575**	**KC297607**	-
Legerioides tumidus	NH-1-M869a	Isopoda	no	United States	JKM	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-		**KC297576**	**KC297608**	-
Legeriomyces minae	PEI-X-6	Ephemeroptera	no	Canada	DBS	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	JX155622	JX155648	**JX155478**	-
Legeriosimilis sp.	CA-10-W15	Ephemeroptera	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297625**	**KC297577**	**KC297609**	-
Legeriomyces sp. nov.	PEI-X-4	Ephemerellidae	no	Canada	DBS	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297626**	**KC297578**	**KC297610**	-
Legeriosimilis sp.	NOR-31-2	Siphloneuridae	no	Norway	RWL	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297627**	**KC297579**	**KC297611**	-
Pennella sp.	NOR-7-W12	Simuliidae	no	Norway	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	**KC297628**	**KC297580**	**KC297612**	-

Pteromaktron sp.	OR-11-W8	Ephemeroptera	no	United States	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	JX155623	JX155649	**JX155479**	-
Smittium culisetae	COL-18-3	*Culiseta impatiens*	yes	United States	GenBank / RWL	-	-	8bf-16r	1492f-2356r	NG_017185.1	NG_027648.1	**AEW26363.1**	**KC297644**
Smittium culisetae	MAL-X-1	*Aedes crinifer*	yes	Malaysia	CLL	-	-	8bf-16r	1492f-2356r	JQ302897	JQ302835	**AEW26370.1**	**KC297645**
Unnamed Trichopteran tricho	ALG-13-W1	Trichoptera	no	Canada	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	1492f-2356r	JX155625	JX155651	**JX155481**	**KC297653**
Unnamed Trichopteran tricho	ALG-10-W3	Trichoptera	no	Canada	MMW	NS1AA-NS8AA	NL1AA-LR7AA	8bf-16r	-	JX155624	JX155650	**JX155480**	-
Smittium culicis	43-1-2	*Chironomus* sp.	yes	Australia	LCF / BH	-	-	8bf-16r	1492f-2356r	JQ302893	DQ367512.1	**AEW26366.1**	**KC297646**
Smittium mucronatum	FRA-12-3	*Psectrocladius sordidellus*	yes	France	KUMYCOL / RWL	-	-	8bf-16r	1492f-2356r	AF277030.1	JQ302833	**AEW26371.1**	**KC297647**
Smittium simulii	41-1-6	*Orthocladius* sp.	yes	Australia	LCF / BH	-	-	8bf-16r	1492f-2356r	JQ302861	JQ302939	**AEW26374.1**	**KC297648**
Austrosmittium sp.	32-1-8	Orthocladiinae	no	Australia	KUMYCOL	-	-	8bf-16r	-	-	DQ367494.1	**KC297613**	-
Coleopteromyces amnicus	ARG-15-6F	Scirtidae	no	Argentina	LCF	-	-	8bf-16r	-	JQ302853	JQ302931	**JX155465**	-
Pseudoharpella arcomylica	LCF#3	Dixidae	no	United States	LCF	-	-	8bf-16r	-	JQ302882	JQ302956	**JX155467**	-
Smittium angustum	AUS-126-30	*Tanytarsus* sp.	yes	Australia	RWL	-	-	8bf-16r	-	AF277005.1	JQ302822	**JX155420**	-
Smittium annulatum	CR-143-8	Simuliidae	yes	Costa Rica	RWL	-	-	8bf-16r	-	AF277024.1	JQ302832	**JX155421**	-
Smittium caudatum	KS-1-2	Chironomidae	yes	United States	KUMYCOL/ RWL	-	-	8bf-16r	-	AF277031.1	JQ302948	**JX155422**	-
Smittium coloradense	RMBL-13-41	*Cricotopus* sp.	yes	United States	RWL	-	-	8bf-16r	-	AF277041.1	JQ302912	**JX155423**	-
Smittium commune	KS-6-6	Chironomidae	yes	United States	RWL	-	-	8bf-16r	-	AF277034.1	JQ302901	**JX155426**	-
Smittium cylindrosporum	CHI-27-1	*Cricotopus* sp.	yes	Chile	RWL	-	-	8bf-16r	-	AF277018.1	JQ302828	**JX155433**	-
Smittium gravimetallum	KS-F1-3	*Dicrotendipes fumidus*	yes	United States	LCF	-	NL1AA-LR7AA	8bf-16r	-	AF277037.1	JX155634	**JX155437**	-
Smittium megazygosporum	SC-DP-2	*Simulium vittatum*	yes	United States	KUMYCOL / CEB	-	-	8bf-16r	-	AF277045.1	JQ302823	**JX155442**	-

Smittium morbosum	AUS-X-1	*Anopheles hilli*	yes	Australia	KUMYCOL / RWL	-	-	8bf-16r	-	AF277014.1	JQ302913	**JX155443**	-
Smittium orthocladii	LCF-BT-1	*Corynoneura* sp.	yes	United States	LCF / MMW	-	-	8bf-16r	-	DQ367446.1	JQ302900	**AEW26378.1**	-
Smittium tipulidarum	RMBL-31-1	*Elliptera astigmatica*	yes	United States	KUMYCOL / RWL	-	-	8bf-16r	-	AF277043.1	JQ302836	**JX155452**	-
Smittium tronadorium	ARG-24-2F	*Paraheptagyia* sp.	yes	Argentina	LCF	-	-	8bf-16r	-	AF277004.1	JQ302904	**JX155454**	-
Stachylina lentica	NOR-58-10	*Chironomus* sp.	no	Norway	RWL	-	NL1AA-LR7AA	8bf-16r	-	JQ302874	JX155628	**JX155468**	-
Stachylina sp.	NS-X-10	Chironomidae	no	Canada	DBS	-	-	8bf-16r	-	-	-	**JX155469**	-
Trichozygospora chironomidarum	TN-3-16	Chironomidae	yes	United States	RWL	-	NL1AA-LR7AA	8bf-16r	-	JQ302841	**KC297568**	**JX155470**	-

1. AS, Amy Slaymaker; AR, Alen Rizzo; BH, Barb Hayford; CEB, Charles "Eddie" Beard; CLL, Claudia Lopez Lastra; DBS, Douglas B. Strongman; GM, Maria Gabriela Mazzucchelli; JKM, JK Misra; JL, Joyce Longcore; LCF, Leonard C. Ferrington, Jr.; LGV, Laia Guàrdia Valle; MCB, Murray Colbo; MJC, Matías J. Cafaro; MMW, Merlin White; NKR, Nicole Reynolds; PVC, Paula Clarke; RWL, Robert W. Lichtwardt; SM, Steve Moss; WKR, Will K. Reeves. Some of the sequences were generated from culturable isolates from the University of Kansas Mycological Culture Collection, represented as KUMYCOL.

2. Accession numbers in bold were generated for this study (or as joint effort with Wang 2012 study). Cells in grey are from genome sequencing projects not yet uploaded to GenBank.

3. Data derived from Origins of Multicellularity Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

4. Data derived from *Rhizopus oryzae* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

5. These sequence data were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community.

6. rDNA was not available from the genome sequencing project, so data from other isolates was used. The isolate used for the SSU rDNA was not specified in GenBank. The LSU rDNA was taken from isolate C13.

7. rDNA was not available from the genome sequencing project, so data from other isolates was used. The SSU rDNA was taken from isolate "MUCL 30488, CBS 445.63". The LSU rDNA was taken from isolate MS 115.

8. Species used for initial primer design and *in silico* testing.

9. This sequence was determined, on the basis of BLAST results and tree placement, to be from a member of the Mucoromycotina, probably *Cokeromyces recurvatus*. This species is frequently used as a host for *D. bacillospora* in laboratory culture.

Table 1.2 Primers used to amplify nuclear (SSU and LSU) rDNA or protein-coding genes (MCM7; TSR1), among the *Kickxellomycotina* and some other early-diverging fungi.

Primer Name	Gene	Source	Direction	Sequence (5' - 3')	Translated amino acid sequence (5' - 3')	Length	Degeneracy
MCM7-709for	MCM7	Schmitt et al. 2009	For	ACIMGIGTITCVGAYGTHAARCC	TRVSDVKP	23 bp	48[1]
MCM7-8bf	MCM7	**New for this study**	For	GTIGCIGCITAYYTITGYGAY	VAAYLCD	21 bp	16
MCM7-8af	MCM7	**New for this study**	For	TGYGGIWSIGARGTITTYCARGA	CGSEVFQ	23 bp	64
MCM7-1348rev	MCM7	Schmitt et al. 2009	Rev	GATTTDGCIACICCIGGRTCWCCCAT	MGDPGVAKS	26 bp	24[2]
MCM7-16r	MCM7	**New for this study**	Rev	GTYTGYTGYTCCATIACYTCRTG	HEVMEQQT	23 bp	32
TSR1-1018f	TSR1	**New for this study**	For	AAYGARCARACITGGCCIACIGA	NEQTWPT(D/E)	23 bp	8
TSR1-1492f	TSR1	**New for this study**	For	TGGGAYCCITWYGARAAYYTICC	WDP(Y/F)ENLP	23 bp	64
TSR1-2356r	TSR1	**New for this study**	Rev	CAYTTCATRTAICCRTGIGTICC	GTHGYMKC	23 bp	8
NS1AA	SSU rDNA	Wang et al. 2013	For	AAGCCATGCATGTCTAAGTATAA	-	23 bp	-
SR1R	SSU rDNA	Vilgalys & Hester 1990	For	TACCTGGTTGATYCTGCCAGT	-	21 bp	2
NS8AA	SSU rDNA	Wang et al. 2013	Rev	TACTTCCTCTAAATGACCAAGTTTG	-	25 bp	-
NS8	SSU rDNA	White et al. 1990	Rev	TCCGCAGGTTCACCTACGGA	-	20 bp	-
ITS1F	LSU rDNA	Gardes & Bruns. 1993	For	CTTGGTCATTTAGAGGAAGTAA	-	22 bp	-
ITS3	LSU rDNA	White et al. 1990	For	GCATCGATGAAGAACGCAGC	-	20 bp	-
NL1	LSU rDNA	O'Donnell 1993	For	GCATATCAATAAGCGGAGGAAAAG	-	24 bp	-
NL1AA	LSU rDNA	Wang et al. 2013	For	GAGTGAAGCGGGAAIAGCTCAAG	-	23 bp	-
NL4	LSU rDNA	O'Donnell 1993	Rev	GGTCCGTGTTTCAAGACGG	-	19 bp	-

LR5	LSU rDNA	Vilgalys & Hester 1990	Rev	TCCTGAGGGAAACTTCG	-	17 bp	-
LR7AA	LSU rDNA	Wang et al. 2013	Rev	CCACCAAGATCTGCACTAGA	-	20 bp	-
LR11	LSU rDNA	Vilgalys lab page[3]	Rev	GCCAGTTATCCCTGTGGTAA	-	20 bp	-

1. Degeneracy given by Schmitt et al. (2009) as 32 (three-fold degeneracies calculated as two-fold).

2. Degeneracy given by Schmitt et al. (2009) as 16 (three-fold degeneracies calculated as two-fold).

3. Available at http://www.biology.duke.edu/fungi/mycolab/primers.htm.

Table 1.3 MCM7 protein-coding gene testing status among early-diverging fungal groups with notes on earlier and newly established primer combinations.

Clade Tested	Recommended primers	Notes
Chytridiomycota	MCM7-709f MCM7-16r	
Blastocladiomycota	MCM7-709f MCM7-16r	
Zoopagales	MCM7-709f MCM7-16r	
Entomophthorales	MCM7-709f, MCM7-8af MCM7-16r	MCM7-709f preferred over MCM7-8af.
Kickxellomycotina		
Harpellales	MCM7-8bf MCM7-16r	MCM7-709f works for a couple of species.
Kickxellales	MCM7-8bf MCM7-16r	MCM7-709f works for some but not all species.
Asellariales	-	Attempted unsuccessfully.
Dimargaritales	MCM7-709f MCM7-16r	MCM7-8bf not tested.
Orphella clade	MCM7-8bf MCM7-16r	MCM7-709f may work, but not as well as 8bf.
Barbatospora clade	MCM7-8bf MCM7-16r	MCM7-709f not tested.
Spiromyces clade	MCM7-8bf MCM7-16r	MCM7-709f amplified an incorrect gene when attempted.
Ramicandelaber clade	MCM7-709f, MCM7-8bf MCM7-16r	MCM7-709f seemed to sequence better.

Table 1.4 TSR1 protein-coding gene testing status among early-diverging fungal groups with notes on earlier and newly established primer combinations.

Clade Tested	Recommended primers	Notes
Chytridiomycota	TSR1-1492f TSR1-2356r	Not sequenced, but amplification product noted.
Blastocladiomycota	TSR1-1018f TSR1-2356r	TSR1-1492f not tested.
Zoopagomycotina	TSR1-1018f TSR1-2356r	TSR1-1492f not tested.
Entomophthoromycotina	TSR1-1018f TSR1-2356r	TSR1-1492f not tested.
Kickxellomycotina		
Harpellales	TSR1-1492f TSR1-2356r	TSR1-1018f does not appear to work.
Kickxellales	TSR1-1018f, TSR1-1492f TSR1-2356r	TSR1-1018f and TSR-1492f both work well.
Asellariales	-	Attempted unsuccessfully.
Dimargaritales	TSR1-1018f TSR1-2356r	TSR1-1492f not tested.
Orphella clade	TSR1-1492f TSR1-2356r	PCR product did not sequence cleanly but was identifiable as fungal TSR1.
Barbatospora clade	TSR1-1492f TSR1-2356r	TSR1-1018f amplified but would not sequence.
Spiromyces clade	TSR1-1018f, TSR1-1492f TSR1-2356r	TSR1-1018f and TSR1-1492f both work well.
Ramicandelaber clade	TSR1-1018f, TSR1-1492f TSR1-2356r	TSR1-1018f and TSR1-1492f both work well.

Table 1.5 Comparative analysis of phylogenetic trees.

Alignment	Figure	ML score (RAxML)	# Taxa in Alignment	# Char in Alignment	# Interior Branches Total	# Interior Branches Supported	% Interior Branches Supported
MCM7 protein	Fig. 1.1	-12111.24453	81	266	72	39	54.17%
TSR1 protein	Fig. 1.2	-8224.179649	39	207	33	21	63.64%
Nuclear SSU + LSU	Fig.1.3	-25369.28207	76	2492	67	40	59.70%
Nuclear SSU + LSU + MCM7 protein	Fig. 1.4	-38458.25623	76	2758	73	51	69.86%
Nuclear SSU + LSU + MCM7 protein + TSR1 protein	Fig. 1.5	-35502.47807	38	2965	35	29	82.86%
MCM7 nucleotide[1]	Fig. 1.7	-34531.25508	81	780	75	41	54.67%
SSU rDNA[1]	Fig. 1.8	-14149.10549	78	1414	66	28	42.42%
LSU rDNA[1]	Fig. 1.9	-11824.38916	77	1078	65	26	40.00%
SSU+LSU (TSR1 Taxa)[1]	Fig. 1.10	-20628.4317	39	2492	35	25	71.43%

1. Not presented in main body of document - see supplementary materials.

2. All alignments and trees published online in TreeBase submission # 13444. See http://purl.org/phylo/treebase/phylows/study/TB2:S13444.

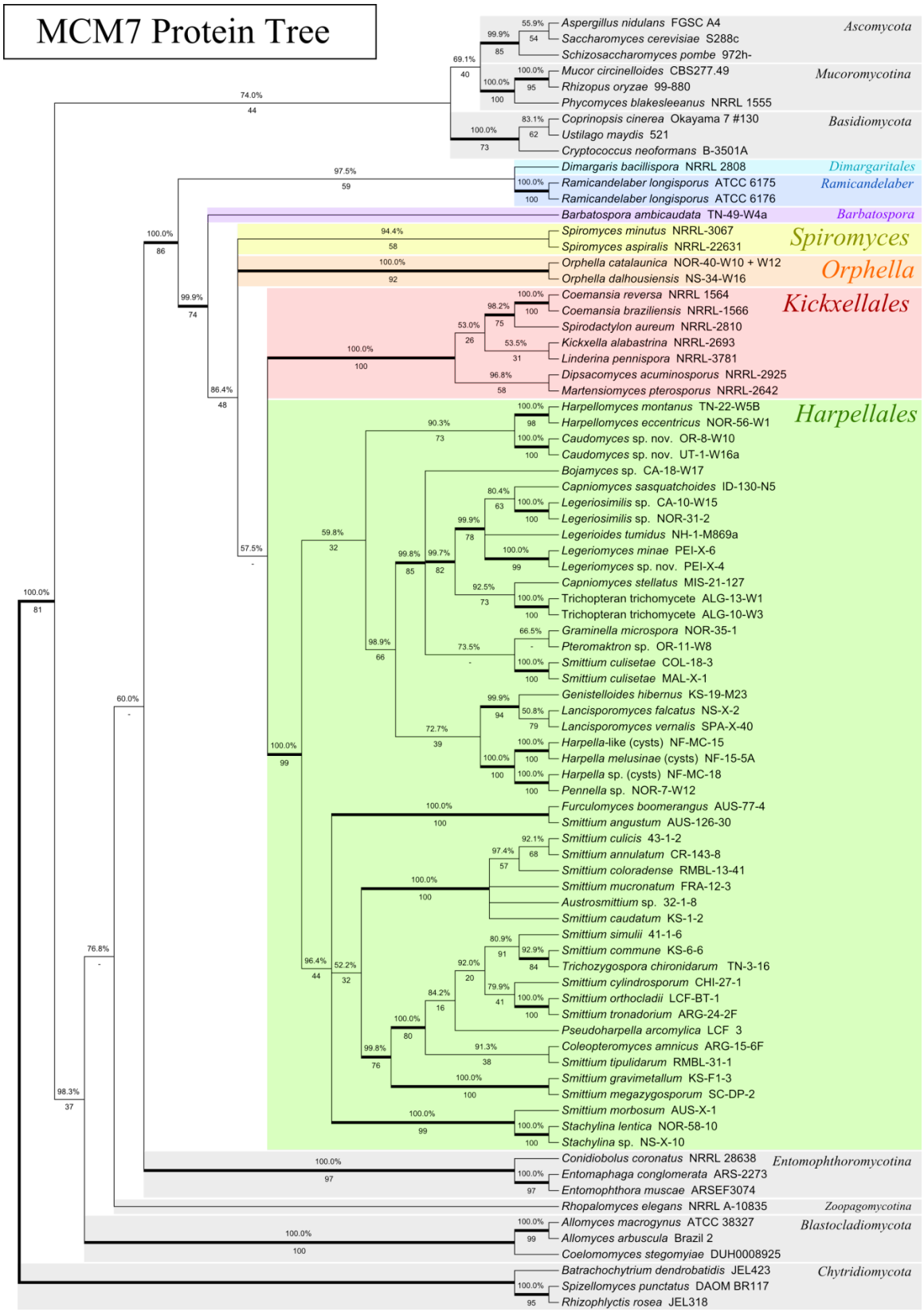

MCM7 Protein Tree
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Ascomycota
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Mucoromycotina
Coprinopsis cinerea Okayama 7 #130
Ustilago maydis 521
Cryptococcus neoformans B-3501A
Basidiomycota
Dimargaris bacillispora NRRL 2808
Dimargaritales
Ramicandelaber longisporus ATCC 6175
Ramicandelaber longisporus ATCC 6176
Ramicandelaber
Barbatospora ambicaudata TN-49-W4a
Barbatospora
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Spiromyces
Orphella catalaunica NOR-40-W10 + W12
Orphella dalhousiensis NS-34-W16
Orphella
Coemansia reversa NRRL 1564
Coemansia braziliensis NRRL-1566
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Kickxellales
Harpellomyces montanus TN-22-W5B
Harpellomyces eccentricus NOR-56-W1
Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Bojamyces sp. CA-18-W17
Capniomyces sasquatchoides ID-130-N5
Legeriosimilis sp. CA-10-W15
Legeriosimilis sp. NOR-31-2
Legerioides tumidus NH-1-M869a
Legeriomyces minae PEI-X-6
Legeriomyces sp. nov. PEI-X-4
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Trichopteran trichomycete ALG-10-W3
Graminella microspora NOR-35-1
Pteromaktron sp. OR-11-W8
Smittium culisetae COL-18-3
Smittium culisetae MAL-X-1
Genistelloides hibernus KS-19-M23
Lancisporomyces falcatus NS-X-2
Lancisporomyces vernalis SPA-X-40
Harpella-like (cysts) NF-MC-15
Harpella melusinae (cysts) NF-15-5A
Harpella sp. (cysts) NF-MC-18
Pennella sp. NOR-7-W12
Furculomyces boomerangus AUS-77-4
Smittium angustum AUS-126-30
Smittium culicis 43-1-2
Smittium annulatum CR-143-8
Smittium coloradense RMBL-13-41
Smittium mucronatum FRA-12-3
Austrosmittium sp. 32-1-8
Smittium caudatum KS-1-2
Smittium simulii 41-1-6
Smittium commune KS-6-6
Trichozygospora chironidarum TN-3-16
Smittium cylindrosporum CHI-27-1
Smittium orthocladii LCF-BT-1
Smittium tronadorium ARG-24-2F
Pseudoharpella arcomylica LCF 3
Coleopteromyces amnicus ARG-15-6F
Smittium tipulidarum RMBL-31-1
Smittium gravimetallum KS-F1-3
Smittium megazygosporum SC-DP-2
Smittium morbosum AUS-X-1
Stachylina lentica NOR-58-10
Stachylina sp. NS-X-10
Harpellales
Conidiobolus coronatus NRRL 28638
Entomaphaga conglomerata ARS-2273
Entomophthora muscae ARSEF3074
Entomophthoromycotina
Rhopalomyces elegans NRRL A-10835
Zoopagomycotina
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Coelomomyces stegomyiae DUH0008925
Blastocladiomycota
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus DAOM BR117
Rhizophlyctis rosea JEL318
Chytridiomycota

Figure 1.1 Phylogeny of the *Kickxellomycotina* and other fungal taxa based on an alignment of MCM7 translated protein sequences.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).

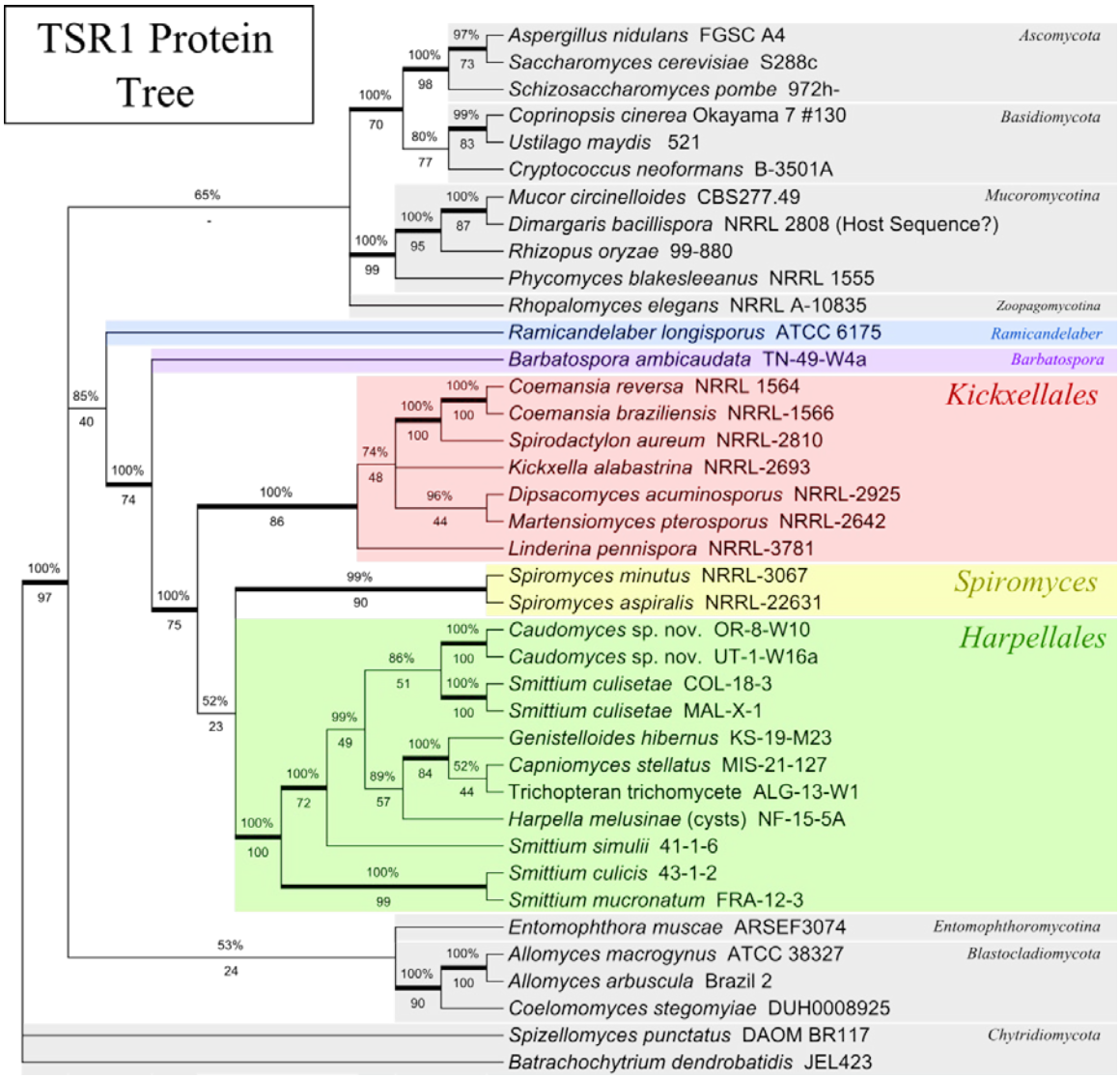


Figure 1.2 Phylogeny of the *Kickxellomycotina* based on an alignment of TSR1 translated protein sequences.

The method of tree calculation and the tree format are the same as Fig. 1.1.

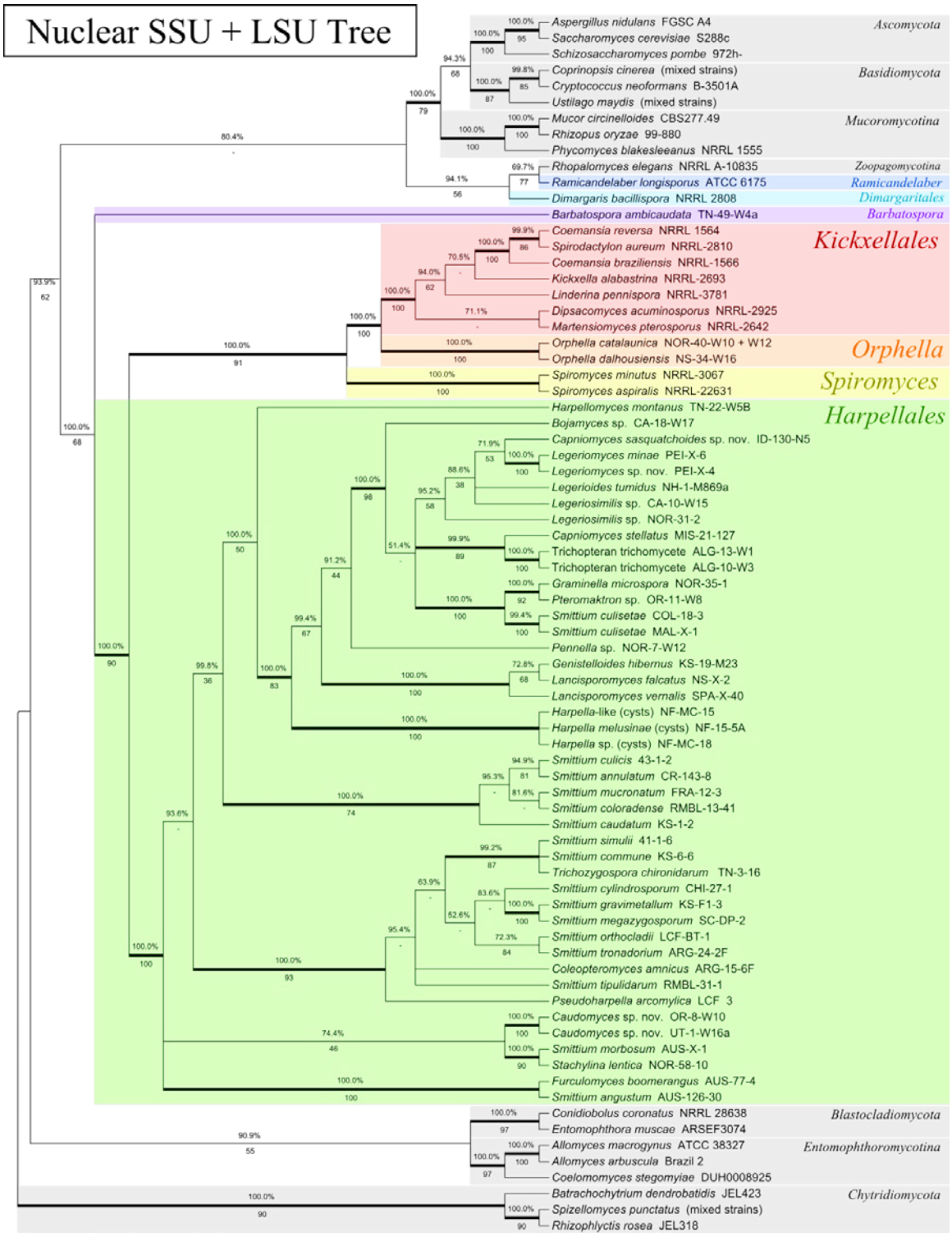

Nuclear SSU + LSU Tree
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Ascomycota
Coprinopsis cinerea (mixed strains)
Cryptococcus neoformans B-3501A
Ustilago maydis (mixed strains)
Basidiomycota
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Mucoromycotina
Rhopalomyces elegans NRRL A-10835
Zoopagomycotina
Ramicandelaber longisporus ATCC 6175
Ramicandelaber
Dimargaris bacillispora NRRL 2808
Dimargaritales
Barbatospora ambicaudata TN-49-W4a
Barbatospora
Coemansia reversa NRRL 1564
Spirodactylon aureum NRRL-2810
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Kickxellales
Orphella catalaunica NOR-40-W10 + W12
Orphella dalhousiensis NS-34-W16
Orphella
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Spiromyces
Harpellomyces montanus TN-22-W5B
Bojamyces sp. CA-18-W17
Capniomyces sasquatchoides sp. nov. ID-130-N5
Legeriomyces minae PEI-X-6
Legeriomyces sp. nov. PEI-X-4
Legerioides tumidus NH-1-M869a
Legeriosimilis sp. CA-10-W15
Legeriosimilis sp. NOR-31-2
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Trichopteran trichomycete ALG-10-W3
Graminella microspora NOR-35-1
Pteromaktron sp. OR-11-W8
Smittium culisetae COL-18-3
Smittium culisetae MAL-X-1
Pennella sp. NOR-7-W12
Genistelloides hibernus KS-19-M23
Lancisporomyces falcatus NS-X-2
Lancisporomyces vernalis SPA-X-40
Harpella-like (cysts) NF-MC-15
Harpella melusinae (cysts) NF-15-5A
Harpella sp. (cysts) NF-MC-18
Smittium culicis 43-1-2
Smittium annulatum CR-143-8
Smittium mucronatum FRA-12-3
Smittium coloradense RMBL-13-41
Smittium caudatum KS-1-2
Smittium simulii 41-1-6
Smittium commune KS-6-6
Trichozygospora chironidarum TN-3-16
Smittium cylindrosporum CHI-27-1
Smittium gravimetallum KS-F1-3
Smittium megazygosporum SC-DP-2
Smittium orthocladii LCF-BT-1
Smittium tronadorium ARG-24-2F
Coleopteromyces amnicus ARG-15-6F
Smittium tipulidarum RMBL-31-1
Pseudoharpella arcomylica LCF 3
Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Smittium morbosum AUS-X-1
Stachylina lentica NOR-58-10
Furculomyces boomerangus AUS-77-4
Smittium angustum AUS-126-30
Harpellales
Conidiobolus coronatus NRRL 28638
Entomophthora muscae ARSEF3074
Blastocladiomycota
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Coelomomyces stegomyiae DUH0008925
Entomophthoromycotina
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus (mixed strains)
Rhizophlyctis rosea JEL318
Chytridiomycota

Figure 1.3 Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of nuclear small subunit (SSU) and nuclear large subunit (LSU) rDNA.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).

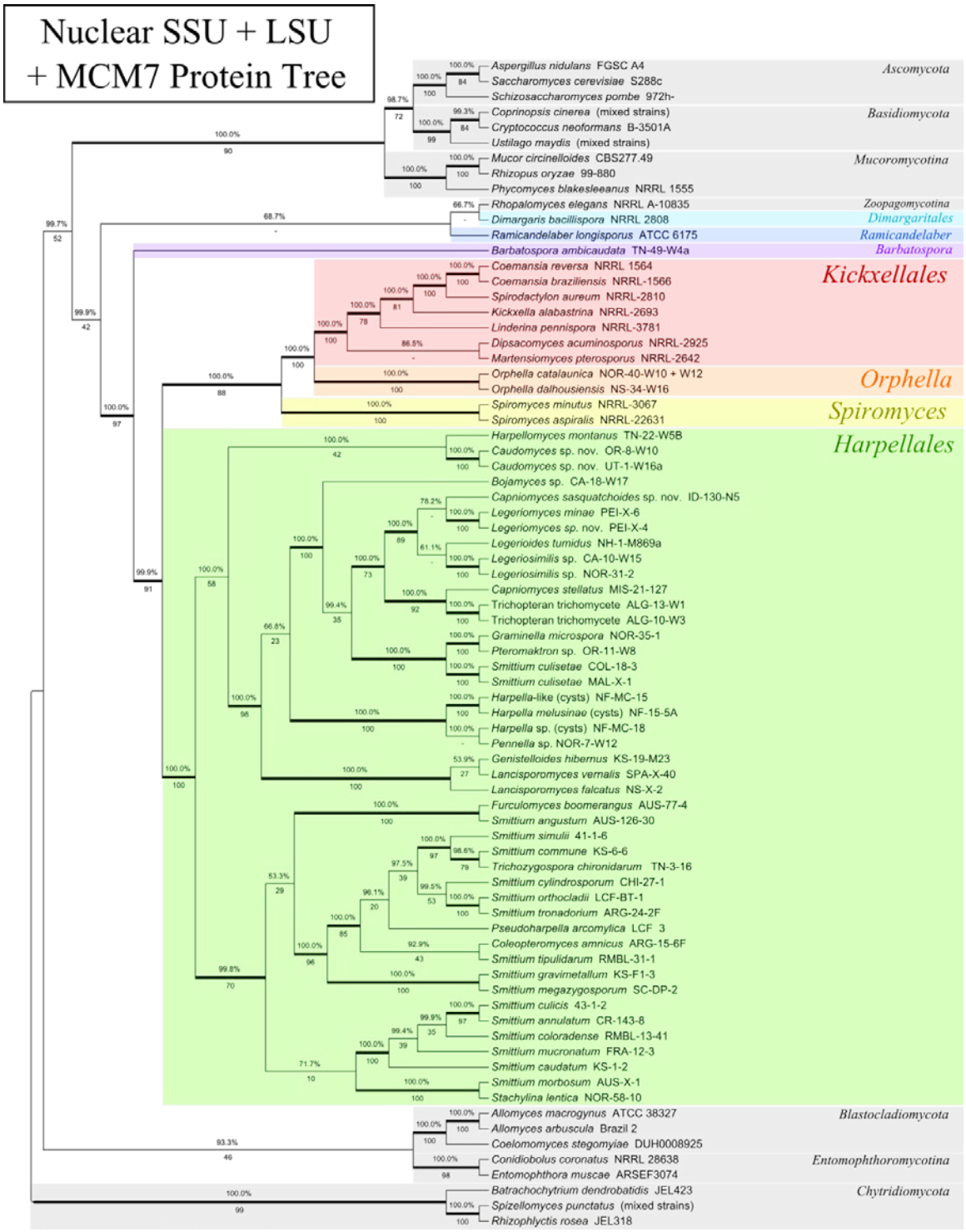

Nuclear SSU + LSU
+ MCM7 Protein Tree
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Ascomycota
Coprinopsis cinerea (mixed strains)
Cryptococcus neoformans B-3501A
Ustilago maydis (mixed strains)
Basidiomycota
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Mucoromycotina
Rhopalomyces elegans NRRL A-10835
Zoopagomycotina
Dimargaris bacillispora NRRL 2808
Dimargaritales
Ramicandelaber longisporus ATCC 6175
Ramicandelaber
Barbatospora ambicaudata TN-49-W4a
Barbatospora
Coemansia reversa NRRL 1564
Coemansia braziliensis NRRL-1566
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Kickxellales
Orphella catalaunica NOR-40-W10 + W12
Orphella dalhousiensis NS-34-W16
Orphella
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Spiromyces
Harpellomyces montanus TN-22-W5B
Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Bojamyces sp. CA-18-W17
Capniomyces sasquatchoides sp. nov. ID-130-N5
Legeriomyces minae PEI-X-6
Legeriomyces sp. nov. PEI-X-4
Legerioides tumidus NH-1-M869a
Legeriosimilis sp. CA-10-W15
Legeriosimilis sp. NOR-31-2
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Trichopteran trichomycete ALG-10-W3
Graminella microspora NOR-35-1
Pteromaktron sp. OR-11-W8
Smittium culisetae COL-18-3
Smittium culisetae MAL-X-1
Harpella-like (cysts) NF-MC-15
Harpella melusinae (cysts) NF-15-5A
Harpella sp. (cysts) NF-MC-18
Pennella sp. NOR-7-W12
Genistelloides hibernus KS-19-M23
Lancisporomyces vernalis SPA-X-40
Lancisporomyces falcatus NS-X-2
Furculomyces boomerangus AUS-77-4
Smittium angustum AUS-126-30
Smittium simulii 41-1-6
Smittium commune KS-6-6
Trichozygospora chironidarum TN-3-16
Smittium cylindrosporum CHI-27-1
Smittium orthocladii LCF-BT-1
Smittium tronadorium ARG-24-2F
Pseudoharpella arcomylica LCF 3
Coleopteromyces amnicus ARG-15-6F
Smittium tipulidarum RMBL-31-1
Smittium gravimetallum KS-F1-3
Smittium megazygosporum SC-DP-2
Smittium culicis 43-1-2
Smittium annulatum CR-143-8
Smittium coloradense RMBL-13-41
Smittium mucronatum FRA-12-3
Smittium caudatum KS-1-2
Smittium morbosum AUS-X-1
Stachylina lentica NOR-58-10
Harpellales
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Blastocladiomycota
Coelomomyces stegomyiae DUH0008925
Conidiobolus coronatus NRRL 28638
Entomophthora muscae ARSEF3074
Entomophthoromycotina
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus (mixed strains)
Rhizophlyctis rosea JEL318
Chytridiomycota

Figure 1.4 Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of SSU and LSU rDNA as well as MCM7 translated protein sequences.

The method used for tree inference and the format of the tree are the same as for Fig. 1.3.

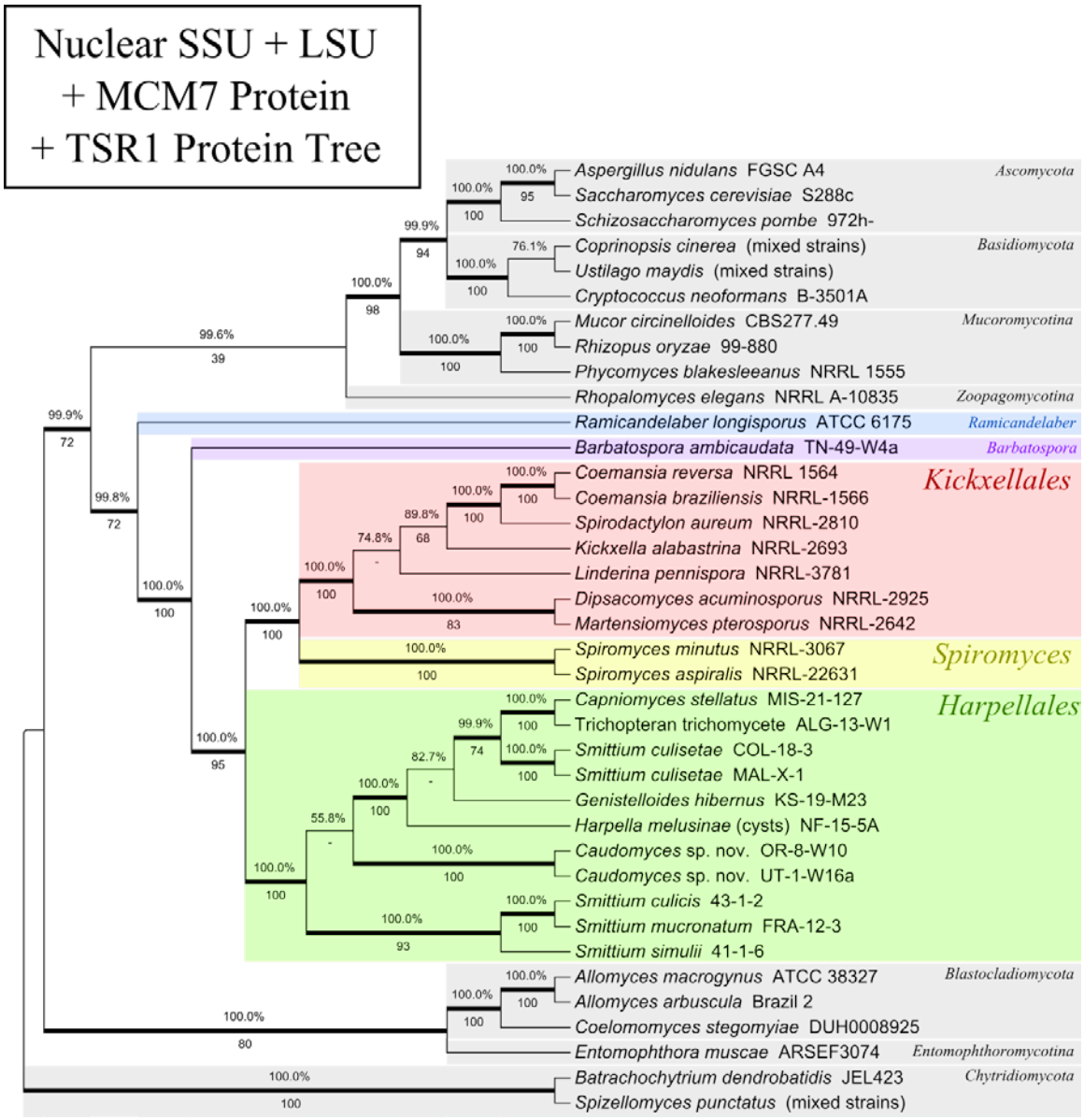


Figure 1.5 Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of SSU and LSU rDNA as well as MCM7 and TSR1 translated protein sequences.

The method used for tree inference and the format of the tree are the same as for Fig. 1.3.

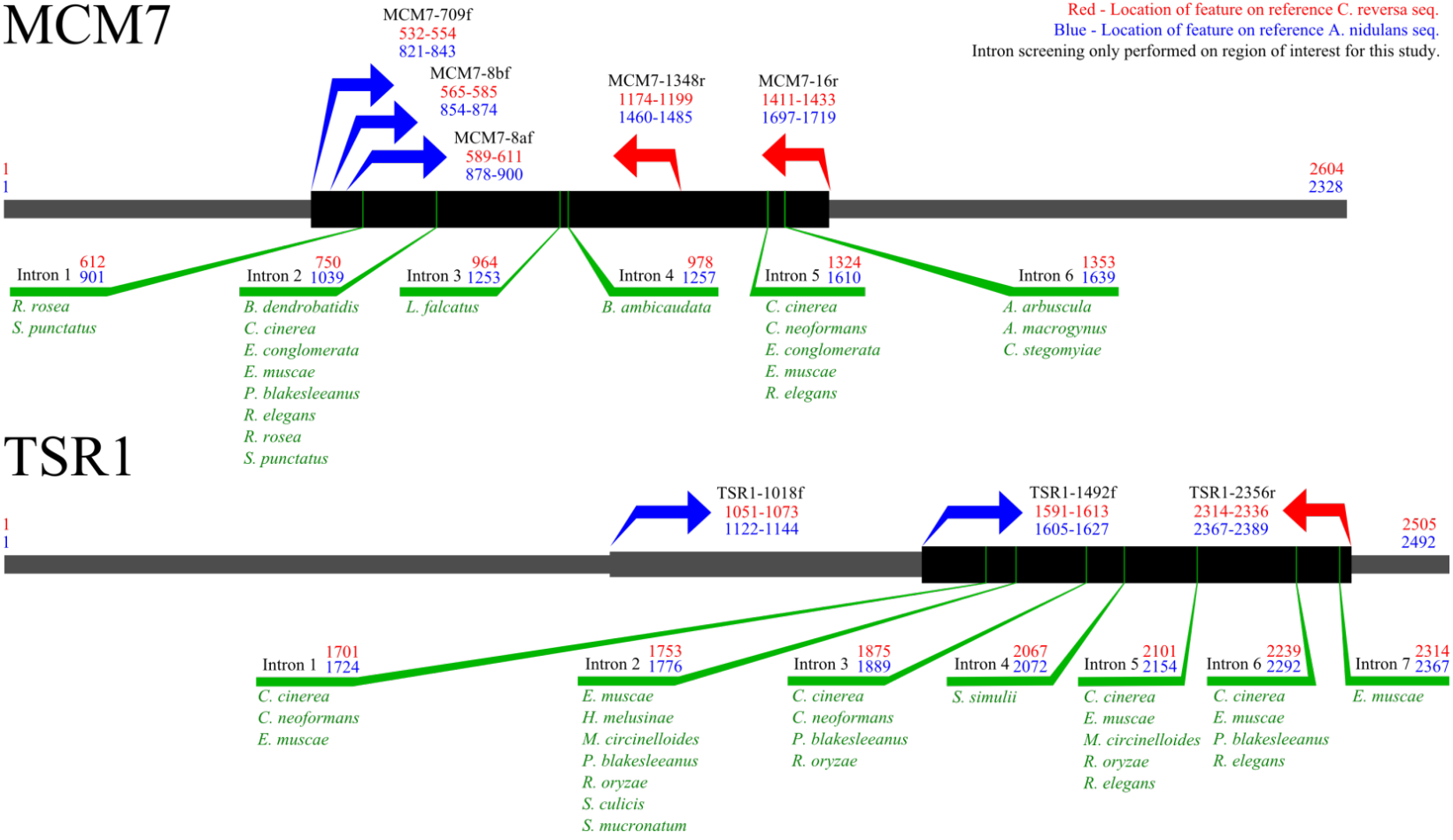

MCM7
Red - Location of feature on reference C. reversa seq.
Blue - Location of feature on reference A. nidulans seq.
Intron screening only performed on region of interest for this study.
MCM7-709f
532-554
821-843
MCM7-8bf
565-585
854-874
MCM7-8af
589-611
878-900
MCM7-1348r
1174-1199
1460-1485
MCM7-16r
1411-1433
1697-1719
1
1
2604
2328
Intron 1 612 901
R. rosea
S. punctatus
Intron 2 750 1039
B. dendrobatidis
C. cinerea
E. conglomerata
E. muscae
P. blakesleeanus
R. elegans
R. rosea
S. punctatus
Intron 3 964 1253
L. falcatus
Intron 4 978 1257
B. ambicaudata
Intron 5 1324 1610
C. cinerea
C. neoformans
E. conglomerata
E. muscae
R. elegans
Intron 6 1353 1639
A. arbuscula
A. macrogynus
C. stegomyiae
TSR1
TSR1-1018f
1051-1073
1122-1144
TSR1-1492f
1591-1613
1605-1627
TSR1-2356r
2314-2336
2367-2389
1
1
2505
2492
Intron 1 1701 1724
C. cinerea
C. neoformans
E. muscae
Intron 2 1753 1776
E. muscae
H. melusinae
M. circinelloides
P. blakesleeanus
R. oryzae
S. culicis
S. mucronatum
Intron 3 1875 1889
C. cinerea
C. neoformans
P. blakesleeanus
R. oryzae
Intron 4 2067 2072
S. simulii
Intron 5 2101 2154
C. cinerea
E. muscae
M. circinelloides
R. oryzae
R. elegans
Intron 6 2239 2292
C. cinerea
E. muscae
P. blakesleeanus
R. elegans
Intron 7 2314 2367
E. muscae

Figure 1.6 Map of the genes MS456 (MCM7) and MS277 (TSR1).

5’ end is at left. Forward primers are marked with blue arrows, reverse primers with red arrows. Introns are labelled in green. Red numbers designate the position of the feature on a reference sequence from *C. reversa*. Blue numbers designate the position of features on a reference sequence from *A. nidulans*. Intron locations are given by the position in the alignment in which those introns would be present, if they existed in the reference species.

MCM7 Nucleotide Tree

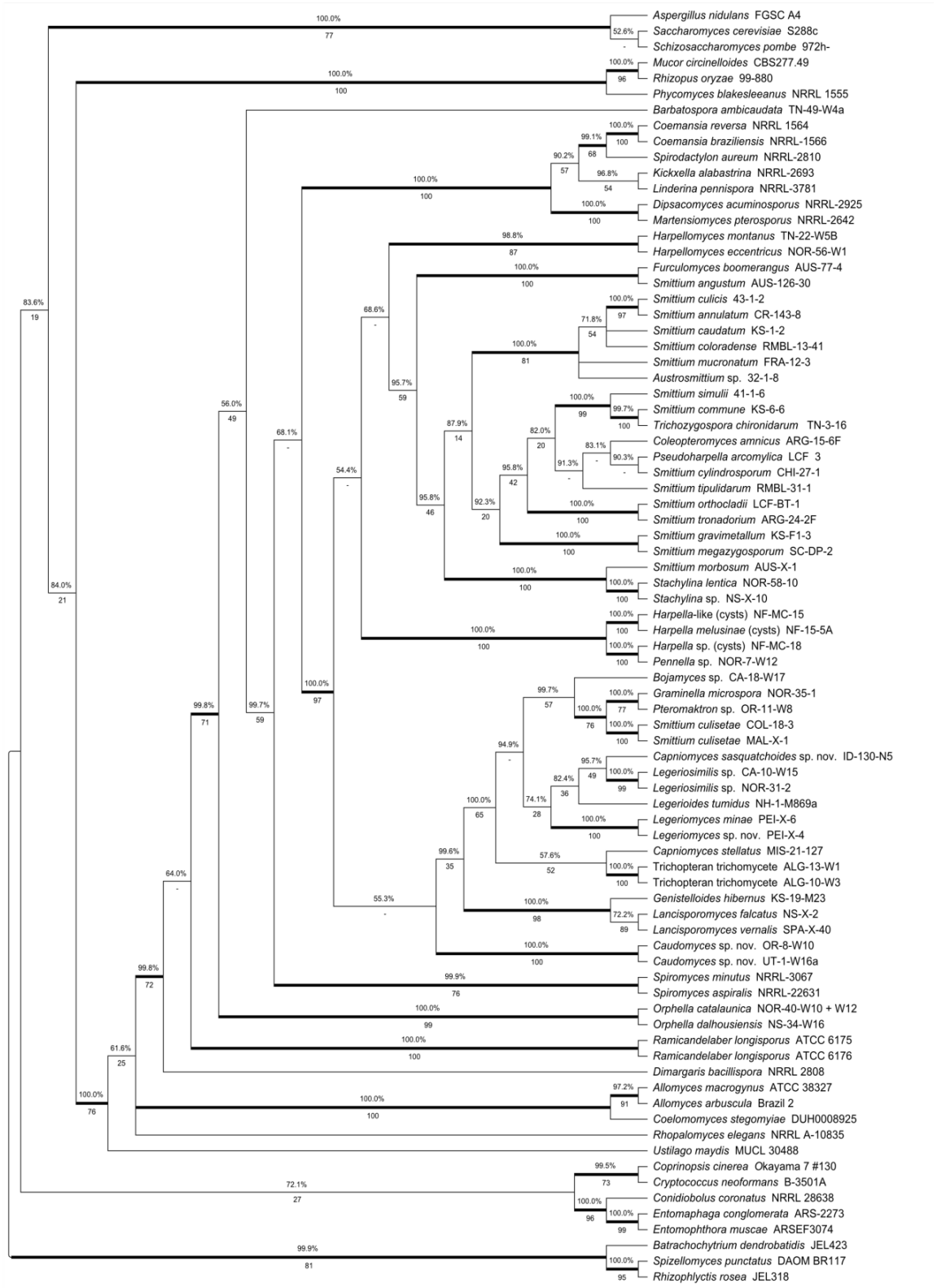


Figure 1.7 Phylogeny of the *Kickxellomycotina* based on an alignment of MCM7 nucleotide sequences.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). The three codon positions were all considered to be on different, unlinked partitions during tree calculation. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).

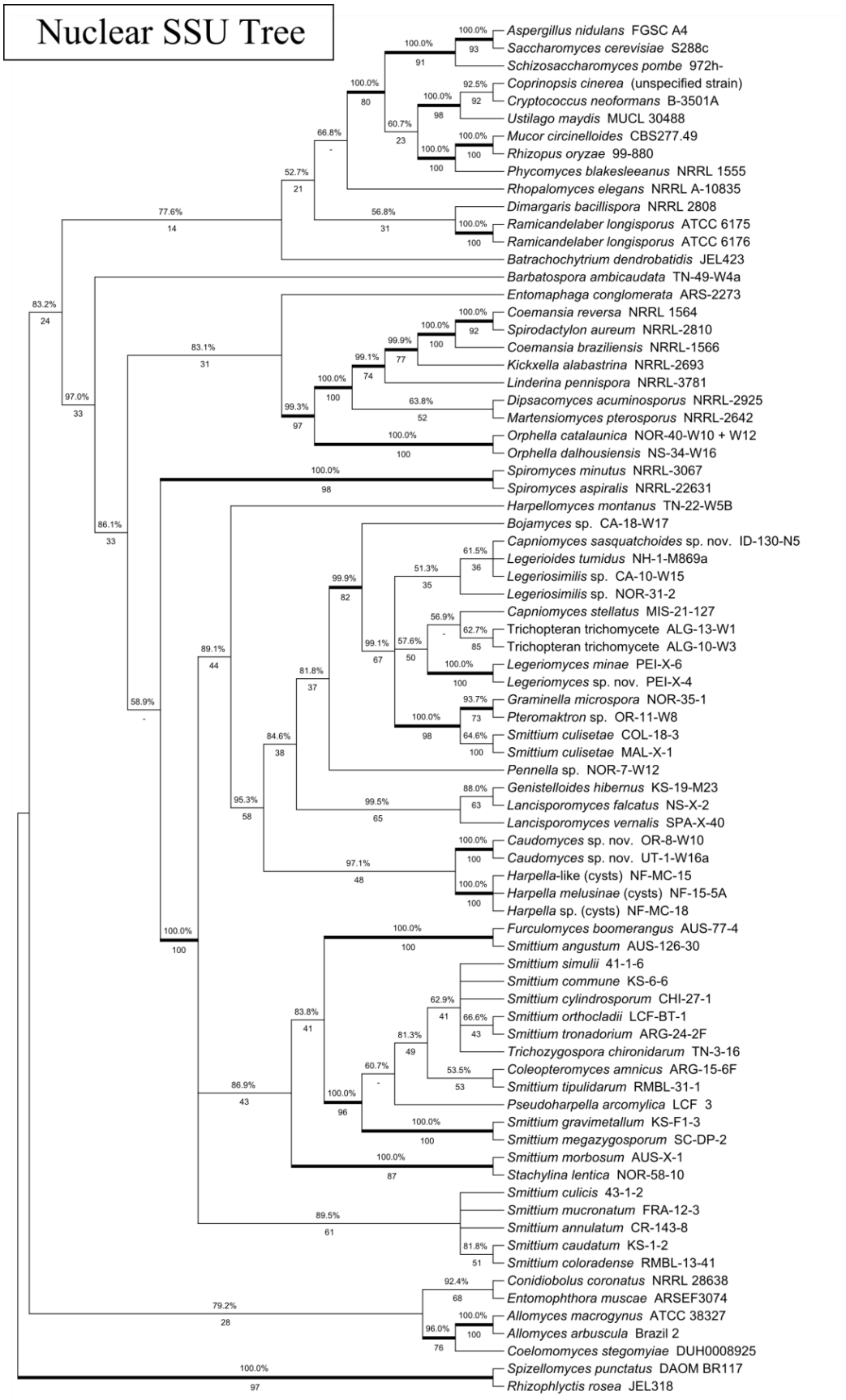
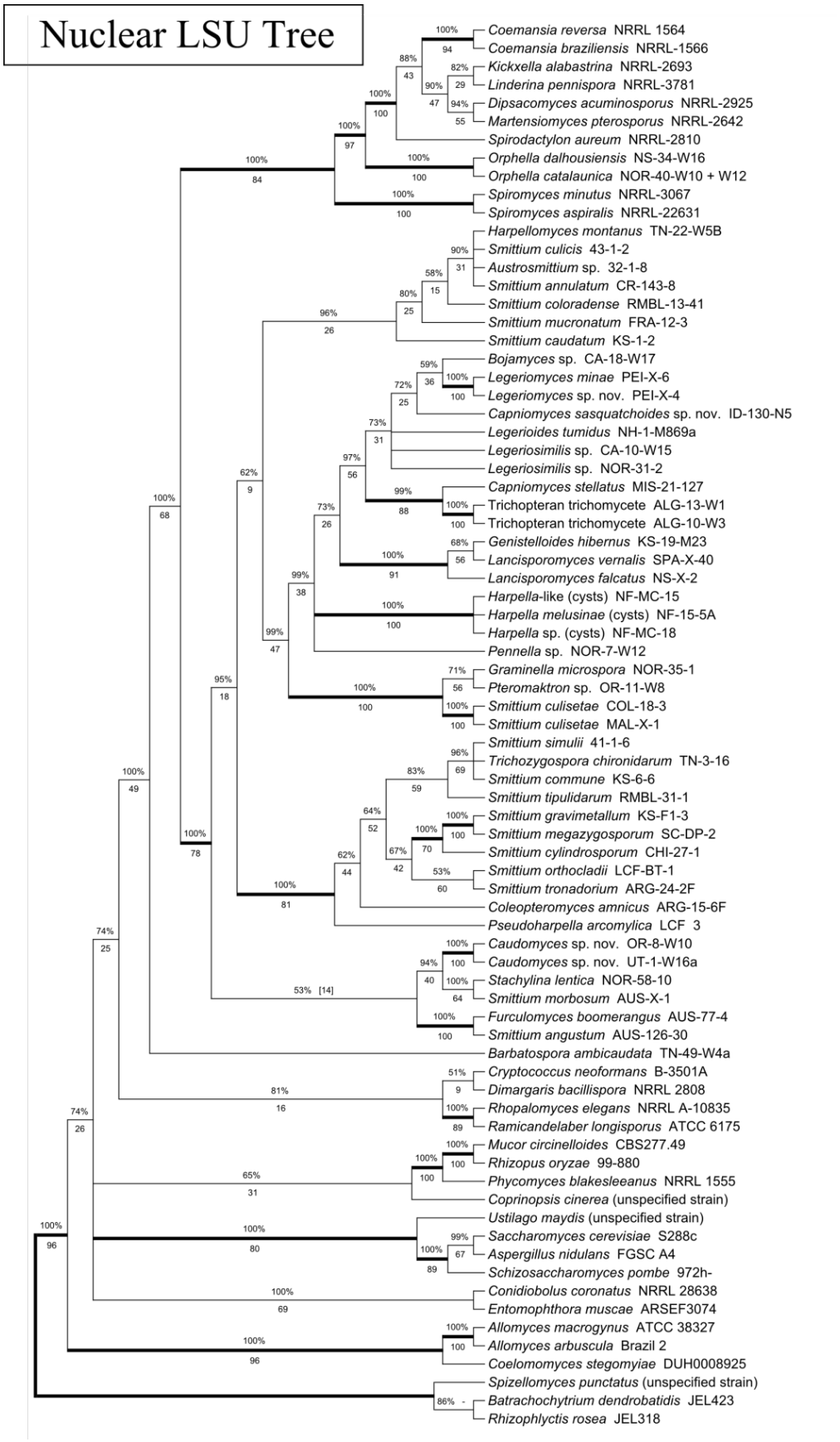

Nuclear SSU Tree
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Coprinopsis cinerea (unspecified strain)
Cryptococcus neoformans B-3501A
Ustilago maydis MUCL 30488
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Rhopalomyces elegans NRRL A-10835
Dimargaris bacillispora NRRL 2808
Ramicandelaber longisporus ATCC 6175
Ramicandelaber longisporus ATCC 6176
Batrachochytrium dendrobatidis JEL423
Barbatospora ambicaudata TN-49-W4a
Entomaphaga conglomerata ARS-2273
Coemansia reversa NRRL 1564
Spirodactylon aureum NRRL-2810
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella catalaunica NOR-40-W10 + W12
Orphella dalhousiensis NS-34-W16
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Harpellomyces montanus TN-22-W5B
Bojamyces sp. CA-18-W17
Capniomyces sasquatchoides sp. nov. ID-130-N5
Legerioides tumidus NH-1-M869a
Legeriosimilis sp. CA-10-W15
Legeriosimilis sp. NOR-31-2
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Trichopteran trichomycete ALG-10-W3
Legeriomyces minae PEI-X-6
Legeriomyces sp. nov. PEI-X-4
Graminella microspora NOR-35-1
Pteromaktron sp. OR-11-W8
Smittium culisetae COL-18-3
Smittium culisetae MAL-X-1
Pennella sp. NOR-7-W12
Genistelloides hibernus KS-19-M23
Lancisporomyces falcatus NS-X-2
Lancisporomyces vernalis SPA-X-40
Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Harpella-like (cysts) NF-MC-15
Harpella melusinae (cysts) NF-15-5A
Harpella sp. (cysts) NF-MC-18
Furculomyces boomerangus AUS-77-4
Smittium angustum AUS-126-30
Smittium simulii 41-1-6
Smittium commune KS-6-6
Smittium cylindrosporum CHI-27-1
Smittium orthocladii LCF-BT-1
Smittium tronadorium ARG-24-2F
Trichozygospora chironidarum TN-3-16
Coleopteromyces amnicus ARG-15-6F
Smittium tipulidarum RMBL-31-1
Pseudoharpella arcomylica LCF 3
Smittium gravimetallum KS-F1-3
Smittium megazygosporum SC-DP-2
Smittium morbosum AUS-X-1
Stachylina lentica NOR-58-10
Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Smittium annulatum CR-143-8
Smittium caudatum KS-1-2
Smittium coloradense RMBL-13-41
Conidiobolus coronatus NRRL 28638
Entomophthora muscae ARSEF3074
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Coelomomyces stegomyiae DUH0008925
Spizellomyces punctatus DAOM BR117
Rhizophlyctis rosea JEL318

Figure 1.8 Phylogeny of the *Kickxellomycotina* based on an alignment of nuclear small subunit (SSU) rDNA.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). The three codon positions were all considered to be on different, unlinked partitions during tree calculation. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).


Nuclear LSU Tree
Coemansia reversa NRRL 1564
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Spirodactylon aureum NRRL-2810
Orphella dalhousiensis NS-34-W16
Orphella catalaunica NOR-40-W10 + W12
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Harpellomyces montanus TN-22-W5B
Smittium culicis 43-1-2
Austrosmittium sp. 32-1-8
Smittium annulatum CR-143-8
Smittium coloradense RMBL-13-41
Smittium mucronatum FRA-12-3
Smittium caudatum KS-1-2
Bojamyces sp. CA-18-W17
Legeriomyces minae PEI-X-6
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Capniomyces sasquatchoides sp. nov. ID-130-N5
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Legeriosimilis sp. CA-10-W15
Legeriosimilis sp. NOR-31-2
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Trichopteran trichomycete ALG-10-W3
Genistelloides hibernus KS-19-M23
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Harpella-like (cysts) NF-MC-15
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Pennella sp. NOR-7-W12
Graminella microspora NOR-35-1
Pteromaktron sp. OR-11-W8
Smittium culisetae COL-18-3
Smittium culisetae MAL-X-1
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Smittium commune KS-6-6
Smittium tipulidarum RMBL-31-1
Smittium gravimetallum KS-F1-3
Smittium megazygosporum SC-DP-2
Smittium cylindrosporum CHI-27-1
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Coleopteromyces amnicus ARG-15-6F
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Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Stachylina lentica NOR-58-10
Smittium morbosum AUS-X-1
Furculomyces boomerangus AUS-77-4
Smittium angustum AUS-126-30
Barbatospora ambicaudata TN-49-W4a
Cryptococcus neoformans B-3501A
Dimargaris bacillispora NRRL 2808
Rhopalomyces elegans NRRL A-10835
Ramicandelaber longisporus ATCC 6175
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Coprinopsis cinerea (unspecified strain)
Ustilago maydis (unspecified strain)
Saccharomyces cerevisiae S288c
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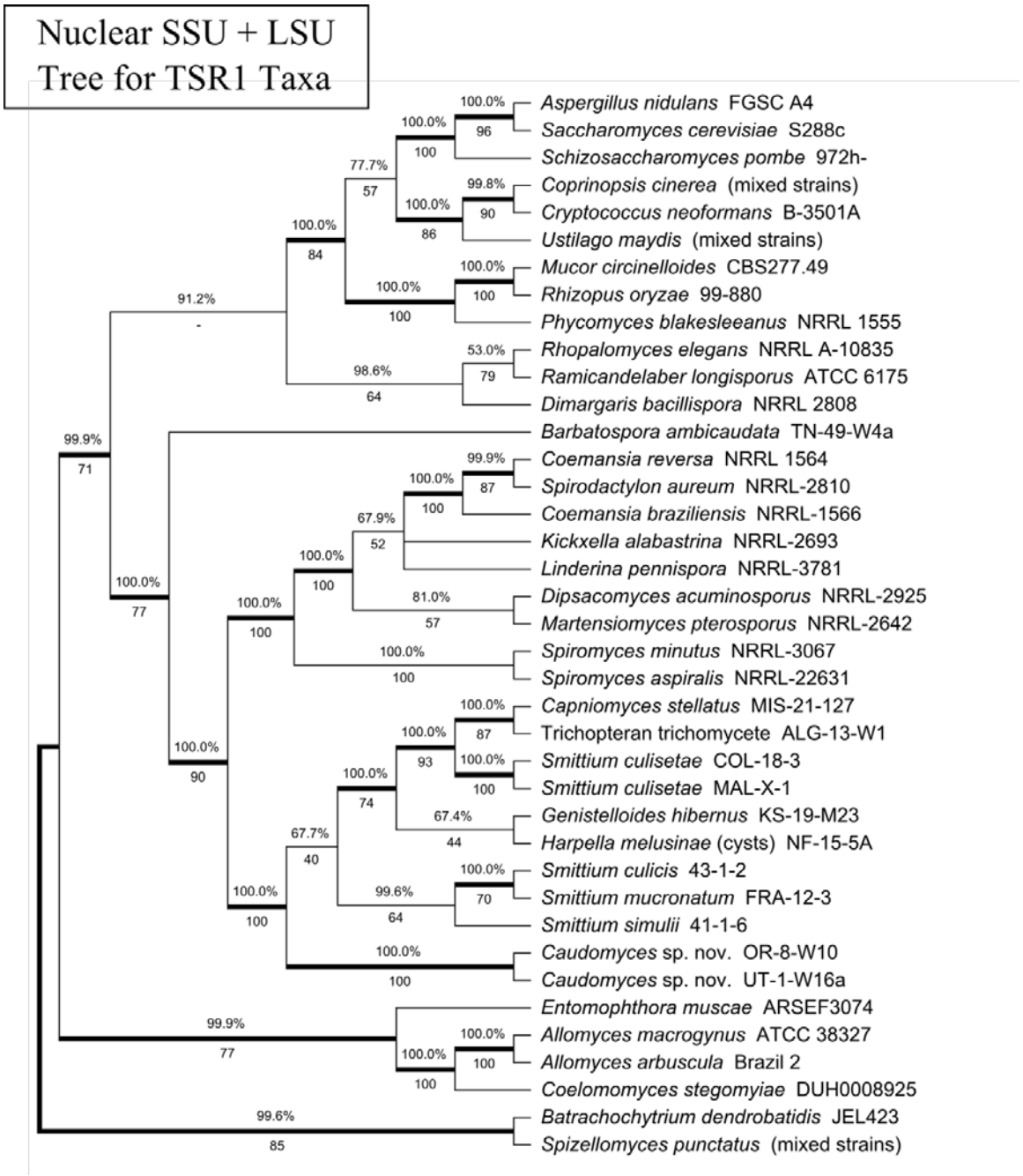
Schizosaccharomyces pombe 972h-
Conidiobolus coronatus NRRL 28638
Entomophthora muscae ARSEF3074
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Coelomomyces stegomyiae DUH0008925
Spizellomyces punctatus (unspecified strain)
Batrachochytrium dendrobatidis JEL423
Rhizophlyctis rosea JEL318
53% [14]

Figure 1.9 Phylogeny of the *Kickxellomycotina* based on an alignment of nuclear large subunit (LSU) rDNA.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). The three codon positions were all considered to be on different, unlinked partitions during tree calculation. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).


Nuclear SSU + LSU
Tree for TSR1 Taxa
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Coprinopsis cinerea (mixed strains)
Cryptococcus neoformans B-3501A
Ustilago maydis (mixed strains)
Mucor circinelloides CBS277.49
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Rhopalomyces elegans NRRL A-10835
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Dimargaris bacillispora NRRL 2808
Barbatospora ambicaudata TN-49-W4a
Coemansia reversa NRRL 1564
Spirodactylon aureum NRRL-2810
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
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Smittium culisetae MAL-X-1
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Smittium mucronatum FRA-12-3
Smittium simulii 41-1-6
Caudomyces sp. nov. OR-8-W10
Caudomyces sp. nov. UT-1-W16a
Entomophthora muscae ARSEF3074
Allomyces macrogynus ATCC 38327
Allomyces arbuscula Brazil 2
Coelomomyces stegomyiae DUH0008925
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus (mixed strains)

Figure 1.10 Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of nuclear small subunit (SSU) and nuclear large subunit (LSU) rDNA, only including samples for which TSR1 data was available.

Only taxa for which we had TSR1 were included in the alignment to provide a basis for comparison to the TSR1 protein tree. Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and > .70 MLBP).

AN EIGHT-GENE MOLECULAR PHYLOGENY OF THE KICKXELLOMYCOTINA, INCLUDING THE FIRST PHYLOGENETIC PLACEMENT OF ASELLARIALES

Abstract

Kickxellomycotina is a recently described subphylum encompassing four orders of zygomycetous fungi distinguished by the formation of disciform septal pores with lenticular plugs. Morphological diversification and life history evolution has made the relationships within and between the four orders difficult to resolve on those grounds alone. Here, we infer the phylogeny of the Kickxellomycotina based on an eight-gene supermatrix including both ribosomal rDNA (18S, 28S, 5.8S) and protein sequences (MCM7, TSR1, RPB1, RPB2, and β-tubulin). The Kickxellomycotina is resolved as monophyletic and with affinity to members of the Zoopagomycotina. Eight unique clades are distinguished within the Kickxellomycotina, including the four defined orders (Asellariales, Dimargaritales, Harpellales, and Kickxellales) as well as four genera previously placed within these orders (*Barbatospora*, *Orphella*, *Ramicandelaber*, and *Spiromyces*). Dimargaritales and *Ramicandelaber* are the earliest diverging members of the subphylum, although the relationship between the two remains uncertain. The remaining six clades form a monophyletic grouping, from which *Barbatospora* diverges first, followed by a split that divides the group into a clade composed of Asellariales and Harpellales, and a clade composed of Kickxellales, *Orphella*, and *Spiromyces*. The comparative morphology and ecology of these clades is discussed in the light of these newly inferred evolutionary relationships and ancestral states are reconstructed for four

potentially informative characters. We also employ and promote a common terminology for the sexual and asexual reproductive features of the Kickxellomycotina.

Introduction

Kickxellomycotina has only recently been described as a formal taxonomic group (Hibbett et al. 2007) although previous studies have hinted at a possible relationship between the four orders that make it up (Benjamin 1979). Members of the Kickxellomycotina are unified by a unique septal morphology that includes a diskiform septal pore and a lenticular plug, both of which have been verified within all four orders (Farr and Lichtwardt 1967, Young 1969, Moss 1975, Moss and Young 1978, Jeffries and Young 1979, Brain et al. 1982, Saikawa et al. 1997b). Other features common to all orders are the production of asexual spores, as either arthrospores or as dehiscent sporangia (referred to as either merosporangia, sporangiola, or trichospores), and the production of zygosporangia.

Beyond these shared features, the group is remarkably diverse, with members occupying ecological niches ranging from saprophytes to haustorial mycoparasites to arthropod endosymbionts. Morphology varies correspondingly. Asexual spore morphology varies between bispored merosporangia in the Dimargaritales (Benjamin 1959, 1961, 1963, 1965), unispored in the Kickxellales (Benjamin 1958, 1966), specialized sporangia with spore extrusion and non-motile appendages in the Harpellales, and arthrospores in the Asellariales (Lichtwardt et al. 2007). Sexual spores vary from spherical to biconical to coiled (Benjamin 1959, Valle and Santamaria 2005, Lichtwardt et al. 2007). Some species possess unique features, such as rhizoids and stolons in *Ramicandelaber* (Ogawa et al. 2001), the multi-celled dissemination units in *Orphella*

(Valle and Santamaria 2005) and possibly *Orchesellaria* (Degawa 2009), or the vegetative reproductive propagules in *Graminella* (Valle et al. 2008).

With all of this diversity, it may not be surprising that the affinities between members of the group have been revealed slowly. The Asellariales and Harpellales were originally placed in a class consisting of hair-like arthropod endosymbionts, the Trichomycetes. This group has traditionally also included two orders later found to be protist, the Amoebidiales and Eccrinales (Cafaro 2005), which later relegated the class name to an ecological grouping (Hibbett et al. 2007). Benjamin (1979) removed the Dimargaritaceae from the Kickxellales and established a new order, Dimargaritales. He also suggested the possibility of a relationship between the four orders (Asellariales, Dimargaritales, Harpellales, and Kickxellales). This idea has been revisited by other authors, but often not including the Dimargaritales, which were considered too morphologically different (Moss and Young 1978) or the Asellariales, due to a lack of sequence data (James et al. 2006). This backdrop set the stage for the formal reclassification as a subphylum in the most recent major reclassification of Fungi (Hibbett et al. 2007).

Several previous studies have used molecular sequences that have contained members of the Kickxellomycotina, but few of them have actually focused upon the group itself. The first (Walker, 1984) used 5S rRNA to examine a number of zygomycetous fungi including Kickxellales and Harpellales species. Notwithstanding the limited resolution provided by the 5S region, it reinforced that the order Amoebidiales was probably not related to the other trichomycete fungi as suspected in Sangar et al. (1972). O'Donnell et al. (1998) focused on the Harpellales and Kickxellales, and demonstrated that both groups were likely monophyletic, with the exception of the Kickxellales and *Spiromyces*,

and that a relationship between the two orders was likely. This is also the only study to date that included both a morphological character matrix as well as a molecular one. Tanabe et al. (2000) used the 18S nuclear rDNA to infer a phylogeny of the parasitic Zygomycota (including Dimargaritales and Zoopagales), but encountered problems due to the unusual sequence divergence of the Dimargaritales nuclear rDNA. Gottlieb and Lichtwardt (2001) used the 18S rDNA to infer a phylogeny of the Harpellales, and also examined the size of the ITS1 and ITS2. Important discoveries included the large size and great divergence of the ITS sequences within these fungi, and the finding that *Smittium culisetae* seemed to be more closely related to other genera with Harpellales than to species of *Smittium*. Keeling (2003) utilized the α- and β-tubulin genes to produce a phylogeny of the Zygomycetes. This study demonstrated the potential utility of these genes within these groups, and called attention to the risk of encountering paralogous copies. Using a pair of protein coding genes, RPB1 and EF1-α to examine the Zygomycota, Tanabe et al. (2004) recovered a monophyletic Kickxellomycotina for the three orders included (with no Asellariales included) using RPB1. The EF1-α tree was poorly resolved, suggesting this gene may be of little use for the Kickxellomycotina.

Numerous studies in 2006 further advanced our understanding of the evolution of the Kickxellomycotina. Assembling the Fungal Tree of Life (AFTOL) was a community-wide effort for a multi-gene based understanding of the evolution of Fungi. This culminated in James et al. (2006), with a six gene (18S, 28S, 5.8S, RPB1, RPB2, and EF1-α) phylogeny of Fungi that included five representatives of the Kickxellomycotina from three of the orders (Asellariales was still not available). The phylogeny suggested that the Kickxellomycotina was monophyletic and most closely related to the

Zoopagomycotina, and that Kickxellales and Harpellales may not be monophyletic. This study, along with Liu et al. (2006), were the first to establish the value of RPB2 for the Kickxellomycotina. White (2006) published the first in-depth phylogeny of the Harpellales, utilizing 18S and 28S rDNA. This study provided additional evidence of the non-monophyly of the Harpellales with respect to *Orphella*, and the non-monophyly of the Kickxellales with respect to *Spiromyces*. It also provided evidence that the two families within the Harpellales, the Harpellaceae and the Legeriomycetaceae, might not be warranted. Finally, White et al. (2006b) used nuclear rDNA (18S, 28S, and 5.8S) to produce a phylogeny of the Zygomycetes. This effort did not recover a monophyletic Kickxellomycotina, but instead placed the Dimargaritales (and the Kickxellales genus *Ramicandelaber*) with the Entomophthoromycota genus *Neozygites* and the Zoopagales. The rest of the Kickxellomycotina were resolved as monophyletic, although Asellariales was still not present due to persistent difficulties in amplifying and sequencing members of this order. The separation of *Orphella* and *Spiromyces* from their current orders was once again reinforced, and the placement of *Ramicandelaber* was uncertain. The incongruities between the White et al. (2006b) and James et al. (2006) trees with respect to the Kickxellomycotina highlighted the need for additional markers and a more in-depth study with greater taxon sampling.

Additional molecular work on the Kickxellomycotina would not appear until 2012. Using an rDNA-based phylogeny of the Harpellales that focused on *Smittium*, Wang et al. (2012a) revealed that *Smittium* may not be monophyletic, as it separates into several well-supported clades under molecular analysis, some of which include other genera. More recently, Tretter et al. (2013) investigated the utility of the genes MCM7 and TSR1

within the Kickxellomycotina. These genes were first reported by Aguileta et al. (2008) as potentially useful phylogenetic markers within Fungi. Schmitt et al. (2009) presented the first published primers for these genes and tested them within the Ascomycota. Within the Kickxellomycotina, MCM7 demonstrated substantial phylogenetic utility, although the utility of TSR1 was less clear. Tretter et al. (2013) was unclear as to the monophyly of the Kickxellomycotina; there was a core clade including all of the Harpellales and Kickxellales (except *Ramicandelaber*) that was monophyletic in all trees, but the rDNA markers combined Dimargaritales and *Ramicandelaber* with the Zoopagales. By contrast for MCM7, the group was monophyletic. MCM7 reinforced the separation of *Orphella*, *Spiromyces*, and *Ramicandelaber* from their current orders, with another genus, *Barbatospora*, also separating from the Harpellales. This study also provided additional evidence that the current family structure of the Harpellales is not phylogenetically supported. The presence of a "Smittium" and "Non-Smittium" clade, first proposed by White (2006) and reinforced in Wang et al. (2012a), was supported instead.

These studies have shed a great deal of light onto the evolutionary history of the Kickxellomycotina, but the need for additional molecular phylogenies of its members remains clear. The purpose of the present study is to examine the evolutionary history of the Kickxellomycotina and provide greater support with a larger number of genes while still maintaining a broad sampling of taxa. Questions we intend to answer include the monophyly of the Kickxellomycotina as currently defined, the relative placement of the four orders including the Asellariales, and whether *Barbatospora*, *Orphella*, *Ramicandelaber*, and *Spiromyces* constitute unique clades that require new taxonomic

designation. Also of interest is the overall placement of the Kickxellomycotina within the Fungi and the evolution of various ecological and morphological characteristics within the group.

Materials and Methods

The majority of the DNA samples for this study had been extracted as part of previous projects (White 2006, James et al. 2006, White et al. 2006b, Wang et al. 2012a, Tretter et al. 2013). We also obtained genomic DNA by growing lyophilized cultures of *Dimargaris bacillispora*, *Dispira cornuta*, *Dispira parvispora*, and *Tieghemiomyces parasiticus* in axenic culture on YGCH media (O'Donnell et al. 1998), followed by extraction according to White (2006).

To overcome the challenges of amplifying a diverse set of genes within a large number of taxa, with which they had not been tested previously (or tested with no success), an array of PCR protocols was used (25 different protocols with 43 different primers). PCR protocol data is summarized in Table 2.1, and primers that were used are summarized in Table 2.2. Four different PCR reagent kits were used: Finnzymes Phusion Hot Start II (Thermo Fisher Scientific, Wilmington, Deleware), Go-Taq Green Master Mix, Go-Taq Green Hot Master Mix (Promega, Madison, Wisconsin), and TaKaRa LA PCR (TaKaRa Bio, Otsa, Shiga, Japan). Reaction chemistry was kept consistent within a given PCR kit with two exceptions: protocols designed to amplify protein-coding genes were run with a higher primer concentration than protocols designed to amplify rDNA, and some protocols included either betaine, DMSO, or BSA (see Table 2.1).

The reaction mix used with the Phusion HS II kit included 1× Phusion HF or GC Buffer, 0.20 mM of each dNTP, 1 mM of additional Mg^{2+} to a total of 2.5 mM, forward

and reverse primers at a concentration of 0.50 µM, and 0.02 U/µL of Phusion HS II TAQ. For the TaKaRa LA PCR kit, the reaction mix included GC Buffer 1 at 1.0×, 0.4 mM of each dNTP, no additional Mg^{2+} to a total concentration of 2 mM, 0.3 µM of each primer, and 0.050 U/µL of TaKaRa LA Taq. For the Promega GoTaq Green Master Mix kit, the master mix was used at 1.0×, primers were used at 0.3 µM, and 1 mM Mg^{2+} was added to a total concentration of 2.5 mM. Finally, for the Promega GoTaq Green Hotstart Master Mix kit, the master mix was once again used at 1.0×, primers were used at 0.3 µM for rDNA or 1.0 µM for protein-coding genes, and 0.5 mM Mg^{2+} was added to a total concentration of 2.5 mM. To each of these mixes, DMSO, betaine, or BSA was added in the concentrations given in Table 2.1. High-purity water was added to a final volume of 20 µL, and either 2 µL of 1/10 diluted genomic DNA (usually used with stocks of DNA obtained from cultures) or 1 µL of full concentration genomic DNA was added. Thermal cycling protocols are provided in Table 2.1. PCR products were analyzed via electrophoresis and sequenced according to Tretter et al. (2013). Products that could not be sequenced directly were cloned with the Promega pGEM-T Easy Vector System utilizing JM109 competent cells according to the manufacturer's instructions. Positive bacterial colonies were picked and added directly to reaction cocktails for PCR, and amplified products sequenced as above.

Additional sequences for analysis were taken from GenBank as well as several Broad Institute and JGI genome sequencing initiatives. Initial alignments of nucleotide sequences for protein-coding genes were made using MUSCLE 3.8.31 (Edgar 2004). Alignments were further adjusted by hand and introns removed. Sequences were translated using Mesquite 2.75 (Maddison and Maddison 2011). The rDNA sequences

were combined with the amino acid sequences into a supermatrix (Table 2.3). Sequences were aligned using MUSCLE again, and alignments were once again adjusted by hand. Ambiguously aligned regions were visually identified and removed.

Gene congruence was assessed via partitioned Bremer supports (Baker and DeSalle 1997). This analysis was performed once in TNT 1.1 (Goloboff et al. 2008) using the script from Peña et al. (2006), and again using PAUP* 4.0b10 (Swofford 2003) and TreeRot v. 3 (Sorenson and Franzosa 2007) with identical results. Model selection was performed using jModelTest2 (Guindon and Gascuel 2003, Darriba et al. 2012) and Prottest 2.4 (Abascal et al. 2005). For all of the protein-coding genes, the best model was LG+ Γ+I (Le and Gascuel 2008). For the 18S and 28S rDNA sequences, the best model was GTR+ Γ+I. Since the 5.8S is not truly independent of the 28S (they bind to one another over a significant portion of the 5.8S sequence's length) they were combined into a single partition.

Three different methods were combined to produce the final 8-gene tree. Mr. Bayes 3.1.2 was used for Bayesian inference (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Altekar et al. 2004) and provided the overall tree topology as well as one form of support. Convergence was assessed using the online version of AWTY (Wilgenbusch et al. 2004). The MCMC run appeared to have converged at 10^6 generations, so this was chosen as the endpoint. Maximum-likelihood and maximum-parsimony analyses were also used to assess tree topology support under an alternate framework. For maximum-likelihood calculations, RAxML 7.2.8 was used with PTHREADS parallelization, the CAT approximation, and the rapid bootstrapping algorithm (Stamatakis 2006a, Stamatakis 2006b, Ott et al. 2007, Stamatakis et al. 2008), with 100

bootstrap replicates performed. For maximum-parsimony calculations, TNT 1.1 was utilized. The new technology search method was used along with the default options to find the best tree. Symmetrical resampling (Goloboff et al. 2003) was used to assess tree support.

To examine the support of various evolutionary hypotheses, Mesquite was used to create constraint trees, and RAxML provided maximum-likelihood phylogenies with per-site likelihoods. Treegraph 2 (Stöver and Müller 2010) utilized to combine support values from multiple trees. CONSEL (Shimodaira and Hasegawa 2001) was used to conduct AU and SH tests of alternative tree topologies (Shimodaira and Hasegawa 1999, Shimodaira 2002). Seven pairs of topologies were tested, with three tests used to examine branches that were incongruent between the trees produced with different methods, and four tests used to examine the topological placement of four genera (*Barbatospora*, *Orphella*, *Spiromyces*, and *Ramicandelaber*) that placed outside of their current order.

Maximum-likelihood phylogenies for each gene, as well as for combined rDNA genes and protein-coding genes, were calculated to examine the relative contribution of each gene and combined analyses of only rDNA or only protein-coding genes. These trees were calculated using RAxML, following the same protocol as above.

Comparative morphological data was compiled from a number of published sources. Information on the evolutionary homology of morphological features was derived from published theories including histological studies, if available, or was hypothesized based on relative position and function. These data are summarized and illustrated in Fig. 2.1.

Results and Discussion

Summary of Results

We report 180 new DNA and amino acid sequences [this includes sequences also listed within Tretter et al. (2013)]. Sequences used within the tree are summarized within Table 2.3. The main phylogeny (Fig. 2.2) is based on the 50% majority-rules consensus tree derived from Bayesian analysis. Alternate supported topologies from the maximum-likelihood and parsimony trees were drawn on the tree with dotted lines. Strongly supported branches were supported by all three methods (Bayesian posterior probability (BPP) > .95, maximum-likelihood boostrap support (MLBP)> .70, parsimony symmetrical resampling (PSR) > .70) and are denoted with bold lines. A cladogram with support values is provided as a supplementary figure (Fig. 2.3). SH and AU tests of seven alternative hypotheses of tree topology are included in Table 2.4.

Partitioned Bremer supports were placed on the single most parsimonious tree (Fig. 2.4) with branches supported by the parsimony symmetrical resampling analysis in bold. To provide an additional analysis of gene congruity, Fig. 2.5 compares the maximum-likelihood topology of the dataset with only rDNA genes to one with only protein-coding ones. Figs. 2.6-2.13 are individual gene trees provided to offer a more detailed examination of gene congruence. Basic statistics for the individual gene and combined phylogenies are provided in Table 2.5.

Overall Tree Topology and Congruence

Congruence between individual genes and the multi-gene tree was generally good, with few strongly supported incongruities. The RPB2 phylogeny (Fig. 2.11) placed

Batrachochytrium dendrobatidis with *Spizellomyces punctatus*, unlike the 8-gene tree (which placed *S. punctatus* with *Rhizophylctis rosea*). This was also reflected in the TSR1 topology (Fig. 2.12). TSR1 also reconstructed a different phylogeny of the Ascomycetes than the 8-gene tree, with *Laccaria*, *Coprinopsis*, *Ustilago*, and *Malassezia* placed together (the 8-gene tree placed *Laccaria* and *Coprinopsis* with *Cryptococcus*, and *Ustilago* and *Malassezia* with *Puccinia*). β-tubulin (Fig. 2.13) placed the Mucoromycotina in a well-supported group with *Piptocephalis* and *Entomophthora*, placed the four Ascomycete species differently, and placed *Smittium simulii* outside of the main *Smittium* group.

Congruence between the protein-coding gene tree and the rDNA tree was also good, with no strongly-supported incongruities. However, the protein-coding gene tree was much closer to the final 8-gene tree, particularly with regard to Dimargaritales and *Asellaria*. These taxa demonstrated unusually long branch length for both rDNA-based genes, which may have resulted in incorrect positioning due to long-branch attraction, which is discussed in greater detail by clade. The MCM7 and β-tubulin phylogenies (Figs. 2.9 and 2.13) were the most similar in overall structure to the 8-gene tree. These two genes had the least sequence length variation and alignment uncertainty (barring 5.8S, but that was too short to contribute much phylogenetic signal).

Relative gene contribution is compared within Table 2.5. Genes with more total characters had better likelihoods, in particular the 18S and 28S rDNA. However, the greater number of protein-coding genes led to a combined score similar to that of the rDNA tree. The combined tree, of course, had the best likelihood.

The three different analysis methods used to produce the 8-gene tree (Fig. 2.2) had three supported incongruities. Bayesian analysis placed the Blastocladiomycota as the first group to diverge after the Chytridiomycota and the Entomophthoromycota as the second, while the maximum-parsimony analysis placed these two groups together on a strongly-supported branch (the maximum-likelihood topology was the same as the Bayesian, but without strong support). For the second, the Bayesian and maximum-parsimony analyses placed Dimargaritales on a branch with *Ramicandelaber* as the first Kickxellomycotina group to diverge, while the maximum-likelihood analysis separated these clades with Dimargaritales as the first group to diverge and *Ramicandelaber* as the second. Thirdly, the Bayesian topology supported *Caudomyces* as the first group of Harpellales to diverge, and *Harpellomyces* as the second (the maximum-likelihood topology was the same but without strong support), but the maximum-parsimony analysis reversed the position of these two branches. Alternate topologies are indicated on Fig. 2.2 via dotted lines.

SH and AU tests were run to assess whether these conflicts indicated irreconcilable differences between the trees. Using maximum-likelihood-based trees, each incongruity was tested, but none of these tests revealed statistical support for either topology. As such, a lack of resolution is likely responsible for these incongruities, and these nodes should be treated as unresolved.

The Broader Fungal Tree

The phylogenic relationships depicted in Fig. 2.2 are slightly different those in the previous multigene trees of Fungi (see James et al. 2006). The topology of the Ascomycota, Basidiomycota, Mucoromycotina, Blastocladiomycota, and

Chytridiomycota are the same. However, the Zoopagomycotina, Entomophthoromycota, and Kickxellomycotina were together (without strong support) on a branch in James et al. (2006), whereas in our study the Entomopthoromycota either diverge alone (with Bayesian and maximum-likelihood) or they are combined with the Blastocladiomycota (with maximum-parsimony). Both the six-gene tree of James et al. (2006) and the 8-gene tree (Fig. 2.2) place the Zoopagomycotina and the Kickxellomycotina together on a branch, but only here is it with strong support. Many shared features support such an affinity, including the presence of merosporangia in both groups, the secretion of adhesives to adhere to arthropods and other animals, and the presence of zygospores. Benjamin (1959) suggested such a possible relationship on morphological grounds, indicating that the Dimargaritales and Kickxellales might descend from a lineage that also included *Piptocephalis* and *Syncephalis.* The Dimargaritales, suggested by the maximum-likelihood analysis to be the first lineage to diverge, do appear to be similar to the mycoparasitic members of the Zoopagomycotina, but many of the shared characters are present in other clades as well. Additionally, taxon sampling among the Zoopagomycotina was insufficient to make any strong conclusions about this, although we promote further studies to resolve this relationship.

Comparative Morphology and Ecology of the Kickxellomycotina

Members of the Kickxellomycotina have hyaline, usually septate hyphae arising either from haustoria, a holdfast cell attached with a secreted glue, or from a substrate (for saprobes). New cells may be either delimited by forming septa within existing hyphae or through budding. Comparative morphology of the sporulating structure, asexual spores, sexual spores, and septal pore and plug are illustrated comparatively, with

generalized sketches of each (Fig. 2.1), for each of the Kickxellomycotina clades (Fig. 2.2).

All known members of this subphylum (with the possible exception of Asellariales, but see below) produce asexual spores within sporangia. These spores have been referred to as either merosporangia, sporangia, sporangiola, or other terms specific within individual orders (such as the trichospores of the Harpellales). Authors attempting to make comparisons between various groups usually have regarded them as merosporangia (Benjamin 1966, Moss and Young 1978, O'Donnell et al. 1998), and this convention is adopted here. Within the Asellariales, the thallus disarticulates to produce arthrospores. However, the arthrospores of *Asellaria* and *Orchesellaria* extrude secondary spores after disarticulation (Lichtwardt 1973, Degawa 2009), and these may actually represent the merosporangia for these genera.

Within the Harpellales (excluding *Orphella*), merosporangia are produced singly upon fertile branches consisting of many 'generative cells.' Fertile thalli may be branched or unbranched within the Harpellales s.s. (defined within this paper as all genera of Harpellales except *Barbatospora* and *Orphella*), depending on the genus, one of the features that delimits the two families of Harpellales s.s.. The Kickxellales s.s. (defined here as all genera of Kickxellales except *Mycoëmilia*, *Ramicandelaber*, and *Spiromyces*) produce a specialized, conidiophore-like structure referred to as a sporocladium upon supporting branches referred to as sporangiophores or sporophores. Each cell of the sporocladium (except for a sterile terminal cell found in many species) supports multiple pseudophialides, each of which produces a single apical merosporangium. This was termed the 'coemansoid pattern' by Moss and Young (1978), who considered the

sporocladium of the Kickxellales to be homologous to the fertile branch of the Harpellales s.s. The structure produced within Asellariales, particularly *Asellaria*, is similar to the Harpellales. However, instead of merosporangia being produced, the fertile branch first breaks apart into arthrospores. In *Orphella*, a sporulating fertile head produces multiple branches, each with many multi-celled dissemination units, which include a single merosporangium (Valle and Santamaria 2005). In Dimargaritales, a sporangiophore supports multiple fertile branches. This can either be in the form of an expanded terminal cell such as in *Dimargaris bacillospora* or a specialized branchlet in *Tieghemiomyces* and *Dispira* (Benjamin 1959, Benjamin 1966). *Ramicandelaber* produces small sporocladia on sporangiophores; each of these sporocladia produces a single large pseudophialide, which produces a merosporangium. *Spiromyces* produces multiple sporangiophores on fertile branches. Each sporophore (perhaps homologous to the sporocladia of Kickxellales s.s. or the fertile branches of Harpellales) supports one to several terminal enlargements that produces multiple spherical merosporangia upon non-septate pedicels.

Many of these eight clades, including all four current orders, are known to produce sexual spores as well. These spores are considered to be zygospores that are released within a zygosporangium. Within the Asellariales, Dimargaritales, Kickxellales s.s., and *Spiromyces*, zygospores are spherical. In the Harpellales s.s., zygospores are spherical in early development but become biconical or uniconical as they mature (Whistler 1963, Moss and Young 1978). Zygospores of *Orphella* are partially or completely coiled (depending on the species) and are released as part of a multi-celled dissemination unit,

similar to the asexual merosporangium (Valle and Santamaria 2005). Zygospores have not yet been observed for any species of *Barbatospora* or *Ramicandelaber*.

The pattern of conjugation and zygospore formation can also differ between and sometimes within clades. In Kickxellales, most species of Dimargaritales, and *Spiromyces*, the zygospores are produced within an enlarged intercalary cell resulting from the conjugation of two visually undifferentiated sexual hyphae. Suspensors can be either opposed or apposed, depending on the species. Within Harpellales, zygospores arise from an enlarged cell (a zygosporophore) that develops from either a conjugating cell or a branch developed from a conjugating cell (Lichtwardt 1972, Moss and Lichtwardt 1977, Benjamin 1979). Within *Orphella*, the zygospore also arises from a zygosporophore, however it is produced as part of a multicellular 'dissemination unit,' similar to the trichospore (Valle and Santamaria 2005). In *Asellaria*, the zygospore forms laterally from either an intercalary cell in the conjugating branches or a terminal cell elsewhere in the thallus (Valle and Cafaro 2008). Some species of Dimargaritales, including all known species of *Dispira* as well as *Dimargaris oblongispora*, produce zygospores terminally on a stalk that forms above the conjugating cells (Benjamin 1979). It is thought that some form of nuclear migration, aided by the specialized septa within the Kickxellomycotina, is utilized by species that produce zygospores away from the point of conjugation. That there are certain features found in the merosporangium within the zygospores of Harpellales, such as the appendage and extrusive spore release, may suggest that the cell the meiotic nuclei migrate into is a modified merosporangium.

Within the Kickxellomycotina, the type of conjugation (homothallic vs. heterothallic) seems to vary within the clades. Most of the Harpellales are heterothallic, but there are

homothallic members such as *Genistellospora homothallica* (Lichtwardt 1972). *Orphella* also has both homothallic and heterothallic members (Dr. Merlin White personal communication). This information has not been consistently reported for Dimargaritales, Kickxellales, or *Spiromyces* although at least some of the Kickxellales are known to be homothallic (Kurihara et al. 2000). Zygospores in Asellariales have only been reported once (Valle and Cafaro 2008) and the homothallic vs. heterothallic nature was not reported.

Septal pore morphology (see Fig. 2.1) has been examined as a potential differentiating character as well. In Dimargaritales, the septal plug has two large, globose protrusions to either side (Saikawa 1977, Jeffries and Young 1979, Brain et al. 1982), which are unique among the Kickxellomycotina. Aside from the globose protrutions, members of the Dimargaritales have a relatively wide and thin septal plug. This is also true in *Ramicandelaber* (Ogawa et al. 2001, Kurihara et al. 2004) and *Spiromyces* as inferred from examination of the putatively related genus *Mycoemilia* (Kurihara et al 2004). In Harpellales s.s. and Kickxellales s.s., the plug is relatively narrow and thick (Farr and Lichtwardt 1967, Young 1969, Benny and Aldrich 1975, Moss and Young 1978, Tanabe et al. 2004). In the Asellariales, the septa of *Asellaria* resemble the Harpellales s.s. and the Kickxellales s.s. (Saikawa et al. 1997b), but the septa of *Orchesellaria* resemble Dimargaritales (minus the protrusions), *Ramicandelaber*, or *Spiromyces* (Moss 1975).

Ecological mode and nutrition type is generally consistent within, but not between, the clades. Asellariales, *Barbatospora*, Harpellales, and *Orphella* are all endosymbionts of arthropods. Kickxellales (except for *Martensella*), *Ramicandelaber*, and *Spiromyces*

are primarily saprobic; *Martensella* is a non-haustorial mycoparasite. Conversely, Dimargaritales are haustorial parasites of fungi.

Phylogeny of the Kickxellomycotina

Within the 8-gene tree, the Kickxellomycotina are supported as monophyletic (Fig. 2.2 clade A. BPP: 100.0%, MLBP: 99/100, PSR: 96/100). This is the first analysis that demonstrates the monophyly of the Kickxellomycotina while including members of all four orders of the subphylum, supporting the evolutionary significance of the unifying feature of the Kickxellomycotina, the lenticular septal pore with the electron-dense plug.

Within the Kickxellomycotina, several genera emerged as not being monophyletic within their orders, specifically, *Barbatospora* and *Orphella* (Harpellales), as well as *Ramicandelaber* and *Spiromyces* (Kickxellales). SH and AU tests were run in order to test the maximum-likelihood tree topology against the constraint tree in which the traditional taxonomy was enforced. All four of these tests determined that the non-constrained tree was significantly more likely than the constrained tree ($p(H_A) < 0.05$ – see Table 2.4). All four were also supported by fully resolved branches within the combined topology (Fig. 2.2) and unique morphological traits (Fig. 2.1 and also discussed later). These clades will be treated individually herein.

Specific sub-clade placement will be discussed within the section for each of the eight clades. However, a few large-scale relationships did emerge. One pairing that was supported within the 8-gene tree (Fig. 2.2 clade B) was a clade consisting of Asellariales, *Barbatospora*, Harpellales s.s., Kickxellales s.s., *Orphella*, and *Spiromyces*. This clade does not include the morphologically distinct Dimargaritales nor the unusual

Ramicandelaber. Another well-resolved clade (Fig. 2.2 clade C) included all of the above except *Barbatospora*.

Clade 1: Harpellales

Harpellales are obligate endosymbionts of arthropods that utilize aquatic insect larvae as hosts (except *Legerioides*, which utilizes aquatic isopods, but morphologically resembles other Harpellales). Common harpellid hosts include Diptera such as black flies (Simuliidae), mosquitos (Culicidae), and midges (Chironomidae), as well as mayflies (Ephemeroptera), and stoneflies (Plecoptera), among others (White 1999). A few species are harbored by biting midges (Ceratopogonidae), crane flies (Tipulidae), and solitary midges (Thaumaleidae), and non-dipterans such as caddis flies (Trichoptera), beetles (Coleoptera), and other taxa. Predaceous species are generally not utilized as hosts (Lichtwardt et al. 2007). The Harpellales form a mass of hair-like, septate, often branched thalli within the hindgut or unbranched thalli if in the midgut of dipteran hosts. Fertile branches, thought to be homologous with sporocladia in the Kickxellales (Moss and Young 1978), consist of septate generative cells that subtend single-celled merosporangia, referred to as “trichospores” within the Harpellales. Often, the trichospore is borne on a non-septate extension of the generative cell, which upon trichospore release may partially remain as a collar. The contents of the generative cell are evacuated into the trichospore, and the non-septate nature of the collar may be patterned by the specific degeneration of the generative cell. Merosporangia may possess one or more non-motile appendages, believed to aid with entanglement in the immediate environment of the host, or otherwise prevent being washed downstream (Lichtwardt et al. 2007). Appendage-like adaptations also exist in *Barbatospora* and *Orphella*, two

unusual members of the Harpellales that are not monophyletic and considered as distinct lineages here (as well as the protist trichomycete clade Eccrinales). Merosporangia are adapted for physiological recognition and rapid rupture to release the sporangiospores once inside the appropriate insect gut. Once released, the young germling immediately forms a temporary holdfast to anchor the developing thallus during its initial growth. The holdfast itself may be amorphous or involve some of the basal cell(s) in the "grasping" of the gut lining.

Sexual spores of the Harpellales are uniquely biconical or uniconical, potentially improving their hydrodynamic properties. These "zygospores," or more accurately, zygosporangia, are produced laterally from a zygosporophore, which may arise from the conjugating thalli (or extensions from it) and are also released with one appendage in most genera. Detached zygospores often include part of the zygosporophore attached as a collar. In some genera, the entire zygosporophore may accompany the detached zygosporangium. Zygosporangia possess the same type of rapid spore extrusion as the merosporangia. This parallel in the ontogeny of both the asexual and sexual spore should not be overlooked for its potential insight into the evolutionary origins of these highly modified sexual spores. Post-conjugation nuclear migration is known to occur in the Harpellales (and possibly other Kickxellomycotina) so it is possible that the zygosporangium is formed from a specialized, modified merosporangium, from which it inherits the similarities in characteristics such as appendage and the extrusive spore release.

The Harpellales include over 20 genera and 200 species (Lichtwardt et al. 2007). Our dataset includes 15 isolates composing 14 species from 11 genera (one of which is

currently not described). The Harpellales s.s. are placed as sister taxon to the Asellariales, corresponding to similarities in life history and morphology, and is represented as a strongly supported clade (branch D, Fig. 2.2). This clade includes the majority of the Kickxellomycotina that live within arthropod guts. It does not, however, include all of them, as *Barbatospora* and *Orphella* are not monophyletic with it. Thus, the trichomycete lifestyle does not fit into a single monophyletic clade, even for true fungal members of the group, and whether the symbiotic habit is an ancestral feature among the later-diverged Kickxellomycotina is an open question. A case could be made that it developed after the Dimargaritales and *Ramicandelaber* diverged and was subsequently lost in the Kickxellales and *Spiromyces*. Alternate possibilities include that it has arisen multiple times, or some combination of the above.

The Harpellales currently have two families; the Harpellaceae, which have unbranched thalli and are found in the midgut, and the Legeriomycetaceae, which have branched thalli and are found in the hindgut. Others have expressed doubt about the monophyly of these families, most recently with the suggestion of a 'Smittium clade,' consisting of *Smittium* and related genera, and a 'non-*Smittium*' clade consisting of most other genera (White et al. 2006b, Wang et al. 2012a). This study partially reinforces this notion; two Harpellaceae, *Harpella melusinae* and *Harpellomyces montanus,* do not form a monophyletic clade, nor do all of the Legeriomycetaceae. Instead, two genera (*Caudomyces* and *Harpellomyces*) have uncertain placement near the base of the Harpellales tree, and the rest of the genera are divided between the Smittium and non-Smittium clades, with *Harpella* in the non-Smittium group. While this tree does not have sufficient taxon sampling to permit taxonomic revision of the family structure by itself,

this can potentially be used to support further efforts to refine the taxonomy within this order.

Pteromaktron has long been sought in a molecular phylogenetic context, as it has an unusual thallus morphology, thought to represent an intermediate between Kickxellales and *Orphella* (Valle and Santamaria, 2005). *Pteromaktron* species have a partially coenocytic main axis as well as a sporulating head reminiscent of *Orphella*, subtending supporting cells, on which merosporangia form and are released. Whistler (1963) referred to these supporting cells as subsidiary cells, and Valle and Santamaria (2005) suggested that they were homologous to pseudophialides. These merosporangia are similar to other Harpellales, particularly *Zancudomyces culisetae* (as well as *Graminella* and *Spartiella*, not included in this tree). This combination of features is particularly unusual for a member of the Harpellales. However, rather than the unusual morphology and size of the thallus, it is the morphology of the asexual spore (Whistler 1963, Williams and Strongman 2012) that is most informative for this genus. Within our sampling, *Pteromaktron* is most closely related to *Zancudomyces culisetae*, which has a different thallus but similar asexual spores. It seems likely that the subsidiary cells of *Pteromaktron* are not pseudophialides, but instead generative cells similar to those of other Harpellales, with individual fertile branches consisting of only a single terminal generative cell. The apparent similarities between *Pteromaktron* and *Orphella* are likely convergent, although the two species inhabit different orders of host (Ephemeroptera and Plecoptera, respectively). It may be notable that both genera extend beyond the anus of the host at maturity.

Clade 2: Asellariales

Asellariales is a smaller group of obligate endosymbionts of arthropods, with three genera; *Asellaria* and *Baltomyces*, both associates of Isopoda, and *Orchesellaria*, associates of springtails (Collembola). All three differ in morphology and general habit, and it is possible that the order is not monophyletic. Thalli of *Asellaria* and *Orchesellaria* closely resembles the hair-like nature of the Harpellales except that the thallus disarticulates into arthrospores at maturity, rather than producing merosporangia. However, in *Asellaria*, the arthrospores may extrude a merosporangium-like structure after release (Lichtwardt 1973). This structure bears a strong resemblance to the disarticulating thalli of *Carouxella* and *Klastostachys*, two genera of Harpellales that present merosporangia bound to the generative cell. Notably, the released arthrospores of *Orchesellaria* develop into a multi-celled dissemination unit with a merosporangium-like secondary cell and a filamentous terminal cell, which Degawa (2009) compared to the dissemination unit of *Orphella*. The morphology of this dissemination unit does not resemble the arthrospores of *Asellaria*, suggesting the possibility that the order may not be monophyletic. Thus far, no further development of the asexual spore has been observed after release for *Baltomyces* (Oman and White 2012).

Baltomyces is morphologically unique with a basal cell that forms multiple septa within it after germination and an asexual spore that may release through a tear in the side-wall of the generative cell (Cafaro 1999, Oman and White 2012). This method of spore release is unknown among the Kickxellomycotina, but is more common among species of the protist clade Eccrinales, which had previously been classified with the fungal trichomycetes. *Baltomyces* also has appendage-like structures on either end of the

asexual spore, a generalized feature only known for *Barbatospora* (among Kickxellomycotina) and also some genera from the Eccrinales (i.e., *Arundinula*, *Astreptonema*, and *Taeniella* [Lichtwardt et al. 2007]).

Asellariales has long remained unsequenced in the literature and we report the first published sequences for the order, although tentative unpublished data was available earlier (Hibbett et al. 2007). Although we attempted to sequence all three genera, we were only able to sequence three samples of *Asellaria ligiae* for five of the eight genes used in our study (18S and 28S rDNA, RPB1, RPB2, and β-tubulin 1). Three sequences of 28S rDNA were secured for *Orchesellaria*, but *Baltomyces* eluded all attempts to amplify and sequence. Using the sequence data we obtained for *Asellaria ligiae*, the group is placed with confidence in the 8-gene tree. *Asellaria* seems to be the sister taxon to the Harpellales s.s., as has been suggested by many authors, based on the similar morphology of the two groups (Moss and Young 1978). Our attempts to place *Orchesellaria* based on 28S rDNA alone were inconclusive.

Asellaria 18S and 28S nuclear rDNA sequences are surprisingly long and highly divergent. Compared to *Zancudomyces culisetae* over a region that included unambiguously aligned start and end regions, the *Asellaria* 18S sequence was 3685 bp vs. 1816 bp for the *Zancudomyces* 18S sequence. Most of the length variation was due to a large region (~1400 bp) near the 3` end of the 18S that could not be aligned and resulted in no BLAST hits when submitted independently. Analysis with RNAFOLD (Gruber et al. 2008) revealed that much of the region may be highly folded, suggesting the possibility that it may have some secondary structure that is of relevance to the ribosome, though we do not rule out that it may be an unidentified intron. Even when this region

was removed, the resulting sequence is still unusually large (~2250 bp). The remaining increase in size appears to be due to some large insertions early in the sequence, and generally expanded variable regions throughout the molecule. Comparatively, some members of Isopoda also have expanded 18S nuclear rDNA (Mattern and Schlegel 2001) suggesting the possibility of an unknown, shared evolutionary pressure on both the endosymbiont and the host. The length of the *Asellaria* 28S rDNA sequence that could be directly compared to *Zancudomyces* was 2047 bp (vs. 1528 for *Zancudomyces*). The 28S appeared similarly enlarged with expanded sequencing length in divergent regions, but without any single large insertions as in the 18S. However, because we only sequenced approximately the first 1/3 of the 28S, other regions may be similarly affected. *Asellaria* nuclear rDNA sequences were also divergent, making them difficult to align. The 28S sequences for *Orchesellaria* did not exhibit unusual length or divergence in comparison with other Kickxellomycotina.

The divergence noted for the 18S and 28S nuclear rDNA within *Asellaria* resulted in unusually high branch length in the rDNA-based tree (Fig. 2.5), which may have altered tree topology due to long-branch attraction. Protein-coding gene sequences (obtained for RPB1, RPB2, and β –tubulin), did not exhibit this high divergence nor length increase and resulted in a branch of relatively typical length within the protein-coding gene tree (Fig. 2.5). For this reason, future molecular phylogenetic studies within the Asellariales should continue to concentrate on protein-coding genes. The placement of *Aselleria* within the 8-gene tree (Fig. 2.2) was the same as the protein-coding gene tree, suggesting that the majority of the signal that placed the order was derived from protein-coding genes. However, the rDNA genes should not be overlooked to better understand the

evolutionary forces that have resulted in their unusual nature, as this may reveal valuable information about the evolution of the group and of rDNA.

Clade 3: Kickxellales

Kickxellales includes 12 genera of primarily saprobic fungi. *Martensella*, a non-haustorial mycoparasite, is the sole exception but it is morphologically typical. The thallus arises from the substrate to form a mass of septate hyphae, which produces sporangiophores – specialized asexual reproductive hyphae. These produce either one or many sporocladia. Sporocladia are mostly multicellular in the Kickxellales, although in *Linderina* the sporocladium is unicellular and multinucleate (Chien 1971). Each sporocladium supports multiple pseudophialides, which each subtend a single merosporangium. Most genera have an elongated 'sterile cell' at the end of the sporocladium. The sporocladium has been considered to be a major taxonomic character of the order, although Moss and Young (1978) considered the basic pattern consistent and termed it the 'coemansoid pattern' and compared it to the fertile branches of the Harpellales and Asellariales. For most species, the merosporangia are released within a droplet of fluid at maturity, except *Spirodactylon* and *Spiromyces,* which are dry-spored. This droplet may be related to the 'labyrinthiforme organelle' or 'abscission vacuole,' an organelle within the pseudophialide thought to be related to spore release (Young 1974, Benny and Aldrich 1975). Moss and Young (1978) also discussed the possibility that this organelle might be related to the one that generates the non-motile appendage in the Harpellales. Zain et al. (2012) observed the presence of a minimal, possibly vestigial appendage in the kickxellid *Linderina pennispora*. Sexual spores have also been observed in several members of the Kickxellales (Benjamin 1958, Benny 2012). These

zygosporangia are spherical and thick-walled, typically produced within the intercalary cell of the conjugating thalli atop suspensors that are either opposed or apposed depending on the species. Two genera of the Kickxellales, *Ramicandelaber* and *Spiromyces*, separate from the core group of the Kickxellales in the 8-gene tree (Fig. 2.2) and are treated separately.

Within the Kickxellales s.s., the two species of *Coemansia* along with *Spirodactylon* form a supported clade and *Kickxella alabastrina* is supported as a sister taxon to this group (Fig. 2.2). Benjamin (1961) reported that in these groups, germ tubes are produced near the middle of the spore, whereas in *Dipsacomyces*, *Linderina*, and *Martensiomyces*, germ tubes are produced basally, suggesting this may be an important taxonomic character within the group. *Dipsacomyces* and *Martensiomyces* are placed together on a supported branch near the base of the Kickxellales s.s.. A less strongly supported branch (BPP: 98.0%, MLBP: 56/100, PSR: 54/100) combines *Linderina* with the *Kickxella* – *Spirodactylon* – *Coemansia* clade and suggests that the *Dipsacomyces* – *Martensiomyces* clade was the first of the sampled Kickxellales s.s. to diverge.

Clade 4 – *Orphella*

Orphella is a clade of stonefly (Plecoptera) gut endosymbionts currently in the Harpellales. *Orphella* produces a septate thallus with a primary central axis and many sterile side branches near the basal cell and holdfast. The main axis splits into many fertile branches near the terminus, which support one to many basal cells. Each basal cell supports one to many asexual or sexual spores, which are released as multi-celled dissemination units.

Valle and Santamaria (2005) revealed the sexual apparatus and provided an excellent description and terminology of sexual and asexual spores. Asexual spores consist of a small supporting cell, a generative cell, a large merosporangium, and a sterile, filamentous terminal cell. Sexual spores are released with some cells derived from the conjugating thalli (a supporting cell, an intercalary cell, and a terminal cell) and some cells that grow from the intercalary cell (a zygosporophore, a zygosporangium, and either one or two additional terminal cells). Merosporangia in *Orphella* may either be straight or curved or coiled, an important taxonomic feature. Zygosporangia exhibit various degrees of curvature, by species.

Homology of the asexual and sexual reproductive features to other members of the Kickxellomycotina is somewhat uncertain. Valle and Santamaria (2005) considered the asexual sporulating structure to be most similar to *Pteromaktron* and the Kickxellales, with the supporting cell being homologous to the pseudophialide of the Kickxellales and the subsidiary cell of *Pteromaktron*. However, *Pteromaktron* does not appear to be related to *Orphella* (Fig. 2.2), and any morphological similarity must be the result of evolutionary convergence. *Orphella* does appear to be related to the Kickxellales. An alternate hypothesis, first proposed here, is that the asexual dissemination unit of *Orphella* constitutes a small, dehiscent Kickxellales-like sporocladium. In this view, the supporting cell and generative cell would be homologous to the sporocladia cells of the Kickxellales, with the sterile terminal cell of *Orphella* being homologous to the sterile cells borne by the sporocladium of most Kickxellales s.s. species. In this view, the basal cell of *Orphella* would be part of the sporangiophore, similar to the central cell of the radial sporocladium of *Kickxella*. Additional study of the ontology of the asexual spore

will be needed to confirm either of these two hypotheses. Valle and Santamaria (2005) also suggested that the zygosporangium formation process was homologous to the formation of sexual spores in the Kickxellales, with a multicellular dissemination unit replacing the zygosporophore and zygosporangium of the Harpellales. This seems plausible and supports our hypothesis that the sexual spore formation process in the Harpellales, *Orphella*, and possibly some of the Dimargaritales may involve nuclear migration into what may be modified asexual reproductive structure. Both the Harpellales and *Orphella* sexual reproductive structures have clear structural similarities with their asexual reproductive units.

Previous authors have suggested the non-monophyly of *Orphella* and Harpellales, either based upon morphological evidence (Valle and Santamaria 2005) or molecular analysis (White 2002, White 2006, White et al. 2006b, James et al. 2006). Molecular analysis either placed *Orphella* as sister to the Kickxellales s.s., or Kickxellales s.s. + *Spiromyces*. Our 8-gene analysis places *Orphella* on a supported branch with the Kickxellales s.s. This placement appears to be somewhat dependant on rDNA. In the protein-coding tree (Fig. 2.5), Kickxellales, *Orphella*, and *Spiromyces* are placed together on an unsupported branch, with *Orphella* diverging first and *Spiromyces* next, although the divergence is weakly supported. Within the rDNA analysis, the placement is the same as the 8-gene tree, and strongly supported. So, while it is clear that *Orphella* and *Spiromyces* represent distinct and well-defined clades, and that *Orphella* is not a member of the Harpellales, the exact relationship between them and the Kickxellales should be considered with a certain degree of caution. Resolution within the protein-coding gene

tree may have been reduced with our inability to sequence β-tubulin or TSR1 for *Orphella*.

Because O*rphella* is placed together with the Kickxellales s.s., a taxonomic revision that places *Orphella* within the Kickxellales, or promotes both *Orphella* and *Spiromyces* to new orders, may be necessary. Based upon the substantial branch length of both the *Orphella* and *Spiromyces* clades, and the morphological differences (as well as different ecological mode of *Orphella*), we consider the second option as preferable.

Clade 5 – Spiromyces

Spiromyces is currently within the Kickxellales, with two species described; *Spiromyces aspiralis* and *S. minutus*. Kurihara et al. (2004) described a new genus with *Mycoëmilia scorparia*, which they considered to be related to *Spiromyces* based upon similarity of the asexual and sexual reproductive morphology, including the lack of pseudophialides. Based upon this, as well as unpublished phylogenetic trees (from both Kurihara et al. and our lab) in which the two genera are monophyletic together, we will consider *Mycoëmilia* as part of the Spiromyces clade.

Members of the Spiromyces clade have somewhat different morphology from the Kickxellales s.s., with a reduced sporulating structure and significant variation between the *Spiromyces* species. In *S. minutus*, a slightly coiled and septate sporangiophore supports multiple subovoid sporocladial cells formed pleurogenously. Each of these has a globose terminal enlargement, which forms by budding and supports multiple spherical merosporangia (Benjamin 1963). In *S. aspiralis*, the structure is similar, except the sporophore does not coil and the globose enlargement of the sporocladial cell is separated with a septum (O'Donnell et al. 1998). In *M. scorparia*, sporocladia are formed primarily

acrogenously. Each terminal sporophore cell supports a number of sporocladia, each of which supports several fusiform spores (Kurihara et al. 2004). In all of these genera, no pseudophialides are formed, but asexual spores are instead supported on non-septate 'pedicels.' This is an important morphological difference between the Spiromyces clade and the Kickxellales s.s., as all members of the latter clade do possess pseudophialides. Both *Spiromyces* species are dry-spored, whereas *Mycoëmilia* releases spores in a droplet of moisture at maturity. Thus, this character seems to vary within the Spiromyces cladem as it does within the Dimargaritales and Kickxellales s.s..

Sexual spores within the Spiromyces clade are spherical and appear somewhat similar to other clades within the Kickxellomycotina. They appear to be pigmented orange-brown in *S. minutus* and brown in *M. scorparia*, in contrast to the usually hyaline spores of most other clades. At maturity, the zygosporangia of the Spiromyces clade have a single eccentric globule visible. This globule appears similar to many of the Dimargaritales observed (Benjamin 1959,1961,1963,1965). However, it is different from the Kickxellales s.s., which normally have many small globules visible (Benjamin 1958). Benjamin (1963) also remarks that the surface sculpturing of the *Spiromyces minutus* sexual spore resembles that of the Dimargaritales.

Ecologically, members of the Spiromyces clade appear to be saprobic. Both species of *Spiromyces* were isolated from rodent dung (Benjamin 1963, O'Donnell et al. 1998). *Mycoëmilia* was isolated from the soil underneath a shrub, possibly associated with the bodies of dead isopods (Kurihara et al. 2004). The Kickxellales s.s. species *Spirodactylon aureum*, similar to *Spiromyces* in some ways but not closely related, was also isolated from rodent dung. Some shared features may be convergent and have evolved with

adaptations for this this ecological niche. Candidate shared features include being dry spored and having ovoid to spherical, ornamented merosporangia. On the other hand, some other Kickxellales s.s. species such as *Coemansia reversa* and *Kickxella alabastrina* were also isolated from rodent dung and do not share these features.

We included both species of *Spiromyces* in our 8-gene tree (Fig. 2.2). As with the placement of *Orphella*, the placement of the Spiromyces clade depends on the signal provided by rDNA (see Figs. 2.4 and 2.5). While some protein-coding genes placed the Spiromyces clade as sister taxon to the Kickxellales s.s., no gene placed the Spiromyces clade within the Kickxellales s.s. (Figs. 2.6-2.13). Given this outcome, the substantial branch length of the Spiromyces clade, and the morphological variation between *Spiromyces* and the Kickxellales s.s., it appears that the genera within the Spiromyces clade (*Spiromyces* and *Mycoëmilia*) might best be considered separate from the Kickxellales with placement in a new order.

Clade 6 – Barbatospora

Barbatospora, known only from a single report, is a monotypic genus currently placed within the Harpellales (White et al. 2006a). Morphologically, *Barbatospora* closely resembles the Harpellales s.s., with a branched, septate thallus originating from a holdfast cell, attached via a secreted glue to the hindgut lining of a host Simuliidae. Numerous fertile branches form, each of which consists of a number of generative cells, that support a single merosporangium upon an enlarged ‘collar region.’

However, the merosporangium of *Barbatospora* varies somewhat from the Harpellales. Within *Barbatospora*, the asexual spores may have multiple, fine non-motile appendages or appendage-like filaments on either the basal end of the spore or both the

basal and apical end. Within the Harpellales s.s., appendages are always basal. White et al. (2006a) reported that the spores varied within the individual. Some spores had appendages on both ends whereas some spores lacked appendages completely. It may be that only the spores formed by terminal generative cells possess the apical appendages. Spores that later demonstrate apical appendages are released with a 'cap' on the apical end that is later lost to reveal them. It is possible that this cap is part of the merosporangial membrane, in which case the appendages would be part of the sporangiospore and likely not homologous to the appendages of Harpellales. Other potentially informative characteristics for *Barbatospora*, such as the sexual spore, the septal pore morphology, and the manner of spore germination, have not been observed. Fortunately, axenic cultures of this isolate exist, so future studies may yet reveal these important details.

Our 8-gene phylogeny (Fig. 2.2) reveals that *Barbatospora* occupies a particularly interesting place in the tree. *Barbatospora* appears to be a sister taxon to the ancestral group (clade C in Fig. 2.2) that later diverged to form the Asellariales, Harpellales s.s., and Kickxellales s.s., as well as *Orphella* and *Spiromyces*. Individual gene trees (Figs. 2.6-13) generally support the conclusion that *Barbatospora* is not within the Harpellales, as no tree places *Barbatospora* within the Harpellales and only the 5.8S tree (Fig. 2.8) even places *Barbatospora* as sister taxon to the Harpellales.

The placement of *Barbatospora* in this position carries strong implications for the evolutionary history of the Kickxellomycotina. The morphological form and life history of *Barbatospora* closely resembles Harpellales s.s. and to some extent the Asellariales (White et al. 2006a). These characteristics may represent retained ancestral characters

from the clade that later split to produce the endosymbiotic Asellariales, Harpellales, and *Orphella*, as well as the saprobic Kickxellales s.s. and *Spiromyces*. However, *Barbatospora* is on a relatively long branch (Fig. 2.2), so it may have diverged significantly from the ancestral form. It is possible that some morphological features may be convergent. However, this seems unlikely given how similar many of these features, such as the growth form, are to the Harpellales s.s.

Clade 7 – Ramicandelaber

Ramicandelaber is an unusual saprobic genus currently within the Kickxellales. Sporophores arise from the substrate, which form several clusters of short verticillate branches near the base. Each branch has several verticillately arranged sporocladia, which subtend a subspherical pseudophialide (Ogawa et al. 2001). Each pseudophialide develops a single merosporangium, either fusiform (in *R. longisporus*, *R. brevisporus*, and *R. taiwenensis*) or fabiform (in *R. fabisporus*) (Chuang et al. 2012). In age, the sporophores of *R. longisporus* will also form many hemispherical pseudophialides, which bear additional spores (Ogawa et al. 2001). Ogawa also noted that the septa of these pseudophialides were ‘obscure’ so it is possible these represent non-septate ‘pedicels’ as in *Spiromyces*. Sexual spores have not yet been observed for any members of this clade.

Ramicandelaber is unique among the Kickxellomycotina in producing rhizoids and stolons. Rhizoids in *Ramicandelaber* are small, root-like projections of the basal cell, which may aid in either nutrient absorption or anchoring of the sporophore to the substrate. Stolons are long, sterile extensions of the apical end of the sporophore, which are repent and form rhizoids at the apex, at least in *R. longisporus* (Ogawa et al. 2001).

The placement of *Ramicandelaber* remains uncertain (Fig. 2.2). Bayesian analysis and maximum-parsimony both strongly support a relationship between *Ramicandelaber* and the Dimargaritales, placing them on a branch together as the first clade among the Kickxellomycotina to diverge. Maximum-likelihood analysis, on the other hand, suggests that both clades diverged separately, with Dimargaritales diverging first and *Ramicandelaber* second. While the potential relationship between *Ramicandelaber* and the Dimargaritales cannot be confirmed by our current phylogeny (likely as a result of long-branch attraction artifacts), it does seem possible to confirm that *Ramicandelaber* is not a member of the Kickxellales s.s.. Ogawa et al. (2005) found that *Ramicandelaber* did not cluster among the Kickxellales for 18S and suggested that it may not be a member of the order. White et al. (2006b) came to a similar conclusion based on a combined nuclear rDNA phylogeny. Chuang et al. (2012) produced a phylogeny of *Ramicandelaber* species utilizing 18S and 28S nuclear rDNA, which also placed *Ramicandelaber* outside of the Kickxellales. Ogawa et al. (2005) and Chuang et al. (2012) both mention the possibility of a potential relationship between *Ramicandelaber* and the Dimargaritales, although Ogawa et al. (2005) considered it unlikely on morphological grounds.

An examination of the morphological similarity between *Ramicandelaber* and the Dimargaritales was difficult because of the substantial variation between them, and the strong uniqueness of both groups. However, we noted some apparent similarity between *Ramicandelaber* and *Tieghemiomyces*, a genus within the Dimargaritales, particularly *T. californicus* (Benjamin 1959). Sporophores of *Tieghemiomyces* tend to branch near the base, forming verticillate clusters of fertile branchlets that support bispored merosporangia. These branches are somewhat similar to those of *Ramicandelaber*,

especially those depicted in Ogawa et al. (2001). Cells in the sporulating structure of *T. californicus* are also known to broaden and become more spherical with age, like the branches of the sporophores in *R. longisporus*. The long, sterile, aerial hyphae of *T. californicus* appear somewhat similar in habit to the stolons of *Ramicandelaber*, except that the stolons of *Ramicandelaber* are repent instead of erect and form rhizoids at their apices. This feature is best illustrated in Kurihara et al. (2004), although they refer to the stolons as aerial hyphae. Finally, it is worth noting that *Tieghemiomyces* thrives in axenic culture compared to other genera of Dimargaritales, suggesting that it may be less obligately parasitic than the others.

Similarity between *Ramicandelaber* and the Dimargaritales is not necessarily indicative of a relationship between the two, even if features are indeed homologous, because they may indicate ancestral states retained by both members but lost by other members of the Kickxellomycotina. Investigating this possible relationship will remain an important goal toward advancing our understanding of the evolution of this group. Additional morphological and genetic data will be needed to clarify the relationship and identify further shared characteristics.

Finally, *Ramicandelaber* appears to be the clade with the longest single branch length within our protein-coding tree (Fig. 2.5). This result is reflected in the branch lengths of the individual protein-coding gene trees (Figs. 2.9-2.13). Because the protein-coding genes should be truly independent (unlike the various rDNA genes and not including RPB1 and RPB2), this seems more likely to indicate a greater true divergence than nuclear rDNA, which seems to have been susceptible to accelerated genetic changes within the Kickxellomycotina (as seen in *Asellaria* and the Dimargaritales). As such,

Ramicandelaber may also be subject to some sort of genome-wide accelerated evolution. The cause for this is uncertain; however, no sexual spores have ever been observed for any species of *Ramicandelaber*. Perhaps it is truly asexual or has extremely limited sexual reproduction and is being affected by Muller's ratchet (Muller 1964, Felsenstein 1974). Alternatively, it may be the only sampled survivor of an ancient clade, with no close relatives within the tree.

Clade 8 – Dimargaritales

Dimargaritales is perhaps the most unusual member of the Kickxellomycotina. There are four known genera of Dimargaritales, and three of these, *Dimargaris*, *Dispira*, and *Tieghemiomyces*, are included in this study. The fourth, *Spinalia*, has not been cultured and is rarely encountered. All known Dimargaritales are haustorial mycoparasites, with most species being parasites of Mucoromycotina and a few being parasites of Ascomycetes (all species present within our study are parasites of Mucoromycotina). The Dimargaritales have the defining feature of the Kickxellomycotina, septal walls with diskiform cavities and lenticular plugs. However, in this order, the plugs have polar protrubences and dissolve in dilute alkali (Benjamin 1979). These characteristics have not been observed in any other Kickxellomycotina for which the septal structure is known.

The Dimargaritales form asexual merosporangia on fertile branchlets that are based upon sporophores. In *Dimargaris*, the sporophores branch frequently and form clusters of fertile branchlets at the apex, which forms a terminal enlargement in some species. In *Tieghemiomyces*, the sporophore is erect and sometimes branching, and supports several side branches near the base which support the fertile branchlets. In *Dispira*, the sporophores may be curved or coiled, and have a sterile apex similar to *Tieghemiomyces*.

Sporophores have clusters of side branches either basally or distributed along its length, which branch many times and often curve strongly inward. The main branch supports a cluster of fertile branchlets at its apex, while the side branches are usually sterile. The Dimargaritales are unique among the Kickxellomycotina in producing bispored merosporangia. Most Dimargaritales are dry spored, with only some species of *Dimargaris* releasing their spores in a drop of liquid (Benjamin 1979). Wet-spored species have smooth-walled spores, but dry-spored species may have ridges or warts present.

Sexual spore formation is similar to that of the Kickxellales s.s. as well as the Spiromyces clade. Zygosporangia are spherical and thick-walled. Mature zygospores have a single large droplet (visible with light microscopy), similar to the Spiromyces clade but not to Kickxellales, which typically presents a large number of smaller droplets. In *Dimargaris* and *Tieghemiomyces*, zygosporangia are produced in the intercalary cell of the conjugating thalli, similar to the Kickxellales s.s. and the Spiromyces clade. In *Dispira*, zygospores are produced on elongated stalks that project above the conjugating thalli (Benjamin 1979).

Attempts to place the Dimargaritales with rDNA sequences have frequently met with difficulty. Tanabe et al. (2000) were unable to place the Dimargaritales with 18S nuclear rDNA. White et al. (2006b) had more success with combined 18S, 28S, and 5.8S, but a large amount of uncertainty remained, particularly concerning the relationship between the Dimargaritales, the rest of the Kickxellomycotina, and the Zoopagomycotina. Both of these studies revealed an anomalously long branch length for the Dimargaritales, both as a group and between the three genera examined. Studies utilizing protein-coding genes,

(e.g., Tanabe et al. 2004, James et al. 2006), had better resolution, recovered a monophyletic Kickxellomycotina, and had more typical branch lengths.

Our 8-gene tree (Fig. 2.2) demonstrates that the Dimargaritales are true members of the Kickxellomycotina. However, due to variation between the phylogenies recovered by different methods, we were not able to precisely place the clade within the Kickxellomycotina. As mentioned previously, we were unable to distinguish between the hypotheses that the Dimargaritales and *Ramicandelaber* are part of a single early diverging clade, or that Dimargaritales diverged first and *Ramicandelaber* later. However, Dimargaritales can be confirmed as one of the early-diverging clades in the Kickxellomycotina, diverging from the common ancestor before the divergence of most of the other clades within the Kickxellomycotina (clade B).

We were able to sequence some additional nuclear rDNA sequences for the Dimargaritales, producing an 18S sequence for *Dispira parvispora*, and 28S sequences for *Dis. cornuta* and *T. parasiticus*. All of these sequences continued the trend observed within the Dimargaritales of both being highly diverged from other fungi as well as from one another. For *Dis. cornuta* and *T. parasiticus*, the 28S nuclear rDNA sequences were substantially different. Both are clearly Dimargaritales in origin, but the 18S sequences available for these two species in GenBank are extremely similar. We were able to obtain an 18S nuclear rDNA sequence for *Dis. cornuta*, and it was nearly identical to the GenBank sequence, so we continued to use it. We were unable to sequence 18S nuclear rDNA from *T. parasiticus*, so we could not confirm the identity of the GenBank sequence for this species. We considered it extremely unlikely that the two 18S sequences would be similar based on the variation within the 28S sequences for these two species.

Therefore, we did not use the GenBank sequence for *T. parasiticus*. Our comparison between the nuclear rDNA tree and the protein-coding gene tree (Fig. 2.5) provides a framework for examining how the unusual rDNA affects the phylogenetic placement of the group. The nuclear rDNA generates long branches for the group and places them in an unsupported clade along with the Entomophthoromycota. The protein-coding gene tree has a more typical branch length for the clade and places them in a supported clade along with the rest of the Kickxellomycotina. Comparing these results, it seems likely that the Dimargaritales have experienced some sort of accelerated evolution of their nuclear rDNA, similar to the Asellariales, although the sequences do not appear similar under examination (*Asellaria* sequences are greatly lengthened while Dimargaritales sequences typically show an unusual number of deletions). Future studies on the Dimargaritales should focus primarily on protein-coding genes.

Within the Dimargaritales, we were unable to resolve any relationships between the four species. This is surprising, as we have two species from the same genus (*Dispira*), a genus that is unique morphologically and clearly different from the other two, but even these two species were not placed in a well-supported clade. A comparison between rDNA and protein-coding trees (Fig. 2.5) and the individual gene trees (Figs. 6-13) helps shed some light on this finding. The protein-coding gene tree, which is the most reliable tree for the Dimargaritales given its unusual nuclear rDNA, suggests the possibility of a rapid early radiation within the group. Rapid early radiation seems likely given the high variation between species seen between the sequences of the various Dimargaritales. The individual gene trees are often in disagreement, leading to a muddled signal that the multi-gene tree could not resolve. Nevertheless, the relationship suggested (but

unsupported) by the multi-gene tree, in which *Dispira* and *Tieghemiomyces* are more closely related to each other than to *Dimargaris* (Fig. 2.3: BPP: 94.0%, MLBP: 64/100, PSR: in conflict but unsupported), seems plausible. The asexual sporulating structures of *Dispira* and *Tieghemiomyces* appear more similar to each other than to those of *Dimargaris*.

Evolution of traits within the Kickxellomycotina

To examine evolution of characters and predict ancestral traits within the Kickxellomycotina, we examined numerous traits to find those that were variable within the subphylum, not autapomorphic within any single clade, and with minimal intra-clade variation. Most traits that we examined lacked variation, were autapomorphic, or were missing data in too many clades to be useful. However, four characters were found that appeared informative. For these characters, ancestral states were reconstructed using the maximum-likelihood tree in Mesquite (Fig. 2.14).

Ecological mode is a key character that is both deeply related to the role of extant species in the environment as well as evolutionary pressures faced by ancestral ones. Note that many morphological features, such as the presence of a secreted holdfast and extrusive spore release, can be essentially reduced to nutritional mode (endosymbiont vs. not endosymbiont). Nutritional mode is almost entirely consistent within the clades, with variation only occurring within the Kickxellales s.s. (i.e., *Martensella* is a non-haustorial mycoparasite but the rest are saprobic). The ancestral state reconstruction, along with the unexpected position of the endosymbiotic *Barbatospora* in the tree, suggests that the ancestor of clade B was likely to have been an arthropod endosymbiont. This possibility is of great interest when considering the early evolution of the clade, as this ancestor may

be more closely related to the common ancestor of the Kickxellomycotina than Dimargaritales or *Ramicandelaber*, which are both on rather long branches. While the placement of Dimargaritales would suggest the possibility of an ancestor that was a haustorial mycoparasite, the reconstruction actually gives a greater probability to an arthropod endosymbiont. However, it was not conclusive.

The second feature examined was the presence or absence of pseudophialides. Pseudophialides are small cells that support a single merosporangium. They were originally only thought to be present within the Kickxellales, but with the separation of the Kickxellales into three clades for this paper, they were re-examined. Of the clades we examined, the Kickxellales s.s. and *Ramicandelaber* are considered to have pseudophialides. Valle and Santamaria (2005) considered the supporting generative cell of the *Orphella* dissemination unit to be homologous to the pseudophialide, so it may have one as well. Accordingly, *Orphella* was designated as uncertain, along with Asellariales. For the purposes of this tree, it was assumed that the subsidiary cell of *Pteromaktron* is not a pseudophialide, but rather a fertile branch consisting of a single generative cell.

The uncertainty regarding Asellariales and *Orphella* makes it difficult to infer ancestral states for the upper levels of the Kickxellomycotina. However, analysis of the tree suggests that true pseudophialides are present in only a minority of the clades within the Kickxellomycotina. Further, the pseudophialides within the two clades that have them (Kickxellales s.s. and *Ramicandelaber*) are not particularly similar. Pseudophialides are always present in the Kickxellales s.s. and are much smaller relative to the sporocladium and merosporangium. In *Ramicandelaber*, they are much larger relative to the

surrounding cells, and may sometimes be absent (in *R. longisporus* when merosporangia form directly from the sporocladium). The potential to be absent, in particular, suggests that the pseudophialides in *Ramicandelaber* may not be homologous to the those in Kickxellales s.s., and may in fact not be pseudophialides at all (being instead part of a fertile branchlet or sporocladium). This could result in pseudophialides being an autapomorphic feature of the Kickxellales s.s. alone.

Zygospore shape was another character examined. Both of the variations from spherical, the biconical zygosporangia of Harpellales s.s. and the curved or coiled zygosporangia of *Orphella*, are autapomorphic. However, this trait was examined in an effort to predict what was likely to be found in *Barbatospora* and *Ramicandelaber*, for which sexual spores are not yet reported. This tree (Fig. 2.14) suggests strongly that the ancestral state for the Kickxellomycotina zygosporangium is spherical. It also suggests that the ancestor of clade C (including Asellariales, Harpellales s.s., Kickxellales s.s. *Orphella*, and the Spiromyces clade) also had spherical zygosporangia. Although the method did not predict the state of the ancestral node for either *Barbatospora* or *Ramicandelaber*, it seems likely from the tree that the zygospores within these clades, if they are produced, would most likely be spherical, based upon the predicted ancestral state for the Kickxellomycotina. Another unique form is also possible.

The last feature examined was the mode of zygospore formation. Within the Kickxellomycotina, zygospores are formed either within the intercalary cell of the conjugating thalli, or as an outgrowth of a supporting cell (which may be part of the conjugating thalli or not depending on the clade and species). We refer to this second mode as lateral formation. Zygospore formation is intercalary in the majority of the

Dimargaritales, the Kickxellales s.s., and the Spiromyces clade. It is lateral in the *Dispira* (Dimargaritales), the Harpellales s.s., and *Orphella*. We describe it as lateral within the Asellariales, but has only been reported once and the morphology was somewhat unclear, appearing similar to species of Kickxellales with apposed suspensors (Valle and Cafaro 2008). The mode of zygospore formation is potentially important because a laterally formed zygospore might suggest homology between the sexual and asexual sporulating structure of a species. In the Harpellales s.s. and *Orphella*, there are important shared characteristics between the merosporangium and zygosporangium. Most Harpellales s.s. have non-motile appendages on both spore types, and in some genera, such as *Trichozygospora* and *Zygopolaris*, they appear to be modified in the same way. Both spore types also exhibit the same rapid sporangiospore extrusion under proper conditions. In *Orphella*, there appears to be clear structural homology between the sexual and asexual dissemination unit (Valle and Santamaria 2005).

The ancestral state reconstruction for this character (Fig. 2.14) is equivocal for lateral and intercalary formation in the ancestor of the group. This outcome is not surprising when one considers that in the Dimargaritales, one of the first diverging clades, both types of development are present. Clades C and E are also split between modes. It may be that this evolutionary transition between the two forms is not difficult in the Kickxellomycotina and may represent an ancestral capability of the group. The unique septal pore structure of the Kickxellomycotina is thought to facilitate nuclear migration, and some species of Harpellales are known to form zygospores far from the location of conjugation (Farr and Lichtwardt 1967). It may be that this migration allows for modifications to the sexual apparatus such as the production of lateral zygospores,

perhaps within a modified merosporangium (allowing for the zygosporangium to inherit properties of the merosporangium such as the appendage and the rapid spore extrusion). Alternately, Moss and Lichtwardt (1977) noted that the Harpellales sexual structure has a total of four nuclei at maturity (two in the conjugants, one in the zygosporophore, one in the zygosporangium) and suggested that the meiotic divisions occur prior to spore release, in order to facility rapid germination by the sexual spore in an appropriate environment.

Conclusion and Future Work

With this study and the multi-gene phylogeny, some long-standing questions about the Kickxellomycotina can start to be answered. The relationship between the Asellariales and the Harpellales has been confirmed with molecular evidence (at least for *Asellaria* as a representative), the relationship between the Kickxellales and Harpellales has been investigated, Dimargaritales has been confirmed as a true member of the clade, and monophyly of each order has been at least partially addressed. Confirming the non-monophyly of *Orphella*, the Spiromyces clade, and *Ramicandelaber* within their current orders and discovering the non-monophyly of *Barbatospora* within the Harpellales, will allow proper taxonomic classification of these clades and future evolutionary studies to better target their morphological or genomic work. Providing both nuclear rDNA and protein-coding gene analyses allows us to examine the relative power of each and also to confirm that the extreme divergence of the Asellariales and the Dimargaritales in rDNA appears to only involve rDNA and not accurately reflect the evolutionary history of the clades as a whole. This should permit future investigators to better determine which

genes to use, particularly if molecular dating is considered (the atypical rapid evolution of the rDNA could easily lead to overestimated ages).

There is further work to be done before the evolutionary history of this group is completely resolved. Two genera previously within the Entomophthoromycota, *Ballocephala* and *Zygnemomyces*, were recently moved into the Kickxellomycotina (Humber 2012). These two genera have the characteristic septal pore and plug of the Kickxellomycotina (Saikawa 1989, Saikawa et al. 1979a), but currently have no published molecular sequences. Obtaining samples of these fungi to place them within a molecular framework should be an important objective of future studies. Investigating the monophyly of the Asellariales by obtaining sequences from *Baltomyces* and *Orchesellaria* remains an important objective. Increasing the number of protein-coding genes used could potentially resolve the relationship between Dimargaritales and *Ramicandelaber*. Finally, improving taxon sampling within the largest order of Kickxellomycotina, the Harpellales, could confirm the monophyly of the other genera and could help clarify the relationship between this order and the Asellariales; including "intermediate" genera with disarticulating thalli accommodating spore release (i.e. *Carouxella* and *Klastostachys*) could be important to help understand the precise nature of this relationship and the evolution of this trait.

Additional morphological and ultrastructural work within the subphylum would also contribute to our understanding of these organisms. Previously, work has been done largely without consideration of the greater relationships within this subphylum, but now that the monophyly has been demonstrated, effort should directed to finding characters that can be compared between clades. Potentially informative characters that have not

been consistently observed include the mode of both asexual and sexual spore germination, which appears to be potentially informative not only between orders but also within the Kickxellales s.s. and Harpellales s.s. Another valuable character may be the mode of sexual conjugation, whether homothallic or heterothallic. This character has historically been noted as difficult to observe due to the complex and often tangled mass of thalli, but we suggest it may be potentially informative within the Harpellales s.s. and within *Orphella*. This trait could also reveal why zygosporangia have not yet been observed within some clades, such as many genera within the Kickxellales. The presence of the labyrinthiforme organelle or abscission vacuole, as noted above, has been confirmed within the Harpellales s.s. and Kickxellales s.s., but would be very informative if observed (or if confirmed to be absent) in any other clade.

Finally, it is hoped that this study might provide some of the groundwork to pursue the use of next-generation sequencing techniques such as pyrosequencing for the study of the Kickxellomycotina. This phylogeny can provide a guide for genomic sequencing for phylogenomic projects such as the upcoming 1000 Fungal Genomes Project (http://1000.fungalgenomes.org/home/). Additionally, the sequences produced for 18S and 28S nuclear rDNA can be used to derive suitable primers for environmental amplification and sequencing of the ITS region, the fungal barcoding gene. This phylum has potentially been undersampled by environmental surveys due to incompatibility of common ITS primers with these taxa (particularly the ones with unusual rDNA such as *Asellaria* or the Dimargaritales). Environmental sampling of either freshwater or soil environments could potentially reveal a great deal of unsampled diversity within the

Kickxellomycotina, particularly considering that difficulty involved in culturing many of its species.

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Table 2.1 PCR protocols used.

PCR Protocol #	Gene	Forward Primer	Reverse Primer	Kit	Cycles	Initial Denature		Denature		Annealing		Extension		Final Extension		Betaine	DMSO	BSA	Notes
37	18S rDNA	SR1R	NS6Z	As per White 2006															
177	18S rDNA	NS1AA	NS8AA	GoTAQ Green MM	45	95°	2:00	95°	0:30	62°	0:45	72°	3:00	72°	10:00	no	no	no[1]	Animal-avoidant 18S amp
170	18S rDNA	SR1R	NS8	GoTAQ Green MM	45	95°	2:00	95°	0:30	52°	0:45	72°	3:00	72°	10:00	no	no	no	Conventional 18S amp
198	18S rDNA	NS3	NS6Z	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	50°	0:45	72°	3:00	72°	10:00	1 M	no	no	Partial 18S for A. ligeae only
199	18S rDNA	NS5Asl	NS8	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	52°	0:45	72°	2:00	72°	10:00	no	no	no	Partial 18S for A. ligeae only
200	18S rDNA	SR1R	NS4Asl	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	52°	0:45	72°	2:00	72°	10:00	no	no	no	Partial 18S for A. ligeae only
204	18S rDNA	NS1Asl	NS8Asl	Phusion II HS (HF Buffer)	45	98°	1:00	98°	0:20	65°	0:30	72°	1:30	72°	10:00	no	no	0.8 µg/µL	18S protocol designed for A. ligeae
183	28S rDNA	NL1AA	LR7AA	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	56°	0:45	72°	3:00	72°	10:00	0.5 M	no	no[1]	Animal-avoidant 28S amp
184	28S rDNA	NL1AA	LR10AA	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	50°	0:45	72°	4:30	72°	10:00	0.5 M	no	0.8 µg/µL	Animal-avoidant 28S long amp
190	28S rDNA	NL1K	NL4	Phusion II HS (GC Buffer)	45	98°	1:00	98°	0:20	63°	0:30	72°	1:30	72°	10:00	no	3%	0.8 µg/µL	Used to amplify A. ligeae
191	28S rDNA	LR0RK	LR12	TaKaRa LA (GC Buffer I)	50	94°	2:00	94°	0:30	45°	0:45	72°	5:00	72°	10:00	no	no	no	Long 28S amp (less successful)
196	28S rDNA	NL1	LR11	TaKaRa LA (GC Buffer I)	50	94°	2:00	94°	0:30	49°	0:45	72°	5:00	72°	10:00	no	no	no	Long 28S amp (most successful)
202	28S rDNA	NL1-Asl	LR7R-Asl	Phusion II HS (HF Buffer)	45	98°	1:00	98°	0:20	64°	0:30	72°	3:00	72°	10:00	1 M	no	no	28S protocol designed for A. ligeae
39	5.8S rDNA	ITS1F	LR5	As per White 2006															
87	5.8S rDNA	ITS1F	NL4	As per White 2006															
182	5.8S rDNA	NS7AA	ITS4AA	Phusion II HS (HF Buffer)	45	98°	1:00	98°	0:20	69°	0:30	72°	2:00	72°	10:00	1 M	no	no	Animal-avoidant ITS/5.8S amp
304	MCM 7	MCM7-709f	MCM7-16r	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	48°	0:45	72°	1:45	72°	10:00	no	no	no	General MCM7 amp
310	MCM 7	MCM7-8bf	MCM7-16r	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	50°	0:45	72°	1:45	72°	10:00	no	no	no	Harpellales and related MCM7 amp
62	RPB1	RPB1-Af	RPB1-Dr	GoTAQ Green MM	50	95°	2:00	95°	0:30	45°	0:30	72°	2:45	72°	10:00	no	no	no	Initial RPB1 Protocol

602	RPB1	RPB1-AfL	RPB1-DrL	GoTAQ Green Hot MM	50	95°	2:00	95°	1:00	57°-47°	1:15	72°	2:45	72°	10:00	no	no	0.8 µg/µL	Seemed more successful than 62
82	RPB2	fRPB2-5f	fRPB2-7cR	GoTAQ Green Hot MM	50	95°	2:00	95°	1:00	53°-43°	1:15	72°	2:45	72°	10:00	no	no	no	General RPB2 protocol
805	RPB2	RPB2-5fM	RPb2-7rM	GoTAQ Green Hot MM	50	95°	2:00	95°	1:00	55°-45°	1:15	72°	2:45	72°	10:00	no	no	no	Only helped with *Orphella* RPB2
501	TSR1	TSR1-1018f	TSR1-2356r	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	54°	0:45	72°	1:45	72°	10:00	no	no	0.8 µg/µL	Longer amp, doesn't work on Harpelalles
502	TSR1	TSR1-1492f	TSR1-2356r	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	54°	0:45	72°	1:45	72°	10:00	no	no	0.8 µg/µL	Shorter amp, better for Harpellales
401	BTUB	BT-K1f	BT-K1r	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	55°	0:45	72°	2:00	72°	10:00	no	no	no	General β -Tubulin amp
402	BTUB	BT-CR1F	BT-CR1R	GoTAQ Green Hot MM	45	95°	2:00	95°	0:30	46°	0:45	72°	2:00	72°	10:00	no	no	no	Specialized β-Tubulin amp for Kickxellales. Doesn't amplify the paralog gene.

1. Tested both ways - didn’t appear to make any difference. Some data may be obtained with or without this reagent.

Table 2.2 Primers used.

Gene	Primer Name	Source	Direction	Sequence (5' - 3')	Translation
18S	SR1R	Vilgalys and Hester 1990	For	TACCTGGTTGATYCTGCCAGT	N/A
18S	NS1AA	Wang et al. 2012a	For	AAGCCATGCATGTCTAAGTATAA	N/A
18S	NS3	White et al. 1990	For	GCAAGTCTGGTGCCAGCAGCC	N/A
18S	NS5Asl	Novel	For	CTGTCTCTTGGAAATGATCGC	N/A
18S	NS1Asl	Novel	For	AAGCCATGCATGTCTAAGTTCAG	N/A
18S	NS6Z	O'Donnell et al. 1998	Rev	GCATCACAGACCTGTTATTGCC	N/A
18S	NS8AA	Wang et al. 2012a	Rev	TACTTCCTCTAAATGACCAAGTTTG	N/A
18S	NS8	White et al. 1990	Rev	TCCGCAGGTTCACCTACGGA	N/A
18S	NS4Asl	Novel	Rev	CACCGCTAGCCATGCCATACAAG	N/A
18S	NS8Asl	Novel	Rev	TACTTCCTCTATGCGCCTAACATTG	N/A
28S	NL1AA	Wang et al. 2012a	For	GAGTGAAGCGGGAAIAGCTCAAG	N/A
28S	NL1K	Novel	For	GCATATTAATAAGCGGAGGAAAAG	N/A
28S	LR0RK	Novel	For	ACCCGCTGAATTTAAGC	N/A
28S	NL1	O'Donnell 1993	For	GCATATCAATAAGCGGAGGAAAAG	N/A
28S	NL1-Asl	Novel	For	GGCGAGTGAAGAGAAGACAATAAG	N/A
28S	LR7AA	Wang et al. 2012a	Rev	CCACCAAGATCTGCACTAGA	N/A
28S	LR10AA	Novel	Rev	GCCACACTTTCATGGTTTGTATTCAC	N/A

28S	NL4	O'Donnell 1993	Rev	GGTCCGTGTTTCAAGACGG	N/A
28S	LR12	Vilgalys lab[1]	Rev	GACTTAGAGGCGTTCAG	N/A
28S	LR11	Vilgalys lab[1]	Rev	GCCAGTTATCCCTGTGGTAA	N/A
28S	LR7R-Asl	Novel	Rev	GTAAGCTGGTCTGGAGCAACC	N/A
5.8S	ITS1F	Gardes and Bruns, 1993	For	CTTGGTCATTTAGAGGAAGTAA	N/A
5.8S	NS7AA	Novel	For	GGAAGTTTGAGGCAATAACAGG	N/A
5.8S	LR5	Vilgalys and Hester 1990	Rev	TCCTGAGGGAAACTTCG	N/A
5.8S	ITS4AA	Novel	Rev	CCGCTTCACTCGCMGTTACTA	N/A
MCM7	MCM7-709f	Schmitt et al. 2009	For	ACIMGIGTITCVGAYGTHAARCC	TRVSDVKP
MCM7	MCM7-8bf	Tretter et al. 2013	For	GTIGCIGCITAYYTITGYGAY	VAAYLCD
MCM7	MCM7-16r	Tretter et al. 2013	Rev	GTYTGYTGYTCCATIACYTCRTG	HEVMEQQT
RPB1	RPB1-Af	Stiller and Hall, 1997	For	GARTGYCCDGGDCAYTTYGG	ECPGHFG
RPB1	RPB1-AfL	Wang et al. 2012b	For	GARTGYCCDGGDCAYTTYGGICA	ECPGHFG(H/Q)
RPB1	RPB1-Dr	Hall lab.	Rev	TTCATYTCRTCDCCRTCRAARTC	DFDGDEM(N/K)
RPB1	RPB1-DrL	Wang et al. 2012b	Rev	TTCATYTCRTCDCCRTCRAARTCIGC	ADFDGDEM(N/K)
RPB2	fRPB2-5f	Liu et al. 1999	For	GAYGAYMGWGATCAYTTYGG	DDRDHFG
RPB2	RPB2-5fM	Novel	For	GAYGAYMGIGATCAYTAYGG	DDRDHYG
RPB2	fRPB2-7cR	Liu et al. 1999	Rev	CCCATRGCTTGYTTRCCCAT	MGKQAMG
RPB2	fRPb2-7rM	Novel	Rev	CCCATIGCYTGYTTICCCAT	MGKQAMG
TSR1	TSR1-1018f	Tretter et al. 2013	For	AAYGARCARACITGGCCIACIGA	NEQTWPT(D/E)

TSR1	TSR1-1492f	Tretter et al. 2013	For	TGGGAYCCITWYGARAAYYTICC	WDP(Y/F)ENLP
TSR1	TSR1-2356r	Tretter et al. 2013	Rev	CAYTTCATRTAICCRTGIGTICC	GTHGYMKC
BTUB	BT-K1f	Keeling et al. 2000	For	GCCTGCAGGICARTGYGGIAAYCA	PAGQCGNQ
BTUB	BT-CR1F	Novel	For	GAYMGIATHGAYGTITAYTTYAA	DRIDVYFN
BTUB	BT-K1r	Keeling et al. 2000	Rev	GGCCTCAGTRAAYTCCATYTCRTCCAT	MDEMEFTEA
BTUB	BT-CR1R	Novel	Rev	AACATIGCIGTRAAYTGRTCRTT	NDQFTAMF

1. Available at http://www.biology.duke.edu/fungi/mycolab/primers.htm.

Table 2.3 List of species/isolates used in the phylogenetic analysis and their GenBank accession numbers or genome sequencing project locations.

Species	Isolate	18S	28S	5.8S	MCM7	RPB1	RPB2	TSR1	BTUB
Batrachochytrium dendrobatidis[1]	JEL423	Supercontig 1.14 188674-190464	NG_027619.1	Supercontig 14 223000-223400	Supercontig 5 1573505-1576449	BDEG_04054.1	BDEG_06085.1	BDEG_02071.1	BDEG_05618.1
Spizellomyces punctatus[1]	Multiple isolates	Supercontig 1.32 115-2731	NG_027618.1	Supercontig 1.30 2400-2600	Supercontig 1.7 1200575-1203751	SPPG_00380.3	Supercontig 1.5 886719-891350	SPPG_02880.3	SPPG_04946.3
Rhizophlyctis rosea	JEL318	AY635829.1	NG_027649.1	AY997078.1	KC297581	DQ294597.1	DQ302786.1	1000-502	AF162078.1
Allomyces macrogynus[1]	ATCC 38327	EF014364.1	Supercontig 3.63 164434-171606	Supercontig 3.15 22619-22766	Supercontig 3.1 1367529-1370128	AMAG_07841.2	AMAG_10446.2	AMAG_17353.1	AMAG_13901.1
Coelomomyces stegomyiae	DUH0008925	NG_017164.1	NG_027644.1	AY997038.1	KC297583	DQ294579.1	DQ302766.1	KC297630	-
Mucor circinelloides[3]	CBS277.49	Scaffold 11 800000-801950	Scaffold 11 804000-809666	Scaffold 3 505429-505570	Scaffold 14 106477-109288	Scaffold 11 255181-260819	Scaffold 09 1025314-1029155	Scaffold 1 4335584-4338189	Scaffold 3 457532-459736
Phycomyces blakesleeanus[3]	NRRL 1555	NG_017190.1	NG_027559.1	Scaffold 15 326590-326758	Scaffold 5 753037-756512	Scaffold 16 1039264-1045117	Scaffold 22 851100-855169	Scaffold 24 361829-364983	Scaffold 2 3043826-3045360
Rhizopus oryzae[2]	99-880	Supercontig 3.6 2079534-2081357	Supercontig 3.6 2074749-2079337	Supercontig 3.6 2079200-2079380	RO3G_11608	RO3G_07354	CH476736.1	RO3G_12091.3	CH476734.1
Puccinia graminis[3]	CRL 75-36-700-3	AY125409.1	Supercontig 2.33 1429-5500	Supercontig 2.33 5050-5250	XM_003327822.2	XM_003334476.1	XM_003321826.1	XM_003320360.2	XM_003330619.2
Coprinopsis cinerea	Multiple isolates	M92991.1	AF041494.1	FN396149.1	XM_001833124.2	XM_001828525.2	XM_001829088.1	XM_001830626.2	XM_001838247.2
Laccaria bicolor[3]	S238N-H82	Scaffold 7 728623-730473	AC156603.2	Scaffold 7 745500-745700	XM_001875086.1	XM_001881359.1	XM_001873347.1	Scaffold 12 695382-698248	XM_001883271.1
Cryptococcus neoformans	B-3501A	BR000310.1	BR000310.1	BR000310.1	XM_769543.1	XM_770004.1	XM_770778.1	XM_770411.1	XM_771811.1
Ustilago maydis	Multiple isolates	X62396	AF453938	AF135431.1	XM_757456.1	XM_754917.1	Contig 1.150 178676-183443	XM_756040.1	XM_756882.1

Malassezia globosa[3]	CBS 7966	Scaffolds 11.1 137082-138856	Scaffold 11.1 139000-142976	Scaffold 11.1 139100-139280	XM_001728742.1	Scaffold 7.1 12763-18312	XM_001731569.1	Scaffold 6.1 83515-86450	XM_001731293.1
Aspergillus nidulans	FGSC A4	U77377.1	EU840227.1	L76746.1	XM_658504.1	XM_653321.1	XM_653321.1	XM_658778.1	XM_653694.1
Aspergillus niger[3]	Multiple isolates	Chromosome 6.1 668060-670020	Chromosome 6.1 664309-668309	AM270052	ACJE01000013.1	ACJE01000004.1	ACJE01000005.1	CJE01000010.1	ACJE01000021.1
Saccharomyces cerevisiae	S288c	NC_001144.5	NC_001144.5	BK006945.2	NM_001178550.2	BK006938.2	BK006948.2	NM_001180119.1	NM_001179929.1
Schizosaccharomyces pombe	972h-	CU329672.1	NC_003421.2	NC_003421.2	NM_001022466.2	NM_001021568.2	NM_001018498.2	NM_001018823.2	NM_001022572.2
Rhopalomyces elegans	NRRL A-10835	NG_017191.1	NG_027654.1	-	KC297584	DQ294596.1	DQ302785.1	KC297631	993-401-C
Piptocephalis corymbifera	NRRL 2385	NG_017192.1	NG_027616.1	AY997073.1	-	DQ294610.1	DQ302788.1	992-501	992-401-1
Entomophthora muscae	ARSEF3074	NG_017183.1	NG_027647.1	AY997047.1	KC297587	DQ294590.1	DQ302778.1	KC297632	996-401
Conidiobolus coronatus	NRRL 28638	NG_017182.1	NG_027617.1	AY997041.1	995-304	DQ294591.1	DQ302779.1	995-502	AF162057.1
Coemansia reversa[3]	NRRL 1564	AY546685.1	NG_027615.1	Scaffold 121 5840-5992	Scaffold 81 3398-5725	Scaffold 2 621744-627434	Scaffold 27 282896-286826	Scaffold 24 40229-42782	Scaffold 7 113928-115952
Barbatospora ambicaudata	TN-49-W4a	KC297614	902-87/184	902-87/184	KC297588	902-602	902-82	KC297633	902-401
Coemansia braziliensis	NRRL-1566	AF007532.1	AF031069.1	416-182	KC297589	416-62	416-82	KC297634	416-402L
Kickxella alabastrina	NRRL-2693	AF007537.1	419-184/196	420-182	JX155485	420-62L	420-82	KC297635	420-402-3
Dipsacomyces acuminosporus	NRRL-2925	AF007534.1	423-184/191	423-182	KC297590	423-602	423-82	KC297636	423-402H-C
Martensiomyces pterosporus	NRRL-2642	AF007539.1	AF031066	425-182	KC297591	425-62L	425-82-C	KC297637	-
Linderina pennispora	NRRL-3781	AF007538	418-196	418-182	JX155486	418-602	418-82	KC297638	-
Spirodactylon aureum	NRRL-2810	AF007541.1	AF031068.1	-	KC297592	426-602-C	426-82	KC297639	-
Spiromyces minutus	NRRL-3067	AF007542.1	DQ273810.1	AY997091.1	KC297593	421-602	421-82	KC297640	421-402

Spiromyces aspiralis	NRRL-22631	AF007543.1	NG_027560.1	991-182	KC297594	DQ294599	DQ302790.1	KC297641	991-402-L
Orphella dalhousiensis	NS-34-W16	DQ322626.1	DQ273830.1	191G-39-1	KC297599	DQ294606.1	191G-805	-	-
Harpellomyces montanus	TN-22-W5B	JQ302887.1	954G-183/184	954G-182	KC297600	-	954G-82-HT-C	954-502	-
Capniomyces stellatus	MIS-21-127	DQ367451.1	EF396194.1	EF396189.1	JX155472	EF014379.1	EF014396.1	KC297643	AF162061.1
Smittium culisetae	COL-18-3	NG_017185.1	NG_027648.1	JN943286.1	JN993375.1	EF014378.1	EF014395.1	KC297644	AF162069.1
Lancisporomyces falcatus	NS-X-2	JQ302865.1	JQ302943.1	520-182H	JX155477	520-602	520-82	-	-
Harpella melusinae (cysts)	NF-15-5A	JX155621	244-183/196	244-182	JX155475	244-602	244-82	KC297652	244-401-C
Trichopteran trichomycetes	ALG-13-W1	JX155625	918-183/196	-	JX155481	918-602-C	918-82	KC297653	918-401-C
Caudomyces sp.	UT-1-W16a	JX155620	JX155646	763-182	JX155473	-	-	KC297650	-
Caudomyces sp.	OR-8-W10	KC297620	1025-183/196	-	KC297603	-	1025-82	KC297649	-
Legeriomyces minae	PEI-X-6	JX155622	JX155648	-	JX155478	930-602	930-82	-	-
Pteromaktron sp.	OR-11-W8	JX155623	983G-183/184	983G-182	JX155479	983G-602H-HT-C	-	983G-502	-
Genistelloides hibernus	KS-19-M23	DQ367456.1	192-183/184	-	JX155474	192-602	192-82	KC297651	192-401
Smittium culicis	43-1-2	JQ302893.1	362-97/191/196	JN943292.1	JN993378.1	JN991279.1	362-82	KC297646	362-401-C
Smittium mucronatum	FRA-12-3	AF277030.1	68-97/184	JN943282.1	JN993383.1	JN991272.1	68-82-1	KC297647	68-401
Smittium simulii	41-1-6	JQ302861.1	JQ302939.1	374-182	JN993386.1	JN991268.1	374-82	KC297648	374-401
Smittium morbosum	AUS-X-1	AF277014.1	70-196	70-182	JX155443	70-62	70-82	-	70-401
Asellaria ligiae	FL-3-W3	877G-SSU	877G-LSU	-	-	877-602	877-82	-	877-401M
Ramicandelaber longisporus	ATCC 6175	987b-170	987a-196	-	KC297595	987a-602-C	987a-82	KC297642	987a-401-4

Ramicandelaber longisporus	ATCC 6176	988a-170	988a-196	-	KC297596	988a-602-C	988b-82	988a-501	988a-401-2
Dimargaris bacillispora	NRRL 2808	NG_017180.1	NG_027650.1	AY997043	KC297597	DQ294588.1	DQ302775.1	1032-501	1032-401-3
Dispira cornuta	Multiple isolates	AB016021.2	1034-196	-	1034-304	-	1034-82	1034-501	1034-401-2
Dispira parvispora	NRRL 3066	1036-170	-	-	1037-304	1036-602-C	1036-82	1036-501	1036-401-4
Tieghemiomyces parasiticus	NRRL 2924	-	1038-196	-	1038-304-1	1038-602-C	1038-82	1038-501	1038-401-2

1. Data derived from Origins of Multicellularity Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

2. Data derived from *Rhizopus oryzae* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

3. These sequence data were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community.

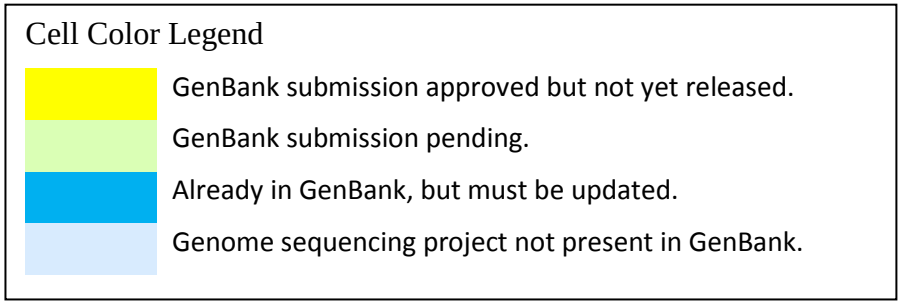


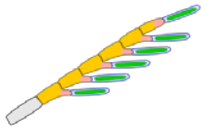
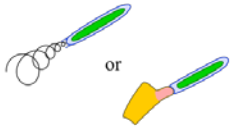
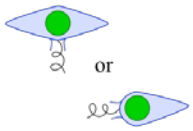
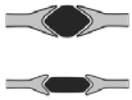
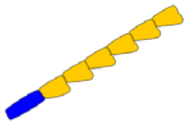
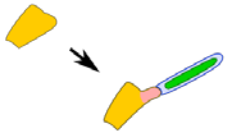
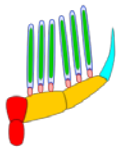
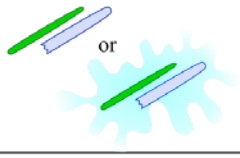
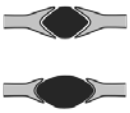
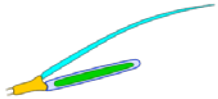
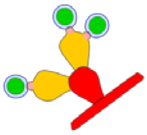
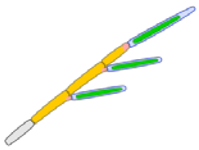
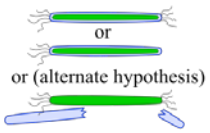
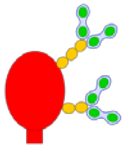
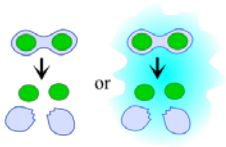
Table 2.4 SH and AU test results for alternative hypotheses involving tree method conflicts and putatively unique lineages.

		Topology ln likelihood		SH		AU		Reject H_A	
	Alternative Hypothesis	**H_0**	**H_A**	**$p(H_0)$**	**$p(H_A)$**	**$p(H_0)$**	**$p(H_A)$**	**SH**	**AU**
1	Dimargaritales and *Ramicandelaber* are joined	-118855.018	-118864.104	0.798	0.202	0.814	0.186		
2	Entomophthoromycotina and Blastocladiomycota are joined	-118855.018	-118862.395	0.722	0.278	0.720	0.280		
3	*Harpellomyces* is the first Harpellales genus to diverge	-118855.018	-118859.226	0.692	0.308	0.692	0.308		
4	*Ramicandelaber* is a member of the Kickxellales	-118855.018	-119095.251	1.000	0.000	1.000	0.000	*	*
5	*Orphella* is a member of the Harpellales	-118855.018	-119053.984	1.000	0.000	1.000	0.000	*	*
6	*Barbatospora* is a member of the Harpellales	-118855.018	-118934.846	1.000	0.000	1.000	0.000	*	*
7	*Spiromyces* is a member of the Kickxellales	-118855.018	-118988.276	1.000	0.000	1.000	0.000	*	*

Table 2.5 Comparative analysis of phylogenetic trees.

Gene(s)	Figure	Taxa	Total Characters	Informative Characters	Tree Likelihood	Supported Branches	Unsupported Branches	Percent Supported
18S rDNA	Fig. 2.6	54	1581	946	-19599.42959	26	25	51%
28S rDNA	Fig. 2.7	54	2678	1575	-35311.03047	30	21	59%
5.8S rDNA	Fig. 2.8	43	148	110	-2053.586036	11	29	28%
MCM7	Fig. 2.9	53	273	231	-10658.40254	25	25	50%
RPB1	Fig. 2.10	51	384	337	-13929.20887	28	20	58%
RPB2	Fig. 2.11	53	353	305	-12992.29199	28	22	56%
TSR1	Fig. 2.12	50	216	212	-11782.39168	26	21	55%
BTUB	Fig. 2.13	44	402	296	-7777.280461	23	18	56%
Combined rDNA	Fig. 2.5	55	4407	2631	-57410.33493	35	17	67%
Protein	Fig. 2.5	55	1628	1381	-58628.44163	40	12	77%
8-gene	Fig. 2.2	55	6035	4012	-118855.0177	44 (41)	8 (9)	84% (79%)

1. Gene abbreviations: MCM7 – DNA replication licensing factor Mcm7, RPB1 – RNA polymerase II, largest subunit. RPB2- RNA polymerase II, second largest subunit. TSR1 – Ribosomal biogenesis protein TSR1. I. BTUB - β –Tubulin.

Clade	Sporulating Structure	Asexual Spore	Sexual Spore	Septal Morphology
Harpellales				
Asellariales				
Kickxellales				
Orphella				?
Spiromyces				
Barbatospora			?	?
Ramicandelaber			?	
Dimargaritales				

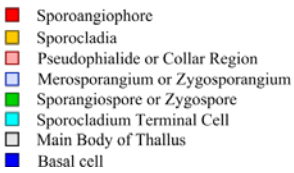
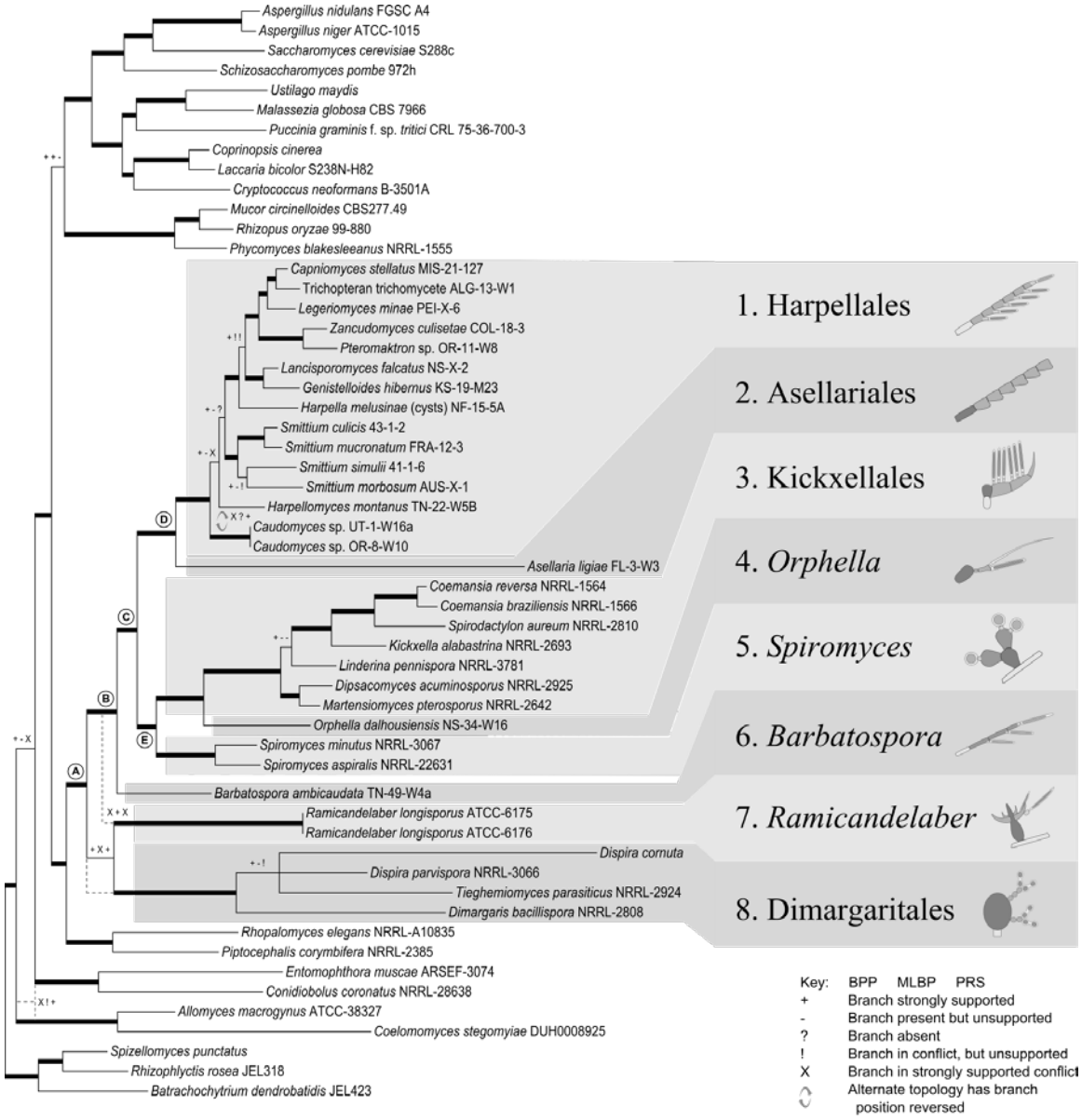


Figure 2.1 Comparative morphology of the Kickxellomycotina.

Designation of homologous features is based upon literature, when available, or inferred by position when not. Question marks refer to features either not known or not currently published. Septal structure of Spiromyces is inferred from *Mycoëmilia*, a closely related species.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Aspergillus niger ATCC-1015
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h
Ustilago maydis
Malassezia globosa CBS 7966
Puccinia graminis f. sp. tritici CRL 75-36-700-3
Coprinopsis cinerea
Laccaria bicolor S238N-H82
Cryptococcus neoformans B-3501A
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL-1555
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Legeriomyces minae PEI-X-6
Zancudomyces culisetae COL-18-3
Pteromaktron sp. OR-11-W8
Lancisporomyces falcatus NS-X-2
Genistelloides hibernus KS-19-M23
Harpella melusinae (cysts) NF-15-5A
Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Smittium simulii 41-1-6
Smittium morbosum AUS-X-1
Harpellomyces montanus TN-22-W5B
Caudomyces sp. UT-1-W16a
Caudomyces sp. OR-8-W10
Asellaria ligiae FL-3-W3
Coemansia reversa NRRL-1564
Coemansia braziliensis NRRL-1566
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Barbatospora ambicaudata TN-49-W4a
Ramicandelaber longisporus ATCC-6175
Ramicandelaber longisporus ATCC-6176
Dispira cornuta
Dispira parvispora NRRL-3066
Tieghemiomyces parasiticus NRRL-2924
Dimargaris bacillispora NRRL-2808
Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
Entomophthora muscae ARSEF-3074
Conidiobolus coronatus NRRL-28638
Allomyces macrogynus ATCC-38327
Coelomomyces stegomyiae DUH0008925
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423
1. Harpellales
2. Asellariales
3. Kickxellales
4. Orphella
5. Spiromyces
6. Barbatospora
7. Ramicandelaber
8. Dimargaritales
Key: BPP MLBP PRS
+ Branch strongly supported
- Branch present but unsupported
? Branch absent
! Branch in conflict, but unsupported
X Branch in strongly supported conflict
Alternate topology has branch position reversed

Figure 2.2 Phylogeny of the Kickxellomycotina and other fungal taxa based on an 8-gene alignment including three sections of rDNA and five translated protein-coding genes.

Tree is based on a 50% majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). Additional support was provided by maximum-likelihood bootstrap inference and maximum-parsimony symmetrical resampling. Branches in bold are highly supported (>95% BPP, 70% MLBP, 70% PSR). Letters within circles refer to monophyletic clades discussed within the text.

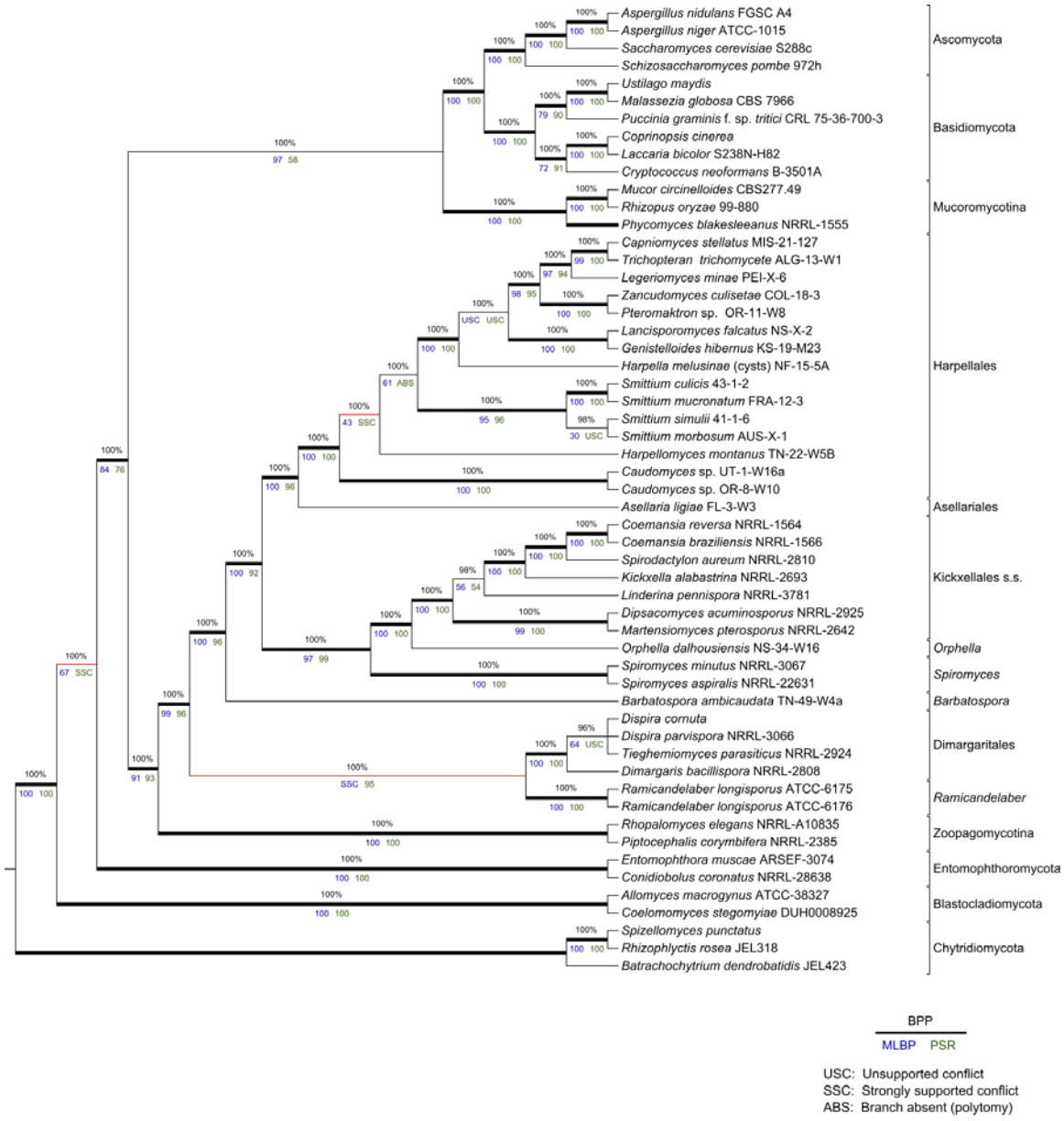
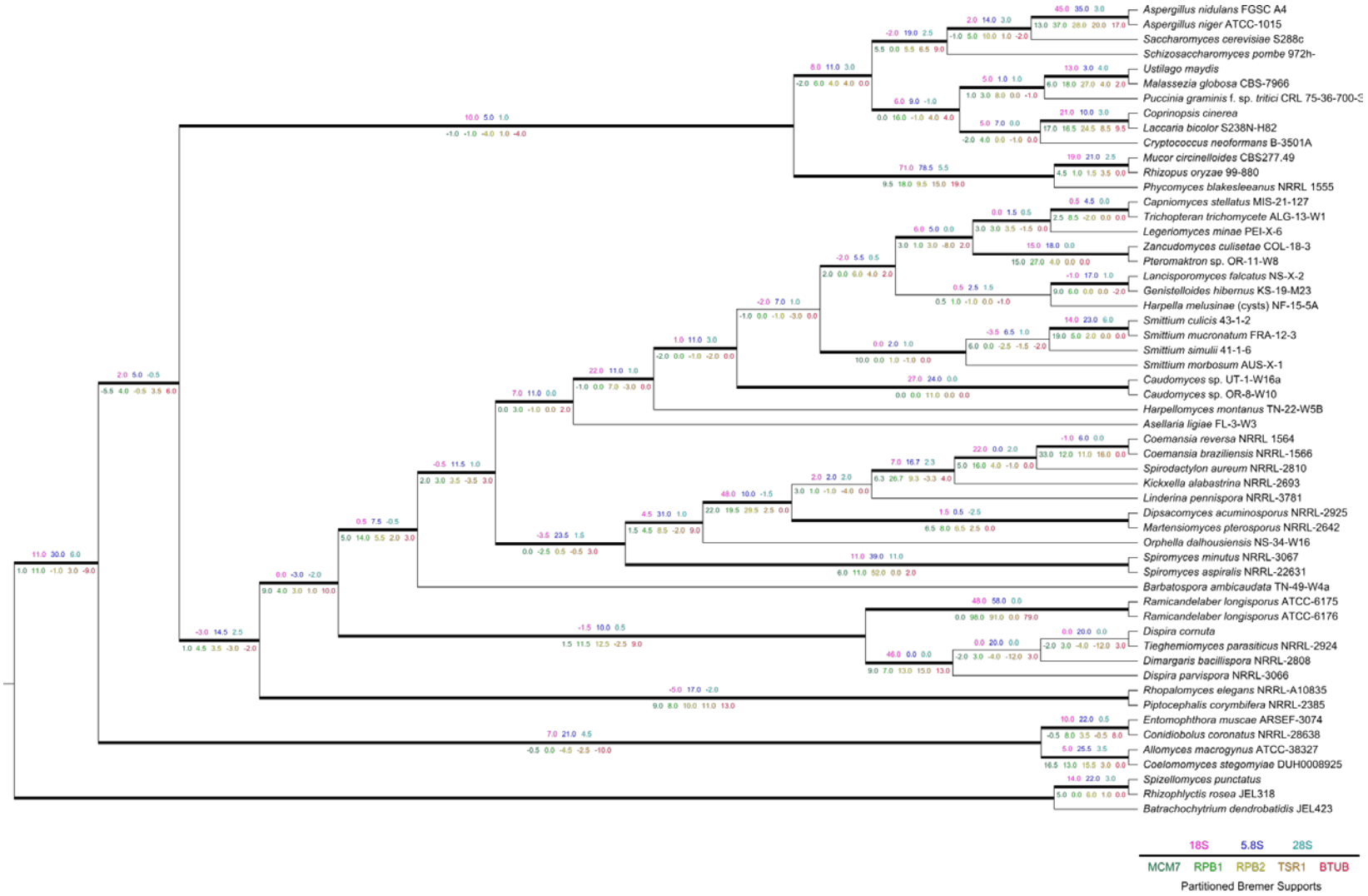


Figure 2.3 Cladogram of 8-gene Kickxellomycotina tree.

Tree produced by same methods as Fig. 2.2 and provided so that numerical supports may be examined. Branches in bold are highly supported (>95% BPP, 70% MLBP, 70% PSR). Branches in red indicate strongly supported conflicts between trees.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Aspergillus niger ATCC-1015
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h-
Ustilago maydis
Malassezia globosa CBS-7966
Puccinia graminis f. sp. tritici CRL 75-36-700-3
Coprinopsis cinerea
Laccaria bicolor S238N-H82
Cryptococcus neoformans B-3501A
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL 1555
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Legeriomyces minae PEI-X-6
Zancudomyces culisetae COL-18-3
Pteromaktron sp. OR-11-W8
Lancisporomyces falcatus NS-X-2
Genistelloides hibernus KS-19-M23
Harpella melusinae (cysts) NF-15-5A
Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Smittium simulii 41-1-6
Smittium morbosum AUS-X-1
Caudomyces sp. UT-1-W16a
Caudomyces sp. OR-8-W10
Harpellomyces montanus TN-22-W5B
Asellaria ligiae FL-3-W3
Coemansia reversa NRRL 1564
Coemansia braziliensis NRRL-1566
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Barbatospora ambicaudata TN-49-W4a
Ramicandelaber longisporus ATCC-6175
Ramicandelaber longisporus ATCC-6176
Dispira cornuta
Tieghemiomyces parasiticus NRRL-2924
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
Entomophthora muscae ARSEF-3074
Conidiobolus coronatus NRRL-28638
Allomyces macrogynus ATCC-38327
Coelomomyces stegomyiae DUH0008925
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423
18S
5.8S
28S
MCM7
RPB1
RPB2
TSR1
BTUB
Partitioned Bremer Supports

Figure 2.4 Partitioned Bremer supports.

These support values were produced with TNT v. 1.1 using scripts provided by Peña et al. 2006. Tree topology was taken from the most parsimonious tree produced with TNT via the new technology bootstrap method.

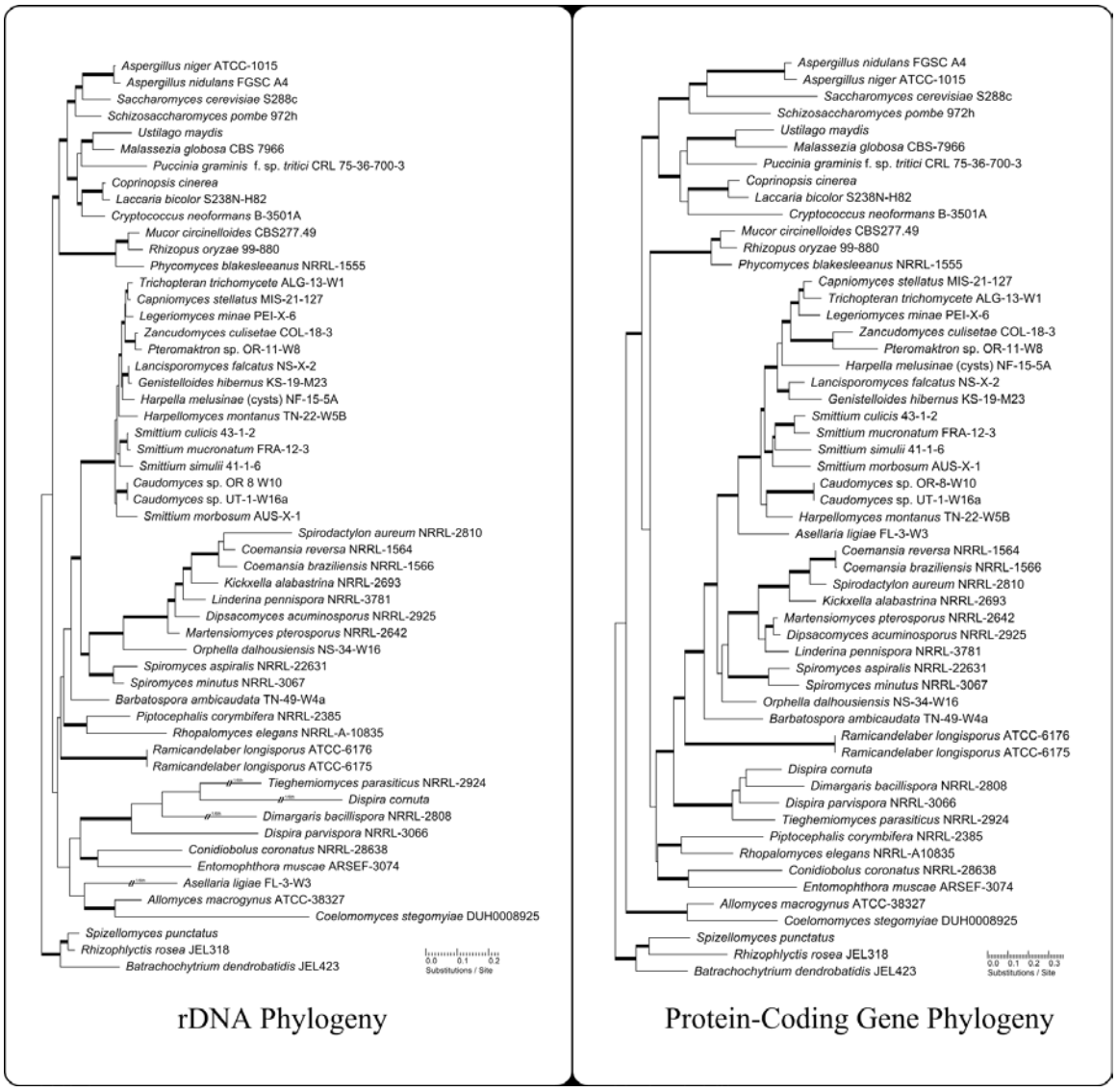
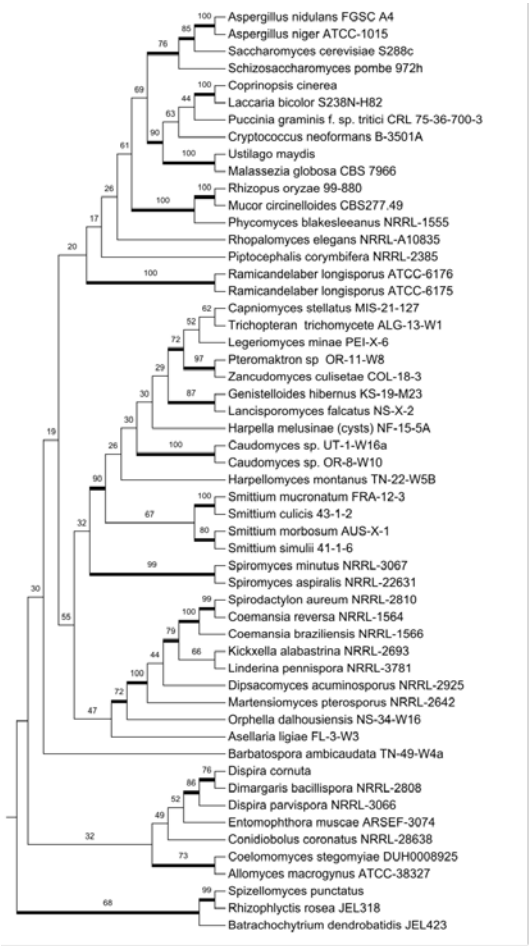


Figure 2.5 Comparison between the phylogeny produced via the three rDNA genes (nuclear 18S, 28S, and 5.8S) and five protein-coding genes (MCM7, RPB1, RPB2, TSR1, β-tubulin).

Trees were taken from the most likely topology produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Cryptococcus neoformans B-3501A
Ustilago maydis
Malassezia globosa CBS 7966
Rhizopus oryzae 99-880
Mucor circinelloides CBS277.49
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Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
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Ramicandelaber longisporus ATCC-6175
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Legeriomyces minae PEI-X-6
Pteromaktron sp OR-11-W8
Zancudomyces culisetae COL-18-3
Genistelloides hibernus KS-19-M23
Lancisporomyces falcatus NS-X-2
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Caudomyces sp. UT-1-W16a
Caudomyces sp. OR-8-W10
Harpellomyces montanus TN-22-W5B
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Smittium culicis 43-1-2
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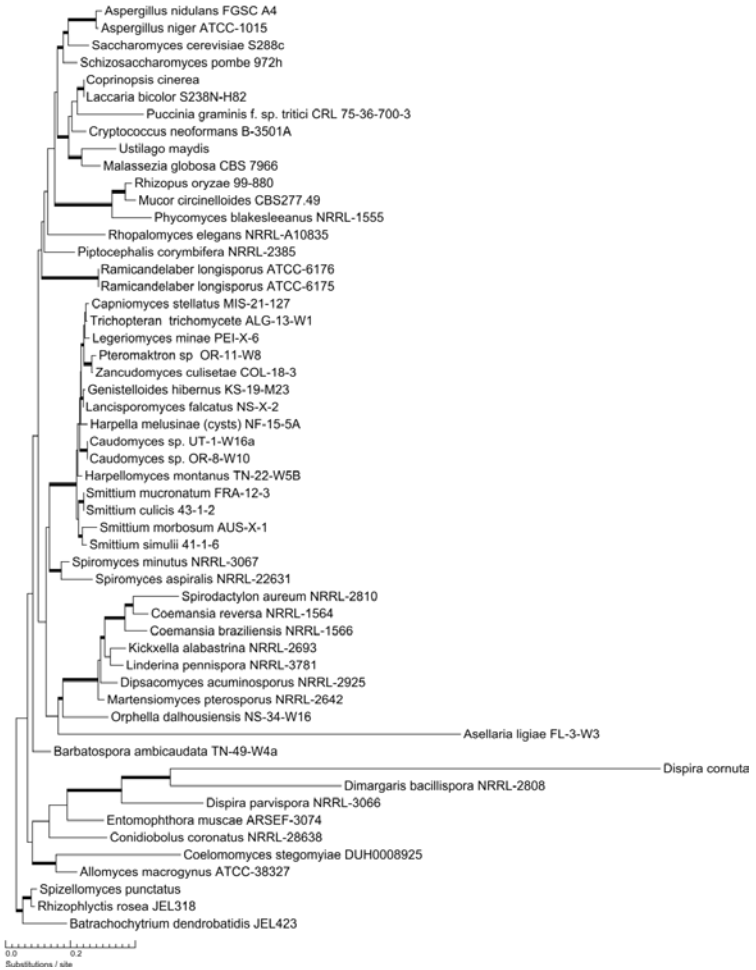
Smittium simulii 41-1-6
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Spirodactylon aureum NRRL-2810
Coemansia reversa NRRL-1564
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Asellaria ligiae FL-3-W3
Barbatospora ambicaudata TN-49-W4a
Dispira cornuta
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Entomophthora muscae ARSEF-3074
Conidiobolus coronatus NRRL-28638
Coelomomyces stegomyiae DUH0008925
Allomyces macrogynus ATCC-38327
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423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Mucor circinelloides CBS277.49
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Piptocephalis corymbifera NRRL-2385
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Trichopteran trichomycete ALG-13-W1
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Pteromaktron sp OR-11-W8
Zancudomyces culisetae COL-18-3
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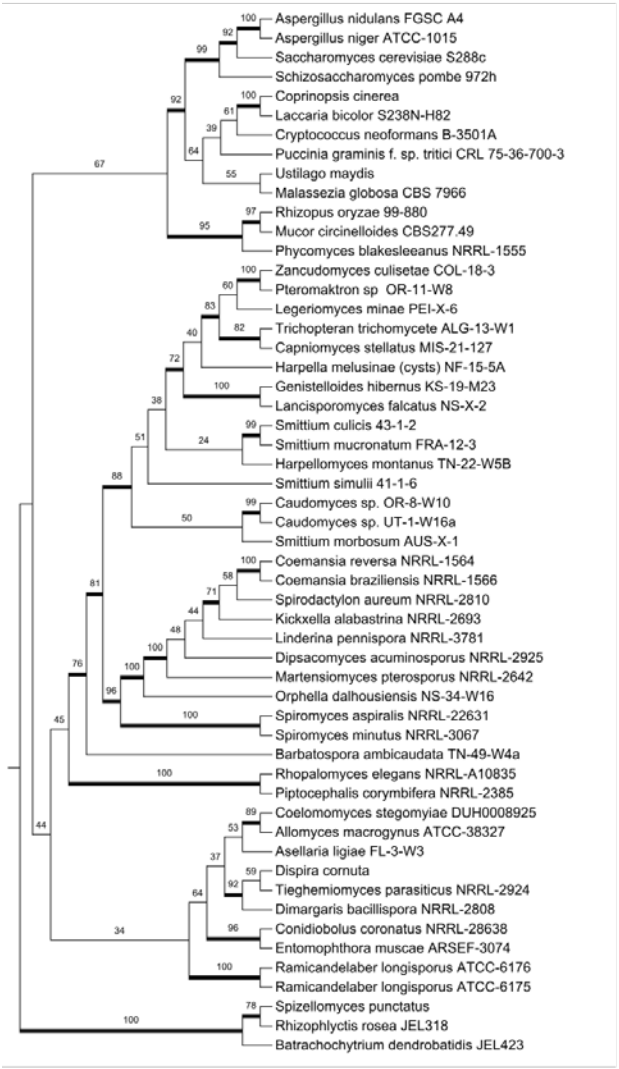
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Asellaria ligiae FL-3-W3
Barbatospora ambicaudata TN-49-W4a
Dispira cornuta
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Entomophthora muscae ARSEF-3074
Conidiobolus coronatus NRRL-28638
Coelomomyces stegomyiae DUH0008925
Allomyces macrogynus ATCC-38327
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423
0.0
0.2
Substitutions / site

Figure 2.6 Individual gene phylogeny produced using nuclear 18S rDNA.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Lancisporomyces falcatus NS-X-2
Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Harpellomyces montanus TN-22-W5B
Smittium simulii 41-1-6
Caudomyces sp. OR-8-W10
Caudomyces sp. UT-1-W16a
Smittium morbosum AUS-X-1
Coemansia reversa NRRL-1564
Coemansia braziliensis NRRL-1566
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Spiromyces aspiralis NRRL-22631
Spiromyces minutus NRRL-3067
Barbatospora ambicaudata TN-49-W4a
Rhopalomyces elegans NRRL-A10835
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Dispira cornuta
Tieghemiomyces parasiticus NRRL-2924
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Conidiobolus coronatus NRRL-28638
Entomophthora muscae ARSEF-3074
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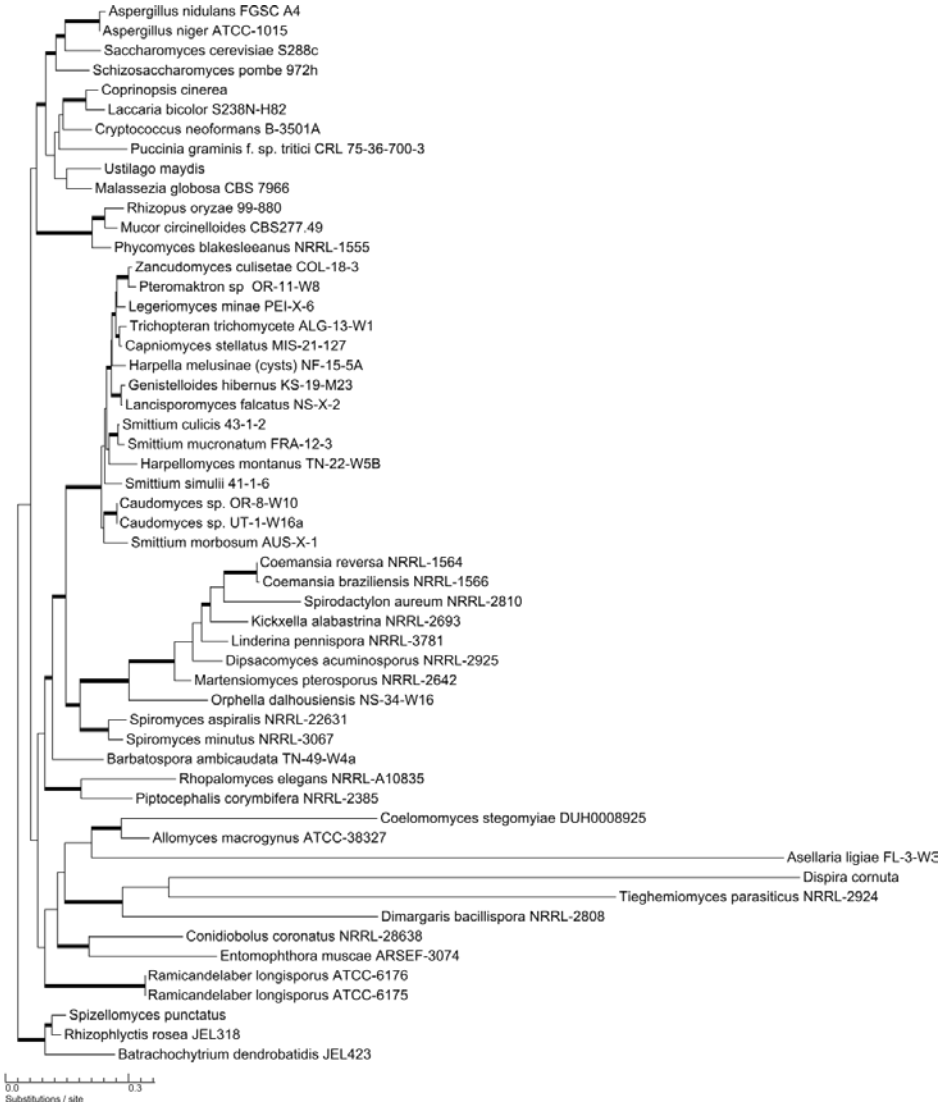
Batrachochytrium dendrobatidis JEL423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Rhizopus oryzae 99-880
Mucor circinelloides CBS277.49
Phycomyces blakesleeanus NRRL-1555
Zancudomyces culisetae COL-18-3
Pteromaktron sp OR-11-W8
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Trichopteran trichomycete ALG-13-W1
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Lancisporomyces falcatus NS-X-2
Smittium culicis 43-1-2
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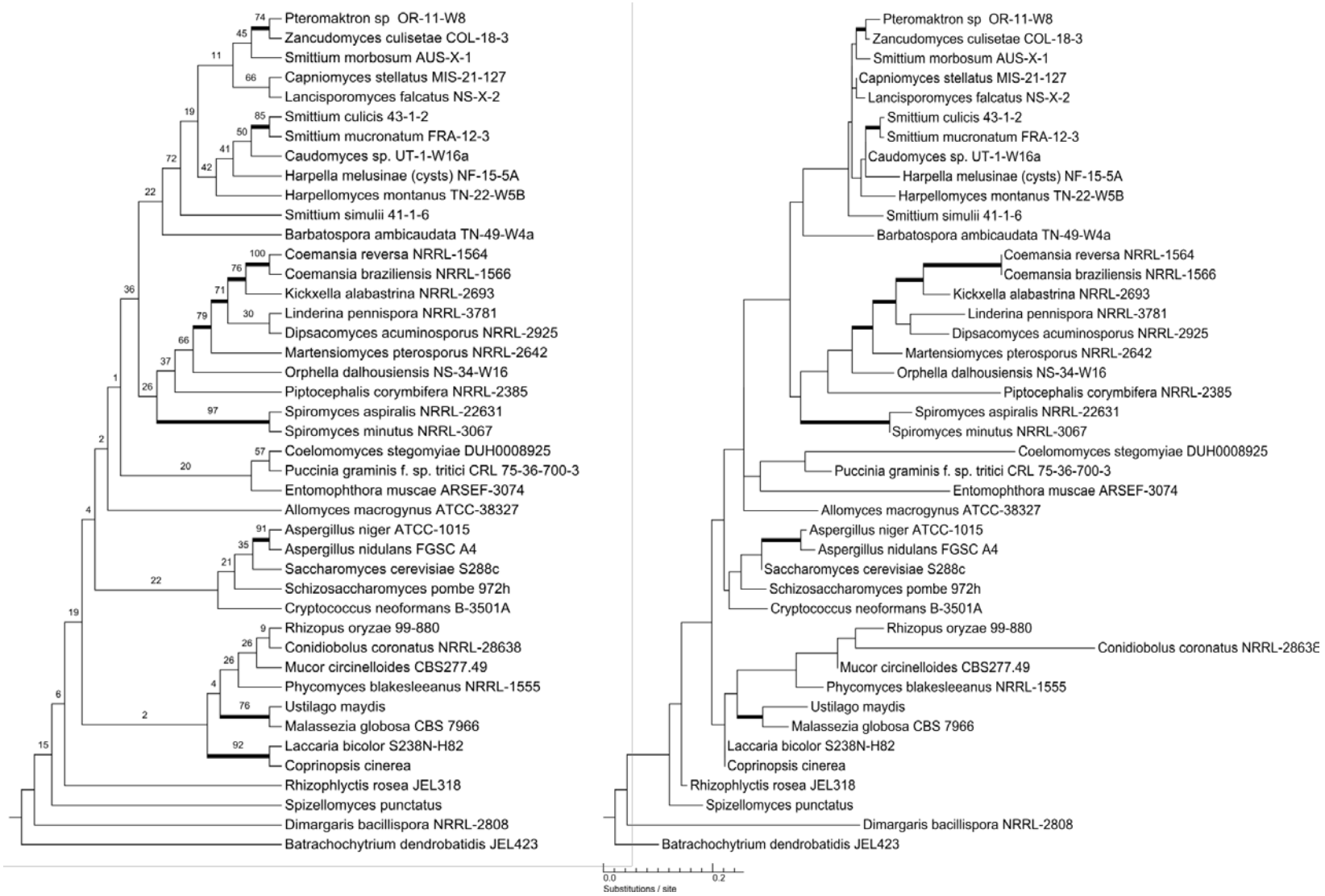
Ramicandelaber longisporus ATCC-6176
Ramicandelaber longisporus ATCC-6175
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423
0.0
0.3
Substitutions / site

Figure 2.7 Individual gene phylogeny produced using nuclear 28S rDNA.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Caudomyces sp. UT-1-W16a
Harpella melusinae (cysts) NF-15-5A
Harpellomyces montanus TN-22-W5B
Smittium simulii 41-1-6
Barbatospora ambicaudata TN-49-W4a
Coemansia reversa NRRL-1564
Coemansia braziliensis NRRL-1566
Kickxella alabastrina NRRL-2693
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Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Orphella dalhousiensis NS-34-W16
Piptocephalis corymbifera NRRL-2385
Spiromyces aspiralis NRRL-22631
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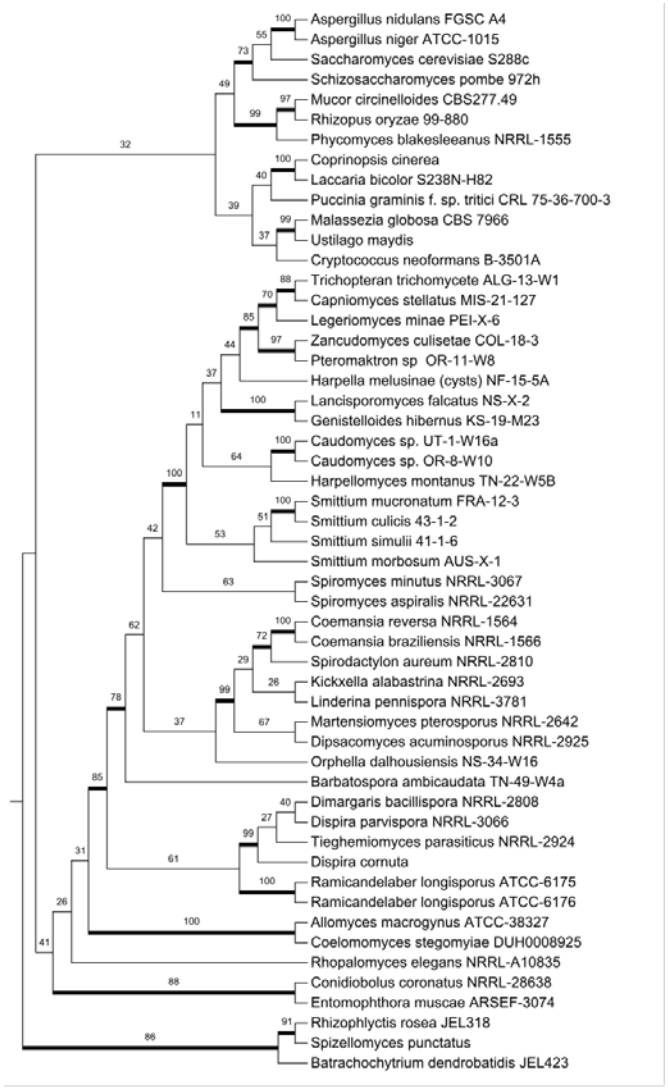
Entomophthora muscae ARSEF-3074
Allomyces macrogynus ATCC-38327
Aspergillus niger ATCC-1015
Aspergillus nidulans FGSC A4
Saccharomyces cerevisiae S288c
Schizosaccharomyces pombe 972h
Cryptococcus neoformans B-3501A
Rhizopus oryzae 99-880
Conidiobolus coronatus NRRL-28638
Mucor circinelloides CBS277.49
Phycomyces blakesleeanus NRRL-1555
Ustilago maydis
Malassezia globosa CBS 7966
Laccaria bicolor S238N-H82
Coprinopsis cinerea
Rhizophlyctis rosea JEL318
Spizellomyces punctatus
Dimargaris bacillispora NRRL-2808
Batrachochytrium dendrobatidis JEL423
0.0
0.2
Substitutions / site

Figure 2.8 Individual gene phylogeny produced using nuclear 5.8S rDNA.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL-1555
Coprinopsis cinerea
Laccaria bicolor S238N-H82
Puccinia graminis f. sp. tritici CRL 75-36-700-3
Malassezia globosa CBS 7966
Ustilago maydis
Cryptococcus neoformans B-3501A
Trichopteran trichomycete ALG-13-W1
Capniomyces stellatus MIS-21-127
Legeriomyces minae PEI-X-6
Zancudomyces culisetae COL-18-3
Pteromaktron sp OR-11-W8
Harpella melusinae (cysts) NF-15-5A
Lancisporomyces falcatus NS-X-2
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Caudomyces sp. OR-8-W10
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Linderina pennispora NRRL-3781
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Dipsacomyces acuminosporus NRRL-2925
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Ramicandelaber longisporus ATCC-6176
Allomyces macrogynus ATCC-38327
Coelomomyces stegomyiae DUH0008925
Rhopalomyces elegans NRRL-A10835
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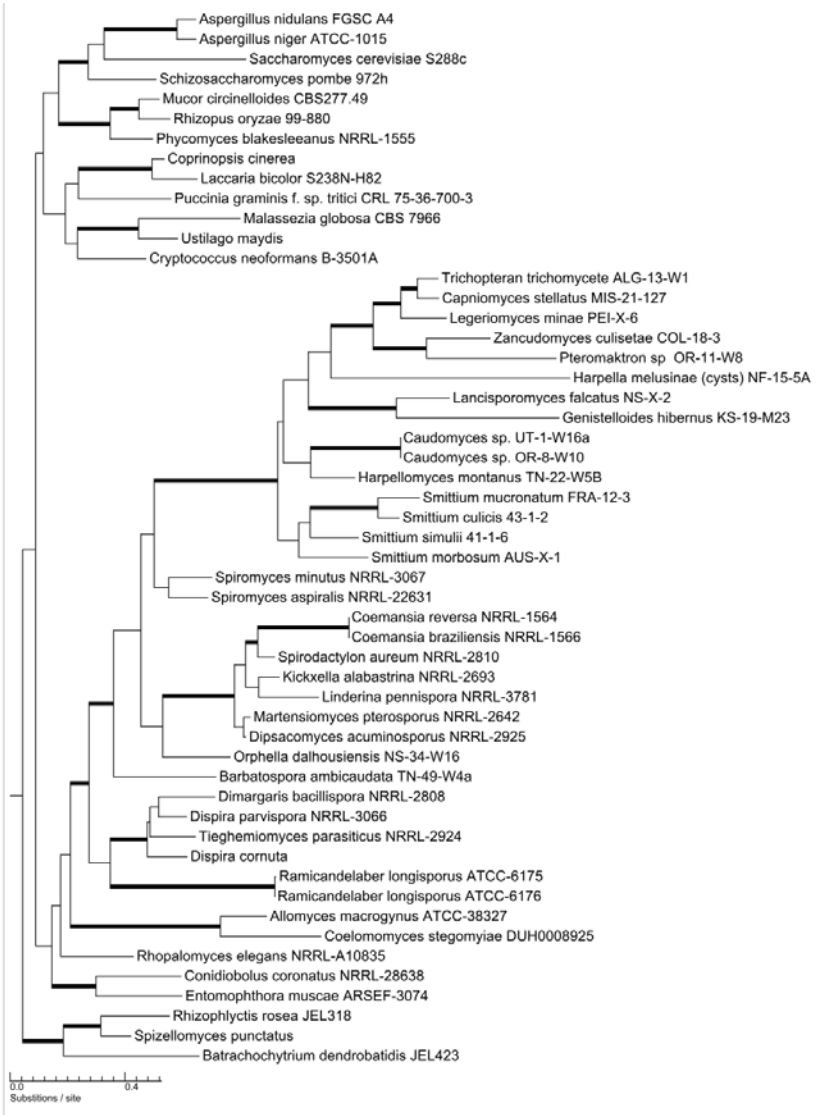
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Linderina pennispora NRRL-3781
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Tieghemiomyces parasiticus NRRL-2924
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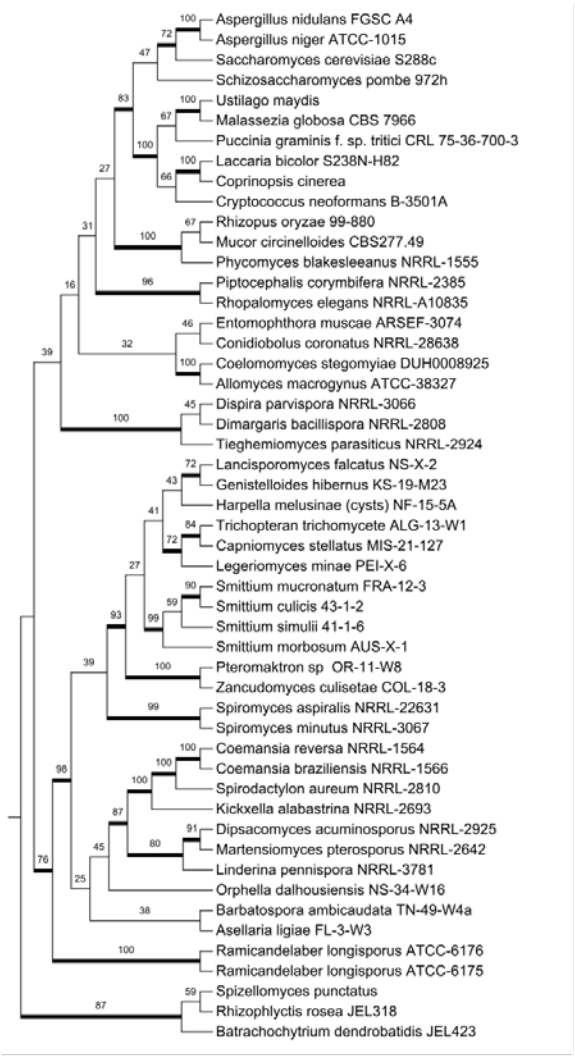
Rhopalomyces elegans NRRL-A10835
Conidiobolus coronatus NRRL-28638
Entomophthora muscae ARSEF-3074
Rhizophlyctis rosea JEL318
Spizellomyces punctatus
Batrachochytrium dendrobatidis JEL423
0.0
0.4
Substitions / site

Figure 2.9 Individual gene phylogeny produced using translated MCM7 protein sequences.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Martensiomyces pterosporus NRRL-2642
Linderina pennispora NRRL-3781
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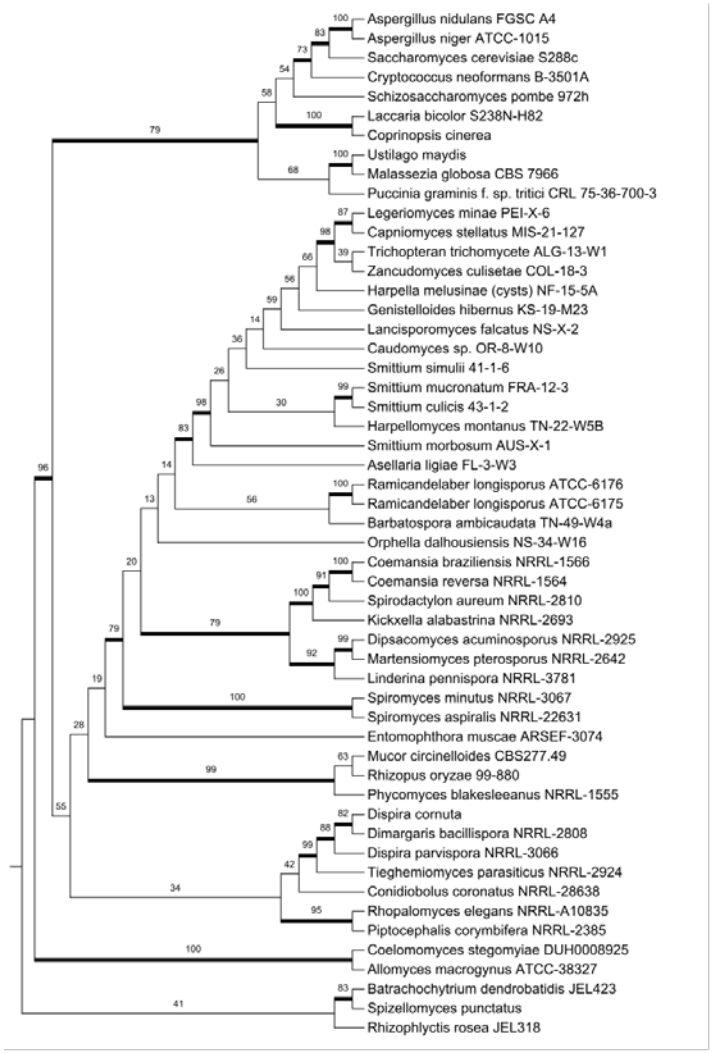
Asellaria ligiae FL-3-W3
Ramicandelaber longisporus ATCC-6176
Ramicandelaber longisporus ATCC-6175
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
Batrachochytrium dendrobatidis JEL423
0.0
0.4
Substitutions / site

Figure 2.10 Individual gene phylogeny produced using translated RPB1 protein sequences.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Smittium morbosum AUS-X-1
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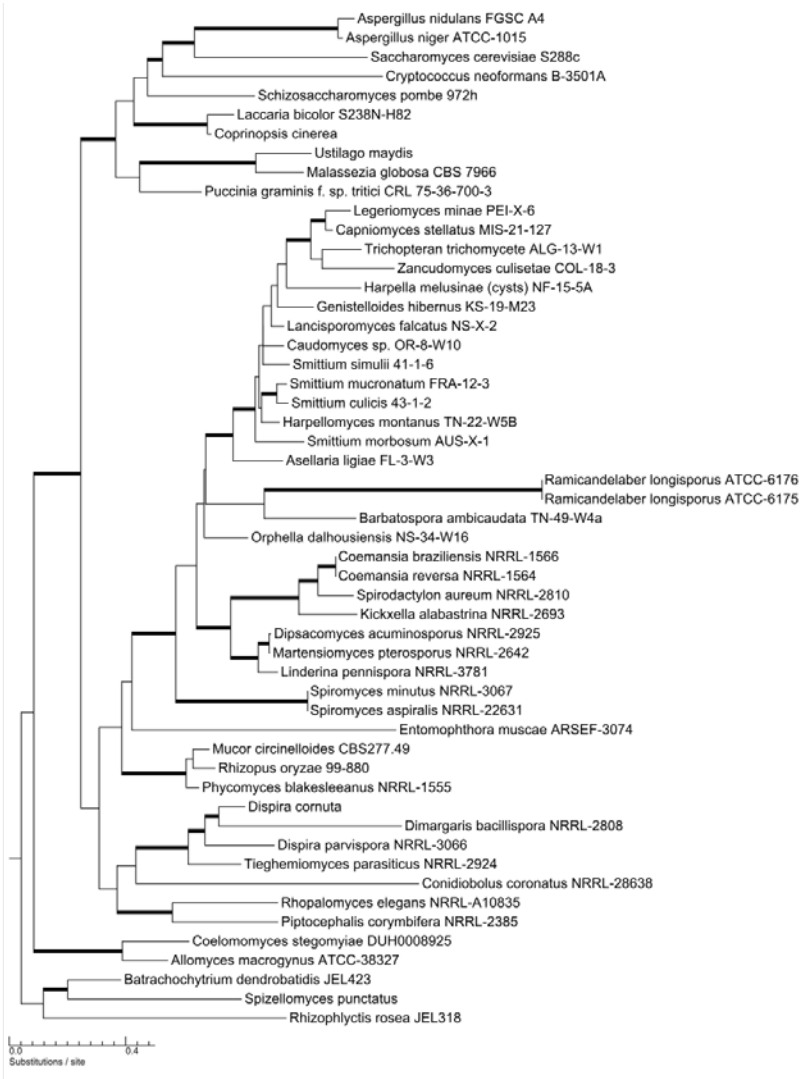
Barbatospora ambicaudata TN-49-W4a
Orphella dalhousiensis NS-34-W16
Coemansia braziliensis NRRL-1566
Coemansia reversa NRRL-1564
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Linderina pennispora NRRL-3781
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Entomophthora muscae ARSEF-3074
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL-1555
Dispira cornuta
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Tieghemiomyces parasiticus NRRL-2924
Conidiobolus coronatus NRRL-28638
Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
Coelomomyces stegomyiae DUH0008925
Allomyces macrogynus ATCC-38327
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus
Rhizophlyctis rosea JEL318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Cryptococcus neoformans B-3501A
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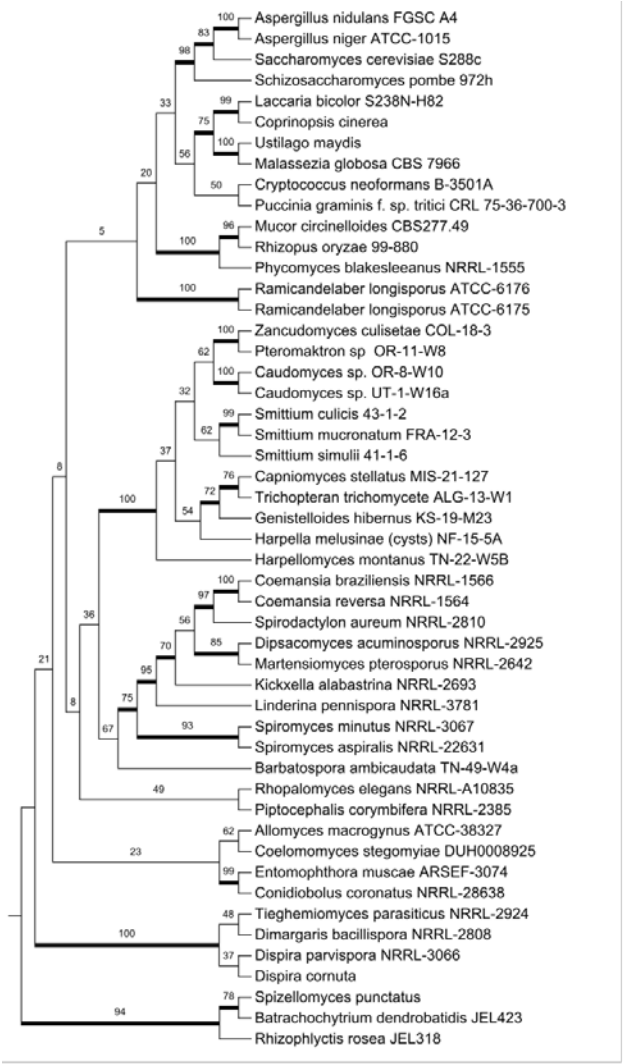
Laccaria bicolor S238N-H82
Coprinopsis cinerea
Ustilago maydis
Malassezia globosa CBS 7966
Puccinia graminis f. sp. tritici CRL 75-36-700-3
Legeriomyces minae PEI-X-6
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Zancudomyces culisetae COL-18-3
Harpella melusinae (cysts) NF-15-5A
Genistelloides hibernus KS-19-M23
Lancisporomyces falcatus NS-X-2
Caudomyces sp. OR-8-W10
Smittium simulii 41-1-6
Smittium mucronatum FRA-12-3
Smittium culicis 43-1-2
Harpellomyces montanus TN-22-W5B
Smittium morbosum AUS-X-1
Asellaria ligiae FL-3-W3
Ramicandelaber longisporus ATCC-6176
Ramicandelaber longisporus ATCC-6175
Barbatospora ambicaudata TN-49-W4a
Orphella dalhousiensis NS-34-W16
Coemansia braziliensis NRRL-1566
Coemansia reversa NRRL-1564
Spirodactylon aureum NRRL-2810
Kickxella alabastrina NRRL-2693
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Linderina pennispora NRRL-3781
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Entomophthora muscae ARSEF-3074
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL-1555
Dispira cornuta
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Tieghemiomyces parasiticus NRRL-2924
Conidiobolus coronatus NRRL-28638
Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
Coelomomyces stegomyiae DUH0008925
Allomyces macrogynus ATCC-38327
Batrachochytrium dendrobatidis JEL423
Spizellomyces punctatus
Rhizophlyctis rosea JEL318
0.0
0.4
Substitutions / site

Figure 2.11 Individual gene phylogeny produced using translated RPB2 protein sequences.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Cryptococcus neoformans B-3501A
Puccinia graminis f. sp. tritici CRL 75-36-700-3
Mucor circinelloides CBS277.49
Rhizopus oryzae 99-880
Phycomyces blakesleeanus NRRL-1555
Ramicandelaber longisporus ATCC-6176
Ramicandelaber longisporus ATCC-6175
Zancudomyces culisetae COL-18-3
Pteromaktron sp OR-11-W8
Caudomyces sp. OR-8-W10
Caudomyces sp. UT-1-W16a
Smittium culicis 43-1-2
Smittium mucronatum FRA-12-3
Smittium simulii 41-1-6
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Genistelloides hibernus KS-19-M23
Harpella melusinae (cysts) NF-15-5A
Harpellomyces montanus TN-22-W5B
Coemansia braziliensis NRRL-1566
Coemansia reversa NRRL-1564
Spirodactylon aureum NRRL-2810
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Barbatospora ambicaudata TN-49-W4a
Rhopalomyces elegans NRRL-A10835
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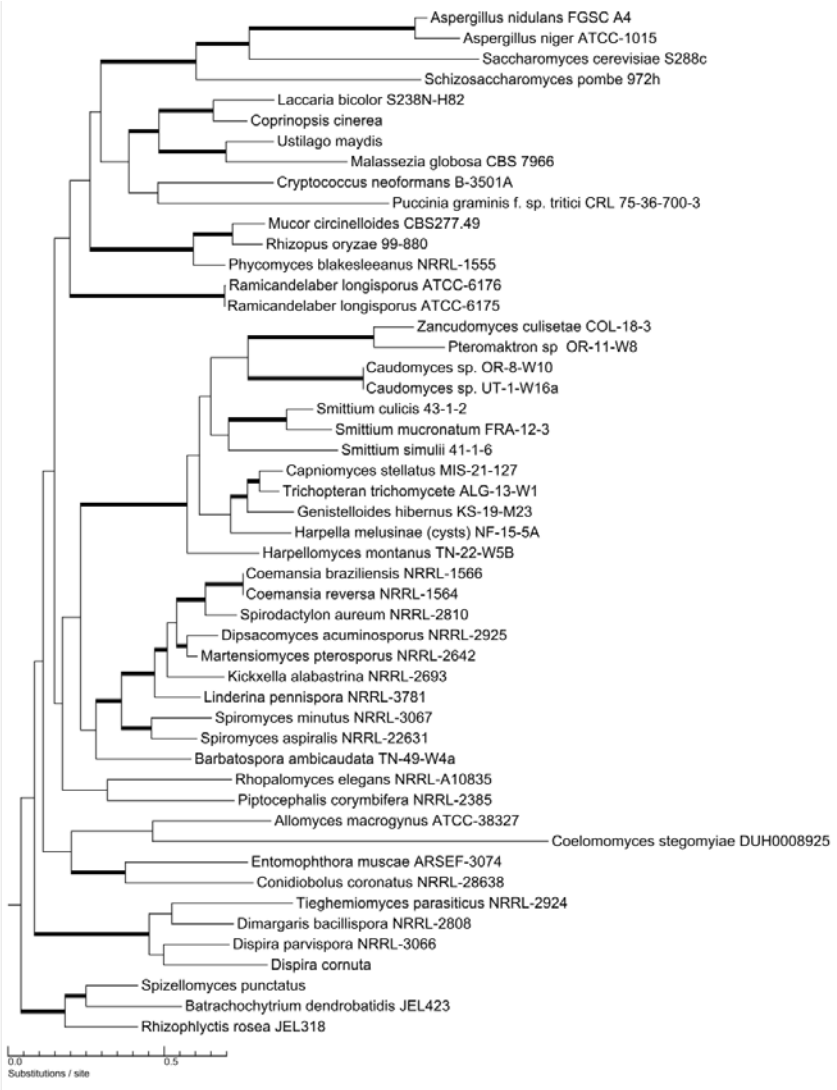
Conidiobolus coronatus NRRL-28638
Tieghemiomyces parasiticus NRRL-2924
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Dispira cornuta
Spizellomyces punctatus
Batrachochytrium dendrobatidis JEL423
Rhizophlyctis rosea JEL318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Coprinopsis cinerea
Ustilago maydis
Malassezia globosa CBS 7966
Cryptococcus neoformans B-3501A
Puccinia graminis f. sp. tritici CRL 75-36-700-3
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Smittium culicis 43-1-2
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Smittium simulii 41-1-6
Capniomyces stellatus MIS-21-127
Trichopteran trichomycete ALG-13-W1
Genistelloides hibernus KS-19-M23
Harpella melusinae (cysts) NF-15-5A
Harpellomyces montanus TN-22-W5B
Coemansia braziliensis NRRL-1566
Coemansia reversa NRRL-1564
Spirodactylon aureum NRRL-2810
Dipsacomyces acuminosporus NRRL-2925
Martensiomyces pterosporus NRRL-2642
Kickxella alabastrina NRRL-2693
Linderina pennispora NRRL-3781
Spiromyces minutus NRRL-3067
Spiromyces aspiralis NRRL-22631
Barbatospora ambicaudata TN-49-W4a
Rhopalomyces elegans NRRL-A10835
Piptocephalis corymbifera NRRL-2385
Allomyces macrogynus ATCC-38327
Coelomomyces stegomyiae DUH0008925
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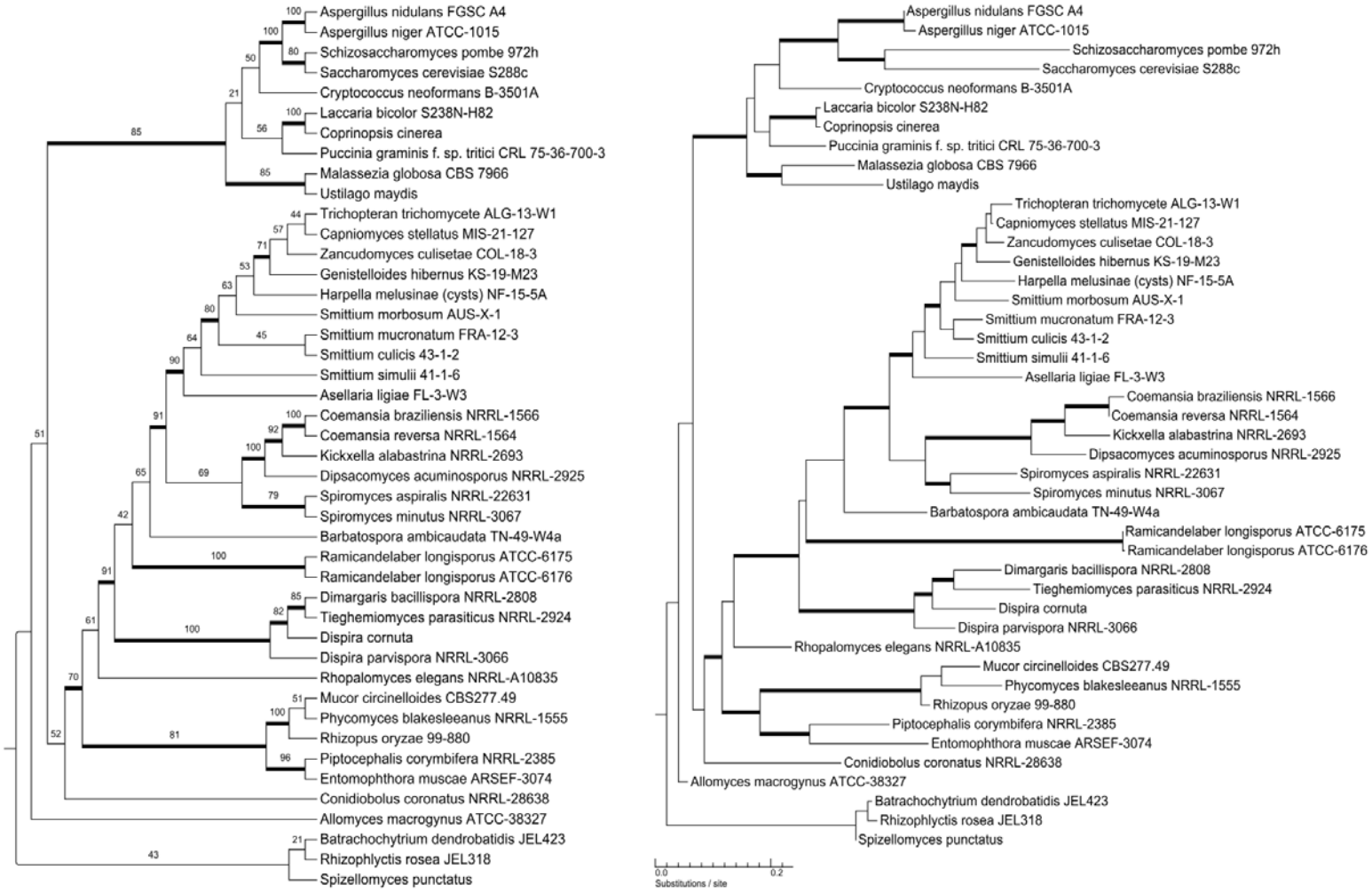
Conidiobolus coronatus NRRL-28638
Tieghemiomyces parasiticus NRRL-2924
Dimargaris bacillispora NRRL-2808
Dispira parvispora NRRL-3066
Dispira cornuta
Spizellomyces punctatus
Batrachochytrium dendrobatidis JEL423
Rhizophlyctis rosea JEL318
0.0
0.5
Substitutions / site

Figure 2.12 Individual gene phylogeny produced using translated TSR1 protein sequences.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).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Aspergillus niger ATCC-1015
Schizosaccharomyces pombe 972h
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Cryptococcus neoformans B-3501A
Laccaria bicolor S238N-H82
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Malassezia globosa CBS 7966
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Entomophthora muscae ARSEF-3074
Conidiobolus coronatus NRRL-28638
Allomyces macrogynus ATCC-38327
Batrachochytrium dendrobatidis JEL423
Rhizophlyctis rosea JEL318
Spizellomyces punctatus
0.0
0.2
Substitutions / site

Figure 2.13 Individual gene phylogeny produced using translated β-tubulin protein sequences.

Topology was taken from the most likely tree produced via RAxML 7.2.8. Branches in bold indicate strong support (≥70% bootstrap support, 100 replicates performed).

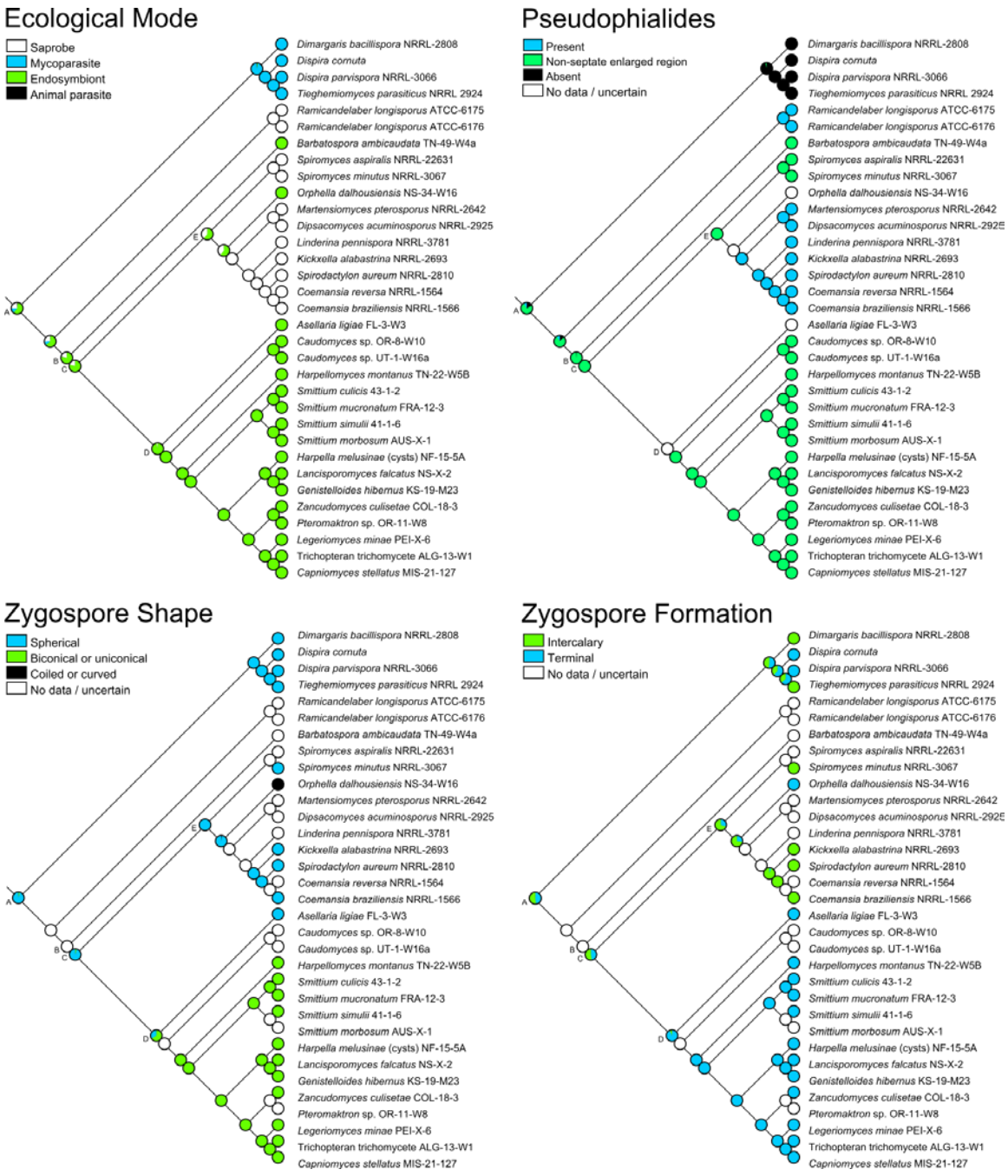

Figure 2.14 Ancestral state reconstruction of four potentially informative morphological characters.

Reconstructions were conducted in Mesquite v. 2.75 using the maximum-likelihood method; characters were mapped on the most likely tree produced via RAxML v. 7.2.8.