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*

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Abstract The recently recognised 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 DNA 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. Phylogenetic inference suggests that *Barbatospora, Orphella, Ramicandelaber* and *Spiromyces* may represent unique lineages. It is suggested that these markers may be more broadly useful for phylogenetic studies among other groups of early-diverging fungi.

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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 recognised, respectively. However, in the early-diverging fungi, the process has been only partially successful. Some monophyletic groups have been broken up. For example, the *Chytridiomycotina* had two other phyla, the *Blastocladiomycota* and the *Neocallimastigomycota*, created for certain taxa previously belonging to it (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 genera 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 techniques and phylogenomics undoubtedly will provide better 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*, *Kickxellomycotina* and *Zoopagomycotina*, each of which has at least one major clade with no member species yet successfully cultured. For this reason, finding powerful single-copy nuclear 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 while also considering project timeframes and budgets.

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 licence 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 (Moss & Young 1978). Some molecular-based studies (James et al. 2006a, Sekimoto et al. 2011) have suggested it is monophyletic, whereas others (White et al. 2006a) have suggested the relationship between the *Kickxellomycotina* and closely related taxa may be more complex. Some studies, such as Tanabe et al. (2004), have been inconclusive, with different genes disagreeing on the monophyly of the clade. 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 congruence and resolution. 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 2).

**PCR amplification**

**MCM7**

Initial attempts to amplify MCM7 were conducted using the primers MCM7-709for and MCM7-1348rev of Schmitt et al. (2009). PCR products from three taxa (*Coemansia braziliensis, Dipsacomyces acuminosporus* and *Smittium culicis*) 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 2), 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). 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

| Primer name | Gene | Source | Direction | Sequence (5’–3’) | Translated amino acid sequence (5’–3’) | Length | Degeneracy |
|-------------|------|--------|-----------|------------------|----------------------------------------|--------|------------|
| MCM7-709for| MCM7 | Schmitt et al. 2009 | For | ACIMGICGTTCVGYAGTHA4RC | TRVSDKVP | 23 bp | 48’ |
| MCM7-7bf   | MCM7 | New for this study | For | GTGGCCGCTATYTTYTGGYAY | VAAYLCD | 21 bp | 16 |
| MCM7-8af   | MCM7 | New for this study | For | TGYYGWSIGARGTITTCYARGA | CGSEFQ | 23 bp | 64 |
| MCM7-1348rev| MCM7 | Schmitt et al. 2009 | Rev | GATTGTCDCACICGCIGRTCWCCCAT | MGDPPAKS | 26 bp | 2’ |
| MCM7-16r   | MCM7 | New for this study | Rev | GYGTTGTYGTTCATCITCRCRTG | HEVMEQQT | 23 bp | 32 |
| TSR1-1018f | TSR1 | New for this study | Rev | AAYGARCARACITGCGCCACIGGA | NEOTWPT(D/E) | 23 bp | 8 |
| TSR1-1492f | TSR1 | New for this study | Rev | TGGGGAYCCCITWYGARAAYYTICC | WDP(Y/F)ENLP | 23 bp | 64 |
| TSR1-2356r | TSR1 | New for this study | Rev | CAYYTCCARTRA1CCTRTGI1TCC | GTHGYMKC | 23 bp | 8 |
| NS1AA      | LSU rDNA | Wang 2012 | For | AAGGCATCGTCATTGCTAGTAA | -- | 23 bp | -- |
| SSR1R      | LSU rDNA | Vilgays & Hester 1990 | For | TACCTGGTIGATYCTGCGACGAG | -- | 21 bp | 2 |
| NS4A       | LSU rDNA | Wang 2012 | Rev | TACCTTCCTTA1AGTACCAAGTTTG | -- | 26 bp | -- |
| NS8        | LSU rDNA | White et al. 1990 | Rev | TCCGAGGTCCTACCCTAGCA | -- | 20 bp | -- |
| ITS1F      | LSU rDNA | Gardes & Bruns 1993 | For | CTTGTCATTCTAGGAGAATGAA | -- | 22 bp | -- |
| ITS3       | LSU rDNA | White et al. 1990 | For | GCATCTGATGAAAGCGACG | -- | 20 bp | -- |
| NL1        | LSU rDNA | O’Donnell 1993 | For | GCATTATGAACGCGAAAAAG | -- | 24 bp | -- |
| NL1A       | LSU rDNA | Wang 2012 | For | GAGTTAAGCCGGGAAAIGTCGCAA | -- | 23 bp | -- |
| NL4        | LSU rDNA | O’Donnell 1993 | Rev | GGTCCATTCTTTAACAGAGGG | -- | 19 bp | -- |
| L5R        | LSU rDNA | Vilgays & Hester 1990 | Rev | TCTGAGGGGAAACTTGC | -- | 17 bp | -- |
| L5R7A      | LSU rDNA | Wang 2012 | Rev | CCAACCAAGATCCTGAAGTA | -- | 20 bp | -- |
| LR1A       | LSU rDNA | Vilgays lab page | Rev | GCGAGATATCCTCGTGGGAA | -- | 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.
| Species | Isolate # | Source / Host | Culture | Country | Coll. | Primer combination | GenBank accession or genome identifier |
|---------|-----------|--------------|---------|---------|-------|--------------------|-----------------------------------|
| Batrachochytrium dendrobatidis<sup>a</sup> | JEL423 | Broad Institute sequencing project<sup>1</sup> | – | – | – | 18S, 28S, MCM7, TSR1 | Supercontig 14: 186674-190464 |
| Spizellomyces punctatus<sup>b</sup> | DAOM BR117 | Broad Institute sequencing project<sup>1</sup> | – | – | – | 18S, 28S, MCM7, TSR1 | Supercontig 32: 115-2731 |
| Rhizophylops rosea | JEL318 | AFTOL DNA sample | – | – | 71-16r | Supercontig 6: 164434-171606 | AMAG_17353.1 |
| Affolomyces macropyga<sup>c</sup> | ATCC 38327 | Broad Institute | – | – | – | Supercontig | AMAG_00422.2 |
| Affolomyces arbuscula | Brazil 2 | AFTOL DNA sample | – | – | 71-16r | Supercontig | 186943-2081357 |
| Coelomomyces punctatus | sequencing project | – | – | – | – | Supercontig | AMAG_17351.1 |
| Rhizopus oryzae | 5 | 753037-756512 | 361829-364983 | USA | – | 7f-16r | 1018f-2356r | Supercontig | RO3G_11608 |
| Ustilago maydis | 8 | 63 | 115-2731 | USA | – | 7f-16r | 1018f-2356r | Supercontig | AMAG_17351.1 |
| Cryptococcus neoformans | 8 | 99-880 | Broad Institute | – | – | – | Scaffold 6 | Supercontig 6: 2079534-207937 | Scaffold 11: 2074749-207937 |
| Trichosporon tropisporum | 8 | 2079534 | 207937 | Supercontig | NRRL A-10835 | AFTOL DNA sample | Scaffold 6 | Supercontig 6: 2079534-207937 | Scaffold 11: 2074749-207937 |
| Conidiobolus coronatus | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | 99-880 | Broad Institute | – | – | – | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | 972 | GenBank | – | – | – | Supercontig | AMAG_17351.1 |
| Conidiobolus coronatus | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Entomophaga conglomerata | NRRL A-10835 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |
| Batrachochytrium dendrobatidis | NRRL 28638 | AFTOL DNA sample | – | – | 71-16r | Supercontig | AMAG_17351.1 |

**Notes:**
- <sup>a</sup> For references, see Table 1.
- <sup>b</sup> For references, see Table 1.
- <sup>c</sup> For references, see Table 1.
Capniomyces stellatus: MIS-21-127

Caudomyces sp.: OR-8-W10

Caudomyces sp.: UT-I-W16a

Furculomyces boomerangus: AUSS-7-47

Genistelloides hibernus: KS-19-M23

Graminella microspora: OR-35-1

Hesperla or Pennella sp.: MF-MC-15

Hesperla or Pennella sp.: MF-MC-18

Landstomycoria sptis: NS-X-2

Landstomycoria sptis: SPA-X-40

Legeriomyces minae: CA-10-W15

Legeriomyces sp. nov.: PEL-X-4

Legeriosemilla sp. NO-31-2

Pnotomon sp.: NR-7-W12

Phaeomyces sp.: OR-11-W8

Smittium culcele: COL-15-3

Smittium culcele: MAL-X-1

Unnamed Trichopteron tricho: AL-13-W1

Unnamed Trichopteron tricho: AL-10-W3

Unnamed genbi: GC-43-1-2

Unnamed genbi: GC-41-1-6

Unnamed genbi: GC-32-1-8

Coleopteromyces amnicus: ARG-15-6F

Pseudohexaperta acromyia: LC-23-1

Smittium angustum: AUS-126-30

Smittium annuelle: OR-14-3-8

Smittium caudatus: KS-1-2

Smittium cylindricus: RMBL-13-41

Smittium commune: KS-6-6

Smittium cyaneus: CS-27-1

Smittium graminis: KSF-3

Smittium megazygosporum: SM-12-2

Smittium simulii: OR-35-1

Smittium simulii: OR-6-10

Trichozospora chironomidarum: TN-3-16

1. Amy Byrnam; Alfie Rizzo; BH; Bar Hayford; CEB; Charles ‘Eddie’ Beard; CLL; Claudio Lopez Lastra; DBS; Douglas B. Strongman; GM; Maria Gabriela Mazzucelli; JKM; JK Mira; J.; Joyce Longo; LCF, Leonard C. Ferrington, Jr.; LGV, Laa Guadilia Valle; MCB, Murray CoBo; MUC; Matus J. Cafero; MMW; Merlin White; NKR, Nicole Reynolds; PVB, Paula Clarke, RWL; Robert W. Lichtwardt, SM, Steve Moss, WKR; WII K. Reeves. Some of these 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).

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

4. 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.

5. 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 "MUCL 30488, CBS 445.63". The SSU rDNA was taken from isolate "MUCL 30488, CBS 445.63".

6. Species used for initial primer design and in silico testing.
concentration, 0.44 µL of 25 mM MgCl₂ (to a total concentration of 2.5 mM), 4.16 µL dH₂O, 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 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min. 15 s, a final extension at 72 °C for 10 min, followed by a final hold step at 4 °C. Reagents for the MCM7-9bf 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 s, annealing at 50 °C for 45 s, 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 2) were used to make this initial assessment. When published primers (Schmitt et al. 2009) did not appear compatible with the early-diverging fungi, we considered based on estimated compatibility with the Blastocladiomycota, Kickxellomycotina but was more broadly compatible within the Mucoromycotina, for which we had data, we considered the development of new primers. Ultimately, three new primers were developed and tested (Table 1). One set (TSR1-1018f with TSR1-2356r) successfully amplified products 1250–1300 bp 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 s, annealing at 50 °C for 45 s, 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. Reagents for the MCM7-9bf 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 s, annealing at 50 °C for 45 s, 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.

rRNA genes

Ribosomal RNA gene sequences were amplified and sequenced as well as obtained from GenBank (Table 2). 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 MgCl₂ (to a final concentration of 2.5 mM), 0.35 µL of 50 µg/µL BSA, 6.45 µL dH₂O, 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 45 s, 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 MgCl₂ (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 dH₂O, 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 s, annealing at 62 °C for 45 s, 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 necessary for bands being cut) and DNA was extracted using a `freeze and `sweep` method. Briefly, pipet tips with excised gel cores were placed in a 1.5 mL microcentrifuge tube, frozen at -20 °C, spun at 14500 G for 10 min, 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 designated and set, 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+ΓI method had the highest AICc score. For the 2nd codon position, the GTR+Γ method was slightly higher; however, for simplicity of analysis, the GTR+ΓI model was used in all cases. The ProtTest programme (Drummond & Stimmer 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+ΓI model, described by Le & Gascuel (2008), consistently received the highest 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, with the Maximum-Likelihood results offered as well, considering possible Bayesian overestimation of branch supports (Suzuki et al. 2002). MrBayes v.3.1.2 was used for Bayesian inference (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003, Altekar et al. 2004). The LG+I 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 mil generations (BI) and 100 bootstrap replicates (ML) were performed, with half of the BI generations treated as burn-in.
Fig. 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).
A total of nine topologies were produced by our analyses (Fig. 1–5 and S1–S4). 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 2). 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 2) 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 3) and TSR1 (Table 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 and therefore useful for placing distantly related taxa. 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 RP1B and RP2B, when used under similar conditions. Schmitt et al. (2009) did not report any size variation or introns within their MCM7 dataset. 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.
Nuclear SSU + LSU Tree

Fig. 3. Phylogeny of the Kickxellomyctina 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 below branches are maximum-likelihood bootstrap supports produced from 100 bootstrap replicates. Bold branches are highly supported (>95% BPP and >.70 MLBP).

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 Chytridomycota, which we found did not have the correct primer site for TSR1-1018f. Within the Kickxellomyctina, 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) did report 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
A method used for tree inference and the format of the tree are the same as for Fig. 3. Phylogeny of the TSR1 during the course of this study. Several hypervariable regions could not be aligned and also needed to be excluded within the dataset.

To assess the congruence of the MCM7 gene to the accepted phylogeny of the trichomycete fungi, the topology of the MCM7 nucleotide and protein trees was compared to a tree based on 18S and 28S rDNA as well as to existing analyses. The MCM7 nucleotide tree had one significant and well-supported incongruity with the rDNA tree as well as the accepted phylogeny: the basidiomycete Ustilago maydis was placed in a well-supported group including the Kickxellales, instead of with the other basidiomycetes. In general, the MCM7 nucleotide tree failed to recover a higher-level classification of the fungi 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. 6. Several hypervariable regions could not be aligned and also needed to be excluded within the dataset. Of the 9 253 characters in the complete alignment, 1 226 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.

Fig. 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. 3.
Coprinopsis cinerea

Intron 2

Intron 6

Intron 4

Intron 5

Intron 3

Intron 5 2101

Intron 3 1875

Fig. 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.
Fig. S1  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).
Fig S2  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).
Fig. S3: Phylogeny of the Kickxellomyctina based on an alignment of nuclear large subunit (LSU) rDNA. Tree is based on a 50% majority-rule 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).
that was congruent with the accepted phylogeny (James et al. 2006a, White et al. 2006a, Hibbett et al. 2007). We suspect that the third codon base is saturated at this level of taxon selection (2006a, White et al. 2006a, Hibbett et al. 2007). 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 also amplified and sequenced other groups of early-diverging fungi, including members of the Blastocladiomycota, Chytridiomycota, Entomophthoromycota and Zoopagomycota for comparative purposes. The Glomera-mycota, Mucoromycota 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 Table 3 and 4.

Phylogenetic utility for the genes varied. The translated MCM7 protein sequences appear to be similar in resolving power to the SSU rDNA (see Table 5), potentially making it a valuable single copy protein-coding gene contribution to multi-gene studies. Congruity with earlier multi-gene trees (Aguliet al.
Table 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  |                                            |
| Kickxellales       | MCM7-8bf, MCM7-16r  |                                            |
| Asellariales       |                     | Attempted unsuccessfully                   |
| Dimargaritales     | MCM7-709f, MCM7-16r |                                            |
| Orphella clade     | MCM7-8bf, MCM7-16r  |                                            |
| Barbatoспорa clade | MCM7-8bf, MCM7-16r  |                                            |
| Spiromyces clade   | MCM7-8bf, MCM7-16r  |                                            |
| Ramicandelaber clade| MCM7-709f, MCM7-8bf, MCM7-16r | MCM7-709f seemed to sequence better |

Table 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 |                                        |
| Blastocladiomycota | TSR1-1018f, TSR1-2356r |                                        |
| Zoopagomycotina    | TSR1-1018f, TSR1-2356r |                                        |
| Entomophthoromycotina | TSR1-1018f, TSR1-2356r |                                         |
| Kickxellomycotina  |                     |                                            |
| Harpellales        | TSR1-1492f, TSR1-2356r |                                        |
| Kickxellales       | TSR1-1018f, TSR1-1492f, TSR1-2356r | TSR1-1018f and TSR1-1492f both work well. |
| Asellariales       | -                   | Attempted unsuccessfully                   |
| Dimargaritales     | TSR1-1018f, TSR1-2356r |                                        |
| Orphella clade     | TSR1-1492f, TSR1-2356r |                                        |
| Barbatoспорa clade | TSR1-1492f, TSR1-2356r |                                        |
| 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. |

2008) suggests that it is resistant to environmental selective pressures 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.

In our view, TSR1 was more challenging (and perhaps less useful) when compared to MCM7, at least at the taxonomic scale of this study. While it was possible to reconstruct a phylogeny of fungi with TSR1 that was congruent on the large scale with previous analyses (James et al. 2006a, White et al. 2006a, Liu et al. 2009) and with the combined rDNA analysis here, it did present more hindrances in this regard than MCM7. Furthermore, introns needed to be removed from further consideration in preparing the alignment file. Nonetheless, at this time, these issues do not seem severe enough to suggest eliminating TSR1 from future consideration. They should, however, be taken into account by those considering its potential utility. TSR1 is likely to be more useful in studies on clades of more closely related species, where its greater variability may be considered an asset.

Combined analyses using both the 3- and 4-gene datasets had greater resolving power than any single-gene analysis. The 3-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). Since this, along with Wang (2012), represent the first multi-gene studies primarily concentrating on the Kickxellomycotina that includes sequences from both rDNA and protein-coding genes, we also present a clade-based phylogenetic perspective on the various clades presented (Fig. 1–5).

Phylogenetic analyses

Kingdom Fungi

Except for the Kickxellomycotina, taxon sampling limits our commentary about the other fungal groups. However, by comparing our trees to evolutionary hypotheses presented by others, we offer our assessment of the power of these genes 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) 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. 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. 2) placed the Dikarya on a well-supported branch (BPP: 100.0 %, MLBP: 70/100). The MCM7 protein tree (Fig. 1) placed the Ascomycota together with the Mucoromycotina, but was not well supported (BPP: 69.1 %, MLBP: 40/100). Multi-gene analyses (Fig. 4, 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 (3-gene: BPP: 100.0 %, MLBP: 90/100; 4-gene: BPP: 100.0 %, MLBP: 98/100).

**Kickxellomycotina**

The *Kickxellomycotina*, the primary focus of this study, are a subphyllum 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*, and has only otherwise been found in the genus *Ballocephala* and *Zygnemomyces* within the Entomophthoromycota (Saikawa 1989, Saikawa et al. 1997). However, no molecular data from these two genera have yet been examined, and the morphology is not conclusive, so the affinity of these genera is uncertain. 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 trichosporangia) as well as zygospores. The sexual spores can vary in shape, being spherical in the *Asellariales*, *Dimargaritales* and *Kickxellales*, biconal (or rarely unicinal) within the *Harpellales*, and coiled within *Orphella* (Moss & Young 1978, Valle & Santamaria 2005, Valle & Cafaro 2008).

The MCM7 protein tree (Fig. 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. 2) also recovered a monophyletic *Kickxellomycotina*, with five of the main subclades represented (the *Asellariales*, *Dimargaritales* and *Orphella* have yet to yield sufficient sequences), although it was only strongly supported below *Ramicandelaber* (BPP: 100.0 %, MLBP: 74/100). The 4-gene analysis (Fig. 5) was also able to recover a monophyletic *Kickxellomycotina* (BPP: 99.8 %, MLBP: 72/100), but the 3-gene analysis (Fig. 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; BPP: 99.9 %, MLBP: 74/100) and TSR1 (Fig. 2; BPP: 100.0 %, MLBP: 74/100) single-gene analyses, and in both the 3-gene (Fig. 4; BPP: 100.0 %, MLBP: 97/100) and 4-gene (Fig. 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 ‘gut fungi’, and can shift between parasitic, commensalsitic and mutualistic roles (Lichtwardt et al. 2007). The *Harpellales* have a unique zygospore, whether biconal or unicinal, that distinguishes them from other orders within the *Kickxellomycotina*. Most species of *Harpellales* also produce unisporous asexual spores (Moss & Young 1978) for asexual reproduction, referred to as trichosporangia (noting that *Caruoxella* and *Klastostachys* spores remain attached to the generative cell, which is dehiscent, similar to the asexual spores of the *Asellariales*). These spores are specialized for the aquatic environment, with many species having mucilaginous non-motile appendages. Moreover, trichosporangia 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 excrated which secures the thallus to the gut lining of the host. Some genera of *Harpellales* (*Genistellospora*, *Harpella* and *Pennisula*) 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. 2006b). 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. (2006a), 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 *Smittium culisetae* and most of the other genera of the *Harpellales*. Wang et al. (2013) are moving *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 *Austromittium*, *Smittium*...
Coeleptomyces, Furculomyces, Pseudoharpella, Stachylinna and Trichogyospora.

Our 3-gene analysis (Fig. 4) provides further evidence of this split, with Coeleptomyces, Furculomyces, Smittium, Stachylinna and Trichogyospora all placed together and well supported (BPP: 99.8 %, MLBP: 70/100). Another well-supported clade includes Bojamyces, Capniomyces, Genistelloides, Graminella, Harpella, Lansiciporomyces, Legerioides, Legeriomycetes, Penella, Pteromakton and Smittium culisetae (BPP: 100.0 %, MLBP: 98/100). Harpellomyces and Caudomycetes were placed as just outside this group, although only strongly supported by the Bayesian analysis (Harpellomyces: BPP: 100.0 %, MLBP: 58/100). The MCMC protein analysis alone is not as well-resolved, but still contains all of the same clades, supporting the conclusion that both of these analyses are underlying the correct species tree. This 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. 2) does not fully agree with the phylogeny provided by the MCMC protein or rDNA tree (Fig. 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, White et al. unpubl. data). 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 4-gene tree incorporating the TSR1 protein (Fig. 5) did have the same topology as the one from the 3-gene analysis (Fig. 4).

Ecological and morphological correlations for these endosymbionts are difficult to place. 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 trichospor dehiscence from the fertile thallus. Trichogyospora is the exception, with its large number of very 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 trichospor. A collar is present in Smittium culisetae and Bojamyces, but for both it is fiared, and perhaps unlike most species of Smittium. Additionally, phylogenetically related genera Graminella and Pteromakton 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 Coeleptomyces, with one species from aquatic Coeleptora), whereas the non-Smittium clade has members that utilize a diverse group of hosts including not only Diptera, but also Ephemeropertera, 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 Colembola). 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 breaking up of the thallus at maturity to produce arthropores. The general similarity in growth form and life history, along with the similarity between the arthropores of Asellaria to the asexual reproductive propagules of Carouellia (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 Kickxellomyccotina (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 sporocidia that produce multiple, unisporous merozoosporangia supported upon small basal cells, the pseudophialides, and also sexually through spherical zygospores (Benny 2005). The sporocidia 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 & Young (1978) as ‘slime spores’, with only the genera Spiromyces and Spirodactylon being dry-spored (Benny 2005). Moss & 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 trichospor appendage produced by the Harpellales. This study also compared the morphology of the reproductive structure of the two groups, describing the structures as having a shared ‘coenomansoid’ 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. 2006a). The Kickxellales have a septal structure similar to the Harpellales and Asellariales, with a lenticular sepal pore with an electron-dense plug (O’Donnell et al. 1998).

All trees inferred for this study revealed a strongly-supported and monophyletic Kickxellales clade that includes Coemansia, Dipascomyces, Kickxella, Linderina, Martensomyces and Spirodactylon (3-gene: BPP: 100.0 %, MLBP: 100/100; 4-gene: BPP: 100.0 %, MLBP: 100/100). This clade never included Ramicandelaberae. 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 3-gene analysis (Fig. 4; BPP: 100.0 %, MLBP: 100/100) and the rDNA-based analysis (Fig. 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 Ramicandelaberae 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 Spiromycetes and Ramicandelafer sections).

Within the monophyletic Kickxellales, relationships are difficult to resolve. The group consisting of Coemansia brazierilensis, Coemansia reversa and Spirodactylon aureum was first shown by O’Donnell et al. (1998) and again by White et al. (2006a).

This relationship is further demonstrated by both the MCM7 (Fig. 1; BPP: 98.2 %, MLB: 75/100) and TSR1 phylogenies (Fig. 2; BPP: 100.0 %, MLB: 100/100), providing multi-gene support. However, in both the MCM7 (Fig. 1; BPP: 100.0 %, MLB: 100/100) and TSR1 (Fig. 2; BPP: 100.0 %, MLB: 100/100) analyses, C. reversa and C. brazierilensis 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 polyphylectic (Fig. 3; BPP: 99.9 %, MLB: 86/100). This may represent a true instance of incomplete lineage sorting within the Kickxellales.

Alternately, it may be due to long-branch attraction related to Spirodactylon, which appears to be unusually diverged from the other Kickxellales with regard to rDNA but not 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, 2) are not well resolved within the Kickxellales clade. The 3-gene tree (Fig. 4) is well resolved with regard to internal members of the group; a poorly-supported group consisting of Dipsacomyces and Martensiomycies is the most early-diverging member (BPP: 86.5 %, MLB: not presented), followed by well-supported individual branches containing Lindenina (BPP: 100.0 %, MLB: 78/100) and Kickxella (BPP: 100.0 %, MLB: 81/100). The 4-gene analysis (Fig. 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 Martensiomycies (BPP: 100.0 %, MLB: 83/100). This relationship is present, although not as strongly supported, in all four individual-gene analyses (Fig. 1. 2, S2, S3) as well as previously by O’Donnell et al. (1998).

**Dimargaritales**

Dimargaritales is an unusual group of Kickxellomycticina. Mycoparasites of Mucorales and Ascomycota, they have several morphological and life history features that differentiate them from other Kickxellomycticina. 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 bisporous merothripsaranga (all other Kickxellomycticina are unisporous) 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 2). 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 be genuine.

The MCM7 analysis reveals a monophyletic Kickxellomycticina that includes Dimargaris (BPP: 100.0 %, MLB: 86/100) as part of an early-diverging group that also contains Ramicandelafer, although the connection between Ramicandelafer and Dimargaritales was only supported by the Bayesian analysis (BPP: 97.5 %, MLB: 59/100). Multi-gene analysis was less clear because the three-gene analysis placed Dimargaris in an unsupported group with Rhopalomyces (BPP: 86.7 %, MLB: not present). The notion that Dimargaritales is one of the most early-diverging members of the Kickxellomycticina 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 merothripsaranga and appear to have a similar growth form. It may be that the Kickxellomyctaina 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.

We suggest that 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 artifacts (White et al. 2006a). 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, Ramicandelafer and Spiromycetes**

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

Barbatospora has not been reported since the type B. ambicauda was described from blackflies in the Great Smoky Mountains National Park, USA (White et al. 2006c). Although the general growth form of Barbatospora resembles the Harpellales, with a branched, septate thallus and a secreted holfast, 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 Leieriomycetaceae.

Phylogenetically, Barbatospora consistently places within a branch that includes the Harpellales, the Kickxellales, Orphella and Spiromycetes. This placement is well-supported in the MCM7 (Fig. 2; BPP: 99.9 %, MLB: 74/100), TSR1 (Fig. 2; BPP: 100.0 %, MLB: 74/100), 3-gene (Fig. 4; BPP: 100.0 %, MLB: 97/100), and 4-gene (Fig. 5; BPP: 100.0 %, MLB: 100/100) analyses, and is present (but not completely supported) in the rDNA (Fig. 3; BPP: 100.0 %, MLB: 68/100) analysis as well. Within this group, the Harpellales, Kickxellales, Orphella and Spiromycetes are together on a strongly-supported branch within the TSR1 (Fig. 2; BPP: 100.0 %, MLB: 75/100), rDNA (Fig. 3; BPP: 100.0 %, MLB: 90/100), 3-gene (Fig. 4; BPP: 99.9 %, MLB: 91/100), and 4-gene analyses (Fig. 5; BPP: 100.0 %, MLB: 95/100), and a branch that is not strongly supported within the MCM7 (Fig. 1; BPP: 86.4 %, MLB: 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 & Santamaría 2005). Orphella is unique among gut fungi in releasing both trichospores and zygospores as multi-celled
CONCLUSIONS

The comparison between the rDNA-based and MCM7-based phylogenies suggest that MCM7 is likely to be 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 be able to place it reliably within 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 separate from the other Harpellales. The 3-gene analysis (Fig. 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. 2006a), 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.

Spiromyces is a member of the Kickxellales, although previous phylogenetic analyses have placed it apart from that order. It is separated from the Kickxellales by Orphella by previous studies (James et al. 2006a), 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 pseudohyphal 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 (Fig. 1, 2). Within the 3-gene tree (Fig. 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. 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, may form supporting branches (Benny 2005). It is also unusual how, in age, Ramicandelaber sporocladia broaden and become covered with more pseudohyphal and become covered with more pseudohyphal, 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. 2006a). On the MCM7 tree (Fig. 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. 2), it was placed as an unsupported branch as the earliest-diverging member of the Kickxellomycotina (recall that the Dimargaris 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 (Fig. 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.

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