



# 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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## Key words

DNA replication licensing factor  
*Harpellales*  
*Kickxellomycotina*  
MCM7  
MS277  
MS456  
ribosomal biogenesis protein  
*Trichomyces*  
TSR1  
*Zygomycota*

**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 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. 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 *Chytridiomycota* 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 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 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, Aguilera 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 (Aguilera 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, Kearsley & Labib 1998). MS277 corresponds to the TSR1 gene, a ribosome biogenesis protein (Gelperin et al. 2001).

Although Aguilera et al. (2008) demonstrated the utility and power of these two genes for phylogenetic analysis, neither primer sequences nor PCR protocols were provided. Schmitt

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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 lichenised 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 oppor-

tunity 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 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 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

**Table 1** 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 <sup>1</sup>
<b>MCM7-8bf</b>	MCM7	<b>New for this study</b>	For	GTIGCIGCITAYTITGYGAY	VAAYLCD	21 bp	16
<b>MCM7-8af</b>	MCM7	<b>New for this study</b>	For	TGYGGIWSIGARGTITTYCARGA	CGSEVFQ	23 bp	64
MCM7-1348rev	MCM7	Schmitt et al. 2009	Rev	GATTTDGCACICCCIGGRTCWCCCAT	MGDPGVAKS	26 bp	24 <sup>2</sup>
<b>MCM7-16r</b>	MCM7	<b>New for this study</b>	Rev	GTYTGYTGYTCCATIACYTCRTG	HEVMEQQT	23 bp	32
<b>TSR1-1018f</b>	TSR1	<b>New for this study</b>	For	AAYGARCARACITGGCCACIGA	NEQTWPT(D/E)	23 bp	8
<b>TSR1-1492f</b>	TSR1	<b>New for this study</b>	For	TGGGAYCCITWYGARAAYYTICC	WDP(Y/F)ENLP	23 bp	64
<b>TSR1-2356r</b>	TSR1	<b>New for this study</b>	Rev	CAYTTCATRTAICCRTGIGTICC	GTHGYMKC	23 bp	8
NS1AA	SSU rDNA	Wang 2012	For	AAGCCATGCATGTCTAAGTATAA	–	23 bp	–
SR1R	SSU rDNA	Vilgalys & Hester 1990	For	TACCTGATGTGATYCTGCCAGT	–	21 bp	2
NS8AA	SSU rDNA	Wang 2012	Rev	TACTTCTCTAAATGACCAAGTTTG	–	25 bp	–
NS8	SSU rDNA	White et al. 1990	Rev	TCCGCAGGTTCCACCTACGGA	–	20 bp	–
ITS1F	LSU rDNA	Gardes & Bruns 1993	For	CTTGGTCATTTAGAGGAAGTAA	–	22 bp	–
ITS3	LSU rDNA	White et al. 1990	For	GCATCCATGAAGAACGCAGC	–	20 bp	–
NL1	LSU rDNA	O'Donnell 1993	For	GCATATCAATAAGCGGAGGAAAAG	–	24 bp	–
NL1AA	LSU rDNA	Wang 2012	For	GAGTGAAGCGGGAAGCTCAAG	–	23 bp	–
NL4	LSU rDNA	O'Donnell 1993	Rev	GGTCCGTGTTTCAAGACGG	–	19 bp	–
LR5	LSU rDNA	Vilgalys & Hester 1990	Rev	TCCTGAGGGAAAACCTTCG	–	17 bp	–
LR7AA	LSU rDNA	Wang 2012	Rev	CCACCAAGATCTGCACTAGA	–	20 bp	–
LR11	LSU rDNA	Vilgalys lab page <sup>3</sup>	Rev	GCCAGTTATCCCTGTGGTAA	–	20 bp	–

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

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

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

Table 2 Fungal species, isolate number, and source, amplified with specific primer combinations.

Species	Isolate #	Source / Host	Culture	Country	Coll.	Primer combination			GenBank accession or genome identifier			
						18S	28S	MCM7	18S	28S	MCM7	TSR1
<i>Batrachomyces dendrobatidis</i> <sup>5</sup>	JEL423	Broad Institute sequencing project <sup>3</sup>	-	-	-	-	-	-	Supercontig 14_188674-190464	NG_027619	BDEG_04439.1	BDEG_02071.1
<i>Spizellomyces punctatus</i> <sup>5</sup>	DAOM BR117	Broad Institute sequencing project <sup>3</sup>	-	-	-	-	-	-	Supercontig 32_115-2731	NG_027618.1	SPPG_04786.3	SPPG_02880.2
<i>Rhizophlyctis rosea</i>	JEL318	AFTOL DNA sample	-	-	-	7f-16r	-	-	AY635829.1	DQ273787.1	KC297581	-
<i>Allomyces macrogynus</i> <sup>5</sup>	ATCC 38327	Broad Institute sequencing project <sup>3</sup>	-	-	-	-	-	-	Supercontig 63_164434-188875	Supercontig 63_164434-188875	AMAG_00422.2	AMAG_17353.1
<i>Allomyces arbuscula</i>	Brazil 2	AFTOL DNA sample	-	-	-	7f-16r	1018f-2356r	-	NG_017166.1	DQ273806.1	KC297582	KC297629
<i>Coelomomyces siegomyiae</i>	DUH0008925	AFTOL DNA sample	-	-	-	7f-16r	1018f-2356r	-	NG_027644.1	NG_027644.1	KC297583	KC297630
<i>Mucor circinelloides</i> <sup>5</sup>	CBS277.49	JGI sequencing project <sup>5</sup>	-	-	-	-	-	-	Scaffold 11	Scaffold 11	106477-109288	Scaffold 1
<i>Phycomyces blakesleeenans</i> <sup>5</sup>	NRRL 1555	JGI sequencing project <sup>5</sup>	-	-	-	-	-	-	800000-801950	804000-809666	4335584-4338189	Scaffold 24
<i>Rhizopus oryzae</i> <sup>5</sup>	99-880	Broad Institute sequencing project <sup>4</sup>	-	-	-	-	-	-	NG_017190.1	NG_027559.1	753037-756512	Scaffold 5
<i>Coprinopsis cinerea</i> <sup>5</sup>	okayama7#130 <sup>5</sup>	GenBank	-	-	-	-	-	-	Supercontig 6	Supercontig 6	RO3G_11608	RO3G_12091.3
<i>Cryptococcus neoformans</i> <sup>5</sup>	B-3501A	GenBank	-	-	-	-	-	-	2079534-2079537	2074749-2079337	-	-
<i>Ustilago maydis</i> <sup>5</sup>	5217	GenBank	-	-	-	-	-	-	M92991	AF041494	AACS02000002.1	AACS02000003.1
<i>Aspergillus nidulans</i> <sup>5</sup>	F GSC A4	GenBank	-	-	-	-	-	-	BR000310.1	BR000310.1	AAEY01000024.1	AAEY01000024.1
<i>Saccharomyces cerevisiae</i> <sup>5</sup>	S288c	GenBank	-	-	-	-	-	-	X62396	AF453938	AACP01000247.1	AACP01000184.1
<i>Schizosaccharomyces pombe</i> <sup>5</sup>	972h-	GenBank	-	-	-	-	-	-	UJ7377.1	EU840227.1	AACDD01000102.1	AACDD01000107.1
<i>Rhopalomyces elegans</i>	NRRL A-10835	AFTOL DNA sample	-	-	-	7f-16r	1018f-2356r	-	NC_001144.5	NC_001144.5	BK006936.2	BK006938.2
<i>Conidiobolus coronatus</i>	NRRL 28638	AFTOL DNA sample	-	-	-	7f-16r	-	-	CU329672.1	NC_003421.2	CU329671.1	CU329670.1
<i>Entomophaga conglomerata</i>	ARS-2273	Adult Chironomidae	yes	-	-	7f-16r	-	-	NG_017191.1	NG_027654.1	KC297584	KC297631
<i>Entomophthora muscae</i>	ARSEF3074	AFTOL DNA sample	-	-	-	7f-16r	-	-	AF368509.1	NG_027617.1	KC297585	-
<i>Barbatospora ambicaudata</i>	TN-49-W4a	<i>Simulium vandalicum</i>	yes	USA	MMW	SR1R-NS8	ITS1F-NL4	-	NG_017183.1	NG_027647.1	KC297587	KC297632
<i>Coemansia reversa</i>	NRRL 1564	JGI sequencing project <sup>5</sup>	yes	-	-	-	-	-	KC297614	KC297566	KC297588	KC297633
<i>Coemansia brasiliensis</i>	NRRL-1566	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	Scaffold 121	Scaffold 121	Scaffold 81	Scaffold 24
<i>Kickxella alabastrina</i>	NRRL-2693	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	3595-5518	7595-11423	3398-5725	40229-42762
<i>Dipsacomyces acuminosporus</i>	NRRL-2925	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007532.1	AF031069.1	KC297589	KC297634
<i>Martensomyces pterosporus</i>	NRRL-2642	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007537.1	AF031064.1	JX155485	KC297635
<i>Linderina pennispora</i>	NRRL-3781	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007534.1	AF031065.1	KC297590	KC297636
<i>Spirodactylon aureum</i>	NRRL-2810	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007538.1	AF031066.1	KC297591	KC297637
<i>Spiromyces minutus</i>	NRRL-3067	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007541.1	AF031068.1	KC297592	KC297639
<i>Spiromyces spiralis</i>	NRRL-22631	ARS Culture Collection	yes	-	-	8bf-16r	1492f-2356r	-	AF007542.1	DO273810.1	KC297593	KC297640
<i>Orphella catalaunica</i>	NOR-40-W10 + W12	Leucidrid	no	Norway	MMW	NS1AA-NS8AA	ITS1F-LR5	-	AF007543.1	NG_027560.1	KC297594	KC297641
<i>Orphella dalhousiensis</i>	NS-34-W16	<i>Paracapia</i> sp.	no	Canada	GenBank/MMW	-	-	-	KC297617	DO322626.1	KC297599	-
<i>Ramicandelaber longisporus</i>	ARSEF 6175	Eggs/cysts, <i>Heterodera glycines</i>	yes	China	-	SR1R-NS8	NL1-LR11	1492f-2356r	KC297615	KC297567	KC297595	KC297642
<i>Ramicandelaber longisporus</i>	ARSEF 6176	Eggs/cysts, <i>Heterodera glycines</i>	yes	China	-	SR1R-NS8	-	-	-	-	KC297596	-
<i>Dimargaris bacillispora</i>	NRRL 2808	AFTOL DNA sample	yes	USA	MMW	7f-16r	1018f-2356r	-	NG_017180.1	NG_027650.1	KC297597	KC456420 <sup>9</sup>
<i>Harpellomyces montanus</i>	TN-22-W5B	Thaumaleidae	no	Norway	MMW	8bf-16r	NL1AA-LR7AA	-	KC297618	KC297570	KC297600	-
<i>Harpellomyces eccentricus</i>	NOR-56-W1	Thaumaleidae	no	Norway	MMW	8bf-16r	-	-	-	-	KC297601	-
<i>Bojamyces</i> sp.	CA-18-W17	Ephemeroptera	no	USA	MMW	8bf-16r	NL1AA-LR7AA	-	JX155619	JX155645	JX155471	-
<i>Capniomyces sasquatchoides</i>	ID-130-N5	Plecoptera	no	USA	NKR	8bf-16r	NL1AA-LR7AA	-	KC297619	KC297571	KC297602	-

Species	Accession	GenBank/RWL	Country	Host	Sex	GenBank/RWL	Accession	Host	Sex	GenBank/RWL	Accession	Host	Sex
<i>Capniomyces stellatus</i>	MIS-21-127	USA	USA	GenBank/RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	DQ367451.1	EF396194.1	JX155472
<i>Caudomyces</i> sp.	OR-8-W10	USA	USA	MMW	no	USA	1492F-2356R	8bf-16r	-	USA	KC297620	KC297572	KC297603
<i>Caudomyces</i> sp.	UT-1-W16a	USA	USA	MMW	no	USA	1492F-2356R	8bf-16r	-	USA	JX155620	JX155646	JX155473
<i>Furculomyces boomerangus</i>	AUS-77-4	Australia	Australia	RWL	no	Australia	AF277013.1	8bf-16r	-	Australia	AF031074.1	AF031074.1	JX155466
<i>Genistelloides hibernus</i>	KS-19-M23	USA	USA	GenBank/JKM	no	USA	1492F-2356R	8bf-16r	-	USA	DQ367456.1	DQ367456.1	JX155474
<i>Graminella microspora</i>	NOR-35-1	Norway	Norway	RWL	no	Norway	1492F-2356R	8bf-16r	-	Norway	JQ302945	JQ302945	KC297604
<i>Harpella</i> or <i>Pennella</i> sp.	NF-MC-15	Canada	Canada	MCB	no	Canada	1492F-2356R	8bf-16r	-	Canada	KC297621	KC297573	KC297605
<i>Harpella melusinae</i> (cysts)	NF-15-5A	Canada	Canada	RWL	no	Canada	1492F-2356R	8bf-16r	-	Canada	JX155621	JX155647	JX155475
<i>Harpella</i> sp.	NF-MC-18	Canada	Canada	MCB	no	Canada	1492F-2356R	8bf-16r	-	Canada	KC297622	KC297574	KC297606
<i>Lancisporomyces falcatulus</i>	NS-X-2	Canada	Canada	DBS	no	Canada	1492F-2356R	8bf-16r	-	Canada	JQ302865	JQ302865	JX155477
<i>Lancisporomyces vernalis</i>	SPA-X-40	Spain	Spain	LGV	no	Spain	1492F-2356R	8bf-16r	-	Spain	KC297623	KC297575	KC297607
<i>Legioides tumidus</i>	NH-1-IM869a	USA	USA	JKM	no	USA	1492F-2356R	8bf-16r	-	USA	JX155622	JX155648	KC297608
<i>Legiomyces minae</i>	PEIX-6	Canada	Canada	DBS	no	Canada	1492F-2356R	8bf-16r	-	Canada	KC297625	KC297577	JX155478
<i>Legiosmillos</i> sp.	CA-10-W15	USA	USA	MMW	no	USA	1492F-2356R	8bf-16r	-	USA	KC297626	KC297577	KC297609
<i>Legiomyces</i> sp. nov.	PEIX-4	Canada	Canada	DBS	no	Canada	1492F-2356R	8bf-16r	-	Canada	KC297627	KC297578	KC297610
<i>Legiosmillos</i> sp.	NOR-31-2	Norway	Norway	RWL	no	Norway	1492F-2356R	8bf-16r	-	Norway	KC297628	KC297579	KC297611
<i>Pennella</i> sp.	NOR-7-W12	Norway	Norway	MMW	no	Norway	1492F-2356R	8bf-16r	-	Norway	KC297629	KC297580	KC297612
<i>Pteromakron</i> sp.	OR-11-W8	USA	USA	MMW	no	USA	1492F-2356R	8bf-16r	-	USA	JX155623	JX155649	JX155479
<i>Smittium culisetae</i>	COL-18-3	USA	USA	GenBank/RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	NG_017185.1	NG_017185.1	AEW26363.1
<i>Smittium culisetae</i>	MAL-X-1	Malaysia	Malaysia	CLL	yes	Malaysia	1492F-2356R	8bf-16r	-	Malaysia	JQ302897	JQ302897	AEW26370.1
<i>Smittium trichopteran tricho</i>	ALG-13-W1	Canada	Canada	MMW	no	Canada	1492F-2356R	8bf-16r	-	Canada	JX155624	JX155650	KC297645
Unnamed Trichopteran tricho	ALG-10-W3	Canada	Canada	MMW	no	Canada	1492F-2356R	8bf-16r	-	Canada	JX155625	JX155651	KC297663
<i>Smittium culcis</i>	43-1-2	Australia	Australia	LCF/BH	yes	Australia	1492F-2356R	8bf-16r	-	Australia	DO367512.1	DO367512.1	JX155480
<i>Smittium mucronatum</i>	FRA-12-3	France	France	KUMYCOL/RWL	yes	France	1492F-2356R	8bf-16r	-	France	JQ302833	JQ302833	AEW26371.1
<i>Smittium simuli</i>	41-1-6	Australia	Australia	LCF/BH	yes	Australia	1492F-2356R	8bf-16r	-	Australia	AF277030.1	AF277030.1	KC297646
<i>Ausrosmittium</i> sp.	32-1-8	Australia	Australia	KUMYCOL	no	Australia	1492F-2356R	8bf-16r	-	Australia	JQ302861	JQ302861	KC297647
<i>Coleopteromyces amnicus</i>	ARG-15-6F	Argentina	Argentina	LCF	no	Argentina	1492F-2356R	8bf-16r	-	Argentina	JQ302853	JQ302853	JX155465
<i>Pseudoharpella arcomylica</i>	LCF#3	USA	USA	LCF	no	USA	1492F-2356R	8bf-16r	-	USA	JQ302882	JQ302882	JX155467
<i>Smittium angustum</i>	AUS-126-30	Australia	Australia	RWL	yes	Australia	1492F-2356R	8bf-16r	-	Australia	AF277005.1	AF277005.1	JX155420
<i>Smittium annulatum</i>	CR-143-8	Costa Rica	Costa Rica	RWL	yes	Costa Rica	1492F-2356R	8bf-16r	-	Costa Rica	AF277024.1	AF277024.1	JX155421
<i>Smittium caudatum</i>	KS-1-2	Chironomidae	USA	KUMYCOL/RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	JQ302948	JQ302948	JX155422
<i>Smittium coloradense</i>	RMBL-13-41	Cricetopus sp.	USA	RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	JQ302912	JQ302912	JX155423
<i>Smittium commune</i>	KS-6-6	Chironomidae	USA	RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	JQ302901	JQ302901	JX155426
<i>Smittium cylindrosporum</i>	CH-27-1	Cricetopus sp.	Chile	RWL	yes	Chile	1492F-2356R	8bf-16r	-	Chile	JQ302828	JQ302828	JX155433
<i>Smittium gravimelalum</i>	KS-F1-3	<i>Dicrotelipipes fumidus</i>	USA	LCF	yes	USA	1492F-2356R	8bf-16r	-	USA	AF277037.1	JX155634	JX155437
<i>Smittium megalosporum</i>	SC-DP-2	<i>Smittium vittatum</i>	USA	KUMYCOL/CEB	yes	USA	1492F-2356R	8bf-16r	-	USA	AF277045.1	JQ302823	JX155442
<i>Smittium morbosum</i>	AUS-X-1	<i>Anopheles hillii</i>	Australia	KUMYCOL/RWL	yes	Australia	1492F-2356R	8bf-16r	-	Australia	AF277014.1	JQ302913	JX155443
<i>Smittium orthocladii</i>	LCF-BT-1	<i>Corynorhiza</i> sp.	USA	LCF/MMW	yes	USA	1492F-2356R	8bf-16r	-	USA	DQ367446.1	JQ302900	AEW26378.1
<i>Smittium ipulidarum</i>	RMBL-31-1	<i>Eliptera asigmatia</i>	USA	KUMYCOL/RWL	yes	USA	1492F-2356R	8bf-16r	-	USA	AF277043.1	JQ302836	JX155452
<i>Smittium tronadorium</i>	ARG-24-2F	<i>Parahelipogonia</i> sp.	Argentina	LCF	yes	Argentina	1492F-2356R	8bf-16r	-	Argentina	AF277004.1	JQ302904	JX155454
<i>Stachylinea lenica</i>	NOR-58-10	<i>Chironomus</i> sp.	Norway	RWL	no	Norway	1492F-2356R	8bf-16r	-	Norway	JQ302874	JX155628	JX155468
<i>Stachylinea</i> sp.	NS-X-10	Chironomidae	Canada	DBS	yes	Canada	1492F-2356R	8bf-16r	-	Canada	-	-	JX155469
<i>Trichozygospora chironomidarum</i>	TN-3-16	Chironomidae	USA	RWL	no	USA	1492F-2356R	8bf-16r	-	USA	JQ302841	KC297568	JX155470

<sup>1</sup> AS, Amy Slaymaker; AR, ALEN RIZZO; BH, Barb Hayward; 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 Vallé; MCB, Murray Colbo; MJC, Matias J. Cáraro; 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 cultured isolates from the University of Kansas Mycological Culture Collection, represented as KUMYCOL.

<sup>2</sup> Accession numbers in bold were generated for this study (or as joint effort with Wang 2012 study).  
<sup>3</sup> Data derived from Origins of Multicellularity Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>).  
<sup>4</sup> Data derived from Rhizopus oryzae Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>).  
<sup>5</sup> 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.  
<sup>6</sup> 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.  
<sup>7</sup> rDNA was not available from the genome sequencing project, so data from other isolates was used. The isolate used for the SSU rDNA was taken from isolate 'MUCL\_30488', CBS 445.63'. The LSU rDNA was taken from isolate MS 115.  
<sup>8</sup> Species used for initial primer design and in silico testing.



concentration, 0.44  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$  (to a total concentration of 2.5 mM), 4.16  $\mu\text{L}$   $\text{dH}_2\text{O}$ , and 2  $\mu\text{L}$  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 with a final hold step at 4 °C. Reagents for the MCM7-8bf and MCM7-16r primer were identical except that 0.35  $\mu\text{L}$  of 50  $\mu\text{g}/\mu\text{L}$  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, based on estimated compatibility with the *Blastocladiomycota*, *Chytridiomycota* 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). 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*. Since 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 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  $\mu\text{L}$  of Promega Go-Taq Green Master Mix, 0.66  $\mu\text{L}$  of each primer at 10  $\mu\text{M}$  concentration, 0.88  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$  (to a final concentration of 2.5 mM), 0.35  $\mu\text{L}$  of 50  $\mu\text{g}/\mu\text{L}$  BSA, 6.45  $\mu\text{L}$   $\text{dH}_2\text{O}$ , and 2  $\mu\text{L}$  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 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  $\mu\text{L}$  of Promega Go-Taq Green Hot Master Mix, 0.66  $\mu\text{L}$  of each primer at 10  $\mu\text{M}$  concentration, 0.44  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$  (to a total concentration of 2.5 mM), 2.20  $\mu\text{L}$  of 5M Betaine, 0.35  $\mu\text{L}$  of 50  $\mu\text{g}/\mu\text{L}$  BSA, 4.69  $\mu\text{L}$   $\text{dH}_2\text{O}$ , and 2  $\mu\text{L}$  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 56 °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 squeeze' 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.

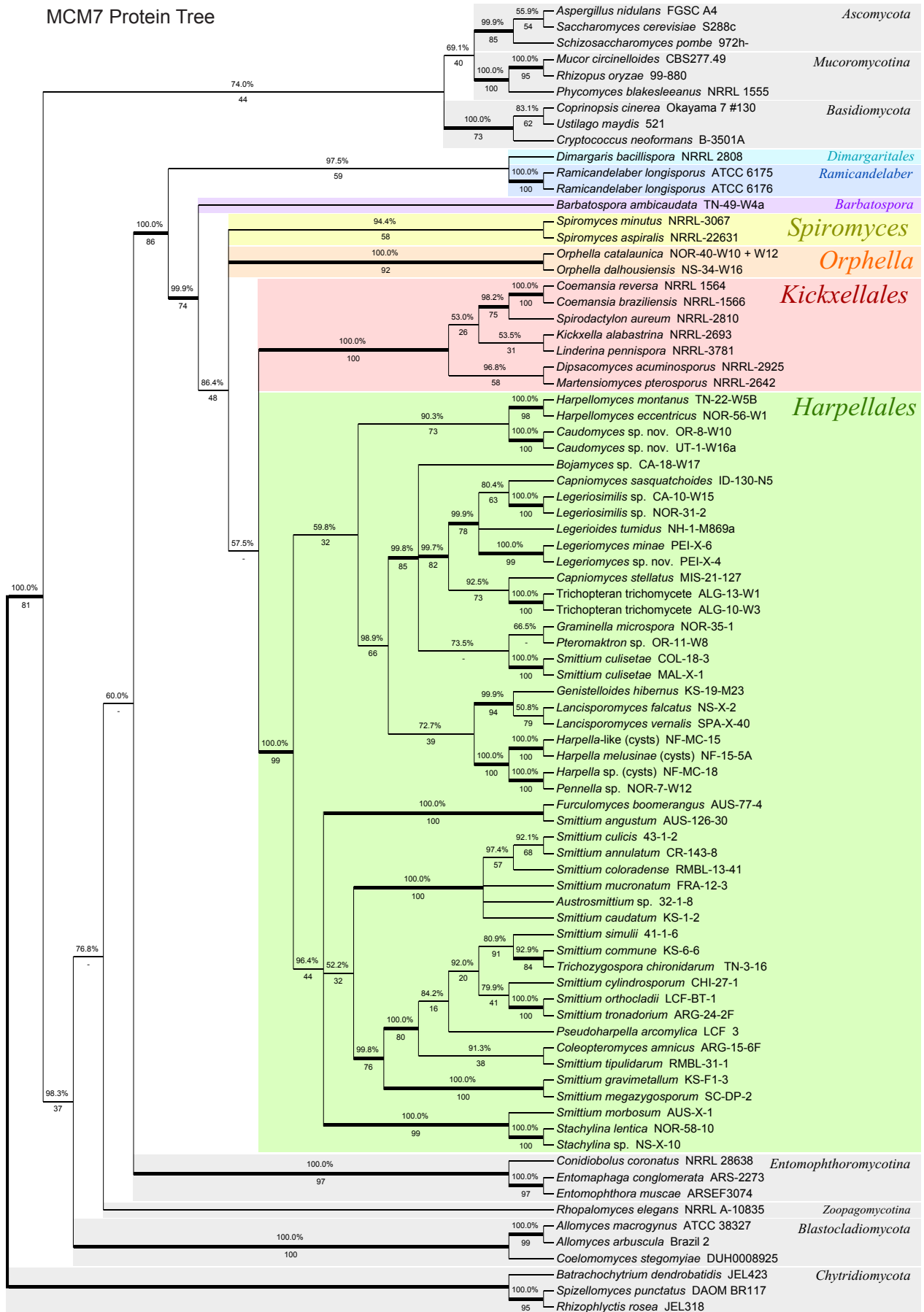
### Phylogenetic analyses

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.

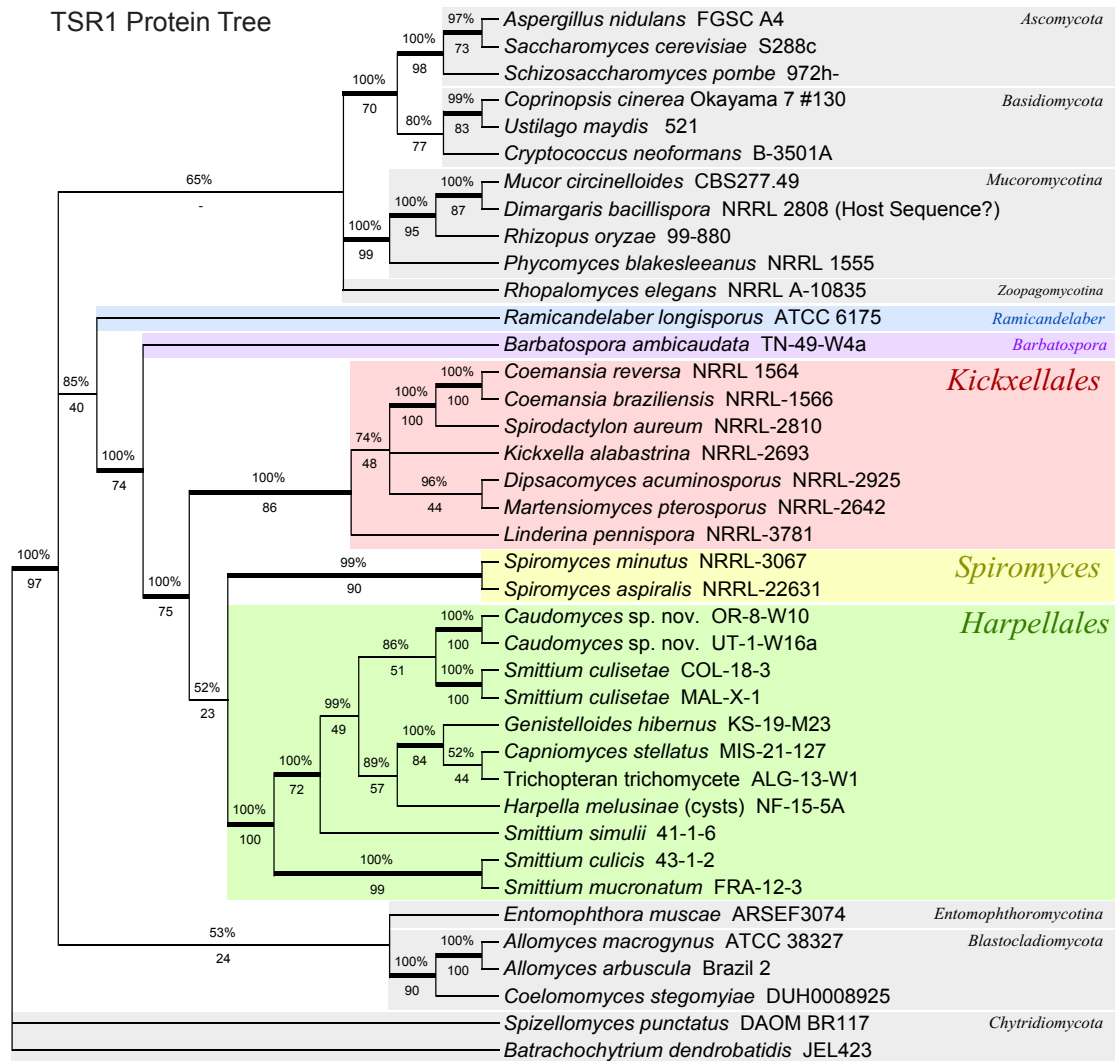
Each of the alignments was tested using an appropriate model selection program. The 18S and 28S nucleotide sequences, as well as each of the three individual codon positions of the MCM7 nucleotide alignment, were tested with jModelTest (Guindon & Gascuel 2003, Posada 2008). Model selection was based on the corrected AIC (AICc) score. For all sequences tested, except for the 2nd codon position of the MCM7 nucleotide alignment, the GTR+  $\Gamma$ +I method had the highest AICc score. For the 2nd codon position, the GTR+  $\Gamma$  model was slightly higher; however, for simplicity of analysis, the GTR+  $\Gamma$ +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+  $\Gamma$ +I 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+  $\Gamma$ +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% PPP and > .70 MLBP).



**Fig. 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.

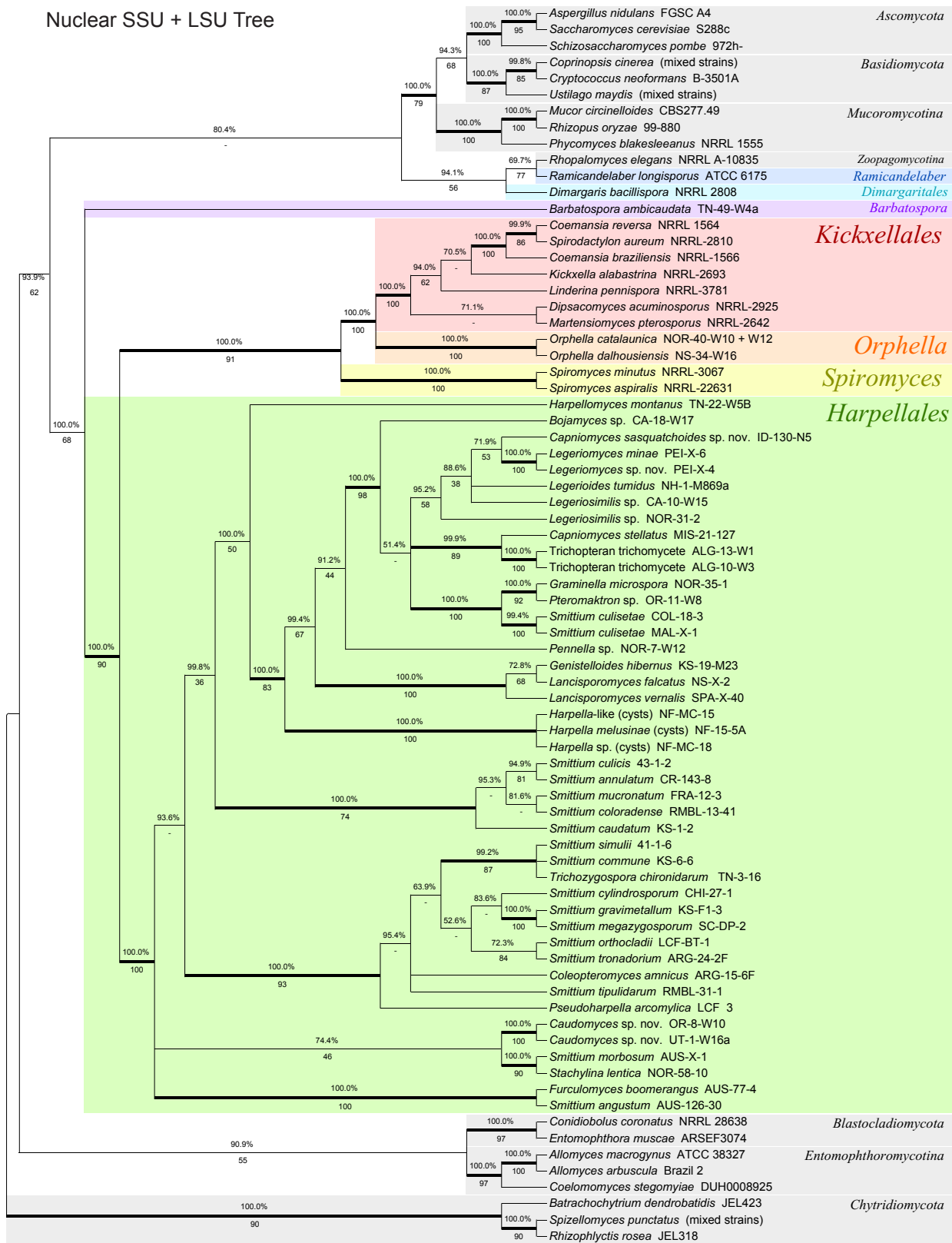
A total of nine topologies were produced by our analyses (Fig. 1–5 and S1–S4). Six of the analyses used a large number of taxa (76–81) and were primarily intended to investigate the use of MCM7, whereas three of the analyses used a smaller number (38–39) and were intended to evaluate the use of TSR1 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 RPB1 and RPB2, 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.



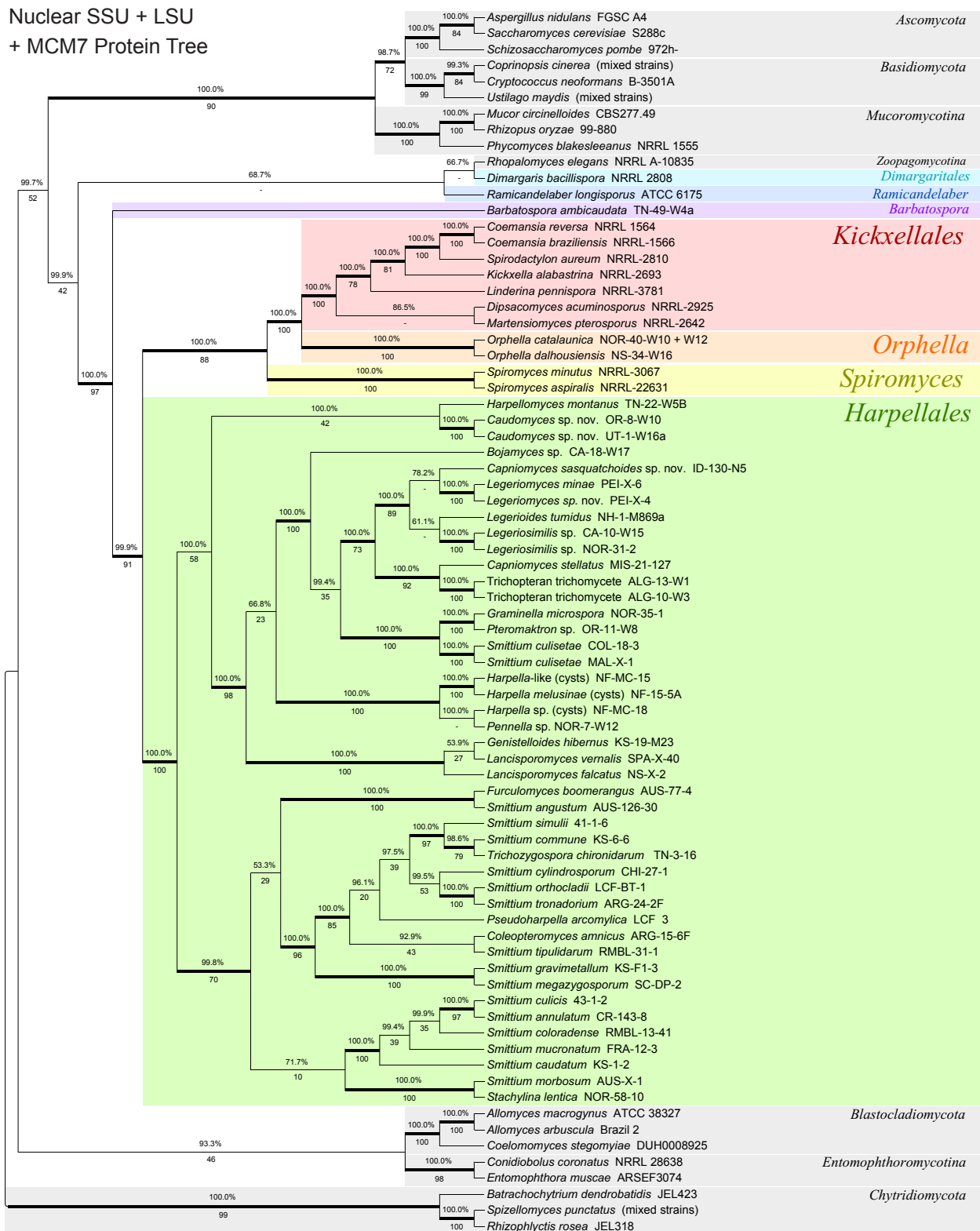
**Fig. 3** Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of nuclear small subunit (SSU) and nuclear large subunit (LSU) rDNA. Tree is based on a 50 % majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). Numbers above branches are Bayesian 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).

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) 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

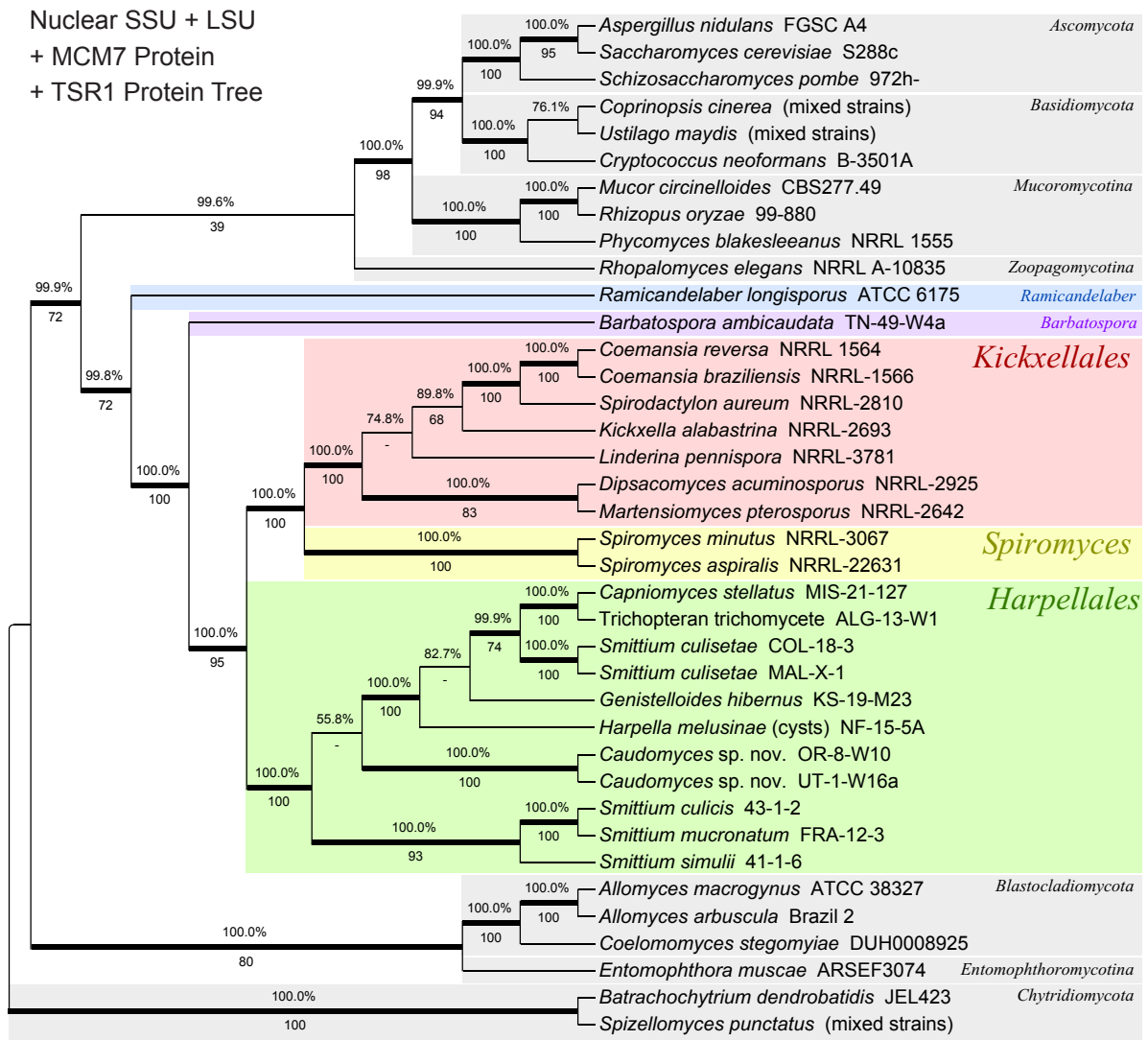




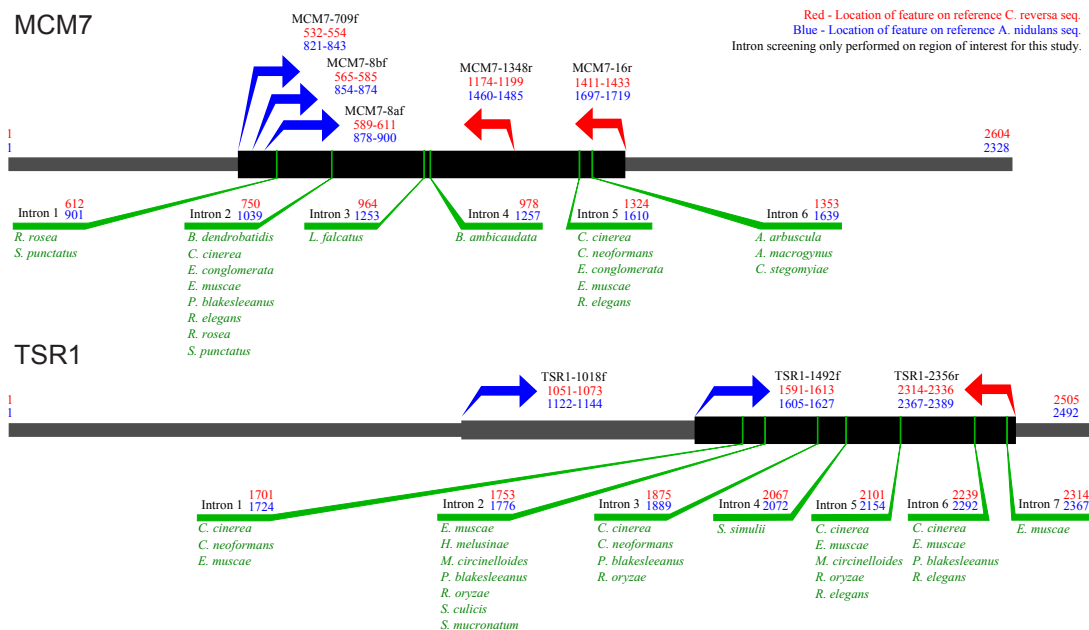
**Fig. 4** Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of SSU and LSU rDNA as well as MCM7 translated protein sequences. The method used for tree inference and the format of the tree are the same as for Fig. 3.

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.

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 *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

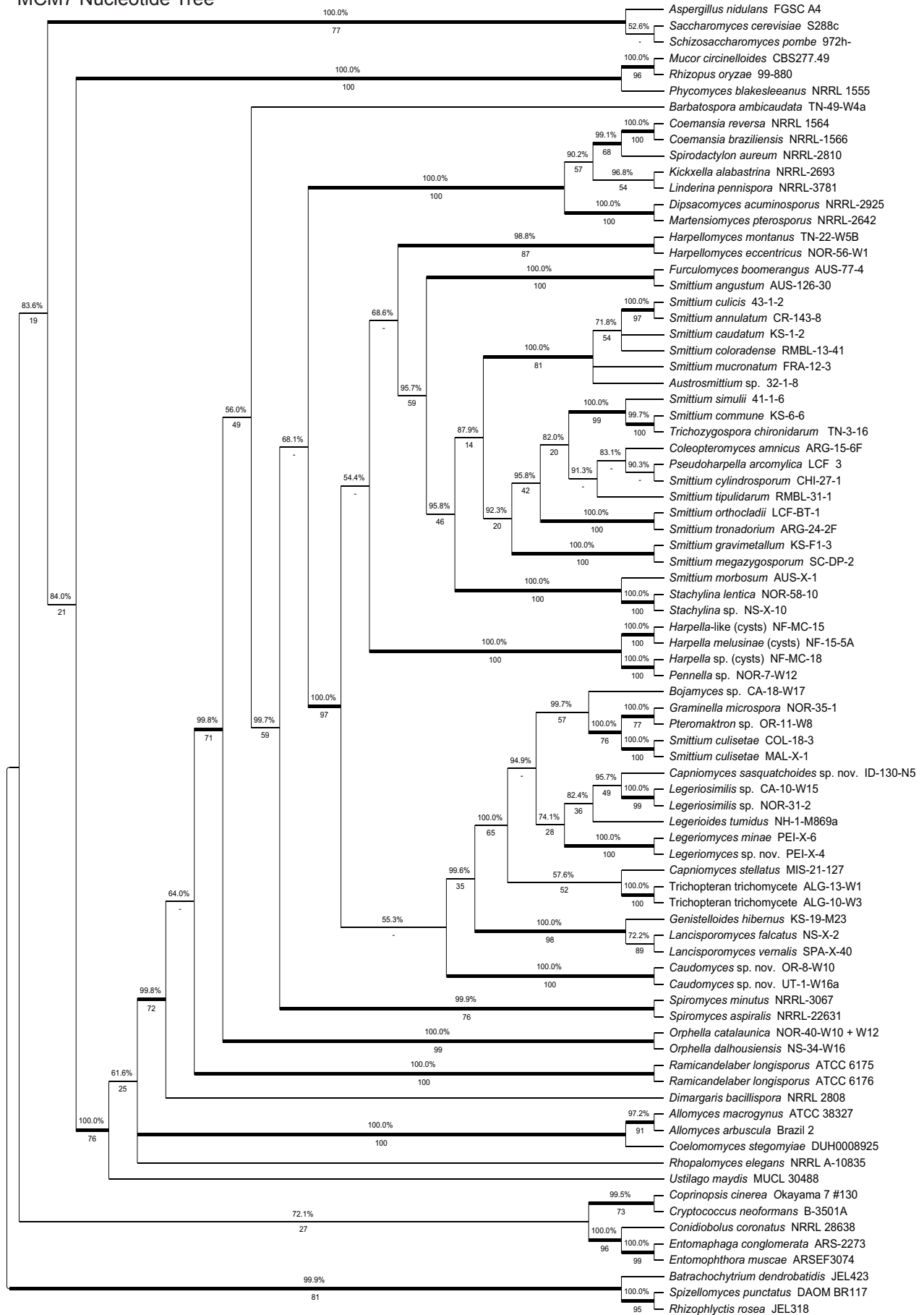


**Fig. 5** Phylogeny of the *Kickxellomycotina* based on a concatenated alignment of SSU and LSU rDNA as well as MCM7 and TSR1 translated protein sequences. The method used for tree inference and the format of the tree are the same as for Fig. 3.

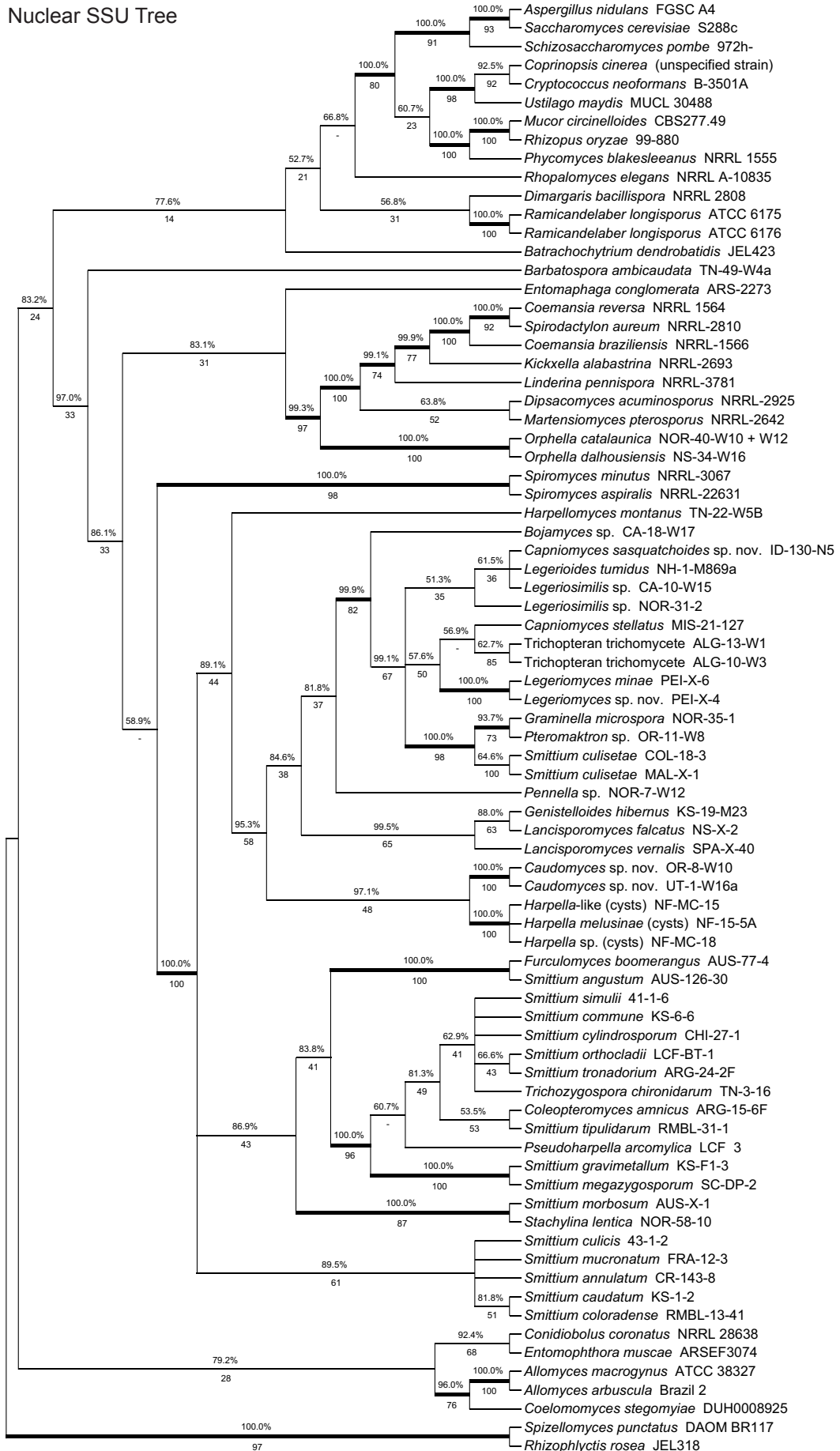


**Fig. 6** Map of the genes MS456 (MCM7) and MS277 (TSR1). 5' end is at left. Forward primers are marked with blue arrows, reverse primers with red arrows. Introns are labelled in green. Red numbers designate the position of the feature on a reference sequence from *C. reversa*. Blue numbers designate 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,

MCM7 Nucleotide Tree



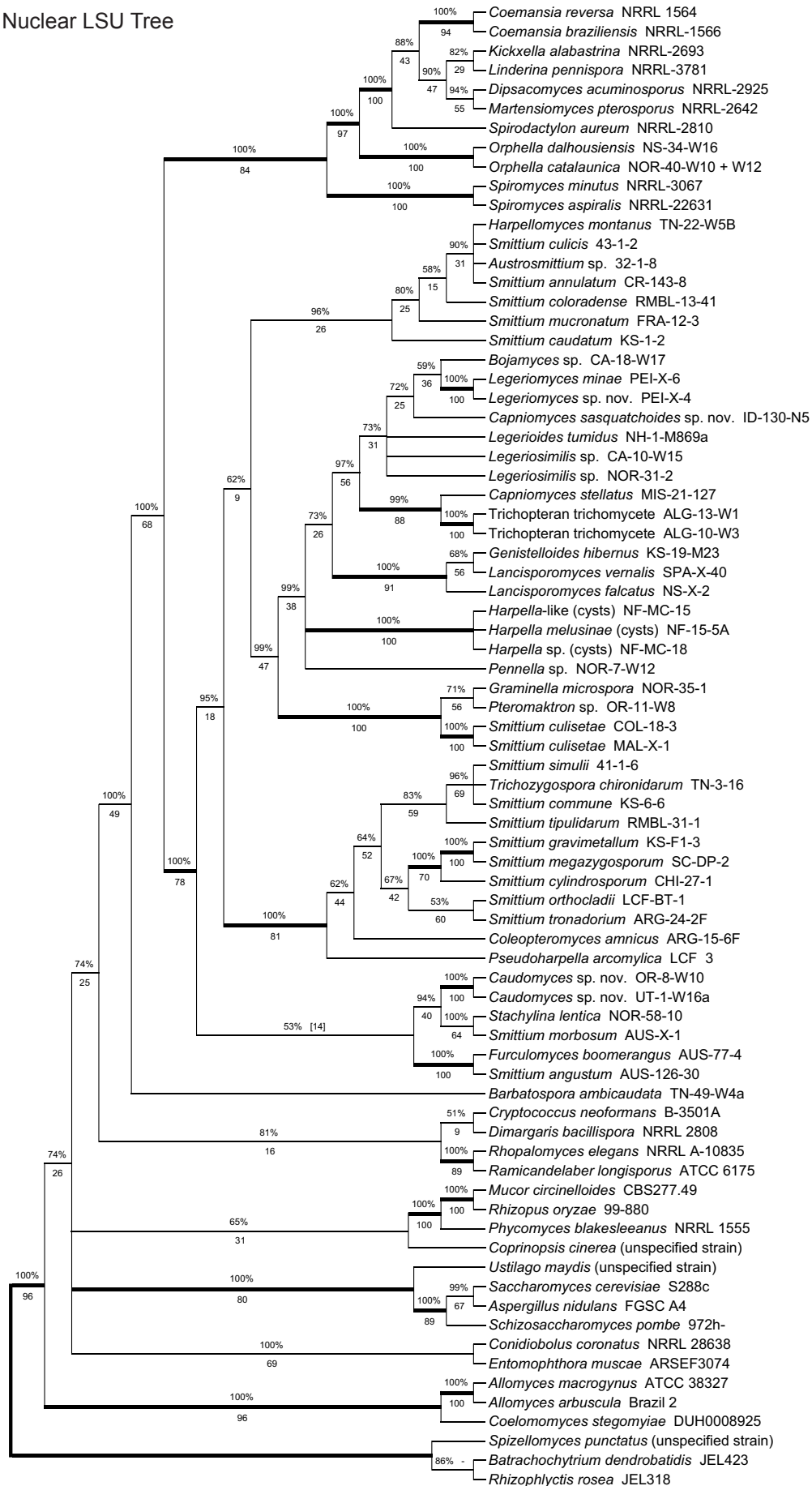
**Fig. S1** Phylogeny of the *Kickxellomycotina* based on an alignment of MCM7 nucleotide sequences. Tree is based on a 50 % majority-rules consensus of 10k trees produced with Bayesian inference (5k used as burn-in). The three codon positions were all considered to be on different, unlinked partitions 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).

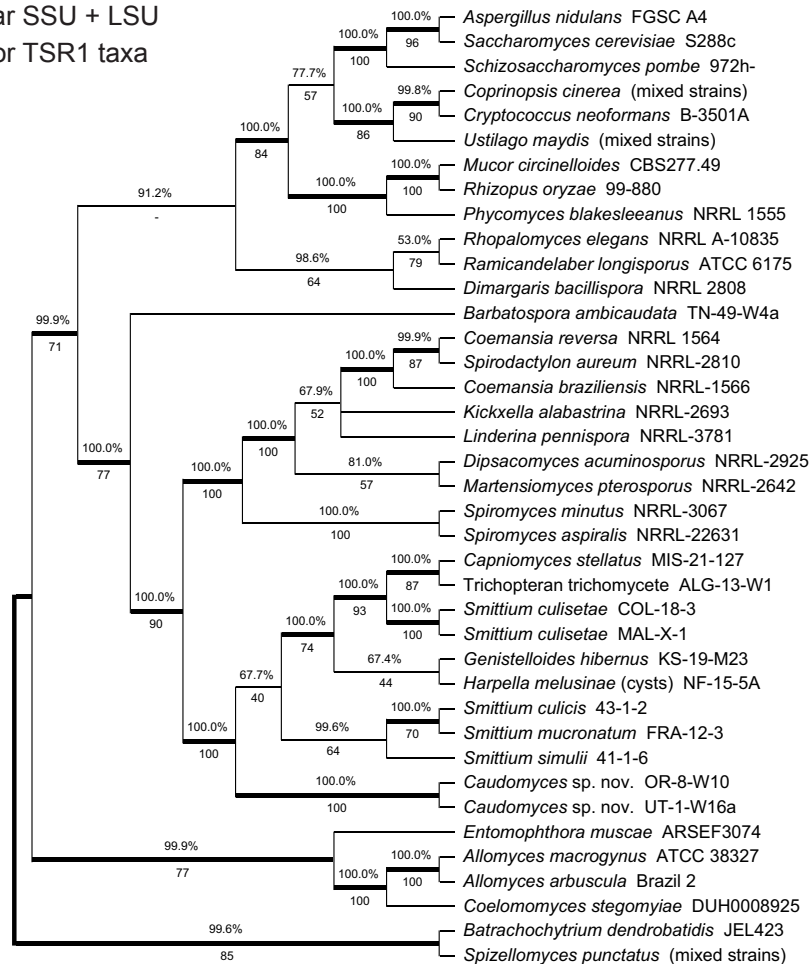


Nuclear LSU Tree



**Fig. S3** 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



**Fig. S4** Phylogeny of the Kickxellomycotina based on a concatenated alignment of nuclear small subunit (SSU) and nuclear large subunit (LSU) rDNA. For this tree, 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).

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 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 was, in general, more congruent with both the rDNA 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 braziliensis*, *Coemansia reversa* 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 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 also 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 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 (Aguileta et 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
<i>Chytridiomycota</i>	MCM7-709f, MCM7-16r	
<i>Blastocladiomycota</i>	MCM7-709f, MCM7-16r	
<i>Zoopagales</i>	MCM7-709f, MCM7-16r	
<i>Entomophthorales</i>	MCM7-709f, MCM7-8af, MCM7-16r	MCM7-709f preferred over MCM7-8af
<i>Kickxellomycotina</i>		
<i>Harpellales</i>	MCM7-8bf, MCM7-16r	MCM7-709f works for a couple of species
<i>Kickxellales</i>	MCM7-8bf, MCM7-16r	MCM7-709f works for some but not all species
<i>Asellariales</i>	–	Attempted unsuccessfully
<i>Dimargaritales</i>	MCM7-709f, MCM7-16r	MCM7-8bf not tested
<i>Orphella</i> clade	MCM7-8bf, MCM7-16r	MCM7-709f may work, but not as well as 8bf
<i>Barbatospora</i> clade	MCM7-8bf, MCM7-16r	MCM7-709f not tested
<i>Spiromyces</i> clade	MCM7-8bf, MCM7-16r	MCM7-709f amplified an incorrect gene when attempted
<i>Ramicandelaber</i> 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
<i>Chytridiomycota</i>	TSR1-1492f, TSR1-2356r	Not sequenced, but amplification product noted.
<i>Blastocladiomycota</i>	TSR1-1018f, TSR1-2356r	TSR1-1492f not tested.
<i>Zoopagomycotina</i>	TSR1-1018f, TSR1-2356r	TSR1-1492f not tested.
<i>Entomophthoromycotina</i>	TSR1-1018f, TSR1-2356r	TSR1-1492f not tested.
<i>Kickxellomycotina</i>		
<i>Harpellales</i>	TSR1-1492f, TSR1-2356r	TSR1-1018f does not appear to work.
<i>Kickxellales</i>	TSR1-1018f, TSR1-1492f, TSR1-2356r	TSR1-1018f and TSR1-1492f both work well.
<i>Asellariales</i>	–	Attempted unsuccessfully.
<i>Dimargaritales</i>	TSR1-1018f, TSR1-2356r	TSR1-1492f not tested.
<i>Orphella</i> clade	TSR1-1492f, TSR1-2356r	PCR product did not sequence cleanly but was identifiable as fungal TSR1.
<i>Barbatospora</i> clade	TSR1-1492f, TSR1-2356r	TSR1-1018f amplified but would not sequence.
<i>Spiromyces</i> clade	TSR1-1018f, TSR1-1492f, TSR1-2356r	TSR1-1018f and TSR1-1492f both work well.
<i>Ramicandelaber</i> 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

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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:



**Table 5** Comparative analysis of phylogenetic trees.

Alignment	Figure	Treebase #	ML score (RAxML)	# Taxa in Alignment	# Char in Alignment	# Interior Branches Total	# Interior Branches Supported	% Interior Branches Supported
MCM7 protein	Fig. 1	13444	-12111.24453	81	266	72	39	54.17 %
TSR1 protein	Fig. 2	13444	-8224.179649	39	207	33	21	63.64 %
Nuclear SSU + LSU	Fig. 3	13444	-25369.28207	76	2492	67	40	59.70 %
Nuclear SSU+LSU+MCM7 protein	Fig. 4	13444	-38458.25623	76	2758	73	51	69.86 %
Nuclear SSU+LSU+MCM7 protein+TSR1 protein	Fig. 5	13444	-35502.47807	38	2965	35	29	82.86 %
MCM7 nucleotide <sup>1</sup>	Fig. S1	13444	-34531.25508	81	780	75	41	54.67 %
SSU rDNA <sup>1</sup>	Fig. S2	13444	-14149.10549	78	1414	66	28	42.42 %
LSU rDNA <sup>1</sup>	Fig. S3	13444	-11824.38916	77	1078	65	26	40.00 %
SSU+LSU (TSR1 taxa) <sup>1</sup>	Fig. S4	13444	-20628.4317	39	2492	35	25	71.43 %

<sup>1</sup> Not presented in main body of document – see supplementary materials.

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 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*, and has only otherwise been found in the genus *Ballocephala* and *Zygnemomyces* within the *Entomophthoromycotina* (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 trichospores) as well as zygospores. The sexual spores can vary in shape, being spherical in the *Asellariales*, *Dimargaritales* 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) 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 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, 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 which 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. 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 *Austrosmittium*,



*Coleopteromyces*, *Furculomyces*, *Pseudoharpella*, *Stachylina* and *Trichozygospora*.

Our 3-gene analysis (Fig. 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*, *Penella*, *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 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 MCM7 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 trichospore dehisces from the fertile thallus. *Trichozygospora* 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 trichospore. A collar is present in *Smittium culisetae* and *Bojamyces*, but for both it is flared, and perhaps unlike most 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 breaking up 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*. Saprobian 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 & 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 trichospores 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 '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. 2006a). 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 for this study revealed a strongly-supported and monophyletic *Kickxellales* clade that includes *Coemansia*, *Dipsacomycetes*, *Kickxella*, *Linderina*, *Martensiomycetes* and *Spirodactylon* (3-gene: BPP: 100.0 %, MLBP: 100/100; 4-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 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 *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 are difficult to resolve. The group consisting of *Coemansia braziliensis*, *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 %, MLBP: 75/100) and TSR1 phylogenies (Fig. 2; BPP: 100.0 %, MLBP: 100/100), providing multi-gene support. However, in both the MCM7 (Fig. 1; BPP: 100.0 %, MLBP: 100/100) and TSR1 (Fig. 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. 3; BPP: 99.9 %, MLBP: 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 *Dipsacomycetes* and *Martensiomycetes* 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 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 *Dipsacomycetes* and *Martensiomycetes* (BPP: 100.0 %, MLBP: 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 *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 bisporous merosporangia (all other *Kickxellomycotina* 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 *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 because the three-gene analysis placed *Dimargaris* in an unsupported group with *Rhopalomyces* (BPP: 66.7 %, MLBP: not present). The notion 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.

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*, *Ramicandelaber* and *Spiromyces***

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. ambicaudata* 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 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 zygosporangia, 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. 2; BPP: 99.9 %, MLBP: 74/100), TSR1 (Fig. 2; BPP: 100.0 %, MLBP: 74/100), 3-gene (Fig. 4; BPP: 100.0 %, MLBP: 97/100), and 4-gene (Fig. 5; BPP: 100.0 %, MLBP: 100/100) analyses, and is present (but not completely supported) in the rDNA (Fig. 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), 3-gene (Fig. 4; BPP: 99.9 %, MLBP: 91/100), and 4-gene analyses (Fig. 5; BPP: 100.0 %, MLBP: 95/100), and a branch that is not strongly supported within the MCM7 (Fig. 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 zygosporangia 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 & 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. 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.

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), noting the possible disproportionate phylogenetic signal from rDNA.

*Spiromyces* is currently a member of the *Kickxellales*, although previous phylogenetic analyses have placed it apart from that order. It is separated from the *Kickxellales* by *Orphella* (White 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 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 (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 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. 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 *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 (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*.

## 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 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 and 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 (White et al. unpubl. data). 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 to place. While the gene regions require some manual adjustment (intron removal, translation, and removal of poorly aligned regions) to be used, 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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