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RESEARCH ARTICLE

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New primers for promising single-copy genes in fungal phylogenetics and systematics

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Key words

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Abstract Developing powerful phylogenetic markers is a key concern in fungal phylogenetics. Here we report degenerate primers that amplify the single-copy genes *Mcm7* (MS456) and *Tsr1* (MS277) across a wide range of *Pezizomycotina* (*Ascomycota*). Phylogenetic analyses of 59 taxa belonging to the *Eurotiomycetes*, *Lecanoromycetes*, *Leotiomycetes*, *Lichinomycetes* and *Sordariomycetes*, indicate the utility of these loci for fungal phylogenetics at taxonomic levels ranging from genus to class. We also tested the new primers in silico using sequences of *Saccharomycotina*, *Taphrinomycotina* and *Basidiomycota* to predict their potential of amplifying widely across the *Fungi*. The analyses suggest that the new primers will need no, or only minor sequence modifications to amplify *Saccharomycotina*, *Taphrinomycotina* and *Basidiomycota*.

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INTRODUCTION

Molecular systematics has revolutionised our view of fungal evolution. Recent large scale sequencing efforts resulted in comprehensive multi-locus phylogenies, which have significantly improved our understanding of phylogenetic relationships within fungi (Binder & Hibbett 2002, Lumbsch et al. 2004, Lutzoni et al. 2004, James et al. 2006). These data led to the first phylogenetic classification of the *Fungi* (Hibbett et al. 2007). However, early events in fungal evolution still remain uncertain because of missing support and resolution at the backbone of the phylogeny. We lack information, for example, about the relationships of the different ascomycete classes to one another, or the evolution within major lineages, such as the lichenised *Lecanoromycetes*, or the basidiomycete clade *Agaricomycetes*. Robust and well-supported phylogenies are essential for a better understanding of fungal evolution, and a prerequisite for studies aiming at reconstructing the evolution of non-molecular characters on the background of a molecular phylogeny.

Commonly used molecular loci in fungal phylogenetics include nuclear and mitochondrial ribosomal rDNA (18S, 28S, ITS, IGS, mtSSU, mtLSU), as well as protein-coding genes, such as RNA polymerases (*RPB1* and *RPB2)*, β-tubulin, γ-actin, ATP synthase (*ATP6*), and elongation factor EF-1α (*TEF1*α). Some single-copy protein-coding genes such as *RPB1* and *RPB2* are promising for yielding well resolved and highly supported phylogenies (Liu & Hall 2004, Reeb et al. 2004, Crespo et al. 2007, Lumbsch et al. 2007). Other protein-coding genes, such as the tubulins, are present in the genome in multiple copies and thus have the potential of being phylogenetically misleading (Landvik et al. 2001). Generally, slow evolving loci are more suitable for reconstruction of deep phylogenetic relationships, while loci with high rates of evolution are better for the reconstruction of more recent evolutionary events. Ribosomal loci with high and heterogeneous rates of change, such as ITS, IGS and mtSSU rDNA, can be used to distinguish taxa at the genus and species level. However, the non-coding regions of these loci are prone to significant length variation, making alignment of distantly related taxa problematic. Fast evolving ribosomal genes are therefore less useful in large scale concatenated analyses involving higher-level phylogenetic relationships. Molecular systematists are constantly searching for loci that are conserved enough to produce reliable alignments, and at the same time have sufficient variability to yield well resolved and well supported phylogenies. Analysing phylogenetic relationships at lower and higher taxonomic levels simultaneously, while using only a few loci, is desirable, because sequencing entire genomes or even multiple loci is not feasible for many phylogenetically interesting taxa. Fungal material suitable for molecular study is often limited, and culturing of many species impossible.

In a recent study Aguileta et al. (2008) used a bioinformatics approach to assess the performance of single-copy protein-coding genes for fungal phylogenetics. Their analyses of 30 published fungal genomes revealed two loci, MS277 and MS456, which outperformed all other single-copy genes in phylogenetic utility. MS277 corresponds to the gene *Tsr1,* required for rRNA

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Table 1 Material and DNA sequences used in this study.

Species	Order	Class	Source	GenBank accession	
				Mcm7 (MS456)	*Tsr1* (MS277)
Ajellomyces capsulatus	*Onygenales*	*Eurotiomycetes*	–	XM_001538714	XM_001541629
Arctomia delicatula	*incertae sedis*	*Lecanoromycetes*	Sweden, 2002, *Palice s.n.* (F)	GQ272388	GQ272430
Arctomia teretiuscula	*incertae sedis*	*Lecanoromycetes*	China (GZU – holotype)	GQ272389	GQ272431
Aspergillus clavatus	*Eurotiales*	*Eurotiomycetes*	–	XM_001275314	XM_001275562
Aspergillus fumigatus	*Eurotiales*	*Eurotiomycetes*	–	XM_750254	XM_750526
Aspergillus nidulans	*Eurotiales*	*Eurotiomycetes*	–	XM_658504	XM_658778
Aspergillus nidulans	*Eurotiales*	*Eurotiomycetes*	–	XM_001213626	XM_001208611
Aspergillus niger	*Eurotiales*	*Eurotiomycetes*	–	XM_001397760	XM_001399262
Aspergillus oryzae	*Eurotiales*	*Eurotiomycetes*	–	XM_001826176	XM_001821764
Aspicilia caesiocinerea	*Pertusariales*	*Lecanoromycetes*	USA, *Lumbsch 19277e* (F)	GQ272390	GQ272432
Aspicilia cinerea	*Pertusariales*	*Lecanoromycetes*	USA, *Lumbsch 19190c* (F)	GQ272391	GQ272433
Botryotinia fuckeliana	*Helotiales*	*Leotiomycetes*	–	XM_001556412	XM_001554531
Bulbothrix apophysata	*Lecanorales*	*Lecanoromycetes*	Costa Rica, *Lücking 16650btu* (F)	GQ272392	GQ272434
Cetrariastrum andense	*Lecanorales*	*Lecanoromycetes*	Peru, *Lumbsch 19334* (MAF)	GQ272429	GQ272471
Cetrariastrum dubitans	*Lecanorales*	*Lecanoromycetes*	Peru, *Lumbsch 19366* (MAF)	GQ272427	GQ272470
Chaetomium globosum	*Sordariales*	*Sordariomycetes*	–	XM_001220296	XM_001225626
Coccidioides immitis	*Onygenales*	*Eurotiomycetes*	–	XM_001240385	XM_001245725
Dermatocarpon intestiniforme	*Verrucariales*	*Eurotiomycetes*	Turkey, 27.7.1997, *John* (F)	GQ272393	GQ272435
Dermatocarpon miniatum	*Verrucariales*	*Eurotiomycetes*	Germany, 17.10.2001, *Zimmermann* (F)	GQ272394	GQ272436
Everniastrum lipidiferum	*Lecanorales*	*Lecanoromycetes*	Peru, *Lumbsch 19309b* (MAF)	GQ272395	GQ272437
Everniopsis trulla	*Lecanorales*	*Lecanoromycetes*	Peru, *Lumbsch 19309b* (F)	GQ272396	GQ272438
Flavoparmelia marchantii	*Lecanorales*	*Lecanoromycetes*	Australia, (MAF-Lich 10492)	GQ272420	GQ272463
Gibberella zeae	*Hypocreales*	*Sordariomycetes*	–	XM_387281	XM_384579
Lecanora allophana	*Lecanorales*	*Lecanoromycetes*	Turkey, *Lumbsch 19618d* (F)	GQ272399	GQ272444
Lecanora carpinea	*Lecanorales*	*Lecanoromycetes*	Turkey, *Lumbsch 19611m* (F)	GQ272400	GQ272443
Lecanora chlarotera	*Lecanorales*	*Lecanoromycetes*	Turkey, *Lumbsch 19622e* (F)	GQ272398	GQ272440
Lecanora margarodes	*Lecanorales*	*Lecanoromycetes*	Australia, *Lumbsch 19086b* (F)	GQ272401	GQ272439
Lecanora pulicaris	*Lecanorales*	*Lecanoromycetes*	Turkey, *Lumbsch 19627c* (F)	GQ272419	GQ272441
Lecanora subcarpinea	*Lecanorales*	*Lecanoromycetes*	Turkey, *Lumbsch 19622a* (F)	GQ272428	GQ272442
Lobothallia radiosa	*Pertusariales*	*Lecanoromycetes*	Switzerland, 9.8.2004, *Lumbsch* (F)	GQ272397	GQ272445
Magnaporthe grisea	*Sordariales*	*Sordariomycetes*	–	XM_364455	XM_368157
Malcolmiella psychotrioides	*Ostropales*	*Lecanoromycetes*	Costa Rica, *Lücking s.n.* (F)	GQ272412	GQ272456
Malcolmiella sp. 1	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37092* (hb. Kalb)	GQ272402	GQ272447
Malcolmiella sp. 2	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 36969* (hb. Kalb)	GQ272411	GQ272455
Malcolmiella sp. 3	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37093* (hb. Kalb)	GQ272405	GQ272450
Malcolmiella sp. 4	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 36858* (hb. Kalb)	GQ272403	GQ272448
Malcolmiella sp. 5	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37060* (hb. Kalb)	GQ272407	GQ272446
Malcolmiella sp. 6	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37072* (hb. Kalb)	GQ272408	GQ272452
Malcolmiella sp. 7	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 36832* (hb. Kalb)	GQ272406	GQ272451
Malcolmiella sp. 8	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37005* (hb. Kalb)	GQ272409	GQ272453
Malcolmiella sp. 9	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 36963* (hb. Kalb)	GQ272404	GQ272449
Malcolmiella sp. 10	*Ostropales*	*Lecanoromycetes*	Thailand, *Kalb 37086* (hb. Kalb)	GQ272410	GQ272454
Neosartorya fischeri	*Eurotiales*	*Eurotiomycetes*	–	XM_001260497	XM_001260746
Neurospora crassa	*Sordariales*	*Sordariomycetes*	–	XM_958785	XM_951859
Ochrolechia parella	*Pertusariales*	*Lecanoromycetes*	Turkey, *Lumbsch 19625g* (MIN)	GQ272421	GQ272464
Ochrolechia subpallescens	*Pertusariales*	*Lecanoromycetes*	USA, *Lumbsch 19900a & Schmitt* (MIN)	GQ272422	GQ272465
Parmeliopsis hyperopta	*Lecanorales*	*Lecanoromycetes*	Spain (MAF-Lich 10181)	GQ272426	GQ272468
Peltula euploca	*Lichinales*	*Lichinomycetes*	USA, *Lumbsch 19923b & Schmitt* (MIN)	GQ272424	GQ272467
Penicillium marneffei	*Eurotiales*	*Eurotiomycetes*	–	XM_002146315	XM_002148793
Pertusaria amara	*Pertusariales*	*Lecanoromycetes*	USA, *Lumbsch 19925a & Schmitt* (MIN)	GQ272423	GQ272466
Pertusaria velata	*Pertusariales*	*Lecanoromycetes*	USA, *Lumbsch 19913c & Schmitt* (MIN)	GQ272425	GQ272469
Podospora anserina	*Sordariales*	*Sordariomycetes*	–	XM_001912857	XM_001909251
Psiloparmelia denotata	*Lecanorales*	*Lecanoromycetes*	Peru, *Lumbsch 19302g* (F)	GQ272413	GQ272457
Pyrenula subpraelucida	*Pyrenulales*	*Eurotiomycetes*	Costa Rica, *Lücking 17550f* (F)	GQ272414	GQ272459
Pyrgillus javanicus	*Pyrenulales*	*Eurotiomycetes*	Australia, *Lumbsch 19115e* (F)	GQ272415	GQ272458
Sclerotinia sclerotiorum	*Helotiales*	*Leotiomycetes*	–	XM_001586126	XM_001593622
Umbilicaria leprosa	*incertae sedis*	*Lecanoromycetes*	Peru, *Lumbsch 19355a* (F)	GQ272416	GQ272460
Usnea endochrysaea	*Lecanorales*	*Lecanoromycetes*	USA, *Buck 51175* (hb. Lendemer)	GQ272417	GQ272461
Verrucaria muralis	*Verrucariales*	*Eurotiomycetes*	Czech Republic, *Palice 6011* (hb. Palice)	GQ272418	GQ272462

Table 2 Primers developed in the current study.

Primer Name	Direction	Sequence (5'-3')	Position in *A. nidulans* mRNA (XM_658504 and XM_658778)	Corresponding amino acid sequence in *A. nidulans* (AN5992 and AN6266)	Length	Degeneracy
Mcm7-709for	For	ACI MGI GTI TCV GAY GTH AAR CC	709	TRVSDVKP	23 bp	32
Mcm7-1348rev	Rev	GAY TTD GCI ACI CCI GGR TCW CCC AT	1348	MGDPGVAKS	26 bp	16
Mcm7-1447rev	Rev	C ATI ACI GCI GCI GTR AGR CC	1447	GLTAAVM	21 bp	4
Tsr1-1453for	For	GAR TTC CCI GAY GAR ATY GAR CT	1453	EFPDEIEL	23 bp	32
Tsr1-1459for	For	CCI GAY GAR ATY GAR CTI CAY CC	1459	PDEIELHP	23 bp	32
Tsr1-2308rev	Rev	CTT RAA RTA ICC RTG IGT ICC	2308	GTHGYFK	21 bp	8

Table 3 Annealing conditions and PCR success rates for primers used in this study.

Gene	Primer combination	Approximate fragment length	Annealing temp.	PCR success (% of attempts)
Mcm7 (MS456)	*Mcm7*-709for/*Mcm7*-1348rev	640 bp	56 °C	80 %
Mcm7 (MS456)	*Mcm7*-709for/*Mcm7*-1447rev	740 bp	56 °C	50 %
Tsr1 (MS277)	*Tsr1*-1459for/*Tsr1*-2308rev	750 bp	49 °C	40 %
Tsr1 (MS277)	*Tsr1*-1453for/*Tsr1*-2308rev	750 bp	49 °C	40 %

accumulation during biogenesis of the ribosome (Gelperin et al. 2001), while MS456 corresponds to the gene *Mcm7*, a DNA replication licensing factor required for DNA replication initiation and cell proliferation (Moir et al. 1982, Kearsey & Labib 1998). Alignments based on these two loci alone recovered phylogenies that had the same topology, resolution power, and branch support as phylogenies based on a concatenated analysis of all 135 orthologous single-copy genes identified from fungal genomes (Aguileta et al. 2008). Strikingly, the authors report that most protein-coding genes commonly used in fungal systematics, such as *RPB1, RPB2, TEF1*α, β-tubulin, and γ-actin are not found among the best performing genes.

In the current study we designed degenerate primers to amplify a 600–800 bp fragment of each, MS277 and MS456, over a wide range of *Pezizomycotina*. We tested variability and phylogenetic utility of these loci at taxonomic levels ranging from genus to class. Our analyses include in silico comparisons of the new primers to sequences of *Saccharomycotina* and *Basidiomycota* to predict primer utility in these phylogenetic groups.

MATERIALS AND METHODS

Material and GenBank sequences used in the current study are listed in Table 1. We designed new degenerate primers based on amino acid alignments of *Mcm7* (MS456) and *Tsr1* (MS277) of euascomycete sequences available in GenBank. These alignments included members of *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes* and *Sordariomycetes*. Primer sequences and annealing conditions are reported in Table 2 and 3. The locations of the fragments amplified by the new primers are indicated in Fig. 1. We used *Aspergillus nidulans* mRNA sequences of *Mcm7* and *Tsr1* as reference sequences (GenBank accession numbers XM_658504 and XM_658778). *Saccharomycotina*, *Taphrinomycotina* and *Basidiomycota* used for in silico analysis of primer fit are listed in Table 4.

Molecular procedures

We extracted total genomic DNA from our samples using the Qiagen Plant Mini Kit (Qiagen). PCR reactions (25 µL) contained PuReTaq Ready-To-Go PCR beads (GE Healthcare), 1.25 µL of each primer (10 mM), 19.5 µL H_2O, and 3 µL DNA template. Alternatively we used 0.125 µL AmpliTaq Gold Taq (Applied Biosystems), 2.5 µL buffer, 2 µL dNTPs, 2.5–4 µL MgCl (20 mM), 0–5 µL BSA, 1.25 µL of each primer, and 3 µL DNA template. We found that increasing the amount of forward primer *Tsr1*-1459for to 2.5 µL, as well as adding *2* µL MgCl (20 mM) to PCR reactions involving PCR beads often improved PCR results. PCR cycling conditions for *Mcm7*-709for/*Mcm7*-1447rev and *Mcm7*-709for/*Mcm7*-1348rev (MS456) were: initial denaturation 94 °C for 10 min, followed by 38 cycles of 94 °C for 45 s, 56 °C for 50 s, 72 °C for 1 min, and final elongation 72 °C for 5 min. PCR cycling conditions for *Tsr1*-1459for/*Tsr1*-2308rev (MS277) were the same as above except with 49 °C annealing temperature. Amplification products were stained with EZ-Vision DNA dye (Amresco) and viewed on 1 % low melt agarose gels. We excised bands of the expected length from the gel and purified them using GELase (Epicentre). Alternatively, PCR products were cleaned using the Bioclean Columns kit (Biotools, Madrid) according to the manufacturer's instructions. We sequenced the fragments using Big Dye v3.1 chemistry (Applied Biosystems) and the same primers as for PCR. Cycle sequencing was executed with the following program: initial denaturation for 1 min at 96 °C followed by 32 cycles of 96 °C for 15 s, 50 °C for 10 s, 60 °C for 4 min. Sequenced products were precipitated with 25 µL of 100 % EtOH mixed with 1 µL of 3 M NaOAC, and 1 µL of EDTA, before they were loaded on an ABI PRISMTM 3730 DNA Analyser (Applied Biosystems). We assembled partial sequences using SeqMan v4.03 (Lasergene) and edited conflicts manually. We aligned the sequences based on amino acid sequence using ClustalW as implemented in the program BioEdit v7.0.9 (Hall 1999) and subsequently translated them back to nucleotides.

Phylogenetic analyses

We assembled two alignments including the same 59 taxa each. For phylogenetic analysis we used a maximum parsimony (MP), maximum likelihood (ML) and a Bayesian approach (B/MCMC) (Larget & Simon 1999, Huelsenbeck et al. 2001). We performed all analyses on the single gene alignments as well as on a combined alignment. We tested for potential conflict between individual datasets by comparing the 75 % MP bootstrap consensus trees.

We used PAUP v4.0 (Swofford 2003), GARLI v0.96 (Zwickl 2006) and MrBayes v3.1.2. (Huelsenbeck & Ronquist 2001) to analyse the alignments. MP analyses included 100 replicates with random sequence additions and TBR branch swapping in effect. MP bootstrapping (Felsenstein 1985) was performed based on 2 000 replicates with the same settings as for the

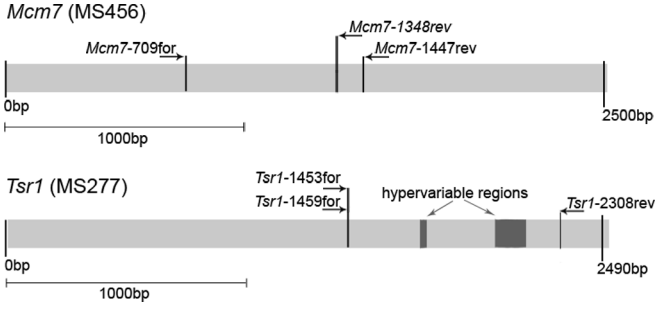

Fig. 1 Locations of the new primers for *Mcm7* and *Tsr1* using *Aspergillus nidulans* mRNA (XM_658504 and XM_658778) as reference sequence. Shaded areas in *Tsr1* indicate regions of high sequence variability.

Table 4 Taxa used to test the fit of the new primers in silico.

Taxon	*Mcm7*	*Tsr1*
Saccharomycotina		
Ashbya gossypii	NP_984137	NP_984911
Kluyveromyces lactis	XP_454998	XP_454177
Saccharomycetes cerevisiae	NP_009761	NP_010223
Yarrowia lipolytica	XP_501070	XP_500653
Taphrinomycotina		
Schizosaccharomyces pombe	NP_596545	NP_593391
Basidiomycota		
Coprinopsis cinerea	EAU88865	EAU91047
Cryptococcus neoformans	XP_571487	XP_570891
Ustilago maydis	EAK87259	EAK85759

MP search. Likelihood analyses were run using the GTR+I+G model and default settings in GARLI. For Bayesian analyses we partitioned the dataset into three parts (each codon position) and each partition was allowed to have its own parameter values (Nylander et al. 2004). No molecular clock was assumed, and no interpartition rate heterogeneity was allowed. Heating of the chains was set to 0.2. A run with 3 000 000 generations starting with a random tree and employing 4 simultaneous chains was executed for the individual datasets. Every 100th tree was saved into a file. The first 300 000 generations (i.e. the first 3 000 trees) were deleted as the 'burn in' of the chain. For the combined alignment dataset we executed a run with 6 000 000 generations and deleted the initial 600 000 generations (i.e. the first 6 000 trees). We plotted the log-likelihood scores of sample points against generation time using TRACER v1.0 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure that stationarity was achieved after the first 300 000 (600 000 for the combined alignment dataset) generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). Additionally, we used AWTY (Nylander et al. 2008) to compare splits frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. We calculated a majority rule consensus tree with average branch lengths of the remaining 54 000 trees (27 000 from each of the parallel runs) using the sumt option of MrBayes. For the combined alignment dataset the majority rule consensus tree consisted of 108 000 ($2 \times 54\,000$) trees from the stationarity phase. Posterior probabilities were obtained for each clade. Clades with posterior probabilities ≥ 0.95 were considered as strongly supported. Phylogenetic trees were visualised using the program Treeview (Page 1996).

RESULTS

We report 84 new sequences of *Mcm7* (MS456) and *Tsr1* (MS277) for 42 lichenised ascomycetes belonging to the classes *Eurotiomycetes*, *Lecanoromycetes* and *Lichinomycetes* (Table 1). PCR success rates for our newly developed primers were highest for the primer combination *Mcm7*-709for/*Mcm7*-

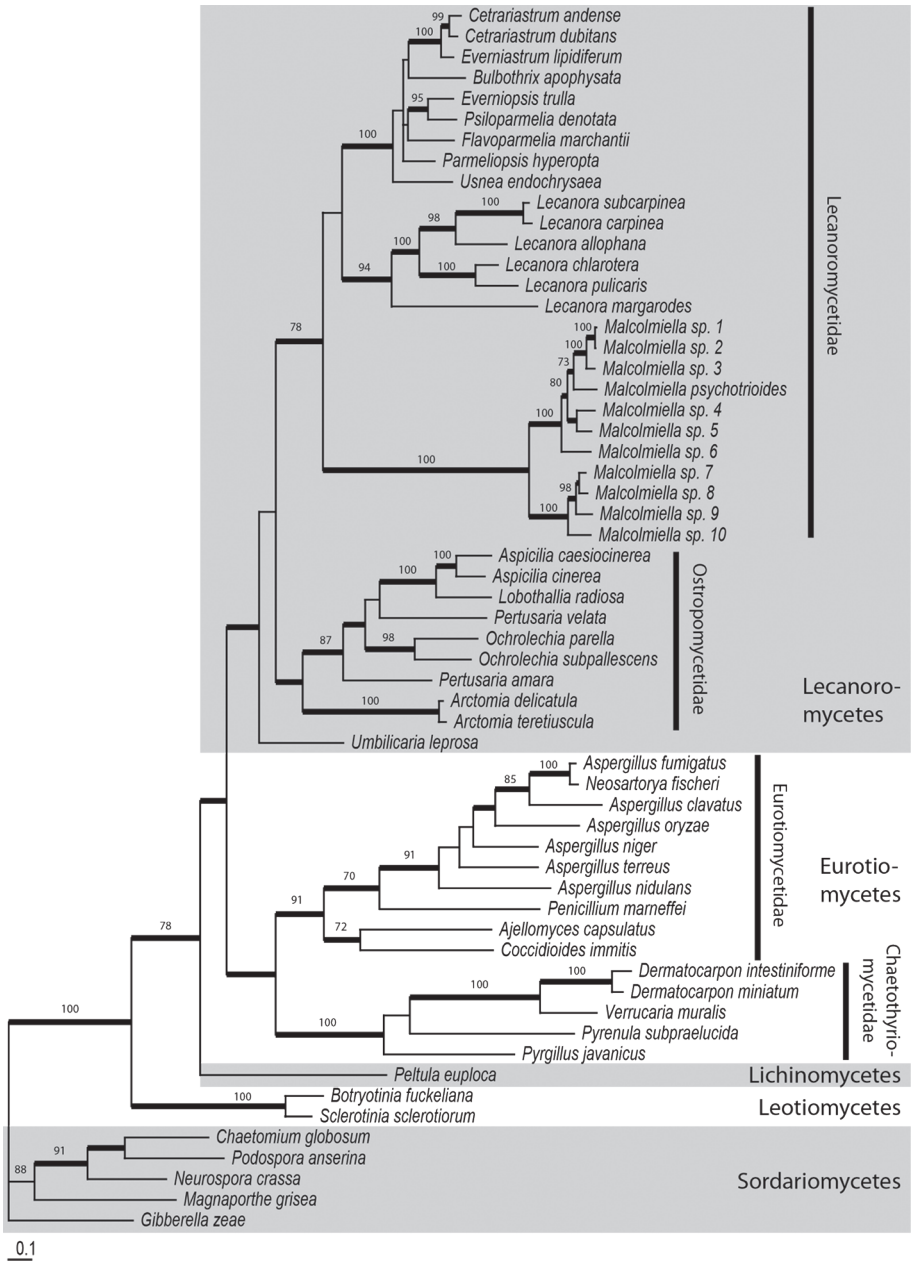

Fig. 2 Phylogeny of *Pezizomycotina* (*Ascomycota*) based on a combined alignment of *Mcm7* (MS456) and *Tsr1* (MS277) sequences. Total alignment length is 1203 bp. This is a 50 % majority rule consensus tree based on a sampling of 108 000 B/MCMC trees. **Bold** branches indicate posterior probabilities ≥ 0.95. Numbers above branches are maximum parsimony bootstrap support values ≥ 70 based on 2 000 random addition replicates.

Table 5 *Mcm7* and *Tsr1* sequence and alignment properties.

	Mcm7 (MS456)	*Tsr1* (MS277)
Introns	None	some (length: 189–272 bp)
Total alignment length (bp)	573	827
Hypervariable (excluded) sites	None	198
Variable sites	357/573 (62.3 %)	489/629 (77.7 %)
Constant sites	216/573 (37.7 %)	140/629 (22.3 %)
Within-genus sequence variation (p-distances) excluding hypervariable sites:		
Malcolmiella (11 OTUs)	0.0055–0.2227	0.0332–0.2193
Aspergillus (7 OTUs)	0.0230–0.2307	0.0357–0.3076
Lecanora (6 OTUs)	0.0377–0.2756	0.0226–0.4148

Table 6 Comparison of phylogenetic analyses (MP, ML, B/MCMC) between single and combined datasets.

	Mcm7 (MS456)	*Tsr1* (MS277)	Combined
MP tree length	3537	4606	8200
Number of MP trees	1	12	8
Consistency Index (CI) excluding uninformative sites	0.195	0.216	0.205
# of nodes supported by bootstrap ≥ 70 in MP analyses (based on 2 000 replicates)	23	30	37
ML score using GTR+I+G (GARLI)	-13732	-18424	-32262
# of nodes supported by PP ≥ 95 in B/MCMC analyses	36	38	44

1348rev (± 80 %), while *Mcm7*-709for/*Mcm7*-1447rev worked in ± 50 % of the attempted PCRs, and the *Tsr1* primers in ± 40 %. Multiple bands were sometimes present when we used the primer combinations *Mcm7*-709for/*Mcm7*-1447rev and *Tsr1*-1459for/*Tsr1*-2308rev. *Tsr1*-1453for is a modification of *Tsr1*-1459for that we used under the same annealing conditions. We used the *Aspergillus nidulans* mRNA sequences of *Mcm7* (XM_658504) and *Tsr1* (XM_658778) as references for the locations of our primers. The full length genomic DNA sequences of *Aspergillus nidulans Mcm7* and *Tsr1* contain 1–2 introns of ± 60 bp length, which, however, do not overlap with the sequence fragments amplified by primers developed in this study. We found introns (length: 189–272 bp) with characteristic GT-intron-AG splice sites near the reverse primer (*Tsr1*-2308rev) in *Tsr1* in three *Lecanora* species. Two hypervariable regions containing many gaps (*Tsr1*: positions 198–221 and 518–628) were excluded from the phylogenetic analysis. The *Mcm7* alignment contained no gaps and no ambiguously aligned regions. Properties of the sequences and alignments are summarized in Table 5. We performed parsimony bootstrap analyses on each individual dataset, and examined 75 % bootstrap consensus trees for conflict (Lutzoni et al. 2004). We used the program Modeltest v3.7 (Posada & Crandall 1998) to determine the nucleotide substitution model that best fit our data. For both datasets the program selected the GTR+I+G model.

The tree topologies obtained from the single gene datasets resulting from MP, ML and Bayesian analyses did not show any strongly supported conflicts. Thus, we present only the B/MCMC tree of the combined analysis (Fig. 2). Statistical values and number of supported nodes obtained by MP, ML and Bayesian analyses of single and combined datasets are summarised in Table 6. The *Sordariomycetes* were used as outgroup. The classes *Sordariomycetes*, *Leotiomycetes*, *Eurotiomycetes* and *Lecanoromycetes* are monophyletic and highly supported (PP ≥ 95). *Lichinomycetes* is only represented by a single species, *Peltula euploca*. The phylogenetic estimate obtained from the combined analysis of *Mcm7* and *Tsr1* agrees with previously published phylogenies (Gargas et al. 1995, James et al. 2006). *Lecanoromycetes* form a supported sister group relationship with *Eurotiomycetes*. Basal to this are *Lichinomycetes* and *Leotiomycetes*. Within *Lecanoromycetes*, the subclasses *Lecanoromycetidae* and *Ostropomycetidae* form supported groups, while the genus *Umbilicaria* is in an unsupported position at the base of *Lecanoromycetes*. Within

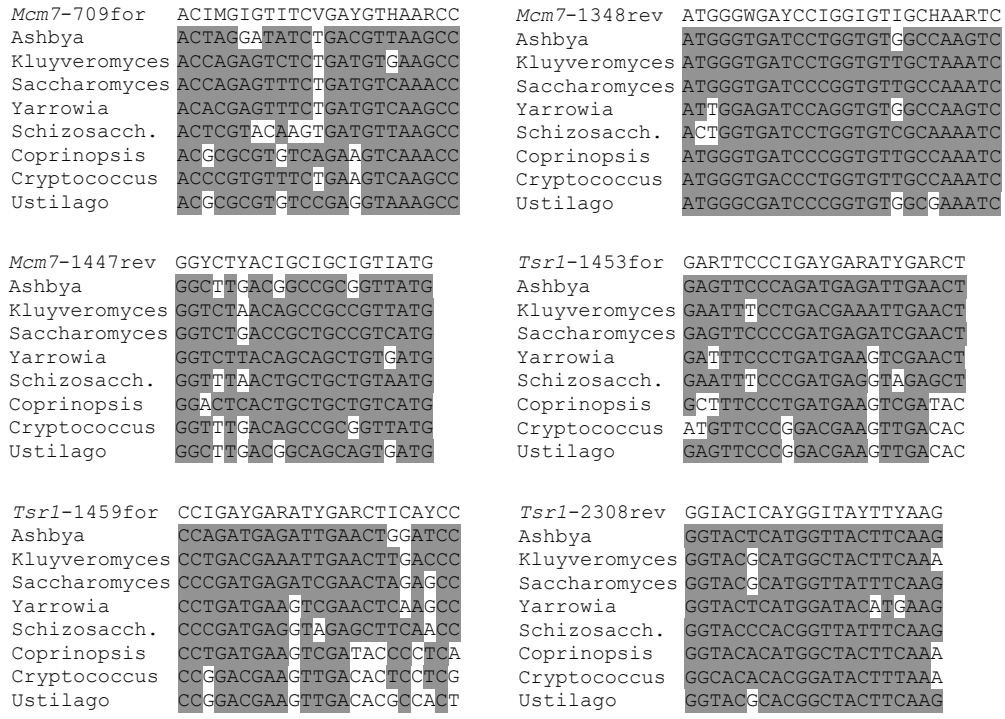

Fig. 3 Comparison of the new primers to homologous sequences in *Saccharomycotina* (*Ashbya, Kluyveromyces, Saccharomyces, Yarrowia*), *Taphrinomycotina* (*Schizosaccharomyces*) and *Basidiomycota* (*Coprinopsis, Cryptococcus, Ustilago*). 100 % matches between primer sequence and gene sequences studied are indicated by grey shading. High sequence similarities indicate that the new primers are likely to fit in members of the analysed groups. Some primer sequences may require slight modifications.

Eurotiomycetes, Eurotiomycetidae and *Chaetothyriomycetidae* form supported clades. We included multiple species/strains of the genera *Aspergillus* (7), *Lecanora* (6), and *Malcolmiella* (11) to assess within-genus variation of the analysed loci, as well as resolution power at low taxonomic levels. Genetic distances within *Aspergillus, Lecanora* and *Malcolmiella* are reported in Table 5. Each of these genera forms a supported monophyletic clade with high internal resolution and support (Fig. 2).

We aligned selected members of *Saccharomycotina, Taphrinomycotina* and *Basidiomycota* (Table 4) with our datasets and compared the new primer sequences to the corresponding positions in these taxa. The low number of mismatches suggests that the new primers will need no adjustments or only minor modifications to also fit these phylogenetic groups (Fig. 3).

DISCUSSION

We developed new degenerate primers, which amplify fragments of the single-copy protein-coding genes *Mcm7* and *Tsr1* in *Pezizomycotina*. Our study confirms that *Mcm7* and *Tsr1* are suitable loci for the reconstruction of phylogenetic relationships among fungi (Aguileta et al. 2008). We were able to obtain sequences from representatives of 5 classes and 11 orders of euascomycetes, demonstrating the ability of the primers to amplify a wide range of unrelated taxa. Additionally we tested primer fit in silico using members of *Saccharomycotina*, *Taphrinomycotina* and *Basidiomycota* and found that the new primers can be used for these groups as well, possibly with slight sequence modifications.

Our analyses within *Pezizomycotina* show that *Mcm7* and *Tsr1* are able to resolve large scale as well as fine scale phylogenetic relationships. The sequences are alignable across a wide range of unrelated taxa and at the same time have sufficient variability to resolve within-genus relationships (Table 5). This property sets the new loci apart from commonly used ribosomal markers, such as ITS or mtSSU, which also have the power to resolve lower level phylogenetic relationships, but may yield ambiguous and saturated alignments, when used to compare distantly related taxa. We predict that *Mcm7* and *Tsr1* have an even higher potential to resolve phylogenetic relationships between fungi when analyzed in combination with other routinely used datasets, such as 18S, 28S, *RPB1* and *RPB2*.

Mcm7 and *Tsr1* are two relatively long (~ 2.5 kb) single-copy genes which can be aligned across major fungal lineages, such as *Ascomycota* and *Basidiomycota* (Aguileta et al. 2008). The fact that *Homo sapiens* sequences can be used as outgroups (Aguileta et al. 2008, www.systematicbiology.org, online Appendix 5) indicates that these loci might also be useful for phylogenetic studies involving fungi as well as non-fungal organisms.

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