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

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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, 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 Saccharomyces, 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 Saccharomyces, 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 (TEF1α). 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
### Table 1: Material and DNA sequences used in this study.

| Species                        | Order             | Class               | Source                          | GenBank accession                  |
|--------------------------------|-------------------|---------------------|---------------------------------|------------------------------------|
| Aejlomyces capsulatus          | Ostropales        | Eurotiomycetes      |                                 |                                    |
| Arctonia delicata              | Lecanorales       | Lecanoromycetes     | Sweden, 2002, Palice s.n. (F)   | XM_001538714 XM_001541629          |
| Arctonia teretiscula           | Lecanorales       | Lecanoromycetes     | China (GZU – holotype)          | XM_007316393 QQ_007316393          |
| Aspergillus clavatus           | Eurotiales        | Eurotiomycetes      |                                 |                                    |
| Aspergillus fumigatus          | Eurotiales        | Eurotiomycetes      |                                 |                                    |
| Aspergillus nidulans           | Eurotiales        | Eurotiomycetes      |                                 |                                    |
| Aspergillus niger              | Eurotiales        | Eurotiomycetes      |                                 |                                    |
| Gibberella zeae                | Hypocreales       | Sordariomycetes     |                                 |                                    |
| Lecanora allophana             | Lecanorales       | Lecanoromycetes     | Costa Rica, Lücking 16500th (F) | QQ_007316393 QQ_007316393          |
| Lecanora carpinea              | Lecanorales       | Lecanoromycetes     | Peru, Lumbsch 19334 (MAF)       | QQ_007316393 QQ_007316393          |
| Lecanora chloraidea            | Lecanorales       | Lecanoromycetes     | Turkey, Lumbsch 19622 (F)       | QQ_007316393 QQ_007316393          |
| Lecanora margarodes            | Lecanorales       | Lecanoromycetes     | Australia, Lumbsch 19066 (F)    | QQ_007316393 QQ_007316393          |
| Lecanora pulcaris              | Lecanorales       | Lecanoromycetes     | Turkey, Lumbsch 19627c (F)      | QQ_007316393 QQ_007316393          |
| Lecanora subcarpinea           | Lecanorales       | Lecanoromycetes     | Turkey, Lumbsch 19622a (F)      | QQ_007316393 QQ_007316393          |
| Lobothallia radiosa            | Sordariomycetes   | –                   |                                 |                                    |
| Magnaportia grisea             | Sordariomycetes   | –                   | XM_364455 XM_368157             |                                    |
| Malcolmiiella psychrothioides  | Ostropales        | Lecanoromycetes     | Costa Rica, Lücking s.n. (F)    | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 1            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37092 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 2            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 36895 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 3            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37093 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 4            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 36658 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 5            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37060 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 6            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37072 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 7            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 36832 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 8            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37005 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 9            | Ostropales        | Lecanoromycetes     | Thailand, Kaib 36963 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Malcolmiiella sp. 10           | Ostropales        | Lecanoromycetes     | Thailand, Kaib 37086 (hb. Kaib) | QQ_007316393 QQ_007316393          |
| Neurosora crassa               | Sordariomycetes   | –                   | XM_001260497 XM_001260746       |                                    |
| Ochrolechia parella            | Pertusarias       | Lecanoromycetes     | Turkey, Lumbsch 19625g (MIN)    | QQ_007316393 QQ_007316393          |
| Ochrolechia subpallescens      | Pertusarias       | Lecanoromycetes     | USA, Lumbsch 1990a & Schmitt (MIN) | QQ_007316393 QQ_007316393       |
| Parmeliopsis hyperopta         | Lecanorales       | Lecanoromycetes     | Spain (MAF-Lich 10181)          | QQ_007316393 QQ_007316393          |
| Peltula euplica                | Lichinorales      | Lichinomycetes      | USA, Lumbsch 19923b & Schmitt (MIN) | QQ_007316393 QQ_007316393 |
| Pseudofusarium mameffii        | Eurotiales        | Eurotiomycetes      |                                 | QQ_007316393 QQ_007316393          |
| Pseudofusarium ampelaster      | Pertusarias       | Lecanoromycetes     | USA, Lumbsch 1925a & Schmitt (MIN) | QQ_007316393 QQ_007316393       |
| Pseudofusarium velatum         | Pertusarias       | Lecanoromycetes     | USA, Lumbsch 19913c & Schmitt (MIN) | QQ_007316393 QQ_007316393 |
| Podospora anserina             | Sordariomycetes   | –                   | XM_001912857 XM_001909251       |                                    |
| Psilocarpella delicata         | Lecanorales       | Lecanoromycetes     | Peru, Lumbsch 19302g (F)        | QQ_007316393 QQ_007316393          |
| Pyrenula subpseudolida         | Pyrenulales       | Lecanoromycetes     | Costa Rica, Lücking 17500f (F)  | QQ_007316393 QQ_007316393          |
| Pyrgillus javanicus            | Pyrenulales       | Lecanoromycetes     | Australia, Lumbsch 1918e (F)    | QQ_007316393 QQ_007316393          |
| Sclerotinia sclerotiorum       | Helotiales        | Leotiomycetes       |                                 | QQ_007316393 QQ_007316393          |
| Umbrillicaria leprea           | incertae sedis    | Lecanoromycetes     | Peru, Lumbsch 19355a (F)        | QQ_007316393 QQ_007316393          |
| Usnea endochrysea              | Lecanorales       | Lecanoromycetes     | USA, Buck 51175 (hb. Lendemer)   | QQ_007316393 QQ_007316393          |
| Vernacularia muralis          | Verrucariales     | Verrucariomycetes   | Czech Republic, Palice 6011 (hb. Palice) | QQ_007316393 QQ_007316393 |

### 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 |
|-------------|-----------|------------------|--------------------------------------------------------|--------------------------------------------------------------------|--------|------------|
| Mmcl7-079for For | ACI MGI GTI TCV GAY GTH AAR CC | 709 | TRVSVDKYP | 23 bp | 32 |
| Mmcl7-1348rev Rev | GAY TTD GCI GCI GCI GGR TCV CCC AT | 1348 | MGDGPVAKS | 26 bp | 16 |
| Mmcl7-1447rev Rev | C ATI GCI GCI GCI GTR AGR CC | 1447 | GLTAAXM | 24 bp | 8 |
| Tar1-1453for For | GAR TTC CCI GAY GAY ATY GAR CT | 1453 | EPDEIELPH | 23 bp | 32 |
| Tar1-1459for For | CCI GAY GAR ATY GAR CII CAY CC | 1459 | PDEIELPH | 23 bp | 32 |
| Tar1-2308rev Rev | CTT RAA RTA ICC RTG IGT ICC | 2308 | GTHGYFK | 21 bp | 8 |
accumulation during biogenesis of the ribosome (Gelpin 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 (Aguilera et al. 2008). Strikingly, the authors report that most protein-coding genes commonly used in fungal systems, such as RPB1, RPB2, TEF1, 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, Eurotiumycetes, Leotiumycetes and Sordariomycetes. Primer sequences and annealing conditions are reported in Table 2 and 3. The locations of the fragments amplified by the new primers to sequences of Aspergillus nidulans mRNA (XM_658504 and XM_658778) as reference sequences. GenBank accession numbers XM_658504 and XM_658778). Saccharomyces cerevisiae, Cryptococcus neoformans, and Schizosaccharomyces pombe (Applied Biosystems), 2.5 μL buffer, 2 μL dNTPs, 2.5–4 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/Mcm7-1144rev 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 (Epitect). Alternatively, PCR products were cleaned using the BioLite 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 PRISM 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

| 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 % |

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

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

| Taxon | Mcm7 | Tsr1 |
|-------|------|------|
| Saccharomyccotina | NP_984137 | NP_984911 |
| Ashbya gossypii | XP_454998 | XP_454177 |
| Kluyveromycyes lactis | XP_570881 | NP_010223 |
| Yarrowia lipolytica | XP_501070 | XP_500653 |
| Taphrinomycotina | NP_595645 | NP_593391 |
| Schizosaccharomycyes pombe | NP_595645 | NP_593391 |

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.
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 × 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/Mcm7-709rev and Tsr1-506for/Tsr1-913rev. Sequence alignments were performed using Clustal X, and were manually adjusted using Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA). Phylogenetic analyses were performed using maximum parsimony (MP) and Bayesian inference (BI). For MP search, 1000 bootstrap replicates were performed using PAUP* version 4.0b10 (Swofford 2002) according to the following settings: stepwise addition, tree bisection reconnection (TBR) branch swapping and 1000 random addition replicates. For BI analyses we used MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001). We ran 8 parallel chains, each for 12 000 000 generations (burn-in = 3 000 000 generations) with increment of 100 trees per generation. The trees were sampled every 100 generations. The combined alignment dataset consisted of 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.

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.
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 Tsr1-1458for/Tsr1-2308rev. Tsr1-1453for is a modification of Tsr1-1458for 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 hydropervariable 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 out-group. The classes Sordariomycetes, Leotiomyces, Eurotiomyces and Lecanoromycetes are monophyletic and highly supported (PP ≥ 95). Lichinomycetes is only represented by a single species, Peitula 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 Eurotiomyces. Basal to this are Lichinomycetes and Leotiomyces. 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

### 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       | 188          |
| 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:

- Malcolmia (11 OTUs): 0.0065–0.2237
- Aspergillus (7 OTUs): 0.0230–0.2219
- Lecanora (6 OTUs): 0.0377–0.2756

Introns None some (length: 189–272 bp)

### 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 | None          | 0.195        | 0.216    |
| # 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       |
EUROTOMYCETES, EUROTOMYCETIDAE AND CHAETOThYRIONYCTIDAE form supported clades. We included multiple species/strains of the genera Aspergillus (7), Lecanora (6), and Malcomiella (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 Malcomiella 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 Saccharomyces, Taphrinomyces and Basidiomyces (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 Pezizomyces. Our studies confirm that Mcm7 and Tsr1 are suitable loci for the reconstruction of phylogenetic relationships among fungi (Aguilera 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 Saccharomyces, Taphrinomyces and Basidiomyces and found that the new primers can be used for these groups as well, possibly with slight sequence modifications.

Our analyses within Pezizomyces 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 (Aguilera et al. 2008). The fact that Homo sapiens sequences can be used as outgroups (Aguilera 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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