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Contributions of *rpb2* and *tef1* to the phylogeny of mushrooms and allies (Basidiomycota, Fungi)

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Abstract

A phylogeny of the fungal phylum Basidiomycota is presented based on a survey of 160 taxa and five nuclear genes. Two genes, *rpb2*, and *tef1*, are presented in detail. The *rpb2* gene is more variable than *tef1* and recovers well-supported clades at shallow and deep taxonomic levels. The *tef1* gene recovers some deep and ordinal-level relationships but with greater branch support from nucleotides compared to amino acids. Intron placement is dynamic in *tef1*, often lineage-specific, and diagnostic for many clades. Introns are fewer in *rpb2* and tend to be highly conserved by position. When both protein-coding loci are combined with sequences of nuclear ribosomal RNA genes, 18 inclusive clades of Basidiomycota are strongly supported by Bayesian posterior probabilities and 16 by parsimony bootstrapping. These numbers are greater than produced by single genes and combined ribosomal RNA gene regions. Combination of nrDNA with amino acid sequences, or exons with third codon positions removed, produces strong measures of support, particularly for deep internodes of Basidiomycota, which have been difficult to resolve with confidence using nrDNA data alone. This study produces

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strong boostrap support and significant posterior probabilities for the first time for the following monophyletic groups: (1) Ustilaginomycetes plus Hymenomycetes, (2) an inclusive cluster of hymenochaetoid, corticioid, polyporoid, Thelephorales, russuloid, athelioid, Boletales, and euagarics clades, (3) Thelephorales plus the polyporoid clade, (4) the polyporoid clade, and (5) the cantharelloid clade. Strong support is also recovered for the basal position of the Dacrymycetales in the Hymenomycetidae and paraphyly of the Exobasidiomycetidae.

Keywords: Basidiomycota; EF1-α; Fungi; Introns; RNA polymerase II; Systematics

1. Introduction

Although the number of multi-gene phylogenetic studies of fungi has increased over the past dozen years, the proportion of such studies that utilize multiple loci has not grown (Lutzoni et al., 2004). PCR primers, however, have been developed for single or low-copy nuclear protein-coding genes for fungal phylogenetic research across a broad spectrum of major fungal groups including the phyla Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota (Frøslev et al., 2005; Helgason et al., 2003; James et al., 2006; Kretzer and Bruns, 1999; Liu et al., 1999; Liu and Hall, 2004; Matheny et al., 2002; Matheny, 2005; O'Donnell et al., 1998; O'Donnell et al., 2001; Thon and Royse, 1999; Voigt and Wostemeyer, 2000, 2001). Of these, several genes have arisen as candidates to complement or rival the suite of nuclear and mitochondrial ribosomal RNA genes (rDNA) that have served as the platform for fungal molecular systematics for almost the past 20 years (Blanz and Unseld, 1987; Bruns et al., 1991; Bruns et al., 1998; Hibbett, 1992). Examples include *atp6*, a mitochondrial protein-coding gene (Kretzer and Bruns, 1999; Robison et al., 2001); *gapdh*, the glyceraldehyde 3-phosphate dehydrogenase gene (Den Bakker et al., 2004; Berbee et al., 1999; Smith, 1989); *rpb2*, which encodes the second largest subunit of RNA polymerase II (Liu et al., 1999; Liu and Hall, 2004; Matheny, 2005; Reeb et al., 2004; Wang et al., 2004; Zhang and Blackwell, 2002); *rpb1*, the gene that encodes the largest subunit of RNA polymerase II (Kropp and Matheny, 2004; Matheny et al., 2002; Matheny, 2005; Tanabe et al., 2002); β-tubulin encoding genes (Ayliffe et al., 2001; Begerow et al., 2004; Einax and Voigt, 2003; Keeling et al., 2000; Thon and Royse, 1999); actin encoding genes (Cox et al., 1995; Helgason et al., 2003; Tarka et al., 2000; Voigt and Wostemeyer, 2000); and *tef1*, which codes for the translation elongation factor 1-α (Baldauf and Palmer, 1993; O'Donnell et al., 2001; Rehner and Buckley, 2005; Roger et al., 1999). The development of alternative markers has been critical in the field of molecular systematics due to inherent limitations of taxon-rich single gene studies (Lipscomb et al., 1998; Rokas and Carroll, 2005; Rosenberg and Kumar, 2001) and limitations of rDNA regions in general (Aanen et al., 2001; Alvarez and Wendel, 2003; Buckler et al., 1997; Hasegawa and Hashimoto, 1993; Kauserud and Schumacher, 2003; Moncalvo et al., 2002; O'Donnell and Cigelnik, 1997; Okabe and Matsumoto, 2003; Sang, 2002; Stiller and Hall, 1999). Here, we present a multi-gene analysis of the Basidiomycota using *rpb2*, *tef1*, and nuclear 18S, 25S, and 5.8S rRNA genes.

The Basidiomycota (basidiomycetes) includes about 30,000 described species (Kirk et al., 2001) distributed across three classes: the Ustilaginomycetes (true smuts and allies and yeast forms), Urediniomycetes (rusts, anther smuts, and diverse yeasts), and Hymenomycetes (mushrooms and allies and other molds). A fourth class, the Wallemiomycetes (Matheny et al., 2006b; Zalar et al., 2005), was recently proposed to accommodate several unusual xerophilic molds. The phylum is important not just because of its taxonomic diversity but also due to various ecological roles as mutualists with vascular plants, bryophytes, green algae, and cyanobacteria; as decomposers primarily in terrestrial ecosystems; and as pathogens of plants, animals, and other fungi. When reproducing sexually, basidiomycetes typically produce four meiotic products (basidiospores) on the exterior of their meiosporangia (basidia). Not all basidiomycetes reproduce sexually, and some are better known by their asexual or anamorphic state (e.g., polyphyletic yeasts), including the medically important *Malassezia*, *Trichosporon*, *Pseudozyma*, and *Cryptococcus* species (Fell et al., 2000; Fell et al., 2001; Guého et al., 1998; Oberwinkler, 1987; Sampaio, 2004; Scorzetti et al., 2002; Sugita et al., 2003; Xu et al., 2000). The rusts alone constitute an order of about 7000 species that are obligate pathogens of ferns, conifers, and flowering plants (Cummins and Hiratsuka, 2003). One thousand five hundred species of smuts, bunts, and allies make up another diverse assemblage of pathogens of mostly flowering plants (Vánky, 2002). The mushroom-forming fungi include roughly 20,000 species (Kirk et al., 2001) that decompose lignin and/or cellulose, in addition to a diverse group of root symbionts that form mycorrhizas with trees, shrubs, and grasses. In addition, some basidiomycetes form diverse and unusual symbioses with insects (Aanen et al., 2002; Chapela et al., 1994; Henk and Vilgalys, 2006).

Few multi-gene phylogenies have addressed higher-level relationships in the Basidiomycota. Though some recent papers have utilized multiple regions of rDNA at a relatively broad taxonomic level (Binder and Hibbett, 2002; Binder et al., 2005; Larsson et al., 2004; Lutzoni et al., 2004), only one study (Begerow et al., 2004) has incorporated a protein-coding gene (β-tubulin) for exemplars across the three major lineages (classes) of the phylum. Previous phylogenetic assessments of the Basidiomycota relied principally on nuclear rDNA gene analyses, which have contributed

considerable insight. Nevertheless, many aspects of the Basidiomycota phylogeny have not been resolved with rDNA, especially at deeper nodes. Many 18S or 25S studies suggest that the Hymenomycetes and Ustilaginomycetes are sister groups but with weak or no bootstrap support (Berbee and Taylor, 1993; Gargas et al., 1995b; Swann and Taylor, 1995a; Swann and Taylor, 1995b; Prillinger et al., 2002; Tehler et al., 2003; Weiss et al., 2004a). A recent analysis of ultrastructural characters also supports this view (Lutzoni et al., 2004). However, other rDNA studies suggest alternative arrangements such as a sister relationship between Hymenomycetes and Urediniomycetes or an unresolved trichotomy between these three classes (Guého et al., 1989; Swann and Taylor, 1993; Berres et al., 1995; McLaughlin et al., 1995a; Nishida et al., 1995; Taylor, 1995; Begerow et al., 1997; Swann et al., 1999; Tehler et al., 2000; Bauer et al., 2001; Swann et al., 2001; Begerow et al., 2004). Gross relationships among major lineages within each class of Basidiomycota are also poorly resolved or not strongly supported by taxon-rich studies using nuclear and/or mitochondrial rDNA regions (Begerow et al., 2000; Binder and Hibbett, 2002; Binder et al., 2005; Hibbett and Thorn, 2001; Lutzoni et al., 2004; Moncalvo et al., 2002; Weiss et al., 2004a). The development of protein-coding genes as phylogenetic markers is thus required to resolve the phylogeny of the Basidiomycota. Availability of primers for orthologous and phylogenetically informative genomic regions should facilitate development of multi-gene genealogies, which provides another impetus for their use (Taylor et al., 2000; Taylor and Fisher, 2003; Xu et al., 2000).

We present a study of 160 species of Basidiomycota for which we have sequenced a 950–1200 bp fragment of *tef1* between exons 4 and 8 (Wendland and Kothe, 1997) and, in most instances, 2200 bp of *rpb2* between conserved domains 5 and 11 (Liu et al., 1999). Both genes are useful for analyses at high taxonomic levels because they are conserved at the amino acid level and easy to align, and because conserved primers sites have been identified (Liu et al., 1999; Rehner and Buckley, 2005). We explore the evolutionary dynamics of these genes at the level of coding sequences and gene structure with respect to spliceosomal intron placement and evaluate each gene phylogenetically. We also combine the protein-coding data with nuclear rDNA sequences (18S, 25S, and 5.8S) in an attempt to resolve nodes that have been unresolved or weakly supported by previous studies. The strongly supported, highly resolved phylogenetic trees resulting from these multi-gene analyses will be useful for revising the classification of Basidiomycota, but that is not the purpose of the present study. Instead, the taxon names used here are derived from prior works, and they include a mixture of formal Linnaean taxa and informal clade names. A revised classification of Basidiomycota (and other fungi) that draws heavily on the present study is in preparation and will be published elsewhere (see http://www.clarku.edu/faculty/dhibbett/AFTOL/AFTOL.htm).

2. Materials and methods

2.1. Taxon sampling

One hundred and forty sequences were produced for *tef1* and one hundred and thirty-two for *rpb2*. We sampled 146 taxa for the nuclear ribosomal DNA (nrDNA) tandem repeat, which includes the 18S, 25S, and 5.8S genes and the two internal transcribed spacers (S1). Taxa from the *tef1* and *rpb2* datasets were merged with those from a matrix of nrDNA for a final total of 146 taxa in the combined data set, including 12 Urediniomycetes, 6 Ustilaginomycetes, 125 Hymenomycetes, and 3 Ascomycota that were used for outgroup purposes. Exemplars were sampled from every subclass of Basidiomycota (Kirk et al., 2001) except for the Entorrhizomycetidae (Bauer et al., 2001), which is composed of a single genus, *Entorrhiza*, in the Ustilaginomycetes, and the Wallemiomycetes, which includes the sole genus, *Wallemia* (Zalar et al., 2005). Taxon sampling was extensive within the Hymenomycetidae or Homobasidiomycetes and allies. Exemplars have been selected to represent every major lineage of Binder et al. (2005), the most exhaustive sampling of Homobasidiomycetes to date, with exception of the *Gloeophyllum* clade (Thorn et al., 2000). The intent of our sampling strategy was to avoid missing data. However, of the taxa included in the combined data set, 11 lack *rpb2* sequences and 18 lack *tef1* sequences. Some *rpb2* sequences from domains 5 to 7 or 7 to 11 were merged in the combined matrix but omitted from the *rpb2* only analysis. Despite these character gaps, almost 90% of the taxa are represented by sequences from the five genes in the combined dataset. Methods of phylogenetic inference generally do not appear sensitive to moderate amounts of missing data for large alignments, thus, we include taxa for which a gene region is missing (Philippe et al., 2004; Wiens, 2006). For outgroup purposes we used the following sequences of Ascomycota: *Schizosaccharomyces pombe* (Z19136, AY046272, AY046223, D13337, NC_003421), *Saccharomyces cerevisiae* (J01355, AF548094, AJ544253, M15693, M10992), *Aleuria aurantia* AY544654, AY544698, AF072090, DQ247785, DQ466085, and *Neurospora crassa* (AY681158, AY046271, AY681193, AF107789, XM_329192).

2.2. DNA extraction

Sources of genomic DNA included fresh fruit bodies or spores, dried herbarium specimens, and live or lyophilized cultures. Most DNA extractions were performed at Clark University following the procedures detailed at http://www.clarku.edu/faculty/dhibbett/HibbettLab.protocols.htm, as well as those outlined in Hughes et al. (1998) and Hofstetter et al. (2002). Extractions at Clark University relied principally on the E.Z.N.A Fungal Miniprep Kit (Omega Bio-tek, Doraville, Georgia) or, in the case of rust and smut spores, the E.Z.N.A. Forensic DNA Extraction Kit (Omega Bio-tek). Genomic DNA was also isolated from several lyophilized cultures at the Centraalbureau voor

Schimmelcutures (CBS) in Amsterdam, the Netherlands using the FastPrep instrument (MPE Biochemicals, Irvine, California), following the protocols recommended by the manufacturer.

2.3. PCR, primers, and sequencing

Primer pairs PNS1/NS41 and NS19b/NS8 or NS51/ITS2 were used to obtain almost the entire 18S rRNA gene (ca. 1800 bp). Primers LR0R/LR7 (or rarely LR0R/LR5) were used to obtain the 5′ end of the 25S rRNA (ca. 1400 bp). ITS1F/ITS4 were used to amplify the 5.8S rRNA gene and the flanking internal transcribed spacers (ITS). Boundaries of the 5.8S gene and ITS regions were identified following Hibbett et al. (1995). ITS products varied in size due to differences in spacer lengths, but the 5.8S gene generally was about 158 bp in length. PCR conditions for 18S, 25S, and ITS are standard and follow protocols outlined in White et al. (1990). NS19b, NS19bc, NS6, and NS51; LR3R, LR3, LR22, LR16, and LR5; and ITS2 and 5.8SR were used as internal sequencing primers for 18S, 25S, and ITS, respectively. Nuclear rDNA PCR and sequencing primers are available in the following references (Vilgalys and Hester, 1990; White et al., 1990; Gardes and Bruns, 1993; Hibbett, 1996; O'Donnell et al., 1998) and online at http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm.

Final products of *tef1* ranged between 300 and 330 amino acid residues in length. The entire gene contains about 460 amino acids (Wendland and Kothe, 1997). A touchdown PCR was used with an initial annealing temperature of 60 °C but thereafter followed the protocol in Rehner and Buckley (2005). Primers 983F and 2218R amplified products between 900 and 1200 bp in size. Primers 2212R, 1953R, and EFcF were used for internal sequencing. 526F and 1567R were used to amplify *Bensingtonia yuccicola* with EFcF and EFiR as internal sequencing primers. Primers *tef1*F and *tef1*R (Morehouse et al., 2003) were used to obtain sequences of *Tricholoma myomyces*, *Asterophora lycoperdoides*, *Calocybe carnea*, and *Lyophyllum leucophaeatum*. Primer sequences for the protein-coding genes are provided in Table 1.

We sequenced about 2200 nucleotides of the *rpb2* gene with the following PCR primer pairs: f5F/b7R for Ustilaginomycetes; f5F/b7R2 for other basidiomycetes; and b6.9F/b11R1 following PCR conditions in Matheny et al. (2002). Primers b6R2, b6F, b8.2R, b10.9R, g7F, and f7cF were used for internal sequencing (Table 1). The region sequenced corresponds to conserved domains 5

Table 1
PCR and sequencing primers for *rpb2* and *tef1*

Primer name	Nucleotide sequence 5′–3′	Protein sequence	Source
bRPB2-3.1F	AARGTYYTKATYGCMCARGAGCG	TAQERMA	http://faculty.washington.edu/benhall/
gRPB2-6R	GCAGGRCARACCAWMCCCCA	WGM/LVCPA	Liu et al. (1999)
bRPB2-6R2	GGRCANACCATNCCCCARTG	HWGMVCP	This study
fRPB2-5F	GAYGAYMGWGATCAYTTYGG	DDRDHFG	Liu et al. (1999)
fRPB2-5R	CCRAARTGATCWCKRTCRTC	DDRDHFG	Liu et al. (1999)
bRPB2-7R	GAYTGRTTRTGRTCRGGGAAVGG	PFPDHNQS	http://faculty.washington.edu/benhall/; Matheny (2005)
bRPB2-7R2	ACYTGRTTRTGRTCNGGRAANGG	PFPDHNQ	This study
bRPB2-6F	TGGGGYATGGTNTGYCCYGC	WGMVCPA	http://faculty.washington.edu/benhall/; Matheny (2005)
bRPB2-6.3F	GTYATYGGTGTNTGGATGGG	WGMVCPA	Liu et al. (1999)
bRPB2-7.1R	CCCATRGCYTGYTTMCCCATDGC	AMGKQAMG	http://faculty.washington.edu/benhall/; Matheny (2005)
bRPB2-6.9F	TGGACNCAYTGYGARATYCAYCC	WTHCEIHP	This study
bRPB2-11R1	TGGATYTTGTCRTCCACCAT	MVDDKIH	This study
bRPB2-10.9R	GTRAASGGYGTGGCRTCYCC	GDATPFT	http://faculty.washington.edu/benhall/
fRPB2-7cF	ATGGGYAARCAAGCYATGGG	MGKQAMG	http://faculty.washington.edu/benhall/
bRPB2-8.2R	CTNCGGAANAGRCCRCGRTC	DRGLFRS	This study
gRPB2-11bR	CAATCWCGYTCCATYTCWCC	GEMERD	Liu et al. (1999)
EF1-526F	GTCGTYGTYATYGGHCAYGT	VVVIGHV	http://ocid.nacse.org/research/aftol/primers.php
EF1-1567R	ACHGTRCCRATACCACCRATCTT	KIGGIGTV	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley (2005)
EF1-cF	ATYGCYGCNGGTACYGGYGARTTCGA	IAAGTGEFE/D	http://ocid.nacse.org/research/aftol/primers.php
EF1-dF	AAGGAYGGNCARACYCGNGARCAYGC	KDGQTREHA	http://ocid.nacse.org/research/aftol/primers.php
EF1-iR	GCRTGYTCNCGRGTYTGNCCRTC	DGQTREHA	http://ocid.nacse.org/research/aftol/primers.php
EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	APGHRDFI	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley (2005)
EF1-2218R	ATGACACCRACRGCRACRGTYTG	QTVAVGVI	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley (2005)
EF1-2212R	CCRACRGCRACRGTYTGTCTCAT	MRQTVAVG	http://ocid.nacse.org/research/aftol/primers.php; Rehner and Buckley (2005)
EF1-1953R	CCRGCRACRGTRTGTCTCAT	CHTAHIAC	http://ocid.nacse.org/research/aftol/primers.php
tef1F	TACAARTGYGGTGGTATYGACA	YKCGGD	Morehouse et al. (2003)
tef1R	ACNGACTTGACYTCAGTRGT	TTEVKSV	Morehouse et al. (2003)

through 11 in Liu et al. (1999). About 30 of the 132 *rpb2* sequences span only the region between domains 5 and 7, which is about 1100 bp (350 amino acid residues) in length. We also sequenced a subset of samples between domains 3 and 5 to score taxa for the presence of *rpb2* introns 1 and 2. To obtain this region, we used primers b3.1F/g6R or b6R2 and internal sequencing primer f5R.

PCR products were typically cleaned with a QIAquick PCR purification kit (QIAGEN, Valencia, California) following the protocol of the manufacturer but with a final elution between 20 and 30 μl. Some products were purified with Pellet Paint NF co-precipitant (Novagen, Darmstadt, Germany) following the ethanol precipitation protocol of the manufacturer or a Geneclean kit (Bio101 Systems, Carlsbad, California). Sequence reactions were prepared in 10 μl volumes with 1.0 μl ABI Prism Terminator BigDye 1.1 (Applied Biosystems, Foster City, California), 1.0 μl DNA template, 1.0 μl of 2 μM primer, 1.5 μl 5× buffer, and 5.5 μl of water. Reactions were shipped in sealed 96-well plates, purified, and sequenced on an ABI 3700 automated DNA sequencer at the DNA sequencing facility of the Duke University Biology Department, R. Vilgalys laboratory.

Small percentages of *rpb2* (11%), *tef1* (14%), and ITS (3%) products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, California) due to intron or spacer length heterogeneity or due to faint or multiple PCR bands. PCR products were first cleaned and then ligated to a pCR 2.1-TOPO plasmid vector and inserted into One Shot MAX efficiency DH5α-T1 chemically competent cells of *Escherichia coli*. Between 50 and 100 μl of cells in liquid SOC medium were incubated for 18 h at 37 °C on Luria–Bertani (LB) agar prepared with 50 μg/ml of kanamycin and 55 μl of 50 mg/ml X-gal in dimethylformamide. Colonies were screened by PCR with primers M13F (5′-GTAAAACGACGGCCAGTGAA-3′) and M13R (5′-CAGGAAACAGCTATGACCAT-3′). Up to three colonies with an expected-size insert were chosen for sequencing.

2.4. Alignments and phylogenetic analyses

ABI chromatograms were assembled into contigs using Sequencher 4.1 software (GeneCodes Corp., Ann Arbor, Michigan). Nucleotide and *rpb2* and *tef1* amino acid sequences were initially aligned with Clustal X 1.83 (Thompson et al., 1997) or assembled manually in MacClade 4.0 (Maddison and Maddison, 2000). Spliceosomal intron regions were exluded from analyses, in addition to positions judged too ambiguous to align. Alignments are available on the AFTOL database (http://aftol.biology.duke.edu/pub/alignments/download_alignments).

Bayesian and maximum parsimony (MP) analyses were conducted for separate and combined data sets. Both protein-coding nucleotides and amino acid sequences were analyzed. Analyses of combined nucleotide data entailed inclusion and exclusion of third codon positions. Bayesian analyses were implemented using mixed models with MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) and the parallel version of MrBayes 3.1.1 (Altekar et al., 2004) on a Linux cluster with AMD Opteron 246 processors. MP analyses were done in PAUP* (Swofford, 2003). A General-Time-Reversible (GTR) model and parameters for rate heterogeneity were estimated for each gene region and codon position. Spliceosomal introns were excised from the *tef1* and *rpb2* nucleotide datasets to facilitate Bayesian analyses that entailed partitioning the data by codon position. We set "aamodelpr" in MrBayes to "mixed", whereby a mixture of models with fixed rate matrices for amino acid sequences was evaluated. We also invoked rate heterogeneity parameters for the amino acid matrices by setting "rates = invgamma". Bayesian analyses of nucleotide data were run between two and five million generations using four chains. Two million generations were run on amino acid data. Stationary phases of likelihood scores and chain swapping were observed in a spreadsheet program. Bayesian posterior probabilities (PP) were determined by computing majority rule consensus trees using the set of trees that reached stationary phase. A probability of 0.95 was considered significant.

Nodal support under parsimony conditions was determined by non-parametric bootstrapping (Felsenstein, 1985). MP bootstrapping used equal weights and entailed 1000 replicates using the SPR or TBR algorithms, 10 random addition sequences, holding one tree per step during stepwise addition, and setting MulTrees 'off' (DeBry and Olmstead, 2000; Salamin et al., 2003). A bootstrap proportion (BP) above 70% was considered a significant value (Hillis and Bull, 1993).

We compared nodes between gene trees using MP bootstrap scores to assess strongly supported conflicts between clades. Conflict was noted if a clade was monophyletic with significant bootstrap support for one gene but non-monophyletic with significant support from another gene (De Queiroz, 1993; Lutzoni et al., 2004). The five datasets were assembled into a single nexus file for combined analysis after conflict assessment.

2.5. Evaluation of codon positions

Twenty-seven sequences were selected from *tef1* and *rpb2* for the same set of taxa for pairwise distance comparisons in PAUP* to assess saturation of codon positions. Three classes of Basidiomycota were represented in the comparisons. Uncorrected ("p") distances were plotted against Kimura-2 parameter (K2P) corrected distances for first, second, and third codon positions. Superimposed substitutions (saturation) were inferred to have occurred if an increase in corrected distances outpaced uncorrected distances. Unsaturated positions exhibit distances that increase in a linear fashion. Saturated distances show a characteristic plateau. Overall mean K2P distances were calculated by codon position for each gene in MEGA 3.1 (Kumar et al., 2004).

2.6. Phylogenetic evaluation of spliceosomal intron placement

39 taxa were chosen to evaluate the phylogenetic utility of spliceosomal intron positions in *rpb2* and *tef1*. Taxon sampling was designed to mitigate against missing data and to be taxonomically inclusive for the phylum. In some instances, data from different species were merged to produce composite taxa. This was done for *Hydnum* (*H. albomagnum* and *H. repandum*), *Dacrymyces* (*Dacrymyces* sp. and *D. chrysospermus*), and *Endocronartium* (*E. harknessii* and *Phragmidium* sp.). Presence of introns was coded as 1 and their absence as 0, similar to gap characters, with a variable ascertainment bias (Ronquist et al., 2005). The matrix was analyzed in MrBayes using "lset coding = variable" and a simple F81-like model for binary data. The matrix was analyzed with independent runs up to 500,000 generations. The intron data set was also analyzed in PAUP* using NJ standard total character differences and bootstrapped 1000 times.

3. Results

3.1. Sequences produced in this study

639 new sequences were produced or, in a few cases, extended for this study. We generated 135 *rpb2* seqences, 128 *tef1* sequences, 122 18S sequences, 112 25S sequences, and 132 ITS sequences including the 5.8S gene. We combined these with 104 previously published *rpb2*, *tef1*, and nrDNA sequences available from GenBank or genome projects (S1). In total about 750 sequences were analyzed.

3.2. Gene duplications, polymorphisms, alleles, and pseudogenes

No gene duplications were inferred for *rpb2*, and generally the number of non-synonymous polymorphic sites in individuals was nil or extremely low. The number of silent polymorphic sites was generally less than 1.0% of the nucleotide sequence length. Direct sequencing and/or cloning of some individuals suggest a strong degree of allelism or possible hybridization in very few instances. For example, a crust fungus, *Stereum hirsutum* (russuloid clade), shows an unusual high degree of polymorphism with 69 polymorphic sites out of 2630 characters (2.6% of all positions). Only two of the polymorphisms code for different amino acids. Conversely, very few polymorphisms were observed in the *tef1* sequence of *S. hirsutum*. No pseudogenes of *rpb2* were observed.

Multiple copies of *tef1* are present in *Auricularia* (Auriculariales), *Heterobasidion* (russuloid clade), and *Polyozellus* (Thelophorales). Duplications of *tef1* are not widespread, however. Pseudogenes, sequences in which the reading frame is disrupted by an insertion or deletion, were identified for *Hydnellum geogenium* (Thelephorales) and the agaric *Mycetinis alliaceus*. An unusual degree of polymorphism was noted in the resupinate *Tulasnella pruinosa* (10 non-synonymous polymorphic sites). The number of silent polymorphic sites in individuals was very low, ranging between zero and eleven positions. Of the 140 *tef1* sequences presented here, none are similar to the EF-like sequences reported by Keeling and Inagaki (2004), which are characterized by unique amino acid insertions.

Intron length heterogeneity within individuals was observed occasionally in both *rpb2* and *tef1*. Typically, a single bp was inserted or deleted but longer indels up to 3 bp in length were recorded. In contrast, spacer length heterogeneity of the internal transcribed spacers (ITS) was observed in about 20% of the taxa sequenced. In four cases, indels in both spacers necessitated cloning in order to obtain the entire 5.8S sequence (e.g., *Coltricia perennis*, *Cotylidia* sp., *Camarophyllus borealis*, *Laccaria ochropurpurea*, and other unpublished sequences). The ITS regions of a California isolate of *Hebeloma velutipes* contained 14 polymorphic positions, which affirms the widespread polymorphism reported for this species by Aanen et al. (2001).

3.3. Distribution of spliceosomal introns in tef1 and rpb2

Fig. 1 illustrates examples for *tef1* and *rpb2* amino acid sequences and the distribution of spliceosomal introns. Supplementary files S2 and S3 score the presence and absence of 22 introns in *tef1* and 7 introns in *rpb2*, respectively, for every taxon sampled. Most of the introns range between 45 and 70 bp in length, though some *rpb2* introns of Ustilaginomycetes and Urediniomycetes (*Ustilago maydis*, *Pseudozyma flocculosa*, *Cintractia sorghi-vulgaris*, and *Rhodotorula hordea*) are longer ranging between 106 and 362 bp.

Intron position in *tef1* often appears lineage-specific (S2). For instance, two orders of Urediniomycetes, the Platygloeales and Uredinales, share several uniquely positioned introns. The class Hymenomycetes is characterized by possession of intron 5, except for the Dacrymycetales, which lacks introns altogether. Intron 5 is the only *tef1* intron present in the Tremellomycetidae sampled (*Cryptococcus*). Species of Homobasidiomycetes are characterized by possession of introns 2 and 11. Only two major homobasidiomycete lineages are missing either of these introns (trechisporoid clade and the Auriculariales). All members of the Agaricaceae (*Coprinus*, *Lycopderon*, *Chlorophyllum*, and *Macrolepiota*) share intron position 19, whereas only members of the Clavariaceae possess intron 21.

Homoplasy of intron positions is evident, however. For example, the euagarics clade and *Leucosporidium scottii* (Urediniomycetes) share intron position 19. Intron loss appears to characterize several species and monophyletic groups. Intron 11, for example, shared by many euagarics, is absent from *Agrocybe praecox*, *Bolbitius vitellinus*, Psathyrellaceae, and Inocybaceae. *Coprinus comatus*, *Peniophora nuda*, and *Pycnoporus* sp. appear to have lost intron 5 independently, unlike most hymenomycete lineages.

a *tef1* intron distribution

IKN<1>MITGTSQADCAILIIATGIGEFEAGISKDGQTREHALLAFTLGVRQLIVACNKMDTCK<2>WSED<3>RF
NEIVKET<4>NGFIKKVGYNPKAVPFVPISGWHGDNMLEETTN<5>MPWYK<6>GWTKE<7>TKSGVSKGKTLLEA
IDASRPPTRPTDKPLRLPLQ<8>DVYKIGGIGTVPVGRVETGVIKA<9>GMVVKFAPTNVTTEVKSVEMHHEQIPE
GLPGDNVGF<10>N<11>VKNVSIKDIRRGNVCGDSKNDPPMEAASFNAQVIVLNHPGQIGAGYTPV<12>LD<13>
CHTA<14>HIACKFS<15>ELIEKIDRRTGKVMEAAPKF<16>VKSGDAAIVK<17>LVSQK<18>PL<19>CV
<20>ETYAD<21>YPPLGRFAVRD<22>MR

b *rpb2* intron distribution

QERMATNHVYVFAKAQPAPINFLAEIRSLVKKGGKTISQFQVKMFHRHQERS<1>LGNVMKATIPYIKVDIPIWVV
FRALGVISDRDILEHICYDMQDAQMLEMLKPCIDDGFVIQDRE<2>VALDFIGNRGTTTGLSRERRIRYAQEILQK
EMLPHVSMSEGSESKKAYFFGYMIHRLLLAALERRELDDRDHLGKNRLDLAGPLLANLFRMLFRKFNRDVYRYLQK
<3>CVETHKEFNVSVGVKHQTITNGLKYSLATGNWGDQKKSMSSKAGVSQVLNRYTYASTLSHLRRCNTPLGREGK
IAKPRQLHNTHWGMVCPAETPEGQACGLVKNLALMSCISVGSYSAPVIEFLEEWGLETLEENAHSATPCTKVFVNG
VWMGVHRDPANLVKTIKKLRRKDDISPEVSVVRDIREKELRLYTDAGRVCRPLFIVENQQLVLQKKHIKWLVRGTN
DDGEEYKWEQLIKGGVIELLDAEEEETVMISMTPEDLENSRLQQSGVDIHANDGDFDPAARLKAGINAHTWTHCEI
HPSMILGICASIIPFPDHNQ<4>SPRNTY<5>QSAMGKQA<6>MGIYLTNFLIRMDTMANILYYPQKPLATTRSME
YLKFRELPAGQNAIVAILCYSGYNQEDSVIMNQSSIDRGLFRSIYYRSYMDVEKNNGIHSMEVFEKPMRESTLRMK
HGTYDKLEDDGLIAPGTGVVGEDIIIGKTAPIPPESEELGQRTRMHTRRDVSTPLKNTERGIVDQVLVTTNYEGQK
FVKVRVRSTRIPQIGDKFASRHGQKGTIGITYRQEDMPFTCEGIVPDIIINPHAIPSRMTIGHLVECLLSKVATLI
GNEGDATPFTDLTVESVSTFLRQKGYQSRGLEVMYHGHTGRKLQAQVYLGPTYYQRL<7>KHMVDDKIH

Fig. 1. Basidiomycete amino acid sequence and intron positions in *tef1* and *rpb2*. Numbers refer to intron positions encountered proceeding upstream. (a) *tef1* amino acid sequence of *Cryptococcus neoformans* between primer sites 983F and 2218R. (b) *rpb2* amino acid sequence of *Armillaria mellea* between conserved domains 3 and 11.

Intron position in *rpb2* is more conserved than in *tef1*. The *rpb2* introns 1, 2, 3, and 4 are shared by many species of Hymenomycetidae and Urediniomycetes (S3). At least two derived clades of Hymenomycetes (euagarics and Boletales) lack intron 2. Exemplars of the Tremellomycetidae share possession of intron 3 only. Species of Ustilaginomycetes lack introns shared by other basidiomycetes, with the exception of *Tilletiaria anomala* (Georgefischeriales), in which only intron 4 is present. In contrast, members of the Ustilaginales and *R. hordea* possess unqiue intron positions in very conserved regions of the gene between domains 7 and 11.

3.4. Basidiomycete tef1 gene phylogeny

The nucleotide data matrix contains 995 characters, of which 535 (54%) are parsimony-informative. The protein data matrix includes 330 amino acid positions; 128 (39%) of these are parsimony-informative. A 50% majority-rule consensus tree from a Bayesian analysis of nucleotides is shown (Fig. 2). Nucleotide posterior probabilities are drawn from the last 12,000 trees sampled from a run of five million generations. Amino acid posterior probabilities were calculated from 10,966 trees sampled from two million generations. The model best-fit to the amino acids by MrBayes is a Rtrev model (Dimmic et al., 2002). Amino acid data did not significantly support any poorly supported nodes produced by the nucleotide analysis.

Some major groups of Basidiomycota are recovered as monophyletic by *tef1* nucleotide sequences with significant posterior probabilities. These include the Hymenomycetes, Hymenomycetidae, Auriculariales, Dacrymycetales, and Thelephorales. All five sequences of the Ustilaginomycetes form a monophyletic group. Nucleotide sequences of *tef1* also support the monophyly of the Uredinales and its sister position to the Platygloeales. Protein sequences recover the monophyly of the Ustilaginomycetes, Hymenomycetidae, Thelephorales, and Dacrymycetales (Table 2). At lower taxonomic levels, nucleotide sequences recover some families such as the Ustilaginaceae, Gomphaceae, Hymenochaetaceae, and Omphalotaceae (euagarics). However, *tef1* resolution is poor overall, and the number of branches that receive significant support values is low. The backbone of the Hymenomycetidae is poorly supported (Fig. 2), and third positions are saturated (S4).

Several clades that are strongly supported as monophyletic by rDNA (Binder and Hibbett, 2002; Hibbett and Thorn, 2001) are not monopohyletic based on *tef1*. These include groups of Homobasidiomycetes such as the hymenochaetoid, euagarics, gomphoid-phalloid, and russuloid clades. Monophyly of a heterobasidiomycete order, the Sebacinales, is also not supported by *tef1*. Nucleotide data suggest the paraphyly of the Urediniomycetes (rusts, various yeasts, and allies) and an inclusive Urediniomycetes and Ustilaginomycetes clade. Towards the tips of the *tef1* tree, closely related genera or species (e.g., Agaricaceae, Inocybaceae, Physalacriaceae, Lyophylleae) are similarly not supported as monophyletic using either nucleotide or amino acid data despite the findings of previous studies.

3.5. Basidiomycete rpb2 gene phylogeny

The phylogeny of the Basidiomycota inferred from *rpb2* nucleotides is well-resolved by Bayesian inference with similar clades recovered by rDNA phylogenies (Fig. 3). Posterior probabilities of nucleotide and amino acid data are drawn, respectively, from 8000 and 3730 trees. 132 taxa were sampled that include 2157 nucleotide sites (1357

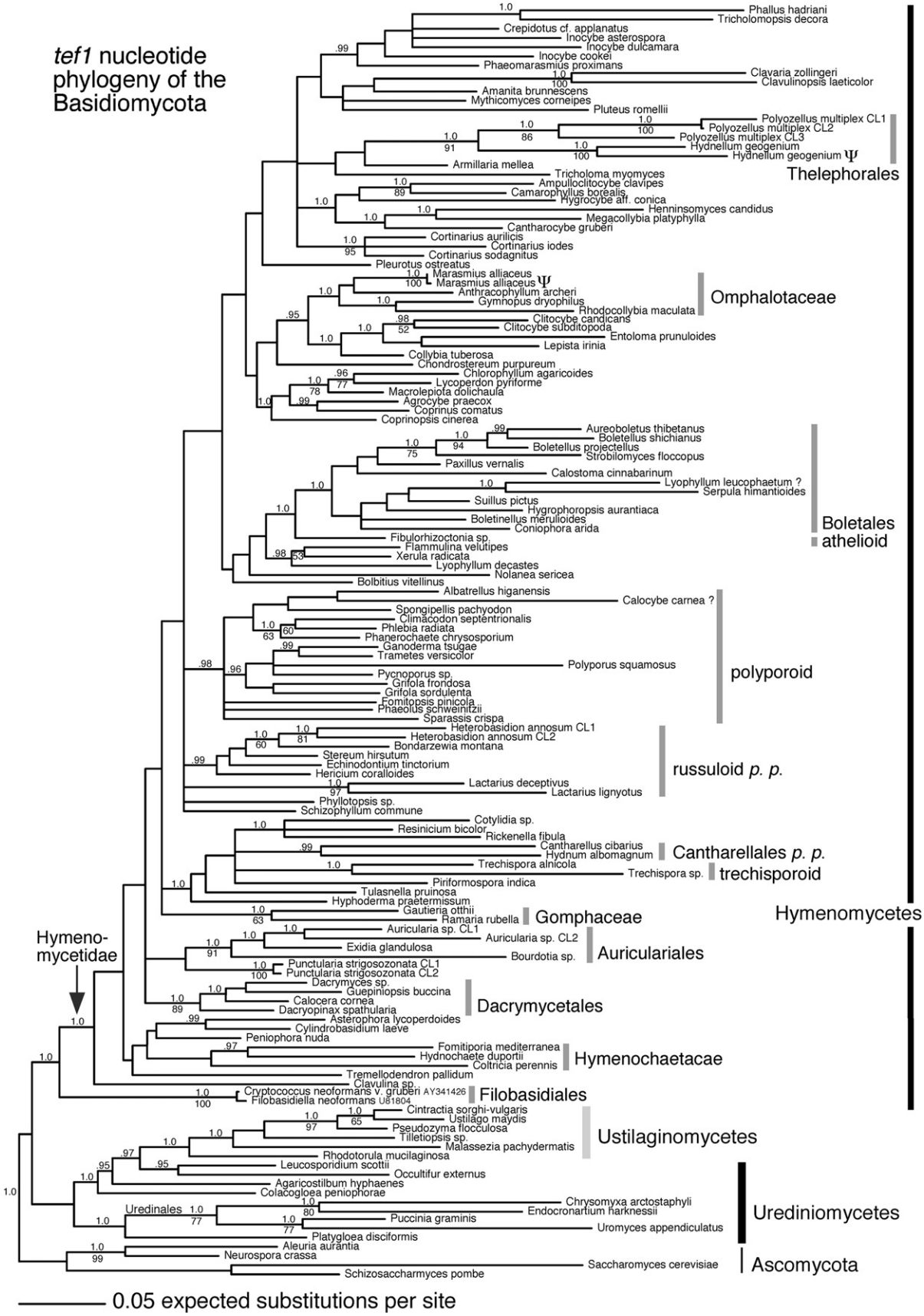

Fig. 2. Phylogeny of the Basidiomycota based on nucleotide sequences of *tef1*. The tree is a Bayesian 50% majority-rule consensus tree and produced from a dataset of 140 sequences and 995 nucleotide characters. The psi symbol (ψ) indicates a pseudogene sequence. Values above and below branches are posterior probabilities ($\geqslant 0.95$) and MP bootstrap ($\geqslant 50\%$) values, respectively.

Table 2
BP/PP support values for major clades of Basidiomycota

Clade	*tef1* Exons	*tef1* Amino acids	*rpb2* Exons	*rpb2* Amino acids	nrDNA	Combined nucleotides BP[a]/BP[b]/PP[c]	Combined rDNA and amino acids (BP)
Hymenomycetes	nm[d] /nm	<50/nm	nm/<.95	<50/nm	91/1.0	nm/81/1.0	95
Hymenomycetidae	nm/1.0	<50/<.95	nm/nm	88/1.0	69/<.95	69/78/1.0	96
Auriculariales	91/nm	nm/nm	—[e]	—	89/1.0	100/96/1.0	90
Boletales	nm/nm	<50/nm	<50/1.0	61/<.95	100/1.0	100/100/1.0	100
Cantharelloid	nm/nm	nm/nm	<50/<.95	<50/1.0	<50/nm	63/87/1.0	69
Corticioid	—	—	62/1.0	71/1.0	100/1.0	100/100/1.0	100
Dacrymycetales	89/1.0	63/1.0	100/1.0	100/1.0	100/1.0	100/100/1.0	100
Euagarics	nm/nm	nm/nm	nm/1.0	<50/nm	70/<.95	<50/80/1.0	76
Gomphoid-phalloid	nm/nm	50/nm	75/1.0	86/1.0	100/1.0	100/100/1.0	100
Hymenochaetoid	nm/nm	nm/nm	nm/nm	nm/nm	82/1.0	<50/90/1.0	59
Polyporoid	nm/nm	nm/nm	nm/1.0	<50/.95	<50/1.0	nm/83/1.0	85
Russuloid	nm/nm	nm/nm	<50/1.0	66/nm	90/1.0	89/100/1.0	99
Sebacinales	nm/nm	nm/nm	99/1.0	100/1.0	100/1.0	100/100/1.0	100
Thelephorales	91/nm	92/<.95	100/1.0	99/1.0	100/1.0	100/100/1.0	100
Tremellomycetidae	—	—	100/1.0	100/1.0	99/1.0	100/100/1.0	100
Urediniomycetes	nm/nm	<50/<.95	70/1.0	89/1.0	100/1.0	99/100/1.0	100
Urediniomycetidae	nm/1.0	<50/<.95	72/1.0	94/1.0	100/1.0	100/100/1.0	100
Uredinales	77/1.0	nm/nm	88/1.0	92/1.0	100/1.0	100/100/1.0	100
Platygloeales	—	—	<50/<.95	98/1.0	100/1.0	99/100/1.0	100
Microbotryomycetidae	nm/nm	nm/nm	nm/1.0	nm/<.95	nm/1.0	nm/nm/1.0	nm
Agaricostilbomycetidae	nm/nm	nm/nm	—	—	100/1.0	100/100/1.0	100
Ustilaginomycetes	<50/1.0	63/1.0	99/1.0	99/1.0	95/1.0	100/100/1.0	100
Ustilaginomycetidae	97/1.0	68/1.0	100/1.0	100/1.0	100/1.0	100/100/1.0	100
Exobasidiomycetidae	nm/nm	nm/nm	nm/nm	nm/nm	nm/nm	nm/nm/nm	nm
Total of Significantly Supported Clades	5/6	1/3	10/16	13/15	19/20	16/22/23	20

Note. Gene regions that produce either a significant BP (⩾70%) or PP (⩾0.95) support value have light gray shaded cells. Gene regions that produce both significant BP/PP values have dark gray shaded cells.

[a] BP analysis with third codon positions included.
[b] BP analysis with third codon positions excluded.
[c] Bayesian analysis with third codon positions included.
[d] nm = not monophyletic.
[e] Monophyly not possible to test.

(63%) parsimony-informative) and 792 amino acids (371 (47%) parsimony-informative). The protein data set is best modeled by the JTT model (Jones et al., 1992). Like *tef1*, third codon positions are saturated (S4). The *rpb2* gene is more variable than *tef1* when mean pairwise K2P distances per codon position and percentages of parsimony-informative sites are compared. The following overall means of K2P distances were recorded by codon position: *tef1*/*rpb2* first (0.087/0.184), *tef1*/*rpb2* second (0.060/0.095), *tef1*/*rpb2* third (0.654/1.459). *rpb2* produces 9 and 8% more parsimony-informative nucleotide and amino acid positions, respectively.

At deep taxonomic levels, each class of Basidiomycota is monophyletic based on *rpb2* although their relationship to each other is not strongly supported. The Dacrymycetales (Hymenomycetidae) clusters with the Tremellomycetidae in the nucleotide tree but without strong support. The Hymenomycetidae, with Dacrymycetales at a basal position, is monophyletic in the amino acid tree (not shown) with significant support values. The Auriculariales, a heterobasidiomycetous order, is nested among the Homobasidiomycetes. One polyphyletic group at roughly the ordinal level includes the hymenochaetoid clade represented here by a xanthochroic group of Hymenochaetaceae (*Coltricia* and *Fomitoporia*) and another group of non-xanthochroic taxa including *Resinicium*, *Rickenella*, *Cotylidia*, and *Hyphoderma*. Both nucleotide and amino acid sequences support the non-monophyly of the hymenochaetoid clade, a result consistent with *tef1*.

Several clades that have been difficult to resolve with significant support by rDNA studies are strongly supported by *rpb2*. These include the polyporoid clade and a combined euagarics, bolete, and atheloid clade. Sequences of *rpb2* also unite Malasseziales (Exobasidiomycetidae) and Ustilaginales (Ustilaginomycetidae), and Platygloeales and Uredinales. Additional strongly supported groups include the Cantharellales (but excluding species of *Tulasnella*), Dacrymycetales, russuloid clade, and gomphoid-phalloid clade. The cantharelloid clade is monophyletic and excludes the Sebacinales.

3.6. Basidiomycete nuclear rDNA phylogeny

The nrDNA phylogeny is based on three nuclear ribosomal RNA genes—25S, 18S, and 5.8S ribosomal RNAs. One hundred and forty-six taxa were sampled in the data

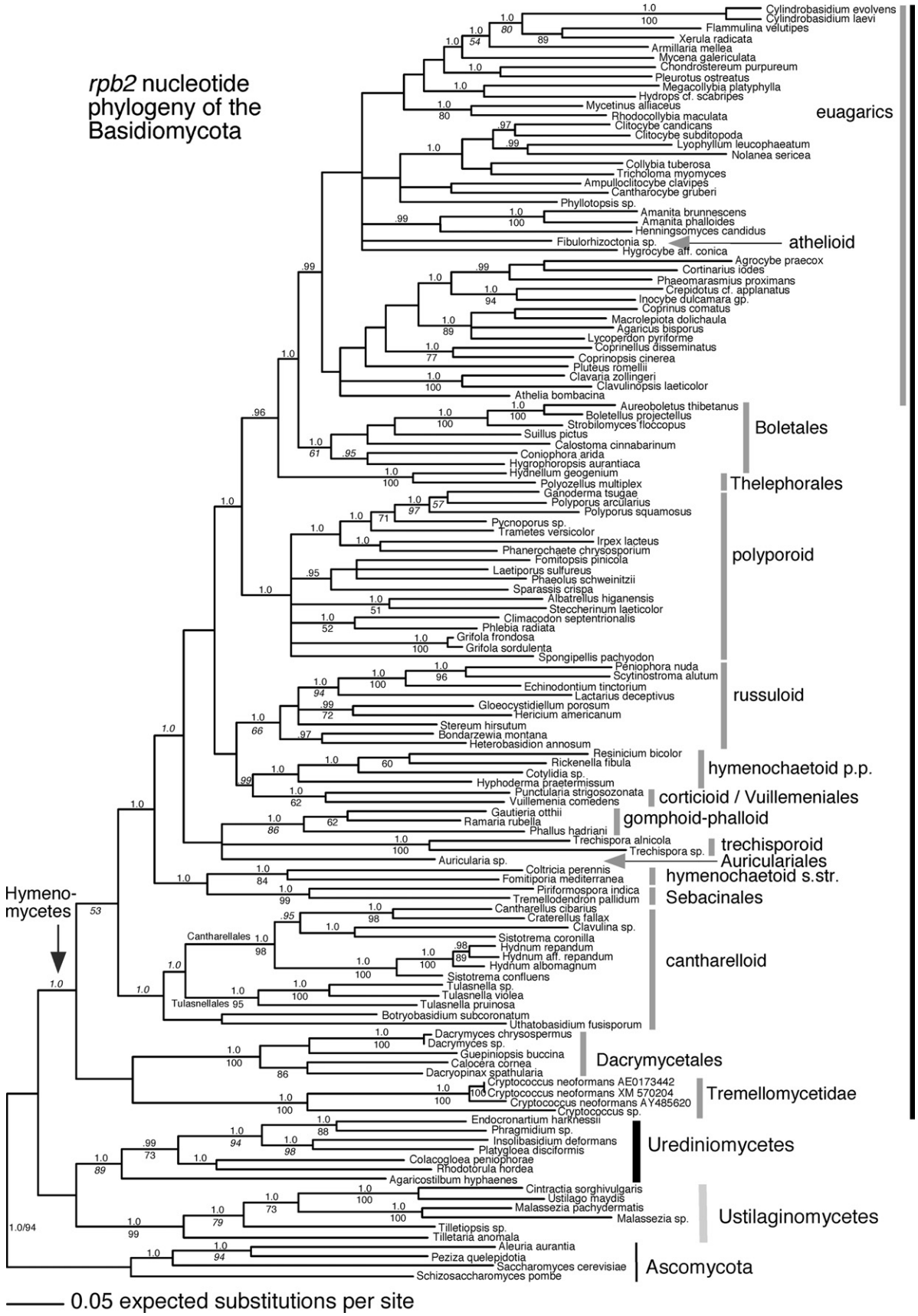

Fig. 3. Phylogeny of the Basidiomycota based on nucleotide sequences of *rpb2*. The tree is a Bayesian 50% majority-rule consensus tree and produced from a dataset of 132 sequences and 2157 characters. Posterior probabilities $\geqslant 0.95$ are indicated above branches and MP bootstrap values $\geqslant 50\%$ below. Italicized support values are produced from the analysis of amino acid sequences.

set, which includes 3457 sites. Of these, 1456 (42%) are parsimony-informative. Table 2 shows various support values for major clades of Basidiomycota. The nrDNA tree is well-resolved and many groups are significantly supported. The class Ustilaginomycetes is suggested as sister to the Hymenomycetes but without significant support (Table 2). Strong support values are produced for a monophyletic Hymenomycetes (91% BP, 1.0 PP) and a significant posterior probability for the Homobasidiomycetes (0.95 and excluding the Auriculariales), unlike *tef1* and *rpb2*. The hymenochaetoid clade is strongly supported with high bootstrap and posterior probability, in contrast to *tef1* and *rpb2*. The eugarics, Boletales, and russuloid clades receive higher support values than when analyzed by the protein-coding genes separately (Table 2). The monophyly of the Boletales and athelioid clade is another srongly supported nrDNA clade.

3.7. Assessments of intergene conflicts

Only two cases of strongly supported conflict were observed between nrDNA and the protein-coding genes, and these occur at relatively low-taxonomic levels. nrDNA unites the resupinate *Tulasnella* spp. (Tulasnellales) with the chanterelle groups *Cantharellus* and *Craterellus* (Cantharellaceae) with significant support values. Yet, *Tulasnella* is excluded from clades containing *Cantharellus*, *Craterellus*, and other taxa by *rpb2*. The *tef1* Bayesian analysis also conflicts strongly with the nrDNA result. Similarly, nrDNA strongly unites the bolete *Strobilomyces* and the boletoid gasteromycete, *Calostoma*. In contrast, both *rpb2* and *tef1* oppose this grouping with significant support values. Because the conflicts concern lower level taxonomic arrangements, we chose to include these data in combined analyses.

3.8. Combination of multiple protein-coding gene sequences with rDNA regions supports 18 inclusive clades of Basidiomycota

The analysis of combined nucleotide data includes 6603 sites, of which 3318 (50%) are parsimony-informative. Combined nucleotides of nrDNA and amino acid sequences of *rpb2* and *tef1* produce 4511 characters for analysis. 1954 (43%) of these are parsimony-informative. When third codon positions are excluded, 5555 total characters are available, of which 2307 (42%) are parsimony-informative. Under parsimony 64 nodes (44%) are strongly supported when nrDNA data are combined with exon sequences including third positions, 74 nodes (51%) are strongly supported when amino acid data are combined with nrDNA, and 85 nodes (59%) receive strong parsimony bootstrap support when all nucleotide data are combined minus third codon positions. Eighteen inclusive clades of basidiomycetes, discussed below, receive significant PP, and almost all receive high bootstrap support (>70%) when multiple protein-coding genes are combined with multiple rDNA regions (Fig. 4). Twelve of these inclusive clades are strongly supported in combined analyses that treat the protein data as amino acid sequences or exclude third codon positions (Table 3). BP (including and excluding third codon positions) and PP support values are shown for each of the clades in Table 3.

3.8.1. Urediniomycetes

Twelve taxa of the Urediniomycetidae, Microbotryomycetidae, and Agaricostilbomycetidae form a monophyletic group. This class is not monophyletic based on single gene treatments using β-tubulin protein sequences (Begerow et al., 2004) and ca. 600 bp fragments of 25S rDNA (Weiss et al., 2004a). Analysis of 25S and 18S sequences for 174 diverse urediniomycete taxa supports the monophyly of Urediniomycetes (Aime et al., 2006). Protein sequences of *tef1* and protein and nucleotide sequences of *rpb2* also support a monophyletic Urediniomycetes. The 12 taxa sampled here form subclades consistent with the subclasses Urediniomycetidae and Agaricostilbomycetidae, but 9 of 13 analyses fail to recover the monophyly of the Microbotryomycetidae. The Urediniomycetes has been proposed recently as the subphylum Pucciniomycotina (Bauer et al., 2006).

3.8.2. Ustilaginomycetes and Hymenomycetes clade

No name exists for the combination of these two classes, which is characterized by similar ultrastructural and biochemical characters (Lutzoni et al., 2004; McLaughlin et al., 1995b; Weiss et al., 2004a). Until this study, MP bootstrap has not supported the clade with strong (>70%) support. Our combined rDNA nucleotide and amino acid data produce this higher-level arrangement with 82% bootstrap support (68% BP when third positions are omitted), and a PP of 1.0 when all nucleotides are analyzed. Inclusion of third positions distorts this topology under parsimony conditions. Of the protein coding genes, only *tef1* protein data recover the topology when analyzed separately. Both classes share a similar septal wall configura-

Fig. 4. Phylogeny of the Basidiomycota based on combined *tef1*, *rpb2*, and nrDNA nucleotide sequences (third positions included). A Bayesian 50% majority-rule consensus tree is shown to the right. Posterior probabilities ⩾0.95 are indicated above branches. MP bootstrap values ⩾50%, produced by nrDNA nucleotides combined with amino acid sequences, are shown below branches. MP bootstrap values ⩾50% of the nucleotides only analysis, with third positions excluded, are italicized; BP values from analyses with third positions included are underlined. Numbers in gray circles refer to clades discussed in the text. The cladogram inserted on the left is the MP bootstrap tree of rDNA nucleotides combined with amino acid sequences. BP values ⩾50% are indicated on the left; BP values from combined nucleotides, but with third positions excluded, are indicated to the right. The 82% BP value for the trechisporoid/gomphoid-phalloid clade is recovered when third positions are included (38% BP with third positions excluded).

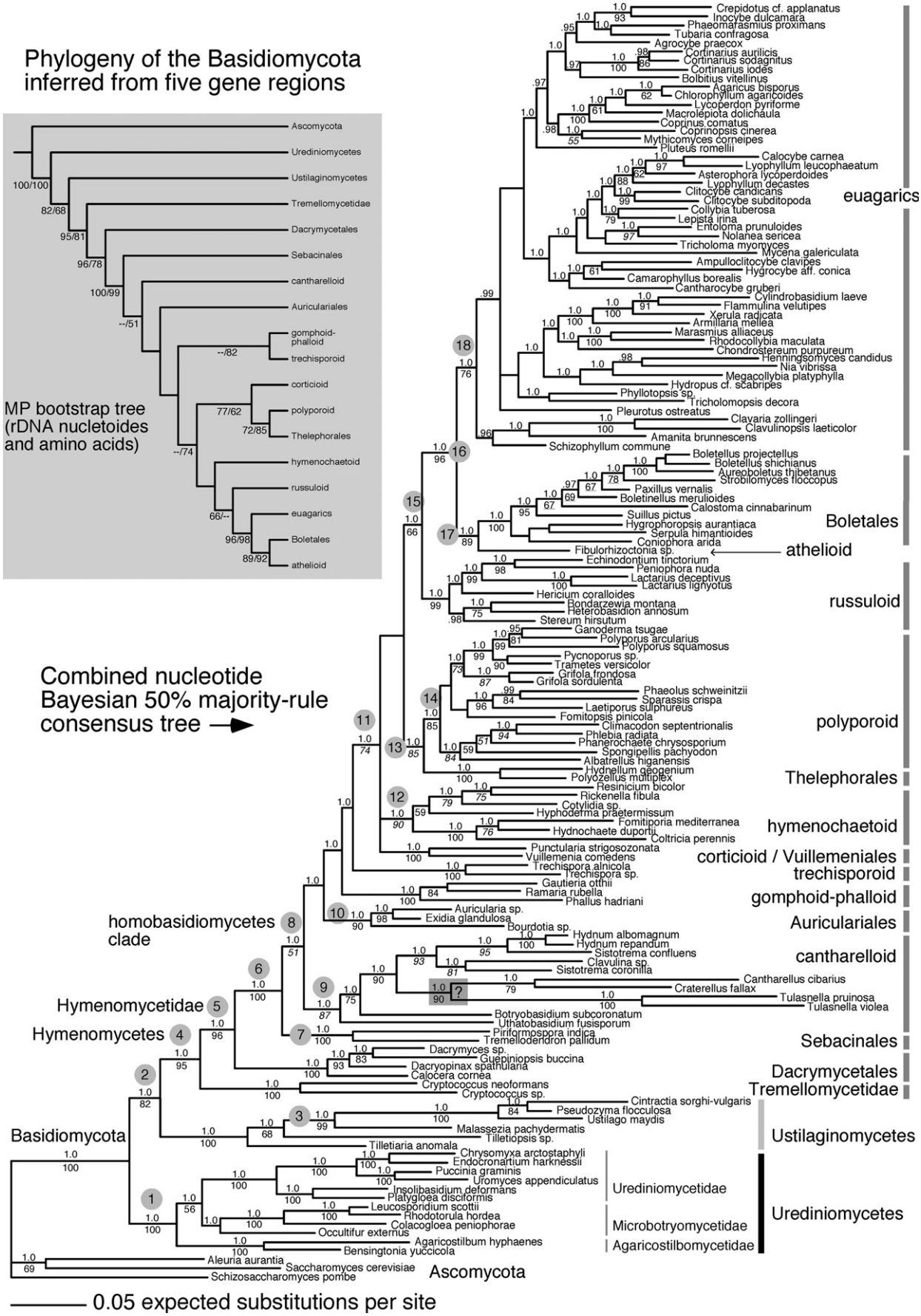
Phylogeny of the Basidiomycota inferred from five gene regions
MP bootstrap tree (rDNA nucletoides and amino acids)
Ascomycota
Urediniomycetes
Ustilaginomycetes
Tremellomycetidae
Dacrymycetales
Sebacinales
cantharelloid
Auriculariales
gomphoid-phalloid
trechisporoid
corticioid
polyporoid
Thelephorales
hymenochaetoid
russuloid
euagarics
Boletales
athelioid
Combined nucleotide Bayesian 50% majority-rule consensus tree
homobasidiomycetes clade
Hymenomycetidae
Hymenomycetes
Basidiomycota
Urediniomycetidae
Microbotryomycetidae
Agaricostilbomycetidae
Ascomycota
0.05 expected substitutions per site

Table 3
Support values by protein-coding gene and nrDNA regions for 18 inclusive clades of Basidiomycota

No	Clade	*tef1* nts[a] BP/PP	*tef1* aa[b] BP/PP	*rpb2* nts BP/PP	*rpb2* aa BP/PP	18S, 25S, 5.8S BP/PP	Combined BP[c]/BP[d]/BP[e]/PP[f]
1	Urediniomycetes	nm[g]/nm	<50/<.95	70/1.0	89/1.0	100/1.0	99/100/100/1.0
2	Ustilaginomycetes + Hymenomycetes	nm/nm	<50/<.95	nm/nm	nm/nm	66/<.95	nm/82/68/1.0
3	Malasseziales + Ustilaginales	<50/nm	65/1.0	73/1.0	91/1.0	90/1.0	99/99/100/1.0
4	Hymenomycetes	nm/1.0	<50/nm	nm/<.95	<50/nm	91/1.0	nm/95/81/1.0
5	Hymenomycetidae	<50/1.0	<50/<.95	nm/nm	88/1.0	69/<.95	69/96/78/1.0
6	Sebacinales + Auriculariales + homobasidiomycetes	nm/nm	nm/nm	nm/nm	53/<.95	98/1.0	94/100/99/1.0
7	Sebacinales	nm/nm	nm/nm	99/1.0	100/1.0	100/1.0	100/100/100/1.0
8	Homobasidiomycetes + Auriculariales	nm/nm	nm/nm	nm/<.95	53/<.95	<50/1.0	nm/<50/51/1.0
9	cantharelloid clade	nm/nm	nm/nm	<50/<.95	<50/<.95	<50/nm	63/69/87/1.0
10	Auriculariales	91/1.0	nm/nm	—[h]	—	89/1.0	100/90/96/1.0
11	Homobasidiomycete group, node 11	nm/nm	nm/nm	nm/nm	nm/nm	<50/nm	nm/50/74/1.0
12	Hymenochaetoid clade	nm/nm	nm/nm	nm/nm	nm/nm	82/1.0	<50/63/90/1.0
13	Thelephorales + polyporoid clade	nm/nm	nm/nm	nm/nm	nm/nm	<50/<.95	nm/72/85/1.0
14	Polyporoid clade	nm/nm	nm/nm	nm/1.0	<50/1.0	<50/1.0	nm/85/83/1.0
15	Russuloid, athelioid, Boletales, and euagarics clade	nm/nm	nm/nm	nm/nm	nm/nm	<50/1.0	<50/66/49/1.0
16	Athelioid, Boletales, euagarics	nm/nm	nm/nm	nm1.0	<50/1.0	81/1.0	73/96/98/1.0
17	Athelioid + Boletales	nm/1.0	nm/nm	nm/nm	nm/nm	80/1.0	66/89/92/1.0
18	Euagarics clade	nm/nm	nm/nm	nm/nm	nm/nm	70/.96	<50/76/80/1.0

Note. Gene regions that produce either a significant BP (⩾70%) or PP (⩾0.95) support value have light gray shaded cells. Gene regions that produce both significant BP/PP values have dark gray shaded cells.
[a]Nucleotides.
[b]Aminoacids.
[c]BP analysis including third codon positions.
[d]BP analysis with amino acid sequences.
[e]BP analysis excluding thrid codon positions.
[f]Bayesian analysis including third codon positions.
[g]nm = not monophyletic.
[h]Monophyly not possible to test.

tion, spindle pole body morphology, cell wall composition, and secondary structure of the 5S RNA (Blanz and Unseld, 1987; Lutzoni et al., 2004; Oberwinkler, 1987; Tu and Kimbrough, 1978; Weiss et al., 2004a). Begerow et al. (2004) produced an alternative topology (Urediniomycetes plus Hymenomycetes) with combined 25S and β-tubulin sequences but with a non-significant PP. Bauer et al. (2006) recently introduced the Ustilaginomycetes and Hymenomycetes at the subphylum rank, the Ustilaginomycotina and Agaricomycotina, respectively.

3.8.3. Malesseziales and Ustilaginales clade

This arrangement stands in contrast to some 25S only studies with dense taxon sampling (Bauer et al., 2001; Begerow et al., 2000; Sampaio, 2004). These studies nest the Malesseziales, an order of animal pathogens and associates (Bauer et al., 1997; Guého et al., 1998; Guillot and Guého, 1995; Sugita et al., 2003), within the Exobasidiomycetidae but without strong support. Our results, however, are consistent with Lutzoni et al. (2004), which produce a paraphyletic Exobasidiomycetidae. Other studies using 25S sequences in Fell et al. (2001) and Weiss et al. (2004a) also depict a paraphyletic or an unresolved Exobasidiomycetidae. Expanded taxon sampling in the Ustilaginomycetes reinforces the monophyly of the Malasseziales with Ustilaginales and allies with strong support (Gossmann et al., unpublished; Matheny et al., 2006b).

3.8.4. Hymenomycetes

This clade (=Agaricomycotina in Bauer et al., 2006) receives up to 95% MP bootstrap support and contains the two subclasses Hymenomycetidae and Tremellomycetidae (Swann and Taylor, 1995b). 18S data support the monophyly of Hymenomycetes but with poor bootstrap support (Swann and Taylor, 1993). Our sampling of the Tremellomycetidae is sparse, but this subclass was shown to be monophyletic by early studies with inclusive taxon sampling (Swann and Taylor, 1995a,b). Sampaio (2004), Weiss et al. (2004a), and Bauer et al. (2006) recover a monophyletic Hymenomycetidae but an unresolved or paraphyletic Tremellomycetidae. The status of the Tremellomycetidae requires more attention. Analyses by Matheny et al. (2006b) show that the addition of protein-coding data support the monophyly of the Tremellomycetidae.

3.8.5. Hymenomycetidae

This strongly supported subclass is characterized by mushroom-forming fungi that are not known to produce a yeast-like stage. It contains the heterobasidiomycete orders Dacrymycetales, Sebacinales, and Auriculariales, in addition to the homobasidiomycete clades of Hibbett and Thorn (2001), Binder and Hibbett (2002), Larsson et al. (2004), and Binder et al. (2005). The Dacrymycetales is strongly supported in a sister position to other members of the subclass in contrast to the nrDNA tree in Lutzoni

et al. (2004). The Homobasidiomycetes is not monophyletic, however, when all data are combined. Results from our nrDNA analyses and those of Larsson et al. (2004) and Binder et al. (2005) show weak to moderate MP support for a monophyletic Homobasidiomycetes (exluding the Auriculariales) using rDNA regions alone.

3.8.6. Sebacinales and the Homobasidiomycetes clade

This group is strongly supported by BP and PP values. The arrangement of the Sebacinales in a position sister to the Homobasidiomycetes clade is strongly supported by Bayesian analysis only. This cluster of taxa will be refered to as the Agaricomycetes by the AFTOL classification group.

3.8.7. Sebacinales

This order of diverse plant symbionts is recognized as a unique clade of heterobasidiomycete taxa within the Hymenomycetidae (Weiss et al., 2004a,b). The group is known for its broad mycorrhizal formation with liverworts, orchids, Ericaceae, and other angiosperms, and gymnosperms (Berch et al., 2002; Selosse et al., 2002a,b). Binder et al. (2005) found weak support for the monophyly of the cantharelloid clade, inclusive of the Sebacinales, the latter characterized by septate basidia. However, the Sebacinales merits recognition as an autonomous clade independent of the cantharelloid clade. The Sebacinales appears to represent the earliest basidiomycete lineage with mycorrhizal members (Weiss et al., 2004a,b).

3.8.8. Homobasidiomycetes clade

The Homobasidiomycetes is monophyletic with the inclusion of the heterobasidiomycete order Auriculariales, but bootstrap support is poor (51%). The backbone of the Homobasiomycetes, a paraphyletic group in Lutzoni et al. (2004) and in this study, is significantly supported by posterior probabilities, but MP bootstrap values remain below 50% for most backbone nodes. Two groups not depicted by the nucleotide Bayesian analysis do receive high BP support. These include a union of the trechisporoid and gomphoid-phalloid clades (82% BP with third positions included) and a separate grouping of the polyporoid clade, Thelephorales, and Vuillemeniales or corticioid clade (77% protein BP). Both alternative arrangements are shown in the insert MP bootstrap tree of Fig. 4. No previous studies have united the trechisporoid and gomphoid-phalloid clades, but both are characterized by many species with hyphal swellings near septa and verrucose or warty basidiospores (Agerer and Iosifidou, 2004; Joost Stalpers, personal communication). Inclusive clades, supported both by high BP and PP, are reported below in more detail.

3.8.9. Cantharelloid clade

This group contains taxa from the orders Cantharellales, Ceratobasidiales, Tulasnellales, and Botryobasidiales. The clade receives a significant PP and 87% BP when third positions are excluded (69% BP with amino acids). The union of *Tulasnella* species with *Cantharellus* and *Craterellus* is an artifact attributed to extreme rate heterogeneity of the nrDNA sequences in these taxa, hence the question mark for the monophlyly of the group depicted in Fig. 4. Sequences of *rpb2* (Fig. 3) strongly support the separation of Tulasnellales in one lineage, and Cantharellales (Cantharellaceae, Clavulinaceae, and Hydnaceae) in another. These three families of Cantharellales are also united by possession of stichic basidia (Donk, 1971; Penancier, 1961). The cantharelloid clade is strongly supported by Bayesian analysis as the sister group to the remaining Homobasidiomycetes plus Auriculariales. Non-rDNA sources of data are required to address evolutionary relationships within the cantharelloid clade due to evolutionary rate acceleration in nuclear rDNA sequences of *Tulasnella* and the Cantharellaceae (Moncalvo et al., 2006).

3.8.10. Auriculariales

This heterobasidiomycetous order is monophyletic in a strict sense as first suggested by Swann and Taylor (1993) using 18S sequences and later by Weiss and Oberwinkler (2001) using 25S data. It corresponds in many respects to the Auriculariales *sensu* Bandoni (Bandoni, 1984; Wells and Bandoni, 2001). The monophyly of the order *sensu* Weiss and Oberwinkler is strongly supported. Binder et al. (2005) reported the Auriculariales as a paraphyletic group. Additional rDNA data, including different isolates of *Pseudohydnum gelatinosum* and *Bourdotia*, support the monophyly of the Auriculariales (Matheny, unpublished; Matheny et al., 2006b). Our results are consistent with those in Prillinger et al. (2002) and Lutzoni et al. (2004) where the Auriculariales is nested among other basal homobasidiomcyete lineages. Wells et al. (2004) recently have assessed family-level relationships in the order.

3.8.11. Homobasidiomycete group, node 11

This inclusive cluster of eight homobasiomycete clades is strongly supported by Bayesian analysis of combined nucleotides and a 74% bootstrap of nucleotide data with third codon positions excluded. It contains the hymenochaetoid, corticioid, polyporoid, Thelephorales, russuloid, athelioid, Boletales, and euagarics clades. Maximum likelihood analyses of multiple rDNA regions in Binder and Hibbett (2002) recovered a similar topology.

3.8.12. Hymenochaetoid clade

Both *rpb2* and *tef1* fail to support the monophyly of this group, which has been poorly to strongly supported by rDNA (Binder and Hibbett, 2002; Binder et al., 2005; Larsson et al., 2004). MP bootstrapping of nrDNA supports the monophyly of the clade with 82% BP and 1.0 PP (Table 2). Combined *rbp2*, *tef1*, and nrDNA sequences (with third positions excluded) also recover the clade with a significant PP and 90% BP, but with reduced bootstrap support (63%) when third positions are included. The clade appears to contain two subgroups, the xanthochroic Hymenochaetaceae or Hymenochaetales *sensu* Oberwinkler (Oberwinkler, 1977; Parmasto, 2001), characterized by the presence of

setae and darkening of flesh after alkalization; and a poorly supported non-xanthochroic arrangement of taxa that lack setae but possess cystidia, viz. *Cotylidia*, *Rickenella*, *Hyphoderma*, and *Resinicium* (Fig. 4). Nucleotide analyses that exclude third positions support *Hyphoderma* as the sister group to the Hymenochaetaceae with 78% BP (not shown). Other bryophilous gilled-mushrooms and clavarioid and resupinate forms belong to the hymenochaetoid clade (Matheny et al., 2006a; Redhead et al., 2002).

3.8.13. Thelephorales and polyporoid clade

The monophyly of this group is recovered with strong support for the first time with 72% BP when amino acids are included and 85% BP when nucleotides are analyzed without third codon positions. Within the clade is a sister arrangement between the Thelephorales, a mycorrhizal group (including orchid symbionts) (Bruns et al., 1998; Hibbett and Thorn, 2001; Stalpers, 1993), and a wood-decomposing group, the polyporoid clade. A three-gene analysis including *rpb2* also recovers a similar topology (Lutzoni et al., 2004) in addition to an 18S only study of aphyllophoralean taxa (Kim and Jung, 2000). MP bootstrapping (77%) of protein sequences combined with nrDNA supports the Vuillemeniales (Boidin, 1998) or corticioid clade (Larsson et al., 2004) sister to an inclusive Thelephorales and polyporoid clade (see insert in Fig. 4).

3.8.14. Polyporoid clade

This group has been difficult to resolve by rDNA data (Binder and Hibbett, 2002; Hibbett and Donoghue, 1995; Wang et al., 2004). However, in our combined analyses the clade is supported as monophyletic with high MP bootstrap and posterior probability. Amino acid and nucleotide sequences of *rpb2* alone also support the monophyly of this group (Fig. 3). It is sister with strong support to the Thelephorales. BP values for several subclades are higher in combined analyses, in which third codon positions are excluded.

3.8.15. Russuloid, athelioid, Boletales, and euagarics clade

This inclusive group of homobasidiomycete clades receives significant PP and 66% BP. The russuloid clade may constitute several orders of taxa (Larsson and Larsson, 2003) and is supported as sister to an arrangement of athelioid, Boletales, and euagarics clades. This topology was also shown by Binder and Hibbett (2002) and Larsson et al. (2004) but without strong support. The position of the russuloid clade is not firmly resolved and other multi-gene studies (Binder et al., 2005; Lutzoni et al., 2004; Wang et al., 2004) depict alternative topologies, but with weak support.

3.8.16. Athelioid, Boletales, and euagarics clade

This relationship is supported by high MP bootstrap (96%) and a significant PP. In the AFTOL classification, the group is referred to as the Agaricomycetidae. Multigene rDNA studies (Binder and Hibbett, 2002; Larsson et al., 2004) support a union of the Boletales and euagarics clade and, where sampled, the athelioid clade.

3.8.17. Athelioid and Boletales clade

One exemplar of the athelioid clade, *Fibulorhizoctonia* sp., occupies a position sister to the Boletales with strong support values. Of the five separate genes in this study, *tef1*, 18S, and 5.8S also support this topology. The relationship was depicted by Larsson et al. (2004) using 25S and 5.8S sequences but without strong support. However, our combined 18S, 25S, and 5.8S sequences produce both significant PP and BP for this cluster (Table 3). The athelioid clade includes taxa with diverse ecologies, including saprotrophs, ectomycorrhiza formers, insect associates, and plant pathogens (Binder et al., 2005; Larsson et al., 2004; Matsuura et al., 2000). Mycorrhizal symbioses in the Boletales and their transitions to mycoparasitic lifestyles (infecting other Boletales) appear to have evolved multiple times from brown-rot saprotrophic ancestors (Binder and Hibbett, 2006).

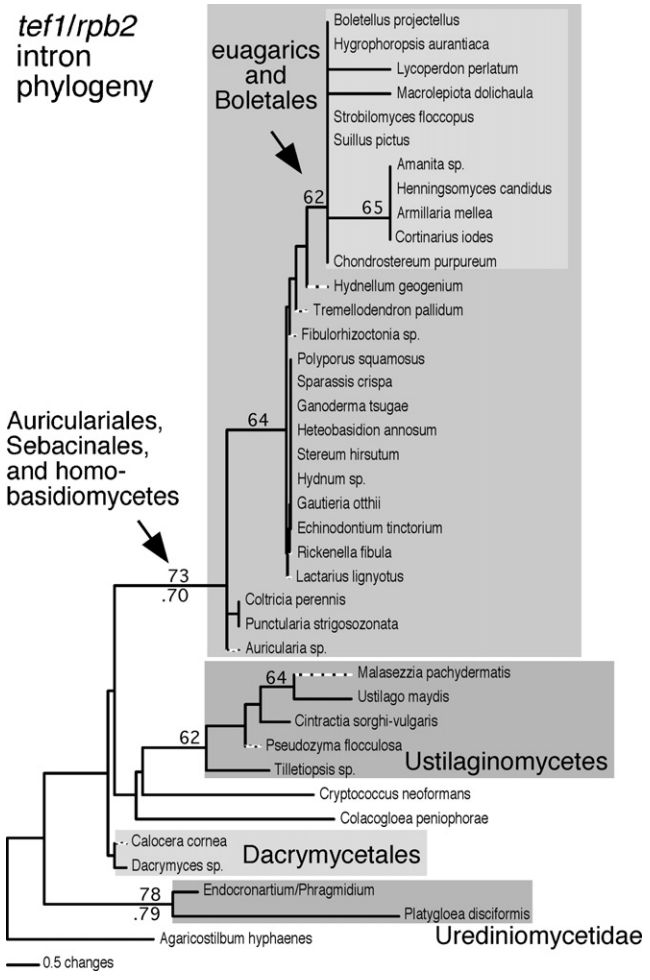

Fig. 5. NJ phylogram of the Basidiomycota inferred from presence/absence of 29 spliceosomal intron positions in *tef1* and *rpb2*. Values above nodes reflect proportions of 1000 bootstrapped datasets. Values below nodes indicate the posterior probability calculated from 9000 trees combined from independent runs for which likelihood scores had reached stationarity. Dashed branch lengths are ambiguous due to missing data for those terminals.

3.8.18. Euagarics

The clade is partially consistent with the suborder Agaricineae in Singer (1986) and dominated by gilled mushrooms, but also includes many noteworthy non-gilled forms (Binder et al., 2005; Bodensteiner et al., 2004; Larsson et al., 2004; Matheny et al., 2006a; Moncalvo et al., 2002). Analyses of combined data (amino acids and nucleotides with third positions excluded) produce strong support for the monophyly of the euagarics. No morphological characters are known that unite the group (Moncalvo et al., 2000). Many previous studies using more taxa with 25S sequences or combined rDNA regions with fewer taxa (Binder et al., 2005; Larsson et al., 2004; Moncalvo et al., 2000; Moncalvo et al., 2002; Thorn et al., 2000) were unable to generate strong support for the monophyly of the euagarics, except for Binder and Hibbett (2002), which combined nuclear and mitochondrial sources of rDNA.

3.9. Phylogenetic utility of spliceosomal introns

A neighbor-joining tree for 39 taxa based on positions of *rpb2* and *tef1* spliceosomal introns is shown in Fig. 5. It is rooted with the urediniomycete *Agaricostilbum hyphaenes*. Gross level phylogenetic structure can be observed for several conserved clades, which is consistent with phylogenetic hypotheses derived from sequence data. These include support for the Urediniomycetidae, Ustilaginomycetes, Dacrymycetales, Homobasidiomycetes clade plus the Sebacinales, and a monophyletic Boletales and euagarics clade.

4. Discussion

4.1. tef1 and rpb2 are conserved protein-coding genes useful to infer basidiomycete phylogeny

No studies across the Basidiomycota have yet included *tef1* sequences except at low taxonomic levels (Geml et al., 2005; Kauserud, 2004; Kauserud and Schumacher, 2003). Yet, amino acid sequences of the gene are strongly conserved across eukaryotes (Baldauf and Palmer, 1993; Cottrelle et al., 1985; Roger et al., 1999; Steenkamp et al., 2006). This feature has enabled the design of degenerate primers in exons conserved among fungi, plants, and animals.

Although basidiomycete amino acid sequences align easily and exhibit few indels, intron placement is highly variable across *tef1* at broad taxonomic levels. We record at least 22 unique spiceosomal intron positions in the *tef1* region sequenced (Fig. 1, S2). More are located upstream of the region we studied. However, the Ustilaginomycetes and Dacrymycetales are relatively depauperate of *tef1* introns in comparison with other Hymenomycetes and Urediniomycetes (S2). This pattern suggests that spliceosomal intron loss and gain is dynamic in fungal *tef1* sequences and is of some phylogenetic importance (Galagan et al., 2005; Nielsen et al., 2004; Zhaxybayeva and Gogarten, 2003).

Several recent papers have reported cases of lateral transfer of *tef1* in eubactaria or hypothesized lateral gene transfer between eukaryotes or homologous recombination between distantly related archaea (Inagaki et al., 2002; Inagaki et al., 2006; Keeling and Inagaki, 2004). This would compromise the use of *tef1* for phylogenetic purposes. Despite these reports, neither lateral transfer nor ancient *tef1* duplications have yet been reported in the Basidiomycota. Two copies that code for elongation factor-1α have been isolated in the ascomycete *Saccharomyces cerevisiae* (Cottrelle et al., 1985), but only a single copy occurs in the basidiomycete *Schizophyllum commune* (Wendland and Kothe, 1997). Our results substantiate the single-gene copy status of *tef1* with few exceptions. In such cases, duplicated copies cluster together in *Auricularia*, *Heterobasidion*, and *Polyozellus* (Fig. 2). This suggests duplications of *tef1* are relatively recent, relatively rare, and sporadic. In other cases, they have degraded into pseudogenes in *Mycetinis* and *Hydnellum*, given the presence of indels that disrupt the reading frame in these sequences.

Regions of *rpb2* are likewise strongly conserved among eukaryotes and groups of archaea (Denton et al., 1998; Liu et al., 1999; Walsh et al., 2004) but are more variable than *tef1* in fungi. Liu et al. (1999) produced a study across the Ascomycota, in which variable and conserved regions of the gene between conserved domains 3 and 11 were analyzed. Variable regions of *rpb2* between domains 6 and 7 subsequently have been applied with increasing popularity to phylogenetic studies of Ascomycota and Basidiomycota but generally below the ordinal rank (Chaverri et al., 2003; Frøslev et al., 2005; Hansen et al., 2005; Matheny, 2005; Matheny and Bougher, 2006; Miller and Huhndorf, 2004; Reeb et al., 2004). Wang et al. (2004) incorporated 55 *rpb2* sequences across the Hymenomycetidae, a subclass of mushrooms and allies. However, these sequences were restricted to the most variable region of the gene between domains 6 and 7, a coding region more variable than the rapidly evolving internal transcribed spacer (ITS) regions in mushrooms such as *Cortinarius* (Frøslev et al., 2005). We did not detect any evidence of *rpb2* duplication in basidiomycetes.

Gene structure within both genes is also imbued with phylogenetic signal. Twenty-two intron positions from *tef1* and seven in *rpb2* were utilized to reconstruct a phylogeny of the Basidiomycota. Several gross-level groupings emerge in this picture (Fig. 5) with support for the Ustilaginomycetes, Urediniomycetidae, and Homobasidiomycetes plus Sebacinales and Auriculariales. Even at fine taxonomic levels, intron characters support, for example, a monophyletic Boletales and euagarics clade. None of the aforementioned groups are significantly supported by Bayesian analysis of the 29 intron characters, but most of the patterns are present. These results indicate that basidiomycete intron positions are conserved enough to distinguish and characterize several higher-level taxonomic

groups as has been reported in other eukaryotes such as land plants (Dombrovska and Qiu, 2004). In contrast, patterns of group I intron insertions in the rDNA of fungi are sporadic and dynamic even at the population level (Gargas et al., 1995a; Hibbett, 1996; Lickey et al., 2003; Nishida and Sugiyama, 1995; Takashima and Nakase, 2001).

The number of nodes that received strong (>70%) parsimony bootstrap support was higher in combined data sets that either incorporated the translation of the protein-coding regions into amino acid data or excluded rapidly evolving third codon positions (Tables 2, 3). However, the combined Bayesian topology (Fig. 4), which included analysis of all codon positions, is largely congruent in terms of branching order and support values with the parsimony bootstrap trees produced from combined analyses with amino acid positions or the first two codon positions (Tables 2, 3). In this data set third positions were modeled separately from first and second positions. Nevertheless, we would encourage future studies of basidiomycete phylogeny, as has been similarly observed for the phylogeny of seed plants (Burleigh and Matthews, 2004) to exclude third codon positions when doing parsimony analyses, or treat the sequences as amino acid data. Indeed, twelve inclusive clades of Basidiomycota were either poorly supported, or not even resolved, in combined nucleotide parsimony analysis that included third codon positions for 146 taxa, and a total of 21 fewer nodes (15%) received non-significant bootstrap support.

4.2. rpb2 resolves major clades of basidiomycetes at high and low taxonomic levels in contrast to tef1

The *rpb2* gene is more variable than *tef1* based on pairwise distance comparisons and percentages of parsimony-informative sites. Bayesian analyses of nucleotide sequences of *rpb2* support ten additional clades that are unsupported by *tef1*. At the amino acid level, *rpb2* significantly supports fifteen major clades compared to three for *tef1*. However, an important difference between our treatments of the two genes is sequence length. Most of our *rpb2* sequences are about twice as long as our *tef1* sequences. Future studies could investigate extending *tef1* sequences upstream.

Despite these differences, both genes recover the monophyly of several taxonomically inclusive clades above the ordinal level. These include the Ustilaginomycetes, Urediniomycetes, Urediniomycetidae, Hymenomycetidae, and the Uredinales plus Platygloeales. Nucleotide sequences of *tef1* suggest the paraphyly of the Urediniomycetes, but *rpb2* and *tef1* protein sequences support its monophyly. Dense taxon sampling within the Urediniomycetes with short fragments of 25S sequences has cast doubt on the monophyly of this class (Weiss et al., 2004a), however, we conclude the phylogenetic results of this earlier study are not accurate. Half of our treatments of *tef1* and *rpb2* recover the monophyly of the Hymenomycetes. Both genes also support a monophyletic Dacrymycetales.

It is within the subclass Hymenomycetidae where *rpb2* supports more rDNA-recognized clades than *tef1*. Such *rpb2* groupings include the Boletales, polyporoid clade, russuloid clade, gomphoid-phalloid clade, cantharelloid clade, and Sebacinales. At or near the family level, the *tef1* gene lacks the signal required to discriminate low-level taxomic groups, for example in the euagarics, such as the Agaricaceae, Inocybaceae, Lyophylleae, and Physalacriaceae. All of these groups have been shown to be monophyletic by other genes.

4.3. Sources of conflict between genes for basidiomycete phylogeny is low

The combination of multiple sources of gene sequence data is a desirable attribute for phylogenetic analysis when significantly supported conflict between genes is low. We observe no strongly supported conflicts between *rpb2* and *tef1*. Two cases of conflict between the protein-coding genes and nrDNA, however, merit attention. We find that nrDNA sequences of species of *Tulasnella*, *Cantharellus*, and *Craterellus* contain several short unique regions that cannot be aligned with other basidiomycetes. Despite recoding these unusual rDNA regions as missing data across both 25S and 18S partitions, and excluding their putative 5.8S gene regions, our rDNA phylogenies, no matter the phylogenetic method, still cluster *Tulasnella* with *Cantharellus* and *Craterellus* with measures of strong support. We interpret their monophyly as an artifact attributed to the nrDNA sequences (Fig. 4). In contrast, *rpb2* sequences (Fig. 3) of *Tulasnella*, *Cantharellus*, and *Craterellus* exhibit nucleotide sites that are straightforward to align and that conflict significantly with their rDNA placment. This result is corroborated by Moncalvo et al. (2006) using sequences of *rpb2* and the mitochondrial small subunit RNA. *Tulasnella rpb2* sequences occur outside the Cantharellales, a clade represented here by *Cantharellus*, *Craterellus*, *Hydnum*, *Clavulina*, and a polyphyletic *Sistotrema*. Of the five genes in our study, *rpb2* is the most reliable marker to investigate phylogeny and evolution in the cantharelloid clade. Conflict was also observed in the Boletales concerning the position of *Strobilomyces*. Morphological characters do not support a close relationship between *Strobilomyces* and *Calostoma*, as strongly indicated by our rDNA data (not shown). Both *rpb2* and *tef1* (when third positions are included) place *Strobilomyces* in a position near the Boletacceae.

4.4. Combined five-gene analyses produce novel and well-supported clades of Basidiomycota

At least five inclusive clades of Basidiomycota receive dual significant BP and PP support when *rpb2* and *tef1* are combined with rDNA—(1) Hymenomycetes plus Ustilaginomycetes; (2) an inclusive clade of eight homobasidiomycete lineages: hymenochaetoid, corticioid, Thelephorales, polyporoid, russuloid, athelioid, Boletales, and

euagarics clade; (3) Thelephorales plus the polyporoid clade; (4) the polyporoid clade; and (5) the cantharelloid clade. These groupings have yet to be supported by high bootstrap values. One additional inclusive group (the russuloid, athelioid, Boletales, and euagarics clade) receives 66% protein BP and a significant posterior probability. Parsimony bootstrap values from combined nucleotides analyses excluding third codon positions were proportional with BP values from combined nrDNA and amino acid sequences (Tables 2, 3).

Several deep nodes of the basidiomycete tree of life are also strongly supported with posterior probabilities and parsimony bootstrap values for the first time by combined five gene analyses. Although ultrastructural traits and similar biochemistry have been used to unite the Ustilaginomycetes with the Hymenomycetes, previous phylogenetic studies of this problem using single genes have either not supported this relationship or supported it without strong bootstrap support. Our results reinforce the hypothesis that septal pore traits, cell wall composition, and some biochemical characters reflect the phylogeny of the Basidiomycota (Lutzoni et al., 2004; Weiss et al., 2004a). However, the Homobasidiomycetes of Binder et al. (2005), Hibbett and Thorn (2001), and Larsson et al. (2004) appears to be paraphyletic. Five-gene data support an inclusive grouping of homobasidiomycete clades including the heterobasidiomycete order Auriculariales. As such, Bayesian analyses support the hypothesis that the Auriculariales is nested among other basal homobasidiomycete groups, rather than subtending them as indicated by previous studies, but bootstrap support for this topology remains weak. Although the monophyly of the Hymenomycetes has not been challenged, the branching order within the class historically has not been robust. Here, the position of the Dacrymycetales as the earliest branch of the Hymenomycetidae is strongly supported (Fig. 4).

Our results raise doubts over several rDNA clades of basidiomycetes (e.g., paraphyly of Exobasidiomycetidae and Homobasidiomycetes, composition of the cantharelloid clade) but produce strong support for previously contentious groups (e.g., Hymenomycetidae, hymenochaetoid clade, polyporoid clade) or novel inclusive clades that are, as of yet, currently undiagnosed (clades 2, 5, 11, 13, and 17 in Fig. 4). Several lineages of Homobasidiomycetes are strongly supported here for which no formal or informal name exists (Table 3, Fig. 4). Classifications and informal proposals of basidiomycete relationships based on rDNA alone are not completely sufficient and should be evaluated or reinforced by other sources of molecular data. Inferences about outgroup choice and character evolution, for example, are tenuous when the phylogenetic trees on which they are based are not robust. Future studies could rely on the use of multigene data sets as scaffolds on which to attach more taxa that are represented by fewer genes (Hibbett et al., 2005; Wiens, 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.08.024.

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