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Molecular phylogenetics of the Gloeophyllales and relative ages of clades of Agaricomycotina producing a brown rot

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Abstract: The Gloeophyllales is a recently described order of Agaricomycotina containing a morphologically diverse array of polypores (*Gloeophyllum*), agarics (*Neolentinus*, *Heliocybe*) and resupinate fungi (*Veluti-Veluticeps*, *Boreostereum*, *Chaetodermella*), most of which have been demonstrated to produce a brown-rot mode of wood decay and are found preferentially on coniferous substrates. Multiple phylogenetic studies have included taxa of Gloeophyllales, but none have sampled the order thoroughly, and so far only ribosomal RNA genes have been used. Consequently the limits and higher level placement of the Gloeophyllales are obscure. We obtained sequence data for three protein-coding genes (*rpb2*, *atp6*, *tef1*) and three rRNA regions (nuc-ssu, nuc-lsu, 5.8S) in 19 species of Gloeophyllales representing seven genera and analyzed them together with a diverse set of Agaricomycotina, emphasizing Polyporales. *Boreostereum*, which is suspected to produce a white rot, is the sister group of the rest of the Gloeophyllales, all of which produce a brown rot. *Gloeophyllum* contains at least two independent clades, one of which might correspond to the genus *Osmoporus*. White rot and resupinate fruiting bodies appear to be plesiomorphic in Gloeophyllales. Relaxed molecular clock analyses suggest that the Gloeophyllales arose in the Cretaceous, after the origin of Pinaceae.

Key words: Agaricomycotina, brown rot, systematics, taxonomy, wood decay

INTRODUCTION

The Gloeophyllales is a recently described order (Thorn in Hibbett et al. 2007) of wood-decaying mushrooms (Agaricomycotina), containing a single family, Gloeophyllaceae (Kirk et al. 2008). *Gloeophyllum* as currently circumscribed includes roughly 13 species, including the model brown-rot species, *Gloeophyllum trabeum* (Pers. : Fr.) Murrill and *G. sepiarium* (Wulfen. : Fr.) P. Karst., which are widely used in experimental studies on wood-decay chemistry (e.g. Jensen et al. 2001, Baldrian and Valásková 2008). A complete genome sequence of *G. trabeum* is in production (http://genome.jgi-psf.org/pages/fungi/home.jsf). Species of *Gloeophyllum* have pileate, effused-reflexed or resupinate fruiting bodies, poroid to lamellate hymenophores and di- to trimitic hyphal construction; all have bipolar mating systems and produce a brown rot (Gilbertson and Ryvarden 1986). This combination of characters is similar to that seen in certain brown-rot taxa of Polyporales, such as *Fomitopsis* P. Karst., *Phaeolus* (Pat.) Pat. and *Daedalea* Pers. It therefore was a surprise when the first molecular phylogenetic study to include *Gloeophyllum* placed it in an isolated position with no obvious close relatives among the Polyporales or other wood-rotting Agaricomycotina (Hibbett and Donoghue 1995). A number of later phylogenetic studies sampled taxa that are closely related to *Gloeophyllum*, contributing to an improved, although still imperfect, understanding of the group's limits. It is now well established that the Gloeophyllales contains, in addition to *Gloeophyllum*, the brown-rot, bipolar agaric genera *Neolentinus* Redhead & Ginns and *Heliocybe* Redhead & Ginns (Binder et al. 2005, Hibbett and Donoghue 2001, Thorn et al. 2000), which were segregated from the white rot *Lentinus* Fr., based largely on decay mode (Redhead and Ginns 1985, Hibbett and Vilgalys 1993). Several studies also have suggested that the resupinate to stereoid *Boreostereum* Parmasto and *Veluticeps* (Cooke) Pat. are closely related to *Gloeophyllum* (Binder et al. 2005, Kim and Jung 2000, Larsson et al. 2007, Lim 2001, Yoon et al. 2003). These results are somewhat surprising because *Veluticeps*, although a brown-rot fungus, is reportedly tetrapolar (Martin and Gilbertson 1973), while *Boreostereum* is possibly a white-rot fungus and homothallic (Chamuris 1988, Nakasone 1990a).

There are conflicting reports in the literature regarding the relationships of *Columnocystis* Pouzar, which has been placed in synonymy with *Veluticeps* (Hjortstam and Tellería 1990, Nakasone 1990b) and Gloeophyllales. Analyses of nuclear large subunit

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(nuc-lsu) and 5.8S rRNA gene sequences by Larsson (2007) strongly supported monophyly of *Columnocystis abietina* (Pers. : Fr.) Pouzar (= *Veluticeps abietinum* [Pers. : Fr.] Hjortstam & Tellería) and *Veluticeps berkeleyi* Cooke, along with the resupinate *Chaetodermella luna* (Romell ex D.P. Rogers & H.S. Jacks.) Rauschert. In contrast analyses of nuclear small subunit rRNA gene sequences by Kim and Jung (2000) and Yoon et al. (2003) suggested that *Columnocystis abietina* and *Columnocystis ambigua* (Peck) Pouzar are closely related to the polypore *Meripilus giganteus* (Pers. : Fr.) P. Karst. (Polyporales). Other controversial reports concern the white-rot polypore *Donkioporia expansa* (Desm.) Kotl. & Pouzar (a well known species that decays wood in service; Ridout 1999), which has been placed in the Gloeophyllales based on nuc-ssu rRNA sequences (Kim and Jung 2000, 2001). Clearly erroneous results that might be due to misidentifications include a placement of *Neolentinus dactyloides* (Cleland) Redhead & Ginns with *Pleurotus eryngii* (DC. : Fr.) Quél. (Agaricales) and *Gloeophyllum abietinum* (Bull.) P. Karst. within a clade of *Antrodia* P. Karst. and *Oligoporus* Bref. species (Polyporales) (Hibbett and Vilgalys 1993, Kim et al. 2003).

In sum, seven genera (*Gloeophyllum*, *Neolentinus*, *Heliocybe*, *Veluticeps*, *Boreostereum*, *Chaetodermella* and *Donkioporia*) have been referred to the Gloeophyllales according to one or more of 10 phylogenetic studies. However, these studies each contain no more than three of the putative genera of Gloeophyllales, which makes it difficult to integrate their results taxonomically and most of these studies use only one of three rRNA genes (no locus is represented in all taxa), which would complicate attempts to combine datasets. The limits of some genera within the Gloeophyllales also are uncertain. *Gloeophyllum* in the current sense (Gilbertson and Ryvarden 1986, Kirk et al. 2008) contains about 13 species, but several of these also were classified in *Osmoporus* Singer (1944); *Heliocybe* was placed in synonymy with *Neolentinus* (Rune 1994); and *Pileodon* P. Roberts & Hjortstam and *Campylomyces* Nakasone are segregates of *Veluticeps* that have yet to be included in a phylogenetic analysis (Hjortstam and Telléria 1990, Nakasone 2004). The higher level placement of the Gloeophyllales also is poorly resolved in the analyses cited above. Depending on the analysis, the Gloeophyllales has been placed close to the Boletales, Polyporales, Corticiales or Thelephorales but never with strong support.

As a complement for rRNA genes, several putatively single-copy protein coding genes have been widely used in fungal phylogenetics. Some of the most promising are *rpb2* encoding for the second largest subunit of the 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, Matheny et al. 2007), the gene coding for the translation elongation factor 1-α *tef1* (Baldauf and Palmer 1993, O'Donnell et al. 2001, Matheny et al. 2007, Rehner and Buckley 2005, Roger et al. 1999) and the mitochondrial gene encoding for subunit 6 of ATPase *atp6* (Kretzer and Bruns 1999, Robison et al. 2001). In the present study we sampled six genes, including *rpb2*, *tef1*, *atp6*, nuc-lsu, nuc-ssu and 5.8S rRNA, in 19 species representing seven genera of Gloeophyllales, and we combined these data with a broad sample of Agaricomycotina, emphasizing Polyporales.

Resolving the relationships of the Gloeophyllales is important to understanding the evolution of the brown-rot mode of wood decay in Agaricomycotina. Hibbett and Donoghue (2001) inferred six independent origins of brown rot, including one in the lineage leading to Gloeophyllales, and suggested that the evolution of a brown rot promoted shifts to specialization on coniferous substrates. Testing this hypothesis requires a robust, time-calibrated phylogenetic tree. Previous attempts to produce a time-calibrated phylogeny for the fungi faced problems related to the scarcity of fossils to be used as calibration points (Simon et al. 1993, Berbee and Taylor 1993). More recent studies (Taylor and Berbee 2006, Hibbett and Matheny 2009) benefit from new approaches to calibration (Peterson et al. 2004), recently discovered fungal fossils (Taylor et al. 2005) and an extensive molecular sampling for several fungal species (Rokas and Carroll 2005). But even under those circumstances, results may be uncertain because fungal fossils are difficult to place as calibration points and variation in molecular evolutionary rates might induce error. To address the pattern of evolution of the brown-rot mode of wood decay in Agaricomycotina, we performed ancestral state reconstructions using parsimony. To estimate the timing of these events, we used relaxed molecular clock analyses that attempt to compensate for rate heterogeneity.

MATERIALS AND METHODS

This study used sequence data from six gene regions, including five nuclear genes, RPB2, EF1, nucLSU, nucSSU and 5.8S, and the mitochondrial ATP6. New sequences for all six regions were generated for 19 species representing six genera that have been proposed to be in the Gloeophyllales. The only genus of Gloeophyllales not directly sequenced was *Chaetodermella*, which Larsson (2007) showed to be closely related to *Veluticeps*. Sequences from an additional 82 species of Agaricomycetes were downloaded from GenBank, including a nucLSU sequence of *Chaetodermella*

TABLE I. Gloeophyllales and *Donkioporia expansa* collections studied and GenBank accessions for sequences

Species	Collection	GenBank accession numbers					
		RPB2	EF1	ATP6	nucLSU	nucSSU	5.8s
Boreostereum radiatum	RLG-9717-sp[a]	HM536101	—	—	HM536050	HM536051	HM536085
Chaetodermella luna	NH 8482	—	—	—	EU118615[f]	—	—
Donkioporia expansa	P188[b]/P185	HM536102	HM536103	—	HM536052	AJ540307[f]	HM536087
Gloeophyllum carbonarium	FP-97972-Sp[a]	HM536104	HM536105	HM536128	HM536054	HM536055	—
Gloeophyllum mexicanum	FP-104133-Sp[a]	—	HM536106	HM536129	HM536056	HM536057	HM536088
Gloeophyllum odoratum	CBS-4461[a]	—	—	—	HM536058	—	HM536089
Gloeophyllum protractum	H-80[a]	HM536107	HM536108	HM536130	HM536059	HM536060	HM536090
Gloeophyllum sepiarium	Wilcox-3BB[a]	HM536109	HM536110	HM536131	HM536062	HM536061	HM536091
Gloeophyllum striatum	ARIZ AN027866[c]	HM640259	HM536111	HM536132	HM536063	HM536064	HM536092
Gloeophyllum subferrugineum	FPRI-508[a]	—	—	—	HM536065	HM536066	—
Gloeophyllum trabeum	1320[a]	HM536112	HM536113	HM536133	HM536067	HM536068	HM536094
Heliocybe sulcata	IBUG 9930[d]	HM536114	HM536115	—	HM536069	HM536070	HM536095
Neolentinus adhaerens	DAOM 214911[e]	HM536116	HM536117	HM536134	HM536071	HM536072	HM536096
Neolentinus kauffmanii	DAOM 214904[e]	—	HM536118	HM536135	HM536073	HM536074	HM536097
Neolentinus lepideus	DAOM 208668[e]	HM536121	HM536122	HM536137	HM536077	HM536078	—
Neolentinus lepideus	DAOM 208724[e]	HM536119	HM536120	HM536136	HM536075	HM536076	HM536098
Osmoporus odoratus	F0015308	—	—	—	EF153195[f]	—	—
Veluticeps abietina	GBB-398[a]	HM536123	HM536124	HM536138	HM536079	HM536080	—
Veluticeps berkeleyi	HHB-8594[a]	HM536125	HM536126	HM536139	HM536081	HM536082	HM536099
Veluticeps fimbriata	L-10628[a]	HM640260	HM536127	HM536140	HM536084	HM536083	HM536100

[a] Strains provided by Center for Forest Mycology Research, Forest Product Laboratory, USDA Forest Service.
[b] Strain provided by Department of Wood Sciences, University of Hamburg.
[c] Specimen provided by ARIZ, Robert L. Gilbertson Mycological Herbarium, University of Arizona.
[d] Specimen provided by IBUG Herbaria, Instituto de Botánica, Universidad de Guadalajara.
[e] Specimens provided by DAOM Herbarium, Agriculture and Agri-Food Canada.
[f] Sequences downloaded from GenBank.

luna and 57 ATP6 sequences emphasizing Polyporales that were generated by Z. Wang (2006) but had not been reported previously. Taxonomic sampling was designed to address the composition and higher level placement of the Gloeophyllales in the Agaricomycetes, as well as the number of origins and phylogenetic placement of the brown-rotting clades of mushrooms. *Cryptococcus neoformans* (San Felice) Vuill. (Tremellales) was selected as outgroup. Herbarium specimens and cultures from which sequence data were obtained are provided (TABLE I).

Molecular methods.—DNA samples were isolated from herbarium specimens and cultures with a SDS extraction buffer with phenol-chloroform extraction and ethanol precipitation, as described in Binder et al. (2005). PC R reactions were performed with primer combinations LR0R/LR7 (nucLSU rRNA), PNS1/NS41 and NS19b/NS8 (nucSSU rRNA), ITS1F/ITS4 (5.8S rRNA), 983F/2218R[2212R] (EF1), 5F/7.1R (RPB2), and ATP6-1[3]/ATP6-2[4] (ATP6). PCR products were cleaned with QIAquick PCR purification spin columns kits (QIAGEN, Valencia, California) following the manufacturer's protocols. Sequencing reactions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosistems, Foster City, California) were performed with

primers LR0R, LR3, LR3R, LR7 (nucLSU); PNS1, NS19b, NS41, NS51, NS8 (nucSSU); ITS1F, ITS4 (5.8s); 983F, 1567R, 1577F, 2212R (EF1); ATP6-1, ATP6-2, ATP6-3, ATP6-4 (ATP6); 6.9F, 8.2R, 11R1, 10.9R, 7cF, 5F, 6F, 6R2, 7.1R (RPB2). Primer sequences can be found in 5.8S, White et al. (1990), Gardes and Bruns (1993); EF1, Rehner and Buckley (2005); ATP6, Kretzer and Bruns (1999); nucSSU, White et al. (1990), Hibbett (1996); nucLSU, Vilgalys and Hester (1990), Moncalvo et al. (2000); RPB2, Liu et al. (1999), Matheny et al. (2007). Sequencing reactions were run on ABI 377 and 3130 automated DNA sequencers. Sequences were edited and assembled with Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan), and spliceosomal introns were excised from RPB2 and EF1 sequences because these regions seemed not to provide relevant phylogenetic information for this taxonomic level of analysis (Matheny et al. 2007). New sequences were deposited in GenBank, accession numbers HM536050-HM536140, HM640259, HM640260 (TABLE I).

Matrix assembly, model selection and compatibility tests.—Two matrices were assembled with 104 species each, one with nucleotides only and the other with amino acid sequences for the protein-coding genes and nucleotide sequences for the ribosomal RNA (rRNA) genes (matrices are available at TreeBASE http://purl.org/phylo/treebase/phylows/study/TB2:S10711). The matrix produced by Matheny et al. (2007) was used as a reference, and the alignment and taxonomic sampling were modified manually in MacClade 4.05 (Maddison and Maddison 2000).

Nine additional matrices were assembled for model selection, six for nucleotides and three for the amino acids. Amino acid models were selected with ProtTest 1.4 (Abascal et al. 2005) through the Web server version (http://darwin.uvigo.es/software/prottest_server.html) with a BioNJ starting tree, optimizing branch lengths, model and topology, and implementing the Akaike information criterion for model selection. Models selected for amino acid data were WAG + gamma (RPB2), DAYHOFF + gamma + invariant sites (EF1) and MtART + gamma (ATP6); this last model is a mitochondrial model specific for arthropods (Abascal et al. 2007), but because it was not possible to implement for maximum likelihood searches this was replaced with MTREV, which is a more general mitochondrial model. Nucleotide models were selected with Modeltest 3.7 (Posada and Crandall 1998, Posada and Buckley 2004), implementing the Akaike information criterion. For all nucleotide partitions the best fitting model was the GTR + gamma + invariant sites model. To assess compatibility between partitions maximum parsimony bootstrap analyses were conducted for all nine matrices with PAUP* 4.0b10 (Swofford 2001) with all characters equally weighted, 1000 replicates and MAXTREES set to auto increase. No support was observed for clades in conflict with the topologies derived from the six single-partition analyses (result not shown).

Phylogenetic analyses and ancestral state reconstruction.—Three methods of phylogenetic analysis were applied, including maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. MP searches were conducted with PAUP* 4.0b10 (Swofford 2001) with nucleotide sequences of both rRNA and protein-coding genes or a combination of nucleotide (rRNA) and amino acid sequences (protein-coding genes). All MP analyses used 10 000 replicates, MAXTREES set to auto increase, and summarized the resulting trees with strict consensus. ML searches were conducted with RAxML 7.0.3 (Stamatakis 2006) with 250 replicates with the GTRMIXI model for six partitions. Searches with the amino acids and nucleotides matrix used the WAG, DAYHOFF and MTREV models for amino acids and a single partition with the GTR model for nucleotides. Bayesian searches were conducted with MrBayes 3.0 (Ronquist and Huelsenbeck 2003); for the nucleotides matrix six partitions were defined with the GTR + gamma + invariant sites model, flat priors for all trees, unlinked parameters and four chains (one cold and three heated). Fifty million generations were run, saving trees every 1000 generations. For the Bayesian amino acid and nucleotide sequences analysis six partitions were defined, model jump for amino acids was used setting aamodelpr = mixed; settings for the other parameters were the same as in the nucleotides analysis.

Character support was measured under the MP and ML criteria with the nonparametric bootstrap (Felsenstein 1985). MP bootstrap was conducted in PAUP* 4b10 with 500 replicates, MAXTREES set to 1000, 100 heuristic searches per replicate, and all characters equally weighted. ML bootstrap was conducted in RAxML 7.0.3 with 300 replicates with the same models and data partitions as in the searches for the optimal trees. The pattern of evolution of wood-decay types was estimated under the parsimony criterion in MacClade 4.05 (wood-decay pattern 0 = white-rotting, 1 = brown-rotting).

Penalized likelihood and relaxed molecular clock analyses.—Penalized likelihood (PL) (Sanderson 2002) and Bayesian relaxed molecular clock analyses (Drummond et al. 2006) were used to explore the timing of origins of the brown-rotting decay type, based on matrices containing amino acids for protein-coding genes and nucleotides for rRNA genes. PL uses an input tree with a known topology and branch lengths estimated without a molecular clock constraint. PL then transforms the input tree into an ultrametric tree, estimating branch lengths and variable rates of evolution, assuming autocorrelation between ancestral and descendant rates along the tree. This method allows every lineage to have a unique rate and restricts strong variation across clades through the use of a penalty function; both parameters are regulated with a smoothing factor (Sanderson 2002). The assumption of autocorrelation has been challenged (Magallón 2004), and some authors also claim that it is preferable to infer the topology along with branch lengths and rates (Drummond et al. 2006). The Bayesian relaxed molecular clock approach involves a simultaneous inference of tree topology and variable evolutionary rates, which are not autocorrelated.

PL analysis was performed with r8s 1.71 (Sanderson 2003), with the optimal tree obtained in ML analyses. The smoothing factor was calculated with two-step cross validation. In the first round initial values were set to cvStart = 0

cvInc = 0.5 cvNum = 8. In the second round values were modified according with results from the first round (cvStart = 1 cvInc = 0.1 cvNum = 10). A smoothing value of 1.4 was selected. Bootstrap was conducted as recommended in the r8s documentation to obtain confidence values a nonparametric (Sanderson 2003). One hundred bootstrap matrices were produced with the seqboot module of PHYLIP 3.6 (Felsenstein 1989) using the original amino acid and nucleotides matrix. For each resampled matrix branch lengths were calculated on the selected topology with RAxML 7.0.3 with the WAG model for amino acids and GTR for nucleotides. A single file with the 100 trees was assembled and analyzed with r8s with a smoothing value of 1.4. Ultrametric trees were summarized with TreeAnnotator 1.4.7 (Drummond and Rambaut 2007) and displayed in FigTree 1.1.2 (Rambaut 2008).

Bayesian relaxed molecular clock analyses used two matrices that were produced with BEAUTi 1.4.7 (Drummond and Rambaut 2007), one for the amino acid sequences with WAG + gamma with four categories, and invariant sites, and a second for the ribosomal nucleotides sequences with GTR + gamma with four categories, invariant sites and empirical base frequencies. Once the basic matrices were produced they were manually edited, including both alignments and model parameters in the same file, following guidelines for the analysis of multilocus data sets provided by Drummond and Rambaut (2006 in http://beast.bio.ed.ac.uk/Tutorial_8). The substitution rate mean was fixed, an uncorrelated log normal relaxed clock was invoked, root height was fixed to 10 with a normal prior distribution with stdev = 1.0, a Yule speciation process tree prior was invoked and the topology used for the penalized likelihood analysis was used as a starting tree. The MCMC chain was set to 5 000 000 generations with sampling every 1000 generations. Five independent chains were run in BEAST 1.4.2, and 10% of samples were discarded from each independent chain. The remaining states were combined in LogCombiner 1.4.7, summarized in TreeAnnotator 1.4.7 and viewed in FigTree 1.1.2

RESULTS AND DISCUSSION

The final dataset contains 506 gene sequences, with approximately 19% missing data (104 nuc-lsu rRNA, 99 nuc-ssu rRNA, 77 5.8S rRNA, 85 *rpb*2, 73 *atp*6, 68 *tef*1). GenBank accession numbers and strain information for Gloeophyllales and *Donkioporia expansa* are provided (TABLE I), as well as information for sequences of other taxa downloaded from GenBank (SUPPLEMENTARY TABLE I). Forty species were represented by all six genes, and 34 species were represented by five genes. Eight of the 19 isolates of Gloeophyllales had all six genes, six had five genes, *Boreostereum radiatum* had four genes and four species had one or two of the rRNA genes (TABLE I). The dataset containing nucleotides only had an aligned length of 7681 base pairs (4081 variable positions, 3057 parsimony informative positions), while the dataset containing nucleotides and amino acids had an aligned length of 5043 characters (2320 variable, 1430 parsimony informative). MP analyses of the nucleotide dataset returned nine trees of 36 962 steps (CI = 0.1728 excluding uninformative characters, RI = 0.3811, RCI + 0.0761), while MP analyses of the dataset containing nucleotides and amino acids returned 11 trees of 12 085 steps (CI = 0.3176 excluding uninformative characters, RI = 0.5236, RCI = 0.1988).

Limits of the Gloeophyllales.—All phylogenetic analyses recovered a monophyletic ''core Gloeophyllales'' group that included *Gloeophyllum*, *Chaetodermella*, *Heliocybe*, *Neolentinus* and *Veluticeps* (FIGS. 1, 2; SUPPLEMENTAL FIGS. 1–3). This assemblage is morphologically diverse, but all members are brown-rotting wood decayers. Five of the six analyses placed *Boreostereum radiatum* as the sister group of the core Gloeophyllales clade. The parsimony tree inferred with nucleotides placed *B. radiatum* within the Corticiales, but character support was lacking (SUPPLEMENTAL FIG. 1). The nucleotide parsimony tree also differed from the other analyses in the placement of the Russulales and Thelephorales (FIG. 3). Disagreements between the nucleotide parsimony analysis and the other analyses could be due to misleading phylogenetic signal from third codon positions in the protein coding genes. In the nucleotide alignment 1228 (40%) of the 3057 parsimony informative characters corresponded with third positions. We speculate that this high proportion of rapidly evolving positions might be responsible for artifacts under parsimony. Due to the lack of character support for the placement of *Boreostereum* within the Corticiales, we conclude that this genus is a member of the Gloeophyllales.

All six analyses suggested that *Donkioporia expansa* is nested within the core polyporoid clade, contrary to the results of Kim and Jung (2001), which placed it in the Gloeophyllales based on nucSSU sequences (with weak support). The closest relatives of *D. expansa* in our analyses are *Ganoderma tsugae* and *Cryptoporus volvatus* (FIG. 2). These results were consistent with those of Vlasák et al. (2010), who analyzed ITS sequences of *D. expansa* and a second species of the genus, *Donkioporia albidofuscus* (Domański) Vlasák & Kout, which they transferred from *Dichomitus* D.A. Reid. *Donkioporia* differs from most of the Gloeophyllales in its production of a white rot, which is consistent with its placement in the core polyporoid clade.

Our results are congruent with the current circumscription for the order (Thorn Hibbett et al. 2007), differing only in the placement of *Donkioporia*. The

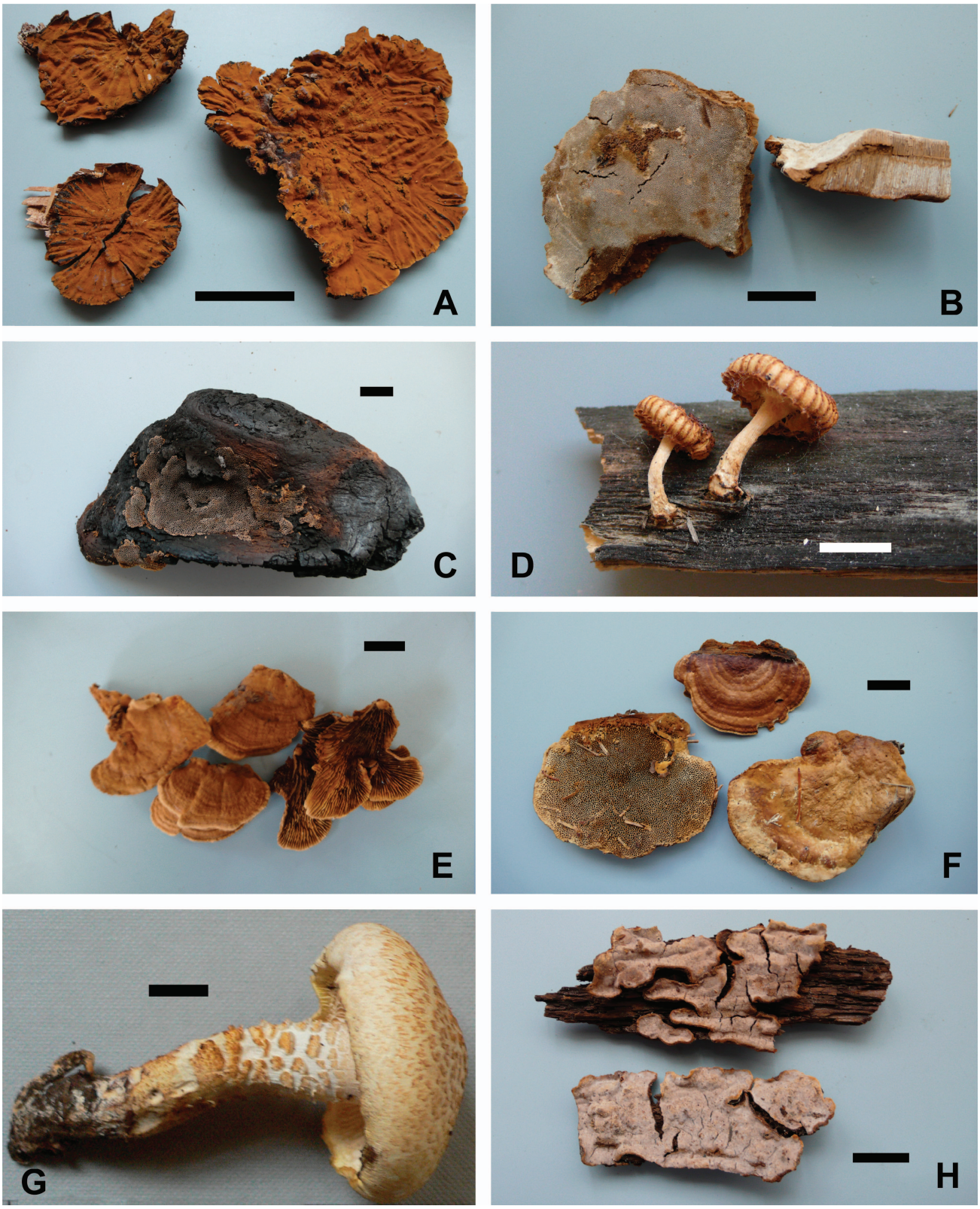

FIG. 1. Fruiting body diversity in Gloeophyllales and *Donkioporia expansa* (Polyporales). A. *Boreostereum radiatum* (Peck) Parmasto DAOM 160898. B. *Donkioporia expansa* (Desm.) Kotl. & Pouzar AN028794. C. *Gloeophyllum carbonarium* (Berk. & M.A. Curtis) Ryvarden AN010068. D. *Heliocybe sulcata* (Berk.) Redhead & Ginns IBUG 9930. E. *Gloeophyllum sepiarium* (Wulfen) P. Karst. AN028562. F. *Gloeophyllum protractum* (Fr.) Imazeki AN032340. G. *Neolentinus lepideus* (Fr.) Redhead & Ginns RGS-DSH s.n. H. *Veluticeps berkeleyi* Cooke SD62-6-4. Bars = 1 cm.

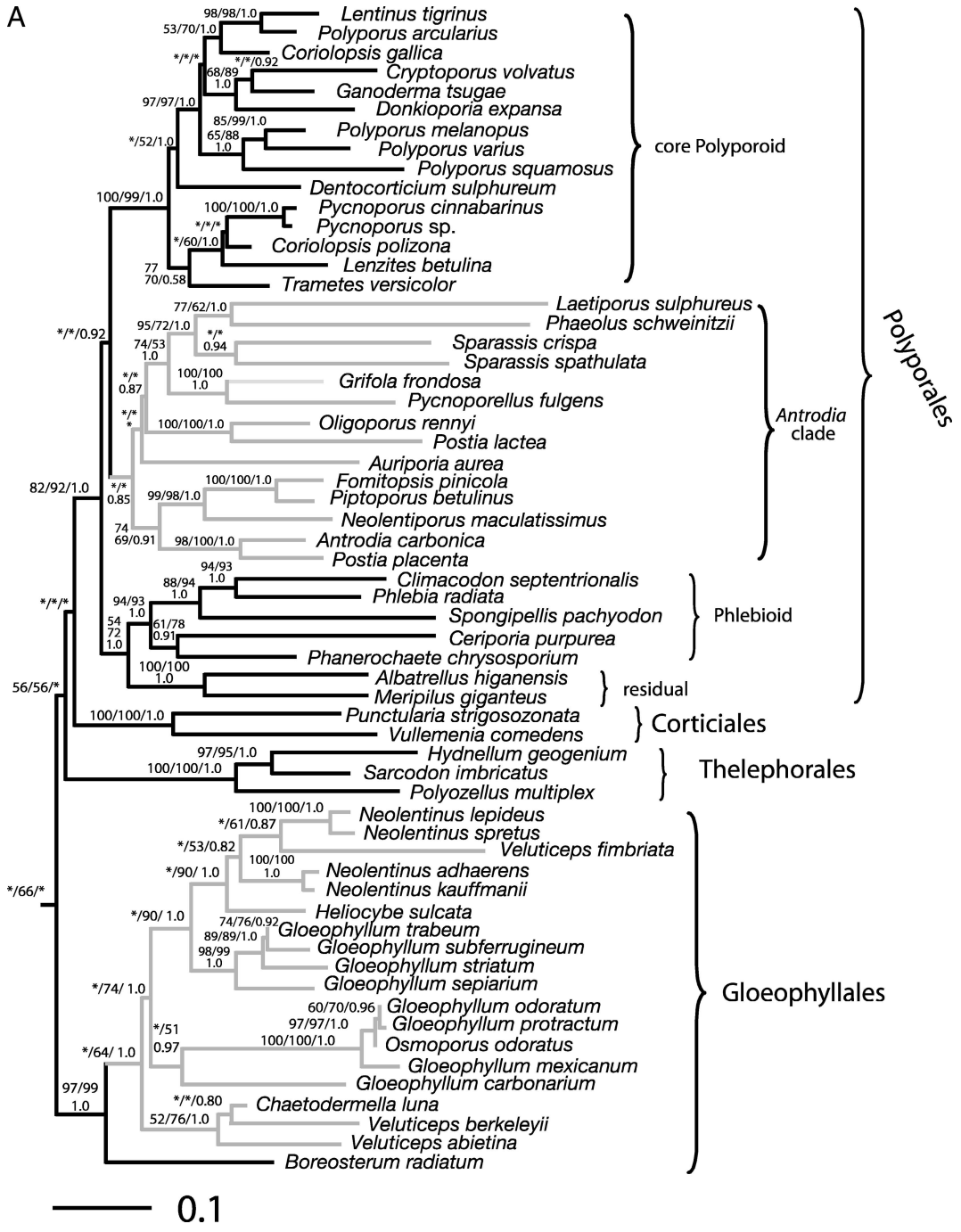

FIG. 2. Phylogenetic relationships of Gloeophyllales inferred with amino acids (*rpb2*, *atp6*, *tef1*) and nucleotide (nuc-lsu, nuc-ssu, 5.8S rRNA) sequences. Topology from maximum likelihood analysis. Support values along branches from maximum parsimony bootstrap, maximum likelihood bootstrap and Bayesian analyses respectively. Red branches indicate brown-rot lineages. Dashed red branches indicate presumed brown-rot lineages. Blue branch indicates a reversion to white rot in a brown-rot linage. Branch lengths are drawn proportional to genetic distances (bar = 0.1 average changes per site), except long branches leading to *Cantharellus* and Agaricomycotina.

Gloeophyllales is highly heterogeneous in fruiting body form, including polypores (*Gloeophyllum*), agarics (*Neolentinus*, *Heliocybe*) and corticioid and stereoid forms (*Boreostereum*, *Chaetodermella*, *Veluticeps*) with dimitic or trimitic construction. Mating systems also are variable; *Neolentinus* and *Gloeophyllum* are bipolar, *Veluticeps* is reportedly tetrapolar, and *Boreostereum* has been suggested to be homothallic (Redhead and Ginns 1985; Gilbertson and Ryvarden 1986; Martin and Gilbertson 1973; Cha-

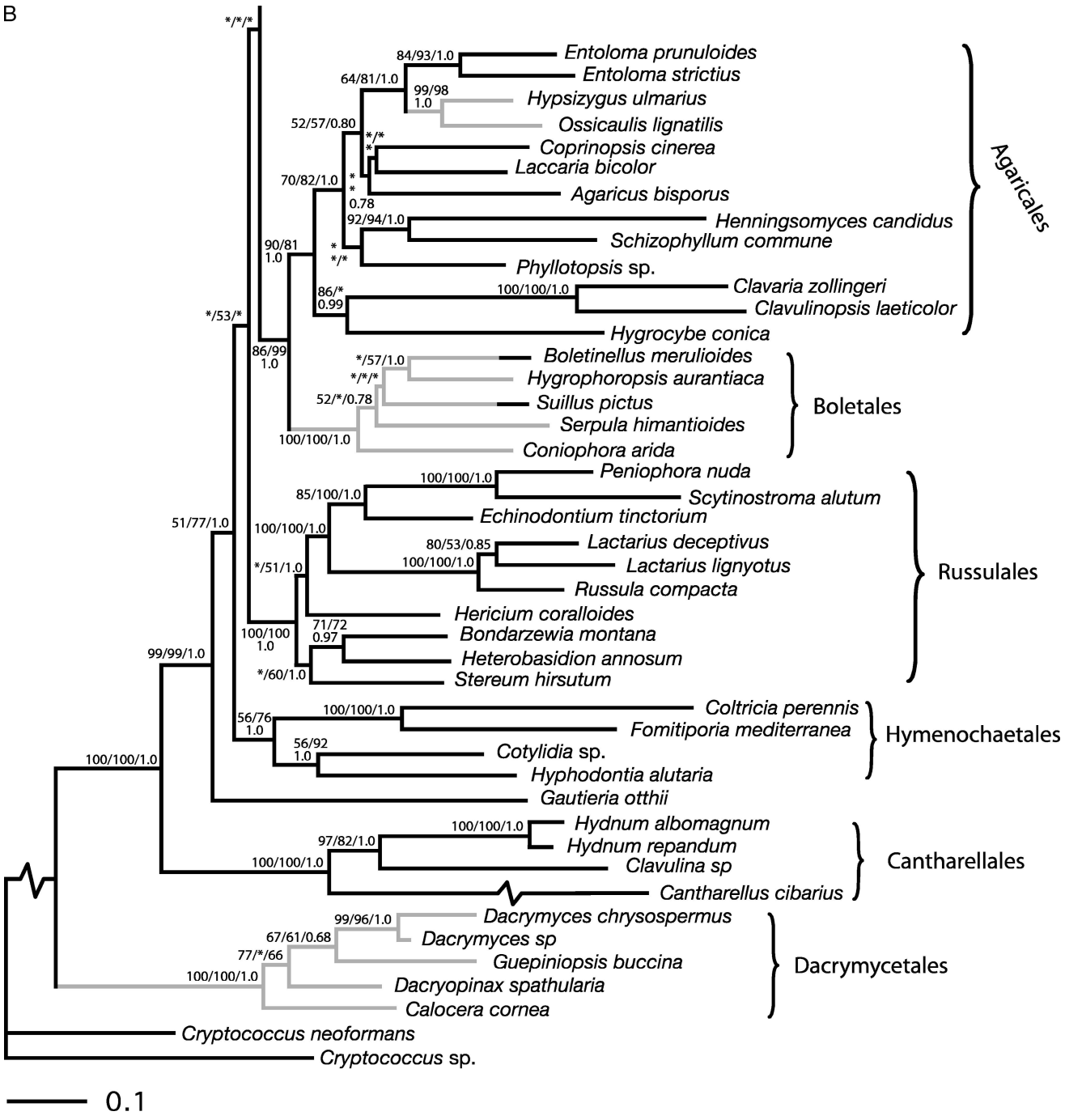

FIG. 2. Continued.

muris 1988; Nakasone 1990a, b). *Boreostereum radiatum* is a stereoid species that has been reported to produce a white-rot type of wood decay, although evidence on its decay mode is somewhat ambiguous (Chamuris 1988). Nakasone (1990a) reported this species as white rot, but Chamuris (1988) reported this species as negative for laccase and tyrosinase, present in most white-rot species. Genomic comparisons between *Boreostereum* and *Gloeophyllum trabeum* could provide insight into the mechanistic basis of transitions between white-rot and brown-rot modes of wood decay.

Isolates of *Gloeophyllum* form at least two clades. One is a strongly supported group that includes *G. sepiarium*, *G. trabeum* and others, while the other is a weakly supported group containing *G. carboniarium* (Berk. & Curt.) Ryv., *G. protractum* (Fr.) Imaz., *G. odoratum* (Fr.) Imaz. and *G. mexicanum* (Mont.) Ryv.

The latter three species are strongly supported as monophyletic, and they include taxa that have been classified in *Osmoporus*, including *O. odoratus* (Wulfen) Singer (type species for the genus) and *O. protractus* (Fr.) Bondartsev, suggesting that that genus needs reinstating. Similarly an alternate generic placement is available for *Gloeophyllum carbonarium*, which has been classified as *Griseoporia carbonaria* (Berk. & M.A. Curtis) Ginns (Ginns 1984).

Chaetodermella luna, which was represented only by a nuc-lsu rRNA sequence, was nested within a paraphyletic assemblage of *Veluticeps* isolates, but support was not strong (FIG. 2, SUPPLEMENTARY FIGS. 1–3). In some analyses (FIG. 2) *Veluticeps fimbriata* was nested within a paraphyletic assemblage of *Neolentinus* species, which would imply a reversal from an agaricoid form to a resupinate form, but in other analyses (FIG. 4, SUPPLEMENTARY FIG. 1) it iwas placed with the other *Veluticeps* species, which presents a more parsimonious scenario for morphological evolution. *Heliocybe sulcata* is placed as the sister group of the *Neolentinus* clade (with or without *V. fimbriata*) with generally strong support from ML and Bayesian analyses (FIG. 2). Thus the transfer of *Heliocybe* into *Neolentinus* suggested by Rune (1994) appears to be unnecessary.

Higher level relationships of Gloeophyllales and Polyporales and number of origins of brown rot.—The higher level placement of the Gloeophyllales was resolved inconsistently in the phylogenetic analyses. Most analyses placed the Gloeophyllales in a larger clade that also contained Polyporales and Thelephorales; MP and ML analyses with nucleotides plus amino acids also placed Corticiales in that group (FIGS. 2, 3). The precise placement of the Gloeophyllales within the Gloeophyllales-Polyporales-Thelephorales-Corticiales clade varied among analyses however, and the parsimony analysis of nucleotide sequences suggested that the Gloeophyllales (excluding *B. radiatum*) is outside that group. Our results echoed those of analyses that grouped the Gloeophyllales with the Thelephorales (Binder et al. 2005, Larsson 2007), Polyporales (Thorn et al. 2000, Binder et al. 2005) and Corticiales (Lutzoni et al. 2004, Binder et al. 2005, Larsson 2007). However the present analysis used many of the same sequences as the studies just cited and should not be considered a truly independent estimate of the phylogenetic placement of the Gloeophyllales.

All analyses recovered the Polyporales as a monophyletic group, which is consistent with the multilocus studies of Binder and Hibbett (2002), Binder et al. (2005), Matheny et al. (2007) and Larsson (2007). The sister group of the Polyporales was resolved variously as the Corticiales (ML nucleotides plus amino acids), Thelephorales (ML and Bayesian analyses of nucleotides), Corticiales-Thelephorales-Gloeophyllales (MP nucleotides plus amino acids) or Russulales-Corticiales-*Boreostereum* (MP nucleotides) (FIG. 3). Thus monophyly of the Polyporales was strongly supported in the present study but its higher level relationships remained poorly resolved.

Binder et al. (2005) identified four groups within the Polyporales, including the ''core polyporoid clade'', ''*Antrodia* clade'', ''phlebioid clade'' and ''residual polyporoid clade''. The *Antrodia* clade contained all brown-rot taxa of the Polyporales as well as the white-rotting *Grifola frondosa*. Binder and Hibbett (2002) analysis and others suggested that the brown-rotting *Wolfiporia cocos* is in the core polyporoid clade, but the isolate used in those studies was misidentified; *Wolfiporia* is actually in the *Antrodia* clade (Linder and Banik 2008). The present analyses provided strong to moderate support for the core polyporoid clade and the phlebioid clade plus residual polyporoid clade (FIG. 2). The *Antrodia* clade was resolved as monophyletic in ML and Bayesian analyses, but it was paraphyletic in the MP analyses with the core polyporoid clade nested within it. The topology obtained in the Bayesian relaxed molecular clock analysis performed with BEAST also showed the *Antrodia* clade to be paraphyletic, but in this case it is the phlebioid clade that is nested within the *Antrodia* clade (FIG. 4).

Under equally weighted parsimony, trees that showed the *Antrodia* clade to be monophyletic imply five origins of brown rot in lineages leading to the core Gloeophyllales (*B. radiatum* retains the plesiomorphic white-rot decay type), Dacrymycetales, *Antrodia* clade, Boletales and Lyophyllaceae (sensu Matheny et al. 2008, including the brown-rotting agarics *Ossicaulis* and *Hypsizygus*) and one reversal from brown rot to white rot in *Grifola frondosa* (FIG. 3). Trees that showed the *Antrodia* clade to be paraphyletic still implied five origins of brown rot but required one additional reversal from brown rot to white rot, leading either to the phlebioid clade (plus residual polyporoid clade) or the core polyporoid clade. The results of the present analysis were similar to those of Hibbett and Donoghue (2001), who inferred 6–7 independent origins for the brown-rotting decay pattern. The reason that Hibbett and Donoghue resolved more origins of brown rot than the present study is that they included the brown-rot *Fistulina hepatica* (Agaricales), which was not sampled here, and they scored the misidentified ''*Wolfiporia cocos*'' isolate as a brown rot (isolate FPL 4198).

Transitions between decay modes are rare in the Agaricomycetes. Based on comparisons of the ge-

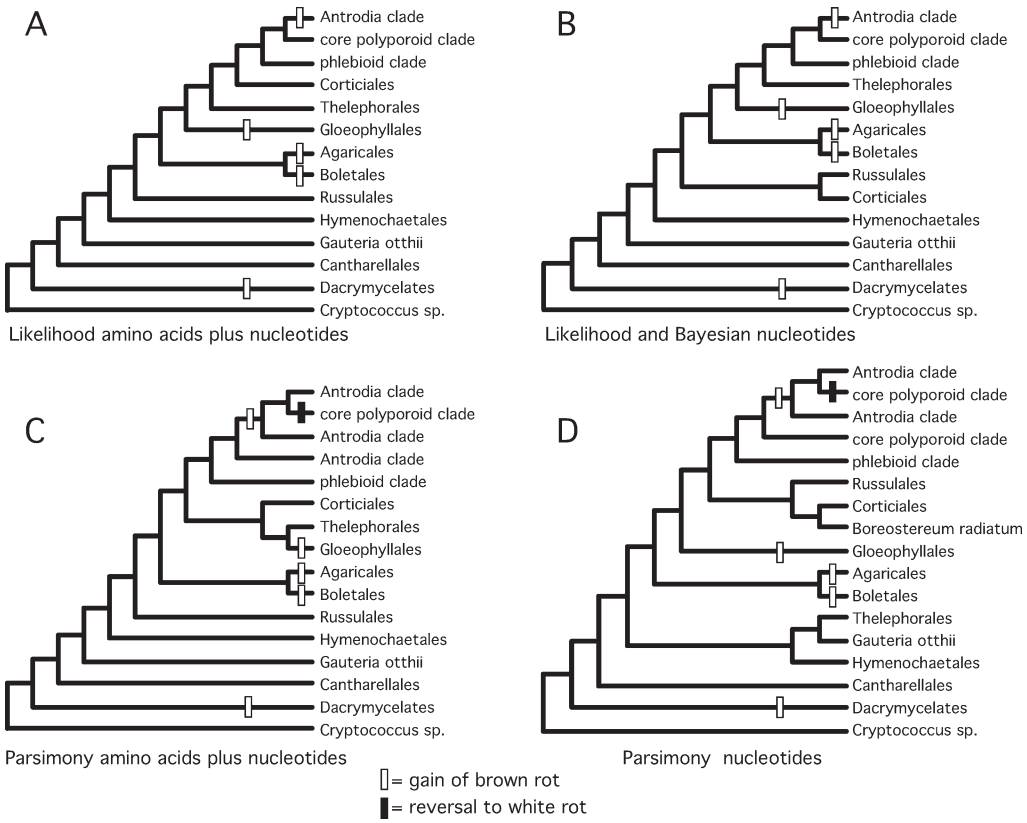

FIG. 3. Simplified phylogenetic topologies and ancestral state reconstructions of the evolution of brown rot. A. Maximum likelihood analysis of amino acids and nucleotides. B. Maximum likelihood and Bayesian analyses of nucleotides. C. Maximum parsimony analyses of amino acids and nucleotides. D. Maximum parsimony analyses of nucleotides. Reconstructions show only gains of brown rot and reversal to white rot in core polyporoid clade (C, D). Reversal to white rot in *Grifola frondosa* and multiple gains and losses of brown rot in Agaricales and Boletales are not indicated.

nomes of the brown-rot polypore *Postia placenta* (*Antrodia* clade) and the model white-rot fungus, *Phanerochaete chrysosporium* (phlebioid clade), it appears that transitions between white rot and brown rot involve large shifts in copy numbers of genes encoding enzymes encoding class II fungal peroxidases (including manganese peroxidase and lignin peroxidase), which putatively are involved in degradation of lignocellulose (gene families encoding certain glycoside hydrolases also are reduced in *P. placenta* vs. *P. chrysosporium*). The *P. chrysosporium* genome contains 15 genes encoding class II fungal peroxidases, but these genes are absent in the *P. placenta* genome, which contains only one ''low redox potential'' peroxidase of uncertain function (Martinez et al. 2009). As yet there are no complete genomes for members of the core polyporoid clade, but cloning studies have demonstrated that there are multiple class II fungal peroxidases in core polyporoid taxa such as *Trametes versicolor*, *Cryptoporus volvatus*, and *Dichomitus squalens* (Morgenstern et al. 2008). If the *Antrodia* clade is paraphyletic with respect to either the phlebioid clade or the core polyporoid clade, then this would imply that there have been parallel expansions or contractions of class II fungal peroxidases. Therefore the topologies showing monophyly of the *Antrodia* clade, which required only one reversal from brown rot to white rot, are more parsimonious than those showing paraphyly of the *Antrodia* clade, requiring two such reversals (FIG. 3).

Relative timing of origins of brown rot.—PL analysis used the topology obtained in the ML analysis of amino acids plus nucleotides, while Bayesian relaxed molecular clock analyses estimated the phylogeny directly with BEAST. Topological differences between the PL tree and the BEAST tree involved the higher level placement for certain non-brown-rotting clades, such as Corticiales and Russulales, as well as the resolution of the *Antrodia* clade as monophyletic (PL) or paraphyletic (BEAST) (FIG. 4, SUPPLEMENTAL FIG. 3). Nonetheless the relative timing of the diversification of the five brown-rotting clades was largely

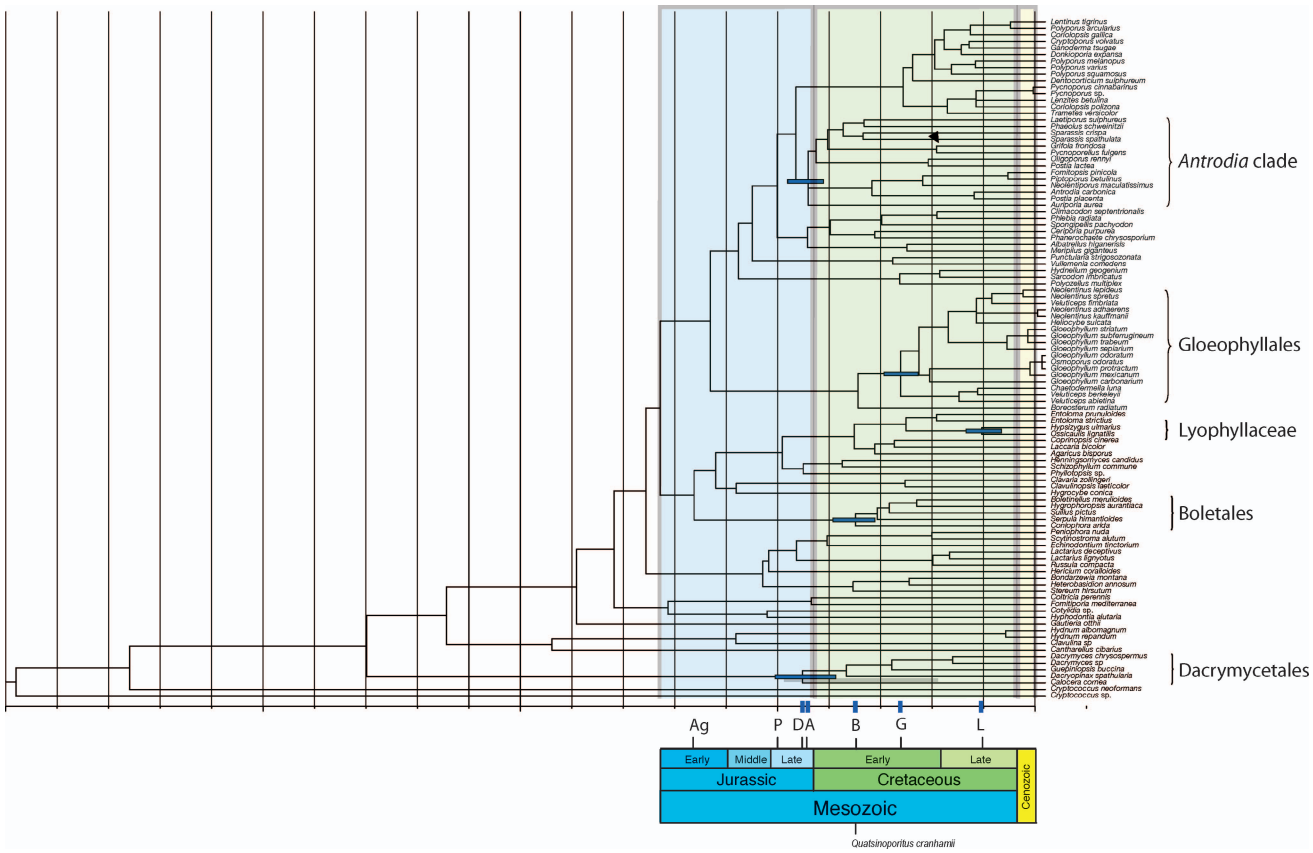

FIG. 4. Chronogram estimated with penalized likelihood analysis implemented in r8s, emphasizing relative ages of brown-rot clades (node height ranges for other clades are not shown). Bars indicate 95% bootstrap node height ranges (node height ranges for other clades are not shown). Triangle indicates putative reversal from brown rot to white rot leading to *Grifola frondosa*. Mean age of origin of groups indicated with letters above timescale: Ag = Agaricomycetidae, P = Polyporales, D = basal split in Dacrymycetales, A = Antrodia clade, B = Boletales, G = Gloeophyllales, L = Lyophyllaceae.

congruent between both methods. In both analyses the oldest nodes that represent brown-rot taxa (based on the ancestral state reconstruction) were the root nodes of the Dacrymycetales and the *Antrodia* clade, followed by the root nodes of the Boletales, core Gloeophyllales and the Lyophyllaceae. In the PL analysis the origins of brown-rot clades occured in three discrete episodes, delimited by non-overlapping node height ranges based on analyses of bootstrapped datasets. The first episode involved diversification of the Dacrymycetales, *Antrodia* clade and Boletales; the second episode involved the core Gloeophyllales; and the third episode involved the Lyophyllaceae (FIG. 4). In the BEAST analysis the 95% highest posterior density (HPD) ranges of node heights for these groups overlapped, so it was not possible strictly speaking to assign the nodes to different episodes (SUPPLEMENTAL FIG. 3). Nevertheless, based on mean node heights, the *Antrodia* clade and the Dacrymycetales were resolved as the oldest nodes, followed by the Boletales, Gloeophyllales and Lyophyllaceae.

To place the origins of brown-rot Agaricomycetes in the context of geologic time and the evolution of their woody plant hosts we compared the results of our PL and BEAST analyses to Bayesian relaxed clock phylogenies for Fungi and plants by Hibbett and Matheny (2009), who sampled many of the same fungal taxa as the present study and used a similar set of genes (nucSSU, nucLSU, RPB1, RPB2). The analysis of Hibbett and Matheny suggested that the Boletales is about the same age as or possibly younger than the Pinaceae and that the Agaricomycetidae (including Agaricales and Boletales) is about as old as the Angiosperms. To obtain absolute age estimates for our trees we assumed that the age of the Pinaceae is 130 000 000 y, based on studies of Gernandt et al. (2008) and Miller (1976), and we assumed that the age of the Angiosperms is 189 000 000 y, following Magallón and Sanderson (2005). An origin of Angiosperms in the lower Jurassic has been controversial, but it is consistent with other molecular clock studies (Wikström et al. 2001, Bell et al. 2005, Soltis et

al. 2009) and is supported by the occurrence of pollen with Angiosperm-like characters in the Triassic and Jurassic (Maheshwari 2007, Zavada 2007).

Extrapolating from the studies cited above we placed an absolute timescale on the PL and BEAST chronograms. Molecular clock analyses have numerous sources of error, including rate heterogeneity and those associated with calibration, so this has to be seen as a tentative and explorative exercise. Nevertheless the age estimates for major groups of Agaricomycetes that we obtained were consistent with the oldest Agaricomycete fossils (the Cretaceous *Quatsinoporites cranhamii* SY Sm., Currah & Stockey and *Archaeomarasmius leggetti* Hibbett, D Grimaldi & Donoghue, which are putative members of the Hymenochaetales and Agaricales respectively; Hibbett et al. 1997, Smith et al. 2004), which were not used as calibration points. Both PL and BEAST analyses placed the root nodes of the Hymenochaetales and Agaricales in the middle to early Jurassic (FIG. 4), according to our calibration.

With this calibration PL analysis placed the earliest origins of brown-rotting clades of Agaricomycetes in the late Jurassic to early Cretaceous. The 95% HPD ranges of node heights in the BEAST analysis were broad, but median node heights place the origin of the *Antrodia* clade in the middle Jurassic with the root node of the Dacrymycetales in the late Jurassic (FIG. 4). Currently the most frequent—but not exclusive—substrates for brown rot Agaricomycetes are conifers (Gilbertson 1980, Hibbett and Donoghue 2001). The middle Jurassic had a large amount of conifer biomass but low species diversity (Burgoyne et al. 2005), while species diversity rose in the early Cretaceous (Philippe et al. 2004). Genera such as *Pseudolarix* were available since the early Cretaceous (LePage and Basinger 1995), and even if this is not a common substrate for brown-rotting fungi today *Pseudolarix* wood is not so different from *Abies* or *Cedrus* wood (Esteban and de Palacios 2009), which are more common substrates. In addition some other Pinaceae-like genera were available in the late Jurassic (Philippe et al. 2004) and could have supported the early diversification of the *Antrodia* clade and Dacrymycetales. In contrast the origin of the Gloeophyllales is placed in the Aptian period of the early Cretaceous in both PL and BEAST analyses and the origin of the Lyophyllaceae is placed in the late Cretaceous, when even Angiosperms (substrate for *Ossicaulis*) were well established. By the time the Gloeophyllales appeared, Pinaceae wood was already available as a substrate.

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