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Molecular phylogeny of *Laetiporus* and other brown rot polypore genera in North America

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Abstract: Phylogenetic relationships were investigated among North American species of *Laetiporus*, *Leptoporus*, *Phaeolus*, *Pycnoporellus* and *Wolfiporia* using ITS, nuclear large subunit and mitochondrial small subunit rDNA sequences. Members of these genera have poroid hymenophores, simple septate hyphae and cause brown rots in a variety of substrates. Analyses indicate that *Laetiporus* and *Wolfiporia* are not monophyletic. All North American *Laetiporus* species formed a well supported monophyletic group (the ''core *Laetiporus* clade'' or *Laetiporus* s.s.) with the exception of *L. persicinus*, which showed little affinity for any genus for which sequence data are available. Based on data from GenBank, the southern hemisphere species *L. portentosus* also fell well outside the core *Laetiporus* clade. *Wolfiporia dilatohypha* was found to represent a sister group to the core *Laetiporus* clade. Isolates of *Phaeolus*, *Pycnoporellus* and members of the core *Laetiporus* clade all fell within the Antrodia clade of polypores, while *Leptoporus mollis* and *Laetiporus portentosus* fell within the phlebioid clade of polypores. *Wolfiporia cocos* isolates also fell in the Antrodia clade, in contrast to previous studies that placed *W. cocos* in the core polyporoid clade. ITS analyses resolved eight clades within *Laetiporus* s.s., three of which might represent undescribed species. A combined analysis using the three DNA regions resolved five major clades within *Laetiporus* s.s.: a clade containing conifer-inhabiting species (''Conifericola clade''), a clade containing *L. cincinnatus* (''Cincinnatus clade''), a clade containing *L. sulphureus* s.s. isolates with yellow pores (''Sulphureus clade I''), a clade containing *L. sulphureus* s.s. isolates with white pores (''Sulphureus clade II'') and a clade containing *L. gilbertsonii* and unidentified isolates from the Caribbean (''Gilbertsonii clade''). Although there is strong support for groups within the core *Laetiporus* clade, relationships among these groups remain poorly resolved.

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INTRODUCTION

The genera *Laetiporus* Murrill, *Leptoporus* Quél., *Phaeolus* (Pat.) Pat., *Pycnoporellus* Murrill and *Wolfiporia* Ryvarden & Gilb. contain species that possess simple septate hyphae, cause brown rots and produce annual, polyporoid fruiting bodies with hyaline spores. These shared morphological and physiological characters have been considered important in traditional polypore taxonomy (e.g. Gilbertson and Ryvarden 1986, Gilbertson and Ryvarden 1987, Ryvarden 1991). However recent molecular work indicates that *Laetiporus*, *Phaeolus* and *Pycnoporellus* fall within the ''Antrodia clade'' of true polypores identified by Hibbett and Donoghue (2001) while *Leptoporus* and *Wolfiporia* fall respectively within the ''phlebioid'' and ''core polyporoid'' clades of true polypores (Binder et al 2005).

Recent molecular and mating studies also have led to a revision of genus *Laetiporus* in North America (Banik et al 1998, Banik and Burdsall 1999, Banik and Burdsall 2000), which formerly contained only *L. sulphureus* (Bull.) Murrill and *L. persicinus* (Berk & M.A. Curtis) Gilbertson. Burdsall and Banik (2001) concluded that genus *Laetiporus* contains at least six morphologically and ecologically distinct species. Despite this recent work relationships remain unresolved among North American *Laetiporus* species and among other brown rot polyporoid species lacking clamps. This study had two goals: (i) to determine relationships among North American species of *Laetiporus* and (ii) to determine whether any North America polyporoid species that cause brown rots and lack clamps are closely related to *Laetiporus*. To accomplish these goals species of *Laetiporus*, *Leptoporus*, *Phaeolus*, *Pycnoporellus* and *Wolfiporia* were sequenced in three regions of rDNA: the intergenic transcribed spacer (ITS), the nuclear large subunit (nLSU) and the mitochondrial small subunit (mtSSU).

Of the genera included in this study the most widely known is *Laetiporus*. As it is currently defined *Laetiporus* includes annual polypore species that produce a brown rot, have dimitic binding hyphae and lack cystidia and clamp connections (Gilbertson

and Ryvarden 1986, Ryvarden 1991). With the exception of *L. persicinus*, all North American *Laetiporus* species produce brightly colored, conspicuous fruiting bodies that have been regarded traditionally as part of the *L. sulphureus* s.l. species complex. Species in the *L. sulphureus* s.l. complex are popular edibles that frequently are collected under the common name ''Sulfur Shelf'' or ''Chicken of the Woods'' (Arora 1986). *Laetiporus sulphureus* s.l. also has been investigated for novel antimicrobial and medicinal compounds (Turkoglu et al 2007) and its bright pigments have been examined for potential as food colorants (Davoli et al 2005). Various authors from the late 19th and early 20th centuries described additional varieties and species within the *L. sulphureus* s.l. complex to account for the wide range of morphological and ecological variation exhibited by this species, although few of these names were used widely or consistently. Burdsall and Banik (2001) recognize five species and an additional variety within the *L. sulphureus* s.l. complex: *L. cincinnatus* (Morgan) Burds., Banik & T.J. Volk, *L. conifericola* Burds. & Banik, *L. gilbertsonii* Burds., *L. gilbertsonii* var. *pallidus* Burds., *L. huroniensis* Burds. & Banik and *L. sulphureus* s.s.

With the exception of *Laetiporus cincinnatus*, all species in the *L. sulphureus* s.l. complex typically produce sessile to laterally substipitate pilei with bright orange upper surfaces and yellow or white pore layers (Burdsall and Banik 2001). *Laetiporus cincinnatus* produces centrally stipitate, rosette-shaped fruiting bodies with a white pore layer; these fruiting bodies arise near the base of large diameter hardwood trees, usually *Quercus* species, in the eastern and midwestern United States. In North America *Laetiporus sulphureus* s.s. (as defined by Burdsall and Banik 2001 and the present study) is found in the eastern and midwestern United States and often occurs on *Quercus* species although specimens occasionally are found on other hardwoods. Preliminary evidence based on a limited number of ITS sequences suggests that North American and European populations of *L. sulphureus* s.s. are conspecific (unpubl data), however further comparative work is needed. If species barriers exist between North American and European populations of *L. sulphureus* s.s., nomenclatural revision of the North American species would be required given that *L. sulphureus* s.s. is typified based on European material.

The recently described species *L. conifericola* and *L. huroniensis* (Burdsall and Banik 2001) are restricted to conifers, with *L. huroniensis* being found in the upper midwestern United States and *L. conifericola* being restricted to western North America. *Laetiporus gilbertsonii* occurs in the southern and western regions of North America and occurs on a wide range of hardwoods including *Eucalyptus*. The upper pileus surface of *L. gilbertsonii* is generally more salmon to pink than other *Laetiporus* species while the pore layer is either yellow or white, which differentiates the two color forms, *L. gilbertsonii* with a yellow pore layer and *L. gilbertsonii* var. *pallidus* with a white pore layer.

Laetiporus persicinus is the one North American *Laetiporus* species not considered part of the *L. sulphureus* s.l. complex. This is due to striking macroscopic differences, including a brown to pinkish-brown pileus surface, a pinkish-cream pore layer and flesh and tubes that bruise blackish-brown. *Laetiporus persicinus* originally was described by Berkeley and Curtis in 1872 as *Polyporus persicinus* but was transferred to *Laetiporus* in 1981 by R. Gilbertson (Gilbertson 1981). *Laetiporus persicinus* is found in the southeastern United States occurring primarily on *Quercus* although it is found occasionally on conifers. Burdsall and Banik (2001) hypothesized that *L. persicinus* might not be closely related to species in the *L. sulphureus* s.l. complex based on the dark pigmentations in the fruiting bodies, the appearance of the binding hyphae and preliminary RFLP data.

Work by Hibbett and Donoghue (1995) with mtSSU rDNA sequences indicated that genus *Phaeolus* is closely related to *Laetiporus sulphureus* s.l. This result has been observed in many analyses (Hibbett and Donoghue 2001, Hibbett and Binder 2002, Binder et al 2005), which confirmed that *Phaeolus* is not aligned with the *Hymenochaetales* despite superficial similarities (Wagner and Fischer 2001). The single North American species of *Phaeolus* treated by Gilbertson and Ryvarden (1987), *P. schweinitzii* (Fr.) Pat., is an important root rot pathogen in conifer ecosystems. Like *L. cincinnatus* and *L. persicinus*, *P. schweinitzii* has a centrally stipitate fruiting body found at the base of trees. Unlike *Laetiporus* species, it possesses a monomitic hyphal system and produces hymenial cystidia. Young *Phaeolus* fruiting bodies are yellow to orange but become predominantly brown with age (Gilbertson and Ryvarden 1987).

Genus *Pycnoporellus* also contains species with monomitic, yellow to orange fruiting bodies, leading Gilbertson and Ryvarden (1987) to suggest a close relationship between *Phaeolus* and *Pycnoporellus*. Gilbertson (1981) included both North American species of *Pycnoporellus*, *P. alboluteus* (Ellis & Everh.) Kotl. & Pouzar and *P. fulgens* (Fr.) Donk, in genus *Phaeolus* (Gilbertson 1981). However Gilbertson and Ryvarden (1987) placed these species in *Pycnoporellus*. Like *Phaeolus* both *Pycnoporellus* species grow primarily on coniferous wood although neither forms the

centrally stipitate fruiting bodies typical of *Phaeolus*. *Pycnoporellus alboluteus* is resupinate and occurs primarily in western North America while *P. fulgens* is a pileate species found predominantly in eastern North America. A distinguishing characteristic of *Pycnoporellus* species is a bright red reaction produced by 2% KOH on fruiting bodies; *Phaeolus schweinitzii* in contrast turns dark brown to black in 2% KOH (Gilbertson and Ryvarden 1987).

Species in genus *Wolfiporia* show microscopic similarities to species of *Laetiporus*, *Phaeolus* and *Pycnoporellus* despite producing macroscopic fruiting bodies that are thin, resupinate and lacking in bright pigmentations. The two species of *Wolfiporia* reported from North America, *W. cocos* (F.A. Wolf) Ryvarden & Gilb. and *W. dilatohypha* Ryvarden & Gilb., are dimitic and possess greatly inflated skeletal hyphae, a defining characteristic of this genus (Ryvarden 1991). Although *Wolfiporia cocos* also has been known as *W. extensa* (Peck) Ginns, Redhead and Ginns (2006) proposed to conserve the basionym *Poria cocos* against *Daedalea extensa* due to a lack of acceptance of the nomenclaturally correct name *W. extensa*. The conservation of the epithet *cocos* should stabilize the nomenclature associated with this species and end years of nomenclatural ambiguity (see Ginns and Lowe 1983, Ginns 1984). *Wolfiporia cocos* is most widely known for producing large, edible sclerotia that have been referred to as ''tuckahoes'' or ''Indian bread'' in North America (Weber 1929). The sclerotia of *W. cocos* have been reported from China, usually under the name *Poria cocos*, where they have been collected and used medicinally (Zhang et al 1997, Wang et al 2004, Wu et al 2004, etc.).

Leptoporus is a monotypic genus in North America containing species *L. mollis* (Pers.) Quélet. Fruiting bodies of *L. mollis* are sessile to effused-reflexed and display reddish-purple to purple-brown coloration (Gilbertson and Ryvarden 1986, Ryvarden 1991). *Leptoporus* is similar to *Ceriporia* microscopically, having a monomitic hyphal system and lacking both clamp connections and cystidia (Gilbertson and Ryvarden 1986). *Ceriporia* has been delimited from *Leptoporus* based solely on the type of decay, with *Ceriporia* species producing a white rot (Gilbertson and Ryvarden 1986). *Leptoporus mollis* is widespread in North America but seems to be collected rarely based on the number of collections in herbaria.

Although the genera examined in this study are known to be a polyphyletic assemblage, they were chosen as a starting point in the search for species that might be closely related to *Laetiporus*. The goal of this study was not to complete an exhaustive evolutionary investigation of all of these genera but to determine whether a realignment of species within these genera might be warranted in light of recent work on *Laetiporus*. Many studies have demonstrated that nonpolyporoid genera, including the spathulate genus *Sparassis* and numerous corticioid genera, are closely related to the genera in this study (Hibbett and Donoghue 2001, Binder et al 2005). This emphasizes the need for future work that includes a wide range of species with diverse morphologies as well as species that produce clamp connections. This work is a first step toward increasing taxon sampling of species that might fall within the Antrodia clade of true polypores while also determining relationships among species in genus *Laetiporus*.

MATERIALS AND METHODS

DNA isolation, PCR and sequencing.—Cultures to be sequenced were obtained from the culture collection of the Center for Forest Mycology Research, U.S.D.A. Forest Service, Madison Field Office of the Northern Research Station, which is housed at the Forest Products Laboratory in Madison, Wisconsin. Each culture was grown on potato-dextrose agar and a small amount of the resulting mycelium without associated agar was prepared for use as template DNA in PCR following the protocol of Volk et al (1996).

Two regions of nuclear rDNA and one of mitochondrial rDNA were amplified in PCR. Except where otherwise noted primer designations are those of White et al (1990). PCR was performed with 5× Green GoTaq reaction buffer and GoTaq DNA polymerase (Promega, Madison, Wisconsin). GoTaq reaction buffer was diluted to a 1× working concentration and 0.025 units of GoTaq DNA polymerase were added per microliter of reaction volume. Each primer had a final concentration of 0.2 μM and each dNTP (Promega, Madison, Wisconsin) had a final concentration of 200 μM. Thermocycler conditions were: initial denaturing at 94 C for 2 min; 30 cycles of denaturing at 94 C for 40 s, annealing at 53 C for 40 s and extension at 72 C for 120 s; and a final extension step of 72 C for 5 min.

A section of nLSU rDNA was amplified with primers LR16/LROR. For most of the isolates a section of ITS was amplified with ITS4/ITS5. Partial ITS sequences from isolates PR-2 and PR-6326 were amplified with primers ITS3/ITS4, while ITS2/ITS5 and ITS3/ITS4 were used for *Wolfiporia cocos* isolates. A section of mtSSU rDNA was amplified with ms-1/ms-2. Attempts at amplification of each of these three regions were made from cultures of these taxa: *Laetiporus cincinnatus*, *L. conifericola*, *L. gilbertsonii*, *L. gilbertsonii* var. *pallidus*, *L. huroniensis*, *L. persicinus*, *L. sulphureus* s.s., *Leptoporus mollis*, *Phaeolus schweinitzii*, *Pycnoporellus alboluteus*, *P. fulgens*, *Wolfiporia cocos*, *W. dilatophya* and the unidentified isolates PR-2, PR-6326, PR-914, GDL-1, EUC-1 and KOA-1. Preliminary morphological data placed all unidentified isolates in genus *Laetiporus*. Collection information for all isolates is provided (TABLE I).

PCR products were purified with QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Sequencing reactions were performed following

TABLE I. Isolates sequenced for this study

Species	Collection Number	Locality	GenBank Accession Numbers		
			ITS	nLSU	mtSSU
Laetiporus cincinnatus	DA-37	Dane Co., Wisconsin, USA	EU402557	EU402521	EU402485
L. cincinnatus	MAS-1	Suffolk Co., Massachusetts, USA	EU402558		
L. cincinnatus	WS-1	Washington Co., Wisconsin, USA	EU402559		
L. cincinnatus	46-1104	Orleans Parish, Lousiana, USA	EU402560	EU402522	EU402484
L. conifericola	AK-1	Municipality of Anchorage, Alaska, USA	EU402574		
L. conifericola	CA-8	Mendocino Co., California, USA	EU402575	EU402523	EU402487
L. conifericola	JAM-1	Ketchikan Gateway Borough, Alaska, USA	EU402577	EU402524	EU402486
L. conifericola	NV-2	Washoe Co., Nevada, USA	EU402578		EU402488
L. conifericola	TREE-5	Kenai Peninsula Borough, Alaska, USA	EU402576		
L. gilbertsonii	CA-13	Napa Co., California, USA	EU402549	EU402527	EU402496
L. gilbertsonii	OR-2	Marion Co., Oregon, USA	EU402550		EU402495
L. gilbertsonii var. *pallidus*	TJV2000-101	Wakulla Co., Flordia, USA	EU402553	EU402528	EU402493
L. gilbertsonii var. *pallidus*	L-12954	San Jose Prov., Costa Rica	EU402551		
L. gilbertsonii var. *pallidus*	FP150268	St. Andrews Parish, Jamaica	EU402552	EU402529	EU402494
L. huroniensis	HMC-1	Marquette Co., Michigan, USA	EU402569		
L. huroniensis	HMC-2	Marquette Co., Michigan, USA	EU402570		EU402490
L. huroniensis	HMC-3	Marquette Co., Michigan, USA	EU402571	EU402540	
L. huroniensis	MI-7	Marquette Co., Michigan, USA	EU402572		
L. huroniensis	MI-14	Gogebic Co., Michigan, USA	EU402573	EU402539	EU402489
L. persicinus	HHB9564	Leon Co., Flordia, USA	EU402579	EU402513	
L. persicinus	HHB9668	Leon Co., Flordia, USA	EU402580		EU402503
L. persicinus	RLG14725	East Baton Rouge Parish, Lousiana, USA	EU402581	EU402512	EU402502
L. persicinus	RLG14739	East Baton Rouge Parish, Lousiana, USA (?)	EU402582		
L. sulphureus	CT-1	Fairfield Co., Connecticut, USA	EU402565	EU402532	EU402479
L. sulphureus	DA-41	Dane Co., Wisconsin, USA	EU402566	EU402533	EU402481
L. sulphureus	ERT-713	Mississippi, USA	EU402564		
L. sulphureus	GR-12	Grant Co., Wisconsin, USA	EU402561	EU402534	EU402480
L. sulphureus	NJ-2	Morsic Co., New Jersey, USA	EU402562		
L. sulphureus	TJV95-84	Dane Co., Wisconsin, USA	EU402563		
L. sulphureus	MAS-2	Middlesex Co., Massachusetts, USA	EU402568	EU402531	EU402491
L. sulphureus	TJV99-150	La Crosse Co., Wisconsin, USA	EU402567	EU402530	EU402492
L. sp.	GDL-1	Guadeloupe, Lesser Antilles, France (Caribbean)	EU402547	EU402525	EU402483
L. sp.	PR-914	Luquillo Mts, Puerto Rico, USA	EU402548	EU402526	EU402482
L. sp.	EUC-1	Maui Co., Hawai'i, USA	EU402545	EU402541	
L. sp.	KOA-1	Hawai'i Co., Hawai'i, USA	EU402546	EU402542	
Leptoporus mollis	RLG7163	Pima Co., Arizona, USA	EU402583	EU402511	EU402506
L. mollis	TJV93-174	Jefferson Co., Washington, USA	EU402584	EU402510	EU402507
Phaeolus schweinitzii	DA-38	Dane Co., Wisconsin, USA	EU402585	EU402514	EU402508
P. schweinitzii	HHB18924	Alaska, USA	EU402586	EU402515	EU402509

TABLE I. Continued

Species	Collection Number	Locality	GenBank Accession Numbers		
			ITS	nLSU	mtSSU
P. schweinitzii	MI-5	Gogebic Co., Michigan, USA	EU402587		
''*Polyporus talpae*''	PR-2	Puerto Rico, USA		EU402543	
''*P. talpae*''	PR-6326	Luquillo Mts, Puerto Rico, USA		EU402544	
Pycnoporellus alboluteus	HHB12816	Municipality of Anchorage, Alaska, USA	EU402588	EU402538	
P. alboluteus	HHB17598	Kenai Peninsula Borough, Alaska, USA	EU402589	EU402537	
P. alboluteus	OKM6406	Flathead Co., Montana, USA	EU402590		
P. fulgens	CA-20	Mariposa Co., California, USA	EU402591		
P. fulgens	FP101689	Vilas Co., Wisconsin, USA	EU402592	EU402536	EU402504
P. fulgens	HHB17342	Kenai Peninsula Borough, Alaska, USA	EU402593	EU402535	EU402505
Wolfiporia cocos	MD-106	Benton Co., Oregon, USA	EU402594	EU402519	EU402501
W. cocos	MD-275	Benton Co., Oregon, USA	EU402595	EU402520	EU402500
W. dilatohypha	CS-63	Clark Co., Kentucky, USA	EU402555	EU402516	EU402497
W. dilatohypha	FP72162	Tennessee, USA	EU402556	EU402517	EU402498
W. dilatohypha	FP94089	Greenbrier Co., West Virginia, USA	EU402554	EU402518	EU402499

the BigDye terminator protocol (ABI Prism). The purified PCR products for each isolate were sequenced from both the 3′ and 5′ direction with the same primers used in the PCR amplification. Sequencing products were analyzed at the University of Wisconsin Biotechnology Center (Madison, Wisconsin) with an ABI Prism 377 sequencer. Complementary sequences for each isolate were compared and discrepancies resolved with the sequence electropherograms. For analysis purposes all sequences were terminated at the base immediately adjacent to the primer attachment point. In addition to the primers used in amplification two primers were synthesized: FPLms-4 (TGTACTTTAGGTCCTAAA) and FPLms-7 (ACAG GATGTGCGACTTGC). These primers were used to sequence long mtSSU amplification products that occurred in *L. persicinus*, *P. fulgens* and two of three isolates of *W. dilatohypha*.

Phylogenetic analyses.—Sequences were aligned manually with Sequence Alignment Editor (Se-Al) v2.0a9. Sequences were deposited in GenBank (see TABLE I) and sequence alignments for nLSU, mtSSU, ITS and a combined dataset of all three regions were deposited in TreeBase (accession # S1994). Maximum parsimony was implemented in PAUP 4.0b10 (Swofford 2002) and Bayesian analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003).

The nLSU sequences were aligned with 15 additional sequences from GenBank: *Antrodia carbonica* AF287844, *Ceriporia purpurea* AF287852, *Dacryobolus sudans* AY293176, *Fomes fomentarius* AF287857, *Grifola frondosa* AY629318, *G. frondosa* AY826982, *Laetiporus portentosus* AY293191, *Parmastomyces transmutans* AF518635, *Pleurotus ostreatus* U04140, *Polyporus squamosus* AF393069, *Sarcodon imbricatus* AY586711, *Sparassis crispa* AY218386, *S. spathulata* AF287889, *S. spathulata* AY218396 and *Wolfiporia cocos* AF393081. The sequences from GenBank represent a selection of species from the three major polyporoid clades (Antrodia, phlebioid and core polyporoid) defined by Binder et al (2005), as well as two sequences from outside the polyporoid clade, which were included as outgroups. Sequences of nLSU from two isolates originally identified as *Laetiporus persicinus* (PR-2 and PR-6326, ''*Polyporus talpae*'' in TABLE I) were excluded from analyses because they could not be aligned with other species; BLAST analyses of these sequences produced no significant matches.

For nLSU data, heuristic searches were conducted in PAUP with characters unordered and of equal weight and gaps treated as missing data. Default settings were used with these exceptions: stepwise-addition option was set to random with 100 replicates, steepest descent was used with the TBR branch swapping option and the number of trees saved was set to automatically increase. Bootstrap support for clades (Felsenstein 1985) was estimated from 1000 heuristic searches with the same settings described above, with the exception that the stepwise-addition option was set to random with 50 replicates. Bayesian inference was implemented in MrBayes using default settings with the GTR model. Two million generations were performed with samples taken in increments of 100. The first 5000 trees

(25%) were considered the burn-in and were excluded from construction of the consensus tree. After running the analysis the standard deviation of the split frequencies was examined to confirm it was below 0.01, the potential scale reduction factor was examined to confirm all parameters were close to 1.0, and the plot of the generations versus the log probability of the data (the log likelihood values) was examined to confirm it had reached the stationary phase (was neither increasing nor decreasing). Three independent Bayesian runs were performed and posterior probabilities were averaged across runs.

For the ITS region, sequences from species in the core *Laetiporus* clade (see FIG. 1) were found to align easily with *Wolfiporia dilatohypha* sequences, while sequences from all other species were found to be more than 25% dissimilar based on sequence identity, making them difficult or impossible to align. Analyses for the ITS region therefore were performed with species in the core *Laetiporus* clade with *W. dilatohypha* as outgroup. Ten *Laetiporus sulphureus* ITS sequences from GenBank (AF229196, AM269785, AM269786, AY089742, AY218417, AY835667, AY835668, DQ221108, DQ450876 and EF088657) were found to align with our dataset and were included in analyses. Maximum parsimony and Bayesian analyses were run on the ITS sequences using the methods described for nLSU data with these exceptions: 1 400 000 generations were run in the Bayesian analysis and the burn-in was set to 3500 (25%).

Maximum parsimony and Bayesian analyses were run on the mtSSU sequences with the methods described for ITS data. All species (TABLE I) were included with the exception of species for which sequence data were not successfully obtained (see RESULTS). *Leptoporus mollis* was used as outgroup based on the results of the nLSU analysis.

A combined analysis was conducted with all isolates for which complete nLSU, mtSSU and ITS data were available. Species were included in this analysis if sequence data could be aligned across all three regions. Potential conflicts among DNA regions were identified by first running an independent parsimony analysis on each of the three regions. The parsimony analysis included running a heuristic search in PAUP using default settings with these exceptions: stepwise-addition option was set to random with 100 replicates, steepest descent was used with the TBR branch swapping option and the number of trees saved was set to automatically increase. A strict consensus tree was constructed for each region, and the trees were examined for potential conflict. Bootstrap analyses were run for each dataset with the methods described for the nLSU analysis. No conflicts were observed among the datasets (i.e. no conflicting branches were observed among the consensus trees and no conflicting branches were supported with a bootstrap value >70), so the three regions were combined into one dataset. Maximum parsimony and Bayesian analyses were run on the combined dataset with the methods described for the ITS region.

RESULTS

PCR and sequencing.—Complete ITS sequences were obtained from 51 isolates (TABLE I) yielding 30 sequence variants. All ITS sequences were 555–720 bp long except for *Wolfiporia cocos*, which had an ITS length of 1863 bp. The extreme length of *W. cocos* ITS was due to an approximately 1000 bp insertion in ITS 1 and an approximately 270 bp insertion in ITS 2 when compared to *Phaeolus schweinitzii*.

Sequences of nLSU were obtained from 35 isolates (TABLE I) yielding 20 sequence variants. Sequences of nLSU were 662–734 bp. Sequences of mtSSU were obtained from 31 isolates; *Pycnoporellus alboluteus* and isolates PR-2 and PR-6326 did not yield mtSSU sequences. Most mtSSU sequences were 491–610 bp. However *L. persicinus*, *P. fulgens* and two of three isolates of *W. dilatohypha* had large insertions that resulted in sequence lengths in excess of 1700 bp; these insertions occurred at the same location within each sequence. Insertions were removed to align sequences. (GenBank accession numbers can be found in TABLE I.)

Phylogenetic analyses.—The heuristic search using nLSU sequences produced 44 equally parsimonious trees (one of which is shown in FIG. 1) (length = 946, CI = 0.517, RI = 0.757). Five of the seven *Laetiporus* species included in the analysis (*L. cincinnatus*, *L. conifericola*, *L. gilbertsonii*, *L. huroniensis* and *L. sulphureus*) fell in the group referred to as the core *Laetiporus* clade or *Laetiporus* s.s., while two *Laetiporus* species (*L. persicinus* and *L. portentosus*) fell outside the core *Laetiporus* clade. The unidentified *Laetiporus* isolates from Hawai'i and the Caribbean fell within the core *Laetiporus* clade. *Wolfiporia dilatohypha* isolates formed a sister group to the core *Laetiporus* clade.

Although a relatively small number of species were included in the nLSU analysis, the tree reflects the overall structure of the polyporoid clade as defined by Binder et al (2005), including Antrodia, phlebioid and core polyporoid clades. *Laetiporus persicinus*, *Phaeolus schweinitzii*, *Pycnoporellus alboluteus*, *P. fulgens* and the isolates of *Wolfiporia cocos* sequenced for this study all fell within the Antrodia clade. The one *W. cocos* sequence taken from GenBank (AF393081) fell within the core polyporoid clade (FIG. 1). *Phaeolus schweinitzii* formed a clade with the *W. cocos* isolates sequenced for this study, although this clade has weak statistical support (bootstrap ≥70 or posterior probability ≥0.95). *Pycnoporellus alboluteus* and *P. fulgens* formed a clade with strong statistical support (bootstrap ≥70 and posterior probability ≥0.95). *Leptoporus mollis*, *Ceriporia purpurea* and *Laetiporus portentosus* formed a strongly supported clade representing the phlebioid clade of polypores.

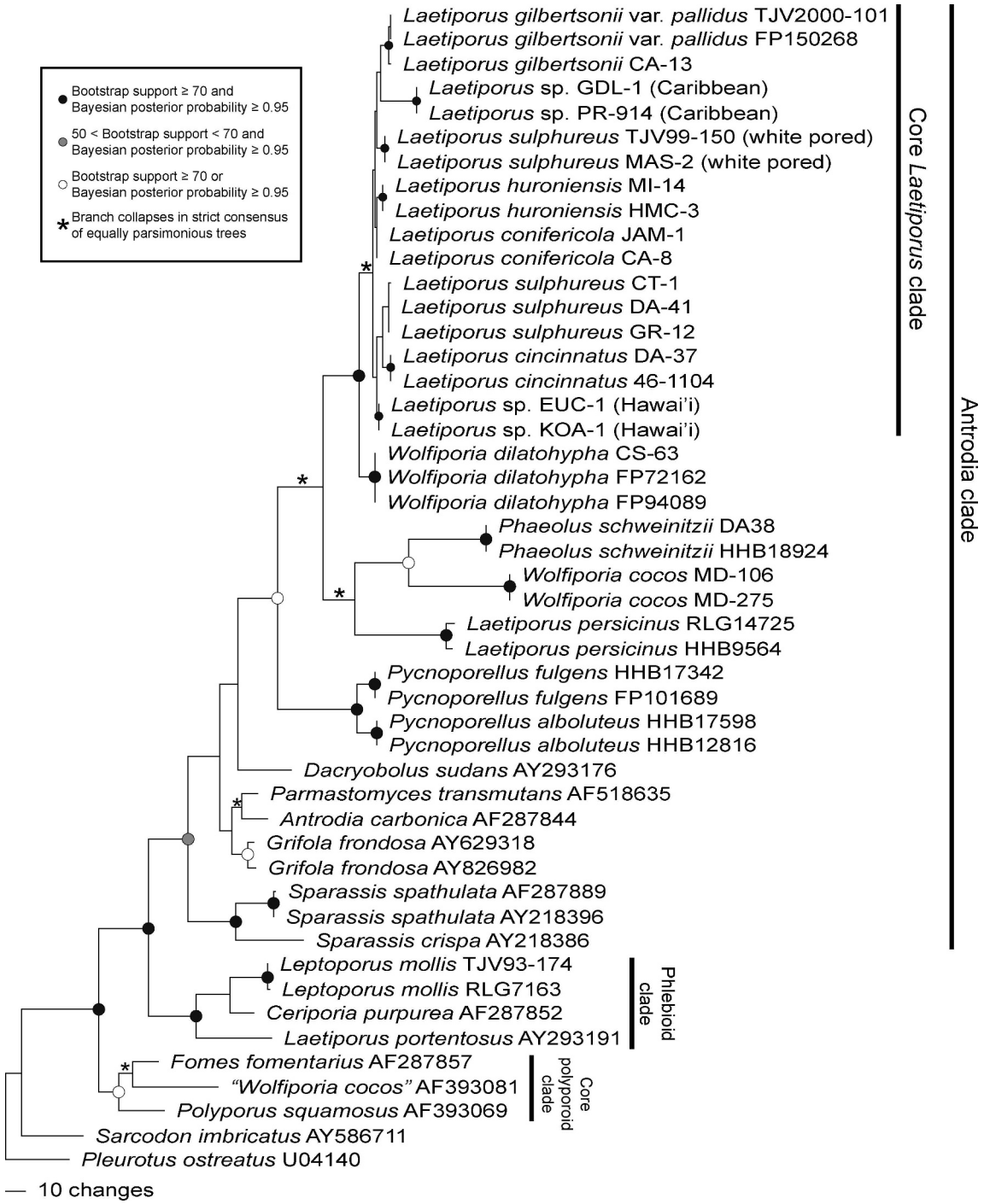

FIG. 1. Analysis based on nLSU sequences. The tree shown is one of 44 equally parsimonious trees (length = 946, CI = 0.517, RI = 0.757). Data were taken from GenBank for isolates not included in TABLE I; these isolates are labeled with GenBank accession numbers. All other isolates were sequenced for this study and are labeled with collection numbers. Quotation marks around a species indicate a suspect identification.

The heuristic search with ITS data produced 98 equally parsimonious trees (one of which is shown in FIG. 2) (length = 191, CI = 0.843, RI = 0.941). Eight clades were found, seven of which have strong statistical support. These eight clades correspond to five described species (*Laetiporus cincinnatus*, *L. conifericola*, *L. gilbertsonii*, *L. huroniensis* and *L. sulphureus* s.s.) as well as three apparently undescribed species of *Laetiporus*. Relationships among *Laetiporus* species were poorly supported, although

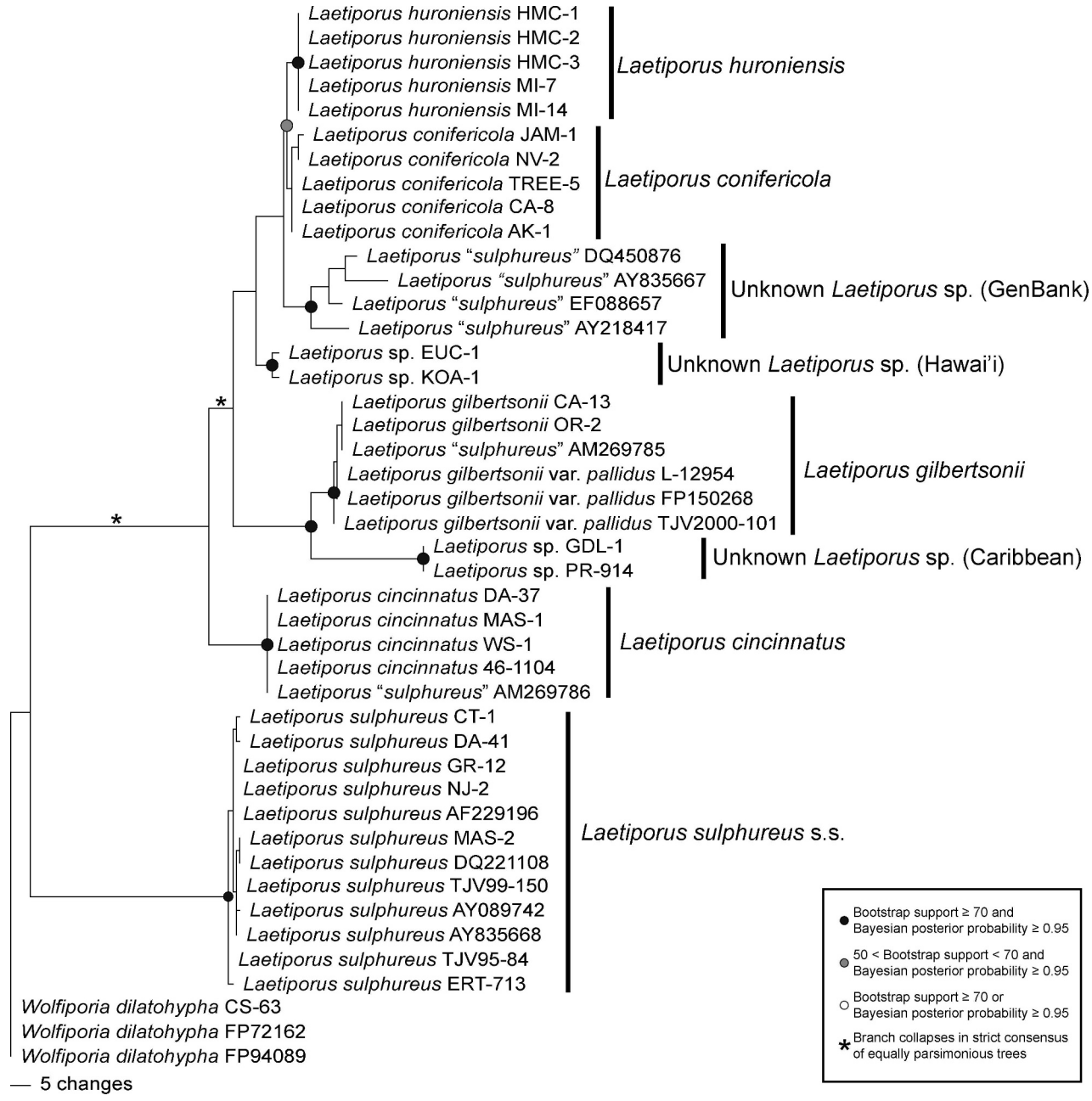

FIG. 2. Analysis based on ITS sequences. The tree shown is one of 98 equally parsimonious trees (length = 191, CI = 0.843, RI = 0.941). Data were taken from GenBank for isolates not included in TABLE I; these isolates are labeled with GenBank accession numbers. All other isolates were sequenced for this study and are labeled with collection numbers. Quotation marks around a species indicate a suspect identification.

there is strong support for a clade containing *L. gilbertsonii* and the apparently undescribed Caribbean isolates. There is moderate support for a clade containing *L. conifericola* and *L. huroniensis*. Two sequences (AM269785 and AM269786) listed in GenBank as *Laetiporus sulphureus* clustered strongly with other *Laetiporus* spp. (*L. gilbertsonii* and *L. cincinnatus* respectively). The GenBank sequence AY835668, which is derived from a *L. sulphureus* isolate collected from *Quercus* in northern Germany, clustered with North American isolates of *L. sulphureus* s.s.

Results from the heuristic search with mtSSU sequences produced few statistically supported clades. The core *Laetiporus* clade was weakly supported, while relationships within the clade were unresolved. Alignment of mtSSU sequences from species outside the core *Laetiporus* clade was difficult due to large

variable regions; alignment within the core *Laetiporus* clade showed little variation among isolates, with the exception of one variable region approximately 30 base pairs long. Within this variable region a 16 bp indel (TTTAATTTTAAATTCA) was found to occur in four isolates: two *L. conifericola* isolates (JAM-1 and CA-8) and two *L. sulphureus* s.s. isolates with white pores (MAS-2 and TJV99-150); this indel did not occur in any of the other isolates sampled.

The heuristic search with the combined dataset produced 31 equally parsimonious trees (one of which is shown in FIG. 3) (length = 244, CI = 0.812, RI = 0.892). Five clades were resolved with weak to strong statistical support. *Laetiporus gilbertsonii* isolates and the *Laetiporus* isolates from the Caribbean formed a weakly supported clade (Gilbertsonii). *Laetiporus cincinnatus* isolates formed a strongly supported clade (Cincinnatus) that did not group consistently with any other species (the branch containing Gilbertsonii and Cincinnatus clades collapses in a strict consensus tree). *Laetiporus sulphureus* s.s. isolates split into two strongly supported clades, one with yellow pores (Sulphureus clade I) and one with white pores (Sulphureus clade II). The two conifer-inhabiting species, *L. conifericola* and *L. huroniensis*, formed a weakly supported clade (Conifericola). The three branches indicating relationships among major clades collapse in a strict consensus tree.

DISCUSSION

Sequence analysis confirms that *Laetiporus sulphureus* s.l. represents a large species complex in North America, which supports work by Burdsall and Banik (2001) using mating compatibility, RFLP, morphology and ecology. Although five major clades encompassing eight species groups were identified within *Laetiporus* s.s., further work is needed to resolve relationships among species and to determine the number of *Laetiporus* species in North America. *Laetiporus sulphureus* s.l. is known from many continents, including Africa, Asia and Europe (Ryvarden and Johansen 1980, Ryvarden and Gilbertson 1993, Núñez and Ryvarden 2001) so additional species undoubtedly remain uncharacterized worldwide.

Of all species in the core *Laetiporus* clade, *Laetiporus sulphureus* s.s. displayed the largest number of sequence variants and also showed variation in pore color, either white or yellow. Burdsall and Banik (2001) describe *Laetiporus sulphureus* s.s. as possessing lemon yellow pores but mention rare specimens with white pores that are indistinguishable from *L. sulphureus* s.s in morphology, habitat and RFLP patterns. The two isolates of this type included in this study (MAS-2 and TJV99-150) cluster with *L. sulphureus* s.s. in the ITS analysis (FIG. 2) and are clearly differentiated from both *L. cincinnatus* and *L. gilbertsonii* var. *pallidus*, the two species that are known to possess white pore layers. ITS data indicate that these white-pored isolates fall within *L. sulphureus* s.s., yet both the nLSU (FIG. 1) and combined datasets (FIG. 3) support separation of white pored forms of *L. sulphureus* s.s. from isolates with yellow pores. Additional isolates are needed to determine whether this differentiation warrants the description of a new species or variety. Unfortunately single spores from white-pored *L. sulphureus* s.s. fruiting bodies have low germination rates across a wide pH range of agar media (unpubl data), which has hampered mating studies. Although names have been introduced to describe white-pored forms of *L. sulphureus* s.l., such as Peck's *L. sulphureus* var. *semialbinus* (Peck 1906), it is likely these names describe the more common and morphologically distinct species *L. cincinnatus* (Burdsall and Banik 2001).

The only other *Laetiporus* species found to have variation in the color of the pore layer was *L. gilbertsonii*. Both color forms of *L. gilbertsonii* are almost indistinguishable in these analyses, with only two base pairs of variations in both the ITS and nLSU regions. This suggests that a change in pore coloration can occur with little additional genetic differentiation in this group. *Laetiporus gilbertsonii* also shows variation in host preference, being found on both *Quercus* spp. and *Eucalyptus* spp. (Burdsall and Banik 2001). The only other isolate in this study collected on *Eucalyptus*, EUC-1, was collected in Hawai'i and clustered with an addition Hawai'ian isolate from a native *Acacia koa* (koa tree). These Hawai'ian isolates, along with the Caribbean isolates in the Gilbertsonii clade and the white-pored isolates of *L. sulphureus* s.s., represent three likely candidates for undescribed species in the core *Laetiporus* clade.

A fourth undescribed *Laetiporus* sp. was identified in the ITS analysis (FIG. 2, *L.* ''*sulphureus*'' AY218417, AY835667, DQ450876, EF088657), although all representatives in this group are GenBank sequences for which little geographic or host data exist. One sequence of this type (AY835667) was obtained from a *Laetiporus sulphureus* s.l. isolate collected on *Picea abies* in southern Germany (Davoli et al 2005), raising the possibility that these isolates are conspecific with the European conifer-inhabiting *Laetiporus sulphureus* s.l. isolates investigated by Rogers et al (1999). Burdsall and Banik (2001) report that *L. monticola* Cerny. was described in 1989 to accommodate European *Laetiporus* specimens found on conifers,

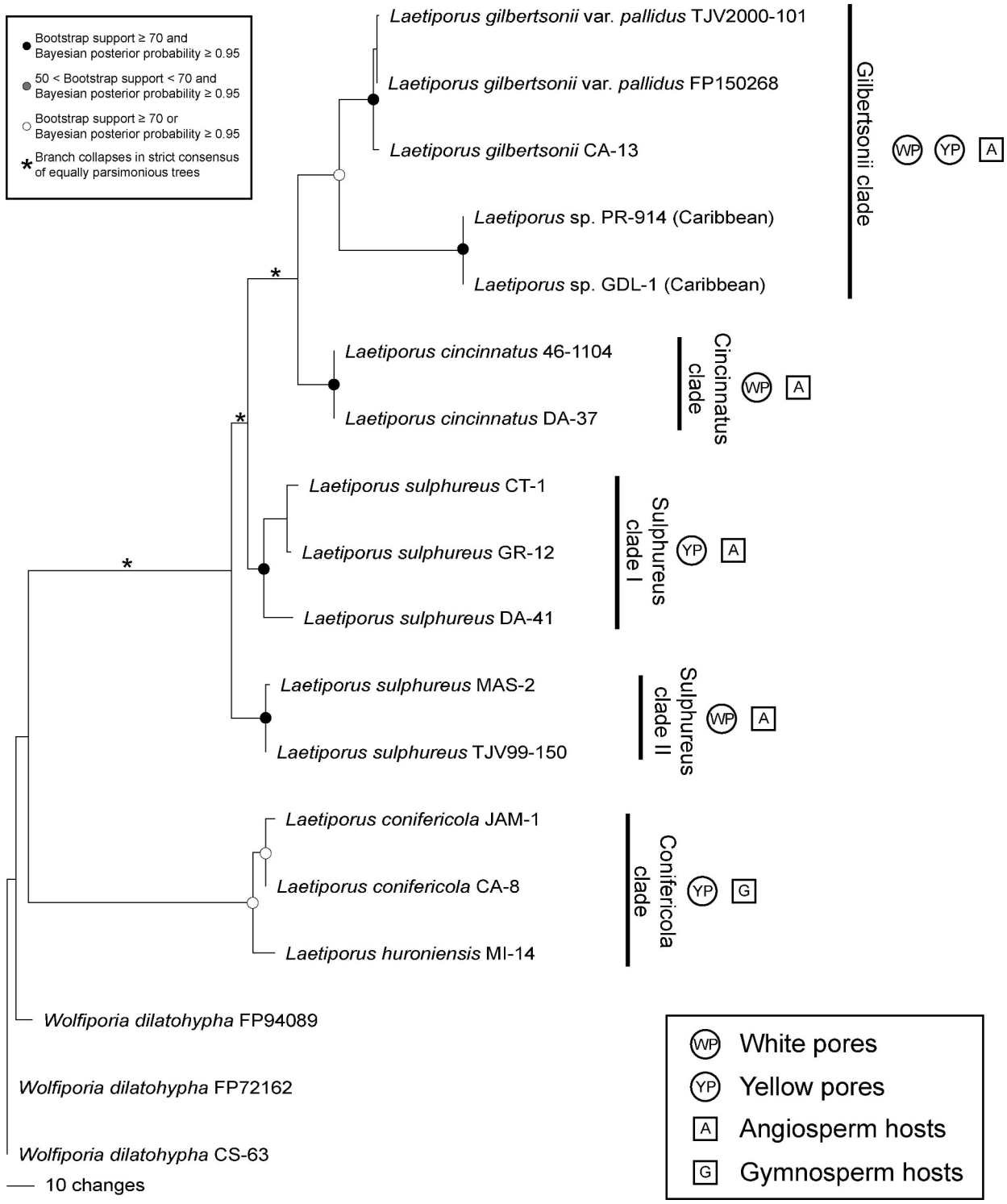

FIG. 3. Combined analysis based on nLSU, mtSSU and ITS sequences. The tree shown is one of 31 equally parsimonious trees (length = 244, CI = 0.812, RI = 0.892). Isolates were included in this analysis only if sequence data were available for all three regions.

although apparently this species was never validly published due to a lack of a Latin diagnosis. Further taxonomic work is needed to determine whether conifer-inhabiting *Laetiporus* isolates from Europe constitute an unrecognized species. GenBank sequence AY835668, which is derived from a *L. sulphureus* isolate collected on *Quercus* in northern Germany (Davoli et al 2005), clustered with North

American *L. sulphureus* s.s. isolates (FIG. 2), supporting the hypothesis that *L. sulphureus* s.s. is restricted to angiosperms and is found in both North America and Europe.

GenBank ITS data also produced two sequences of *Laetiporus* ''*sulphureus*'' (AM269785 and AM269786) that clustered strongly with known *Laetiporus* isolates identified independently with mating compatibility and fruiting body morphology (Banik and Burdsall 2000, Burdsall and Banik 2001). *Laetiporus* ''*sulphureus*'' sequence AM269785 is from a Californian isolate associated with an unknown host (Guglielmo et al 2007) and clustered with *L. gilbertsonii* sequences; *L.* ''*sulphureus*'' sequence AM269786 is from an isolate collected on *Quercus* in Wisconsin (Guglielmo et al 2007) and clustered with *L. cincinnatus* sequences (FIG. 2). The phylogenetic placement of AM269785 and AM269786, taken together with the host and geographic data associated with their parent isolates, indicate the species names associated with these GenBank sequences are in need of revision.

The discovery of *Wolfiporia dilatohypha* as a sister group to the core *Laetiporus* clade was fortuitous and might help to define the generic limits of *Laetiporus* as sequence data accumulate from other regions of the world. Two species of *Laetiporus*, *L. portentosus* and *L. persicinus*, fell well outside the core *Laetiporus* clade, indicating that *Laetiporus* as it is currently defined represents a polyphyletic assemblage. The other North American polypore species sequenced for this study, including *Leptoporus mollis*, *Phaeolus schweinitzii*, *Pycnoporellus alboluteus* and *P. fulgens*, also do not appear to be closely allied with the core *Laetiporus* clade; however, with the exception of *L. mollis*, all these species fall in the Antrodia clade of polypores. The *Wolfiporia cocos* isolates sequenced for this study also fell within the Antrodia clade, although this is in contrast to earlier studies that placed *W. cocos* in the core polyporoid clade (this point is addressed in detail later in the discussion). Further taxon sampling and additional sequences analyses are needed to confirm the placement of these brown rot polypore species in broader basidiomycete phylogenies.

A lack of monophly in *Laetiporus* first was demonstrated by Hibbett and Donoghue (2001) with sequence analysis of two rDNA regions from the southern hemisphere species *L. portentosus*. Using a single isolate of *L. sulphureus* s.l., Hibbett and Donoghue (2001) found that *L. portentosus* is not closely related to *L. sulphureus* s.l. Although both *L. portentosus* and *L. persicinus* show macroscopic, microscopic and physiological similarities to other *Laetiporus* species, sequence data suggest these are convergent characters and that neither species is closely related to any of the species in the core *Laetiporus* clade. Although *L. portentosus* was placed in *Piptoporus*, Rajchenberg (1995) transferred it to *Laetiporus* based on morphological and cultural characteristics. Unfortunately these characters appear to be phylogenetically uninformative at the generic level for these species groups. Hibbett and Donoghues' (2001) analysis indicated that *L. portentosus* falls within the ''Antrodia clade'' of true polypores, although the nLSU analysis in this study placed it in the phlebioid clade. More gene regions from additional *L. portentosus* isolates need to be sequenced to resolve this issue.

Like *Laetiporus portentosus*, *L. persicinus* falls outside the core *Laetiporus* clade in the nLSU analysis. This result is consistent with *L. persicinus*' divergent morphological traits, including a lack of bright orange coloration in the pileus surface and flesh and tubes that stain blackish-brown. BLAST analyses of the GeneBank (NCBI) databases using ITS, nLSU and mtSSU regions from *L. persicinus* revealed no significant similarity to any known genus, suggesting that placement in a new genus may be warranted. However further work is needed to confirm that an appropriate genus does not already exist. Since being described as *Polyporus persicinus* by Berkeley and Curtis in 1872, this species has been placed in numerous genera, including *Meripilus* (Ryvarden 1972) and *Buglossoporus* (Corner 1984), before being transferred to *Laetiporus* by Gilbertson (1981).

Wolfiporia dilatohypha, which formed a sister group to the core *Laetiporus* clade, was the only species outside genus *Laetiporus* with an ITS sequence that aligned easily with core *Laetiporus* species. *Wolfiporia dilatohypha* isolates were so similar to species in the core *Laetiporus* clade that future work must address whether *W. dilatohypha* is congeneric with species in the core *Laetiporus* clade. The *Wolfiporia cocos* isolates sequenced for this study were found to be distantly related to both *W. dilatohypha* and all *Laetiporus* species examined. This indicates that *Wolfiporia*, like *Laetiporus*, is polyphyletic. Although five species are presently found in genus *Wolfiporia*, only two were included in this study. Further taxon sampling is needed to determine whether any of the three remaining *Wolfiporia* species, *W. cartilaginea* Ryvarden, *W. curvispora* Y.C. Dai, and *W. sulphurea* (Burt) Ginns, are closely allied with other *Wolfiporia* species or genus *Laetiporus*.

The close relationship between *Laetiporus* and *Wolfiporia dilatohypha* was surprising given that *W. dilatohypha* fruiting bodies are macroscopically unlike *Laetiporus* fruiting bodies. *Wolfiporia dilatohyphae* fruiting bodies are effused, thin (3–4 mm) and white

to buff colored with relatively large pores (1–5 per mm), which is in contrast to the large, brightly colored, pileate fruiting bodies of *Laetiporus*. Three cultural isolates of *W. dilatohypha* were sequenced for this study and all had identical LSU sequences and nearly identical ITS regions (two base pairs of variation). However no sequences have yet been obtained from fruiting body material and the most recent cultural isolate was collected in 1963. Fresh fruiting bodies of *W. dilatohypha* are needed to confirm that the sequences in this study are consistent with sequences derived from fruiting structures. Unfortunately fruiting bodies of this species seem to be rare or are rarely collected.

Although *Wolfiporia dilatohypha* was found to be closely related to the core *Laetiporus* clade based on ITS and nLSU data, it is interesting to note that isolates of *W. cocos*, the type species of *Wolfiporia*, do not appear to be closely related to *W. dilatohypha*. The two *W. cocos* isolates sequenced for this study (MD-106 and MD-275) both had identical ITS, nLSU and mtSSU regions. An additional 13 isolates from the Center for Forest Mycology Research (CFMR) cultural collection (Madison, Wisconsin) were sequenced in the nLSU region and all displayed sequences that matched the two original isolates almost identically (unpubl data). However nLSU and mtSSU data for *W. cocos* in this study differ strongly from those reported by Hibbett and Donoghue (2001) and Binder and Hibbett (2002). Hibbett and Donoghue (2001) sequenced the nuclear and mitochondrial SSU of *W. cocos* (isolate No. FPL4198), while Binder and Hibbett (2002) sequenced the nuclear and mitochondrial LSU of the same isolate (the GenBank nLSU sequence of this isolate is included in FIG. 1 as ''*Wolfiporia cocos*'' AF393081). Sequences from isolate FPL4198 subsequently have been included in many analyses by various authors (Hibbett and Binder 2002, Hibbett 2004, Binder et al 2005). Isolate FPL4198 has shown strong affinities for the core polypore clade, clustering with white rot genera such as *Polyporus* and *Trametes* (see FIG. 1).

Kim and Jung (2000) however sequenced the nuclear SSU of *W. cocos* (isolate ATCC13490) and found that it clustered with other brown rot genera such as *Laetiporus*, *Phaeolus* and *Sparassis*. As proposed by Binder et al (2005), this disparity might be due to the mislabeling or contamination of isolate FPL4198, which does not appear to represent *W. cocos*. Unfortunately the original isolate of FPL4198 housed at CFMR died in culture and is not available for further study. Additional cultural isolates, fruiting bodies and sclerotia of *W. cocos* are needed to clarify the position of this species and to determine the extent of variability within this species. Unfortunately such an analysis might be hampered by the size of the ITS region in *W. cocos*, which was found to be greater than 1800 base pairs due to insertion events. In addition to making the ITS difficult to amplify, these insertions make it difficult to align the ITS of *W. cocos* with other species.

Although previous investigations have suggested that *Phaeolus schweinitzii* is closely related to *Laetiporus sulphureus* s.l., the ITS of *P. schweinitzii* is difficult to align with *Laetiporus* spp. and the nLSU of *P. schweinitzii* shows only 89% sequence identity to *L. sulphureus* s.s. The closest relative of *P. schweinitzii* in the nLSU analysis was *Wolfiporia cocos*, although this finding has weak statistical support. Further sampling is needed to determine whether close relatives of *P. schweinitzii* exist and whether there is variability within this widespread species. The proposed link between *Phaeolus* and *Pycnoporellus* (Gilbertson and Ryvarden 1987) was not borne out by this study because *Phaeolus schweinitzii* and *Pycnoporellus* isolates shared only 85% sequence identity for the nLSU and alignments of ITS regions were impossible between these genera. The two *Pycnoporellus* species however showed similar nLSU sequences (97% sequence identity) and the ITS regions were aligned easily. This evidence, together with the similar microstructures and chemical staining reactions in KOH, suggests that *Pycnoporellus* consists of a natural grouping of species.

Unlike the other genera investigated, genus *Leptoporus* seems to fall outside the Antrodia clade as defined by Binder et al (2005). The nLSU sequences of *Leptoporus* generated for this study match almost identically (99% identity over 643 base pairs) those of Binder et al (2005), who found that *Leptoporus* fell near *Ceriporia* in the phlebiod clade. Genus *Leptoporus* and *Ceriporia* share similar microscopic characters and Gilbertson and Ryvarden (1987) state that the production of brown rot is the only differentiating character for *Leptoporus*. If this placement is correct it would indicate that *Leptoporus* arose within a clade of white rot species. Isolates of *Leptoporus* were found to display two ITS types that differed by 18 base pairs, indicating that further work is needed to determine whether *L. mollis* represents a species complex.

In this study two isolates (PR-2 and PR-6326) were encountered that possessed extremely divergent nLSU sequences. These isolates came from collections that had been classified as *Laetiporus persicinus*, although preliminary sequence analysis indicated these isolates varied strongly from *L. persicinus* and all other species included in this study. Although ITS data could not be obtained, the nLSU region from these isolates was amplified successfully from cultures and fruiting bodies and had no significant BLAST

matches in GenBank. Preliminary parsimony analysis places this species in the core polyporoid clade, although an insertion of approximately 150 base pairs makes alignment of these sequences difficult (Karl-Henrik Larsson pers comm). This species has dark brown fruiting bodies that grow as basal rosettes from tree roots in a fashion similar to *L. persicinus* and *Phaeolus schweinitzii*; however, unlike *L. persicinus* or *P. schweinitzii*, the fruiting bodies can attain tremendous dimensions (0.5–0.8 m diam) and have a thick, black pileus cuticle. Preliminary evidence based on excavating tree roots close to fruiting bodies suggests this species produces a white rot rather than the brown rot associated with *L. persicinus* (D. Jean Lodge pers comm). The nLSU data confirms that this species is highly divergent from all other polypore species for which sequence data are available despite its morphological and microscopic similarities to *L. persicinus*. Gross morphology suggests this taxon might represent *Polyporus talpae* Cooke (= *Meripilus talpae* [Cooke] D.A. Reid) or possibly *Meripilus tropicalis* Guzmán & Pérez-Silva (Guzmán and Silva 1975). This species also might have been described by Murrill from Jamaica in 1910 (Murrill 1910) as *Amauroderma brittonii* Murrill. Both *Polyporus talpae* and *Amauroderma brittonii* long have been considered synonyms of *Laetiporus persicinus* but further work is needed to determine the legitimacy of this synonymy.

It is clear that many species within the Antrodia clade of polypores remain poorly characterized. Additional isolates of *Laetiporus*, *Leptoporus*, *Phaeolus*, *Pycnoporellus* and *Wolfiporia* are needed from wider geographic regions to clarify the positions of these brown rot polypores in larger basidiomycete phylogenies. *Laetiporus sulphureus* s.l. in particular requires further attention on a worldwide basis because this species complex is one of the larger and more economically important groups in the Antrodia clade. The sequences provided by this study hopefully will provide a framework that aids further phylogenetic and taxonomic investigations.

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