Comparison of general fungal and basidiomycete-specific ITS primers for identification of wood decay fungi

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Abstract

The identity of the fungi associated with and responsible for wood decay could lead to the development of more environmentally benign wood preservative systems. In this study we have reconstructed the phylogenetic tree of a select group of basidiomycete fungi using nucleotide sequences of the internal transcribed spacer (ITS) region. We then compared this to trees generated from restriction fragment length polymorphism (RFLP) analysis using two different primers to see if the latter procedures can correctly identify multiple isolates of select wood decay fungi. The phylogenetic tree using maximum likelihood analysis revealed three well-supported genera, *Trametes*, *Phanerochaete*, and *Gloeophyllum*, with bootstrap values of 75 or greater. *Trametes* and *Phanerochaete* were sister taxa, and *Gloeophyllum* was a sister taxon to the *Trametes/Phanerochaete* clade. Neither set of RFLP data could resolve the three genera into monophyletic groups. The RFLP tree based on general fungal primers also did not resolve species, while the basidiomycete-specific data could resolve species. In the basidiomycete-specific tree, all isolates of both *G. striatum* and *G. trabeum* comprised monophyletic groups. Eight of nine *T. versicolor* isolates, 10 of 11 *G. sepiarium* isolates, and nine of 10 *T. hirsuta* isolates comprised monophyletic groups. *Phanerochaete* could not be consistently resolved into monophyletic groups at either the generic or specific level. Our studies indicate that RFLP analysis using general fungal primers are not likely to be useful in identifying species or reconstructing phylogenetic relationships. RFLP analysis using basidiomycete-specific primers may be useful in identifying some species but not in reconstructing phylogenetic relationships although it is a simpler procedure than sequencing.

More than 95 percent of the 1.5 million homes constructed in the United States each year are framed with wood (Smith and Wu 2005) and wood composite panels, such as OSB and plywood, which are the most common wall siding materials. Wood frequently exposed to moisture will be degraded by insects and microorganisms, thereby losing its strength and ability to serve as a construction material. In the United States, homeowners spend more than 5 billion dollars annually in replacement costs not including labor (Dost and Botsai 1990). Traditional methods to protect wood from decay have focused on the use of chemical preservatives as nonspecific, broad spectrum pesticides (Eaton and Hale 1993). Effective wood preservatives often generate environmental concerns, and many have been banned or their use restricted after they have been in service for a number of years because of unintended consequences to nontarget organisms, including humans. Wood preservatives that affect only organisms responsible for wood decay have the potential to be both more environmentally innocuous and effective in the long run. Basidiomycete fungi are the principle microbial decomposers of

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wood, and in order to better control and understand wood decomposition, a method to rapidly identify decay fungi and an understanding of their physiological functions in wood decay are necessary.

Fungi represent a diverse and widespread group of microorganisms whose numbers are estimated at more than 1.6 million species (Gardes and Bruns 1996). There are approximately 1,600 wood decay species (Bennet et al. 2002). Traditional methods for identifying decay fungi are difficult and time consuming. Substantial expertise is required because these fungi are traditionally classified by their basidiocarp, which is rarely present on wood products or in culture. Culture methods to identify decay fungi are also unable to differentiate mycelia of closely related species and have low sensitivity in detecting early stages of decay (Kim et al. 2005, Schmidt and Moreth 1999a). Because of their increased sensitivity and selectivity, molecular methods are currently being used to identify decay fungi. These methods include Rapid Amplified Polymorphic DNA (RAPD) (Hseu et al.1996), Internal Transcribed Spacer-Restriction Fragment Length Polymorphism (ITS-RFLP) (Adair et al. 2002, Fischer and Wagner 1999, Schmidt and Moreth 1999b, Jasalavich et al. 2000), species-specific priming PCR (polymerase chain reaction) (SSPP) (Moreth and Schmidt 2000, Schmidt and Moreth 2000), Amplified Fragment Length Polymorphism (AFLP) (Parrent et al. 2004), Sequence-Specific Oligonucleotide Probe (SSOP) (Oh et al. 2003), rDNA-ITS region sequence analysis (White et al. 2001, Schmidt and Moreth 2002, Moreth and Schmidt 2005) and Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Schmidt and Kallow 2005). Each of these methods has its own advantages and limitations including ease of application, equipment required, technical expertise, and consistency of results.

Regardless of procedures, most taxonomic analysis focuses on ribosomal DNA (rDNA). rDNA is a nuclear, multicopy gene family arranged in tandem arrays that codes for the rRNA subunits of the ribosome molecule. Within each array are genes coding for the small (18S) and large (28S) subunits. To the outside of the small and large rRNA sequences are the external transcribed spacer (ETS) and the intergenic spacers (IGS). Between the small and large rRNA sequences are the internal transcribed spacer regions (ITS1 and ITS2). The 5.8S nuclear rDNA gene lies between ITS1 and ITS2. The ITS regions are highly conserved within most species (with intraspecific similarities >99%) but are variable between species, making it suitable for use in taxonomy (Gomes et al. 2002, O'Brien et al. 2005). Many primer sets are designed to target the conserved regions of fungal rRNA (Turenne et al. 1999) increasing the sensitivity and selectivity for species identification. The earliest PCR primer sets to be routinely used to amplify the fungal ITS regions were the fungal specific primer ITS1, and the fungal general primer ITS4 (White et al. 1990). These primers work well on DNA from individual organisms but do not perform well on DNA extracts containing both host plant and microbial DNA. The plant-excluding primers, ITS1F and ITS4B, were developed to be specific for fungi and basdiomycetes, respectively (Gardes and Bruns 1996)

Numerous studies have used PCR-based methods to identify fungi, but the specific primers and the number of isolates per species used in each study have varied (Diehl et al. 2004, Guidot et al. 1999, Johnston and Aust 1994, McElroy et al. 2003, Schmidt and Moreth 1999a,1999b, Walsh et al. 1995, Zaremski et al. 1999). If the identification is focused on basidiomycetes then the ITS4B primer is often used. However, the question of intraspecific variation within the ITS region for numerous wood decay basidiomycetes is unknown. Adair et al. (2002) tested 26 wood decay basidiomycetes and 20 wood inhabiting ascomycetes by ITS-RFLP using several different primer pairs including ITS4B. Of the 26 basidiomycetes evaluated, 15 were single isolate representations of the species. No intraspecific variation was detected by ITS-RFLP among the 11 basidiomycete species represented by two to four isolates. The ITS4B gene was amplified in three of the ascomycetes. RFLP of the ITS fragment (amplified with fungal general primers ITS1 and ITS4) was also able to distinguish species of *Armillaria borealis, A. ostayeae, A. epistipes, A. gallica, A. medlea, A. tubescens,* and *A. ectypa* (Chillali et al. 1998). This study used two to four isolates of each species. A comparison of ITS sequences from 27 isolates of *Serpula lacrymans* and four isolates of *S. himantioides* using the ITS4B primer found very little sequence variation within *S. lacrymans,* while the *S. himantioides* showed polymorphisms among the isolates (Hogberg and Land 2004). Kim et al. (2005) isolated 132 basidiomycete decay fungi colonizing wood playground equipment. From these cultures the fungal general primers LROR and LR3 were used to amplify the 28S region. Unique sequences were compared to GenBank sequences and 30 different fungal taxa were identified with 13 of these belonging to the polyporoid clade of the basidiomycetes. Only three species were identified based on culture morphology. Some of these studies have examined a very limited number of isolates of each species, and thus the variation within some species has not been adequately assessed. Additionally, we do not know if RFLP data and sequence data are comparable.

In this study we have reconstructed the phylogenetic tree of a select group of basidiomycete fungi using nucleotide sequences of the ITS region of the rDNA suite of genes and have compared this to trees generated from RFLP analysis using general fungal and basidiomycete-specific primers to see if the latter procedures can correctly identify multiple isolates of select wood decay fungi.

Materials and methods

Fungal cultures

Fungal cultures, their sources, and use in this research project are listed in **Table 1**. Cultures were grown initially on Sabauraud Dextrose Agar (Difco Laboratories) and subsequently in Sabauraud Dextrose Broth (Difco Laboratories) for 3 to 7 days each at 28 °C. Mycelia was filtered from the broth culture through Whatman 541 filter paper, rinsed three times with distilled water, blotted to remove excess water and stored at −70 °C until DNA extraction.

DNA isolation and PCR amplification

DNA was isolated from fungal mycelia (0.02 g) by grinding with quartz sand, and extraction following the Qiagen DNeasy Plant kit protocol. The ITS region of fungal DNA was amplified using the fungal specific primer set: ITS1-F (CTT GGT CAT TTA GAG GAA GTA A) and ITS4 R (TCC TCC GCT TAT TGA TAT GC) or the basidiomycete specific primer set: ITS1-F (CTT GGT CAT TTA GAG GAA GTA A) and ITS4B (CAG GAG ACT TGT ACA CGG TCC AG) as

Table 1. — Fungal species and codes used in generating phylogenetic trees. Checks indicate which isolate was used in which analysis. RFLP BASDIO indicates the RFLP tree generated using the basidiomycete specific primers. RFLP GEN indicates the RFLP tree generated using the general fungal primers. SEQUENCE indicates the tree generated using sequence data.

Species	Source	Code	RFLP BASDIO	RFLP GEN	Sequence
T. hirsuta 66131	a	THF	√	√	√
T. hirsuta 46211	a	THE	√	√	√
T. hirsuta 34679	b	THD	√	√	√
T. hirsuta 10666	a	THG	√	√	√
T. hirsuta DR277	a	THA	√	√	√
T. hirsuta RLG	a	THB	√		
T. hirsuta 8591	a	THC	√		
T. hirsuta 10700	b	THH	√		
T. hirsuta 105981	b	THI	√		
T. hirsuta 125074	b	THJ	√		
T. versicolor 64311	a	TVF	√	√	√
T. versicolor 60985	a	TVE	√	√	√
T. versicolor 12679	a	TVH	√	√	√
T. versicolor 32745	a	TVA	√		
T. versicolor 34578	a	TVB	√		
T. versicolor 34584	a	TVC	√		
T. versicolor 42462	a	TVD	√		
T. versicolor 11235	a	TVG	√		
T. versicolor DR-EST	c	TVI	√		
*P. chrysosporium*102169	a	PCG	√	√	
P. chrysosporium 34541	b	PCB	√	√	√
P. chrysosporium 48747	a	PCD	√	√	
P. chrysosporium 32629	a	PCA	√	√	√
P. chrysosporium 62777	a	PCE	√	√	
P. chrysosporium 48746	a	PCC	√		
P. chrysosporium 62778	a	PCF	√		
P. sanguinea 7524	b	PSGA		√	
P. sanguinea 9865	b	PSGB		√	
P. sanguinea 102375	b	PSGC		√	
G. striatum 32520	a	GSTH		√	
G. striatum 102563	b	GSTI		√	
G. striatum 32521	a	GSTA	√	√	√
G. striatum 7305	b	GSTE	√	√	√
G. striatum 64699	b	GSTF	√	√	√
G. striatum BKW-003	b	GSTB	√		
G. striatum CR04	b	GSTD	√		
G. sepiarium 28094	a	GSEPE	√	√	
G. sepiarium 12677	a	GSEPD	√	√	
G. sepiarium 9419	a	GSEPA	√	√	√
G. sepiarium 60231	a	GSEPB	√	√	√
G. sepiarium 64312	a	GSEPC	√	√	
G. sepiarium 32892	a	GSEPH	√	√	
G. sepiarium 32518	a	GSEPF	√		
G. sepiarium 32519	a	GSEPG	√		
G. sepiarium 46278	a	GSEPI	√		
G. sepiarium 14159	a	GSEPK	√		
G. sepiarium DR275	c	GSEPJ	√		
G. trabeum 105470	b	GTE		√	
G. trabeum 101508	b	GTC	√	√	
G. trabeum Dietz	b	GTF		√	
G. trabeum 32084	a	GTG		√	
G. trabeum 32743	a	GTH		√	
G. trabeum 8715	a	GTI		√	
G. trabeum 11539	a	GTA	√		
G. trabeum 13021	a	GTB	√		
G. trabeum 274	c	GTD	√		

[a]American Type Culture Collection (ATCC).
[b]USDA Forest Products Laboratory, Madison, Wisconsin.
[c]Michigan Technological University, Dr. Dana Richtor.

described by White et al. (1990). Amplifications were performed in 100µl PCR reaction tubes containing 5µl DNA, 10µl 10X thermophilic buffer, 8 µl $MgCl_2$, (25mM) 4 µl dNTP (10mM), 10µl (10µM) ITS1-F, 10µl (10µM) ITS4, 52 µl ddH_2O and 1 µl (10u/µl) *Taq (*Promega*)*. Amplifications occurred in an Eppendorf Mastercycler Thermal Cycler with the following program for 40 cycles: initial denaturation temperature 94 °C for 1 minute 30 seconds, melt temperature 95 °C for 35 seconds, annealing temperature 55 °C for 55 seconds, extending temperature of 72 °C for 1 minute, final extension temperature 72 °C for 10 minutes, hold temperature 4 °C (Jasalavich et al. 2000). The ITS bands were identified by gel electrophoresis on a 2 percent agarose. Molecular weights of each ITS fragment were determined using GelPro Express software and a 100 bp ladder.

Restriction digestion of PCR products

The fungal ITS fragments were digested with four restriction enzymes: *Hinf*1, *HaeIII*, *Alu,* and *TaqI* (Promega) following manufacturers recommendations of 37 °C for 4 hours (*Hinf*1, *Hae*III and *Alu*) and 65 °C for 2 hours for *Taq*I. Digested bands were separated by gel electrophoresis using 2.5 percent high resolution agarose gel in 1X TBE running buffer amended with 0.002 percent ethidium bromide. Bands were visualized under a transillumninator box with UV light. Gels were photographed using a Polaroid camera and visualized with PhotoMax Pro™ software. Molecular weights of the digested bands were calculated using GelPro Express software and a 50 bp ladder. At least three replications per fungal isolate were run.

RFLP data analyses

RFLP profiles for each restriction enzyme were converted to binary data (presence or absence of a fragment for each sample) in Excel using 50bp binning. A simple matching similarity matrix for each restriction enzyme was calculated for samples and analyzed with hierarchical cluster analysis in SYSTAT (Wilkinson 1983) and PHYLIP (Felsenstein 2005). Trees were produced with TreeView (Page 1996).

DNA sequencing

Excess primers and buffers were removed from the amplified ITS fragment according to procedures given in the Mo Bio PCR DNA purification kit. DNA concentration, as determined by fluorescence, was done according to procedures in the Sigma DNA Quantification Kit and ranged between 2 ng/µl and 100 ng/µl. The fungal ITS fragment was prepared for sequencing according to the Beckman CEQ DTCS kit. The target DNA concentration was 45 ng /50 fmol. Fragments

were sequenced with a Beckman CEQ 2000XL capillary sequencer.

Sequence data analysis and creation of phylogenic tree

ITS sequence data were analyzed by the CEQ™ 8000 Genetic Analysis System (Beckman Coulter) software. The forward and reverse sequences for each fungal species were aligned with Clustal W (Kumar et al. 2001) validated visually and a consensus sequence was generated also with Clustal W. The identity of the consensus sequence was confirmed using BLAST search to known sequences in NCBI Genbank. A phylogenetic tree based on maximum likelihood with 100 bootstrap replications was constructed using DAMBE (Xia 2000, Xia and Xie 2001). *Paecilomyces* sp. was used as an out-group. Neighbor-joining and maximum parsimony trees were also constructed for comparison but not shown.

Results and discussion

Sequence data of ITS fragments of selected wood decay fungi were used to generate a phylogenetic tree for comparison of relatedness among these fungi to relatedness in phylogenetic trees generated from two restriction fragment length polymorphism (RFLP) methods on the fungal ITS fragments. There were 15 wood decay fungal samples used to generate sequence data, 33 fungal samples to generate RFLP-GEN data, and 47 fungal samples to generate RFLP-B. Some of the fungal isolates would not adequately sequence. Several different sequencing preparations were attempted, but all failed to provide an acceptable sequence. The sequence of several isolates from cultures did not correspond to the expected species and these were omitted from the study. *Trametes hirsuta* 46211 (THE) appeared as an outlier from the other *T. hirsuta* isolates in the three phylogenetic trees. This isolate was identified through GenBank as *T. hirsuta*. The other *T. hirsuta* isolates were identified through GenBank as a basidiomycete. *P. chrysosporium* 62777 (PCE) was identified as *P. sordia*.

The ITS-based phylogenetic tree using maximum likelihood revealed three well-supported genera, *Trametes*, *Phanerochaete*, and *Gloeophyllum*, with bootstrap values of 75 or greater (**Fig. 1**). *Trametes* and *Phanerochaete* were sister taxa, and *Gloeophyllum* was a sister taxon to the *Trametes/Phanerochaete* clade. Other phylogenetic analyses showed similar patterns with some variation. These three genera were well supported by neighbor-joining analysis (data not shown), but the clade containing both *Trametes* and *Phanerochaete* was not supported. Maximum parsimony analysis showed well-supported *Trametes* and *Gloeophyllum* genera, but a monophyletic *Phanerochaete* genus was not supported since one *Phanerochaete* species was a sister taxon to the *Trametes* genus and the other *Phanerochaete* species was a sister taxon to the *Gloeophyllum* genus (data available from corresponding author).

Within *Trametes* one species, *T. versicolor*, was well supported by maximum likelihood analysis with a bootstrap value of 100 (**Fig. 1**). *T. hirsuta* could not be resolved into a single clade. Isolates A, D, G, and F constituted one monophyletic group, but isolate E was a sister taxon to the rest of the entire *Trametes* clade. Within the *Gloeophyllum* genus, neither species (*G. sepiarium, G. striatum*) could be separated into monophyletic clades. Regardless of phylogenetic analysis employed, basidiomycete species could not be resolved

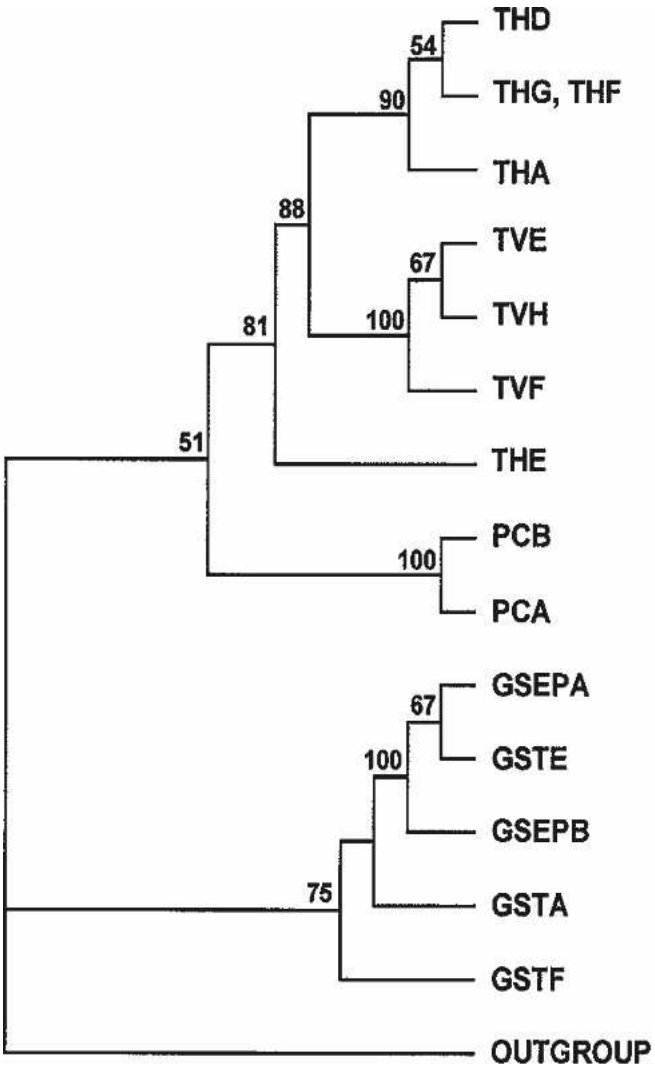

Figure 1. — Maximum likelihood phylogenetic tree derived from Internal Transcribed sequence data of select basidiomycete fungi. Sample codes are listed in Table 1. Bootstrap support values above 50 percent are indicated above the branches.

from ITS sequences alone, but maximum likelihood and neighbor-joining analyses successfully resolved genera.

RFLP-based cluster analyses of trees generated from general fungal primers and basidiomycete-specific primers are shown in **Figures 2** and **3**, respectively. Neither set of cluster analyses could resolve the three genera into monophyletic groups. The tree based on general fungal primers (**Fig. 2**) also did not resolve species well. The tree based on Basidiomycete-specific primers (**Fig. 3**) resolved species much better. All isolates of both *G. striatum* and *G. trabeum* comprised monophyletic groups. Eight of nine *T. versicolor* isolates, 10 of 11 *G. sepiarium* isolates, and 9 of 10 *T. hirsuta* isolates comprised monophyletic groups. *Phanerochaete* could not be consistently resolved into monophyletic groups at either the generic or specific level. In contrast to Hogberg and Land (2004) only two of the species tested in this study were resolved by sequence data as monophyletic groups (*T. versicolor* and *P. chrysosporium*). Identification of unknowns from environmental samples, such as in the study by Kim et al. (2005), assumes each species resolves as monophyletic

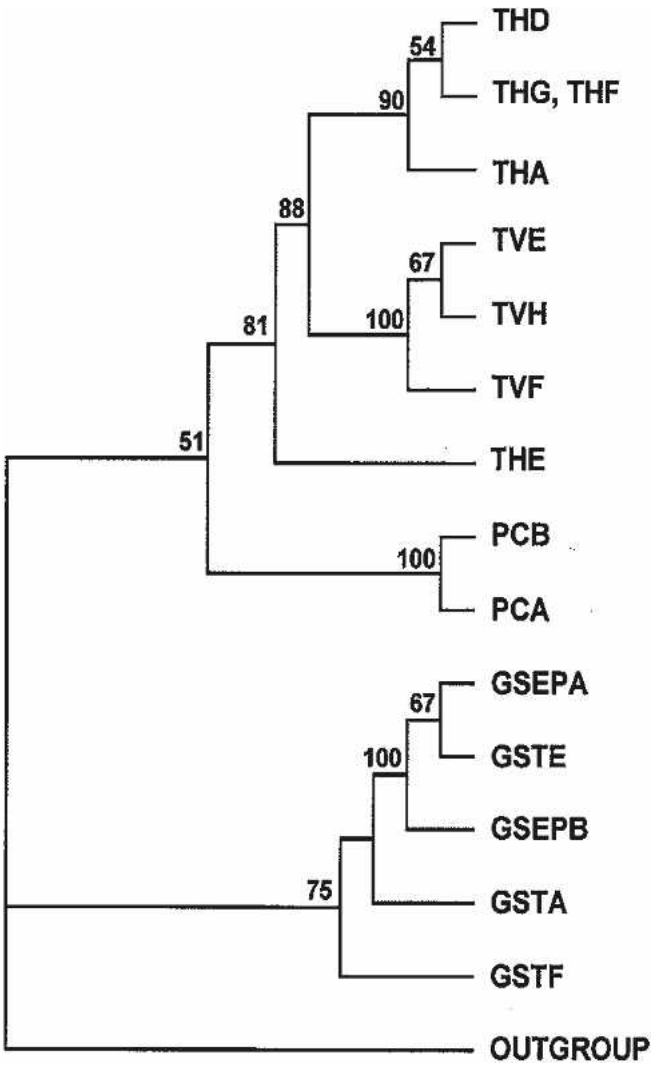

Figure 2. — RFLP-GEN phylogenetic tree generated from restriction digest of Internal Transcribed Spacer Region amplified by general fungal primers, ITS1F-ITS4 and digested by Hinf1, HaeIII, Alu, and TaqI.

groups. Our data do not support this assumption for all species.

Based on RFLP-GEN and RFLP-B phylogenetic trees compared to the sequence (SEQ) tree, results indicated that a very similar clustering pattern exists for *Trametes* among the three trees. *Trametes hirsuta* isolates THA, THD and THG clustered together in the same pattern in RFLP-Gen, RFLP-B, and SEQ trees. *T. hirsuta*, THE appeared as a sister group to the other *Trametes* in all three phylogenetic trees. *T. hirsuta* THF grouped with the other *T. hirsuta* in the RFLP-B and sequence trees but grouped with isolates of *T. versicolor* in the RFLP-GEN tree. The three isolates of *Trametes versicolor* (TVE, TVF, TVH) clustered in the same clade in all three trees.

There were seven isolates of *Phanerochaete chrysosporium* (PC) evaluated in the RFLP-B and five in the RFLP-GEN phylogenetic trees but only two isolates in the sequence phylogenetic tree because of a lack of acquiring usable sequence data in spite of several attempts. *Phanerochaete chrysosporium* clustered separately from *Trametes* in all trees. In the

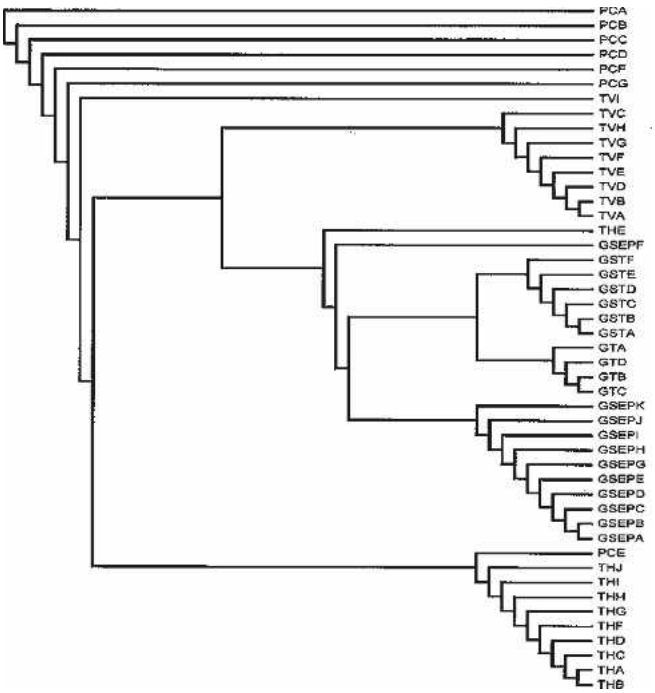

Figure 3. — RFLP-B phylogenetic tree generated from restriction digest of Internal Transcribed Spacer Region amplified by basidiomycete- specific primers and digested by Hinf1, HaeIII, Alu, and TaqI.

RFLP-GEN tree, four of the five PC isolates clustered together while the fifth, PCE, clustered more with *P. sanguinea*, PSGA and PSGB. PSGC clustered as a sister clade within the *Phanerochaete* clade and was determined to be 99.8 percent similar to *P. sordida* (AY219381.1) by GenBank Blast analysis. However, no *P. sorida* isolates were sequenced to confirm this observation. *Gloeophyllum sepiariu*m 32892 (GSEPH) appeared with the *Phanerochaete* clade but was later determined to be *P. chrysosporium* AFTOL 776 (92.2% similarity) by sequence analysis. In the RFLP-B phylogenetic tree, all PC isolates except PCE (which clustered in the same clade as *Trametes versicolor*), appeared unrelated to each other or any other isolates evaluated. The only two PC isolates, PCA and PCB, successfully sequenced did cluster in the same clade and was well supported by a bootstrap value of 100.

The data for *Gloeophyllum striatum* and *G. sepiarium* did not result in a distinct clustering pattern in the RFLP-GEN or SEQ phylogenetic trees. There was an insufficient number of isolates of *G. trabeum* analyzed by either RFLP-GEN or sequence methods. In the RFLP-B tree, the three species of *Gloeophyllum* did form a distinct clade with each other with the exception of isolate GSEPF which formed a sister clade with other *Gloeophyllums*.

Identification of wood decay fungi has challenged researchers for many years. Successful identification of wood decay fungi will increase as more researchers add to the databases. For this to occur there are research needs for 1) additional nucleotide sequences to generate more robust phylogenetic trees with greater potential to resolve both genera and species, and 2) inclusion of multiple isolates of each species. Our studies indicate that RFLP analysis using general fungal primers are not likely to be useful in identifying species or reconstructing phylogenetic relationships. RFLP analysis using Basidiomycete-specific primers may be useful in identifying some

species but not in reconstructing phylogenetic relationships although it has an advantage that it is a simpler procedure compared to sequencing. RFLP may be more useful if additional basidiomycete-specific primers are incorporated.

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