Detection and Identification of Decay Fungi in Spruce Wood by Restriction Fragment Length Polymorphism Analysis of Amplified Genes Encoding rRNA†

CLAUDIA A. JASALAVICH, ANDREA OSTROFSKY, AND JODY JELLISON*

Department of Biological Sciences, University of Maine, Orono, Maine 04469-5735

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We have developed a DNA-based assay to reliably detect brown rot and white rot fungi in wood at different stages of decay. DNA, isolated by a series of CTAB (cetyltrimethylammonium bromide) and organic extractions, was amplified by the PCR using published universal primers and basidiomycete-specific primers derived from ribosomal DNA sequences. We surveyed 14 species of wood-decaying basidiomycetes (brown-rot and white-rot fungi), as well as 25 species of wood-inhabiting ascomycetes (pathogens, endophytes, and saprophytes). DNA was isolated from pure cultures of these fungi and also from spruce wood blocks colonized by individual isolates of wood decay basidiomycetes or wood-inhabiting ascomycetes. The primer pair ITS1-F (specific for higher fungi) and ITS4 (universal primer) amplified the internal transcribed spacer region from both ascomycetes and basidiomycetes from both pure culture and wood, as expected. The primer pair ITS1-F (specific for higher fungi) and ITS4-B (specific for basidiomycetes) was shown to reliably detect the presence of wood decay basidiomycetes in both pure culture and wood; ascomycetes were not detected by this primer pair. We detected the presence of decay fungi in wood by PCR before measurable weight loss had occurred to the wood. Basidiomycetes were identified to the species level by restriction fragment length polymorphisms of the internal transcribed spacer region.

Wood is an important renewable and biodegradable natural resource with a multitude of uses. Wood is used extensively as a structural material for buildings, wharves, telephone poles, and furniture due to its high strength per unit weight, its versatility, and its variety. Wood also serves as the industrial raw material for the manufacture of paper and paper products, wood composites, and other products made from cellulose, such as textiles and cellophane. In many parts of the world wood is used as a fuel for heating and cooking.

The primary biotic decomposers of wood are basidiomycete decay fungi, which can attack and degrade both wood in the forest and wood in service. In the forest ecosystem wood decay fungi play an important role in carbon and nitrogen cycling and help to convert organic debris into the humus layer of the soil. Some fungi attack living trees; others invade downed timber and slash on the forest floor, lumber, and wood in service. Wood decay basidiomycetes colonize and degrade wood using enzymatic and nonenzymatic processes. Brown-rot fungi preferentially attack and rapidly depolymerize the structural carbohydrates (cellulose and hemicellulose) in the cell wall leaving the modified lignin behind. White-rot fungi can progressively utilize all major cell wall components, including both the carbohydrates and the lignin. As decay progresses the wood becomes discolored and loses strength, weight, and density. Decay and discoloration caused by fungi are major sources of loss in both timber production and wood use, with losses of 15 to 25% marketable wood volume in standing timber and of 10 to 15% in wood products during storage and conversion. Each year ca. 10% of the timber cut in the United States is used to replace wood in service that has decayed, resulting in the expenditure of hundreds of millions of dollars for raw materials, labor, and liability (22).

Brown rots rapidly and drastically reduce wood strength early in the decay process, while white rots cause a slower progressive decrease in wood strength. Brown-rot fungi can reduce wood strength by as much as 75% at less than 5% weight loss of the wood (22). For this reason it is important to develop methods which can detect wood decay very early, at the incipient stage prior to the occurrence of significant strength loss. Techniques which have been used to detect incipient decay include isolation and culturing of fungi, chemical staining, nuclear magnetic resonance, and electrical resistance, as well as serological methods, such as immunoblotting and enzyme-linked immunosorbent assay (ELISA) (3). ELISA has been found to be a sensitive method for detecting incipient decay (4, 11), but the assay sensitivity can be inhibited by wood extractives (12). Optimal methods for early detection of decay for wood in service have not been developed.

The development of the DNA-based PCR (14) and taxon-specific primers (2, 6, 7, 16, 17) is making it increasingly feasible to detect and study fungi in their natural substrates. A DNA-based method to detect the presence of wood decay fungi would potentially use only small amounts of wood, thus allowing for nondestructive sampling. The extreme sensitivity and potential specificity of the assay would theoretically allow for the detection of fungal decay agents at an incipient stage enabling remedial biocidal treatments to be applied before significant strength loss had occurred. Detection of specific decay agents is also a necessary prerequisite to allow evaluation of fungal colonization and proliferation in preservative-treated woods undergoing remediation. Specific and sensitive assay procedures would aid in the monitoring and development of successful fungus-based bioremediation technologies.

For our DNA-based detection method, we selected the internal transcribed spacer (ITS) region (ITS1, the 5.8S ribosomal DNA [rDNA], and ITSII) as the target sequence for
amplification for three reasons. The ITS region is present at a very high copy number in the genome of fungi, as part of the tandemly repeated nuclear rDNA; this, coupled with PCR amplification, should produce a highly sensitive assay. The DNA sequences of the ITS1 and ITS2 are highly variable; this feature can be exploited to generate restriction fragment length polymorphism (RFLP) patterns to identify wood decay fungi or to design taxon-specific primers. The European Armillaria species Armillaria cepistipes, A. gallica, A. borealis, A. ostoyae, and A. mellea are clearly delimited by RFLPs of rDNA (17), to identify ectomycorrhizal fungi to the genera and/or species level (5, 7, 8, 9, 10), and to identify intersterility groups of Heterobasidion annosum (6). In designing an assay to detect fungi by PCR amplification using total DNA isolated from infected plant material as the template, it is important to be able to discriminate between DNAs of fungal and plant origin. Primers which specifically amplify the ITS region from only fungal DNA (7) and not plant DNA are available. These fungus-specific primers were originally designed to identify fungal symbionts directly from ectomycorrhizae and to identify rusts, which are obligate parasites, in the host tissue (7). More recently, these primers have been used to study the community structure of ectomycorrhizal fungi in a pine forest (8) and the genetic structure of a natural population of Suillus punctus (2).

The objectives of our study were (i) to rigorously test the specificity of the basidiomycete-specific primer (7) by surveying a large number of wood decay basidiomyces, as well as wood-inhabiting ascomycetes (pathogens, endophytes, and saprophytes); (ii) to optimize the PCR assay conditions for specific detection of brown-rot and white-rot fungi in wood; (iii) to identify the PCR-detected fungi to species via RFLPs of the amplified internal transcribed spacer region; and (iv) to develop a DNA-based method to detect incipient stages of wood decay, thus allowing remedial treatments to be applied to wooden structural members before substantial strength loss has occurred.

MATERIALS AND METHODS

Fungal culture. The fungi used in this study and their sources are given in Table 1. Cultures were grown on plates of malt agar at room temperature for use in DNA isolation or as inocula for soil block jars.

Soil block culture. Modified ASTM soil block jars (1) were set up as follows. A soil mix (1:1:1 by volume) was prepared by mixing equal volumes of potting soil, sphagnum moss, and vermiculite and then moistened with deionized distilled water. About 1 cup of the mix was placed in each pint-sized Mason jar, and water was allowed to absorb overnight. The next day, 20 ml of water was added per jar so that the soil mix was moist and a little free water was present. Two days later the jars were again fed with an additional 20 ml of water to allow fungal colonization of the soil mix from fresh mycelia that had been taken from plate cultures, from lyophilized mycelia, or from infected wood by extraction with cetyltrimethylammonium bromide (CTAB) in the presence of β-mercaptoethanol, followed by organic extractions and isopropanol precipitation of the DNA. Our method is based on those of Taylor et al. (19) and Wilson (21). For fresh mycelia a 2X CTAB extraction buffer (2% [wt/vol] CTAB; 100 mM Tris HCl, pH 8.0; 1 M NaCl; 20 mM EDTA; 0.2% [vol/vol] β-mercaptoethanol) was used, with the β-mercaptoethanol being added just prior to use. For lyophilized mycelia or dry tissues such as wood samples, a 1X CTAB extraction buffer (diluted 2X β-mercaptoethanol was used. It is especially important to use the 1X CTAB extraction buffer for wood samples; otherwise, the aqueous and organic phases invert due to dehydration of the wood when the 2X CTAB extraction buffer is used.

Wood blocks were sampled by drilling through noninoculated wood surfaces. Precautions were observed during drilling of the wood blocks to prevent contamination of samples. Both the work table and gloves were swabbed with 70% ethanol and to surface sterilize them and to collect any sawdust before and after drilling each sample. A rechargeable cordless drill was used because it has less surface area, fewer crevices, and no cord to collect dirt and sawdust, and a molded housing which can be easily wiped clean with 70% ethanol. Drill bits were carefully cleaned with laboratory detergent, rinsed, soaked in 95% ethanol, and flame sterilized. A drill bit was inserted through a cone of filter paper (new for each sample), positioned so as to cover the chuck and prevent sawdust from entering it. We drilled through each wood block, on a line perpendicular to the face of the block that had contacted the colonized feeder strip, with a 1/8-in. diameter bit to generate a fine sawdust from which DNA could be isolated directly; no further grinding of the sawdust was needed to achieve good DNA extraction. Once a prepared drill bit was used to drill a wood sample, it was not reused until it had been re-cleaned and resterilized by the procedure described above. Fresh or lyophilized mycelia was simply ground to a fine powder with liquid nitrogen in a mortar and pestle for use in DNA extraction.

Grund or drilled material (100 to 200 μl) was transferred to a sterile microfuge tube. Then, 600 μl of the appropriate CTAB extraction buffer was added, and the sample was mixed to resuspend the powdered tissue in the buffer and incubated at 65°C for 2 h. The sample was extracted with 1 volume of chloroform-isooamyln alcohol (24:1, vol/vol) and centrifuged at 10,000×g for 10 min at room temperature. The aqueous phase was transferred to a new tube and 0.8 volume of 10% (wt/vol) CTAB in 0.7 M NaCl was added. After mixing, the sample was incubated at 65°C for 1 h. Once again the sample was extracted with chloroform-isooamyln alcohol (24:1, vol/vol) and centrifuged as described above. The aqueous phase was transferred to a new tube and 0.6 volume of ice-cold isopropanol was added and after standing at −20°C, the precipitate was collected by centrifugation at 12,000×g for 15 min. The pellet was washed twice with ice-cold 70% (vol/vol) ethanol and dried. The pellet was resuspended in DNA storage buffer (6 mM Tris HCl, 0.1 mM EDTA; pH 7.5); 100 μl was used for DNA isolated from mycelia, and 50 μl was used for DNA isolated from wood samples. Incubation at 65°C speeded up resuspension of the DNA.

PCR amplification. The ITS region was amplified by PCR from DNA isolated from pure cultures of each of the fungi listed in Table 1 and from wood blocks colonized by individual isolates of wood decay basidiomyces or wood-inhabiting ascomycetes. Primers ITS1-F (CTT GGT CAT TTA GAG GAA GTA A), which is specific for the higher fungi (7), and ITS4 (TCC TCC GAT TAT GCA G), the universal primer (20), were used together as a positive control for amplification, since they would be expected to amplify the ITS region from both ascomycetes and basidiomyces. The primer pair ITS1-F and ITS4-B (CAG GAG ACT TGT ACA CGG TCC AG), which is specific for basidiomycetes (7), were used to specifically amplify the ITS region from only basidiomyces. Amplifications were performed in 50-μl reactions of PCR buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; 0.001% [wt/vol] gelatin [Perkin-Elmer], 200 μM concentrations of each deoxyribonucleotide triphosphate, and 200 mM concentrations of each of the appropriate primers, with nonacetylated bovine serum albumin (BSA; Sigma-A7906) at 250 ng/μl, total DNA isolated from a pure fungal culture or from a wood decay sample, 0.056 μM TaqStart antibody (Clontech), and 0.002 μM AmpliTaq DNA polymerase (Perkin-Elmer), i.e., 2 U per reaction. The TaqStart antibody and AmpliTaq were mixed together and preincubated prior to being added to the rest of the reaction components as per the manufacturer’s instructions (Clontech). Samples were

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### TABLE 1. Amplification of ITS region from DNA isolated from pure fungal cultures

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<th>Species</th>
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<th>Source</th>
<th>Ecology</th>
<th>PCR amplification(^a) with primer pair:</th>
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**Ascomycetes**  

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\(^a\) a, American Type Culture Collection; b, Forest Products Laboratory, Madison, Wis.; c, Gerard Adams, Michigan State University; d, Barry Goodell, University of Maine, Orono; e, Dilip Lakshman, University of Maine, Orono; f, David Lambert, University of Maine, Orono; g, Kevin Smith, University of New Hampshire; h, Swedish Agricultural University, Uppsala, Sweden; i, Paul I. Morris, Forintek Canada Corp., Vancouver, British Columbia, Canada.

\(^b\) C, conifers; H, hardwoods; R, Rosaceae; S, wood in service.

\(^c\) Primer ITS1-F is specific for higher fungi, primer ITS4 is a universal primer, and primer ITS4-B is specific for basidiomycetes.
Hormonema dematiodes

and 19, no template DNA (i.e., negative controls). (B) Lanes 1 and 20, PCR

TAE (40 mM Tris-2.5% (wt/vol) Sepharose Gel Matrix (Gibco-BRL) in 1

buffer. DNA bands were visualized by fluorescence under UV light and photo-

acetate, 1 mM sodium EDTA) with EtBr at 100 ng/ml in the gel and running

then incubated for 6 h at 37°C for the

appropriate restriction reaction buffer and 10 U of the appropriate enzyme

m

RFLPs; each sample was digested with

Restriction fragments were separated by electrophoresis in 2% (wt/vol) agarose gels

FIG. 1. PCR amplification of nuclear rDNA from total DNA isolated from

culture of basidiomycetes (A) and ascomycetes (B). Electrophoresis in 2% (wt/vol) agarose in 1× TBE. The two outer lanes contain molecular weight

markers. Inner even-numbered lanes contain samples amplified by the primer pair ITS1-F and ITS4-B, and the odd-numbered lanes contain samples amplified

by primers ITS1-F and ITS4. (A) Lanes 2 to 9 contain brown-rot fungi, and lanes

10 to 17 contain white-rot fungi. Lanes 1 and 20, PCR markers (Promega); lanes

2 and 3, Coniophora puteana Fp-90099-Sp; lanes 4 and 5, Gloeophyllum trabeum

Mad-517; lanes 6 and 7, Phanerochaete chrysosporium Fp-686-B; lanes 8 and 9, Serpula

lactinans Harm-888-R; lanes 10 and 11, Lentinula edodes 117-1t(d); lanes 12

and 13, Russula bicolor ATCC 64987; lanes 14 and 15, Sarcoscypha coccinea

ATCC 64986; lanes 16 and 17, Trametes versicolor Fp-101664-Sp; lanes 18

and 19, no template DNA (i.e., negative controls). (B) Lanes 1 and 20, PCR

markers (Promega); lanes 2 and 3, Aureobasidium pullulans ATCC 34621; lanes

4 and 5, Hormonema dematioides; lanes 6 and 7, Pestalotiopsis sp.; lanes 8 and 9,

Leucostoma kanzlei; lanes 10 and 11, Scleroderris lagerbergii 1877; lanes 12 and 13,

Serpula lacrymans; lanes 14 and 15, Sphaeropsis sapinea Fp-117; lanes 16 and 17,

Xenomera abietis; lanes 18 and 19, no template DNA (i.e., negative controls).

DNA isolation from decayed wood. CTAB extraction in the presence of β-mercaptoethanol followed by organic extractions

and isopropanol precipitation of the DNA yielded DNA clean

eufloidby PCR regardless of whether the starting material was fungal mycelia or decayed wood. The more
decayed the wood, the more pigmented was the DNA-containing aqueous phase. Subsequent extractions with chloroform-

isoamyl alcohol and phenol-chloroform-isoamyl alcohol removed some of the pigmented by-products of wood decay, and

more remained behind in the aqueous isopropanol phase upon precipitation of the DNA. However, substances inhibitory to

PCR could carry through the purification procedure. For example, in preliminary experiments when DNA was isolated from

replicate sets of drilled samples from highly decayed wood blocks (60% plus weight loss), the aqueous phase of

samples in which the initial CTAB extraction had lasted overnight were much more strongly pigmented than those initially

extracted for only 2 h, as in the standard protocol (see Materials and Methods); we would expect more by-products of wood decay to be extracted in an overnight versus a 2-h incubation.

All of the 2-h CTAB-extracted DNA preparations were amplified by PCR; however, several of the overnight CTAB-

extracted DNA preparations did not amplify, probably due to a higher concentration of compounds inhibitory to PCR re-

maining after purification.

Optimization of PCR assay conditions for detection of basidiomycetes. The primers ITS1-F (higher fungus specific) and

ITS4 (universal primer) amplified only one band (500 to 1,300 bp, depending on the fungal species) from DNA isolated from

pure cultures of both ascomycetes and basidiomycetes via an ordinary PCR protocol, i.e., no hot start was needed. However,

when we amplified the ITS region with the primers ITS1-F and ITS4-B (basidiomycete specific) from total DNA isolated from

pure cultures, we obtained a number of minor amplification bands in both basidiomycetes and ascomycetes with the pub-

lished amplification protocol that used an annealing temperature of 55°C (7). Although the main product (850 to 1,460 bp, depending on the fungal species) was not amplified from as-

overlaid with mineral oil and amplified in a MJ Research Thermocycler Model

PTC-100. PCR reactions consisted of an initial denaturation at 94°C for 1 min

25 s, 35 cycles of amplification, and a final extension at 72°C for 10 min; each

cycle of amplification consisted of denaturation at 95°C for 35 s, annealing for

5 s (at 55°C for reactions with ITS1-F and ITS4 and at 60°C for reactions with

ITS1-F and ITS4-B), and extension at 72°C for 1 min.

Weakly positive or negative amplifications were reconfirmed as positive or

negative by taking an aliquot of the PCR reaction and reamplifying it with the

primer pair used in the original reaction. Aliquots of the PCR reaction using

template DNA isolated from wood and the primers ITS1-F and ITS4-B were also

amplified by PCR; however, several of the overnight CTAB-

extracted DNA preparations did not amplify, probably due to a higher concentration of compounds inhibitory to PCR re-

maining after purification.

RESULTS

FIG. 2. PCR amplification of nuclear rDNA from total DNA isolated from

wood blocks colonized by wood decay fungi or endophytes. Electrophoresis in 2% (wt/vol) agarose in 1× TBE. The two outer lanes contain molecular weight

markers. Inner even-numbered lanes contain samples amplified by the primer

pair ITS1-F and ITS4-B, and the odd-numbered lanes contain samples amplified

by primers ITS1-F and ITS4. Lanes 2 to 9 contain brown-rot basidiomycetes; lanes

8 to 13, white-rot basidiomycetes; lanes 14 to 17, endophytic ascomycetes. Lanes 1

and 20, PCR markers (Promega); lanes 2 and 3, Postia placenta Mad-698-R; lanes

4 and 5, Gloeophyllum trabeum Mad-617-R; lanes 6 and 7, Leucogyrophana

pinastri; lanes 8 and 9, Lentinula edodes 117-1t(d); lanes 10 and 11, Trametes

vericolor; lanes 12 and 13, Sarcoscypha coccinea ATCC 64986; lanes 14 and 15,

Hormonema dematioides; lanes 16 and 17, Pestalotiopsis sp.; lanes 18 and 19, no

template DNA (i.e., negative controls).
comycetes, a small band amplified very strongly in certain species of ascomycetes, e.g., a 210-bp band from *Phialocephala fusca* and a 330-bp band from *Phialocephala* species of ascomycetes, e.g., a 210-bp band from *comycetes*, a small band amplified very strongly in certain 

### TABLE 2. Wood decay and detection of fungal species in wood blocks after 8 months of colonization

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Mean % wt loss of wood ± SD&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR amplification&lt;sup&gt;b&lt;/sup&gt; with primer pair:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS1-F–ITS4 ITS1-F–ITS4-B</td>
</tr>
<tr>
<td><strong>Uninoculated control wood</strong></td>
<td></td>
<td></td>
<td>+ + + +</td>
</tr>
<tr>
<td><strong>Brown-rot basidiomycetes</strong></td>
<td></td>
<td></td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Contiophora puteana</em></td>
<td>Fp-90099-Sp</td>
<td>0.1 ± 0.3</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Postia placenta</em></td>
<td>Mad-698-R</td>
<td>65.5 ± 1.2</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Postia placenta</em></td>
<td></td>
<td>65.8 ± 1.4</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Gloeophyllum trabeum</em></td>
<td>Mad-617-R</td>
<td>67.3 ± 3.3</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Gloeophyllum trabeum</em></td>
<td></td>
<td>69.6 ± 1.8</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Gloeophyllum sepiarium</em></td>
<td>10-BS2-2</td>
<td>68.1 ± 0.9</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Leucogyrophana pinastri</em></td>
<td></td>
<td>68.1 ± 4.8</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Serpula lacrimans</em></td>
<td>Harm-888-R</td>
<td>67.5 ± 1.9</td>
<td>+ + + +</td>
</tr>
<tr>
<td><strong>White-rot basidiomycetes</strong></td>
<td></td>
<td></td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Lentinula edodes</em></td>
<td>117=lt(d)</td>
<td>3.0 ± 1.7</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Trametes versicolor</em></td>
<td></td>
<td>35.1 ± 13.6</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Trametes versicolor</em></td>
<td>Fp-101664-Sp</td>
<td>0.0 ± 0.1</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Inxip luteus</em></td>
<td>KTS 003</td>
<td>40.1 ± 12.1</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Resinicum bicalcor</em></td>
<td>HHB-8850-sp</td>
<td>10.4 ± 0.9</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Resinicum bicalcor</em></td>
<td>ATCC 44175</td>
<td>3.7 ± 3.6</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Resinicum bicalcor</em></td>
<td>ATCC 64897</td>
<td>12.1 ± 5.7</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Scytinostroma galactinum</em></td>
<td>MB-1880-sp</td>
<td>−0.8 ± 0.5</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Scytinostroma galactinum</em></td>
<td>ATCC 64896</td>
<td>1.1 ± 0.3</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Scytinostroma galactinum</em></td>
<td>ATCC 44178</td>
<td>−0.2 ± 0.7</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Phanerochaete chrysosporium</em></td>
<td>ATCC 24725</td>
<td>9.9 ± 11.8</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Phanerochaete chrysosporium</em></td>
<td></td>
<td>14.2 ± 5.7</td>
<td>+ + + +</td>
</tr>
<tr>
<td><strong>Wood-inhabiting ascomycetes</strong></td>
<td></td>
<td></td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Phialophora mutabilis</em></td>
<td>ATCC 42792</td>
<td>0.4 ± 0.2</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Trichoderma reesi</em></td>
<td>ATCC 26921</td>
<td>−0.2 ± 0.4</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>ATCC 32630</td>
<td>−0.1 ± 0.1</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Hormonema dematiodes</em></td>
<td></td>
<td>0.4 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Pestalotiopsis sp.</em></td>
<td></td>
<td>0.6 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Xenomos isabietis</em></td>
<td></td>
<td>0.5 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Aureobasidium pullulans</em></td>
<td>ATCC 34621</td>
<td>0.2 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Phialocephala fusca</em></td>
<td>ATCC 62326</td>
<td>−0.2 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Ceratocystis pallida</em></td>
<td>ATCC 60758</td>
<td>2.7 ± 0.0</td>
<td>+ + + +</td>
</tr>
<tr>
<td><em>Ophiostoma ulmi</em></td>
<td>ATCC 32439</td>
<td>0.1 ± 0.0</td>
<td>+ + + +</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean of two replicate spruce wood blocks.

<sup>b</sup> Primer ITS1-F is specific for higher fungi, primer ITS4 is a universal primer, and primer ITS4-B is specific for basidiomycetes. Each plus or minus sign represents the amplification results for an individual wood block.

**In order to achieve specific amplification of DNA isolated from decayed wood, further adjustments to the PCR protocol were needed. Addition of nonacetylated BSA to PCR reactions, which is known to relieve inhibition of amplification by humic acids, fulvic acids, and organic components of soils and manure (13), allowed some amplification to occur from samples containing inhibitory wood decay by-products; but this amplification was often nonspecific. Additionally, a hot-start protocol, either the traditional method or the TaqStart antibody system, was required to obtain specific amplification of DNA isolated from wood decay samples.

We adopted as our standard PCR amplification conditions the inclusion of nonacetylated BSA at 250 ng/µl and the use of a hot start, the TaqStart antibody system for all reactions regardless of the tissue source of template DNA or primers used. These conditions are described in detail in Materials and Methods.

**Fungal species survey.** We surveyed a total of 43 species (60 isolates) of fungi. These included 16 species of basidiomycetes, 14 of which are wood decay fungi, both brown rot and white rot, and 27 species of ascomycetes, 25 of which are wood inhabitants (pathogens, endophytes, and saprophytes). For the initial survey (Table 1), PCR amplifications were performed using total DNA isolated from pure cultures of the fungi as the DNA template. Primers ITS1-F and ITS4 amplified the ITS region from all of these fungi, both ascomycetes and basidio-
mycetes, as expected. Primers ITS1-F and ITS4-B amplified the ITS region from only the basidiomycetes (Fig. 1, Table 1).

**Detection of decay fungi in wood.** The next step was to see if we could detect fungi in spruce wood by PCR amplification (Fig. 2, Table 2) using total DNA isolated from colonized wood blocks as the DNA template. We surveyed 30 species of fungi colonizing spruce wood. Two replicate jars were set up and inoculated, as described in Materials and Methods, for each of the wood-decaying ascomycete species listed in Table 1 (excluding *Fomitopsis pinicola* and *Trichaptum abietinum*) and for each of the following wood-inhabiting ascomycetes: *Ceratocystis pilifera* ATCC 60758, *Opitutia ulmi* ATCC 32439, *Phialophora fusca* ATCC 62326, *Phialophora mutabilis* ATCC 42792, *Trichoderma reesi* ATCC 26921, *Trichoderma viride* ATCC 32630, *Aureobasidium pullulans*, *Hormonema dematioides*, *Postia placenta*, and *Xenomeres abietis*. Wood blocks cut from radial sections of spruce wood were added to the colonized feeder strips and harvested after 8 months of colonization.

Wood blocks with ascomycetes had a negligible weight loss, the majority by less than 0.5%, and seemed unchanged in appearance. The weight losses of wood blocks with white-rot fungi were very variable and ranged from negligible to approximately 40%; there was little to no change in the color of the wood, but some of the more decayed ones, e.g., replicate blocks colonized by one of the isolates of *Trametes versicolor* had become stringy in texture. Wood blocks with brown-rot fungi had decayed the most and were very brown in color; all but one isolate had caused a weight loss of 65 to 70%, the exception being *Contiophora puteana* isolate Fp-90099-Sp. In all of the wood block treatments, we had weight losses ranging between 0 and 70%. Primers ITS1-F and ITS4 amplified DNA from all of the samples, including the uninoculated control blocks. Primers ITS1-F and ITS4-B amplified DNA from wood blocks that had been inoculated with only basidiomycetes, i.e., the brown-rot isolates and white-rot isolates; ITS1-F and ITS4-B did not amplify DNA from uninoculated control blocks nor from any of the wood blocks inoculated with wood-inhabiting ascomycetes. The unknown contaminant fungus detected in the uninoculated control blocks is probably an ascomycete, since no amplification occurs when the basidiomycete-specific primer ITS4-B is present in the PCR reaction; we suspect it may be a mold known to survive in wood upon repeated autoclaving. We could reliably detect the presence of wood decay fungi by PCR with the primers ITS1-F and ITS4-B in spruce blocks exhibiting a range of degradation states.

**Fungal identification.** In order to identify the basidiomycetes detected by PCR, we generated RFLPs of the ITS region, the product amplified by primers ITS1-F and ITS4-B, by restriction digestion with *RsaI*, *AluI*, *HaeIII*, *TagI*, or *TaqI*. *HaeIII* was not very useful because it did not cut the amplon from 10 out of the 14 species of wood-decaying basidiomycetes tested nor that from the ectomycorrhizal *Pisolithus tinctorius* and the soil-borne *Rhizoctonia solani*, which do not decay wood. The other restriction endonucleases generated more fragments per digest, so that each basidiomycete could be identified to the species level from the combination of its RFLP profiles (Fig. 3, Table 3). The majority of RFLP profiles generated for any given enzyme were unique for each fungal species. The two *Gloeophyllum* species, however, had identical *AluI* RFLP profiles and identical *HaeIII* RFLP profiles; *G. trabeum* and *G. sepiarium* could be separated by their *TagI* RFLP profiles and their *TagI-HaeIIII* RFLP profiles. Different isolates of a given fungal species usually had identical RFLP profiles for a particular restriction endonuclease; this was true for each enzyme for isolates of *G. trabeum*, *Irpex lacteus*, *Postia placenta*, *Resinicium bicolor*, and *Serpula lacrimans*. For other fungi, some enzymes would generate RFLP profiles which separated isolates at the species level and other enzymes would generate RFLP profiles which separated isolates at the subspecies level. For example, isolates of *Scytinostroma galactinum* had identical *AluI* RFLP profiles and identical *HaeIII* RFLP profiles, but the isolates could be distinguished from each other by their respective *TagI* RFLP profiles and *TagI HaeIIII* RFLP profiles.

The identities of basidiomycetes detected by PCR from colonized spruce wood blocks were confirmed by comparing the RFLPs of the product amplified by primers ITS1-F and ITS4-B from DNA isolated from wood blocks to that from the respective pure culture of the fungus. Figure 4 demonstrates that the *TaqI* RFLP profile for any one wood block matches that of the *TagI* digest of the amplon obtained from DNA for each of the following fungal species: *G. trabeum*, *I. lacteus*, *P. placenta*, *R. bicolor*, and *S. lacrimans*. For other fungi, analogous results were also obtained with *AluI*, *HaeIII*, and *TagI-HaeIIII* digests.

**Time course studies.** In order to determine how early we could detect wood decay fungi in wood, we ran two time course studies with the brown-rot fungi *Postia placenta* isolate Mad-698-R and *Gloeophyllum trabeum* isolate Mad-617-R. Soil block jars were set up and inoculated as described in Materials and Methods. For each time course experiment, three replicate jars were inoculated for each combination of time and fungal isolate, as well as for a time-zero uninoculated control and an 8-month-incubated uninoculated control. The first time course used wood blocks cut from radial sections of spruce sapwood, and the second time course used wood blocks cut from longitudinal sections. Wood blocks were harvested after 1, 2, 4, and 8 weeks and after 4 and 8 months of colonization.

Wood decay progressed more rapidly in wood blocks cut from radial versus longitudinal sections of spruce sapwood, as evidenced by the change in percent weight loss of the wood over time (Table 4). A few samples from the first time course and several from the second time course amplified weakly or not at all with primers ITS1-F and ITS4-B; the positive or negative nature of each was confirmed by reamplification of an aliquot of the original PCR reaction. *Gloeophyllum trabeum*
TABLE 3. RFLPs of ITS regions for basidiomycetes

<table>
<thead>
<tr>
<th>Species and isolate(s)</th>
<th>RFLPs (bp) obtained with:</th>
<th>Species and isolate(s)</th>
<th>RFLPs (bp) obtained with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Alu</strong></td>
<td><strong>HaeIII</strong></td>
<td><strong>TaqI</strong></td>
</tr>
<tr>
<td>Brown-rot basidiomycetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coniophora puteana Fp-90099-Sp</td>
<td>500 470 530 247</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fp-90099-Sp</td>
<td>187 242 185 197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>948 908 786 813 970</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fomitopsis pinicola</td>
<td>380 545 310 248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K8Sp</td>
<td>200 127 203 165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gloeophyllum traabeam</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>This lab</td>
<td>155 107 318 215</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mad-617-R</td>
<td>105 72 72 107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 11539</td>
<td>86 72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>781 849 890 814 890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gloeophyllum sepiarum</td>
<td>435 670 500 420</td>
<td></td>
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<tr>
<td>This lab</td>
<td>155 107 318 215</td>
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<tr>
<td>Mad-617-R</td>
<td>105 72 72 107</td>
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<tr>
<td>Total</td>
<td>781 849 890 814 890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucogyrophana pinastri</td>
<td>580 320 400 245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>This lab</td>
<td>300 245 180 127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 170 127 96</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>980 993 943 639 1,050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poria placenta</td>
<td>448 610 530 445</td>
<td></td>
<td></td>
</tr>
<tr>
<td>This lab</td>
<td>240 110 110 110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mad-698-R</td>
<td>100 72 72 72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>781 849 882 739 890</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucogyrophana pinastri</td>
<td>580 320 400 245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>This lab</td>
<td>300 245 180 127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 170 127 96</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>980 993 943 639 1,050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resinicium bicolor</td>
<td>380 800 270 270</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HHB-8850-sp</td>
<td>150 50 245 245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 44175</td>
<td>98 215 215</td>
<td></td>
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</tr>
<tr>
<td>ATCC 64897</td>
<td>69 60 60</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>697 850 790 790 850</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All isolates of a species which have identical RFLP profiles per restriction endonuclease for all enzymes are listed together. If different isolates of a species have different RFLP profiles for at least one of the restriction endonucleases, then those isolates are listed separately.

b Amplicon of the ITS region amplified by primers ITS1-F (specific for higher fungi) and ITS4-B (specific for basidiomycetes).

c This fragment is a triplet, as reflected in the fragment total.

d This fragment is a doublet, as reflected in the fragment total.
and *P. placenta*, both brown-rot basidiomycetes, could be detected in wood by PCR amplification using primers ITS1-F (higher fungus specific) and ITS4-B (basidiomycete specific) after 1 week of colonization, the shortest colonization period used in the study. *G. trabeum* was detected in all replicates of all samples from all colonization times in both cuts of wood and could be detected at a 0.3% mean weight loss of the wood. *P. placenta* was detected in all of the samples from all of the wood blocks cut from radial sections but not in all of those cut from longitudinal sections. After 1 week of colonization (0.5% mean weight loss), *P. placenta* could be detected in only one of three of the wood blocks cut from longitudinal sections, but by 2 weeks (3.0% mean weight loss), it could be detected in three of three blocks; detection was also variable at later time points.

**DISCUSSION**

Although our procedure for DNA isolation and purification may be longer than desired to routinely screen large numbers of wood samples, we thought it best to begin the process of assay development with a method highly likely to yield DNA amplifiable by PCR, since many by-products of wood decay, if present at too high a concentration in the reaction, would inhibit amplification of the DNA template. When setting up PCR reactions with wood samples that have very low DNA concentrations, diluting out the inhibitors could also mean diluting out the DNA past the threshold of detection. So it is better to start with a DNA preparation from which one has removed as much of the inhibitory materials as possible. With the minipreparation procedure described in the Materials and Methods, one person can drill and isolate DNA from 24 wood samples in one work day, observing all the necessary precautions both during drilling of the wood and DNA isolation to prevent any cross-contamination of samples.

Avoiding cross-contamination of samples is critical. Early on, we found that preparation of the wood for DNA isolation is the step at which cross-contamination can most easily occur due to the inherent properties of sawdust. For example, a Wiley mill is not a good choice for grinding samples for PCR work. It is very difficult to clean out all of the crevices in which sawdust can be caught and, even after disassembly, careful brushing out of remaining debris, reassembly, and running through several volumes of clean fungus-free wood, and re-cleaning all the surfaces and crevices with a cotton swab, there is still carryover from one wood decay sample to the next; furthermore, this whole process takes an unacceptably long time. A drill is a much better choice. A rechargeable cordless drill has fewer crevices and surfaces to collect dirt and debris and can be more easily cleaned than a Wiley mill. Drill bits are easy to clean and flame sterilize and are relatively inexpensive, so one can have many of them ready to use. One can prepare wood samples for DNA isolation very rapidly with a drill and at far less risk of sample cross-contamination via sawdust. It is also important to wear gloves and to keep the work area clean, i.e., it is advisable to swab both your gloves and work surface with 70% ethanol to collect any bits of sawdust between drilling each sample. By observing these precautions, as described in detail in Materials and Methods, we have not detected any cross-contamination in samples prepared by drilling and so have adopted this procedure for routine use.

We have developed a DNA-based method to reliably detect brown-rot and white-rot fungi in spruce wood using the published (7) primers ITS1-F (higher fungus specific) and ITS4-B (basidiomycete specific) to amplify the ITS region. We have optimized the reaction conditions for PCR with these primers for template DNA isolated from both pure culture and spruce wood and can detect brown-rot and white-rot fungi from incipient through advanced stages of wood decay. Some late-stage brown-rot samples appeared to have weaker amplification signals than less-decayed samples (data not shown). This could be due to carryover of by-products of wood decay inhibitory to PCR, degradation of DNA in the late stages of wood decay, or a combination of the two. Currently, our assay is only qualitative; more work needs to be done to make it quantitative. The ability to detect decay fungi in other species of wood, preservative-treated wood, and wood composites should also be examined. The differing chemical compositions of both the undecayed and decayed forms of these substrates could introduce new kinds of PCR-inhibitory compounds that may or may not be eliminated or neutralized by our current methodology.

While the primer pair ITS1-F and ITS4-B will detect only basidiomycetes, it will detect any basidiomycete present. For example, if the wood sample were taken from a root, there might be mycorrhizae present that would also be detected. Identity of the basidiomycete present can be achieved by restriction digestion of the PCR product. We could distinguish wood decay basidiomycetes at the species level by comparing the RFLP profiles obtained by *TaqI* digestion of the ITS region amplified by ITS1-F and ITS4-B or by comparing the combination of different RFLP profiles generated from this amplicon.
by a number of different restriction endonucleases. Gardes et al. (8) identified 20 taxa of ectomycorrhizal fungi to the species or species group level from the RFLP profiles of the ITS region amplified by these primers using DNA from mycorrhizae and basidiocarps. Using this method to identify all of their samples, these researchers were able to create a snapshot of the community structure of these ectomycorrhizal fungi both above and below ground in natural stands of *Pinus michurina*.

Although PCR amplification followed by digestion with restriction endonucleases worked fine for samples containing only one fungus, field samples could pose a greater challenge and contain more than one species of wood decay basidiomycete. As the number of different wood decay basidiomycetes contained in a wood sample increases, it would become correspondingly more difficult to identify them all to the species level based on RFLPs. For a more specific and one-step assay in which a number of different species could be identified concurrently in one PCR reaction, Schmidt and Moreth (16) developed species-specific primers based on the DNA sequence of ITSII for the indoor rot fungi *Serpula lacrimans* and *Serpula huitantioides*. We are currently designing species-specific primers for other brown-rot fungi.

We are also looking at DNA sequences of enzymes thought to be involved in wood decay to see if it is possible to design primers that would specifically detect only wood decay basidiomycetes and not other basidiomycetes. It would be useful to be able to detect several wood decay species concurrently in samples where other non-wood-decaying species are likely to occur, e.g., tree roots and forest soils. However, for the purposes of detecting wood decay fungi in branches, tree trunks, harvested timber, or wood in service, where the probability of nondecay basidiomycetes colonizing the internal wood is very low, the assay we developed using the published primers ITS1-F and ITS4-B is potentially very useful. The very lack of specificity which limits the direct identification of the fungus to species can be an advantage in developing a broad-based assay. Previous workers have used PCR amplification in conjunction with RFLP analysis to identify wood decay fungi (15, 23), but their work has focused on identification of the fungi in culture or fungal material present on the wood versus the direct identification of early stages of decay within the wood. By focusing our efforts on the development of an assay that can sample the or fungal material present on the wood versus the direct identification of early stages of decay within the wood. By focusing our efforts on the development of an assay that can sample the

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**Table 4. Time courses of wood decay and detection of brown-rot basidiomycetes**

<table>
<thead>
<tr>
<th>Expt and species</th>
<th>Time</th>
<th>Mean % wt loss of wood ± SD</th>
<th>PCR amplification with primer pair:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Expt 1 (wood blocks cut from radial sections of spruce)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>0</td>
<td>0.3 ± 0.4</td>
<td>−−−</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>8 mo</td>
<td>0.4 ± 0.3</td>
<td>+ + +</td>
</tr>
<tr>
<td><em>Gloeophyllum trabeum</em> Mad-617-R</td>
<td>1 wk</td>
<td>0.7 ± 1.6</td>
<td>+ + +</td>
</tr>
<tr>
<td>2 wk</td>
<td>15.5 ± 4.2</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td>34.1 ± 2.0</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>8 wk</td>
<td>64.7 ± 1.9</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>70.1 ± 1.0</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>8 mo</td>
<td>69.3 ± 2.3</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td><em>Postia placenta</em> Mad-698-R</td>
<td>1 wk</td>
<td>2.6 ± 0.4</td>
<td>+ + +</td>
</tr>
<tr>
<td>2 wk</td>
<td>11.7 ± 2.8</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>4 wk</td>
<td>44.2 ± 9.7</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>8 wk</td>
<td>61.9 ± 0.8</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>66.3 ± 0.5</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>8 mo</td>
<td>67.6 ± 0.6</td>
<td>+ + +</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4 continued**

| *Expt 2 (wood blocks cut from longitudinal sections of spruce)* |      |                           |                                  |
| *Uninoculated control* | 0    | 0.3 ± 0.2                 | −−−                              |
| *Uninoculated control* | 8 mo | 0.03 ± 0.1                | + + +                            |
| *Gloeophyllum trabeum* Mad-617-R | 1 wk  | 0.3 ± 0.4                | + + +                            |
| 2 wk  | 1.9 ± 1.2              | + + +                            |
| 4 wk  | 13.5 ± 2.1             | + + +                            |
| 8 wk  | 34.5 ± 2.7             | + + +                            |
| 4 mo  | 63.7 ± 8.0             | + + +                            |
| 8 mo  | 70.7 ± 6.5             | + + +                            |
| *Postia placenta* Mad-698-R | 1 wk  | 0.5 ± 0.4                | −−−                              |
| 2 wk  | 3.0 ± 0.7              | + + +                            |
| 4 wk  | 14.8 ± 3.1             | + + +                            |
| 8 wk  | 38.7 ± 8.1             | + + +                            |
| 4 mo  | 59.8 ± 3.7             | + + +                            |
| 8 mo  | 60.7 ± 6.1             | + + +                            |

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*Mean of three replicate spruce wood blocks.*

*Primer ITS1-F is specific for higher fungi, primer ITS4 is a universal primer, and primer ITS4-B is specific for basidiomycetes. Each plus or minus sign represents the amplification results for an individual wood block.*
ACKNOWLEDGMENTS

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REFERENCES
