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A multiplex PCR-based method for the detection and early identification of wood rotting fungi in standing trees

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Running headline: molecular diagnosis of decay fungi

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ABSTRACT

Aims: The goal of this research was the development of a PCR-based assay to identify important decay fungi from wood of hardwood tree species in northern temperate regions.

Methods and Results: Eleven taxon-specific primers were designed for PCR amplification of either nuclear or mitochondrial ribosomal DNA regions of *Armillaria* spp., *Ganoderma* spp., *Hericiium* spp., *Hypoxylon thouarsianum* var. *thouarsianum*, *Inonotus/Phellinus*-group, *Laetiporus* spp., *Perenniporia fraxinea*, *Pleurotus* spp., *Schizophyllum* spp., *Stereum* spp. and *Trametes* spp. Multiplex PCR reactions were developed and optimized to detect fungal DNA and identify each taxon with a sensitivity of at least one pg of target DNA in the template. This assay correctly identified agents of decay in 82% of tested wood samples.

Conclusions: The development and optimization of multiplex PCRs allowed for reliable identification of wood rotting fungi directly from wood.

Significance and Impact of the Study: Early detection of wood decay fungi is crucial for assessment of tree stability in urban landscapes. Furthermore, this method may prove useful for prediction of the severity and the evolution of decay in standing trees.

Keywords: ITS, mt SSU, nuc LSU, molecular diagnostic, wood decay.

INTRODUCTION

One of the unique traits of wood decay fungi is their ability to decompose lignified cell walls (Blanchette 1991). Based on their enzymatic capabilities, wood decay fungi can be classified into either brown rot or white rot fungi. While the former can progressively degrade both carbohydrates and lignin, the latter preferentially attack and rapidly depolymerize mostly hemicelluloses and celluloses (Blanchette 1991; Worrall *et al.* 1997). Decay caused by either type of wood rot fungi leads to structural deterioration of the woody tissues. While only a few groups of decay fungi are directly responsible for tree mortality, the loss of wood mechanical strength caused by these organisms is nonetheless inherently linked to hazardous situations including tree wind throws or limb failures. In urban environments, or at the interface between urban and rural environments, tree or limb failures can lead to significant damage of property and/or to tragic injuries.

The detection of potentially hazardous trees is mainly based on Visual Tree Assessment (VTA), an approach consisting of a visual inspection of signs and symptoms linked to the presence of imperfections in the structure of trees (Mattheck and Breloer 1992). While the VTA approach is useful for the diagnosis of decay at an advanced stage, it rarely allows the detection of incipient decay or the identification of the rotting fungi involved. Although modern technologies are improving our ability to detect internal wood decay (Tomikawa *et al.* 1990; Habermehl *et al.* 1999; Müller *et al.* 2001; Sambuelli *et al.* 2003), the identification of the agents responsible for such decay is not always feasible without the presence of fungal fruit bodies, which are only sporadically visible, usually in advanced stages of infection. Because the biology and ecology of different decay fungi is varied, the identification of the taxa involved in each instance is important for prediction of the severity of the fungal infection (Lonsdale 1999). An accurate and early identification of the causal agent is crucial for rapidly progressing decay fungi, because infections caused by such fungi can turn a sound tree into a hazard in a short period of time. Current diagnostic methods can be employed only in late stages of decay, when trees may have already become hazardous. A method for the early identification of decay fungi is not available, but it is necessary for a timely detection of hazardous trees and to establish a preventive hazard management plan for trees in urban environments.

In the absence of unequivocal signs, it is currently necessary to isolate the decay fungus and characterize it by comparison of growth rates, enzymatic capabilities and biochemical and immunological traits (Nobles 1965; Stalpers 1978; Anselmi and Bragaloni 1992; Jellison and Jasalavich 2000; Clausen 2003). The main drawback of culture diagnoses based on cultures is that fungal isolation is time-consuming and, in some cases, impossible. Moreover, the identification of closely related taxa based on the examination of cultural characters is complicated and often impractical. Biochemical and immunological techniques were largely developed for the detection and the identification of common brown rot fungi from lumber (Jellison and Jasalavich 2000; Clausen 2003), but have limited applications in standing trees.

Techniques based on fungal DNA detection are a promising alternative for specific, sensitive and rapid routine diagnoses directly from wood samples. PCR-based methods using nuclear or mitochondrial ribosomal DNA (rDNA) loci have proven valuable for fungal identification at different taxonomic levels (Johannesson and Stenlid 1999). The Internal Transcribed Spacers (ITS I and II) are useful for delimiting species whereas the structural ribosomal genes, such as the nuclear small and large subunit rDNA (nuc SSU and nuc LSU), are more useful for identification at higher taxonomic levels (Bruns and Shefferson 2004). The mitochondrial small subunit (mt SSU) rDNA includes both conserved and variable domains, providing molecular markers to resolve phylogenetic relationships of both higher and lower ranks among taxa (Hong *et al.* 2002). Furthermore, multicopy arrangement and highly conserved priming sites, typical of both nuclear and mitochondrial rDNA, permit amplification from virtually all fungi, even if the starting sample is lacking in quantity or quality (Jasalavich *et al.* 2000). The amplification of rDNA genes with universal fungal primers followed by restriction endonucleases digestion (RFLP) proved to be suitable for taxon-specific identification from cultures of decay fungi (Harrington and Wingfield 1995; Fischer and Wagner 1999; Johannesson and Stenlid 1999; Adair *et al.* 2002). However, when DNA extraction is performed from environmental matrices like wood samples, where more than one fungal taxon could be present, PCR-RFLP is appropriate in its automated version (i.e., terminal-RFLP), or following a preliminary cloning step (Kennedy and Clipson 2003). These methods, as well as other techniques commonly applied to fungal communities fingerprinting, such as direct sequencing of cloned amplicons, denaturing gradient gel electrophoresis

(DGGE), and hybridization of immobilized sequence-specific oligonucleotide probes (SSOP) with amplified rDNA (Vainio and Hantula 2000; Buchan *et al.* 2002; Oh *et al.* 2003) are, in general, costly and time-consuming procedures that are not suitable for routine analysis. Taxon-specific primers have been already used for the identification and the detection of fungal decay directly from wood (Moreth and Schmidt 2001; Bahnweg *et al.* 2002; Gonthier *et al.* 2003) without the necessity of automatic electrophoretic systems, expensive fluorescent dyes, restriction enzyme digests and blotting procedures. As an added benefit, the simultaneous application of taxon-specific primers in multiplex PCR reactions has been reported in clinical and food microbiology to increase the diagnostic capacity of PCR (Elnifro 2000; Corbiere Morot-Bizot 2004) and thus saves time and efforts without compromising the specificity of the analysis.

The objectives of this study were (i) to design taxon-specific primers for wood rotting fungi which can cause mechanical failures in urban trees; (ii) to develop multiplex PCR assays by combining taxon-specific primers with similar annealing temperature and test their sensitivity under simulated natural conditions; (iii) to validate the diagnostic protocol by evaluating the efficiency and the specificity of such method on wood samples collected from trees showing decay symptoms and/or signs.

MATERIALS AND METHODS

Target decay fungi, sampling and culturing

The design and the initial validation of ten genera- and two species-specific diagnostic PCR assays was based on 80 fungal collections. The collections were selected because of their prevalence in the northern temperate areas, their aggressive role in the deterioration of wood in standing trees and their broad range of hosts (Table 1) (Hickman and Perry 1997; Lonsdale 1999; Nicolotti *et al.* 2004; Bernicchia 2005). The taxa selected included *Armillaria* spp., *Ganoderma* spp., *Hericiium* spp., *Hypoxyylon thouarsianum* var. *thouarsianum*, *Inonotus* spp., *Laetiporus* spp., *Perenniporia fraxinea*,

Phellinus spp., *Pleurotus* spp., *Schizophyllum* spp., *Stereum* spp., *Trametes* spp. Collections were either pure cultures or fruit bodies specimens (Table 1).

Cultures were either provided by CABI Bioscience National Centre of Wood Rotting Fungi, CAS Institute of Microbiology, USDA Forest Products lab, USDA Agricultural Service and WRL Swiss Federal Research Institute, or obtained from fruit bodies collected in California and Italy, and identified through analytical keys (Hickman and Perry 1997; Bernicchia 2005) (Table 1). Pure fungal cultures were isolated from the context of the fruit bodies by using potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) medium with 0.2 g l⁻¹ of streptomycin sulphate. Prior to DNA extraction, isolates were sub-cultured in a 2% (w/v) liquid malt extract (ME; AppliChem GmbH, Darmstadt, Germany) medium for approximately two weeks at room temperature. Liquid cultures were harvested by filtration and lyophilized. Alternatively, mycelia were harvested from plugs inoculated on 16 cm² discs of gel-drying film (Promega, Madison, WI USA) incubated at room temperature on PDA plates for two weeks.

DNA extractions, PCR and sequencing conditions

DNA was extracted from lyophilized mycelia or dried fruit bodies specimens using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Hayden *et al.* 2004). Fifty mg of lyophilized tissue was pulverized in a 2-ml screw cap tube by shaking for 30 s at 4.5 m s⁻¹ in a FastPrep FP120 Cell Disrupter (Qbiogene, Irvine, CA USA) with 6.35 or 2.00 mm-diameter glass beads. Pulverized tissue was subjected to two repetitions of freezing (on dry ice for two min) and thawing (at 75°C for two min) in 500 µl of CTAB. DNA was purified using 500 µl of phenol/chloroform/isoamyl alcohol 25/24/1 (v/v/v) followed by further purification using the GeneClean Turbo kit (Qiagen, Valencia, CA, USA). DNA was extracted directly from wood chips using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) after the pulverization step previously described. The concentration of nucleic acid extracts was estimated by comparison of the genomic DNA with the quantified bands of GeneRuler 100 bp DNA ladder (Fermentas GmbH,

St.Leon-Rot, Germany) visualized on a 0.8% (w/v) standard agarose (AppliChem GmbH, Darmstadt, GE) gel.

Ribosomal DNA amplifications of the 5'-end portion of the nuc LSU and of the portion including ITS1 and ITS2 were performed using fungal-specific primers. The nuc LSU with two variable domains (D1, D2) was amplified with primers *ctb6* and *tw13* (White *et al.* 1990; O'Donnell 1993). The ITS1, 5.8S, ITS2 were amplified with primers ITS1-F and ITS4 (White *et al.* 1990; Gardes and Bruns 1993). For taxa lacking suitable portions for taxon-specific primer design in the above regions, a portion of mt SSU was amplified using primers MS1 and MS2 (White *et al.* 1990). PCR was performed in a 25 µl volume containing 1X PCR buffer, 1.5 mmol l⁻¹ of MgCl₂, 0.2 mmol l⁻¹ of dNTPs, 0.5 µmol l⁻¹ of each primer, 0.025 U µl⁻¹ of *Taq* polymerase (Invitrogen Corporation, Carlsbad, CA, USA) and at least one ng of genomic DNA. Thermocycling was conducted as follows: 94°C for one min, 35 cycles of 93°C for 45 s, 58°C for 50 s, 72°C for 45 s with a one s increment every cycle, and 72°C for 10 min. Detection and quantification of PCR products were carried out after electrophoresis on a 1.5% (w/v) standard agarose gel. PCR products were cleaned by using Qia-quick purification kit (Qiagen, Valencia, CA, USA), and cycle-sequenced with a BigDye Terminator v. 3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA) in a reaction mix with 0.8 µmol l⁻¹ of reverse or forward primers. Cycle-sequencing was performed using the following cycling parameters: 25 cycles of 96°C 10 s, 50 °C for five s, 60 °C for four min. Sequenced products were precipitated with 0.8 mol l⁻¹ of sodium acetate at pH 4.8, 3.4 mmol l⁻¹ of EDTA and 25 µl of 100% (v/v) ethanol. Products were washed in 70% (v/v) ethanol. Sequencing reactions were loaded on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequences alignment, taxon-specific primer design for multiplex PCR

The quality of each sequence and the congruity of sense and anti-sense DNA strands were compared with Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Additional sequences available in the European Bioinformatics Institute nucleotide sequences database (EMBL-EBI; <http://www.ebi.ac.uk/>) were used to increase the sample size of target taxa and ensure specificity of

primers by including as many representatives as possible of the target taxon and closely related non-target taxa (Table 1). Domains conserved within a taxon, but variable among taxa were chosen as target regions for taxon-specific primer design. To identify these regions, all sequences from a given taxon were aligned, using CLUSTALW (Thompson *et al.* 1994), with one sequence of a closely related species used for outgroup comparison. Sequences of outgroup taxa were obtained either by sequencing PCR products of pure culture or fruit bodies DNA extracts, as formerly described, or from nucleotide resources of EMBL-EBI (Table 1).

Taxon-specific primers to be used in multiplex PCR were designed on the selected regions using the software PRIMER3 (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3>). Taxon-specific reverse primers were designed according to the following guidelines: (i) lack of complementation with the outgroup sequences especially for base substitutions at the 3'-end and/or for the presence of INDELS (insertions or deletions); (ii) similar melting temperatures; (iii) no formation of secondary structures either for self or primer complementation; (iv) amplified DNA fragments of different lengths for visualization and scoring. The likelihood of secondary structures formation was estimated by the calculation of the change of Gibbs free energy (ΔG) from single strand oligonucleotides to reduce the chance of heteroduplex formation using the software OLIGOANALYZER 3.0 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). Significant matches of taxon-specific primers with sequences derived from other organisms were further investigated by using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST/>).

Taxon-specific primer testing and multiplex PCR development

The specificity and the efficiency of PCR-based DNA amplification using taxon-specific primers were evaluated on DNA extracts obtained from all the above listed fungal collections (Table 1), and on further DNA extracts obtained from other fungal taxa known to be saprophytes or fungi responsible for decay commonly found on wood substrates (Table 4). The annealing temperature of each primer pair was optimized using a thermocycling gradient in order to improve PCR efficiency with the highest stringency. The PCR conditions were the same used for the amplification of rDNA regions

except for the use of a $0.25 \mu\text{mol l}^{-1}$ forward primer fluorescently labelled at the 5'-end with 6-FAM. Fragment analysis was performed in an ABI PRISM 3100 Genetic Analyzer using the GeneScan-500 ROX sizing standard. The fragment intensity and size were estimated with the ABI PRISM 3100 GeneScan analysis software v. 3.7 (Applied Biosystems, Foster City, CA, USA).

Multiplex PCR was performed by combining taxon-specific reverse primers with similar annealing temperatures and variable amplicon sizes. In order to evaluate the PCR efficiency and specificity of the combination of taxon-specific primers, multiplex PCR were performed on DNA extracts from all fungal isolates after optimization of annealing temperature. Multiplex PCR assays were tested by adjusting concentration of MgCl_2 (1.5 mmol l^{-1} and 3.0 mmol l^{-1}) and by adding $0.5 \mu\text{g } \mu\text{l}^{-1}$ of Bovin Serum Albumin (BSA). In the case of simultaneous amplification of DNA fragments of highly different size, the primers corresponding to the smaller amplicons were tested either with the same concentration of the primers flanking the longer amplicons, or with a lower primer concentrations ($0.5 \mu\text{mol l}^{-1}$ or $0.25 \mu\text{mol l}^{-1}$, respectively). Amplified DNA fragments were visualized on a gel containing 1% (w/v) of high resolution MetaPhor (Cambrex Bio Science Inc., Rockland, ME, USA) and 1% (w/v) of standard agarose, after electrophoretic migration (4 V cm^{-1}).

Sensitivity of the multiplex PCR assays

We tested the sensitivity of detection of taxon-specific primers under simulated natural conditions by adding known amounts of pure fungal DNA (10 pg to 10^{-4} pg) to a $100 \mu\text{l}$ DNA extracts solution (concentration about $200 \text{ ng } \mu\text{l}^{-1}$) obtained from 100 mg (dry weight) of both *Quercus agrifolia* and *Platanus hybrida* wood using the QIAamp DNA Stool Mini Kit. Multiplex PCR were performed for each fungal DNA dilution in a $25 \mu\text{l}$ volume containing 1X PCR buffer, 1.5 mmol l^{-1} of MgCl_2 , 0.2 mmol l^{-1} of dNTPs, $0.5 \mu\text{mol l}^{-1}$ of each primer, $0.025 \text{ U } \mu\text{l}^{-1}$ of *Taq* polymerase (Invitrogen Corporation, Carlsbad, CA, USA), $0.5 \mu\text{g } \mu\text{l}^{-1}$ of BSA and $1 \mu\text{l}$ of DNA dilution. The presence and the intensity of the multiplex PCR amplicons were estimated after electrophoretic migration on agarose gels as previously described.

Validation of the multiplex PCR assay on field samples

In order to test the sensitivity and the reliability of the developed assay, 114 samples from symptomatic trees were analysed. Fifty-eight wood samples were collected from *Quercus* spp. in central California. Using a scalpel, small portions of sapwood and/or heartwood with evidence of decay were cut out from recent tree failures, placed in paper envelopes and held in a dessicator at –19°C (Swiecki *et al.* 2005). The remaining 56 samples were collected in northern Italy from broadleaved trees belonging to 19 different species and 15 genera including *Acer*, *Aesculus*, *Cedrus*, *Celtis*, *Fagus*, *Juglans*, *Malus*, *Platanus*, *Populus*, *Prunus*, *Quercus*, *Robinia*, *Sophora*, *Tilia* and *Ulmus*. Trees were selected on the basis of the presence of a visible fruit body (Nicolotti *et al.* 2004). Twenty-seven samples were wood cores extracted using a swedish increment borer, and 29 samples were portions of decayed sapwood and/or heartwood excised as described above near the fruit body. In order to avoid DNA contaminations, the scalpel and the borer were cleaned with a 0.5 % NaClO solution (w/v) between each sample. A visual identification of the decay fungus involved was performed by analysing the macroscopic features of the fruit bodies (Hickman and Perry 1997; Bernicchia 2005).

Approximately 100 mg of fresh wood was lyophilized, homogenized and extracted with the QIAamp DNA Stool Mini Kit following the protocol previously described. Each DNA extract was diluted 100-fold and amplified through the multiplex PCR-based diagnostic assay to detect and identify the decay fungi. The ability of our assay to correctly identify decay fungi known to be present in each sample was interpreted as level of diagnostic efficiency. When the PCR-based assay detected a fungus other than the visible ones, fungal rDNA operons were sequenced directly from wood and the “unknown” fungus was identified using a BLAST search analysis.

RESULTS

Taxon-specific primers

Eleven taxon-specific reverse PCR primers and two forward primers were designed to detect the target taxa (Table 2). A single universal forward primer (25sF; Table 2) was designed in conjunction with reverse primers that amplified *Hericium* spp., *Laetiporus* spp. and *Pleurotus* spp. DNA fragments ranging from 146 bp to 200 bp on the nuc LSU. DNA sequences obtained from PCR amplification of the ITSII were used to design the reverse primers specific for *Armillaria* spp., *H. thouarsianum* var. *thouarsianum*, *P. fraxinea*, *Schizophyllum* spp. and *Stereum* spp. (Table 2). Used in conjunction with the universal forward primer ITS3 (White *et al.* 1990), these reverse primers amplified the target DNA fragments. The *Armillaria* reverse primer did not perfectly match all sequences, but included one substitution in the 3rd bp (*A. gallica*) and 11th bp (*A. nabsnona*) positions of the priming region. Since the mismatches were not positioned in the 3' priming region, no inefficiency in PCR amplification was observed. The size of PCR products amplified for *Stereum* ranged from 231 to 236 bp. The ITS1 was suitable for the design of a *Ganoderma* spp.-specific primer to be used in conjunction with the universal forward primer ITS1-F. Although the primer was not a perfect match for one isolate of *Ganoderma lucidum* from North America (SP26; Table 1) because of two substitutions in the 6th and 14th bp positions and one insertion in the 7th bp position, successful amplification of the expected 226 bp amplicon from all isolates was obtained with a 55°C annealing temperature. A reverse primer for *Trametes* spp. was designed on the mt SSU in combination with MS1 to amplify a diagnostic 220 bp fragment.

It was not possible to design taxon-specific primers for *Inonotus* spp. and for *Phellinus* spp. on nuc LSU, since this region lacks sequences that are homologous within these two taxa and variable between them. Similarly, the ITS region was not suitable for taxon-specific primer design due to the significant sequence divergence within these two taxa. Instead, a reverse primer specific to the *Inonotus/Phellinus*-group and a forward universal primer were designed on the nuc LSU. This primer combination amplified a 111 bp PCR product from all DNA extracts of *Inonotus/Phellinus*-group.(Table 2).

Multiplex PCR development

Primers for taxon-specific priming were combined in three multiplex PCR reactions (Table 3). Since the reverse primer HypoR was highly specific to *H. thouarsianum* var. *thouarsianum*, species with a limited geographic distribution, this primer was excluded from the multiplex reactions. The universal fungal primers ITS1-F and ITS4 were used in one multiplex (M1) to evaluate the efficiency of fungal DNA extraction, avoiding, thus, possible false negatives due to either undetectable DNA quantities or to PCR inhibitory compounds.

PCR efficiency was comparable between simplex and multiplex reactions and PCR amplification was not significantly affected by changing the concentration of MgCl₂ in PCR reactions (Fig. 1). Using the optimized reaction parameters (Table 3), no cross-reactivity with non-target DNA was found and multiplex reactions did not produce any ambiguous or extra amplicons (Table 4).

The amplified fragments in multiplex reactions were easily differentiated and scored according to the size using standard agarose gels (Fig. 2). Our results showed that amplicons of similar molecular size, such as *Pleurotus* spp. and *Laetiporus* spp.-specific DNA fragments in M2 or *Stereum* spp. and *Trametes* spp.-specific DNA fragments in M3, were easily separated and scored (Fig. 2).

Sensitivity of the method under simulated natural conditions

The sensitivity assay performed by spiking DNA obtained from the host with DNA of the target organisms determined the threshold of DNA detection ranged from 10⁻² pg to 1 pg depending on the set of primers (Fig. 3). In some multiplex reactions, lower concentrations (10⁻² to 10⁻¹ pg) of DNA were detected in *P. hybrida* wood than in *Q. agrifolia* wood (1 to 10⁻¹ pg), indicating that for some target taxa the sensitivity of the multiplex PCR assay was affected by the host tissues (Table 5). Nonetheless, sensitivity of the assay was extremely high for both tree species tested in this study.

Validation of the method on field samples

The fungal rDNA of 92% of 114 samples was successfully amplified. In 82% of the samples at least one of our target decay fungi was detected (Fig. 4). In 65% of the samples we found consistent matches between multiplex PCR-based and visual-based diagnosis of decay fungi. Most of the missed target fungi included *Stereum* spp., *Trametes* spp. and *H. thouarsianum* var. *thouarsianum*. (Fig. 4). BLAST search analysis based on fungal DNA directly sequenced from wood confirmed all but one diagnoses not matched by a visible fungal fruit body (Fig.5).

Fungal taxa using universal primers were identified in 93% and 89% of decayed wood and wood core samples, respectively. Target decay fungi were detected in 64% and 67% of decayed wood and wood core samples, respectively.

DISCUSSION

Taxon-specific primers were successfully designed and were confirmed to be highly sensitive and specific for their target organisms. The combination of primer pairs in multiplex PCR reactions allowed for the development of a reliable, rapid and sensitive protocol for the identification of some wood decay fungi known to be responsible for tree failures in the temperate regions of the northern hemisphere. The validation of the assay on field samples collected from decay-affected trees proved the assay can be successfully performed directly from wood DNA extracts, thus bypassing the difficult and laborious step of culturing.

In order to design an assay that would detect selected wood decay agents in the northern temperate regions, we opted to work at the 'generic' rather than at the individual species rank. The resulting assay allows for the reliable detection of decay fungi based on the size determination of 11 PCR amplicons. The need for assays based on individual fungal groups arises from the impossibility to design a single assay encompassing all known species of decay fungi. Furthermore, species-level diagnosis is complicated not only by the large number of species involved, but also by the presence of

intra-specific DNA sequence variations among samples of different provenances. Finally, closely related species within a genus often share similar decay characteristics and thus, are similar in terms of overall effects on the structural integrity and type of decay.

Although the assay here described was mostly based on genus-level specific primers, there were some exceptions. On one hand, species-level primers were developed for two important decay fungi: *H. thouarsianum* var. *thouarsianum* and *P. fraxinea*; on the other hand, *Inonotus* spp. and *Phellinus* spp. were considered as a unique target. Species-level diagnoses for the above two taxa is justified by their important role in wood decay processes. *Hypoxylon thouarsianum* var. *thouarsianum* has been reported to be significantly associated to bole failures of oaks in California (Swiecki *et al.* 2005). Similarly, *P. fraxinea* is one of the most widespread species causing butt rot on several broadleaved trees in northern Italy, as confirmed by a recent study conducted by Nicolotti *et al.* (2004).

A unique primer specific to both *Inonotus* spp. and *Phellinus* spp. was designed out of necessity because of the complex evolutionary history and non-monophyletic nature of the two genera belonging to Hymenochaetales (Wagner and Fischer 2002).

The nuc LSU was targeted for primer design at the 'generic' rank since it is often used for resolving phylogenetic relationships at higher taxonomic level (Moncalvo *et al.* 2000; Binder and Hibbett 2002). One of the two divergent domains amplified (D2) showed sufficient inter-generic heterogeneity for taxon-specific priming of *Hericium* spp., *Laetiporus* spp., and *Pleurotus* spp. Thus, it was possible to develop multiplex PCR reactions with a common forward oligonucleotide and taxon-specific reverse primers for selective amplification of rDNA fragments. The divergent domain D3, not included in the nuc LSU region sequenced in this study but available from EMBL-EBI accessions, was used to design a reverse primer specific to the *Inonotus/Phellinus*-group. Conversely, in four of the target decay fungi (*Armillaria* spp., *Ganoderma* spp., *Schizophyllum* spp. and *Stereum* spp.), the taxon-specific primers designed on the nuc LSU cross-reacted and amplified non-target fungal taxa under highly stringent PCR conditions (data not shown). Reverse primers both at the generic (*Armillaria* spp., *Schizophyllum* spp. and *Stereum* spp.) and at the specific rank (*H. thouarsianum* var. *thouarsianum* and *P. fraxinea*) were designed in the ITSII. A *Ganoderma* spp.-specific primer was

designed in the ITS1, instead. Because both intra-specific variation and heterotype variation in ITS1 and ITS2 among fungal isolates have been proved in other studies (O'Donnell 1992; Kausserud and Schumacher 2001), the investigation on the efficiency in PCR amplification of different isolates within a same taxon was particularly significant for taxon-specific primers designed in such region. When a capillary electrophoretic system was used to more precisely estimate amplicon size obtained by the assay, the size of the amplicons matched the expected size for each target taxon in most instances. Slight size variations though were detected for *Stereum* spp. and *Ganoderma* spp. owing to INDELS (insertions or deletions), such limited variations did not result in any misinterpretation of the multiplex PCR results. It was also observed that the amplicon from North American *G. lucidum* isolates was two bases smaller than European *G. lucidum* isolates. However such level of sequence polymorphisms among the two groups is not surprising, since ITS and nuc LSU sequence analyses have shown that North American *G. lucidum* isolates collected from hardwood and European *G. lucidum* are included in different phyletic groups (Moncalvo *et al.* 1995a). For *Trametes* spp., no suitable priming site could be found on the nuclear rDNA; however, the mt SSU was suitable for the design of a taxon-specific primer.

Although each primer pair can be used individually, we designed and tested optimal conditions for multiplex PCR assays, in order to simultaneously detect multiple taxa in a single reaction. The use of different primers that anneal in the same region will favor priming of the primer with the best sequence match by potentially increasing the reliability of the PCR amplification due to the competition among primers for the same priming site (Garbelotto *et al.* 1996). However, by multiplexing, PCR sensitivity may be reduced. In our multiplex reactions, a detection threshold of 10^{-1} pg of target DNA per one mg wood DNA extracts was found. Reactions targeting *Armillaria* spp., *Inonotus/Phellinus*-group, and *Trametes* spp. had a threshold one order of magnitude higher. Differences in sensitivity among primer pairs may be explained by differences in melt temperatures, GC content, degree of mismatch between primer and priming site, and by the differences including copy numbers between priming sites in the nuclear and the mitochondrial regions.

Differences in detection thresholds between *Q. agrifolia* and *P. hybrida* wood samples are most likely due to the presence of different inhibitory compounds and warrants the need to test the

sensitivity assay on each plant species of interest. Moreover, this method was successfully used on 19 tree species, thus proving its potential for a broad application on a wide range of hosts. It may be noteworthy to highlight the assay was also successfully employed on samples at advanced stages of decay, in spite of the high concentration of inhibitory compounds normally present in such substrates (Jasalavich *et al.* 2000). In 10% of the samples a fungus was detected by the amplification of rDNA fragments with the fungal universal primers ITS1-F and ITS4, but there was no detection of target decay taxa. DNA amplicons from these samples were sequenced and BLAST search analysis indicated that the amplified organisms displayed high DNA sequence homology with anamorphic ascomycetes, such as *Penicillium* spp. and *Phialemonium* spp., or with secondary wood rotting basidiomycetes. The presence of anamorphic ascomycetes, known to be wood saprophytes or soft rot agents, may thus reduce the efficiency of the detection of wood decay basidiomycetes (Adair *et al.* 2002).

The molecular analysis detected wood decay basidiomycetes other than the one producing the fruit body in 35% of samples. The reason for this lack of concordance may be due to misidentification of the fruit body and/or patchy patterns of wood localization of the target decay fungus. The most frequently missed organism was the ascomycete *H. thouarsianum* var. *thouarsianum*. The difficulty in detecting such species from sapwood and heartwood samples of trees displaying typical fruit bodies may be explained by the fact that *Hypoxylon* spp. are reported as colonizers of living bark tissue and soft rot agent of peripheral sapwood (Tainter and Baker 1996), and their presence may be limited in the wood, where *Hypoxylon* may be out competed by more aggressive decay organisms. The presence of aggressive decay fungi in trees with *H. thouarsianum* var. *thouarsianum* fruit bodies were typically identified as those assigned to the *Inonotus/Phellinus*-group and *Stereum* spp. *P. fraxinea* and *Ganoderma* spp. were detected in the same trees both through multiplex-PCR and through a combination of visual and molecular diagnosis. The simultaneous presence of such two taxa has been already observed and deemed to be responsible for hazardous butt and root rots in urban broadleaved trees (Nicolotti, unpublished).

The fact that the success of PCR-based identification from wood cores extracted with a Swedish increment borer was comparable to that obtained from samples taken from decayed wood portions, suggests that this multiplex PCR-based method may be employed by arborists as a

complement to VTA analysis. The method here described can be easily performed in disease diagnostic clinics equipped with basic molecular biology instruments.

The ability to detect and identify the genus of fungal agents during early stages of decay enhances the ability to predict the rate of decay progression both within and between trees (Lonsdale 1999). However, in some cases, closely related species may form associations with different hosts and affect their hosts in substantially different ways. For example, *Ganoderma* includes species, known to be butt and root rot agents (*Ganoderma adspersum*, *G. resinaceum*), as well as species known to be less aggressive stem decay agents, unlikely to affect the structural deterioration of woody tissues (European *G. lucidum*) (Bernicchia 2005). Within the Hymenochaetales, *Pseudoinonotus dryadeus* and *Phellinus torulosus* should be identified at the species level, since they are reported to be active butt and root rot agents of *Quercus* spp. and responsible for rapid and aggressive decay (Lonsdale 1999; Bernicchia 2005). The development of species-level assays for these relevant cases is in progress, and will be the subject of a further publication.

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1 **Table 1** List of fungal taxa collections and sequences used for primer design and/or testing and GenBank accession numbers. The geographic origin and host is included for cultures and fruit bodies obtained in this
 2 study.

Species	ID collection	Host/ Geographic origin	Source*	GenBankAccession numbers		Mt SSU
				Nuc LSU (5')	ITS	
<i>Armillaria cepistipes</i> Velen.†			EMBL-EBI		AJ250053	
<i>Armillaria gallica</i> Marxm. & Romagn.	Napa-141	<i>Quercus agrifolia</i> Née/ USA (CA)	USDA-ARS	AM269818	AM269760	
<i>A. gallica</i>	Napa-144	Unidentified hardwood/ USA (CA)	USDA-ARS	AM269817	AM269759	
<i>A. gallica</i> †			EMBL-EBI	AY213570	AJ250054; AY190247	
<i>Armillaria mellea</i> (Vahl) P. Kumm§	DP26	<i>Aesculus hippocastanum</i> L./ Italy	Di.Va.P.R.A.			
<i>A. mellea</i>	Mar-001	Unidentified hardwood/ USA (CA)	USDA-ARS	AM269820	AM269761	
<i>A. mellea</i>	Mar-016	<i>Quercus agrifolia</i> Née/ USA (CA)	USDA-ARS	AM269819		
<i>A. mellea</i>	T4D	<i>Vitis vinifera</i> L./ Switzerland	WSL	AM269821	AM269762	
<i>A. mellea</i> †			EMBL-EBI		AJ250051; AF163578; AF163584; AF163589	
<i>Armillaria nabsnona</i> Volk & Burds	Men-017	<i>Alnus rubra</i> Bong./ USA (CA)	USDA-ARS	AM269822	AM269763	
<i>A. nabsnona</i>	Men-023	<i>Alnus rubra</i> Bong./ USA (CA)	USDA-ARS	AM269823	AM269764	
<i>A. nabsnona</i> †			EMBL-EBI		AY213574	
<i>Armillaria ostoyae</i> (Romagn.) Herink†			EMBL-EBI	AY207145	AJ250055	
<i>Armillaria tabescens</i> (Scop.) Emel†			EMBL-EBI	AF042593; AY213590	AY213588	
<i>Baeospora myosura</i> (Fr.) Singer**	OKM-3708-Sp	<i>Picea abies</i> (L.) H. Karst./ USA (ME)	USDA-FPL	AM269824	AM269765	
<i>Daedaleopsis confragosa</i> (Bolton) J. Schröt.†**			EMBL-EBI	AF261542		AF039576
<i>Daldinia concentrica</i> (Bolton) Ces. & De Not.†**			EMBL-EBI	U47828	AY616683	
<i>Fomes fasciatus</i> (Sw.) Cooke**	FP-1061048-T	<i>Carya</i> sp./ USA (MS)	USDA-FPL	AM269825	AM269766	
<i>Ganoderma adpersum</i> (Schulz.) Donk	DP60	<i>Aesculus hippocastanum</i> L./ Italy	Di.Va.P.R.A.	AM269828	AM269770	
<i>G. adpersum</i>	DP87	<i>Aesculus hippocastanum</i> L./ Italy	Di.Va.P.R.A.	AM269826	AM269767	
<i>G. adpersum</i>	FGA1	<i>Pterocarya fraxinifolia</i> Wingnut./ Italy	Di.Va.P.R.A.	AM269829	AM269771	
<i>G. adpersum</i> †			EMBL-EBI		AJ006685	
<i>Ganoderma applanatum</i> (Pers.) Pat.†			EMBL-EBI	AJ406526; AY515339	AY787672	
<i>Ganoderma gibbosum</i> (Blume & T. Nees) Pat.†			EMBL-EBI		AY593854	
<i>Ganoderma lucidum</i> (Curtis) P.Karst‡	GITO99	<i>Aesculus hippocastanum</i> L./ Italy	Di.Va.P.R.A.	AM269830	AM269773	
<i>G. lucidum</i> ‡	SP5	<i>Umbellularia californica</i> (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269831		
<i>G. lucidum</i> ‡	SP26	Unknown/ USA (CA)	UC Berkeley		AM269772	
<i>G. lucidum</i> †			EMBL-EBI	AX78776	AF506372; AY456341	
<i>Ganoderma orbiforme</i> (Fr.) Ryvarden†			EMBL-EBI	AX78777		
<i>Ganoderma Pfeifferi</i> Bres.	G2/11	Unknown/ Italy	Di.Va.P.R.A.	AM269832	AM269774	
<i>Ganoderma resinaceum</i> Boudier	DP1	<i>Platanus acerifolia</i> (Ait.) Willd./ Italy	Di.Va.P.R.A.	AM269833	AM269775	
<i>G. resinaceum</i>	FGR1	<i>Fagus sylvatica</i> L./ Italy	Di.Va.P.R.A.	AM269834	AM269776	
<i>G. resinaceum</i>	FGR3	<i>Populus nigra</i> L./ Italy	Di.Va.P.R.A.	AM269835		
<i>G. resinaceum</i>	FGR5	<i>Aesculus hippocastanum</i> L./ Italy	Di.Va.P.R.A.	AM269836	AM269777	
<i>G. resinaceum</i>	G4/13	Unknown/ Italy	Di.Va.P.R.A.	AM269837	AM269778	
<i>Ganoderma</i> sp.‡	SP11	<i>Umbellularia californica</i> (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269827	AM269768	
<i>Ganoderma</i> sp.‡	SP13	<i>Umbellularia californica</i> (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269838		
<i>Ganoderma</i> sp.‡	SP16	<i>Quercus agrifolia</i> Née/ USA (CA)	UC Berkeley		AM269769	
<i>Ganoderma</i> sp.†			EMBL-EBI	AF255149		
<i>Ganoderma tsugae</i> Murril†			EMBL-EBI	AX78778; AY684163		
<i>Gloeocystidiellum porosum</i> (Berk. & M.A. Curtis) Donk†**			EMBL-EBI	AF310101		
<i>G. porosum</i> †**			EMBL-EBI		AY048881	
<i>Hericium coralloides</i> (Scop.) Pers.	HHB-9082-Sp	<i>Acer saccharum</i> Marsh/ USA (MI)	USDA-FPL	AM269840		
<i>Hericium erinaceum</i> (Bull.) Pers.	654	Unknown/ Czech Republic	CAS-IM	AM269839	AM269779	
<i>H. erinaceum</i>	JHO-62-149	<i>Acer saccharum</i> Marsh/ USA (MI)	USDA-FPL	AM269841		
<i>H. erinaceum</i> †			EMBL-EBI	AJ406493	AY534601	
<i>Hohenbuehelia tristis</i> G. Stev.†**			EMBL-EBI	AF135171		

<i>Hypoxyylon thouarsianum</i> var. <i>thouarsianum</i> (Lév.) Lloyd‡	SP106	<i>Lithocarpus densiflorus</i> (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley	AM269842	AM269780
<i>H. thouarsianum</i> var. <i>thouarsianum</i> ‡,§	SP8	<i>Lithocarpus densiflorus</i> (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley		
<i>Inonotus andersonii</i> (Ellis & Everh.) Nikol.§	557	Unknown/ Czech Republic	CAS-IM		
<i>I. andersonii</i>	L(61)11-14-C	<i>Quercus velutina</i> Lam./ USA (OH)	USDA-FPL		AM269781
<i>I. andersonii</i> ‡	SP23	<i>Quercus wislizeni</i> A. DC./ USA (OH)	Phyt. Res.	AM269843	
<i>I. andersonii</i>	T1545	Unknown/ USA (OH)	USDA-FPL	AM269844	
<i>I. andersonii</i> †			EMBL-EBI	AY059041	AY558599
<i>Inonotus dryophilus</i> (Berk.) Murril	703	Unknown/ Czech Republic	CAS-IM	AM269847	
<i>I. dryophilus</i>	L(61)5-20-A	<i>Quercus prinus</i> L./ USA (OH)	USDA-FPL	AM269846	AM269783
<i>I. dryophilus</i> ‡	SP25	<i>Quercus agrifolia</i> Née/ USA (CA)	Phyt. Res.	AM269845	AM269782
<i>I. dryophilus</i> †			EMBL-EBI	AF311012	
<i>Inonotus hispidus</i> (Bull.) P. Karst. ‡ §	FIH2	<i>Malus</i> sp./ Italy	Di.Va.P.R.A.		
<i>I. hispidus</i>	FP-106082-T	<i>Quercus phellos</i> L./ USA (MS)	USDA-FPL	AM269848	AM269784
<i>I. hispidus</i> †			EMBL-EBI	AF311014	
<i>Inonotus radiatus</i> (Sowerby) P. Karst. §	276	Unknown/ Czech Republic	CAS-IM		
<i>I. radiatus</i> †			EMBL-EBI	AF237732	
<i>Inonotus rheades</i> (Pers.) Bondartsev & Singer†			EMBL-EBI	AF311019	
<i>Inonotus tamaricis</i> (Pat.) Maire†			EMBL-EBI	AF311021	
<i>Laetiporus sulphureus</i> (Bull.) Murril§	FLS1	<i>Prunus cerasus</i> L./ Italy	Di.Va.P.R.A.		
<i>L. sulphureus</i>	FLS2	<i>Prunus cerasus</i> L./ Italy	Di.Va.P.R.A.	AM269849	
<i>L. sulphureus</i>	FP-101671-T	<i>Quercus</i> sp./ USA (WI)	USDA-FPL	AM269851	AM269786
<i>L. sulphureus</i> ‡	SP35	Unknown/ USA (CA)	USDA-FPL	AM269850	AM269785
<i>L. sulphureus</i> †				AY218414; AY684162	AF229196; AY835668
<i>Laxitextum bicolor</i> (Pers.) Lentz**	NO-7316-Sp	<i>Salix</i> sp./ USA (LA)	USDA-FPL		AM269787
<i>L. bicolor</i> †**			EMBL-EBI	AF287871	
<i>Perenniporia fraxinea</i> (Bull.) Ryvarden	DP6	<i>Fagus sylvatica</i> L./ Italy	Di.Va.P.R.A.	AM269852	AM269788
<i>P. fraxinea</i>	DP83	<i>Robinia pseudoacacia</i> L./ Italy	Di.Va.P.R.A.	AM269853	AM269789
<i>P. fraxinea</i>	FPF1	<i>Platanus hybrida</i> Brot./ Italy	Di.Va.P.R.A.	AM269854	AM269790
<i>P. fraxinea</i>	FPF2	<i>Robinia pseudoacacia</i> L./ Italy	Di.Va.P.R.A.	AM269855	AM269791
<i>P. fraxinea</i>	FPF3	<i>Robinia pseudoacacia</i> L./ Italy	Di.Va.P.R.A.		AM269792
<i>P. fraxinea</i>	FPF5	<i>Ulmus pumila</i> L./ Italy	Di.Va.P.R.A.	AM269856	AM269793
<i>P. fraxinea</i>	FPF6	<i>Robinia pseudoacacia</i> L./ Italy	Di.Va.P.R.A.		AM269794
<i>Perenniporia ochroleuca</i> (Berk.) Ryvarden†**			EMBL-EBI	AY515330	
<i>Perenniporia subacida</i> (Peck) Donk†**			EMBL-EBI		AY089739
<i>Phaeolus schweinitzii</i> (Fr.) Pat.†**	SP39	<i>Picea sitchensis</i> (Bong.) Carr./ USA (CA)	UC Berkeley	AM269857	
<i>Phellinus gilvus</i> (Schwein.) Pat.‡	SP18	<i>Quercus agrifolia</i> Née/ USA (CA)	UC Berkeley	AM269858	AM269795
<i>P. gilvus</i> ‡	SP20	<i>Quercus agrifolia</i> Née/ USA (CA)	UC Berkeley	AM269859	AM269796
<i>P. gilvus</i> †			EMBL-EBI	AY059025	AY089739
<i>Phellinus igniarius</i> var. <i>cinereus</i> L. (Qué.)	575	Unknown/ Czech Republic	CAS-IM	AM269860	AM269797
<i>P. igniarius</i> var. <i>cinereus</i> †			EMBL-EBI	AF287884	AF110991
<i>Phellinus pini</i> (Brot.) Bondartsev & Singer	578	Unknown/ Czech Republic	CAS-IM	AM269861	AM269798
<i>Phellinus punctatus</i> (Fr.) Pilát	262	Unknown/ Czech Republic	CAS-IM	AM269863	AM269800
<i>P. punctatus</i>	DP25	<i>Tilia x vulgaris</i> Heyne/ Italy	Di.Va.P.R.A.	AM269862	AM269799
<i>P. punctatus</i> †			EMBL-EBI	AF311007	
<i>Phellinus robustus</i> P. Karst (Bourdot & Galzin)	587	Unknown/ Czech Republic	CAS-IM		AM269802
<i>P. robustus</i>	RLG-9585-T	<i>Salix</i> sp./ USA (MN)	USDA-FPL	AM269864	AM269801
<i>P. robustus</i> †			EMBL-EBI	AF311008	
<i>Phellinus torulosus</i> Pers. (Bourdot & Galzin)	759	Unknown/ Czech Republic	CAS-IM	AM269865	AM269803
<i>P. torulosus</i> †			EMBL-EBI	AF311041	
<i>Phellinus tremulae</i> (Bondartsev) Bondartsev & Borissov	243	Unknown/ Czech Republic	CAS-IM		AM269804
<i>P. tremulae</i> †			EMBL-EBI	AF311042	
<i>Phellinus tuberculosus</i> (Baumg.) Niemelä	265	Unknown/ Czech Republic	CAS-IM	AM269866	AM269805
<i>P. tuberculosus</i>	DP40	<i>Prunus pissardi</i> Carrière/ Italy	Di.Va.P.R.A.		AM269806
<i>P. tuberculosus</i> †			EMBL-EBI	AF311043	

<i>Pleurotus ostreatus</i> (Jacq.) P. Kumm.	2470	Unknown/ Italy	Di.Va.P.R.A.	AM269868	
<i>P. ostreatus</i> §	FP-101798-Sp	<i>Populus tremuloides</i> Michx./ USA (WI)	USDA-FPL		
<i>P. ostreatus</i> ‡§	SP29	<i>Alnus rubra</i> Bong./ USA (CA)	UC Berkeley		
<i>P. ostreatus</i> ‡	SP37	<i>Alnus rubra</i> Bong./ USA (CA)	UC Berkeley	AM269867	
<i>P. ostreatus</i> †			EMBL-EBI	AY645052	AY636055
<i>Pleurotus populinus</i> Hilber & Miller†			EMBL-EBI		U04080
<i>Pleurotus pulmonarius</i> (Fr.) Quéf.**	JPL-531-Sp	<i>Abies lasiocarpa</i> (Hook.) Nutt./ USA (AZ)	USDA-FPL	AM269869	AM269807
<i>P. pulmonarius</i> †			EMBL-EBI	AY450349	AY368669
<i>Pseudoinonotus dryadeus</i> (Pers.:Fr.) Murr.	FP-105836-4	<i>Quercus alba</i> L./ USA (OH)	USDA-FPL	AM269870	AM269808
<i>P. dryadeus</i> †			EMBL-EBI	AF311011	
<i>Pycnoporus sanguineus</i> (L.) Murril†**			EMBL-EBI		AF363759
<i>Schizophyllum commune</i> Fr.	DP61	<i>Acer pseudoplatanus</i> L./ Italy	Di.Va.P.R.A.	AM269871	AM269809
<i>S. commune</i>	Jacquot	<i>Carya</i> sp./ France	USDA-FPL	AM269872	
<i>S. commune</i> †			EMBL-EBI	AJ406555	AF249379; AF249386; AY573544; AY636062
<i>Schizophyllum radiatum</i> (Sw.) Fr.	CBS-301.32	Unknown/ USA (CA)	USDA-FPL	AM269873	
<i>S. radiatum</i> †			EMBL-EBI	AY571023	
<i>Stereum hirsutum</i> (Willd.) Pers.	DP49	<i>Prunus pissardi</i> Carrière/ Italy	Di.Va.P.R.A.	AM269874	AM269810
<i>S. hirsutum</i> ‡	SP9	<i>Lithocarpus densiflorus</i> (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley	AM269875	
<i>S. hirsutum</i> †			EMBL-EBI	AY039330	AY854063
<i>Stereum rugosum</i> Pers.	388020	Unknown/ UK	CABI-NCWRF	AM269876	AM269811
<i>Stereum sanguinolentum</i> (Alb. & Schwein.)†			EMBL-EBI		AY618670; AF533962
<i>Stereum</i> sp.†			EMBL-EBI	AF506483	AY207328
<i>Stereum subtomentosum</i> Pouzar†			EMBL-EBI		AF506482
<i>Trametes cervina</i> (Schwein.) Bres. §	FP105490-Sp	<i>Quercus</i> sp./ USA (MD)	UC Berkeley		
<i>Trametes gibbosa</i> (Pers.) Fr.†			EMBL-EBI	AF291371	
<i>Trametes hirsuta</i> (Wulfen) Pilát†			EMBL-EBI		AY534110; AY787683
<i>Trametes pubescens</i> (Schumach.) Pilát†			EMBL-EBI	AY515341	AF042154
<i>Trametes suaveolens</i> (L.) Fr.†			EMBL-EBI	AF261537	U27079
<i>Trametes versicolor</i> L. Loyd	2473	Unknown/ Switzerland	Di.Va.P.R.A.	AM269878	AM269814
<i>T. versicolor</i> §	DP37	<i>Platanus acerifolia</i> Willd./ Italy	Di.Va.P.R.A.		AM269880
<i>T. versicolor</i>	Mad-697	<i>Fagus grandifolia</i> Ehrh./ USA (VT)	USDA-FPL		AM269813
<i>T. versicolor</i> ‡§	SP27	<i>Picea sitchensis</i> (Bong.) Carr./ USA (CA)	UC Berkeley		
<i>T. versicolor</i> ‡	SP33	<i>Alnus rubra</i> Bong./ USA (CA)	EMBL-EBI	AM269877	AM269812
<i>T. versicolor</i> †			EMBL-EBI	AY684159	AY636060
<i>Trametes villosa</i> (Sw.) Kreisel†			EMBL-EBI		AF042324; U27080
<i>Trametes zonatella</i> Ryvarden§	FTZ1	<i>Quercus robur</i> L./ Italy	Di.Va.P.R.A.		AF042325
<i>Trichaptum abietinum</i> (Dicks.) Ryvarden†**			EMBL-EBI	AY059063	
<i>Trichaptum biforme</i> (Fr.) Ryvarden**	L-15822-Sp	Unknown/ USA (NY)	USDA-FPL		AM269815
<i>Trichaptum fuscoviolaceum</i> (Ehrenb.) Ryvarden**	L-15378-Sp	<i>Abies balsamea</i> (L.) P. Mill./ USA (NY)	USDA-FPL	AM269879	AM269816

3

4 *CABI-NCWRF National Collection of Wood Rotting Fungi, Garston (UK); CAS-IM Academy of Sciences of Czech Republic Institute of Microbiology Department of Experimental Mycology, Videnska (Czech
5 Republic); Di.Va.P.R.A. Department of Exploitation and Protection of the Agricultural and Forestry resources, University of Torino, Grugliasco (Italy); EMBL-EBI European Bioinformatics Institute nucleotide
6 sequences database; Phyt. Res. Phytosphere Research, Vacaville, CA; UC Berkeley, Department of Environmental Science, Policy, and Management Berkeley (USA); USDA-ARS, United States Department of
7 Agriculture, Agricultural Research Service, Davis, CA (collection from Kendra Baumgartner); USDA-FPL United States Department of Agriculture Forest Products Lab, Madison WI (USA); WSL Swiss Federal
8 Institute for Forest, Snow and Landscape Research, Birmensdorf (CH).

9 †Species not included in the fungal collections but whose rDNA sequences were used for primer design.

10 ‡Collections from fruit bodies (no pure fungal cultures obtained).

11 §Collections exclusively used for taxon-specific primers testing.

12 **Species used as outgroups for target taxon. *B. myosura* for *Armillaria* spp.; *D. confragosa* and *P. sanguineus* for *Trametes* spp.; *D. concentrica* for *H. thouarsianum* var. *thouarsianum*; *F. fasciatus* for *Ganoderma*
13 spp.; *G. porosum* for *Stereum* spp.; *H. tristis* for *Pleurotus* spp.; *L. bicolor* for *Hericium* spp.; *P. ochroleuca* and *P. subacida* for *P. fraxinea*; *P. schweinitzii* for *Laetiporus* spp.; *Pleurotus pulmonarius* for *Schizophyllum*
14 spp.; *T. biforme* and *T. fuscoviolaceum* for *Inonotus/Phellinus*-group

15

16 **Table 2** Primer sequences, priming regions, melt temperatures, and target amplicon size for taxon-specific PCR of wood rot fungi.

Primer name	Nucleotide sequence (5'-3')	Tm*	Gene Region	Use in taxon-specific PCR	PCR product size (bp)	Identified taxon	Reference
Armi2R	AAACCCCATATCCAATCC	56°C	ITS II	Reverse primer with the forward ITS3	185	<i>Armillaria</i> spp.	This study
Gano2R	TATAGAGTTTGATGATAACGCA	55°C	ITS I	Reverse primer with the forward ITS1-F	226-228	<i>Ganoderma</i> spp.	This study
Heri2R	CAGCCCTTGTCGGCAGT	61°C	nuc LSU	Reverse primer with the forward 25sF	200	<i>Hericium</i> spp.	This study
Hyme2R	TGCDCCCCTYGCGGAG	60/64°C	nuc LSU	Reverse primer with the forward F115	111	<i>Inonotus/Phellinus</i> -group	This study
HypoR	GCTACGCTTAGGGATGCTA	60°C	ITS II	Reverse primer with the forward ITS3	219	<i>H. thouarsianum</i> var. <i>thouarsianum</i>	This study
LaetR	CCGAGCAAACGAATGCAA	54°C	nuc LSU	Reverse primer with the forward 25sF	146	<i>Laetiporus</i> spp.	This study
PerR	ATCTGCAAAGACCGTAAGGT	60°C	ITS II	Reverse primer with the forward ITS3	152	<i>P. fraxinea</i> .	This study
Pleu2R	AACCAGGAAGTACGCCTCAC	60°C	nuc LSU	Reverse primer with the forward 25sF	158	<i>Pleurotus</i> spp.	This study
Schi2R	CTCCAGCAGACCTCCACTTC	63°C	ITS II	Reverse primer with the forward ITS3	190	<i>Schizophyllum</i> spp.	This study
Ste2R	GTCGCAACAAGACGCACTAA	58°C	ITS II	Reverse primer with the forward ITS3	231-236	<i>Stereum</i> spp.	This study
TraR	TTCATAGTCTTATGGAACCGC	58°C	mt SSU	Reverse primer with the forward MS1	220	<i>Trametes</i> spp.	This study
25sF	TGGCGAGAGACCGATAGC	58°C	nuc LSU	Forward			This study
F115	TAAGCGACCCGTCTTGAAAC	58°C	nuc LSU	Forward			This study
ITS1-F	CTTGGTCATTAGAGGAAGTAA	55°C	nuc LSU	Forward			Gardes and Bruns 1993
ITS3	GCATCGATGAAGAACGCAGC	60°C	5.8S	Forward			White <i>et al.</i> 1990
ITS4	TCCTCCGCTTATTGATATGC	56°C	nuc LSU	Reverse			White <i>et al.</i> 1990
MS1	CAGCAGTCAAGAATATTAGTCAATG	61°C	mt SSU	Forward			White <i>et al.</i> 1990

17

18 *Tm indicate the salt-adjusted melt temperature (see Howley *et al.* 1979).

19

20 **Table 3** Multiplex PCR primers combination, conditions and diagnostic purposes.

Multiplex PCR name	Primers combination		Diagnostic purpose	Cycling Parameters
	Forward	Reverse		
M1	ITS1-F	ITS4	Fungi	5 min denaturation at 95°C; 35 cycles of: 45s at 95°C, 45s at 55°C, 45s at 72°C; 10 min final extension at 72°C
		Gano2R	<i>Ganoderma</i> spp.	
	F115	Hyme2R	<i>Inonotus/Phellinus</i> -group	
M2	ITS3	Armi2R	<i>Armillaria</i> spp.	5 min denaturation at 95°C; 35 cycles of: 45s at 95°C, 45s at 60°C, 45s at 72°C; 10 min final extension at 72°C
		LaetR	<i>Laetiporus</i> spp.	
	25sF	Pleu2R	<i>Pleurotus</i> spp.	
		Heri2R	<i>Hericium</i> spp.	
M3	ITS3	PerR	<i>P. fraxinea</i>	5 min denaturation at 95°C; 35 cycles of: 45s at 95°C, 45s at 63°C, 45s at 72°C; 10 min final extension at 72°C
		Schi2R	<i>Schizophyllum</i> spp.	
		Ste2R	<i>Stereum</i> spp.	
	MS1	TraR	<i>Trametes</i> spp.	

21
22
23 **Table 4** Summary of the diagnostic efficiency assays of the M1, M2, M3 reactions on DNA extracts from target fungal taxa and from other
24 common wood rotting or saprophytic fungi.

Species	ID collection	M1*	M2	M3	Species	ID collection	M1*	M2	M3
<i>Agrocybe aegerita</i> (V. Brig.) Singer†	DP12	-	-	-	<i>Omphalotus olearius</i> (DC.) Singer†	HHB-7441-Sp	-	-	-
<i>Armillaria gallica</i>	Napa-144;-141	-	185	-	<i>Omphalotus olivascens</i> H.E. Bigelow, O.K. Mill. & Thiers†	KPC-CA-1	-	-	-
<i>Armillaria mellea</i>	DP26; Mar-001;-016; T4D	-	185	-	<i>Oxyporus corticola</i> (Fr.) Ryvarden†	RLG-4894-Sp	-	-	-
<i>Armillaria nabsnona</i>	Men-017;-023	-	185	-	<i>Oxyporus latemarginatus</i> (Durieu & Mont.) Donk†	FP-101894-Sp	-	-	-
<i>Baeospora myosura</i> †	OKM-3708-Sp	-	-	-	<i>Oxyporus</i> sp.†	SP32	-	-	-
<i>Bjerkandera adusta</i> (Willd.) P. Karst.†	DP66	-	-	-	<i>Perenniporia fraxinea</i>	DP6; 83; FPF1; 2; 3; 5; 6	-	-	152
<i>Crepidotus mollis</i> (Schaeff.) Staude†	SP36	-	-	-	<i>Phaeolus schweinitzii</i> †	SP39	-	-	-
<i>Fistulina hepatica</i> (Schaeff.) With.†	FP-103444-T	-	-	-	<i>Phellinus gilvus</i>	SP18; 20	111	-	-
<i>Fomitopsis cajanderi</i> (P. Karst.) Kotl. & Pouzar†	SP34	-	-	-	<i>Phellinus igniarius</i> var. <i>cinereus</i>	575	111	-	-
<i>Fomes fasciatus</i> †	FP-1061048-T	-	-	-	<i>Phellinus pini</i>	578	111	-	-
<i>Fomitopsis pinicola</i> (Sw.) P. Karst.†	SP6	-	-	-	<i>Phellinus punctatus</i>	262; DP25	111	-	-
<i>Fomitopsis rosea</i> (Alb. & Schwein.) P. Karst.†	SP7	-	-	-	<i>Phellinus robustus</i>	587; RLG-9585-T	111	-	-
<i>Ganoderma adpersum</i>	DP87; DP60; FGA1	228	-	-	<i>Phellinus torulosus</i>	759	111	-	-
<i>Ganoderma lucidum</i>	GITO99	228	-	-	<i>Phellinus tremulae</i>	243	111	-	-
<i>Ganoderma lucidum</i>	SP26	226	-	-	<i>Phellinus tuberculosus</i>	265; DP40	111	-	-
<i>Ganoderma pfeifferi</i>	G2/11	228	-	-	<i>Pleurotus ostreatus</i>	2470; FP-101798-Sp; SP29; 37	-	158	-
<i>Ganoderma resinaceum</i>	DP1; FGR1; 3; 5; G4/13	228	-	-	<i>Pleurotus pulmonarius</i>	JPL-531-Sp	-	158	-
<i>Ganoderma</i> sp.	SP11; SP13; SP16	228	-	-	<i>Pseudoinonotus dryadeus</i>	FP-105836-4	111	-	-
<i>Hericium coralloides</i>	HHB-9082-Sp	-	200	-	<i>Ramaria</i> sp.†	SP31	-	-	-
<i>Hericium erinaceum</i>	654; JHO-62-149	-	200	-	<i>Schizophyllum commune</i>	DP61; Jacquirot	-	-	190
<i>Hohenbuehelia atrocoerulea</i> (Fr.) Singer†	FP-102477-Sp	-	-	-	<i>Schizophyllum radiatum</i>	CBS-301.32	-	-	190
<i>Hypoxyylon thouarsianum</i> var. <i>thouarsianum</i>	SP8; SP106	-	-	-	<i>Stereum hirsutum</i>	DP49; SP9	-	-	236
<i>Inonotus andersonii</i>	557; L(61)11-14-C; SP23	111	-	-	<i>Stereum rugosum</i>	388020	-	-	231
<i>Inonotus dryophilus</i>	703; L(61)5-20-A; SP25	111	-	-	<i>Trametes cervina</i>	FP105490-Sp	-	-	220
<i>Inonotus hispidus</i>	FIH2; FP-106082-T	111	-	-	<i>Trametes versicolor</i>	2473; DP37; Mad-697; SP27;33	-	-	220
<i>Inonotus radiatus</i>	276	111	-	-	<i>Trametes zonatella</i>	FTZ1	-	-	220
<i>Laetiporus sulphureus</i>	FP-101671-T; FLS1; 2; SP35	-	146	-	<i>Trichaptum bifforme</i> †	L-15822-Sp	-	-	-
<i>Laxitextum bicolor</i> †	NO-7316-Sp	-	-	-	<i>Trichaptum fuscoviolaceum</i> †	L-15378-Sp	-	-	-
<i>Lentinula edodes</i> (Berk.) Pegler†	Ra-3-2E	-	-	-	<i>Tricholoma robustum</i> (Alb. & Schwein.) Ricken†	DP65	-	-	-
<i>Lenzites betulina</i> (L.) Fr. †	SP4	-	-	-					

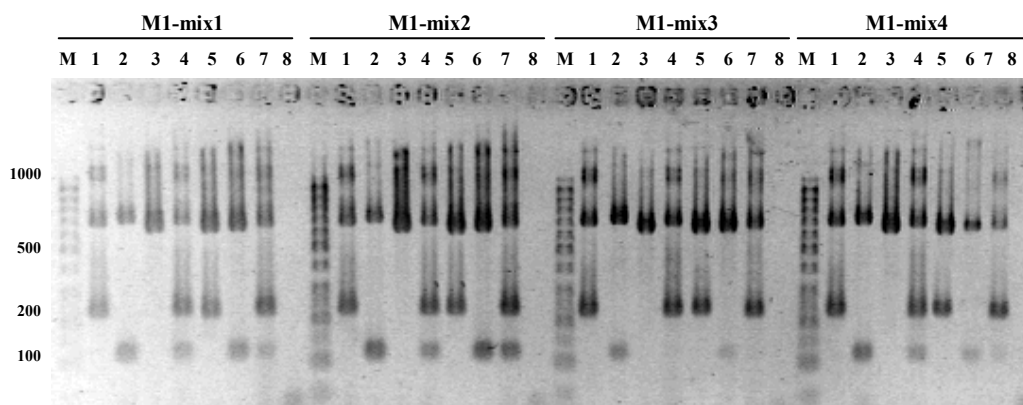
25
26 *All samples, after M1, were successfully amplified with the fungal-specific primers ITS1-F and ITS4. Values indicate the size (bp) of the
27 taxon-specific amplified fragment. No amplification product with any taxon-specific primers is indicated with -.

28 †Fungal collections including other common wood rotting or saprophytic species used to test the specificity of the multiplex PCR assays.

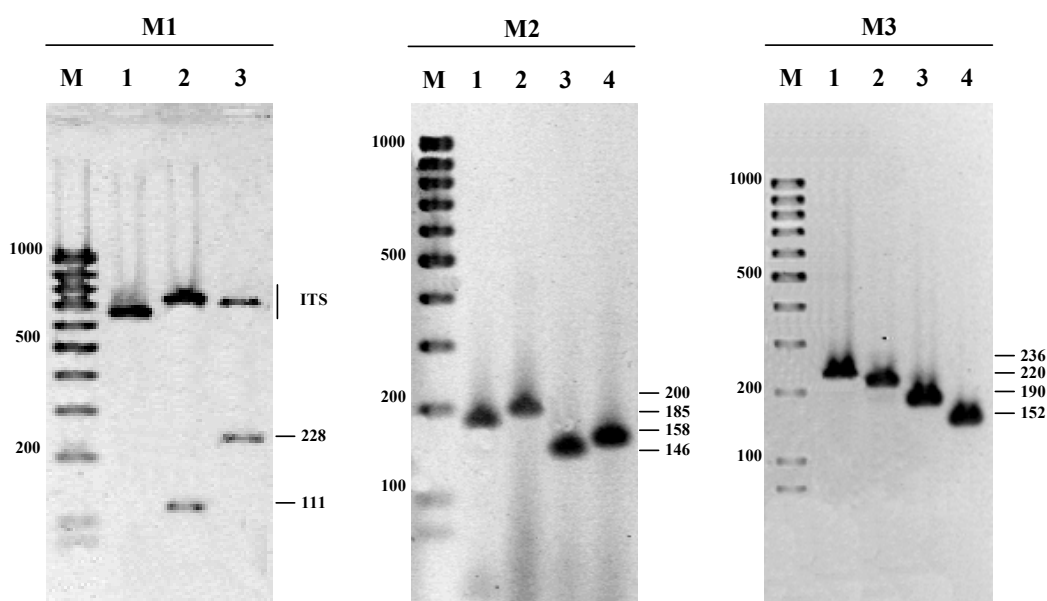
30 **Table 5** Minimum DNA amount threshold detectable by multiplex PCR 1, 2, 3 and by *H. thouarsianum*-specific PCR.

PCR protocol tested	Species and related specimen used	DNA amount threshold*	
		<i>Quercus agrifolia</i>	<i>Platanus hybrida</i>
M1	<i>Ganoderma resinaceum</i> -FGR1	10 ⁻¹ pg	10 ⁻² pg
	<i>Phellinus tuberculatus</i> -FPT1	1 pg	1 pg
	<i>Inonotus hispidus</i> -FIH2	1 pg	1 pg
M2	<i>Hericium flagellum</i> -654	10 ⁻¹ pg	10 ⁻² pg
	<i>Pleurotus ostreatus</i> -2470	10 ⁻¹ pg	10 ⁻¹ pg
	<i>Laetiporus sulphureus</i> -FLS2	10 ⁻¹ pg	10 ⁻¹ pg
	<i>Armillaria</i> sp.-T4D	1 pg	1 pg
M3	<i>Stereum hirsutum</i> -DP49	10 ⁻¹ pg	10 ⁻¹ pg
	<i>Trametes versicolor</i> -2473	1 pg	1 pg
	<i>Schizophyllum commune</i> -DP61	10 ⁻¹ pg	10 ⁻¹ pg
	<i>Perenniporia fraxinea</i> -FPF5	10 ⁻¹ pg	10 ⁻¹ pg
	<i>H. thouarsianum</i> -specific PCR <i>Hypoxyylon thouarsianum</i> var. <i>thouarsianum</i> -P1	10 ⁻¹ pg	10 ⁻¹ pg

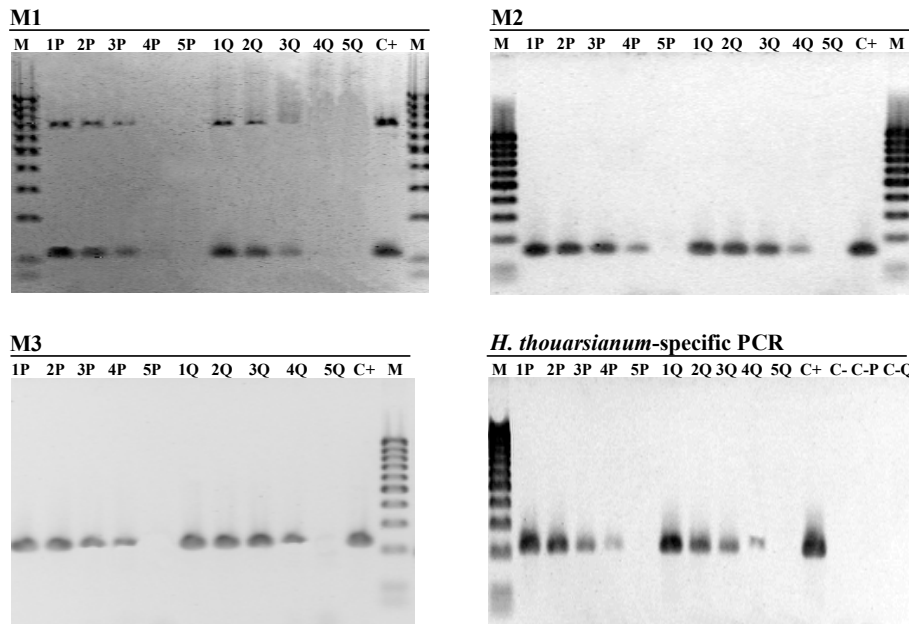
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 32 *The reported values indicate the minimum amount of target fungal DNA in wood DNA solution obtained from one mg of either *Q. agrifolia*
 33 or *P. hybrida* wood (details in the text).



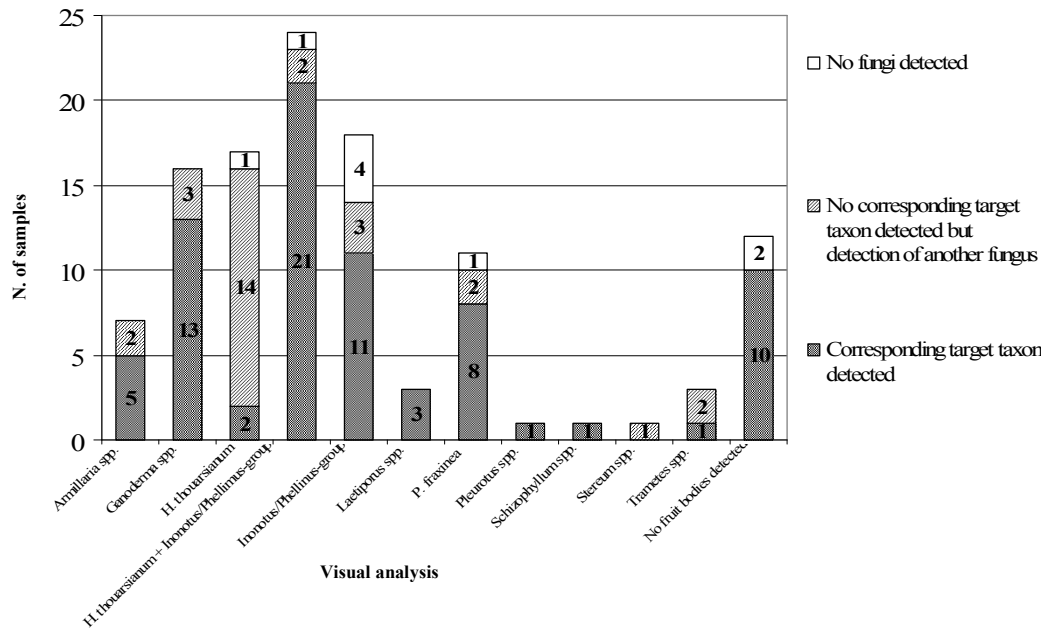
34
 35 **Fig. 1** M1 PCR conditions assay. Comparison of PCR outcomes by using different primers and MgCl₂ concentrations. In the first and second
 36 reactions (M1-mix1 and M1-mix2), each primer is 0.5 μmol l⁻¹, whereas in the mix 3 and 4 primers ITS1f and ITS4 are 0.5 μmol l⁻¹, primers
 37 Gano2R, Hyme2R and F115 are 0.25 μmol l⁻¹. MgCl₂ concentration in mix1 and mix3 was 1.5 mmol l⁻¹; in the mix2 and 4 the MgCl₂
 38 concentration was 3.0 mmol l⁻¹. The PCR assay was performed on 0.5 ng of *Ganoderma resinaceum* (lane 1), *Phellinus tuberculatus* (lane 2)
 39 and *Trametes versicolor* (lane 3) genomic DNA as well as on artificial mix containing 0.5 ng of *G. resinaceum* and *P. tuberculatus* (lane 4),
 40 of *G. resinaceum* and *T. versicolor* (lane 5), of *P. tuberculatus* and *T. versicolor* (lane 6) and of *G. resinaceum*, *P. tuberculatus* and *T.*
 41 *versicolor* (lane 7) genomic DNA. The negative controls of PCR reactions are loaded in the lane 8. M= Molecular weight marker GeneRuler
 42 50 bp DNA ladder (DNA Ladder, Fermentas GmbH, St.Leon-Rot, Germany).
 43



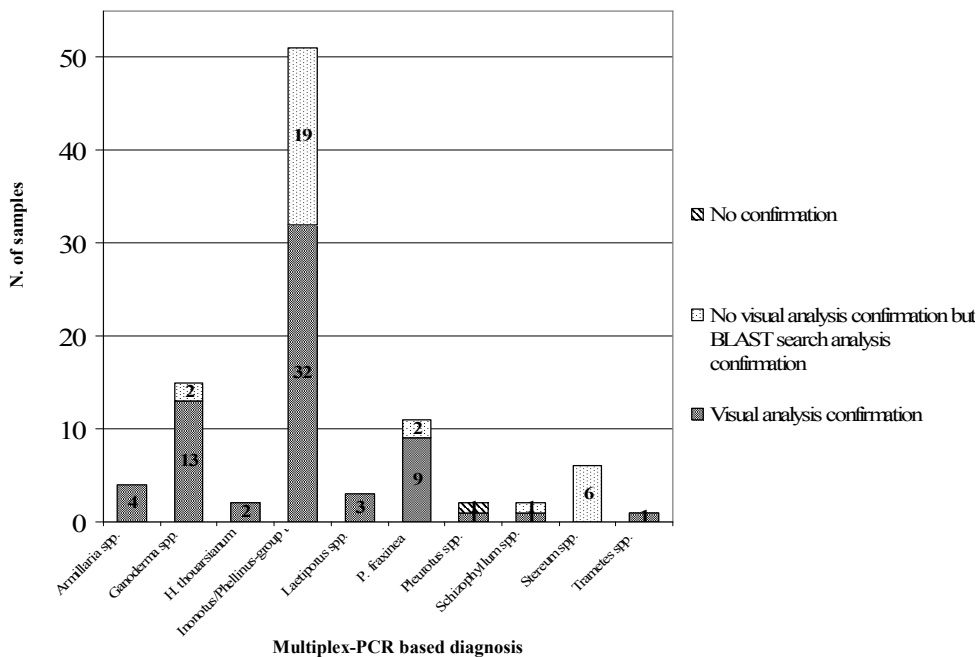
44 **Fig. 2** The results of M1, M2, M3 visualized on a UV-Gel documentation system after a 2 h electrophoresis at 4 V cm⁻¹ on a 1% Metaphor
 45 1% Standard agarose gel. M1: PCR products from DNA extracts of *Trametes* sp. (ITS band), of *Phellinus* sp. (ITS band + 111 bp) and
 46 *Ganoderma* sp. (ITS band + 228 bp) were loaded in the lanes 1, 2 and 3, respectively. M2: PCR products from DNA extracts of *Armillaria*
 47 sp. (185 bp), of *Hericium* sp. (200 bp), of *Laetiporus* sp. (146 bp) and of *Pleurotus* sp. (158 bp) were loaded in the lanes 1, 2, 3 and 4,
 48 respectively. M3: PCR products from DNA extracts of *Stereum* sp. (236 bp), of *Trametes* sp. (220 bp), of *Schizophyllum* sp. (190 bp) and of
 49 *P. fraxinea* (152 bp) were loaded in the lanes 1, 2, 3 and 4, respectively. M= Molecular weight marker 100bp DNA ladder are shown



50 **Fig. 3** Outcomes from sensitivity assays performed for M1, M2, M3 and *H. thouarsianum*-specific-PCR on 10-fold dilutions of *Phellinus*
 51 *tuberculosis*, *Pleurotus ostreatus*, *Stereum hirsutum* and *Hypoxylon thouarsianum* var. *thouarsianum* DNA extracts, respectively. The
 52 dilutions were carried out in wood DNA extracts either from *Platanus hybrida* or from *Quercus agrifolia*. 1P= 100 pg fungal DNA in wood
 53 DNA extract solution of *P. hybrida*; 2P= 10 pg fungal DNA in wood DNA extract solution of *P. hybrida*; 3P= 1 pg fungal DNA in wood
 54 DNA extract solution from *P. hybrida*; 4P= 10^{-1} pg fungal DNA in wood DNA extract solution from *P. hybrida*; 5P= 10^{-2} pg fungal DNA in
 55 wood DNA extract solution from *P. hybrida*; 1Q= 100 pg fungal DNA in wood DNA extract solution from *Q. agrifolia*; 2Q= 10 pg fungal
 56 DNA in wood DNA extract solution from *Q. agrifolia*; 3Q= 1 pg fungal DNA in wood DNA extract solution from *Q. agrifolia*; 4Q= 10^{-1} pg
 57 fungal DNA in wood DNA extract solution from *Q. agrifolia*; 5Q= 10^{-2} pg fungal DNA in wood DNA extract solution from *Q. agrifolia*;
 58 C+= 1 ng fungal DNA; C-= no DNA; C-P= DNA extract solution from *P. hybrida*; C-Q= DNA extract solution from *Q. agrifolia* M=
 59 Molecular weight marker of 100bp DNA ladder.



61 **Fig. 4** Efficiency of the method on wood samples from decay-affected trees. Comparison of the results from visual analysis to the multiplex
 62 PCR-based diagnosis. No fungi detected means no fungal rDNA amplification. No corresponding target taxon detected means no rDNA
 63 amplification of the target taxon visually identified. The corresponding target taxon detected for trees where no fruit bodies were detected
 64 was obtained from BLAST search analysis of the sequenced rDNA amplicon.



65 **Fig. 5** Specificity of the method on wood samples from decay-affected trees. Comparison of the results obtained from the multiplex PCR-
 66 based diagnosis to the outcomes from visual analysis and BLAST search analysis of the sequenced rDNA amplicon.
 67