RESEARCH LETTER

# A PCR-based method for the identification of important wood rotting fungal taxa within *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l.

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## Abstract

Two multiplex PCRs, based on 10 taxon-specific primers designed on rRNA gene regions, were developed for the identification of taxa within the lignivorous genera *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l., each comprising both secondary and primary aggressive decay fungi. Each multiplex PCR proved to correctly identify $1 \times 10^{-2}$ pg of fungal target DNA directly from wood. This method can be helpful in detecting decay in standing trees independent of its stage of advancement, and to identify the associated decay agents.

## Introduction

A tree management plan based on the timely detection of hazardous trees is essential to prevent wind throws or limb failures, whose impact can be dangerous especially in urban environments. The loss of mechanical strength in wood caused by decay fungi is one of the major causes of tree instability (Lonsdale, 1999). Moreover, fungal taxa differ in their pattern of spread within the tree, and consequently they affect the structural integrity of wood differently (Lonsdale, 1999). When visual inspection of signs and symptoms is complemented by instrumental analyses, decay in standing trees can be detected even at incipient stages (Tomikawa *et al.*, 1990; Mattheck & Breloer, 1993; Habermehl *et al.*, 1999; Müller *et al.*, 2001; Nicolotti *et al.*, 2003). Recently, a PCR-based method was developed for the early identification of several decay fungi from the wood of broadleaved trees (Guglielmo *et al.*, 2007). This method allows the identification of *Ganoderma* at a generic rank and fungi belonging to *Inonotus sensu lato* (s.l.) and *Phellinus* s.l. at a super generic rank that includes both genera. Within these genera are reported several of the most hazardous fungi affecting urban tree stability (Lonsdale, 1999; Terho *et al.*, 2007). However, hundreds of species characterized by different biological and ecological traits are described within *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. (Adaskaveg & Gilbertson, 1995; Wagner & Fischer, 2002), making it necessary to identify decay agents at a lower taxonomic rank in order to differentiate primary aggressive decay taxa from secondary or saprophytic ones (Lonsdale, 1999; Schwarze & Ferner, 2003; Bernicchia, 2005; Swiecki *et al.*, 2005).

The internal transcribed spacers 1 and 2 (ITS1 and 2) of the rRNA gene regions have been shown to be useful for sub-generic classification within *Ganoderma* (Moncalvo *et al.*, 1995a, b). Similarly, DNA sequence divergence at the 5′-end of the nuclear large subunit (25S) of rRNA gene has been used to support the generic status of each monophyletic group within *Inonotus* s.l. and *Phellinus* s.l. (Wagner & Fischer, 2001, 2002). While fungal identification based on sequence homology may be possible using these divergent

and diagnostic DNA loci, this approach may be unfeasible from environmental samples that are often characterized by the presence of several fungal taxa. Although it may be possible to clone the sequence and possibly identify all the coexisting fungal taxa, a faster, cheaper and more direct approach involves PCR-based diagnostic assays that employ primers designed to specifically amplify only the target taxa. For instance, taxon-specific primers have allowed for the development of sensitive diagnostic protocols for a restricted number of *Ganoderma* species on oil palms and for *Inonotus tomentosus* on conifers (Utomo & Niepold, 2000; Germain *et al.*, 2002). A comparable method for diagnosis of the most widespread and hazardous *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. species of the north temperate area is still lacking.

The objectives of this work were: (1) the design of taxon-specific primers to be used in multiplex PCR for the identification, at a subgeneric and a generic rank, of common and hazardous wood-rotting fungi of broadleaved trees within *Ganoderma* and within *Inonotus* s.l. and *Phellinus* s.l., respectively; (2) the testing of multiplex PCRs for reliability in the detection and identification of fungi directly from wood and for sensitivity in simulated field assays.

## Materials and methods

### Fungal isolates and DNA extraction

Fungal isolates used to design and test taxon-specific primers belonged to those species of *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. noted in the northern temperate areas for their aggressiveness in the deterioration of wood from broadleaved trees (Tables 1 and 2). The isolates were either provided by European and North American institutes or obtained from fruit bodies collected in California and Italy, as described by Guglielmo *et al.* (2007), and identified through analytical keys (Breitenbach & Kranzin, 1986; Hickman & Perry, 1997; Bernicchia, 2005).

DNA was extracted using a CTAB-based method (Guglielmo *et al.*, 2007) from fungal cultures grown at room temperature for 2 weeks in a 2% (w/v) liquid malt extract medium (AppliChem), harvested by filtration and lyophilized.

### PCR and sequencing conditions

The DNA region including ITS1, 5.8S and ITS2 was amplified with primers ITS1-F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) from isolates of *Ganoderma*, whereas the 5′-end portion of the 25S DNA region, including the variable domain D2, was amplified with primers CTB6 and TW13 (White *et al.*, 1990; O'Donnell, 1993) from isolates of *Inonotus* s.l. and *Phellinus* s.l. PCR reactions were performed in a 25 μL volume containing 1 × PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 0.5 μM of each primer, 0.025 U $\mu L^{-1}$ of Taq polymerase (Invitrogen) and at least 1 ng of genomic DNA. PCR reactions were conducted using an initial cycle with a 94 °C denaturation for 5 min, followed by 35 cycles, with each cycle consisting of a 94 °C denaturation for 45 s, a 58 °C annealing for 50 s and a 72 °C extension for 1 min, and one final cycle with a 72 °C extension for 10 min. The concentration of PCR products was estimated through comparison with the quantified DNA fragments of a GeneRuler$^{TM}$ 100 bp DNA ladder (Fermentas) after electrophoresis on a 1.5% (w/v) standard agarose gel (AppliChem).

PCR products were cleaned using Qia-quick purification kits (Qiagen), and cycle-sequenced with a BigDye Terminator (BDT) v. 3.1 cycle-sequencing kit (Applied Biosystems) in a 5 μL volume containing 0.5 × sequencing buffer, 1 μL of BDT reaction mix, 0.8 μM of the reverse or forward primer and about 10 ng of DNA template. Primers were the same as described above for PCR amplification. Cycle sequencing was performed using the following cycling parameters: 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The sequenced products were precipitated with 0.8 M of sodium acetate at pH 4.8, 3.4 mM 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). The quality of each sequence and the congruity of sense and antisense DNA strands were compared with the Sequencher 4.1.4 (Gene Codes Corporation).

### Taxon-specific primers design and testing

Sequences obtained from the isolates and additional sequences available in EMBL–EBI (http://www.ebi.ac.uk/) were employed for taxon-specific primer design (Tables 1 and 2). Sequences were aligned using CLUSTALW (Thompson *et al.*, 1994) to highlight rRNA gene portions conserved within species but displaying inter specific variability for *Ganoderma*. In the poorly described and extensive *Inonotus* s.l. and *Phellinus* s.l., each including hundreds of species, sequence alignments were employed to identify rRNA gene portions conserved among species within the newly described genera *Fomitiporia*, *Fuscoporia*, *Inocutis*, *Inonotus sensu stricto* (s.s.), *Phellinus* s.s. and *Pseudoinonotus* (Wagner & Fischer, 2002), but displaying an intergeneric variability.

Taxon-specific reverse primers to be used in multiplex PCR reactions in combination with a common forward primer were designed on the selected portions with the following features: (1) lack of complementation with sequences of the other taxa; (2) similar melting temperatures; (3) resulting DNA amplicons of length specific and unique for the target

**Table 1.** Isolates of *Ganoderma* used in this study for primer design and/or testing

| Species | ID collection | Host/Geographical origin | Sources* | GenBank accession number† |
|---|---|---|---|---|
| *Ganoderma adspersum*‡ | CBS351.74 | *Salix* sp./Belgium | EMBL-EBI | X78742/78763 |
| *Ganoderma adspersum*§ | DP38 | *Juglans regia*/Italy | Di.Va.P.R.A | AM906054 |
| *Ganoderma adspersum*§ | DP60 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A. | AM269770 |
| *Ganoderma adspersum*§ | DP102 | *Cercis siliquastrum*/Italy | Di.Va.P.R.A. | AM906055 |
| *Ganoderma adspersum*§ | FGA1 | *Pterocarya fraxinifolia*/Italy | Di.Va.P.R.A. | AM269771 |
| *Ganoderma adspersum*§ | GaGe99 | *Fagus sylvatica*/Italy | Di.Va.P.R.A. | AM906056 |
| *Ganoderma adspersum*§ | GaTo00 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A | AM906057 |
| *Ganoderma adspersum*‡ | ITA42 | *Morus* sp./Italy | EMBL-EBI | EF060011 |
| *Ganoderma applanatum*§ | FIN131R610 | *Tilia* sp./Finland | METLA | EF060004 |
| *Ganoderma applanatum*¶ | Gap1 | *Fraxinus excelsior*/Czech Republic | MUAF | |
| *Ganoderma applanatum*¶ | Gap2 | *Tilia platyphyllos*/Czech Republic | MUAF | |
| *Ganoderma applanatum*¶ | Gap3 | *Fagus* sp./Czech Republic | MUAF | |
| *Ganoderma applanatum*‡ | K(M)120829 | N.a./England | EMBL-EBI | AY884179 |
| *Ganoderma applanatum*‡ | NOR53-1143 | *Betula* sp./Norway | EMBL-EBI | EF060005 |
| *Ganoderma applanatum*‡ | Olrim 925 | *Fraxinus excelsior*/Lithuania | EMBL-EBI | AY787672 |
| *Ganoderma australe*‡‖ | HMAS86595 | N.a./UK | EMBL-EBI | AY884184 |
| *Ganoderma australe*‡‖ | K(M)120819 | N.a./UK | EMBL-EBI | AY884182 |
| *Ganoderma lucidum*¶ | 754 | N.a./Czech Republic | MUAF | |
| *Ganoderma lucidum*‡ | CBS176.30 | N.a./UK | EMBL-EBI | AF094511 |
| *Ganoderma lucidum*‡ | CBS270.81 | N.a./France | EMBL-EBI | Z37049/Z37099 |
| *Ganoderma lucidum*‡ | CBS430.84 | *Quercus hypoleucoides*/USA | EMBL-EBI | Z37051/Z37075 |
| *Ganoderma lucidum*§ | GlCN04 | *Platanus acerifolia*/Italy | Di.Va.P.R.A. | AM906058 |
| *Ganoderma lucidum*§ | GlTO99 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A. | AM269773 |
| *Ganoderma lucidum*‡ | HMAS86597 | N.a./UK | EMBL-EBI | AY884176 |
| *Ganoderma lucidum*‡ | RYV33217 | *Betula* sp./Norway | EMBL-EBI | Z37096/Z37073 |
| *Ganoderma lucidum*¶ | SP5 | *Umbellularia californica*/USA (CA) | UC Berkeley | |
| *Ganoderma lucidum*§ | SP26 | N.a./USA (CA) | UC Berkeley | AM269772 |
| *Ganoderma pfeifferi*§ | 874 | *Fagus sylvatica*/Czech Republic | MUAF | AM906059 |
| *Ganoderma pfeifferi*‡ | K(M)120818 | N.a./England | EMBL-EBI | AY884185 |
| *Ganoderma pfeifferi*‡ | K(M)120821 | N.a./England | EMBL-EBI | AY884181 |
| *Ganoderma resinaceum*¶ | 853 | *Salix fragilis*/Czech Republic | MUAF | |
| *Ganoderma resinaceum*¶ | 883 | *Quercus robur*/Czech Republic | MUAF | |
| *Ganoderma resinaceum*‡ | CBS152.27 | N.a./UK | EMBL-EBI | Z37062/Z37085 |
| *Ganoderma resinaceum*‡ | CBS194.76 | *Fagus sylvatica*/Netherlands | EMBL-EBI | X78737/X78758 |
| *Ganoderma resinaceum*‡ | CH160999 | *Quercus rubra*/Germany | EMBL-EBI | EF060007 |
| *Ganoderma resinaceum*§ | DP1 | *Platanus acerifolia*/Italy | Di.Va.P.R.A. | AM269775 |
| *Ganoderma resinaceum*§ | DP2 | *Celtis australis*/Italy | Di.Va.P.R.A. | AM906060 |
| *Ganoderma resinaceum*§ | DP23 | *Platanus acerifolia*/Italy | Di.Va.P.R.A. | AM906061 |
| *Ganoderma resinaceum*§ | DP63 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A. | AM906062 |
| *Ganoderma resinaceum*§ | DP107 | *Acer platanoides*/Italy | Di.Va.P.R.A. | AM906064 |
| *Ganoderma resinaceum*§ | FGA5 | *Celtis australis*/Italy | Di.Va.P.R.A. | AM906063 |
| *Ganoderma resinaceum*§ | FGR1 | *Fagus sylvatica*/Italy | Di.Va.P.R.A. | AM269776 |
| *Ganoderma resinaceum*¶ | FGR3 | *Populus nigra*/Italy | Di.Va.P.R.A. | |
| *Ganoderma resinaceum*§ | FGR5 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A. | AM269777 |
| *Ganoderma resinaceum*§ | G4/13 | N.a./Italy | Di.Va.P.R.A. | AM269778 |
| *Ganoderma resinaceum*§ | GrTo96 | *Aesculus hippocastanum*/Italy | Di.Va.P.R.A. | AM906065 |
| *Ganoderma resinaceum*‡ | HMAS86599 | N.a./UK | EMBL-EBI | AY884177 |
| *Ganoderma* sp.§** | SP11 | *Umbellularia californica*/USA (CA) | UC Berkeley | AM269768 |
| *Ganoderma* sp.¶** | SP13 | *Umbellularia californica*/USA *(CA)* | UC Berkeley | |
| *Ganoderma* sp.§** | SP16 | *Quercus agrifolia*/USA (CA) | UC Berkeley | AM269769 |

*METLA, Finnish Forest Research Institute, Vantaa (Finland); MUAF, Mendel University of Agronomy and Forestry, Faculty of Forestry and Wood technology, Brno (Czech 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 sequences database; UC Berkeley, Department of Environmental Science, Policy, and Management Berkeley, USA.

†GeneBank accession numbers are referred to sequences of rRNA gene region including ITS1, 5.8S and ITS2.

‡Isolates whose sequences were obtained through EMBL-EBI and used for primer design.

§Isolates used for primer design and testing.

¶Isolates used for primer testing.

‖From the analysis of rRNA gene sequence these isolates, named *G. australe*, correspond to *G. adspersum*.

**From fruit bodies analysis and from rRNA gene analysis these north American isolates are supposed to belong to the *G. applanatum* group.

**Table 2.** Isolates of *Inonotus* s.l. and *Phellinus* s.l. used in this study for primer design and/or testing

| Species/Genera* | ID collection | Host/Geographical origin | Source† | GenBank accession number‡ |
|---|---|---|---|---|
| *Inonotus andersonii*/*Inonotus* s.s.§ | 557 | *N.a./Czech Republic* | CAS-IM | |
| *Inonotus andersonii*/*Inonotus* s.s.¶ | CBS 312.35 | *Quercus* sp./USA | EMBL-EBI | AY059041 |
| *Inonotus andersonii*/*Inonotus* s.s.§ | L(61)11-14-C | *Quercus velutina*/USA (OH) | USDA-FPL | |
| *Inonotus andersonii*/*Inonotus* s.s.‖ | SP23 | *Quercus wislizeni*/USA (OH) | Phyt Res | AM269843 |
| *Inonotus andersonii*/*Inonotus* s.s.‖ | T1545 | N.a./USA (OH) | USDA-FPL | AM269844 |
| *Inonotus dryadeus*/*Pseudoinonotus*¶ | 93-929 | *Quercus* sp./Germany | EMBL-EBI | AF311011 |
| *Inonotus dryadeus*/*Pseudoinonotus*‖ | FP-105836-4 | *Quercus alba*/USA (OH) | USDA-FPL | AM269870 |
| *Inonotus dryadeus*/*Pseudoinonotus*§ | IdRa | *Fraxinus excelsior*/Italy | Di.Va.P.R.A. | AM910615 |
| *Inonotus dryophilus*/*Inocutis*‖ | 703 | N.a./Czech Republic | CAS-IM | AM269847 |
| *Inonotus dryophilus*/*Inocutis*‖ | L(61)5-20-A | *Quercus prinus*/USA (OH) | USDA-FPL | AM269846 |
| *Inonotus dryophilus*/*Inocutis*¶ | MF 87-918 | *Platanus* sp./France | EMBL-EBI | AF311012 |
| *Inonotus dryophilus*/*Inocutis*‖ | SP25 | *Quercus agrifolia*/USA (CA) | Phyt Res | AM269845 |
| *Inonotus hispidus*/*Inonotus* s.s.‖ | FIH2 | *Malus* sp./Italy | Di.Va.P.R.A. | AM906066 |
| *Inonotus hispidus*/*Inonotus* s.s.‖ | FP-106082-T | *Quercus phellos*/USA (MS) | USDA-FPL | AM269848 |
| *Inonotus hispidus*/*Inonotus* s.s.¶ | FPL-3597 | N.a./USA | EMBL-EBI | AF518623 |
| *Inonotus hispidus*/*Inonotus* s.s.¶ | MF 92-829 | *Fraxinus excelsior*/Germany | EMBL-EBI | AF311014 |
| *Inonotus obliquus*/*Inonotus* s.s.§ | IoTo96 | *Prunus avium*/Italy | Di.Va.P.R.A. | |
| *Inonotus obliquus*/*Inonotus* s.s.¶ | TW 705 | *Betula* sp./Germany | EMBL-EBI | AF311017 |
| *Inonotus rheades*/*Inocutis*¶ | TW 385 | *Populus tremulae*/Germany | EMBL-EBI | AF311019 |
| *Phellinus contiguus*/*Fuscoporia*§ | PcTo98 | *Corylus avellana*/Italy | Di.Va.P.R.A. | |
| *Phellinus contiguus*/*Fuscoporia*¶ | TW699 | N.a./Germany | EMBL-EBI | AF311029 |
| *Phellinus gilvus*/*Fuscoporia*¶ | FPL-5528 | N.a./USA | EMBL-EBI | AF518636 |
| *Phellinus gilvus*/*Fuscoporia*¶ | MF 91-42e | *Quercus agrifolia*/USA (CA) | EMBL-EBI | AY059025 |
| *Phellinus gilvus*/*Fuscoporia*‖ | SP18 | *Quercus agrifolia*/USA (CA) | UC Berkeley | AM269858 |
| *Phellinus gilvus*/*Fuscoporia*‖ | SP20 | *Quercus agrifolia*/USA (CA) | UC Berkeley | AM269859 |
| *Phellinus igniarius*/*Phellinus* s.s.‖ | 575 | N.a./Czech Republic | CAS-IM | AM269860 |
| *Phellinus igniarius*/*Phellinus* s.s.¶ | FPL5599 | N.a./USA | EMBL-EBI | AF287884 |
| *Phellinus igniarius*/*Phellinus* s.s.¶ | MF 83-1110a | *Salix fragilis*/Germany | EMBL-EBI | AF311033 |
| *Phellinus igniarius*/*Phellinus* s.s.§ | PiTo98 | *Salix* sp./Italy | Di.Va.P.R.A. | |
| *Phellinus lundelii*/*Phellinus* s.s.§ | PlCN97 | *Prunus* sp./Italy | Di.Va.P.R.A. | |
| *Phellinus lundelii*/*Phellinus* s.s.¶ | TN5760 | *Betula* sp./Finland | EMBL-EBI | AF311015 |
| *Phellinus punctatus*/*Fomitiporia*‖ | 262 | N.a./Czech Republic | CAS-IM | AM269863 |
| *Phellinus punctatus*/*Fomitiporia*§ | DP21 | *Platanus acerifolia*/Italy | Di.Va.P.R.A. | |
| *Phellinus punctatus*/*Fomitiporia*‖ | DP25 | *Tilia x vulgaris*/Italy | Di.Va.P.R.A. | AM269862 |
| *Phellinus punctatus*/*Fomitiporia*‖ | DP96 | *Tilia x vulgaris*/Italy | Di.Va.P.R.A. | AM906067 |
| *Phellinus punctatus*/*Fomitiporia*¶ | MF85-74 | *Salix caprea*/Germany | EMBL-EBI | AF311007 |
| *Phellinus punctatus*/*Fomitiporia*¶ | MUCL34101 | N.a./Germany | EMBL-EBI | AY618200 |
| *Phellinus robustus*/*Fomitiporia*‖ | 587 | Unknown/Czech Republic | CAS-IM | AM906068 |
| *Phellinus robustus*/*Fomitiporia*‖ | RLG-9585-T | *Salix* sp./USA (MN) | USDA-FPL | AM269864 |
| *Phellinus robustus*/*Fomitiporia*¶ | TW242 | *Quercus robur*/Germany | EMBL-EBI | AF311008 |
| *Phellinus torulosus*/*Fuscoporia*‖ | 759 | N.a./Czech Republic | CAS-IM | AM269865 |
| *Phellinus torulosus*/*Fuscoporia*¶ | Pt4 | *Olea europaea*/France | EMBL-EBI | AF311041 |
| *Phellinus torulosus*/*Fuscoporia*§ | PtTo98 | *Robinia Pseudoacacia*/Italy | Di.Va.P.R.A. | |
| *Phellinus tremulae*/*Phellinus* s.s.‖ | 243 | N.a./Czech Republic | CAS-IM | AM906069 |
| *Phellinus tremulae*/*Phellinus* s.s.¶ | MF 89-826c | *Populus tremula*/Estonia | EMBL-EBI | AF311042 |
| *Phellinus tuberculosus*/*Phellinus* s.s.‖ | 265 | N.a./Czech Republic | CAS-IM | AM269866 |
| *Phellinus tuberculosus*/*Phellinus* s.s.‖ | DP39 | *Prunus pissardi*/Italy | Di.Va.P.R.A. | AM906070 |
| *Phellinus tuberculosus*/*Phellinus* s.s.‖ | DP40 | *Prunus pissardi*/Italy | Di.Va.P.R.A. | AM906071 |
| *Phellinus tuberculosus*/*Phellinus* s.s.§ | FPT1 | *Malus* sp./Italy | Di.Va.P.R.A. | |
| *Phellinus tuberculosus*/*Phellinus* s.s.¶ | TW114 | *Prunus* sp./Germany | EMBL-EBI | AF311043 |

*Genera of *Inonotus* s.l. and *Phellinus* s.l. described by Wagner & Fischer (2001, 2002).

†CAS-IM, Academy of Sciences of Czech Republic Institute of Microbiology Department of Experimental Mycology, Videnska, Czech Republic; Phyt Res, Phytosphere Research, Vacaville, CA; USDA-FPL, United States Department of Agriculture Forest Products Lab, Madison, WI, USA.

‡GeneBank accession numbers are referred to sequences of the rRNA gene region including D2-25S.

§Isolates used for primer testing.

¶Isolates whose sequences were obtained through EMBL-EBI and used for primer design.

‖Isolates used for primer design and testing.

taxon. The softwares PRIMER3 (http://www.broad.mit.edu/) and OLIGOANALYZER 3.0 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) were used to design primers with the above-described features. To further evaluate the specificity of taxon-specific primers, they were aligned with sequences of the following outgroup species phylogenetically close to the target taxa: *Fomes fasciatus* (accession number: AM269766) was used for *Ganoderma*, *Hymenochaete rubiginosa* (AF323741), *Phylloporia ribis* (AF311040), *Fulvifomes robiniae* (AY059038), *Phellinus cavicola* (AY059052) were used for *Inonotus* s.l. and *Phellinus* s.l. Finally, using nucleotide BLAST (BLASTN) analysis (http://www.ncbi.nlm.nih.gov/BLAST/), any significant matches of taxon-specific primers with sequences derived from other organisms were further investigated.

The specificity and efficiency of PCR amplifications were evaluated on DNA extracts of the isolates listed in Tables 1 and 2, both in uniplex and multiplex PCR assays. Annealing temperatures were optimized using a thermocycling gradient and adjusted to the highest stringency in order to improve PCR efficiency. PCR conditions were the same as those used for the amplification of rRNA gene regions, except for the use of a 0.25 μM 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 size and intensity of each amplicon were estimated with the ABI PRISM 3100 GENESCAN ANALYSIS software v. 3.7 (Applied Biosystems). Amplicons were also visualized on a gel containing 1% (w/v) of high-resolution MetaPhor (Cambrex) and 1% (w/v) of standard agarose, after electrophoretic migration.

### Reliability of multiplex PCR assays on wood

Multiplex PCRs were performed on two groups of 23 and 56 wood samples, respectively, diagnosed as infected by *Ganoderma* and *Inonotus* s.l. or *Phellinus* s.l. using the method described by Guglielmo *et al.* (2007). Wood samples were collected in Californian and Italian landscapes from different broadleaved trees with evident decay symptoms and/or signs. DNA was extracted from 100 mg of each wood sample using a QIAamp DNA Stool Mini Kit (Qiagen). The M1 reaction described by Guglielmo *et al.* (2007) was performed on a 1/100 and a 1/500 dilution of each DNA extract to detect *Ganoderma* and *Inonotus* s.l. or *Phellinus* s.l.

The ability to correctly identify *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. species using this approach was assessed by comparing results of multiplex PCR with results expected either through the analysis of the fruit bodies emerging from the tree or through BLASTN of rRNA gene fragments amplified by uniplex PCR reactions.

### Sensitivity of multiplex PCR assays

For each target taxon, the sensitivity of multiplex PCRs was tested by adding $100–10^{-3}$ pg of fungal DNA to DNA extracts obtained from 100 mg (dry weight) of *Platanus hybrida* wood. The concentration of genomic fungal DNA was estimated by comparison with the quantified DNA fragments of a GeneRuler$^{TM}$ 100 bp DNA ladder after electrophoresis on a 0.8% (w/v) standard agarose gel. Wood DNA extract was obtained using the QIAamp DNA Stool Mini Kit and diluted in 100 μL of sterile water. Multiplex PCRs were performed, for each fungal DNA dilution, in a 25 μL volume containing 1 × PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.5 μM of each primer, 0.025 U $\mu L^{-1}$ of Taq polymerase (Invitrogen), 0.5 mg $mL^{-1}$ of bovine serum albumin and 1 μL of DNA dilution. The presence of the multiplex PCR amplicons was estimated after electrophoretic migration on agarose gels.

## Results

### Multiplex PCR development

Two multiplex PCRs, named Mgano and Mhyme, were developed by combining the universal fungal forward primer ITS1-F with four primers for subgeneric identification of *Ganoderma*, and the common forward primer 25sF (Guglielmo *et al.*, 2007) with six taxon-specific primers designed for *Inonotus* s.l. and *Phellinus* s.l., respectively (Table 3). The site of hybridization of reverse primers is shown in Fig. 1. DNA fragment analysis showed that both uniplex and multiplex PCR with optimized thermocycling parameters allowed DNA amplifications of the appropriate taxon-specific length from each of the selected fungal species (Table 3). It should be noted that the same primer amplified *Ganoderma* taxa named differently in North America and Europe. For instance, GadR is specific for *Ganoderma adspersum* and *Ganoderma pfeifferi* in Europe and *Ganoderma applanatum* in North America; likewise, GrR is specific for *Ganoderma resinaceum* in Europe and *Ganoderma lucidum* in North America (Table 3). Nonetheless, primers effectively discriminated among *Ganoderma* taxa present in the same continent.

Mgano and Mhyme taxon-specific amplicons were discernible after a 2 h 30 min-gel electrophoresis at 6 V $cm^{-1}$ (Fig. 2). No cross-reactivity occurred for any taxon-specific primers when used in multiplex reactions.

### Reliability and sensitivity of multiplex PCRs

Mgano and Mhyme allowed the correct identification of the fungal taxon from each of the 79 wood samples. Among the 23 *Ganoderma* samples, we identified *G. resinaceum* and *G. adspersum* in Italian collections. Among the 56 *Inonotus*

**Table 3.** Multiplex PCR primers combination, diagnostic purpose and cycling parameters

| Multiplex PCR name | Primers combination | | Taxon-specific amplicon size† (bp) | Corresponding taxa identified‡ | Cycling parameters |
|---|---|---|---|---|---|
| | Forward | Reverse* | | | |
| Mgano | ITS1-F | GadR (5′-CAGGCAACAA GTGCGCTC-3′) | 211 | *G. adspersum*, *G. pfeifferi*, *G. applanatum* (from North America) | 5 min denaturation at 95 °C; 35 cycles of: 45 s at 95 °C, 45 s at 56 °C, 45 s at 72 °C; 10 min final extension at 72 °C |
| | | GapR (5′-GACACGCTTCA CAAGCTCC-3′) | 200 | *G. applanatum* (from Europe) | |
| | | GlR (5′-TTCACGAAGCCC CGCAAG-3′) | 193 | *G. lucidum* (from Europe) | |
| | | GrR (5′-AAGAGCCCGCTT CACAACG-3′) | 178 | *G. resinaceum*, *G. lucidum* (from North America) | |
| Mhyme | 25sF | FomR (5′-CCCAGCCCATG TATACAATAG-3′) | 258 | *Fomitiporia* (*P. punctatus*, *P. robustus*) | 5 min denaturation at 95 °C; 35 cycles of: 45 s at 95 °C, 45 s at 62 °C, 45 s at 72 °C; 10 min final extension at 72 °C |
| | | FuscR (5′-CACACTCCGAA GAGTGCC-3′) | 225 | *Fuscoporia* (*P. contiguus*, *P. gilvus*, *P. torulosus*) | |
| | | IdryaR (5′-ACCGACGCATA CAACAAAGG-3′) | 254 | *I. dryadeus* | |
| | | InocuR (5′-CCTCAGTCCCC GACGGT-3′) | 265 | *Inocutis* (*I. dryophilus*) | |
| | | InssR (5′-GATGTTGACCC GTCCGAC-3′) | 214 | *Inonotus* s.s. (*I. andersonii*, *I. hispidus*, *I. obliquus*) | |
| | | PhssR (5′-GGCGCTACATTC CCTCTG-3′) | 173 | *Phellinus* s.s. (*P. igniarius*, *P. lundelii*, *P. tremulae*, *P. tuberculosus*) | |

*Corresponding oligonucleotidic sequences are indicated in parentheses.

†Taxon-specific amplicon sizes are indicated in base pairs (bp).

‡The taxa indicated have been positively tested in uniplex and multiplex PCR assays performed in this study.

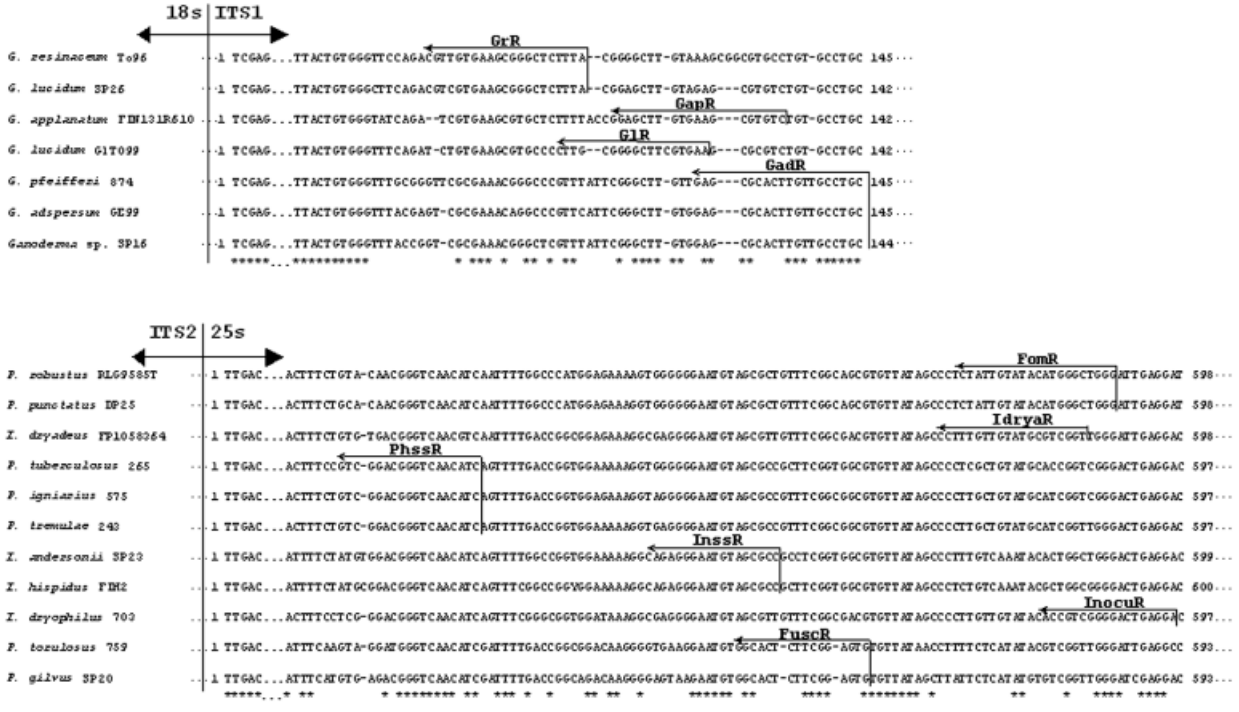


**Fig. 1.** ITS and 25S sequence alignments displaying the priming sites complementary to the 10 reverse taxon-specific primers designed.

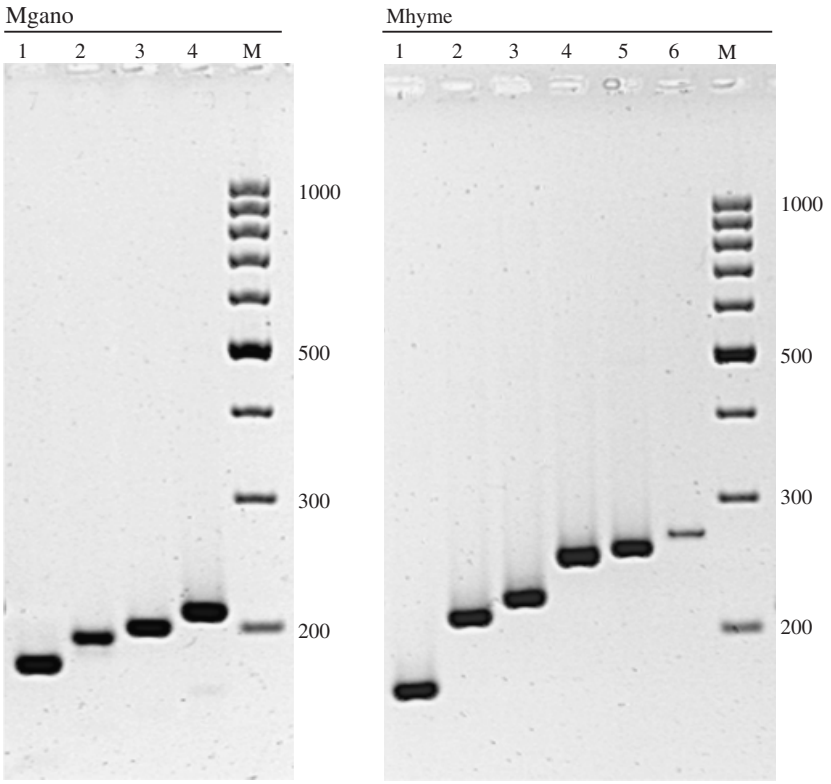


**Fig. 2.** The results of Mgano and Mhyme visualized on a UV-gel documentation system after a 2 h 30 min electrophoresis at 6 V cm$^{-1}$ on a 1% Metaphor 1% standard agarose gel. Mgano: PCR products of *Ganoderma resinaceum* (178 bp), European *Ganoderma lucidum* (193 bp), European *Ganoderma applanatum* (200 bp) and *Ganoderma adspersum* (211 bp) were loaded in the lanes 1, 2, 3 and 4, respectively. Mhyme: PCR products of *Phellinus* s.s. sp. (173 bp), *Inonotus* s.s. sp. (214 bp), *Fuscoporia* sp. (225 bp), *Inonotus dryadeus* (254 bp), *Fomitiporia* sp. (258 bp) and *Inocutis* sp. (265 bp) were loaded in the lanes 1, 2, 3, 4, 5 and 6, respectively. M, molecular weight marker 100-bp DNA ladder.

s.l. or *Phellinus* s.l. samples, Mhyme identified *Inonotus andersonii* and *Phellinus gilvus* in Californian collections and *Inonotus dryadeus*, *Inonotus hispidus*, *Phellinus punctatus*, *Phellinus torulosus* and *Phellinus tuberculosus* in Italian collections.

The threshold of sensitivity in simulated field assay was at least $10^{-1}$ pg of DNA for each primer, except for FuscR, whose detection threshold was 1 pg (Fig. 3).

## Discussion

The taxon-specific primers designed in this study proved to be appropriate for the development of two multiplex PCRs allowing for the specific and sensitive identification of widespread decay agents of broadleaved trees in European and North American landscapes.

With respect to the diagnostic method previously developed (Guglielmo *et al*., 2007), the PCR-based assay here described allows to identify at a lower taxonomic rank *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l., each comprising hundreds of species. The chance to better define, within these three taxa, the decay agent involved makes the molecular method more appropriate for tree risk assessment purpose.

A worldwide species identification within *Ganoderma* is still unclear and often controversial (Hong & Jung, 2004). In this context, our approach has primarily focused on identifying groups of species with different decay characteristics. Through primers designed on the ITS 1 region, we were able to distinguish *G. resinaceum*, reported in Europe as one of the most common and harmful decay fungi (Lonsdale, 1999; Nicolotti *et al*., 2004), from European *G. lucidum*, described as a widespread but poorly active decay agent (Bernicchia, 2005). Likewise, the two common and similar *G. applanatum* and *G. adspersum* can now be distinguished; this is noteworthy because the two species differ in terms of invasiveness in the standing tree: while *G. applanatum* is regarded as predominantly saprotrophic, being confined to trees with a dysfunctional xylem, *G. adspersum*, due to its ability to breach the reaction zone, is reported as a very aggressive decay agent on high-vitality trees (Schwarze & Ferner, 2003). Although the specific primer for *G. adspersum* proved to prime the DNA amplification of *G. pfeifferi* the molecular differentiation of these two species was deemed

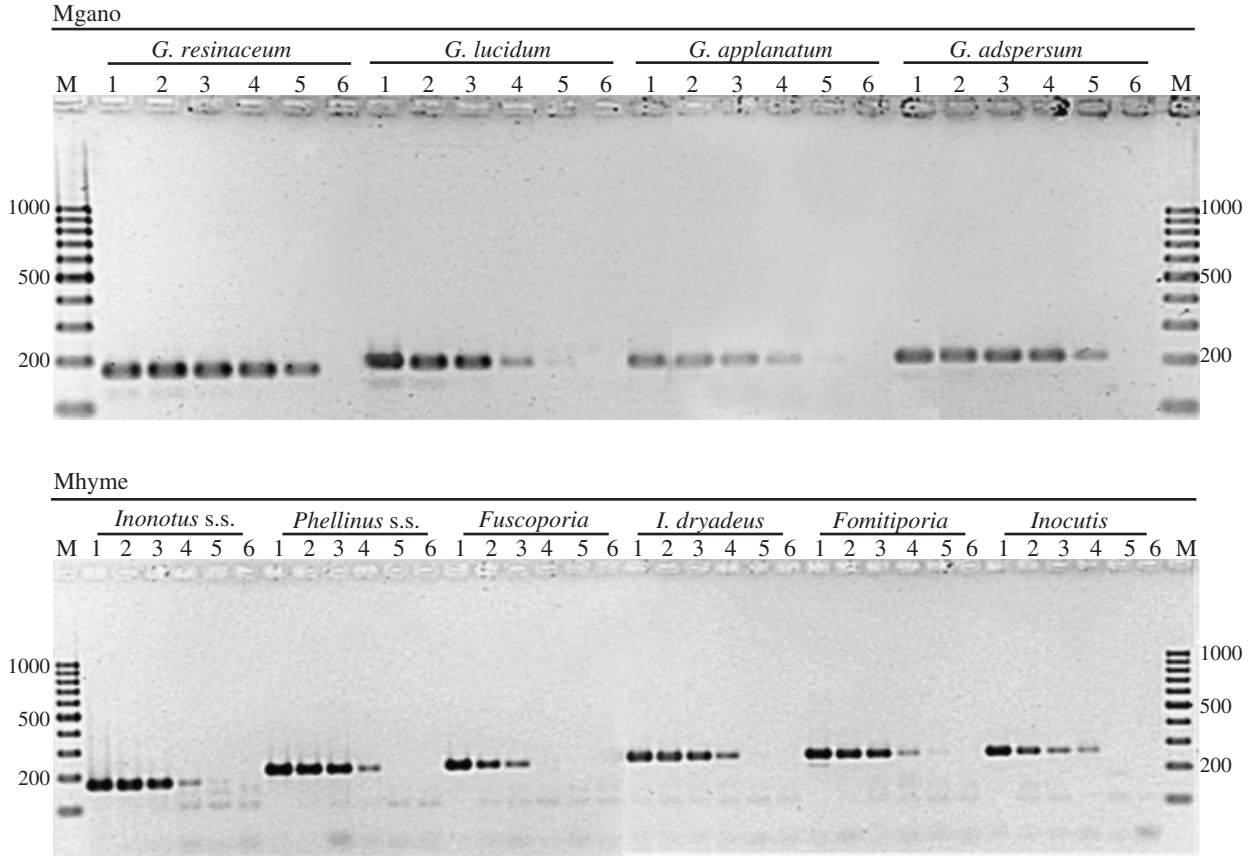


**Fig. 3.** Outcomes from sensitivity assays performed for Mgano and Mhyme. On each lane a different fungal DNA dilution, in wood DNA extracts from *Platanus hybrida*, has been loaded: 1, 100 pg fungal DNA; 2, 10 pg; 3, 1 pg; 4, $10^{-1}$ pg; 5, $10^{-2}$ pg; 6, $10^{-3}$ pg. M, Molecular weight marker of 100-bp DNA ladder. Isolates used in Mgano were *Ganoderma adspersum* DP60, *Ganoderma applanatum* Gap1, *Ganoderma lucidum* GlTo99 and *Ganoderma resinaceum* GrTo96. Isolates used in Mhyme were *Inonotus andersonii* 557, *Inonotus dryadeus* FP-105836-4, *Inonotus dryophilus* 703, *Phellinus igniarius* 575, *Phellinus punctatus* DP96 and *Phellinus torulosus* 759.

not to be necessary because of the narrow range of the hosts of *G. pfeifferi*, mainly reported on *Fagus* spp. (Lonsdale, 1999). In North America, the identification of the two most common and hazardous *Ganoderma* species, *G. applanatum* and *G. lucidum* (Hickman & Perry, 1997), may be achieved through the primers designed for *G. adspersum* and *G. resinaceum*, respectively. Although apparently controversial, this supports the hypothesis that *G. applanatum* and *G. lucidum* complexes are polyphyletic and include allopatric sister taxa, as previously reported (Hseu *et al.*, 1996; Smith & Sivasithamparam, 2000; Hong & Jung, 2004).

A species-level diagnosis for the *Inonotus* s.l. and *Phellinus* s.l. species is complicated not only by the large number of species concerned, but also by the presence of intraspecific DNA sequence variations among isolates of different provenances (Fischer & Binder, 2004). Through taxon-specific primers designed on the D2 of 25S rRNA gene, *Inonotus* s.l. and *Phellinus* s.l. may be identified at a generic rank, with the exception of *I. dryadeus*, which can be diagnosed at a species level. The possibility of identifying this fungus at the species level is significant because *I. dryadeus* is reported as a hazardous rotting agent of the innermost roots whose decay, even at an advanced stage, is often overlooked (Lonsdale, 1999). Moreover, because several species of *Inonotus* s.l. and *Phellinus* s.l. display a high degree of host preference and a limited geographic distribution, the knowledge of the decay agent at the genus level may be useful to infer its nature at the species level. For instance, if a *Fuscoporia* is detected on oaks in North America, it is likely to be *P. gilvus* (Swiecki *et al.*, 2005). On the other hand, the same result obtained from broadleaved trees in southern Europe is likely to indicate the presence of *P. torulosus* or *P. contiguus* (Bernicchia, 2005). Within *Inocutis*, while *Inonotus dryophilus* is common on different broadleaved tree species, especially *Quercus* spp. and *Fraxinus* spp., *I. rheades* is most frequent on *Populus* spp. Within *Phellinus* s.s., *P. tuberculosus* is commonly associated with rosaceous trees, whereas *Phellinus igniarius* with *Salix* spp. and *Phellinus tremulae* with *Populus* spp. (Lonsdale, 1999; Bernicchia, 2005).

Multiplex PCRs displayed the same efficiency as uniplex PCRs. Furthermore, by multiplexing, the competition of primers for close-priming sites improves the reliability of the PCR reaction (Garbelotto *et al.*, 1996). This was evident for IdryaR, whose 3′-end high complementary with sequences of *I. andersonii* and *I. hispidus* led to aspecific DNA amplifications from isolates of these species in uniplex PCR (data not shown) but not in multiplex PCR. Finally, Mgano and Mhyme proved to be as sensitive as the multiplex PCRs developed by Guglielmo *et al.* (2007), displaying a detection threshold of at least $10^{-1}$ pg of fungal DNA in wood DNA extracts for all primers but FuscR. The lower sensitivity of FuscR may be explained by the presence of a self-annealing site leading to potential hairpin formation, thus reducing the primer ability to anneal the complementary sequence of DNA template.

The results of PCR amplification with taxon-specific primers were detected both through a capillarity electrophoretic system, to estimate the precise size of each amplicon, and through an agarose gel electrophoresis. The former assay allowed to exclude the presence of polymorphisms in the amplicon length within each target taxon, due to nucleotide insertions or deletions (INDELS). This may frequently occur in ITS regions, as already reported (Guglielmo *et al.*, 2007). The latter assay proved that taxon-specific amplicons are discernible through basic electrophoretic systems commonly available in any diagnostic laboratory.

These newly developed multiplex PCRs correctly identified decay fungi directly from wood substrates. They can complement the molecular approach recently developed for the early identification of wood decay agents in standing trees (Guglielmo *et al.*, 2007) by providing a more accurate identification of important decay fungi within *Ganoderma*, *Inonotus* s.l. and *Phellinus* s.l. Furthermore, the method may be used to improve our knowledge of the biology and ecology of these fungi.

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