Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

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

F. Guglielmo[1], S.E. Bergemann[2], P. Gonthier[1], G. Nicolotti[1] and M. Garbelotto[2]

1 Department of Exploitation and Protection of Agricultural and Forestry Resources, Plant Pathology, University of Torino, Grugliasco , Italy
2 Ecosystem Sciences Division, Department of Environmental Science, Policy and Management, University of California Berkeley, California, USA

Keywords
internal transcribed spacers, mitochondrial small subunit, nuclear large subunit, molecular diagnostic, wood decay.

Correspondence
Matteo Garbelotto, University of California at Berkeley, Department of Environmental Science, Policy and Management, Ecosystem Sciences Division, 137 Mulford Hall, Berkeley, CA 94720, USA.
E-mail: matteo@nature.berkeley.edu

2006/1023: received 14 July 2006, revised 22 December 2006, and accepted 21 February 2007

doi:10.1111/j.1365-2672.2007.03378.x

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., *Hericium* 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 1 pg of target DNA in the template. This assay correctly identified the 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.

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 the 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 the 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 comparing 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 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 (ITSI and ITSII) 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 and are not suitable for routine analysis. Taxon-specific primers have been already used for the identification and 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 *et al.* 2000; Corbiere Morot-Bizot *et al.* 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 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 initial validation of ten genera- and two species-specific diagnostic PCR assays were 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., *Hericium* spp., *Hypoxylon thouarsianum* var. *thouarsianum*, *Inonotus* spp., *Laetiporus* spp., *Perenniporia fraxinea*, *Phellinus* spp., *Pleurotus* spp., *Schizophyllum* spp., *Stereum* spp. and *Trametes* spp. Collections were either pure cultures or fruit body specimens (Table 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 study

Species	ID collection	Host/geographic origin	Source*	GenBank Accession numbers		
				Nuc LSU (5′)	ITS	Mt SSU
Armillaria cepistipes Velen.†			EMBL–EBI		AJ250053	
Armillaria gallica Marxm. & Romagn.	Napa-141	*Quercus agrifolia* Née/ USA (CA)	USDA–ARS	AM269818	AM269760	
A. gallica	Napa-144	Unidentified hardwood/ USA (CA)	USDA–ARS	AM269817	AM269759	
A. gallica†			EMBL–EBI	AY213570	AJ250054; AY190247	
Armillaria mellea (Vahl) P. Kumm§	DP26	*Aesculus hippocastanum* L./ Italy	Di.Va.P.R.A.			
A. mellea	Mar-001	Unidentified hardwood/ USA (CA)	USDA–ARS	AM269820	AM269761	
A. mellea	Mar-016	*Quercus agrifolia* Née/ USA (CA)	USDA–ARS	AM269819		
A. mellea	T4D	*Vitis vinifera* L./ Switzerland	WSL	AM269821	AM269762	
A. mellea†			EMBL–EBI		AJ250051; AF163578; AF163584; AF163589	
Armillaria nabsnona Volk & Burds	Men-017	*Alnus rubra* Bong./ USA (CA)	USDA–ARS	AM269822	AM269763	
A. nabsnona	Men-023	*Alnus rubra* Bong./ USA (CA)	USDA–ARS	AM269823	AM269764	
A. nabsnona†			EMBL–EBI		AY213574	
Armillaria ostoyae (Romagn.) Herink†			EMBL–EBI	AY207145	AJ250055	
Armillaria tabescens (Scop.) Emel†			EMBL–EBI	AF042593; AY213590	AY213588	
Baeospora myosura (Fr.) Singer¶	OKM-3708-Sp	*Picea abies* (L.) H. Karst./ USA (ME)	USDA–FPL	AM269824	AM269765	
Daedaleopsis confragosa (Bolton) J. Schröt.†¶			EMBL–EBI	AF261542		AF039576
Daldinia concentrica (Bolton) Ces. & De Not.†¶			EMBL–EBI	U47828	AY616683	
Fomes fasciatus (Sw.) Cooke¶	FP-1061048-T	*Carya sp.*/ USA (MS)	USDA–FPL	AM269825	AM269766	
Ganoderma adspersum (Schulz.) Donk	DP60	*Aesculus hippocastanum* L./ Italy	Di.Va.P.R.A.	AM269828	AM269770	
G. adspersum	DP87	*Aesculus hippocastanum* L./ Italy	Di.Va.P.R.A.	AM269826	AM269767	
G. adspersum	FGA1	*Pterocarya fraxinifolia* Wingnut./ Italy	Di.Va.P.R.A.	AM269829	AM269771	
G. adspersum†			EMBL–EBI		AJ006685	
Ganoderma applanatum (Pers.) Pat.†			EMBL–EBI	AJ406526; AY515339	AY787672	
Ganoderma gibbosum (Blume & T. Nees) Pat.†			EMBL–EBI		AY593854	
Ganoderma lucidum (Curtis) P.Karst‡	GlTO99	*Aesculus hippocastanum* L./ Italy	Di.Va.P.R.A.	AM269830	AM269773	
G. lucidum‡	SP5	*Umbellularia californica* (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269831		
G. lucidum‡	SP26	Unknown/ USA (CA)	UC Berkeley		AM269772	
G. lucidum†			EMBL–EBI	AX78776	AF506372; AY456341	
Ganoderma orbiforme (Fr.) Ryvarden†			EMBL–EBI	AX78777		
Ganoderma pfeifferi Bres.	G2/11	Unknown/ Italy	Di.Va.P.R.A.	AM269832	AM269774	
Ganoderma resinaceum Boudier	DP1	*Platanus acerifolia* (Ait.) Willd./ Italy	Di.Va.P.R.A.	AM269833	AM269775	
G. resinaceum	FGR1	*Fagus sylvatica* L./ Italy	Di.Va.P.R.A.	AM269834	AM269776	

Table 1 Continued

Species	ID collection	Host/geographic origin	Source*	GenBank Accession numbers		
				Nuc LSU (5′)	ITS	Mt SSU
G. resinaceum	FGR3	*Populus nigra* L./ Italy	Di.Va.P.R.A.	AM269835		
G. resinaceum	FGR5	*Aesculus hippocastanum* L./ Italy	Di.Va.P.R.A.	AM269836	AM269777	
G. resinaceum	G4/13	Unknown/ Italy	Di.Va.P.R.A.	AM269837	AM269778	
Ganoderma sp.‡	SP11	*Umbellularia californica* (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269827	AM269768	
Ganoderma sp.‡	SP13	*Umbellularia californica* (Hook. & Arn.) Nutt./ USA (CA)	UC Berkeley	AM269838		
Ganoderma sp.‡	SP16	*Quercus agrifolia* Née/ USA (CA)	UC Berkeley		AM269769	
Ganoderma sp.†			EMBL–EBI	AF255149		
Ganoderma tsugae Murril†			EMBL–EBI	AX78778; AY684163		
Gloeocystidiellum porosum (Berk. & M.A. Curtis) Donk†¶			EMBL–EBI	AF310101		
G. porosum†¶			EMBL–EBI		AY048881	
Hericium coralloides (Scop.) Pers.	HHB-9082-Sp	*Acer saccharum* Marsh/ USA (MI)	USDA–FPL	AM269840		
Hericium erinaceum (Bull.) Pers.	654	Unknown/ Czech Republic	CAS–IM	AM269839	AM269779	
H. erinaceum	JHO-62-149	*Acer saccharum* Marsh/ USA (MI)	USDA–FPL	AM269841		
H. erinaceum†			EMBL–EBI	AJ406493	AY534601	
Hohenbuehelia tristis G. Stev.†¶			EMBL–EBI	AF135171		
Hypoxylon thouarsianum var. *thouarsianum* (Lév.) Lloyd‡	SP106	*Lithocarpus densiflorus* (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley	AM269842	AM269780	
H. thouarsianum var. *thouarsianum*‡§	SP8	*Lithocarpus densiflorus* (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley			
Inonotus andersonii (Ellis & Everh.) Nikol.§	557	Unknown/ Czech Republic	CAS–IM			
I. andersonii	L(61)11-14-C	*Quercus velutina* Lam./ USA (OH)	USDA–FPL		AM269781	
I. andersonii‡	SP23	*Quercus wislizeni* A. DC./ USA (OH)	Phyt. Res.	AM269843		
I. andersonii	T1545	Unknown/ USA (OH)	USDA–FPL	AM269844		
I. andersonii†			EMBL–EBI	AY059041	AY558599	
Inonotus dryophilus (Berk.) Murril	703	Unknown/ Czech Republic	CAS–IM	AM269847		
I. dryophilus	L(61)5-20-A	*Quercus prinus* L./ USA (OH)	USDA–FPL	AM269846	AM269783	
I. dryophilus‡	SP25	*Quercus agrifolia* Née/ USA (CA)	Phyt. Res.	AM269845	AM269782	
I. dryophilus†			EMBL–EBI	AF311012		
Inonotus hispidus (Bull.) P. Karst.‡ §	FIH2	*Malus sp.*/ Italy	Di.Va.P.R.A.			
I. hispidus	FP-106082-T	*Quercus phellos* L./ USA (MS)	USDA–FPL	AM269848	AM269784	
I. hispidus†			EMBL–EBI	AF311014		
Inonotus radiatus (Sowerby) P. Karst. §	276	Unknown/ Czech Republic	CAS–IM			
I. radiatus†			EMBL–EBI	AF237732		
Inonotus rheades (Pers.) Bondartsev & Singer†			EMBL–EBI	AF311019		

Table 1 Continued

Species	ID collection	Host/geographic origin	Source*	GenBank Accession numbers		
				Nuc LSU (5′)	ITS	Mt SSU
Inonotus tamaricis (Pat.) Maire†			EMBL–EBI	AF311021		
Laetiporus sulphureus (Bull.) Murril§	FLS1	*Prunus cerasus* L./ Italy	Di.Va.P.R.A.			
L. sulphureus	FLS2	*Prunus cerasus* L./ Italy	Di.Va.P.R.A.	AM269849		
L. sulphureus	FP-101671-T	*Quercus sp.*/ USA (WI)	USDA–FPL	AM269851	AM269786	
L. sulphureus‡	SP35	Unknown/ USA (CA)	USDA–FPL	AM269850	AM269785	
L. sulphureus†				AY218414; AY684162	AF229196; AY835668	
Laxitextum bicolor (Pers.) Lentz¶	NO-7316-Sp	*Salix sp.*/ USA (LA)	USDA–FPL		AM269787	
L. bicolor†¶			EMBL–EBI	AF287871		
Perenniporia fraxinea (Bull.) Ryvarden	DP6	*Fagus sylvatica* L./ Italy	Di.Va.P.R.A.	AM269852	AM269788	
P. fraxinea	DP83	*Robinia pseudoacacia* L./ Italy	Di.Va.P.R.A.	AM269853	AM269789	
P. fraxinea	FPF1	*Platanus hybrida* Brot./ Italy	Di.Va.P.R.A.	AM269854	AM269790	
P. fraxinea	FPF2	*Robinia pseudoacacia* L./ Italy	Di.Va.P.R.A.	AM269855	AM269791	
P. fraxinea	FPF3	*Robinia pseudoacacia* L./ Italy	Di.Va.P.R.A.		AM269792	
P. fraxinea	FPF5	*Ulmus pumila* L./ Italy	Di.Va.P.R.A.	AM269856	AM269793	
P. fraxinea	FPF6	*Robinia pseudoacacia* L./ Italy	Di.Va.P.R.A.		AM269794	
Perenniporia ochroleuca (Berk.) Ryvarden†¶			EMBL–EBI	AY515330		
Perenniporia subacida (Peck) Donk†¶			EMBL–EBI		AY089739	
Phaeolus schweinitzii (Fr.) Pat.‡¶	SP39	*Picea sitchensis* (Bong.) Carr./ USA (CA)	UC Berkeley	AM269857		
Phellinus gilvus (Schwein.) Pat.‡	SP18	*Quercus agrifolia* Née/ USA (CA)	UC Berkeley	AM269858	AM269795	
P. gilvus‡	SP20	*Quercus agrifolia* Née/ USA (CA)	UC Berkeley	AM269859	AM269796	
P. gilvus†			EMBL–EBI	AY059025	AY089739	
Phellinus igniarius var. *cinereus* L. (Quél.)	575	Unknown/ Czech Republic	CAS–IM	AM269860	AM269797	
P. igniarius var. *cinereus*†			EMBL–EBI	AF287884	AF110991	
Phellinus pini (Brot.) Bondartsev & Singer	578	Unknown/ Czech Republic	CAS–IM	AM269861	AM269798	
Phellinus punctatus (Fr.) Pilát	262	Unknown/ Czech Republic	CAS–IM	AM269863	AM269800	
P. punctatus	DP25	*Tilia x vulgaris* Heyne/ Italy	Di.Va.P.R.A.	AM269862	AM269799	
P. punctatus†			EMBL–EBI	AF311007		
Phellinus robustus P. Karst (Bourdot & Galzin)	587	Unknown/ Czech Republic	CAS–IM		AM269802	
P. robustus	RLG-9585-T	*Salix sp.*/ USA (MN)	USDA–FPL	AM269864	AM269801	
P. robustus†			EMBL–EBI	AF311008		
Phellinus torulosus Pers. (Bourdot & Galzin)	759	Unknown/ Czech Republic	CAS–IM	AM269865	AM269803	
P. torulosus†			EMBL–EBI	AF311041		
Phellinus tremulae (Bondartsev) Bondartsev & Borissov	243	Unknown/ Czech Republic	CAS–IM		AM269804	
P. tremulae†			EMBL–EBI	AF311042		
Phellinus tuberculosus (Baumg.) Niemelä	265	Unknown/ Czech Republic	CAS–IM	AM269866	AM269805	

Table 1 Continued

Species	ID collection	Host/geographic origin	Source*	GenBank Accession numbers		
				Nuc LSU (5′)	ITS	Mt SSU
P. tuberculosus	DP40	*Prunus pissardi* Carrière/ Italy	Di.Va.P.R.A.		AM269806	
P. tuberculosus†			EMBL–EBI	AF311043		
Pleurotus ostreatus (Jacq.) P. Kumm.	2470	Unknown/ Italy	Di.Va.P.R.A.	AM269868		
P. ostreatus§	FP-101798-Sp	*Populus tremuloides* Michx./ USA (WI)	USDA–FPL			
P. ostreatus‡§	SP29	*Alnus rubra* Bong./ USA (CA)	UC Berkeley			
P. ostreatus‡	SP37	*Alnus rubra* Bong./ USA (CA)	UC Berkeley	AM269867		
P. ostreatus†			EMBL–EBI	AY645052	AY636055	
Pleurotus populinus Hilber & Miller†			EMBL–EBI		U04080	
Pleurotus pulmonarius (Fr.) Quél.¶	JPL-531-Sp	*Abies lasiocarpa* (Hook.) Nutt./ USA (AZ)	USDA–FPL	AM269869	AM269807	
P. pulmonarius†			EMBL–EBI	AY450349	AY368669	
Pseudoinonotus dryadeus (Pers.:Fr.) Murr.	FP-105836-4	*Quercus alba* L./ USA (OH)	USDA–FPL	AM269870	AM269808	
P. dryadeus†			EMBL–EBI	AF311011		
Pycnoporus sanguineus (L.) Murril†¶			EMBL–EBI		AF363759	
Schizophyllum commune Fr.	DP61	*Acer pseudoplatanus* L./ Italy	Di.Va.P.R.A.	AM269871	AM269809	
S. commune	Jacquiot	*Carya sp.*/ France	USDA–FPL	AM269872		
S. commune†			EMBL–EBI	AJ406555	AF249379; AF249386; AY573544; AY636062	
Schizophyllum radiatum (Sw.) Fr.	CBS-301·32	Unknown/ USA (CA)	USDA–FPL	AM269873		
S. radiatum†			EMBL–EBI	AY571023		
Stereum hirsutum (Willd.) Pers.	DP49	*Prunus pissardi* Carrière/ Italy	Di.Va.P.R.A.	AM269874	AM269810	
S. hirsutum‡	SP9	*Lithocarpus densiflorus* (Hook. & Arn.) Rehder/ USA (CA)	UC Berkeley	AM269875		
S. hirsutum†			EMBL–EBI	AY039330	AY854063	
Stereum rugosum Pers.	388020	Unknown/ UK	CABI–NCWRF	AM269876	AM269811	
Stereum sanguinolentum (Alb. & Schwein.)†			EMBL–EBI		AY618670; AF533962	
Stereum sp.†			EMBL–EBI	AF506483	AY207328	
Stereum subtomentosum Pouzar†			EMBL–EBI		AF506482	
Trametes cervina (Schwein.) Bres. §	FP105490-Sp	*Quercus sp.*/ USA (MD)	UC Berkeley			
Trametes gibbosa (Pers.) Fr.†			EMBL–EBI	AF291371		
Trametes hirsuta (Wulfen) Pilát†			EMBL–EBI		AY534110; AY787683	AF042154
Trametes. pubescens (Schumach.) Pilát†			EMBL–EBI	AY515341		
Trametes suaveolens (L.) Fr.†			EMBL–EBI	AF261537		U27079
Trametes versicolor L Loyd	2473	Unknown/ Switzerland	Di.Va.P.R.A.	AM269878	AM269814	AM269880
T. versicolor§	DP37	*Platanus acerifolia* Willd./ Italy	Di.Va.P.R.A.			
T. versicolor	Mad-697	*Fagus grandifolia* Ehrh./ USA (VT)	USDA–FPL		AM269813	
T. versicolor‡§	SP27	*Picea sitchensis* (Bong.) Carr./ USA (CA)	UC Berkeley			
T. versicolor‡	SP33	*Alnus rubra* Bong./ USA (CA)	EMBL–EBI	AM269877	AM269812	
T. versicolor†			EMBL–EBI	AY684159	AY636060	AF042324; U27080
Trametes villosa (Sw.) Kreisel†			EMBL–EBI			AF042325

Table 1 Continued

Species	ID collection	Host/geographic origin	Source*	GenBank Accession numbers		
				Nuc LSU (5′)	ITS	Mt SSU
Trametes zonatella Ryvarden§	FTZ1	*Quercus robur* L./ Italy	Di.Va.P.R.A.			
Trichaptum abietinum (Dicks.) Ryvarden†¶			EMBL–EBI	AY059063		
Trichaptum biforme (Fr.) Ryvarden¶	L-15822-Sp	Unknown/ USA (NY)	USDA–FPL		AM269815	
Trichaptum fuscoviolaceum (Ehrenb.) Ryvarden¶	L-15378-Sp	*Abies balsamea* (L.) P. Mill./ USA (NY)	USDA–FPL	AM269879	AM269816	

*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 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 Molecular Biology Laboratory – European Bioinformatics Institute nucleotide sequences database; Phyt. Res., Phytosphere Research, Vacaville, CA (USA); UC Berkeley, Department of Environmental Science, Policy and Management, Berkeley (USA); USDA–ARS, United States Department of 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 Institute for Forest, Snow and Landscape Research, Birmensdorf (Switzerland).

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

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

§Collections exclusively used for taxon-specific primers testing.

¶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* 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.; *P. pulmonarius* for *Schizophyllum* spp.; *T. biforme* and *T. fuscoviolaceum* for *Inonotus/Phellinus*-group

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 WSL 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 fruit bodies by using potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) medium with 0·2 g l^{-1} of streptomycin sulfate. Prior to DNA extraction, isolates were subcultured in a 2% (w/v) liquid malt extract (ME; AppliChem GmbH, Darmstadt, Germany) medium for approximately 2 weeks at room temperature. Liquid cultures were harvested by filtration and lyophilized. Alternatively, mycelia were harvested from plugs inoculated on 16 cm^2 discs of gel-drying film (Promega, Madison, WI, USA) and incubated at room temperature on PDA plates for 2 weeks.

DNA extractions, PCR and sequencing conditions

DNA was extracted from lyophilized mycelia or dried fruit body specimens using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Hayden *et al.* 2004). Fifty milligrams of lyophilized tissue were 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 2 min) and thawing (at 75°C for 2 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) after the pulverization step previously described. The concentration of nucleic acid extracts was estimated by comparing 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 gel (AppliChem GmbH, Darmstadt, Germany).

Ribosomal DNA amplifications of the 5′-end portion of the nuc LSU and of the portion including ITSI and ITSII 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 ITSI, 5·8S, ITSII 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 $MgCl_2$, 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 1 ng of genomic DNA. Thermocycling was conducted as follows: 94°C for 1 min, 35 cycles of 93°C for 45 s, 58°C for 50 s, 72°C for 45 s with a 1 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), 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 for 10 s, 50°C for 5 s, 60°C for 4 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).

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 nontarget 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 structure 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 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 μmol 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).

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 the concentration of $MgCl_2$ (1·5 mmol l^{-1} and 3·0 mmol l^{-1}) and by adding 0·5 μg μl^{-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 primers flanking the longer amplicons or with a lower primer concentration (0·5 μmol l^{-1} or 0·25 μmol l^{-1}, respectively). Amplified DNA fragments were visualized on a gel containing 1% (w/v) of high resolution MetaPhor (Cambrex Bio Science Inc., Rock-

land, 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 μl DNA extracts solution (concentration about 200 ng μl^{-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 μl volume containing 1x PCR buffer, 1·5 mmol l^{-1} of $MgCl_2$, 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), 0·5 μg μl^{-1} of BSA and 1 μl of DNA dilution. The presence and intensity of 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 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.* 2006). 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 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 a 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. Because 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 ITSI 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 226–228 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, because 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 as a result of 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).

Table 2 Primer sequences, priming regions, melting 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	AAACCCCCATAATCCAATCC	56°C	ITS II	Reverse primer with the forward ITS3	185	*Armillaria* spp.	This study
Gano2R	TATAGAGTTTGTGATAAACGCA	55°C	ITS I	Reverse primer with the forward ITS1-F	226–228	*Ganoderma* spp.	This study
Heri2R	CAGCCCTTGTCCGGCAGT	61°C	nuc LSU	Reverse primer with the forward 25sF	200	*Hericium* spp.	This study
Hyme2R	TGCDCCCCCTYGCGGAG	60/64°C	nuc LSU	Reverse primer with the forward F115	111	*Inonotus/ Phellinus*-group	This study
HypoR	GCTACGCTTAGGGGATGCTA	60°C	ITS II	Reverse primer with the forward ITS3	219	*H. thouarsianum* var. *thouarsianum*	This study
LaetR	CCGAGCAAACGAATGCAA	54°C	nuc LSU	Reverse primer with the forward 25sF	146	*Laetiporus* spp.	This study
PerR	ATCTGCAAAGACCGGTAAGGT	60°C	ITS II	Reverse primer with the forward ITS3	152	*P. fraxinea*	This study
Pleu2R	AACCAGGAAGTACGCCTCAC	60°C	nuc LSU	Reverse primer with the forward 25sF	158	*Pleurotus* spp.	This study
Schi2R	CTCCAGCAGACCTCCACTTC	63°C	ITS II	Reverse primer with the forward ITS3	190	*Schizophyllum* spp.	This study
Ste2R	GTCGCAACAAGACGCACTAA	58°C	ITS II	Reverse primer with the forward ITS3	231–236	*Stereum* spp.	This study
TraR	TTCATAGTCTTATGGAAACCGC	58°C	mt SSU	Reverse primer with the forward MS1	220	*Trametes* 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 *et al*. (1990)
ITS4	TCCTCCGCTTATTGATATGC	56°C	nuc LSU	Reverse			White *et al*. (1990)
MS1	CAGCAGTCAAGAATATTAGTCAATG	61°C	mt SSU	Forward			White *et al*. (1990)

*Tm indicates the salt-adjusted melting temperature (see Howley *et al.* 1979).

Multiplex PCR development

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

PCR efficiency was comparable between simplex and multiplex reactions, and PCR amplification was not significantly affected by changing the concentration of $MgCl_2$ in PCR reactions (Fig. 1). Using optimized reaction parameters (Table 3), no cross-reactivity with nontarget 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 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 that the threshold of DNA detection ranged from 10^{-2} pg to 1 pg depending on the set of primers (Fig. 3).

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: 45 s at 95°C, 45 s at 55°C, 45 s at 72°C; 10 min final extension at 72°C
		Gano2R	*Ganoderma* spp.	
	F115	Hyme2R	*Inonotus/Phellinus*-group	
M2	ITS3	Armi2R	*Armillaria* spp.	5 min denaturation at 95°C; 35 cycles of: 45 s at 95°C, 45 s at 60°C, 45 s at 72°C; 10 min final extension at 72°C
	25sF	LaetR	*Laetiporus* spp.	
		Pleu2R	*Pleurotus* spp.	
		Heri2R	*Hericium* spp.	
M3	ITS3	PerR	*P. fraxinea*	5 min denaturation at 95°C; 35 cycles of: 45 s at 95°C, 45 s at 63°C, 45 s at 72°C; 10 min final extension at 72°C
		Schi2R	*Schizophyllum* spp.	
		Ste2R	*Stereum* spp.	
	MS1	TraR	*Trametes* spp.	

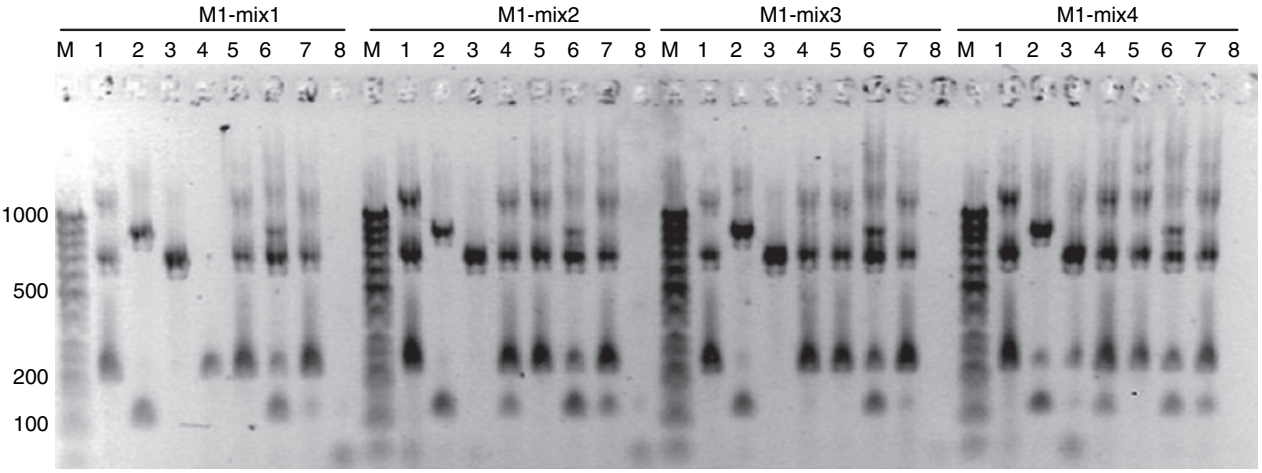

Figure 1 M1 PCR conditions assay. Comparison of PCR outcomes by using different primers and $MgCl_2$ concentrations. In the first and second reactions (M1-mix1 and M1-mix2), each primer is 0·5 μ mol l^{-1}, whereas in the mix 3 and 4 primers ITS1f and ITS4 are 0·5 μmol l^{-1}, primers Gano2R, Hyme2R and F115 are 0·25 μmol l^{-1}. $MgCl_2$ concentration in mix1 and mix3 was1·5 mmol l^{-1}; in the mix2 and 4 the $MgCl_2$ concentration was 3·0 mmol l^{-1}. The PCR assay was performed on 0·5 ng of *Ganoderma resinaceum* (lane 1), *Phellinus tuberculosus* (lane 2) and *Trametes versicolor* (lane 3) genomic DNA as well as on artificial mix containing 0·5 ng of *G. resinaceum* and *P. tuberculosus* (lane 4), of *G. resinaceum* and *T. versicolor* (lane 5), of *P. tuberculosus* and *T. versicolor* (lane 6) and of *G. resinaceum*, *P. tuberculosus* and *T. versicolor* (lane 7) genomic DNA. The negative controls of PCR reactions are loaded in the lane 8. M = Molecular weight marker GeneRuler 50 bp DNA ladder (DNA Ladder, Fermentas GmbH, St.Leon-Rot, Germany).

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 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 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

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

Species	ID collection	M1*	M2	M3	Species	ID collection	M1*	M2	M3
Agrocybe aegerita (V. Brig.) Singer†	DP12	–	–	–	*Omphalotus olearius* (DC.) Singer†	HHB-7441-Sp	–	–	–
Armillaria gallica	Napa-144;-141	–	185	–	*Omphalotus olivascens* H.E. Bigelow, O.K. Mill. & Thiers†	KPC-CA-1	–	–	–
Armillaria mellea	DP26; Mar-001;-016; T4D	–	185	–	*Oxyporus corticola* (Fr.) Ryvarden†	RLG-4894-Sp	–	–	–
Armillaria nabsnona	Men-017;-023	–	185	–	*Oxyporus latemarginatus* (Durieu & Mont.) Donk†	FP-101894-Sp	–	–	–
Baeospora myosura†	OKM-3708-Sp	–	–	–	*Oxyporus* sp.†	SP32	–	–	–
Bjerkandera adusta (Willd.) P. Karst.†	DP66	–	–	–	*Perenniporia fraxinea*	DP6; 83; FPF1; 2; 3; 5; 6	–	–	152
Crepidotus mollis (Schaeff.) Staude†	SP36	–	–	–	*Phaeolus schweinitzii*†	SP39	–	–	–
Fistulina hepatica (Schaeff.) With.†	FP-103444-T	–	–	–	*Phellinus gilvus*	SP18; 20	111	–	–
Fomitopsis cajanderi (P. Karst.) Kotl. & Pouzar†	SP34	–	–	–	*Phellinus igniarius* var. *cinereus*	575	111	–	–
Fomes fasciatus†	FP-1061048-T	–	–	–	*Phellinus pini*	578	111	–	–
Fomitopsis pinicola (Sw.) P. Karst.†	SP6	–	–	–	*Phellinus punctatus*	262; DP25	111	–	–
Fomitopsis rosea (Alb. & Schwein.) P. Karst.†	SP7		–	–	*Phellinus robustus*	587; RLG-9585-T	111	–	–
Ganoderma adspersum	DP87; DP60; FGA1	228	–	–	*Phellinus torulosus*	759	111	–	–
Ganoderma lucidum	GlTO99	228			*Phellinus tremulae*	243	111	–	–
Ganoderma lucidum	SP26	226	–	–	*Phellinus tuberculosus*	265; DP40	111	–	–
Ganoderma pfeifferi	G2/11	228	–	–	*Pleurotus ostreatus*	2470; FP-101798-Sp; SP29; 37	–	158	–
Ganoderma resinaceum	DP1; FGR1; 3; 5; G4/13	228	–	–	*Pleurotus pulmonarius*	JPL-531-Sp	–	158	–
Ganoderma sp.	SP11; SP13; SP16	228	–	–	*Pseudoinonotus dryadeus*	FP-105836-4	111	–	–
Hericium coralloides	HHB-9082-Sp	–	200	–	*Ramaria* sp.†	SP31	–	–	–
Hericium erinaceum	654; JHO-62-149	–	200	–	*Schizophyllum commune*	DP61; Jacquiot	–	–	190
Hohenbuehelia atrocoerulea (Fr.) Singer†	FP-102477-Sp	–	–	–	*Schizophyllum radiatum*	CBS-301·32	–	–	190
Hypoxylon thouarsianum var. *thouarsianum*	SP8; SP106	–	–	–	*Stereum hirsutum*	DP49; SP9	–	–	236
Inonotus andersonii	557; L(61)11–14-C; SP23	111	–	–	*Stereum rugosum*	388020	–	–	231
Inonotus dryophilus	703; L(61)5-20-A; SP25	111	–	–	*Trametes cervina*	FP105490-Sp	–	–	220
Inonotus hispidus	FIH2; FP-106082-T	111	–	–	*Trametes versicolor*	2473; DP37; Mad-697; SP27;33	–	–	220
Inonotus radiatus	276	111	–	–	*Trametes zonatella*	FTZ1	–	–	220
Laetiporus sulphureus	FP-101671-T; FLS1; 2;SP35	–	146	–	*Trichaptum biforme*†	L-15822-Sp	–	–	–
Laxitextum bicolor†	NO-7316-Sp	–	–	–	*Trichaptum fuscoviolaceum*†	L-15378-Sp	–	–	–
Lentinula edodes (Berk.) Pegler†	Ra-3-2E	–	–	–	*Tricholoma robustum* (Alb. & Schwein.) Ricken†	DP65	–	–	–
Lenzites betulina (L.) Fr. †	SP4	–	–	–					

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

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

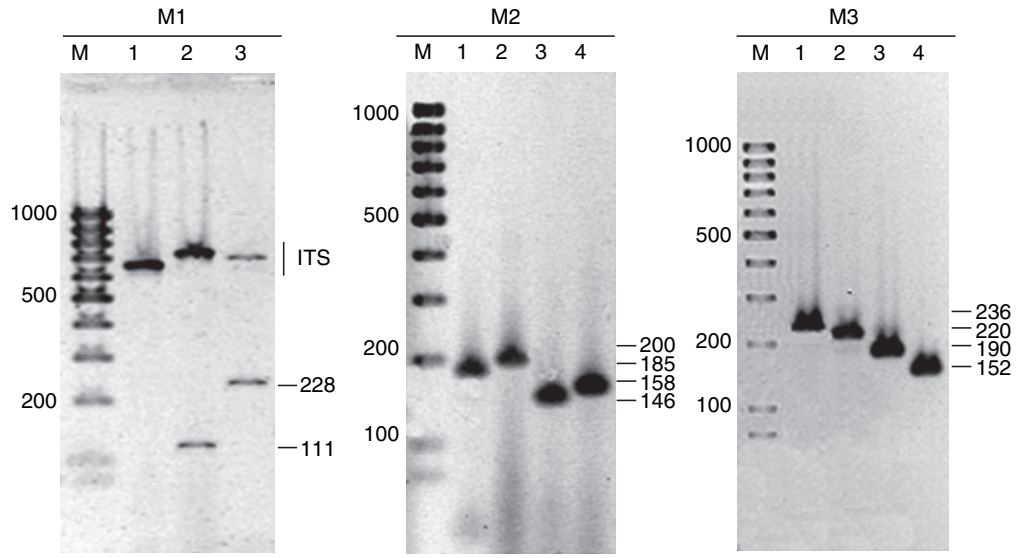

Figure 2 The results of M1, M2, M3 visualized on a UV-Gel documentation system after a 2 h electrophoresis at 4 V cm^{-1} on a 1% Metaphor 1% Standard agarose gel. M1: PCR products from DNA extracts of *Trametes* sp. (ITS band), of *Phellinus* sp. (ITS band + 111 bp) and *Ganoderma* sp. (ITS band + 228 bp) were loaded in the lanes 1, 2 and 3, respectively. M2: PCR products from DNA extracts of *Armillaria* 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, respectively. M3: PCR products from DNA extracts of *Stereum* sp. (236 bp), of *Trametes* sp. (220 bp), of *Schizophyllum* sp. (190 bp) and of *P. fraxinea* (152 bp) were loaded in the lanes 1, 2, 3 and 4, respectively. M = Molecular weight marker 100 bp DNA ladder are shown.

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 that 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 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 described here 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 contrary, *Inonotus* spp. and *Phellinus* spp. were considered as a unique target. Species-level diagnoses for the above two taxa are 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.* 2006). 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 because it is often used for resolving phylogenetic relationships at higher taxonomic level (Moncalvo *et al.* 2000; Binder and Hibbett 2002). One of the two

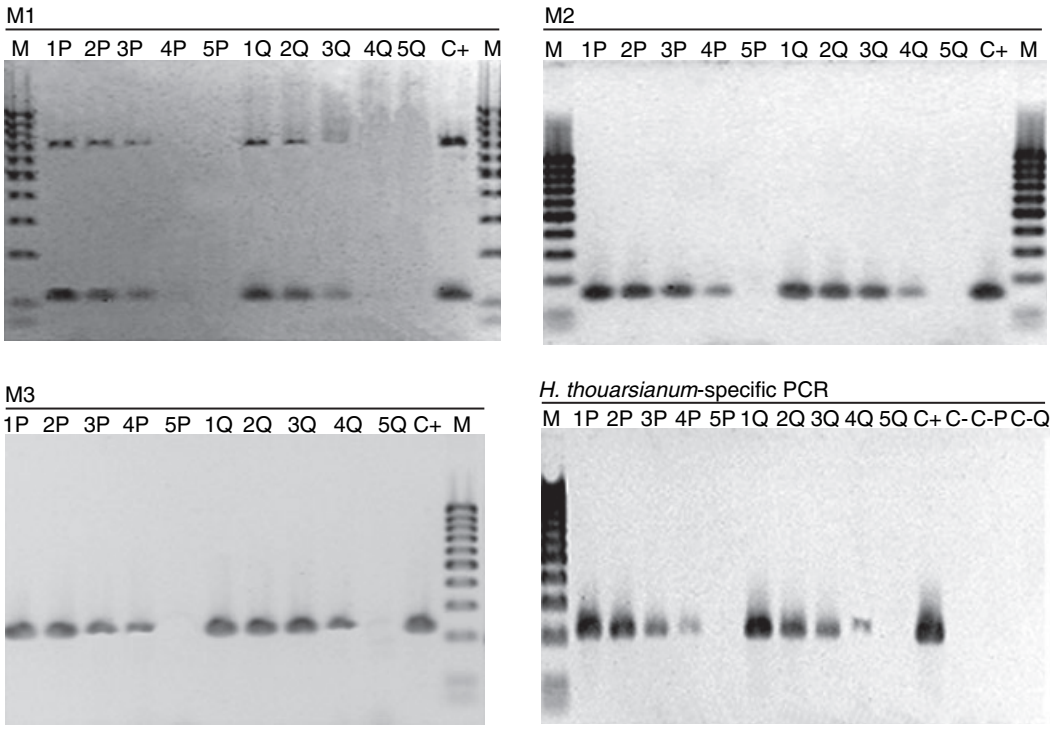

Figure 3 Outcomes from sensitivity assays performed for M1, M2, M3 and *H. thouarsianum*-specific-PCR on 10-fold dilutions of *Phellinus tuberculosus*, *Pleurotus ostreatus*, *Stereum hirsutum* and *Hypoxylon thouarsianum* var. *thouarsianum* DNA extracts, respectively. The dilutions were carried out in wood DNA extracts either from *Platanus hybrida* or from *Quercus agrifolia*. 1P = 100 pg fungal DNA in wood 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 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 wood DNA extract solution from *P. hybrida*; 1Q = 100 pg fungal DNA in wood DNA extract solution from *Q. agrifolia*; 2Q = 10 pg fungal 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 fungal DNA in wood DNA extract solution from *Q. agrifolia*; 5Q = 10^{-2} pg fungal DNA in wood DNA extract solution from *Q. agrifolia*; 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 = Molecular weight marker of 100 bp DNA ladder.

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

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

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

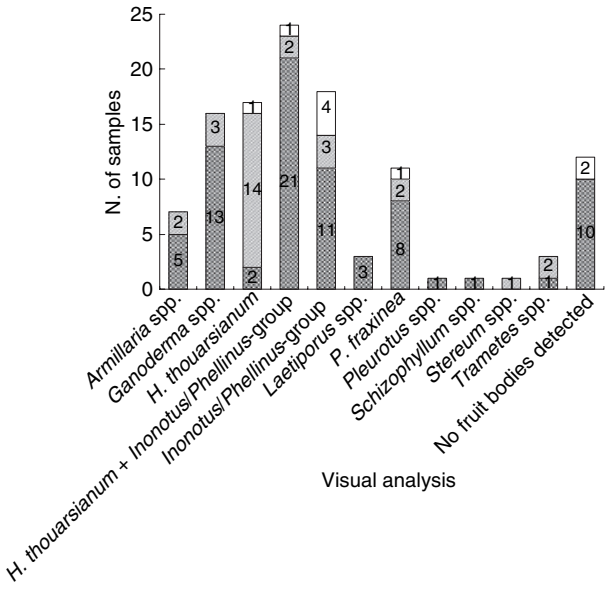

Figure 4 Efficiency of the method on wood samples from decay-affected trees. Comparison of the results from visual analysis to the multiplex PCR-based diagnosis. No fungi detected means no fungal rDNA amplification (□). No corresponding target taxon detected means no rDNA amplification of the target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

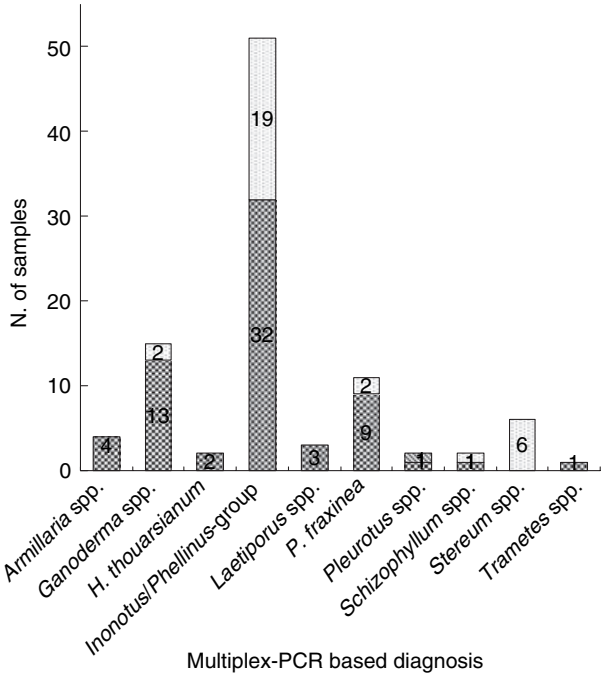

Figure 5 Specificity of the method on wood samples from decay-affected trees. Comparison of the results obtained from the multiplex PCR-based diagnosis to the outcomes from visual analysis and BLAST search analysis of the sequenced rDNA amplicon. ▧ No confirmation; □ no visual analysis confirmation but BLAST search analysis confirmation; ■ visual analysis confirmation.

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 nontarget 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 ITSI, instead. Because both intra-specific variation and heterotype variation in ITSI and ITSII among fungal isolates have been proved in other studies (O'Donnell 1992; Kauserud 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 amplicons matched the expected size for each target taxon in most instances. Although slight size variations were detected for *Stereum* spp. and *Ganoderma* spp. owing to INDELS , 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 a level of sequence polymorphism among the two groups is not surprising, because 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.* 1995). 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 favour the priming of primer with

the best sequence match by potentially increasing the reliability of PCR amplification as a result of 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 milligram of 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 melting 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 that 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 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 as wood saprophytes or soft rot agents, may thus reduce the efficiency of 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 the misidentification of 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 wood where *Hypoxylon* are 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 two such 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 of decayed wood portions suggests that this multiplex PCR-based method may be employed by arborists as a complement to VTA analysis. The method described here 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, because 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.

Acknowledgements

We gratefully acknowledge Jessie Micales at the USDA Forest Products Lab and Kendra Baumgartner at USDA Agricultural Research Service for the generous donation of several cultures. We also thank Ted Swiecki for assistance with identification of several collections and his support throughout the duration of this project. Particular thanks to Chiara Billi for her contribution to this study. This research was supported by a grant of the Comune di Torino-Settore Verde Pubblico and by a grant from the California Department of Forestry and Fire Protection.

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