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Summary

Ten taxon-specific primers were designed to amplify the Internal Transcribed Spacer of the rRNA operon of several important decay fungi of coniferous wood, including *Armillaria* spp., *Echinodontium* spp., *Fomitopsis pinicola*, *Fuscoporia torulosa*, *Heterobasidion annosum sensu lato* (*s.l.*), *Onnia* spp., *Phaeolus schweinitzii*, *Phellinus weirii s.l.*, *Pholiota* spp. and *Porodaedalea* spp. Primers designed in this study and in a previous one for the identification of *Laetiporus sulphureus* and *Stereum* spp. were combined in two multiplex PCRs, which were tested for efficiency and specificity, and detected at least 1 pg of fungal target DNA. Target DNA at concentrations of 10^{-1} pg or lower can be detected with this assay applying SYBR® Green Real Time PCR. Validation assays performed on 129 naturally infected wood samples or fruiting bodies confirmed the reliability of the multiplex PCR-based diagnostic method. This method represents a simple and rapid diagnostic tool for the detection of a number of destructive wood decay fungi in conifer wood.

1 Introduction

Wood decay fungi represent a functionally important component of forest ecosystems, due to their involvement in nutrient cycles, in the formation of specialized but important ecological niches, in the regeneration of forests, and in the improvement of soil quality (Garbelotto 2004; Odling-Smee et al. 2003; Laiho and Prescott 2004). Fungal diversity, species richness, as well as the presence of certain species, may all be used as indicators of forest health (Glaeser and Lindner 2011). A number of wood decay fungi have been identified as positive indicators for the creation and conservation of habitats for wildlife (Vasaitis 2013).

Conversely, several wood decay fungi are reported as aggressive pathogens causing losses in the productivity of commercial forests. To limit such damage, some species are regulated and even quarantined in some areas of the worlds (Williams et al. 1986; Hansen and Goheen 2000; Sinclair and Lyon 2005; Lannenpaa et al. 2008; Gonthier 2010; Garbelotto and Gonthier 2013). Although a range of management strategies can be implemented to minimize the damages caused by these fungi (Edmonds 2013), the effectiveness of such strategies is largely dependent on the infection biology and epidemiology of the fungal pathogen involved, hence the need for an accurate diagnosis (Glaeser and Lindner 2011). In addition to loss in productivity, structural deterioration of wood caused by decay fungi is deemed as one of the main factors leading to tree and limb failures (Lonsdale 1999; Giordano et al. 2013a), hence timely detection of potentially hazardous trees may allow prevention of such failures with a significant reduction of associated damage to properties and/or people. Knowledge of the fungal species involved in the decay process can be valuable in association with the Visual Tree Assessment (VTA) protocol (Mattheck and Breloer 1997) routinely employed to assess tree stability. Indeed, since different fungi may differ in their ability to colonize a tree, a correct diagnosis can be useful to predict, to some extent, the severity of fungal infection (Lonsdale 1999; Guglielmo et al. 2007). Fungi in fact differ in their rate of decay, in their ability to colonize the root system besides aerial portions of the tree, and some species can indiscriminately colonize both the sapwood and the heartwood of a tree: faster decayers, root colonizers, and

indiscriminate decay agents obviously represent greater threats to the stability of a tree (Lonsdale 1999; Guglielmo et al. 2007).

The detection and identification of wood decay fungi traditionally has been based on the analysis of macro- and micro-morphological features of either fruiting bodies, or pure cultures (Nicolotti et al. 2010). However, this diagnostic approach is often unreliable because fruiting bodies may be ephemeral or absent and because of the difficulties with fungal isolation from woody tissues (Nicolotti et al. 2010; Giordano et al. 2013b). Molecular methods based on DNA analyses can provide efficient, sensitive and rapid diagnostic tools for the detection and identification of wood decay fungi without requiring a prior fungal isolation step (Glaeser and Lindner 2011). DNA assays based on multiplex taxon-specific priming PCRs have been already developed for the detection and identification of several wood decay fungi commonly associated with broadleaved trees (Guglielmo et al. 2007; 2008; Nicolotti et al. 2009). These diagnostic assays, combined with an appropriate sampling method (Guglielmo et al. 2010), are suitable for rapid detection of several wood decay fungi for urban tree monitoring (Michelotti et al. 2012).

Although several studies have addressed the development of diagnostic PCR assays for wood decay fungi of conifers, most of them have been focused on the identification of a single species (Germain et al. 2002; Lim et al. 2005), whereas others may have been developed to differentiate related congeneric species (Hantula and Vainio 2003; Sicoli et al. 2003). Thus, a comprehensive assay for the diagnosis of the most widespread and hazardous wood decay fungi of conifers is still lacking. This study was aimed at developing a comprehensive and rapid molecular diagnostic tool for the detection and identification of twelve amongst the most widespread and harmful wood decay fungi of conifers in the northern hemisphere. More specifically, our aims included: (i) to design and/or test taxon-specific primers; (ii) to assess their sensitivity in both multiplex end-point PCRs and in SYBR® Green Real Time PCR assays; and (iii) to validate the diagnostic protocol based on these multiplex PCRs on environmental samples comprising either fruiting bodies or decayed wood samples collected from conifer trees.

2 Material and methods

2.1 Target wood decay fungi and fungal collections

The following twelve fungal taxa were selected as targets of the molecular diagnostic assay: *Armillaria* spp. (i.e. *A. borealis*, *A. cepistipes*, *A. mellea* and *A. ostoyae*), *Echinodontium* spp. (i.e. *E. tinctorium* and *E. tsugicola*), *Fomitopsis pinicola*, *Fuscoporia torulosa*, *Heterobasidion annosum sensu lato* (*s.l.*), *Laetiporus sulphureus*, *Onnia* spp. (i.e. *Onnia leporina* and *O. tomentosa*), *Phaeolus schweinitzii*, *Phellinus weirii s.l.* (i.e. *P. sulphurascens* and *P. weirii s.s.*), *Pholiota* spp. (i.e. *P. lenta*, *P. lubrica*, *P. scamba*, *P. spumosa* and *P. squarrosa*), *Porodaedalea* spp. (i.e. *P. cancriformans*, *P. chrysoloma*, *P. gilbertsonii*, *P. laricis*, *P. pini* and *P. yamanoi*) and *Stereum* spp. (i.e. *Stereum hirsutum* and *Stereum sanguinolentum*).

Fungal isolates belonging to the target taxa as well as to other fungal species commonly associated with wood of conifers were used to design and/or to test the taxon-specific primers (Table 1). Fungal isolates were obtained from fruiting bodies as previously described (Guglielmo et al. 2007). Fruiting bodies were collected in Italy and North America and identified through analytical keys (Breitenbach and Kränzlin 1986, 1995; Gilbertson and Ryvarden 1986; Allen et al. 1996; Bernicchia 2005). For several isolates identification was confirmed through nucleotide Basic Local Alignment Search Tool (BLASTn) analysis of the ribosomal DNA (rDNA) region comprising the Internal Transcribed Spacers (ITS) I and II. Further isolates were kindly provided by different European institutes (Table 1).

2.2 DNA extraction, PCR and sequencing

DNA extraction was performed through the CTAB-based method described by Guglielmo et al. (2007) on lyophilized fungal cultures that were grown at room temperature for at least one week in a 2% (w/v) malt extract liquid medium (AppliChem GmbH). The concentration and purity of DNA extracted from fungal mycelia were estimated through the analysis of absorbance at 260 and 280

nm using a spectrophotometer GENESYS 10 UV (Thermo Electron Scientific Instruments) as well as by comparing, through the Image Lab™ Software (Bio-Rad Laboratories), the genomic DNA with the quantified bands of GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific) after a 0.8% (w/v) standard agarose (Applichem GmbH) gel electrophoresis.

PCR assays aimed at amplifying the rDNA region comprising the ITS were performed in a 25 µl volume containing 1x of PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 0.5 µM each of the primers ITS1f and ITS4 (White et al. 1990; Gardes and Bruns 1993), 0.025U μl^{-1} of GoTaq® polymerase (Promega) and at least 0.1 ng of genomic DNA. PCR reactions were conducted as follows: an initial cycle with a 95°C denaturation step of 5 min, followed by 35 cycles, with each cycle consisting of a 95°C denaturation step of 45 s, a 55°C annealing step of 50 s and a 72°C extension step of 1 min, and one final cycle with a 72°C extension step of 10 min. The concentration of PCR products was estimated after a 0.8% (w/v) standard agarose (Applichem GmbH) gel electrophoresis as described for the quantification of genomic DNA.

PCR products were cleaned using Qia-quick purification kits (Qiagen), and cycle-sequenced with a Big Dye Terminator (BDT) v. 3.1 cycle-sequencing kit (Applied Biosystems) in a 5 µl volume containing 0.5x of 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 assays. Cycle sequencing was performed as follows: 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 adjusted to 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 through the software Sequencher 4.1.4 (Gene Codes Corporation). GenBank accession numbers of the ITS sequences obtained in this study are reported in Table 1.

2.3 Taxon-specific primer design and testing

Sequences obtained from the fungal isolates reported in table 1 as well as ITS sequences available in GenBank EMBL (http://www.ebi.ac.uk/) (Supplementary table 1) were used to design the taxon-specific primers. Outgroup species, i.e. phylogenetically closely related non target species, were: *Amylostereum chailletii* for *Echinodontium* spp. (Tabata et al. 2000); *Bondarzewia berkleyi* for *H. annosum s.l.* (Worrall et al. 2010); *Flammulina velutipes* for *Armillaria* spp. (Ronikier and Ronikier 2011); *Hypholoma fasciculare* for *Pholiota* spp. (Moncalvo et al. 2000); *Laetiporus persicinus* for *P. schweinitzii* (Lindner and Banik 2008); *Onnia leporina* for *Porodaedalea* spp. (Tomšovský et al. 2010); *Phellinidium fragrans* for *Phellinus weirii s.l.* (Wagner and Fischer 2002); *Phellinus senex* for *F. torulosa* (Jeong et al. 2005); *Piptoporus betulinus* for *F. pinicola* (Kim et al. 2005); *Porodaedalea chrysoloma* for *Onnia* spp. (Wagner and Fischer 2002). ITS sequences of the outgroup species were obtained from GenBank (Supplementary table 1).

Sequence alignments were performed through BioEdit v. 7.2.3 (Hall 1999) to detect ITS portions conserved within target taxa but displaying differences with respect to the outgroup species. On the basis of these multiple alignments, taxon-specific reverse primers were designed through the software Amplicon v. b09 (Jarman 2004) with the following features: (1) lack of homology with outgroup sequences, especially for differences at their 3'-end and/or presence of INDELs (insertions or deletions), and (2) no high self-complementarity and likelihood of hairpin loop formation. Additionally, taxon-specific reverse primers were developed to be used in multiplex PCR with a common forward primer, which was either ITS1f or ITS3 (White et al. 1990). For this purpose, primers within a multiplex PCR were designed to have similar melting temperatures, low likelihood of hetero-dimer formation and to amplify DNA fragments of different taxon-specific size.

The software PRIMER3 (Koressaar and Remm 2007; Untergrasser et al. 2012) and OLIGOANALYZER 3.0 (Integrated DNA Technologies, Inc.) were further used to assess the above-reported features. Nucleotide BLASTn analysis was performed to determine whether taxon-

specific primers displayed any significant homology with sequences derived from other non-target organisms.

In addition to these sets of primers, primers developed in a previous study (Guglielmo et al. 2007) were included in the assay to detect *Laetiporus sulphureus* and *Stereum* spp.

The specificity and efficiency of each primer were assessed in both uniplex and multiplex PCR assays performed on DNA extracts of the isolates listed in Table 1. Annealing temperatures were optimized using a thermocycling gradient and adjusted to the highest stringency. PCR conditions were the same as those used for the amplification of ITS. The size of any DNA fragment amplified after PCR was estimated, after electrophoretic migration (6 V cm^{-1}) on a gel containing 1% (w/v) of high-resolution MetaPhor (Cambrex) and 1% (w/v) of standard agarose, through the Image Lab™ Software on the basis of the GeneRuler 100 bp DNA ladder.

2.4 Sensitivity assays

The sensitivity of multiplex PCRs was estimated for each taxon-specific primer on known amounts of fungal DNA (ten-fold dilution series from 100 to 10^{-3} pg) diluted in a DNA extract solution (1 ng μl^{-1}) obtained from lyophilized wood of *Pinus pinea* through the QIAamp DNA Stool Mini Kit (Qiagen). Multiplex PCRs were performed on each fungal DNA dilution, as well as on wood DNA extract without fungal DNA as negative control. Sensitivity was assessed on one isolate per target taxon. The presence and intensity of multiplex PCR products were estimated after gel electrophoresis as described above.

The sensitivity of each taxon-specific primer was additionally estimated in SYBR® Green Real Time PCR assays on the same DNA dilutions. PCR reactions were performed in a 20 µl final volume containing 1x of the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Labratories, Inc.) and primers at a final concentration of 0.5 µM. Real time amplifications were carried out in a Connect™ Real Time PCR Detection System (Bio-Rad Laboratories, Inc) using the CFX Manager™ Software with the following parameters: initial denaturation and activation at 98°C

for 2 min followed by 39 cycles at 95°C for 5 s and at an annealing temperature optimized for each primer for 15 s. Fluorescence was determined at the end of each extension step. Parameters for the melting curve analysis were as follows: ramp from 65°C to 93°C with a temperature increment of 0.5°C and a read plate every 2 s. Melting curves were visualized by using the Precision Melt Analysis™ software (Bio-Rad Laboratories, Inc). To determine the PCR sensitivity and efficiency of the real time assays, a six-point standard curve was generated and the amplification results plotted with DNA dilution towards threshold cycle (Ct). Each fungal DNA dilution was tested in triplicate. The PCR efficiency was calculated from the slope of the line using the equation $E = 10^{-1/slope} - 1$.

2.5 Validation assay

Multiplex PCRs were validated on 129 environmental samples collected in Italian and North American woodlands and comprising 29 fungal fruiting bodies and 100 wood portions from live conifer trees displaying symptoms of advanced decay and belonging to the following species: *Abies concolor*, *A. magnifica*, *Calocedrus decurrens*, *Cedrus atlantica*, *C. deodara*, *Cupressus* sp., *Pinus halepensis*, *P. muricata*, *P. pinaster*, *P. pinea*, *P. ponderosa*, *P. sylvestris*, *Pseudotsuga menziesii* and *Thujopsis* sp.

DNA was extracted from 100 mg of lyophilized fungal fruiting body or wood sample through the QIAamp DNA Stool Mini Kit as described by Guglielmo et al. (2007). Before DNA extraction, lyophilized samples were frozen in liquid nitrogen and pulverized in 2 ml screw cap tubes by shaking for 30 s at 4.5 m s^{-1} in a FastPrep FP 120 Cell Disrupter (Qbiogene) with 6.35 and 2.00 mm-diameter glass beads. Multiplex PCRs were performed on a 1/100 dilution of each DNA extract. The detection of a target species was determined by the presence of unambiguous multiplex PCR products of the expected size after gel electrophoresis.

Taxon-specific amplicons obtained after multiplex PCRs were subjected to sequencing and BLASTn analysis, as described above, to exclude the occurrence of cross-reaction with other

species. When analysis of fungal fruiting bodies resulted in the presence of ITS amplicons but in the absence of amplicons specific to any target fungal taxa, sequencing of the ITS regions was performed as described above to exclude the presence of false negatives.

3 Results

3.1 Taxon-specific primers

Five taxon-specific reverse primers were designed on the ITS I region to amplify, in combination with the forward primer ITS1f, rDNA fragments of *Echinodontium* spp., *Fomitopsis pinicola*, *Phaeolus schweinitzii*, *Phellinus weirii s.l.* and *Porodaedalea* spp. (Table 2). Except for *Porodaedalea* spp., for which amplicon sizes ranged from 220 to 222 owing to inter- and intra-specific INDELS, each fungal taxon was characterized by an amplicon of a specific size (Table 2). These primers were combined in a multiplex PCR, named MC-1, which also included the universal reverse primer ITS 4 as a positive control to assess the efficiency of fungal DNA extraction (Table 2). The MC-1 also included the two primers 25sF and Laet2R designed by Guglielmo et al. (2007) to specifically amplify rDNA fragments of *Laetiporus sulphureus*. Using the optimized cycling parameters reported in Table 2, MC-1 produced taxon-specific amplicons, which were easily distinguished after a 1 h 45 min gel electrophoresis (Fig. 1).

Likewise, five taxon-specific reverse primers were developed to amplify, with the common forward primer ITS3, DNA fragments of the ITS II region of *Armillaria* spp., *Fuscoporia torulosa*, *Heterobasidion annosum s.l.*, *Onnia* spp. and *Pholiota* spp. (Table 2). These primers, along with the primer Ste2R previously designed for the detection of *Stereum* spp. (Guglielmo et al. 2007), were included in the multiplex PCR named MC-2 and allowed amplifying, with the optimized cycling parameters, taxon-specific DNA fragments easily distinguishable after a 1 h 45 min gel electrophoresis (Fig. 1). Except for *Pholiota* spp. and *Stereum* spp., for which amplicon sizes ranged from 308 to 315 and from 234 to 236 bp, respectively, each fungal taxon was characterized by an amplicon of a specific size (Table 2).

Taxon-specific primers allowed the correct identification, in both uniplex and multiplex PCRs, of the fungal target isolates collected in this study without cross-reacting with any of the other non-target fungal species (Table 1).

3.2 Sensitivity assays

The threshold of fungal DNA detection in a *Pinus pinea* wood DNA extract solution ranged from 10^{-1} to 1 pg depending on the set of primers used in both multiplex end-point PCRs MC-1 and MC-2 (Table 3).

The same taxon-specific primers used in SYBR® Green Real Time PCR assays allowed the detection of the amplified products, with a threshold cycle (Ct) ≤35, down to an initial fungal DNA amount ranging from 10^{-1} to 10^{-3} pg, depending on the taxon-specific primer (Table 3). PCR efficiency estimated from the best fit line calculated by linear regression analysis of standard curves (see Fig. 2 as an example) ranged from 99.3 % to 147.4 % (data not shown). Melting curve analysis supported the absence of any non-specific products after each taxon-specific priming PCR (Fig. 3). The resulting melting peaks were: 82.5°C for *O. leporina* OT-Slu; 83.0°C for *H. annosum s.s.* Cal1; 83.5°C for *A. ostoyae* Vald and *P. spumosa* Mut-1238; 84.0°C for *F. torulosa* isolate 759; 84.5°C for *F. pinicola* P2/118 and *L. sulphureus* isolate 2073-XIV; 85.0°C for *P. pini* 28; 85.5°C for *P. sulphurascens* Pa-22r and *S. sanguinolentum* 13-27; 87.0°C for *E. tinctorium* Aho-60-88-R; 87.5°C for *P. schweinitzii* TM2.1.

3.3 Validation assay

Multiplex PCRs allowed identification of target fungal taxa in 15 out of the 29 fruiting bodies and in 52 out of the 100 wood samples. Among the target fungal taxa, we detected *Armillaria* spp., *F. torulosa*, *F. pinicola*, *H. annosum s.l.*, *L. sulphureus*, *Onnia* spp., *P. schweinitzii*, *Porodaedalea* spp. and *Stereum* spp. (Fig. 4). BLASTn analysis of the sequence of taxon-specific amplicons confirmed the multiplex PCR identification in all the 67 samples that amplified target fungal taxa. Fungal

DNA not belonging to any of the fungal targets was detected in 58 samples, among which, after BLASTn analysis of fungal ITS, 13 fruiting bodies were confirmed as belonging to other taxa, i.e. *Fomitopsis cajanderi*, *Oligoporus guttulatus*, *Pholiota limonella*, *Pleurotus ostreatus* and *Sistotrema brinkmannii*. No fungal DNA amplification occurred from one fruiting body and from three wood samples.

4 Discussion

The taxon-specific primers designed in this study were developed to detect and identify, at different taxonomic ranks, ten important and/or widespread wood decay fungi of conifers from the northern hemisphere, including serious root and butt rot agents and comprising both white rot and brown rot fungi. These primers can be used in two multiplex end-point PCRs (MC-1 and MC-2) as well as in SYBR® Green Real Time PCR assays. Validation of multiplex PCRs on decayed wood samples indicated that this method can be an effective diagnostic tool with applicability ranging from forest disease management to ecological monitoring.

Depending on the fungus, we opted to design primers targeting either a higher, i.e. generic or sub-generic level, or a lower taxonomic rank, i.e. species level. Primers were developed at highest taxonomic ranks for taxa, i.e. *Armillaria* spp., *Heterobasidion annosum s.l.*, *Onnia* spp. and *Porodaedalea* spp. These genera include important wood rotting fungi for which identification at a species level can be inferred from the host, thanks to their host preference and/or geographical distribution, as well as by PCR-based methods validated in previous studies.

Armillaria is a complex genus encompassing about 40 biological species with a worldwide distribution and a broad host range, including shrubs, conifers and broadleaved trees (Pegler 2000). Since the *Armillaria* spp.-specific primer previously developed for the diagnosis of wood decay fungi of broadleaves didn't match completely the ITS sequences of *A. ostoyae* (Guglielmo et al. 2007), a novel taxon-specific primer, named Armi3R, was designed in this study to amplify the ITS sequences of *Armillaria* spp. commonly associated with conifers. Early detection of trees infected

by this pathogen can be important to strategies for limiting the spread of the disease in woodlands as well as to reduce hazardous windthrow in parkland and urban settings. Indeed, *Armillaria* spp. have been frequently reported as associated with uprooted conifer trees (Lonsdale 1999). Depending on the purpose of the diagnosis, since within *Armillaria* some species, i.e. *A. mellea* and *A. ostoyae*, have been reported as aggressive root rot pathogens, whereas others, i.e. *A. cepistipes* and *A. borealis*, can figure as secondary pathogens or weak parasites (Guillaumin et al. 1985), a further identification at a specific level may be advisable. The recent PCR-based diagnostic assay targeting elongation factor-1 alpha (EF-1 a) gene and allowing distinction of six European *Armillaria* species, including the two closely related species *A. ostoyae* and *A. borealis* (Mulholland et al. 2012), could be used for this purpose.

The reverse primer Het1r was designed for targeting to the species complex *Heterobasidion annosum s.l.*, which consists of three European, i.e. *H. abietinum*, *H. annosum sensu stricto* (*s.s.*) and *H. parviporum*, and two North American species, i.e. *H. irregulare* and *H. occidentale* (Niemelä and Korhonen 1998; Otrosina and Garbelotto 2010). *Heterobasidion annosum s.s.* and *H. irregulare* are mainly associated with root rot and mortality of *Pinus* spp., whereas *H. abietinum* is a pathogen of *Abies* spp., *H. parviporum* may be found almost exclusively on *Picea abies* and *H. occidentale* shows a preference for *Abies* spp., *Pseudotsuga* spp. and *Tsuga* spp. (reviewed by Garbelotto and Gonthier 2013). The management of such pathogenic species is similar as they share similar infection biology and epidemiological traits (Garbelotto and Gonthier 2013), hence an identification with the assay here described might be sufficient for decision-making. However, an accurate identification at a species level may be required when dealing with reforestation of heavily infested areas, where planting of tree species tolerant to the *Heterobasidion* species present in the site may be needed (Gonthier and Thor 2013). Several PCR-based assays are available and can be used to refine the diagnosis at a species level (Garbelotto et al. 1996; Gonthier et al. 2001; Hantula and Vainio 2003; Gonthier et al. 2007; Gonthier and Thor 2013). The primer designed in this study

to detect *H. annosum s.l.* may also amplify the Asian species *H. insulare* because ITS sequence didn't display sufficient divergence to discriminate the two taxa (data not shown).

The reverse primer Onn2r was developed for *Onnia*, which includes *O. tomentosa*, a root and butt rot pathogen distributed in the northern hemisphere and causing major economic losses in *Picea* spp. and *Pinus* spp. stands especially in North America, and *O. leporina* (= *O. circinata*), a related species mainly reported on *Pinus* spp. (Lewis 2013). Since symptoms of the disease and fruiting bodies of the fungus can take several years to appear, this pathogen is often detected at advanced stage of infection, when management options are limited (Germain et al. 2002). A timely detection of *Onnia* spp. in a forest stand may thus facilitate the management of this disease. Identification at the species level might be useful for stands of certain tree species, such as *Picea glauca*, where *O. tomentosa* has been reported as more aggressive than *O. leporina* (Germain et al. 2002). These two species can be easily distinguished by using the PCR-based approach developed by Germain et al. (2002).

A primer was designed for *Porodaedalea*, which includes pathogens causing white pocket rots of conifers that are economically important diseases in some areas of the world (Tomšovský et al. 2010). Species identified by the primer Por1r include: the European *P. chrysoloma*, *P. laricis* and *P. pini*, whose host preferences are *Picea* spp., *Larix* spp. and *Pinus* spp., respectively (Niemelä et al. 2005); the North American *P. cancriformans*, *P. gilbertsonii* and *P. pini*, mainly reported on *Abies* spp., *Pseudotsuga menziesii* and *Pinus* spp., respectively (Fischer 1996; Larsen 2000); the Asian *P. himalayensis*, *P. laricis*, *P. pini* and *P. yamanoi*, commonly associated with *Picea likiangensis*, *Larix olgensis*, *Pinus* spp. and *Picea jezoënsis*, respectively (Dai 1999). Depending on the purpose of the forest stand, i.e. timber production versus preservation of wildlife habitat, different management strategies have been described in case of detection of *Porodaedalea* spp. (Tainter and Baker 1996). An identification at an intermediate taxonomic rank using primers specific to a relevant group of congeneric taxa was deemed appropriate in tree cases, namely for *Phellinus weirii s.l.*, *Echinodontium* spp., and *Pholiota* spp. Primers were designed to an intermediate taxonomic rank

either because the fungi represented species complexes (i.e *P. weirii s.l.*) or because they were developed to target only species within the genera clearly associated with wood decay of conifers (for *Echinodontium* and *Pholiota*). The primer Pwe2r allows identifying *Phellinus sulphurascens* and *P. weirii s.s.*, previously known as the Douglas fir and the red cedar forms of *P. weirii s.l.* (Larsen et al. 1994). Both species are reported as aggressive pathogens of conifer trees causing the laminated root rot, an economically important disease in North America and eastern Asian countries (Lewis 2013). Since *P. weirii s.l.* is reported to spread very efficiently from tree to tree through root contacts and as fruiting bodies of the fungus are quite uncommon (Lewis 2013), the PCR-based diagnostic assay developed in this study can be useful to make more effective exclusion and eradication strategies, which are common methods used to prevent the introduction, and to limit the spread and the severity of the disease (Lewis 2013). It is worth noting that *P. weirii s.l.* is listed as an A1 pest recommended for quarantine in the area covered by the European and Mediterranean Plant Protection Organization (EPPO).

Within the genus *Echinodontium*, the primer Ech1r was developed to selectively amplify ITS sequences of *E. tinctorium* and *E. tsugicola*, two species causing white heart rot of living *Pinaceae* in North America and Japan, respectively. Within the wide and heterogeneous genus *Pholiota*, a primer was designed to detect a phylogenetically close group based on their ITS sequences (data not shown), and comprising several species reported as important wood decomposers of conifer stumps and root systems, i.e. *P. lenta*, *P. lubrica*, *P. mixta*, *P. scamba*, *P. squarrosa* and *P. spumosa*.

Finally, primers at a species level were developed for *Fomitopsis pinicola*, *Fuscoporia torulosa* and *Phaeolus schweinitzii* due to their relevance in tree stability and/or for their ecological role as saprobes. *Fomitopsis pinicola* has been reported as a mortality agent in pristine forests of north Eurasia and/or as an efficient wood decomposer (Vasaitis 2013). In fact, the fungus has been suggested as a bio-indicator of the recycling efficiency of nutrients (Glaeser and Lindner 2011). Although *F. torulosa* has been mainly found on broadleaved trees (Kotlaba 1975), it has been also reported to cause white root rot and stem rot of conifers (Panconesi et al. 1994), and it has been

frequently detected in uprooted *Cedrus* spp. in Italian parklands (Gonthier and Guglielmo, unpublished). Finally, *P. schweinitzii* has been reported as frequently associated with failures of conifer trees in parkland and urban settings (Lonsdale 1999; Lindner and Banik 2008), besides being an important root rot agent in forest ecosystems and a valuable indicator of forest value for conservation purposes (Bernicchia 2005; Vasaitis 2013).

Primers previously designed for the detection of *Laetiporus sulphureus* and *Stereum* spp. were successfully tested in this study on samples collected from conifers. It should be noted that both fungal taxa include populations or species, e.g. *Stereum sanguinolentum*, that are reported as destructive wound pathogens in either old natural or managed coniferous forests (Vasaitis et al. 2009; Vasaitis 2013).

Since this study aimed at developing a rapid diagnostic tool, taxon-specific primers were designed and/or tested to be used in multiplex PCRs allowing a simultaneous detection of twelve taxa in two reactions. In order to reduce the likelihood of hetero-duplex formation, which can compromise PCR efficiency and sensitivity, the number of primers was minimized by using common forward oligonucleotides in each multiplex PCR assay. In MC-1 a further reverse primer, i.e. ITS4, was added to amplify fungal ITS, thus providing an internal positive control for DNA extraction from fungal tissues. Moreover, in case of DNA extraction from environmental substrates, such as wood, ITS1f and ITS 4 allow detecting other fungal taxa not identified by taxon-specific primers (Guglielmo et al. 2007).

Multiplex PCR assays performed on fungal DNA diluted in *Pinus pinea* wood DNA extracts proved to be as sensitive as the multiplex PCRs developed for wood decay fungi of broadleaved trees (Guglielmo et al. 2007), with a minimum threshold of DNA amount varying from 10^{-1} to 1 pg depending on the target fungal DNA. Regardless of the efficiency of taxon-specific primers, these results suggested that QIAamp DNA Stool Mini Kit allows for an effective DNA extraction from wood of conifers, which can contain high amounts of extractives, such as resin acids, potentially inhibiting PCR reaction (Bahnweg et al. 1998). It should be noted that the wood of pines and in

particular of *P. pinea* is among the most resinous of all conifer tree species (Giordano 1986), hence our experimental conditions should be regarded as extreme with respect to inhibitors of PCR. The efficiency of the DNA extraction method was further confirmed by the low frequency of wood samples, i.e. three out of 100 analyzed during the validation assay, where no ITS amplification occurred.

Sensitivity assays performed in SYBR® Green Real Time PCRs proved taxon-specific primers can be appropriate for quantitative diagnostic analysis. Whilst quantification of wood decay fungi may not be crucial when analyzing woody substrates for diagnostic purposes, a real time PCR-based approach can be useful to determine the amount of fungal airborne inoculum. This approach, combined with a simple spore trapping method as described by Schweigkofler et al. (2004), can be relevant for the management of abundantly sporulating wood decay pathogens, such as *H. annosum s.l.*, and for epidemiological studies on wood decay fungi. Since melting curve analysis showed taxon-specific amplicons of different species to be characterized by similar and, in few cases, the same melting temperatures, SYBR® Green Real Time PCR should be used only in uniplex assays to avoid misidentifications.

The reliability of the multiplex PCRs developed in this study was further demonstrated through the validation assay performed on environmental samples, comprising either fruiting bodies or decayed wood collected from different tree species. Specificity of the molecular assay was inferred from the absence of amplicons of non-specific size as well as from the confirmation that all the taxon-specific amplicons belonged to the corresponding target taxon after BLASTn analysis. Moreover, efficiency of the method was verified by the absence of false negatives, determined by BLASTn analysis of ITS amplicons from fruiting bodies identified as not belonging to any of the target taxa. It should be noted, however, that the presence and frequency of false negatives has not been checked on decayed wood, since a laborious cloning step prior to sequencing would have been necessary and such approach may not have ensured the detection of all fungi present in the sample.

The multiplex PCR-based method developed for wood decay fungi of broadleaved trees (Guglielmo et al. 2007; 2008; Nicolotti et al. 2009) has been successfully used in urban tree monitoring (Michelotti et al. 2012) as well as for inferring the infection and colonization patterns of wood decay fungi (Guglielmo et al. 2012). The novel taxon-specific primers developed in this study aimed at providing a similarly comprehensive diagnostic tool specifically addressed to wood decay fungi of conifer trees. As suggested by the results of the validation assay, although a relevant number of important and widespread wood decay fungi are detectable by using this molecular method, several other fungi are not. Since the addition of new taxon-specific primers can reduce the multiplex PCR efficiency and make the diagnostic assay more laborious and time-consuming, alternative methods based on padlock probe technology can be developed to detect and identify a broader range of fungal taxa (Szemes et al. 2005). Nonetheless, this latter diagnostic approach requires costly instruments such as scan array systems and hence it may not be appropriate for all plant disease diagnostic laboratories.

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Table 1. Fungal isolates used for taxon-specific primer design and/or testing, and results of specificity assays.

Species	ID collection	Host-Geographical provenance	Source	GenBank accession number[1]	PCR – product size (bp)[2]	
					MC-	MC-2
Isolates used for taxon-specific primer design and/or testing						
Armillaria mellea	Cedro CN	*Cedrus* sp.-Italy	DISAFA		-	253
Armillaria mellea	T4D	*Vitis vinifera*-Switzerland	DISAFA	AM269762	-	253
Armillaria ostoyae	Vald	N/A-Italy	DISAFA		-	253
Echinodontium tinctorium	Aho-60-88-R	N/A-USA	ESPM	KF996509	254	-
Echinodontium tinctorium	FP-47304-T	N/A-USA	ESPM	KF996510	254	-
Echinodontium tinctorium	HHB-12844-5p	N/A-USA	ESPM	KF996511	254	-
Fomitopsis pinicola	DR-EST-011	N/A-USA	ESPM	KF996512	193	-
Fomitopsis pinicola	C-Joux	*Picea abies*-Italy	DISAFA		193	-
Fomitopsis pinicola	FFP2	*Abies alba*-Italy	DISAFA		193	-
Fomitopsis pinicola	FOMI 1	*Picea abies*-Italy	DISAFA		193	-
Fomitopsis pinicola	G-es	N/A-Spain	DISAFA	KF996513	193	-
Fomitopsis pinicola	M124	*Pinus sylvestris*- Sweden	SLU		193	-
Fomitopsis pinicola	P2/118	N/A-Italy	DISAFA		193	-
Fuscoporia torulosa	759	N/A-Czech Republic	DISAFA	AM269803	-	278
Heterobasidion abietinum	Ferrara 38EF	N/A-Italy	DISAFA		-	162
Heterobasidion abietinum	P137r	N/A-Italy	DISAFA		-	162
Heterobasidion annosum s.s.	Cal1	*Fagus* sp.-Italy	DISAFA		-	162
Heterobasidion annosum s.s.	Sib2	*Pinus sylvestris*-Russia	DISAFA		-	162
Heterobasidion irregulare	1116-1	N/A-USA	ESPM		-	162
Heterobasidion irregulare	38NA	N/A-Italy	DISAFA		-	162
Heterobasidion irregulare	Conk1	N/A-USA	ESPM		-	162
Heterobasidion parviporum	Cep7	*Picea abies*-Italy	DISAFA		-	162
Heterobasidion parviporum	EuS430.1	N/A-Finland	ESPM		-	162
Laetiporus sulphureus	A5a	*Picea abies*-Italy	DISAFA		146	-
Laetiporus sulphureus	A5b	*Larix decidua*-Italy	DISAFA		146	-
Laetiporus sulphureus	2073-XIV	*Quercus robur*-Italy	DISAFA		146	-
Onnia leporina	ICT	N/A-Italy	DISAFA		-	183
Onnia leporina	Phaeo1	*Pinus sylvestris*-Italy	DISAFA	KF996514	-	183
Onnia tomentosa	COLO-57-68-R	N/A-USA	ESPM	KF996515	-	183
Onnia tomentosa	FP-100585-5p	N/A-USA	ESPM	KF996516	-	183
Onnia tomentosa	LOO-13789-Q	N/A-USA	ESPM	KF996517	-	183
Onnia tomentosa	OT-Slu	*Picea abies*-Sweden	SLU	KF996518	-	183
Phaeolus schweinitzii	CeMiCa	*Cedrus* sp.-Italy	DISAFA	KF996519	242	-
Phaeolus schweinitzii	3-09	*Pinus pinea*-Italy	DISAFA	KF996520	242	-
Phaeolus schweinitzii	Bud-23-A	N/A-USA	ESPM	KF996521	242	-
Phaeolus schweinitzii	FP-94474-R	N/A-USA	ESPM	KF996522	242	-
Phaeolus schweinitzii	HHB-2054-5p	N/A-USA	ESPM	KF996523	242	-
Phaeolus schweinitzii	PCVV	*Pinus cembra*-Italy	DISAFA	KF996524	242	-
Phaeolus schweinitzii	PG4	*Pinus pinea*-Italy	DISAFA	KF996525	242	-
Phaeolus schweinitzii	PhaeoCi	N/A-Italy	DISAFA	KF996526	242	-
Phaeolus schweinitzii	TM2.1	*Pinus sylvestris*-Sweden	SLU	KF996527	242	-
Phellinus sulphurascens	Mat-9-2	N/A-USA	ESPM	KF996528	175	-
Phellinus sulphurascens	Pa-22r	N/A-USA	ESPM	KF996529	175	-
Phellinus chrysoloma	M175	*Picea abies*-Sweden	SLU	KF996530	220	-
Porodaedalea pini	14	*Pinus halepensis*-Italy	BPV	KF996531	220	-
Porodaedalea pini	28	*Pinus halepensis*-Italy	BPV	KF996532	220	-
Porodaedalea pini	578	N/A-Czech Republic	DISAFA		220	-
Porodaedalea pini	74-64-Z	N/A-USA	ESPM		220	-
Porodaedalea pini	PT4	N/A-Italy	DISAFA		220	-

Porodaedalea pini	FP-59059-T	N/A-USA	ESPM		220	-
Pholiota spumosa	Mut-1238	*Larix* sp.-Italy	DISAFA		-	313
Stereum hirsutum	DP49	*Prunus pissardi*-Italy	DISAFA		-	236
Stereum sanguinolentum	13-27	*Picea abies*-Italy	DISAFA	KF996533	-	234
Isolates used to test primer specificity						
Amylostereum areolatum	Oph2	*Picea abies*-Italy	DISAFA	KJ093491	-	-
Antrodia sp.	FP-100031-T	N/A-USA	ESPM	KF996534	-	-
Bjerkandera adusta	LG3	*Pinus sylvestris*-Italy	DISAFA	KJ093490	-	-
Chondrostereum purpureum	CP	N/A-Italy	DISAFA		-	-
Coniophora sp.	PN2	*Picea abies*-Italy	DISAFA		-	-
Fomitopsis officinalis	OKM-3807-T	N/A-USA	ESPM		-	-
Ganoderma carnosum	811	*Pinus sylvestris*-Czech Republic	DISAFA		-	-
Irpex lacteus	LG5	*Pinus sylvestris*-Italy	DISAFA	KJ093494	-	-
Lecanicillium lecanii	LG4	*Pinus sylvestris*-Italy	DISAFA	KJ093501	-	-
Marasmius sp.	Vald17a	*Fagus sylvatica*-Italy	DISAFA	KJ093495	-	-
Mycena aff. *murina*	D1.1c	*Picea abies*-Italy	DISAFA	KJ093496	-	-
Oudemansiella radicata	Vald20a	N/A-Italy	DISAFA		-	-
Peniophorella praetermissa	LG7	*Pinus sylvestris*-Italy	DISAFA	KJ093493	-	-
Phlebiopsis gigantea	13A	*Picea abies*-Italy	DISAFA		-	-
Resinicium bicolor	Ayma2C	*Picea abies*-Italy	DISAFA	KJ093498	-	-
Rhodonia placenta	B36	*Picea abies*-Italy	DISAFA	KJ093497	-	-
Schizophyllum commune	LG8	*Pinus sylvestris*-Italy	DISAFA	KJ093499	-	-
Thanatephorus cucumeris	LG6	*Pinus sylvestris*-Italy	DISAFA	KJ093500	-	-
Trametes trogii	LG1	*Pinus sylvestris*-Italy	DISAFA	KJ093492	-	-

BPV, Department of Biology and Plant Pathology of the University of Bari (Italy); DISAFA, Department of Agricultural, Forest and Food Sciences of the University of Torino (Italy); ESPM, Department of Environmental Science, Policy and Management of the University of California in Berkeley (USA); N/A, data not available; SLU, Department of Forest Mycology and Pathology of the Swedish University of Agricultural Sciences in Uppsala (Sweden).

[1]GenBank accession numbers are referred to sequences of ribosomal DNA including ITS1, 5.8S and ITS2. Empty rows refer to isolates used for primer testing only.

[2]We reported the size (in base pairs) of the taxon-specific amplified DNA fragments after the multiplex PCR 1 and multiplex PCR 2 assays for wood decay of conifer (MC-1 and MC-2, respectively). No amplification product with any taxon-specific primers is indicated with -.

Table 2. Multiplex PCR primers combination, diagnostic purpose and cycling parameters.

Multiplex PCR name	Primer combination		Taxon-specific amplicon size (bp)[4]	Target fungi	Cycling parameters
	Forward	Reverse[3]			
MC-1	ITS1f[1]	ITS4[1]	-	Fungi	5 min denaturation at 95°C; 35 cycles of: 30 s at 95°C, 45 s at 55°C, 45 s at 72°C; 10 min final extension at 72°C
		Ech1r (AGTTGTATTAAATGCGTGCG)	254	*Echinodontium tinctorium, E. tsugicola*	
		Psch2r (TTGTACGACATGCGTTAC)	242	*Phaeolus schweinitzii*	
		Pwe2r (ACGACAGACTTCGAAAAG)	175	*Phellinus sulphurascens, P. weirii s.s.*	
		Fpi1r (GAAGACCCCACTCCAATC)	193	*Fomitopsis pinicola*	
		Por1r (CACTACTAACAAAGTCAACC)	220-222	*Porodaedalea cancriformans, P. chrysoloma, P. gilbertsonii, P. himalayensis, P. laricis, P. pini, P. yamanoi*	
	25sF[2]	Laet2R[2]	146	*Laetiporus sulphureus*	
MC-2	ITS3[1]	Het1r (GCGCTTTCACAAGAAAAGC)	162	*Heterobasidion abietinum, H. annosum s.s., H. irregulare, H. occidentale, H. parviporum*	5 min denaturation at 95°C; 35 cycles of: 30 s at 95°C, 45 s at 62°C, 45 s at 72°C; 10 min final extension at 72°C
		Onn2r (AAGTCCCTTTTCCTTTCAGT)	183	*Onnia leporina, O. tomentosa*	
		Fto1r (GCAAGCTGCATCGGTGAA)	278	*Fuscoporia torulosa*	
		Armi3r (GCCTAGCAGCCARAGTCA)	253	*Armillaria borealis, A. cepistipes, A. mellea, A. ostoyae*	
		Phsq1r (GACAGTTAGAAGCGRTGCTA)	308-315	*Pholiota lenta, P. lubrica, P. mixta, P. scamba, P. squarrosa, P. spumosa*	
		Ste2R[2]	234-236	*Stereum hirsutum, S. sanguinolentum*	

[1]Nucleotidic sequences of primers ITS1f, ITS3 and ITS4 are reported in Gardes and Bruns (1993) and White et al. (1990).
[2]Nucleotidic sequences of primers 25sF, Laet2R and Ste2R are reported in Guglielmo et al. (2007).
[3]Nucleotidic sequences (5'→3') of reverse primers designed in this study are reported in brackets.
[4]Amplicon sizes are indicated in base pairs (bp); no amplicon size is reported for ITS4 since this primer allows amplifying, in combination with the primer ITS1f, fungal rDNA of different size depending on the taxon.

Table 3. Sensitivity assays performed on MC-1 and MC-2.

Multiplex PCR assay	**Species**	**ID isolate**	**DNA amount threshold[1]**	
			End-point PCR	**SYBR® Green Real-Time PCR[2]**
MC-1	*E. tinctorium*	Aho-60-88-	1 pg	10^{-1} pg
	P. schweinitzii	TM 2.1	1 pg	10^{-1} pg
	P. pini	28	1 pg	10^{-1} pg
	F. pinicola	P2/118	10^{-1} pg	10^{-2} pg
	P. sulphurascens	Pa-22r	10^{-1} pg	10^{-2} pg
	L. sulphureus	2073-XIV	1 pg	10^{-1} pg
MC-2	*P. spumosa*	Mut-1238	10^{-1} pg	10^{-2} pg
	F. torulosa	759	10^{-1} pg	10^{-2} pg
	A. ostoyae	Vald	1 pg	10^{-2} pg
	O. leporina	OT-Slu	1 pg	10^{-2} pg
	H. annosum s.s.	Cal1	10^{-1} pg	10^{-3} pg
	S. sanguinolentum	13-27	10^{-1} pg	10^{-2} pg

[1]Minimum amount of target fungal DNA diluted in wood DNA extract solution from *Pinus pinea* (details in the text).
[2]Only PCR products with a threshold cycle (Ct) ≤35 were regarded as positive.

Figure legends

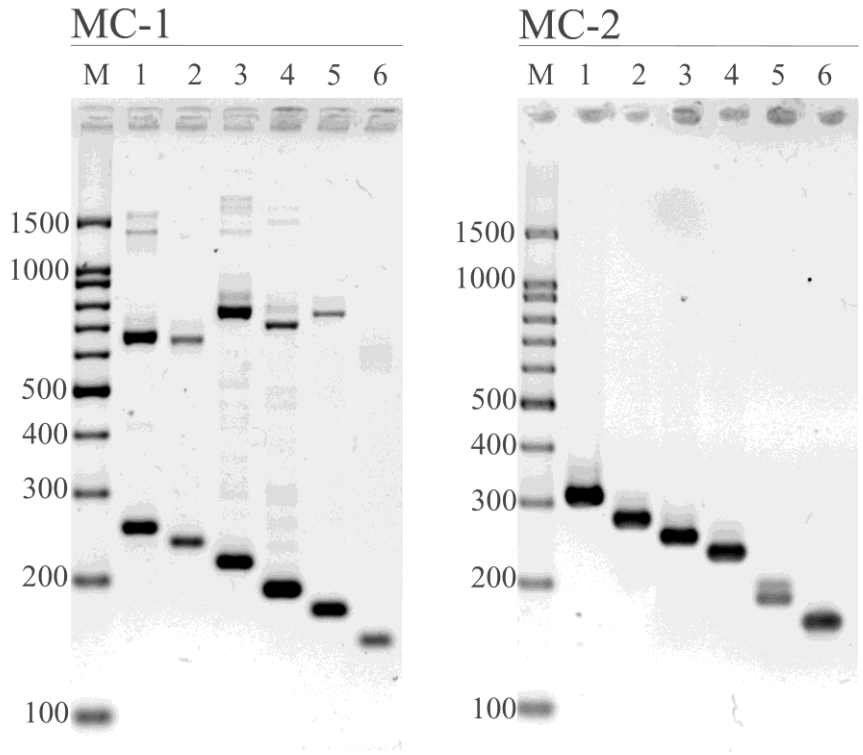
Fig. 1. MC-1 and MC-2 products visualized on a UV-gel documentation system after 1 h 45 min electrophoresis at 6 V min^{-1} on a 1% Metaphor-1% Standard agarose gel. MC-1: taxon-specific amplicons from DNA extract of *Echinodontium tinctorium* (254 bp), *Phaeolus schweinitzii* (242 bp), *Porodaedalea pini* (220 bp), *Fomitopsis pinicola* (193 bp), *Phellinus sulphurascens* (175 bp) and *Laetiporus sulphureus* (146 bp) were loaded on lanes 1, 2, 3, 4, 5 and 6, respectively. The upper bands present in each lane are PCR products deriving from the amplification of the fungal rDNA through the primers ITS1f and ITS4. MC-2: taxon-specific amplicons from DNA extract of *Pholiota spumosa* (313 bp), *Fuscoporia torulosa* (278 bp), *Armillaria ostoyae* (253 bp), *Stereum sanguinolentum* (234 bp), *Onnia leporina* (183 bp) and *Heterobasidion annosum s.s.* (162 bp) were loaded on lanes 1, 2, 3, 4, 5 and 6, respectively. M is the molecular weight marker 100-bp DNA Ladder.

Fig. 2. Standard curve displaying the linear relationship between the log 10 of known amount (pg) of genomic DNA of *Heterobasidion annosum s.s.* isolate Cal1 and Ct value obtained after SYBR® Green Real time PCR assay with the taxon-specific primer Het1r. Each DNA dilution was tested in triplicate (details in the text).

Fig. 3. Melting curves (A, B) and melting curve difference plots (after normalization and overlay) (C, D) of fungal isolates tested in MC-1 and MC-2. In detail, in (A) and (C) melting curves of *E. tinctorium* (□), *P. schweinitzii* (●), *P. pini* (♦), *F. pinicola* (△), *P. sulphurascens* (▼) and *L. sulphureus* (■) are shown; in (B) and (D) melting curves of *O. leporina* (●), *H. annosum s.s.* (△), *P. spumosa* (■), *A. ostoyae* (□), *F. torulosa* (▼) and *S. sanguinolentum* (♦) are shown. DNA amplicons are measured after SYBR® Green Real Time PCR. Melting curves in (A) and (B) are displayed as negative first derivative of the fluorescence-versus-temperature plot over the temperature (-d(RFU)/dT versus T). Differences in terms of relative fluorescence are easily visualized in plots (C) and (D) obtained by using the Precision Melt Analysis™ software.

Fig. 4. Results of multiplex PCR assays performed on 129 environmental samples collected in Italy and in North America. The number of samples positive to each target taxon is reported. The term "Other fungi" means absence of any taxon-specific amplicon but presence of fungal rDNA amplified through primers ITS1f and ITS4. "No fungi" means absence of any fungal rDNA amplified.

Fig. 1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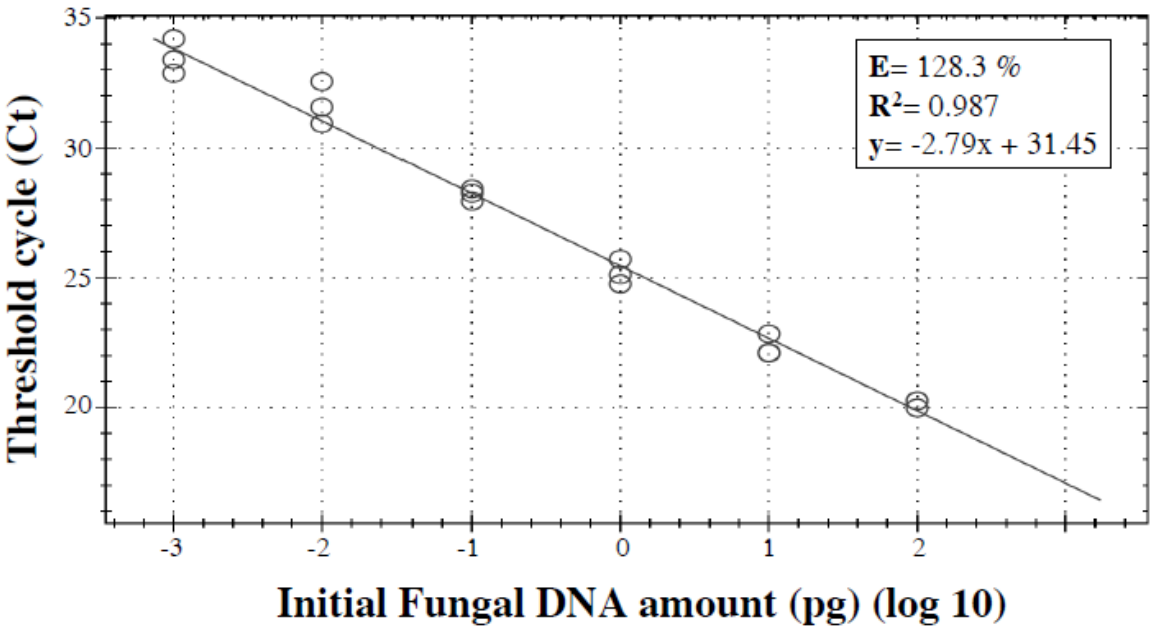
M 1 2 3 4 5 6
1500
1000
500
400
300
200
100
MC-2
M 1 2 3 4 5 6
1500
1000
500
400
300
200
100

42 Fig. 2

43

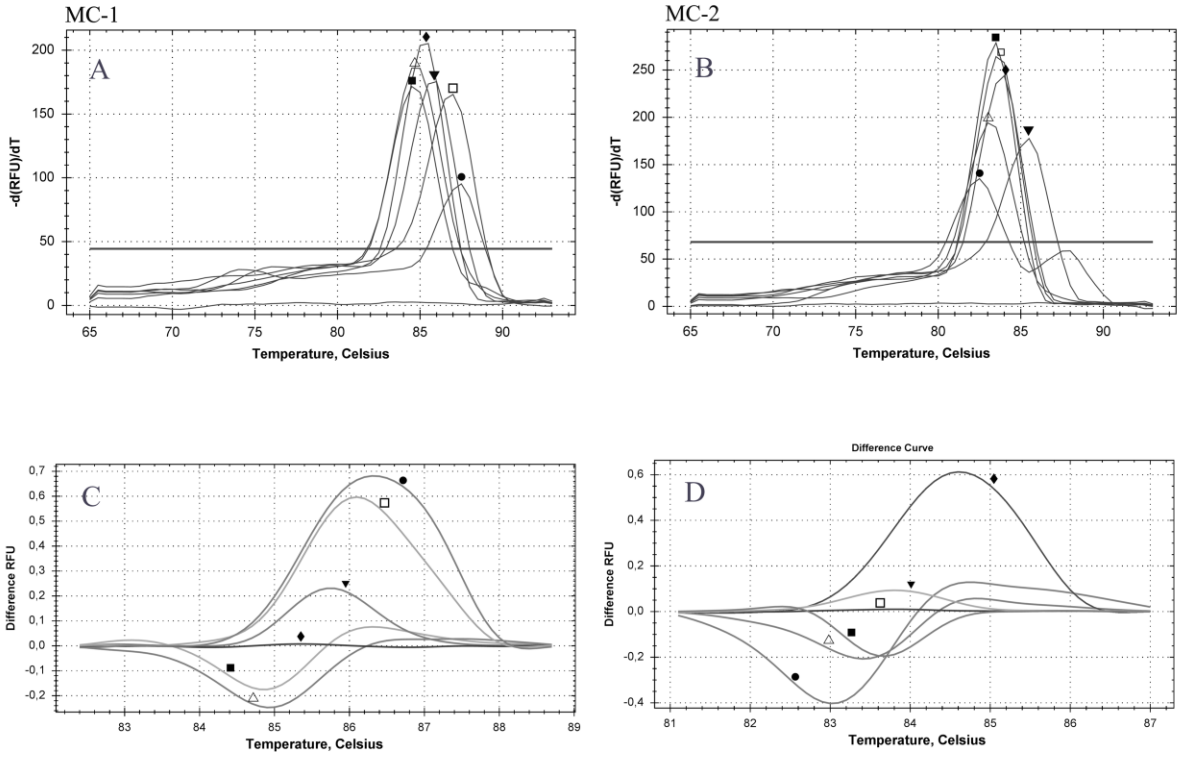


44

45

46

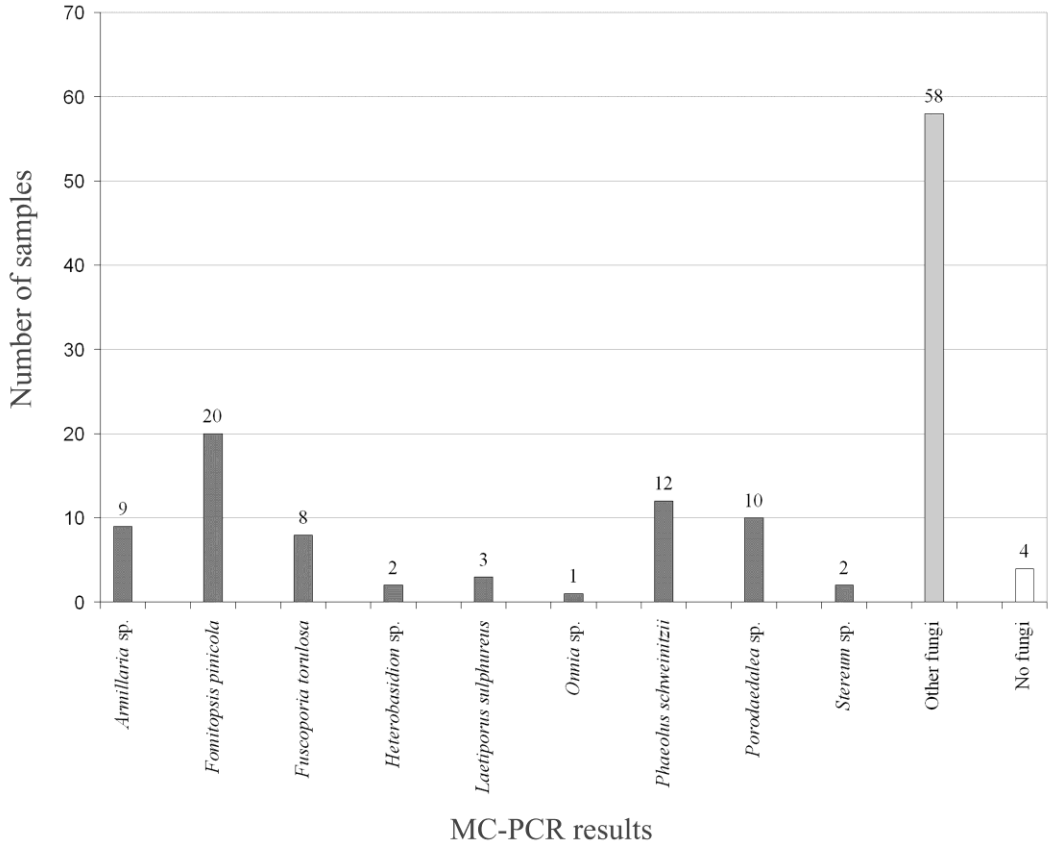
47 Fig. 3


MC-1
A
-d(RFU)/dT
Temperature, Celsius
MC-2
B
-d(RFU)/dT
Temperature, Celsius
C
Difference RFU
Temperature, Celsius
Difference Curve
D
Difference RFU
Temperature, Celsius

48

49

Fig. 4


Number of samples
70
60
50
40
30
20
10
0
9
20
8
2
3
1
12
10
2
58
4
Armillaria sp.
Fomitopsis pinicola
Fuscoporia torulosa
Heterobasidion sp.
Laetiporus sulphureus
Omnia sp.
Phaeolus schweinitzii
Porodaedalea sp.
Stereum sp.
Other fungi
No fungi
MC-PCR results