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Multiplex real-time PCR assays for the detection and identification of *Heterobasidion* species attacking conifers in Europe.

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Summary.

Four species of the destructive forest pathogen *Heterobasidion annosum sensu lato* (s.l.) are present in Europe: *H. annosum sensu stricto* (*s.s.*), *H. abietinum*, and *H. parviporum* are native species, while *H. irregulare* is a non-native invasive species currently reported only in Italy, yet recommended for regulation throughout Europe. In this study, we developed real-time PCR detection tests for each of the four species, which can be used simultaneously or individually thanks to probes labeled with species-specific fluorescent dyes. We evaluated the different performance criteria of each assay, and determined that they were theoretically capable of detecting amounts of DNA corresponding to 311, 29 and 29 cell nuclei in *H.*

annosum s.s., *H. irregulare*, and *H. parviporum*, respectively. The specificity of each assay was assessed with a wide set of strains. Real-time PCR tests successfully detected *Heterobasidion* species from 36 fruiting bodies taken from the forest, as well as from artificially inoculated or naturally infected wood samples. The multiplex real-time PCR assays developed in this study could have practical applications both in forest management and in phytosanitary monitoring.

Introduction

Many cryptic species (i.e. species from the same genus that are difficult to identify by morphological criteria) are described in fungi, and our knowledge on the number, biology and evolution of such species is still limited (Hawksworth & Lücking, 2017). However, the rapid and recent development of various molecular tools has considerably improved species identification, and consequently shed light on their geographic distribution and ecology (Desprez-Loustau et al., 2018). The genus *Heterobasidion* encompasses 13 fungal species, of which 12 can be grouped into two major species complexes. The first species complex is *H. insulare sensu lato* (s.l.), comprising the saprotrophic species *H. amyloideum*, *H. australe*, *H. ecrustosum*, *H. insulare* sensu typi, *H. linzhiense*, *H. orientale*, and *H. tibeticum* (Chen et al., 2015). The complex *H. annosum* s.l. comprises the phytopathogenetic species *H. abietinum*, *H. annosum sensu stricto* (s.s.), *H. irregulare*, *H. parviporum,* and *H. occidentale*, which are responsible for severe economic and environmental losses, and are deemed major threats for conifers in the Northern Hemisphere (Garbelotto & Gonthier, 2013). Depending on the host species, losses caused by *H. annosum* s.l. are associated either with root rot leading to tree mortality or with the development of heartwood decay in the roots, bole and stem, impairing the quality of wood and the stability of trees (Garbelotto & Gonthier, 2013). The different *Heterobasidion* species have been reported to show host preferences (Garbelotto & Gonthier, 2013). However, their ecology and geographic distribution overlap to a large extent (Garbelotto & Gonthier, 2013) and can co-occur in the same stands (Gonthier, 2019, Gonthier et al., 2001, Sedlák & Tomšovský, 2014). In addition, several studies have shown incomplete species barriers between some species with rare hybrids being found in forests (Sedlák & Tomšovský, 2014, Garbelotto et al., 1996, Gonthier & Garbelotto, 2011, Sillo et al., 2019).

In Europe, only species from *H. annosum* s.l. are present. *H. annosum* s.s. attacks mostly pines (*Pinus* spp.), although it may also be found on other hosts, including Norway spruce

(*Picea abies*) and even occasionally broadleaves (Garbelotto & Gonthier, 2013). *H. parviporum* and *H. abietinum* are mostly found associated with Norway spruce and firs (*Abies* spp.) respectively (Garbelotto & Gonthier, 2013). These three Eurasian species have been present for a long time in European forests (Dalman et al., 2010). Several *Heterobasidion* species are able to infect certain tree species like *Larix decidua*, *Pinus* spp., *Picea abies* and *Pseudotsuga menziesii* (Garbelotto & Gonthier, 2013). Occasionally co-infections of a tree by two *Heterobasidion* species have even been observed (Gonthier et al., 2003). The host specificity of these fungi is not as pronounced during saprobic growth as when they infect living trees, and several *Heterobasidion* species are able to colonize the stumps of a given tree species (Garbelotto & Gonthier, 2013). Besides these indigenous *Heterobasidion* species, the North American *H. irregulare* was introduced into a single site in Europe, probably during World War II (Gonthier et al., 2004, Garbelotto et al., 2013). Since then it has become invasive, spreading to both pine and oak stands along the Tyrrhenian coast in central Italy, often in association with significant mortality in Italian stone pine (*P. pinea*) (Gonthier et al., 2007, Gonthier et al., 2014, Gonthier et al., 2012). Furthermore, *H. irregulare* has been documented to hybridize with *H. annosum s.s.* in the central Italian invasion area (Gonthier & Garbelotto, 2011). In 2015, the threat posed by this species led the European Plant Protection Organization (EPPO) to add *H. irregulare* to the A2 list of pests recommended for regulation, needing the development of a robust, rapid and accurate method of detection in the field.

To our knowledge, several molecular tools have already been designed to target pathogenic species or groups of species in the *Heterobasidion* genus. Gonthier et al. (2015) designed an ITS rDNA-based primer pair to be used in conventional end point PCR or in a SYBR-Green real-time PCR, that enabled direct detection of all five species of *H. annosum* s.l. from environmental samples but that did not discriminate between the species. Hietala et al. (2003) developed a real-time assay to study the colonization of *H. annosum* s.l. in spruce based on polymorphisms occurring in a gene coding laccase. Their assay targeted indistinctly *H.*

parviporum and *H. abietinum*, but this test also amplified DNA from *H. annosum s.s.*, *H. araucariae* and *H. insulare*, although with higher Ct values. In addition, PCR-based assays targeting mitochondrial DNA have been developed to discriminate European species *H. abietinum*, *H. annosum s.s.* and *H. parviporum* (Gonthier et al., 2001, Gonthier et al., 2003). PCR-based assays based on the nuclear genome have also been developed to distinguish between *H. annosum s.s*. and *H. irregulare* (Gonthier et al., 2007). The ITS region was selected by Lamarche et al. (2017) to design a set of real-time PCR assays using hydrolysis probes with different levels of specificity to detect the species of *H. annosum* s.l. occurring in North America, namely *H. irregulare* and *H. occidentale,* and the *H. annosum* s.s. species. They also developed an additional test to amplify DNA from all five species of the *H. annosum* species complex. This tool has been used on some samples, such as airborne particles captured by silicone-made spore traps for monitoring purposes but not on woody samples (Lamarche et al., 2017). Recently, Sillo et al. (2018) focused on *H. irregulare*, which is emerging in Europe, and developed a LAMP assay that targets a specific region identified through comparative genomics, and that is not only suitable for a wide range of sample materials but is also fit for direct use in field.

Most of the above assays can be used for typing isolates and fruiting bodies, but are either unsuitable for or not validated on environmental (wood) samples. When *Heterobasidion* fruiting bodies are present, which is seldom the case when the trees are still alive (Giordano et al., 2015), macro- and micromorphological features overlap among species and hamper reliable species identification (Garbelotto & Gonthier, 2013). It is therefore necessary to isolate the fungus from woody tissues or fruiting bodies for identification, which is usually done by combining analyses of microscopic features in pure culture and barcode sequence analysis. However, isolating and culturing *Heterobasidion* from these types of tissue is time-consuming and not always successful, this depending on the freshness of the sample and on the presence of culture-competing and fast-growing saprotrophic species. Therefore, the

development of species-specific molecular markers that can be directly applied to environmental samples would be of great help for the rapid characterization of *Heterobasidion* populations. It should be noted that the identification of *Heterobasidion* species is of practical importance. After logging an infested forest, *Heterobasidion* species can persist in stumps for several decades and infect the next generation of trees when their root systems come into contact with the infected roots of a tree from the previous generation (Piri, 2003). A strategy deemed effective in stopping the accumulation of *Heterobasidion* inoculum in heavily-infested stands consists in changing the tree species, i.e. replacing susceptible species by a resistant one upon regeneration (Garbelotto & Gonthier, 2013). For this and other control strategies to be implemented, *Heterobasidion* spp. must be accurately diagnosed. No single assay allowing species-specific detection of all four *Heterobasidion* species present in Europe has been available until our study. Indeed, we have now developed and validated on a range of sample materials a new set of real-time PCR primers and probes to simultaneously and individually detect all four species of *Heterobasidion* found on the European continent.

Materials and methods

Fungal isolates

A total of 108 *Heterobasidion* isolates representing seven distinct species and two interspecific hybrids, were included in this study (Table 1). The first set of 67 isolates was cultured in ANSES on Potato Dextrose Broth (Difco) for 10 days at 21°C under constant shaking. The pellet of mycelium was then blotted onto sterile Whatman paper to eliminate the broth before being transferred into a 2-mL lysing matrix A microtube (MP Biomedicals) with 400 µL of lysis buffer AP1 (DNeasy Plant mini kit, Qiagen) and ground by shaking using a FastPrep 24 5G orbital shaker (MP Biomedicals) at 6.5 unit for 20 sec. The DNA was

extracted following the manufacturer's recommendations and eluted in 100 µL of AE buffer (Qiagen). The DNA concentration of each extract was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific), adjusted to 0.5 ng μL^{-1} and these work solutions were then kept at -20°C until analysis. The identity of the *Heterobasidion* isolates was confirmed by partial *RPB1* gene sequencing according to Chen et al. (2015) in addition to BLAST analysis with reference sequences of each of the four species available in GenBank.

A second set of 17 *Heterobasidion* isolates was tested by the University of Torino (Table 1). Approximately 200 mg of mycelium for each isolate, previously grown in 2% malt extract liquid medium at 25°C for seven days, was collected using a vacuum pump, lyophilized overnight and ground using two glass beads 0.4 mm in diameter in a FastPrepTM Cell Disrupter (FP220-Qbiogene). DNA was then extracted from mycelia using the EZNA Stool DNA Kit (Omega Bio-Tek, USA) according to Gonthier et al. (2015).

A third set of 24 *Heterobasidion* isolates was tested by the NIBIO-Norwegian Institute of Bioeconomy Research (Table 1). Approximately 20 mg of mycelium for each isolate, previously grown for ten days at 21°C on cellophane-coated 2% malt extract agar in Petri dish conditions, was harvested with a sterile surgical knife and ground manually in an Eppendorf tube with the aid of a plastic pestle and quartz sand. DNA was extracted from mycelia using protocol #8 ("Isolation of DNA from Mouse Tails") of the Easy-DNA Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The DNA concentration of each extract was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific), adjusted to 0.5 ng μL^{-1}, then kept at -20°C until analysis.

Nine fungal species that are frequently isolated from conifer roots and the collar were also included (Table 1). DNA was extracted from the mycelia of these isolates using the EZNA Stool DNA Kit (Omega Bio-Tek, USA) according to Gonthier et al. (2015).

179

180 **Environmental samples**

181 Fruiting bodies were collected in different regions of France and Italy. The DNA from
182 fruiting bodies obtained in France was extracted using the DNEasy Plant minikit as described
183 above for fungal isolates. The DNA from Italian fruiting bodies was extracted using the
184 EZNA Stool DNA Kit (Omega Bio-Tek, USA), as described by Sillo et al. (2018).
185 Environmental-like samples, composed of wood chips from three artificially inoculated *Pinus*
186 *sylvestris* logs, were included in the study: one had been inoculated with *H. annosum* (isolate
187 49SA), one with *H. irregulare* (isolate CP15) and one had not been inoculated (control).
188 Wood chips were obtained by drilling logs eight weeks post-inoculation (Sillo et al., 2018),
189 two biological replicates were prepared for each treatment.
190 Naturally infected environmental samples were also collected and tested. Wood chips were
191 sampled from five infected *P. pinea* trees located in the Gallinara pine plantation (Anzio,
192 Rome, IT). The presence of *Heterobasidion* spp. in these samples was confirmed through
193 molecular analysis performed using the tool developed by Gonthier et al. (2015). Three out of
194 five samples had tested positive to *H. irregulare* based on the LAMP assay described by Sillo
195 et al. (2018). DNA was extracted from wood chips using the EZNA Stool DNA Kit (Omega
196 Bio-Tek, USA) according to Gonthier et al. (2015). The final elution step was carried out in
197 150 µL of sterile water.

198

199 **Design of primers and hydrolysis probes**

200 Several phylogenetic markers useful for discriminating between *Heterobasidion* species were
201 assessed, such as *RPB1, GAPDH, ITS, EFA, GST1, ATP5* and *mtSSU* (Chen et al., 2015).
202 Other single-copy genes with high potential in fungal phylogenetics were also evaluated, such
203 as *Mcm7* and *Tsr1* (Schmitt et al., 2009) or *GH63* (Pérez-Izquierdo et al., 2017). Orthologous
204 sequences for all the genes used by Chen et al. (2015) and for the four target species were

retrieved from GenBank and aligned using Muscle (Edgar, 2004) implemented in Geneious software version R9 (https://www.geneious.com). A search for regions rich in species-specific single nucleotide polymorphisms (SNPs), and thus potentially suitable for the design of species-specific primers and probes, was conducted. Sequences of *Mcm7*, *RPB1*, *Tsr1* and *GH63* were generated by PCR using the sequencing primers described in Table 2, for a representative panel of *H. abietinum* (four isolates), *H. annosum s.s.* (five isolates), *H. irregulare* (six isolates) and *H. parviporum* (four isolates), as indicated in Table 1. Partial gene sequences were amplified by PCR in the conditions described by Matheny et al. (2002) for *RPB1*, Schmitt et al. (2009) for *Mcm7* and *Tsr1*, and Pérez-Izquierdo et al. (2017) for *GH63*. The sequences of the partial *Mcm7* and *RPB1* genes generated during this study were deposited in GenBank (Table 1).

A series of candidate primers / probe sets specific to each of the four target species was designed from polymorphic DNA regions using PRIMER 3 in Geneious software. The melting temperature, potential formation of secondary structures, and interactions among the oligonucleotide sequences were evaluated *in silico* using the same software and PriDimerCheking software (Shen et al., 2010). A BLASTN analysis of the NCBI database was used to evaluate primer and probe specificity against other closely related genome sequences.

Candidate primers and probes were first assessed using DNA extracts from a restricted panel of isolates, i.e. a representative set of four isolates of the four target species from different geographic origins (see Table 1), then retained for further validation using the whole set of DNA extracts available.

Construction of stabilized plasmid controls and limit of detection

To obtain stabilized positive controls for the real-time PCR reactions, the specific amplicon of each of the four target species was inserted in a plasmid using the TOPO TA cloning kit

(Invitrogen) according to the manufacturer's recommendations. For *H. abietinum*, *H. annosum s.s.*, and *H. irregulare*, the amplicon targeting a specific region of *Mcm7* was inserted, whereas for *H. parviporum*, the *RPB1* region was used as DNA insert. The plasmid solutions were purified, and the DNA concentration was measured using a Nanodrop 2000 spectrophometer (Thermo Scientific, Wilmington, DE, USA). The number of plasmid copies (pc) of DNA inserts could be determined from the DNA concentration measured and the molecular weight of the amplicon plus the plasmid sequence. For each specific plasmid, the raw plasmid solution was diluted in a ten-fold series, and the limit of detection for each test was determined as the lowest concentration of pc consistently yielding a positive result in a real-time PCR replicated ten times.

Verification of test specificity

Specificity, i.e. the ability to generate positive results with DNA from target species and negative results with DNA from non-target species, was assessed with the panel of strains from all *Heterobasidion* species presented in Table 1. Preliminary attempts to use all four primer/probe sets in a single PCR tube (quadruplex PCR) showed unacceptable loss of sensitivity for one or more of the species-specific tests. However, a triplex real-time PCR of *H. irregulare* and *H. parviporum* plus the Fungi-Quant test targeting the 18S rDNA of a broad range of fungal species (Liu et al., 2012), and a duplex real-time PCR of *H. abietinum* and *H. annosum s.s.* could be successfully achieved without compromising the sensitivity of each assay (data not shown). In France, the tests were conducted according to the following conditions using triplicate reactions for each template DNA. For *H. abietinum* ([Cy5] reporting dye) and *H. annosum* s.s. ([FAM] reporting dye), the duplex real-time PCR were performed in a final volume of 20 µL using the Core kit No Rox (Eurogentec, Seraing, Belgium) and containing 1× Polymerase buffer, 5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each forward and reverse primer, 0.1 µM of probe, 0.025 U/µL of HotGold Star DNA

polymerase (Eurogentec), 2 µL of template DNA (0.5 ng μL^{-1}), and molecular-grade water to 20 µL. For *H. parviporum* ([ROX] reporting dye) and *H. irregulare* ([JOE] reporting dye), the test included the FungiQuant primers/probe combination to be used as a DNA quality control ([FAM] reporting dye). The triplex real-time reactions were performed as described above for duplex, with the addition of the FungiQuant F/-R/-Prb primers/probe at the same concentration as for the target *Heterobasidion* species. Primer and probe characteristics are indicated in Table 2.

Real-time PCR was performed under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/elongation at 65°C for 55 s. In each reaction, the cycle threshold (Ct) value was determined using Rotor-Gene software version 1.7.75, and the threshold line was fixed manually at 0.02.

In Italy, a singleplex real-time PCR assay was performed. Primers and probes were synthesized by Eurofins Genomics. All the probes were labeled with [FAM] and [BHQ1]. The reaction mixture for real-time PCR was as described above in France except that another brand of master mix was used (GoTaq® Probe qPCR Master Mix, Promega). The final concentration of species-specific primers and probes was 0.3 µM and 0.1 µM, respectively, with the exception of the primers and probe for the FungiQuant assay, for which the concentration was 2 µM and 0.2 µM respectively. Real-time amplifications were carried out in a Connect Real-Time PCR Detection System (Bio-Rad Laboratories) using the CFX manager software (Bio-Rad Laboratories) with the same PCR cycling parameters described above. Ct values are the mean of two technical replicates.

In Norway, all the real-time PCR assays were conducted in singleplex conditions with primers and probes synthesized by Eurogentec. Probes were labeled with reporter dyes [FAM] (*H. annosum*, FungiQuant), [TAMRA] (*H. parviporum*, *H. irregulare*) or [ROX] (*H. abietinum*) and appropriate quenchers, [BHQ1] or [BHQ2]. The reaction mixture for real-time PCR was as described above in France except that Takyon Low Rox Probe MasterMix dTTP Blue

(Eurogentec) was used for assays with FAM-or TAMRA-labeled probes and the *qPCR Core kit No ROX (Eurogentec) was used for the ROX labeled assay*. Real-time amplifications were carried out in an Applied Biosystems ViiA 7 system (ThermoFisher) with standard, instead of fast, cycling and the same PCR cycling parameters as described above. Two technical replicates were prepared for each sample.

Assessment of performance criteria.

ANSES assessed the performance criteria for the real-time PCR assays in duplex for *H. abietinum*/*H. annosum* s.s. and in triplex for *H. irregulare*/*H. parviporum*/FungiQuant.

The analytical sensitivity was assessed using a 10-fold dilution series of the plasmid DNA (pDNA) positive controls diluted in 1× Tris-EDTA (10 mM Tris, 1 mM EDTA). The dilution series ranged from 112 10^6 to 112 pc mL^{-1}, 112 10^6 to 112 pc mL^{-1}, 88 10^6 to 88 pc mL^{-1}, and 82 10^6 to 82 pc mL^{-1} for *H. abietinum*, *H. annosum* s.s., *H. irregulare*, and *H. parviporum*, respectively. Each target was tested alone in the TE buffer. The limit of detection (LOD) was determined as the minimal quantity of target DNA that could be amplified by real-time PCR in 100% of cases. For each real-time PCR assay, a standard curve was constructed and the corresponding amplification efficiency was computed. The limit of detection was also determined with ten-fold serial dilutions starting from 0.5 ng μL^{-1} genomic DNA extracts from strains 1987-1661/4 (*H. parviporum*), FOM0132 (*H. irregulare*), LSVM975 (*H. abietinum*), and 1960/56/4 (*H. annosum s.s.*). The genome size of *H. annosum* s.s. and *H. irregulare* was estimated to be approximately 31 and 33 Mb respectively (Choi et al., 2017) and 33 Mb on average for *H. parviporum* (Zeng et al., 2018), whereas the genome size of *H. abietinum* remains to be determined. Since one pg of DNA corresponds to 965 Mpb (Bennett & Smith, 1976), it is therefore possible to estimate the quantity of DNA in one nucleus of *H. annosum* s.s. (0.0321 pg), *H. irregulare,* and *H. parviporum* (0.0341 pg).

The repeatability of each species-specific real-time PCR assay was evaluated with 10 replicates of different pDNA concentrations set at 10× LOD and 100× LOD, as well as a 1 ng mL^{-1} solution of the target species gDNA, all diluted in TE. The reproducibility was tested with one replicate of the same DNA concentrations during an individual run, plus three different runs over 2 weeks by three different operators and using two Rotor-Gene thermal cyclers. For each combination, the intra- and interassay coefficient of variation (CV) was computed.

To examine the robustness of the real-time PCR assay, i.e. its ability to withstand experimental variations without compromising sensitivity and specificity, several reaction parameters were deliberately modified and the assay was carried out using 10 replicates of different pDNA concentrations close to the limit of detection, i.e. 10× LOD and 100× LOD, as well as with 10 replicates of 1 ng mL^{-1} target species gDNA diluted in TE. To verify the effect on specificity, gDNA from the different target species was also included for testing. The robustness of the real-time PCR assay was challenged with a ±10% variation in the reaction volume or DNA template volume, and slight variations in the hybridization temperature (±2°C).

Statistical analyses were performed with R version 3.5.2. The normal distribution of the Ct values was tested by the Shapiro-Wilks normality test. Data were subjected to an ANOVA and pairwise differences between the mean Ct values were compared using Tukey's honestly significant difference test. Differences were considered statistically significant at $P < 0.01$.

Results

Design of species-specific oligonucleotides

Eleven phylogenetic markers were assessed for their interspecific polymorphisms, and alignments were scrutinized for the design of species-specific primers and probe. Based on *in*

silico predictions and analyses, regions *Tsr1, GH63, RPB2, GST1* and *GAPDH* were promising, but wet lab analyses using the restricted set of isolates showed either cross-amplification of non-target DNA or non-specific PCR products, and these regions were therefore discarded. Partial sequences of the *Mcm7* gene and of the *RPB1* gene showed numerous polymorphic regions for all four target species. For *RPB1* and *Mcm7,* the levels of intraspecific similarity observed were above 98.2% and 98.5 %, respectively, while the levels of interspecific similarity ranged from 95.7 to 98.4% and from 94.4 to 97.1%, respectively (Supplementary Table 1).

In addition, some of the single nucleotide polymorphisms observed were concentrated in certain regions of these genes making them of particular interest for the design of species-specific primers and probes. The design of primers and probes was manually adjusted to amplify short fragments (ca 150-300 pb) and the melting temperature, potential secondary structures, and interhybridization within and between all primers and probes were evaluated *in silico* using Geneious software. Finally, regions within *Mcm7* were chosen to design three primers/probe combinations specific to *H. abietinum*, *H. annosum* s.s. and *H. irregulare*, whereas a region within the *RPB1* gene was retained to design a primers/probe set specific to *H. parviporum* (Table 2). According to the sequencing results, the DNA target regions for each primer and probe were 100% conserved within the target species.

Verification of assay specificity

Specificity of the assays was verified with a wide panel of DNA from target and non-target species yielded in real-time PCR assays run in multiplex format or when performed individually by the University of Torino and NIBIO (Table 1).

The tests targeting *H. annosum* s.s., *H. irregulare* and *H. parviporum* yielded positive results with DNA from all the target strains included in the study (33, 14, and 29 strains tested, respectively), thus supporting their inclusivity and specificity. A double positive signal was

also obtained with the two hybrid *H. annosum* s.s. × *H. irregulare* strains from Italy, as expected (Table 1). These three assays neither cross-reacted with DNA from *H. araucariae* or *H. insulare*, nor with the nine other fungal species frequently associated with conifers (Table 1). However, a very late Ct value (39.33) was recorded with DNA from *H. occidentale* isolate II1A when running the test targeting *H. parviporum*, despite the occurrence of five mismatches between *H. parviporum* specific primers and probe, and the *H. occidentale RPB1* sequence (data not shown).

The test targeting *H. abietinum* successfully amplified DNA from the 24 target strains, regardless of origin (Table 1). For this test, negative results were obtained with DNA from all but one of the non-target *Heterobasidion* species. However, DNA from seven *H. parviporum* strains, i.e. isolates P162r and 2004-714 from Italy; 2004-676 from Montenegro; and 1998-1616/1, 1998-1622/2, 2004-676, and 2007/166/1 from Norway yielded unexpected positive results with the *H. abietinum* assay. These results were not anticipated based on the initial *Mcm7* sequencing since the *H. parviporum Mcm7* sequence displayed two, three to five, and two SNPs in the regions targeted by the *H. abietinum* specific forward primer (Habi For 4) , reverse primer (Habi Rev 14), and probe (Habi P 7), respectively. Yet the cross-detection was consistent and confirmed after repetitions. Following these unexpected results, genomic DNA was extracted from isolates P162r and 1998-1616/1, and the *RPB1* and *Mcm7* genes were sequenced using the primers sets described in Table 2. Sequencing results showed that the two isolates harbored an *RPB1* sequence 100% consistent with *H. parviporum*, whereas the *Mcm7* sequence showed double peaks at the interspecific polymorphic sites, suggesting the presence of two different alleles related to *H. parviporum* and *H. abietinum*.

Performance values

The real-time PCR tests successfully yielded 100% repeatable positive results with as little as 112, 112, 88 and 82 plasmid copies of target DNA per reaction for *H. abietinum*, *H. annosum*

386 *s.s*., *H. irregulare,* and *H. parviporum*, respectively. Therefore, these DNA concentrations
387 were considered as the LOD for each test and target species, and were subsequently used as
388 benchmarks for the experiments addressing repeatability, reproducibility, and robustness of
389 the real-time assay. The R^2 computed showed that all Ct values followed a linear regression
390 when plotted against the target concentration. The R^2 values for *H. abietinum*, *H. annosum*
391 s.s., *H. irregulare*, and *H. parviporum* were all 0.99, while the PCR efficiency (% E)
392 calculated from the slope ranged between 94.0 and 100.1%. The intra-assay and interassay
393 CVs indicated that the duplex (*H. annosum s.s*. and *H. abietinum*) and triplex (*H. irregulare,*
394 *H. parviporum*, and FungiQuant) real-time PCRs were highly repeatable and reproducible
395 with a mean Ct value coefficient of variation always below 3.5% (Table 3). The limit of
396 detection with genomic DNA from target species was estimated at 1, 10, 1, and 1 pg per PCR
397 tube for *H. abietinum*, *H. annosum* s.s., *H. irregulare*, and *H. parviporum* respectively.
398 According to the genome size of the three latter fungi, the limit of detection corresponds to
399 311 (*H. annosum* s.s.), 29 (*H. irregulare*), and 29 (*H. parviporum*) nuclei per PCR tube.
400 Unfortunately, the limit of detection could not be computed for *H. abietinum*, since the
401 genome size of this species remains unknown. However, assuming that it is close to those of
402 the three other species, the limit of detection of *H. abietinum* could be around 30 nuclei per
403 PCR tube.
404 The robustness of all four real-time tests was supported by the little variation in mean Ct
405 values observed with the modified volume and temperature settings. For each of the four
406 targets, the mean Ct values were sometimes significantly affected by a deliberate ±10%
407 variation in the reaction volume or the template DNA volumes, or by a ±2°C of the
408 hybridization temperature (Supplementary Table 2). However, under our experimental
409 conditions, artificially modifying the PCR parameters never increased the Ct values by more
410 than 3.5 cycles, while the maximum mean Ct values that were reached by modifying the real-
411 time PCR parameters always stayed below 40. These results meant that each pathogen would

still be detected under these disturbed conditions, even at the lowest concentration levels. In addition, for each primer/probe combination, no amplification was observed with DNA from the other three non-target *Heterobasidion* species tested, regardless of the modified conditions. No cross-reactions were observed between any of the four target species primer-probe combinations (data not shown) even under the conditions potentially decreasing specificity (10% reduction in reaction volume, 10% increase in template DNA volume, or −2°C decrease in hybridization temperature).

Lastly, when testing 0.5 ng L^{-1} genomic DNA extracts for the specificity assays (Table 1), variation in Ct values were observed within each species. Although it was not possible to assess the differences statistically because the sample size was too small and uneven, mean Ct values differed between laboratories: the delay was up to 7.5 cycles for *H. abietinum*, 7.7 cycles for *H. annosum* s.s., 10.5 cycles for *H. irregulare*, and 11.2 cycles for *H. parviporum* (data not shown). This delay in Ct value was even higher with the FungiQuant assay, with mean Ct values varying by up to 14.3 cycles between laboratories, thus suggesting a combined effect of the DNA extraction procedure, PCR master mix brand or real-time PCR platform on assay sensitivity.

Detection / identification of *Heterobasidion* species from fruiting bodies and from wood

It was possible to detect and identify the *Heterobasidion* species with all the types of material tested (Table 4). The expected species were successfully detected by real-time PCR for all four wood logs artificially inoculated with either *H. irregulare* (two) or *H. annosum* s.s. (two). In addition, *H. irregulare* and *H. annosum* s.s. were successfully detected in respectively three and two wood chip samples taken from symptomatic trees in Italy. A total of 36 fruiting bodies collected in French and Italian pine stands were also tested, and it was possible to identify the presence of either *H. abietinum*, *H. annosum s.s.* or *H. irregulare* for all of them. *H. abietinum* was identified from five fruiting bodies from France (four sampled

438 on *P. abies* and one on *Pseudotsuga menzeii*), whereas *H. annosum* s.s. was identified from
439 23 fruiting bodies from France (all of them except one sampled on *P. abies*). The eight
440 fruiting bodies from Italy that were previously assigned to *H. irregulare* by the LAMP test of
441 Sillo et al. (2018) were confirmed by the real-time PCR developed in our study.
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445 **Discussion**
446 To the best of our knowledge, no test was previously available to specifically and individually
447 detect the four *Heterobasidion* species attacking conifers in Europe, including the invasive *H.*
448 *irregulare*. In this work, ten phylogenetic markers were screened to find polymorphisms that
449 were specific to each of the four species of *Heterobasidion*. Two genes were finally found to
450 be suitable for the design of primers and probes to be used in real-time PCR, targeting DNA
451 from *H. annosum* s.s., *H. irregulare*, and *H. abietinum* (*Mcm7*) on the one hand, and from *H.*
452 *parviporum* (*RPB1*) on the other hand. The tests worked well either in singleplex or multiplex
453 reactions. They were validated on a variety of biological material: pure fungal cultures, wood
454 chips from colonized trees and fruiting bodies. The *RPB1* gene was used by Chen et al. (2015)
455 as a nuclear phylogenetic marker for *Heterobasidion.* Although the present study confirmed
456 its potential for species identification, it only enabled the successful design of
457 oligonucleotides specific to *H. parviporum.* The design of real-time PCR primers and probes
458 specific to *H. annosum* s.s., *H. irregulare*, and *H. abietinum* was possible using *Mcm7*,
459 another single-copy nuclear gene that was not previously used to study *Heterobasidion.*
460 However, *Mcm7*, referred to previously as *MS456,* had already proved to be a good
461 discriminator for fungal phylogeny (Aguileta et al., 2008). Schmitt et al. (2009) confirmed the
462 potential of this *Mcm7* gene for designing PCR or real-time PCR primers for a wide range of
463 fungi, especially in Ascomycota and it was recently used to design molecular tools targeting

closely related plant pathogenic species (Ahmed et al., 2018). Our study confirmed the great potential of this gene for the discrimination of species complexes from the phylum Basidiomycota. This gene also features clusters of single-nucleotide polymorphisms that are required for the design of specific primers and probes used in real-time PCR.

The two regions targeted by the multiplex real-time PCR assay developed here are present as single copy genes in the *Heterobasidion* genome. Standard curve interpolation may be used to quantify the DNA pools of each *Heterobasidion* species, which serve as a proxy for their biomass in environmental samples. The analysis of a DNA extract from a wood sample, for instance, would provide quantitative data and help us to study the competition between the different species when they co-occur on a similar substrate. However, we sometimes noticed a large variation between mean Ct values with DNA templates obtained from pure fungal cultures and tested at a standardized DNA concentration by the different laboratories involved in this work. This variation may be due to several factors, such as a non-standardized master mix chemistry, or differences in the real-time PCR platform and associated analysis software influencing assay sensitivity (Grosdidier et al., 2017). Other contributing factors could be the imprecise quantification of total DNA in the template solution, or a variation in the quality of DNA obtained with different DNA extraction kits (Bustin & Huggett, 2017, Ebentier et al., 2013). The quantitative values should therefore only be used and compared all other things being equal, which requires using an identical analysis protocol from DNA extraction to real-time PCR data analysis, coupled with the application of standard curves established by the same analytical chemistry and equipment.

The assays developed in this study can also be useful for detection of heterokaryotic hybrids between different species, since they can simultaneously detect DNA from different species of *H. annosum sensu lato*. As expected therefore, DNA from the two artificial *H. annosum* s.s. × *H. irregulare* hybrids generated by Giordano et al. (2018) yielded positive results with both the *H. annosum* s.s. and *H. irregulare* real-time PCR assays. Interestingly, in our analyses, a

few *H. parviporum* isolates previously identified as pure genotypes of this species tested positive in both *H. parviporum* and *H. abietinum* real-time PCR assays. The *RPB1* sequence obtained from two of these isolates confirmed that they shared 100% identity with a *H. parviporum RPB1*. Still, their genome also harbors at least a partial *H. abietinum Mcm7* sequence, according to the results of our tests and sequencing. Considering that isolate P162r was isolated from wood disks exposed in the forest in Charvensod (north western Italy) (Gonthier et al., 2001), where spores of *H. abietinum* may also be present, we could have been dealing with a heterokaryotic hybrid isolate harboring nuclei from both species. The interfertility rate between strains from sympatric populations of *H. parviporum* and *H. abietinum* actually ranges between 0.20-0.25 in this part of Europe but it has been proved that under laboratory conditions, the interfertility between *H. parviporum* and *H. abietinum* can be as high as 0.75 when pairing strains from allopatric populations (Garbelotto & Gonthier, 2013,). Therefore, some of the *H. parviporum* isolates showing signs of *H. abietinum* DNA may either be heterokaryotic isolates or offspring of interspecific crosses with traces of introgression, although we cannot completely exclude this outcome could be the result of contaminations occurred in the laboratories, where isolates and DNA of *H. abietinum* are manipulatedAs a little introgression may naturally occur within European *Heterobasidion* populations, our new molecular tools could help to clearly identify first-generation hybrids and accurately quantify their frequency within populations. We cannot exclude These results, and the weak cross reaction observed for *H. occidentale* DNA with the *H. parviporum* test, reflect the close phylogenetic relation between these species that diverged quite recently (Chen et al., 2015). Future studies should therefore clarify the frequency of hybridization among *Heterobasidion* species and shed some light on their viability in natural populations, with the help of other nuclear markers, such as the ones used for recently described *H. occidentale* × *H. irregulare* hybrids (Sillo et al., 2019). However, the observation of double positive signals following the *H. abietinum* and *H. parviporum* real-time PCR assays with

some DNA from some of our *H. parviporum* pure cultures suggests caution, and we recommend running all four species-specific assays for the analysis of environmental samples. In practice, double signals with DNA extracts from fruiting body or wood samples collected in the field, should be interpreted with care, although such cases were never encountered during our proof-of-concept experiments with infected wood and fruiting bodies. These new species-specific assays targeting each of the four European *Heterobasidion* species will have relevant practical applications, because they may be used to discriminate between fungal pathogens that are virtually indistinguishable from a macro- and micro-morphological perspective in the field (Garbelotto & Gonthier, 2013). Our multiplex tool may therefore have applications in forest management when considering change in tree species for stands heavily infested by *Heterobasidion.* These new species-specific assays may also be useful for studying the ecology of the different *Heterobasidion* species in sympatric areas, without needing any prior and sometimes uncertain isolation of the fungi. All the fruiting bodies or naturally infected wood chips from infected trees were successfully analysed and the causal species identified in this study, showing that the sensitivity of the assays was sufficient for working with real-life samples. The assays have been successfully used by ANSES over the last few months and have been shown to work with environmental samples of poor quality and unsuitable for morphological identification. As a proof of concept, the multiplex real-time PCR assays are currently being used in France as part of a project aiming to obtain a clear and reliable picture of the current situation by mapping the natural distribution of each species in mainland France. We detected in this first sampling mostly *H. annosum s.s.* on *P. abies* which is not usually the main host for this *Heterobasidion* species, *P. abies* being more often infected by *H. parviporum* (Gonthier et al., 2001). We also recently identified fruiting bodies of *H. abietinum* on *Betula* pendula, surrounded by severely infected Douglas firs, and probably reflecting a saprobic association (J. Hubert, ANSES, unpublished). This preliminary study illustrated the potential of the molecular tools developed here for screening the host

range of each *Heterobasidion* species in forests formed by different tree species. At the distribution margin, or under atypical or disturbed environments, fungal pathogens may colonize new hosts, and extend their ecological niches (Ennos, 2014). Central France - where most of the *Heterobasidion* fruiting bodies were sampled during our proof-of-concept study - is an interesting area where a change of forest composition has occurred as a result of the intensive plantation of conifer species during the 1950s. This human impact on forest ecosystem may explain the results observed here. Among the native European species, *H. annosum* s.s. is the most polyphagous and the most aggressive on pine, *Pinus sylvestris* having been one of the dominant conifer species in this French area before these massive plantations. Results obtained from environmental samples in Italy also showed the potential for describing the frequency of each species within stands without depending on fructification, which is less frequent during the first stages after infection and while the trees are still alive. Finally, with respect to the emerging species *H. irregulare*, our test will be of great benefit for quick identification of foci of infection, and for adoption of eradication and containment measures to prevent the further spread of this pathogen that is currently recommended for regulation in Europe.

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Table 1: Characteristics of the fungal strains used in the study and results of the real-time PCR tests carried out on the DNA extracts (Habi, *H. abietinum*; Hann, *H. annosum s.s.*; Hirr, *H. irregulare*; Hpar, *H. parviporum*, FQ, FungiQuant). For each strain, a standardized 0.5 ng µL^{-1} DNA concentration was used as a template for all experiments.

Species	Code	Origin	Real-time PCR test (mean Ct value ± SD)					GenBank accession	
			Habi	Hann	Hirr	Hpar	FQ	*RPB1*	*Mcm7*
Heterobasidion abietinum	16-562/3F[a]	-	22,33 ± 0,27	> 40	> 40	> 40	15,16 ± 0,10		
	2002-146[ad]	Austria	22,17 ± 0,03	> 40	> 40	> 40	15,73 ± 0,05	MK654910	MK729093
	17/058[a]	France	22,02 ± 0,36	> 40	> 40	> 40	14,74 ± 0,32	MK654910	
	17/059[a]	France	22,87 ± 0,66	> 40	> 40	> 40	15,72 ± 0,23	MK654910	
	Cou067[a]	France	21,51 ± 0,32	> 40	> 40	> 40	13,85 ± 0,15		
	FOM195[a]	France	21,54 ± 0,41	> 40	> 40	> 40	14,23 ± 0,36		
	LSV M975[ad]	France	20,96 ± 0,41	> 40	> 40	> 40	13,09 ± 0,13	MK654910	MK729093
	2004-729[a]	Italy	21,64 ± 0,64	> 40	> 40	> 40	13,38 ± 0,05	MK654913	
	2004-722[a]	Italy	22,84 ± 0,71	> 40	> 40	> 40	16,03 ± 0,19	MK654911	
	38ef[a]	Italy	29,01 ± 0,22	> 40	> 40	> 40	21,09 ± 0,03	MK654910	
	B9A[a]	Italy	28,04 ± 0,08	> 40	> 40	> 40	21,77 ± 0,08	MK654909	
	P137r[a]	Italy	27,49 ± 0,26	> 40	> 40	> 40	19,53 ± 0,01	MK654910	
	RB1A[a]	Italy	27,43 ± 0,55	> 40	> 40	> 40	20,14 ± 0,25	MK654911	
	SvA9i[a]	Italy	21,05 ± 0,04	> 40	> 40	> 40	11,49 ± 0,15	MK654912	
	SvB3a[d]	Italy	23,87 ± 0,41	> 40	> 40	> 40	10,89 ± 0,66	MK654910	
	Val2S1[ad]	Italy	23,08 ± 0,27	> 40	> 40	> 40	13,45 ± 0,15		MK729093
	VPS (V2) [a]	Italy	34,22 ± 0,46	> 40	> 40	> 40	28,11 ± 0,31		
	2003-185[a]	Montenegro	22,63 ± 0,35	> 40	> 40	> 40	14,33 ± 0,19	MK654910	
	2002-143[a]	Poland	23,25 ± 0,71	> 40	> 40	> 40	14,66 ± 0,15	MK654914	
	CF_9_3[a]	Italy	23,74 ± 0,23	> 40	> 40	> 40	10,08 ± 0,02		
	NUS 1_8_D[b]	Italy	26,11 ± 0,31	> 40	> 40	> 40	11,32 ± 0,15		
	CF_15_1[b]	Italy	20,01 ± 0,03	> 40	> 40	> 40	9,63 ± 0,70		
	2004-673[c]	Montenegro	30,80 ± 0,39	> 40	> 40	> 40	26,09 ± 0,05		
	2004-692 [c]	Montenegro	29,80 ± 0,10	> 40	> 40	> 40	25,05 ± 0,22		
H. annosum s.s.	1960/56/4[a]	UK	> 40	20,62 ± 0,27	> 40	> 40	14,43 ± 0,07	MK688980	
	Läy1[ad]	Finland	> 40	21,31 ± 0,16	> 40	> 40	15,31 ± 0,33		MK729095
	Cou06S2[a]	France	> 40	21,80 ± 0,16	> 40	> 40	14,43 ± 0,07		
	LSV M1138[ad]	France	> 40	19,72 ± 0,04	> 40	> 40	15,31 ± 0,33	MK688978	MK729097
	LSV M344[ad]	France	> 40	21,20 ± 0,25	> 40	> 40	15,11 ± 0,09	MK688978	MK729096
	LSV M345[a]	France	> 40	22,71 ± 0,27	> 40	> 40	14,07 ± 0,30	MK688978	
	LSV M346[a]	France	> 40	21,15 ± 0,20	> 40	> 40	13,61 ± 0,31	MK688981	
	LSV M347[a]	France	> 40	21,53 ± 0,17	> 40	> 40	14,21 ± 0,10	MK688978	
	LSV M394[a]	France	> 40	21,20 ± 0,48	> 40	> 40	13,61 ± 0,31	MK688978	
	TRE09W1[a]	France	> 40	20,93 ± 0,49	> 40	> 40	13,21 ± 0,03		
	1960-156[a]	Italy	> 40	21,09 ± 0,22	> 40	> 40	13,84 ± 0,15	MK688978	
	109SA (V2) [a]	Italy	> 40	28,08 ± 0,25	> 40	> 40	20,81 ± 0,39	MK688984	
	B32a[a]	Italy	> 40	27,92 ± 0,54	> 40	> 40	21,82 ± 0,29	MK688978	
	BM42NG[a]	Italy	> 40	29,29 ± 0,64	> 40	> 40	22,47 ± 0,10	MK688978	
	BM42NG(V2)[a]	Italy	> 40	26,59 ± 0,24	> 40	> 40	20,03 ± 0,42		
	CAL1[a]	Italy	> 40	29,59 ± 0,09	> 40	> 40	23,19 ± 0,31	MK688978	
	Con2S2[ad]	Italy	> 40	20,65 ± 0,13	> 40	> 40	13,32 ± 0,19		MK729094
	FOM131[a]	Italy	> 40	21,16 ± 0,20	> 40	> 40	13,71 ± 0,04		
	SvA5d[a]	Italy	> 40	26,91 ± 0,28	> 40	> 40	20,59 ± 0,29	MK688979	
	1961/44/22[a]	Norway	> 40	20,37 ± 0,11	> 40	> 40	14,05 ± 0,10	MK688979	

	1937/1080/1[a]	Norway	> 40	21,82 ± 0,21	> 40	> 40	14,42 ± 0,09	MK688983	
	1993-334/6[a]	Norway	> 40	22,22 ± 0,12	> 40	> 40	16,02 ± 0,14	MK688982	
	1991-345/1[a]	Norway	> 40	21,40 ± 0,38	> 40	> 40	14,44 ± 0,08	MK688978	
	Ha carp[b]	Italy	> 40	30,08 ± 0,49	> 40	> 40	9,36 ± 0,76		
	St.D.alta 4.2b[b]	Italy	> 40	23,97 ± 0,21	> 40	> 40	11,40 ± 0,76		
	3A HET 31[b]	Italy	> 40	31,40 ± 0,04	> 40	> 40	10,81 ± 0,36		
	137OC[b]	Italy	> 40	28,26 ± 0,66	> 40	> 40	9,00 ± 0,22		
	1993-375/1[c]	Norway	> 40	29,94 ± 0,41	> 40	> 40	24,89 ± 0,07		
	2003-9/1[c]	Norway	> 40	29,12 ± 0,20	> 40	> 40	25,21 ± 0,11		
	2003-10/1[c]	Norway	> 40	30,42 ± 0,10	> 40	> 40	26,22 ± 0,13		
	2014-167[c]	Norway	> 40	30,71 ± 0,27	> 40	> 40	26,25 ± 0,14		
	2004-684[c]	Montenegro	> 40	31,82 ± 0,22	> 40	> 40	26,54 ± 0,04		
	2004-689[c]	Montenegro	> 40	32,24 ± 0,23	> 40	> 40	27,50 ± 0,10		
H. irregulare	FOM0132[ad]	-	> 40	> 40	18,75 ± 0,11	> 40	13,03 ± 0,05		MK729099
	1961-2[a]	Canada	> 40	> 40	22,79 ± 0,08	> 40	16,70 ± 0,07	MK688987	MK729100
	2008-21[a]	USA	> 40	> 40	21,59 ± 0,31	> 40	15,27 ± 0,28	MK688988	MK729100
	38NA[a]	Italy	> 40	> 40	27,48 ± 0,04	> 40	21,67 ± 0,16	MK688985	MK729098
	48NB[ad]	Italy	> 40	> 40	26,85 ± 0,36	> 40	18,96 ± 0,53	MK688985	
	91NA[ad]	Italy	> 40	> 40	25,61 ± 0,11	> 40	20,68 ± 0,20	MK688986	
	LSV M1121[a]	Italy	> 40	> 40	20,40 ± 0,28	> 40	15,49 ± 0,10		MK729100
	RF3s[ad]	Italy	> 40	> 40	20,73 ± 0,16	> 40	15,19 ± 0,09		MK729100
	1116-1[b]	USA	> 40	> 40	19,83 ± 0,04	> 40	10,89 ± 0,39		
	CP15[b]	Italy	> 40	> 40	22,99 ± 0,05	> 40	14,09 ± 0,20		
	CONK1[b]	USA	> 40	> 40	21,69 ± 0,18	> 40	13,17 ± 0,99		
	9OA[b]	Italy	> 40	> 40	24,76 ± 0,01	> 40	11,41 ± 0,33		
	1960-152[c]	USA	> 40	> 40	32,63 ± 0,66	> 40	26,63 ± 0,30		
	1961-3[c]	Canada	> 40	> 40	33,02 ± 0,11	> 40	27,06 ± 0,20		
H. parviporum	16-562/2F[a]	-	> 40	> 40	> 40	17,44 ± 0,13	13,49 ± 0,17		
	FOM190[a]	-	> 40	> 40	> 40	18,02 ± 0,05	14,11 ± 0,05		
	FOM191[a]	-	> 40	> 40	> 40	18,28 ± 0,05	14,65 ± 0,10		
	5/A[ad]	Finland	> 40	> 40	> 40	18,40 ± 0,06	15,48 ± 0,25		MK729103
	K7R39[a]	Finland	> 40	> 40	> 40	17,52 ± 0,11	14,62 ± 0,14		
	16-596/F[a]	France	> 40	> 40	> 40	17,61 ± 0,34	14,04 ± 0,18		
	2004-721[a]	Italy	> 40	> 40	> 40	18,34 ± 0,10	14,65 ± 0,14	MK729090	
	CEP7[a]	Italy	> 40	> 40	> 40	26,50 ± 0,46	24,49 ± 0,33		
	LSV M1155[ad]	Italy	> 40	> 40	> 40	17,93 ± 0,10	15,61 ± 0,53		MK729104
	P162r[bd]	Italy	35,3 ± 0,16	> 40	> 40	24,65 ± 0,88	14,57 ± 0,09	MK729090	
	SvA1c[b]	Italy	> 40	> 40	> 40	24,29 ± 0,06	10,78 ± 0,23	MK729090	
	SvA5a[a]	Italy	> 40	> 40	> 40	23,47 ± 0,12	19,95 ± 0,07	MK729090	
	1987-164/2[a]	Norway	> 40	> 40	> 40	18,62 ± 0,19	14,46 ± 0,28	MK729090	
	1987-1661/4[a]	Norway	> 40	> 40	> 40	18,53 ± 0,16	14,86 ± 0,17	MK729090	
	1992-523/10[ad]	Norway	> 40	> 40	> 40	18,51 ± 0,04	14,82 ± 0,28	MK729090	MK729102
	1998-1616/1[a]	Norway	21,86 ± 0,28	> 40	> 40	17,60 ± 0,20	13,34 ± 0,06	MK729090	MK729101
	13A HET 32[b]	Italy	> 40	> 40	> 40	36,80 ± 0,08	14,45 ± 0,59		
	7.3 HET 54[b]	Italy	> 40	> 40	> 40	26,41 ± 0,04	16,04 ± 0,04		
	1987-1675/1[c]	Norway	> 40	> 40	> 40	30,75 ± 0,21	24,38 ± 0,20		
	1998-1616/1[c]	Norway	30,35 ± 0,33	> 40	> 40	28,19 ± 0,21	23,99 ± 0,04		
	1998-1622/2[c]	Norway	31,26 ± 0,11	> 40	> 40	28,97 ± 0,36	24,63 ± 0,02		
	2004-352/1[c]	Norway	> 40	> 40	> 40	30,49 ± 0,22	25,05 ± 0,04		
	2004-353/1[c]	Norway	> 40	> 40	> 40	30,77 ± 0,23	25,44 ± 0,04		
	2004-676[c]	Montenegro	34,19 ± 0,98	> 40	> 40	31,81 ± 0,39	26,54 ± 0,16		
	2004-714[c]	Italy	32,94 ± 0,99	> 40	> 40	31,55 ± 0,02	27,67 ± 0,22		
	2005-842/2[c]	Norway	> 40	> 40	> 40	29,72 ± 0,02	25,05 ± 0,06		

	2005-851/1[c]	Norway	> 40	> 40	> 40	30,52 ± 0,04	27,91 ± 0,13	
	2007-166/1[c]	Norway	32,66 ± 0,16	> 40	> 40	28,99 ± 0,20	27,13 ± 0,11	
	2014-44[c]	Norway	> 40	> 40	> 40	31,84 ± 0,04	29,33 ± 0,60	
H. irregulare × *H. annosum* s.s. [e]	MUT00005668[b]	Italy	> 40	20,25 ± 0,04	19,01 ± 0,43	> 40	14,35 ± 0,46	
	MUT00005669[b]	Italy	> 40	18,93 ± 0,47	19,56 ± 0,44	> 40	13,88 ± 0,25	
H. occidentale	II1A[b]	USA	> 40	> 40	> 40	39,33 ± 0,25	19,98 ± 0,06	
H. araucariae	1965-13[c]	New Zealand	> 40	> 40	> 40	> 40	29,26 ± 0,19	MK729092
H. insulare	2002-149[a]	Japan	> 40	> 40	> 40	> 40	13,42 ± 0,34	
	2002-152[a]	China	> 40	> 40	> 40	> 40	14,53 ± 0,26	MK729091
	2002-148[c]	Japan	> 40	> 40	> 40	> 40	24,85 ± 0,20	
	2002-154[c]	China	> 40	> 40	> 40	> 40	24,11 ± 0,18	
Echinodontium tinctorium	Aho-60-88-R[b]	Italy	> 40	> 40	> 40	> 40	28,99 ± 0,88	
Armillaria ostoyae	Vald[b]	Italy	> 40	> 40	> 40	> 40	13,90 ± 0,23	
Stereum sanguinolentum	12[b]	Italy	> 40	> 40	> 40	> 40	15,23 ± 0,12	
Phaelous schweinitzii	574[b]	Italy	> 40	> 40	> 40	> 40	24,71 ± 0,08	
Onnia tomentosa	OT-Slu[b]	Sweden	> 40	> 40	> 40	> 40	13,66 ± 0,08	
Fuscoporia torulosa	DP39[b]	Italy	> 40	> 40	> 40	> 40	11,16 ± 0,33	
Fomitopsis pinicola	C-Joux[b]	Italy	> 40	> 40	> 40	> 40	10,60 ± 0,56	
Porodaedalea pini	14[b]	Italy	> 40	> 40	> 40	> 40	22,22 ± 0,59	
Phellinus sulphurascens	Pa-22r[b]	USA	> 40	> 40	> 40	> 40	21,00 ± 1,24	

[a] DNA sample analyzed by ANSES.
[b] DNA sample analyzed by the University of Torino.
[C] DNA sample analyzed by NIBIO
[d] Isolate included in the panel for the first screening step of candidate species-specific PCR primers
[e] Artificial heterokaryotic F1 hybrids generated by Giordano et al. (2018)

685
686 **Table 2: Primers and probes used in the study**
687

Target (gene)	Name	Sequence (5' – 3')	Product size
Heterobasidion sp. (*RPB1*)	RPB1-Af[a]	GAR TGY CCD GGD CAY TTY GG	1393 bp
	RPB1-Cf[a]	CCN GCD ATN TCR TTR TCC ATR TA	
Heterobasidion sp. (*Mcm7*)	Mcm7-709for[b]	ACN MGN GTN TCV GAY GTH AAR CC	640 bp
	Mcm7-1348rev[b]	GAY TTD GCN ACN CCN GGR TCW CCC AT	
Heterobasidion sp. (*Tsr1*)	Tsr1-1453for[b]	GAR TTC CCN GAY GAR ATY GAR CT	750 bp
	Tsr1-2308rev[b]	CTT RAA RTA NCC RTG NGT NCC	
Heterobasidion sp. (*GH63*)	GH63IF[c]	AGG GAY GAR GGN TTC CAY YT	400-650 bp
	GH63IR[c]	CGN CGG AAC CAN TCR TAR TG	
H. abietinum (Mcm7)	Habi For 4[d]	TCG TTT CAG CCC TTT CCA A	291 bp
	Habi Rev 14[d]	TTG ATG AAT ATA GTG CGC CTC G	
	Habi P 7[d]	Cy5- GG TGC GTC GTC GCC TTC ATT ATT TT-BHQ2	
H. annosum s.s. (Mcm7)	Hann For 14[d]	CGT CGC CTT AAT GAT TTC ATA AG	267 bp
	Hann Rev 10[d]	TGT CAC TGT ACT GTT TCT TTA GC	
	Hann P 11[d]	FAM-ACC ATA CAY GTT GGC GGG AAC CTC-BHQ1	
H. irregulare (Mcm7)	Hirr For 1[d]	CGT CGT CTC CAT GAT CTC AA	202 bp
	Hirr Rev 5[d]	TTG ATG AAT ATA GTG CGC TTC A	
	Hirr P 5[d]	JOE-CCA TWC ACG TTG GCG GGA ACC TT-BHQ1	
H. parviporum (RPB1)	Hpar For 4[d]	CAA TCG TAT GGG GTC ATT GTA A	110 bp
	Hpar Rev 6[d]	CAC ATC CGC CAT GTC CC	
	Hpar P 8[d]	ROX-GAT CTG CGA GCC CGA CGA ACC G-BHQ2	
Any fungal genus (18S rDNA)	FungiQuant-F[e]	GGR AAA CTC ACC AGG TCC AG	
	FungiQuant-R[e]	GSW CTA TCC CCA KCA CGA	
	FungiQuant-Prb[e]	FAM-TGG TGC ATG GCC GTT-BHQ1	

688 [a] Primers for conventional PCR developed by MATHENY et al. (2002)
689 [b] Primers for conventional PCR developed by SCHMITT et al. (2009)
690 [c] Primers for conventional PCR developed by PÉREZ-IZQUIERDO et al. (2017)
691 [c] Primers and probe for real-time PCR developed in this study
692 [e] Primers and probe for real-time PCR developed by LIU et al. (2012)
693

694
695 **Table 3: Repeatability and reproducibility for the *Heterobasidion* real-time assays**
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Target	Concentration	Intra-assay		Inter-assay	
		Mean Ct ± SD[a]	CV (%)	Mean Ct ± SD	CV (%)
H. abietinum	100× LOD [b]	28.3±0.1	0,1	28.4±0.5	1.7
	10× LOD	30.5±0.2	0,3	32.1±0.9	2.8
	1 ng μL^{-1} gDNA[c]	31.5±0.4	0,5	31.7±1.1	3.5
H. annosum	100× LOD	25.4±0.2	2.0	25.8±0.4	1.7
	10× LOD	29.7±0.5	2.2	29.2±0.8	2.7
	1 ng μL^{-1} gDNA[d]	22.9±0.5	2.8	22.5±0.7	3.1
H. irregulare	100× LOD	23,2 ± 0,4	1,6	22.6±0.3	1.2
	10× LOD	25,9 ± 0,3	1,2	25.4±0.8	3.2
	1 ng μL^{-1} gDNA[e]	19,9 ± 0,6	3,2	19.5±0.1	0.3
H. parviporum	100× LOD	21,1 ± 0,2	1,2	21.0±0.5	2.6
	10× LOD	24,4 ± 0,1	0,4	24.2±0.1	0.5
	1 ng μL^{-1} gDNA[f]	17,7 ± 0,3	1,5	17.7±0.2	1.1

697 [a] The mean Ct value and standard deviation were calculated with 10 replicates
698 [b] LOD (Limit Of Detection) corresponds to 112, 112, 88 and 82 of pc μL^{-1} for *H. abietinum, H. annosum, H. irregulare,* and *H. parviporum* respectively.
699 [c] *H. abietinum* strain 17/058
700 [d] *H. annosum* s.s. strain LSVM1138
701 [e] *H. irregulare* strain 38NA
702 [f] *H. parviporum* strain LSVM1155
703

704
705 **Table 4: Environmental samples tested during the study (Habi, *H. abietinum*; Hann, *H. annosum s.s.*; Hirr, *H. irregulare*; Hpar, *H.***
706 ***parviporum*, FQ, FungiQuant).**
707

				Real-time PCR test (mean Ct value ± SD)				
Species	**Code**	**Origin**	**Host**	**Habi**	**Hann**	**Hirr**	**Hpar**	**FQ**
Wood chips from artificially-inoculated logs								
Logs inoculated with *H. irregulare*	LOG1-CP15/a	Italy	*Pinus sylvestris*	> 40	> 40	37.43±1.54	> 40	26.22±0.46
Logs inoculated with *H. annosum s.s.*	LOG2-49SA/a	Italy	*Pinus sylvestris*	> 40	34.82±2.94	> 40	> 40	23.59±0.57
Logs inoculated with *H. irregulare*	LOG1-CP15/b	Italy	*Pinus sylvestris*	> 40	> 40	37.06±0.24	> 40	24.52±0.25
Logs inoculated with *H. annosum s.s.*	LOG2-49SA/b	Italy	*Pinus sylvestris*	> 40	30.24±0.31	> 40	> 40	23.32±0.83
Control log	LOG5-mock/a	Italy	*Pinus sylvestris*	> 40	> 40	> 40	> 40	29.56±0.6
Control log	LOG5-mock/b	Italy	*Pinus sylvestris*	> 40	> 40	> 40	> 40	27.11±1.1
Wood chips from infected trees								
Wood chips from tree infected by *H. irregulare**	PT15S	Italy	*Pinus pinea*	> 40	> 40	36.99±0.14	> 40	33.33±1.5
Wood chips from tree infected by *Heterobasidion* spp.*	PT17S	Italy	*Pinus pinea*	> 40	31.36±0.06	> 40	> 40	29.32±0.95
Wood chips from tree infected by *Heterobasidion* spp.*	PT20S	Italy	*Pinus pinea*	> 40	35.70±0.74	> 40	> 40	29.01±0.48
Wood chips from tree infected by *H. irregulare**	7_50	Italy	*Pinus pinea*	> 40	> 40	33.94±0.86	> 40	24.17±1.18
Wood chips from tree infected by *H. irregulare**	14_50	Italy	*Pinus pinea*	> 40	> 40	32.28±0.37	> 40	29.75±0.52
Fruiting bodies								
Heterobasidion irregulare *	IV1	Italy	*Pinus pinea*	> 40	> 40	32.12±0.08	> 40	19.24±0.07
Heterobasidion irregulare *	IV2	Italy	*Pinus pinea*	> 40	> 40	23.70±0.09	> 40	14.50±0.38
Heterobasidion irregulare *	IV3	Italy	*Pinus pinea*	> 40	> 40	21.30±0.08	> 40	13.52±0.58
Heterobasidion irregulare *	IV5	Italy	*Pinus pinea*	> 40	> 40	21.28±0.06	> 40	13.13±0.18
Heterobasidion irregulare *	IV-Dep	Italy	*Pinus pinea*	> 40	> 40	21.41±0.06	> 40	13.11±0.35
Heterobasidion irregulare *	IVX	Italy	*Pinus pinea*	> 40	> 40	23.53±0.66	> 40	14.26±0.01
Heterobasidion irregulare *	IVY	Italy	*Pinus pinea*	> 40	> 40	20.81±0.08	> 40	13.42±0.42
Heterobasidion irregulare *	Gan-Gall	Italy	*Pinus pinea*	> 40	> 40	23.53±0.15	> 40	13.92±0.22
Heterobasidion sp.	17-00500 10701	France	*Pseudotsuga* sp.	> 40	16,17±0,01	> 40	> 40	9,02±0,03
Heterobasidion sp.	17-00501 10695	France	*Pseudotsuga* sp.	15,93±0,08	> 40	> 40	> 40	9,57±0,01
Heterobasidion sp.	17-00571/1 dil FOMORCAAA	France	*Picea abies*	> 40	20,31±0,08	> 40	> 40	16,51±0,02
Heterobasidion sp.	17-00571/2 dil FOMORCBBB	France	*Picea abies*	> 40	19,48±0,09	> 40	> 40	17,46±0,03
Heterobasidion sp.	17-00571/3 dil FOMORC001	France	*Picea abies*	> 40	17,97±0,03	> 40	> 40	14,71±0,03
Heterobasidion sp.	17-00571/4 dil FOMORC002	France	*Picea abies*	> 40	18,38±0,15	> 40	> 40	15,23±0,19
Heterobasidion sp.	17-00571/5 dil FOMORC003	France	*Picea abies*	> 40	19,53±0,12	> 40	> 40	15,10±0,42
Heterobasidion sp.	17-00571/6 dil FOMORC004	France	*Picea abies*	> 40	19,02±0,12	> 40	> 40	15,43±0,07
Heterobasidion sp.	17-00571/7 dil FOMORC005	France	*Picea abies*	> 40	19,12±0,75	> 40	> 40	16,29±0,38
Heterobasidion sp.	17-00571/8 dil FOMORC006	France	*Picea abies*	> 40	19,53±0,10	> 40	> 40	16,85±0,02
Heterobasidion sp.	17-00571/9 dil FOMORC007	France	*Picea abies*	> 40	19,52±0,01	> 40	> 40	17,15±0,81
Heterobasidion sp.	17-00573/1 dil FOMCLM008	France	*Abies grandis*	20,94±0,29	> 40	> 40	> 40	16,07±0,08
Heterobasidion sp.	17-00573/2 dil FOMCLM009	France	*Abies grandis*	20,72±0,62	> 40	> 40	> 40	18,16±0,52
Heterobasidion sp.	17-00573/3 dil FOMCLM010	France	*Abies grandis*	20,20±0,23	> 40	> 40	> 40	17,69±0,25
Heterobasidion sp.	18-00068/1 dil	France	*Picea abies*	> 40	18,71±0,34	> 40	> 40	12,32±0,11
Heterobasidion sp.	18-00068/2 dil	France	*Picea abies*	> 40	30,60±0,49	> 40	> 40	23,56±0,10
Heterobasidion sp.	18-00068/3 dil	France	*Picea abies*	> 40	25,35±0,13	> 40	> 40	18,36±0,20
Heterobasidion sp.	18-00104 dil 10985/1	France	*Picea abies*	> 40	19,00±0,23	> 40	> 40	11,14±0,00
Heterobasidion sp.	18-00104 dil 10985/2	France	*Picea abies*	> 40	21,11±0,04	> 40	> 40	13,61±0,02
Heterobasidion sp.	18-00104 dil 10985/3	France	*Picea abies*	> 40	23,33±0,07	> 40	> 40	16,26±0,04
Heterobasidion sp.	18-00105 dil 10987	France	*Picea abies*	> 40	32,00±0,34	> 40	> 40	20,07±0,01
Heterobasidion sp.	18-00244/1 dil 11175	France	*Picea abies*	> 40	23,39±0,02	> 40	> 40	18,27±0,00

Heterobasidion sp.	18-00244/2 dil 11176	France	*Picea abies*	> 40	21,04±0,08	> 40	> 40	15,61±0,40
Heterobasidion sp.	18-00397/1 dil 11427A	France	*Picea abies*	> 40	27,88±0,20	> 40	> 40	20,94±0,10
Heterobasidion sp.	18-00397/2 dil 11427B	France	*Picea abies*	> 40	17,80±0,18	> 40	> 40	12,96±0,61
Heterobasidion sp.	18-00397/3 dil 11427C	France	*Picea abies*	> 40	20,74±0,22	> 40	> 40	24,50±1,21
Heterobasidion sp.	18-00397/4 dil 11427D	France	*Picea abies*	> 40	17,02±0,02	> 40	> 40	10,65±0,19
Heterobasidion sp.	18-00582 dil 11635	France	*Abies alba*	20,37±0,39	> 40	> 40	> 40	14,60±0,12

*based on outcomes of molecular analyses performed by Sillo et al. (2018); DNA extracted and tested by the University of Torino