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

19

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31

32 **Key words:** molecular assay, conifer root rot, monitoring.

33

34 **Summary.**

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

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

49

50

51 **Introduction**

52 Many cryptic species (i.e. species from the same genus that are difficult to identify by
53 morphological criteria) are described in fungi, and our knowledge on the number, biology and
54 evolution of such species is still limited (Hawksworth & Lücking, 2017). However, the rapid
55 and recent development of various molecular tools has considerably improved species
56 identification, and consequently shed light on their geographic distribution and ecology
57 (Desprez-Loustau et al., 2018). The genus *Heterobasidion* encompasses 13 fungal species, of
58 which 12 can be grouped into two major species complexes. The first species complex is *H.*
59 *insulare sensu lato* (s.l.), comprising the saprotrophic species *H. amyloideum*, *H. australe*, *H.*
60 *ecrustosum*, *H. insulare sensu typi*, *H. linzhiense*, *H. orientale*, and *H. tibeticum* (Chen et al.,
61 2015). The complex *H. annosum* s.l. comprises the phytopathogenetic species *H. abietinum*,
62 *H. annosum sensu stricto* (s.s.), *H. irregulare*, *H. parviporum*, and *H. occidentale*, which are
63 responsible for severe economic and environmental losses, and are deemed major threats for
64 conifers in the Northern Hemisphere (Garbelotto & Gonthier, 2013). Depending on the host
65 species, losses caused by *H. annosum* s.l. are associated either with root rot leading to tree
66 mortality or with the development of heartwood decay in the roots, bole and stem, impairing
67 the quality of wood and the stability of trees (Garbelotto & Gonthier, 2013). The different
68 *Heterobasidion* species have been reported to show host preferences (Garbelotto & Gonthier,
69 2013). However, their ecology and geographic distribution overlap to a large extent
70 (Garbelotto & Gonthier, 2013) and can co-occur in the same stands (Gonthier, 2019, Gonthier
71 et al., 2001, Sedlák & Tomšovský, 2014). In addition, several studies have shown incomplete
72 species barriers between some species with rare hybrids being found in forests (Sedlák &
73 Tomšovský, 2014, Garbelotto et al., 1996, Gonthier & Garbelotto, 2011, Sillo et al., 2019).
74 In Europe, only species from *H. annosum* s.l. are present. *H. annosum* s.s. attacks mostly
75 pines (*Pinus* spp.), although it may also be found on other hosts, including Norway spruce

76 (*Picea abies*) and even occasionally broadleaves (Garbelotto & Gonthier, 2013). *H.*
77 *parviporum* and *H. abietinum* are mostly found associated with Norway spruce and firs (*Abies*
78 spp.) respectively (Garbelotto & Gonthier, 2013). These three Eurasian species have been
79 present for a long time in European forests (Dalman et al., 2010). Several *Heterobasidion*
80 species are able to infect certain tree species like *Larix decidua*, *Pinus* spp., *Picea abies* and
81 *Pseudotsuga menziesii* (Garbelotto & Gonthier, 2013). Occasionally co-infections of a tree by
82 two *Heterobasidion* species have even been observed (Gonthier et al., 2003). The host
83 specificity of these fungi is not as pronounced during saprobic growth as when they infect
84 living trees, and several *Heterobasidion* species are able to colonize the stumps of a given tree
85 species (Garbelotto & Gonthier, 2013). Besides these indigenous *Heterobasidion* species, the
86 North American *H. irregulare* was introduced into a single site in Europe, probably during
87 World War II (Gonthier et al., 2004, Garbelotto et al., 2013). Since then it has become
88 invasive, spreading to both pine and oak stands along the Tyrrhenian coast in central Italy,
89 often in association with significant mortality in Italian stone pine (*P. pinea*) (Gonthier et al.,
90 2007, Gonthier et al., 2014, Gonthier et al., 2012). Furthermore, *H. irregulare* has been
91 documented to hybridize with *H. annosum* s.s. in the central Italian invasion area (Gonthier &
92 Garbelotto, 2011). In 2015, the threat posed by this species led the European Plant Protection
93 Organization (EPPO) to add *H. irregulare* to the A2 list of pests recommended for regulation,
94 needing the development of a robust, rapid and accurate method of detection in the field.
95 To our knowledge, several molecular tools have already been designed to target pathogenic
96 species or groups of species in the *Heterobasidion* genus. Gonthier et al. (2015) designed an
97 ITS rDNA-based primer pair to be used in conventional end point PCR or in a SYBR-Green
98 real-time PCR, that enabled direct detection of all five species of *H. annosum* s.l. from
99 environmental samples but that did not discriminate between the species. Hietala et al. (2003)
100 developed a real-time assay to study the colonization of *H. annosum* s.l. in spruce based on
101 polymorphisms occurring in a gene coding laccase. Their assay targeted indistinctly *H.*

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

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

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

143

144

145 **Materials and methods**

146 **Fungal isolates**

147 A total of 108 *Heterobasidion* isolates representing seven distinct species and two
148 interspecific hybrids, were included in this study (Table 1). The first set of 67 isolates was
149 cultured in ANSES on Potato Dextrose Broth (Difco) for 10 days at 21°C under constant
150 shaking. The pellet of mycelium was then blotted onto sterile Whatman paper to eliminate the
151 broth before being transferred into a 2-mL lysing matrix A microtube (MP Biomedicals) with
152 400 µL of lysis buffer AP1 (DNeasy Plant mini kit, Qiagen) and ground by shaking using a
153 FastPrep 24 5G orbital shaker (MP Biomedicals) at 6.5 unit for 20 sec. The DNA was

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

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

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

176 Nine fungal species that are frequently isolated from conifer roots and the collar were also
177 included (Table 1). DNA was extracted from the mycelia of these isolates using the EZNA
178 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 μ L 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

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

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

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

227

228 **Construction of stabilized plasmid controls and limit of detection**

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

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

241

242 **Verification of test specificity**

243 Specificity, i.e. the ability to generate positive results with DNA from target species and
244 negative results with DNA from non-target species, was assessed with the panel of strains
245 from all *Heterobasidion* species presented in Table 1. Preliminary attempts to use all four
246 primer/probe sets in a single PCR tube (quadruplex PCR) showed unacceptable loss of
247 sensitivity for one or more of the species-specific tests. However, a triplex real-time PCR of
248 *H. irregulare* and *H. parviporum* plus the Fungi-Quant test targeting the 18S rDNA of a broad
249 range of fungal species (Liu et al., 2012), and a duplex real-time PCR of *H. abietinum* and *H.*
250 *annosum s.s.* could be successfully achieved without compromising the sensitivity of each
251 assay (data not shown). In France, the tests were conducted according to the following
252 conditions using triplicate reactions for each template DNA. For *H. abietinum* ([Cy5]
253 reporting dye) and *H. annosum s.s.* ([FAM] reporting dye), the duplex real-time PCR were
254 performed in a final volume of 20 μ L using the Core kit No Rox (Eurogentec, Seraing,
255 Belgium) and containing 1 \times Polymerase buffer, 5 mM MgCl₂, 0.2 mM of each dNTP, 0.3 μ M
256 of each forward and reverse primer, 0.1 μ M of probe, 0.025 U/ μ L of HotGold Star DNA

257 polymerase (Eurogentec), 2 μL of template DNA ($0.5 \text{ ng } \mu\text{L}^{-1}$), and molecular-grade water to
258 20 μL . For *H. parviporum* ([ROX] reporting dye) and *H. irregulare* ([JOE] reporting dye), the
259 test included the FungiQuant primers/probe combination to be used as a DNA quality control
260 ([FAM] reporting dye). The triplex real-time reactions were performed as described above for
261 duplex, with the addition of the FungiQuant F/-R/-Prb primers/probe at the same
262 concentration as for the target *Heterobasidion* species. Primer and probe characteristics are
263 indicated in Table 2.

264 Real-time PCR was performed under the following conditions: initial denaturation at 95°C for
265 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/elongation at
266 65°C for 55 s. In each reaction, the cycle threshold (Ct) value was determined using Rotor-
267 Gene software version 1.7.75, and the threshold line was fixed manually at 0.02.

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

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

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

288

289 **Assessment of performance criteria.**

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

292 The analytical sensitivity was assessed using a 10-fold dilution series of the plasmid DNA
293 (pDNA) positive controls diluted in 1× Tris-EDTA (10 mM Tris, 1 mM EDTA). The dilution
294 series ranged from 112 10⁶ to 112 pc mL⁻¹, 112 10⁶ to 112 pc mL⁻¹, 88 10⁶ to 88 pc mL⁻¹, and
295 82 10⁶ to 82 pc mL⁻¹ for *H. abietinum*, *H. annosum* s.s., *H. irregulare*, and *H. parviporum*,
296 respectively. Each target was tested alone in the TE buffer. The limit of detection (LOD) was
297 determined as the minimal quantity of target DNA that could be amplified by real-time PCR
298 in 100% of cases. For each real-time PCR assay, a standard curve was constructed and the
299 corresponding amplification efficiency was computed. The limit of detection was also
300 determined with ten-fold serial dilutions starting from 0.5 ng µL⁻¹ genomic DNA extracts
301 from strains 1987-1661/4 (*H. parviporum*), FOM0132 (*H. irregulare*), LSVM975 (*H.*
302 *abietinum*), and 1960/56/4 (*H. annosum* s.s.). The genome size of *H. annosum* s.s. and *H.*
303 *irregulare* was estimated to be approximately 31 and 33 Mb respectively (Choi et al., 2017)
304 and 33 Mb on average for *H. parviporum* (Zeng et al., 2018), whereas the genome size of *H.*
305 *abietinum* remains to be determined. Since one pg of DNA corresponds to 965 Mpb (Bennett
306 & Smith, 1976), it is therefore possible to estimate the quantity of DNA in one nucleus of *H.*
307 *annosum* s.s. (0.0321 pg), *H. irregulare*, and *H. parviporum* (0.0341 pg).

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

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

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

328

329

330 **Results**

331 **Design of species-specific oligonucleotides**

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

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

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

352

353 **Verification of assay specificity**

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

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

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

367 The test targeting *H. abietinum* successfully amplified DNA from the 24 target strains,
368 regardless of origin (Table 1). For this test, negative results were obtained with DNA from all
369 but one of the non-target *Heterobasidion* species. However, DNA from seven *H. parviporum*
370 strains, i.e. isolates P162r and 2004-714 from Italy; 2004-676 from Montenegro; and 1998-
371 1616/1, 1998-1622/2, 2004-676, and 2007/166/1 from Norway yielded unexpected positive
372 results with the *H. abietinum* assay. These results were not anticipated based on the initial
373 *Mcm7* sequencing since the *H. parviporum* *Mcm7* sequence displayed two, three to five, and
374 two SNPs in the regions targeted by the *H. abietinum* specific forward primer (Habi For 4) ,
375 reverse primer (Habi Rev 14), and probe (Habi P 7), respectively. Yet the cross-detection was
376 consistent and confirmed after repetitions. Following these unexpected results, genomic DNA
377 was extracted from isolates P162r and 1998-1616/1, and the *RPBI* and *Mcm7* genes were
378 sequenced using the primers sets described in Table 2. Sequencing results showed that the
379 two isolates harbored an *RPBI* sequence 100% consistent with *H. parviporum*, whereas the
380 *Mcm7* sequence showed double peaks at the interspecific polymorphic sites, suggesting the
381 presence of two different alleles related to *H. parviporum* and *H. abietinum*.

382

383 **Performance values**

384 The real-time PCR tests successfully yielded 100% repeatable positive results with as little as
385 112, 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 $\pm 10\%$
407 variation in the reaction volume or the template DNA volumes, or by a $\pm 2^\circ\text{C}$ 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

412 still be detected under these disturbed conditions, even at the lowest concentration levels. In
413 addition, for each primer/probe combination, no amplification was observed with DNA from
414 the other three non-target *Heterobasidion* species tested, regardless of the modified
415 conditions. No cross-reactions were observed between any of the four target species primer-
416 probe combinations (data not shown) even under the conditions potentially decreasing
417 specificity (10% reduction in reaction volume, 10% increase in template DNA volume, or
418 -2°C decrease in hybridization temperature).

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

428

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

430 It was possible to detect and identify the *Heterobasidion* species with all the types of material
431 tested (Table 4). The expected species were successfully detected by real-time PCR for all
432 four wood logs artificially inoculated with either *H. irregulare* (two) or *H. annosum* s.s.
433 (two). In addition, *H. irregulare* and *H. annosum* s.s. were successfully detected in
434 respectively three and two wood chip samples taken from symptomatic trees in Italy. A total
435 of 36 fruiting bodies collected in French and Italian pine stands were also tested, and it was
436 possible to identify the presence of either *H. abietinum*, *H. annosum* s.s. or *H. irregulare* for
437 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.

442

443

444

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* (*RPBI*) 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 *RPBI* 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

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

468 The two regions targeted by the multiplex real-time PCR assay developed here are present as
469 single copy genes in the *Heterobasidion* genome. Standard curve interpolation may be used to
470 quantify the DNA pools of each *Heterobasidion* species, which serve as a proxy for their
471 biomass in environmental samples. The analysis of a DNA extract from a wood sample, for
472 instance, would provide quantitative data and help us to study the competition between the
473 different species when they co-occur on a similar substrate. However, we sometimes noticed a
474 large variation between mean Ct values with DNA templates obtained from pure fungal
475 cultures and tested at a standardized DNA concentration by the different laboratories involved
476 in this work. This variation may be due to several factors, such as a non-standardized master
477 mix chemistry, or differences in the real-time PCR platform and associated analysis software
478 influencing assay sensitivity (Grosdidier et al., 2017). Other contributing factors could be the
479 imprecise quantification of total DNA in the template solution, or a variation in the quality of
480 DNA obtained with different DNA extraction kits (Bustin & Huggett, 2017, Ebentier et al.,
481 2013). The quantitative values should therefore only be used and compared all other things
482 being equal, which requires using an identical analysis protocol from DNA extraction to real-
483 time PCR data analysis, coupled with the application of standard curves established by the
484 same analytical chemistry and equipment.

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

490 few *H. parviporum* isolates previously identified as pure genotypes of this species tested
491 positive in both *H. parviporum* and *H. abietinum* real-time PCR assays. The *RPB1* sequence
492 obtained from two of these isolates confirmed that they shared 100% identity with a *H.*
493 *parviporum* *RPB1*. Still, their genome also harbors at least a partial *H. abietinum* *Mcm7*
494 sequence, according to the results of our tests and sequencing. Considering that isolate P162r
495 was isolated from wood disks exposed in the forest in Charvensod (north western Italy)
496 (Gonthier et al., 2001), where spores of *H. abietinum* may also be present, we could have
497 been dealing with a heterokaryotic hybrid isolate harboring nuclei from both species. The
498 interfertility rate between strains from sympatric populations of *H. parviporum* and *H.*
499 *abietinum* actually ranges between 0.20-0.25 in this part of Europe but it has been proved that
500 under laboratory conditions, the interfertility between *H. parviporum* and *H. abietinum* can be
501 as high as 0.75 when pairing strains from allopatric populations (Garbelotto & Gonthier,
502 2013.). Therefore, some of the *H. parviporum* isolates showing signs of *H. abietinum* DNA
503 may either be heterokaryotic isolates or offspring of interspecific crosses with traces of
504 introgression, although we cannot completely exclude this outcome could be the result of
505 contaminations occurred in the laboratories, where isolates and DNA of *H. abietinum* are
506 manipulated. As a little introgression may naturally occur within European *Heterobasidion*
507 populations, our new molecular tools could help to clearly identify first-generation hybrids
508 and accurately quantify their frequency within populations. We cannot exclude These results,
509 and the weak cross reaction observed for *H. occidentale* DNA with the *H. parviporum* test,
510 reflect the close phylogenetic relation between these species that diverged quite recently
511 (Chen et al., 2015). Future studies should therefore clarify the frequency of hybridization
512 among *Heterobasidion* species and shed some light on their viability in natural populations,
513 with the help of other nuclear markers, such as the ones used for recently described *H.*
514 *occidentale* × *H. irregulare* hybrids (Sillo et al., 2019). However, the observation of double
515 positive signals following the *H. abietinum* and *H. parviporum* real-time PCR assays with

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

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

558

559

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575 The authors declare to have no conflict of interest.

576

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673 spruce pathogen (Heterobasidion parviporum) and identification of its potential virulence factors.
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- 675

676
677
678**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 \pm SD)					GenBank accession			
			Habi	Hann	Hirr	Hpar	FQ	<i>RPB1</i>	<i>Mcm7</i>		
<i>Heterobasidion abietinum</i>	16-562/3F ^a	-	22,33 \pm 0,27	> 40	> 40	> 40	> 40	15,16 \pm 0,10			
	2002-146 ^{ad}	Austria	22,17 \pm 0,03	> 40	> 40	> 40	> 40	15,73 \pm 0,05	MK654910	MK729093	
	17/058 ^a	France	22,02 \pm 0,36	> 40	> 40	> 40	> 40	14,74 \pm 0,32	MK654910		
	17/059 ^a	France	22,87 \pm 0,66	> 40	> 40	> 40	> 40	15,72 \pm 0,23	MK654910		
	Cou067 ^a	France	21,51 \pm 0,32	> 40	> 40	> 40	> 40	13,85 \pm 0,15			
	FOM195 ^a	France	21,54 \pm 0,41	> 40	> 40	> 40	> 40	14,23 \pm 0,36			
	LSV M975 ^{ad}	France	20,96 \pm 0,41	> 40	> 40	> 40	> 40	13,09 \pm 0,13	MK654910	MK729093	
	2004-729 ^a	Italy	21,64 \pm 0,64	> 40	> 40	> 40	> 40	13,38 \pm 0,05	MK654913		
	2004-722 ^a	Italy	22,84 \pm 0,71	> 40	> 40	> 40	> 40	16,03 \pm 0,19	MK654911		
	38ef ^a	Italy	29,01 \pm 0,22	> 40	> 40	> 40	> 40	21,09 \pm 0,03	MK654910		
	B9A ^a	Italy	28,04 \pm 0,08	> 40	> 40	> 40	> 40	21,77 \pm 0,08	MK654909		
	P137r ^a	Italy	27,49 \pm 0,26	> 40	> 40	> 40	> 40	19,53 \pm 0,01	MK654910		
	RB1A ^a	Italy	27,43 \pm 0,55	> 40	> 40	> 40	> 40	20,14 \pm 0,25	MK654911		
	SvA9i ^a	Italy	21,05 \pm 0,04	> 40	> 40	> 40	> 40	11,49 \pm 0,15	MK654912		
	SvB3a ^d	Italy	23,87 \pm 0,41	> 40	> 40	> 40	> 40	10,89 \pm 0,66	MK654910		
	Val251 ^{ad}	Italy	23,08 \pm 0,27	> 40	> 40	> 40	> 40	13,45 \pm 0,15		MK729093	
	VPS (V2) ^a	Italy	34,22 \pm 0,46	> 40	> 40	> 40	> 40	28,11 \pm 0,31			
	2003-185 ^a	Montenegro	22,63 \pm 0,35	> 40	> 40	> 40	> 40	14,33 \pm 0,19	MK654910		
	2002-143 ^a	Poland	23,25 \pm 0,71	> 40	> 40	> 40	> 40	14,66 \pm 0,15	MK654914		
	CF_9_3 ^a	Italy	23,74 \pm 0,23	> 40	> 40	> 40	> 40	10,08 \pm 0,02			
	NUS 1_8_D ^b	Italy	26,11 \pm 0,31	> 40	> 40	> 40	> 40	11,32 \pm 0,15			
	CF_15_1 ^b	Italy	20,01 \pm 0,03	> 40	> 40	> 40	> 40	9,63 \pm 0,70			
	2004-673 ^c	Montenegro	30,80 \pm 0,39	> 40	> 40	> 40	> 40	26,09 \pm 0,05			
	2004-692 ^c	Montenegro	29,80 \pm 0,10	> 40	> 40	> 40	> 40	25,05 \pm 0,22			
	<i>H. annosum</i> s.s.	1960/56/4 ^a	UK	> 40	20,62 \pm 0,27	> 40	> 40	> 40	14,43 \pm 0,07	MK688980	
		Läy1 ^{ad}	Finland	> 40	21,31 \pm 0,16	> 40	> 40	> 40	15,31 \pm 0,33		MK729095
		Cou0652 ^a	France	> 40	21,80 \pm 0,16	> 40	> 40	> 40	14,43 \pm 0,07		
		LSV M1138 ^{ad}	France	> 40	19,72 \pm 0,04	> 40	> 40	> 40	15,31 \pm 0,33	MK688978	MK729097
		LSV M344 ^{ad}	France	> 40	21,20 \pm 0,25	> 40	> 40	> 40	15,11 \pm 0,09	MK688978	MK729096
		LSV M345 ^a	France	> 40	22,71 \pm 0,27	> 40	> 40	> 40	14,07 \pm 0,30	MK688978	
LSV M346 ^a		France	> 40	21,15 \pm 0,20	> 40	> 40	> 40	13,61 \pm 0,31	MK688981		
LSV M347 ^a		France	> 40	21,53 \pm 0,17	> 40	> 40	> 40	14,21 \pm 0,10	MK688978		
LSV M394 ^a		France	> 40	21,20 \pm 0,48	> 40	> 40	> 40	13,61 \pm 0,31	MK688978		
TRE09W1 ^a		France	> 40	20,93 \pm 0,49	> 40	> 40	> 40	13,21 \pm 0,03			
1960-156 ^a		Italy	> 40	21,09 \pm 0,22	> 40	> 40	> 40	13,84 \pm 0,15	MK688978		
1095A (V2) ^a		Italy	> 40	28,08 \pm 0,25	> 40	> 40	> 40	20,81 \pm 0,39	MK688984		
B32a ^a		Italy	> 40	27,92 \pm 0,54	> 40	> 40	> 40	21,82 \pm 0,29	MK688978		
BM42NG ^a		Italy	> 40	29,29 \pm 0,64	> 40	> 40	> 40	22,47 \pm 0,10	MK688978		
BM42NG(V2) ^a		Italy	> 40	26,59 \pm 0,24	> 40	> 40	> 40	20,03 \pm 0,42			
CAL1 ^a		Italy	> 40	29,59 \pm 0,09	> 40	> 40	> 40	23,19 \pm 0,31	MK688978		
Con252 ^{ad}		Italy	> 40	20,65 \pm 0,13	> 40	> 40	> 40	13,32 \pm 0,19		MK729094	
FOM131 ^a		Italy	> 40	21,16 \pm 0,20	> 40	> 40	> 40	13,71 \pm 0,04			
SvA5d ^a		Italy	> 40	26,91 \pm 0,28	> 40	> 40	> 40	20,59 \pm 0,29	MK688979		
1961/44/22 ^a		Norway	> 40	20,37 \pm 0,11	> 40	> 40	> 40	14,05 \pm 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		
	1370C ^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		
<i>H. irregulare</i>	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
	RF35 ^{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		
	90A ^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		
<i>H. parviporum</i>	1961-3 ^c	Canada	> 40	> 40	33,02 ± 0,11	> 40	27,06 ± 0,20		
	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		

Running title: Detection of Heterobasidion species.

	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	
<i>H. irregulare</i> × <i>H. annosum</i> 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	
<i>H. occidentale</i>	II1A ^b	USA	> 40	> 40	> 40	39,33 ± 0,25	19,98 ± 0,06	
<i>H. araucariae</i>	1965-13 ^c	New Zealand	> 40	> 40	> 40	> 40	29,26 ± 0,19	MK729092
<i>H. insulare</i>	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	
<i>Echinodontium tinctorium</i>	Aho-60-88-R ^b	Italy	> 40	> 40	> 40	> 40	28,99 ± 0,88	
<i>Armillaria ostoyae</i>	Vald ^b	Italy	> 40	> 40	> 40	> 40	13,90 ± 0,23	
<i>Stereum sanguinolentum</i>	12 ^b	Italy	> 40	> 40	> 40	> 40	15,23 ± 0,12	
<i>Phaelous schweinitzii</i>	574 ^b	Italy	> 40	> 40	> 40	> 40	24,71 ± 0,08	
<i>Onnia tomentosa</i>	OT-Slu ^b	Sweden	> 40	> 40	> 40	> 40	13,66 ± 0,08	
<i>Fuscoporia torulosa</i>	DP39 ^b	Italy	> 40	> 40	> 40	> 40	11,16 ± 0,33	
<i>Fomitopsis pinicola</i>	C-Joux ^b	Italy	> 40	> 40	> 40	> 40	10,60 ± 0,56	
<i>Porodaedalea pini</i>	14 ^b	Italy	> 40	> 40	> 40	> 40	22,22 ± 0,59	
<i>Phellinus sulphurascens</i>	Pa-22r ^b	USA	> 40	> 40	> 40	> 40	21,00 ± 1,24	

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^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)

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Table 2: Primers and probes used in the study

Target (gene)	Name	Sequence (5' – 3')	Product size
<i>Heterobasidion</i> sp. (<i>RPB1</i>)	RPB1-AP ^a	GAR TGY CCD GGD CAY TTY GG	1393 bp
	RPB1-CP ^a	CCN GCD ATN TCR TTR TCC ATR TA	
<i>Heterobasidion</i> sp. (<i>Mcm7</i>)	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	
<i>Heterobasidion</i> sp. (<i>Tsr1</i>)	Tsr1-1453for ^b	GAR TTC CCN GAY GAR ATY GAR CT	750 bp
	Tsr1-2308rev ^b	CTT RAA RTA NCC RTG NGT NCC	
<i>Heterobasidion</i> sp. (<i>GH63</i>)	GH63IF ^c	AGG GAY GAR GGN TTC CAY YT	400-650 bp
	GH63IR ^c	CGN CGG AAC CAN TCR TAR TG	
<i>H. abietinum</i> (<i>Mcm7</i>)	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	
<i>H. annosum</i> s.s. (<i>Mcm7</i>)	Habi P 7 ^d	Cy5- GG TGC GTC GTC GCC TTC ATT ATT TT-BHQ2	267 bp
	Hann For 14 ^d	CGT CGC CTT AAT GAT TTC ATA AG	
	Hann Rev 10 ^d	TGT CAC TGT ACT GTT TCT TTA GC	
<i>H. irregulare</i> (<i>Mcm7</i>)	Hann P 11 ^d	FAM-ACC ATA CAY GTT GGC GGG AAC CTC-BHQ1	202 bp
	Hirr For 1 ^d	CGT CGT CTC CAT GAT CTC AA	
	Hirr Rev 5 ^d	TTG ATG AAT ATA GTG CGC TTC A	
<i>H. parviporum</i> (<i>RPB1</i>)	Hirr P 5 ^d	JOE-CCA TWC ACG TTG GCG GGA ACC TT-BHQ1	110 bp
	Hpar For 4 ^d	CAA TCG TAT GGG GTC ATT GTA A	
	Hpar Rev 6 ^d	CAC ATC CGC CAT GTC CC	
Any fungal genus (18S rDNA)	Hpar P 8 ^d	ROX-GAT CTG CGA GCC CGA CGA ACC G-BHQ2	
	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	

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^a Primers for conventional PCR developed by MATHENY et al. (2002)

^b Primers for conventional PCR developed by SCHMITT et al. (2009)

^c Primers for conventional PCR developed by PÉREZ-IZQUIERDO et al. (2017)

^d Primers and probe for real-time PCR developed in this study

^e Primers and probe for real-time PCR developed by LIU et al. (2012)

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Table 3: Repeatability and reproducibility for the *Heterobasidion* real-time assays

Target	Concentration	Intra-assay		Inter-assay	
		Mean Ct ± SD ^a	CV (%)	Mean Ct ± SD	CV (%)
<i>H. abietinum</i>	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
<i>H. annosum</i>	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
<i>H. irregulare</i>	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
<i>H. parviporum</i>	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

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^a The mean Ct value and standard deviation were calculated with 10 replicates

^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.

^c *H. abietinum* strain 17/058

^d *H. annosum* s.s. strain LSVM1138

^e *H. irregulare* strain 38NA

^f *H. parviporum* strain LSVM1155

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

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

Running title: Detection of *Heterobasidion* species.

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

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