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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/140 (1)

Diagnostics

PM 7/140 (1) *Heterobasidion irregulare*

Specific scope

This Standard describes a diagnostic protocol for *Heterobasidion irregulare*.[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

Approved in 2019-09.

1. Introduction

The root rot agent of conifers *Heterobasidion irregulare* is native to North America, where it mainly attacks pines (*Pinus* spp.) but also junipers (*Juniperus* spp.) and incense cedar (*Calocedrus decurrens*) (Garbelotto & Gonthier, 2013). However, it was introduced in Europe in the 1940s (Gonthier *et al*., 2004), where three native *Heterobasidion* species are also present, namely *H. abietinum*, *H. annosum sensu stricto* (*s.s*.) and *H. parviporum* (Garbelotto & Gonthier, 2013). These species are included in the *Heterobasidion annosum sensu lato* (*s.l*.) species complex. They are clearly distinct based on their geographical origin, morphology, biochemical, phylogenetic and genomics traits, and host preference (Otrosina *et al*., 1993; Linzer *et al*., 2008; Dalman *et al*., 2010; Garbelotto & Gonthier, 2013; Sillo *et al*., 2015). After its introduction, *H. irregulare* has spread to pine stands and urban parks over a 103 km stretch of Tyrrhenian coast around Rome, in association with significant mortality of *Pinus pinea* (Gonthier *et al*., 2007; Garbelotto *et al*., 2013; Gonthier *et al*., 2014). Although very similar morphologically and causing a similar disease with a comparable virulence towards the same hosts (i.e. pines), *H. irregulare* and *H. annosum s.s*. differ in their saprobic and sporulating potential, the former being characterized by a much higher transmission rate compared to the latter (Garbelotto *et al*., 2010; Giordano *et al*., 2014; Gonthier *et al*., 2014). *H. irregulare* × *H. annosum s.s*. are also widespread in the invasion area of *H. irregulare* in Italy (Gonthier & Garbelotto, 2011). Spores of *H. irregulare* have also been detected in pure oak forests in Italy (Gonthier *et al*., 2012), although there the fungus has never been isolated from oak tissues. Hence, testing on oak is consequently not included in this protocol.

A flow diagram describing the diagnostic procedure for *Heterobasidion irregulare* is presented in Fig. 1.

2. Identity

Name: *Heterobasidion irregulare* Garbel. & Otrosina.
Synonyms: *Polyporus irregularis* Underw.
Taxonomic position: Fungi, Basidiomycota, Agaricomycotina, Russulales, Bondarzewiaceae.
EPPO Code: HETEIR.
Phytosanitary categorization: EPPO A2 List n° 389.

3. Detection

Host plants may carry the fungus in the wood of roots and of the basal portions of the stem, in this case especially the sapwood. Host commodities potentially harbouring the pathogen include squared and non-squared wood, bark, plants for planting and packaging material (EPPO, 2018).

As for other *Heterobasidion* species, the detection of *H. irregulare* in the forest is based on the examination of symptoms and the finding of fruiting bodies (Greig, 1998; Gonthier & Thor, 2013). Detection should be complemented by the analysis of samples collected in the field (see below) with molecular tests, which provide the only reliable way to accurately identify the pathogen.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

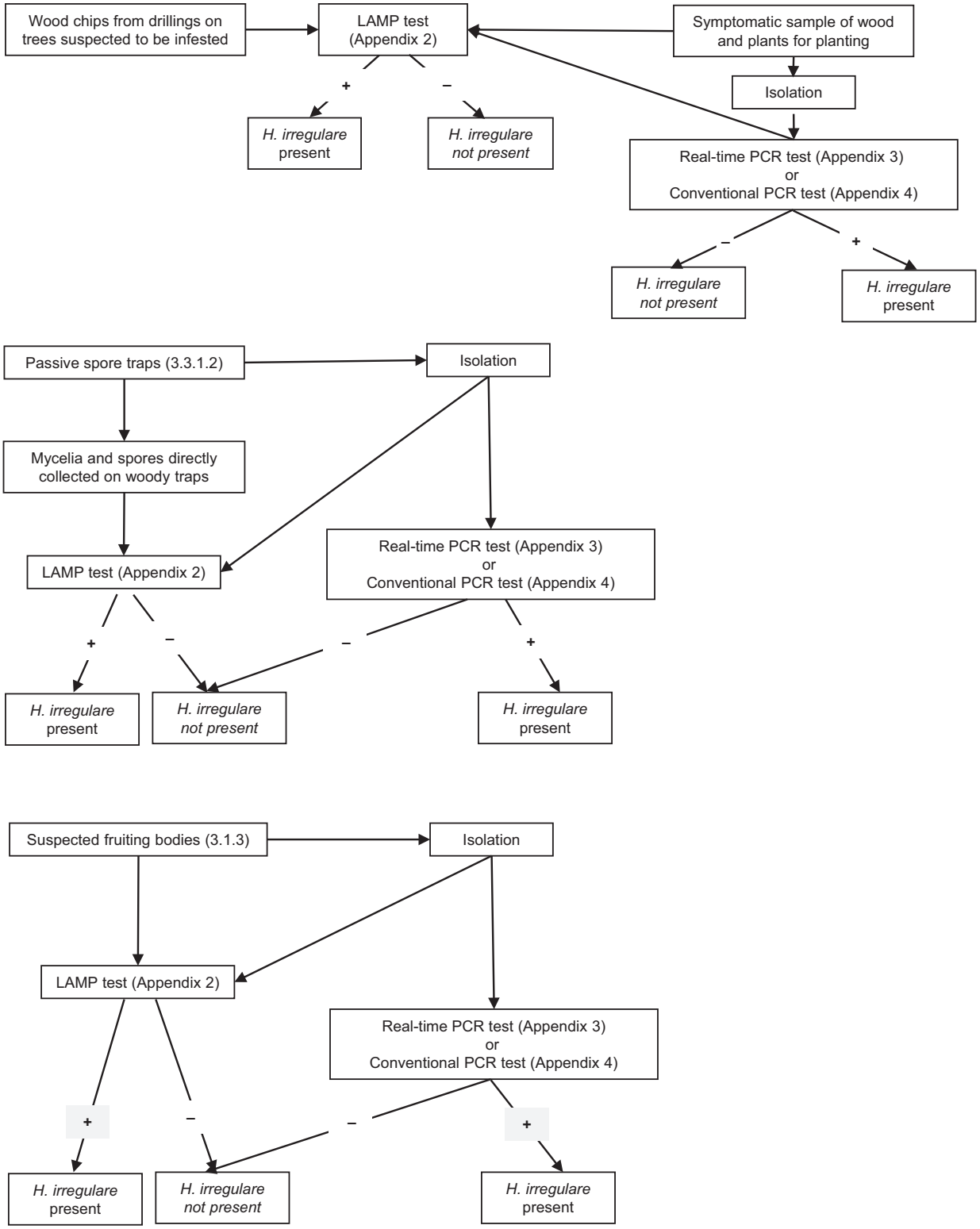

Fig. 1 Flow diagrams of the diagnostic procedure for the detection and the identification of *H. irregulare*.

3.1. Disease symptoms

3.1.1. Symptoms on trees

General symptoms caused on host trees by *Heterobasidion* species have been previously reviewed (Greig, 1998; Gonthier & Thor, 2013). In tree species characterized by a resinous heartwood like *Pinus* spp., which are the main hosts of *H. irregulare*, both young and adult trees are susceptible to a white root rot and may be killed by the fungus (Fig. 2). Mortality occurs in a short period of time (i.e. one season) in young trees, with the foliage turning first red then brown, and progresses more slowly in older trees (Greig, 1998). In older trees, symptoms before death may include a decrease in annual shoot growth and the shedding of old needles, resulting in the so-called lion-tailing phenomenon and in a thinner crown (Gonthier & Thor, 2013). Needle length may also be shorter in infected trees. It should be noted that up to two-thirds of a root system may be destroyed by the pathogen before symptoms appear in the crown (Greig, 1998). This stage of obvious decline may last up to 10 years before a tree dies. Gaps in the forest resulting from tree mortality are often colonized by broadleaf trees or herbaceous weeds: this trait is characteristic of the disease (Gonthier & Thor, 2013). In *Juniperus* spp., symptoms are similar to those described above for pines, eventually resulting in plant death (Fig. 2).

• Confusion with other species

Attacks by *H. irregulare* may not be easily distinguished from those of other root rot agents like *H. annosum s.s.* and *Armillaria* spp. Pine-infecting *Heterobasidion* spp., including *H. irregulare*, produce sheets of a white, paper-thin mycelium beneath the bark of infected roots or at the tree collar (Greig, 1998). Although this cannot be regarded as a reliable diagnostic feature of the disease, it is generally sufficient to discriminate between Heterobasidion root rots and Armillaria root rots, whose signs consist of a thicker mycelium and/or the presence of rhizomorphs (Guillaumin & Legrand, 2013) (Fig. 3). Conversely, the features of the mycelium beneath the bark do not allow a distinction to be made between *H. irregulare* and other root rot fungi, including *H. annosum s.s.*

3.1.2. Symptoms on wood

Symptoms on wood include the development of a white stringy dry decay (Otrosina & Garbelotto, 2010).

3.1.3. Fruiting bodies

As for other *Heterobasidion* species, fruiting bodies of *H. irregulare* commonly develop at ground level, at the base of stumps or dead trees, and occasionally of diseased trees, and are often partially covered by litter (Fig. 4). Characteristically, they may envelop litter debris. They may also form on the underside of the root plates of fallen trees. In dry forests of western USA, fruiting bodies have been found under the intact top surface of pine stumps (Otrosina & Garbelotto, 2010). Fruiting bodies may be produced more intensively in the rainy and humid periods of the year such as in autumn.

3.2. Samples for laboratory testing

Guidance on sampling for laboratory testing is provided in PM 9/28 (EPPO, 2020).

3.3. Isolation

It is recommended that the samples are incubated in damp condition to maximize the success of isolation.

3.3.1. Sample preparation: Incubation of samples in damp conditions

3.3.1.1. Pieces of wood. Pieces of wood of at least 10 × 10 × 5 mm collected from the outer sapwood of roots or the tree collar of suspected trees (PM 9/28 [EPPO, 2020]), or from stumps/logs are incubated in a humid chamber at about 20–24°C for 10–15 days. After 10 days samples are inspected twice a week for a period of 3 weeks for the presence of colonies of *Heterobasidion* spp. These colonies can be easily identified at 65–160 × magnification under a dissecting microscope due to the presence of conidiophores of the unequivocally diagnostic asexual stage of *Heterobasidion* spp., formerly referred to as *Spiniger meineckellus* (A.J. Olson) (Fig. 5). The conidiophores appear as a mass of whitish 'pinheads' on stalks (Fig. 5).

Fig. 2 Mortality of *Pinus pinea* (left) and *Juniperus hemisphaerica* (right) caused by *Heterobasidion* spp.

Fig. 3 Mycelia of *Heterobasidion* spp. (left), mycelia (white arrow) and rhizomorphs (black arrow) of *Armillaria* spp. (right) beneath the bark.

Fig. 4 Examples of fruiting bodies of *Heterobasidion irregulare*.

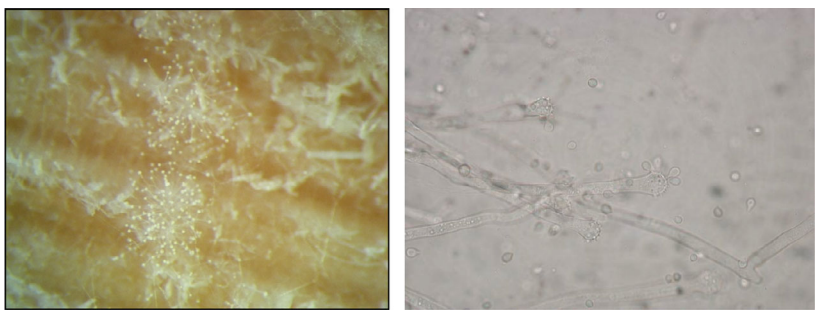

Fig. 5 *Heterobasidion* spp. conidiophores on a piece of wood after incubation in damp conditions at 160 × magnification (left). *H. irregulare* conidiophores and conidia at the microscope at 400 × magnification (right).

3.3.1.2. Passive spore traps. Passive spore traps consist of *Pinus* spp. or *Picea abies* wood disks, approximately 12 cm in diameter and 0.5 cm in thickness, exposed for 24 h in the field inside open Petri dishes containing sterile pieces of filter paper dampened with sterile water (see PM 9/28 [EPPO, 2020]). After field exposure of woody spore traps (see PM 9/28 [EPPO, 2020]), filter papers on which wood disks were placed (Fig. 6) should be replaced by new filter papers dampened with sterile water in the laboratory, and disks should be incubated at about 20–24°C for 10–15 days. It may be possible to recover mycelium after 6 days, although it is preferable to wait for the development of conidiophores (see above). Mycelia and/or conidiophores grown on wood disk surfaces should be collected using an adhesive tape for subsequent DNA extraction (see Appendix 2, Section 2.1.2.) (Fig. 6).

For isolation, disks should be inspected as described above (see Section 3.3.1.1) to identify individual colonies of *Heterobasidion* spp. When *Heterobasidion* spp. colonies are present, a sample of 3–5 colonies per disk should be isolated as described below.

3.3.2. Isolation procedure

Isolation may be attempted from fruiting bodies or from infected wood samples incubated as described in Section 3.3.1. In the case of wood samples, isolation without prior incubation may be attempted but experience shows that this is less successful. Pieces of tissue of approximately 2 × 2 × 5 mm in size are excised using a sterile scalpel from the context of fruiting bodies or from wood. Before being transferred onto Petri dishes filled with a PCNB-

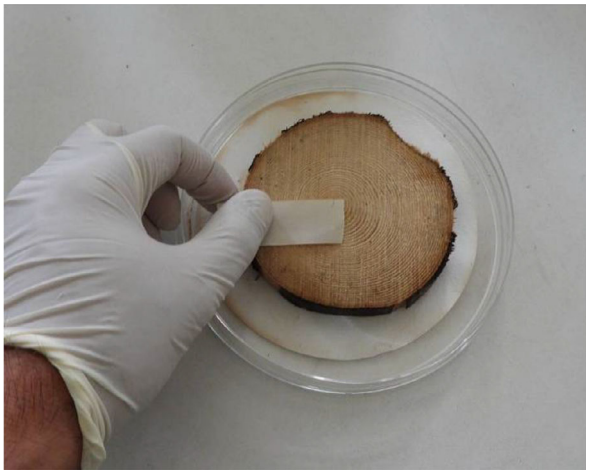

Fig. 6 Procedure to collect mycelia growing on the surface of a wood disk using adhesive tape. Mycelia are collected by sticking the adhesive tape several times on the whole surface of the woody spore trap.

based selective medium for *Heterobasidion* spp. (see Appendix 1), the pieces of tissue are surface sterilized in a 30% solution of hydrogen peroxide (H_2O_2) or in a 5% solution of sodium hypochlorite (NaOCl) for approximately 8 s, and subsequently rinsed three times in sterile water for approximately 10 s (Giordano *et al*., 2009). Hyphae and conidiophores from colonies grown on woody samples can instead be directly transferred onto Petri dishes filled with a PCNB-based selective medium (see Appendix 1) without the need for a disinfection step. Petri dishes are incubated at room temperature (Kuhlman & Hendrix, 1962) for at least 4–5 days in the light. Subcultures of pure *Heterobasidion* spp. mycelia can be carried out on malt extract agar (MEA). Although successful isolation can sometimes be achieved with generic agar-based substrates (e.g. MEA, potato dextrose agar [PDA]) amended with antibiotics, a higher rate of isolation success has been achieved with the PCNB-based selective medium (Gonthier, pers. comm.).

For an optimal DNA extraction from fungal isolates, it is recommended that isolates are cultured in flasks containing a liquid medium, e.g. malt extract 2% (w/v), at room temperature for at least 1 week. Mycelia are subsequently collected from flasks through a vacuum pump.

3.4. Screening tests

H. irregulare can be detected and identified using the loop-mediated isothermal amplification (LAMP) test for *H. irregulare* (Sillo *et al*., 2018) on any type of sample, including suspected fruiting bodies, infected wood samples, wood chips resulting from drillings conducted on suspected trees, mycelia collected on woody traps using adhesive tape. This test is described in Appendix 2.

For the LAMP test, samples collected by drilling should be transferred into 2-mL screw-cap microtubes, kept at −20°C for at least 24 h, and placed in a lyophilizer for an additional period of 24 h or overnight. This process is described by Guglielmo *et al*. (2010).

Both a real-time PCR test (Lamarche *et al*., 2017; Appendix 3) and a conventional PCR test (Gonthier *et al*., 2007; Appendix 4) have been developed for the identification of *H. irregulare*. While these tests have been validated on isolates, no validation data on fruiting bodies and wood-based samples is available. However, based on the fact that the risk of cross-reaction with other fungi is very limited when testing fruiting bodies and the analytical sensitivity will not be an issue, the two tests are considered appropriate for fruiting bodies as well.

4. Identification

Morphological characteristics alone do not allow identification of *Heterobasidion* at species level, consequently molecular tests are required.

4.1. Morphological characters of *Heterobasidion* spp.

Colonies are typical and easy to distinguish from other wood-decaying fungi. A description of fruiting bodies, conidiophores, asexual spores and mycelia is provided below.

Abundant and characteristic conidiophores and asexual spores (conidia) are produced in culture (Stalpers, 1978), making it easy to distinguish *Heterobasidion* spp. from other fungi (Fig. 5). However, macromorphological and micromorphological characters of cultures are not sufficiently divergent to discriminate *H. irregulare* from other *Heterobasidion* species (Mitchelson & Korhonen, 1998).

4.1.1. Fruiting bodies

H. irregulare fruiting bodies are 1–30 cm across (Otrosina & Garbelotto, 2010), perennial, typically in the form of brackets (shelf-shaped) or resupinate, and rubbery in texture (Fig. 3). The top surface, if present, is reddish or dark brown (tobacco brown) in colour and becomes darker with age. The margin is distinct and white. The lower surface is characterized by numerous small pores and is white or cream coloured. Incipient fruiting bodies may form under the bark or on roots of windblown trees. These are small in size (0.5–1 cm in diameter) and are referred to as 'pustules' or 'popcorns' because of their appearance (Greig, 1998; Otrosina & Garbelotto, 2010). Slight differences among the different species of *Heterobasidion* occur in the macromorphology and micromorphology of fruiting bodies, especially in the size of pores, in the length of brown hairs on the upper margin of fruiting bodies and, to a lesser extent, in the size of spores (Mitchelson & Korhonen, 1998; Otrosina & Garbelotto, 2010). As differences are small and morphological traits are partially overlapping

among species, identification of *H. irregulare* based on morphology of fruiting bodies is not reliable.

4.1.2. Conidiophores, asexual spores and mycelia

In culture, *Heterobasidion* spp., including *H. irregulare*, develop club-like conidiophores with conidiogenous vesicles 7.5–18(–22) µm diameter (Stalpers, 1978) (Fig. 5). Asexual spores, 4–8(–10) × 2.5–5(–6) µm in size, are subglobose to ovoid or lacrymoid, smooth, without vacuoles (Stalpers, 1978). Colonies are white or cream to light buff, ivory yellow or honey yellow. Marginal and aerial hyphae are 1.5–5.5(–8) µm wide, with clamp connections typically present but rare (Stalpers, 1978).

4.2. Molecular tests

Identification of *H. irregulare* is possible with the following molecular tests:

- LAMP (Sillo *et al*., 2018), see Appendix 2
- real-time PCR (Lamarche *et al*. 2017), see Appendix 3
- conventional PCR (Gonthier *et al*., 2007), see Appendix 4.

This last approach, combined with a PCR test on a mitochondrial locus according to Gonthier *et al*. (2003, 2007), may allow hybrids between *H. irregulare* and *H. annosum s.s*. to be identified.

Sequencing of target loci (e.g. translation elongation factor 1-alpha) and BLASTn analysis against sequences published in sequence databases (e.g. GenBank) may be used for identification. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2016).

5. Reference material

Selected *H. irregulare* isolates are deposited in culture collections of the University of Torino.

For reference material, contact the University of Torino, Department of Agricultural, Forest and Food Sciences, Largo Paolo Braccini 2, I-10095 Grugliasco, Turin (IT). paolo.gonthier@unito.it.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended that this database is consulted as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on this organism can be obtained from:

P. Gonthier & F. Sillo (see contact details below).

M. Garbelotto, UC Berkeley (US). matteog@berkeley.edu.

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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The Standard was reviewed by the Panel on Diagnostics in Mycology in October 2018.

References

Chomczynski P & Rymaszewski M (2006) Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. *BioTechniques* **40**, 454–458.

Dalman K, Olson Å & Stenlid J (2010) Evolutionary history of the conifer root rot fungus *Heterobasidion annosum sensu lato*. *Molecular Ecology* **19**, 4979–4993.

EPPO (2016) EPPO Diagnostic Standard PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bulletin* **46**(3), 501–537.

EPPO (2018) EPPO Global Database. European and Mediterranean Plant Protection Organization. https://gd.eppo.int [accessed in September 2018].

EPPO (2020) PM 9/28 *Heterobasidion irregulare*: Procedures for official control. *EPPO Bulletin* **50**(1), 142–147.

Garbelotto M & Gonthier P (2013) Biology, epidemiology, and control of *Heterobasidion* species worldwide. *Annual Review of Phytopathology* **51**, 39–59.

Garbelotto M, Guglielmo F, Mascheretti S, Croucher PJP & Gonthier P (2013) Population genetic analyses provide insights on the

introduction pathway and spread patterns of the North American forest pathogen *Heterobasidion irregulare* in Italy. *Molecular Ecology* **22**, 4855–4869.

Garbelotto M, Linzer R, Nicolotti G & Gonthier P (2010) Comparing the influences of ecological and evolutionary factors on the successful invasion of a fungal forest pathogen. *Biological Invasions* **12**, 943–957.

Giordano L, Gonthier P, Varese GC, Miserere L & Nicolotti G (2009) Mycobiota inhabiting sapwood of healthy and declining Scots pine (*Pinus sylvestris* L.) trees in the Alps. *Fungal Diversity* **38**, 69–83.

Giordano L, Gonthier P, Lione G, Capretti P & Garbelotto M (2014) The saprobic and fruiting abilities of the exotic forest pathogen *Heterobasidion irregulare* may explain its invasiveness. *Biological Invasions* **16**, 803–814.

Gonthier P, Garbelotto M & Nicolotti G (2003) Swiss stone pine trees and spruce stumps represent an important habitat for *Heterobasidion* spp. in subalpine forests. *Forest Pathology* **33**, 191–203.

Gonthier P, Warner R, Nicolotti G, Mazzaglia A & Garbelotto MM (2004) Pathogen introduction as a collateral effect of military activity. *Mycological Research* **108**, 468–470.

Gonthier P, Nicolotti G, Linzer R, Guglielmo F & Garbelotto M (2007) Invasion of European pine stands by a North American forest pathogen and its hybridization with a native interfertile taxon. *Molecular Ecology* **16**, 1389–1400.

Gonthier P & Garbelotto M (2011) Amplified fragment length polymorphism and sequence analyses reveal massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into its introduced congener *H. irregulare*. *Molecular Ecology* **20**, 2756–2770.

Gonthier P & Thor M (2013) Annosus root and butt rots. In *Infectious Forest Diseases* (eds. Gonthier P & Nicolotti G), pp. 128–158. CAB International, Wallingford (UK).

Gonthier P, Lione G, Giordano L & Garbelotto M (2012) The American forest pathogen *Heterobasidion irregulare* colonizes unexpected habitats after its introduction in Italy. *Ecological Applications* **22**, 2135–2143.

Gonthier P, Anselmi N, Capretti P, Bussotti F, Feducci M, Giordano L, *et al*. (2014) An integrated approach to control the introduced forest pathogen *Heterobasidion irregulare* in Europe. *Forestry* **87**, 471–481.

Greig BJW (1998) Field recognition and diagnosis of *Heterobasidion annosum*. In *Heterobasidion annosum. Biology, Ecology, Impact and Control* (eds. Woodward S, Stenlid J, Karjalainen R & Hüttermann A), pp. 35–41. CAB International, Wallingford (UK).

Guglielmo F, Gonthier P, Garbelotto M & Nicolotti G (2010) Optimization of sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees. *Letters in Applied Microbiology* **51**, 90–97.

Guillaumin JJ & Legrand P (2013) Armillaria root rots. In *Infectious Forest Diseases* (eds. Gonthier P & Nicolotti G), pp. 159–178. CAB International, Wallingford (UK).

Kuhlman EG & Hendrix FF (1962) Selective medium for isolation of *Fomes annosus*. *Phytopathology* **52**, 1310.

Lamarche J, Potvin A, Stewart D, Blais M, Pelletier G, Shamoun SF, *et al*. (2017) Real-time PCR assays for the detection of *Heterobasidion irregulare*, *H. occidentale*, *H. annosum sensu stricto* and the *Heterobasidion annosum* complex. *Forest Pathology* **47**, e12321.

Linzer RE, Otrosina WJ, Gonthier P, Bruhn J, Laflamme G, Bussières G, *et al*. (2008) Inferences on the phylogeography of the fungal pathogen *Heterobasidion annosum*, including evidence of interspecific horizontal genetic transfer and of human-mediated, long-range dispersal. *Molecular Phylogenetics and Evolution* **46**, 844–862.

Mitchelson K & Korhonen K (1998) Diagnosis and differentiation of intersterility groups. In *Heterobasidion annosum. Biology, Ecology, Impact and Control* (eds. Woodward S, Stenlid J, Karjalainen R & Hüttermann A), pp. 71–92. CAB International, Wallingford (UK).

Olson A, Aerts A, Asiegbu F, Belbahri L, Bouzid O, Broberg A, *et al*. (2012) Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. *New Phytologist* **194**, 1001–1013.

Otrosina WJ, Chase TE, Cobb FW Jr & Korhonen K (1993) Population structure of *Heterobasidion annosum* from North America and Europe. *Canadian Journal of Botany* **71**, 1064–1071.

Otrosina WJ & Garbelotto M (2010) *Heterobasidion occidentale* sp. nov. and *Heterobasidion irregulare* nom. nov.: a disposition of North American *Heterobasidion* biological species. *Fungal Biology* **114**, 16–25.

Sillo F, Garbelotto M, Friedman M & Gonthier P (2015) Comparative genomics of sibling fungal pathogenic taxa identifies adaptive evolution without divergence in pathogenicity genes or genomic structure. *Genome Biology and Evolution* **7**, 3190–3206.

Sillo F, Giordano L & Gonthier P (2018) Fast and specific detection of the invasive forest pathogen *Heterobasidion irregulare* through a Loop-mediated isothermal AMPlification (LAMP) assay. *Forest Pathology* **48**, e12396.

Stenlid J & Rayner ADM (1991) Patterns of nuclear migration and heterokaryosis in pairings between sibling homokaryons of *Heterobasidion annosum*. *Mycological Research* **95**, 1275–1283.

Stalpers JA (1978) *Identification of wood-inhabitating Aphyllophorales in pure culture. Studies in Mycology No. 16*, 248 pp. Centraalbureau voor Schimmelcultures, Baarn (NL).

Staroscik A (2004) dsDNA copy number calculator. URI Genomics & Sequencing Center. https://cels.uri.edu/gsc/cndna.html [accessed in September 2018].

Tomlinson JA, Dickinson MJ, & Boonham N (2010) Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device, *Phytopathology* **100**(2), 143–149.

Appendix 1 – Buffers and media (if relevant)

PCNB-based selective medium (Kuhlman & Hendrix, 1962)

Bacto-peptone	5 g
Agar	20 g
$MgSO_4$	0.25 g
KH_2PO_4	0.5 g
Streptomycin	78 500 IU
Pentachloronitrobenzene	190 ppm
Distilled water to	1 L
Autoclave for 20 min at 121°C	
After sterilization, add	
ethyl alcohol 95%	20 mL
lactic acid 50%	2 mL

Store at approximately 4°C in the dark for not more than 15 days.

Buffer for the rapid DNA extraction for the LAMP test for *H. irregulare*

Alkaline polyethylene glycol (PEG) buffer (Chomczynski & Rymaszewski, 2006).

Poly(ethylene glycol) average Mn 4600	2.5 g
KOH 2M	0.93 mL
Distilled water	39 mL
Adjust pH to 13.0–13.5 with KOH 0.5M	

Store at room temperature.

Tris-borate-EDTA (TBE) 5X

Tris base	54 g
Boric acid	27.5 g
EDTA (pH 8.0) 0.5M	20 mL
Distilled water to	1 L
Autoclave for 20 min at 121°C.	

Store at room temperature.

Appendix 2 – LAMP (Sillo *et al.*, 2018)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1 Detection of *H. irregulare* in samples derived from fruiting bodies, wood samples, wood chips resulting from drillings conducted on suspected trees, adhesive tape from woody traps and isolates.

1.2 The targeted gene is cytochrome P450 monooxygenase with haem-binding activity (Transcript ID 472015; NCBI Reference Sequence: XM_009545194.1) of *H. irregulare* TC 32-1 genome (Olson *et al.*, 2012).

1.3 Oligonucleotides:

Name	Sequence (5′→3′)	Amplicon length (bp)
Forward primer HirrSC3_F3	GCCACCAAAACTGGTTGT	198
Reverse primer HirrSC3_B3	TGAAGATGTCAATGGAGGT	
Forward primer HirrSC3_FIP	TCACTAGAACCGATTTCATG GGTAAAGGTGCTAGAGCATAGC	
Reverse primer HirrSC3_BIP	AGTGGAGAATCGTTGTTACAG TCCACTGTCGACATAAGTGCA	
Forward primer HirrSC3_FL	ACATGGCGTACGTATGCTTG	
Reverse primer HirrSC3_BL	GAGGTTGAAGACAAAAAC TTACGTG	

Recommended purification method: HPLC.

1.4 Equipment: Real-time PCR system such as CFX Connect™ Real-time PCR detection system equipped with FAM reading channel (Bio-Rad, USA) or Genie® II (OptiGene, UK).

1.5 Software and settings (automatic or manual) for data analysis.Bio-Rad CFX Manager™ software V.3.0 (Bio-Rad, USA). Automatic settings for fluorescence (FAM) detection and melting curves analysis. Other detection instruments equipped with a fluorophore (FAM) reading channel can also be used.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Tissue source: Wood chips resulting from drillings on suspected trees, fruiting bodies, infected wood samples. About 200 mg of lyophilized wood chips or fruiting bodies per sample should be homogenized using four glass beads (3 mm and 5 mm in diameter) in a FastPrep FP120 Cell Disrupter (Qbiogene, Irvine, CA, USA) with the following settings: 30 s of homogenization at speed 5.5, repeated twice.For DNA extraction use of the EZNA® Stool DNA Kit (Omega Bio-Tek, USA) is recommended, following the manufacturer's instructions. Final elution should be preferably done in 150 µL of the elution buffer provided in the kit.

2.1.2 Tissue source: Mycelia collected from woody spore traps (see Section 3.3.1.2). The rapid DNA extraction method for mycelia collected from woody spore traps is based on the use of alkaline PEG (Chomczynski & Rymaszewski, 2006). The samples (pieces of adhesive tape) are homogenized as a crude macerate using a 10-mm stainless steel bead in 5-mL plastic tubes containing 2 mL alkaline PEG lysis buffer (see Appendix 2). Tubes are shaken by hand for 2 min, and 1 µL of the ten-fold dilution of the crude macerate should be used in the LAMP test. This rapid DNA extraction method can be also used on small amounts (approximately 50–150 mg) of biological material from fruiting bodies, infected wood samples and wood chips resulting from drillings. However, the test performed using this rapid extraction method can show a lower analytical sensitivity compared to that performed following the standard method described above (Sillo *et al.*, 2018).

2.1.3 Tissue source: Cultures (isolates). Extraction of DNA from isolates can be performed by using available kits, e.g. Qiagen's DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

2.1.4 DNA should preferably be stored at approximately −20°C.

2.2 LAMP

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water *	NA	2.75	NA
Isothermal Mastermix ISO-001 (OptiGene)	1.3×	15	0.975×
Forward Primer F3 (HirrSC3_F3)	40 µM	0.125	250 nM
Reverse Primer B3(HirrSC3_B3)	40 µM	0.125	250 nM
Forward Primer FIP(HirrSC3_FIP)	40 µM	0.25	500 nM
Reverse Primer BIP(HirrSC3_BIP)	40 µM	0.25	500 nM
Forward Primer FL(HirrSC3_FL)	40 µM	0.25	500 nM
Reverse Primer BL(HirrSC3_BL)	40 µM	0.25	500 nM
Subtotal		19	
DNA dilution		1	
Total		20	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free. NA, not applicable.

2.2.2 LAMP conditions: The temperature condition for the LAMP test, which is an isothermal test, is 65°C for 40 min with a read plate (fluorescence detection) every minute. The programme for the calculation of melting curves is a ramp from 65°C to 95°C with a temperature increment of 0.1°C and a read plate every 10 s. Amplification and melting curves can be analysed using Bio-rad-CFX manager software (Bio-Rad, USA).

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control.[2] For LAMP reactions not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- For plant samples, the test should be combined with the COX LAMP test developed by Tomlinson *et al*. (2010) in order to confirm the successful DNA extraction.

3.2 Interpretation of results

In order to assign results from the test the following criteria should be followed:

Verification of the controls

- NIC and NAC should give no amplification
- PIC and PAC (and if relevant IPC) amplification curves (real-time measurement with melting curve analysis) should be exponential. A positive reaction is defined by exponential fluorescence signal as well as T_m (85.50°C ± 0.5°C SD).

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve and a melting curve with a peak at 85.50°C ± 0.5 as defined for PIC and PAC (see above).
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve but with T_m different from that expected (85.50°C ± 0.5°C).
- Tests should be repeated if any contradictory or unclear results are obtained.

Comparison of melting curves among samples and positive controls should be carried out in order to exclude false-positive signals (Fig. 7).

4. Performance characteristics available

The test was evaluated according to PM 7/98 (EPPO, 2018).

4.1 Analytical sensitivity data

Based on published data (Sillo *et al*., 2018), the minimum limit of detection (LOD) of the assay is 19.9 pg/µL, corresponding to about 20 pg of target DNA per reaction. Based on the size of the nuclear genome of *H. irregulare* (33 Mb) (Olson *et al*., 2012), the number of nuclei in 20 pg of DNA may be estimated at 553 using appropriate formulas (Staroscik, 2004). Based on the average number

[2]Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole genome amplicons).

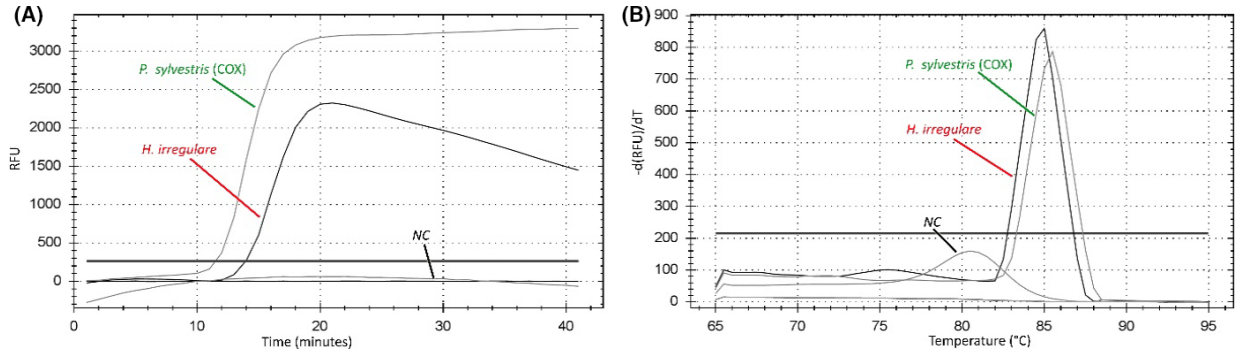

Fig. 7 Example of an infected *Pinus sylvestris* sample positive to COX (internal positive controls [IPC]) and *H. irregulare* (target) LAMP tests. (A) Amplification plot of plant DNA as detected by the COX LAMP test (grey line) and of *H. irregulare* DNA as detected by the specific test (black line). (B) Melting curves displayed as negative first derivative of the fluorescence versus temperature plot over the temperature ($-$d(RFU)/dT versus T). NC, negative amplification control. [Colour figure can be viewed at wileyonlinelibrary.com]

of nuclei contained in *Heterobasidion* spp. cells (Stenlid & Rayner, 1991), the above threshold of 20 pg DNA would correspond to about 50 hyphal cells.

4.2 Analytical specificity data

The analytical specificity-inclusivity was evaluated on:

9 isolates of *H. irregulare* of different origins.

All isolates were detected.

The analytical specificity-exclusivity was evaluated on:

12 isolates of other *Heterobasidion* spp. (non-target species most closely related in the genus)

14 isolates of seven fungal species often found in pines.

The LAMP test did not show cross-reactivity with DNA of other *Heterobasidion* species, including *H. abietinum, H. annosum s.s.* and *H. parviporum*, nor with the other seven wood decay fungal species often found in pine trees (Sillo *et al.*, 2018).

4.3 Data on repeatability

Repeatability was assessed by performing the standard DNA extraction and LAMP assay twice, and the rapid DNA extraction and LAMP assay by two different operators. Five positive and five negative DNA samples were used (Sillo *et al.*, 2018). There were no significant differences in terms of time of detection of the pathogen when analyses were performed by different operators (*t*-test; P value < 0.05).

4.4 Data on reproducibility

The value of diagnostic specificity was 1.0 (100%) and the value of diagnostic sensitivity was 1.0 (100%), considering a set of 40% of positive samples extracted with standard DNA extraction protocol.

The value of diagnostic specificity was 1.0 (100%) and the value of diagnostic sensitivity was 0.964 (96.4%), considering a set of 40% of positive samples extracted with rapid DNA extraction protocol (Sillo *et al.*, 2018).

Appendix 3 – Real-time PCR (Lamarche *et al.*, 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 The tests are designed for the detection and identification of *H. irregulare* and *Heterobasidion annosum s.l.* in isolates and fruiting bodies in simplex reactions.

1.2 The targetted gene is ITS

1.3 Oligonucleotides:

Name	Sequence (5′→3′)	Amplicon length (bp)
Heterobasidion annosum s.l.		
Forward primer Hannosum_sl_F388	GAGATCCATTGTTG AAAGTTGTATA	204
Reverse primer Hannosum_sl_R591	GAATATCGTGCRRGGT TGAA	
Probe Hannosum_ sl_T512RC	6-Fam/CCATCTCAC/ZEN/ ACCTGTGCACACTC/ 3IABkFQ	
Heterobasidion irregulare		
Forward primer Hirregulare_4A/ G_F435	CATTCTGAAGACAT ACGAGGGA	66
Reverse primer Hirregulare_R500	GGTCGGGTTCTTTTGAT	
Probe Hannosum_ ss_irregulare_ T464RC	6-Fam/TTCCGAGCC/ZEN/ GCGTCTTCT/3IABkFQ	

1.4 Cycler or real-time PCR system or other equipment name, producer name. The test requires appropriate instruments for fluorescence detection, e.g. Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).

1.5 Software and settings (automatic or manual) for data analysis. Data analysis requires commercial and freeware standard software for real-time PCR data analysis,

e.g. Applied Biosystems® 7500 Real-Time PCR Software.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Tissue source: isolates

Extraction of DNA from isolates can be performed by using available kits. The Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) is used in the test described in Lamarche *et al*. (2017) according to the manufacturer's instructions.

Storage temperature and conditions of DNA: DNA should preferably be stored at approximately −20°C.

2.2 Real-time PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	1.8	NA
QuantTect™ Multiplex PCR NoROX Master Mix (Qiagen)	2×	5	1×
Forward primer	10 µM	0.6	0.6 µM
Reverse primer	10 µM	0.6	0.6 µM
Probe	10 µM	1	1 µM
Subtotal		9	
DNA dilution		1	
Total		10	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free. NA, not applicable.

2.2.2 Real-time PCR thermocycling conditions: 95°C for 15 min, followed by 50 cycles at 95°C for 15 s and 60°C for 90 s.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product[3] For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) should be used to monitor each individual sample separately and to check for potential PCR inhibition. IPCs can be genes either present in the matrix DNA or added to the DNA solutions.

IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. 18S rDNA).

3.2 Interpretation of results

In order to assign results from a PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Performance characteristics available

The data presented below is from Lamarche *et al*. (2017).

4.1 Analytical sensitivity dataAnalytical sensitivity was determined as the lowest target concentration yielding at least 95% of positive results. It was estimated to nine copies of the ITS region of target DNA, corresponding approximately to one binucleate spore per microlitre.

4.2 Analytical specificity-inclusivity

The analytical specificity-inclusivity was evaluated on 17 isolates of *H. irregulare* of different origins and different hosts. All isolates were detected.

The analytical specificity-(exclusivity) was evaluated on:

7 isolates *Heterobasidion abietinum*

14 isolates of *H. annosum s.s.*

8 isolates of *H. occidentale*

9 isolates of *H. parviporum*

5 isolates of *H. orientale*

[3]Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons.).

1 isolate of *H. araucariae*
5 isolates of *H. ecrustosum*
1 isolate of *H. insulare*

Using the real-time PCR test, no cross-reaction with the DNA of these isolates and species was observed.

4.3 Data on repeatabilityA high degree of repeatability was shown (see Lamarche *et al*., 2017).

4.4 Data on reproducibility
Data not available.

Appendix 4 – Conventional PCR test (Gonthier *et al*., 2007)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1 The test is a duplex conventional PCR test and is used for the identification of *H. irregulare* isolates and fruiting bodies. This test can be coupled with a PCR test on a mitochondrial locus according to Gonthier *et al*. (2003, 2007) in order to detect hybrids between *H. irregulare* and *H. annosum s.s*.

1.2 The targeted gene is the elongation factor 1-α (EF1-α) (GenBank Accession Number *H. irregulare* CP15: DQ916085.1).

1.3 Primer sequences and amplicon sizes for *H. irregulare*

Name	Sequence (5′→ 3′)	Amplicon length (bp)
Forward primer EFaHaFor	CTATGTCGCGGTACA GCTTG	169 bp
Reverse primer EFaHaRev	GCGAGGA(T/C)AAGAA GTAATCAGCA	
Forward primer EFaNAPFor	GTACATGGTCACTGTA CGTAGATGC	71 bp

The primers EFaHaFor and EFaHaRev amplify the target gene of *H. annosum* s.l., while EFaNAPFor in combination with EFaHaRev amplify a portion of the target gene of *H. irregulare*.

1.4 PCR system or other equipment name, producer name

The test requires standard instruments for PCR (i.e. thermocycler) and equipment for gel electrophoresis. PCR products should be electrophoresed in a 0.5 × Tris-borate buffer (TBE; see Appendix 2 for recipe) gel containing 1% standard agarose and 1% metaphor agarose (FMC Bioproducts) at 4 V/cm for 2 h.

1.5 Software and settings (automatic or manual) for data analysis.

Data analysis requires commercial and freeware standard software for gel documentation system, e.g. Image Lab™ software (Bio-Rad).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Tissue source: isolates

Extraction of DNA from isolates can be performed by using available kits, e.g. Qiagen's DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

2.1.2 Storage temperature and conditions of DNA: DNA should preferably be stored at approximately −20°C.

2.2 Conventional PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water*	NA	7.875	NA
PCR buffer (Promega)	5×	5	1×
$MgCl_2$ (Promega)	25 mM	1.5	1.5 mM
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer (EFaHaFor)	10 μM	1.25	0.5 μM
Forward primer (EFaNAPFor)	10 μM	1.25	0.5 μM
Reverse primer (EFaHaRev)	10 μM	1.25	0.5 μM
GoTaq DNA polymerase (Promega)	5 U/μL	0.125	0.625 U
Subtotal		23	
Genomic DNA extract		2	
Total		25	

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 μm filtered) and nuclease-free. NA, not applicable.

2.2.2 PCR thermocycling conditions

PCR conditions for amplification: 94°C for 5 min, followed by 35 cycles at 95°C for 45 s, 62°C for 45 s and 72°C for 45 s. Final extension step is required (72°C for 7 min).

3.Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product.[3] For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Internal positive controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

When generic primers are used on isolated specimens, this could be considered as an alternative to the PIC.

3.2 Interpretation of results

In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce two amplicons of 169 and 71 bp after PCR.

When these conditions are met

- A test will be considered positive if two amplicons of 169 and 71 bp are produced after PCR (Fig. 8).

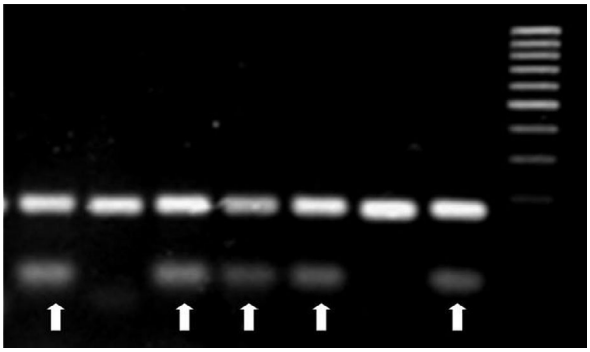

Fig. 8 Visualization on agarose/metaphor gel after electrophoresis of amplicons produced by the conventional PCR test for *H. irregulare*. Two bands of 169 and 71 bp are produced when the PCR is performed on *H. irregulare* DNA (white arrows). Ladder is 100 bp.

- A test will be considered negative if it produces no band or bands of a different size, or if only the amplicon of 169 bp is produced. Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

Data not available

4.2 Analytical specificity data

The analytical specificity-inclusivity was evaluated on 490 isolates of *H. irregulare*. All isolates were detected (Gonthier *et al*., 2007).

The analytical specificity-exclusivity was evaluated on:

86 isolates of *H. annosum s.s*.

No cross-reaction between species was noted (Gonthier *et al*., 2007).

4.3 Data on repeatability

Data not available.

4.4 Data on reproducibility

Data not available.

[3]Laboratories should take additional care to prevent risks of cross-contamination when using high-concentration positive controls (e.g. cloned products, gBlocks and whole genome amplicons)