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ISPM 27
Diagnostic protocols for regulated pests

DP 34: *Heterobasidion annosum sensu lato*

Adopted 2025; published 2025

CONTENTS

1. Pest information

Heterobasidion annosum sensu lato (*s.l.*) (Fr.) Bref., 1888 is a complex of species (also referred to as the *H. annosum* complex) that includes the most common and destructive fungal pathogens of conifers causing root and butt rots. They are widely distributed in the coniferous forests of the northern hemisphere (Korhonen *et al*., 1998; Dai *et al*., 2003; Worrall *et al*., 2010). *Heterobasidion annosum s.l.* is a major concern, especially in intensively managed forests, where the decrease in wood production and the reduction in wood quality caused by the pathogens can result in high economic losses.

The fungus had long been regarded as a single species until mating experiments revealed the occurrence of intersterile groups within *H. annosum s.l.* (Korhonen, 1978; Chase and Ullrich, 1988; Capretti *et al.*, 1990). A formal description was published for each intersterile group, with the species being named according to their host preferences. Five phytopathogenic species are therefore currently included within *H. annosum s.l.*: *H. abietinum* Niemelä & Korhonen, 1998, *H. annosum sensu stricto* (*s.s.*) (Fr.) Bref., 1888, *H. irregulare* Garbel. & Otrosina, 2010, *H. occidentale* Otrosina & Garbel., 2010 and *H. parviporum* Niemelä & Korhonen, 1998. They are characterized based on their geographical distribution, host preference, morphology, and biochemical, phylogenetic and genomic traits (Otrosina *et al*., 1993; Linzer *et al*., 2008; Dalman, Olson and Stenlid, 2010; Garbelotto and Gonthier, 2013; Chen *et al*., 2015; Sillo *et al*., 2015; Yuan *et al*., 2021). An additional species, *H. subparviporum* Y.C. Dai, Jia J. Chen & Yuan Yuan, 2021, seems to be a saprotroph (Yuan *et al*., 2021).

Three of the phytopathogenic species – *H. abietinum*, *H. annosum s.s.* and *H. parviporum* (Niemelä and Korhonen, 1998) – are found in some countries in Europe and Asia. They are mainly associated with *Abies alba*, *Pinus* spp., and *Picea abies*, respectively (Garbelotto and Gonthier, 2013), but also found on most *Picea* and *Abies* species and on other conifers, such as *Pseudotsuga* spp. and *Larix* spp. (Korhonen *et al*., 1998). Depending on tree species composition and structure, these three species of *Heterobasidion* can co-exist in the same conifer stand (e.g. Gonthier, Garbelotto and Nicolotti, 2003). The remaining two species occur naturally in North America: *H. occidentale* (Otrosina and Garbelotto, 2010), mainly associated with *Picea* spp., *Tsuga* spp., *Pseudotsuga* spp. and *Sequoiadendron* spp.; and *H. irregulare*, mainly associated with *Pinus* spp., *Juniperus* spp., *Calocedrus decurrens* and *Abies* spp. Garbelotto and Gonthier (2013) describe the susceptibility of some tree species to *H. annosum s.l.* species and the main host tissue colonized.

The presence of *H. irregulare* in Europe is a relatively recently discovered example of a biological invasion, which occurred after the accidental introduction of this North American species into central Italy during the Second World War (Gonthier *et al*., 2004). *Heterobasidion irregulare* is now associated with significant mortality of *Pinus pinea*, in monocultural pine stands, in mixed oak–pine natural forests and in urban parks (Gonthier *et al*., 2007, 2014). In addition, it has been recently reported that *H. irregulare* is replacing *H. annosum s.s.* along the Tyrrhenian coast of Italy (Garbelotto *et al*., 2022), therefore threatening some other parts of Europe. In September 2015, *H. irregulare* was included in the A2 list of pests recommended for regulation by the European and Mediterranean Plant Protection Organization (EPPO). More information about *H. irregulare* is available in the EPPO Global Database (EPPO, 2024a).

Although *H. annosum s.l.* species are characterized by partial interfertility in laboratory experiments, hybrids between the two sympatric North American species, *H. irregulare* and *H. occidentale*, have rarely been found in North America (Garbelotto *et al*., 1996; Lockman *et al*., 2014). Nevertheless, high hybridization rates have been observed between the two naturally allopatric species, *H. annosum s.s.* and *H. irregulare*, in Italian coastal pine woodlands (Gonthier *et al*., 2007). Phenotypic experiments have been performed to assess the fitness (virulence on host plants and saprobic growth) of artificial and natural *Heterobasidion* hybrids relative to the fitness of pure genotypes of each one of the two species (*H. annosum s.s.* and *H. irregulare*) (Giordano *et al*., 2018; Sillo *et al*., 2021).

Species of *H. annosum s.l.* have a varied infection biology (Rishbeth, 1950, 1951a, 1951b). Airborne propagules (almost exclusively basidiospores) infect freshly exposed wood surfaces, such as stump tops

or stem and root wounds (primary infection). Once established, the fungus may spread vegetatively via root contacts and grafts (secondary infection) (Stenlid and Redfern, 1998).

Heterobasidion annosum s.l. commonly develops fruiting bodies producing basidiospores at ground level: at the base of stumps or dead trees, on the roots of fallen trees, on decayed logs left in the forest, or inside the internal cavities of old decayed stumps. It also produces conidia developing from conidiophores; however, they play an active role only in short-distance dispersal of the fungus (Redfern and Stenlid, 1998) or when accidentally carried by root-feeding insects (Kadlec, Starý and Zumr, 1992).

For more general information about *H. annosum s.l.* (Heterobasidion root rot), see the CABI Compendium (CABI, 2022). Information on its distribution, updated regularly, is available in the EPPO Global Database (EPPO, 2024b).

2. Taxonomic information

The species complex *H. annosum s.l.* comprises six species (Chen *et al*., 2015; Yuan *et al*., 2021), in which five are phytopathogens responsible for severe economic and environmental losses (Garbelotto and Gonthier, 2013): *H. abietinum*, *H. annosum s.s.*, *H. irregulare*, *H. occidentale* and *H. parviporum*. A sixth species in the complex, *H. subparviporum*, seems to be a saprotroph (Yuan *et al*., 2021).

From a phytosanitary point of view, diagnostic protocols are available only for the five phytopathogenic species. Therefore, the following taxonomic information, and the whole of this diagnostic protocol, refers exclusively to the phytopathogenic members of the *H. annosum s.l.* species complex.

Name: *Heterobasidion annosum sensu lato* (Fr.) Bref., 1888

Other scientific names: *Polyporus annosus* Fr., 1821
Fomes annosus (Fr.) Cooke, 1885
Spiniger meineckellus (A.J. Olson) Stalpers, 1974 (anamorph or asexual stage)

Taxonomic position: Fungi, Basidiomycota, Russulales, Bondarzewiaceae, *Heterobasidion*

Common names: annosum root rot, annosus root rot, butt rot of conifers, heart rot of conifers, Heterobasidion root rot, root rot of conifers

MycoBank: MB#17745

An updated account of the global species diversity of the genus *Heterobasidion*, including species within the Asian *H. insulare* complex, is available in Yuan *et al*. (2021).

3. Detection

The detection of *H. annosum s.l.* species in the field is based on the examination of symptoms and the finding of fruiting bodies (Greig, 1998; Gonthier and Thor, 2013). Detection should be complemented by the collection of samples in the field and their subsequent analysis with molecular methods. Molecular methods provide the most reliable way to accurately identify different species, and the best way of distinguishing closely related pathogen species. Detection of these pathogens can be achieved by biological (isolation and culture) or molecular methods, or by combining both types of methods (isolation and culture followed by molecular methods).

Serological tests cannot be used to detect *Heterobasidion* infection on site (forest) or from fungal hyphae in wood (Mitchelson and Korhonen, 1998).

This diagnostic protocol describes well-established methods for the detection and identification of different species within the complex *H. annosum s.l.* (except for *H. subparviporum*). It is not a comprehensive review of all methods available for the diagnosis of the species within *H. annosum s.l.*

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated. Notably, any changes to the polymerase enzyme used would need to be complemented with adjustments in the polymerase chain reaction (PCR) conditions.

3.1 Symptoms

Two different kinds of symptoms may be observed inside infected trees, depending on tree species and age: root rot and stem rot. Stumps and dead trees should also be examined for the presence of fruiting bodies. Symptoms and fruiting bodies are described below and are illustrated in Figure 1 to Figure 6. At the forest-stand scale, the occurrence of windthrown trees (see section 3.1.4.) or disease centres (zones of dead and dying trees) can be an indication of *Heterobasidion* infection in *Picea abies* and *Pinus sylvestris* stands (e.g. Piri *et al*., 2021).

3.1.1 Root rot

Tree species characterized by a resinous heartwood (e.g. *Pinus* spp., *Juniperus* spp.) are susceptible to a deadly root rot because the cambium of roots and root collars is attacked (Figure 1); the pathogen also causes a white, stringy, dry decay of wood. In young trees, mortality occurs within a short period (i.e. one season); in older trees, mortality proceeds more slowly. Disease symptoms may include the yellowing or browning of foliage, the decline of annual shoot growth, the shedding of old needles (also called the "lion-tailing phenomenon"; Figure 2) and crown transparency. Up to two-thirds of a root system may be killed by the pathogen before symptoms appear in the crown (Greig, 1998).

Symptoms of infection may not be easily distinguished from those of other root-rot agents such as *Armillaria* spp. *Heterobasidion annosum s.l.* produces sheets of white, paper-thin mycelium beneath the bark of infected roots or at the tree collar (Figure 3; 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 or the presence of rhizomorphs (Figure 3; Guillaumin and Legrand, 2013).

3.1.2 Stem rot

Species characterized by a non-resinous heartwood (e.g. *Picea* spp., *Abies* spp.) are susceptible to extensive heart rot in the roots, the butt (bole) and the stem (Figure 4). Often the decay is confined to the butt. However, in *Picea abies*, decay columns may extend several metres into the stem. In this case, the sheets of white, paper-thin mycelium beneath the bark are absent. Even when heart rot develops, in some cases resulting in cavities (Figure 4), external symptoms are rarely visible and mortality does not occur. However, symptoms may include resin flow, bulging of the lower stem, and crown deterioration. Mortality has been observed in saplings and in very young trees. Foliage yellowing can be observed in cases of advanced decay (Greig, 1998).

Heterobasidion species cause a stringy white rot and decayed wood often has the characteristic appearance of "pocket rot" (Figure 5) and stays rather light in colour for a long time. Fresh rot in the heartwood of spruce trees is often surrounded by a violet or aniline-coloured ring (the so called "reaction zone" between the colonized heartwood and healthy sapwood). *Armillaria* spp. typically cause a darker, clearly delimited rot, leading to the hollowing of spruce trees earlier than if infected by *H. parviporum*. In *Pinus sylvestris*, stem rot from *Heterobasidion* infection is confined to the base of the tree, and a typical symptom is the occurrence of resin patches visible on the stump surface when the tree is felled.

3.1.3 Fruiting bodies

Fruiting bodies of *H. annosum s.l.* most commonly develop at ground level: at the base of stumps or dead trees, inside the internal cavities of old decayed stumps, on the roots of windthrown trees, or enveloping litter debris (Figure 6). Occasionally, in sheltered conditions, tiers of brackets may be found on dead trees up to 2 m above the ground. In very dense, moist forests, fruiting bodies may be seen on the ground some distance away from standing trees, but these fruiting bodies are always attached to

infected roots just below the surface litter. In exceptional cases, fruiting bodies can also grow at the base of living trees in which stem rot is at a very advanced state (Greig, 1998). Brackets frequently form on the cut ends and the ground-facing side of pieces of timber left for several months in the forest (Figure 6). Fruiting bodies may be produced more intensively in the rainy and humid periods of the year.

Unfortunately, identifications in the field may be erroneous because morphological traits partially overlap between species and small differences occur. In general, fruiting bodies are perennial, pileate, resupinate or effused-reflexed (partly pileate, partly resupinate), rubbery in texture, and 1–40 cm across. The top surface is reddish or dark brown in colour (Figure 7) and becomes darker with age. The margin is distinct and white. The lower surface is white or cream coloured and is characterized by numerous small pores. Incipient fruiting bodies are small white resupinate pads. These are smaller in size (0.5–1 cm in diameter) and are referred to as "popcorn" because of their appearance (Figure 7; Otrosina and Garbelotto, 2010).

3.1.4 Windthrown trees

The uprooting of trees, for example in disease centres, can be used as an indication of *H. annosum s.l.* root and stem rot and can direct the closer examination of trees for sample collection.

Heterobasidion annosum s. l. root and stem rots increase the vulnerability of tree stands to wind damage because of the consequential decrease in tree anchorage (by wood decay in roots) and stem strength (by wood decay in stem wood). The risk mainly concerns tree species with a shallow root system and no tap-root, such as *Picea abies* (Figure 1). The loss of wood strength resulting from *H. annosum s.l.* infection has been experimentally demonstrated in *Picea sitchensis* (Pratt, 1979) and *Picea abies* (Giordano *et al*., 2013; Krisans *et al*., 2020). In the forest, this is manifested as uprooted trees (Figure 1).

Few studies of the strength properties of decayed wood from naturally infected trees have been reported and there is no standard method of estimating strength loss in these circumstances. Most studies on the loss of wood strength through fungal decay have been done on small samples of known physical properties, artificially inoculated, and assessed after varying periods of incubation.

The average rupture modulus (bending strength before rupture) and elasticity modulus (stiffness) of *Heterobasidion*-decayed wood in *P. sitchensis* trees can be 26% and 39% lower, respectively, than for healthy trees (Pratt, 1979).

Infected *Picea abies* trees are more susceptible to uprooting than healthy trees: 20% more susceptible in the case of trees with a diameter at breast height (DBH) of ≥21.3 cm and up to 33% in smaller trees (Giordano *et al*., 2013). More recently, Krisans *et al*. (2020) obtained similar results confirming the effect of *H. annosum s.l.* root and stem rots on the stability of trees. Based on a modelling study by Honkaniemi *et al.* (2017), an increase in the amount of wood decay reduces the predicted wind speeds needed for both uprooting and stem breakage of *Picea abies* trees and increases the predicted amount of wind damage. The probability of uprooting is predicted to be higher than that of stem breakage for infected trees.

3.2 Sampling and sample preparation

Different techniques for sampling and sample preparation as described below are recommended depending on the material being tested. Except for wood-disc traps for spores, samples should be kept cool and sent to the laboratory in closed plastic or paper bags or containers for isolation and culture by the following day at the latest. However, in sealed self-closing plastic bags, excessive moisture can hasten tissue degradation and saprophytic activity. Storage at low temperatures (2–8 °C) is highly recommended to prolong sample life. Even then, cultures should be established within a few days.

3.2.1 Fruiting bodies

Fruiting bodies and incipient fruiting bodies ("popcorn") of *H. annosum s.l.* should be placed in a sealed container or in closed plastic or paper bags and stored at 2–8 °C until laboratory analyses.

For the subsequent isolation of *H. annosum s.l.* from fruiting bodies, see section 3.3.1.

3.2.2 Wood samples from symptomatic or suspected trees, or wood residues

Wood samples (at least 10 × 10 × 5 mm) from living trees that are symptomatic or suspected of being infected should be collected, after bark removal, with a hammer and a chisel from the outer sapwood of roots or the tree collar or from stumps or logs. Depending on their state of decay, stumps and logs can also be sampled using an axe to expose the inner parts, or by using a drill that produces coarse woody chips. The chisel or axe must be disinfected with 70–95% ethanol (v⁄v) at the start of sample collection and after every sampling. Wood samples should be placed in a sealed container or in closed plastic or paper bags and stored at 2–8 °C until laboratory analyses. Subsequently, they should be incubated at room temperature (18–24 °C) under moist conditions for 7–15 days to promote development of mycelium and conidia (the anamorph stage).

Wood samples can also be collected from living trees by extracting wood cores by means of an increment borer. The increment borer must be disinfected with ≥70% ethanol (v⁄v) at the start of sample collection and after every drilling. The boring hole in living trees is typically sealed using garden wax to protect the tree from subsequent infections by wood-decay fungi. Wood cores may be incubated in sterile plastic bottles in the dark at room temperature (18–24 °C) for up to one month to induce growth of mycelia and conidiophores. Wood pieces harvested using an increment borer can also be used directly for establishing cultures on agar plates (see section 3.3.2).

It should be noted that there is a risk of missing the infected tissue when collecting wood samples from trees. This should be considered especially when sampling large trees and tree species where *Heterobasidion* spp. can cause extensive resin accumulation (*Pinus* spp.) that may hamper or prevent successful isolation of cultures from the affected wood (Piri *et al.*, 2021). In those cases, it is recommended that the number of samplings from each tree be increased. When sampling dead wood (e.g. stumps, logs, fallen trees), a more invasive investigation of the material can be carried out, and sampling can then be targeted at wood patches showing the typical appearance of Heterobasidion rot.

Sampling of symptomatic or suspected trees may be performed at any time of the year.

3.2.3 Wood sawdust from drillings of symptomatic (or suspected) and asymptomatic trees

Wood sawdust should be taken from drillings conducted at the base of symptomatic, suspected and asymptomatic trees, with a drill long enough to reach the heartwood, to obtain wood decayed by *Heterobasidion* spp. The procedure published by Guglielmo *et al.* (2010) is described here. After bark removal, four holes at 90° from one another should be drilled with a 4-mm-diameter, 43-cm-long drill bit at the base of the trunk (approximately 5 cm above ground). Two samples should be obtained by combining opposite drillings in 9-cm-diameter plastic Petri dishes (Figure 8) or in tubes and used for the molecular detection of the pathogen; alternatively, all the wood sawdust generated (from the four drillings) can be pooled together in one sample. After each sampling, the drill bit must be disinfected with 0.5% (w⁄v) sodium hypochlorite (NaClO) solution, rinsed with sterile distilled water, and wiped with 70% or 95% ethanol (v⁄v).

The diagnostic efficiency is higher in smaller trees (DBH 1.30 m above ground <80 cm) than in larger ones (DBH >80 cm) (Guglielmo *et al.*, 2010). In the latter case, or in the presence of monumental specimens, it may be useful to increase the number of drillings and to analyse the sawdust from each drilling separately to maximize the diagnostic efficiency. However, the choice of the most appropriate sampling method may vary depending on considerations such as tree diameter, acceptable injury levels, cost and the diagnostic sensitivity that is needed (Guglielmo *et al.*, 2010).

Samples should be stored at –20 °C until laboratory analyses.

Sampling of symptomatic, suspected and asymptomatic trees may be performed at any time of the year. Drilling may also be used to sample timber or wood in service.

Wood sealant should not be used to fill in the hole left by the drilling tool. However, thiophanate-methyl fungicide 38.5% (w/v) should be sprayed on holes to prevent fungal infections (Guglielmo *et al*., 2010).

3.2.4 Wood-disc traps for spores

Spores of *H. annosum s.l.* can be trapped using the modified version of the wood-disc exposure method (Rishbeth, 1959; James and Cobb, 1984; Gonthier *et al*., 2007). Freshly cut wood discs, approximately 11–13 cm in diameter, 0.5–1.5 cm in thickness and without bark, cut the day before their exposure in the forest from living and healthy *Picea abies* or *Pinus* spp., should be sprayed with 65% ethanol (v/v) and exposed for 24 hours in the field. Discs should be placed singly in open, 15-cm diameter plastic Petri dishes containing sterile pieces of filter paper dampened with 3.5 mL of sterile distilled water (Figure 8) to prevent drying during exposure. Alternatively, the discs can be placed on top of aluminium foil pieces. The Petri dishes or foil are used to prevent soil-borne contaminants. Discs in closed Petri dishes should also be included as controls to check for possible contamination with *H. annosum s.l.* already present in the wood discs used as traps or airborne spores that landed on the wood during trap preparation. After field exposure, the filter papers on which the wood discs are placed should be replaced by new filter papers dampened with sterile distilled water in the laboratory. The wood discs are then incubated at room temperature (18–24 °C) for 7–15 days. The discs should be regularly checked for possible *H. annosum s.l.* colonies after a week in case such colonies subsequently become overgrown by other fungi (e.g. *Ophiostoma* spp., *Penicillium* spp., *Trichoderma* spp.).

For the subsequent isolation of *H. annosum s.l.* from wood-disc traps for spores (hereafter referred to as "wood-disc traps"), see section 3.3.2.

3.3 Isolation and culture

Isolation of *H. annosum s.l.* may be performed directly from fresh fruiting bodies or from infected wood samples or wood-disc traps incubated in moist conditions as described above (see sections 3.2.2 and 3.2.4, respectively). Isolation of *H. annosum s.l.* is not feasible from fine wood sawdust.

3.3.1 Isolation from fruiting bodies

Pieces of tissue approximately 2 × 2 × 5 mm in size should be excised using a sterile scalpel from the context of fruiting bodies, surface-disinfected in a 30% (w/v) hydrogen peroxide (H_2O_2) solution or in a 5% (w/v) sodium hypochlorite (NaOCl) solution and rinsed three times in sterile distilled water (Giordano *et al*., 2009). Then, the pieces of tissue are transferred onto plastic Petri dishes filled with pentachloronitrobenzene (PCNB)-based selective culture medium for *H. annosum s.l.* (Table 1; Kuhlman and Hendrix, 1962) or with the semi-selective culture medium modified from Legrand and Guillaumin (1993) by Bendel *et al*. (2006) (Table 2). Another common procedure is to directly pick freshly exposed context tissue from the fruiting body and transfer it to plastic Petri dishes filled with selective, semi-selective or generic culture medium. Fresh context tissue between the hymenium and cuticle can be exposed by tearing the fruiting body in parts (by hand) or by making surface cuts with a disinfected scalpel to a fruiting body sprayed with 70% ethanol. Clean fruiting-body tissue can then be picked using disinfected, pointed tweezers. Petri dishes are incubated at room temperature (18–24 °C) or in fungal-growth incubators for at least 4–5 days.

Cultures can also be obtained from spore prints or individual basidiospores. To accomplish this, the lid of a Petri dish is propped at an angle over the Petri dish, with the fruiting body wedged between the lid and the edge of the Petri dish (but without touching the agar medium). This assembly is then incubated until spores are released on the agar surface. Individual germinating spores can be detected under a light microscope and picked from the agar surface using a modified Pasteur pipette sterilized by ethanol and flaming or using a sterile medical needle.

Subcultures can be maintained on generic agar-based media (e.g. malt extract agar, potato dextrose agar). Despite a lower success rate because of the more abundant presence of contaminant colonies (e.g. *Penicillium* spp., *Trichoderma* spp.), isolation of *H. annosum s.l.* can also be performed on these generic agar-based media.

Table 1. PCNB-based selective culture medium

Reagents and autoclaving parameters	Quantity
Initial mixture:	
- Bacto peptone	5 g
- Agar	20 g
- $MgSO_4$	250 mg
- KH_2PO_4	500 mg
- Pentachloronitrobenzene (PCNB)	190 ppm
- Distilled water to a final volume of 978 mL	
Autoclave for 20 min at 121°C	
After sterilization*, add:	
- Streptomycin	100 mg
- Ethanol (95%)	20 mL
- Lactic acid (50%)	2 mL

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

* After the medium is cooled to 41–45 °C in a water bath, the streptomycin, ethanol and the lactic acid are added; the medium is shaken before being poured to resuspend the relatively insoluble PCNB.

Source: Modified from Kuhlman and Hendrix (1962) to incorporate the streptomycin after autoclaving according to current practices.

Kuhlman, E.G. & Hendrix Jr, F.F. 1962. A selective medium for the isolation of *Fomes annosus*. *Phytopathology*, 52: 1310–1312.

Table 2. Semi-selective culture medium

Reagents and autoclaving parameters	Quantity
Initial mixture:	
- Malt extract	20 g
- Agar	15 g
- Distilled water to a final volume of 1 L	
Autoclave for 20 min at 121 °C	
After sterilization, add:	
- Thiabendazole*	230 mg
- Streptomycin	100 mg
- Polymyxin sulphate	50 mg
- Sodic benzylpenicillin	100 mg

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

* Added in 1 mL concentrated lactic acid, 85–90%.

Source: Adapted from Legrand and Guillaumin (1993) by Bendel ***et al***. (2006):

Bendel, M., Kienast, F., Bugmann, H. & Rigling, D. 2006. Incidence and distribution of *Heterobasidion* and *Armillaria* and their influence on canopy gap formation in unmanaged mountain pine forests in the Swiss Alps. *European Journal of Plant Pathology*, 116: 85–93. https://doi.org/10.1007/s10658-006-9028-1.

Legrand, P. & Guillaumin, J.J. 1993. *Armillaria* species in the forest ecosystems of the Auvergne (Central France). *Acta Oecologica*, 14: 389–403.

3.3.2 Isolation from wood samples and wood-disc traps for spores

For isolation from wood samples and wood-disc traps, individual colonies can be easily recognized using a 10–40× lens or under a dissecting microscope based on the presence of conidiophores of the anamorphic stage of the fungus. The conidiophores appear as a mass of whitish “pinheads” on stalks (Figure 8). Hyphae and conidiophores should be directly transferred onto plastic Petri dishes filled with

either the selective or semi-selective culture medium described above (see section 3.3.1). In the case of wood-disc traps, when *Heterobasidion* spp. colonies are present, 3–5 colonies per disc should be isolated. It is also possible to pick only the conidiophore heads and plate them directly on generic agar-based media using disinfected, pointed tweezers under a dissecting microscope. Petri dishes are incubated at room temperature (18–24 °C) or in fungal-growth incubators for at least 4–5 days. Subcultures can be maintained on generic agar-based media as described above (see section 3.3.1).

In the case of wood samples, isolation without the prior incubation described in section 3.2.2 may be attempted, but experience shows that this is less successful.

3.4 Detection by molecular methods

Molecular methods have been developed to detect pathogenic species or groups of species in *H. annosum s.l.* using conventional PCR, real-time PCR, multiplex real-time PCR or loop-mediated isothermal amplification (LAMP)[1] (Table 3).

For this diagnostic protocol, six methods (or sets of methods) have been selected based on laboratories' experience of using them and the availability of validation data: one method for the detection of *H. annosum s.l.* (species complex) and five species-specific methods (or sets of methods) to detect different *H. annosum s.l.* species. These methods are described below.

For practical purposes, Bahnweg *et al*. (2002) developed specific PCR primers for *H. annosum s.l.* allowing simultaneous detection of *Armillaria* spp. in multiplex PCR. This method can be applied to detect these two pathogens causing tree root rot, particularly in the early stages of infection in which symptoms are not yet clearly expressed (see sections 3.1.1 and 3.1.2). Both *H. annosum s.l.* and *Armillaria* spp. are economically important pathogens of conifers that can co-exist in the same stand and sometimes co-infection is possible. In the absence of signs of these two pathogens (e.g. fruiting bodies, rhizomorphs for *Armillaria* spp.), *H. annosum s.l.* symptoms may not be easily distinguished from those of *Armillaria* spp. or of other root-rot agents.

Methods based on PCR targeting mitochondrial DNA have been developed to discriminate the species *H. abietinum*, *H. annosum s.s.* and *H. parviporum* (Gonthier *et al*., 2001; Gonthier, Garbelotto and Nicolotti, 2003). A duplex PCR-based method targeting nuclear and mitochondrial DNA has also been developed to distinguish between *H. annosum s.s.* and *H. irregulare* (Gonthier *et al*., 2007) and to detect hybrids between these two species. In addition, real-time PCR methods have been developed. The internal transcribed spacer (ITS) region was selected by Lamarche *et al*. (2017) to design a set of real-time PCR methods using hydrolysis probes with different levels of specificity to detect the species of *H. annosum s.l.* occurring in North America, *H. irregulare* and *H. occidentale*, and *H. annosum s.s.* Ioos *et al*. (2019) developed multiplex real-time PCR methods for the detection and identification of *H. annosum s.l.* species infecting conifers in Europe (*H. abietinum*, *H. annosum s.s.*, *H. irregulare* and *H. parviporum*), which can be used simultaneously or individually thanks to probes labelled with species-specific fluorescent dyes. Sillo, Giordano and Gonthier (2018) focused on *H. irregulare* and developed a LAMP[1] method that targets a specific region (cytochrome P450 monooxygenase with haem-binding activity) identified through comparative genomics

The molecular methods included in this protocol (Table 3) have been successfully used on pure fungal cultures, fruiting bodies, and environmental (wood) samples; however, validation may have only been completed on pure fungal cultures. The molecular detection directly from environmental materials represents a powerful tool for a rapid and reliable monitoring of these pathogenic species.

[1] When using LAMP on a regular basis in an area which has a patent system such as Japan (Patent Nos. 3,313,358, 3,974,441 and 4,139,424), the United States of America (US6,410,278, US6,974,670 and US7,494,790), the European Union (Nos. 1,020,534, 1,873,260, 2,045,337 and 2,287,338), China (ZL008818262), the Republic of Korea (Patent No, 10-0612551), Australia (No. 779160), and the Russian Federation (No. 2,252,964), it is necessary for users to receive a licence from Eiken Chemical Co., Ltd. before use.

As previously indicated, this diagnostic protocol does not provide a comprehensive review of all molecular methods available for the diagnosis of the species of *H. annosum s.l.* Other molecular tests are available in the literature and other equipment, kits or reagents may be used provided that a verification is carried out.

Table 3. Molecular methods for detection of the pathogenic species or groups of species of *Heterobasidion annosum sensu lato* included in this diagnostic protocol

Section	Method (source)	Species targeted						Other *
		H. annosum s.l.	*H. abietinum*	*H. annosum s.s.*	*H. irregulare*	*H. occidentale*	*H. parviporum*	
3.4.3.1	cPCR[1]	+	-	-	-	-	-	+[8]
3.4.3.2	cPCR[2, 3]	-	+	+	-	-	+	-
3.4.3.3	cPCR[4]	+	-	+	+	-	-	-
3.4.4.1	real-time PCR[5]	+	-	+	+	+	-	-
3.4.4.2	real-time PCR[6]	-	+	+	+	-	+	-
3.4.4.3	LAMP[†, 7]	-	-	-	+	-	-	-

Notes: * Detection of *Armillaria* spp.

[†] See page footnote 1.

cPCR, conventional PCR; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

Sources: [1] Bahnweg, G., Möller, E.M., Anegg, S., Langebartels, C., Wienhaus, O. & Sandermann Jr, H. 2002. Detection of *Heterobasidion annosum s.l.* [(Fr.) Bref.] in Norway spruce by polymerase chain reaction. *Journal of Phytopathology*, 150: 382–389. https://doi.org/10.1046/j.1439-0434.2002.00772.x

[2] Gonthier, P., Garbelotto, M., Varese, G.C. & Nicolotti, G. 2001. Relative abundance and potential dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. *Canadian Journal of Botany*, 79: 1057–1065. https://doi.org/10.1139/b01-090

[3] 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. https://doi.org/10.1046/j.1439-0329.2003.00323.x

[4] 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. https://doi.org/10.1111/j.1365-294X.2007.03250.x

[5] Lamarche J., Potvin, A., Stewart, D., Blais, M., Pelletier, G., Shamoun, S.F., Hamelin, R.C. & Tanguay, P. 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. https://doi.org/10.1111/efp.12321

[6] Ioos, R., Chrétien, P., Perrault, J., Jeandel, C., Dutech, C., Gonthier, P., Sillo, F., Hietala, A.M., Solheim, H. & Hubert, J. 2019. Multiplex real-time PCR assays for the detection and identification of *Heterobasidion* species attacking conifers in Europe. *Plant Pathology*, 68: 1493–1507. https://doi.org/10.1111/ppa.13071

[7] 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(2): e12396. https://doi.org/10.1111/efp.12396

3.4.1 Preparation of material

Depending on the tissue source (wood samples, wood sawdust from drillings, fruiting bodies, mycelium from cultures), different methods may be used for homogenization or disruption of the tissue.

About 100 mg of wood tissue (including sawdust) or fruiting bodies may be disrupted by a tissue pulverizer; ready-to-use lysing matrix tubes or 2-mL screw-cap tubes containing two steel beads are recommended. Various grinding methods can be used, provided they produce a homogenously ground sample (e.g. FastPrep FP120 Cell Disrupter (Qbiogene), TissueLyser (QIAGEN)).[2] Freeze-drying overnight (lyophilization), or pre-freezing in liquid nitrogen or at −80 °C (overnight), or the combination of lyophilization followed by freezing in liquid nitrogen can be beneficial for disruption.

[2] The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable.

DNA can be extracted from fungal cultures (obtained by isolation from wood samples, fruiting bodies or wood-disc traps; see section 3.3) that have been grown on agar plates supplemented with cellophane membranes to facilitate the collection of mycelia. DNA can also be extracted from fungal mycelia cultured in flasks containing a liquid medium (e.g. 2% malt extract (w/v)) at room temperature (18–24 °C) for at least one week. The fungal mycelium is separated from the culture medium (supernatant) by vacuum filtration to dryness on a filter paper, freeze-dried overnight, and subsequently ground in a FastPrep FP120 Cell Disrupter (Qbiogene) or TissueLyser (QIAGEN).[2]

3.4.2 DNA extraction

Extraction of DNA from wood tissue (including sawdust), fruiting bodies or fungal cultures grown in a liquid medium or on agar plates (see section 3.4.1) can be performed using different commercial kits (e.g. DNeasy Plant Mini Kit (QIAGEN), EZNA Stool DNA Kit (Omega Bio-Tek)), following the manufacturers' instructions.[2]

The DNA from fungal cultures grown on agar Petri dishes can be directly extracted by the "hyphal tip isolation" method (Schweigkofler, O'Donnell and Garbelotto, 2004), modified as described here. Briefly, the fungal mycelium is collected with the tip of a micropipette and suspended in 100 µL of sterile distilled water, frozen in liquid nitrogen for three minutes, thawed at 75 °C, vortexed for one minute, and finally microcentrifuged for five minutes at 19 000 *g*. The freezing and thawing are repeated three times, with the last thaw extended to 15 minutes. Samples are then centrifuged for five minutes at 19 000 *g* and the supernatant is used as template DNA for PCR.

The LAMP[1] method of Sillo, Giordano and Gonthier (2018) targeting *H. irregulare* can be coupled with a rapid DNA extraction method based on the use of alkaline polyethylene glycol (PEG) (Chomczynski and Rymaszewski, 2006). Briefly, the samples (approximately 50–150 mg of biological material from fruiting bodies, wood samples or wood chips resulting from drillings) are homogenized into a crude macerate using a 10-mm stainless-steel bead in 5 mL plastic tubes containing 2 mL alkaline PEG lysis buffer (Table 4). Tubes are shaken by hand for two minutes, and 1 µL of the ten-fold dilution of the crude macerate is used in the LAMP[1] method (Sillo, Giordano and Gonthier, 2018; EPPO, 2020a).

Table 4. Alkaline polyethylene glycol (PEG) buffer

Reagents	Quantity
Polyethylene glycol average Mn 4 600	2.5 g
KOH 2 M	0.93 mL
Distilled water	39 mL
Adjust pH to 13.0−13.5 with KOH 0.5 M	

Note: Store at room temperature.

Source: 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. https://doi.org/10.2144/000112149

For all extraction methods, DNA should be stored at −20 °C or below until use.

3.4.3 Detection by conventional PCR

There are several conventional taxon-specific PCR methods for the detection of *Heterobasidion* species. Two of these methods (or sets of methods) are described below. For practical purposes, an additional multiplex PCR method targeting *H. annosum s.l.* and *Armillaria* spp. (Bahnweg *et al*., 2002) is also described.

3.4.3.1 Multiplex PCR of Bahnweg et al. *(2002) targeting* Heterobasidion annosum s.l. *and* Armillaria *spp.*

The *Heterobasidion*-specific primers HET-7a and HET-8a were developed by Bahnweg *et al*. (2002) and combined in a multiplex PCR with the *Armillaria*-specific primers ARM-1 and ARM-2 (Schulze *et al*., 1997) for the simultaneous detection of these two economically important pathogens of conifers.

All four primers (HET-7a, HET-8a, ARM-1 and ARM-2) were derived from ITS sequences. The HET-7a and HET-8a primers are considered genus-specific because differences in ITS sequences between species within *H. annosum s.l.* are very small (Kasuga *et al*., 1993). The specificity of the method was cross-checked against a wide panel of pure cultures from target (*Armillaria* spp. and *Heterobasidion* spp.) and non-target species, including other root- and butt-rot fungi (e.g. *Ilyonectria destructans* (Zinssm.) Rossman, L. Lombard & Crous, 2015), wood decomposers (e.g. *Pholiota squarrosa* (Vahl) P. Kumm., 1871, *Stereum sanguinolentum* (Alb. & Schwein.) Fr., 1838, *Trichaptum abietinum* (Pers. ex J.F. Gmel.) Ryvarden, 1972, *Tyromyces* spp.), ectomycorrhizal fungi (e.g. *Hebeloma mesophaeum* (Pers.) Quél., 1872, *Russula ochroleuca* Fr., 1838) and parasitic Peronosporales (e.g. *Phytophthora* spp., *Pythium* spp.) that may occur in the same environment. The PCR method was verified on environmental wood samples. As little as 1 pg of *Armillaria* DNA in a mixture with 100 ng of *Heterobasidion* DNA still yielded a visible amplicon (Bahnweg *et al*., 2002).

The master mix and cycling parameters for the PCR are described in Table 5 and the primers are as follows:

HET-7a (forward): 5′-GCT GCT TCT CAC AAA CTC TTC G-3′ (Bahnweg *et al*., 2002)

HET-8a (reverse): 5′-AAG CAG GTC CCC CAC AAT CG-3′ (Bahnweg *et al*., 2002)

ARM-1 (forward): 5′-AGG GTA TGT GCA CGT TCG AC-3′ (Schulze *et al*., 1997)

ARM-2 (reverse): 5′-GGA AAG CTA AGC TCG CGC TA-3′ (Schulze *et al*., 1997)

Table 5. Master mix composition, cycling parameters and amplicons for multiplex PCR targeting *Heterobasidion annosum sensu lato* and *Armillaria* spp.

Reagents	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	2.0 mM
dNTPs	0.1 mM
Primer HET-7a (forward)	0.2 µM
Primer HET-8a (reverse)	0.2 µM
Primer ARM-1 (forward)	0.2 µM
Primer ARM-2 (reverse)	0.2 µM
Bovine serum albumin (DNase-free)	0.4 mg
DNA polymerase (Pharmacia) [‡]	1 U
DNA (volume)	1 µL
Cycling parameters	
Initial denaturation	94 °C for 3 min
Annealing	68 °C for 1 min
Elongation	72 °C for 2 min
Number of cycles	30
- Denaturation	94 °C for 30 s
- Annealing	68 °C for 1 min
- Elongation	72 °C for 2 min
Final elongation	72 °C for 5 min
Expected amplicons	
Heterobasidion annosum sensu lato	400 bp
Armillaria spp.	660 bp

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Sources: Bahnweg, G., Möller, E.M., Anegg, S., Langebartels, C., Wienhaus, O. & Sandermann Jr, H. 2002. Detection of *Heterobasidion annosum s.l.* [(Fr.) Bref.] in Norway spruce by polymerase chain reaction. *Journal of Phytopathology*, 150: 382–389. https://doi.org/10.1046/j.1439-0434.2002.00772.x

Schulze, S., Bahnweg, G., Möller, E.M. & Sandermann Jr, H. 1997. Identification of the genus *Armillaria* by specific amplification of an rDNA-ITS fragment and evaluation of genetic variation within *A. ostoyae* by rDNA-RFLP and RAPD analysis. *European Journal of Forest Pathology*, 27: 225–239. https://doi.org/10.1111/j.1439-0329.1997.tb00865.x

The PCR products can be visualized by standard agarose gel electrophoresis.

3.4.3.2 Taxon-specific, competitive-priming PCR of Gonthier et al. *(2001) and Gonthier, Garbelotto and Nicolotti (2003) targeting* Heterobasidion abietinum, H. annosum s.s. *and* H. parviporum

A taxon-specific, competitive-priming PCR (Garbelotto *et al.*, 1996, 1998), combined with a PCR-mediated detection of species-specific DNA insertions in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA gene (Gonthier *et al.*, 2001), was modified by Gonthier, Garbelotto and Nicolotti (2003) to target three species, *H. abietinum*, *H. annosum s.s.* and *H. parviporum*, by a single PCR amplification and gel electrophoresis. The method utilizes a mix of four primers in a single PCR followed by the analysis of amplicon sizes after gel electrophoresis. The specificity of the method was verified with a wide panel of pure fungal cultures from all three target species and was also verified

on environmental samples including both fruiting bodies and naturally infected wood samples. Samples were collected in two subalpine stands of natural mixed spruce (*Picea abies*), larch (*Larix* spp.) and Swiss stone pine (*Pinus cembra*) in the western Italian Alps where all three target species can co-exist.

The master mix and cycling parameters for the PCR are described in Table 6 and the primers are as follows:

MLS (forward): 5′-AAA TTA GCC ATA TTT TAA AAG-3′ (Garbelotto *et al*., 1998)

Mito 5 (reverse): 5′-TAA GAC CGC TAT A(T/A)A CCA GAC-3′ (Garbelotto *et al*., 1998)

MLF (forward): 5′-TAA AAA TTT AAA TTG CCT AA-3′ (Garbelotto *et al*., 1998)

Mito 7 (reverse): 5′-GCC AAT TTA TTT TGC TAC C-3′ (Gonthier *et al*., 2001)

Table 6. Master mix composition, cycling parameters and amplicons for conventional PCR targeting *Heterobasidion abietinum*, *H. annosum sensu stricto* and *H. parviporum*

Reagents	**Final concentration**
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Primer MLS (forward)	0.5 µM
Primer Mito 5 (reverse)	0.5 µM
Primer MLF (forward)	0.5 µM
Primer Mito 7 (reverse)	0.5 µM
DNA polymerase (Promega)[‡]	0.625 U
DNA (volume)	6.25 µL
Cycling parameters	
Initial denaturation	94 °C for 1.5 min
Numbers of cycles	35
- Denaturation	92 °C for 1 min
- Annealing	53 °C for 1 min
- Elongation	72 °C for 3 min
Final elongation	72 °C for 10 min
Expected amplicons	
Heterobasidion abietinum	195 bp
Heterobasidion annosum sensu stricto	230 bp
Heterobasidion parviporum	185 bp

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Sources: Garbelotto, M., Otrosina, W.J., Cobb, F.W. & Bruns, T.D. 1998. The European S and F intersterility groups of *Heterobasidion annosum* may represent sympatric protospecies. *Canadian Journal of Botany*, 76: 397–409. https://doi.org/10.1139/b97-185

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W. & Otrosina, W.J. 1996. Use of taxon-specific competitive-priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum*. *Phytopathology*, 86: 543–551. https://doi.org/10.1094/Phyto-86-543

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. https://doi.org/10.1046/j.1439-0329.2003.00323.x

Gonthier, P., Garbelotto, M., Varese, G.C. & Nicolotti, G. 2001. Relative abundance and potential dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. *Canadian Journal of Botany*, 79: 1057–1065. https://doi.org/10.1139/b01-090

The PCR products can be visualized by a high-resolution 2.5% agarose gel electrophoresis.

3.4.3.3 Conventional PCR of Gonthier et al. *(2007) targeting* Heterobasidion annosum s.l., H. annosum s.s. *and* H. irregulare

A taxon-specific conventional PCR was developed by Gonthier *et al*. (2007) targeting *H. annosum s.s.* and *H. irregulare*; this PCR can also be used to detect hybrids between *H. annosum s.s.* and *H. irregulare*. The DNA was characterized by three sets of PCR primers specifically designed to target one nuclear locus (two sets) and one mitochondrial locus in *Heterobasidion* (Gonthier *et al*., 2001). The primers EFaNAPFor and EFaEuPFor were designed on the nuclear elongation factor 1-α (EFA) to exclusively amplify either *H. irregulare* or *H. annosum s.s.*, respectively, when each of them is used in combination with the two *H. annosum* universal primers EFaHaFor and EFaHaRev. The use of both EFa primer sets allows the results obtained by each primer set to be confirmed. The primers Mito 5, Mito 7 and Mito 8 amplify a 121 base pair (bp) amplicon in the mitochondrial ribosomal operon for *H. irregulare* and one of 158 bp for *H. annosum s.s.* The specificity of the two resulting PCR methods (one targeting the nuclear locus and the other targeting the mitochondrial locus) was verified on 490 isolates of *H. irregulare*, 86 isolates of *H. annosum s.s.* and 6 hybrid isolates between the two species. Hybrid isolates are typed as EU (*H. annosum s.s.*) at the mitochondrial locus and as NA (*H. irregulare*) at the nuclear locus, or vice versa as NA at the mitochondrial locus and as EU at the nuclear locus (see section 1 for hybridization between *H. annosum s.s.* and *H. irregulare*). The methods are 100% specific to the target species; no cross-reaction between species was noted (Gonthier *et al*., 2007).

The master mix and cycling parameters for the two PCR methods are described in Table 7 and Table 8, and the primers are as follows:

Mito 8 (forward): 5′-GCG GTG TAA TAA AAT CGG-3′ (Gonthier *et al*., 2001)

Mito 5 (reverse): 5′-TAA GAC CGC TAT A(T/A)A CCA GAC-3′ (Garbelotto *et al*., 1998)

Mito 7 (reverse): 5′-GCC AAT TTA TTT TGC TAC C-3′ (Gonthier *et al*., 2001)

EFaHaFor (forward): 5′-CTA TGT CGC GGT ACA GCT TG-3′ (Gonthier *et al*., 2007)

EFaNAPFor (forward): 5′-GTA CAT GGT CAC TGT ACG TAG ATG C-3′ (Gonthier *et al*., 2007)

EFaEuPFor (forward): 5′-ATG GTC ACT GTA CGT AGA TCA TGC-3′ (Gonthier *et al*., 2007)

EFaHaRev (reverse): 5′-GCG AGG A(T/C)A AGA AGT AAT CAG CA-3′ (Gonthier *et al*., 2007)

Table 7. Master mix composition, cycling parameters and amplicons for conventional PCR to target the nuclear locus of *Heterobasidion annosum sensu lato*, *H. annosum sensu stricto* and *H. irregulare*

Reagents	**Final concentration**
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Primer EFaHaFor (forward)	0.5 µM
Primer EFaNAPFor or EFaEuPFor (forward)	0.5 µM
Primer EFaHaRev (reverse)	0.5 µM
DNA polymerase (Promega)[‡]	0.625 U
DNA (volume)	2 µL
Cycling parameters	
Initial denaturation	94 °C for 5 min
Numbers of cycles	35
- Denaturation	95 °C for 45 s
- Annealing	62 °C for 45 s
- Elongation	72 °C for 45 s
Final elongation	72 °C for 7 min
Expected amplicons	
Heterobasidion annosum sensu lato	169 bp
Heterobasidion annosum sensu stricto	69 bp
Heterobasidion irregulare	169 bp and 71 bp

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Source: 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. https://doi.org/10.1111/j.1365-294X.2007.03250.x

Gonthier, P. & Thor, M. 2013. Annosus root and butt rots. In: P. Gonthier & G. Nicolotti, eds. *Infectious forest diseases*, pp. 128–158. Wallingford, UK, CABI. 641 pp. https://doi.org/10.1079/9781780640402.0128

Table 8. Master mix composition, cycling parameters and amplicons for conventional PCR to target the mitochondrial locus of *Heterobasidion annosum sensu stricto* and *H. irregulare*

Reagents	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Primer Mito 8 (forward)	0.5 µM
Primer Mito 5 (reverse)	0.5 µM
Primer Mito 7 (reverse)	0.5 µM
DNA polymerase (Promega)[‡]	0.625 U
DNA (volume)	6.25 µL
Cycling parameters	
Initial denaturation	94 °C for 1.5 min
Numbers of cycles	35
- Denaturation	92 °C for 1 min
- Annealing	53 °C for 1 min
- Elongation	72 °C for 3 min
Final elongation	72 °C for 10 min
Expected amplicons	
Heterobasidion annosum sensu stricto	158 bp
Heterobasidion irregulare	121 bp

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 2.

bp, base pairs; PCR, polymerase chain reaction.

Sources: Garbelotto, M., Otrosina, W.J., Cobb, F.W. & Bruns, T.D. 1998. The European S and F intersterility groups of *Heterobasidion annosum* may represent sympatric protospecies. *Canadian Journal of Botany*, 76: 397–409. https://doi.org/10.1139/b97-185

Gonthier, P., Garbelotto, M., Varese, G.C. & Nicolotti, G. 2001. Relative abundance and potential dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. *Canadian Journal of Botany*, 79: 1057–1065. https://doi.org/10.1139/b01-090

The PCR products can be visualized by a high-resolution 2.5% agarose gel electrophoresis.

3.4.4 Detection by real-time PCR

There are several *Heterobasidion*-specific real-time PCR methods available in the literature. Some of these are described in sections 3.4.4.1 to 3.4.4.3.

3.4.4.1 Real-time PCR of Lamarche et al. *(2017) targeting* Heterobasidion annosum s.l., H. annosum s.s., H. irregulare *and* H. occidentale

Lamarche *et al*. (2017) developed four real-time PCR primer–probe sets for the detection of *H. annosum s.l., H. annosum s.s., H. irregulare* and *H. occidentale*. The primer–probe sets were all designed to work with the same chemistry under a unique, standardized set of thermocycling parameters. Therefore, they can be used in microwell plates arrayed in any machine format to suit individual users' needs and to increase throughput. Reactions for multiple samples, targeting multiple pathogens can be performed in a single real-time PCR run.

Lamarche *et al*. (2017) assessed the performance of the real-time PCR methods using these primer–probe sets. The specificity of the methods was verified with a wide panel of pure fungal cultures from

target and phylogenetically closely related species of *Heterobasidion*: *H. abietinum* (7 isolates); *H. annosum s.s.* (14); *H. araucariae* P.K. Buchanan, 1988 (1); *H. ecrustosum* Tokuda, T. Hatt. & Y.C. Dai, 2009 (5); *H. insulare* (Murrill) Ryvarden, 1972 (1); *H. irregulare* (17 isolates of different origins and hosts); *H. occidentale* (8); *H. orientale* Tokuda, T. Hatt. & Y.C. Dai, 2009 (5); and *H. parviporum* (9) (Tokuda *et al*., 2009; Jang *et al*., 2014; EPPO 2020a). All four methods developed are 100% specific to their target species; no cross-reaction was observed. Sensitivity, evaluated by assessing the limit of detection (with a threshold of 95% of positive results), was found to be between 1 and 100 copies of the ITS region of the target DNA, corresponding to one binucleate spore per µL. The methods showed a high degree of repeatability; the coefficient of variation for each method, using the mean Ct value and the standard deviation, ranged from 1.2% for the *H. occidentale* method to 5.2% for the *H. annosum s.s.* method (DNA samples containing 5 000 copies of the target gene region were used). The real-time PCR methods were also verified on environmental samples collected through a trial examining the aerial density of *Heterobasidion* spores in a forest nursery.

The master mix and cycling parameters for the multiplex real-time PCR are described in Table 9. The primers and probes (Lamarche *et al*., 2017) are as follows:

For *Heterobasidion annosum s.l.*:

Hannosum_sl_F388 (forward primer): 5′-GAG ATC CAT TGT TGA AAG TTG TAT A-3′

Hannosum_sl_R591 (reverse primer): 5′-GAA TAT CGT GCR RGG TTG AA-3′

Hannosum_sl_T512RC (probe): 6FAM-5′-CCA TCT CAC/ZEN/ACC TGT GCA CAC TC-3′-3IABkFQ

For *Heterobasidion annosum s.s.*:

Hannosum_ss_4A/T_F435 (forward primer): 5′-CCA TTC TAA AGA CAT ACG ATG AG-3′

Hannosum_ss_R501 (reverse primer): 5′-GTC GGG TTC TTT TGA C-3′

Hannosum_ss_irregulare_T464RC (probe): 6FAM-5′-TTC CGA GCC/ZEN/GCG TCT TCT-3′-3IABkFQ

For *Heterobasidion irregulare*:

Hirregulare_4A/G_F435 (forward primer): 5′-CAT TCT GAA GAC ATA CGA GGG A-3′

Hirregulare_R500 (reverse primer): 5′-GGT CGG GTT CTT TTG AT-3′

Hannosum_ss_irregulare_T464RC (probe): 6FAM-5′-TTC CGA GCC/ZEN/GCG TCT TCT-3′-3IABkFQ

For *Heterobasidion occidentale*:

Hoccidentale_2C/T_F60 (forward primer): 5′-TCT TTC GAC GGT TGG AAG TG-3′

Hoccidentale_3C/G_R195 (reverse primer): 5′-GAG GCT TTG CTG GTC GTT-3′

Hoccidentale_T96RC (probe): 6FAM-5′-CGT GGG+T+A+C GCC-3′-3IABkFQ

Table 9. Master mix composition and cycling parameters for multiplex real-time PCR targeting *Heterobasidion annosum sensu lato, H. annosum sensu stricto, H. irregulare* and *H. occidentale*

Reagents	Final concentration
PCR-grade water	–[†]
QuantiTect Multiplex PCR NoROX master mix (QIAGEN)[‡]	1×
Forward primer	0.6 µM
Reverse primer	0.6 µM
Probe	1 µM
DNA (volume)	1 µL
Cycling parameters	
Initial denaturation	95 °C for 15 min
Numbers of cycles	50
- Denaturation	95 °C for 15 s
- Annealing and elongation	60 °C for 90 s

Notes: [†] For a final reaction volume of 10 µL.

[‡] See page footnote 2.

PCR, polymerase chain reaction.

Source: Lamarche J., Potvin, A., Stewart, D., Blais, M., Pelletier, G., Shamoun, S.F., Hamelin, R.C. & Tanguay, P. 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. https://doi.org/10.1111/efp.12321

3.4.4.2 Multiplex real-time PCR of Ioos et al. *(2019) targeting* Heterobasidion abietinum, H. annosum s.s., H. irregulare *and* H. parviporum

Ioos *et al*. (2019) developed real-time PCR detection methods for *H. abietinum, H. annosum s.s., H. irregulare* and *H. parviporum*, which can be used individually or simultaneously by using probes labelled with species-specific fluorescent dyes. The primers and probes target a specific region of the DNA replication licensing factor (*Mcm7*) for *H. abietinum*, *H. annosum s.s.* and *H. irregulare*, and the RNA polymerase II large subunit (*RPB1*) for *H. parviporum*. Preliminary attempts to use all four primer–probe sets in a single PCR tube (quadruplex PCR) showed an unacceptable loss of sensitivity. However, it was found that a triplex real-time PCR, using primers and probes for *H. irregulare* and *H. parviporum* plus the FungiQuant primer–probe set of Liu *et al*. (2012), together with a duplex real-time PCR for *H. abietinum* and *H. annosum s.s.*, could be successfully achieved without compromising the sensitivity of each method. The FungiQuant primer–probe set is used as the internal positive control and targets the 18S ribosomal (r)DNA of a broad range of fungal species.

Ioos *et al*. (2019) assessed the performance of the real-time PCR methods in triplex for *H. irregulare*, *H. parviporum* and FungiQuant and in duplex for *H. abietinum* and *H. annosum s.s.* The limit of detection with genomic DNA from target species was estimated at 1 pg, 10 pg, 1 pg and 1 pg for *H. abietinum, H. annosum s.s., H. irregulare* and *H. parviporum*, respectively. The specificity of the methods was verified with a wide panel of pure fungal cultures from target and non-target species; in addition to other non-target *Heterobasidion* species (e.g. *H. araucariae*, *H. insulare*, *H. occidentale*), non-target species included *Armillaria ostoyae* (Romagn.) Herink, 1973, *Coniferiporia sulphurascens* (Pilát) L.W. Zhou & Y.C. Dai, 2016, *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh., 1900, *Fomitopsis pinicola* (Sw.) P. Karst., 1881, *Fuscoporia torulosa* (Pers.) T. Wagner & M. Fisch., 2001, *Onnia tomentosa* (Fr.) P. Karst., 1889, *Phaeolus schweinitzii* (Fr.) Pat., 1900, *Porodaedalea pini* (Brot.) Murrill, 1905 and *Stereum sanguinolentum* (Ioos *et al*., 2019).

These methods can also be used to detect heterokaryotic hybrids between species, because they can simultaneously detect DNA from different species of *H. annosum s.l.* Ioos *et al*. (2019) obtained positive results with the DNA from two artificial *H. annosum s.s.* × *H. irregulare* hybrids generated by Giordano *et al*. (2018): both the *H. annosum s.s.* and *H. irregulare* real-time PCR methods yielded positive results.

The real-time PCR methods were verified on environmental samples, including both fruiting bodies and artificially inoculated or naturally infected wood samples (Ioos *et al*., 2019).

The master mix and cycling parameters for the multiplex real-time PCR are described in Table 10. The primers and probes are as follows:

For *Heterobasidion abietinum*:

Habi For 4 (forward primer): 5′-TCG TTT CAG CCC TTT CCA A-3′ (Ioos *et al*., 2019)

Habi Rev 14 (reverse primer): 5′-TTG ATG AAT ATA GTG CGC CTC G-3′ (Ioos *et al*., 2019)

Habi P 7 (probe): Cy5-5′-GGT GCG TCG TCG CCT TCA TTA TTT T-3′-BHQ2 (Ioos *et al*., 2019)

For *Heterobasidion annosum s.s.*:

Hann For 14 (forward primer): 5′-CGT CGC CTT AAT GAT TTC ATA AG-3′ (Ioos *et al*., 2019)

Hann Rev 10 (reverse primer): 5′-TGT CAC TGT ACT GTT TCT TTA GC-3′ (Ioos *et al*., 2019)

Hann P 11 (probe): FAM-5′-ACC ATA CAY GTT GGC GGG AAC CTC-3′-BHQ1 (Ioos *et al*., 2019)

For *Heterobasidion irregulare*:

Hirr For 1 (forward primer): 5′-CGT CGT CTC CAT GAT CTC AA-3′ (Ioos *et al*., 2019)

Hirr Rev 5 (reverse primer): 5′-TTG ATG AAT ATA GTG CGC TTC A-3′ (Ioos *et al*., 2019)

Hirr P 5 (probe): JOE-5′-CCA TWC ACG TTG GCG GGA ACC TT-3′-BHQ1 (Ioos *et al*., 2019)

For *Heterobasidion parviporum*:

Hpar For 4 (forward primer): 5′-CAA TCG TAT GGG GTC ATT GTA A-3′ (Ioos *et al*., 2019)

Hpar Rev 6 (reverse primer): 5′-CAC ATC CGC CAT GTC CC-3′ (Ioos *et al*., 2019)

Hpar P 8 (probe): ROX-5′-GAT CTG CGA GCC CGA CGA ACC G-3′-BHQ2 (Ioos *et al*., 2019)

For 18S rDNA (internal positive control):

FungiQuant-F (forward primer): 5′-GGR AAA CTC ACC AGG TCC AG-3′ (Liu *et al*., 2012)

FungiQuant-R (reverse primer): 5′-GSW CTA TCC CCA KCA CGA-3′ (Liu *et al*., 2012)

FungiQuant-Prb (probe): FAM-5′-TGG TGC ATG GCC GTT-3′-BHQ1 (Liu *et al*., 2012)

Table 10. Master mix composition and cycling parameters for multiplex real-time PCR targeting *Heterobasidion abietinum, H. annosum sensu stricto, H. irregulare* and *H. parviporum*

Reagents	Final concentration
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	5.0 mM
dNTPs	0.2 mM
Primer (forward)	0.3 µM
Primer (reverse)	0.3 µM
Probe	0.1 µM
HotGold Star DNA polymerase (Eurogentec)‡	0.025 U
DNA (volume)	2 µL
Cycling parameters	
Initial denaturation	95 °C for 10 min
Numbers of cycles	40
- Denaturation	95 °C for 15 s
- Annealing and elongation	65 °C for 55 s

Notes: † For a final reaction volume of 20 µL.

‡ See page footnote 2.

PCR, polymerase chain reaction.

Source: Ioos, R., Chrétien, P., Perrault, J., Jeandel, C., Dutech, C., Gonthier, P., Sillo, F., Hietala, A.M., Solheim, H. & Hubert, J. 2019. Multiplex real-time PCR assays for the detection and identification of *Heterobasidion* species attacking conifers in Europe. *Plant Pathology*, 68: 1493–1507. https://doi.org/10.1111/ppa.13071

3.4.4.3 LAMP[1] of Sillo, Giordano and Gonthier (2018) targeting **Heterobasidion irregulare**

Sillo, Giordano and Gonthier (2018) developed a LAMP[1] method for the early detection of *H. irregulare*. The targeted gene is the gene for a cytochrome P450 monooxygenase with haem-binding activity in the genome of *H. irregulare* strain TC 32-1 (Olson *et al*., 2012).

Sillo, Giordano and Gonthier (2018) assessed the performance of the method, reporting the limit of detection to be about 20 pg of target DNA per reaction and the time taken to achieve a detection result to be less than 40 minutes. No cross-reactivity was observed with 12 isolates of non-target phylogenetically closely related species, including *H. abietinum*, *H. annosum s.s.* and *H. parviporum*, or with 14 isolates of seven other wood-decay hymenomycetes found in pine trees (*Echinodontium tinctorium*, *Fomitopsis pinicola*, *Fuscoporia torulosa*, *Onnia* spp., *Phaeolus schweinitzii*, *Porodaedalea pini* and *Stereum* spp.). Nine isolates of *H. irregulare* of different origins were also used.

The LAMP[1] method was successfully tested on a variety of different samples, including fungal fruiting bodies, infected plants and colonized wood. A survey on environmental samples collected in the field was also performed using the LAMP[1] method coupled with a rapid DNA extraction method (Sillo, Giordano and Gonthier, 2018).

The master mix and incubation conditions for the LAMP[1] method are described in Table 11 and the primers (Sillo, Giordano and Gonthier, 2018) are as follows:

HirrSC3_F3 (forward external primer): 5′-GCC ACC AAA ACT GGT TGT-3′

HirrSC3_B3 (reverse external primer): 5′-TGA AGA TGT CAA TGG AGG T-3′

HirrSC3_FIP (forward internal primer): 5′-TCA CTA GAA CCG ATT TCA TGG GTA AAG GTG CTA GAG CAT AGC-3′

HirrSC3_BIP (reverse internal primer): 5′-AGT GGA GAA TCG TTG TTA CAG TCC ACT GTC GAC ATA AGT GCA-3′

HirrSC3_FL (forward loop primer): 5′-ACA TGG CGT ACG TAT GCT TG-3′
HirrSC3_BL (reverse loop primer): 5′-GAG GTT GAA GAC AAA AAC TTA CGT G-3′

Table 11. Master mix composition and incubation conditions for LAMP* method targeting *Heterobasidion irregulare*

Reagents	Final concentration
PCR-grade water	–†
Isothermal Mastermix ISO-001 (OptiGene)‡	0.975×
HirrSC3_F3 (forward primer)	0.25 µM
HirrSC3_B3 (reverse primer)	0.25 µM
HirrSC3_FIP (forward primer)	0.5 µM
HirrSC3_BIP (reverse primer)	0.5 µM
HirrSC3_FL (forward primer)	0.5 µM
HirrSC3_BL (reverse primer)	0.5 µM
DNA (volume)	1 µL
Incubation conditions	
Isothermal amplification	65 °C for 40 min
- Fluorescence detection	Every minute
Melting curve	From 65 °C to 95 °C
- Temperature increment	0.1 °C
- Fluorescence detection	Every 10 s

Notes: * See page footnote 1.

† For a final reaction volume of 20 µL.

‡ See page footnote 2.

LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

Source: 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(2): e12396. https://doi.org/10.1111/efp.12396

The LAMP[1] reactions are performed using an instrument equipped with a fluorophore (FAM) reading channel and melting curve analysis. Depending on the equipment, the peak value may vary and should be verified for each laboratory.

3.4.5 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of method used and the level of certainty required – should be considered for each series of nucleic acid extractions and amplification of the target pests or target nucleic acid. For PCR-based methods (conventional PCR and real-time PCR) and LAMP,[1] a positive nucleic acid control, a negative amplification control and either an internal positive control or positive extraction control, are the minimum controls to be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the amplification of nucleic acid of the target pest. This can include amplification of nucleic acid extracted from the target pest, total nucleic acid extracted from infected host tissue, a whole genome or a synthetic control. For reactions not performed on isolated pests, the positive nucleic acid control should preferably be near to the limit of detection.

Negative amplification control (no template control). This control is necessary to rule out false positives resulting from contamination (e.g. with the target DNA) during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor both contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified.

Alternatively, extraction blanks (e.g. sterile water, clean extraction buffer) can be processed with the samples to be tested if sufficient uninfected host tissue is not available. This will allow contamination of extraction reagents and cross-contamination between samples to be identified. It is recommended that multiple controls be included when large numbers of positives are expected.

Positive extraction control. This control is used to ensure that the target nucleic acid extracted is of sufficient quantity and quality and that the target is detected. Nucleic acid is extracted from infected host tissue or, if suitable infected material is not available, healthy plant tissue that has been spiked with the target pest.

Internal positive control. As an alternative (or in addition) to the external positive controls (positive extraction control and positive nucleic acid control), an internal positive control can be used to monitor each individual sample separately and to check for potential PCR inhibitors. Internal positive controls can be genes either present in the matrix DNA or added to the DNA solution.

In Lamarche *et al.* (2017) and Gonthier *et al*. (2007), internal positive controls can include specific amplicons or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-organism target nucleic acid that is also present in the sample (e.g. plant *COX* gene or eukaryotic 18S rDNA).

In the multiplex real-time PCR of Ioos *et al.* (2019), the FungiQuant primer–probe set of Liu *et al.* (2012), which targets the 18S rDNA of a broad range of fungal species, is used as an internal positive control. This fungus-specific internal positive control monitors for the presence of fungal DNA in the nucleic acid sample.

When using the method of Sillo, Giordano and Gonthier (2018) on plant samples, the test should be combined with the cytochrome oxidase (COX) LAMP[1] method developed by Tomlinson, Dickinson and Boonham (2010) in order to confirm successful DNA extraction. The primers (Tomlinson, Dickinson and Boonham, 2010) are as follows:

COX-F3 (forward primer) 5′-TAT GGG AGC CGT TTT TGC-3′

COX-B3 (reverse primer): 5′-AAC TGC TAA GRG CAT TCC-3′

COX-FIP (forward primer): 5′-ATG GAT TTG RCC TAA AGT TTC AGG GCA GGA TTT CAC TAT TGG GT-3′

COX-BIP (reverse primer): 5′-TGC ATT TCT TAG GGC TTT CGG ATC CRG CGT AAG CAT CTG-3′

COX-FL (forward primer): 5′-ATG TCC GAC CAA AGA TTT TAC C-3′

COX-BL (reverse primer): 5′-GTA TGC CAC GTC GCA TTC C-3′

3.4.6 Interpretation of results

3.4.6.1 Interpretation of conventional PCR results

A conventional PCR will be considered valid only if the controls produce the expected results:

- the negative amplification control produces no band corresponding to the expected amplicon size; and
- the positive nucleic acid control, the positive extraction control (if used), and the internal positive control (if used), produce bands that correspond to the expected amplicon size.

When these conditions are met:

- a sample will be considered negative if it produces no band or if it produces a band that corresponds to an amplicon size that is different than expected; and

- a sample will be considered positive if it produces a band corresponding to the expected amplicon size.

In the conventional PCR of Gonthier *et al*. (2007) to target the nuclear locus, a sample will be considered positive for *H. irregulare* if two amplicons – one of 169 bp and the other of 71 bp – are produced and positive for *H. annosum s.s.* if only one amplicon of 69 bp is produced.

In the same conventional PCR, pure isolates of *H. annosum s.s.* are typed as EU (*H. annosum s.s.*) at both loci (nuclear and mitochondrial); pure isolates of *H. irregulare* are typed as NA (*H. irregulare*) at both loci. Isolates that are hybrids between *H. annosum s.s.* and *H. irregulare* are typed as EU at the mitochondrial locus and as NA at the nuclear locus, or vice versa as NA at the mitochondrial locus and as EU at the nuclear locus.

3.4.6.2 Interpretation of real-time PCR results

A real-time PCR will be considered valid only if the controls produce the expected results:

- the negative amplification control produces no amplification curve; and
- the positive nucleic acid control, the positive extraction control (if used), and the internal positive control (if used), produce an exponential amplification curve.

When these conditions are met:

- a sample will be considered negative if it produces no exponential amplification curve or if it produces a curve that is not exponential; and
- a sample will be considered positive if it produces an exponential amplification curve.

A Ct cut-off value may be applied according to laboratory validation data.

3.4.6.3 Interpretation of LAMP[1] results

The LAMP[1] reaction will be considered valid only if the controls produce the expected results:

- the negative amplification control produces no amplification curve; and
- the positive nucleic acid control, the positive extraction control (if used) and the internal positive control (if used), produce an exponential amplification curve (i.e. an exponential fluorescence signal) and a melting curve with a peak at 85.50 °C ± 0.5 °C (Sillo, Giordano and Gonthier, 2018).

When these conditions are met:

- a sample will be considered negative if it produces no amplification curve or if it produces a curve that has a melting temperature different from that expected (85.50 °C ± 0.5 °C); and
- a sample will be considered positive if it produces an exponential amplification curve and a melting curve with a peak at 85.50 °C ± 0.5 °C as defined for positive controls.

Comparison of melting curves between samples and positive controls should be carried out to exclude false-positive signals. Depending on the equipment, the peak value may vary and should be verified for each laboratory.

4. Identification

Heterobasidion annosum s.l. can be identified by the macro- and micromorphological characteristics of its fruiting bodies, or by its growth characteristics and morphology in pure culture, but experience with the identification of *Heterobasidion* species is required. Even with experience, however, the slight differences and the partial overlapping of morphological traits between *H. annosum s.l.* species may lead to erroneous identifications. Molecular methods, therefore, provide the most reliable way to accurately identify the pathogen at species level. In some cases, the traditional method using mating experiments with confirmed homokaryotic strains for each species (Korhonen, 1978; Stenlid, 1985; Mitchelson and Korhonen, 1998) can be used as an alternative to molecular methods, but it requires expertise in the interpretation of the results and more time. The conventional PCR and real-time PCR

methods described in sections 3.4.3 and 3.4.4 are species specific and are used for detection of the pathogen in infected material, wood-disc traps or pure fungal cultures. DNA sequencing (see section 4.2) can also be performed to confirm the identity of pure fungal cultures of the pathogen.

4.1 Morphological identification of fruiting bodies

The fruiting bodies of *H. annosum s.l.* are 1–40 cm across, perennial, very irregular in shape, pileate, resupinate or effused-reflexed, and rubbery in texture (Figure 6, Figure 7 and Figure 9). The top surface (when present) is reddish or dark brown in colour (Figure 7) and becomes darker with age. The margin is distinct, thin and white. The context is 0.2−1 cm thick, whitish, and corky to woody. The hyphal system is dimitic and non-agglutinated, with generative hyphae and skeletal hyphae. The lower surface (hymenophore) is labyrinthine or daedaleoid, white or cream coloured, with numerous small irregular pores; there are (1–)2–3(–5) pores per mm, with individual pores being 150–500(–1000) µm in diameter (Figure 9). Tubes are unevenly or distinctly stratified and are 2–10 mm long in each layer. Basidia are 9−13 × 5−7 µm, clavate, four-spored, and without a basal clamp. Cystidia are absent. Basidiospores are 3.5−5.5 × 3−4 µm, ovoid to broadly ellipsoid, hyaline, thin-walled and slightly asperulate, with a few guttules (CABI, 2022).

Slight differences between *H. annosum s.l.* species occur in the macromorphology and micromorphology of fruiting bodies, especially in the size of pores, the length of brown hairs on the upper margin and, to a lesser extent, the size of spores (Mitchelson and Korhonen, 1998; Otrosina and Garbelotto, 2010). The differences are small and morphological traits partially overlap between species, which may lead to erroneous identifications in the field. Some diagnostic differences between species occurring in Europe and Asia (*H. abietinum*, *H. annosum s.s.* and *H. parviporum*) and species occurring in North America (*H. irregulare* and *H. occidentale*) that may be of use in the field where fruiting bodies are found are available in Table 12 (modified from Otrosina and Garbelotto, 2010).

Isolation of *H. annosum s.l.* may be performed directly from fresh fruiting bodies (see section 3.3.1).

Table 12. Some diagnostic differences between species of *Heterobasidion annosum sensu lato* occurring in Europe and Asia, and those occurring in North America, that may be of use in the field where fruiting bodies are found

Species	Location of fruiting bodies	Length of fruiting bodies (cm)	Mean pore density in mm^2 (SD) and % of irregular pores
H. abietinum	Root collar, along roots, and in decay pockets	In colder areas (central-eastern Europe) up to 30 cm, in mesic warm areas up to 40–45 cm	12.5 (0.3) (no % data available)
H. annosum s.s.	Root collar and primary roots of infected or dead trees, on stem pieces left in the forest and in decay pockets in stumps	Up to 30 cm	8 (0.3) (no % data available)
H. irregulare	Root collar (eastern North America, Italy), under bark of buttress and under intact stump surface (western United States of America)	Up to 30 cm in North America In central Italy, fruiting body can be 30–40 cm, and larger than those of *H. annosum s.s.*	7.3 (0.12) 11%
H. occidentale	Decay pockets in stumps and fallen trees, and under intact surface of pine stumps	Up to 40 cm	8.6 (0.07) 6%
H. parviporum	Root collar, along roots and in decay pockets within the butt, and also on decayed tree stumps and stem pieces left in the forest	Up to 30 cm Gaitnieks *et al.* (2021) observed larger fruiting bodies than earlier studies: the largest one had a total area of 1 784 cm^2 and a length of 97 cm	13.4 (0.4) (no % data available)

Notes: SD, standard deviation.

Gaitnieks, T., Bruna, L., Zaluma, A., Burnevica, N., Klavina, D., Legzdina, L., Jansons, J. & Piri, T. 2021. Development of *Heterobasidion* spp. fruit bodies on decayed *Piecea abies*. *Forest Ecology and Management*, 482: 118835. https://doi.org/10.1016/j.foreco.2020.118835

Source: Adapted from Otrosina, W.J. & 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. https://doi.org/10.1016/j.mycres.2009.09.001

4.1.1 Cultural characteristics and morphology

In culture, *H. annosum s.l.* can be easily distinguished from other fungi based on its club-like conidiophores with conidiogeneous vesicles 7.5–18(–22) µm diameter (Figure 10; Stalpers, 1978). Asexual spores (conidia), 4–8(–10) × 2.5–5(–6) µm in size, are subglobose to ovoid or lacrymoid, smooth, hyaline, non-septate, thick-walled and without vacuoles (Figure 10; Stalpers, 1978). Colonies grow rapidly on generic agar-based media, reaching 6–8 cm in 7 days; they are white or cream to light buff, ivory yellow or honey yellow, and are sometimes pulverulent because of conidial production (Figure 11). In aged heterokaryotic cultures, brownish-yellow coloration may develop. Marginal and aerial hyphae are 1.5–5.5(–8) µm wide. Most septa in the hyphae are simple; clamp connections are formed in cell divisions of heterokaryotic hyphae, but their occurrence is irregular and they are not numerous (Figure 11; Stalpers, 1978; Korhonen and Stenlid, 1998). Clamps are most frequent on long, straight hyphae with non-vacuolated cytoplasm (Korhonen and Stenlid, 1998). Mycelia originating from germination of single basidiospores are haploid and lack clamp connections.

Macromorphological and micromorphological characters of colonies of *H. annosum s.l.* are not sufficiently divergent to allow a reliable identification of species within the complex (Mitchelson and Korhonen, 1998). Instead, the identification of species within *H. annosum s.l.* was traditionally based

on the use of mating experiments (i.e. sexual compatibility experiments), through which an unknown isolate is paired on agar medium with confirmed homokaryotic strains of the different species. Details on how to perform mating experiments are provided by Korhonen (1978), Stenlid (1985) and Mitchelson and Korhonen (1998). However, the partial *in vitro* interfertility among species may occasionally lead to an ambiguous diagnosis.

Cultures of *H. annosum s.l.* can be stored on malt extract agar or potato dextrose agar slopes or cryogenic vials at 5 °C. DNA can be stored at −20 °C or lower temperatures until use.

As previously indicated, molecular methods (including DNA sequencing, see section 4.2) provide the only reliable way to accurately identify closely related species of the pathogen.

4.2 Molecular identification

The conventional PCR and real-time PCR methods described in sections 3.4.3 and 3.4.4 are species specific and can be used for identification of the pathogen in infected material, wood-disc traps or pure fungal cultures. For *Heterobasidion* isolated in pure culture, sequence analysis of the ITS and EFA regions (see section 4.2.1) is an alternative that can be used for sequence-based, species-level identification.

4.2.1 ITS and EFA sequencing for species-level identification

Heterobasidion isolated in pure culture can be identified by the amplification and sequencing of the ITS DNA region (including ITS1, 5.8S and ITS2) with two different primer pairs: ITS1-F and ITS4 or ITS1-F and ITS4-B (White *et al*., 1990; Gardes and Bruns, 1993). The first primer pair, which includes the fungal-selective primer ITS1-F, efficiently amplifies the DNA of ascomycetes and basidiomycetes; the second one, which also includes the phylum-selective (basidiomycetes) primer ITS4-B, efficiently amplifies the DNA of basidiomycetes but results in either no product or an extremely faint product for the DNA of ascomycetes (Gardes and Bruns, 1993). The EFA region (Johannesson and Stenlid, 2003) can be used to further confirm the identification of close species, such as when distinguishing between *H. abietinum* and *H. parviporum*. These primer pairs can be used to generate amplification products for sequencing from all species of *Heterobasidion*. The expected amplicon produced using these primers can be generated only from DNA extracted from a pure fungal culture (for the preparation of material and DNA extraction, see sections 3.4.1 and 3.4.2).

The master mix and cycling parameters for the PCR are described in Table 13 and the primers are as follows:

ITS1-F (forward primer): 5′-CTT GGT CAT TTA GAG GAA GTA A-3′ (Gardes and Bruns, 1993)

ITS4 (reverse primer): 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al*., 1990)

ITS4-B (reverse primer): 5′-CAG GAG ACT TGT ACA CGG TCC AG-3′ (Gardes and Bruns, 1993)

EFA-F (forward primer): 5′-TCA ACG TGG TCG GTG AGC AGG TA-3′ (Johannesson and Stenlid, 2003)

EFA-R (reverse primer): 5′-AAG TCA CGA TGT CCA GGA GCA TC-3′ (Johannesson and Stenlid, 2003)

Table 13. Master mix composition, cycling parameters and amplicons for ITS and EFA sequencing

Reagents	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
$MgCl_2$	1.5 mM
dNTPs	0.2 mM
Primers:	
- ITS1-F (forward)	0.5 µM
- ITS4 or ITS4-B (reverse)	0.5 µM
or	
- EFA-F (forward)	0.5 µM
- EFA-R (reverse)	0.5 µM
DNA polymerase (Promega)[‡]	0.025 U
DNA (volume)	6.25 µL
Cycling parameters	
Initial denaturation	95 °C for 5 min
Numbers of cycles	35
- Denaturation	95 °C for 30 s
- Annealing	55 °C for 30 s
- Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
ITS	600 bp
EFA	400 bp

Notes: [†] For a final reaction volume of 25 µL.

[‡] See page footnote 2.

bp, base pairs; EFA, elongation factor 1-α; ITS, internal transcribed spacer; PCR, polymerase chain reaction.

Sources: Gardes, M. & Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2: 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Johannesson, H. & Stenlid, J. 2003. Molecular markers reveal genetic isolation and phylogeography of the S and F intersterility groups of the wood-decay fungus *Heterobasidion annosum*. *Molecular Phylogenetics and Evolution*, 29: 94–101. https://doi.org/10.1016/S1055-7903(03)00087-3

White, T.J., Bruns, T.D., Lee, S.B. & Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: N. Innis, D.H. Gelfand, J.J. Sninsky & T.J. White, eds. *PCR protocols – A guide to methods and applications*, pp. 315–322. Academic Press, San Diego, USA. 482 pp.

The PCR products can be visualized by standard agarose gel electrophoresis. The remaining amplification product can be purified using a suitable PCR purification kit following the manufacturer's instructions and the purified amplicon can be bidirectionally sequenced with ITS1-F and ITS4, ITS1-F and ITS4-B, or EFA-F and EFA-R primer pairs.

4.2.2 Controls for molecular tests

The minimum controls for a sequence-based identification are a positive nucleic acid control and a negative amplification control for the PCR.

Positive nucleic acid control. This control is used to monitor the efficiency of the amplification of nucleic acid from the target pest. The nucleic acid extracted from a pure fungal culture of the target pest can be used.

Negative amplification control. This control is necessary to rule out false positives resulting from contamination (e.g. with the target DNA) during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

4.2.3 Interpretation of results

A test is considered valid if the negative amplification control does not produce a band corresponding to the expected amplicon size (see 3.4.6.1) and the positive nucleic acid control does produce a band that corresponds to the expected amplicon size.

After sequencing, the quality of the resulting sequence should be checked by visual assessment of the electropherograms. Consensus sequences may be built from the forward and reverse reads. The final edited sequence should cover at least 99% of the expected length for data interpretation and should be compared with published sequences using the Basic Local Alignment Search Tool (BLAST), available at the National Center for Biotechnology Information, United States of America (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

There are three additional, useful databases for sequence comparison:

- Barcode of Life Data System (BOLD): the BOLD Identification System for ITS is a default identification tool for fungal barcodes (https://www.boldsystems.org).
- UNITE: this is a database and sequence management environment centred on the eukaryotic nuclear ribosomal ITS region (https://unite.ut.ee).
- MycoBank: this is an online database documenting mycological nomenclatural novelties and providing pairwise sequence alignments and polyphasic identifications of fungi and yeasts against curated reference databases (https://www.mycobank.org/).

For species identification, the sequence should be at least a 99% match to published reference sequences (preferably from type specimens, if available). Dalman, Olson and Stenlid (2010) provide GenBank accession numbers for reliable specimens of all *Heterobasidion* species: GenBank accession numbers from FJ627520 to FJ627596 for ITS and FJ627434 to FJ627364 for EFA. Examples are: *H. abietinum* isolate Faf4-2, GenBank accession number FJ627561.1 for ITS and FJ627400.1 for EFA; *H. annosum s.s.* isolate W15, GenBank accession number FJ627596.1 for ITS and FJ627434.1 for EFA; *H. irregulare* isolate MON 111, GenBank accession number FJ627580.1 for ITS and FJ627418.1 for EFA; *H. occidentale* isolate MON 108, GenBank accession number FJ627578.1 for ITS and FJ627416.1 for EFA; and *H. parviporum* isolate Fas1, GenBank accession number FJ627567.1 for ITS and FJ627406.1 for EFA.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be adversely affected by the results of the diagnosis, records and evidence of the results (e.g. cultures, slides, photos of fungal structures, photos of symptoms and signs, photos of separation gels) should be retained for at least one year in a manner that ensures traceability.

6. Contact points for further information

Further information on this protocol can be obtained from:

Plant Protection Service Lombardy Region, Viale Raimondi 54, 22070 Vertemate con Minoprio, Como, Italy (Luana Giordano; email: luana_giordano_cnt@regione.lombardia.it; tel.: (+39) 031 320583).

Natural Resources Institute Finland, Latokartanonkaari 9, FI-00790 Helsinki, Finland (Eeva J. Vainio; email: eeva.vainio@luke.fi; tel.: (+358) 29 5325410).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), who will forward it to the Technical Panel on Diagnostic Protocols (TPDP).

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This protocol is partially adapted, with permission, from EPPO (2020a, 2020b).

8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

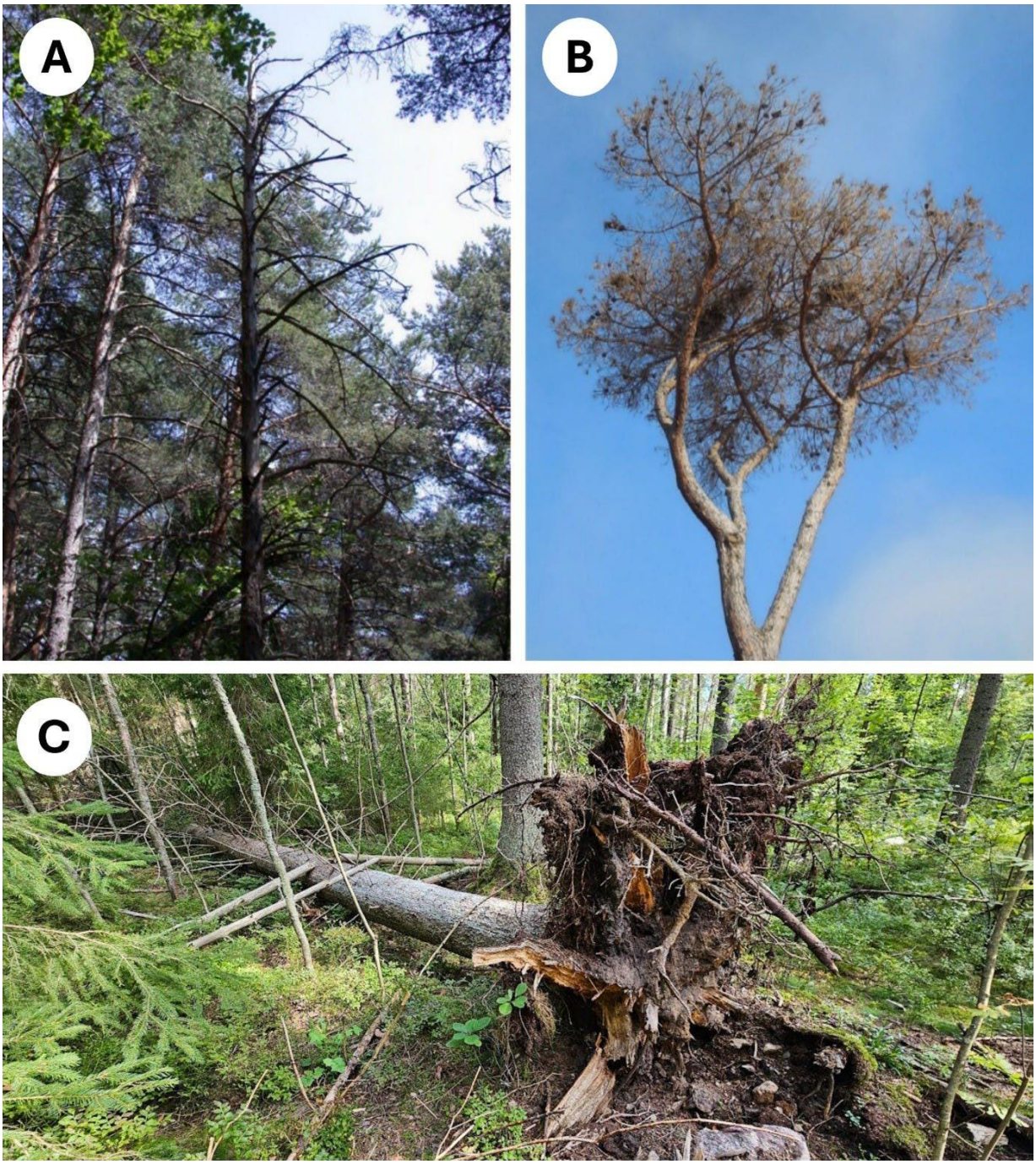

Figure 1. Mortality of *Pinus sylvestris* (A) and *Pinus pinea* (B) caused by *Heterobasidion annosum sensu stricto* and *Heterobasidion irregulare*, respectively. Fallen *Picea abies* with *Heterobasidion* infection (C).

Sources: L. Giordano, Plant Protection Service Lombardy Region, Italy (A and B); E.J. Vainio, Natural Resources Institute Finland (C).

Figure 2. The lion-tailing phenomenon of *Pinus pinea* caused by *Heterobasidion irregulare*.
Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

Figure 3. Paper-thin mycelium of *Heterobasidion* sp. (A). *Pinus sylvestris* cambium necrosis beneath the bark at the tree collar (B). Thicker white mycelium of *Armillaria* sp. beneath the bark at the tree collar of *Pinus sylvestris* (C).

Sources: EPPO, 2020 (A); L. Giordano, Plant Protection Service Lombardy Region, Italy (B and C).

EPPO (European and Mediterranean Plant Protection Organization). 2020. *Heterobasidion irregulare*. PM 7/140(1). *EPPO Bulletin*, 50(1): 19–31. https://doi.org/10.1111/epp.12618. Reproduced with permission, with modified caption.

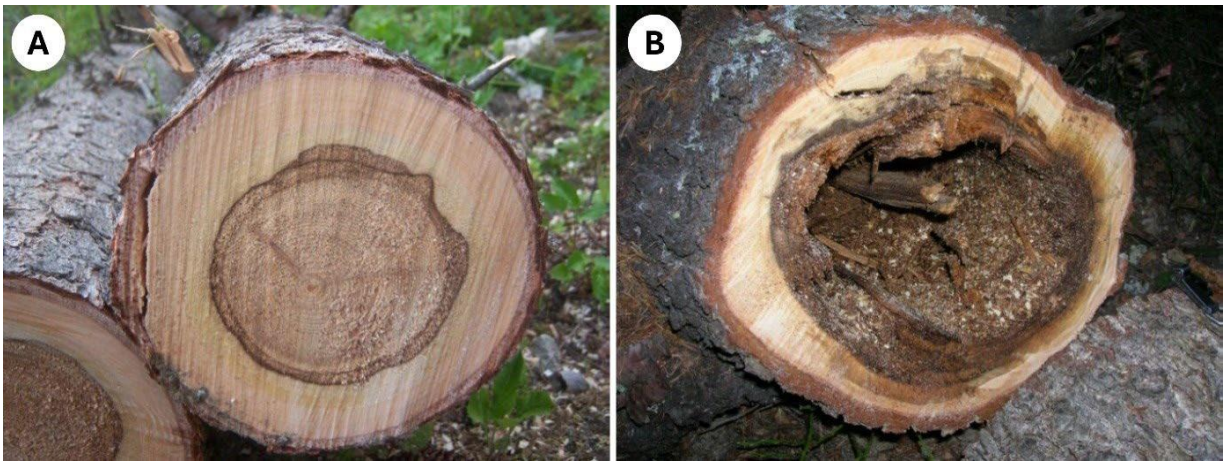

Figure 4. Stem rot of *Picea abies* caused by *Heterobasidion parviporum* (A). When stem rot develops, it can result in cavities (B).

Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

Figure 5. Pocket rot caused by *Heterobasidion* infection.

Source: E. Vainio, Natural Resources Institute Finland.

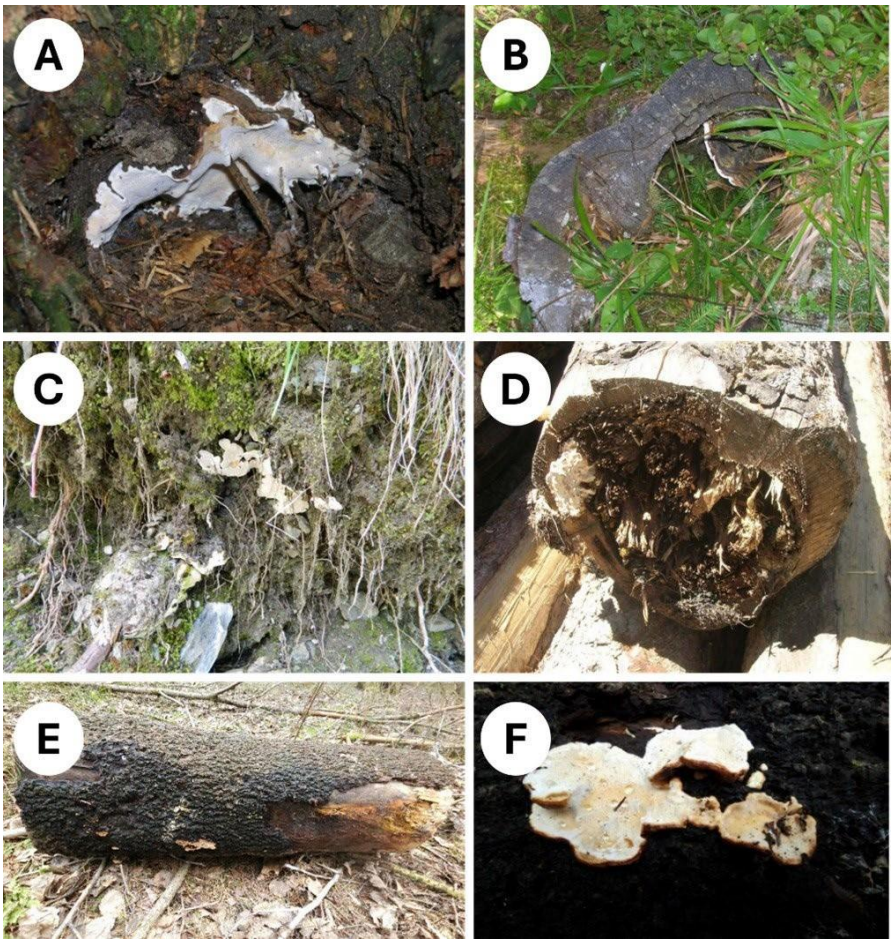

Figure 6. Examples of *Heterobasidion annosum sensu lato* fruiting bodies. They may envelop litter debris (A) and develop inside the internal cavities of old decayed stumps (B), on infected roots of living trees (C), and on the cut ends (D) and the ground-facing side of pieces of timber left for several months in the forest (E, F).

Sources: L. Giordano, Plant Protection Service Lombardy Region, Italy (A, B, C and D); E.J. Vainio, Natural Resources Institute Finland (E and F).

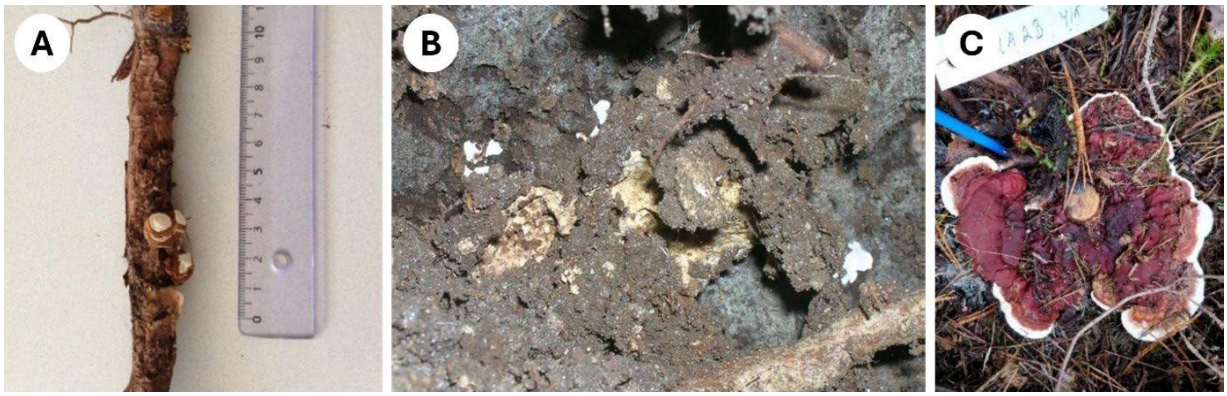

Figure 7. Incipient fruiting bodies of *Heterobasidion annosum sensu lato*, referred to as “popcorn” because of their appearance in pine roots (A, B). Large fruiting body surrounding a stump of a very small *Pinus sylvestris* seedling that has been cut after being killed by *H. annosum sensu stricto* (C): the reddish-brown top surface of the fruiting body is visible.

Sources: E.J. Vainio, Natural Resources Institute Finland (A and C); L. Giordano, Plant Protection Service Lombardy Region, Italy (B).

Figure 8. Conidiophores of the anamorphic stage of *Heterobasidion annosum sensu lato* that appear as a mass of whitish “pinheads” on stalks (A). Wood sawdust from drilling at the base of a tree (B). Wood-disc trap for spores exposed in the field inside an open, 15-cm diameter, plastic Petri dish containing a sterile piece of filter paper dampened with sterile distilled water (C).

Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

Figure 9. Fruiting bodies of *Heterobasidion annosum sensu lato* (A, B); pores of the lower surface of fruiting bodies (C).

Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

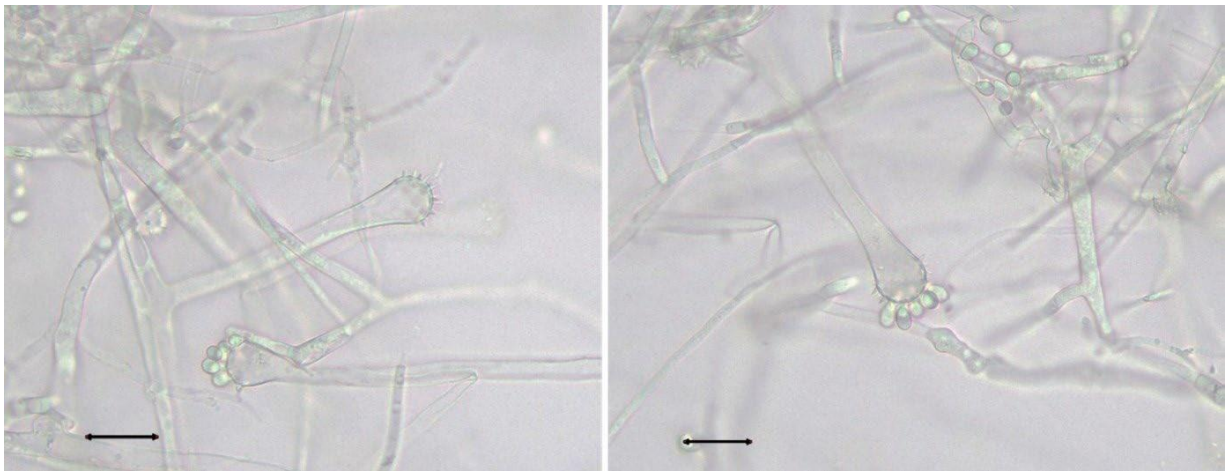

Figure 10. Conidiophores and conidia of the asexual stage of *Heterobasidion annosum sensu lato* (40× magnification; scale bar 20 µm).

Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

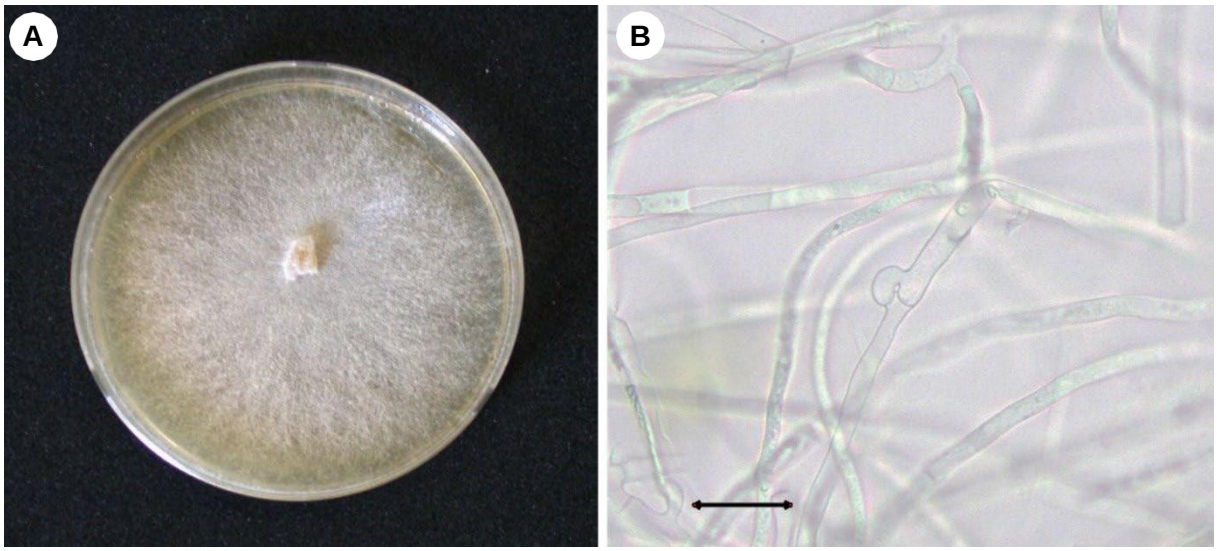

Figure 11. White-cream colony of *Heterobasidion annosum sensu lato* on malt extract agar (age of culture ca. three weeks) (A) and clamp connection (40× magnification; scale bar 20 µm) (B).

Source: L. Giordano, Plant Protection Service Lombardy Region, Italy.

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Required citation:
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Publication history
This is not an official part of the standard

2021-11 Standards Committee (SC) added subject *Heterobasidion annosum* (2021-015) to work programme, priority 3.

2023-04 Expert consultation.

2023-11 Technical Panel on Diagnostic Protocols (TPDP) revised and recommended to SC for consultation.

2024-01 SC approved for consultation via e-decision (2024_eSC_May_02).

2024-07 Consultation.

2024-11 TPDP revised and recommended to SC for adoption.

2025-01 SC approved for 45-day DP notification period (2025_eSC_May_05).

2025-03 SC adopted DP on behalf of CPM (no objections received).

ISPM 27. **Annex 34**. *Heterobasidion annosum sensu lato*. IPPC Secretariat. Rome, FAO.

2025-05 Secretariat, with approval from discipline lead, corrected computer errors introduced when draft DP posted for notification (deleted "is available in Garbelotto and Gonthier (2013)" from the end of third paragraph of section 1; inserted footnote 1 text; omitted *Heterobasidion annosum sensu lato* from Table 8 caption).

Publication history last updated: 2025-05