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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/117 (1)

Diagnostics
Diagnostic

PM 7/117 (1) *Hymenoscyphus pseudoalbidus*

Specific scope

This Standard describes a diagnostic protocol for *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*)[1].

Specific approval and amendment

Approved as an EPPO Standard in 2013-09.

Introduction

Chalara fraxinea, the asexual form of the fungus *Hymenoscyphus pseudoalbidus*, was identified for the first time in Poland in 2006 (Kowalski, 2006). This emerging pathogen affects tree species of the genus *Fraxinus (*Kowalski & Holdenrieder, 2009a), mainly common ash (*Fraxinus excelsior*) and narrow-leafed ash (*F. angustifolia*; Kirisits *et al.*, 2009). The disease is of major concern in nurseries, urban areas and forests, causing decline and mortality of trees of all ages.

Initially, *Chalara fraxinea* was suspected to be the anamorphic stage of *Hymenoscyphus albidus*, a widespread saprotrophic fungus native to Europe (Kowalski & Holdenrieder, 2009b). However, DNA-based molecular studies revealed that *C. fraxinea* was a new fungus genetically close to *H. albidus* and given the name *H. pseudoalbidus* (Queloz *et al.*, 2010; Husson *et al.*, 2011), which possibly originates from Asia (Zhao *et al.*, 2012).

Hymenoscyphus pseudoalbidus forms ascospores on infected fallen petioles/rachises in the leaf litter, which are air-dispersed (Kowalski & Holdenrieder, 2009b; Timmermann *et al.*, 2011). The fungus enters the host through the young parts (e.g. stalks, leaves, epicormic shoots) and progresses from there to reach the woody tissue. The fungus can also be carried over long distances by the movement of infected plant material, notably from nurseries (Kirisits *et al.*, 2012). According to Gross *et al.* (2012), conidia are not infectious and might act as spermatia.

Ash dieback caused by *H. pseudoalbidus* has been reported in numerous countries in Europe, including Austria (Cech, 2006; Halmschlager & Kirisits, 2008), Belgium (Chandelier *et al.*, 2011), the Czech Republic (Jankovsky & Holdenrieder, 2009), Croatia (Barić *et al.*, 2012) Denmark (Thomsen *et al.*, 2007), France (Ioos *et al.,* 2009b), Finland (Rytkönen *et al.*, 2010), Germany (Schumacher *et al.*, 2007), Hungary (Szabo, 2009), Italy (Ogris *et al.*, 2010), Latvia, Estonia, Romania and Kaliningrad (Russia; Kirisits *et al.*, 2009), Lithuania (Juodvalkis & Vasiliauskas, 2002), Norway (Talgø *et al.*, 2009), Slovakia (Kunca *et al.*, 2011), Slovenia (Ogris *et al.*, 2009), Sweden (Bakys *et al.*, 2009), Switzerland (Engesser *et al.*, 2009), Netherlands (EPPO, 2010), England and Scotland (EPPO, 2012a), Guernsey (EPPO, 2012b) and Ireland (EPPO, 2013).

A flow diagram describing the diagnostic procedure for *Hymenoscyphus pseudoalbidus* is presented in Fig. 1.

Identity

Name: *Hymenoscyphus pseudoalbidus* V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber & O. Holdenrieder sp. nov.
Anamorph: *Chalara fraxinea* T. Kowalski.
Taxonomic position: Fungi: Ascomycota: Ascomycetes: Heliotales: Heliotiaceae: *Hymenoscyphus.*
Phytosanitary categorization: EPPO Alert List.
EPPO Code: CHAAFR.

Detection

Symptoms

On natural regeneration, saplings or coppice sprouts, necroses can be observed on leaves, rachises and shoots/

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

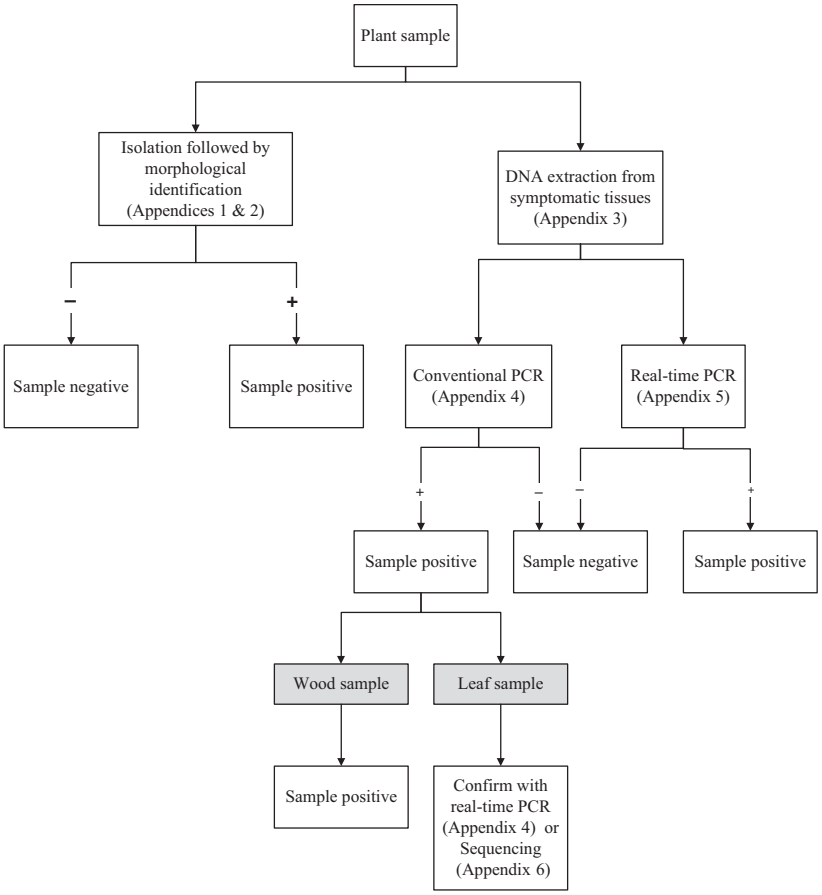

Fig. 1 Flow diagram for diagnosis of *Hymenoscyphus pseudoalbidus*.

branches. Infected leaves wilt, turn black, become dry and often remain attached to the stem in summer (Fig. 2A). The pathogen also causes elongated bark necroses that often have an angular form and brownish to orange coloration (Fig. 2B,C). These necroses may lead to dieback of shoots and branches. Wood discoloration can be seen in cross-sections of affected branches and stems (Fig. 2D,E).

On adult trees, healed cankers are visible on the trunk (Fig. 2F). In active cankers, necrotic lesions frequently extend into the xylem beyond the margin of the superficial lesion. Necroses may occur at the base of the tree in the most severely affected stands (Fig. 2G). These necroses are often invaded by *Armillaria* spp. infecting from the ground (Husson *et al.*, 2012). The crowns of declining trees exhibit wilting and premature shedding of leaves, shoot dieback (Fig. 2H) and prolific epicormic shoots, giving the tree a particular shape. Necrotic lesions can also extend into the roots, but are usually restricted to the aerial parts of the plant (Kirisits *et al.*, 2009).

In the leaf litter, white or cream coloured apothecia (1.5–5 mm in diameter) containing ascospores can be seen from June through to October on the blackened area of ash rachises from the previous year (Fig. 3), or less frequently on the shoots of 1–3-year-old dead ash seedlings (Kowalski & Holdenrieder, 2009b).

Sampling procedure

There are several possibilities for sampling plant material.

If fruiting bodies are visible on lesions (Fig. 4), affected plant parts should be sampled.

Leaves, leaf rachises and petioles showing dark necrosis can be taken from diseased ash of all ages.

For saplings, natural regeneration or adult trees showing dieback, a portion of a cankered shoot about 10–20 cm in length is taken at the junction between healthy and infected areas (leading edge).

For adult trees with older cankers on the trunk, the bark must be removed and a piece of wood dissected from the leading edge.

If diagnostic work is targeted to a confirmation of the pathogen's presence in the stand only, discoloured petioles and rachises can be collected from the litter. If felling of diseased mature trees has to be avoided, it could be helpful to look for symptoms on natural regeneration of ash, which is generally abundant in ash stands and is very susceptible to the disease.

Samples are placed in sealed plastic bags. They can be stored at 2–8°C prior to analysis for 24–48 h (if isolation is performed) or for an extended period (several days if molecular detection is selected). For molecular analysis, samples can also be frozen.

Fig. 2 Symptoms of ash dieback caused by *Hymenoscyphus pseudoalbidus.* (A) Leaf wilting, (B, C) cortical necrosis on young branches; (D, E) wood discoloration, (F) canker on trunk, (G) collar necrosis, (H) crown dieback.

Fig. 3 Apothecia on leaf petioles collected in the leaf litter.

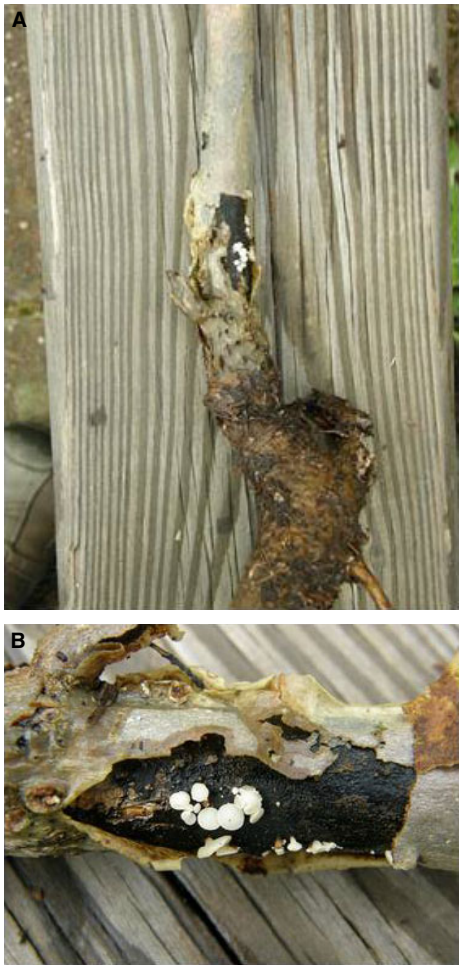

Fig. 4 (A, B) Ascomata of *Hymenoscyphus pseudoalbidus* on a stem-base necrosis of a common ash plant. Photos by T. Kirisits, Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Austria.

Isolation

As only the anamorph form of *H. pseudoalbidus* is observed in pure culture after isolation, morphological identification aims to identify *C. fraxinea*.

Isolation from infected plant tissues is carried out on malt extract agar (MEA) supplemented with antibiotic, as recommended by Kowalski (2006), or cherry decoction agar (CDA; Appendix 1).

For successful isolation, it is essential to use host tissues in which the pathogen is active, as bark necroses are colonized quickly by other microfungi, which are able to suppress the growth of *C. fraxinea* on artificial media. Isolation is most easily carried out from necrotic leaves, leaf rachises or petioles.

If the isolation is made from stems or shoots, thin slices approximately 0.5 cm thick are taken from the symptomatic wood and/or bark, preferentially at the leading edge. If isolation is made from cankers on the trunk of mature trees, wood chips are excised with a scalpel from the area with wood discoloration.

Plant samples (inner bark, outer xylem, leaves and petioles) are surface-disinfected (Appendix 2) and plated onto the medium. The agar plates should be incubated in the dark at 2–8°C for 3–4 weeks. Subcultures are made onto MEA without antibiotics for the morphological identification.

Chalara fraxinea forms hyaline cotton white or light orange colonies, often darkening to light brown or dark green in some areas (Fig. 5A–C). On ready-to-use MEA, mycelium is more cottony with intense orange or brown pigments (Fig. 5D,E).

Direct detection *in planta* using molecular techniques

There are several PCR tests available for the detection of *H. pseudoalbidus*. These tests are described in Appendices 4 and 5. The two real-time PCR tests described in Appendix 5 are specific and allow the distinction from *H. albidus*, a non-pathogenic fungal species closely related to *H. pseudoalbidus*. Unlike isolation, molecular tests can be carried out on 'non-fresh' plant material (maintained for several days at 2–8°C or frozen). See Identification for a detailed description of methods.

Identification

The procedures for the identification of *H. pseudoalbidus* on *Fraxinus* spp. are described in the flow diagram in Fig. 1.

Morphological characteristics in pure culture

For morphological identification, the isolates are grown on MEA for 2–3 weeks at 20–22°C in the dark. The growth rate of the fungus is very slow (0.3–1.7 mm per day; Hauptman *et al.*, 2013). *Chalara fraxinea* is characterized by cylindrical hyaline or pale brown phialoconidia (3.2–4.0 × 2.0–2.5 µm; Fig. 5G) which are extruded from pigmented phialides (16–24 µm long, Fig. 5H) in chains or in slimy droplets (Fig. 5F) The first-formed conidium is shortly clavate 6–7 × 2.2–2.5 µm. Stromata composed of cells with thickened, dark brown walls can be visible in 2- or 3-week-old cultures. All isolates are confirmed as *C. fraxinea* based on the morphological features described by Kowalski (2006).

Molecular methods

The following molecular methods are available to confirm the identity of *H. pseudoalbidus* or to detect the pathogen directly *in planta*.

(1) Conventional PCR targeting the ITS rDNA operon (Johansson *et al.*, 2010) – see Appendix 4.
(2) Real-time PCR targeting the ITS rDNA operon (Ioos *et al.,* 2009b; Chandelier *et al.,* 2010) – see Appendix 5.

To confirm the identity of *C. fraxinea* in pure culture, the ITS rDNA operon can also be sequenced (see Appendix 6).

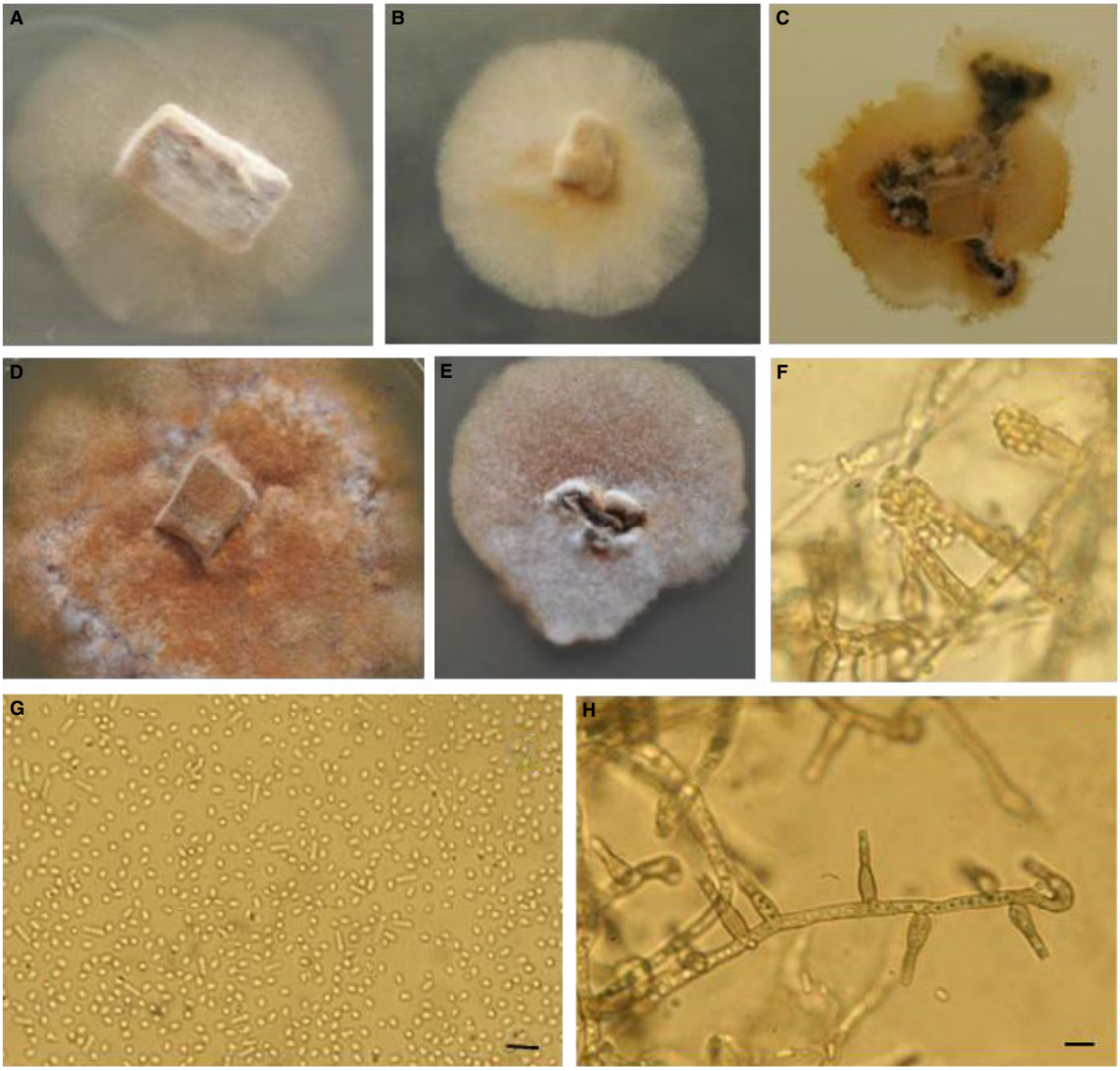

Fig. 5 (A, B) Pure cultures of *Chalara fraxinea* on MEA, (C) old culture (with stroma) on MEA, (D, E) Pure culture of *Chalara fraxinea* on ready-to-use MEA (Fluka Analytical Sigma-Aldrich, Germany), (F) conidia in droplets, (G) conidia (elongated conidia correspond to first-formed conidia), (H) phialides (500× magnification; scale bar = 10 μm).

DNA extraction from pure culture

Fungal DNA should be extracted using an appropriate standard method for DNA extraction from fungi (commercial plant DNA extraction kits) and analysed using any of the tests presented in Appendices 4, 5 or 6.

DNA extraction from plant tissue

Total DNA from potentially infected plant tissue should be extracted as described in Appendix 3 and analysed using any of the tests presented in Appendices 4 or 5.

Reference cultures

The type strain of *H. pseudoalbidus* (CBS 122505 from Poland; CBS 122193 from Austria) is available from the CBS–KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.

Reporting and documentation

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

Performance criteria

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

Further information

Further information on this organism can be obtained from: A. Chandelier, Life Sciences Department, Walloon Agricultural Research Centre, Rue de Liroux 4,B-5030 Gembloux, Belgium; R. Ioos, French National Agency for Food Environmental and Occupational Health & Safety (Anses), Plant Health Laboratory, Mycology Unit, Domaine de Pixérécourt – Bât. E, BP 90059, F54220 Malzéville, France.

Feedback on this diagnostic protocol

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

Protocol revision

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

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

Acknowledgements

This protocol was originally drafted by: A. Chandelier, Walloon Agricultural Research Centre, Gembloux, Belgium; T. Cech, Federal Research and Training Centre for Forests, Natural Hazards and Landscape (BFW). Department of Forest Protection, Unit of Phytopathology and Biochemistry, Vienna, Austria; R. Ioos, French National Agency for Food Environmental and Occupational Health & Safety (Anses), Plant Health Laboratory, Malzeville, France.

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Appendix 1 – Composition of culture media

Except when stated otherwise; all media are sterilized by autoclaving at 121°C for 15 min, poured at 20 mL per Petri dish and stored at room temperature for approximately 2 months or kept for 3 months at 2–8°C.

Malt extract agar (MEA)

Malt extract	20.0 g
Microbiological grade agar	15.0 g
Distilled water to	1 L

The basal medium is autoclaved for 20 min and poured into Petri dishes. It can be stored at room temperature for approximately 2 months.

For MEA supplemented with antibiotics, the medium is cooled after sterilization (48–52°C) before adding streptomycin sulphate (100 mg L^{-1}). This medium should be stored at 2–8°C for a maximum of 1 month.

Ready-to-use MEA (Fluka Analytical, Sigma-Aldrich, Germany – composition: malt extract 30 g L^{-1}; mycological peptone 5 g L^{-1}; agar 5 g L^{-1}) can also be used. This medium should be prepared according to the manufacturer's instructions and autoclaved. It can be stored at room temperature for approximately 2 months.

Cherry decoction agar (CDA), (KCHM Rosendahl, pers. comm.)

Organic (biological) cherry juice (in glass bottles)	100 mL
Microbiological grade agar	15.0 g
Distilled water	900 mL

The medium is sterilized for 5 min at 102–105°C. The final pH is 4.4.

Petri dishes and tubes can be stored for 6 months at 2–10°C.

Appendix 2 – Disinfection techniques for isolation

(a) Twig, stem, shoot

Large sections including canker wounds (minimum 10–20 cm long) are sent to the laboratory. In the laboratory, slices approximately 0.5 cm thick are cut from the leading edge of the infection. Tissues are soaked in ethanol 96% for 1 min, then soaked in 4% sodium hypochlorite (NaOCl) for 5 min and again soaked in ethanol 96% for 30 s. Spraying the plant parts with ethanol and flaming briefly may be used (Jankovsky & Holdenrieder 2009). The outer bark is then removed and pieces of approximately 5 × 2 × 2 mm comprising wood, phloem and cambium are taken aseptically from the samples and plated on MEA supplemented with streptomycin sulphate.

(b) Leaf, rachis, petiole

Pieces of leaves (approximately 1–2 cm) are cut from the edge of a necrosis or a necrotic spot and dipped into 2% sodium hypochlorite (NaOCl) for approximately 30 s, rinsed twice in sterile distilled water, drained and plated onto MEA supplemented with streptomycin sulphate. From rachises the epidermis is peeled off and then they are treated as described above (T. Kirisits, pers. comm.).

Appendix 3 – DNA extraction for *in planta* detection

Small pieces of plant tissue are excised from the leading edge of suspect lesions and homogenized. Different grinding methods can be used, such as mortar and pestle with liquid nitrogen, bead mills or the Homex grinder from Bioreba, providing they produce a homogeneous ground sample. For branches with a large diameter (>1 cm), sampling with a drill (diameter of drill bit = 3 mm) in the wood area displaying discoloration can also be done without further grinding (Chandelier *et al.*, 2010a). DNA extraction can be performed using commercial kits, for example the NucleoSpin Plant extraction kit (Macherey & Nagel, Düren, Germany) or the DNeasy Plant kit (Qiagen, Hilden, Germany), following the manufacturer's instructions; alternatively, DNA can be extracted using the CTAB DNA extraction protocol (Lodhi *et al.*, 1994). For DNA isolation from cultured isolates, the same procedure can be followed.

Appendix 4 – Identification to species level by conventional PCR (Johansson *et al.*, 2010)

1. General information

1.1 This protocol was developed by Johansson *et al.* (2010).
1.2 Nucleic acid source is infected plant tissues.
1.3 Name of targeted gene is the 18S gene (forward primer) and the ITS-2 region of the rDNA operon (reverse primer).
1.4 Amplicon size with these primers is 456 bp.
1.5 Oligonucleotides:
Forward primer: 5′-AGC TGG GGA AAC CTG ACT G-3′
Reverse primer: 5′-ACA CCG CAA GGA CCC TAT C-3′

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 3
2.2 PCR reaction (see Table 1).
2.3 Thermocycler conditions:
PCR is carried out in a thermocycler equipped with a heated lid. Reaction conditions include an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and elongation at 72°C for 1 min. A final elongation step is done at 72°C for 8 min. The PCR product is kept below −18°C until separation by electrophoresis in an agarose gel (1–2%) followed by gel staining (with ethidium bromide or similar product).

Table 1 PCR method

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	14.44	N.A.
PCR buffer Y	10 X	2	1 X
$MgCl_2$	50 mM	0.3	0.75 mM
dNTP	10 mM	0.4	0.2 mM
Forward primer	1 µM	0.4	0.02 µM
Reverse primer	1 µM	0.4	0.02 µM
DNA Taq polymerase (ThermoRed)	5 U/µL	0.06	0.3 U/20 µL
Subtotal		18	
DNA	2.5 ng/µL	2	0.25 ng/µL
Total		20	

3. Essential procedural information

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

- *Negative isolation control (NIC)* to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- *Positive isolation control (PIC)* to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- *Negative amplification control (NAC)* to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix.
- *Positive amplification control (PAC)* to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately by amplification of a duplicate sample spiked with the target nucleic acid.

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

Verification of controls:
- NIC and NAC should produce no amplicons.
- PIC, PAC and IPC (if used) should produce amplicons of 456 bp.

When these conditions are met:
- A test will be considered positive if amplicons of 456 bp are produced.
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

Confirmation with another method is recommended due to possible cross-reaction with *H. albidus,* especially in cases where discoloured leaf rachises are tested (see flow diagram, Fig. 1).

4. Performance criteria available

A total of 78 isolates from various countries including Sweden (34), Finland (3), Lithuania (12), Denmark (22), Germany (1), Poland (4), Austria (1) and Czech Republic (1) were included in the validation.

4.1 Analytical sensitivity data
The analytical sensitivity has been established by the authors at 2.5 pg $\mu L^{-1.}$

4.2 Analytical specificity data
The test specificity has been checked against different fungi, notably *Hymenoscyphus scutula* and *H. caudatus*. According to *in silico* analysis, cross-reactions with *H. albidus* might occur (forward primer: 100% homology between both species, reverse primer: only one mismatch close to the 5′end).

Appendix 5 – Identification at species level by real-time PCR using dual-labelled probes

(A) Real-time PCR (Ioos *et al.*, 2009b; Ioos & Fourrier, 2011)

1. General information

1.1 This protocol was developed by Ioos *et al.* (2009b) and further improved by Ioos and Fourrier (2011).

1.2 Nucleic acid source is infected plant tissues or pure fungal cultures.

1.3 Name of targeted gene is ITS rDNA operon.

1.4 Oligonucleotides: Primer sequences for *Hymenoscyphus pseudoalbidus*:

Cfrax-F: 5′-ATT ATA TTG TTG CTT TAG CAG GTC-3′
Cfrax-R: 5′-TCC TCT AGC AGG CAC AGT C-3′
Cfrax-P: 5′-FAM- CTC TGG GCG TCG GCC TCG-BHQ1-3′

Primer sequences for the plant internal control (18S rDNA gene)

18S-UniF: 5′-GCA AGG CTG AAA CTT AAA GGA A-3′
18S-UniR: 5′-CCA CCA CCC ATA GAA TCA AGA-3′
18S-UniP: 5′- JOE-ACG GAA GGG CAC CAC CAG GAG T- BHQ1-3′

1.5 The duplex test was developed with the qPCR Mastermix No ROX (Eurogentec, Seraing, Belgium) and a Rotor-Gene 6500 (Corbett Research, Mortlake, Australia) set with an autogain optimization for each channel, which was performed before the first fluorescence acquisition. The Ct value for each reaction was determined using the Rotor-Gene software, version 1.7.75, setting the threshold line at 0.02.

2. Methods

2.1 Nucleic acid extraction and purification: see Appendix 3

2.2 PCR reaction

2.2.1 Mastermix (see Table 2).

2.2.2 PCR conditions

The PCR reaction conditions are carried out in a thermocycler adapted for real time reactions and includes an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 15 s at 95°C and 55 s at 65°C respectively. The Ct value for each reaction is determined using the software provided with the thermocycler.

3. Essential procedural information

3.1 Controls
For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively:

– *Negative isolation control (NIC)* to monitor cross-reactions with the host tissue and/or contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer
– *Positive isolation control (PIC)* to ensure that nucleic acid of sufficient quantity and quality is isolated: implementation of a 18S uni-F/-R/-P test, systematically used in duplex with Cfrax-F/-R/-P in order to check the

Table 2 Mastermix method (A, real-time PCR: Ioos *et al.*, 2009b; Ioos & Fourrier, 2011)

Reagent	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	2.4	N.A.
qPCR Mastermix No ROX (Eurogentec, Seraing, Belgium)	2 X	10	1 X
Primer Cfrax-F	5 µM	1.2	0.3 µM
Primer Cfrax-R	5 µM	1.2	0.3 µM
Dual-labelled probe Cfrax-P	5 µM	0.4	0.1 µM
Primer 18S-UniF	5 µM	1.2	0.3 µM
Primer 18S-UniR	5 µM	1.2	0.3 µM
Dual-labelled probe 18S-UniP	5 µM	0.4	0.1 µM
Subtotal		18	
DNA	0.4–15 ng/µL	2	0.04–1.5 ng/µL
Total		20	

*These figures are indicative. They can be modified provided the final concentration in the PCR reaction is respected.

quality of DNA extraction. This 18S uni-F/-R/-P combination targets a conserved region within the 18S rDNA gene from a wide range of plants (Ioos *et al.* 2009a).

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

3.2 Interpretation of results

The cycle cut-off value for target *H. pseudoalbidus* is set at 34, and was obtained using the equipment/materials and chemistry used as described in this appendix. When necessary, the Ct cut-off value should be determined for the required control. The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls:

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct > 34).
- PAC should have a Ct value below the target cut-off value.
- PIC should have a Ct value below the 18S uni cut-off value, which was set to 26.6, using the equipment/materials and chemistry used as described in this appendix.

When these conditions are met:

- a test will be considered positive if it produces an exponential amplification curve and a Ct value below the cut-off value
- a test will be considered negative if it produces no exponential amplification curve and a Ct value equal or above the cut-off value.

4. Performance criteria available

Validation data in a context of accreditation ISO 17025 are available (Ioos & Fourrier, 2011).

4.1 Analytical sensitivity

The detection limit has been established by the authors at 20 fg *H. pseudoalbidus* DNA per PCR reaction.

4.2 Analytical specificity

The test specificity has been checked using 20 strains of the target organisms and 34 non-target fungal taxa isolated from ash tissue (Table 1 in Ioos *et al.*, 2009b). No cross-reactions were observed.

4.3 Data on repeatability

The coefficient of variation (%CV) based on mean Ct values is:

0.96% for target concentration of 4.8×10^4 copies of the target DNA.
1.70% for a target concentration of 4.8×10^3 copies of the target DNA.
2.19% for a target concentration of 4.8×10^2 copies (LOD) of the target DNA.
0.89% for a naturally infested ash sample.

4.4 Data on repeatability

The coefficient of variation (%CV) based on mean Ct values is:

1.08% for target concentration of 4.8×10^4 copies of the target DNA.
1.63% for a target concentration of 4.8×10^3 copies of the target DNA.
3.32% for a target concentration of 4.8×10^2 copies (LOD) of the target DNA.
2.56% for a naturally infested ash sample.

(B) Real-time PCR (Chandelier *et al.*, 2010)

1. General information

1.1 This protocol was developed by Chandelier *et al.* (2010a).

1.2 Nucleic acid source is infected plant tissues or pure fungal cultures.

1.3 Name of targeted gene region is the ITS rDNA operon.

1.4 Oligonucleotides: primer sequences:
Cf-F: 5′-CCC TTG TGT ATA TTA TAT TGT TGC TTT AGC -3′
Cf-R: 5′-GGG TCC TCT AGC AGG CAC AGT -3′
Cf-P 5′-FAM- TCT GGG CGT CGG CCT CGG-BHQ1-3′

1.5 Amplifications were done in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Life Technologies).

2. Method

2.1 Nucleic acid extraction and purification: see Appendix 3.

2.2 PCR reaction

2.2.1 Mastermix (see Table 3).

2.2.2 PCR conditions

PCR is carried out in a thermocycler adapted to real-time reactions. Reaction conditions include initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation and annealing/elongation, 15 s at 95°C and 60 s at 60°C, respectively. The Ct value for each reaction is determined using the software provided with the thermocycler.

Table 3 Mastermix method (B, real-time PCR: Chandelier *et al.*, 2010)

Reagents	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	7.05	N.A.
Reaction buffer (qPCR Core Kit, Eurogentec, Liege, Belgium)	10 X	2	1 X
$MgCl_2$	50 mM	2	5 mM
dNTP mix	5 mM	0.8	0.2 mM
Primer Cf-F	5 µM	1	0.25 µM
Primer Cf-R	5 µM	1	0.25 µM
Dual-labelled probe Cf-P	5 µM	1	0.25 µM
HotGoldStar Taq DNA polymerase (qPCR Core Kit, Eurogentec)	5 U/µL	0.15	0.75 U/20 µL
Subtotal		15 µL	
DNA	0.5–10 ng/µL	5	0.125–2.5 ng/µL
Total		20	

*These figures are indicative. They can be modified provided the final concentration in the PCR reaction is respected.

3. Essential procedural information

3.1 Controls

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

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

In addition to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately by amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

The cycle cut-off value for target *H. pseudoalbidus* is set at 35, and was obtained using the equipment/materials and chemistry used as described in this appendix (ABI7000 Sequence Detection System, qPCR core kit (Eurogentec, Belgium) and a threshold value set manually at 0.5). When necessary, the Ct cut-off value should be determined for the required control. The cycle cut-off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls:
- The PIC, PAC (and IPC if used) amplification curves should be exponential.
- NIC and NAC should be negative (Ct > cut off).
- PIC (and IPC if used) should have a Ct value below the cut-off value.
- PAC should have a Ct value within the limits (mean ± 3 standard deviations) of a control chart (Shewart, 1939). These limits have to be calculated previously from 30 Ct values of the PAC obtained from at least three PCR runs.

When these conditions are met:

- a test will be considered positive if it produces an exponential amplification curve and a Ct value below the cut-off value.
- a test will be considered negative if it produces no exponential amplification curve and/or a Ct value equal or above the cut-off value.
- tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data

The detection limit has been established by the authors at 5 pg DNA per PCR reaction.

4.2 Analytical specificity data

Detection of *H. pseudoalbidus* isolates from three different origins: Poland, France and Belgium. *In silico* analysis also revealed 100% homology of the targeted sequences (primers and probes) with sequences of isolates from Norway, Sweden and Lithuania. No cross-reaction, notably with the closely related species *H. albidus*. No cross-reaction with the tested organisms (several fungi isolated from ash trees).

4.3 Data on repeatability

Repeatability standard deviation = 0.67 Ct.

4.4 Data on reproducibility

Reproducibility standard deviation = 2.02 Ct.

Appendix 6 – Confirmation of *Hymenoscyphus pseudoalbidus* by DNA sequencing

1. General information

The identity of *H. pseudoalbidus* isolates can also be confirmed by DNA sequencing. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction. This method targets the ITS rDNA operon (White *et al.*, 1990). Care should be taken in sequence analysis due to the high sequence homology between *H. pseudoalbidus* and *H. albidus* for the targeted region. It is especially important to check correctly the sequence if the isolation was made from petioles where both species can occur (see paragraph on DNA sequencing of amplicons below).

Primer sequences:

ITS 1: 5′-TCC GTA GGT GAA CCT GCG G-3′.

ITS 4: 5′-TCC TCC GCT TAT TGA TAT GC-3′.

2. Method

2.1 Nucleic acid extraction and purification: see Appendix 3.

2.2 PCR reaction (according to Husson *et al.*, 2011: see Table 4).

2.3 Thermocycler conditions

PCR is carried out in a thermocycler equipped with a heated lid. Reaction conditions include an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 30 s and elongation at 72°C for 80 s. A final extension is carried out at 72°C for 10 min. The PCR product is kept below −18°C until electrophoresis [on 10 µL in agarose gel (1–2%) followed by gel staining (to check the efficiency of the PCR)] and sequencing on the remaining 10 µL.

3. Essential procedural information

3.1 Controls

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

- *Negative isolation control (NIC)* to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- *Positive isolation control (PIC)* to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a target sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- *Negative amplification control (NAC)* to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.

Table 4 PCR reaction according to Husson *et al.* (2011)

Reagent	Working concentration*	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	13.26	N.A.
Reaction buffer	10 X	2	1 X
MgCl2	50 mM	0.6	1.5 mM
dNTP	10 mM	0.4	0.2 mM
Primer ITS1	5 µM	0.8	0.2 µM
Primer ITS4	5 µM	0.8	0.2 µM
DNA polymerase – (high-fidelity Taq polymerase	5 U/µL	0.14	0.7 U/20 µL
Subtotal		18	
DNA	~25 ng/µL	2	~2.5 ng/µL
Total		20	

*These figures are indicative. They can be modified provided that the final concentration in the PCR reaction is respected.

– *Positive amplification control (PAC)* to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).

4. Sequencing of PCR amplicons

The following steps have to be carried out[2]:

– The remaining 10 µL from positive test reactions are sent for two-way sequencing with forward primer ITS1 and reverse primer ITS4 according to the manufacturer's instructions.
– The retrieved sequences are compared by BLAST (Basic Local Alignment Search Tool, available at http://blast.ncbi.nlm.nih.gov) against those DNA sequences deposited in GenBank for *H. pseudoalbidus* (accession number HM193468).
– Due to the close proximity between *H. pseudoalbidus* and *H. albidus* (a saprotrophic species), the level of homology between the analysed sequence and the sequence available in GenBank for *H. pseudoalbidus* should be close to 100% [according to Husson *et al.* (2011) the percentage of identity between both species is 98.4% for ITS sequences obtained with primers ITS1/ITS4 while 100% identity is found within ITS sequences from different isolates of *H. pseudoalbidus*].
– In case of uncertainty, other gene regions can be sequenced (for details see Husson *et al.*, 2011)

[2]An EPPO Standard PM 7/XX *DNA barcoding as an identification tool for plant pests* is in preparation and will replace the sequencing description once adopted.