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EPPO STANDARD ON DIAGNOSTICS

PM 7/45(2) *Cryphonectria parasitica*

SPECIFIC SCOPE

This Standard describes a diagnostic protocol for *Cryphonectria parasitica*.[1]

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

SPECIFIC APPROVAL AND AMENDMENT

Approved in 2004–09. Revised in 2024–08.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Cryphonectria parasitica is a bark-inhabiting fungus causing blight of chestnut (*Castanea* spp.) and other susceptible tree genera and species (mostly *Quercus* spp.) (EPPO, 2022a). It is native from Eastern Asia, where it was reported in China, Japan and the Korean peninsula, but by the end of the nineteenth century, the disease spread to North America and was reported in Europe in the late 1930s. There is variation in susceptibility between host tree species. The most susceptible are the American chestnut (*Castanea dentata*) and the European chestnut (*Castanea sativa*). The virulent form of the disease develops quickly in these species causing necrosis of bark and mortality of the distal part of the tree (Heiniger & Rigling, 1994). Hypovirulence due to infection of the fungus by the RNA virus, *Cryphonectria* hypovirus 1 (CHV 1), has however, enabled the regrowth of chestnut trees and stands in many regions of Europe. Virulent and hypovirulent strains of the fungus give rise to different types of cankers and this may, in some cases, make detection and identification difficult. In more tolerant hosts (in Europe, mostly *Quercus petraea* and less often *Quercus robur*, *Quercus ilex* and other oaks, as well as hybrids between the European chestnut and Asian chestnut species) or in its hypovirulent form, chestnut blight appears as perennial 'healing' cankers with superficial infections of the bark that rarely causes the death of branches, sprouts or the whole tree. Further information on biology and geographical distribution can be found in EFSA (2014) and EPPO (2022a). A datasheet providing more information on the biology is also available in EPPO Global Database EPPO (2022b). See also Fulbright (1999), Heiniger & Rigling (1994), Rigling & Prospero (2018), Roane et al. (1986).

A flow diagram describing the diagnostic procedure for *C. parasitica* is presented in Figure 1.

2 | IDENTITY

Name: *Cryphonectria parasitica* (Murrill) M.E. Barr.

Other names: *Endothia parasitica* (Murrill) P.J. Anderson & H.W. Anderson.

Taxonomic position: *Fungi: Ascomycota: Diaporthales: Cryphonectriaceae.*

EPPO Code: ENDOPA.

Phytosanitary categorization: EPPO A2 list: no. 69, EU PZ Quarantine pest (Annex III) & EU-RNQP (Annex IV).

3 | DETECTION

Host plants may carry the fungus in the bark (to the depth of the cambium).

3.1 | Disease symptoms and sign of presence

3.1.1 | Symptoms and fungal structure of virulent strains

Chestnut blight in its typical virulent form is associated with extensive necrosis of the bark. When an infection by a virulent strain occurs in the crown of a tree and the canker has completely girdled the affected branch, leaves distal to the infection point wilt, desiccate and remain attached to the branches for a long time (Figure 2).

In *Castanea sativa* (seedlings, young trees and recently grafted trees), the first symptoms appear as a discoloration of the outer bark ranging from yellowish-brown to

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

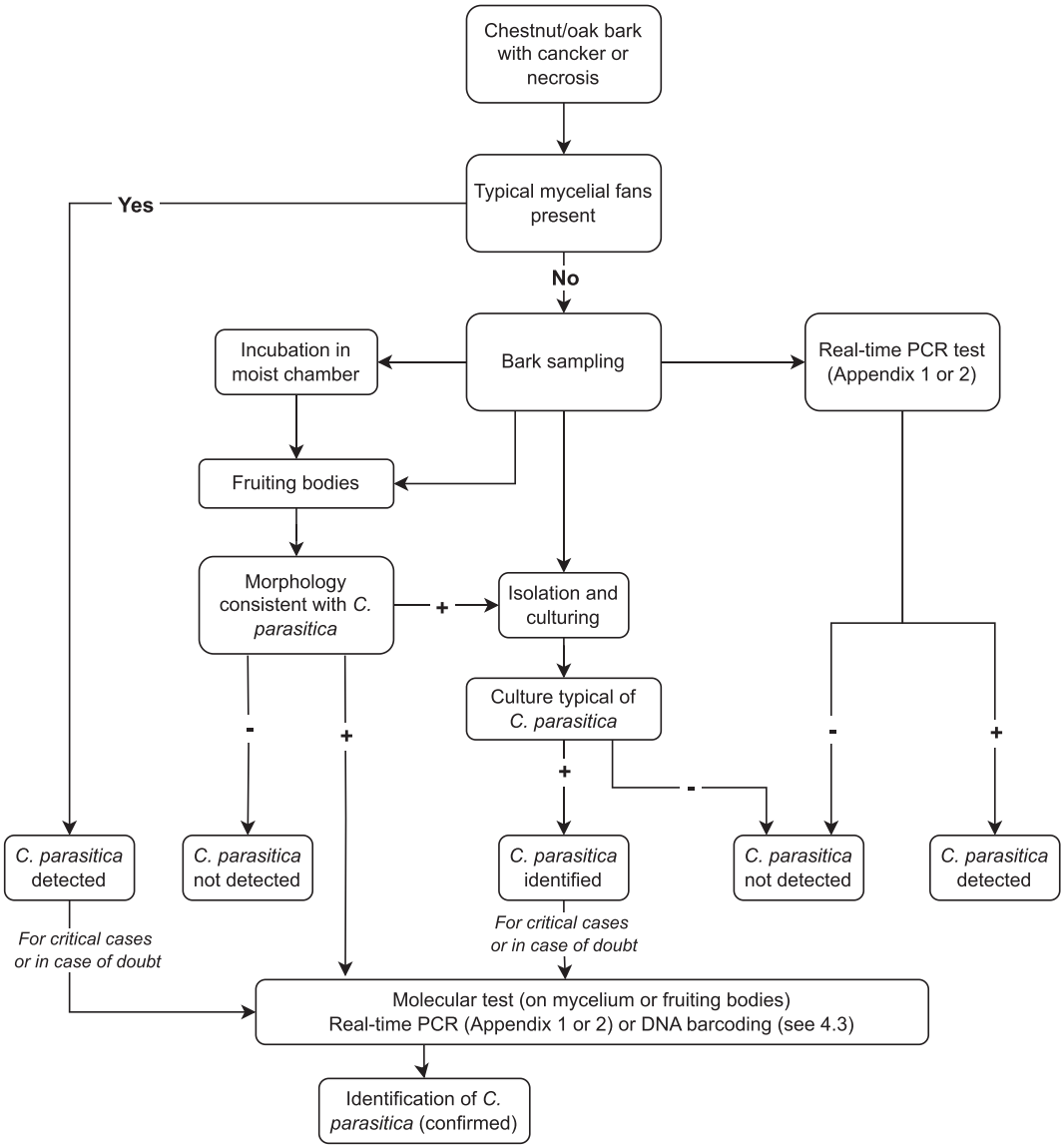

FIGURE 1 Flow diagram describing the diagnostic procedure for *Cryphonectria parasitica*. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

FIGURE 2 Wilting and death of a branch due to chestnut blight. Copyright: Rigling et al. (2014).

red-brown due to necrosis (Figure 3a). On grafted trees, infections are most frequently found in the region of the graft, where graft callusing occurs. In both coppices and orchards, infections are often located at the base of the stem (collars or insertion points). The cambium under the infected bark is killed (Figure 3b) and the bark appears sunken or swollen. Over the time, cracks can also form in the necrotized bark as a consequence of dehydration (Figure 3c), then patches of bark slough off thus exposing the underlying wood resulting in a typical bark canker. Epicormic shoots are produced below the canker. Characteristic creamy-yellowish coloured, flat mycelial fans are normally present throughout the symptomatic bark and the underlying cambium layer (Figure 3d,e). On the surface of the bark cankers, yellow to yellow-orange stromata may develop, each containing one, rarely more, conidiomata (locular pycnidia). In moist conditions, these may extrude twisted yellow tendrils (Figure 3f) consisting of sticky conidia. Tendrils can be more than 1 cm long and their production can

FIGURE 3 Symptoms caused by *Cryphonectria parasitica* (virulent strain) in chestnut bark and fungal structures. (a) Active canker on a young tree characterized by an orange to red brown coloured outer bark; (b) necrosis of inner bark and mycelial fans; (c) longitudinal crack in the cankered bark; (d) creamy-yellowish coloured mycelial fans developing in the inner bark; (e) close up on mycelial fans; (f) yellow-orange stromata; (g) old brick-red stromata; (h) stromata formed in bark cracks; (i) partially debarked canker on a chestnut pole (orange stromata visible on necrotized bark patches still attached to the wood). Courtesy: Photos A-C, G, I, CRAW (BE); photo D, Matsiakh I, UNFU (UA) & SLU (SE); photo E Crossley D, ©Fera Science Ltd. and FBIPP (GB), photos F-H Robin C, INRAE (FR).

be triggered artificially by incubating infected bark in moist chambers. Ascomata may be formed in the same stromata. Old stromata are brick-red to red-brown (Figure 3g) and may contain numerous flask-shaped perithecia with long necks, deeply embedded in the stroma. Ostioles of perithecia can be observed using a hand-magnifying lens and appear as black dots on papillate protuberances on the surface of the stroma. Stromata with conidiomata and ascomata are also formed in bark cracks (Figure 3h). Remnants of dried, light-brown mycelial mats are often seen on the exposed wood after bark fall. Both fungal structures and symptoms of the disease may be seen on chestnut poles where bark remains attached to the wood (Figure 3i).

The fungus is also able to infect chestnut via the abandoned galls of the gall wasp *Dryocosmus kuriphilus* causing cankers and subsequent death of the twig (Figure 4) (Prospero & Forster, 2011).

Another type of symptom caused by virulent strains is associated with the crown and includes wilting of the current year's shoots with green leaves remaining attached and eventually turning brown later in the season. The wilting symptom is associated in early summer with the presence of *C. parasitica* in cryptic dried buds on stems from the previous year's growth and evident necrotic areas surrounding the bud (Vannini et al., 2018) (Figure 5). Because the green wilted leaves are hanging on the shoots, it is considered flagging.

FIGURE 4 Canker on a young twig which most likely developed following the colonization of an abandoned gall of *Dryocosmus kuriphilus* by *Cryphonectria parasitica* (copyright: Rigling et al., 2014).

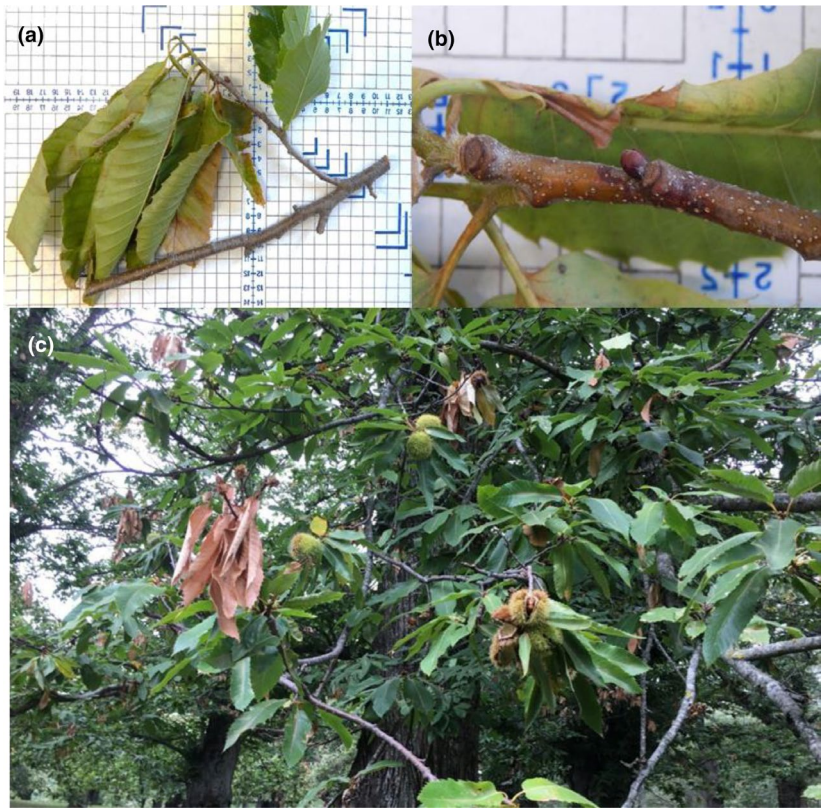

FIGURE 5 Wilting-like-a-flag; (a) wilting-like-a-flag in early summer; (b) A necrotizing cryptic dried bud at the base of shoots with a wilting ongoing. (c) wilting-like-a-flag in late summer-early fall. Courtesy: Vannini et al. (2018) (a–c).

FIGURE 6 Symptoms caused by *Cryphonectria parasitica* on sessile oaks (*Quercus petraea*). Copyright: Szabo et al. (2007).

Quercus spp. are rarely infected. Symptoms consist of slow-developing cankers. Exposed wood is present in the centre of the canker due to detachment of the necrotized bark beginning from the infection point and it is surrounded by a prominent callus ridge as a sign of the host defence reaction (Figure 6). Generally, *C. parasitica* is not lethal to oak trees.

Wood in trade of infected *C. sativa* and *Quercus* spp. may show the symptoms as described above. Fungal structures can also be found including mycelial fans (in the inner and outer layers of the living bark and in the cambium), stromata or bare conidiomata (in the sapwood, most commonly at the cut end but also on the sides), and mycelium (in the cambium and in the sapwood of the debarked wood). Annual rings of sapwood closest to vascular cambium can be also infected, although mycelial fans do not form there. Bark fragments and chips of wood can contain mycelium and stromata.

3.1.2 | Symptoms and fungal structures of hypovirulent strains

Hypovirulence in *C. parasitica* is defined as the inability of the fungus to cause the disease in its virulent form. The virulence and sporulation capacity of the fungus is reduced, and infection of the bark leads to milder symptoms. Hypovirulence is the basis for natural disease control.

Stems of young *C. sativa* trees infected with hypovirulent strains may exhibit early symptoms similar to virulent forms and the whole plant or single twigs may be killed by the hypovirulent strains. Older infections are limited to superficial bark layers and callus production can be triggered. Infections are localized and gradually stop expanding.

On stems and branches of adult trees, there are two distinctive types of hypovirulent cankers (also called healing, healed or passive cankers): superficial and callused cankers. Superficial cankers are alterations restricted to the outer part of the bark and are initially characterized by a reddish-orange surface with more or less pronounced swelling. Later, the bark is divided into small angular scales of uniform size that originate from bark cracking (Figure 7a). This part of the stem differs markedly from the smooth appearance of adjacent uninfected bark. The cambium under the infected bark is viable. There may be extensive sloughing off of colonized tissues and the exposed underlying bark may appear reddish-brown or black. These areas may be restricted to a few centimetres in diameter and oval in shape; alternatively, the whole circumference of the stem may be extensively affected. Stromata are rarely formed in the cracks of the bark: conidiomata are sometimes produced; ascomata are almost never formed. Mycelial fans are not easily found and are smaller, paler, and thinner than in the virulent form of the disease. They are located only in certain dead bark tissues but can sometimes be revealed by peeling off the bark in thin slices.

Callused cankers expose wood in the centre of the infection and are surrounded by a prominent callus ridge (Figure 7b).

The symptoms of the hypovirulent form of the disease are principally seen on the callus, although nearby bark can also be colonized extensively. This colonization is characterized by the sloughing away

FIGURE 7 Type of cankers caused by hypovirulent isolates of *Cryphonectria parasitica* in chestnut. (a) Passive, superficial canker; (b) Callused canker. Copyright: Rigling & Prospero (2018)

of the outermost bark tissues and by the formation of cracks in the remaining bark. Logs of *Castanea* and *Quercus* spp. with thick bark seldom show symptoms of colonization by hypovirulent strains of the fungus.

Canker morphology can vary considerably from the above described types, as intermediate or mixed forms are often observed, depending on the dynamics and severity of the hypovirus infection, but also on the degree of susceptibility/resistance of the host tree. For example, virulent strains can cause very similar symptoms on resistant hosts as hypovirulent strains do on susceptible hosts.

3.2 | Test sample requirements

For isolation and real-time PCR, bark samples (inner and outer bark) are collected from the advancing edge of the necrosis (canker) on stems, branches or trunk of *Castanea* spp. or *Quercus* spp.

Samples are taken with a disinfected axe, knife, leather borer (a few cm in diameter) or cork borer.

Microscopic examination of the stromata present in the canker area can be performed either immediately after sampling or after incubation of the sample in a moist chamber.

3.3 | Incubation in moist chamber

Pieces of bark, if possible with typical stromata, are placed in a Petri dish or in a larger plastic transparent box on several sheets of blotting paper. After dampening with distilled water, this moist chamber is kept at room temperature under fluorescent light with an alternate cycle of 12 h daylight and 12 h darkness (or in a light place in the laboratory, not directly exposed to the sun). The sample is regularly observed through stereomicroscope for up to 8 weeks and fruiting bodies are removed for microscopic examination.

3.4 | Isolation

3.4.1 | Isolation from wood chips

In the laboratory, small chips of the collected sample (see Section 3.2) (a few millimetres in size) are removed from the inner portion of the infected tissue with a scalpel, or a borer. The sample can be surface-sterilized for up to 2 min in a solution of sodium hypochlorite (diluted commercial bleach, 1% active chlorine) or 70% ethanol for a few seconds, and rinsed in sterile distilled water. It is then aseptically transferred onto potato dextrose agar (PDA) (Appendix 1) and incubated at approximately 22–25°C for approximately 3–5 days. Alternatively, the chips may be dipped in 70% ethanol, flamed and then plated on water agar with or without streptomycin (1.5% water agar with 100 $\mu g\,mL^{-1}$ streptomycin sulphate). Any sparse mycelium growing from the edge of the chips and resembling a *C. parasitica* colony is subcultured to PDA. Cultures are incubated at room temperature (an alternating cycle of 12 h fluorescent light and 12 h darkness or 1 week under day light followed by 1 week in darkness may be used) to induce colony pigmentation and pycnidia formation.

3.4.2 | Isolation from oozing conidia or entire pycnidia

Isolation can also be carried out from oozing conidia or entire pycnidia (if conidia tendrils are not present) collected using a stereomicroscope. No disinfection is needed. Regular medium such as Malt Extract Agar (MEA) (Appendix 1) supplemented with chloramphenicol at 100 mg/L can be used.

3.5 | Molecular tests

Two real-time PCR tests are recommended for the detection of *C. parasitica* from DNA of infected bark tissues.

- The test of Chandelier et al. (2019) targeting the ITS region of the rDNA gene (multi copy gene) – (see Appendix 2).
- The test of Rubio et al. (2017) targeting the translation elongation factor 1-alpha (EF1-α) gene (single copy gene) (see Appendix 3).

4 | IDENTIFICATION

The fungus can be identified either from its fruiting bodies formed on chestnut or oak cankers or after incubation under moist conditions using morphology and molecular tests or from its growth characteristics in culture after isolation. Distinction of *C. parasitica* from closely related species requires morphological expertise. Confirmation using a molecular test on mycelium or fruiting bodies is recommended in critical cases or in case of doubt (PM 7/76, EPPO, 2018).

4.1 | Growth characteristics in culture

4.1.1 | *Cryphonectria parasitica*

Mycelial growth rate on PDA can be up to 5 mm per day at approximately 20°C although much lower growth rates can be observed in some hypovirulent isolates. Mycelium is white when young, becoming light yellow and then orange-yellow. In hypovirulent isolates, it can remain white. Some isolates can exhibit any of the transitional colours from white to yellow to orange. Yellow to orange tints in such isolates are confined to the central part of the colony (Figure 8). About 5 days after subculturing, the primordial conidiomata are seen in culture initially appearing as a dense, globose mat. Only conidiomata are formed in culture; ascomata are not produced. In virulent isolates, conidiomata are produced abundantly in diurnal concentric rings. Hypovirulent isolates are characterized by a decreased ability to form conidiomata and by irregular distribution over the surface of the medium.

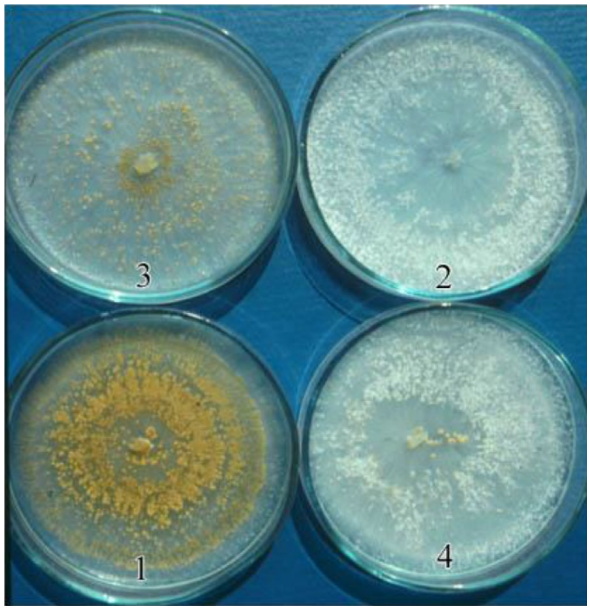

FIGURE 8 Cultures of *C. parasitica* isolates on PDA (1 – virulent, 2 – hypovirulent, isolates; 3 and 4 show an intermediate phenotype). Courtesy: SFI (Ljubljana, SI).

4.1.2 | Other fungi with orange pigmentation of the colony

Two other *Cryphonectria* species can colonize chestnut bark and produce an orange pigmentation of the colony on PDA: *Cryphonectria naterciae* M.H. Bragança, E. Diogo, & A.J.L. Phillips and *C. radicalis* (Fries) Barr. Both species have been found in Europe on the bark of chestnut and oak species.

C. radicalis can be distinguished from *C. parasitica* by the production of purple droplets on the hyphae (visible under the dissecting microscope) when grown on PDA plates for 1 week in the dark (Hoegger et al., 2002). On corn meal medium (10 g corn meal per 20 mL water in 100 mL Erlenmeyer flasks), *C. naterciae* and *C. radicalis* modify the colour of the medium from beige to reddish purple while *C. parasitica* does not cause any colour change (Bragança et al. 2011) (Figure 9).

Cryphonectria carpinicola, recently described in Europe, also shows an orange culture, but is mainly associated with *Carpinus* sp. (Cornejo et al., 2021).

4.2 | Microscope examination of samples

Morphological structures produced by *C. parasitica* on bark can be confused with those of *C. radicalis*. As these two fungal species can infect chestnut bark, molecular tests on DNA extracted from fruiting bodies (see

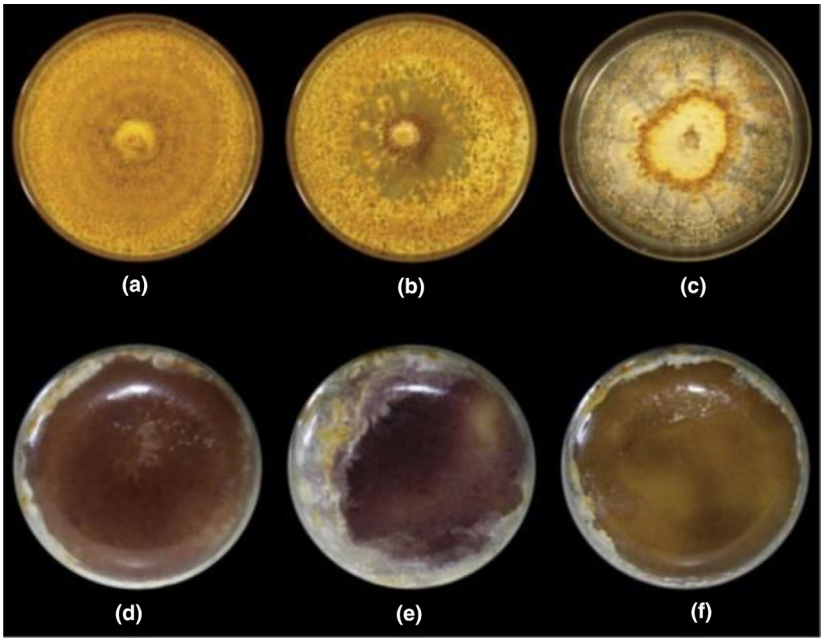

FIGURE 9 Culture morphology on PDA (top row), and coloration of corn meal medium (bottom row): (a, d) *Cryphonectria naterciae*; (b, e) *C. radicalis*; (c, f) *C. parasitica*. Copyright: Bragança et al. (2011).

FIGURE 10 Pycnidial stromata in the bark, conidial tendrils are excreted. Courtesy: ANSES (Nancy, FR).

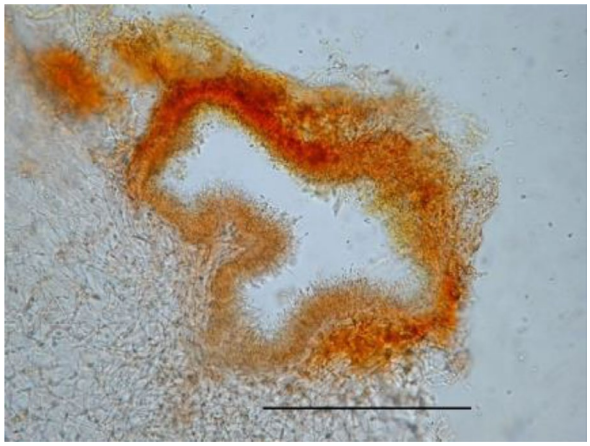

FIGURE 11 Pycnidium from culture, inner wall is covered with conidiogenous cells (bar = 50 µm). Courtesy: SFI, Ljubljana, SI.

Section 4.3) or isolation (see Sections 3.4 and 4.1) are recommended to confirm the identification of *C. parasitica* (see flow diagram).

The reproductive structures of *C. parasitica* conform to the following description (Ellis & Ellis, 1985): stromata yellow to dark orange, 0.5–3–4 mm in diameter and 2.5 mm high (smaller, if dry), embedded in the bark with the upper parts protruding from the surface (Figure 10); conidiomata (pycnidia) 100–300 µm in diameter are formed as irregular locules within stromatic tissue (Figure 11). Moreover, composite stromata are sometimes found where several pycnidia have coalesced to form a large labyrinthiform conidioma more than 1 mm in diameter. In moist conditions, conidiomata extrude twisted yellow tendrils of sticky conidia (Figure 10); conidiophores are branched, with lateral and terminal branches bearing conidiogenous cells; conidia are 3–5 × 1.5–2 µm in size (mean 3.6 × 1.8 µm), ellipsoidal to bacilliform, occasionally slightly curved, hyaline and aseptate; ascomata (perithecia), are 300–400 µm in diameter, globose and deeply immersed in the stroma (Figures 12 and 13); perithecia have long cylindrical periphysate neck (up to 300–600 µm long and 200 µm in diameter) converging through the external stromatic disc to form a small black papillate structure; asci are 32–55 × 7–8.5 µm in size, clavate to cylindric clavate, short-stalked, thin-walled, and 8-spored; ascospores are usually arranged in two rows, 7–12 × 3.5–5.0 µm (mean 8.6 × 4.5 µm), ellipsoidal, two-celled, smooth; sometimes with minor constriction at the septum (Figure 12).

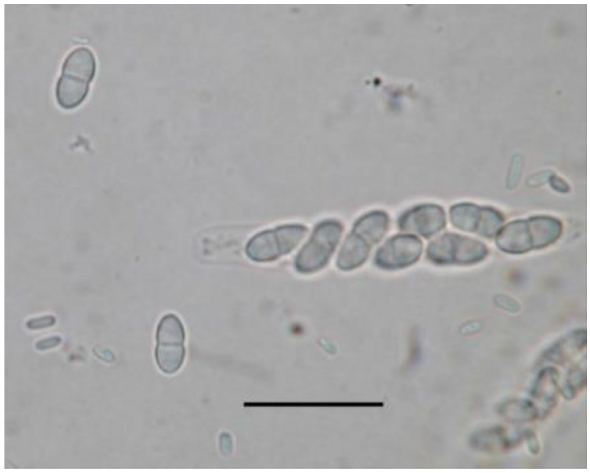

FIGURE 12 Ascus, ascospores and conidia (bar = 20 µm) Courtesy: SFI (Ljubljana, SI).

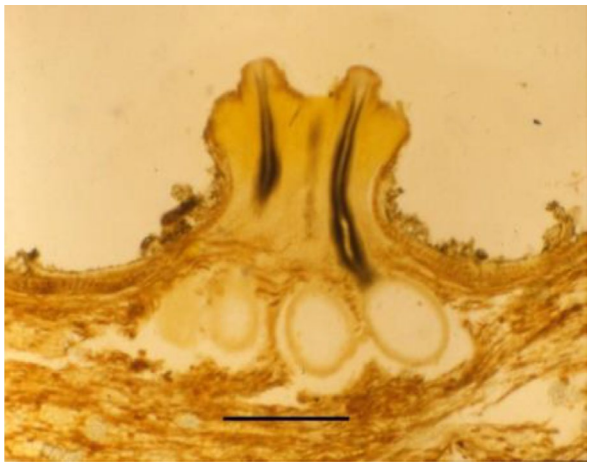

FIGURE 13 Perithecia in a stroma, necks and ostioles on papillate protuberances of the stroma (bar = 500 µm) Courtesy: SFI (Ljubljana, SI).

4.3 | Molecular tests

4.3.1 | Real-time PCR

Two real-time PCR tests are available for the identification of *C. parasitica* from DNA of mycelium (see Appendices 2 and 3).

4.3.2 | Sequencing

Sequencing of target loci (e.g. ITS barcode, see EPPO (2021)) and BLAST analysis against sequences published in sequence databases (e.g. GenBank) can be used for identification. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021).

5 | REFERENCE MATERIAL

Reference material can be obtained from Westerdijk collection (NL)

ITS reference sequences (e.g. accession numbers EU442645; GU993820) and TEF reference sequences (e.g. KC879168, KP824763) can be found in GenBank.

6 | REPORTING AND DOCUMENTATION

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

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics 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. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Università degli Studi della Tuscia, Dipartimento di Protezione delle Piante, v.s. Camillo de Lellis, I-01100 Viterbo (Italy); E-mail: vannini@unitus.it

INRAE, UMR BIOGECO 69, route d'Arcachon 33612 Cestas Cedex; C Robin E-mail: cecile.robin@inrae.fr

Swiss Federal Research Institute WSL, CH-8903 Birmensdorf (Switzerland); E-mail: simone.prospero@wsl.ch

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

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

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified

as needing revision are marked as such on the EPPO website.

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

ACKNOWLEDGEMENTS

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APPENDIX 1 - MEDIA

All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.

Potato dextrose agar (PDA)

Potato dextrose agar	39 g*
Distilled water to	1 L

* Or according to manufacturer's instructions.

Malt extract agar (MEA)
Commercially available or

Malt extract	20 g
Microbiological grade agar	15 g
Distilled water to	1 L

APPENDIX 2 - REAL-TIME PCR (CHANDELIER ET AL., 2019)

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

1. General Information

1.1 This real-time PCR test is used for detection of *Cryphonectria parasitica* directly from bark/wood samples of *Castanea sativa*. The test was also evaluated on DNA extracted from oak (*Quercus robur*)

bark spiked with genomic DNA from *C. parasitica*. It can also be used for identification of *Cryphonectria parasitica* from DNA extracted from mycelium.

1.2 The protocol for the TaqMan® based real-time-PCR was published by Chandelier et al. (2019)

1.3 The PCR primers and hydrolysis (TaqMan®) probe target the ITS region of the rDNA.

1.4 Oligonucleotides.

	Name	Sequence	Amplicon size
Forward primer	Cp-F4	5′-GATACCCTT TGTGAACTTATAA-3′	92 bp
Reverse primer	Cp-R3	5′-GGGGAGAA GGAAGAAAATC-3′	
Probe	Cp-S3	5′-FAM-TTTATC GTTGCCTCGG CGCTGA-BHQ1-3′	

1.5 PCR reactions and analyses have been carried out in a real-time PCR detection system StepOne Plus in combination with the manufacturer´s software (ThermoFisher).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Tissues source:

- Plant tissue: Bark samples (inner and outer bark) are cut into small pieces and ground in a mortar with liquid nitrogen. For DNA extraction using NucleoSpin Plant II kit (Macherey Nagel, Germany) an aliquot (~100 mg) is mixed with 400 µL lysis buffer and 10 µL RNase A (10 mg/mL) and DNA is extracted following the manufacturer's instructions. An alternative lysis buffer (Tex buffer) was used for the validation of the protocol by Chandelier et al. (2019). This buffer (400 µL) is also mixed with 10 µL RNase for the lysis step.
- Culture of *C. parasitica*: mycelium (~100 mg) is collected in a 2 mL tube and frozen before grinding. A stainless-steel bead (5 mm) is added to the tube, which is then mixed in a mixer mill MM200 (2 × 30 s at 30 Hz). The DNA extraction is carried out on the ground material as for plant tissue.

Different commercial kits can be used for DNA isolation. However, bark tissues contain potential PCR inhibitors. It is therefore recommended to validate the extraction step if using a different DNA extraction kit.

2.1.2 The samples are used immediately or stored at approximately −20°C

2.2 Real-time Polymerase Chain Reaction (real-time PCR)

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	NA	5	NA
Maxima Probe/ Rox qPCR master mix (ThermoFisher Scientific)*	2x	10	1x
Forward Primer (Cp-F4)	5 µM	1	250 nM
Reverse Primer (Cp-R3)	5 µM	1	250 nM
Probe (Cp-S3)	5 µM	1	250 nM
Subtotal		18	
DNA extract		2	
Total		20	

* For real-time PCR thermocyclers not using Rox passive reference, use a master mix without Rox.

When using DNA extract from bark, BSA (1 mg/mL final concentration) can be added to the mastermix to prevent inhibition.

2.2.2 PCR conditions

Real-time PCR amplifications include an initial activation step of the polymerase at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

3. Essential procedural information

3.1Controls

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

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

procedure to molecular grade water that was used to prepare the reaction mix.

- **Positive amplification control (PAC)** to monitor the efficiency of the 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 the detection limit of the method. For PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA; Ioos et al., 2009)
- Specific amplification or co-amplification of nucleic acid control from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same sample spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

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

Comments:

For samples with Ct value higher than 36 (i.e. above the limit of detection of the method according to Chandelier et al. 2019), a new analysis (DNA extraction and real-time PCR) should be carried out. If the same situation is observed (amplification curve with Ct value above 36), the sample is considered as negative.

Ct values mentioned in this comment section were obtained by using the specific material, equipment and chemicals described here and should be re-checked and adapted in each laboratory when implementing this test.

4. Performance characteristics available

Validation data were produced by Chandelier et al. (2019) (see Section 4.1). The test was further validated in the framework of two TPS (see Section 4.2.). The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validation list).

4.1 Intra laboratory validation (Chandelier et al., 2019, unless indicated otherwise)

The validation was not carried out in accordance with PM 7/98.

4.1.1 Analytical sensitivity data:

The analytical sensitivity (limit of detection) of the TaqMan® assay as determined by Chandelier et al. (2019) for detecting *C. parasitica* was 2 fg of *C. parasitica* gDNA per PCR reaction.

4.1.2 Analytical specificity data

Inclusivity was evaluated with 5 different *C. parasitica* isolates originating from different countries in Europe. Inclusivity was 100%.

Exclusivity was evaluated with 26 non-target microorganisms (23 fungal species and 3 *Phytophthora* species), most of them isolated from healthy chestnut bark. No cross reaction was noted, even with *C. radicalis*, *C. naterciae* and *Holocryphia eucalypti* (species which are closely related to *C. parasitica*).

In addition, no cross reaction was observed with *Cryphonectria carpinicola* (D. Rigling, Pers. Comm.).

4.1.3 Repeatability and reproducibility

- Repeatability: the analysis was carried out on 12 replicates of a standard DNA (recombinant plasmid containing the amplification target previously amplified with primers CpF4 and CpR3) at three concentration levels (10 000, 1000 and 100 copies per PCR reaction). The test was 100% repeatable (relative repeatability standard deviation lower than 1% for the three concentrations tested)

- Reproducibility: the test was carried out on 3 replicates of a standard DNA at six concentrations (from 10^7 to 100 copies per PCR) in 4 different PCR runs carried out on different days with different primer preparations. The relative reproducibility standard deviation ranged from 1.8 to 2.7% for the six concentration levels. The test was also evaluated on 3 replicates of a standard DNA at six concentrations (from 10^7 to 100 copies per PCR) on three thermocyclers with an automatic setting of the fluorescence threshold in all cases. PCR efficiency and the correlation coefficient were calculated from standard curves established on three instruments. The determination coefficient (R^2) was higher than 0.999 in all cases.

4.2 Test Performance study (TPS)

A TPS was organized by Fera; GB in 2019 in the framework of an Euphresco project (2017-A-249 'Early detection of *Cryphonectria parasitica* in planting material'), and in 2020 in the framework of VALITEST. A total of 8 European laboratories participated in the Euphresco TPS and 8 in the VALITEST TPS.

For the VALITEST TPS, the panel of samples consisted of 2 controls (NAC and PAC) and 8 DNA extracts (from pure fungal cultures) including:

- 2 non-target samples.
- 6 target samples (3 isolates × 2 dilutions).

The performance of the DNA extraction step was not evaluated.

	DNA extract panel
Diagnostic sensitivity	92.7%
Diagnostic specificity	93.8%
Accuracy	98.4%
Repeatability	98.4%
Reproducibility	86.8%

For the Euphresco TPS, the 2 panels of samples consisted of 15 samples including:

- 10 samples of bark material (8 target samples and 2 non-target).
- 5 DNA extracts (3 target samples and 2 non-target from pure culture).

	Bark sample panel	DNA extract panel
Diagnostic sensitivity	98.1%	65.2%
Diagnostic specificity	92.9%	100%
Accuracy	97.1%	79.8%

APPENDIX 3 - REAL-TIME PCR (RUBIO ET AL., 2017)

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

1. General information

1.1 This real-time PCR method is used for detection of *Cryphonectria parasitica* directly from bark samples of *Castanea sativa*. The method is also applicable to DNA extracted from oak bark and can be used for identification on DNA extracted from mycelium.
1.2 The real-time-PCR test was published by Rubio et al. (2017).
1.3 The PCR primers and the dual-labelled, minor groove binder probe (MGB) were designed in the translation elongation factor 1-alpha (EF1-α) gene.
1.4 Oligonucleotides.

	Name	Sequence
Forward primer	*C. parasitica*-F	5′-GGAAGGTATG TATCAATCAGCTGCA-3′
Reverse primer	*C. parasitica* -R	5′-GCTGCTGATG GTATGGGAAGTT-3′
Probe	*C. parasitica* Pr	5′-FAM-CCCCAT CCCCAAATG-MGB-NFQ-3′

1.5 PCR reactions and analyses have been carried out in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 Tissues source:

- Plant material: Bark tissue (200–500 mg) is cut into small pieces and homogenized using a Homex 6 (BIOREBA, Reinach, Switzerland) in 3 mL of CTAB buffer [2% CTAB, 1.5 M NaCl, 120 mM sodium phosphate buffer pH 8, 2% antifoam B emulsion (Sigma, Gillingham, GB)]. The sample is then centrifuged for 5 min at 10 000*g*. Supernatant (700 µL) is mixed with

200 µL of chloroform by briefly vortexing, followed by centrifugation for 5 min at 13 000*g*. The aqueous layer (500 µL) is mixed with 500 µL of isopropanol and 50 µL of MagneSil Paramagnetic Particles (PMPs) (Promega, Southampton, GB). DNA is then extracted using a KingFisher mL magnetic particle processor (Thermo Fisher Scientific, Loughborough, GB). The sample is incubated with magnetic particles for 10 min. The PMPs are moved sequentially through 1 mL GITC lysis buffer (5.25 M Guanidinium thiocyanate, 50 mM Tris-HCl pH 6.4, 20 mM EDTA, 1.3% Triton X-100) for 2 min; two washes of 1 mL of 70% ethanol for 2 min each; and DNA is eluted in 200 µL of 1× TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) for 2 min after a drying time of 10 min.

- Culture of *C. parasitica*: see Appendix 1

2.1.2 The samples are used immediately or stored at approximately −20°C

2.2 Real-time Polymerase Chain Reaction (real-time PCR)

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	NA	8.0	NA
2X iTaq™ Universal Probes Supermix (BioRad, GB)	2×	12.5	1×
Forward Primer (*C. parasitica*-F)	5 µM	1.5	300 nM
Reverse Primer (*C. parasitica*-R)	5 µM	1.5	300 nM
Probe (*C. parasitica*-Pr)	5 µM	0.5	100 nM
Subtotal		24	
DNA extract		1	
Total		25	

2.2.2 PCR conditions

Real-time PCR amplifications include an initial activation step of the polymerase at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 65°C for 1 min.

3. Essential procedural information

3.1 Controls

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

- **Negative isolation control (NIC)** to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected host tissue or if not available clean extraction buffer.
- **Positive isolation control (PIC)** to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- **Negative amplification control (NAC)** to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to 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). The PAC should preferably be near the detection limit of the method. For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. The plant cytochrome oxidase I (COX) gene assay of Weller et al. (2000) as modified by Hughes et al. (2006) was used by Rubio et al. (2017) to confirm successful DNA extraction from plant samples.

3.2 Interpretation of results

Verification of the controls

- The PIC, PAC and IPC amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

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

4. Performance characteristics available

Validation data were produced by Rubio et al. (2017) (see Section 4.1). The test was further validated in the framework of a TPS (see Section 4.2).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data https://dc.eppo.int/validation_data/validationlist).

4.1 Intra laboratory validation (Rubio et al., 2017)

The validation was carried out in accordance with PM 7/98.

4.1.1 Analytical sensitivity data

The analytical sensitivity (limit of detection) of the assay as determined by Rubio et al. (2017) for detecting *C. parasitica* was 2 pg gDNA /PCR reaction.

4.1.2 Analytical specificity data

Inclusivity was evaluated with 9 different *C. parasitica* isolates originated from three countries (1 from the United Kingdom, 7 from France and 1 from Japan). Inclusivity was 100%.

Exclusivity was evaluated with 36 non-target isolates of 26 fungal species: *Alternaria mali*, *Botryosphaeria laricina*, *Botrytis cinerea*, *B. narcissicola*, *Cercospora beticola*, *Ciborinia camelliae*, *Colletotrichum* sp., *C. cubensis* (×2), *C. gyrosa* (×2), *C. havanensis* (×2), *C. macrospora*, *C. nitschkei* (×2), *C. radicalis* (×2), *C. buxicola*, *Eutypa lata* (×2), *Fusarium oxysporum*, *Gnomoniopsis* sp., *Phomopsis* sp., *Phytophthora cambivora*, *P. cinnamomi*, *P. kernoviae* (×3), *P. ramorum* (×3), *Plectosporium tabacinum*, *Verticillium albo-atrum*, *V. dahliae*, *V. tricorpus*. No cross reactions were noted, even with *C. radicalis*, which is closely related to *C. parasitica* and found in chestnut bark. Not yet evaluated in *C. carpinicola*.

4.1.3 Repeatability and reproducibility

Repeatability: the test was carried out on 6 replicates of DNA extracts from pure culture at two concentrations (undiluted DNA extract and diluted DNA extract at the limit of detection). The test was 100% repeatable.

Reproducibility: the test was carried out on 8 replicates of DNA extracts from pure culture by 2 users, on four different instruments. The test was 100% reproducible.

4.2 Test Performance study (TPS)

A TPS was organized in 2020 in the framework of the EU project VALITEST. The results of 9 laboratories were considered.

The panel of samples consisted of 2 controls (NAC and PAC) and 8 DNA extracts (from pure fungal cultures) including:

- 2 non-target samples.
- 6 target samples (3 isolates ×2 dilutions – undiluted and 20 times diluted DNA extract).

The performance of the DNA extraction step was not evaluated.

	DNA extract panel
Diagnostic sensitivity	91.7%
Diagnostic specificity	88.9%
Accuracy	98.6%
Repeatability	98.6%
Reproducibility	82.6%