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Diagnostics
Diagnostic

PM 7/4 (3) *Bursaphelenchus xylophilus*

Specific scope

This standard describes a diagnostic protocol for *Bursaphelenchus xylophilus*[1].

Specific approval and amendment

First approved in 2000-09. Revised on 2009-09.
Second revision approved on 2012-09

Introduction

Bursaphelenchus xylophilus, the pine wood nematode, is an EPPO A2 pest. The nematode is native to North America [Canada, US, Mexico (unconfirmed)] and is thought to have been carried to Japan at the beginning of the 20th century on timber exports. In Japan, the nematode has been causing massive mortality of native pine trees (*Pinus densiflora*, *P. thunbergii*, *P. luchuensis*), where it is the most damaging forest pest. It has also spread to China, Korea, Portugal and Taiwan. More recently, it has also killed pines in the USA, but only exotic species including *P. sylvestris*, in the warmer southern areas of the country. Regardless of the limited occurrence of pine wilt disease in North America, *B. xylophilus* as such is widely dispersed in native conifer stands. In 1999, *B. xylophilus* was detected on *P. pinaster* in continental Portugal, and in 2009 in the island of Madeira. Limited outbreaks in Spain, in 2008, 2010 and 2012 are under eradication. Updated information on geographical distribution is available in the EPPO PQR database[2].

Pinus spp. are the most susceptible host trees, but species of *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* have also been reported as hosts of the nematode (Evans *et al.*, 1996).

In nature, *B. xylophilus* is spread from tree to tree through activity of the adult stages of wood-inhabiting longhorn beetles of the genus *Monochamus* (Coleoptera: Cerambycidae). These can transmit the nematode either to the shoots of living trees during the feeding of the adult insects (maturation feeding by either sex), or, depending on *Monochamus* species, to the trunks or larger branches, including wood waste or debris, weakened trees or trees that recently died, during oviposition by the females. Transmission of *B. xylophilus* to the live trees during feeding by the adult beetles can result in the development of wilt disease in the tree, but only in susceptible species of *Pinus* under suitable climatic and edaphic conditions. Nevertheless, transmission of *B. xylophilus* by maturation feeding may occur also to *Pinus* and other tree genera but without resulting in wilt development. Transmission during oviposition can occur on most if not all coniferous species, provided the trees are weakened, dying from any cause or which have recently died, thus making them suitable for *Monochamus* oviposition. Transmission at oviposition can also occur on timber and cutting waste. A known exception is *Thuja plicata*, which is considered to be immune to attack by *Monochamus* spp. and so to entry of the nematode. Thus, *B. xylophilus* can be found in wood of *Pinus* spp. expressing wilt disease after *B. xylophilus* infestation or in wood of trees of any coniferous species (except *T. plicata*) that have been weakened enough to allow *Monochamus* spp. to oviposit and transmit the nematode, which then lives on living plant cells and or hyphae of wood fungi. The nematode is very easily carried by wood moving in trade, either as a commodity (live plants, round wood, sawn wood etc.), or as wood packaging material being transported with other commodities, other than wood packaging material meeting the requirements of ISPM No. 15 'Regulation of Wood Packaging Material in International Trade' (FAO, Rome, revised 2009). More details about its biology, distribution and economic importance can be found in EPPO/CABI (1997).

The diagnostic procedure for *Bursaphelenchus xylophilus* is presented in Fig. 1.

[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.

[2]http://www.eppo.org/DATABASES/pqr/pqr.htm.

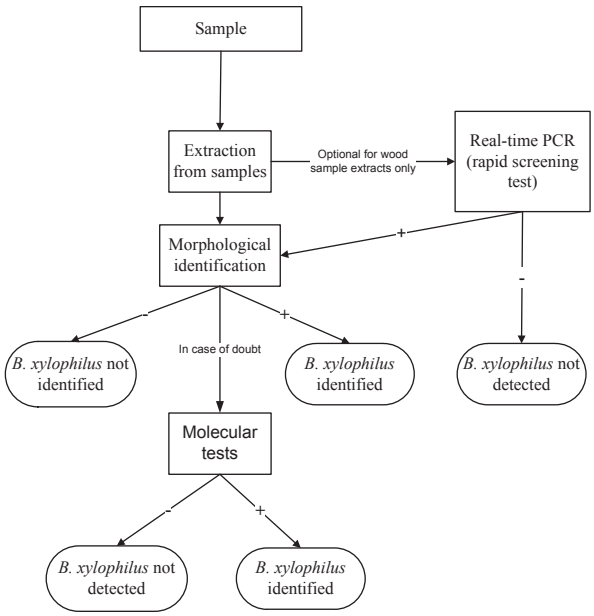

Fig. 1 Flow diagram for the detection and identification of *Bursaphelenchus xylophilus.*

Identity

Name: *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970
Synonyms: *Aphelenchoides xylophilus* Steiner & Buhrer, 1934
*Bursaphelenchus lignicolu*s Mamiya & Kiyohara, 1972
Taxonomic position: Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Parasitaphelenchinae.
EPPO code: BURSXY
Phytosanitary categorization: EPPO A2 list no. 158, EU Annex designation II/A1

Detection

Bursaphelenchus xylophilus can be present in coniferous plants for planting, cut branches, wood, isolated bark and wood shavings, but not in needles, cones or seeds. As symptoms caused by its presence are not specific, samples of imported wood and standing conifer trees should be taken. Guidance on sampling is given in Appendix 3 of PM 9/1 *Procedures for official control of* Bursaphelenchus xylophilus *and its vector*s. *Bursaphelenchus xylophilus* can also be detected in adult vector insects (*Monochamus* spp).

Extraction from wood

Wood samples should reach an incubation temperature of approximately 25°C for at least 14 days to allow any nematodes present to breed and maximise the likelihood of detection.

Live nematodes can be extracted from infested wood using the Baermann funnel technique involving immersion of small pieces of wood (not larger than 1 cm in width using a cutting method that does not generate heat) or wood shavings in water for 48 h. Nematodes migrate from the chopped wood into the water and can be collected from the closed bottom of the funnel. Other methods involving immersion of wood in water or a mistifier are also suitable (see PM 7/XXX on *Nematode extraction*, in preparation). Using a stereo microscope, the nematodes can be transferred with the help of a pipette or a needle from a small Petri dish to a glass slide for microscopic examination and morphological identification. Molecular tests can be used for screening on wood extract (e.g. Appendix 4) but should

Table 1 The identification of genus *Bursaphelenchus* (extracted from wood and bark)

1	Tylenchid stylet, pharynx with a metacorpus	2
	Dorylaimid stylet no metacorpus	NBS
2	Metacorpus with metacorpus plates	3
	No metacorpus plates in metacorpus	NBS
3	One gonad vulva posterior	4
	Two gonads vulva median	NBS
4	Metacorpus strongly developed, distinct at lower magnifications; especially clear in fixed specimens, ovoid-rounded-rectangular in shape; in lateral perspective no sign of a dorsal pharyngeal gland opening or a ventral curvature of pharyngeal lumen behind stylet knobs	5
	Metacorpus smaller, fusiform to rounded; in lateral perspective dorsal pharyngeal gland opening and a ventral curvature of pharyngeal lumen behind stylet knobs	NBS
5	Pharyngeal gland overlaps intestine dorsally	6
	Pharyngeal gland bulb abuts intestine	NBS
6	Stylet knobs present (knobs may be small)	7
	Stylet knobs absent	NBS
7	Male tail tip enveloped by a small bursa (best seen in the dorso-ventral aspect, and even visible using a stereomicroscope)	8
	Bursa absent	NBS
8	Vulva 70–80% of body length from anterior end; male tail tip strongly recurved	*9*
	Vulva 85–90% of body length from anterior end; male tail tip not strongly recurved	NBS
9	Lateral field with 4 lines; vulva with prominent flap; spicules strongly arcuate (Figs 2C and 3C–D)	*Bursaphelenchus xylophilus* group (BXG)
	Characters different	Non *Bursaphelenchus xylophilus* group

NBS, not *Bursaphelenchus* species.

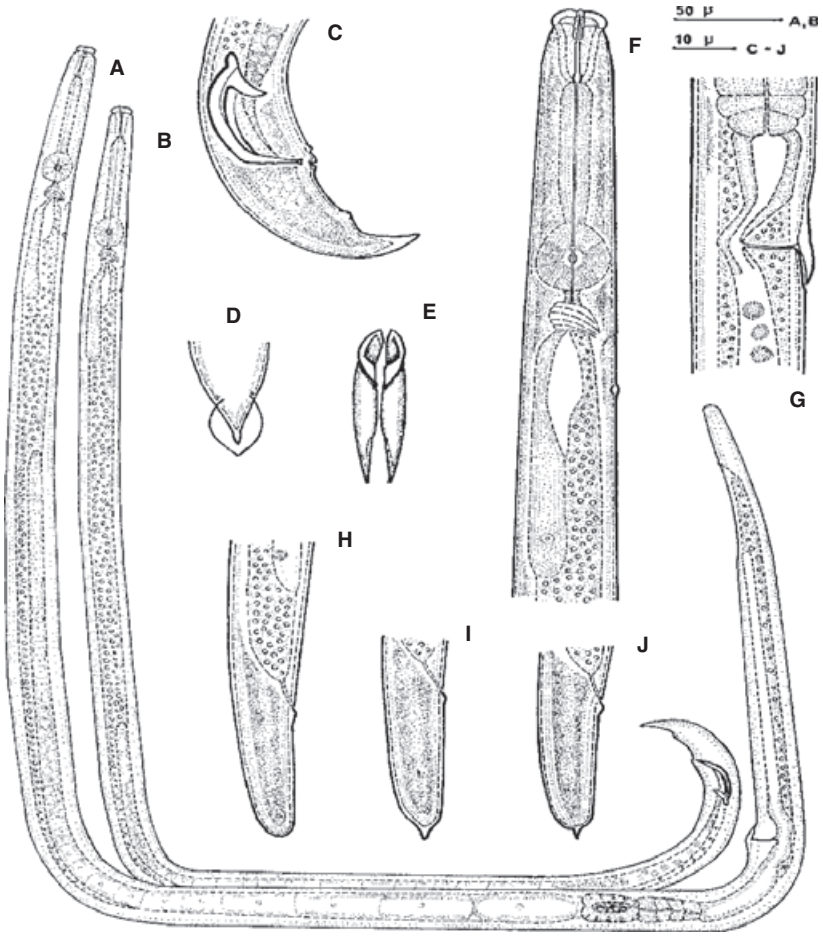

Fig. 2 *Bursaphelenchus xylophilus* n.sp. (from Mamiya & Kiyohara, 1972). (A) female; (B) male; (C) male tail; (D) ventral view of mail tail, tip with bursa; (E) ventral view of spicules; (F) female, anterior portion; (G) female vulva; (H–J) female tail.

be confirmed by morphological identification. When only juveniles are present, they should be cultured on *Botryotinia fuckeliana* to produce adult specimens for identification (Shroeder *et al.*, 2009).

Extraction from vectors

The fourth dispersal juvenile stage can be extracted from the body of the vector insect. The insects should be cut into pieces and the nematode extracted over 24 h by the Baermann funnel technique. For morphological identification nematodes collected from the base of the funnel should be placed on mycelium of *Botryotinia* (syn. *Botrytis cinerea*), where they will moult to adults and multiply.

Identification

Identification on the basis of morphological features

For a positive morphological identification of *B. xylophilus* adult specimens, of both sexes should be available. The analysis requires the preparation of good quality microscope slides, access to a high-powered microscope and considerable experience in nematode taxonomy, especially to differentiate the small group of species closely related to *B. xylophilus*.

Many nematode species can be present in wood samples, especially if decay of the tissues has begun. Some of these will be saprophytic species, which lack the solid mouth spear (stylet) typical of plant parasitic nematodes; the others will mainly belong to the order Aphelenchida, which have the dorsal pharyngeal gland opening in the metacorpus (in contrast to the Tylenchida where the gland opens into the lumen of the pharynx behind the stylet knobs). Adults of nematodes of the *Bursaphelenchus xylophilus* group can be distinguished by using the key given in Table 1.

Many species of *Bursaphelenchus* inhabit wood, living on fungal contaminants and fungi growing in the frass produced by wood-living insects such as bark beetles and beetles in the family cerambycidae. Within the genus *Bursaphelenchus* several species-groups are recognised. Species belonging to the '*xylophilus*-group' are characterised by four lateral lines, the presence of a vulval flap, large

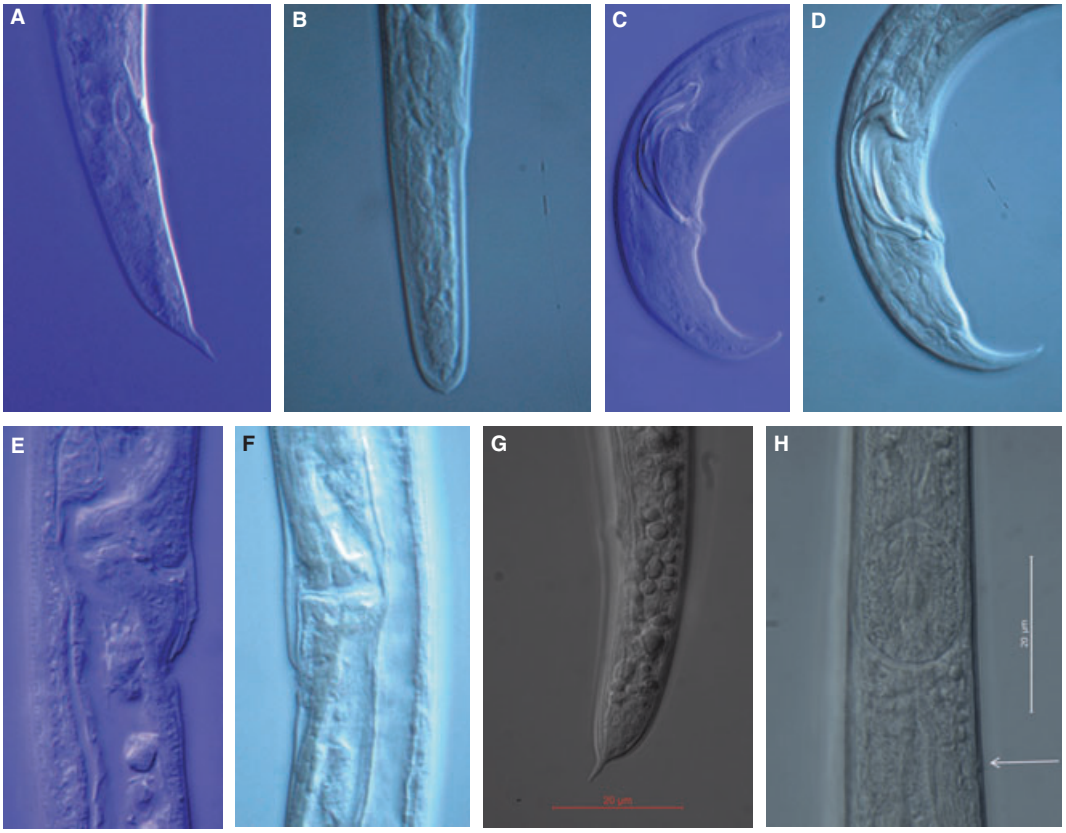

Fig. 3 Morphological characters of *Bursaphelenchus* spp. (A) *B. mucronatus* 'East-Asian type' showing the conical tail with mucro; (B) *B. xylophilus*, female tail of the 'Round-tailed form'; (C) *B. trypophloei* spicule shape; (D) *B. xylophilus* spicule shape; (E) *B. populi*, showing the curved vulval flap ending in a depression in body wall; (F) *B. xylophilus* showing the straight vulval flap; (G) *B. xylophilus* mucronated form (US 10). (H) *B. xylophilus* mucronated form (US 10) showing a posterior position of the excretory pore (arrow). Photos: A, C, E (Prof. Malek Tomalak, Institute of Plant Protection, Poznan, Poland); B, D, F, G, H (Christer Magnusson Bioforsk, Norway).

strongly arcuate spicules (Figs 2C and 3B) and the arrangement of seven caudal papillae (best seen using a scanning electron microscope; Braasch, 2008). *Bursaphelenchus xylophilus* can be separated from other species of the '*xylophilus*- group' by using the key in Table 2.

The characters presented in Table 2 are typical and will be present in the majority of cases. Due to a certain variation in characters between populations, especially in the shape of the female tail, position of excretory pore etc., it is essential to perform a molecular test (see below) in case of doubt.

Morphology

Bursaphelenchus xylophilus shows the general characters of the genus *Bursaphelenchus* (Nickle, 1970; Hunt, 1993): small to long and slender nematodes; cephalic region high and offset by a constriction, with six lips; stylet well developed, usually with small basal thickenings (Fig. 3F); metacorpus well developed (Fig. 3F).

Female

Vulva with a conspicuous overlapping anterior lip (vulval flap) not ending in a depression (Figs 2G and 3F); vulva usually at 70–80% of the body length; post-uterine sac usually three to six body widths in length; female tail subcylindrical and, in most populations, with a broadly rounded tip (Figs 2H and 3B), but occasionally the tip may have a terminal nipple-like extension or short mucro (Fig. 2I–J) The mucronated form of *B. xylophilus* has a mucro (Fig. 3G) resembling the mucro of *B. mucronatus*. The mucronated form is rare in North America and elsewhere.

Male

Tail with a strong dorsal curvature (Figs 2C and 3D); a small terminal bursa is present at the tail tip, which can be seen readily in dorso-ventral position (Fig. 2D); spicules robust, strongly arcuate with large rounded apex and a prominent sharply pointed rostrum, and spicule tip with a disc-like projection (cucullus); gubernaculum absent (Figs 2C and 3D); caudal papillae occur as an adanal pair just anterior to the anus, two post-anal pairs just anterior to the origin of the bursa origin and a single median papillae just preanal.

Measurements of morphological characters of *B. xylophilus* are given in Table 3. It should be noted that measurements

Table 2 Short key for the identification of *Bursaphelenchus xylophilus* in the '*xylophilus*- group' (extracted from wood and bark)

1	Female tail conical (Fig. 3A) or strongly tapering, with or without Mucro	Not *B. xylophilus*
	Female tail broadly sub-cylindrical with or without mucro) (Figs 2H and 3B)	2
2	Spicule length >30 µm	Not *B. xylophilus*
	Spicule length <30 µm	3
3	Spicule with short and pointed rostrum; limbs of spicule with a rounded curvature (Fig. 3C)	Not *B. xylophilus*
	Spicule with long and pointed rostrum; limbs of spicule with an angular curvature (Figs 2C and 3D)	4
4	Female vulval flap curved ending in a deep depression (Fig. 3E)	Not *B. xylophilus*
	Female vulval flap straight not ending in a deep depression (Figs 2G and 3F)	5
5	Female tail without mucro (Figs 2H and 3B) or with a small projection (Fig. 2I–J)	*B. xylophilus* (round-tailed form)
	Female tail with mucro (Fig. 3G)	6
6	Excretory pore anterior to median bulb	Not *B. xylophilus*
	Excretory pore at or behind median bulb (3 H)	*B. mucronatus kolymensis* and *B. xylophilus* (mucronated form)*
	Position of excretory pore cannot be observed	Identification based on morphological characters impossible. Molecular tests should be performed

*The mucronated form of *B. xylophilus* is mainly found in North America and molecular tests (Gu *et al.*, 2011) are recommended for a reliable separation of this form from the 'European type' of *B. mucronatus*, i.e. *Bursaphelenchus mucronatus kolymensis* (Braasch *et al.,* 2011).
NB: this key is only aimed at the identification of *B. xylophilus*, for a key to other species refer to Ryss *et al.*, 2005). Rearing nematodes on agar plates with fungi may increase the variability of the female tail.

of the same species and strain may differ whether the nematodes are grown in natural substrate (e.g. wood) or on artificial medium (e.g. agar plates with fungi).

Identification by molecular biological methods

Tests available for the identification of *B. xylophilus* include the use of DNA hybridization probes (Abad *et al.*, 1991; Tares *et al.*, 1994) and various PCR procedures (Harmey & Harmey, 1993; Braasch *et al.*, 1995, 1999; Hoyer *et al.*, 1998; Iwahori *et al.*, 1998; Mota *et al.*, 1999; Zheng *et al.*, 2003; Kang *et al.*, 2004; Matsunaga & Togashi, 2004; Burgermeister *et al.*, 2005; Cao *et al.*, 2005; Castagnone *et al.*, 2005; Jiang *et al.*, 2005; Leal *et al.*, 2005; Takeuchi *et al.*, 2005). When molecular tests are used for quarantine purposes to detect *B. xylophilus* in wood products, it is essential to recognise that both live and dead nematodes can be detected by these tests. Several phytosanitary measures will kill *B. xylophilus* in the wood, but dead nematodes are still present and depending on the extraction method may be detected by molecular techniques.

Molecular tests used in the EPPO region are described in Appendices:

- Burgermeister *et al.* (2009) an ITS RFLP PCR used for differentiating *B. xylophilus* from 44 other *Bursaphelenchus* species (Appendix 1).
- Castagnone *et al.* (2005) a species-specific test to identify *B. xylophilus* using a satellite DNA-based PCR technology (Appendix 2).
- François *et al.* (2007) (Appendix 3) a real time PCR test to identify *B. xylophilus* targeting satellite DNA and an adaptation of this real-time PCR for direct detaction on wood extracts (Appendix 4) developed by Anses-LSV (FR).

Reference material

Reference cultures of *Bursaphelenchus* spp. are available in the *Bursaphelenchus* culture collection at the Julius Kühn Institute, Institute for National and International Plant Health, in Braunschweig, Germany. Many *Bursaphelenchus* species can be cultured on *Botryotinia fuckeliana* or other fungi on agar (malt agar, PDA) in the laboratory.

Reporting and documentation

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

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.

Table 3 Measurements of *Bursaphelenchus xylophilus* characters

	Males					Females				
Character	Nickle *et al.* (1981) (n = 5)	Mamiya & Kiyohara (1972) (n = 30)	Mota *et al.* (1999) (n = 12) (Portugal)	Penas *et al.* (2008) (Portugal)	Penas *et al.* (2008) (Portugal) mucronate	Nickle *et al.* (1981) (n = 5)	Mamiya & Kiyohara (1972) (n = 40)	Mota *et al.* (1999)0 (n = 12) (Portugal)	Penas *et al.* (2008) (Portugal)	Penas *et al.* (2008) (Portugal) mucronate
Length (L) mm	0.56 (0.52–0.60)	0.73 (0.59–0.82)	1.03 (0.80–1.30)	0.57 (0.45–0.69)[a]	0.85 (0.70–0.99)	0.52 (0.45–0.61)	0.81 (0.71–1.01)	1.05 (0.89–1.29)	0.59 (0.51–0.66)	0.97 (0.81–1.15)
a	40.8 (35–45)	42.3 (36–47)	49.4 (44–56)	46.0 (40.2–58.5)	54.3 (38.7–63.7)	42.6 (3 7–48)	40.0 (33–46)	50.0 (41–58)	41.9 (32.8–50.6	53.9 (49.0–58.8)
b	9.4 (8.4–10.5)	9.4 (7.6-11.3)	13.3 (11.1–14.9)	9.6 (8.2–10.7)	12.4 (10.4–13.9)	9.6 (8.3–10.5)	10.3 (9.4–12.8)	13.8 (12.7–16.4)	10.1 (9.1–11.2)	13.3 (12.1–14.3)
c	24.4 (21–29)	26.4 (21–31)	28.0 (24–32)	21.6 (19.1–24.6)	25.3 (20.4–29.0)	27.2 (23–31)	26.0 (23–32)	26.6 (22–32)	25.4 (20.2–29.0)	24.4 (18.8–28.0)
Stylet, µm	13.3 (12.6–13.8)	14.9 (14–17)	12.6 (11–16)	11.0 (10.0–14.0)	14.6 (11.0–18.0)	12.8 (12.6–13.0)	15.9 (14–18)	12.3 (11–15)	11.2 (10.0–12.5)	14.7 (12.0–17.0)
Spicules, µm	21.2 (18.8–23.0)	27.0 (25–30)	24 22–25	19.3 (16.5–24.0)	26.3 (23.0–28.0)	–	–	–	–	–
Vulva position, % of L	–	–	–	–	–	74.7 (73–78)	72.7 (67–78)	73.3 (70–76)	71.5 (70.1–72.9)	72.6 (71.5–73.5)

It should be taken into account that specimens cultured for a long time on *Botrytis* plates grow much bigger than those freshly extracted from infested trees and therefore measurements may differ.
(a) Nematode body length divided by greatest width (usually at mid-body); (b) Nematode body length divided by pharynx length from the lips to pharyngo-intestinal valve; (c) Nematode body length divided by tail length.

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.

Further information

Further information on this organism can be obtained from:
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Appendix 1 – ITS RFLP PCR Burgermeister *et al.* (2009)

1. General information

1.1 This test was described by Burgermeister *et al.* in 2005, 2009.

1.2 The target region of the primer set is located in the 18S – 26S rDNA region.

1.3 Nucleic acid source is different life stages of nematodes.

1.4 The amplicon size for the 7 species of the *B. xylophilus* group included in the study varies from 950 to 1030 bp.

1.5 Primer set:
forward: 5′-CGT-AAC-AAG-GTA-GCT-GTA-G-3′ (Ferris *et al.*, 1993)
26S: 5′-TTTCAC- TCG-CCG-TTA-CTA-AGG-3′ (Vrain, 1993)

1.6 Taq DNA polymerase (Stratagene or Fermentas) used for the amplification.

1.7 Nucleotides are used at a final concentration of 0.2 µM each.

1.8 Molecular grade water (MGW) is used to make reaction mixes.

1.9 The test was initially developed on a 9600 Perkin Elmer thermocycler.

2. Methods

2.1 Nucleic acid extraction

DNA is extracted from mixed life stages of nematodes (adult females and males, juveniles).

Several extraction methods can be used and are described below:

• QIAamp DNA Micro Kit, Qiagen.

Nematode samples (1–30 specimens) are placed in 5 µL of water using Eppendorf tubes and frozen at – 20°C until extraction. Before extraction, the sample is thawed, mixed with 10 µL of buffer ATL (Qiagen) and homogenized in the Eppendorf tube using a micropestle (Eppendorf). Buffer ATL (170 µL) and 20 µL proteinase K solution (>600 mAU/mL) are added on rinsing the pestle, the sample is mixed and incubated at 56°C for 3 h. Then 200 µL buffer AL (Qiagen) containing 1 µg carrier RNA (Qiagen) are added and the sample is mixed by pulse-vortexing for 15 s. Ethanol (200 µL) is added, the sample is pulse-vortexed for 15 s, transferred to a QIAamp MinElute Column and centrifuged at 6000 *g* for 1 min. The flow-through is discarded, and the column washed twice, first by adding 500 µL buffer AW1 (Qiagen) and centrifuging at 6000 *g* for 1 min, then by adding 500 µL buffer AW2 (Qiagen) and centrifuging at 6000 *g* for 1 min. Then the column is centrifuged at 20 000 *g* for 3 min to dry the membrane. For elution of adsorbed DNA, the column is placed in a clean Eppendorf tube, and 20 µL (for single nematode extraction) to 100 µL (for extraction of up to 30 nematodes) of buffer AE (Qiagen) is applied to the membrane. The sample is then incubated for 10 min at room temperature and centrifuged at 20 000 *g* for 1 min. The eluate containing extracted DNA is stored at −20°C until use. DNA concentration can be determined fluorometrically using a DyNA Quant 200 fluorometer (Hoefer/Pharmacia) and the fluorescent dye, Hoe 33258.

• Lysis (modified from Stanton *et al.* 1998)

Nematodes (1–30 specimens) were incubated in 20 µL 0.25 M NaOH at 25°C for 16 h and subsequently heated to 99°C for 2 min. The sample was cooled to room temperature, and 20 µL 0.25 M HCl, 5 µL Tris-HCl pH 8.0 and 5 µL 2% Triton X-100 were added with mixing. The final sample was pH 8. In the original description of Stanton *et al.* (1998), the NaOH containing sample is only partially neutralised by addition of 10 µL instead of 20 µL 0.25 M HCl and the final sample is therefore strongly alkaline with pH of approximately 12. DNA concentration of extracts obtained with either method was determined fluorimetrically using a DyNA Quant 200 fluorometer (Hoefer/Pharmacia/GE Healthcare, Munich, Germany) and the fluorescent dye, Hoe 33258.

With both DNA extraction methods, sufficient DNA for PCR could be obtained from single nematodes (see also the section on performance criteria).

Other methods of DNA extraction from single nematodes without a DNA purification step have also been described.

• DNA extraction according to Iwahori *et al.* (2000)

A *Bursaphelenchus* specimen is placed into 1 µL of water, left to dry and crushed with a filter paper chip. The filter paper chip with the nematode remains acting as the DNA template is immediately transferred to a PCR tube and mixed with the PCR solution or extracted with PCR buffer and the extract used as the PCR template.

• 'worm lysis buffer'

A single *Bursaphelenchus* specimen is placed in 5 µL of lysis buffer, frozen at −70°C for 10 min, heated at 60°C for 1 h and then at 95°C for 15 min to obtain the DNA template for PCR.

2.2 Polymerase Chain Reaction and RFLP

2.2.1 Master mix (concentration per 50-µL single reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR buffer (including 10 mM Tris-HCl pH 8.8, 50 mM KCl; same provider as for the DNA polymerase)	10×	5	1×

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
$MgCl_2$	10 mM	12.5*	2.5 mM*
dNTPs (*Roche*)	25 mM	0.4*	0.2 mM*
Primers (for each)	50 µM	0.6	0.6 µM
DNA polymerase (*Stratagene or Fermentas*)	5 U μL^{-1}	0.4	2 U
DNA		2 ng (volume depending on concentration of DNA solution)	
Molecular grade water	N.A.	To make up to 50	N.A.
Total		50	

*When using Taq DNA polymerase from Fermentas (Burgermeister *et al.*, 2009).

2.2.2 PCR cycling conditions: Initial denaturation at 94°C for 2.5 min, 40 reaction cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min.

2.3 RFLP procedure: Suitable aliquots of the amplified DNA are digested with 3 units of the restriction endonucleases *Alu*I, *Hae*III, *Hin*fI, *Msp*I and *Rsa*I, following the manufacturer's instructions.

2.4 Analysis of DNA fragments: DNA fragments are separated by electrophoresis on agarose gel (1.8% and 2.5% respectively for PCR and RFLP) and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

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: 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.
- 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, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

Table 4 RFLP restriction fragment length polymorphism (from Burgermeister *et al.*, 2005 except when specified otherwise)

Bursaphelenchus species	PCR product (bp)	Restriction fragments (bp)				
		RsaI	HaeIII	MspI	HinfI	AluI
B. conicaudatus	980	510	550	290	270	380
		450	160	200	190	310
				120	90	
*B. doui**	981	435	640	328	283	616
		296	205	264	228	365
		228	83	165	209	
		22	53	114	154	
				110	83	
					24	
B. fraudulentus	1030	560	340	340	310	470
		470	290	290	260	390
			150	130	160	180
			110			
B. luxuriosae	950	500	750	450	270	600
		420	160	240	240	320
			50	130	170	
B. mucronatus european type	950	410	620	370	410	700
		290	220	310	250	250
		230	110	280	130	
					90	
B. mucronatus asiatic type	950	500	620	370	410	700
		410	310	310	250	250
				280	130	
					90	
*B. singaporensis**	914	474	800	299	494	357
		418	532	254	261	209
		22	268	237	135	195
			114	124	24	153
B. xylophilus	950	500	730	570	270	460
		420	200	380	260	250
					140	140
						100

*From Burgermeister *et al.* (2009) bp numbers have been calculated from sequence data and consequently some bands might not be visible after electrophoresis (e.g. smaller bands or bands of close size).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. These can include: co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (e.g. eukaryotic 18S rDNA) amplification of samples spiked with exogenous nucleic acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results:

Verification of the controls:

- NIC and NAC should produce no amplicons.

Table 5 List of species tested during the validation

ID	Species	Geographical origin	ID	Species	Geographical origin
04-415-1	*Bursaphelenchus xylophilus*	Canada	04-421-1	*B. mucronatus*	France
08-1063-1 (J10)	*B. xylophilus*	Asia	05-948-1	*B. mucronatus*	France
08-104-1	*B. xylophilus*	China	04-1245-1	*B. mucronatus*	France
05-54-1	*B. xylophilus*	Portugal	09-376-1 (J13)	*B. mucronatus*	Asia
08-746-1	*B. xylophilus*	China	08-767-1	*B. mucronatus*	China
08-747-1	*B. xylophilus*	Japan	08-770-1	*B. mucronatus*	Japan
09-374-1	*B. xylophilus*	Canada	06-1284-1	*B. sexdentati*	France
09-85-1	*B. doui*		06-1285-1	*B. sexdentati*	France
09-89-1	*B. fraudulentus*		07-1052-1	*B.* sp.	France
09-90-1	*B. singaporensis*		06-1280-1	*B.* sp.	France
09-91-1	*B. macromucronatus*		06-1674-1	*B.* sp.	China

- PIC and PAC should produce restricted fragment lengths as given in Table 4.
- When relevant the IPC should produce the expected amplicon.

When these conditions are met:

- A sample will be considered positive if it produces the the restriction fragment lengths as given in Table 4.
- A sample 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

4. Performance criteria available

The following performance criteria were provided by Anses – Plant Health Laboratory (FR) May 2011

4.1 Analytical sensitivity data
Five nematodes.

4.2 Analytical specificity data
In Burgermeister *et al.* (2009) the test was evaluated with 44 *Bursaphelenchus* species, including 7 of the 9 species of the *B. xylophilus* group: *B. conicaudatus*, *B. doui*, *B. fraudulentus*, *B. luxuriosae*, *B. mucronatus*, *B. singaporensis* and *B. xylophilus.* French validation data: Seven target populations and 15 non target populations were tested (see Table 5). No cross reaction was noted.

4.3 Data on repeatability
100%.

4.4 Data on reproducibility
100% for 5 individuals.

Appendix 2 – Satellite DNA-based PCR technique Castagnone *et al.* (2005)

1. General information

1.1 This method was developed by Castagnone *et al.* in 2005.

1.2 The primer set targets one family of satellite DNA of *Bursaphelenchus xylophilus*.

1.3 Nucleic acid source is different life stages of nematodes.

1.4 The amplicon is a ladder of multimers of the 160-bp monomer unit (160; 320; 480 bp ….).

1.5 Primer set:

J10-1: 5′-GGT-GTC-TAG-TAT-AAT-ATC-AGA-G-3′

J10-2Rc: 5′-GTG-AAT-TAG-TGA-CGA-CGG-AGT-G-3′

1.6 Taq DNA polymerase (MP Biomedicals, ex Qbiogene, France) used for the amplification.

1.7 Nucleotides are used at a final concentration of 0.2 mM each.

1.8 Molecular grade water (MGW) is used to make reaction mixes.

1.9 The test was initially developed on a TRIO-Thermoblock thermocycler (Biometra).

2. Methods

2.1 Nucleic acid extraction

Amplification is performed on individual nematodes, prepared according to a PCR procedure modified from Williams *et al.* (1992). Briefly, single nematodes are transferred to a dry thin walled PCR tube, covered with 2.5 µL lysis buffer (50 mM KCl, 10 mM Tris pH 8.2, 2.5 mM $MgCl_2$, 60 mg mL^{-1} proteinase K, 0.45% NP40, 0.45% tween 20, 0.01% gelatin) and overlaid with mineral oil. Tubes are put at −80°C for 45 min, and immediately transferred to 60°C for 60 min and then 95°C for 15 min in the thermal cycler. The resulting DNA extract is then used as template in a specific PCR.

2.2 Polymerase Chain Reaction

2.2.1 Master mix (concentration per 25 µL single reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR buffer (including 10 mM Tris-HCl pH 8.2, 50 mM KCl)	10×	2.5	1×
$MgCl_2$	10 mM*	6.25	2.5 mM

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
dNTPs (*Roche*)	25 mM*	0.2	0.2 mM
Primers (for each)	50 µM*	0.74	250 ng equivalent to 1.48 µM
Polymerase (*MP Biomedicals, ex Qbiogene*)	5 U μL^{-1}	0.2	1 U
DNA		10 ng (volume depending on concentration of DNA solution)	
Molecular grade water		To make up to 25	
Total		25	

*Example, given from laboratory experience.

2.2.2 PCR cycling conditions

Initial denaturation at 94°C for 5 min, 25 reaction cycles of 94°C for 30 s, 64°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min.

2.2.3 Analysis of DNA fragments:

DNA fragments are separated by electrophoresis on agarose gel (2.5%) and visualized under UV light according to standard procedures (e.g. Sambrook *et al.*, 1989).

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: 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
- 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.

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

Alternative internal positive controls 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. eukaryotic 18S rDNA) amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce an amplification ladder of multimers of the 160-bp monomer after PCR reaction.
- When relevant the IPC should produce the expected amplicon.

When these conditions are met

- A sample will be considered positive if the amplification of a ladder of multimers of the 160-bp monomer is obtained after a PCR reaction.
- A sample will be considered negative, if it produces no band or a band not associated with a ladder pattern.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

The following performance criteria were provided by Anses – Plant Health Laboratory (FR) (May, 2011)

4.1 Analytical sensitivity data

2–5 nematodes.

4.2 Analytical specificity data

In Castagnone *et al.* (2005), it is reported that the test was evaluated with four *Bursaphelenchus* species, *B. leoni*, *B. mucronatus*, *B. tusciae*, *B. xylophilus* (3 isolates).

In the validation study of ANSES-LSV (2011) 7 target populations have been tested and 15 non-target organisms (see Table 5). No cross reaction was noted.

4.3 Data on repeatability

100%.

4.4 Data on reproducibility

95.8% for 2 nematodes.100% for 5 nematodes.

Appendix 3 – Real-time PCR protocol (François *et al.*, 2007)

1. General information

1.1 This method was developed by François *et al.* in 2007.

1.2 The test was evaluated with eleven *Bursaphelenchus* species, *B. antoniae*, *B. conicaudatus*, *B. fraudulentus*, *B. hofmani*, *B. glochis*, *B. luxuriosae*, *B. mucronatus*, *B. pinophilus*, *B. sexdentati*, *B. tusciae*, *B. xylophilus* (13 isolates).

1.3 The primer set targets a 77 bp long amplicon of the target sequence from *Bursaphelenchus xylophilus* MspI satellite DNA monomeric unit (accession number L09652).

1.4 The amplicon's size is 77 bp long.

1.5 Primer set:

BSatF: 5′-TGA-CGG-AGT-GAA-TTG-ACA-AGA-CA-3′

BSatRV: 5′-AAG-CTG-AAA-CTT-GCC-ATG-CTA-AA3′

Probe:

BsatS: 5′-FAM-ACA-CCA-TTC-GAA-AGC-TAA-TCG-CCT-GAG-A-TAMRA-3′ (Eurogentec)

1.6 Taq DNA polymerase is included in the qPCR Core Kit (Eurogentec, Belgium) used for the amplification.

1.7 Nucleotides are used at a final concentration of 0.2 mM each.

1.8 Molecular grade water (MGW) is used to make reaction mixes.

1.9 The test was initially developed on a DNA engine Opticon 2 (MJ Research) and on SmartCycler II (Cepheid, Sunnyvale, CA, US) for test on wood extracts and ringtest on individual nematodes.

1.10 With Opticon 2 (MJ Research), data were analysed using the Opticon 2 Monitor software version 3.1 according to the manufacturer's instructions (MJ Research).

The test has been performed with nematodes, and also directly on artificially infested wood. Complementary information on DNA extraction, real time PCR master mix is available in the original article regarding use of this test as a detection tool. However, in this protocol this test is only recommended for identification of isolated nematodes.

2. Methods

2.1 Nucleic acid extraction from isolated nematodes

Genomic DNA from pooled nematodes of each isolate using the phenol/polymerase method (Sambrook *et al.*, 1989), quantified spectrophotometrically and aliquoted and stored at −80°C. Alternatively, DNA from a defined number of nematodes (1–2000 individuals) was extracted as a simplified procedure, as previously described (Castagnone *et al.*, 2005, see Appendix 2), with a slight modification. Unlike the original protocol, the volume of lysis buffer used was not constant but proportionate to the number of nematodes, i.e. 3 µL for 1–4 nematodes and 20 µL for higher numbers of nematodes.

2.2 Real Time Polymerase Chain Reaction

For isolated nematode identification, the MJ Research equipment is used according to the following conditions.

2.2.1 Master mix (concentration per 25-µL single reaction on MJ Research equipment).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		17.6	
PCR buffer (including 10 mM Tris-HCl pH 8.2, 50 mM KCl; qPCR core kit – Eurogentec)	10×	2.5	1×
$MgCl_2$ (*Eurogentec*)	50 mM	2.5	5 mM
dNTPs (*Eurogentec*)	5 mM	1	0.2 mM
Primers (for each)	50 µM*	0.1	200 nM
Probe BsatS	50 µM*	0.1	200 nM
HotGoldStar DNA polymerase (Eurgentec qPCR core kit)	5 U μL^{-1}	0.1	0.5 U
Subtotal		24	
DNA (genomic DNA template)		1	
Total		25	

*Example, given from laboratory experience.

2.2.2 PCR cycling conditions MJ Research equipment

Initial denaturation at 95°C for 10 min, 30 reaction cycles of 95°C for 15 s, 59°C for 30 s, the measure of fluorescence is performed in the annealing/elongation step (59°C).

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: 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.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results

The cycle cut off value for *B. xylophilus* is set at 25.58 ± 2.35 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 internal control. The cycle cut off value needs to be verified in each laboratory when implementing the test for the first time.

Verification of the controls:

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative (Ct >cut off).
- PIC and PAC should have a Ct value below the cut off value.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, 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.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

The following performance criteria were provided in the original article (François et al., 2007).

4.1 Analytical sensitivity data

Analytical sensitivity/limit of detection: 1 pg of DNA (3 repetitions in separate experiments).

4.2 Analytical specificity data

Specificity: achieved with the ten other *Bursaphelenchus* species, and also with mixture of nematode (1% *B. xylophilus* nematodes mixed with *B. mucronatus* positively detected; even 0.01% *B. xylophilus* DNA template among *B. mucronatus* DNA template positively detected).

4.3 Data on Repeatability

100% from the sensitivity experiment repeated 3 times.This protocol has been subjected to a test performance study within the EU PORTCHECK project on SmartCycler equipment and with specific master mix (Takara mix).

Appendix 4 – Real time PCR protocol for direct detection on wood extracts

1. General Information

1.1 This protocol has been developed by Anses-LSV (FR) from 2009 to 2011, based on the test developed by François *et al.* (2007).

1.2 The test was evaluated with *B. xylophilus* (7 populations), *B. mucronatus* (6 populations), *B. doui*, *B. fraudulentus*, *B. macromucronatus*, *B. singaporensis*, *B. sexdentati* (2 populations), *Bursephelenchus* sp. (3 populations).

1.3 The primer set targets a 77 bp long amplicon of the target sequence from *Bursaphelenchus xylophilus* MspI satellite DNA monomeric unit (accession number L09652) Additionally a universal primer set, targeting 18SrDNA from eukaryotes, is included in the test as a positive control.

1.4 The amplicon's size for the target nematode is 77 bp long and 150 bp long for universal control.

1.5 Nematode primer set and probe (Eurogentec):

Primers: BSatF: 5′-TGA-CGG-AGT-GAA-TTG-ACA-AGACA-3′; BSatRV: 5′-AAG-CTG-AAA-CTT-GCC-ATG-CTA-AA-3′;

Probe: BsatS: 5′-FAM-ACA-CCA-TTC-GAA-AGC-TAA-TCGCCT-GAG-A-BHQ1-3′

Universal primer set and probe (Eurogentec):

Primers: 18 S uni-F 5′-GCA-AGG-CTG-AAA-CTT-AAA-GGA-A-3′; 18S uni-R 5′-CCA-CCA-CCC-ATA-GAA-TCA-AGA-3′

Probe: 18S uni-P 5′-JOE-ACG-GAA-GGG-CAC-CAC-CAG-GAG-T-BHQ1-3′

1.6 Taq DNA polymerase: Lightcycler® 480 probes master (2× concentrated, ready-tu-use hot-start PCR mix; Roche diagnostics), contains FastStart Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 6.4 mM $MgCl_2$.

1.7 Molecular grade water (MGW) is used to make reaction mixes.

1.8 The test was developed and evaluated on Lightcycler® 32 capillaries (Roche) and Lightcycler® LC480 wells (Roche). Robustness has also been evaluates on other PCR machines (see point 4).

1.9 For Lightcycler® 32 capillaries (Roche), the Lightcycler® software 4.1 is used with automatic settings for data analysis. For Lightcycler® LC480 wells (Roche), Lightcycler® 480software release 1.5.0 is used with automatic settings for data analysis.

2. Methods

2.1 Nucleic Acid Extraction and Purification

The DNA extraction is performed with QIAamp® DNA mini kit Qiagen

The DNA extraction is performed on nematode extract obtained from nematodes extraction from wood. Several steps are to be followed before DNA extraction as such:

- Nematodes extract is processed in tubes with conical bottom (at least 30 mL) for at least 3 h, in order to form a deposit including nematodes. Approximately 1.5 mL is carefully removed from the pellet and transferred into a 2 mL microtube. This sample is centrifuged at 15 000 *g* for 10 min, and then the supernatant is discarded. At this step, the samples can be stored in the freezer until further process.
- A mechanical extraction is performed with glass beads (1 bead of 3 mm and several beads of 1 mm, Sigma);

these beads are added to each sample. Furthermore 180 µL of lysis buffer (labelled ATL in the extraction kit) and 20 µL of proteinase K (reagents provided in QIAmp Qiagen kit) are also added before bead beating treatment (30 beats per second for 40 s).

DNA extraction procedure is then performed by transferring the microtube's content to the provided column in QIAmp Qiagen kit. The DNA extraction is then processed according to the recommended procedure for QIAmp Qiagen kit (Handbook provided by QiagenThird version April 2010; Protocol: DNA Purification from Tissues, QIAamp DNA Mini Kit).

2.2 Real-time Polymerase Chain Reaction

2.2.1 Master mix (concentration per 20 µL single reaction on Lightcycler LC480 (Roche Diagnostics).

The test for each sample should preferably be duplicated (2 wells per sample in one test performed).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		4.44	
Real time PCR mix including $MgCl_2$, hot start DNA polymerase, DNTPs (Roche)	2×	10	1×
BsatF	50 µM	0.12	0.3 µM
BsatR	50 µM	0.12	0.3 µM
BsatS	50 µM	0.04	0.1 µM
18SuniF	50 µM	0.12	0.3 µM
18SuniR	50 µM	0.12	0.3 µM
18SuniP	50 µM	0.04	0.1 µM
Subtotal		15	
DNA		5	
Total		20	

2.2.2 PCR cycling parameters.

Initial denaturation at 95°C for 10 min, 35 reaction cycles of 95°C for 15 s, 60°C for 1 min, the measure of fluorescence is performed at the annealing/elongation step (60°C).

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 contamination with target DNA from *B. Xylophilus,* during nucleic acid extraction: nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue (when working with plant material) or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: in our case, the use of conserved primers that amplify conserved non-target nucleic acid that is also present in the sample (here eukaryotic 18S rDNA) confirms the efficacy of DNA extraction.
- 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, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results

The cycle cut off value for target *Bursaphelenchus xylophilius* is set at 25, 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 the controls:

- NIC and NAC should be negative (Ct > cut off).
- The PIC and PAC amplification curves should be exponential.
- The PIC and PAC should have a Ct value below the cut off value.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, and it produces a Ct value of <25 for the *B. xylophilus* primer set and probe.
- A sample will be considered negative, if it produces a Ct of 25 or more for the *B. xylophilus* amplification and a Ct value <30 for the internal control/18S universal primer set and probe.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

The following performance criteria results were provided by Anses – Plant Health Laboratory (FR) May 2011

4.1 Analytical sensitivity data

One nematode (whatever the stage).

4.2 Analytical specificity data

Seven target populations and 15 non-target populations (see Table 5) were tested and no cross reaction was noted. Furthermore, approximately 500 wood routine samples (certified free from target nematode) were tested and no false positive result was obtained.

4.3 Data on repeatability

100% for 1 individual.

4.4 Data on reproducibility

100% for 1 individual.