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EPPO STANDARD ON DIAGNOSTICS

PM 7/4 (4) *Bursaphelenchus xylophilus*

Specific scope: This Standard describes a diagnostic protocol for *Bursaphelenchus xylophilus*.[1] This Standard should be used in conjunction with PM 7/76 Use of EPPO diagnostic protocols. Terms used are those in the EPPO Pictorial Glossary of Morphological Terms in Nematology (EPPO, 2020).

Specific approval and amendment: first approved in 2000–09. Revised on 2009–09. Second revision approved on 2012–09, Third revision approved on 2023–01. Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Bursaphelenchus xylophilus, the pine wood nematode, is an EPPO A2 pest. It is presumed that *B. xylophilus* originated in North America and was transported from there to the southern Japanese island of Kyushu in infested timber in the beginning of the 20th century (Malek & Appleby, 1984; Mamiya, 1983; Nickle et al., 1981). The fact that native American conifers are mostly resistant, whereas Japanese species are susceptible, tends to support this view. From Japan, *B. xylophilus* has spread to other Asian countries (Li et al., 1983) and then, at the end of the 20th century, it was introduced in Europe, in Portugal (mainland and Madeira island) and Spain (isolated outbreaks under eradication; Abelleira et al., 2011; Fonseca et al., 2012; Mota et al., 1999). Updated information on geographical distribution is available in the EPPO Global Database (EPPO, 2022).

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 North America the nematode has caused mortality in the exotic pine species *P. sylvestris*, *P. densiflora*, *P. thunbergii* and *P. nigra* (Dropkin et al., 1981; Malek & Appleby, 1984), but rarely in endemic species such as *Pinus elliottii* (Dwinell & Nickle, 1989). In Japan, the nematode has caused massive mortality of native pine trees (*Pinus densiflora*, *P. thunbergii*, *P. luchuensis*) and is the most damaging forest pest.

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 also occur to *Pinus* and other tree genera but without resulting in wilt development. Transmission during oviposition can occur on almost all coniferous species, provided the trees are weakened, dying from any cause or 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 unsuitable for oviposition. Thus, *B. xylophilus* can be found in wood of *Pinus* spp. expressing wilt disease after *B. xylophilus* infection 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 plants or wood moving in trade, either as a commodity (live plants, round wood, sawn wood etc.), or as wood packaging material carrying other commodities. Wood packaging material should meet the requirements of ISPM No. 15 'Regulation of Wood Packaging Material in International Trade' (FAO, Rome, revised 2009). More details about the biology, distribution and economic importance of *B. xylophilus* can be found in the EPPO Datasheet (EPPO, 2022).

The diagnostic procedure for *Bursaphelenchus xylophilus* is presented in Figure 1a,b.

2 | IDENTITY

Name: *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970

Other scientific names: *Aphelenchoides xylophilus* Steiner & Buhrer, 1934

[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.

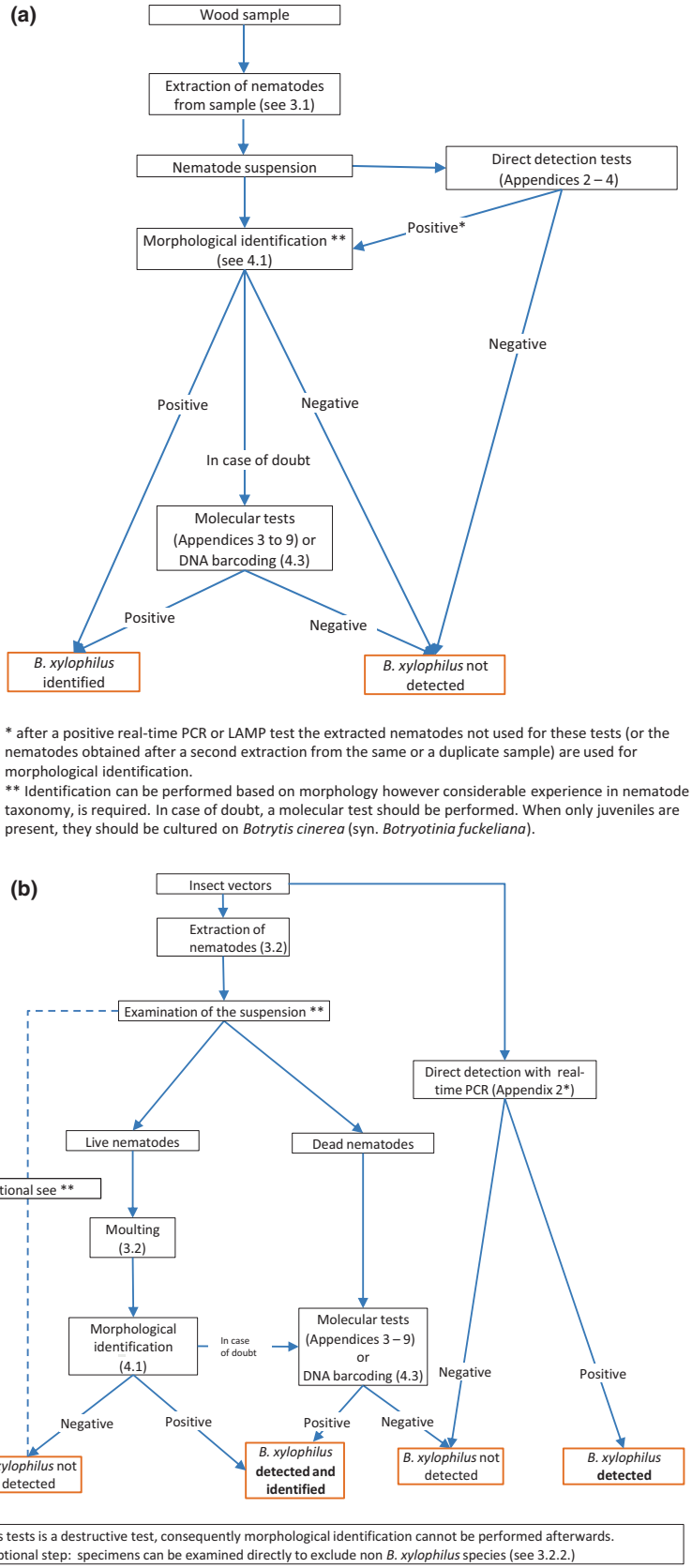

FIGURE 1 Flow diagrams for the detection and identification of *Bursaphelenchus xylophilus* from (a) wood samples and (b) insect vectors.

Bursaphelenchus lignicolus Mamiya & Kiyohara, 1972
Taxonomic position: Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Parasitaphelenchinae.
EPPO Code: BURSXY
Phytosanitary categorization: EPPO A2 list no. 158, EU Annex II/B

3 | DETECTION

Bursaphelenchus xylophilus can be present in coniferous plants for planting, cut branches, wood, isolated bark and wood chips/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 2 of PM 9/1 *Procedures for official control of Bursaphelenchus xylophilus and its vectors*. *Bursaphelenchus xylophilus* can also be detected in adult vector insects (*Monochamus* spp.). Photographs of disease symptoms and vectors are available in EPPO Global Database (EPPO, 2022).

3.1 | Detection from wood material

3.1.1 | Incubation of wood material

Nematodes may occur in very low numbers in a sample, so detection can be difficult. Incubation of wood samples is consequently highly recommended to allow the nematodes to multiply before extraction. Cases where incubation should be performed are:

- Samples collected in the framework of surveillance of pine stands[2] (nematode density may be low in such samples)
- Samples collected in the framework of import inspection for wood, bark, wood packaging material on which:
  - Damage by beetles is visible [e.g. on wood the typical Lamiinae larvae of *Monochamus* beneath the bark or the oval larval galleries ('grub holes') or round exit holes of adults, or holes in wood packaging material] see Figure 2
  - Blue fungal stain is visible in the wood.
- Wood (except wood packaging material) or bark coming from countries where *B. xylophilus* is present.

When incubation is performed, wood samples should be kept humid and reach an incubation temperature of approximately 25°C for at least 14 days to allow any nematodes present to breed and maximize the likelihood of detection. The nematodes are then extracted from the wood samples prior to *B. xylophilus* detection and/or identification.

3.1.2 | Extraction from wood material

Live nematodes can be extracted from infested wood using the Baermann funnel or Oostenbrink dish technique (see PM 7/119 on Nematode extraction; EPPO, 2013). Small pieces of wood (not larger than 1 cm in width using a cutting method that does not generate heat) or wood shavings, chips or disks are immersed in water for approximately 24–48 h. Nematodes migrate from the chopped wood into the water.

3.1.3 | Preparation of samples for morphological identification or molecular testing of specimens

Using a stereo microscope, the nematodes can be transferred with the help of a pipette or a needle from the suspension in a small Petri dish or counting dish to a glass slide for microscopic examination and morphological identification, or for molecular tests to an eppendorf tube.

For morphological identification, adult specimens are needed. Consequently, when only juveniles are present, they should be cultured on *Botrytis cinerea* (syn *Botryotinia fuckeliana*) as described in Appendix 3 of PM 7/148 (EPPO, 2021).

FIGURE 2 Damage from beetles in a consignment of bark (mixed with twigs). Courtesy N Viaene ILVO, BE.

[2]For details on surveillance see PM 9/1 *Bursaphelenchus xylophilus* and its vectors: procedures for official control.

3.1.4 | Direct molecular testing of nematode suspension

The molecular tests listed below can be used to test nematode suspensions obtained after extraction. Nucleic acid extraction should be performed as described in Appendix 1 point 2.

- Real-time PCR for the detection on wood extracts based François et al. (2007) described in Appendix 2
- LAMP test adapted from Kikuchi et al. (2009) described in Appendix 3
- Real-time PCR test based on the LSU rDNA region (ClearDetections) described in Appendix 4.

Morphological identification is required to confirm positive results obtained with molecular testing.

3.2 | Detection from vectors

3.2.1 | Extraction from insect vectors

The fourth dispersal juvenile stage is the most frequent stage found in the body (thorax and abdomen) of the insect vector. This stage was described by Kanzaki et al. (2020) and photos can be seen in EPPO Global Database (https://gd.eppo.int/taxon/BURSXY/photos). However, juveniles at other stages, and adults of species belonging to the *B. xylophilus* group may also be present (Kanzaki et al., 2013).

The insects should be cut into pieces and the nematodes extracted over 24 h using the Baermann funnel technique (see PM 7/119 on Nematode extraction; EPPO, 2013). For morphological identification, nematodes collected from the base of the funnel should either be placed on mycelium of *B. cinerea* (syn. *B. fuckeliana*; see Appendix 3 of PM 7/148 (EPPO, 2021)), where they will moult into adults and multiply, or be placed in water (as described below) where they will moult. Baermann funnel or Oostenbrink dish technique (requiring nematodes to be mobile) should not be used for insects from traps containing preservation liquids as nematodes are immobilized. Instead, direct examination (see 3.2.2.) or direct detection (see 3.2.3.) should be performed.

Direct examination is possible (see PM 7/119 section 2.1 direct examination). Insects are transferred into a Petri dish with water and cut into pieces with a scalpel to release the nematodes. Both motile and dead nematodes will be released from the insect body within 30–60 min. Sieving of the suspension can be performed, if necessary, to discard insect debris. A large sieve (e.g. 500 µm) is used and nematodes are collected on a 38 µm sieve. Live nematodes can be left in a water suspension where they will moult to adults in a few days.

3.2.2 | Examination of suspensions

In the suspension obtained from the insect extraction, (dauer) juveniles or adults of *Bursaphelenchus* can be present. In addition to *Bursaphelenchus*, many other taxa can be found, for example Hexatylina.

Aphelenchoidea is the only superfamily within the suborder Tylenchina without a dorsal gland orifice (DGO) close to the stylet, this orifice is located in the metacorpus (see Figure 2 in the Pictorial Glossary of Morphological Terms in Nematology, EPPO (2020)).

The following key can be used to determine if any suspected *Bursaphelenchus* spp. are present in the suspension. This is an optional step.

1	DGO close to stylet	*Bursaphelenchus* not suspected	
	DGO in metacorpus (not visible if no stylet present)	2	
2	No stylet present	Dauer juvenile of Aphelenchoidea	*Bursaphelenchus* sp. suspected
	Stylet present	3	
3	Spiculae or vulva present	Aphelenchoidea adult	*Bursaphelenchus* sp. suspected
	Spiculae or vulva absent	Aphelenchoidea juvenile	*Bursaphelenchus* sp. suspected

For morphological identification, adult specimens are needed. Consequently, when only juveniles are present, they should be cultured on *Botrytis cinerea* (syn *Botryotinia fuckeliana*) as described in Appendix 3 of PM 7/148 (EPPO, 2021).

When insects harbour dead nematodes only, the identification of the dauer juveniles should be made by molecular tests as described in Figure 1b. Both live and dead nematodes can be detected with these molecular tests. Use of universal primers in molecular tests can monitor possible inhibition caused by chemicals used in traps. It should be noted that preservatives used in traps such as monoethylene glycol will alter certain morphological features of nematodes impeding their morphological identification (Berkvens et al., 2017).

3.2.3 | Direct testing of insect vectors

Insect vectors can also be tested directly with the real-time PCR based on François et al. (2007) described in Appendix 2. This is a destructive test, consequently unlike for wood, morphological identification cannot be performed after this.

4 | IDENTIFICATION

Identification can be performed based on morphology. However, considerable experience in nematode

taxonomy is required, especially to differentiate species within *B. xylophilus* group. In case of doubt, a molecular test should be performed.

4.1 | Identification on the basis of morphological features

For a positive morphological identification of *B. xylophilus*, adult specimens of both sexes are required. The analysis requires the preparation of good quality microscope slides and access to a high-resolution microscope (microscope fitted with 100× oil immersion objective, and preferably with differential interference contrast).

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 super family Aphelenchoidea, which have the dorsal pharyngeal gland opening in the metacorpus (in contrast to the Tylenchoidea where the gland opens into the lumen of the pharynx behind the stylet knobs).

4.1.1 | Identification at genus level

Adult nematodes of the '*B. xylophilus*-group' extracted from wood and bark can be distinguished by using the key given in Table 1.

4.1.2 | Identification at species level

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 recognized. Species belonging to the '*xylophilus*-group' are characterized by four lateral lines, the presence of a vulval flap, large strongly arcuate spicules (Figures 3c and 4d) and the arrangement of seven caudal papillae (best seen using a scanning electron microscope; Braasch, 2008). Adults of *B. xylophilus* extracted from wood and bark can be separated from other species of the '*xylophilus*-group' by using the key in Table 2.

The rearing conditions of *B. xylophilus* (e.g. on agar plates with fungi or in wood) over several generations may change or increase the variability of the female tail as sometimes observed in Portugal (M.L. Inacio, *pers. comm.*).

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.

4.1.3 | Species description

Bursaphelenchus xylophilus shows the general characters of the genus *Bursaphelenchus* (Hunt, 1993; Nickle, 1970):

TABLE 1 The identification of adults of the 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 NBS
	Metacorpus smaller, fusiform to rounded; in lateral perspective dorsal pharyngeal gland opening and a ventral curvature of pharyngeal lumen behind stylet knobs	
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 NBS
	Bursa absent	
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 four lines; vulva with prominent flap; spicules strongly arcuate (Figures 3c and 4c,d)	*Bursaphelenchus xylophilus* group (BXG)
	Characters different	Non-*Bursaphelenchus xylophilus* group

Abbreviation: NBS, not *Bursaphelenchus* species.

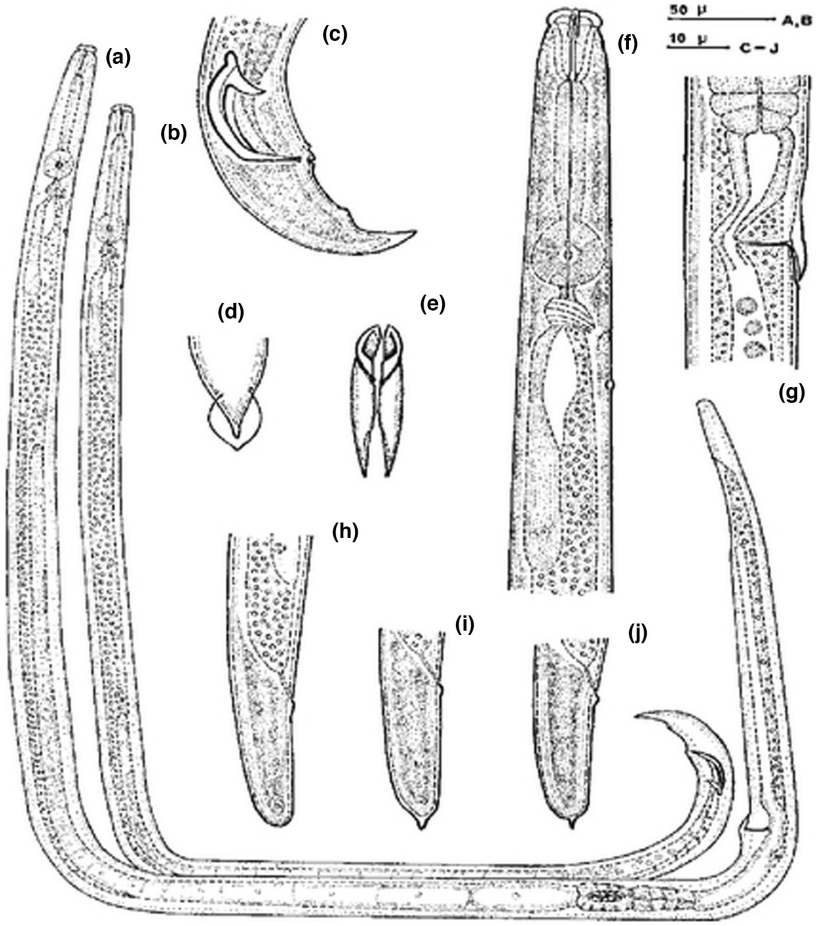

FIGURE 3 *Bursaphelenchus xylophilus* n. sp. (from Mamiya & Kiyohara, 1972). (a) female; (b) male; (c) male tail; (d) ventral view of male tail, tip with bursa; (e) ventral view of spicules; (f) female, anterior portion; (g) female vulva; (h–j) female tail.

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 (Figure 3f); metacorpus well developed (Figure 3f).

Female

Vulva with a conspicuous overlapping anterior lip (vulval flap) not ending in a depression (Figures 3g and 4f); vulva usually at 70%–80% of the body length; post-uterine sac usually three to six body widths in length; female tail sub-cylindrical and, in most populations, with a broadly rounded tip (Figures 3h and 4b), but occasionally the tip may have a terminal nipple-like extension or short mucro (Figure 3i,j). The mucronated form ("M-form") of *B. xylophilus* has a mucro (Figure 4g) which may be similar or less prominent than in the *B. mucronatus* 'European type' i.e. *B. mucronatus kolymensis* (Gu et al., 2011). The "M-form" is rare in North America and elsewhere.

Male

Tail with a strong dorsal curvature (Figures 3c and 4d); a small terminal bursa is present at the tail tip, which can be seen readily in dorso-ventral position (Figure 3d); 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 (Figures 3c and 4d); caudal papillae occur as an adanal pair just anterior to the anus, two post-anal pairs just anterior to the origin of the bursa 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 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).

4.2 | Identification by molecular tests

Many molecular tests are available for the identification of *B. xylophilus*. When molecular tests are used to detect *B. xylophilus* in wood products for quarantine purposes, it is essential to note that both live and dead nematodes can be detected by these tests. Several phytosanitary measures will kill *B. xylophilus* in the wood, as well as insect preservation liquids in traps (Berkvens et al., 2017), but dead nematodes are still present and depending on the extraction method may be detected by molecular techniques.

Molecular tests used for identification in the EPPO region are:

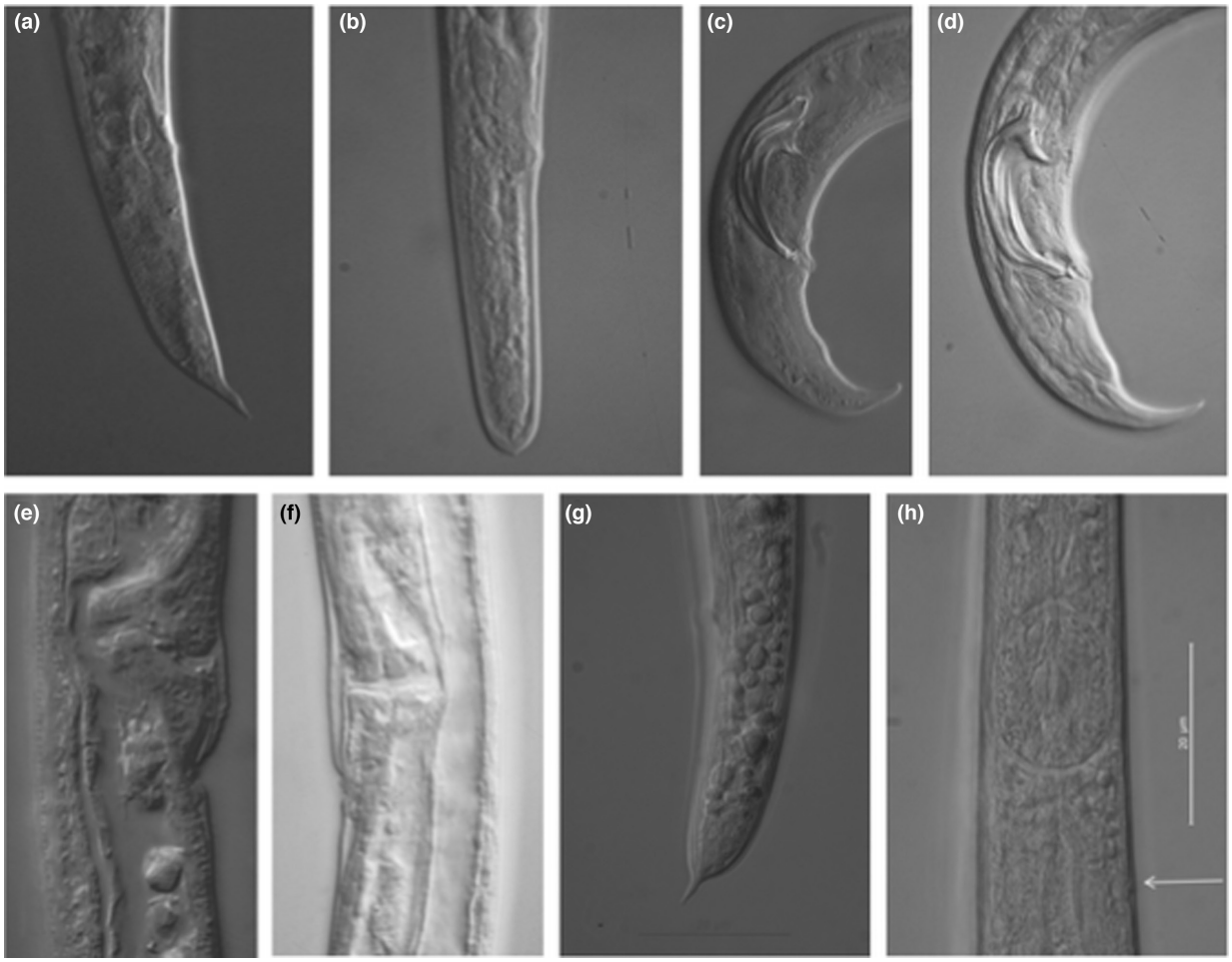

FIGURE 4 Morphological characters of *Bursaphelenchus* spp. (a) *B. mucronatus* 'East-Asian type' *B. mucronatus mucronatus* showing the conical tail with mucro; (b) *B. xylophilus*, female tail of the 'Round-tailed form'; (c) *B. trypophloei* spicule shape; (d) *B. xylophilus* spicule shape; (e) *B. populi*, showing the vulval flap ending in a depression in body wall; (f) *B. xylophilus* showing the straight vulval flap; (g) *B. xylophilus* mucronated form (population US10). (h) *B. xylophilus* mucronated form (population US10) showing a posterior position of the excretory pore (arrow). Photos: a, c, e (Tomalak M, Institute of Plant Protection, Poznan, Poland); b, d, f, g, h (Magnusson C, Bioforsk, Norway).

- LAMP test adapted from Kikuchi et al. (2009) described in Appendix 3,
- Realtime PCR test based on the LSU rDNA region (ClearDetections) described in Appendix 4,
- Conventional PCR (Matsunaga & Togashi, 2004) described in Appendix 5,
- ITS RFLP PCR test (Burgermeister et al., 2009) described in Appendix 6,
- Conventional PCR (Castagnone et al., 2005) described in Appendix 7,
- Real-time PCR (François et al., 2007) described in Appendix 8,
- Multiplex real-time PCR (Filipiak et al., 2019) described in Appendix 9.

4.3 | DNA barcoding

A protocol for DNA barcoding based on 18S rDNA and 28S rDNA is described in Appendix 5 of PM 7/129 DNA barcoding as an identification tool for a number of regulated pests: DNA barcoding of nematodes (EPPO, 2021) and can support the identification of *B. xylophilus*. Sequences are available in databases including EPPO-Q-bank (https://qbank.eppo.int/nematodes/).

5 | REFERENCE MATERIAL

Reference cultures of *Bursaphelenchus* spp. are available at INIAV, Portugal or at the Forest Quarantine Laboratory (AGQF) at Julius Kühn Institut, Institute for National and International Plant Health, in Braunschweig, Germany. Many *Bursaphelenchus* species can be cultured on *Botryotinia fuckeliana* or other fungi on media such as malt or potato dextrose agar in the laboratory. In the framework of their activities and under specific conditions the EURL for plant-parasitic nematodes may supply reference material

TABLE 2 Short key for the identification of adults of *Bursaphelenchus xylophilus* in the '*xylophilus*-group' (extracted from wood and bark). Please note this key is only aimed at the identification of *B. xylophilus*, for a key to other species refer to Braasch & Schönfeld (2015).

1	Female tail conical (Figure 4a) or strongly tapering, with or without mucro Female tail broadly sub-cylindrical with or without macro (Figures 3h and 4b)	Not *B. xylophilus* 2
2	Spicule length > 30 µm Spicule length < 30 µm	Not *B. xylophilus* 3
3	Spicule with short and pointed rostrum; limbs of spicule with a rounded curvature (Figure 4c) Spicule with long and pointed rostrum; limbs of spicule with an angular curvature (Figures 3c and 4d)	Not *B. xylophilus* 4
4	Female vulval flap ending in a deep depression (Figure 4e) Female vulval flap straight not ending in a deep depression (Figures 3g and 4f)	Not *B. xylophilus* 5
5	Female tail without mucro (Figures 3h and 4b) or with a small projection (Figure 3i,j) Female tail with mucro (Figure 4g)	*B. xylophilus* (roundtailed form) 6
6	Excretory pore anterior to median bulb Excretory pore at or behind median bulb (Figure 4h)	Not *B. xylophilus* *B. xylophilus* (mucronated form) or *B. mucronatus kolymensis*[a] Molecular tests according to Matsunaga & Togashi, 2004 (Appendix 5) should be performed to differentiate the mucronated *B. xylophilus* from *B. mucronatus kolymensis*
	Position of excretory pore cannot be observed	Identification based on morphological characters impossible. Molecular tests should be performed

[a]The mucronated form of *B. xylophilus* is mainly found in North America (exact global distribution is not known) and molecular tests (Matsunaga & Togashi, 2004; Appendix 5) are recommended for a reliable separation of this form from the species *Bursaphelenchus mucronatus kolymensis*, which is present in Europe.

TABLE 3 Measurements of *Bursaphelenchus xylophilus* characters.

	Males					**Females**				
Character	**Nickle et al. (1981) (*n* = 5)**	**Mamiya and Kiyohara (1972) (*n* = 30)**	**Mota et al. (1999) (Portugal) (*n* = 12)**	**Penas et al. (2008) (Portugal) (*n* = 20)**	**Penas et al. (2008) (Portugal) mucronate *n* = 10**	**Nickle et al. (1981) (*n* = 5)**	**Mamiya and Kiyohara (1972) (*n* = 40)**	**Mota et al. (1999) (Portugal) (*n* = 12)**	**Penas et al. (2008) (Portugal) (*n* = 20)**	**Penas et al. (2008) (Portugal) mucronate *n* = 10**
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)	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 (37–48)	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)

Note: 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 midbody); (b) Nematode body length divided by pharynx length from the lips to pharyngo-intestinal valve; (c) Nematode body length divided by tail length.

6 | REPORTING AND DOCUMENTATION

Guidance on reporting and documentation is 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 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

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.

9 | STANDARD 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.

10 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Karssen G, National Plant Protection Organization, National Reference Centre, P.O. Box 9102, 6700 HC Wageningen (NL).

Magnusson C, Norwegian Institute of Agricultural and Environmental Research (Bioforsk), Høgskoleveien 7, NO-1432 Ås (NO).

Prior T, Food and Environment Research Agency, Sand Hutton, York YO41 1LZ (GB).

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APPENDIX 1 - DNA EXTRACTION

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

1. **DNA extraction from isolated specimen(s)**

Several DNA extraction methods can be used and are described below:

Qiagen QIAamp DNA kits can be used following the manufacturer's instructions (Protocol: DNA Purification from Tissues).

Methods of DNA extraction without a DNA purification step are described below.

- Lysis (modified from Stanton et al., 1998)

Nematodes (1–30 specimens) are 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 is cooled to room temperature, and 20 µL 0.25 M HCl, 5 µL Tris–HCl 0.5 M, pH 8.0 and 5 µL 2% Triton X-100 added with mixing. The final sample should be at pH 8. In the original description of Stanton et al. (1998), the NaOH containing sample is only partially neutralized 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 extraction according to Iwahori et al. (2000)

A *Bursaphelenchus* specimen is placed into 1 µL of water, left to dry and crushed with a small piece of filter paper. The filter paper piece, 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' modified from Williams et al. (1992)

Nematodes are individually placed in 5 µL lysis buffer (50 mM KCl, 10 mM Tris–HCl (pH 8.2), 2.5 mM $MgCl_2$, 0.45% (w/v) Nonidet P-40, 0.45% (w/v) Tween 20, 0.01% (w/v) gelatin and 0.06 mg/mL proteinase-K) in 0.2 mL MicroAmp reaction tubes (Applied Biosystems) and placed at −70°C or below for 10 min (DNA extraction adapted from Barstead et al., 1991). After thawing at room temperature, the DNA solution is heated at 60°C for 1 h and then at 95°C for 15 min. The resulting crude DNA extract is used as a template for PCR.

- Lysis modified from Ibrahim et al. (1994)

Nematodes (1–30 specimens) are crushed in 100 µL of lysis buffer (Buffer Tris-EDTA [pH 8.0], 1% Nonidet P-40, and 100 µg/mL proteinase K). The mechanical grinding is performed with glass beads (1 3 mm bead and several 1 mm beads, Sigma); these beads are added to each sample before bead beating treatment (30 bps for 40 s).

Tubes containing the DNA solution are heated at 56°C for 1 h and then placed at 95°C for 10 min.

- ClearDetections kit

The nematode DNA extraction kit for individual nematodes & single cysts (ClearDetections) can be used following the manufacturer's instructions (validation data for this kit (and other kits) are available on the EPPO validation database https://dc.eppo.int/validation_data/validationlist).

2. **DNA extraction with the QIAamp DNA Mini Kit following nematode extraction from wood**

After extraction with the Bearmann funnel or Oostenbrink dish methods (see 3.1.2.), the nematode suspension is transferred into a tube (of at least 30 mL) with a conical bottom for at least 3 h, in order to form a deposit including nematodes. Approximately 1.5 mL including the deposit is carefully removed and transferred into a 2 mL microtube. This sample is centrifuged at 15 000 *g* for 10 min, and the supernatant discarded. At this step, the samples can be stored in the freezer until further processing.

A mechanical extraction is performed with glass beads (one 3 mm bead and several 1 mm beads, Sigma); these beads are added to each sample. Then 180 µL of lysis buffer (labelled ATL in the extraction kit) and 20 µL of proteinase K (reagents provided in Qiagen QIAamp DNA mini kit) are also added before bead beating treatment (30 pulse per s for 40 s). Tubes are capped and incubated at 56°C for 1 h.

DNA extraction is then performed according to the recommended procedure (Handbook provided by Qiagen; Protocol: DNA Purification from Tissues, QIAamp DNA Mini Kit).

3. **Direct DNA extraction from insects with the QIAamp DNA mini kit**

The DNA extraction is performed on *Monochamus* mesothoraxes, metathoraxes and abdomens which are prepared from trapped insects. The parts (Figure A1) from up to 20 individuals can be grouped together for DNA extraction.

Several steps are to be followed before DNA extraction as follows:

- Insect parts are processed in one or more tubes (at least 50 mL) with a conical bottom. A maximum of 20 insects can be included per tube. If needed, at this stage of the process, the tubes can be stored at −10°C for later analysis.

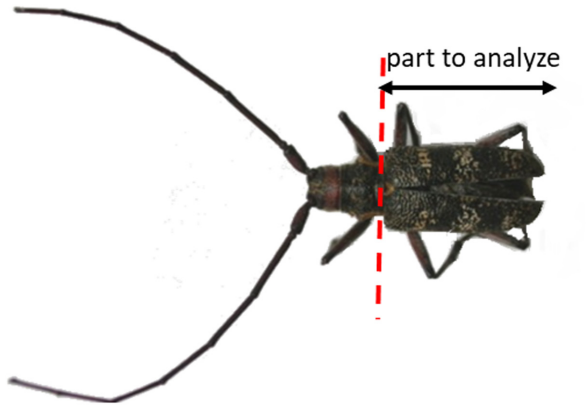

FIGURE A1 Preparation of *Monochamus* individuals for DNA extraction.

- In each tube, add:
  - 1 steel bead of approximately 1 cm
  - approximately 5 mL of PBS buffer[3] 1×
- Position the tubes in the racks of a rotating stirrer (e.g. Reax stirrer from HEIDOLPH) and shake at low speed (30 to 40 rpm) for approximately 1 h.
- Carry out a quick centrifugation before opening the tubes.
- Add 500 µL of protease solution (Qiagen).
- Add 12 mL of AL buffer (Qiagen).
- Vortex to homogenize.
- Incubate the tubes for 10 min at 70°C in a (pre-heated) heat bath.
- Briefly centrifuge the tubes and add 10 mL of 96%–99% ethanol.
- Homogenize well by vortexing and let the tube stand for a few minutes.
- Transfer 700 µL of the tube's content to the column provided in the QIAamp DNA mini kit (QIAGEN).
- The final steps of DNA extraction are carried out according to the recommended procedure for QIAamp DNA mini and Blood mini kit (QIAGEN protocol: DNA purification from tissues). The elution is performed with 100 µL of elution buffer.

APPENDIX 2 - REAL-TIME PCR FOR THE DETECTION ON WOOD EXTRACTS OR INSECT VECTORS BASED ON FRANçOIS ET AL. (2007) (ANSES, 2017, 2018)

The test below differs from the one described in the original publication.

The test below is described as it was carried out during the VALITEST project 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.

[3]10× Phosphate buffered saline (PBS), pH 7.2 (dilute 1:10 before use) NaCl 80.0 g, $NaH_2PO_4.2H_2O$ 4.0 g, $Na_2HPO_4.12H_2O$ 27.0 g, Distilled water 1000 mL.

1. General Information

1.1. This protocol has been developed by Anses-LSV (FR), based on the test developed by François et al. (2007). The test is published as an official test for wood extracts (Anses, 2017) and vectors (Anses, 2018).

1.2. The test is used for direct detection on nematode suspensions from wood extracts or insect vectors.

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

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

1.5. Nematode primer set and probe:

BSatF	5′-TGA-CGG-AGT-GAA-TTG-ACA-AGACA-3′
BSatR	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 (Ioos et al., 2009):

18S 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-to-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. The test was developed and evaluated on Lightcycler® 32 capillaries (Roche) and Lightcycler® LC480 wells (Roche). Robustness has also been evaluated on other PCR machines (see point 4).

1.8. 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

2.1.1. Tissue source: nematode suspensions from wood extracts or insect vectors

2.1.2. DNA extraction See Appendix 1 (point 2 for wood extracts and 3 for insects)

2.2. Real-time Polymerase Chain Reaction

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

Reagent	Working Concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	4.44	N.A.
Lightcycler® 480 probes master mix (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
18S uni-F	50 µM	0.12	0.3 µM
18S uni-R	50 µM	0.12	0.3 µM
18S uni-P	50 µM	0.04	0.1 µM
Subtotal		15	
DNA		5	
Total		20	

1.5. 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/vector or 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; conserved primers that amplify conserved non-target nucleic acid that is also present in the sample.
- 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).

Other control

- In this test, primers and probe targeting eukaryotic 18S rDNA are used as Internal Positive Control (IPC) to confirm the efficacy of DNA extraction and amplification.

3.2. Interpretation of results

The cycle threshold (C_t) cut off value for target *Bursaphelenchus xylophilus* is set at 25 for wood samples and 28 for insects vectors (Anses), and was obtained using the equipment/materials and chemistry used as described in this Appendix. The C_t cut off value for the required 18S rDNA IPC was set at 30. The C_t cut off values need to be verified in each laboratory when implementing the test for the first time.

Verification of the controls:

- The PIC, IPC and PAC amplification curves should be exponential. The C_t value of the IPC should be <30.
- NIC and NAC should give no amplification. However, when using uninfected host tissue/vector as NIC, an exponentional amplification curve should be observed with eukaryotic 18S rDNA primer set and probe.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve, and a C_t value below 25 for wood extracts (or 28 in the case of insects vectors) for the *B. xylophilus* primer set and probe.
- A test will be considered negative, if it produces no exponential curve.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. **Performance characteristics available**

The following performance characteristics results were provided by Anses – Plant Health Laboratory (FR) May 2011 and completed in the framework of the H2020 EU funded project, VALITEST. Data were obtained either from Preliminary Studies (PS) performed by the Test Performance Study (TPS) organizer (Anses – Plant Health Laboratory) or from the TPS involving 14 laboratories. For the TPS, two panels of samples were used: the first panel was composed of DNA extracts and the second of spiked wood extracts. For the evaluation of analytical specificity (exclusivity), two additional species were included.

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. *Validation data for specimen and wood extracts (VALITEST)*

4.1.1. Analytical sensitivity data (PS and TPS)

One nematode (any stage) evaluated with individual specimen added to wood extracts.

During the TPS, 100% of the participants detected pine wood nematode in the sample of DNA of pure culture at the limit of detection and in the sample of wood extract spiked with 5 *B. xylophilus* nematodes.

4.1.2. Analytical specificity data (PS)

Inclusivity

100% evaluated with 5 populations of *B. xylophilus* (originating from China, Portugal and Canada)

Exclusivity

100% evaluated with 19 populations of the following species included: *B. doui* (1), *B. fraudulentus* (1) *B. hoffmanni* (1), *B. kolymensis* (5), *B. macromucronatus* (1), *B. mucronatus* (3), *B. sexdentati* (1), *B. singaporensis* (1), *B. vallesianus* (1), *B. willibaldi* (1), *B.* sp. (3 from China, France and Vietnam).

Additional data from 2011 are available from Anses (see EPPO Database on diagnostic expertise).

4.1.3. Data on repeatability (PS)

100% for 1 individual.

4.1.4. Data on reproducibility (PS)

100% for 1 individual (using 8 replicates of DNA extracts tested in 3 independent runs performed on the same day by two operators on 2 thermocyclers).

4.1.5. Diagnostic sensitivity and diagnostic specificity (TPS)

Diagnostic sensitivity: 100%.
Diagnostic specificity: 100%.

4.1.6. Other information (Anses, 2011)

Selectivity: approximately 500 routine wood samples (samples collected in France in the framework of surveillance and import inspection and certified free from target nematode) were tested and no false positive results were obtained.

4.2. *Validation data for insect extracts (Anses, 2011)*

4.2.1. Analytical sensitivity data

Two nematodes (92% repeatability), five nematodes (100% repeatability) in extracts from 20 (non-infested) insects.

4.2.2. Data on repeatability

100% (for 5 nematodes in 20 non infected vectors).

4.2.3. Data on reproducibility

100% (eight times for five nematodes in 20 non infected vectors tested in three independent runs performed by two operators on two different days and using two different thermocyclers).

4.2.4. Selectivity

No cross-reaction was observed from DNA extracts derived from non-infested *Monochamus* species (not identified to species level).

APPENDIX 3 - LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) (KIKUCHI ET AL., 2009)

The test below differs from the one described in the original publication.

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

1. **General Information**

1.1. The test is used for the detection of *B. xylophilus* in plant material. The test also performs well on extracted nematodes from wood material, isolated nematodes or DNA. The publication specifies that it can be used directly with wood material after incubation of wood in extraction buffer but there is no experience in the EPPO region with this procedure which was not evaluated in the VALITEST project.

1.2. The test was developed by Kikuchi et al. (2009).
1.3. The test targets the internal transcribed spacer (ITS) region.
1.4. Oligonucleotides: Primer sequences are given in 5′–3′ orientation.

Name	Sequence 5′–3′
F3	GCA GAA ACG CCG ACT TGT T
B3	TCA TCC GAA CGT CCC TGA C
FIP	CGC GGA ACA AAC CGC GTA AAA C-CG TTG TGA CAG TCG TCT CG
BIP	AGA GGG CTT CGT GCT CGA TTGGCC GTT GAA ACA ACA TCA CC
LoopF	AGA TGG TGC CTA ACA TTG CG

The sequences are available in the DNA Data Bank of Japan (DDBJ) database under the accession numbers AB500146 and AB500156.

The validation data has been produced using standard purified primers.

1.5. In the framework of the VALITEST project, the test has been successfully performed on four different machines: Mx3000P (Stratagene/Agilent technologies, Singapore), Genie II (Optigene Ltd, Horsham, UK), CFX-96 (Bio-Rad, Hercules, California) and Light Cycler 480 (Roche Applied Science, Penzberg, Germany) with comparable results. The experimental melting temperature (T_M) value observed with these machines varies between 88.26 and 91.40°C.
1.6. Software allowing fluorescence acquisition in real-time and melting curve analysis should be used. The specific instrument manual should be consulted. Note: software for cycling (real-time PCR devices) should be programmed to measure the fluorescence during the amplification and the melting curve analysis.

2. **Methods**

2.1. Nucleic Acid Extraction and Purification

2.1.1. Tissue source: *B. xylophilus* in plant material, isolated nematodes
2.1.2. DNA extraction

- Wood extracts: In the VALITEST project, nucleic extraction was performed as described in Appendix 1 point 2.
- Isolated nematodes see Appendix 1 point 1.

2.2. LAMP

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	4	N.A.
Isothermal Master Mix including DNA binding dye[a] (*Optigene Ltd., Horsham, UK*)		15	1×
Primers:			
F3	100 µM	0.05	0.2 µM
B3	100 µM	0.05	0.2 µM
FIP	100 µM	0.4	1.6 µM
BIP	100 µM	0.4	1.6 µM
LoopF	100 µM	0.1	0.4 µM
Subtotal		20	
DNA extract		5	
Total		25	

[a] DNA binding dye must be read at the same wavelength as FAM.

2.2.2. LAMP conditions: 63°C for 30 min, i.e. 60 cycles of 30 s with measurement of the fluorescence (use of FAM detection channel) at the end of each cycle; melting curve analysis: 98 to 80°C, 0.05°C per second.

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 of a sample of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation

of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

3.2. Interpretation of results: in order to assign results from the LAMP test the following criteria should be followed:

Verification of the controls

- NIC and NAC should produce no fluorescence.
- The PAC (and PIC, if relevant) amplification curve should be exponential and the T_M value should reach the expected value (see point 1.5).

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve and if its T_M value is comparable with the value obtained for the PAC or for the PIC when relevant.
- A test will be considered negative, if it produces no fluorescence or fluorescence with a different T_M
- Tests should be repeated if any contradictory or unclear results are obtained.

4. **Performance characteristics available**

The performance characteristics provided below were produced in the framework of the H2020 EU funded project, VALITEST (grant 773139). Data were obtained either from preliminary studies (PS) performed by the Test Performance Study (TPS) organizer (Anses – Plant Health Laboratory) or from the TPS involving four laboratories. For the TPS, two panels of samples were used: the first panel was composed of DNA extracts and the second of spiked wood extracts. For the evaluation of analytical specificity (exclusivity), two additional species were included.

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. Analytical sensitivity data

One nematode (PS).

During the TPS, 100% of the participants detected pine wood nematode in the DNA sample at the limit of detection and in the sample of wood extract spiked with 5 *B. xylophilus* nematodes.

4.2. Analytical specificity data (PS)

Inclusivity
100% evaluated with 5 population of *B. xylophilus* (originating from China, Canada and Portugal).
Exclusivity
100% evaluated with 19 populations of the following species included: *B. doui* (1), *B. fraudulentus* (1) *B. hoffmanni* (1), *B. kolymensis* (5), *B. macromucronatus* (1), *B. mucronatus* (3), *B. sexdentati* (1), *B. singaporensis* (1), *B. vallesianus* (1), *B. willibaldi* (1), *B.* sp. (3 from China, France and Vietnam).

4.3. Data on repeatability (PS)

100% (for 1 nematode spiked in wood)

4.4. Data on reproducibility (PS and TPS)

100% for 1 individual (using 8 replicates of DNA solution tested in 3 independent runs performed on the same day by 2 operators on 2 thermocyclers).

4.5. Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity: 100%.
Diagnostic specificity: 100%.

4.6. Other data

During the TPS, it was noted that C_t and T_M values differed depending on the sample and on the equipment and reagents used (see details in the validation sheet in the EPPO Database on diagnostic expertise).

4.7. Selectivity

No cross-reaction observed from DNA extracted from healthy wood extracts.

APPENDIX 4 - REAL-TIME PCR TEST BASED ON THE LSU RDNA REGION (CLEARDETECTIONS)

The test below is described as it was carried out in the VALITEST project 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. Scope of the test: identification of *B. xylophilus* specimen(s) by real-time PCR.

1.2. This test is available as an all-inclusive real-time PCR diagnostic kit (ClearDetections, the Netherlands; http://www.cleardetections.com).

1.3. This test targets the LSU rDNA gene.
1.4. The amplicon is over 340 base pairs long.
1.5. Oligonucleotide sequences are not disclosed.
1.6. This test was developed on a Bio-Rad CFX Connect thermal cycler (Bio-Rad, Hercules, CA).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Tissue source isolated specimen(s) or wood extracts.
2.1.2. Nucleic acid extraction for isolated specimen see Appendix 1 point 1, for wood extracts see Appendix 1 point 2.

2.2. Real-time PCR

Real-time PCR test components included in the real-time PCR kit:

- Real-time PCR primer set (species-specific, forward and reverse)
- Lyophilized Positive amplification control (PAC)
- Lyophilized PCR mix with fluorescent DNA-binding dye
- Resuspension buffer
- Bench-side protocol

Separate Real-time PCR diagnostic kit for the detection of general nematode DNA:

- Real-time PCR diagnostic kit for general nematode DNA (for troubleshooting purposes).

2.2.1. PCR cycling conditions: 3 min 95°C, 40 cycles of 10 s 95°C, 1 min 63°C, 30 s 72°C, 0.5°C steps 72–95°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 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 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, included in the kit) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism.

3.2. Interpretation of results: in order to assign results from PCR-based tests the following criteria should be followed

Verification of the controls:

- The PAC and PIC 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 resulting in a Ct value <35*.
- A test will be considered negative if it does not produce an exponential amplification curve. A melt curve analysis is performed and the obtained TM value equals the TM value of the PAC (e.g. 90.5 ± 1 °C).
- Tests should be repeated if any contradictory or unclear results are obtained.

*The C_t cut-off value given above is as established by ClearDetections (NL). As a C_t cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

4.1. Data from ClearDetections

The validation was carried out by ClearDetections (Wageningen, the Netherlands) in accordance with PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*.

DNA extraction performed with the kit Appendix 1 point 1.

4.1.1. Analytical sensitivity data

One individual of *B. xylophilus*.

4.1.2. Analytical specificity data

The test was demonstrated to be 100% specific when applying the prescribed C_t cut-off value and meeting the melt curve analysis requirements.

Inclusivity.

100% The analytical specificity was evaluated with one population of *B. xylophilus* obtained from INIAV and three *B. xylophilus* populations (F0426, FR-04-415 and F4757-2) obtained from the Dutch NPPO (ref. Prof. Gerrit Karssen).

Exclusivity.

100% no cross reaction noted, evaluated with non-target *Bursaphelenchus* samples included: single *B. tusciae*, single *B. conicaudatus*, single *B. luxuriosae*, 1–10 *B. mucronatus*, and 10 *B. fungivorus* In addition, 9–10 *Cryptaphelenchus* sp., 10 *Aphelenchoides* sp., 10 *A. blastophthorus*, 10 *A. saprophilus*, 10 *A. subtenuis*, and 10 *A. besseyi* were included as non-target samples.

4.1.3. Data on robustness

Robustness was assessed using annealing temperatures ranging from 61.4 to 65.5°C. The tested annealing temperatures did not affect the obtained C_t values or T_M values for the tested target specimens.

4.2. Data from the VALITEST project

Performance characteristics provided in the framework of the H2020 EU funded project, VALITEST are provided below. Data were obtained either from preliminary studies (PS) performed by the TPS organizer (Anses – Plant Health Laboratory) or from the Interlaboratory Comparison (TPS) involving 11 laboratories. For the TPS, two panels of samples were used: the first panel was composed of DNA extracts and the second of spiked wood extracts. For the evaluation of analytical specificity (exclusivity), two additional species were included. The DNA extraction was done with a QIAamp DNA Mini kit.

The performance characteristics of the test were determined by diluting the DNA extracts 1/20 as recommended by the kit provider at the time of the TPS. Note that the instructions have been revised after the TPS.

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.2.1. Analytical sensitivity data

The analytical sensitivity was assessed as 10 nematodes when following the recommendations of the kit provider, i.e by diluting the DNA solution to 1/20 (PS).

During the TPS, when following the recommendations of the kit provider, i.e by diluting the DNA solution to 1/20, 91% of the participants detected pine wood nematode in the sample of DNA of pure culture at the maximum dilution (1/100) and 100% of the participants detected pine wood nematode in the sample of wood extract spiked with 5 *B. xylophilus* nematodes.

4.2.2. Analytical specificity data (PS)

Inclusivity

100% was evaluated with 5 *B. xylophilus* populations (from China, Canada and Portugal).

Exclusivity

100% evaluated with 19 populations of the following species included: *B. doui* (1), *B. fraudulentus* (1), *B. hoffmanni* (1), *B. mucronatus kolymensis* (5), *B. macromucronatus* (1), *B. mucronatus* (3), *B. sexdentati* (1), *B. singaporensis* (1), *B. vallesianus* (1), *B. willibaldi* (1), *B.* sp. (3 from China, France and Vietnam).

4.3. Data on repeatability (PS)

100% for 10 individuals following the recommendations of the kit provider at the time of the TPS.

4.4. Data on reproducibility (PS)

100% for 10 individuals following the recommendations of the kit provider at the time of the TPS (using eight replicates of DNA solution tested in three independent runs performed on the same day by two operators on two thermocyclers).

4.2.3. Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity: 98% following the recommendation of the kit provider, i.e. by diluting DNA solution to 1/20. Note that the instructions have been revised after the TPS.

Diagnostic specificity: 98%

APPENDIX 5 - CONVENTIONAL PCR TEST MATSUNAGA AND TOGASHI (2004)

The test below differs from the one described in the original publication.

The test below is described as it was carried out in the VALITEST project 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 test was developed by Matsunaga and Togashi (2004).

1.2. The primer sets target the ITS1–ITS2 region of rDNA of *B. xylophilus* and *B. mucronatus*.

1.3. This reaction produces a DNA amplicon of 557 base pairs (bp) from all *B. xylophilus* populations and an amplicon of 210 bp from all *B. mucronatus* tested.

1.4. Primer set:

		Amplicon size (bp)
For *B. xylophilus*		
X-F	5′-ACG ATG ATG CGA TTG GTG AC-3′	557
X-R	5′-TAT TGG TCG CGG AAC AAA CC-3′	
For *B. mucronatus*		
M-R	5′-TCC GGC CAT ATC TCT ACG AC-3′	210
M-F	5′-GTT TCA ACC AAT TCC GAA CC-3′	

1.5. The test was initially developed on a GeneAmp PCR system 9600, (ABI).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Tissue source: different life stages of nematodes.
2.1.2. Nucleic acid extraction see Appendix 1 point 1.

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
Molecular grade water	N.A.	16.8	
PCR buffer	10×	2.5	1×
dNTPs	25 mM	0.2	0.2 mM
X-F	50 mM	0.1	0.2 µM
X-R	50 mM	0.1	0.2 µM
M-R	50 mM	0.1	0.2 µM
M-F	50 mM	0.1	0.2 µM
Taq DNA Polymerase (MP Biomedical)	5 U/µL	0.1	0.5 U
DNA extract		5	
Total		25	

2.2.2. PCR cycling conditions.

Initial denaturation at 94°C for 5 min, 35 reaction cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 6 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, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

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:

- 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

3.2. Interpretation of results for *B. xylophilus*

Verification of the controls

- NIC and NAC no band is visualized.
- PIC and PAC a band of the expected size 557 bp is visualized.
- When relevant the IPC should produce the expected amplicon.

When these conditions are met

- A sample will be considered positive for *B. xylophilus* if a band of 557 bp is visualized.
- A test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

A band of a 210 bp monomer is observed if *B. mucronatus* is present in the sample.

4. **Performance characteristics available**

The performance characteristics provided below were produced in the framework of the H2020 EU funded project, VALITEST (grant 773139). Data were obtained either from preliminary studies (PS) performed by the TPS organizer (Anses – Plant Health Laboratory) or from the interlaboratory comparison (TPS) involving 15 laboratories and using one panel of samples composed of 6 non-target and 4 target DNA samples of pure culture. Data from the original publication and by Anses-LSV (2011) are also provided.

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. Analytical sensitivity data

One nematode (PS).
During the TPS, 80% of the participants detected pine wood nematode in the sample of DNA of pure culture at the maximum dilution (1/100) (TPS).

4.2. Analytical specificity data

Inclusivity
100%
Evaluated by Matsunaga and Togashi (2004) with 5 populations of *B. xylophilus*.
Evaluated in framework of VALITEST (PS), with 5 populations of *B. xylophilus* including populations originating from China, Portugal and Canada.
Exclusivity
100% no cross reaction noted
Evaluated by Matsunaga and Togashi (2004) with 4 populations of *B. mucronatus*
Evaluated in framework of VALITEST (PS) with 19 populations of the following species included: *B. doui* (1), *B. fraudulentus* (1) *B. hoffmanni* (1), *B. mucronatus kolymensis* (5), *B. macromucronatus* (1), *B. mucronatus* (3), *B. sexdentati* (1), *B. singaporensis* (1), *B. vallesianus* (1), *B. willibaldi* (1), *B.* sp. (3 from China, France and Vietnam).

4.3. Data on repeatability (PS)

100% for 1 individual.

4.4. Data on reproducibility (PS)

100% for 1 individual (using 8 replicates of DNA solution tested in 3 independent runs performed by the same operator on 2 different days and using 2 thermocyclers).

4.5. Diagnostic sensitivity and diagnostic specificity (TPS)

Diagnostic sensitivity: 93%.
Diagnostic specificity: 100%.

APPENDIX 6 - ITS RFLP PCR BURGERMEISTER ET AL. (2009)

The test below differs from the one described in the original publication.

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 test was described by Burgermeister et al. (2005, 2009).
1.2. The target region of the primer set is located in the 18S–28S[4] rDNA region.
1.3. Different life stages of nematodes can be used as nucleic acid source.
1.4. Primer set:

ITS1 F (F194)	5′-CGT-AAC-AAG-GTA-GCT-GTA-G-3′	Ferris et al. (1993)
ITS2 R (26S primer)	5′-TTT-CAC- TCG-CCG-TTA-CTA-AGG-3′	Vrain (1993)

1.5. The amplicon size for the 7 species of the *B. xylophilus* group included in the study varies from 914 to 1030 bp.
1.6. The test was initially developed on a 9600 Perkin Elmer thermocycler.

[4]Wrongly refered to as 26S in Vrain (1993).

2. Methods

2.1. Nucleic acid extraction and purification

2.1.1. Tissue source: adult females and males, juveniles
2.1.2. DNA extraction see Appendix 1 point 1.

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
Molecular grade water	N.A	31.1	N.A.
PCR buffer (MP Biomedicals buffer includes 1.5 mM of $MgCl_2$)	10×	5	1×
$MgCl_2$[a]	10 mM	2.5	2 mM[a]
dNTPs (Eurogentec)	25 mM	0.4	0.2 mM
ITS1 F (F194)	50 µM	0.3	0.6 µM
ITS2 R (26S primer)	50 µM	0.3	0.6 µM
Taq DNA polymerase (MP Biomedicals)	5 U/µL	0.4	2 U
DNA extract		10	
Total		50	

[a]This final concentration takes into account that the PCR buffer contains $MgCl_2$.

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).

RFLP restriction fragment length are given in Table A1.

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

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:

- 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

3.2. Interpretation of results

Verification of the controls:

- NIC and NAC no band is visualized.
- PIC and PAC should produce restricted fragments as given in Table A1.
- When relevant the IPC should produce the expected amplicon.

When these conditions are met:

- A test will be considered positive for *B. xylophilus* if restriction fragments of the expected size as given in Table A1. *for B. xylophilus* is visualized.

TABLE A1 Restriction fragment length polymorphism (from Burgermeister et al., 2005 except when specified otherwise).

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

[a]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).

- A sample will be considered negative for *B. xylophilus* if restriction fragments different from those expected for *B. xylophilus* are visualized.
- Tests should be repeated if any contradictory or unclear results are obtained

4. **Performance characteristics available**

The performance characteristics provided below were produced in the framework of the H2020 EU funded project, VALITEST. Data were obtained either from preliminary studies (PS) performed by the TPS organizer (Anses – Plant Health Laboratory) or from a Test Performance Study (TPS) involving 14 laboratories and using one panel of samples composed of 6 non-target and 4 target DNA samples of pure culture.

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 description and validation data can be found in the validation section of the EPPO Database on diagnostic expertise.

4.1. Analytical sensitivity data

One nematode (extraction method: lysis buffer from Ibrahim et al., 1994) (PS).

During the TPS, 86% of the participants detected pine wood nematode in the sample of DNA of pure culture at the maximum dilution (1/100) (participants were asked to use Taq polymerase from MP Biomedicals or equivalent) (TPS).

4.2. Analytical specificity data (PS)

Inclusivity.

100% evaluated with 5 populations of *B. xylophilus* originating from China, Portugal and Canada.

Exclusivity

100% evaluated with 19 populations of the following species included: *B. doui* (1), *B. fraudulentus* (1) *B. hoffmanni* (1), *B. mucronatus kolymensis* (5), *B. macromucronatus* (1), *B. mucronatus* (3), *B. sexdentati* (1), *B. singaporensis* (1), *B. vallesianus* (1), *B. willibaldi* (1), *B.* sp. (3 from China, France and Vietnam).

4.3. Diagnostic sensitivity (TPS)

96%.

4.4. Diagnostic specificity (TPS)

100%.

4.5. Data on repeatability (PS)

100% for 1 individual.

4.6. Data on reproducibility (PS)

100% for 1 individual (using 8 replicates of DNA solution tested in 3 independent runs performed by the same operator on 2 different days and using 2 thermocyclers).

4.7. Other information

Additional information on analytical specificity is available 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*.

APPENDIX 7 - SATELLITE DNA-BASED PCR TEST CASTAGNONE ET AL. (2005)

The test below differs from the one described in the original publication.

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 test was developed by Castagnone et al. (2005).
1.2. The primer set targets one family of satellite DNA of *B. xylophilus*.
1.3. The amplicon is a ladder of multimers of the 160-bp monomer unit (160; 320; 480 bp ….).
1.4. Primer set:

Forward primer	J10-1	5′-GGT-GTC-TAG-TAT-AAT-ATC-AGA-G-3′
Reverse primer	J10-2Rc	5′-GTG-AAT-TAG-TGA-CGA-CGG-AGT-G-3′

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

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Tissue source: different life stages of nematodes
2.1.2. Nucleic acid extraction

The publication refers to an extraction with the lysis buffer modified from Williams et al. (1992) described in Appendix 1.

The validation data presented in section 4 of this Appendix was obtained with Lysis modified from Ibrahim et al., 1994.

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
Molecular grade water		13.86	
PCR buffer (including 1.5 mM $MgCl_2$)	10×	2.5	1×
$MgCl_2$[a]	10 mM	2.5	2.5 mM[a]
dNTPs (Eurogentec)	25 mM[b]	0.2	0.2 mM
J10-1	50 µM[b]	0.37	250 ng equivalent to 1.48 µM
J10-2Rc	50 µM[b]	0.37	250 ng equivalent to 1.48 µM
Taq Polymerase (MP Biomedical, ex Qbiogene)	5 U/µL	0.2	1 U
DNA extract		5	
Total		25	

[a]Final concentration takes into account that the PCR buffer contains $MgCl_2$.
[b]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).

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:

- 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)
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC no band is visualized
- 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 test will be considered positive if the amplification of a ladder of multimers of the 160-bp monomer is visualized.
- A test will be considered negative, if no ladder or a ladder with bands of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. **Performance characteristics available**

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

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. Analytical sensitivity data

2–5 nematodes.

4.2. Analytical specificity data

Inclusivity

100% evaluated by Castagnone et al. (2005), with 3 isolates of *B. xylophilus* (Castagnone et al., 2005), and by Anses-LSV (2011) with 7 populations of *B. xylophilus*.

Exclusivity

100% No cross reaction was noted evaluated by Castagnone et al. (2005) with *B. leoni*, *B. mucronatus*, *B. tusciae*, and by Anses-LSV (2011) with 15 non-target organisms (see Table A2).

4.3. Data on repeatability

100%

4.4. Data on reproducibility

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

TABLE A2 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

APPENDIX 8 - REAL-TIME PCR PROTOCOL (FRANçOIS ET AL., 2007)

The test below differs from the one described in the original publication.

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 test was developed by François et al. (2007).
1.2. The primer set targets a 77 bp long amplicon of the target sequence from *Bursaphelenchus xylophilus* MspI satellite DNA monomeric unit (GenBank accession number L09652, Tarès et al., 1993).
1.3. The amplicon size is 77 bp long.
1.4. Primer set:

Primer names	Sequence	Amplicon size
BSatF	5′-TGA-CGG-AGT-GAA-TTG-ACA-AGA-CA-3′	77 bp
BSatRV	5′-AAG-CTG-AAA-CTT-GCC-ATG-CTA-AA-3′	
BsatS probe	5′-FAM-ACA-CCA-TTC-GAA-AGC-TAA-TCG-CCT-GAG-A-TAMRA-3′	

1.5. The test was initially developed on a DNA engine Opticon 2 (MJ Research) and on SmartCycler II (Cepheid, Sunnyvale, CA, US) for tests on wood extracts and a test performance study on individual nematodes.
1.6. 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 is purified using the phenol/chloroform method (Sambrook et al., 1989), quantified spectrophotometrically, aliquoted and stored at −80°C. Alternatively, DNA from a defined number of nematodes (1–2000 individuals) is extracted using a simplified procedure, as previously described (Castagnone et al., 2005, referring to an extraction with the lysis buffer modified from Williams et al. (1992) described in Appendix 1.1), 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
BSatF	50 µM[a]	0.1	200 nM
BSatRV	50 µM[a]	0.1	200 nM
Probe BsatS	50 µM[a]	0.1	200 nM
HotGoldStar DNA polymerase (Eurgentec qPCR core kit)	5 U/µL	0.1	0.5 U
Subtotal		24	
DNA extract		1	
Total		25	

[a] 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, annealing/elongation simultaneously at 59°C. The measure of fluorescence is performed at the end of annealing/elongation step.

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

Verification of the controls:

- The PIC and PAC 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

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

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. Analytical sensitivity data

One nematode (the test was evaluated with 10-fold dilutions of purified genomic DNA and down to 1 pg of DNA could be detected).

4.2. Analytical specificity data

Inclusivity.

100% evaluated with 13 populations of *B. xylophilus*.

Exclusivity.

100% no cross reaction observed with *B. antoniae*, *B. conicaudatus*, *B. fraudulentus*, *B. hofmanni*, *B. glochis*, *B. luxuriosae*, *B. mucronatus*, *B. pinophilus*, *B. sexdentati*, *B. tusciae*.

4.3. Data on repeatability

100% (at down to 1 pg based on three separate experiments).

4.4. Other information

B. xylophilus and *B. mucronatus* individual nematodes were mixed in varying proportions (*B. xylophilus* representing from 50% down to 1% of the sample). One single *B. xylophilus* was detected when mixed with 99 *B. mucronatus*. gDNA of *B. xylophilus* spiked in DNA extract derived from *B. mucronatus* (ratio 1:10.000) was also detected.

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 9 - REAL-TIME PCR TEST FROM FILIPIAK 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. Multiplex real-time PCR test for simultaneous identification of *Bursaphelenchus xylophilus, B. mucronatus* and *B. fraudulentus*.
1.2. This test was developed by Filipiak et al. (2019).
1.3. The target region of the primer set is located in the 18S – ITS1 rDNA region.

Universal primers and species-specific TaqMan probes used in the multiplex real-time PCR reactions with *Bursaphelenchus* spp.

Primer/ Probe name	Primer/probe sequence (5′–3′)	Location (nt)
For-univ	AACCTTCGGCTGGATCATTA	4–23[a]
Rev-univ	CTCGGGCTTTTCAATCCTAC	259–278[a]
Bx probe	ROX-CGATTGGTGACTTC GGTTG-BHQ	65–83[a]
Bm probe	HEX-ATGATGTGGGTTCG ATTCGT-BHQ	107–126[b]
Bf probe	6-FAM-CTTGCCGCTTAATT GTTCGT-BHQ	294–313[c]

[a]Corresponding to the sequence of *B. xylophilus* isolate T4 (GenBank accession no. AB277207).
[b]Corresponding to the sequence of *B. mucronatus* isolate Wro-01 (GenBank accession no. JF912332).
[c]Corresponding to the sequence of *B. fraudulentus* isolate PL-01 (GenBank accession no. EU543693).

1.4. Amplicon size in base pairs (including primer sequences)
The amplicon size is 275 bp long for *B. xylophilus,* 270 bp for *B. mucronatus,* and 318 bp for *B. fraudulentus*
1.5. The test was initially developed on a rotor gene 6000 cycler (Corbett research)
Rotor-Gene Q Series Software was used for data analysis.

2. Methods

2.1. Nucleic Acid Extraction and Purification

2.1.1. Tissue source: Isolated nematodes (from 1 to 100 individuals at various developmental stages, i.e. females, males and juveniles)
2.1.2. DNA isolation
see Appendix 1

2.2. Real-time PCR

2.2.1. Master Mix (concentration per 10 µL single reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	1.5	N.A.
Forward Primer (For-univ)	10 µM	0.5	0.5 µM
Reverse Primer (Rev-univ)	10 µM	0.5	0.5 µM
Probe 1 (Bx probe)	10 µM	0.5	0.5 µM
Probe 2 (Bm probe)	10 µM	0.5	0.5 µM
Probe 3 (Bf probe)	10 µM	0.5	0.5 µM
FastGene® Probe qPCR Universal Mix (Nippon Genetics)	2×	5	1×
Subtotal		9	
DNA extract		1	
Total		10	

2.2.2. PCR conditions: Initial denaturation at 95°C for 2 min, 40 reaction cycles of 95°C for 5 s, 58°C for 30 s, 72°C for 30 s, the measure of fluorescence is performed at the end of each 72°C incubation.

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 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: 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).

3.2. Interpretation of results:

Verification of the controls

- The PIC and PAC 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**

The validation was carried out in accordance with PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*.

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 description and validation data can be found in the validation section of the EPPO database on diagnostic expertise.

4.1. Analytical sensitivity data

100% for one single nematode. (the test can detect down to 30 fg of DNA).

4.2. Analytical specificity data

Inclusivity.

100%, with 11 populations of *B. xylophilus* (Filipiak et al., 2019).

Exclusivity.

100% no cross reaction noted, evaluated by Filipiak et al. (2019), with, 11 of *B. mucronatus*, 7 of *B. fraudulentus*, *B. pinophilus, B. piniperdae, B. populi* and *Parasitaphelenchus papillatus*.

4.3. Data on repeatability

100% for 1 individual.

4.4. Data on reproducibility

100% for 1 individual.