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Protecting the world's plant resources from pests

ISPM 27
ANNEX 10

ENG

DIAGNOSTIC PROTOCOLS INTERNATIONAL STANDARD FOR PHYTOSANITARY MEASURES 27

DP 10:
Bursaphelenchus xylophilus

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 10: *Bursaphelenchus xylophilus*

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1. Pest Information

The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle 1970, is the causal agent of pine wilt disease. *B. xylophilus* is believed to be native to North America, where it is widely distributed in Canada and the United States (Ryss *et al.*, 2005) and is apparently of limited distribution in Mexico (Dwinell, 1993). North American pine species are resistant or at least tolerant to *B. xylophilus*, but exotic species planted in North America, especially in the warmer southern areas of the United States, are killed when attacked by the nematode.

B. xylophilus was carried to Japan at the beginning of the twentieth century, presumably on timber exported from North America, and it became one of the most damaging forest pests in the country, where it still causes remarkable losses of pine trees (*Pinus densiflora*, *P. thunbergii* and *P. luchuensis*) today. *B. xylophilus* was also introduced to China (including Taiwan) and Korea; it was found there in the mid to late 1980s. In 1999, *B. xylophilus* was found for the first time in Europe (Portugal) on *P. pinaster*, which is killed by the nematode within a few months after infestation (Mota *et al.*, 1999; Fonseca *et al.*, 2012). *B. xylophilus* has also been detected on *P. nigra* and *P. radiata* in Portugal and Spain, respectively (Inácio *et al.*, 2014; Zamora *et al.*, 2015). In 2008, *B. xylophilus* was found for the first time in Spain (Abelleira *et al*., 2011).

B. xylophilus is transmitted from tree to tree by wood-inhabiting beetles of the genus *Monochamus* (Coleoptera: Cerambycidae) (Linit, 1990; Evans *et al.*, 1996). The nematodes enter the bodies of the insects shortly after the latter emerge from pupation and just before they bore out of the host tree (Wingfield, 1987). The beetles fly to the crown of healthy trees and feed on the young shoots and leaves (maturation feeding). They then mate and the females search for a weakened tree or one that has died recently, or for trunks or bigger branches (including felling debris), depending on the *Monochamus* species, where they lay their eggs through the bark. The beetle larvae that hatch from the eggs feed in the cambial tissues just below the bark for several months. On reaching maturity, they bore deeper into the wood to pupate, and thus their life cycle is completed. *B. xylophilus* takes advantage of this life cycle to obtain transport to new host trees (Wingfield, 1987). Their introduction into the new tree may take place during oviposition by the beetle (this appears to be the only means of transmission for several species of *Bursaphelenchus* that colonize dead trees) (Edwards and Linit, 1992). *B. xylophilus*, however, seems to be unique among these species in that it can also be transmitted to a new tree during maturation feeding by beetles, and the development of pine wilt disease can occur as a consequence of transmission through the young shoots (Wingfield, 1987).

When *B. xylophilus* is transmitted during oviposition, the nematodes remain relatively close to the site of introduction. But when transmission occurs through the young shoots and when the tree succumbs to pine wilt disease, the nematodes distribute throughout the whole tree, destroying wood tissues such as epithelial cells, parenchyma cells of axial and radial resin canals, cambium and phloem. *B. xylophilus* can also be found in roots, even when the above-ground part of the tree is already dead, dried out or felled. Whether the tree develops pine wilt disease depends on the tree species (in general only *Pinus* spp. of non-American origin are affected), its state of health and the climatic conditions (particularly temperature and water supply). These factors also influence the distribution of nematodes throughout the tree: their distribution can be localized or irregular and this needs to be taken into account in sampling strategies (Schröder *et al*., 2009).

B. xylophilus can also be found in dead trees of *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* and other conifers (except *Thuja* spp.), but none of these genera is known to be affected by pine wilt disease, although pathogenicity tests on seedlings show remarkable reactions, including death (Evans *et al*., 1996).

B. xylophilus is almost exclusively vectored by *Monochamus* species, with the vector species varying among the geographic regions; for example, *M. alternatus* in China and Japan, *M. saltuarius* in Japan, *M. carolinensis* in North America and *M. galloprovincialis* in Portugal. Occasionally, other beetles of the family Cerambycidae or other Coleoptera have been found to carry "dauer" juveniles of the

nematode on their bodies, but there is no evidence that they play a role as vectors in the dissemination of the nematode (Evans *et al*., 1996).

Human activity is known to be the principal route for dispersal of *B. xylophilus* over greater distances and *B. xylophilus* and its vectors have been intercepted on a number of occasions in the international trade of wood, wood products and, most notably, solid wood packaging made from conifers. Therefore, the risk of further international spread is high.

Though *B. xylophilus* associated with the vector beetles poses the highest risk of spread, movement of *B. xylophilus* from infested wood to non-infested wood or to uninfested trees can occur under specific circumstances: direct contact from donor to receiving wood, high moisture content of receiving wood or wounds on receiving trees (Sousa *et al.*, 2011; Hopf and Schroeder, 2013).

More details about the biology of *B. xylophilus*, its vectors, pine wilt disease, geographical distribution, trade and economic impacts, and management strategies can be found in the following comprehensive books: Kishi (1995); Mota and Vieira (2004); Mota and Vieira (2008); and Zhao *et al.* (2008).

2. Taxonomic Information

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

Synonyms: *Aphelenchoides xylophilus* Steiner and Buhrer, 1934
Paraphelenchoides xylophilus (Steiner and Buhrer, 1934) Haque, 1967
*Bursaphelenchus lignicolu*s Mamiya and Kiyohara, 1972

Taxonomic position: Nematoda, Rhabditida, Tylenchina, Aphelenchoidea, Aphelenchoididae, Parasitaphelenchinae, *Bursaphelenchus*

Common name: Pine wood nematode

3. Detection

B. xylophilus has six life stages: the egg and four juvenile stages preceding the adult. The first juvenile stage (J1) moults to the second juvenile stage (J2) in the egg. J2 hatches from the egg, and there are two more juvenile stages (J3 and J4) preceding the adult. Different types of juvenile stages appear under different conditions. Under favourable conditions at 25 °C *B. xylophilus* develops from the egg through four propagative juvenile stages (J1 to J4) to reach the adult stage within four days (Hasegawa and Miwa, 2008) (Figure 1).

Under unfavourable conditions, the J_{III} dispersal stage develops in place of the J3 stage. J_{III} is probably a non-feeding stage. It has lipids accumulated in the intestinal cells (Kondo and Ishibashi, 1978) and can survive unfavourable conditions such as drought, low temperature or lack of nutrition. Normally this stage moults into the J_{IV} dispersal stage (dauer juveniles), which is transmitted by vector beetles to new trees. Nevertheless, if the conditions become suitable for nematode development, for example by putting the J_{III} stage on fungal culture plates, the nematodes develop to the J4 propagative juvenile stage (Wingfield *et al*., 1982).

Living *B. xylophilus* can be found in various types of wood of host species, including standing or fallen trees, round wood, sawn wood, and wood products such as coniferous wood packaging material, as well as in saw dust, wood chips and particles, wood waste, untreated furniture and handicrafts. The following sections give specific information on detection of *B. xylophilus* in trees, wood and wood products as well as in its vector beetles. Although correct sampling is essential for obtaining material with the highest likelihood of being infested with *B. xylophilus*, guidance on sampling is not part of the current protocol. General guidance on sampling with reference to the European *Monochamus* species as vectors was published by Schröder *et al.* (2009) and EPPO (2012).

3.1 Detection in trees

If it is not known whether *B. xylophilus* occurs in an area, sampling should be focused on trees near high-risk sites; for example, ports handling imports from countries with known *B. xylophilus* infestation, airports, sawmills, wood processing facilities, places where wood is stored, and areas where forest fires have occurred (*Monochamus* is attracted by forest fires).

To have the best chance of detecting *B. xylophilus* in an area, it is advisable to concentrate sampling on pine trees that are dying or have died recently (Figures 2 and 3), both of which may be standing or fallen. Trees and cut waste from a recent felling season (i.e. one to two year old logging sites) that have been colonized after the felling by *Monochamus* beetles may also be used as sampling material. The following symptoms should be searched for: discoloration (e.g. yellowing) of needles, wilting, evidence of insect attack (e.g. wood shavings on the ground or protruding from cracks in the bark, flat-headed larvae of *Monochamus* beneath the bark, surface galleries beneath the bark with oval entrance holes oriented in the longitudinal direction of the stem, the round exit holes of adults), blue stain fungal growth in the wood and lack of oleoresin flow from wounds. The rate of oleoresin flow should be checked while the trees are still green by removing part of the bark from the cambial layer. Healthy trees will cover the wood surface with resin within one hour while no or reduced resin flow will occur in infested trees. However, these symptoms vary between species of pine and are non-specific for *B. xylophilus* as they may be caused by other pathogens or by physical factors. There is currently no method to visually distinguish between trees that are dying from pine wilt disease and those dying for other reasons. Trees to be sampled preferably should be associated with *Monochamus* attack, either maturation feeding or breeding, but at the least, it should be known that *Monochamus* species occur in the area where samples are to be taken.

The distribution of the nematodes can be localized within the trees, especially shortly after they have been introduced by oviposition or by the maturation feeding of the beetle vector. In cases of pine wilt disease, nematodes can spread rapidly to produce large numbers in all parts of the tree except the needles, cones and seeds. *B. xylophilus* also invades the root system and can survive there for a certain period when the tree is already dead and desiccated or has been felled. However, in non-susceptible trees, under unfavourable climatic conditions or in particular physiological states of the tree, *B. xylophilus* attack can remain limited in distribution within the trees; for example, an infestation of *B. xylophilus* may already be established in the crown or parts of the crown without further spread to other tree parts.

3.2 Detection by the use of insect traps, trap logs and in samples from sawmills and timber yards

Insect traps with lures for attracting *Monochamus* species have been developed in recent years and can be used for monitoring as well (Sanchez-Husillos *et al.*, 2015). When using *Monochamus* traps to collect beetles to be investigated for potential *B. xylophilus* infestation, the beetles need to be caught alive and not in a liquid killing agent.

In areas with a known population of *Monochamus* beetles, logs felled during the flight period of the beetles may be used as trap logs. Beetles are attracted to them for oviposition and it has been proven that nematode transmission will take place in such cases (Dwinell, 1997; Luzzi *et al.*, 1984). The wood or the emerged beetles can be sampled to monitor the presence of *B. xylophilus* in a limited area. Beetles can complete their life cycle in such material. It is also possible to accelerate beetle development by taking the trap logs to the laboratory in autumn: beetles will emerge several weeks before they would have emerged under natural conditions.

Collection of wood samples, shavings or wood chips from sawmills and wood yards might be more successful than sampling standing trees. Such samples may have come from a very wide area because large sawmills might obtain their wood from far away and process both domestic and imported wood. But this is also a disadvantage in that a correlation between a positive sample and the area of origin might be difficult to determine.

3.3 Direct detection in wood, wood products and solid wood packaging

All types of coniferous wood, especially solid wood packaging, particularly from countries in which *B. xylophilus* occurs, can be sampled by low-speed drill, borer, saw, axe, hook and so forth. Sampling should be concentrated on pieces with circular grub holes (i.e. the emergence holes of beetles) and oval entrance holes and larval tunnels, which are sometimes blocked with wood particles. Removal of bark when present may help detect galleries. In the case of sawn wood, normally no exit holes will be seen, but larval tunnels may be seen, which are sometimes difficult to detect because they are blocked with shavings. Pieces with fungal growth, especially blue stain fungus, should be sampled. Nevertheless, several interceptions have shown that living *B. xylophilus* can be detected in samples without the above-mentioned indications (EPPO, 2012).

Solid wood packaging (e.g. pallets) can come into contact with soil during service. This may lead to surface contamination with soil and soil-inhabiting nematodes, which can survive desiccation. To avoid a contamination of the extracted wood sample with those nematodes, the sample should be investigated after removal of the outer part of the wood (Schröder *et al*., 2009).

3.4 Extraction of nematodes from wood samples

Living nematodes can be extracted from infested wood using the Baermann funnel technique or the modified Baermann funnel technique (Penas *et al.*, 2002; EPPO, 2013c). In the Baermann funnel technique, a glass or plastic funnel with the narrow tube at the base closed by means of a rubber tube and a clamp is filled with water. The sample consisting of small pieces of wood or wood shavings is supported on a sieve in the funnel. A paper tissue permeable for nematodes is placed on the sieve to avoid contamination of the water with wood debris. The funnel is then filled with water to cover the sample. The sample is left for 24 to 48 h at room temperature or in an incubator (both at approximately 25 °C), during which time nematodes migrate from the wood into the water and fall to the base of the funnel from where they can be collected by releasing a small quantity of the water (approximately 10 ml) into a small dish.

The principle of the Baermann funnel technique is as described above, but several modifications are used in practice (EPPO, 2013c). For instance, wood chips can be directly submerged in water or they can be placed on a cotton wool filter laid in a plastic basket for extraction of nematodes. In addition, each method described in EPPO (2013c) can be combined with a mistifier spray apparatus.

Under a stereoscopic microscope and using a pipette or a needle the nematodes can be transferred from the small Petri dish to a glass slide for examination under a high power microscope.

Nematodes may occur in very low numbers in the sample, so detection might be difficult. It is recommended to allow the nematodes to multiply before extraction. To do this, the moistened wood sample without any bark is sealed in a plastic bag and incubated at approximately 25 °C for two to three weeks. The nematodes are then extracted with the Baermann funnel technique.

The principle of the Baermann funnel technique is based on detecting living nematodes when they exit the wood sample, but within the recommended 24 to 48 h some nematodes die (Baermann, 1917). Nevertheless, one can be sure that those were alive when the extraction was started. This has to be kept in mind when analysing imported wooden material. Some other extraction methods – for example a centrifugation method (not described here; much faster than the Baermann funnel technique) – will also extract nematodes that were already dead in the wood (Moens, 2000). The centrifugation method can be used to monitor an area with *B. xylophilus* infestation but not to prove that wood has undergone a successful phytosanitary treatment (Moens, 2000).

3.5 Extraction of nematodes from vector insects

Beetles of the genus *Monochamus* caught by traps (Pajares *et al*., 2004; Ibeas *et al*., 2007) or trap logs can be assessed for the presence of nematodes (section 3.2). The beetles need to be caught alive and not in a liquid killing liquid agent, unless they are to be used for direct molecular detection.

Nematode juveniles are usually present as J_{IV} dispersal stage (dauer juveniles) in the tracheae and on the body of the beetles. J_{IV} dauer juveniles do not have a stylet. To isolate the nematodes, the beetles are dissected and crushed in an appropriate dish and kept in water for 24 to 48 h at approximately 25 °C (Sousa *et al.*, 2001; EPPO, 2013c). Dauer juveniles will leave the beetles. J_{IV} dauer juveniles need to be transferred to fungal mats of *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea)* grown on malt agar (section 4.1.1) to enter the propagative life cycle because further morphological identification can only be done on adult nematodes. Alternatively they can be used directly for molecular identification. The Baermann funnel technique may also be used to extract the nematodes from the beetles.

Nematodes extracted from wood or insect vectors as described above can be morphologically examined, or molecular testing for *B. xylophilus* can be performed directly on the extracts. EPPO (2013b) reports a screening procedure based on a modified Baermann extraction method followed by a real-time polymerase chain reaction (PCR) test (adapted from François *et al*., 2007).

There are also several reports of molecular detection methods for which DNA from *B. xylophilus* is extracted directly from wood before amplification (Takeuchi *et al*., 2005; François *et al*., 2007; Kikuchi *et al*., 2009; Hu *et al*., 2011; Kanetani *et al*., 2011; Cardoso *et al*., 2012). However, in these reports, the amount of wood used for the DNA extraction ranges from 5 to 120 mg, which is very small compared with the size of the wood samples that are routinely analysed. In addition, this direct detection approach by molecular assay would detect any target nematode, alive or dead. Consequently, users of this approach should have defined procedures in place to confirm the presence of living nematodes in the sample, if appropriate for the aim of the analysis.

4. Identification

To date, about 110 species of the genus *Bursaphelenchus* have been described (Futai, 2013). The latest overviews can be found in Ryss *et al.* (2005), Hunt (2008), Braasch *et al.* (2009) and Futai (2013). *B. xylophilus* can be positively identified by either one of two methods: that based on morphological features and that based on molecular biology techniques. Although the number of *Bursaphelenchus* species described in recent years has increased and some of them have similar morphological characters, a determination based on morphology is possible in most cases. However, identification of the mucronate form of *B. xylophilus* based on morphological characters may be difficult.

Identification based on morphological features requires preparation of good quality microscope slides, access to a high power microscope and considerable experience in nematode taxonomy, especially in the small group of species closely related to *B. xylophilus* (*B. mucronatus mucronatus, B. mucronatus kolymensis, B. fraudulentus* and others). Identification methods based on molecular biology require expensive equipment and reagents, but can be applied with less technical experience (and very little nematological training). Adequate experience is, however, needed to ensure that the limited nematode material is not lost during the procedure. While morphological identification is based on adult specimens, molecular identification can be made even if only juvenile stages or one sex of adults are available, which is an advantage. While DNA-based PCR methods fail to differentiate between dead and living nematodes, new methods based on mRNA can clarify whether the positive detection originates from living nematodes (Leal *et al.*, 2013).

B. xylophilus can be identified by a nematologist or an experienced phytopathologist with a nematological background using morphological features if the specimens are available as male and female adults and in good condition. However, there may be situations where a combination of morphological features and molecular information is recommended to obtain a higher degree of certainty on the identification; for example, when *B. xylophilus* has been detected in a new area, when *B. xylophilus* has been found by a laboratory for the first time, as quality assurance for compliance with certification schemes, and when *B. xylophilus* is found in consignments during import inspection, especially when the exporting country has been declared to be free from *B. xylophilus*. In addition, *B. xylophilus* can show morphological variations that may make the use of molecular biology techniques necessary; for example, round or mucronate tail tip of females (Figure 4) or the position of

the excretory pore. When only a small number of nematodes have been isolated, multiplying them on *B. fuckeliana* before identification is recommended to obtain enough material for a reliable identification (section 4.1.1).

4.1 Morphological identification

Numerous nematode species may be present in an aqueous extract from coniferous wood, especially if decay of the tissues has begun. Some of these will be saprophagous species where adult nematodes lack the stylet that is typical for nematodes of the orders Tylenchida, Aphelenchida and Dorylaimida. *Bursaphelenchus* species belong to the Aphelenchida, which have the dorsal pharyngeal gland opening into the metacorpus, in contrast to the Tylenchida, where the gland opens into the lumen of the pharynx between the bulb and the stylet (Figure 4). If the extract contains only juveniles, morphological identification of *B. xylophilus* will not be possible. In such cases, aphelenchoide species that fall in the range of *B. xylophilus* juvenile size (see, e.g., Penas *et al.*, 2008) should be separated and either multiplied on a culture plate or used directly for molecular identification.

For identification under a light microscope, a magnification of 400× to 1 000× (oil immersion lens) is recommended. Differential interference contrast (DIC) may facilitate observations.

4.1.1 Preparation of specimens

It may be necessary to multiply the extracted nematodes to obtain enough material for identification. Most *Bursaphelenchus* species can be cultured on the sporulating form of the fungus *B. fuckeliana*. Some species, especially those belonging to the *sexdentati* group, require culture on the non-sporulating form. Both fungal forms are cultured on 2% malt extract agar (MEA) medium (15 g agar-agar, 15 g malt extract, 750 ml water; pH 7.0). Petri dishes (90 mm diameter) are filled with 25 ml sterilized MEA. Either fungal spores or pieces of agar with fungal growth are transferred to the Petri dishes in a clean bench unit. Incubation of the fungal plates is recommended at room temperature (approximately 25 °C). Nematodes to be reared are transferred in a small droplet placed on the mycelium using a pipette or other means. Nematode incubation is recommended at approximately 25 °C (based on its biology), which leads to a sufficient reproduction rate to obtain enough adult and juvenile individuals.

4.1.1.1 Temporary preparations

Temporary preparations for quick identification or study of features best seen in unfixed specimens are prepared as follows. Living specimens are transferred to a small drop of water on a glass slide. The slide is briefly heated over a spirit flame, checking frequently for nematode movement. Heating should be stopped as soon as the specimens stop twitching. A coverslip is applied and the slide is ready for study. Fixing the coverslip is not recommended as the body of the male nematodes may have to be moved subsequently into the dorso-ventral position to see the bursa.

4.1.1.2 Permanent preparations

Permanent preparations for identification under light microscopy are prepared as follows. Living nematodes extracted from plant material or nematode rearing are killed by gentle heat, fixed in FAA fixative (35% distilled water, 10% of 40% formalin, 5% glacial acetic acid, 50% of 95% alcohol) (Andrássy, 1984) or triethanolamine and formalin (TAF) fixative (7 ml formalin (40% formaldehyde), 2 ml triethanolamine, 91 ml distilled water), processed to anhydrous glycerine (for long-term storage) and mounted on slides in anhydrous glycerine as described by Seinhorst (1959) and Goodey (1963). A more rapid method (1–1.5 h) to prepare permanent slides was described by Ryss (2003) based on killing the nematodes in hot 4% formaldehyde solution. Fixation then takes place at different temperatures in a programmable thermal controller, followed by processing to glycerine. More details on preparing nematode specimens and permanent slides, including recipes for fixatives, can be found in van Bezooijen (2006), which is freely available on the Internet.

4.1.2 Key to species level

The following key, partly derived from Bongers (1989), is used to determine the subfamily of female specimens. The key within the subfamily Parasitaphelenchinae to determine the genus *Bursaphelenchus* is adapted from Hunt (2008). The key within the genus *Bursaphelenchus* for the *xylophilus* group is cited from Braasch *et al.* (2009). Alternatively, a simple key, which has been established by consensus in the European and Mediterranean Plant Protection Organization (EPPO) region and is widely used, is available in the EPPO diagnostic protocol for *B. xylophilus* (EPPO, 2013b).

Definitions of terminology used in the following sections can be found in EPPO's *Diagnostic protocols for regulated pests: Pictorial glossary of morphological terms in nematology* (EPPO, 2013a).

4.1.2.1 Key to families or subfamilies

1. Nematode with spear or stylet **2**

– Nematode without spear or stylet ..... **NBS**

2. Mouth with tylenchid stylet, pharynx with metacorpus **3**

– Mouth with dorylaimid stylet, pharynx cylindrical or bottle-shaped, without metacorpus ..... **NBS**

3. Metacorpus with metacorpal plates **4**

– Metacorpus without conspicuous metacorpal plates ..... **NBS**

4. Procorpus clearly separated from metacorpus by a constriction **5**

– Procorpus and metacorpus not separated by a constriction, basal bulb strongly reduced, cuticle conspicuously annulated ..... **NBS**

5. One gonad (vulva posterior) **6**

– Two gonads ..... **NBS**

6. Lip region without setae **7**

– Lip region with setae ..... **NBS**

7. Metacorpus strongly muscular and conspicuously well developed, clearly visible at low magnification, ovoid to rounded rectangular, dorsal pharyngeal gland opens into lumen of pharynx within metacorpus **8**

– Metacorpus normal, dorsal pharyngeal gland opens into lumen of pharynx just behind stylet ..... **NBS**

8. Pharyngeal glands overlap intestine dorsally **9**

– Pharyngeal glands within abutting bulb ..... **NBS**

9. Male tail tip enveloped by a small, bursa-like flap of cuticula (seen only when nematode is lying in the dorso-ventral position) **10**

– No bursa-like flap of cuticula ..... **NBS**

10. Stylet knobs usually present, female with anus ..... **Parasitaphelenchinae**

– Stylet knobs usually not present, female without anus ..... **NBS**

4.1.2.2 Key to subfamily Parasitaphelenchinae

11. In most species, J_{III} or J_{IV} dauer juveniles phoretically associated with insects; vulva posterior (usually 60–80% of body length); spicules partially fused or separated; male tail strongly recurved; bursa present in most species ..... ***Bursaphelenchus***

– J_{IV} dauer juveniles; vulva very posterior (80–90% of body length); spicules partially fused; male tail not strongly recurved; bursa present..... **NBS**

4.1.2.3 Key to genus **Bursaphelenchus**

12. Vulva with prominent flap; spicules long, slender and semicircular with angular lamina in posterior third, capitulum flattened with small condylus and distinct rostrum, cucullus usually present; lateral field with four lines ..... ***xylophilus* group**

– Characters different ..... **Not *xylophilus* group**

4.1.2.4 Key to **xylophilus** *group*

Within the *xylophilus* group the following key (amended according to EPPO (2013b, 2014)) can be used to distinguish *B. xylophilus* extracted from wood and bark from other *Bursaphelenchus* species of the same group. More details concerning the other species belonging to the *xylophilus* group can be found in Braasch and Schönfeld (2015). The *xylophilus* group also contains species that do not originate from coniferous wood (e.g. *B. populi*); these can be excluded simply by determining the species of the wood. Rearing nematodes on agar plates with fungi may increase the variability of the female tail.

13. Female tail broadly subcylindrical, with or without mucro (Figures 4 and 5) **14**

– Female tail conical (Figure 6) or strongly tapering, with or without mucro ..... **Not *B. xylophilus***

14. Spicule length <30 µm (measured from condylus to distal end)..... **15**

– Spicule length >30 µm ..... **Not *B. xylophilus***

15. Spicule with long and pointed rostrum, limbs of spicule with an angular curvature (Figures 5(C) and 7)..... **16**

– Spicule with short and pointed rostrum, limbs of spicule with a rounded curvature . **Not *B. xylophilus***

16. Female vulval flap straight, not ending in a deep depression (Figures 5(G) and 8)..... **17**

– Female vulval flap ending in a deep depression (Figure 9(A)) ..... **Not *B. xylophilus***

17. Female tail with mucro >3 µm (Figures 4(c) and 10(d))..... **18**

– Female tail without mucro (Figures 5(H) and 4(a)) and with or without a small projection <2 µm* (Figures 4(b) and 5(I)–(J))..... ***B. xylophilus* (round-tailed form)**

18. Excretory pore at or behind metacorpus..... ..... ***B. mucronatus kolymensis* and *B. xylophilus* (mucronated form**)**

– Excretory pore anterior to metacorpus ..... **Not *B. xylophilus***

NBS, not *Bursaphelenchus* species.

* In some populations of *B. fraudulentus*, females with a small projection or even without mucro may be found (Figure 9(B)). If the wood species where nematodes occur is not certain (*B. fraudulentus* occurs in deciduous wood but has also been found in larch, though not in pine) molecular testing is recommended.

** 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*; that is, *B. mucronatus kolymensis* (Braasch *et al.*, 2011).

If the position of the excretory pore is not discernible, an identification based on morphological characters may be incorrect. In such cases, molecular tests should be performed.

B. xylophilus has the general characters of the genus *Bursaphelenchus* (Nickle, 1970; Hunt 2008): about 1 mm in length, slender; cephalic region high, offset by a constriction, and with six lips; stylet well developed, usually with small basal thickenings; metacorpus well developed (Figures 11 and 5(F)); male tail terminus strongly curved ventrally, conoid, with a small terminal bursa that can be seen in the dorso-ventral position (Figure 12); spicules robust, rose thorn-shaped, usually with a prominent apex and rostrum; gubernaculum absent (Figures 7 and 10); vulva 70–80% of the body length; post-uterine sac well developed (Figure 5(A)).

Most populations of *B. xylophilus* are round-tailed and can be distinguished from other *Bursaphelenchus* species by the presence of the following three characters (Figure 10). (1) Males of *B. xylophilus* (Figure 7) have relatively large spicules, evenly arcuate, with a sharply pointed prominent rostrum and cucullus (disc-like projection) at the distal ends of the spicules. (2) The tail of the females is subcylindrical with a broadly rounded terminus (Figure 4(a)), normally without a mucro (small projection), but occasionally females of round-tailed populations have a mucro on their tail terminus, which is usually less than 2 μm (Figure 4(b)). (3) The vulva has a long, overlapping anterior lip (Figure 8).

However, females of the mucronate populations generally have a mucro (1.5–4.2 μm) at the tail terminus (Figure 4(c)).

Characters best seen by scanning electron microscopy are four incisures (Figure 13) in the lateral field, and the number and position of caudal papillae in males (Figure 14): an adanal pair just before the anus, two post-anal pairs just before the origin of the bursa, and a single median papilla just preanal. These characters sometimes can barely be seen by light microscopy. Figures 13 and 14 are electron micrographs illustrating these two characters as they are cited in section 4.1.3 for grouping *Bursaphelenchus* species in the *xylophilus* group.

Measurements of morphological characters of *B. xylophilus* are given in Table 1.

Table 1. Measurements (mean, and range in parentheses) of *Bursaphelenchus xylophilus* characters

Males					
Author Character	Nickle *et al.* (1981) (*n* = 5) (United States)†	Mamiya and Kiyohara (1972) (*n* = 40) (Japan)†	Mota *et al.* (1999) (*n* = 12) (Portugal)†	Penas *et al.* (2008) (*n* = 20) (Portugal)†	Penas *et al.* (2008) (*n* = 20) (Portugal)‡
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)	1.04 (0.87–1.17)
a (body length / greatest body diameter)	40.8 (35–45)	42.3 (36–47)	49.4 (44–56)	46.0 (40.2–58.5)	45.7 (41.3–48.9)
b (body length / distance from anterior to pharyngo-intestinal valve)	9.4 (8.4–10.5)	9.4 (7.6–11.3)	13.3 (11.1–14.9)	9.6 (8.2–10.7)	13.7 (11.6–15.4)
c (body length /	24.4 (21–29)	26.4 (21–31)	28.0 (24–32)	21.6 (19.1–24.6)	26.8 (23.6–31.4)

Males					
Author Character	Nickle *et al.* (1981) (*n* = 5) (United States)†	Mamiya and Kiyohara (1972) (*n* = 40) (Japan)†	Mota *et al.* (1999) (*n* = 12) (Portugal)†	Penas *et al.* (2008) (*n* = 20) (Portugal)†	Penas *et al.* (2008) (*n* = 20) (Portugal)‡
tail length)					
Stylet, µm	13.3 (12.6–13.8)	14.9 (14–17)	12.6 (11–16)	11.0 (10–14)	14.0 (12–15)
Spicules, µm	21.2 (18.8–23.0)	27.0 (25–30)	24 (22–25)	19.3 (16.5–24.0)	30.4 (25.0–33.5)

Females					
Author Character	Nickle *et al.* (1981) (*n* = 5) (United States)†	Mamiya and Kiyohara (1972) (*n* = 30) (Japan)†	Mota *et al.* (1999) (*n* = 12) (Portugal) †	Penas *et al.* (2008) (*n* = 20) (Portugal) †	Penas *et al.* (2008) (*n* = 20) (Portugal)‡
Length (L), mm	0.52 (0.45–0.61)	0.81 (0.71–1.01)	1.05 (0.89–1.29)	0.58 (0.51–0.66)	1.13 (0.91–1.31)
a (body length / greatest body diameter)	42.6 (37–48)	40.0 (33–46)	50.0 (41–58)	41.9 (32.8–50.6)	45.6 (39.4–50.3)
b (body length / distance from anterior to pharyngo-intestinal valve)	9.6 (8.3–10.5)	10.3 (9.4–12.8)	13.8 (12.7–16.4)	10.1 (9.1–11.2)	14.7 (11.6–16.8)
c (body length / tail length)	27.2 (23–31)	26.0 (23–32)	26.6 (22–32)	25.4 (20.2–29.0)	28.1 (21.9–34.4)
Stylet, µm	12.8 (12.6–13.0)	15.9 (14–18)	12.3 (11–15)	11.2 (10.0–12.5)	14.4 (12–16)
Vulva position (V), % of L	74.7 (73–78)	72.7 (67–78)	73.3 (70–76)	71.5 (70.1–72.9)	72.6 (70.4–74.5)

† Nematodes after extraction from natural host substrate.

‡ Nematodes grown on fungal culture for one year.

4.1.3 Comparison of *Bursaphelenchus xylophilus* with similar species

Keys for the determination of *Bursaphelenchus* species are available (e.g. Ryss *et al.*, 2005), but both of those in Ryss et al. (2005) suffer from the disadvantage that early descriptions of *Bursaphelenchus* species are incomplete or based on few specimens. See Vieira *et al.* (2003) for the original descriptions of 74 *Bursaphelenchus* species.

B. xylophilus is one species of the *xylophilus* group *sensu* Braasch (2001). Although there is current debate among taxonomists on the number of species within this group, at least 15 species or subspecies (as at April 2015) belong to the *xylophilus* group based on the number of lateral lines (Figure 9), the number and position of caudal papillae and spicule characteristics, and the large vulval flap (Gu *et al*., 2005; Ryss *et al*., 2005; Braasch *et al*., 2009; Braasch and Schönfeld, 2015). At least two *Bursaphelenchus* species (*B. trypophloei* Tomalak & Filipiak, 2011 and *B. masseyi* Tomalak, Worrall & Filipiak, 2013) were recently proposed to be added to the *xylophilus* group; however, this protocol follows the last grouping of Braasch and Schönfeld (2015), who did not consider these species to be valid members of the group because of their spicule morphology. Therefore, the members of the *xylophilus* group are:

- *B. xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970
- *B. fraudulentus* Rühm, 1956 (Goodey, 1960)
- *B. mucronatus mucronatus* (Mamiya & Enda, 1979) Braasch, Gu & Burgermeister, 2011
- *B. mucronatus kolymensis*, Braasch, Gu & Burgermeister, 2011
- *B. conicaudatus* Kanzaki, Tsuda & Futai, 2000
- *B. baujardi* Walia, Negi, Bajaj & Kalia, 2003
- *B. luxuriosae* Kanzaki & Futai, 2003
- *B. doui* Braasch, Gu, Burgermeister & Zhang, 2004
- *B. singaporensis* Gu, Zhang, Braasch & Burgermeister, 2005
- *B. macromucronatus* Gu, Zheng, Braasch & Burgermeister, 2008
- *B. populi* Tomalak & Filipiak, 2010
- *B. paraluxuriosae* Gu, Wang & Braasch, 2012
- *B. firmae* Kanzaki, Maehara, Aikawa & Matsumato, 2012
- *B. koreanus* Gu, Wang & Chen, 2013
- *B. gillanii* Schönfeld, Braasch, Riedel & Gu, 2013

B. xylophilus can be separated into two forms or populations: round-tailed and mucronated (Gu *et al.*, 2011) (Figure 4). Mucronated populations are mainly found in North America and are very similar to *B. mucronatus kolymensis*.

The 15 species or subspecies of the *xylophilus* group can be distinguished from all other *Bursaphelenchus* species by the shape of the male spicules and by the presence in the female of a vulval flap with a characteristic shape. To separate *B. xylophilus* from the 14 other species in the group, the female tail shape (subcylindrical to cylindrical with a normally round terminus, and absence of a mucro) can be used. A detailed key to all species of the *xylophilus* group, including drawings of the main characters, can be found in Braasch and Schönfeld (2015). All other species of the *xylophilus* group have either a conical or a mucronate female tail. However, a few mucronate populations of *B. xylophilus* exist in North America and are difficult to differentiate morphologically from other mucronate species (Figure 4). In addition, *B. xylophilus* females from laboratory cultures normally show a typical round tail terminus, whereas strains obtained from infested or artificially inoculated trees may contain females with mucros of variable length beside round-tailed females (Figure 4). More details on this subject can be found in Gu *et al.* (2011).

The most widespread species in the *xylophilus* group are *B. mucronatus mucronatus* and *B. mucronatus kolymensis*. They are distributed throughout Europe and Asia and also occur in Canada

(Ryss *et al*., 2005). Therefore, it is probable that the most frequent differentiation will be between *B. xylophilus* and *B. mucronatus mucronatus* or *B. mucronatus kolymensis* (Figures 6 and 10).

Reference cultures of 50 *Bursaphelenchus* species, including 41 *B. xylophilus* strains from different origins across the world, are available in the *Bursaphelenchus* culture collection at the Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for National and International Plant Health, Braunschweig, Germany.

4.2 Molecular identification

This section provides information on molecular tests that allow the identification of *B. xylophilus* from isolated nematodes. The tests are generally performed following a morphological examination in order to confirm the results obtained. In the following subsections different types of tests are presented that address specific issues, as described at the beginning of each section.

Many methods are available for the identification of *B. xylophilus*. The molecular tests described hereafter are those recommended at the time of drafting the protocol. Other tests may be performed. Molecular identification can be performed by conventional PCR (section 4.2.2) or by real-time PCR (section 4.2.3) methods. All these techniques, particularly internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) (section 4.2.1), have been used successfully in laboratories throughout the world, but have not, so far, been evaluated by a ring test. A loop-mediated isothermal amplification (LAMP) test (section 4.2.5) was developed for direct detection and identification of the target nematode from wood.

The most recent approach for molecular identification relies on sequencing and barcoding analysis (section 4.2.8). This approach requires access to sequencing facilities and to reliable sequences (such as those found in Q-bank, (http://www.q-bank.eu/Nematodes/) as well as highly skilled staff to analyse the sequences in such a way as to avoid false results.

When molecular techniques are used to detect *B. xylophilus* in wood products for quarantine purposes, it is critical to distinguish between living and dead nematodes. Several phytosanitary treatments kill *B. xylophilus* in wood, and current DNA-based detection methods are unable to differentiate whether a positive result is due to living nematodes or DNA remnants of dead nematodes. The use of molecular methods based on RNA that can distinguish between living and dead nematodes present in wood is preferable for questions of quarantine regulation (Leal *et al*., 2013) (section 4.2.4). This problem needs to be taken into account when choosing the nematode extraction method (e.g. the Baermann funnel technique relies on living nematodes; see sections 3.4 and 3.5) and the molecular technique for determination. Whenever possible, a positive molecular result should be validated by morphological identification.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 ITS-PCR RFLP

Burgermeister *et al*. (2005, 2009) used a PCR-based ITS-RFLP technique for differentiating *B. xylophilus* from 43 other *Bursaphelenchus* species. Almost all descriptions of new *Bursaphelenchus* species published after 2009 contain the ITS-PCR RFLP patterns on the basis of the method developed by Burgermeister *et al*. (2009). Of all the molecular techniques in this protocol, this is the one that has been shown to be effective for the widest range of *Bursaphelenchus* species.

DNA is extracted from mixed life stages of nematodes (adult females and males, juveniles) using the QIAamp DNA Micro Kit (Qiagen[1]). Nematode samples (1 to 30 specimens) are placed in 5 µl water in Eppendorf[1] tubes and frozen at –20 °C until extraction. Before extraction, the sample is thawed, mixed with 10 µl ATL buffer (Qiagen[1]) and homogenized in the Eppendorf[1] tube using a micropestle (Eppendorf[1]). Then the DNA extraction process is conducted according to the manufacturer's recommendations (QIAamp DNA Micro Kit Handbook, Qiagen: "Isolation of genomic DNA from tissues"[1]), except for the following steps. For step 4, the incubation lasts 3 h. For step 12 (elution), 20 µl (for single nematode extraction) to 100 µl (for extraction of up to 30 nematodes) of AE buffer (Qiagen[1]) is applied to the membrane. The eluate containing extracted DNA is stored at –20 °C until use.

ITS-PCR RFLP analysis is carried out by performing PCR on the extracted DNA followed by RFLP on the PCR product. A segment of nematode ribosomal (r)DNA containing the ITS regions ITS1 and ITS2 is amplified by PCR using the following primer pair:

ITS1-forward (F): 5′-CGT AAC AAG GTA GCT GTA G-3′ (Ferris *et al*., 1993)

ITS2-reverse (R): 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain, 1993)

The PCR mixture (50 µl) contains 0.6 µM of each primer, 2 U Taq DNA polymerase (Stratagene[1] or Fermentas[1]), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs and 2 ng DNA template. Amplification is carried out using a thermal cycler, with the following cycling parameters: denaturation at 94 °C for 2.5 min, 40 reaction cycles of (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min) and a final extension at 72 °C for 5 min. After completion of the PCR, 5 µl aliquots of the PCR product are analysed by gel electrophoresis. Suitable aliquots of the amplified DNA are digested with 3 U restriction endonucleases *Alu*I, *Hae*III, *Hinf*I, *Msp*I and *Rsa*I, following the manufacturer's instructions.

B. xylophilus is identified on the basis of the species-specific DNA restriction fragment patterns (Figure 15). Numbers and sizes of DNA restriction fragments at least for the following *Bursaphelenchus* species have been described (Gu, 2014): *B. abietinus*, *B. abruptus*, *B. africanus*, *B. anamurius*, *B. andrassyi*, *B. antoniae*, *B. arthuri*, *B. arthuroides*, *B. braaschae*, *B. burgermeisteri*, *B. chengi*, *B. conicaudatus*, *B. corneolus*, *B. doui*, *B. eggersi*, *B. eremus*, *B. fraudulentus*, *B. fuchsi*, *B. fungivorus*, *B. gerberae*, *B. gillanii*, *B. hellenicus*, *B. hildegardae*, *B. hofmanni*, *B. hylobianum*, *B. koreanus*, *B. leoni*, *B. luxuriosae*, *B. macromucronatus*, *B. masseyi*, *B. mucronatus mucronatus* (previously *B. mucronatus* East Asian type), *B. mucronatus kolymensis* (previously *B. mucronatus* European type), *B. obeche*, *B. paraburgeri*, *B. paracorneols*, *B. paraluxoriosae*, *B. paraparvispicularis*, *B. parathailandae*, *B. parvispicularis*, *B. pinasteri*, *B. pinophilus*, *B. poligraphi*, *B. populi*, *B. posterovolvus*, *B. rainulfi*, *B. seani*, *B. sexdentati*, *B. silvestris*, *B. sinensis*, *B. singporensis*, *B. thailandae*, *B. tusciae*, *B. vallesianus*, *B. willibaldi*, *B. xylophilus*, *B. yongensis* and *B. yuyaoensis*.

B. hunanensis and *B. lini* are proposed to be regrouped and therefore no longer belong to the genus *Bursaphelenchus*. Burgermeister *et al*. (2009) give a comprehensive summary of the patterns and ITS-RFLP DNA fragment sizes for 44 *Bursaphelenchus* species. An example of species differentiation by ITS-RFLP restriction fragment patterns for *B. xylophilus*, *B. mucronatus mucronatus* and *B. mucronatus kolymensis* isolates is provided in Table 2.

[1] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Table 2. Restriction fragment length polymorphism (RFLP) patterns of *Bursaphelenchus* species

Species	PCR product (base pairs)	Restriction fragments (base pairs) produced by restriction enzyme				
		***Rsa*I**	***Hae*III**	***Msp*I**	***Hinf*I**	***Alu*I**
B. mucronatus East Asian type = *B. mucronatus mucronatus*	920	486 412 12	621 299	355 302 263	408 232 121 86 49 24	674 246
B. mucronatus European type = *B. mucronatus kolymensis*	925	413 263 227 22	625 195 105	356 303 266	412 232 121 87 49 24	678 247
B. xylophilus	925	483 420 22	728 197	562 363	263 232 142 139 125 24	433 256 142 96

Source: Burgermeister *et al.* (2009).

4.2.2 Conventional PCR

The following PCR tests allow the species-specific identification of *B. xylophilus* but will not determine whether any other *Bursaphelenchus* species are present.

4.2.2.1 Conventional PCR targeting ITS rDNA

A species-specific method to identify *B. xylophilus* targeting the ITS1–ITS2 region of rDNA was described by Matsunaga and Togashi (2004). This method was evaluated against five and four Japanese populations of *B. xylophilus* and *B. mucronatus*, respectively. The experimental protocol is as follows.

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[1]) 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 in a specific PCR. PCR is performed using the following primer pair:

X-F: 5′-ACG ATG ATG CGA TTG GTG AC-3′

X-R: 5′-TAT TGG TCG CGG AAC AAA CC-3′

PCR is carried out in a 10 µl reaction mixture containing the previously prepared template DNA (5 µl crude DNA extract), 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM each dNTP, 5 pmol each primer and 0.25 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems[1])

using a Perkin Elmer GeneAmp PCR System 9600 thermal cycler (Applied Biosystems[1]). After denaturation at 94 °C for 5 min, cycling is performed for 35 cycles of (94 °C for 30 s, 55.9 °C for 30 s and 72 °C for 1 min), with a final extension at 72 °C for 6 min.

This reaction produces a DNA amplicon of 557 base pairs (bp) from all *B. xylophilus* isolates tested.

4.2.2.2 Conventional PCR targeting satellite DNA

A species-specific method to identify *B. xylophilus* using a satellite DNA-based PCR technology was described by Castagnone *et al.* (2005). Its specificity was evaluated against non-target *Bursaphelenchus* species (*B. leoni*, *B. mucronatus* and *B. tusciae*) as well as one Japanese and two Canadian populations of *B. xylophilus.*

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 PCR tube and covered with 2.5 µl lysis buffer (50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 60 mg/ml proteinase-K, 0.45% Nonidet P-40, 0.45% Tween 20 and 0.01% gelatin). Tubes are placed at –80 °C for 45 min, and immediately transferred to 60 °C for 60 min and then 95 °C for 15 min in a thermal cycler. The resulting crude DNA extract is used as a template in a specific PCR.

PCR primers used in the reaction are designed close to both ends of the sequence of the 160 bp monomer of the satellite DNA family previously characterized in *B. xylophilus* (Tarès *et al.*, 1993; GenBank accession number L09652):

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

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

PCR is carried out in a 25 µl reaction mixture containing the previously prepared template DNA (5 µl crude DNA extract), 50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 200 µM each dNTP, 250 ng each primer and 1 U Taq DNA polymerase (QBiogene[1]). After denaturation at 94 °C for 5 min, cycling is performed for 25 cycles of (94 °C for 30 s, 64 °C for 1 min and 72 °C for 1 min), with a final extension at 72 °C for 5 min.

Because the satellite DNA family has been shown to be constituted of repeats organized in tandem arrays (Tarès *et al.*, 1993), the amplification of a ladder of multimers of the 160 bp monomer is obtained after a PCR containing *B. xylophilus* DNA as template. Conversely, in the case of other *Bursaphelenchus* species, no amplification is detected, which provides a simple and reliable result of either clearly positive or clearly negative for *B. xylophilus* (Castagnone *et al.*, 2005).

4.2.3 Real-time PCR

Real-time PCR tests can be performed for specific identification of *B. xylophilus*. This type of test is generally more sensitive and less time-consuming than the conventional PCR techniques described in sections 4.2.1 and 4.2.2.

4.2.3.1 Real-time PCR targeting satellite DNA sequences

A species-specific method to identify *B. xylophilus* using satellite DNA sequences was described by François *et al*. (2007). This method is highly sensitive, detecting as little as 1 pg genomic DNA and single nematodes in mixed samples in which *B. xylophilus* was associated with the closely related species *B. mucronatus*, up to the limit of 0.01% and 1% of the mixture, respectively. This method also detected *B. xylophilus* directly from 100 mg wood.

DNA is extracted from isolated nematodes originating from pure cultures using a simplified procedure, as previously described (Castagnone *et al.*, 2005), with a slight modification: the volume of the lysis buffer used is not constant but adapted to the number of nematodes (i.e. 3 µl for one to four nematodes and 20 µl for a larger number of nematodes).

DNA extraction from *B. xylophilus*-infested wood is performed using a ChargeSwitch genomic DNA Plant Kit (Invitrogen[1]). Approximately 0.1 g infested wood is cut into small pieces and placed in a plastic bag with 5 ml CST Lysis Buffer containing 1% polyvinylpyrrolidone and 20 mM calcium chloride. The sample is lightly disrupted using a hammer, then 1 ml lysate is removed and processed according to the manufacturer's instructions. Briefly, 100 µl sodium dodecyl sulphate is added to the lysate after which it is incubated at room temperature for 5 min, then 400 µl precipitation buffer is added and it is centrifuged at maximum speed (approximately 18 000 *g*) for 5 min. Approximately 1 ml supernatant is removed, and 100 µl CST detergent and 40 µl CST beads are added to the supernatant. A PickPen 8-M (Bio-Nobile[1]) is used to transfer the CST beads and bound DNA through two washing steps (each with 1 ml CST Wash Buffer) and into 150 µl CST Elution Buffer in a 2.2 ml deep-well plate. The magnetic particles are then removed. The DNA is either tested immediately or stored at –20 °C for future analysis.

The primers and TaqMan probe used in this method are:

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

BSatRV: 5′-AAG CTG AAA CTT GCC ATG CTA AA-3′

Fluorogenic TaqMan probe BSatS: 5′-FAM-ACA CCA TTC GAA AGC TAA TCG CCT GAG A-TAMRA-3′

PCR is carried out in a total volume of 25 µl containing 1 µl genomic DNA. Each reaction contains 2.5 µl of 10× reaction buffer (qPCR Core Kit, Eurogentec[1]), 5 mM $MgCl_2$, 200 µM each dNTP, 0.5 U Taq polymerase (qPCR Core Kit[1]) and 200 nM each primer and probe. Real-time PCR tests are performed in a DNA Engine Opticon 2 thermal cycler (MJ Research[1]). Cycling parameters are 95 °C for 10 min, followed by 30 cycles of (95 °C for 15 s and 59 °C for 30 s). Data are analysed using the Opticon 2 Monitor software version 3.1[1] according to the manufacturer's instructions. Extracts are tested undiluted and diluted 1:10 in nuclease-free water.

Real-time PCR testing of wood extracts is performed on a SmartCycler II (Cepheid[1]). Each reaction consists of 0.025 U/µl Hot Taq (Biogene[1]), 1× PCR buffer, 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 5% trehalose (w/v), 300 nM each primer and 100 nM probe. Cycling conditions are 95 °C for 10 min, followed by 40 two-step cycles of (95 °C for 15 s and 60 °C for 1 min). Data are analysed using the default threshold setting of the SmartCycler II software[1] (30 fluorescence units). Extracts are tested undiluted and diluted 1:10 in nuclease-free water.

4.2.3.2 Real-time PCR test targeting a **hsp70** *gene sequence*

A real-time PCR method based on a heat shock protein gene (*hsp70*) was developed by Leal *et al.* (2007). This method was shown to be specific for *B. xylophilus* (it was tested on five isolates of *B. xylophilus*), with no amplification observed for seven non-target *Bursaphelenchus* species. This *hsp70* PCR is sensitive enough to detect at least 0.005 ng *B. xylophilus* genomic DNA, as well as DNA extracted from single nematodes.

For DNA extraction, the method of Burgermeister *et al.* (2005) is used with the following changes: (1) incubation of sample homogenate is at 56 ºC overnight instead of for 3 h; (2) carrier RNA is used only when DNA is extracted from single nematodes; (3) elution buffer (10 mM Tris-HCl, pH 8.0) is applied to the membrane of the mini-column and incubated for 5 min before centrifugation to elute the sample DNA; (4) DNA extracts are heated at 55 ºC for 5 min to remove any residual ethanol that could later affect the measurement of DNA quantity and quality and PCR amplification; and (5) samples are eluted in 30 µl (for single nematodes) and 50 µl (for samples containing more than one nematode).

The primers and TaqMan probe used in this method are (lower case letters indicate the locked nucleic acids):

BxLNAF: 5′-TAA GAT GTc TTT tAc AGA TGc CAA G-3′

BxLNAR: 5′-GCc TGG ACG AcC TTG AAT-3′

Dual-labelled TaqMan probe BxLNAP: 5′-FAM-AtT GgC CGC AAA TtC GaT GAa CC-IAblkFQ-3′

PCR is carried out in a 20 µl reaction volume containing 5 µl template, 50 mM Tris (pH 8.3), 0.25 mg/ml non-acetylated bovine serum albumin (BSA) (Sigma[1]), 0.1 µM probe, 0.7 µM forward primer, 0.5 µM reverse primer, 0.4 mM each dNTP (Roche[1]), 5.0 mM $MgCl_2$ and 1.0 U FastStart Taq DNA Polymerase (Roche[1]). Amplification is performed in the LightCycler 1.5 thermal cycler (Roche Diagnostics[1]), using the following parameters: initial denaturation and activation of the FastStart Taq DNA Polymerase (Roche Diagnostics[1]) at 95 ºC for 10 min, followed by 45 cycles of (denaturation at 94 ºC for 5 s, annealing at 62 ºC for 20 s and extension at 72 ºC for 10 s). Data are analysed using LightCycler version 3.5 software[1].

To confirm the quality of the purified nematode genomic DNA used in this test, amplification with the control primers ITS1-F and ITS2-R (primers described in section 4.2.1) is performed by conventional PCR. The 25 µl PCR reaction mixture consists of 5 µl template, 2.5 µl of 10× reaction buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH_4)_2SO_4$; pH 8.3), 1.5 mM $MgCl_2$, 1 µM each primer, 1.6 µg BSA, 0.2 mM each dNTP and 1 U FastStart Taq DNA Polymerase (Roche[1]). The cycling parameters include an initial denaturation at 94 ºC for 5 min, followed by 40 cycles of (94 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 2 min), with a final elongation at 72 ºC for 5 min.

4.2.4 RNA-based molecular tests for detection of living *Bursaphelenchus xylophilus*

The following tests detect only living nematodes. Options are given for conventional and real-time reverse transcription (RT)-PCR.

4.2.4.1 Conventional RT-PCR targeting a hsp70 *DNA sequence*

A conventional RT-PCR method for the detection of living *B. xylophilus* based on an *hsp70* gene sequence was described by Leal *et al*. (2013). In this test, the forward and reverse primers are placed on either side of the *hsp70* intron so that genomic DNA can be easily differentiated from cDNA by amplicon size. The specificity of the test was evaluated against six non-target *Bursaphelenchus* species and six isolates of *B. xylophilus*. The limit of detection of this test is 0.4 nematodes per reaction, measured in three of three replicates.

The RNA and genomic DNA are extracted from at least 20 nematodes. The simultaneous extraction of RNA and genomic DNA is performed using the AllPrep DNA/RNA Mini Kit (Qiagen[1]) following the manufacturer's protocol with the following modifications: nematode pellets that had been stored at –80 °C are ground using a Kontes Pellet Pestle (Kimble Chase Life Science and Research Products[1]), and 350 µl lysis buffer RLT (from the Qiagen[1] extraction kit) is added to each pellet containing the nematodes. The homogenization step is completed using QIAshredder Mini Spin Columns (Qiagen[1]). The RNA is eluted from a column using 20 µl RNase-free water and the DNA is eluted using 50 µl pre-warmed EB buffer (from the Qiagen[1] extraction kit). The eluate is allowed to sit on the column membrane for approximately 3 min to facilitate maximum elution with a single centrifugation.

B. xylophilus-specific primers used in this test are as follows, and the amplicon produced from cDNA template is 473 bp:

Hsp23F1: 5′-ACC CAA GTT TGA GTT GTA TTG TTT-3′

Hsp19R2: 5′-ACG GTA ACA ACG GCA TCC T-3′

The following control primers target the actin gene and can be included to ensure the test performs as expected when testing isolated genomic DNA. They produce an amplicon of 228 bp:

BxActF3: 5′-TCG TCA CCA ACT GGG ATG ATA-3′

BxActR3: 5′-CAC CAG TGG TAC GAC CG-3′

A two-step RT-PCR protocol is employed. The RT reaction is completed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics[1]) with the anchored-oligo(dT)18 primer protocol. For

cDNA synthesis, 12 µl RNA is used as starting material. The optional step suggested by the manufacturer of the kit to denature the RNA and the primers at 65 °C for 10 min is included, followed by immediate cooling on ice. After cDNA synthesis is complete, samples are stored at –20 °C for later use as template.

The 25 µl PCR reaction mixture contains 2 µl cDNA as template, 19 µl GoTaq Flexi PCR buffer (Promega[1]), 1.5 mM $MgCl_2$, 0.20 mM each dNTP (Roche Diagnostics[1]), 1.25 U GoTaq Flexi DNA Polymerase (Promega[1]) and 0.4 µM each primer (Hsp23F1 and Hsp19R2). Amplification is performed according to the following cycling parameters: initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min) and a final elongation at 72 °C for 5 min. For the amplification with control primers, the 25 µl PCR reaction mixture is the same as above, except that 1 µl genomic DNA (40 ng/µl) and 1 µM each primer (BxActF3 and BxActR3) are used. Amplification is performed with the following cycling parameters: initial denaturation at 95 °C for 5 min, followed by 35 cycles of (95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min), with a final elongation at 72 °C for 5 min.

4.2.4.2 Real-time RT-PCR targeting a **hsp70** *cDNA sequence*

A SYBR Green real-time RT-PCR test to identify living *B. xylophilus* exclusively by detecting the presence of *hsp70* mRNA as a viability marker was described by Leal *et al.* (2013). This test detects the specific amplification of reverse transcribed *B. xylophilus hsp70* cDNA as the reverse primer binds across an exon–intron junction, thereby eliminating the amplification of genomic DNA. Its specificity was evaluated against six non-target *Bursaphelenchus* species and six isolates of *B. xylophilus.* The limit of detection of this test is 0.25 nematodes per reaction, measured in three of three replicates.

The protocol for the simultaneous extraction of RNA and genomic DNA is carried out as in the conventional PCR method (section 4.2.4.1).

Primers used in this test are:

HspexF3: 5′-AGA ACC ACT CCC TCG TAT GTC-3′

HspexR3: 5′-TCA AAC GCT TGG CAT CAA-3′

The following internal control primers may be included to ensure the test performs as expected:

BxActF3: 5′-TCG TCA CCA ACT GGG ATG ATA-3′

BxActR3: 5′-CAC CAG TGG TAC GAC CG-3′

A two-step RT-PCR protocol is used, and the cDNA synthesis is performed as for the conventional PCR method (section 4.2.4.1), with the exception that either the anchored-oligo(dT)18 primer or the sequence-specific primer (HspexR3) is used. After cDNA synthesis is complete, samples are stored at –20 °C for later use as template.

The 20 µl PCR reaction mixture is composed of 5 µl cDNA template (diluted 1:10 in 10 mM Tris, pH 8.0), 0.6 µM forward primer (HspexF3) and 0.4 µM reverse primer (HspexR3), and 4 µl of 5× LightCycler FastStart DNA MasterPLUS SYBR Green 1 Mix (Roche Diagnostics[1]). Real-time amplification is carried out in a LightCycler 2.0 (Roche Diagnostics[1]) using LightCycler version 4.1 software[1] with the following parameters: initial denaturation and activation at 95 °C for 10 min followed by 40 cycles of (95 °C for 15 s, 66 °C for 10 s and 72 °C for 15 s). For the amplification with control primers, the 20 µl PCR reaction mixture is the same as above, except that 0.5 µM each primer (BxActF3 and BxActR3) is used. Amplification is performed with the following cycling parameters: initial denaturation and activation at 95 °C for 10 min followed by 45 cycles of (95 °C for 15 s, 52 °C for 10 s and 72 °C for 15 s).

4.2.5 LAMP

A method for detecting *B. xylophilus* from wood samples by LAMP was described by Kikuchi *et al.* (2009). These authors developed the method to detect *B. xylophilus* faster and with higher sensitivity

than a TaqMan probe real-time PCR test also developed by their group. Specificity of the primers and the LAMP test was confirmed using DNA from non-target material: ten nematode species related to *B. xylophilus*, six non-target nematode genera, *P. thunbergii*, *P. densiflora* and *B. fuckeliana*. The sensitivity of the LAMP test was defined as ten copies of target gene (ITS) and as 2.5×10^{-5} of a nematode isolated from pure culture.

Wood samples (approximately 0.12 g wood in the experimental procedure) are incubated at 55 °C for 20 min in 800 µl extraction buffer, which contains proteinase-K and dithiothreitol supplied with the *B. xylophilus* detection kit (Nippon Gene[1]), followed by incubation at 95 °C for 10 min.

This method uses the following LAMP primers:

ITS(ID19) F3: 5′-GCA GAA ACG CCG ACT TGT-3′

ITS(ID19) B3: 5′-TCA TCC GAA CGT CCC TGA C-3′

ITS(ID19) FIP: 5′-CGC GGA ACA AAC CGC GTA AAA C-CG TTG TGA CAG TCG TCT C G-3′

ITS(ID19) BIP: 5′-AGA GGG CTT CGT GCT CGA TTGGCC GTT GAA ACA ACA TCA CC-3′

ITS(ID19) LF: 5′-AGA TGG TGC CTA ACA TTG CG-3′

The LAMP reaction is performed as described by Notomi *et al*. (2000) with the Loopamp DNA Amplification Kit (Eiken Chemical[1]). The 25 µl reaction mixture contains 2 µl extracted DNA, 5 pmol each F3 and B3 primers, 40 pmol each FIP and BIP primers, 20 pmol LF primer, 12.5 µl of 2× reaction mix, 1 µl Bst DNA polymerase and 1 µl fluorescent detection reagent (Eiken Chemical[1]). The reaction mixture is incubated at 63 °C for 60 to 120 min and terminated by incubation at 80 °C for 2 min. LAMP amplicons are detected by colour changes of the reaction solution under ultraviolet light.

Amplified products can be evaluated optionally with a probe-based detection system. The 5′-biotinylated form of the FIP primer is used for the LAMP reaction. After the LAMP reaction, 10 µl fluorescein isothiocyanate (FITC)-labelled probe (10 pmol/µl; 5′-GGC GAG AGG GCT TCG TGC TCG ATT GTC GTG C-3′) designed to hybridize to an internal region of the target sequence is added to the reaction mixture and incubated at 95 °C for 5 min, then slowly cooled to 25 °C. The reaction mixture is diluted with 100 µl running buffer (phosphate-buffered saline with 3% Tween) and applied directly to HybriDetect strips (Milenia Biotec[1]) according to the manufacturer's instructions. HybriDetect strips detect fragments containing both biotin and FITC resulting from specific amplification. In contrast, when non-specific amplification has occurred, no signal is observed at the test band line.

4.2.6 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest nucleic acid. For molecular tests, a positive nucleic acid control, a negative amplification control (no template control) and, when relevant (e.g. direct detection of the nematode), an internal control are the minimum controls that should be used. For RT-PCR (conventional or real-time), a positive RT control should be included.

Positive nucleic acid control. This control is used to monitor whether or not the test performed as expected under the experimental conditions and parameters. A positive control can be any nucleic acid that contains the target sequence of the test; that is, *B. xylophilus* nucleic acid that has previously tested positive; a plasmid containing the cloned target sequence; *in vitro* transcribed RNA; a product from a previous amplification reaction; or synthetic double stranded (ds)DNA or a long oligonucleotide.

Negative amplification control (no template control). This control is necessary for PCR to rule out false positives due to contamination during preparation of the reaction mixture or non-specific amplification. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Internal control. For conventional PCR, real-time PCR and LAMP, endogenous controls such as the ITS region, 18S rRNA, or β-actin or COX genes can be used to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

For RT-PCR, a no reverse transcriptase control should be included to verify that RNA samples are not contaminated with genomic DNA. This control contains all the RT-PCR reagents except the reverse transcriptase enzyme. In the absence of genomic DNA contamination, this control should generate no signal after amplification.

For RT-PCR, a positive reverse transcriptase control should be included to verify that the reverse transcriptase enzyme operates correctly. This control contains all the RT-PCR reagents and a RNA extract that includes the target sequence of the test (e.g. an RNA extract prepared by the laboratory and confirmed previously as positive). This control should generate a signal after amplification.

For both PCR and LAMP, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples.

4.2.7 Interpretation of results from PCR

4.2.7.1 Conventional PCR

The pathogen-specific PCR test is considered valid only if:

- the positive control produces an amplification product of the expected size for the target nematode
- the negative extraction control and the negative amplification control do not produce an amplification product of the expected size for the target nematode.

If internal control primers are used, for simplex reactions, positive controls, as well as each of the test samples, should produce an amplification product of the expected size. For multiplex reactions, all negative samples should produce an amplification product of the expected size. In some cases positive samples for the nematode can also produce an amplification product of the expected size with the internal control primers.

The test on a sample will be considered positive if it produces an amplification product of the correct size.

4.2.7.2 Real-time PCR

The real-time-PCR is considered valid only if:

- the positive control produces an amplification curve with the target nematode-specific primers
- the negative controls do not produce an amplification curve.

If internal control primers are used, the positive control and each of the test samples should produce an amplification curve.

4.2.8 Sequencing

Several genomic regions have been directly sequenced from isolated nematodes (single for Wu *et al*. (2013) or bulk from cultures on fungus for Ye *et al.* (2007)) for the purpose of species identification of *B. xylophilus* and differentiation of different *Bursaphelenchus* species. These regions include internal transcribed spacers (ITS-1, ITS-2, 5.8S) of rDNA (Abelleira *et al*., 2011; Wu *et al*., 2013) or the D2–D3 region of the 28S rRNA gene (Ye *et al.*, 2007). The targeted region is amplified by PCR, and the amplicons are sequenced either directly or after they are cloned. Sequence data can then be analysed

using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and compared with *Bursaphelenchus* sequences available in the NCBI database (e.g. accession numbers HQ646254 and KC460340 for the above-mentioned ITS region and AY508105 to AY508109 for the 28S rRNA region).

For the ITS gene, if the sample's pairwise sequence divergence compared with known *B. xylophilus* sequences is less than 2% but more than 2% with all other species, it is identified as *B. xylophilus*. For the 28S gene, if the sample's pairwise sequence divergence compared with known *B. xylophilus* sequences is less than 0.5% but more than 0.5% with all other species, it is identified as *B. xylophilus*. Any other results should be further investigated.

The Cytochrome Oxidase Subunit I COI region can also be used for species identification. Guidance on methodology and a reference sequence obtained from reference material (sequence Q38) is available at Q-bank (http://www.q-bank.eu/Nematodes/), including BLAST.

5. Records

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

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance (ISPM 13 (*Guidelines for the notification of non-compliance and emergency action*)) and where *B. xylophilus* is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- A sample of nematodes either mounted as a permanent slide, or fixed in TAF fixative or in a glycerine solution. For cases where *B. xylophilus* is found in an area for the first time, it would be helpful for further investigations of the pathway to establish a culture of living *B. xylophilus* multiplied on *B. cinerea*. Keeping specimens or DNA for molecular testing at a later stage may also be useful, even in the case of morphological identification.
- If the identification was based on molecular techniques, extracted DNA may be kept at –20 °C and extracted RNA at –80 °C.
- For cases of occurrence of *B. xylophilus* in wood or wood products, including wood packaging, instead of the geographical information on sampling, data concerning the origin, material (e.g. round wood, wood packaging) and import conditions (e.g. simultaneous occurrence of vector beetles) should be kept. Note that wood packaging is not necessarily of the same origin as the consignment. According to ISPM 15 (*Regulation of wood packaging material in international trade*), wood packaging in international trade should bear a mark in which the two first letters represent the ISO code of the country where the wood packaging was produced.

6. Contact Points for Further Information

Further information on this organism or this protocol can be obtained from:

Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for National and International Plant Health, Messeweg 11-12, D-38104 Braunschweig, Germany (Thomas Schröder; e-mail: thomas.schroeder@jki.bund.de).

Technical Center, Ningbo Entry-Exit Inspection and Quarantine Bureau, No. 9 Mayuan Road, Ningbo, 315012 China (Jianfeng Gu; e-mail: jeffgu00@qq.com).

ANSES Plant Health Laboratory, 7 rue Jean Dixméras, 49044 Angers Cedex 01, France (Geraldine Anthoine; e-mail: geraldine.anthoine@anses.fr).

Canadian Forest Service, 506 West Burnside Road, Victoria, BC V8Z 1M5, Canada (Isabel Leal; e-mail: ileal@nrcan.gc.ca).

Canadian Food Inspection Agency, 3851 Fallowfield Road, Ottawa, ON K2H 8P9, Canada (Fencheng Sun; e-mail: sunfc@inspection.gc.ca).

In addition to the experts mentioned above, regional experts on this nematode are listed in Table 3.

Table 3. List of regional and national experts on *Bursaphelenchus xylophilus* (not exhaustive)

Region or country	Contact details of expert
Africa	Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa (Michael J. Wingfield; e-mail: mike.wingfield@fabi.up.ac.za)
Australia	CSIRO Ecosystem Sciences-Black Mountain Laboratories, Clunies Ross Street, Black Mountain, ACT 2601, Australia (Mike Hodda; e-mail: Mike.Hodda@csiro.au)
China	Department of Forest Protection, Nanjing Forestry University, No. 159 Longpan Road, Nanjing, 210037 China (Boguang Zhao; e-mail: 13505186675@126.com)
European Union	NemaLab-ICAM, Departamento Biologia, Universidade de Évora, 7002-554 Évora, Portugal (Manuel Mota; e-mail: mmota@uevora.pt)
Japan	Forest Pathology Laboratory, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan (Mitsuteru Akiba; e-mail: akiban@ffpri.affrc.go.jp)
Republic of Korea (South Korea)	Division of Forest Insect Pests and Disease, Korea Forest Research Institute, 207 Cheongnyangni 2-dong, Dongdaemun-gu, Seoul 130-712, Korea (ROK) (Hyerim Han; e-mail: hrhan@forest.go.kr)

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

7. Acknowledgements

This diagnostic protocol was written by Thomas Schröder (JKI, Federal Research Centre for Cultivated Plants, Institute for National and International Plant Health, Germany (see preceding section)), Geraldine Anthoine (ANSES Plant Health Laboratory, France (see preceding section)), Isabel Leal (Canadian Forest Service, Canada (see preceding section)), Jianfeng Gu (Technical Center, Ningbo Entry-Exit Inspection and Quarantine Bureau, China (see preceding section)) and Fengcheng Sun (Canadian Food Inspection Agency, Canada (see preceding section)).

Vladimir Gaar (Diagnostic Laboratory, State Phytosanitary Administration, Czech Republic) and David McNamara (formerly EPPO) contributed to the protocol at an early stage.

The description of the ITS-RFLP technique was initially prepared by Wolfgang Burgermeister (Institut für Pflanzenvirologie, Mikrobiologie und Biologische Sicherheit, JKI, Germany). The initial description of the conventional PCR targeting satellite DNA method to identify *B. xylophilus* was provided by Philippe Castagnone-Sereno (UMR1064 INRA/UNSA/CNRS, Interactions Plantes-Microorganismes et Sante Vegetale, France).

The text of this diagnostic protocol is based partly on the EPPO diagnostic protocol for *B. xylophilus* (EPPO, 2001, 2013b).

8. References

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

Abelleira, A., Picoaga, A., Mansilla, J.P. & Aguin, O. 2011. Detection of *Bursaphelenchus xylophilus*, causal agent of pine wilt disease on *Pinus pinaster* in northwestern Spain. *Plant Disease*, 95(6): 776–776.

Andrássy, I. 1984. Klasse Nematoda (Ordnungen Monhysterida, Desmoscolecida, Araeolaimida, Chromadorida, Rhabditida). *In Bestimmungsbücher zur Bodenfauna Europas*, pp. 24–25. Stuttgart, Germany, Gustav Fischer Verlag. 509 pp.

Baermann, O. 1917. [A simple method of discovering ankylostomo (nematode) larvae in faeces.] *Med. Geneeslc. Lab. Weltevreden*, 1917: 41–47 (in German).

Barstead, R.J., Kleiman, L. & Waterston, R.H. 1991. Cloning, sequencing and mapping of an alpha-actinin gene from the nematode *Caenorhabditis elegans*. *Cell Motility and the Cytoskeleton*, 20: 69–78.

van Bezooijen, J. 2006. *Methods and techniques for nematology*. Wageningen, Netherlands, Department of Nematology, Wageningen University. 112 pp. Available at https://www.wageningenur.nl/upload_mm/4/e/3/f9618ac5-ac20-41e6-9cf1-c556b15b9fa7_MethodsandTechniquesforNematology.pdf.

Bongers, T. 1989. [*The nematodes of the Netherlands*] (translated from the Dutch by J. van de Haar). Wageningen, Netherlands, Wageningen Agricultural University. 83 pp.

Braasch, H. 2001. *Bursaphelenchus* species in conifers in Europe: Distribution and morphological relationships. *EPPO Bulletin*, 31: 127–142.

Braasch, H., Burgermeister, W. & Gu, J. 2009. Revised intra-generic grouping of *Bursaphelenchus* Fuchs, 1937 (Nematoda: Aphelenchoididae). *Journal of Nematode Morphology and Systematics*, 12(1): 65–88.

Braasch, H., Gu, J. & Burgermeister, W. 2011. *Bursaphelenchus mucronatus kolymensis* comb. n.: New definition of the "European type" of *B. mucronatus*. *Journal of Nematode Morphology and Systematics*, 14(2): 77–90.

Braasch, H. & Schönfeld, U. 2015. Improved morphological key to the species of the *xylophilus* group of the genus *Bursaphelenchus* Fuchs, 1937. *EPPO Bulletin*, 45(1): 73–80.

Burgermeister, W., Braasch, H., Metge, K., Gu, J., Schröder, T. & Woldt, E. 2009. ITS-RFLP analysis, an efficient tool for identification of *Bursaphelenchus* species. *Nematology*, 11: 649–668.

Burgermeister, W., Metge, K., Braasch, H. & Buchbach, E. 2005. ITS-RFLP patterns for differentiation of 26 *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) and observations on their distribution. *Russian Journal of Nematology*, 13(1): 29–42.

Cardoso, J., Fonseca, L. & Abrantes, I. 2012. Direct detection of the pinewood nematode, *Bursaphelenchus xylophilus*, from pine wood, bark and insect vector. *European Journal of Plant Pathology*, 133: 419–425.

Castagnone, C., Abad, P. & Castagnone-Sereno, P. 2005. Satellite DNA-based species-specific identification of single individuals of the pinewood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *European Journal of Plant Pathology*, 112: 191–193.

Dwinell, L.D. 1993. First report of pinewood nematode (*Bursaphelenchus xylophilus*) in Mexico. *Plant Disease*, 77: 846.

Dwinell, L.D. 1997. The pinewood nematode: Regulation and mitigation. *Annual Review of Phytopathology*, 35: 153–166.

Edwards, O.R. & Linit, M.J. 1992. Transmission of *Bursaphelenchus xylophilus* through oviposition wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae*). Journal of Nematology*, 24(1): 133–139.

EPPO (European and Mediterranean Plant Protection Organization). 2001. *Bursaphelenchus xylophilus*. Diagnostics PM 7/4 (1). *EPPO Bulletin*, 31(1): 61–69.

EPPO (European and Mediterranean Plant Protection Organization). 2012. *Bursaphelenchus xylophilus* and its vectors: Procedure for official control. National regulatory control systems PM 9/1 (5). *EPPO Bulletin*, 42(3): 477–485.

EPPO (European and Mediterranean Plant Protection Organization). 2013a. *Diagnostic protocols for regulated pests: Pictorial glossary of morphological terms in nematology.* EPPO Technical Document No. 1056 (rev. 4). Paris, EPPO. 21 pp.

EPPO (European and Mediterranean Plant Protection Organization). 2013b. *Bursaphelenchus xylophilus*. Diagnostics PM 7/4 (3). *EPPO Bulletin*, 43(1): 105–118.

EPPO (European and Mediterranean Plant Protection Organization). 2013c. Nematode extraction. Diagnostics PM 7/119 (1). *EPPO Bulletin*, 43(3): 471–496.

EPPO (European and Mediterranean Plant Protection Organization). 2014. *Bursaphelenchus xylophilus*. Addendum to Diagnostics PM 7/4 (3). *EPPO Bulletin* 44(1): 105.

EPPO/CABI. 1996. *Bursaphelenchus xylophilus*. *In* I.M. Smith, D.G. McNamara & P.R. Scott, eds. *Quarantine pests for Europe*, 2nd edn. Wallingford, UK, CABI. 1425 pp.

Evans, H.F., McNamara, D.G., Braasch, H., Chadoeuf, J. & Magnusson, C. 1996. Pest risk analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*. *EPPO Bulletin*, 26: 199–249.

Ferris, V.R., Ferris, J.M. & Faghihi, J. 1993. Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes. *Fundamental and Applied Nematology*, 16: 177–184.

Fonseca, L., Cardoso, J.M.S., Lopes, A., Pestana, M., Abreu, F., Nunes, N., Mota, M. & Abrantes, I. 2012. The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island. *Helminthologia*, 49(2): 96–103.

François, C., Castagnone, C., Boonham, N., Tomlinson, J., Lawson, R., Hockland, S., Quill, J., Vieira, P., Mota, M. & Castagnone-Sereno, P. 2007. Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus*. *Molecular Plant Pathology*, 8: 803–809.

Futai, K. 2013. Pine wood nematode, *Bursaphelenchus xylophilus*. *Annual Review of Phytopathology*, 51: 61–83.

Goodey, J.B. 1963. *Laboratory methods for work with plant and soil nematodes.* Technical Bulletin No. 2 of the Ministry of Agriculture, Fisheries and Food. London, HMSO. 72 pp.

Gu, J., ed. 2014. [*Identification of* Bursaphelenchus xylophilus *and its closely related species.*] Xiamen, China, Xiamen University Press (in Chinese).

Gu, J., Wang, J., Braasch, H., Burgermeister, W. & Schröder, T. 2011. Morphological and molecular characterisation of mucronate isolates (M form) of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). *Russian Journal of Nematology*, 19(2): 103–120.

Gu, J., Zhang, J., Braasch, H. & Burgermeister, W. 2005. *Bursaphelenchus singaporensis* sp. n. (Nematoda: Parasitaphelenchidae) in packaging wood from Singapore: A new species of the *B. xylophilus* group. *Zootaxa*, 988: 1–12.

Hasegawa, K. & Miwa, J. 2008. Embryology and cytology of *Bursaphelenchus xylophilus*. *In* B.G. Zhao, K. Futai, J.R. Sutherland & Y. Takeuchi, eds. *Pine wilt disease*, pp. 81–104. Tokyo, Springer. 459 pp.

Hopf, A. & Schroeder, T. 2013. Non vector spread of *Bursaphelenchus xylophilus* via wood chips. *In* T. Schroeder, ed. Pine Wilt Disease Conference, 15–18 October 2013, Braunschweig, Germany. *Berichte aus dem Julius Kühn Institut*, 169: 46–47.

Hu, Y.Q., Kong, X.C., Wang, X.R., Zhong, T.K., Zhu, X.W., Mota, M.M., Ren, L.L., Liu, S. & Ma, C. 2011. Direct PCR-based method for detecting *Bursaphelenchus xylophilus*, the pine wood nematode in wood tissue of *Pinus massoniana*. *Forest Pathology*, 41: 165–168.

Hunt, D.J. 2008. A checklist of the Aphelenchoidea (Nematoda: Tylenchina). *Journal of Nematode Morphology and Systematics*, 10(2): 99–135.

Ibeas, F., Gallego, D., Diez, J.J. & Pajares, J.A. 2007. An operative kairomonal lure for managing pine sawyer beetle *Monochamus galloprovincialis* (Coleoptera: Cerymbycidae). *Journal of Applied Entomology*, 131(1): 13–20.

Inácio, M.L., Nóbrega, F., Vieira, P., Bonifácio, L., Naves, P., Sousa, E. & Mota, M. 2014. First detection of *Bursaphelenchus xylophilus* associated with *Pinus nigra* in Portugal and in Europe. *Forest Pathology*, 45(3): 235–238.

Kanetani, S., Kikuchi, T., Akiba, M., Nakamura, K., Ikegame, H. & Tetsuka, K. 2011. Detection of *Bursaphelenchus xylophilus* from old discs of dead *Pinus armandii* var *amamiana* trees using a new detection kit. *Forest Pathology*, 41: 387–391.

Kikuchi, T., Aikawa, T., Oeda, Y., Karim, N. & Kanzaki, N. 2009. A rapid and precise diagnostic method for detecting the pinewood nematode *Bursaphelenchus xylophilus* by loop-mediated isothermal amplification. *Phytopathology*, 99: 1365–1369.

Kishi, Y. 1995. *The pine wood nematode and the Japanese pine sawyer.* Forest Pests in Japan No. 1. Tokyo, Thomas Company Limited. 302 pp.

Kondo, E. & Ishibashi, N. 1978. Ecological significance of dormancy in plant parasitic nematodes. 7. Ultrastructural differences between propagative and dispersal forms in pine wood nematode, *Bursaphelenchus-lignicolus*, with reference to survival. *Applied Entomology and Zoology*, 13: 1–11.

Leal, I., Foord, B., Allen, E., Campion, C., Rott, M. & Green, M. 2013. Development of two reverse transcription-PCR methods to detect living pinewood nematode, *Bursaphelenchus xylophilus*, in wood. *Forest Pathology*, 43: 104–114.

Leal, I., Green, M., Allen, E., Humble, L. & Rott, M. 2007. Application of a real-time PCR method for the detection of pinewood nematode, *Bursaphelenchus xylophilus*, in wood samples from lodgepole pine. *Nematology*, 9: 351–362.

Linit, M.J. 1990. Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). *Journal of Nematology*, 22(2): 231–236.

Luzzi, M.A., Wilkinson, R.C. & Tarjan, A.C. 1984. Transmission of the pinewood nematode *Bursaphelenchus xylophilus*, to slash pine trees and log bolts by a cerambycid beetle, *Monochamus tilliator*, in Florida. *Journal of Nematology*, 16(1): 37–40.

Mamiya, Y. & Kiyohara, T. 1972. Description of *Bursaphelenchus lignicolus* n.sp. from pine wood and histopathology of nematode-infested trees. *Nematologica*, 18: 120–124.

Matsunaga, K. & Togashi, K. 2004. Among-tree difference in the inhibition of systemic dispersal of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) by *Pinus densiflora*. *Applied Entomology and Zoology*, 39(2): 271–277.

Moens, M. 2000. The pinewood nematode: Development of a sampling, extraction and identification method. Final report FAIR1-CT95-0034 EU research project. 102 pp.

Mota, M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K. & Sousa, E. 1999. First record of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology*, 1: 727–734.

Mota, M. & Vieira, P., eds. 2004. The pinewood nematode *Bursaphelenchus xylophilus*. Proceedings of an international workshop, University of Évora, Portugal, 20–22 August 2001. *In Nematological monographs and perspectives*, Vol. 1. Leiden, Netherlands, Brill. 291 pp.

Mota, M. & Vieira, P., eds. 2008. *Pine wilt disease: A worldwide threat to forest ecosystems.* Berlin, Springer Verlag. 405 pp.

Nickle, W.R. 1970. A Taxonomic review of the genera of the Aphelenchoidae (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). *Journal of Nematology*, 2(4): 375–392.

Nickle, W.R., Golden, A.M., Mamiya, Y. & Wergin, W.P. 1981. On the taxonomy and morphology of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle (1970). *Journal of Nematology*, 13: 385–392.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28: e63.

Pajares, J.A., Ibeas, F., Diez, J.J. & Gallego, D. 2004. Attractive responses by *Monochamus galloprovincialis* (Col., Cerambycidae) to host and bark beetle semiochemicals. *Journal of Applied Entomology*, 128(9/10): 633–638.

Penas, A.C., Bravo, M.A., Valads, V. & Mota, M. 2008. Detailed morphometric studies of *Bursaphelenchus xylophilus* and characterisation of other *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) associated with *Pinus pinaster* in Portugal. *Journal of Nematode Morphology and Systematics*, 10(2): 137–163.

Penas, A.C., Dias, L.S. & Mota, M.M. 2002. Precision and selection of extraction methods of aphelenchoid nematodes from maritime pine wood, *Pinus pinaster* L. *Journal of Nematology*, 24(1): 62–65.

Ryss, A.Y. 2003. Express technique to prepare permanent collection slides of nematodes. *Zoosystematica Rossica*, 11: 257–260.

Ryss, A., Viera, P., Mota, M. & Kulinich, O. 2005. A synopsis of the genus *Bursaphelenchus* Fuchs, 1937 (Aphelenchida: Parasitaphelenchidae) with keys to species. *Nematology*, 7(3): 393–458.

Sanchez-Husillos, E., Etxebeste, I. & Pajares, J. 2015. Effectiveness of mass trapping in the reduction of *Monochamus galloprovincialis* Olivier (Col.: Cerambycidae) populations. *Journal of Applied Entomology*, doi:10.1111/jen.12219.

Schröder, T., McNamara, D.G. & Gaar, V. 2009. Guidance on sampling to detect pine wood nematode *Bursaphelenchus xylophilus* in trees, wood and insects. *EPPO Bulletin*, 39: 179–188.

Seinhorst, J.W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica*, 4: 67–69.

Sousa, E., Bravo, M.A., Pires, J., Naves, P., Penas, A.C., Bonifácio, L. & Mota, M.M. 2001. *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. *Nematology*, 3(1): 89–91.

Sousa, E., Naves, P., Bonifácio, L., Henriques, J., Inácio, L. & Evans, H. 2011. Assessing risks of pine wood nematode *Bursaphelenchus xylophilus* transfer between wood packaging by simulating assembled pallets in service. *EPPO Bulletin*, 41: 423–431.

Takeuchi, Y., Kanzaki, N. & Futai, K. 2005. A nested PCR-based method for detecting the pine wood nematode, *Bursaphelenchus xylophilus*, from pine wood. *Nematology*, 7: 775–782.

Tarès, S., Lemontey, J.M., de Guiran, G. & Abad, P. 1993. Cloning and characterization of a highly conserved satellite DNA sequence specific for the phytoparasitic nematode *Bursaphelenchus xylophilus. Gene*, 129: 269–273.

Tomalak, M. & Filipiak, A. 2011. *Bursaphelenchus trypophloei* sp. n. (Nematoda: Parasitaphelenchinae): An associate of the bark beetle, *Trypophloeus asperatus* (Gyll.) (Coleoptera: Curculionidae, Scolytinae), in aspen, *Populus tremula* L. *Nematology*, 13: 619–636.

Tomalak, M., Worall, J. & Filipiak, A. 2013. *Bursaphelenchus masseyi* sp. n. (Nematoda: Parasitaphelenchinae): A nematode associate of the bark beetle, *Trypophloeus populi* Hopkins (Coleoptera: Curculionidae: Scolytinae), in aspen, *Populus tremuloides* Michx. affected by sudden aspen decline in Colorado. *Nematology*, 15: 907–921.

Vieira, P.R., Mota, M. & Eisenback, J.D. 2003. Pinewood Nematode Taxonomic Database. CD-ROM. Blacksburg, VA, Mactode Publications.

Vrain, T.C. 1993. Restriction fragment length polymorphism separates species of the *Xiphinema americanum* group. *Journal of Nematology*, 25: 361–364.

Williams, B.D., Schrank, B., Huynh, C., Shownkeen, R. & Waterston, R.H. 1992. A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics*, 131: 609–624.

Wingfield, M.J. 1987. A comparison of the mycophagous and the phytophagous phases of the pine wood nematode. *In* M.J. Wingfield, ed. *Pathogenicity of the pine wood nematode.* Symposium Series, pp. 81–90. St Paul, MN, APS Press. 122 pp.

Wingfield, M.J., Blanchette, R.A., Nicholls, T.H. & Robbins, K. 1982. The pine wood nematode: A comparison of the situation in the United States and Japan. *Canadian Journal of Forest Research*, 12: 71–75.

Wu, H.Y., Tan, Q.Q. & Jiang, S.X. 2013. First report of pine wilt disease caused by *Bursaphelenchus xylophilus* on *Pinus thunbergii* in the inland city of Zibo, Shandong, China. *Plant Disease*, 97(8): 1126.

Ye, W., Giblin-Davis, R.M., Braasch, H., Morris, K. & Thomas, W.K. 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution*, 43: 1185–1197.

Zamora, P., Rodriguez, V., Renedo, F., Sanz, A.V., Domínguez, J.C., Pérez-Escolar, G., Miranda, J., Álvarez, B., González-Casas, A., Mayor, E., Duenas, M., Miravalles, A., Naves, A., Robertson, L., Gutiérrez Abascal, C.J. & Martín, A.B. 2015. First report of *Bursaphelenchus xylophilus* causing pine wilt disease on pinus radiata in Spain. *Disease Notes*, http://dx.doi.org/10.1094/PDIS-03-15-0252-PDN.

Zhao, B.G., Futai, K., Sutherland, J.R. & Takeuchi, Y. 2008. *Pine wilt disease*. Berlin, Springer. 459 pp.

9. Figures

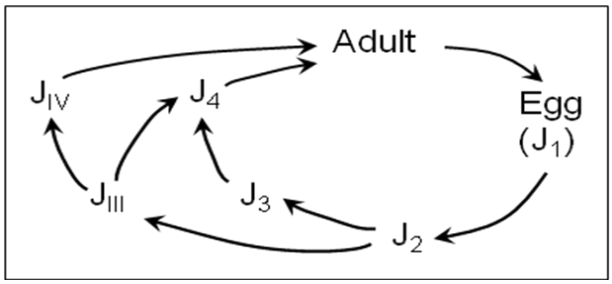

Figure 1. Life cycle of *Bursaphelenchus xylophilus* from egg to adult nematodes.
JX, juveniles of X-stage.
Source: Modified from Wingfield et al*. (1982).*

Figure 2. Evolution of symptoms of pine (*Pinus pinaster*) infested by *Bursaphelenchus xylophilus*, from healthy tree to dead.
Photos courtesy T. Schröder, Julius Kühn-Institut, Germany.

Figure 3. Symptoms of pine wilt disease on *Pinus pinaster* caused by *Bursaphelenchus xylophilus*.
Photo courtesy T. Schröder, Julius Kühn-Institut, Germany.

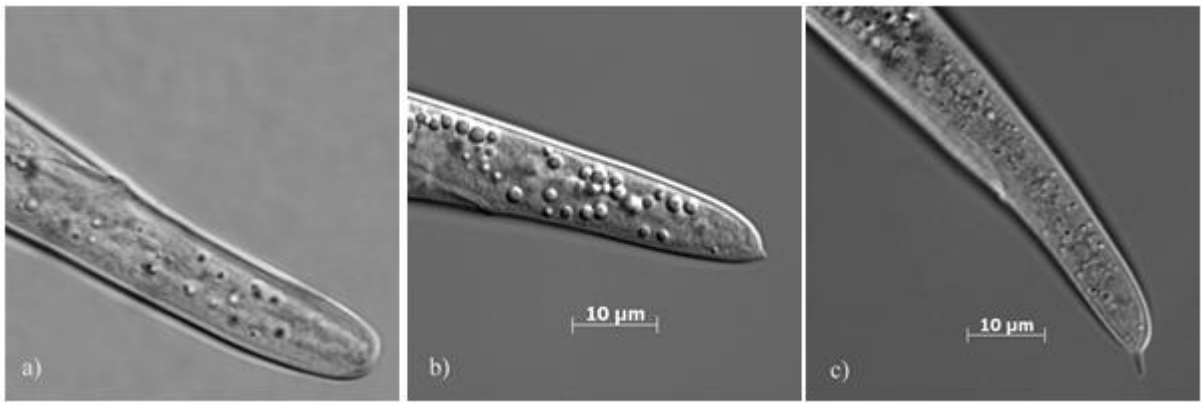

Figure 4. *Bursaphelenchus xylophilus* female tails: (a) round (×1 000 magnification); (b) with small projection; and (c) mucronate form.
Photos courtesy (a) T. Schröder, Julius Kühn-Institut, Germany and (b, c) J. Gu, Ningbo Entry-Exit Inspection and Quarantine Bureau, China.

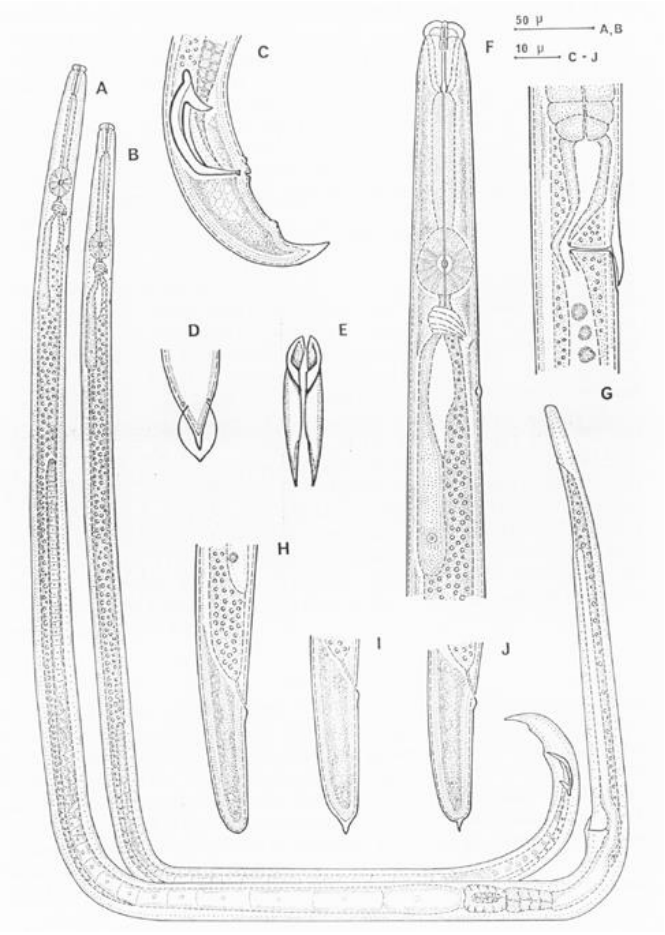

Figure 5. *Bursaphelenchus xylophilus*: (A) female; (B) male; (C) male tail; (D) ventral view of male tail, tip with bursa; (E) ventral view of spicules; (F) female, anterior portion; (G) female vulva; and (H), (I) and (J) female tail. *Source: Mamiya and Kiyohara (1972).*

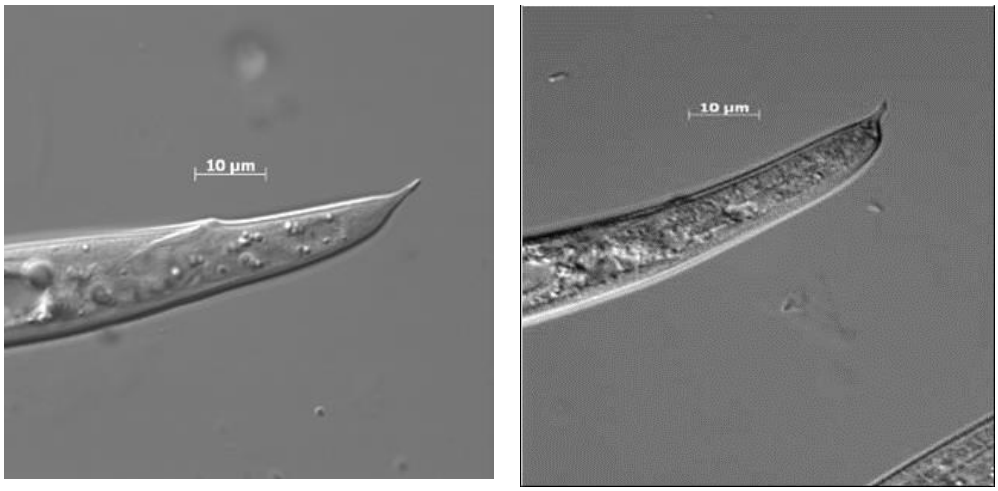

Figure 6. Female tail of *Bursaphelenchus mucronatus mucronatus* (left) and *B. mucronatus kolymensis* (right). *Photos courtesy J. Gu, Ningbo Entry-Exit Inspection and Quarantine Bureau, China.*

Figure 7. *Bursaphelenchus xylophilus* male tail with spicules (×1 000 magnification).
Photo courtesy T. Schröder, Julius Kühn-Institut, Germany.

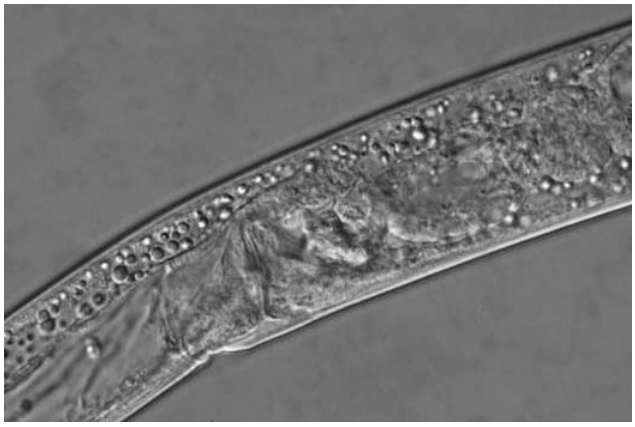

Figure 8. *Bursaphelenchus xylophilus* female with vulval flap (×640 magnification).
Photo courtesy T. Schröder, Julius Kühn-Institut, Germany.

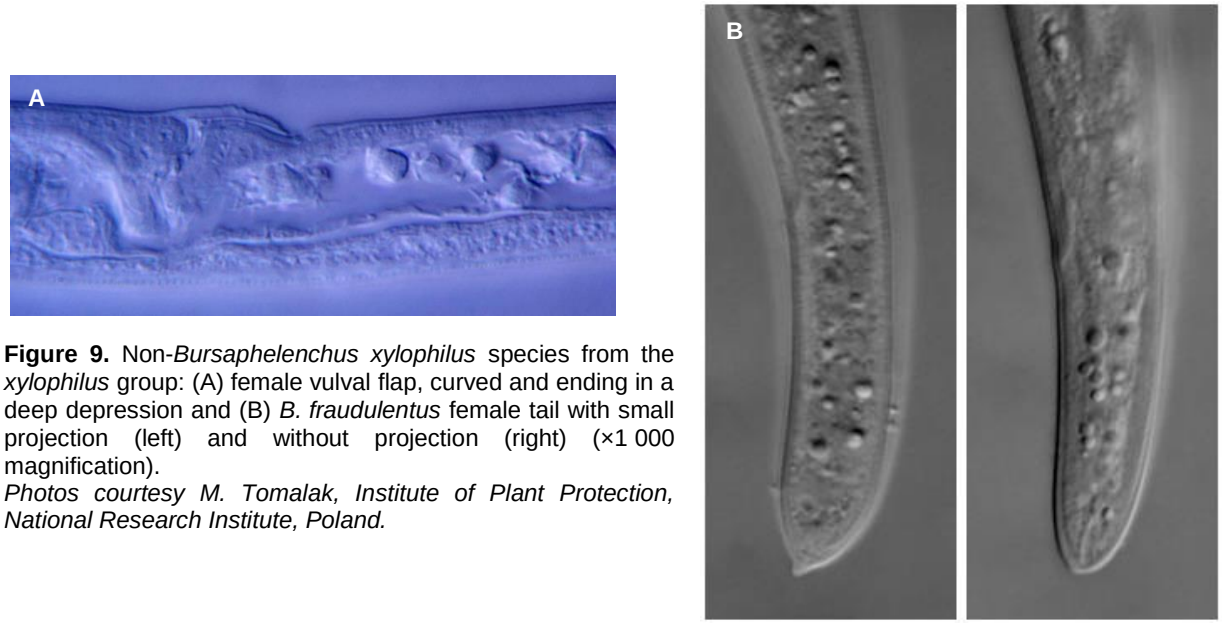

Figure 9. Non-*Bursaphelenchus xylophilus* species from the *xylophilus* group: (A) female vulval flap, curved and ending in a deep depression and (B) *B. fraudulentus* female tail with small projection (left) and without projection (right) (×1 000 magnification).
Photos courtesy M. Tomalak, Institute of Plant Protection, National Research Institute, Poland.

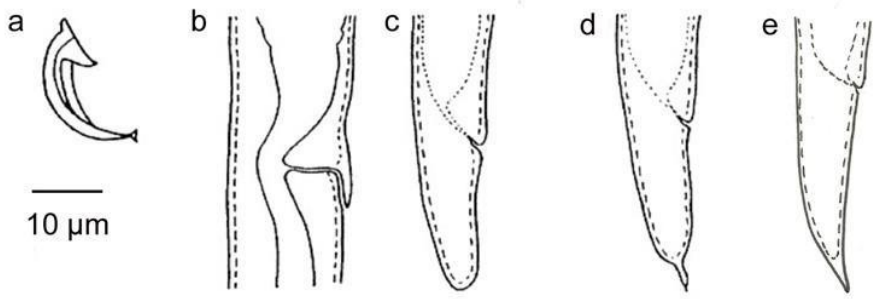

Figure 10. Diagnostic characters of *Bursaphelenchus xylophilus*, *B. mucronatus mucronatus* and *B. mucronatus kolymensis*: (a) spicules of all three species; (b) vulval flap of all three species; (c) female tail terminus of *B. xylophilus*, round form; (d) female tail terminus of *B. mucronatus kolymensis*; and (e) female tail terminus of *B. mucronatus mucronatus.*
Source: Modified from EPPO/CABI (1996).

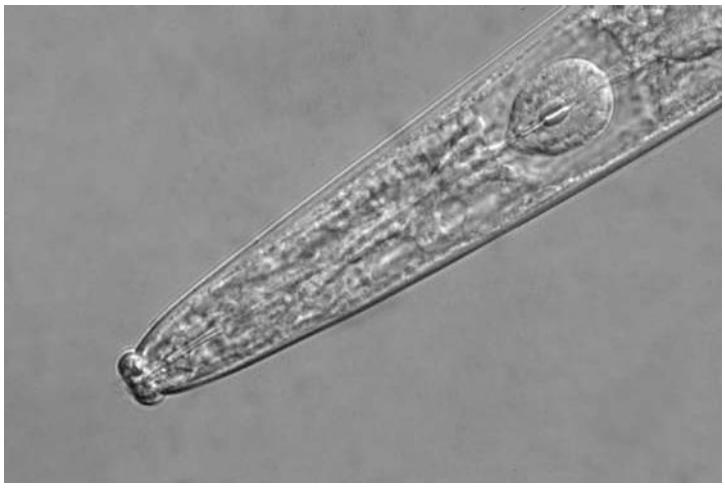

Figure 11. *Bursaphelenchus xylophilus* anterior region with stylet and metacorpus (×640 magnification).
Photo courtesy T. Schröder, Julius Kühn-Institut, Germany.

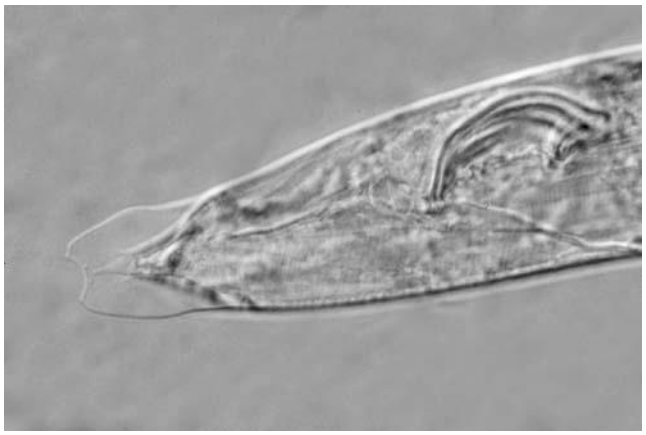

Figure 12. *Bursaphelenchus xylophilus* view of male tail in dorso-ventral position with bursa (×1 000 magnification).
Photo courtesy T. Schröder, Julius Kühn-Institut, Germany.

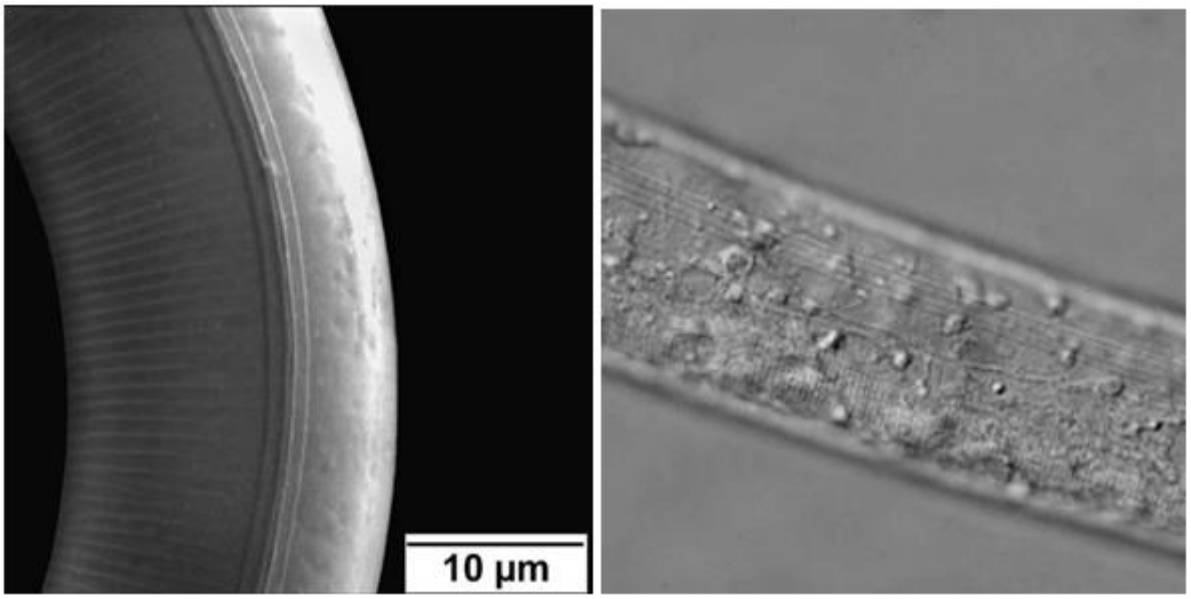

Figure 13. *Bursaphelenchus xylophilus* under lateral field scanning electron microscope (left) and light microscope (right (×1 600 magnification)).
Photos courtesy (left) M. Brandstetter, Austrian Research Centre for Forests, Austria and (right) T. Schröder, Julius Kühn-Institut, Germany.

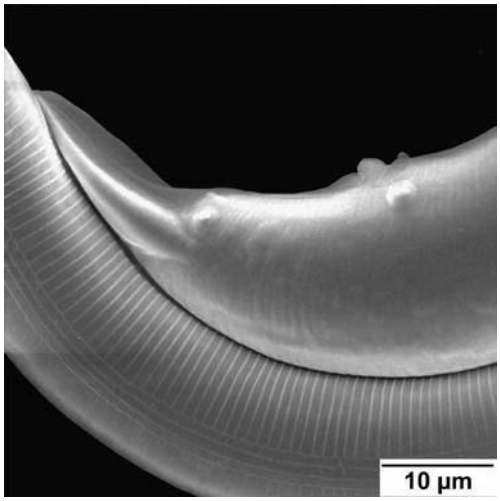

Figure 14. *Bursaphelenchus xylophilus* caudal papillae, scanning electron micrograph.
Photo courtesy M. Brandstetter, Austrian Research Centre for Forests, Austria.

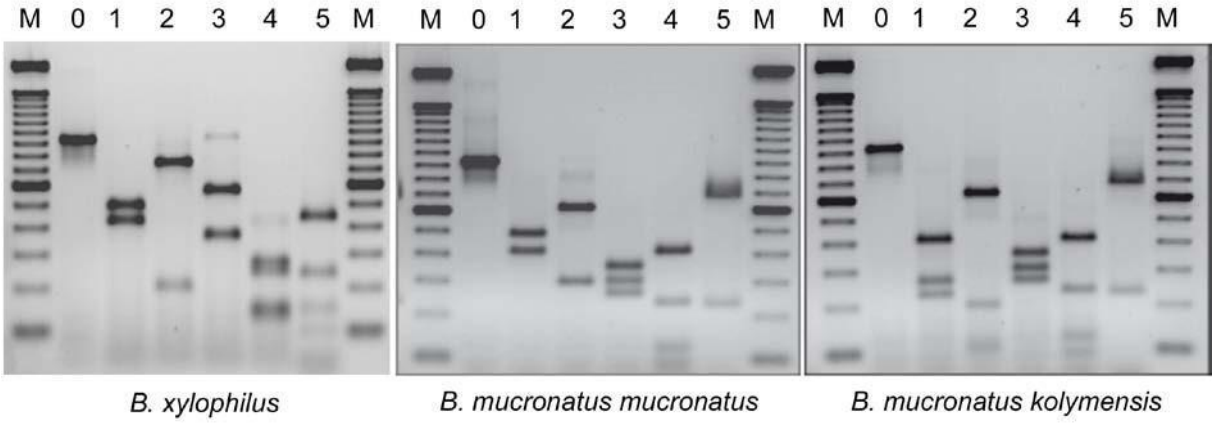

Figure 15. Internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) patterns of *Bursaphelenchus xylophilus* (left), *B. mucronatus mucronatus* (middle) and *B. mucronatus kolymensis* (right). Restriction fragments were obtained by digestion of the amplified ribosomal (r)DNA fragment (0) with *Rsa*I (1), *Hae*III (2), *Msp*I (3), *Hinf*I (4) and *Alu*I (5).
M, DNA marker (100 base pair ladder).
Photos courtesy W. Burgermeister, Julius Kühn-Institut, Germany.

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This is not an official part of the standard

2004-11 SC added subject: *Bursaphelenchus xylophilus* (2004-016).

2006-04 CPM-1 (2006) added work programme topic: Nematodes (2006-008).

2007-09 TPDP discussed draft.

2008-06 TPDP discussed draft with lead author.

2013-09 New DP drafting group formed.

2014-03 Expert consultation.

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2015-02 Member consultation.

2015-10 TPDP approved to submit to SC for adoption e-decision (eTPDP_Oct_02).

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ISPM 27. Annex 10. *Bursaphelenchus xylophilus* (2016). Rome, IPPC, FAO.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

International Plant Protection Convention (IPPC)

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