National Diagnostic Protocol

Pine wood nematode
(*Bursaphelenchus xylophilus*)

NDP 51 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists, it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for pine wood nematode (*Bursaphelenchus xylophilus*) is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this protocol should be sent to: SPHD Secretariat sphd@aff.gov.au

Contents

1 INTRODUCTION

The scope of this diagnostic protocol is limited to the detection and reliable morphological and molecular identification of one species of emergency plant pest, the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle, 1970 the causative agent of pine wilt disease (PWD).

Pine wood nematode is one of the most significant and devastating invasive pathogens affecting *Pinus* spp. worldwide (Webster and Mota 2008, Zhao *et al*. 2008). Pine wilt disease manifests as a rapid wilt of susceptible pine species and causes extensive mortality in softwood plantations and forest environments. It typically kills affected trees within a few weeks to a few months. *Bursaphelenchus xylophilus* is most pathogenic to pines, but species of *Abies* (fir), *Chamaecyparis* (false cypress), *Cedrus* (cedar), *Larix* (larch), *Picea* (spruce) and *Pseudotsuga* (Douglas fir) have also been reported as hosts of the nematode (Evans *et al*. 1996). PWN is carried from an infested tree to a new host tree by longhorn beetles (some of which are also known as pine sawyer beetles) in the genus *Monochamus* (Coleoptera: Cerambycidae). The nematode can be transmitted to living trees during the feeding of adult beetles, or to wood of living or recently dead trees during female beetle oviposition. The nematode can then feed on both living plant cells and hyphae of wood fungi.

A full list of hosts, information on the effects on hosts, and a list of common Australian species of the genus *Pinus* and their susceptibility to *B. xylophilus* can be found in Appendix 8.1. Information on transmission and vectors of *B. xylophilus* is located in Appendix 8.2 and its life cycle in Appendix 8.3.

The IPPC (International Plant Protection Convention) has published a diagnostic protocol for *B. xylophilus* (IPPC 2016). Other published diagnostic protocols for *B. xylophilus* are available in the EPPO (European and Mediterranean Plant Protection Organization) Diagnostic Protocols for Regulated Pests document PM 7/4 (3) (EPPO 2013, 2014), the French Official Gazette of the Ministry of Agriculture of Agrifood and Forestry (Anonymous 2011a,b), and the document ANSES/LSV/MA 051 - Version 2 of ANSES (French Agency for Food, Environmental and Occupational Health and Safety) (Anonymous 2019). Information on diagnostic morphology of *B. xylophilus* can be found in the Pest and Disease Image Library (Walker 2006, Zhao and Crosby 2011).

This protocol provides diagnostic images to morphologically identify *B. xylophilus* which are not included in the IPPC protocol. Additionally, this diagnostic protocol includes diagnostic imagery of other nematode species, including some Australian endemics, which are likely to be confused with *B. xylophilus*. The molecular tests are the same in both protocols. Molecular tests have not been verified in Australia due to lack of molecular-grade material except for DNA barcoding method where desktop verification has been performed. This protocol will be reviewed and updated when molecular-grade specimens become available.

2 TAXONOMIC INFORMATION

Phylum Nematoda
Order: Panagrolaimida
Superfamily: Aphelenchoidoidea
Family: Parasitaphelenchidae
Subfamily: Bursaphelenchinae
Genus: *Bursaphelenchus*

Species 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 lignicolus Mamiya and Kiyohara, 1972

Common names: English: pine wilt nematode, pine wilt disease, pine wood nematode, PWN

Dutch: dennenhoutnematode

French: nématode du bois de pin, nématode des pins, dépérissement des pins

German: kiefernholznematode

Italian: nematode del pino

Portuguese: nemátode da madeira do Pinheiro

Spanish: nematodo de la madera del pino

Swedish: tallvedsnematod

The taxonomy of the genus *Bursaphelenchus* is not well defined. *B. xylophilus* is one of about 174 species of *Bursaphelenchus* (Kanzaki 2008, Futai 2013, d'Errico *et al*. 2015, Kanzaki and Giblin-Davis 2016, Hodda 2022), most of which are mycophagous and have a phoretic relationship with insects, particularly bark beetles and woodborers (Dwinell and Mota 2001). *B. xylophilus* and *B. mucronatus* are considered to be closely related and both are members of the pinewood nematode species complex (PWNSC) (de Guiran and Bruguier 1989, Webster *et al*. 1990, Tares *et al*. 1992, Bolla and Boschert 1993). However, only *B. xylophilus* is associated with pine wilt disease.

Notes: The species now recognised as *B*. *xylophilus* was first described as a species of *Aphelenchoides* in the USA, where it was found on Louisiana pine (*Pinus palustris* Mill.) from Texas, Louisiana and Virginia (Steiner and Buhrer 1934). At the time, most species and genera of what is now the Sub-Order Aphelenchina were placed in this genus. Later, the genus *Bursaphelenchus* Fuchs 1937 was created to contain two new species with bursae in adult males; a bursa was not mentioned in the original description of *Aphelenchoides xylophilus*, so the species was not included. The genus *Bursaphelenchus* was then changed to a subgenus of *Aphelenchoides* by Rühm (1956). The genus *Bursaphelenchus* was subsequently reinstated (Goodey 1960, Meyl 1961) and has been enlarged considerably. The present

concept of the genus includes the synonymy of *Deviburs aphelenchus* Kakuliya 1967, *Huntaphelenchoides* Nickle 1970, *Omemeea* Massey 1971, *Teragramia* Massey 1974, and *Ipsaphelenchus* Lieutier and Laumond 1978 (Hodda 2003, Hunt 1993). *Bursaphelenchus xylophilus* was briefly placed in the genus *Paraphelenchoides* (Haque 1967), although this was not widely recognised, and *Paraphelenchoides* was subsequently synonymised with *Aphelenchoides* (Hodda 2003). The species was ultimately transferred from *Aphelenchoides* into *Bursaphelenchus* by Nickle (1970) when the presence of a bursa in *A. xylophilus* was recognised.

A nematode morphologically similar to *B. xylophilus*, which caused pine wilt disease in *Pinus thunbergii* Parl. in Japan, was described as a different species, *Bursaphelenchus lignicolus* Mamiya and Kiyohara, 1972, and differentiated from *B. xylophilus* by the shape of the spicules in adult males. Another morphologically very similar species, *Bursaphelenchus mucronatus* Mamiya and Enda, 1979 from *Pinus densiflora* Sieb. et Zucc. in Japan, was differentiated from both *B. xylophilus* and *B. lignicolus* by the constant presence and size of a mucro on the tail of adult females and third stage juveniles. Subsequently, *B. lignicolus* was synonymized with *B. mucronatus* (Baujard 1980). This act was not accepted widely, and later *B. lignicolus* was instead synonymised with *B. xylophilus* (Nickle *et al*. 1981). There was also discussion about the possible synonymy of *B. xylophilus* and *B. mucronatus*, because of variability discovered in the morphological characters differentiating the two species, but artificial crossing experiments, pathogenicity trials, biochemical and molecular studies have now added to the evidence separating the two species (Abad *et al*. 1991, Beckenbach *et al*. 1999, de Guiran and Bruguier 1989, de Guiran and Ritter 1984, Fukushige and Futai 1985, Mamiya 1986, Riga *et al*. 1992, Schauer-Blume 1990, Webster *et al*. 1990). A '*xylophilus*' group is now recognised within the genus *Bursaphelenchus*, containing the following 15 closely-related species: *B. acaloleptae*, *B. baujardi*, *B. conicaudatus*, *B. doui*, *B. firmae*, *B. fraudulentus*, *B. gillanii*, *B. koreanus*, *B. luxuriosae*, *B. macromucronatus*, *B. mucronatus* (including the two subspecies *B. m. kolymensis* and *B. m. mucronatus*), *B. paraluxuriosae*, *B. populi*, *B. singaporensis*, and *B. xylophilus* (including two forms, 'M' and 'R') (Giblin and Kaya 1983, Braasch 2001, 2004, 2008, Ryss *et al*. 2005, d'Errico *et al*. 2015, Gu 2018; Kanzaki et al. 2020). This is also known as the pinewood nematode species complex (PWNSC). It should be noted that some degree of interbreeding between *B. xylophilus* and some populations of *B. mucronatus* has been shown experimentally (Bolla and Boschert 1993, Braasch 1994, de Guiran and Boulbria 1986, 1987, de Guiran and Bruguier 1989, Riga *et al*. 1992, Taga *et al.* 2011). However, interbreeding has never been reported in the field where the *B. xylophilus* and *B. mucronatus* co-occur (Evans *et al*. 1993).

Within *B. xylophilus* from North America, two forms are recognised by some authorities. One is a pathogenic form with a round tail, usually occurring in *Pinus* spp. and known as the 'R' form. Under natural conditions, this is the form most associated with disease. The other form has a more pointed or mucronate tail. It occurs mostly on fir or spruce and occasionally on pine and other conifers and is known as the 'M' form (Gu *et al*. 2011).

Populations maintained as laboratory cultures can change in pathogenicity and biochemical composition over time (Bolla *et al*. 1986, Kiyohara and Bolla 1990). DNA profiles of PWN indicate considerable plasticity in the genome, and geographic differences in pathogenicity are also known (Evans *et al*. 1996). It is highly likely that this species is particularly adept at changing to exploit different circumstances.

Other species of the genus *Bursaphelenchus* have been demonstrated as being pathogenic to trees in some circumstances. These species include *B. leoni*, *B. sexdentati* and *B. hellenicus* (Coroppo *et al*. 2000,

Michalopoulos-Skarmoutsos *et al*. 2004, Skarmoutsos and Michalopoulos-Skarmoutsos 2000). There is debate over reports on the pathogenicity of *B. mucronatus* in some circumstances (Giblin-Davis pers. comm., Kruglik 2001, Kulinich 2004).

A taxonomic description of *B. xylophilus* can be found in Appendix 8.4, taxonomic illustrations in Appendix 8.5 (Figures 3–5) and morphometrics in Appendix 8.6 (Table 3).

3 DETECTION

Diagnosis of pine wilt disease relies on microscopic and/or molecular identification of *B. xylophilus*. The nematode can be isolated from cross-sections of small symptomatic branches or by drilling into larger branches or the trunk to remove samples which are then soaked in water for 24 to 48 hours (Donald *et al*. 2016). The nematodes may not be well distributed throughout the tree, so it may be necessary to take several samples from different areas of the tree to find them.

3.1 Symptoms

Pine wilt disease usually kills affected trees within a few weeks to a few months (Donald *et al*. 2016). Needle discoloration is often the first symptom; needles change from their normal colour to a greyish-green to yellowish-green and finally to yellowish-brown in colour (Figure 1). Fading may occur uniformly throughout the crown of the tree or may progress from one branch or section of the crown to another. Sometimes, a branch may die, and the disease will not progress until the next growing season. A diagnostic feature of this disease is that needles do not fall from affected branches.

Figure 1. A forest of Japanese red pine (*Pinus densiflora* Siebold & Zucc.) showing an epidemic of pine wilt disease caused by PWN (*Bursaphelenchus xylophilus*) (from USDA Forest Service - Region 2 - Rocky Mountain Region, USDA Forest Service, Bugwood.org).

3.1.1 Symptoms caused by PWN

PWN can occur on insects, in wood or bark, and in dead, dying or living trees. Only in the latter are there particular symptoms. The presence of symptoms depends on the susceptibility of the host and climatic conditions, but the mechanism remains unknown. In the most severe manifestations of infestation, the syndrome is called pine wilt disease, with symptoms first appearing about 3 weeks after infection, in the form of 'drying out': reduced oleoresin exudation. This is thought to trigger an increased attractiveness of the tree to adult insects, which may gather in large numbers on the trunk to mate. Transpiration from the leaves decreases and soon stops completely. Wilting and yellowing of

needles follows rapidly, and the tree dies 30–40 days after infection (Mamiya 1983). Wilting may appear simultaneously throughout the whole canopy, or it may appear initially on only one branch ('flag') and later spread (Malek and Appleby 1984). In the final stages of the disease, the trunk, branches and roots of the tree contain huge numbers of nematodes, which remain for a considerable time after the tree dies (Malek and Appleby 1984).

PWN also occurs in trees which are dead or dying from fungal attack; thus, symptoms of PWN are easily mistaken for other infections (Wingfield 1987). PWN may also occur in wood of almost any conifer, particularly wood which has not been thoroughly dried. (Note that it is the high temperature associated with kiln drying, not the drying itself, which is lethal to the nematode: see below.) In wood, PWN is mostly associated with 'bluestain' fungi (*Ceratocystis* spp.), but the presence of fungi is not always symptomatic of PWN, nor is fungal presence always associated with the presence of PWN (see life cycle). Similarly, PWN is mostly associated with the holes of the wood-boring larvae of insect vectors, but the tunnels may be present without PWN, and PWN may be present in wood without the beetle larval tunnels.

3.2 Sampling

There are numerous scientific publications on *B. xylophilus* which include sampling methods, but the methods described vary considerably between different authors. After reviewing the relevant literature, Schroder *et al*. (2009) provided guidance on sampling for *B. xylophilus* in an attempt to create a common, agreed, methodology in this field.

There is no practical method for detection of PWN during visual inspection of suspect wood (Schroder *et al*. 2009). The highest risk is posed by wood with evidence of blue-stain fungi and beetle attack which has not been completely kiln dried and which comes from a region where PWN is known to occur. If there is bark present or kiln-dried wood has come into contact with unprocessed wood, this also increases the risk of infection. Wood which has been completely kiln dried and has no evidence of fungi or beetle attack is low risk.

3.2.1 Methods

Samples of wood from around the holes made by the insects or in stained areas should be collected. A total of about 200 g of wood shavings or chips should be collected from at least 5 different places if possible. The wood should be placed in plastic bags and stored out of the sun, avoiding temperature extremes. An insulated container, such as an icebox, without any ice or coolant works well.

For live trees, at least 3 trunk cores should be taken if the whole tree is showing symptoms (EPPO 2003). If only one or a few branches are affected, then at least 3 disks from the branches should be cut. At least 50 g of wood should be used for extraction.

3.2.2 Extraction

As soon as possible after sampling, nematodes should be extracted by the Whitehead Tray method (also known as modified Baermann Funnel) (Thomas 1959, Whitehead and Hemming 1965, Hooper 1986). A thin layer of wood is placed into one or more plastic—not metal—colanders, sieves or perforated trays, sitting in other containers which are filled with tap water to just above the surface of the wood. A thin layer of tissue may be used if the wood material is likely to fall through the sieve or tray. Another layer of towelling or similar paper may be placed over the top if evaporation is likely to

be a problem. The whole apparatus should be left for at least 3 days at ambient temperature. At the end of this time nematodes should be at the bottom of the water if they are present. Next, the nematodes should be concentrated. This can be achieved by passing the water through a 20–40 µm sieve at least 3 times, because some nematodes will pass through the sieve at each pass. The nematodes can also be concentrated by very carefully drawing off the upper layers of water when it is still, using a pipette or similar device. Disturbance of the water or currents will suspend the nematodes, so there needs to be at least a couple of minutes between moving a tray and drawing off any water.

Note that the distribution of nematodes in wood may be extremely patchy and the rate of nematode recovery is considerably less than 100%: failure to recover nematodes by this method does not guarantee absence of nematodes.

4 IDENTIFICATION

Overviews of the genus *Bursaphelenchus* can be found in Ryss *et al*. (2005), Hunt (2008), Braasch *et al*. (2009) and Futai (2013). *B. xylophilus* can be identified by either of two methods: that based on morphological features or that based on molecular techniques.

Morphological identification relies on the presence of adult nematodes. If Aphelenchina are detected, specialist expertise is required to positively identify the genus and species using morphological methods.

Molecular techniques are used mainly for confirmation purposes, but have the advantage when there are only juveniles, or one sex of adult.

4.1 Morphological methods

Analysis of morphological characters remains the standard method for routine identification of the species in the genus *Bursaphelenchus*. 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. Identification based on morphological features requires preparation of good quality microscope slides, access to a high-power microscope and considerable experience in nematode taxonomy. Microscopical observation is recognised as the basic approach for identification of species but an experienced taxonomist is needed in order to distinguish *Bursaphelenchus* species from other nematodes. Morphometric measurements under the microscope can provide substantial amounts of information used for species identification. In addition, scanning electron microscopy (SEM) can provide more detailed information, such as the number of incisures in the lateral field, the spicule shape and the arrangement of caudal papillae, which are considered as important diagnostic features of *Bursaphelenchus* species. Microscope based approaches are still considered the most important tools used for the identification of *Bursaphelenchus* species. However, variation in, and the overlapping range of, some morphological characters makes identification to the species level difficult for some members of the genus (Li 2008). For identification under a light microscope, a magnification of 400X to 1000X (oil immersion lens) is recommended. Differential interference contrast (DIC) may facilitate observations.

Diagnostic images of PWN are presented in Appendix 8.8.

4.1.1 Microscopy

The concentrate recovered from the extraction procedure should be checked for nematodes with a dissecting microscope using transmitted light. A dark field is best if available because unfixed nematodes are opalescent translucent white. If a dark field is unavailable, the light should not be too bright or if there is a mirror, adjusted so that the light is oblique. If nematodes are present, it should be possible at this stage to identify whether any are in the Sub-Order Aphelenchina or they are entirely from other Sub-Orders. To do this, at least 30 nematodes should be transferred in water to microscope slides. Aphelenchina are identified by the presence of an oval median oesophageal bulb (Figures 3E, 4A, B, F, 6–8). Aphelenchina can also be identified by a characteristic body posture of heat killed adult males (Figures 3D, 4B, C, 5B, 7): note that this life stage may not always be present. To heat kill nematodes, they should be heated in excess water to about 60°C, then cooled quickly.

Sample nematodes can be prepared on either temporary or permanent slide mounts. Temporary slides are used for short-term observations and are very simple and quick to prepare; nematodes may be mounted in water or fixative. Good quality permanent slides take more time, with nematodes generally mounted in glycerol.

Temporary slide preparation

Temporary preparations for quick identification or study of features best seen in unfixed specimens are prepared as follows:

- Transfer living specimens to a small drop of water on a glass slide.
- Briefly heat slide over a spirit flame, checking frequently for nematode movement. Stop heating as soon as the specimens stop twitching.
- Apply coverslip – do not fix.

Note: 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.

Permanent slide preparation

Permanent preparations for identification under light microscopy are prepared as follows:

- Kill living nematodes by gentle heat.
- Fix in 4–5% formalin
- Process to anhydrous glycerine (for long-term storage).
- Mount on slides in anhydrous glycerine as described by Seinhorst (1959) and Hooper (1986).

A more rapid method (1–1.5 h) to prepare permanent slides was described by Ryss (2003) based on killing the nematodes in hot 4–5% 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).

4.1.2 Morphological identification

If there are Aphelenchina present, and good microscopic facilities are available, the tails of adult females should be viewed to see whether they are rounded or otherwise in shape (Figures 11, 19, 20). This procedure will eliminate many species with tails of different shapes, but there are many species with tails of similar shape, and so an apparently rounded tail does not identify an Aphelenchina nematode as PWN.

If Aphelenchina are present, specialist expertise is required to positively identify the genus and species. Identification of *B. xylophilus* requires a high level of expertise as it is morphologically difficult to distinguish from other, similar species of *Bursaphelenchus* (Bolla and Wood 2004, Braasch 2004). Special attention should be given to those species belonging to the PWNSC, such as *B. xylophilus* and *B. mucronatus*, which may be capable of genetic exchange, either directly or via intermediate forms (Rutherford *et al*. 1990). In addition, several other species of *Bursaphelenchus* are morphologically similar to *B. xylophilus* and share a combination of characters, including the distinctive angular shape of spicules, presence of four lateral lines and the large vulval flap in females (Braasch 2001, Ryss *et al*. 2005). Initially, photographs of the head, tail and vulva should be sent electronically to the appropriate State diagnostic laboratory or the Australian National Insect Collection (ANIC) at CSIRO National

Research Collections in Canberra for a preliminary diagnosis (see Figures 8–12). For further processing, the sample should be divided into four parts. One part should be fixed using 2% formaldehyde. Note the precautions required and SDS for formaldehyde. To fix specimens, the sub-sample should be transferred first to a container with lid, then an excess of 2% formaldehyde at 60°C should be added. The sample should be swirled, then a similar amount of cold (room temperature) formaldehyde at the same concentration added. This sample should be placed in a sealed container, further sealed using parafilm, and sent by overnight road freight to the appropriate State diagnostic laboratory or ANIC. One of the other parts should be sent in water, and the final part in 70% ethanol, double sealed the same way. Information on where and how the nematode was collected, the collector/s and date, the tree or wood symptoms, the overseas source, the host species, and any associated fungi or insects should be included if possible. The final part should be kept in tap water as a back-up in case of problems with transport of the other sub-samples.

Positive identification to species can be made morphologically on specimens transferred to glycerol and viewed under high-power microscopy. The characteristics of Sub-Order Aphelenchina are: oval median oesophageal bulb and dorsal oesophageal gland orifice opening in median oesophageal bulb (Figures 6–8). Additional characteristics of the superfamily Aphelenchoidoidea are: nerve ring circum-intestinal and the postcorpus, being extremely short (Figure 8). There are no characters which will reliably differentiate the family to which PWNs belong, Parasitaphelenchidae, from other members of the superfamily, nor characters to reliably differentiate the genus *Bursaphelenchus*. It has been claimed that the presence of a bursa in adult males unequivocally diagnoses the genus (Ryss *et al*. 2005), but there are species in other genera (e.g. *Laimaphelenchus*) with this feature (Zhang 2006). This is significant when considering PCR-based identifications (below). The characters supposedly uniting the genus are: high offset lips, stylet with weakly developed knobs, and presence of a terminal bursa in adult males (Figures 9, 12). At least a few of the putative species in the genus lack one or more of these characters.

The PWNSC has been differentiated from other members of the Aphelenchoidoidea as being characterized by: high offset lips, stylet 12–14 µm long with small knobs, bursa present in adult males, spicules slender with cucullus, and vulval flap present in adult females (Figures 9, 10, 12) (Braasch 2001, 2004, Giblin and Kaya 1983, Ryss *et al*. 2005). A rounded tail is an additional useful differentiating character which applies to most populations of *B*. *xylophilus*, and which differentiates this species from others in the pinewood nematode species complex (Figure 8). However, some populations of *B*. *xylophilus*, particularly from North America, have a proportion of the population with mucronate tails, so this character should be used with caution. Chinese populations of *B*. *xylophilus* have been experimentally manipulated to produce mucronate tails (Zhao and Yang 2004). Furthermore, two *Bursaphelenchus* species found in aspen, *B. trypophloei* and *B. masseyi*, partially share characteristics (lateral field with four incisures, vulval flap, similar spicule shape) with species of the pinewood nematode species complex. Their subcylindrical tail with broadly rounded terminus and usual lack of a mucro let them superficially appear similar to *B. xylophilus*. They may be morphologically separated from PWNSC members by spicule morphology, having shorter condyles and rostrum, and the distal third of the dorsal spicule contour is usually straight and not ungular as in species of the PWNSC (Braasch and Schonfeld 2015).

To identify suspect PWN to genus and species level, refer to the simplified morphological key in Table 1. Further keys to are available to the genus *Bursaphelenchus* (Thorne (1961), Yin *et al*. (1988), Hunt (1993) and Mai and Mullin (1996)), to *Bursaphelenchus* species groups Braasch *et al*. (2009), and species Ryss *et al*. (2005). Keys to species of the '*xylophilus*' group (PWNSC) of *Bursaphelenchus* can be

found in Braasch (2008), Braasch and Schonfeld (2015) and Gu (2018). Simplified keys to *B. xylophilus* can be found in EPPO (2013, 2014), Sarniguet *et al*. (2013), Braasch and Schonfeld (2015) and IPPC (2016). In their evaluation of a key to isolate the *xylophilus* group among the genus *Bursaphelenchus* (Braasch *et al*. 2009) and a key to identify the species *B. xylophilus* (EPPO 2009), Sarniguet *et al*. (2013) found questionable for routine use of some of the morphological characteristics for species specific identification. They found that the presence of male caudal papillae could not be observed with a light microscope and require scanning electron microscopy to observe them, that the position of the median bulb is highly variable so that the relative position of the excretory pore was sometimes not as described in the key, and that due to vagaries of the mounting procedure the vulval flap could sometimes not be seen. As a result of these observations, the EPPO Secretariat revised the Standard for *B. xylophilus* (EPPO 2013) to take account of its shortcomings. An additional species of *Bursaphelenchus acaloleptae*, was described from Japan in 2020 (Kanzaki et al. 2020). This species does not appear in the keys described above, however, it is distinguishable from *B*. *xylophilus* following the Key presented in Table 1.

Table 1. Simplified dichotomous morphological key to *Bursaphelenchus xylophilus* (PWN) (after EPPO 2013, 2014, Braasch and Schonfeld 2015, IPPC 2016).

1	Nematode with spear or stylet	**2**
	Nematode without spear or stylet	**not PWN**
2	Mouth with tylenchid stylet, pharynx with metacorpus	**3**
	Mouth with dorylaimid stylet, pharynx cylindrical or bottle-shaped, without metacorpus	**not PWN**
3	Metacorpus with metacorpal plates	**4**
	Metacorpus without conspicuous metacorpal plates	**not PWN**
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	**not PWN**
5	One gonad (vulva posterior)	**6**
	Two gonads	**not PWN**
6	Lip region without setae	**7**
	Lip region with setae	**not PWN**
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	**not PWN**
8	Pharyngeal glands overlap intestine dorsally	**9**
	Pharyngeal glands within abutting bulb	**not PWN**
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	**not PWN**

10	Stylet knobs usually present, female with anus	**(Parasitaphelenchidae) 11**
	Stylet knobs usually not present, female without anus	**not PWN**
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*) 12**
	J_{IV} dauer juveniles phoretically associated with insects; vulva very posterior (80–90% of body length); spicules partially fused; male tail not strongly recurved; bursa present	**not PWN**
12	Female tail broadly subcylindrical, with or without a mucro	**13**
	Female tail conoid or strongly tapering, with or without mucro	**not PWN**
13	Spicule length < 30 µm (measured from condyles to distal end)	**14**
	Spicule length > 30 µm	**not PWN**
14	Spicule with long and pointed rostrum, limbs of spicule with an angular curvature	**15**
	Spicule with short and pointed rostrum, limbs of spicule with a rounded curvature	**not PWN**
15	Female vulval flap straight, not ending in a deep depression	**16**
	Female vulval flap ending in a deep depression	**not PWN**
16	Female tail with mucro > 3 µm	**17**
	Female tail without mucro and with or without a small projection < 2 µm	**PWN (*B. xylophilus* round-tailed form)**
17	Excretory pore at or behind metacorpus	**18**
	Excretory pore anterior to metacorpus	**not PWN**
18	Mucro digitate, mean 2.2–3.0 µm long; spicule condylus slightly offset from dorsal spicule line, capitulum not distinctly concave	**PWN (*B. xylophilus* mucronate-tailed form)**
	Mucro variably shaped, 4 (3–5) µm long, offset from tail; spicule condylus offset from dorsal spicule line, no capitulum depression	***B. mucronatus kolymensis***

4.1.3 Nematodes in Australia likely to be confused with PWN

The most common nematodes occurring in Australia which are likely to be confused with *Bursaphelenchus* are members of the genera *Aphelenchoides*, *Laimaphelenchus* and *Ptychaphelenchus*. All are found in wood and bark, as well as associated with a similar range of insects to *Bursaphelenchus* (Hodda 2009, Huston *et al*. 2022, Zhang 2006). Over 20 species in these genera have been formally recorded from Australia (Appendix 8.7) (Hodda 2003).

In addition, there are other, undescribed species of aphelenchoid nematodes known which may be confused with PWN (Zhang 2006). PWN is distinguishable from species of other genera by the overhanging vulval lip in adult females (Figure 10 vs 18), and mostly by the presence of a bursa in adult males (Figure 12 vs 16). A few species of *Laimaphelenchus* have bursae in males. All *Laimaphelenchus* species have four tubercles on the tail tip (Figure 20), and the species of *Ptychaphelenchus* has three tubercles (Hodda, 2009). Tubercles are absent from the genus

Bursaphelenchus (e.g. Figures 6, 7, 11, 12, 13, 15). Many, but not all, species of *Aphelenchoides* have a single projection on the tail (Figure 19), which is absent from many populations of *B. xylophilus*, except those from the USA and China (Ryss *et al*. 2005, Zhao and Yang 2004).

Note also that a few species of *Bursaphelenchus*—including at least one in the PWNSC—have non-overlapping vulval lips. Diagnostic images of nematodes likely to be confused with PWN are in Appendix 8.9.

There have been few confirmed records of species from the genus *Bursaphelenchus* in Australia. A species of *Bursaphelenchus* described as close to *B. sexdentati* Ruhm was found on the five-spined bark beetle (*Ips grandicollis* (Eichhoff) which were attacking loblolly pine (*Pinus taeda* Linnaeus) in pine plantations in north-east New South Wales (Stone 1990; Stone and Simpson 1990). Stone and Simpson (1991) also reported *Bursaphelenchus* sp. associated with *I. grandicollis* attacking slash pine (*P. elliottii* Engelmann) in the same area of New South Wales. Another species of *Bursaphelenchus* reported from Australia is *B*. aff. *vallesianus*/*sexdentati*, from *Pinus* spp. in SE Queensland in 2014 (Cobon *et al*. 2017, Carnegie *et al*. 2018) and in New South Wales in 2017 (Carnegie *et al*. 2017a, b, 2018). Suspected vectors of this nematode were the burnt pine longhorn beetle *Arhopalus syriacus* (Reitter) and *Ips grandicollis* (Eichhoff) (Carnegie *et al*. 2017b).

Identification of *Bursaphelenchus* can be confused with other genera, even by experienced nematologists. For example, nematodes collected from *Hyleops glabratus* Schedl., the hoop-pine stitch beetle (Coleoptera: Curculionidae) in 1972 by RC Colbran, a senior nematologist at the Queensland Department of Primary Industries were initially determined as an unidentified species of *Bursaphelenchus* (Queensland Museum, Brisbane specimen nos. G201587-9, G202928), butare almost certainly from the genus *Aphelenchoides* (Figures 16–19)(Hodda *et al*. 2008). *Bursaphelenchus hunanensis* Yin, Fang and Tarjan 1988, subsequently recognised by Braasch (2009) as *Deviburs*aphelenchus *hunanensis* (Yin, Fang and Tarjan, 1988), was collected from a dying Aleppo pine (*Pinus halepensis* Miller) at Williamstown, Victoria in 2000 (Figure 15) (Ridley *et al*. 2001, Hodda *et al*. 2008, Smith 2003, Smith *et al*. 2008, Weiss *et al*. 2019). No primary vector was found, but the rusty longhorn beetle, *Arhopalus rusticus* (Linnaeus) (Coleoptera: Curculionidae), a known secondary vector of *Bursaphelenchus* spp., was found. The nematode was subsequently eradicated. *D. hunanensis* had previously been recorded only from dead Chinese red pine (*Pinus massoniana* Lambert) in China (Yin *et al*. 1988).

4.1.4 Reference material

The Australian National Insect Collection (ANIC) at CSIRO Black Mountain Canberra ACT has specimens of many of the species of the genus *Bursaphelenchus*, including *B. xylophilus*, *B. mucronatus* and most of the PWNSC. The collection also has specimens of many of the known Australian species of *Aphelenchoides*, *Laimaphelenchus* and *Ptychaphelenchus*. CSIRO will maintain these specimens and the expertise to identify nematodes within this group. Vouchers of any specimens suspected of being PWN should be deposited in ANIC for future reference, and any submitted for identification will be included in the collection.

4.2 Molecular identification

The methods described in this section were not able to be verified due to the lack of fresh specimens or positive DNA of PWN. This section will be reviewed and updated when molecular-grade specimens become available.

Significant progress in molecular identification methods for *B. xylophilus* has continued in recent years. Initial methods relied on identification based on RFLP analysis of the ITS region (see below under RFLP Fingerprinting) and this was adopted as an international standard. However, developments soon allowed rapid detection using conventional PCR (see below under Species-specific probe and DNA barcoding) or real-time PCR (Cao *et al*. 2005, Francois *et al*. 2007) (not described here). As an alternative to these approaches, a rapid and precise diagnostic method for detecting *B. xylophilus* has been applied in Japan with the use of Loop-Mediated Isothermal Amplification (LAMP) (Kikuchi *et al*. 2009). This LAMP test for detecting *B. xylophilus* from wood samples is also described in the IPPC Diagnostic Protocol on *Bursaphelenchus xylophilus* (IPPC 2016). LAMP uses a set of four to six primers and a DNA polymerase that amplifies with high specificity a selected DNA (ITS) region under isothermal conditions in less than 1 hr without the need for a thermocycler. The products can be visualised by a simple addition of a fluorescent dye that changes colour in the presence of a positive LAMP reaction that allows detection of pine wood nematode with a naked eye. An additional advantage of the LAMP protocol is that DNA extraction does not require specific isolation of nematodes. The LAMP protocol involves two steps: 1. DNA extraction, and 2. LAMP reaction. Both steps are briefly described below.

Note that other molecular methods specified below have only been proven to differentiate species of the PWNSC (the '*xylophilus*' group of the genus *Bursaphelenchus*) reliably, so it is highly desirable to sort taxonomically as far as possible using other means before using these molecular methods.

4.2.1 DNA extraction for LAMP reaction

Incubate wood samples of ~0.12 g at 55 °C for 20 min in 800 µL of extraction buffer that contains proteinase K (an enzyme that breaks keratin, the main component of nematode cuticle) and dithiothreitol (IUPAC name: (2S,3S)-1,4-bis(sulfanyl)butane-2,3-diol), also known as Cleland's Reagent, obtainable from chemical suppliers, or supplied with the commercial detection kit for *B. xylophilus* sold by Nippon Gene). Follow this by incubation at 95 °C for 10 min. Two µL of the substrate should be then used for LAMP reaction (below). This method has been assessed to be more successful and more efficient than the extraction of nematodes from wood samples with the Baermann method or extraction of DNA by crushing wood, followed by phenol/chloroform extraction, ethanol precipitation, and Tris-EDTA purification method (Takeuchi *et al*. 2005). The only necessary equipment is a water bath. Note that the extraction of DNA for other molecular assays is different (below).

4.2.2 LAMP reaction

The LAMP reaction should be performed according to the method described by Kikuchi et al. (2009) with the Loopamp DNA amplification kit (Eiken Chemical). Briefly, the reaction is performed in 25 µL of reaction mixture containing 2 µL of extracted DNA solution, 5 pmol each of F3 (GCA GAA ACG CCG ACT TGT T) and B3 (GTC AGGGACGTT CGG ATG A) primers , 40 pmol each of FIP (F2: CGTTGT GAC AGT CGT CTC G, F1c: TTT TAC GCG GTT TGT TCC GCG) and BIP (B1c: AGA GGG CTT CGT GCT CGA TTG, B2: GGT GAT GTT GTT TCAACG GC) primers, 20 pmol of Loop-F (CGC AAT GTT AGG CAC CAT CTG) primer (Kikuchi *et al*. 2009, Fig. 1B), 12.5 µL of 2× reaction mix, 1 µL of Bst DNA polymerase, and 1 µL of fluorescent detection reagent (Eiken Chemical). The reaction mixture should be incubated at 63 °C for 60–120 min and terminated by incubation at 80 °C for 2 min. LAMP amplicons can be detected by colour changes of the reaction solution under UV light. Alternatively, a probe-based detection system can be used as well, in which case it is necessary to design a 5'-biotinylated form of the FIP primer. Ten

µL of FITC-labelled probe is added to amplicons after the LAMP reaction (10 pmol/µL) to hybridize to an internal region of the target sequence and incubated at 95 °C for 5 min, then slowly cooled to 25 °C. The reaction mixture, diluted with 100 µL of running buffer (phosphate-buffered saline + 3% Tween), is applied directly to HybriDetect strips (Milenia Biotec, Germany) according to the manufacturer's instructions.

HybriDetect strips detect fragments containing both biotin and FITC resulting from specific amplification. In contrast, when nonspecific amplification occurs, no signal can be observed at the detection line (Kikuchi *et al*. 2009).

4.2.3 Species-specific probe

The most reliable method currently available uses species-specific PCR primers for the 160bp monomer of satellite genomic DNA between J10-1 5'-GGTGTCTAGTATAATATCAGAG-3' and J10-2Rc 5' GTGAATTAGTGACGACGGAGTG-3' (Tares *et al*. 1993, Castagnone *et al*. 2005). This method has been tested for DNA extracted by the phenol/chloroform method (Sambrook *et al*. 1989). A modification of this extraction method (Zheng *et al*. 2002), however, is suggested as it avoids the use of the toxic chemicals. It has been successfully used in New Zealand for almost a decade and is as follows. Place a single nematode into 15 µL of double distilled water on a glass slide and cut it into two or three fragments. Transfer 10 µL of water with nematode fragments into a 0.5 mL Eppendorf tube, with 8 µL of nematode lysis buffer (125 mM KCl, 25 mM Tris-Cl pH 8.3, 3.75 mM $MgCl_2$, 2.5 mM DTT, 1.125% Tween 20) and 2 µL of proteinase K (600 µg/mL). Hold the tube at –80 °C for at least 30 mins, and then incubate them at 65 °C for 60 min, then at 95 °C for 10 min. Once DNA is extracted, PCR is carried out in 25 µl reaction mixtures containing 10 ng template DNA, 50 mM KCl, 10 mM tris (pH 8.2), 2.5 mM $MgCl_2$, 200 mM dNTP, 250 ng of each primer and 1 unit of Taq DNA polymerase. Denaturation is at 94 °C for 5 min, followed by 25 cycles of 30 s at 94 °C, 1 min at 64 °C and 1 min at 72 °C, with a post-cycling extension at 72 °C for 5 min (Castagnone *et al*. 2005). An alternative for single worms is to transfer the nematode to a dry thin-walled PCR tube, cover with 2.5 µL lysis buffer (50 mM KCl, 10 mM Tris (pH 8.2), 2.5 mM $MgCl_2$, 60 mg mL^{-1} proteinase K, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin), and overlay with mineral oil. Tubes are then 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 (Williams *et al*. 1992). To avoid the possibility of false negatives, the PCR should be run for a minimum of 5 samples (where more than one nematode is used for each sample) or 10 samples (where a single nematode is used for each sample). The primer pair listed above has not been tested for specificity outside the genus *Bursaphelenchus*, nor even on all species within the genus, and so restriction of the range of possible species to only those in the genus will avoid the possibility of false positives.

4.2.4 DNA barcoding

DNA barcoding has the advantage over the LAMP and species-specific probe methods in that it is likely to provide a species-level identification for nematodes which may resemble, but are not *B. xylophilus*, rather than the binary yes or no answer provided by the other methods. DNA barcoding does, however, require more detailed interpretation of results.

DNA sequences for DNA barcoding identification can be obtained following the procedures outlined in Ye *et al.* 2007 and Gu *et al.* 2011. Single nematodes are obtained by washing in ddH_2O under a dissecting microscope and transferred to a 200 µL Eppendorf tube (containing 8 µL ddH_2O and 1 µL 10× PCR buffer) using a small picking needle. This tube is stored at -70 °C for at least 30 min, after which it is heated at 85 °C for 2 min. Subsequently, 1 µL proteinase K (1 mg mL^{-1}) is added into the

tube, and incubated at 56 °C for 30 min and 95 °C for 10 min. DNA samples are stored at -20 °C until they are used as PCR template.

Four sets of PCR primers are used to target four gene regions, respectively. These are a partial fragment of the 18S (SSU) region, the ITS (1 and 2) region, a partial fragment of the 28S (D2D3 LSU) region of rDNA, and the partial mitochondrial COI gene. Primers for amplification of partial 18S are K4f (5'-ATG CAT GTC TAA GTG GAG TAT TA -3') and K1r (5'- TTC ACC TAC GGC TAC CTT GTT ACG ACT -3'). Primers for amplification of ITS are F194 (5'- CGT AAC AAG GTA GCT GTA G -3') and 5368r (5'-TTT CAC TCG CCG TTA CTA AGG -3'). Primers for amplification of partial 28S are D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3Br (5'-TCG GAA GGA ACC AGC TAC TA-3'). Primers for amplification of mtCOI are COI-F1 (5'- CCT ACT ATG ATT GGT GGT TTT GGT AAT TG -3') and COI-R2 (5'- GTA GCA GCA GTA AAA TAA GCA CG -3').

PCR conditions for the 28S and COI gene regions are as described by Li *et al.* (2008) and Ye *et al.* (2007), with denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (28S) or 51 °C (COI) for 45 s, and extension at 72 °C for 2 min; with a final extension at 72 °C for 10 min. The thermal cycling program for 18S is as follows: denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 42 °C for 30 s, and extension at 72 °C for 3 min; with a final extension at 72 °C for 10 min. While ITS PCR amplification employs an initial denaturation step 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 step at 72 °C for 5 min.

PCR amplification is visually confirmed on a 1–2% Agarose gel run for 30 minutes at 100V. Sanger sequencing is performed by a commercial service provider. The sequenced data received as electropherogram are visually examined, curated, and aligned using a DNA alignment software (e.g., MEGA11, Geneious). Forward and reverse sequences are paired to generate a consensus sequence.

Finally, the consensus sequence obtained can be compared with those available on public databases, such as GenBank. In order to confirm a species-level identification of the nematode, genetic similarity of the sequence obtained to sequences publicly available needs to be assessed, with a suitable (different) threshold of similarity % considered for each genetic marker.

DNA sequences obtained following the process outlined above should be compared to published reference sequences available on GenBank using the "Blastn" option to match with DNA sequences of *B. xylophilus*. If the genetic divergence between the sequence generated and the accession numbers mentioned below is higher than the thresholds reported below, the query sequence should not be considered a match at species level:

- DNA sequences of **ITS** of *B. xylophilus* are expected to match most closely with *B. xylophilus* GenBank accession number ON496984 (van de Vossenberg *et al.* 2023), with less than 4% difference.
- DNA sequences of **28S** of *B. xylophilus* are also expected to match most closely with *B. xylophilus* GenBank accession number ON496984 (van de Vossenberg *et al.* 2023), with less than 1% difference.
- DNA sequences of **COI** of *B. xylophilus* are expected to match most closely with *B. xylophilus* GenBank accession number JF317257 (Gu *et al.* 2011), with less than 5% difference.

On the other hand, the **18S** DNA sequences of *B. xylophilus* currently available on GenBank (accessed 17th Oct 2023) appear to be within 1% difference in multiple species of the xylophilus-group within

the genus, suggesting they are not useful for providing species-level identification for these *Bursaphelenchus* species (Gu 2018).

Interestingly, the EPPO Standard PM 7/129 (EPPO 2016) provides information on DNA-barcode based identification of *B. xylophilus* through conventional PCR and Sanger sequencing and relies on the use of sequence fragments of both the small (18S) and large (28S) subunit ribosomal RNA for positive identification of *B. xylophilus*. However, the standard does not mention COI or ITS, which are recommended here, as a much better choice than 18S (which has been shown in multiple studies to be unable to provide a species-level identification), based on the literature cited above.

4.2.5 RFLP Fingerprinting

Identification is also possible using RFLP analysis of ITS, subject to similar provisos as with PCR of satellite DNA (see Burgermeister *et al*. 2005, 2009). This is the internationally accepted method (IPPC, 2016).

The method recommended for isolation of DNA for RFLP involves from one to 30 nematodes being placed in 5 µL of distilled water in an Eppendorf tube and frozen at -20 °C. To extract DNA, samples are thawed, mixed with 10 µl of buffer ATL (Qiagen), then homogenized using a micro pestle. An additional 170 µl of Buffer ATL and 20 µl of proteinase K solution (>600 mAU/ml) are then added, the sample is mixed and then incubated at 56°C for 3 hours. After incubation 200 µl of Buffer AL (Qiagen) containing 1 µg carrier RNA (Qiagen) is added to the sample, followed by 15 seconds of mixing via pulse-vortexing. 200 µl of 96–100% ethanol is then added, followed by 15 seconds of pulse-vortexing, then transfer of the sample to a QIAamp MinElute column. The column is then centrifuged at 6000 g for 1 minute. Flow-through solution is discarded and the column is washed twice. For the first wash 500 µl buffer AW1 (Qiagen) is added to the spin column followed by centrifuging at 6000 g for 1 minute; flow through is discarded. For the second wash 500 µl buffer AW2 (Qiagen) is added to the spin column and centrifuged at 6000 g for 1 minute; flow through is discarded. The membrane of the spin column is then dried by centrifuging at 20000 g for 3 minutes. After drying, the spin column is transferred to a clean Eppendorf tube and 20 µl of nuclease-free water (for single nematodes) or up to 100 µl of nuclease-free water (for up to 30 nematodes) is added to the membrane of the spin column. The column is then centrifuged at 20000 g for 1 minute. Eluted DNA is stored at -20 °C until use in PCR.

PCR conditions prior to RFLP analysis are as follows: a 50 µl master mix is made using 0.6 µM of the forward primer ITS1-forward (F): 5′- CGT AAC AAG GTA GCT GTA G G-3′ (Ferris et al. 1993), 0.6 µM of the reverse primer ITS2-reverse (R): 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain 1993), 2 units of Taq DNA polymerase, 10 mM Tris-HCL (at pH 8.8), 50 mM KCL, 2 mM MgCl2, 0.2 mM dNTPs and 2 ng DNA template. Amplification with a thermocycler uses the following conditions: an initial denaturation step at 94°C for 2.5 minutes, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for two minutes, then a final extension at 72°C for 5 minutes. After PCR amplification, 5 µl of PCR product per sample are digested with the required restriction endonucleases (AluI, HaeIII, HinfI, MspI and RsaI) following the instructions provided by the manufacturer of the endonucleases (e.g., New England Biolabs®). Restriction fragments are visualised by gel electrophoresis in a 2.5% agarose gel stained.

The pattern expected for *Bursaphelenchus xylophilus* differs from all other species tested (Figure 2). The pattern from the closely-related species *B. mucronatus* is shown as an example. More examples of other species of *Bursaphelenchus* are shown in Braasch *et al*. (1999, 2004), Braasch and Burgermeister (2002), Gu *et al*. (2008, 2012, 2013, 2014), Han *et al*. (2008a, b), Li *et al*. (2008), Burgermeister *et al*. (2009), Tomalak *et al*. (2013), Maria *et al*. (2015), Wang *et al*. (2018) and Tomalak and Filipiak (2019).

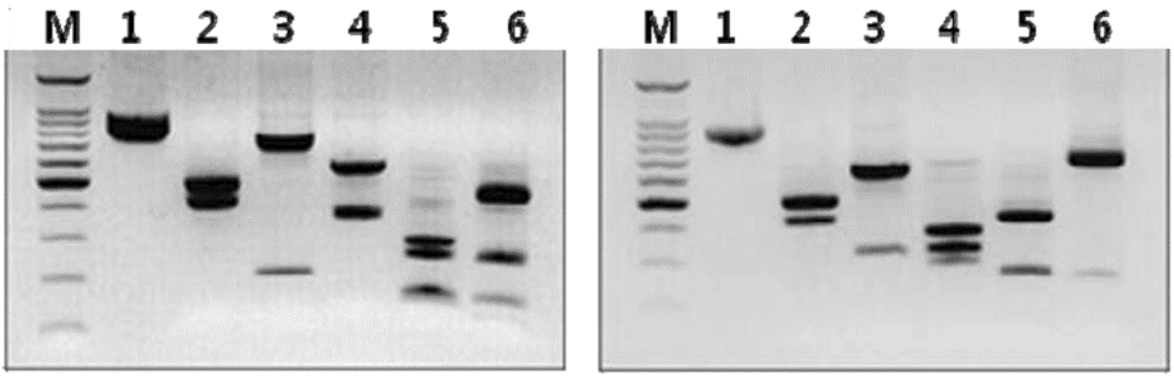

Figure 2. Banding pattern for RFLP analysis of ITS for *Bursaphelenchus xylophilus* (A) and *B. mucronatus* from east Asia (B). Channels are (left to right) M: 1 kb molecular marker, 1: total PCR product, 2: *Rsa* I, 3: *Hae* III, 4: *Msp* I, 5: *Hinf* I, and 6: *Alu* I. (Han *et al*. 2008a).

4.2.6 Reference material

There is no live material currently in Australia. The main laboratories holding reference material for molecular work are:

Location	**Current contact**
Department of Entomology and Nematology, University of Florida, Fort Lauderdale, 3205 College Avenue, Fort Lauderdale, FL 33314-7799	Emeritus Professor RM Giblin Davis
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Manda Khudhir (CSIRO) updated some of the diagnostic imagery in section 8.

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8 APPENDICES

8.1 Hosts of PWN

8.1.1 Host range

Bursaphelenchus xylophilus is pathogenic to a number of pines (*Pinus* spp.), as well as larch (*Larix* spp.), spruce (*Picea* spp.) and fir (*Abies* spp.). It may also infect or be associated with North American pines without causing disease. Susceptible species include Japanese red pine (*P. densiflora*), Japanese black pine (*P. thunbergii*), Masson's pine (*P. massoniana*), Luchu pine (*P. luchuensis*) *and* Scots pine (*P. sylvestris*) (Dwinell and Mota 2001). Conifer species which are rarely colonised by the pinewood nematode include white fir (*Abies concolor*), coast redwood (*Sequoia sempervirens*), western red cedar (*Thuja plicata*), eastern hemlock (*Tsuga canadensis*), and western hemlock (*T. heterophylla*) (Cram and Hanson 2006).

The nematode can develop on live or dead wood and fungi. Beetles are vectors, and there is no evidence of the beetles being used for anything other than transport. The wood of all species tested from the genus *Pinus* will support development of *B. xylophilus*, as will at least some *Larix*, *Abies*, *Picea* and *Pseudotsuga* (EPPO 2013). Living trees vary in susceptibility with species and environmental conditions, and there are also intra-specific differences in susceptibility (Rutherford and Webster 1987, Rutherford *et al*. 1990).

Fungal hosts of *B. xylophilus* include *Ceratostomella ips*, *Ceratostomella pini*, *Ceratocystis* spp., *Diplodia* sp., *Fusarium* sp., *Macrophoma* sp., *Pestalotia* sp., *Rhizosphaera* sp. and *Trichosporium* sp. (Steiner and Buhrer 1934, Kobayashi *et al*. 1974, 1975).

8.1.2 Effect on hosts

Parts of the plants affected are the stem, branches and wood.

Once the nematodes are introduced, they feed on the epithelial cells and resin ducts in susceptible host trees and can become distributed throughout the sapwood of the branches, trunk and roots. Under favourable conditions, vascular dysfunction occurs rapidly, resulting in wilt and death of the susceptible host.

8.1.3 Susceptibility of common species of the genus *Pinus* in Australia to *B. xylophilus* (Evans *et al*. 1996)

Species	Resistance
Pinus caribaea	partial
Pinus caribaea var *hondurensis*	?
Pinus elliotii	resistant
Pinus halepensis	partial
Pinus pinaster	susceptible
Pinus ponderosa	partial
Pinus radiata	partial

8.2 Transmission of PWN

Since PWN is transmitted during oviposition by the adult female *Monochamus* spp., the nematode is found wherever dying or recently killed conifers are colonised by *Monochamus* spp. Worldwide, species of *Bursaphelenchus* phoretic on *Monochamus* spp. are co-distributed with their insect hosts (Dwinell and Mota 2001).

The PWN and its vector *Monochamus* spp. can be dispersed in coniferous wood chips, logs, unseasoned timber, wooden pallets, crates, packing-case material and dunnage (Biosecurity Australia 2006, Dwinell 1986, 1987, 1997, Gu *et al*. 2005, 2006, 2008, 2013). In the field, transmission for short distances through the soil has been demonstrated experimentally, but the significance of this pathway under non-experimental conditions remains unknown. It is thought that this is a relatively minor pathway under natural conditions but may operate where fresh timber is stored adjacent to old timber or wood chip piles. Infection from a pile of wood chips to adjacent trees has been shown to occur (Braasch 1996). PWN has been shown to survive in wood for at least one year and possibly up to six years (Halik and Bergdahl 1990, 1992, 1994, Tomminen and Akar 1990, Tomminen *et al.* 1991), so transmission by contamination in artificial situations where wood is stored is a real possibility.

8.2.1 Vectors

All known and suspected vectors are listed in Table 2. In the native range of PWN (USA) *Monochamus carolinensis* is the major efficient vector. In Japan, the major efficient vector is *Monochamus alternatus*. *Monochamus saltuarius* has been found to carry up to 10,000 nematodes per beetle, but efficiency of transmission is probably low due to the behaviour of the beetle (Sato *et al.* 1987).

Note that dispersal for short distances in wood and soil has also been demonstrated (Braasch 1996, Halik and Bergdahl 1992, Kiyohara and Tokushige 1971). Movement this way is thought unimportant in nature but may assume increasing importance due to human activities such as concentrated or centralized processing or storage of various timber products (Evans *et al.* 1996).

Known efficient vectors are currently absent from Australia, however the suitability of local vectors for carrying PWN is unknown. Local borers of the Family Cerambycidae and Scolytidae carry local species of nematodes from the same superfamily as PWN and an unidentified insect vector carried another species of the genus, *Bursaphelenchus hunanensis* (now recognised as *Deviburs aphelenchus hunanensis*) up to 55 km (Hodda *et al.* 2008). Many genera of Cerambycidae and some genera from other families of Coleoptera have been found to carry PWN, but there is no evidence they have any role as vectors in nature. Within the genus *Monochamus*, several species transmit PWN in nature, and many more are likely to do so, where they to occur in the same place as the nematodes. There is thus considerable plasticity in the species of vector which may be used.

Table 2. Known and likely vectors for *Bursaphelenchus xylophilus*.

Family, species	Range	Host	Transmission of PWN proven [a]	Reference
Cerambycidae				
Acalolepta fraudatrix Bates	Japan	unk.	-	Kobayashi *et al.* 1984, Mamiya 1976
Acanthocinus griseus (Fabricius).	Japan	unk.	-	Kobayashi *et al.* 1984, Mamiya and Enda 1972
Astylopsis sexguttata (Say, 1827) [*Amniscus sexguttatus* (Say)]	N. Am.	*Pinus*, *Picea*	-	Linit *et al.* 1983, Wingfield and Blanchette 1983
Arhopalus rusticus Linnaeus	Japan	unk.	-	Mamiya 1972, Mamiya and Enda 1972
Arhopalus rusticus obsoletus (Randall)	N. Am.	*Pinus*	-	Linit *et al.* 1983
Asemum striatum (Linnaeus)	N. Am.	*Pinus*, *Picea*	-	Linit *et al.* 1983
Aredolpona succedanea (Lewis) [= *Corymbia succedanea* Lewis]	Japan	unk.	-	Kobayashi *et al.* 1984, Mamiya 1972, Mamiya and Enda 1972
Monochamus alternatus Hope	Japan	*Pinus*, *Picea*, *Abies*, *Larix*, *Cedrus*	+	Evans *et al.* 1996, Kobayashi *et al.* 1984, Mamiya and Enda 1972, Togashi 1985
Monochamus carolinensis (Olivier)	N. Am.	*Pinus*	+	Evans *et al.* 1996, Linit *et al.* 1983, Wingfield and Blanchette 1983
Monochamus clamator (LeConte)	N. Am.	*Pinus*	-	Evans *et al.* 1996
Monochamus galloprovincialis (Olivier)	N Asia, Europe, N Africa	*Pinus*	-	Evans *et al.* 1996
Monochamus grandis Waterhouse	Japan	*Abies*, *Picea*	-	Evans *et al.* 1996
Monochamus marmorator Kirby	N. Am.	*Abies*, *Picea*	+	Evans *et al.* 1996, Wingfield and Blanchette 1983
Monochamus mutator (LeConte)	N. Am.	*Pinus*	+	Evans *et al.* 1996, Wingfield and Blanchette 1983
Monochamus nitens Bates	Japan	unk.	+	Evans *et al.* 1996, Kobayashi *et al.* 1984
Monochamus notatus (Drury)	N. Am.	*Pinus*	-	Evans *et al.* 1996
Monochamus obtusus Casey	N. Am.	*Pinus*, *Picea*, *Pseudotsuga*, *Abies*	+	Evans *et al.* 1996, Holdeman 1980
Monochamus rubigeneus Bates [= *Monochamus clamator rubigineus* (Bates)]	N. Am.	*Pinus*		Evans *et al.* 1996

Family, species	Range	Host	Transmission of PWN proven [a]	Reference
Monochamus sartor (Fabricius)	Europe	*Pinus*, *Picea*		Evans *et al.* 1996
Monochamus scutellatus (Say)	N. Am.	*Pinus*, *Abies*, *Picea*, *Larix*	+	Evans *et al.* 1996, Holdeman 1980, Wingfield and Blanchette 1983
Monochamus saltuarius Gebler	Japan, Europe	*Pinus*, *Picea*	+	Evans *et al.* 1996, Kobayashi *et al.* 1984
Monochamus subfasciatus Bates	Japan	*Gingko*	-	Evans *et al.* 1996
Monochamus sutor (Linnaeus)	China, N Asia, Europe	*Pinus*, *Picea*, *Larix*	-	Evans *et al.* 1996
Monochamus tesserula White [= *M. alternatus* Hope]	Japan, China	*Pinus*	-	Evans *et al.* 1996
Monochamus titillator (Fabricius)	N. Am.	*Pinus*, *Picea*, *Abies*	+	Carling 1984, Evans *et al.* 1996, Kinn 1986, Luzzi *et al.* 1984, Williams 1980
Monochamus urussovii (Fischer)	Japan, China, N Asia, Europe	*Pinus*, *Picea*, *Abies*, *Larix*	-	Evans *et al.* 1996
Neacanthocinus obsoletus (Olivier)	N. Am.	*Pinus*, *Abies*	-	Carling 1984, Kinn 1987
Neacanthocinus pusillus (Kirby)	N. Am.	*Pinus*, *Abies*, *Picea*	-	Wingfield and Blanchette 1983
Spondylis buprestoides Linnaeus	Japan	*Pinus*	-	Kobayashi *et al.* 1984, Mamiya 1972, Mamiya 1976
Uraecha bimaculata Thomson	Japan	*Pinus*	-	Kobayashi *et al.* 1984, Mamiya 1976
Xylotrechus saggitatus (Germar)	N. Am.	*Pinus*	-	Wingfield and Blanchette 1983
Buprestidae				
Chysobothris spp.	N. Am.	*Pinus*	-	Linit *et al.* 1983, Wingfield and Blanchette 1983
Curculionidae				
Hylobius pales (Herbst)	N. Am.	*Pinus*	-	Linit *et al.* 1983
Pissodes approximatus Hopkins	N. Am.	*Pinus*, *Picea*	-	Linit *et al.* 1983
Dendroctonus frontalis Zimmermann			+	Kinn 1986, Kinn and Linit 1992
Ips spp.			-	Lieutier and Vallet 1982

[a] - Note that lack of transmission, or inability to successful transmit PWN, has not been proven.

8.3 Life cycle of PWN

Pine wilt disease is the result of a complex interaction between the PWN (*B. xylophilus*), the longhorn beetle (*Monochamus* spp.), a host coniferous tree and sometimes 'blue-stain fungi' (e.g. *Ceratocystis* spp., *Ophiostoma piceae*) (Donald *et al*. 2016). A bacterium or bacterial strain may also be involved in the disease (Vicente *et al.* 2011).

The PWN is transmitted to coniferous trees either when the longhorn beetle feeds on the bark and phloem of twigs of susceptible live trees (primary transmission) or when the female beetle lays eggs (oviposition) in freshly cut timber or dying trees (secondary transmission) (Cram and Hanson 2006). Once the nematodes are introduced, they feed on the epithelial cells and resin ducts in susceptible host trees and can become distributed throughout the sapwood of the branches, trunk and roots. Under favourable conditions or perhaps when a certain bacterium is present, vascular dysfunction occurs rapidly, resulting in wilt and death of the susceptible host.

The presence of PWN from secondary transmission can confound the diagnosis of pine wilt disease. The PWN may be deposited by the longhorn beetle during oviposition into dead or dying trees, which may or may not have been affected by pine wilt disease. Thus, trees that were not killed by the PWN may still hold a significant population of *B. xylophilus*. The nematode is a secondary invader in these cases and does not contribute to tree mortality (Dwinell and Mota 2001). The PWNs feed on blue-stain fungi (*Ceratocystis* spp.) and other fungi that typically invade cut timber and dead and dying softwoods (Cram and Hanson 2006).

The life cycle of the PWN involves a propagative cycle and a dispersal cycle (Cram and Hanson 2006). The propagative cycle occurs in the sapwood and involves six life stages: the egg, four larval stages and the adult. The sequence of egg to adult takes 4 to 5 days under favourable conditions of adequate wood moisture, temperature and nutrient availability. The first stage occurs within the egg followed by hatching to the second stage, which soon moults into the third stage. There are two forms of the third stage: 1) larvae that change into fourth stage larvae, which eventually change into adults that remain in infested trees; and 2) non-feeding disease stage larvae. The development of the nematode switches to dispersal mode in the late stages of tree infection after tree death and occurs only in the presence of *Monochamus* pupae within the wood. These third-stage larvae aggregate on the wall of the pupal chamber of the longhorn beetle in the xylem, and then moult to dauer-juveniles. The dauer-juveniles are the non-feeding larval stage that is specialised for survival during the transport phase of the life cycle. These fourth-stage larvae enter the respiratory system of the young adult beetle and are vectored by the beetle to new hosts. The dauer-juveniles can moult into adults within 48 hours after transmission to a new host.

Duration of life cycle

Duration of the life cycle depends very much on temperature. The time from egg to egg is 12 days at 15 °C, 6 days at 20 °C and 3 days at 30 °C. Development does not occur below 9.5 °C.

Experimentally, PWN can remain in one phase of the life cycle for long periods. They have been maintained for long periods, through many generations, on a range of fungal mycelia, as well as yeasts associated with vector beetles (Fukushige 1991, Kobayashi *et al.* 1974, 1975, Ogura and Tamura 1989). In susceptible trees, PWN will feed on living cells of the tree as long as the cells are viable (Mamiya 1983). The maximum longevity of the dispersal stages may be up to 6 years (Halik and Bergdahl 1994).

8.4 Taxonomic description

(after Hunt 1993, Nickle *et al*. 1981, Ryss *et al*. 2005)

Adult female. Small nematode 400–700 µm long, slender. Die ventrally arcuate on heat relaxation. Cuticle finely annulated. Lateral incisures four. Cephalic region high, offset, with six lips. Stylet 12–18 µm long, with small knobs, conus pointed, shaft robust. Procorpus cylindroid. Median bulb large, elongate ovoid. Oesophageal glands overlapping intestine dorsally, free in body cavity, long. Nerve ring within one body diameter of median bulb. Excretory pore near nerve ring. Hemizonid at about 60–70% of a body diameter behind median bulb. Vulva at 68–78% of body length, with anterior lip directed posteriorly to form a flap; flap directed slightly anteriorly. Genital tract monoprodelphic, outstretched, with developing oocytes mostly in single file. Post-uterine sac long, extending for more than 75% of the distance to anus. Tail subcylindroid with broadly rounded terminus and no mucro. A few populations have a small mucro less than 2 µm long.

Adult male. Similar to female except for reproductive structures and the following. Body posture strongly ventrally curved on heat relaxation ('walking stick'). Tail terminus pointed, with small bursa-like structure situated terminally. Spicules long, strongly arcuate, apex bluntly rounded, rostrum prominent and pointed, tip expanded into cucullus. Seven caudal papillae, one preanal ventromedian, one pair ventrolateral adanal, one pair ventrolateral just anterior to anterior end of bursa, one pair ventrolateral near tail spike.

Juvenile. Similar to adult female except for reproductive structures.

8.5 Taxonomic illustrations

Figures 3–5 present drawings of *B. xylophilus*.

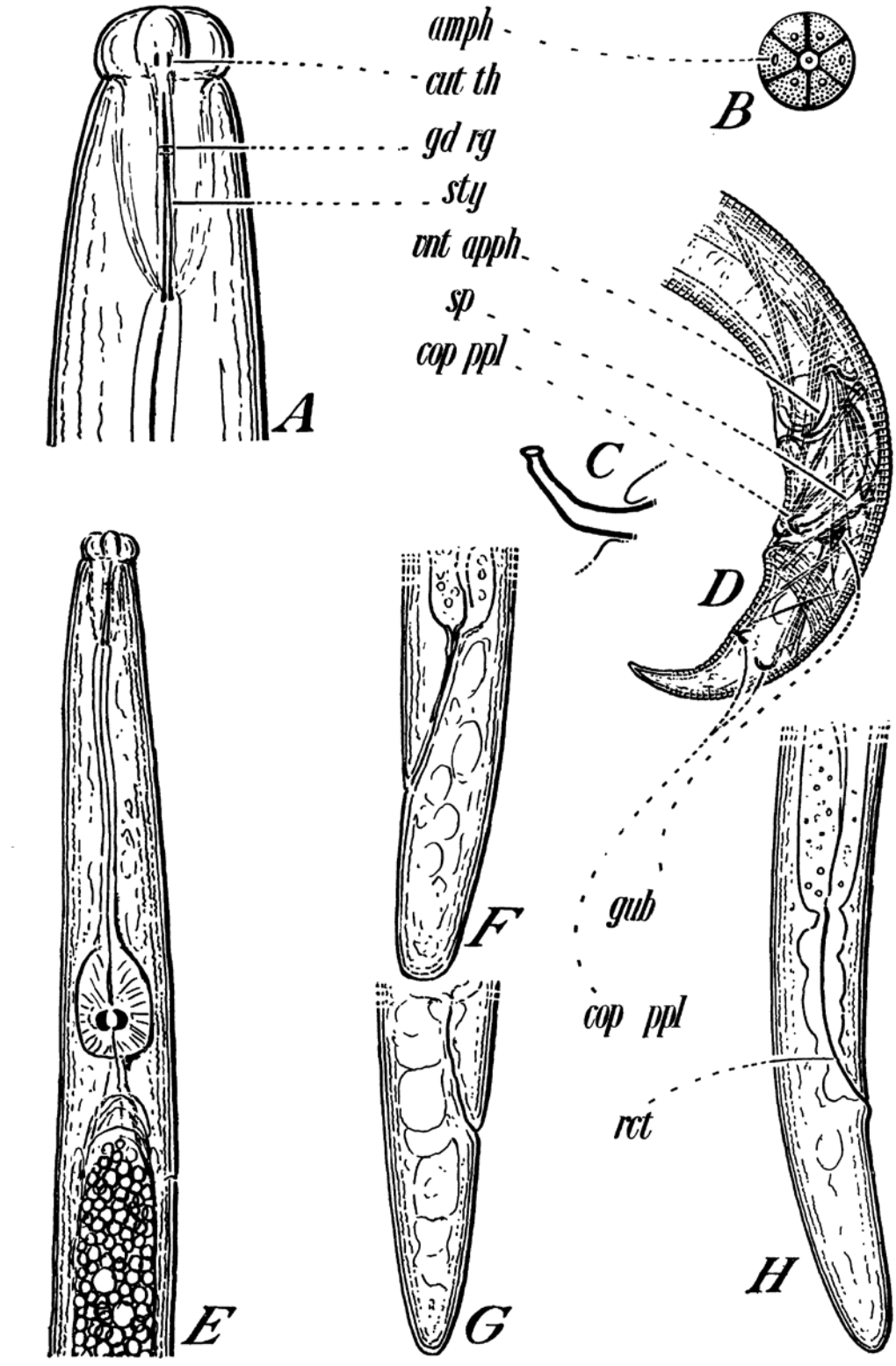

Figure 3. *Bursaphelenchus xylophilus*. A–head of female: *cut th*, cuticular thickening in cephalic portion of alimentary tract; *gd rg*, guiding rings of stylet; *sty*, stylet. B–front view of head: *amph*, amphid. C–extruded spicula showing circular expansion. D–tail of male: *vnt apph*, ventral apophysis; *sp*, spicula; *cop ppl*, copulatory papillae (three pairs); *gub*, gubernaculum. E–anterior end of larva. F and G–tails of larvae, showing variation in shape. H–tail of female: *rct*, rectum. (from Steiner and Buhrer 1934).

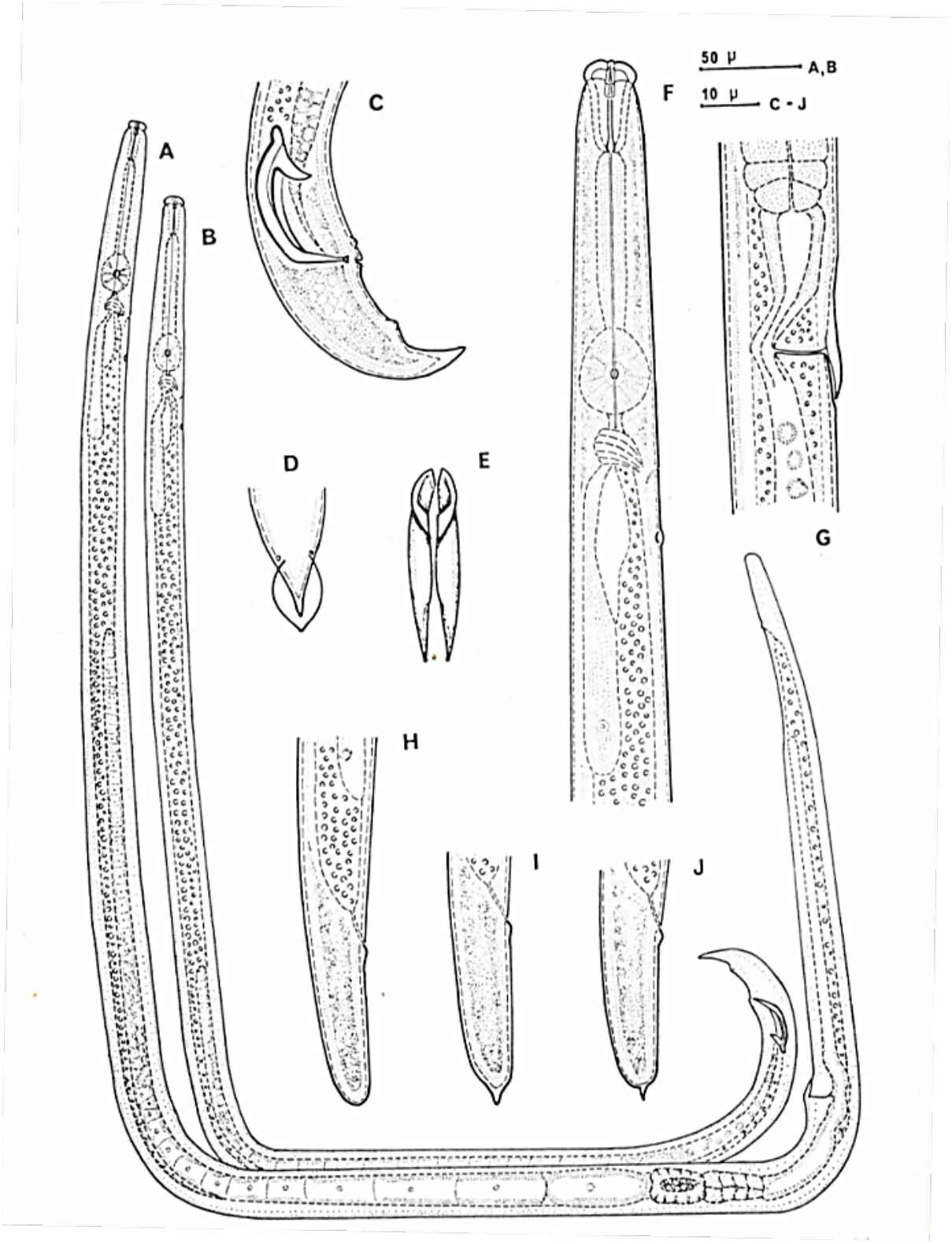

Figure 4. *Bursaphelenchus xylophilus*. A–female. B–male. C–male tail. D–ventral view of male tail, tip with caudal alae. E–ventral view of spicules. F–female, anterior portion. G–female vulva. H–J–female tail. (from Mamiya and Kiyohara 1972).

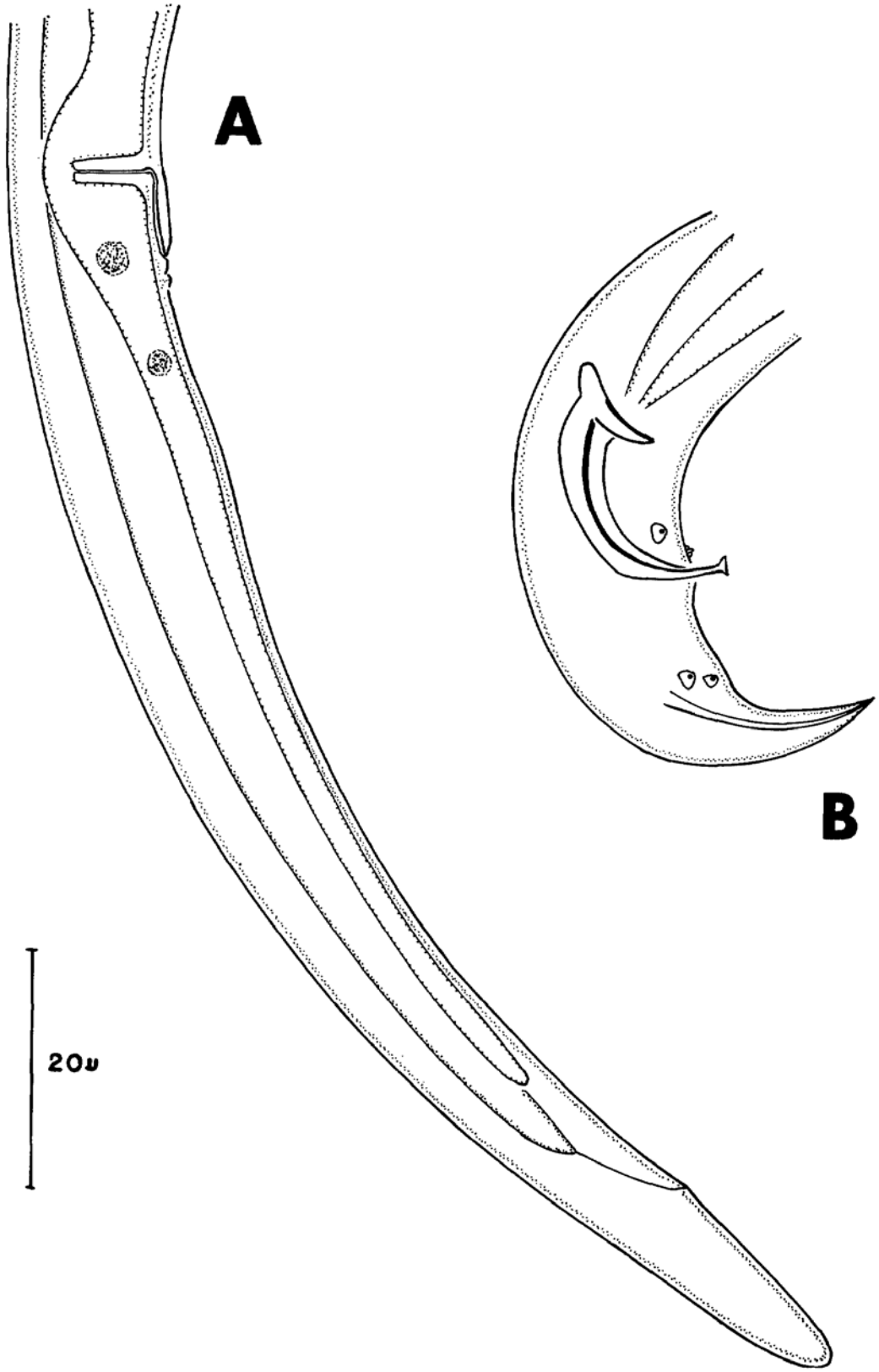

Figure 5. Drawings of the female and male of original *Bursaphelenchus xylophilus* material. A–female tail showing large vulvar flap and long post uterine sac. B–male tail showing typical spicule with expanded tip, caudal alae, and tail papillae. (from Nickle *et al*. 1981).

8.6 Morphometrics

Table 3 presents morphological measurements of female and male *B. xylophilus*.

Table 3. Mean and range (in parentheses) measurements of morphological characters of *Bursaphelenchus xylophilus*.

	author(s)				
	Steiner and Buhrer (1934)	Mamiya and Kiyohara (1972)	Nickle *et al.* (1981)	Mota *et al.* (1999)	Penas *et al.* (2008)
females					
L = body length (mm)	0.9	0.81 (0.71–1.01)	0.52 (0.45–0.61)	1.05 (0.89–1.29)	0.58 (0.51–0.66)
a = body length/greatest body diameter	~59	40.0 (33–46)	42.6 (37–48)	50.0 (41–58)	41.9 (32.8–50.6)
b = body length/distance from anterior end to oesophageal-intestinal valve	11.5	10.3 (9.4–12.8)	9.6 (8.3–10.5)	13.8 (12.7–16.4)	10.1 (9.1–11.2)
c = body length/tail length	26.3	26.0 (23–32)	27.2 (23–31)	26.6 (22–32)	25.4 (20.2–29.0)
V = vulva position, % of L	74	72.7 (67–78)	74.7 (73–78)	73.3 (70–76)	71.5 (70.1–72.9)
stylet length (µm)		15.9 (14–18)	12.8 (12.6–13.0)	12.3 (11–15)	11.2 (10.0–12.5)
males					
L = body length (mm)	0.77	0.73 (0.59–0.82)	0.56 (0.52–0.60)	1.03 (0.80–1.30)	0.57 (0.45–0.69)
a = body length/greatest body diameter	~50	42.3 (36–47)	40.8 (35–45)	49.4 (44–56)	46.0 (40.2–58.5)
b = body length/distance from anterior end to oesophageal-intestinal valve	10.0	9.4 (7.6–11.3)	9.4 (8.4–10.5)	13.3 (11.1–14.9)	9.6 (8.2–10.7)
c = body length/tail length	24.4	26.4 (21–31)	24.4 (21–29)	28.0 (24–32)	21.6 (19.1–24.6)
spicule length (µm)		27.0 (25–30)	21.2 (18.8–23.0)	24 (22–25)	19.3 (16.5–24.0)
stylet length (µm)		14.9 (14–17)	13.3 (12.6–13.8)	12.6 (11–16)	11.0 (10–14)

8.7 Species from the genera *Bursaphelenchus*, *Aphelenchoides*, *Laimaphelenchus* and *Ptychaphelenchus* recorded from Australia

Genus and species	State	Notes
Aphelenchoides besseyi	QLD, NT	
Aphelenchoides bicaudatus	NSW, QLD, VIC, WA	
Aphelenchoides blastophthorus	NSW	
Aphelenchoides brevicaudatus	NSW, QLD	
Aphelenchoides composticola	NSW, QLD, VIC, WA	
Aphelenchoides dactylocercus	VIC	
Aphelenchoides fragariae	NSW, QLD, SA, VIC, WA	
Aphelenchoides goodeyi	QLD, WA	
Aphelenchoides helophilus	VIC	
Aphelenchoides hylurgi	QLD, SA	
Aphelenchoides limberi	NSW	
Aphelenchoides obtusus	NSW	
Aphelenchoides parietinus	NSW, QLD	
Aphelenchoides saprophilus	NSW, QLD	
Aphelenchoides subtenuis	QLD	
Aphelenchoides sp. e	NSW	undescribed
Aphelenchoides sp. f	NSW, QLD	sp. inq. = ?*Aphelenchoides coffeae* (Zimmerman 1898) Filipjev 1934
Aphelenchoides sp.	QLD	Originally misidentified as *Bursaphelenchus sp*.
Bursaphelenchus aff. *vallesianus/sexdentati*	NSW, QLD	
Bursaphelenchus sp.	NSW	associated with *Pinus taeda* and *P. elliottii*
Devibursaphelenchus hunanensis	VIC	apparently eradicated
Laimaphelenchus australis	VIC	described 2006
Laimaphelenchus preissii	SA	described 2006
Laimaphelenchus sp. b	SA	undescribed as yet
Ptychaphelenchus eucalypticola	ACT	described 2008

8.8 Diagnostic images of PWN

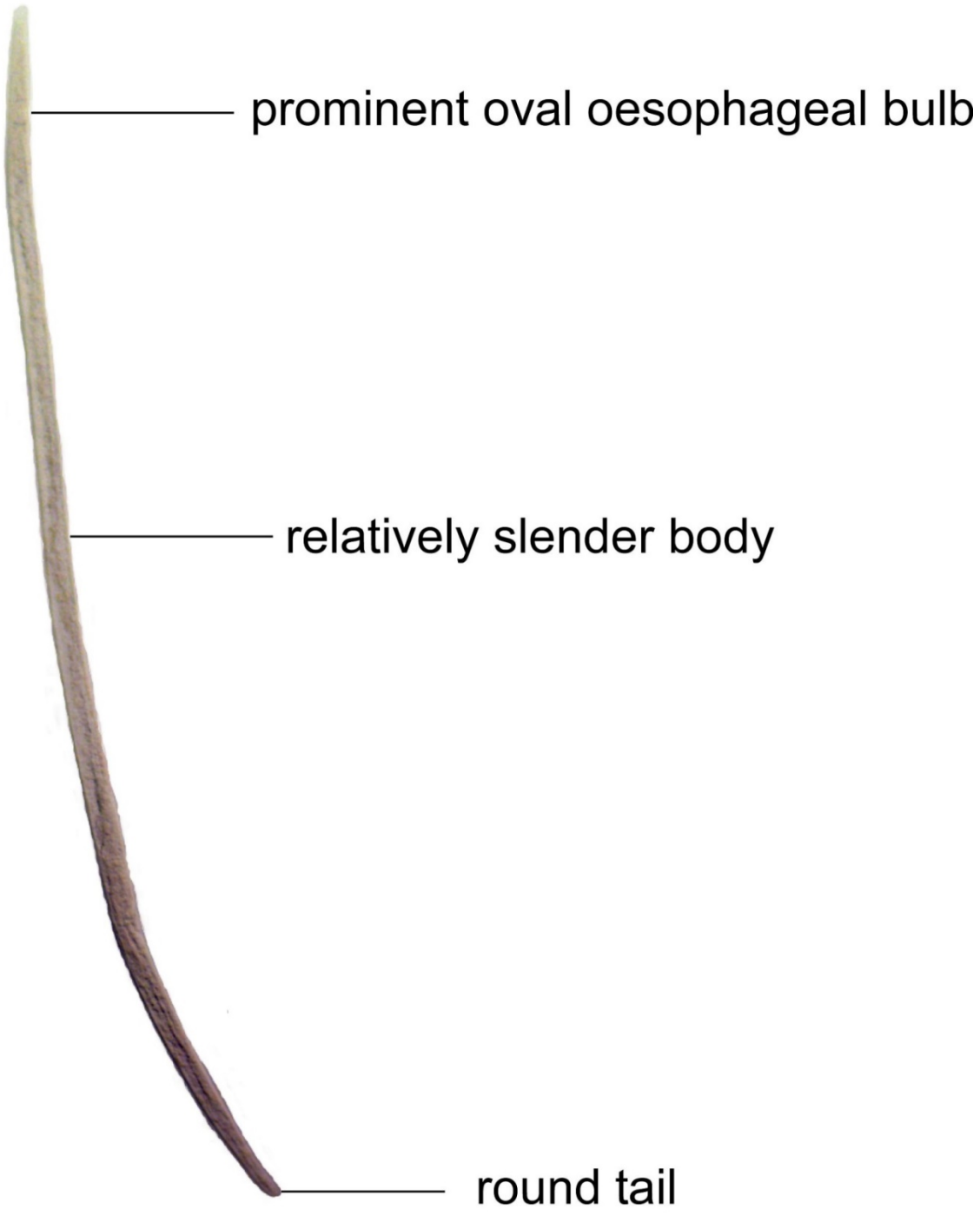

Figure 6. *Bursaphelenchus xylophilus* adult female (photo by Mike Hodda).

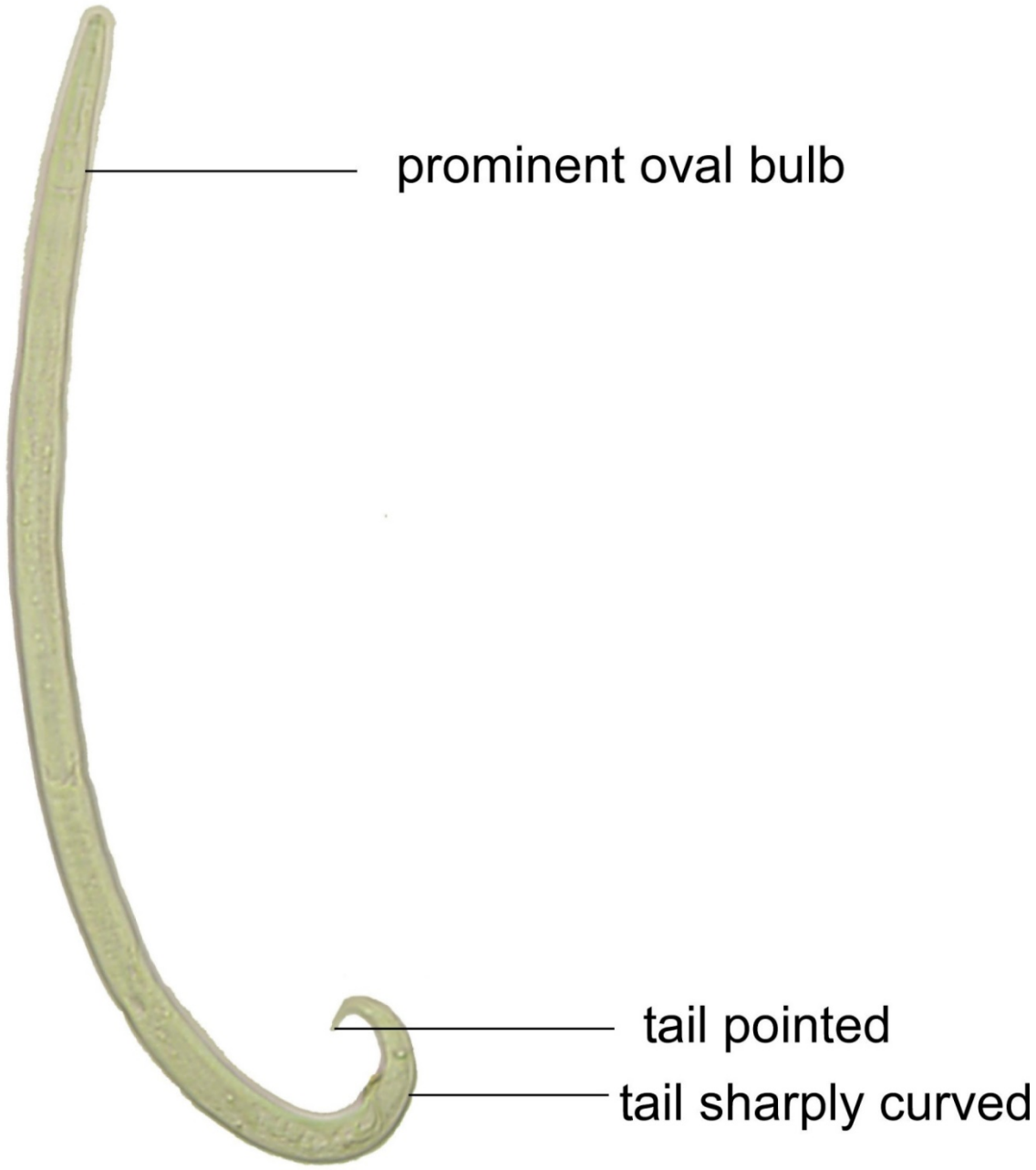

Figure 7. *Bursaphelenchus xylophilus* adult male (photo by Mike Hodda).

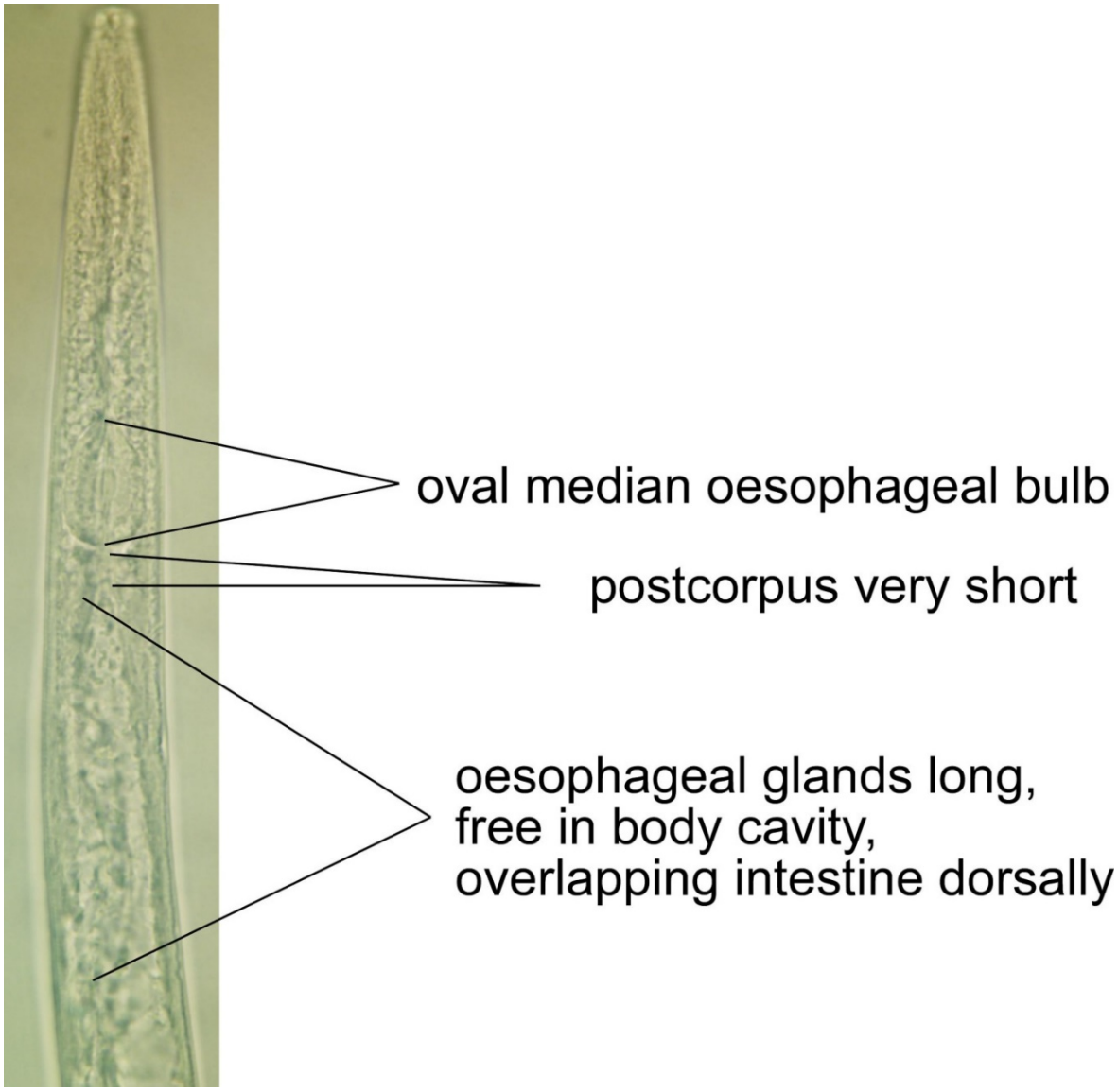

Figure 8. *Bursaphelenchus xylophilus* adult female head and oesophageal region (photo by Mike Hodda).

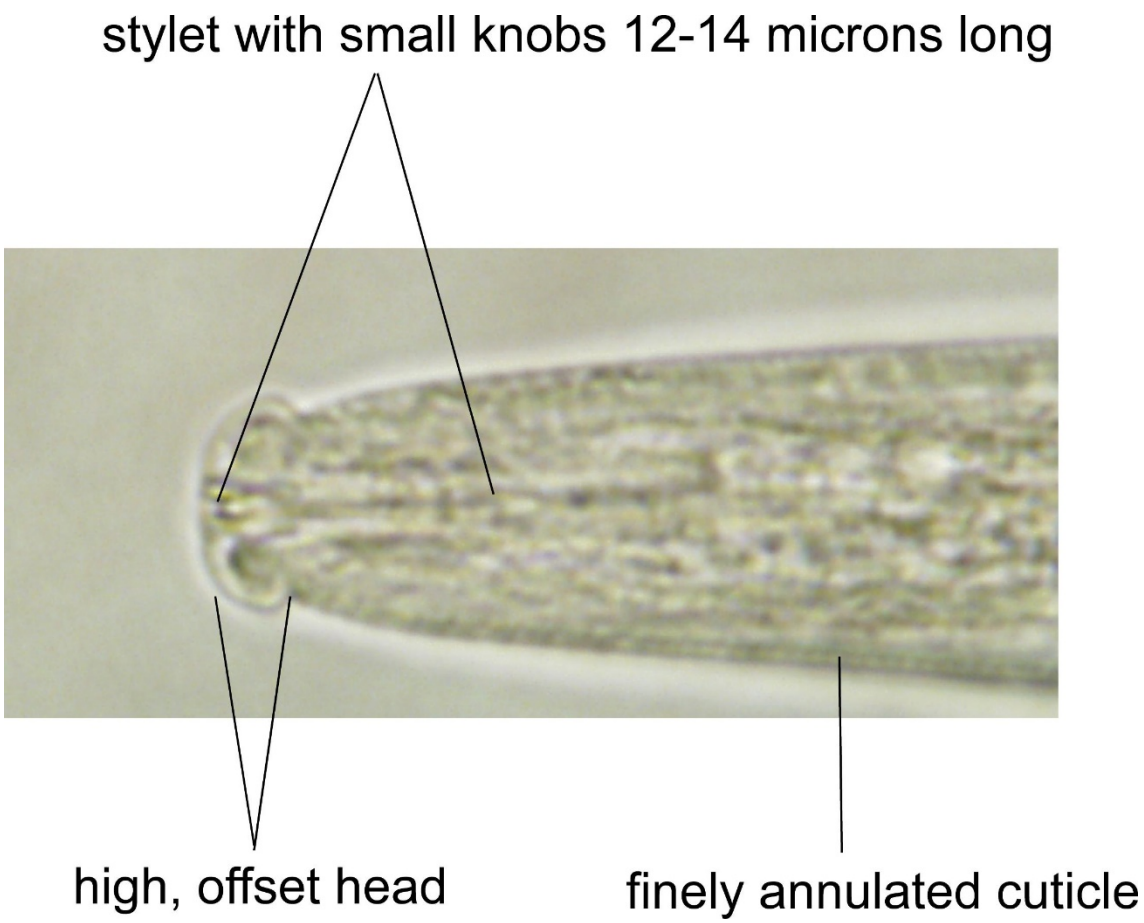

Figure 9. *Bursaphelenchus xylophilus* adult female head detail (photo by Mike Hodda).

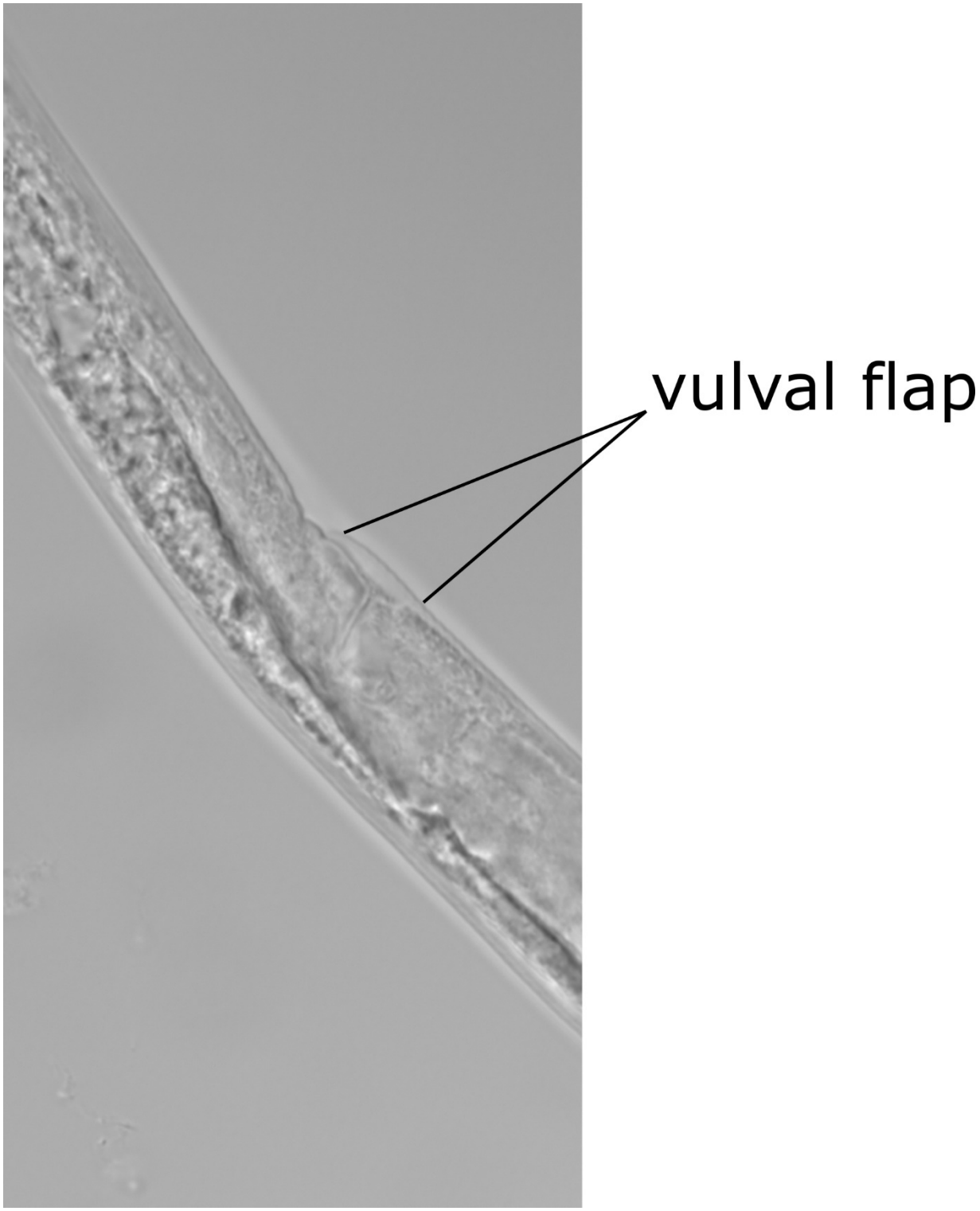

Figure 10. *Bursaphelenchus xylophilus* adult female vulval region (photo by Manda Khudhir).

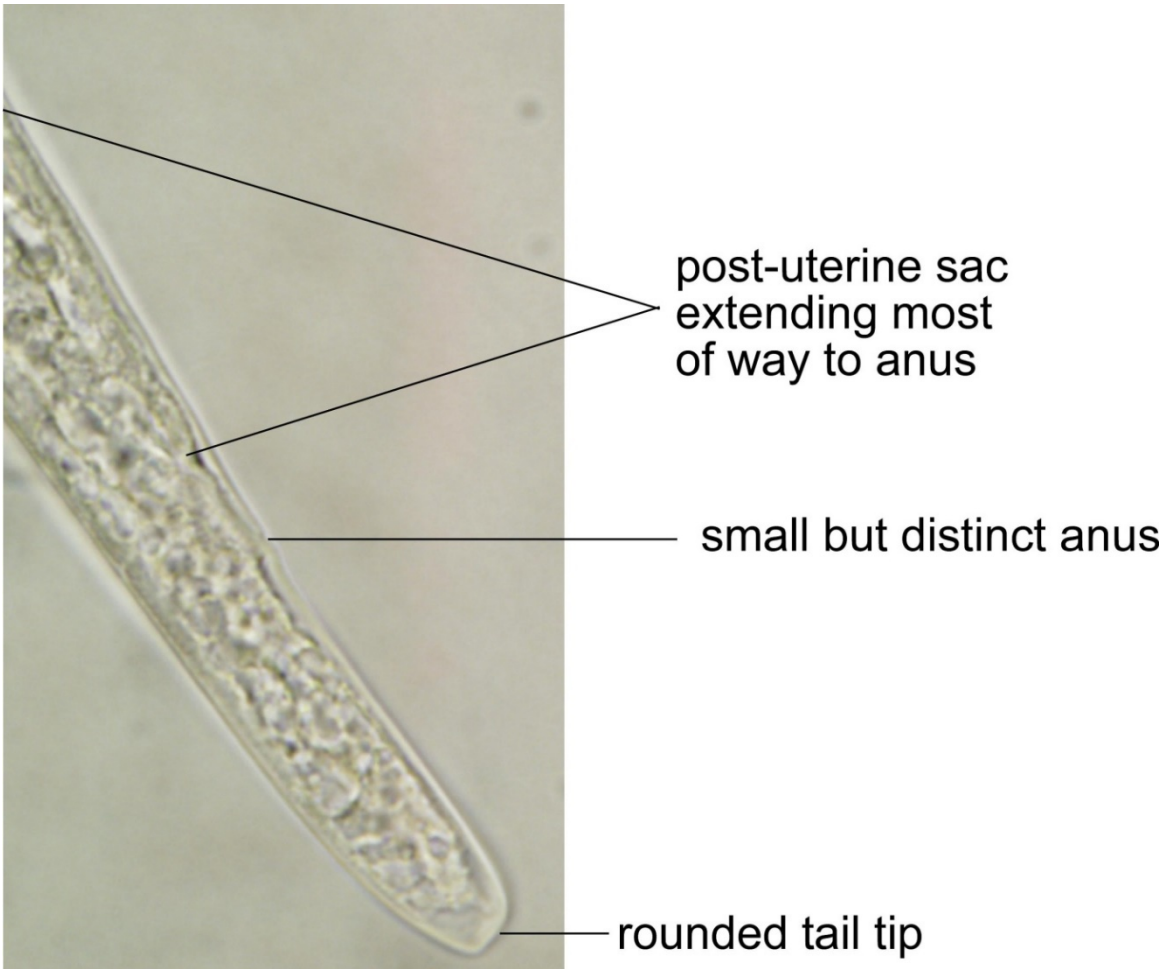

Figure 11. *Bursaphelenchus xylophilus* adult female tail (photo by Mike Hodda).

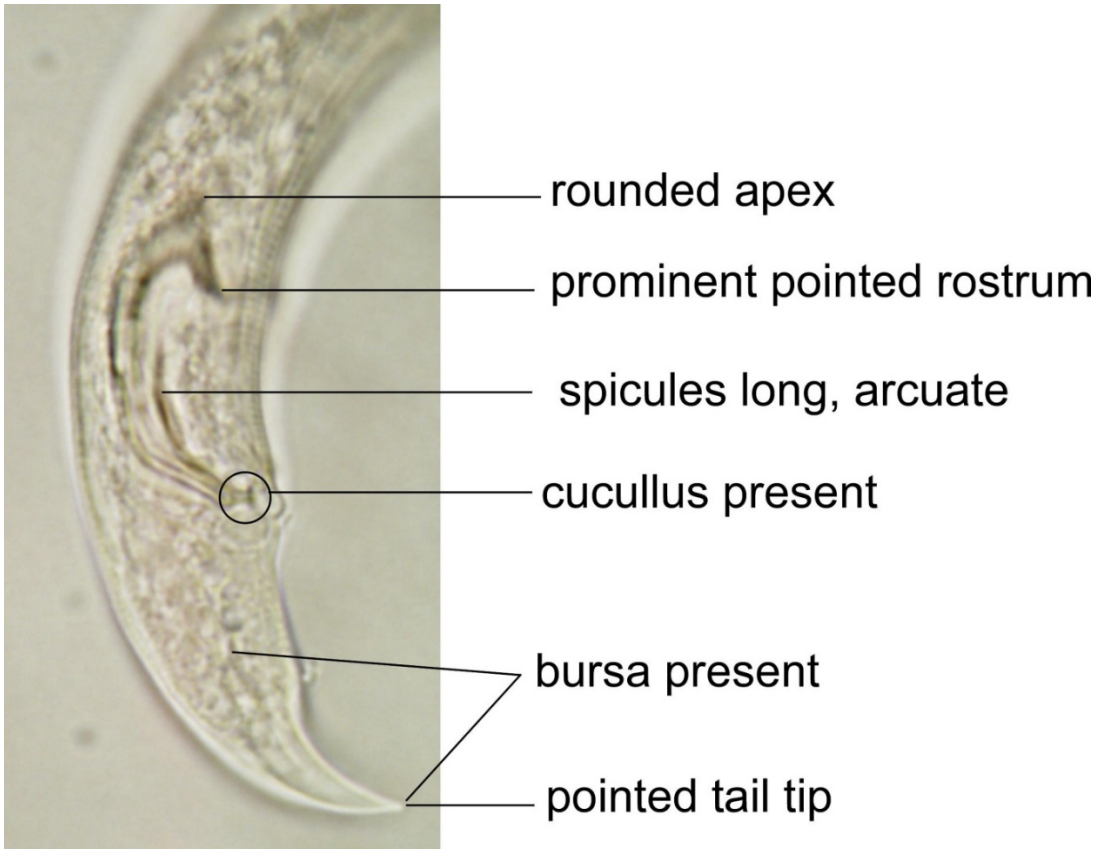

Figure 12. *Bursaphelenchus xylophilus* adult male tail (photo by Mike Hodda).

8.9 Diagnostic images of nematodes likely to be confused with PWN

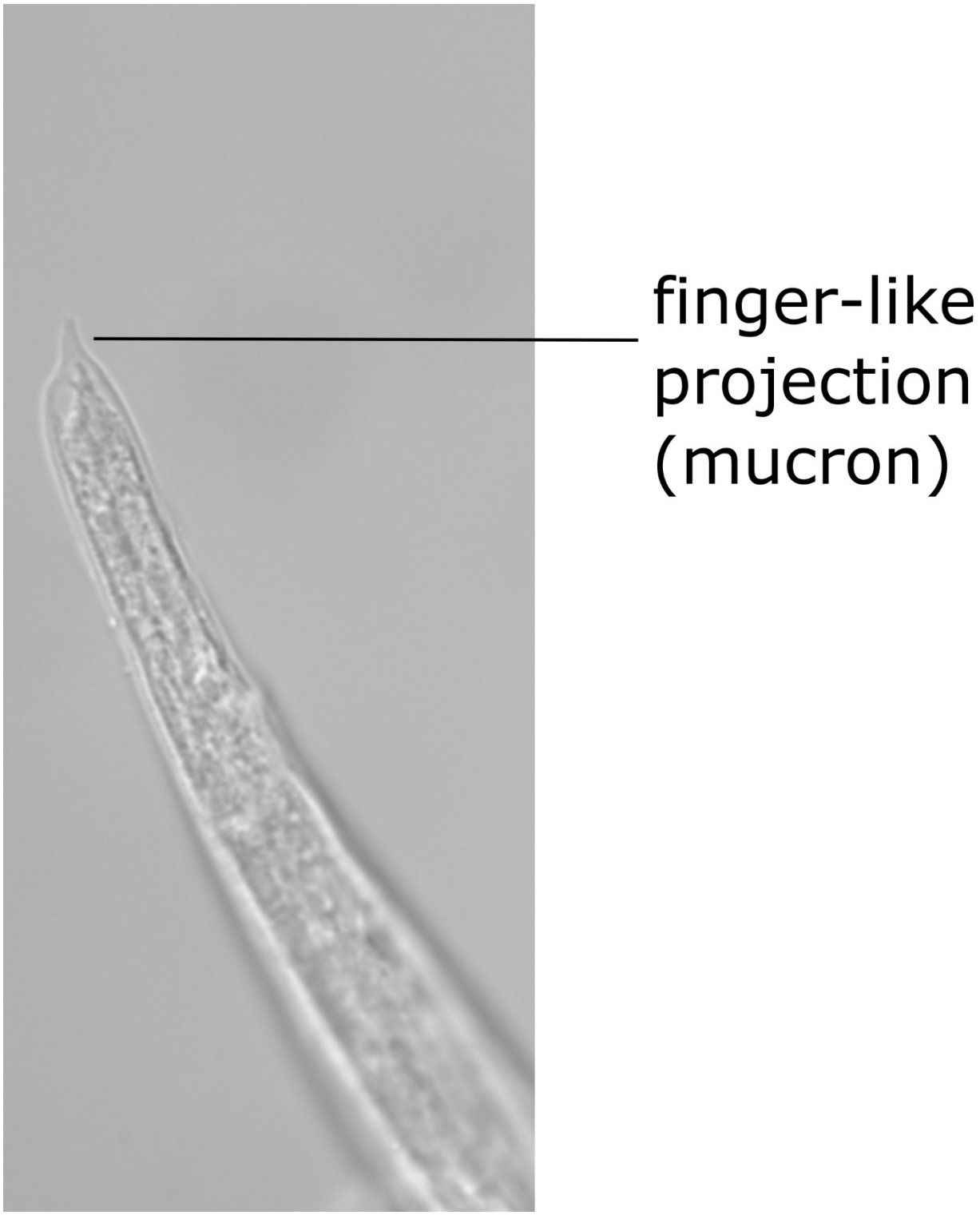

Figure 13. *Bursaphelenchus mucronatus* adult female tail (photo by Manda Khudhir).

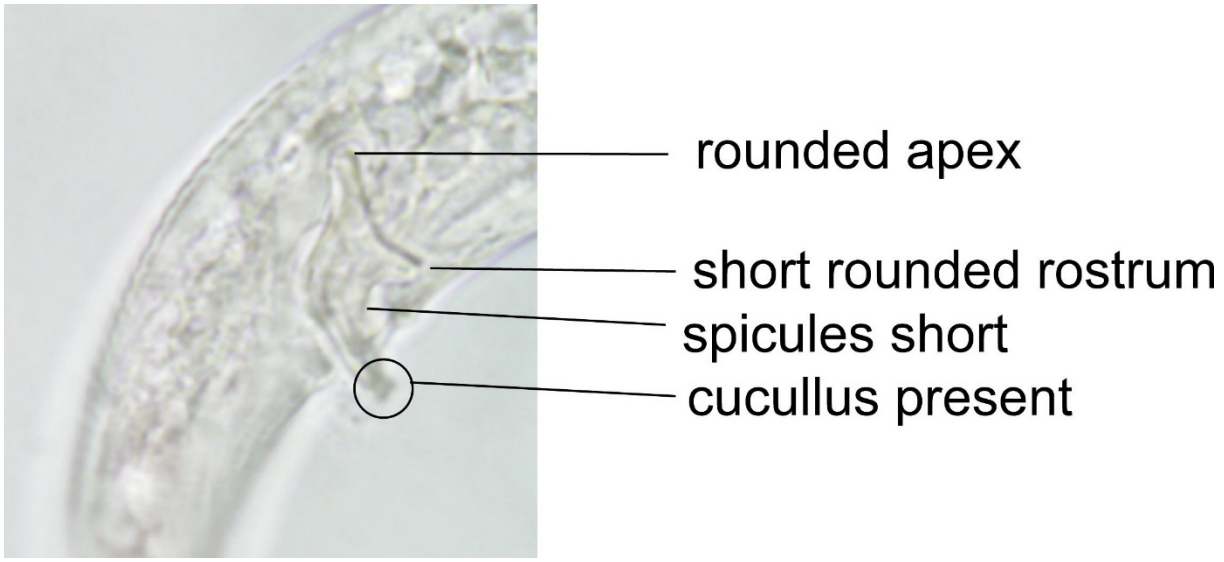

Figure 14. *Bursaphelenchus hellenicus* adult male tail (photo by Mike Hodda).

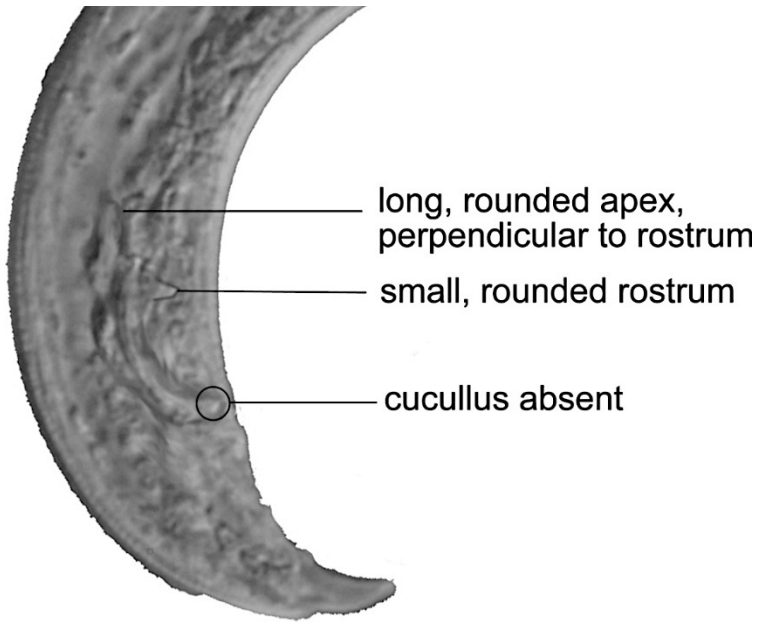

Figure 15. *Deviburs aphelenchus hunanensis* adult male tail (photo by Mike Hodda).

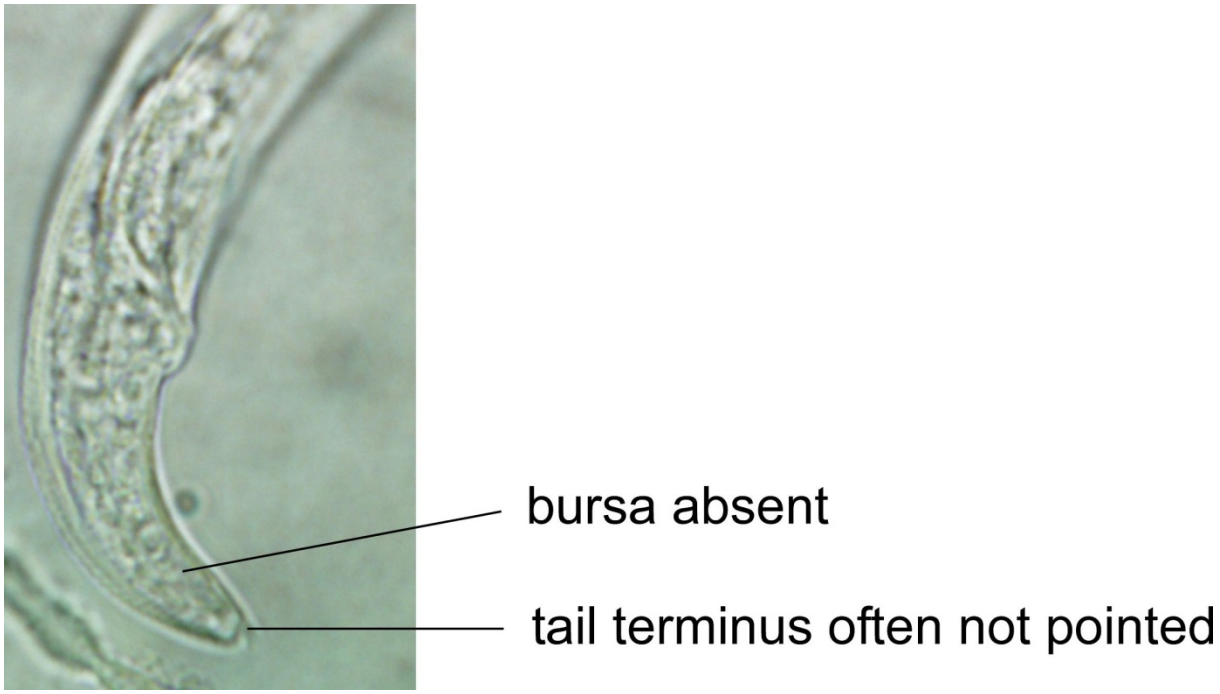

Figure 16. *Aphelenchoides* sp. adult male tail (photo by Mike Hodda).

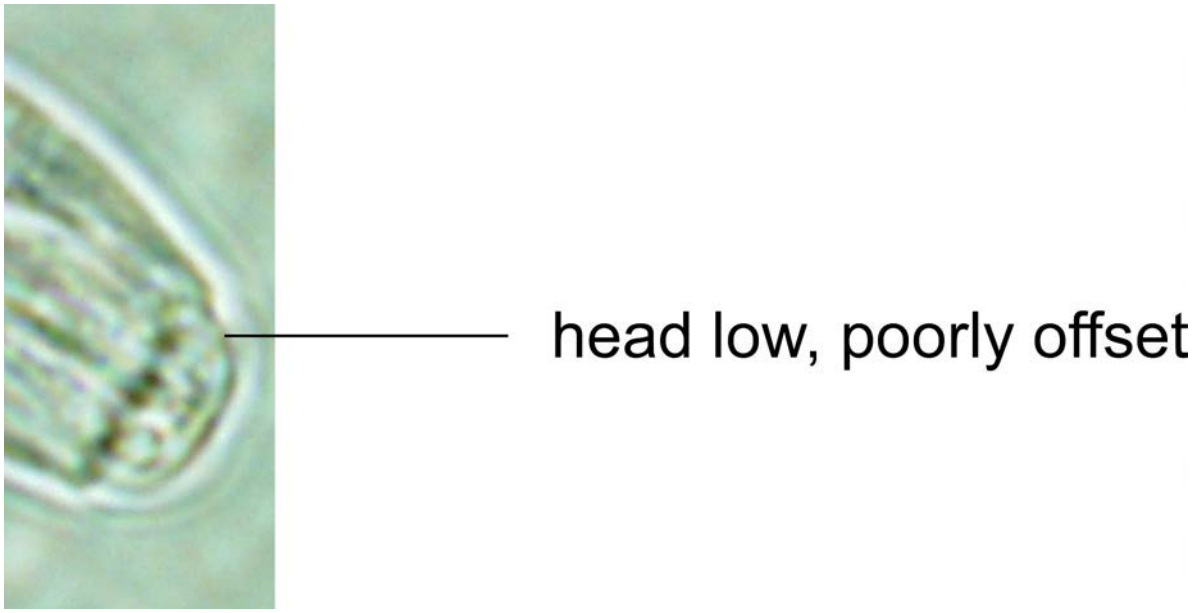

Figure 17. *Aphelenchoides* sp. female head (photo by Mike Hodda).

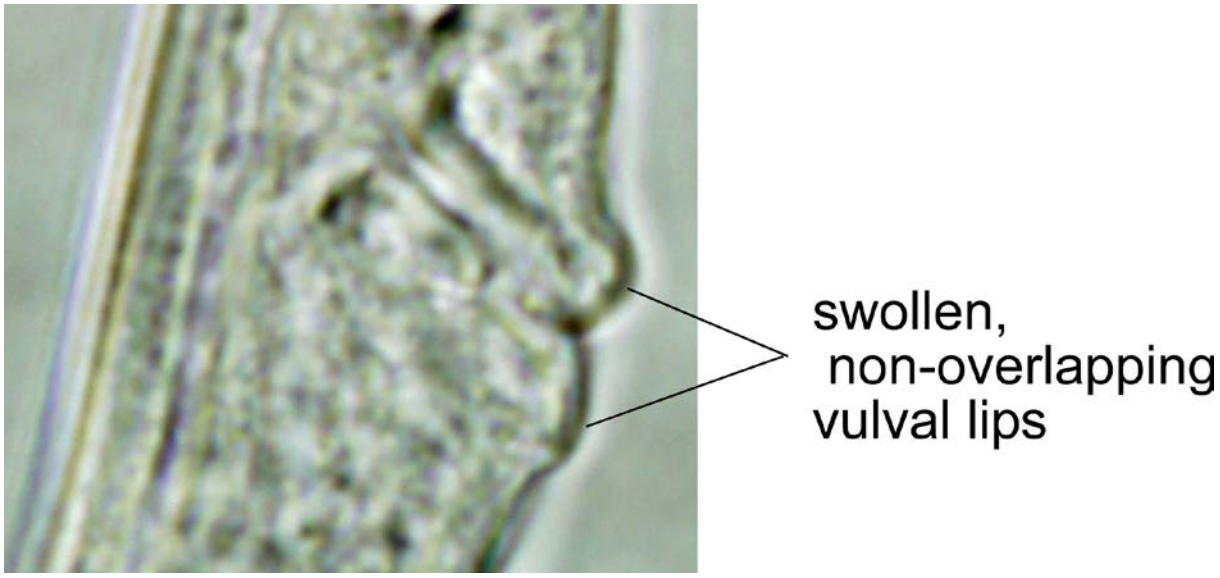

Figure 18. *Aphelenchoides* sp. vulval lips (photo by Mike Hodda).

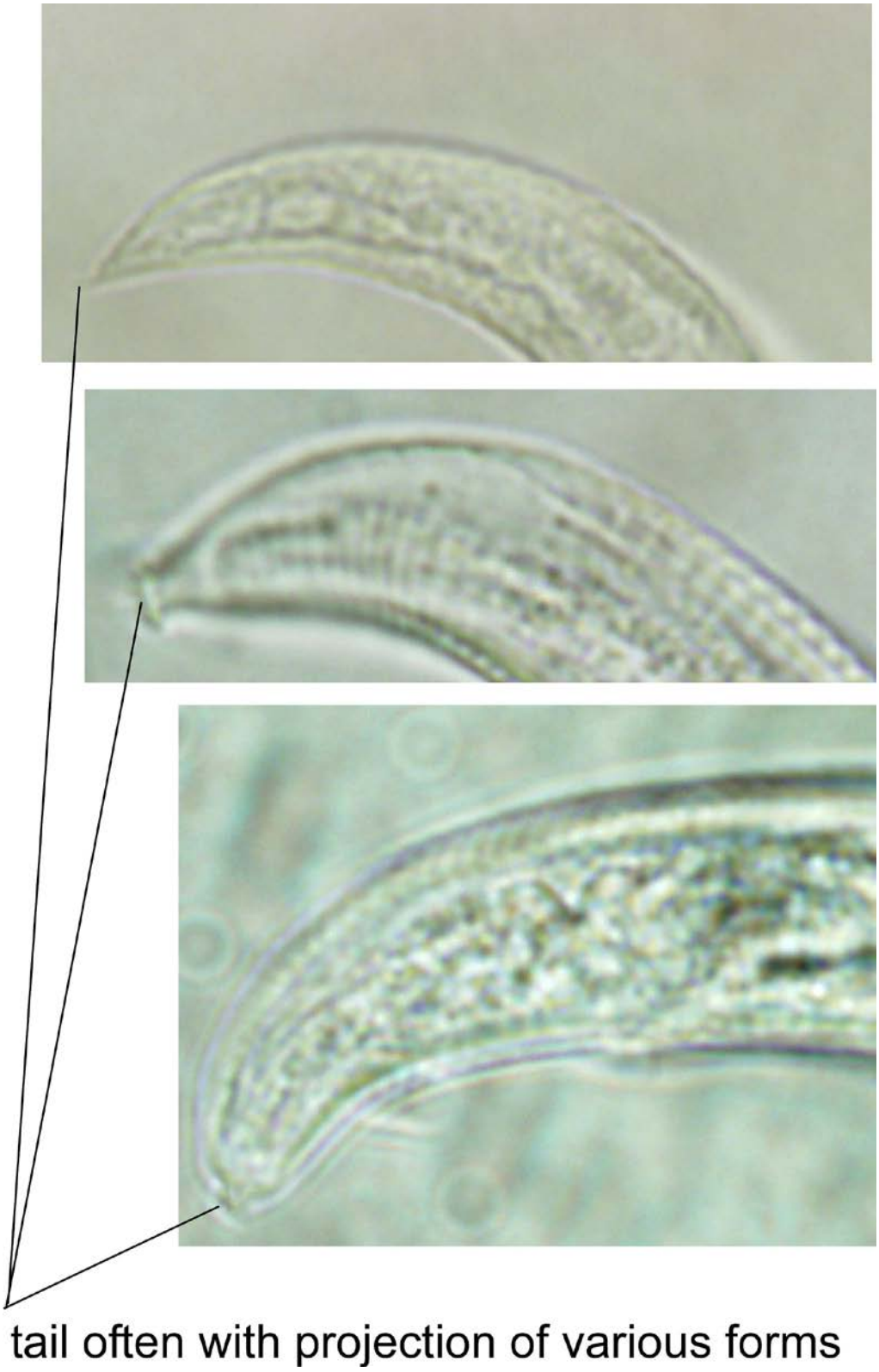

Figure 19. *Aphelenchoides* spp. examples of adult female tail shapes (photo by Mike Hodda).

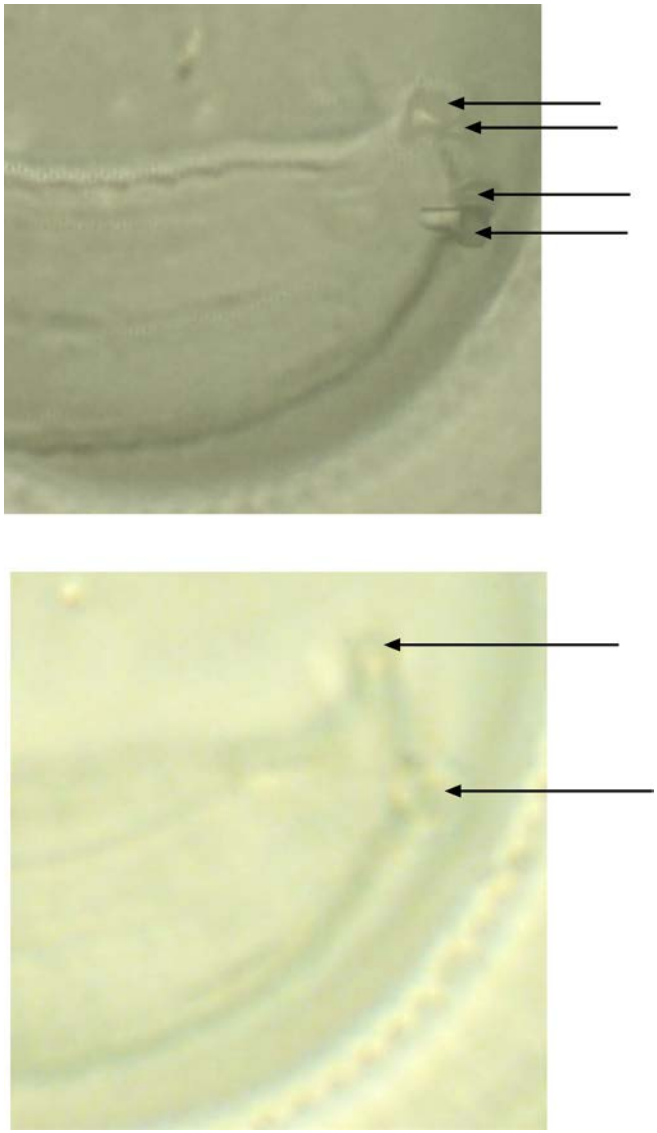

Figure 20. *Laimaphelenchus* sp. adult female tail, showing tubercles (arrowed). Top: modified photo showing all 4 tubercles, montaged from several originals. Bottom: view in one focal plane, showing upper tubercles only (photo by Mike Hodda).

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

9.1 Introduction

This section on diagnostics to support surveillance provides information on procedures to be utilised in the field and by diagnostic laboratories and diagnosticians in the screening, sampling, detection and identification of PWN in a surveillance situation.

If it is not known whether *B. xylophilus* occurs in an area, sampling should be focused on trees near risk sites. Trees in non-forest locations (e.g. parks, botanical gardens, trees in urban or industrial areas) should also be included in the survey if they are within risk areas. Surveys should be concentrated on weakened standing trees (e.g. wind-blown, snow-damaged, drought-stressed, beetle-infested). Cutting waste, broken or cut stumps from a recent felling season (1–2-year-old logging sites) which would be attractive to exotic *Monochamus* beetles, may also be used as sampling material. To plan the survey, logging history should be kept in mind (Schroder *et al*. 2009).

Nematodes can be found throughout the tree except the needles, cones and seeds; this is the case when the tree is infected by pine wilt disease. But more commonly, the distribution of the nematodes will be more localised within the trees, depending on tree susceptibility, physiological state of the tree and climatic conditions; for example, an infestation of *B. xylophilus* may be established in the crown or parts of the crown without further spread to other tree parts. Nematode distribution is especially localised after they have been introduced by oviposition in standing trees, when they will stay near the oviposition site (Schroder *et al*. 2009).

The best use of each test, confidence of identification, time from receipt of sample to result, and number of samples able to be tested at one time for identification of PWN in a surveillance situation are presented in Table 4.

Table 4. The best use of each test, confidence of identification, time from receipt of sample to result, and number of samples able to be tested at one time for identification of PWN in a surveillance situation.

Test	Outcome	Identification confidence	Deployment	Time	Throughput
Microscopic	To genus	High (99%+)	Laboratory	< 1 day	Low (10s)
Microscopic	To species	High (99%+)	Laboratory	< 1 day	Low (10s)
Molecular (PCR)	To species	High (99%+)	Laboratory	< 1 day – 1 week	Medium (10-100)

9.2 Sampling

9.2.1 Sampling timber

There is no practical method for detection of PWN during visual inspection of suspect wood. The highest risk is posed by wood with evidence of blue-stain fungi and beetle attack which has not been completely kiln dried and which comes from a region where PWN is known to occur. If there is bark present or kiln-dried wood has come into contact with unprocessed wood, this also increases the risk of infection. Wood from areas where PWN does not occur is low risk (but note that the range of PWN may be expanding in Asia (Thu 2003)). Wood which has been completely kiln dried and has no evidence of fungi or beetle attack is low risk. Other combinations of circumstances have intermediate levels of risk. The intensity of sampling should reflect the level of risk.

Where sampling is justified by the risk, samples of wood from around the holes made by the insects or in stained areas should be collected. A total of about 200 g of wood shavings or chips should be collected from at least 5 different places if possible. The wood should be placed in plastic bags and stored out of the sun, avoiding temperature extremes. An insulated container such as an ice-box without any ice or coolant works well.

All types of imported coniferous wood, especially solid wood packaging, and particularly from countries in which *B. xylophilus* occurs, should be randomly sampled by low-speed drill, borer, saw, axe, cant hook etc. Sampling should be concentrated on pieces with circular grub holes (i.e. the emergence holes of beetles) or those in which flat-headed *Monochamus* larval stages or pupae are detected in galleries with oval diameter and, sometimes, blocked with wood particles. In the case of sawn wood, no exit holes will normally be seen, but oval larval galleries (which are sometimes difficult to detect because larval galleries are blocked with shavings) can be found. Also, pieces with fungal growth, especially blue-stain fungi, should be sampled. However, several interceptions have shown that live *B. xylophilus* can also be detected in the absence of the above-mentioned indications (Schroder *et al*. 2009).

9.2.2 Sampling live trees

There are also sampling methods for live trees, but these are likely to apply only in the case of an outbreak of pine wilt disease in Australia. If the disease is suspected, at least 3 trunk cores should be taken if the whole tree is showing symptoms (EPPO 2003). If only one or a few branches are affected, then at least 3 disks from the branches should be cut. At least 50 g of wood should be used for extraction. Any apparently stressed or diseased tree within a radius of 5 km from a detection should be sampled (EPPO 2003). If there are any detections within this radius, the area should be expanded so that a 5 km radius from all detections is checked. If the vector is known, the radius sampled should take the flight range into account.

A static sampling procedure which, for example, samples all trees only at chest height may lead to false negative results. For this reason, it is recommended that samples are taken from several different parts of the tree, including the upper part of the trunk, in the canopy area, and preferably at places with evidence of *Monochamus* activity (e.g. signs of maturation feeding, grub holes, larval galleries). To avoid contamination with other, saprophagous nematode species, for the most part, the bark should be removed before sampling. However, it should be noted that *B. xylophilus* does also occur in the bark and experienced nematologists will be able to distinguish saprophytic and aphelenchoid nematodes from *B. xylophilus* (Schroder *et al*. 2009).

The best method for detecting nematodes in dead trees and to obtain a sufficient amount of wood material is to cut wood disks from the tree parts mentioned above. For standing trees and for cutting waste, the use of a drilling machine with bits of at least 17 mm or Forstner bits is a useful tool. The diameter of the drill is not critical but smaller drills may generate more heat than bigger ones. Drill slowly to avoid heat and drill to a depth of up to 4 cm. Depending on the diameter of the drill used, several bore holes may be necessary to gather the required amount of wood material. Sawdust created by a hand- or a chainsaw may also be used. Using a chainsaw might be convenient for obtaining sawdust, but the cleaning of the machine between samples is time consuming. An important requirement for all sampling methods is to avoid heat in a way that may negatively influence the nematodes (Schroder *et al*. 2009).

The total amount of wood sampled from a single tree should be at least 50 g. A tree sample should represent the whole tree; this means wood from the entire length of the tree trunk as well as the crown area should be included. At each site, samples from at least 1 tree, but preferably 5 trees should be taken. This will depend on the numbers of dead or dying trees present. It is important to take a representative sample from the site; if there are many dead or dying trees, then, clearly, the more trees sampled the better (Schroder *et al*. 2009).

In all cases, it is important to avoid cross contamination between samples from different sites; use alcohol (> 70%) or a mini burner to sterilize extraction instruments between samples. Storage and transport of samples must be carried out in cleaned containers or new plastic bags. Care should be taken to avoid desiccation of the samples (Schroder *et al*. 2009).

9.2.3 Sampling insect vectors

All *Monochamus* vectors are exotic to Australia, however if present, insects can be sampled to determine the presence of nematodes. As yet, no effective system for mass trapping of *Monochamus* spp. using a genus- or species-specific attractant or pheromone exists (Schroder *et al*. 2009). However, panel traps with semi-targeted commercial lures (such as Monalt, MonaltPlus, and P-333) may be used for monitoring purposes (Ikeda *et al*. 1981, Nakamura 2008). Alternatively, trap logs may be used to obtain beetles for investigation for the presence of nematodes. Nematode larvae are usually present as 4th instar dispersal stage (dauer-juveniles) in the tracheae and/or on other parts of the body of the beetles (Kondo *et al*. 1982, Lai 2008). To isolate the nematodes, beetles are dissected and crushed in appropriate dishes and then kept in water or on a Bearmann funnel or Whitehead Tray for 24–48 h at 24 °C (Mamiya and Enda 1972, Bowers *et al*. 1992, Sousa *et al*. 2001). Dauer-juveniles will leave the beetles and can be cultured on *Botryotinia fuckeliana* (anamorph: *Botrytis cinerea*) grown on malt agar for further identification (Schroder *et al*. 2009).

9.3 In-field tests

The LAMP test developed by Kikuchi et al. (2009) appears applicable as a very convenient in-field test for PWN.

9.4 Laboratory tests

For laboratory testing procedures for the identification of PWN, please refer to section 4 above.

9.5 Acknowledgements

This section was prepared by John Wainer (Agriculture Victoria).

9.6 References

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