European Union Reference Laboratory

for Plant Parasitic Nematodes

EURL diagnostic protocol

REFERENCE: EURL–*B. xylophilus*-Identification (BXI)_Version 01

February/ 2023

Protocol for *Bursaphelenchus xylophilus*: Morphological & Molecular methods

Foreword

These methods are recommended by:

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The consortium between both laboratories is designated the

European Union Reference Laboratory (EURL) for Plant Parasitic Nematodes

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Contents

1. Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934; Nickle, 1970), is the only plant-parasitic nematode (PPN) listed among the 20 priority quarantine organisms in the European Union (Commission Delegated Regulation (EU) 2019/1702), and is also classified as an EU quarantine organism (listed in Appendix II, Part B, of the Commission Implementing Regulation (EU) 2021/2285 amending Implementing Regulation (EU) 2019/2072 as regards the listing of pests). The totality of EU countries must conduct official controls at EU borders and perform annual surveys for this nematode species. If detected, eradication measures should be taken to avoid their spread within the EU territory (Commission Implementing Decision (EU) 2012/535 and Regulation (EU) 2016/2031). This PPN causes the pine wilt disease (PWD) (Figure 1A), which in general only affects *Pinus* spp., known as the most susceptible host; however, it can also be found in other trees species of *Abies*, *Chamaecyparis*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* (Evans *et al*., 1996). After *B. xylophilus* was exported from North America through infected timber to Japan at the beginning of the 20th century, the nematode spread further to other Asian countries (China and Korea). It was detected for the first time in Europe, in Portugal, in 1999, and 2008 in Spain (Mota *et al*. 1999; Abelleira *et al*. 2011; Fonseca *et al*. 2012). Check the EPPO Global Database (EPPO, 2022) for more updated information on geographical distribution.

As common among Aphelenchid species, the *B. xylophilus* feeds on both fungal (mycophagous) and plant cells, and is transmitted to dead or dying trees during oviposition by insect vectors from the genus *Monochamus* (Coleoptera: Cerambycidae) or to healthy trees during maturation feeding by the vector beetle (Mota & Vieira. 2008). The life cycle of *B. xylophilus* involves two forms: the propagative form (J1 to J4 and adult stages), which is present under suitable conditions, and the dispersal form (dauer juveniles J_{III} and J_{IV}) that is induced under unsuitable conditions, such as desiccation, food shortage, or environmental deterioration due to overpopulation. The dispersal form is intimately related to the vector beetle (Futai 2013). Thus, *B. xylophilus* can be found and detected in adult insects of *Monochamus* spp. (Figure 1B). When *B. xylophilus* is transmitted during oviposition, the nematodes remain relatively close to the introduction site. However, when transmission occurs through the young shoots and when the tree succumbs to PWD, the nematodes are distributed throughout the whole tree, destroying wood tissues such as epithelial cells, parenchyma cells of axial and radial resin canals, cambium and phloem. *Bursaphelenchus xylophilus* can also be found in roots, even when the above-ground part of the tree is already dead, dried out or felled. Symptoms may first appear on one or a few branches but often develop quickly throughout the crown, and trees may die only 1 or 2 months after symptoms appear. Symptoms start with needle discolouration, progressing rapidly from a greyish green to yellow and brown. *Bursaphelenchus xylophilus* feeds on fungi in the wood, including the bluestain fungi that are transmitted by engravers and other bark beetles.

Next to their occurrence in vector insects (*Monochamus* spp.), *B. xylophilus* can be present in several coniferous products: wood, wood products and packaging, wood chips/shavings, plants for planting, branches, isolated bark, but not in needles, cones or seeds. Therefore, samples of imported wood and standing conifer trees should be taken and verified for the presence of the nematode.

Figure 1 – Symptoms of Pine wild disease on *Pinus* caused by *Bursaphelenchus xylophilus* (A), and adult insect of *Monochamus* spp., vector of *B. xylophilus* (B). Pictures from INRAE-Biogeco and INRAE Orléans, France.

2. Terms, abbreviations and definitions

- Container: bowl, beaker, vial, tube, pot, jar, device, etc
- D: Detected
- ND: Not Detected
- EU: European Union
- EPPO: European and Mediterranean Plant Protection Organization
- IPPC: International Plant Protection Convention
- J1: first second-stage juveniles
- J2: second-stage juveniles
- J3: third-stage juveniles
- J4: fourth-stage juveniles
- J_{III}: Dauer juvenile (dispersal third-stage)
- J_{IV}: Dauer juvenile (dispersal fourth-stage)
- NRLs: National Reference Laboratories
- PPN(s): Plant-Parasitic Nematodes
- PWN: pine wood nematode
- PWD: pine wood disease

3. Purpose and scope

The EURL recommended protocols are based on literature reviews and IPPC and EPPO standards[1], when available. These recommended methods, including operational procedures, were performed with the scientific experience and technical expertise of the EURL team. These methods have been adapted, optimised and further validated by the EURL laboratory.

The purpose of this EURL diagnostic protocol[2] is to assist the National Reference Laboratories (NRLs) of EU Member states in carrying out their diagnostic analyses by providing them with details on analytical operating procedures for the detection and identification of *B. xylophilus*.

This document describes a EURL diagnostic protocol for the identification of *Bursaphelenchus xylophilus* that is recommended for use in diagnostic laboratories in the EU. This protocol require a morphological method based on adult specimens' morphology combined with the conventional PCR method. The molecular Matsunaga & Togashi (2004) test proposed in this protocol can distinguish the mucronated form of *B. xylophilus* from the species *B. mucronatus kolymensis*, which is present in Europe.

This protocol can be applied to juveniles and adult stages extracted from wood and bark (nematode extraction detailed in protocol EURL- BXE_Version 01, internal document, 2022).

The universal PCR test from Burgermeister et al., 2009 enables to show the presence of amplifiable DNA in the case of a negative result with the specific PCR test Matsunaga & Togashi (2004).

The performance criteria and validation of the described morphological and molecular methods are available in **Appendix §8.**

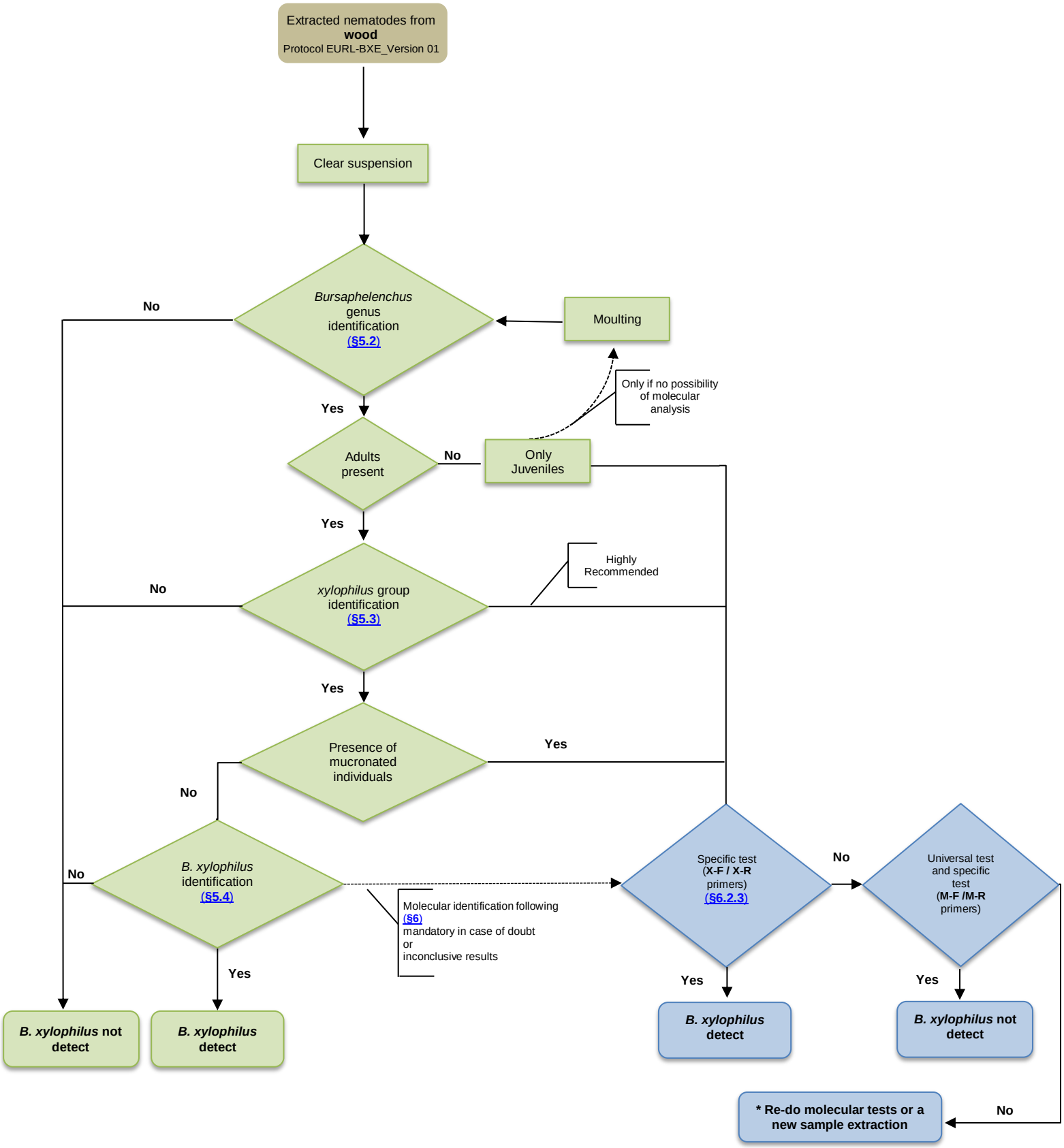
The material linked to this diagnostic protocol, such as slide presentations, technical videos & media, can be found on the EURL Plant Parasitic Nematodes website.

Warning and safety precautions: The user of these methods should be closely familiar with standard laboratory practices. It is the responsibility of the user to establish suitable health and safety practices and ensure compliance with the current regulations. All actions taken in accordance with this method must be performed by employees who have attended relevant training.

[1] For more information on *B. xylophilus* biology, detection, and identification, view the standards EPPO PM 7/4 (4) and IPPC ISPM27 (DP10).

[2] The use of chemical brands or equipment in this recommended protocol does not imply the exclusion of others, which may also be appropriate.

4. Schematic procedure of the methods



* If a final result is not possible, a new sample and/or juveniles are analysed according to another specific test or method, either from the remaining material or after a new extraction from the matrix

5. Morphological identification

The *Bursaphelenchus xylophilus* identification by morphological-based analysis is carried out on nematodes extracted and isolated from wood (detailed in protocol EURL- BXE_Version 01, internal document, 2022).

Morphological identification can only be performed on adult specimens of both sexes; thus, if only juveniles are present and extracted from wood samples, the juveniles collected must be placed on mycelium of *Botryotinia fuckeliana* to moult into adults and multiply. However, if laboratories apply molecular analysis, direct molecular analysis on the collected juveniles should be performed instead (see **§6**).

5.1 Material and consumables

5.1.1 Equipment

- Stereoscopic microscope with episcopic and diascopic illumination (magnification to a minimum of 50X)
- High-definition microscope with DIC (Differential interference contrast) (observations at 1000X magnification)

5.1.2 Consumables and small materials

- Counting dish
- Fishing tool or any other instrument suitable for handling filiform nematodes
- Small scalpel or a syringe needle
- Immersion oil
- microscope slides and coverslips
- Petri dishes
- Microtubes
- Heat source (to kill nematodes)
- A small container such as Syracuse watch glass
- Varnish (e.g. nail polish)

5.2 Identification of *Bursaphelenchus* genus

The first step in the morphological procedure is to identify the nematodes belonging to the **Aphelenchoidea** super family using a stereoscopic and/or upright microscope. The analysis should cover all nematodes present in the extract (suspension) or isolated specimens.

1) Transfer the suspension into a counting dish.
2) Detect if specimens of the **Aphelenchoidea** super family are present. The main morphological criteria[3] are in Figure 2:
   - Tylenchid stylet, pharynx and metacorpus (Figure 2C)
   - Procorpus clearly separated from metacorpus by a constriction (Figure 2B)
   - Both females and males are filiform (Figure 2A)
   - Oesophageal glands overlap the intestine dorsally (2A)
   - One gonad (vulva posterior) (Figure 2F – 2G)
   - Large and developed metacorpus (Figure 2D)

[3] Figures and drawings of these features can be found in the EPPO's Diagnostic protocols for regulated pests: Pictorial glossary of morphological terms in nematology (EPPO 2020)

- Dorsal oesophageal gland opening into the lumen of pharynx within metacorpus (and not as the Tylenchoidea in the lumen of the pharynx behind the basal knobs) (Figure 2B).

3) After identifying the Aphelenchoidea nematodes, select, if possible, 5 males and 5 females for observation under a high-definition microscope, and detect nematodes belonging to the genus ***Bursaphelenchus.*** The main morphological criteria are described and can be observed as well in Figure 2:

- Basal knobs present (but usually small) (Figure 2E)
- Terminal end of the male's tail strongly curved and presenting a small bursa (Figure 2A - 2I)
- Robust spicules in males (Figure 2H)

Note: *If only identification of the genus is possible, the nematodes are subject to molecular identification when the following criteria are observed: offset head, large and developed metacorpus and tail tip sub-cylindrical, with rounded end with or without mucro. In the absence of adult nematodes and molecular identification, the detection of the genus will state:* ***Bursaphelenchus sp detected.***

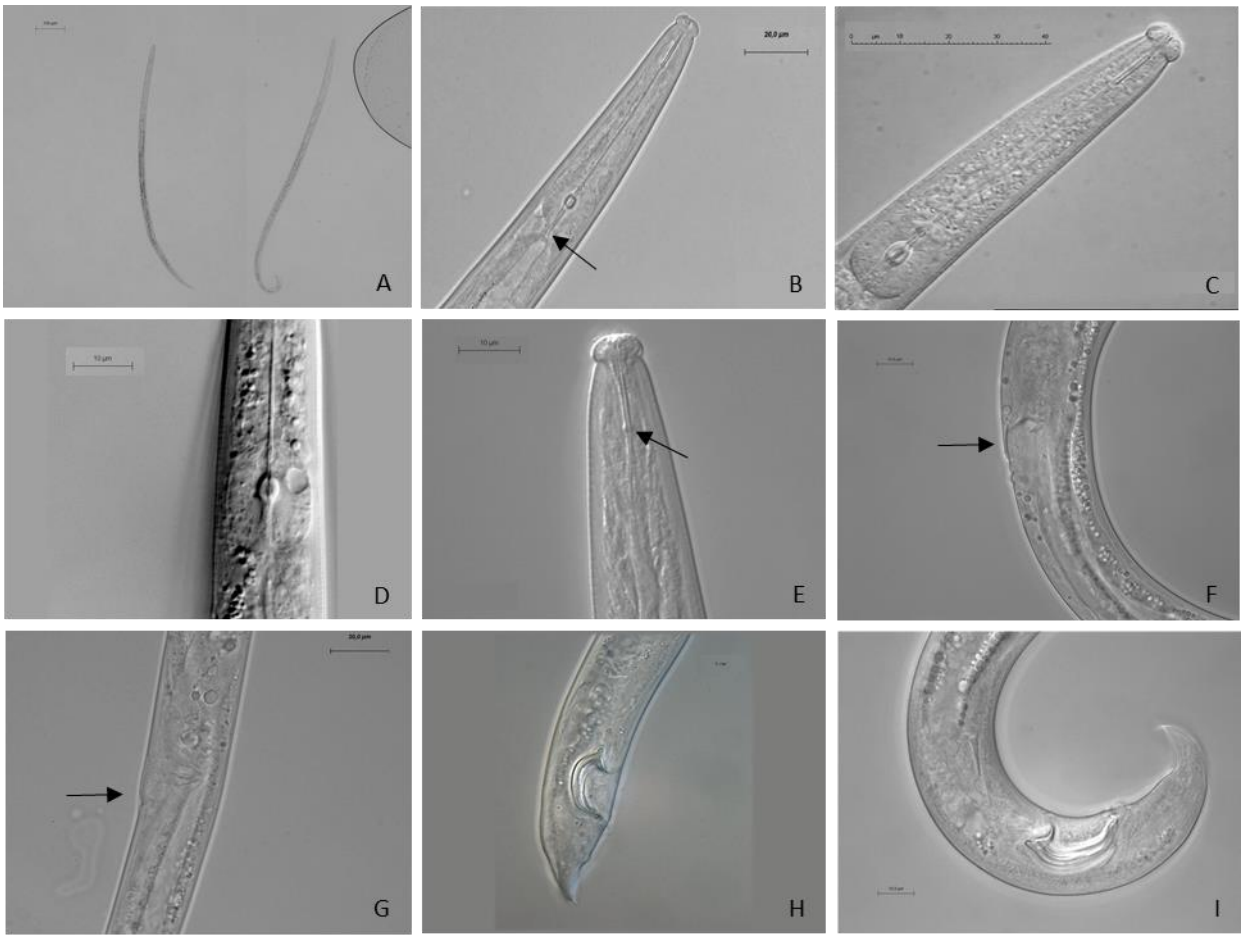


Figure 2 – Morphological criteria of the Aphelenchoidea super-family and *Bursaphelenchus* genus. (A, I) Male's tail strongly curved and presenting a small bursa; (B) dorsal oesophageal gland opening into the lumen of pharynx within metacorpus; (C) Tylenchid stylet, pharynx and metacorpus; (D) metacorpus from *Bursaphelenchus mucronatus*; (E) small basal knobs; (F,G) vulva posterior; (H) Robust spicules. Pictures: C, E, G are from *Bursaphelenchus* sp., and A, B, F, H, I are from *Bursaphelenchus xylophilus*. Picture ANSES-LSV, France.

5.3 Identification of *xylophilus* group

To distinguish the species of the **"*xylophilus*" group** requires, at minimum, the observation of an adult male and female of the population to be determined. The identification can be performed using the following key:

Four lateral lines (Figure 3A); vulva with prominent flap (Figure 3B); (Figure 3C) robust and arcuate spicules with a cucullus (disc-shape projection at the end.....***xylophilus* group**

- Characters different .....**not *xylophilus* group**

Note: Molecular analysis is highly recommended for reliable identification of B. xylophilus when the xylophilus group is identified.

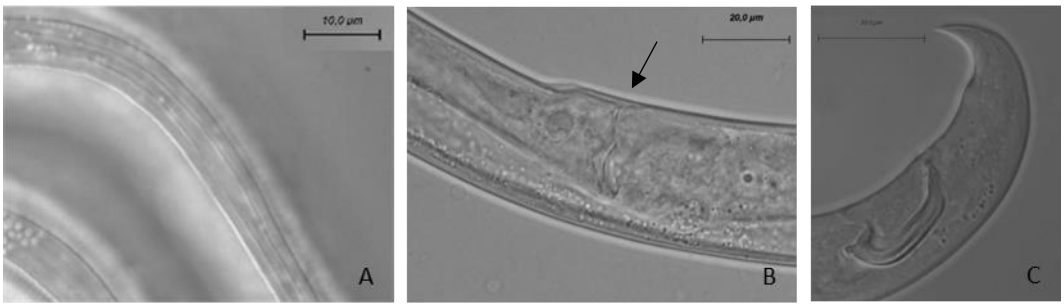


Figure 3 - Morphological criteria of *B. xylophilus* group. (A) lateral lines; (B) vulva with prominent flap and (C) robust and arched spicules with a cucullus. The criteria "vulval with prominent flap" and "robust and arcuate spicules with a cucullus at the end" must be observed to distinguish the group. Sometimes, the vulval with prominent flap cannot be observed on females that are not well positioned (e.g. on the back); therefore, it is advisable to prepare several individuals. Picture ANSES-LSV, France.

5.4 Identification of *B. xylophilus*

Morphological identification of the species *B. xylophilus* requires, as a minimum, the observation of one female of the species. The following key (Sarniguet *et al*. 2013) can be used to distinguish *B. xylophilus* from other *Bursaphelenchus* species of the same group.

1 Female tail conical or tapered with or without mucro (Figure 5D-I) .....**not *B. xylophilus***

Female tail tip sub-cylindrical..... **2**

2 Female tail tip sub-cylindrical with rounded end without mucro (Figure 4D-I) .....***B. xylophilus* (round-tailed form)**

Female tail tip sub-cylindrical, rounded end with a terminal mucro (Figure 4J-L) .....**Not *B. xylophilus* or *B. xylophilus* (mucronated form*)**

Note: * To differentiate the B. xylophilus mucronated form from B. mucronatus* (Figure 5J-L) *and B. fraudulentus, which are present in Europe, a molecular analysis should be performed.

5.5 Morphological identification results

Morphological identification leads to one of the following three results:

1- *Bursaphelenchus xylophilus* **detected***

when at least one of the females observed is identified as belonging to the species.

2- *Bursaphelenchus xylophilus* **not detected***

when none of the specimens observed can be identified as belonging to the *xylophilus* group, or none of the females observed belongs to the species *B. xylophilus*.

3- *Bursaphelenchus* from *xylophilus* group **detected****, but no distinction from the *B. xylophilus* was possible.

when at least one of the females observed is of the *xylophilus* group and has a mucronate subcylindrical tail.

Note:

Regarding **results 1 and 2* - *leads to the end of the analysis. For the expression of the final result, see **§7*

***Regarding **result 3** – *Molecular analysis **should be performed** if the number of individuals is sufficient. Otherwise, only morphological identification is carried out. For the expression of the final result, see **§7**.*

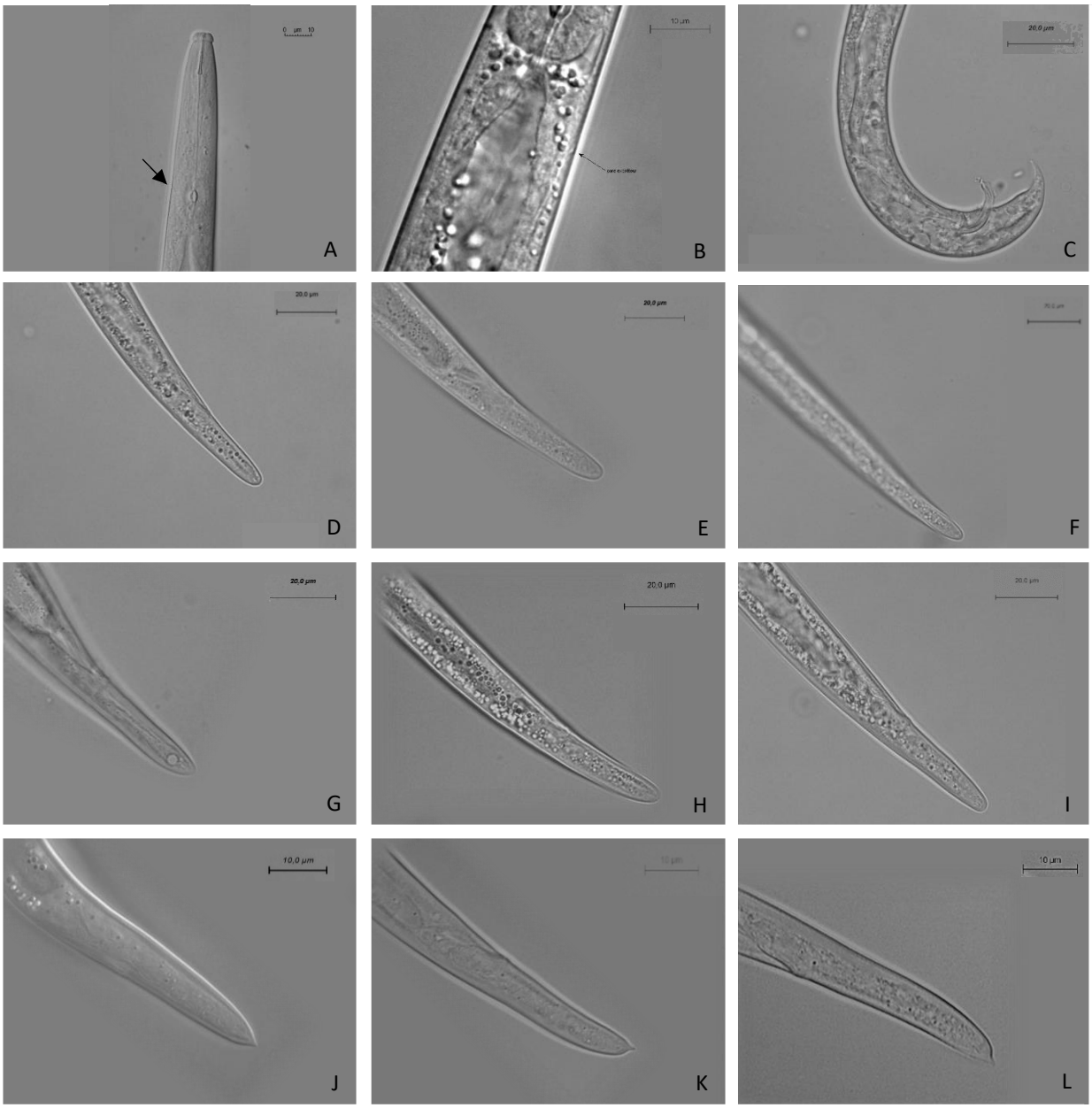


Figure 4 - Morphological criteria of *B. xylophilus.* (A) excretory pore at the metacorpus; (B) excretory pore behind metacorpus; (C) long and pointed spicule rostrum, spicule limbs with an angular curvature; (D-I) female tail tip sub-cylindrical, rounded end without mucro; (J-L) female tail tip sub-cylindrical, rounded end with mucro. Picture ANSES-LSV, France.

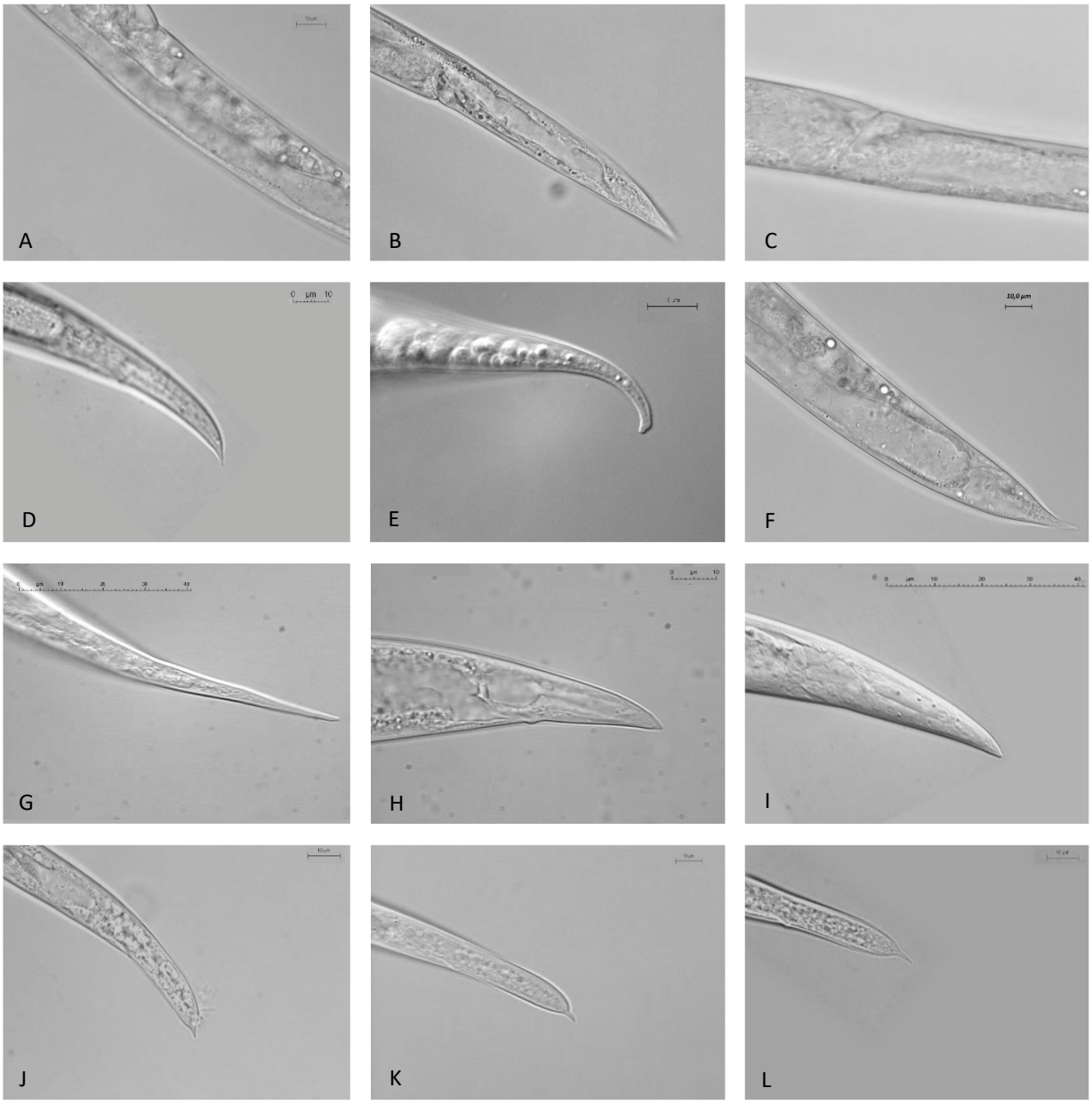


Figure 5- Morphological criteria of other *Bursaphelenchus* spp. (A-C) female vulval flap ending in a deep depression ; (D-I) female tail tip conical or tapered with or without mucro ; (J-L) *B. mucronatus* tail tip sub-cylindrical, with rounded end with mucro. Picture ANSES-LSV, France

6. Molecular identification

Sampling for molecular analysis can be performed on juveniles and adult stages. At least 5 microtubes containing 10 nematodes each are prepared for molecular analysis when available. The nematodes are fished out with the help of a fishing needle and conditioned in the microtubes containing 100 µL of DNA extraction lysis buffer (Ibrahim et al., 1994). A short centrifugation is carried out to place the juveniles at the bottom of the tube. If the analysis is performed on the same day, specimens should be processed directly for DNA extraction (see **§6.2.2**). Otherwise, the tubes can be frozen.
The molecular test described in this protocol allows both live and dead nematodes to be detected.

6.1 Material and consumables

6.1.1 Equipment and small materials

In addition to the standard equipment for molecular biology (pipettes, centrifuges, shaker, water bath, electrophoresis, etc.), the following equipment are necessary for certain steps of the analysis:

- Glass beads (e.g. 3 mm and 1 mm)
- Shaking tissue grinder for approximately 2 mL microtubes (e.g., Tissulyser, Qiagen®) or equivalent equipment
- Conventional or real-time PCR thermal cycler

6.1.2 Consumables

In general, the manipulator must ensure (either by the use of consumables known as molecular biology quality or by cleaning, sterilisation or other appropriate treatment) that the water and products or consumables used are free of contamination with DNA/RNA, nuclease, inhibitor or any other element that may interfere with the molecular analysis and the result.

Any commercial DNA extraction kit and/or PCR mix suitable for conventional PCR application can be used as long it has been found to meet the requirements in an in-lab validation study.

Suppliers' recommendations regarding storage conditions before use should be followed as well as conservation during usage. If impossible, the laboratory should define the most optimal conditions.

The reagent or brands[4] and solution preparations are the following:

- Use pipette tips with filter plug during manipulation for PCR reactions.
- Molecular biology grade water
- **DNA extraction lysis buffer** + metal/glass beads and as alternative freezing and defrosting the juveniles**:** 10mM Tris-HCl pH8.0, 1mM EDTA; Nonidet P40 1%; and proteinase K 100 µg mL^{-1} following Ibrahim *et al.* (1994)
- **DNA polymerase and supplied buffer:**
- The conventional PCR tests were evaluated and can be performed with MP Biomedicals DNA Taq polymerase and associated buffer (ref. 11EPTQD925 or 11EPTQD025 depending on the format/size)
- **Other reagents: single reagents** (dNTP, MgCl2, etc.) or ready-to-use PCR mixes marketed by several suppliers

[4] Brand names or suppliers may be mentioned in the description of the products necessary to implement this method. This information does not mean that the EURL recommends the exclusive use of these products. Equivalent products may be used if their performance is validated, leading to the same results.

- **Primers:**

Test references	Target PPNs	Primers conventional PCR	Sequence 5'- 3'
Mastunaga and Togashi (2004)	***B. xylophilus***	**X-F**	ACG ATG ATG CGA TTG GTG AC
		X-R	TAT TGG TCG CGG AAC AAA CC
	B. mucronatus	**M-F**	TCC GGC CAT ATC TCT ACG AC
		M-R	GTT TCA ACC AAT TCC GAA CC
Burgermeister et al. (2009)	**All nematodes**	**ITS1 F (F194)**	CGT-AAC-AAG-GTA-GCT-GTA-G
		ITS2 R (26S primer)	TTT-CAC- TCG-CCG-TTA-CTA-AGG

Note: *the specific primers B. xylophilus (X-F and X-R) can be used in a duplex reaction with the specific primers B. mucronatus (M-R and M-F) described in Matsunaga & Togashi (2004).*

6.2 Molecular test

6.2.1 Controls and their purpose

Except for the PPC, which is optional, the following controls are mandatory to check the correct performance of DNA extraction and PCR steps.

Controls	Description and Purpose	Expected result *
Negative process control (NPC)	DNA extraction buffer alone conditioned and tested the same way as the tested sample. Verify the absence of contamination during the DNA extraction process.	Negative
Positive process control (PPC)	DNA extracted from the matrix (isolated juveniles) conditioned and tested similarly to the tested sample. Verify the absence of any flaws during the analytical process.	Positive
Positive PCR control (PC)	It contains all the elements of the PCR reaction mix, including a DNA extract from *B. xylophilus* (viable juveniles). This control verifies that the PCR reaction has proceeded correctly, allowing the amplification of the samples containing the target.	Positive
No Template Control (NTC)	It contains all the elements of the PCR reaction mix, but no DNA is added. This control checks the absence of contamination during the PCR preparation and reaction.	Negative
Negative specificity control (NSC)	It contains all the elements of the PCR reaction, including non-target DNA. This allows checking the absence of cross-reaction during the PCR. This type of control is not required for the universal Burgermeister *et al.* (2009) test.	Negative

* The results are only valid if the expected results are met.
If available, the target species may be supplied by the EURL.

6.2.2 DNA extraction

DNA extraction results from the successive action of mechanical grinding (glass or metal beads) and chemical treatment (proteinase K). As an alternative, freezing and defrosting the juveniles can be applied, or nematodes can be sliced into pieces using a scalpel. The analysis is carried out on juveniles and adult stages collected and conditioned previously according to the methodology described in **§6.**

1) Allow specimens conditioned in **§6** to defrost.
2) Add glass or metal beads of different diameters (e.g. 1 bead of 3 mm and a few beads of 1 mm) to the tube containing the isolated nematodes previously conditioned in the lysis buffer.
3) Place the tube on a tissue grinder rack ([e.g. using a Tissulyser II (Qiagen®) shake at a frequency 30 Hz for 40 sec.

4) Place the tube in a water bath at approximately 55°C for at least one hour.
5) Briefly centrifuge at the highest speed to precipitate the cell debris.
6) Take at least 50 µl from the solution and place in a new tube.
7) Incubate for 10 minutes at 95 °C to denature the proteinase K.
8) The DNA can be used immediately or stored at -20 °C until the use.

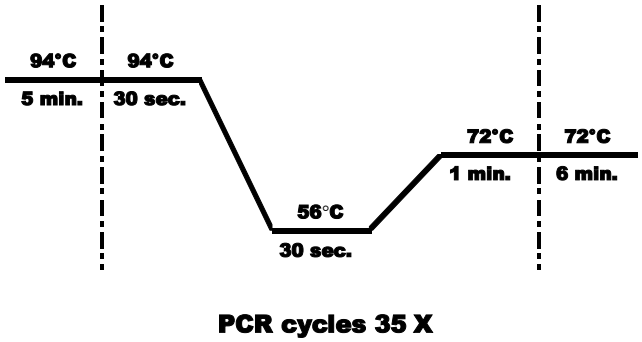
6.2.3 Specific conventional PCR test (Mastunaga and Togashi, 2004)

This conventional multiplex PCR test can be performed on DNA extracted with lysis buffer from isolated specimens. Each DNA sample is tested at least in duplicate. DNA amplification is carried out with the following reagents and under the following conditions:

- **PCR reaction**

	Species-specific PCR test
	Mastunaga and Togashi, 2004
Reagents	**Final concentration per reaction tube**
Total volume	25 µL
Buffer Taq DNA polymerase with $MgCl_2$	1 X
For each primer	0.2 µM
dNTPs	0.2 mM
Taq DNA polymerase	0.5 U /reaction
Molecular grade water water	Adjust to 20 µL
Add DNA to 20 µL of reaction mix	5 µL

- **PCR program:**



PCR cycles 35 X

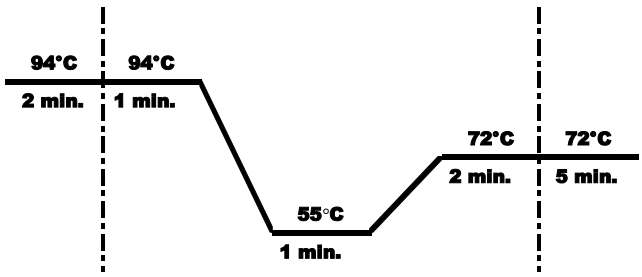
6.2.4 Universal PCR test (Burgermeister et al., 2009)

This conventional PCR test is performed when no amplification is observed (negative result samples) with the specific Mastunaga and Togashi, 2004 test. This test can be performed on DNA extracted with lysis buffer from isolated specimens. Each DNA sample is tested at least in duplicate. DNA amplification is carried out under the following conditions:

- **PCR reaction**

	Species-specific PCR test
	Burgermeister *et al*., 2009
Reagents	**Final concentration per reaction tube**
Total volume	25 µL
Buffer Taq DNA polymerase	1 X
$MgCl_2$ (take into account the $MgCl_2$ that may be present in the Taq buffer)	1.5 mM
For each primer	0.6 µM
dNTPs	0.1 mM
Taq DNA polymerase	2U /reaction
Molecular grade water water	Adjust to 20 µL
Add DNA to 20 µL of reaction mix	5 µL

- **PCR program**



PCR cycles 40 X

6.3 Evaluation and reporting the results

The result of the molecular analysis is a synthesis of the results obtained from each of the tubes analysed. The analysis for the conventional PCR is qualitative. The correct interpretation of the results is carried out by observing the amplicon generated by the PCR tests and verification of the controls. The analysis is valid if, and only if, all of the following conditions are met:

Checking the controls:

- The NPC, NTC and NSC: no amplification is observed in any of the replicates
- All replicates of PC: amplification of the amplicon in accordance with the requirements.

If the results of one or more controls do not comply with those expected (as defined above), the analysis is not valid and, depending on the non-compliance observed, all or part of the analysis must be repeated.

Checking the samples:

The expected fragment sizes of the amplicons are as follows:

	Mastunaga and Togashi (2004) **in duplex**	Universal test Burgermeister ***et al.*, 2009***
B. xylophilus	~557 bp	~ 925 bp
B. mucronatus	~210 bp	~920-925 bp

*The amplicon size for the species of the *B. xylophilus* group varies depending on the species.

For all PCR tests mentioned, for each PCR reaction and after verification of the controls, the result should be as follow:

- **negative** when no amplification is observed;
- **negative** when no amplification at the expected size is observed;
- **positive** when a fragment of the expected size is observed.

The PCR test result is obtained by following the indications mentioned in the following tables:

In the case of the **species-specific Mastunaga and Togashi (2004) PCR test**:

Analysis		**Test Results**
Tube 1	**Tube 2**	
+	+	*Bursaphelenchus xylophilus* **detected**
+	-	The PCR is redone. After a second PCR test, if 1 out of 2 is still positive, the result is interpreted as positive.
-	-	Universal PCR test to be performed. If universal PCR is positive, the result is: *Bursaphelenchus xylophilus* **not detected**

Note: + and - correspond respectively to the presence and absence of the amplificon

In the case of the **Burgermeister et al., 2009 universal test**:

Analysis		Test Result
Tube 1	**Tube 2**	
+	+	POSITIVE, presence of amplifiable DNA, *B. xylophilus* **not detected**
+	-	POSITIVE, presence of amplifiable DNA, *B. xylophilus* **not detected**
-	-	NEGATIVE, non-amplifiable DNA, **the molecular analysis is inconclusive**

Note: + and - correspond respectively to the presence and absence of the amplification

7. Final result

		Morphological identification results				
		*Bursaphelenchus sp** **(see note §7.2)**	*"xylophilus" group* ***D*** **(see note §7.3)**	*Bursaphelenchus* mucronated form** **(see note §7.4)**	*B. xylophilus* **D**	*B. xylophilus* **ND (see §7.3)**
Molecular identification results	*B. xylophilus* **D**	*B. xylophilus* **D**	*B. xylophilus* **D**	*B. xylophilus* **D**	B. xylophilus **D**	
	B. xylophilus **ND**	*B. xylophilus* **ND**	*B. xylophilus* **ND**	*B. xylophilus* **ND**	*B. xylophilus* [3] **D**	
	Non-amplified DNA	*Bursaphelenchus* sp [1, 4] **D**	*"xylophilus"*group [2, 4] **D**	*"xylophilus"*group [2, 4] **D**	*B. xylophilus*[3] **D**	
No molecular identification					*B. xylophilus* **D**	*B. xylophilus* **ND**

D: detected; ND: not detected

If only identification of the genus is possible, the nematodes are subjected to molecular identification or kept to moult. In the absence of adult nematodes and, hence, no further possible identification, the detection of the genus will state:* ***Bursaphelenchus sp. detected.*

***If a Bursaphelenchus mucronated form is present in the sample, molecular identification is mandatory.*

(1) The final result is: *Bursaphelenchus* sp. **detected.**

(2) The final result is: *Bursaphelenchus* sp. of the *xylophilus* group **detected.**

(3) If the morphological results are discordant with the molecular analysis results, or if no amplification is obtained (absence of amplifiable DNA), new specimens (if possible) are re-analysed morphologically and molecularly. If there is no remaining material or if the discrepancy persists, only the **morphological analyses' results** are considered.

(4) New morphological and molecular analyses are undertaken depending on the remaining material, or new sampling is requested.

If you have any question about this protocol, please, send an e-mail to eurl.nematode@anses.fr

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8. Appendix: Performance evaluation and validation of methods

All tests in this protocol were evaluated and validated according to the following criteria:

- Specificity (= analytical specificity-exclusivity): Ability of the method to not detect the target from a range of non-target populations (absence of false positives);
- Sensitivity (= analytical specificity-inclusivity): Ability of the method to detect the target from a range of target populations (absence of false negatives);
- Reproducibility: the ability of the method to reproduce identical results under different conditions (equipment, operators, etc.) from samples at low concentrations.

Other criteria were evaluated for molecular tests:

- Repeatability: Ability of the method to reproduce identical results under identical analytical conditions from samples at low concentrations;
- Accuracy: Ability of the method to detect the target species from a range of populations from the target species and not to detect the target species from a range of populations from the non-target species;
- Limit of detection (= analytical sensitivity): the smallest amount of target species that gives a positive result in all replicates.

8.1. The morphological method

Summary of the evaluation results for the identification of *B. xylophilus* by morphology

Performance criteria	Group identification key	Species identification key
Specificity	100%	100%
Sensitivity	100%	100%
Reproducibility	100%	100%

The full details of these results are described in Sarniguet *et al.* 2013.

8.2 The conventional molecular test

The data below are a compilation of the results of a comparative evaluation of different molecular tests to identify *B. xylophilus* and the performance characteristics obtained within the framework of the molecular test validation by ANSES laboratory.

Concerning the specificity, the molecular tests were applied to the following species/populations:

- 7 populations of *Bursaphelenchus xylophilus*
- 4 populations of *B. mucronatus* ;
- 2 populations of *B. sexdentati ;*
- 1 population of *B. doui;*
- 1 population of *B. fraudulentus;*
- 1 population of *B. singaporensis ;*
- 1 population of *B. macromucronatus;*
- 1 population of *B. hoffmani*;
- 1 population of *B. vallesianus*;
- 1 population of *B. willibaldi*
- 3 populations of *B. sp* from China, France and Vietnam.

Summary of the evaluation results for the identification of *B. xylophilus* by conventional PCR.

Performance criteria	Matsunaga & Togashi (2004)	Burgermeister et al. (2005)
Detection limit (LOD)	1 specimen	1 specimen
Repeatability *****	100%	100%
Reproducibility *****	100%	100%
Analytical Specificity (inclusivity and exclusivity)	100%	100%
Sensitivity	100%	100%
Accuracy	100%	100%

*: At the LOD

The molecular test to be used for the identification: the LOD is globally 1 specimen to reach 100% of repeatability and reproducibility of the test. The specificity of the tests is 100%.

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