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Diagnostics

PM 7/141 (1) *Philaenus spumarius, Philaenus italosignus* and *Neophilaenus campestris*

Specific scope

This Standard describes a diagnostic protocol for the detection and identification of species in the genera *Philaenus* and *Neophilaenus*. [1]

This Standard should be used in conjunction with PM 7/76(5) Use of EPPO diagnostic protocols.

Specific approval and amendment

Approved in 2019-06.

1. Introduction

Insects belonging to the order Hemiptera, sub-order Auchenorrhyncha (Redak *et al*., 2004), that feed on xylem sap (Chatterjee *et al*., 2008) are considered as potential vectors of *Xylella fastidiosa*. In the EPPO region, *Philaenus spumarius* has been confirmed as a vector of *X. fastidiosa* to olive, and probably other host plants, in the Southern Italian outbreak of the bacterium (Saponari *et al*., 2014; Cornara *et al*., 2017a, b). More recently, *Philaenus italosignus* and *Neophilaenus campestris* have also been confirmed to be vectors of *X. fastidiosa* subsp. *pauca* ST53 to olive plants under experimental conditions (Cavalieri *et al*., 2018). These three species are consequently the focus of this diagnostic protocol, even though other xylem sap-feeding insects may transmit the bacterium; this diagnostic protocol will be updated when new scientific evidence becomes available. An EPPO Diagnostic Protocol on *Xylella fastidiosa* (PM 7/24) is available from the EPPO website at https://www.eppo.int/RESOURCES/eppo_standards/pm7_diagnostics.

Philaenus spumarius is widely distributed, very common through most of Europe and present in North Africa, several parts of the former Soviet Union, Afghanistan, Japan, the USA, Canada, the Azores, Hawaii and New Zealand (Cornara *et al*., 2018). The species has never been considered a major threat to agriculture, but it has been regarded as a minor pest of a few crops such as strawberry in the USA after its introduction (Mundinger, 1946; Weaver & King, 1954).

Philaenus italosignus is endemic to Southern mainland Italy and Sicily; *Neophilaenus campestris* has a western Palearctic distribution and is commonly found on grasses. The role of *Philaenus italosignus* and *Neophilaenus campestris* in *X. fastidiosa* transmission to olives in Salento's outbreak in field conditions is probably very limited. Nevertheless, the two species could still play a role in pathosystems other than the Salento's/ST53/olive, or in inoculum maintenance outside of the cultivated groves (Cornara *et al*., 2018).

2. Identity

Identity of *Philaenus* species covered and *Neophilaenus campestris*

Taxonomic position (of the different species): Family Aphrophoridae, Genus *Philaenus* and *Neophilaenus*.

Name: *Philaenus spumarius* (Linnaeus, 1758).
Synonyms: Due to its polymorphism, more than 50 synonyms had been given to *P. spumarius,* as reported by Nast (1972).
EPPO Code: PHILSU.
Phytosanitary categorization: n.a.

Name: *Philaenus italosignus* (Drosopoulos & Remane, 2000).
EPPO Code: PHILIT.
Phytosanitary categorization: n.a.

Name: *Neophilaenus campestris* (Fallén, 1805).
EPPO Code: NEOPCA.
Phytosanitary categorization: n.a.

3. Detection

In this section, descriptions of different life stages are provided. As limited information is available on *Philaenus italosignus* and *Neophilaenus campestris* this section

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

focuses on *P. spumarius*. However, when available, information will be provided on the other species.

Philaenus spumarius is highly polyphagous, consequently nymphs and adults can be detected on various plants, in habitats such as meadows, abandoned fields, waste grounds, roadsides, stream sides, hayfields, marshlands, parks, gardens, vineyards, orchards and other cultivated fields (Yurtsever, 2000). For a list of *P. spumarius'* host plants refer to Weaver & King (1954) and Dongiovanni *et al*. (2019).

3.1. Eggs

Philaenus spumarius

Eggs are laid starting from the end of summer, in concomitance with a decrease in daily average temperature and photoperiod (Witsack, 1973; Morente *et al*., 2018a).

Eggs are laid in masses of one to 30, with an average number of seven, held together by a hardened frothy cement. According to field observations carried out in Southern Italian and Spanish olive orchards, the masses are placed at the base of herbaceous plants close to sheltered sites such as stone walls. The masses are laid in between two apposed surfaces, i.e. the stem and the leaf sheath, or on stubble, dead parts of plants, plants residues, cracks and tree trunk barks, or in the litter (Cornara *et al*., 2018). Under laboratory conditions, females oviposit on dry pine needles covering the pot surface of the host plant (Morente *et al*., 2018a).

An egg is 1 mm long and is approximately a third as wide as long (Weaver & King, 1954). It is elongated, ovoid and tapering in shape, yellowish white with a dark pigmented orange spot at one end. If the egg is fertilized, the orange spot gets bigger and a black lid-like formation develops on it (Yurtsever, 2000).

3.2. Nymphs

Philaenus spumarius

The first instar nymph is approximately 1.35 mm long, beige/light orange; during the development, the colour gradually becomes greenish yellow and is always uniform, in contrast to nymphs of *Neophilaenus* spp. that show a beige/light brown/orange colour with black pattern on the dorsum. Nymphs begin forming the spittle as soon as they start feeding on plants. Several nymphs can aggregate and share the same spittle mass, and several spittles can be found on the same plant. At the time of the last moult, the nymph ceases to form the spittle, which progressively dries up and forms the chamber where the adult stage will appear (Weaver & King, 1954). The spittle of the last three instars is easily spotted; on the contrary, first instar nymphs tend to settle on the basal part of the host plant, making their detection more difficult.

3.3. Adults

Philaenus spumarius

Adults appear in spring and live until autumn, although in Mediterranean conditions they may survive throughout the successive spring in case of mild winters (Saponari *et al*.,

Table 1. Identification of *Philaenus spumarius*, *Philaenus italosignus* and *Neophilaenus campestris*; key starting at Cicadomorpha level. The key was prepared by JF Germain based on Biedermann & Niedringhaus (2009) and personal observations for species from the Southern part of Europe

1	Pronotum extending over abdomen and hiding the scutellum	Membracidae	
	Pronotum not extending over abdomen and not hiding the scutellum		2
2	Three ocelli	Cicadidae	
	Two ocelli		3
3	Hind tibia with laterally one to several stout fixed spines	Cercopidae and Aphrophoridae	4
	Hind tibia with row of bristles	Other family: Cicadellidae	
4	Forewings never with red colouring and Pronotum as wide as the head	Aphrophoridae	5
	Forewings with red colouring and Pronotum wider the head	Other family: Cercopidae	
5	Pronotum and vertex without median keel	Other Aphrophoridae	6
	Pronotum and vertex with median keel	*Aphrophora* spp. and *Peuceptyelus coriaceus*	
6	Body shape compact, fore wings rounded	*Lepyronia coleoptrata*	
	Body shape more slender	*Philaenus* and *Neophilaenus*	7
7	Outer margin of forewing convex and frons plate without median keel and apex of the hind tibia with 8 spurs without distinct row	*Philaenus* spp.	8
	Outer margin of forewing with the first third concave and frons plate with median keel and apex of the hind tibia with 12 spurs on a double row	*Neophilaenus* spp.	9
8	Male aedeagus as illustrated in Fig. 1, apically with six antler-like appendages, circle-shaped for the upper one	*Philaenus spumarius*	
	Male aedeagus illustrated as Fig. 2. Absence of a meddle pair of projections of the frontal part. The shape of the lower pair, which is canon-like in shape, becoming more gradually slender towards the tip and the upper projections, which are shorter in length and point backwards from the main stem of the aedeagus	*Philaenus italosignus*	
	Male aedeagus different	Other *Philaenus*	
9	Male aedeagus illustrated as Fig. 3, hooklike in lateral view, lateral lobe in elongate triangle, shortened side serrated, ear-shaped in lateral view	*Neophilaenus campestris*	
	Males aedeagus different	Other *Neophilaenus*	

2014). Effective methods for quantitative *P. spumarius* sampling have been developed by Morente *et al*. (2018b). Adult *P. spumarius* should preferably be collected with sweep nets or aspirators. A video on insect collection has been published by EFSA and is available at https://www.youtube.com/watch?v=Rjh7FFQCtg8. Sticky traps are usually not as effective as active sampling for xylem feeders, but remain valuable (Cornara *et al*., 2018) and insects may be trapped accidentally, and specimens collected from sticky traps can be used for testing.

Vectors can be removed from the traps using small forceps/pincers and a suitable solvent such as vegetal xylene, Bio-Clear (Bio-Optica, Milano, Italy), kerosene, regular fuel (Purcell *et al*., 2014) or rapeseed oil can be used. After removal from the traps, insects should be rinsed in ethanol/acetone (95–99%). Traps should be serviced on a weekly basis.

4. Identification

4.1. Morphological identification

Characteristics such as colour and length may be subject to post-mortem changes, vary according to insect collection and storage method used, and do not allow reliable species identification. Taxonomical identification for Auchenorrhyncha should be based on the examination of morphological characteristics, some externally visible, some visible through dissections. A reliable morphological diagnosis is based on the observation of genitalia (Holzinger *et al*., 2003).

The dichotomous key presented in Table 1 can be used for the identification of male adult specimens. Observation of genitalia is required to be able to distinguish *Philaenus spumarius* from *Philaenus italosignus*. For the preparation of male genitalia see Appendix 1. Identification at species level of nymphal instars is difficult, thus molecular methods are recommended for immature specimens.

4.1.1. Description of Philaenus spumarius *adults*
Males are smaller than females; male size is 5.3 to 6.0 mm, while female size is 5.4 to 6.9 mm. Body shape is more rounded than in *Neophilaenus*. Colour is very variable, from whitish yellow to black. Several colour morphs have been described (Kunz *et al*., 2011); for a complete list refer to Yurtsever (2000) (Fig. 4a).

4.1.2. Description of Philaenus italosignus adults
Restricted to the Southern part of mainland Italy and Sicily. Similar to *P. spumarius*; only genitalia observation can permit a certain discrimination between the two species. *P. spumarius* and *P. italosignus* occur sympatrically. Males are smaller than females; male size is approximately 6.4 to 7.2 mm, while female size is 7.0 to 8.1 mm (Fig. 4b).

4.1.3. Description of Neophilaenus campestris *adults*
Males are smaller than females; male size is 5.0 to 5.3 mm, while female size is 5.4 to 5.7 mm. Body shape more slender than in *Philaenus*. Base colour greyish yellow to greyish brown, often with reddish undertone, generally with a dark longitudinal streak extending from vertex towards the scutellum. Forewing outer margin with two light spots (Fig. 4c).

Fig. 1 Aedeagus of *Philaenus spumarius* (courtesy of Streito JC, INRA CBGP).

Fig. 2 Aedeagus of *Philaenus italosignus* (courtesy of Streito JC, INRA CBGP).

Fig. 3 Aedeagus of *Neophilaenus campestris*: front and lateral views (courtesy of Streito JC, INRA CBGP).

4.1.4. Key to Philaenus spumarius, Philaenus italosignus *and* Neophilaenus campestris *adult males*
A visual key describing the diagnostic procedure is presented in Fig. 5.

4.2. Molecular methods

Identification is commonly based on the examination of adult specimens; however, molecular identification on all

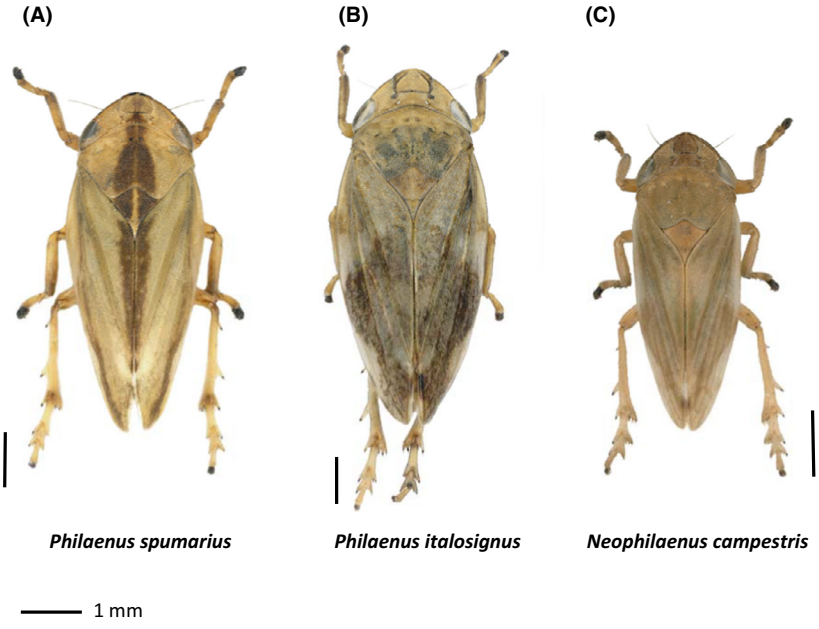

Fig. 4 (a) *Philaenus spumarius*, (b) *Philaenus italosignus* and (c) *Neophilaenus campestris* adults (courtesy of Streito J.C., INRA CBGP).

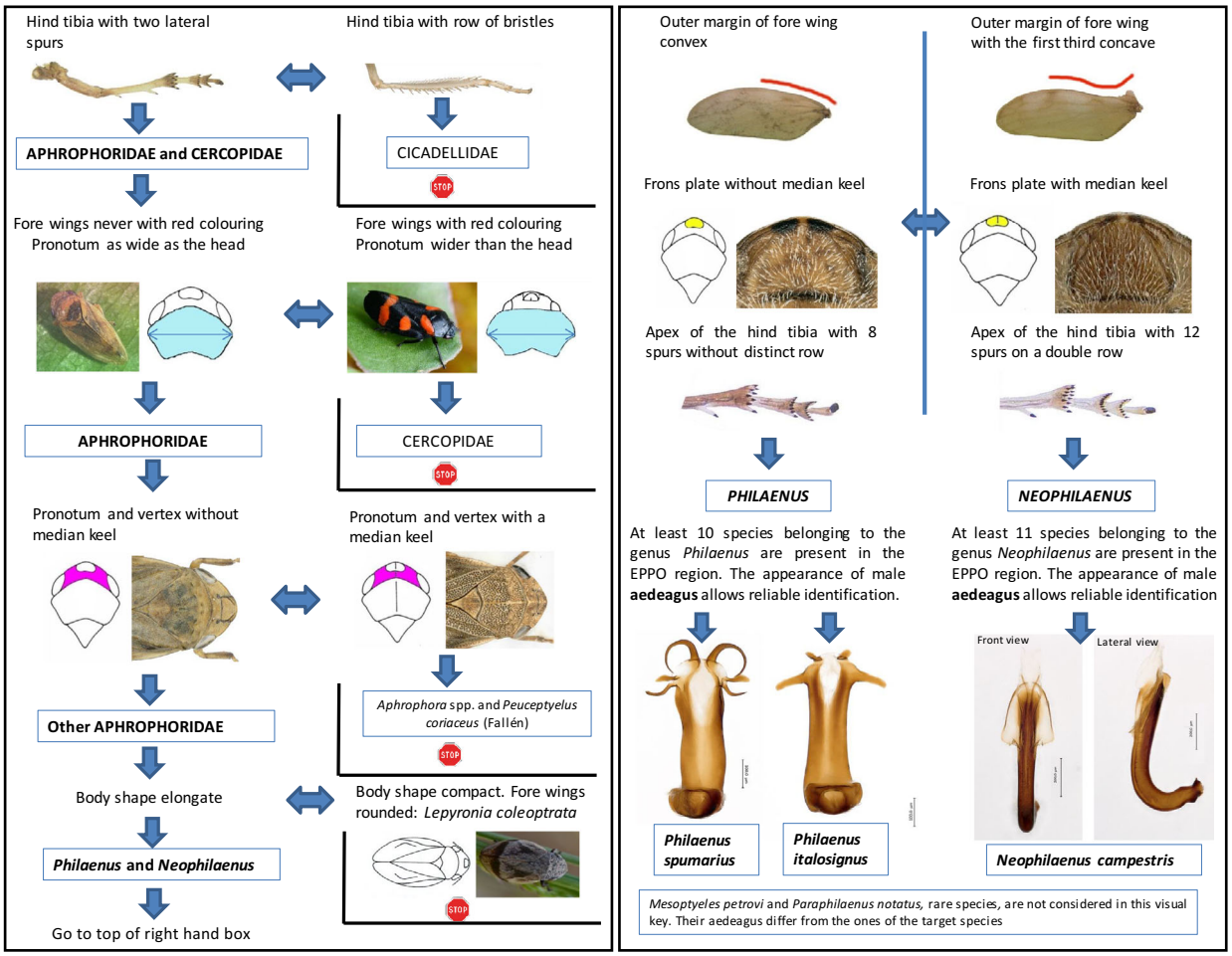

Fig. 5 Visual key of established vectors of *Xylella fastidiosa* in the EPPO region: *Philaenus spumarius*, *Philaenus italosignus* and *Neophilaenus campestris* (courtesy of Streito J.C., INRA CBGP). Follow the arrows in the left hand box and if you reach the bottom of the box (below Philaenus and Neophilaenus) then go to the top of the right hand box

life stages can be carried out using conventional PCR followed by Sanger sequencing analysis. A protocol for DNA barcoding based on COI gene is described in PM 7/129 DNA *barcoding as an identification tool for a number of regulated pests* (EPPO, 2016).

Other molecular tests have been developed for *Philaenus spumarius*; they were evaluated on *P. italosignus* but not against other *Philaenus* spp. The details of a conventional PCR adapted from Lantero *et al*. (2018) and based on the mitochondrial CO1 gene is described in Appendix 2 and a real-time PCR based on the internal transcribed spacer (ITS) is described in Appendix 3. Information on tests specificity is provided in these appendices.

5. Reference material

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6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/77 *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on these organisms can be obtained from:

Mr Fereres A. and Mr Cornara D., ICA-CSIC, calle Serrano 115 dpdo, 28006 Madrid (ES). E-mail: a.fereres@csic.es; danielecornara@gmail.com.

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

We would like to dedicate this Standard to the memory of one of its main authors, Mr Jean-François Germain, who passed away on 5 May 2019. Mr Germain has been a major contributor to EPPO's Diagnostics Standards in Entomology over the last 20 years and the EPPO Secretariat would like to express our sincere gratitude for all his work.

This protocol was originally drafted by Mr Germain J.F., ANSES - LSV - Unité Entomologie et Plantes Invasives, 755 avenue du campus d'Agropolis CS30016, 34988 Montferrier sur Lez (FR), Ms Lester K., SASA, Roddinglaw Road, Edinburgh, EH12 9FJ (GB). E-mail: Katherine.Lester@sasa.gsi.gov.uk, Mr Cornara D., ICA-CSIC, calle Serrano 115 dpdo, 28006 Madrid (ES). E-mail: danielecornara@gmail.com. It was reviewed by the Panel on Diagnostics in Entomology.

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Appendix 1 – Preparation of male genitalia under high power microscope (×200)

If the specimen is dry, soften in warm water for approximately 30 min until the abdomen can be moved. Remove the apical segment of the abdomen under a binocular microscope with forceps and place in warm potassium hydroxide solution (approximately 10%) at approximately 50°C for approximately 20 min to macerate muscles and fat tissues. Rinse and dissect the abdomen in 70–80% ethanol under a binocular microscope (×20), open the abdominal segment along one side with fine scissors or pins, clean out remaining tissues and carefully sever the genitalia. Rinse abdomen and genitalia in distilled water or ethanol and place into glycerine on a cavity slide for study and temporary storage. For permanent storage, place genitalia (aedeagus) in a drop of Canada balsam on a slide.

Alternatively, the specimen could be prepared following the procedure described by Pizza & Porcelli (1993) slightly modified. Macerate the sample, either dry or fresh, in SLS fluid (25 g sodium laureth sulphate, 25 g NaOH, 25 mL Tween 80, in 500 mL distilled H_2O) at 50°C and continuous gentle stirring for 24 h. This procedure allows the complete distensions of insect tissues making the genitalia dissection easier. Detach the genitalia using two pairs of fine scissors or pins, rinse them twice in water at 50°C for approximately 10 min, and dehydrate in ethanol 95%. Further macerate the sample in Essig's Aphid Fluid for 24 h at room temperature. Finally mount the genitalia on a tick slide (place a gum o-ring diameter 1 cm filled with mountant in between the slide and the cover) with polyvinyl alcohol (PVA) mounting medium. The gum o-ring permits the genitalia to be moved just by gently sliding the cover, allowing sample observations from different angles. For Cercopidae and Aphrophoridae, genitalia can be observed without severing them from the abdomen directly after warm SLS maceration, since the genitalia tend to become everted (D. Cornara, personal observations).

Appendix 2 – Conventional PCR (adapted from Lantero *et al.*, 2018)

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

1. General information

1.1 This PCR can be used for the detection of *Philaenus spumarius/P. italosignus*. See section 4 for more details on test specificity.
1.2 The test is adapted from Lantero *et al*. (2018).
1.3 The target sequence is located within the mitochondrial CO1 region.
1.4 Oligonucleotides:

Primer name	Sequence	Amplicon size
Forward primer Phi 2F	5′-GCT TCC TCC TTC ATT AAC GCT T-3′	128 bp
Reverse primer Phi 1R	5′-TAG CTA AAT CAA CAC ATG CAC CAG-3′	

2. Methods

2.1 DNA extraction from both adult and immature insects can be performed using commercially available kits such as the Blood & Tissue kit (Qiagen); an extraction protocol is described in PM 7/129 DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2016). The extracted DNA should be used immediately or stored at approximately −20°C until use.
2.2 Conventional PCR
2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	2.5	NA
Taq PCR Master mix (Qiagen)	2×	6.25	1x
$MgCl_2$ (or alternatives) (Qiagen)	25 mM	0.75	1.5 mM†
Forward primer (Phi 2F)	20 µM	0.5	0.8 (M
Reverse primer (Phi 1R)	20 µM	0.5	0.8 (M
Subtotal		10.5	
DNA stock (5 ng μL^{-1})		2	
Total		12.5	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

†The concentration displayed is not the actual final concentration of $MgCl_2$, as the *Taq* PCR Master Mix (Qiagen) also contains $MgCl_2$.
NA, not applicable.

2.2.2 PCR conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 45 s, 62°C for 1 min and 72°C for 1 min).

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

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

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce an amplicon of 128 bp.

When these conditions are met

- A test will be considered positive if an amplicon of 128 bp is produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

A small experiment was conducted in SASA (GB) and showed that this test does not allow *Philaenus spumarius* to be distinguished from *P. italosignus*.

Philaenus spumarius and *P. tesselatus* are closely related taxa with uncertain taxonomic position in the light of

morphological, cytological and molecular data (Maryanska-Nadachowska *et al*., 2011). From the information available from GenBank and BOLD, *P. spumarius* and *P. tesselatus* are identical within the CO1 region. The test may not discriminate between the two species.

Appendix 3 – Real-time PCR (Lester *et al*., unpublished)

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

1. General information

1.1 This PCR can be used for the identification of *Philaenus spumarius*. See section 4 for more details on test specificity.

1.2 The test is based on a protocol developed by Lester *et al*. (SASA, GB unpublished).

1.3 The target sequence is the internal transcribed spacer 2 (ITS2) region.

1.4 Oligonucleotides:

Primer's name	Sequence
PhiSpu_ITS2_F	5′-TCA TAA CCC CAC GTT TGT CC-3′
PhiSpu_ITS2_R	5′-CAA TTG TTC CGC ATC GTA CG-3′
PhiSpu_ITS2_P (HPLC purified)	5′-FAM-GCC CAC AAC CGC CAC GAC CA BHQ1-3′

1.5 This test has been successfully performed on QuantStudio™ Flex 6 machine. Analyses were performed using QuantStudio™ real-time PCR software.

2. Methods

2.1 DNA extraction from both adult and immature insects can be performed using commercially available kits such as the Blood & Tissue kit (Qiagen); an extraction protocol is described in PM 7/129 DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2016). The extracted DNA should be used immediately or stored at approximately –20°C until use.

2.2 Real-time polymerase chain reaction

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water*	NA	5.75	NA
Real-time PCR buffer (JumpStart((*Taq* ReadyMix((	2×	10	1×
Forward Primer (PhiSpu_ITS2_F)	10 µM	0.5	250 nM
Reverse Primer (PhiSpu_ITS2_R)	10 µM	0.5	250 nM
Probe 1 (PhiSpu_ITS2_P)	10 µM	0.25	125 nM
18S Endogenous control (Applied Biosystems ®)	20×	1	1×
Subtotal		18	
DNA dilution		2	
Total		20	

*Molecular grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.
NA, not applicable.

2.2.2 PCR cycling conditions: 2 min at 95°C, followed by 40 cycles of denaturation and annealing/elongation for 15 s at 95°C and 1 min at 60°C, respectively. Heating ramp speed: 1.6°C s^{-1}.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results: in order to assigning results from PCR-based test the following criteria should be followed:

Verification of the controls

- The PIC and PAC amplification curves should be exponential.

- NIC and NAC should give no amplification

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

4.1 Analytical specificity data

The analytical specificity was checked against target and non-target species of eight Auchenorrhyncha species, including *Aphrophora alni, Balclutha punctata, Cicadella viridis, Elymana sulphurella, Evacanthus interruptus, Neophilaenus campestris, Neophilaenus lineatus* (adult and nymph specimens) and *Philaenus spumarius* (adult and nymph specimens). No cross-reaction was observed in non-target species. This test was evaluated against *Philaenus italosignus* and no cross-reaction was observed, but not against other *Philaenus* species. *Philaenus spumarius* and *P. tesselatus* are closely related taxa with uncertain taxonomic position in the light of morphological, cytological and molecular data (Maryanska-Nadachowska *et al*., 2011). From the information available from GenBank and BOLD, *P. spumarius* and *P. tesselatus* are identical within the ITS2 region. The test may not discriminate between the two species.

4.2 Data on repeatability and on reproducibility

The test was checked for repeatability and reproducibility. *Philaenus spumarius* material (50 pg μL^{-1} and 5 pg μL^{-1}) and non-target species (15 ng μL^{-1}) were tested in triplicate over three separate runs. A consistent positive signal was achieved. No amplification was observed for non-target species. The same test was also performed using Takyon™ Rox Probe MasterMix (Eurogentec) using different equipment (7900 Applied Biosystems®) and results were consistent with previous tests.