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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/129 (1)

Diagnostics
Diagnostic

PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests

Specific scope

This Standard describes the use of DNA barcoding protocols in support of the identification of a number of regulated pests and invasive plant species comparing DNA barcode regions with those deposited in publically available sequence databases.[1] It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

2016-09

1. Introduction

DNA barcoding is a generic diagnostic method that uses a short standardized genetic marker in an organism's DNA to aid identification at a certain taxonomic level. The chosen marker region should reflect the group taxonomy of the target species. Therefore, the marker region should provide a high interspecific variability and low intraspecific differences and should enable the identification of as many species as possible belonging to a shared higher taxonomical level such as genus, family or order (e.g. Chen *et al.*, 2013). An organism is identified by finding the closest matching reference record. The first genetic marker to be described as a 'barcode' was the mitochondrial cytochrome *c* oxidase I (*COI*) gene which is used for species identification in the animal kingdom (Hebert *et al.*, 2003). Later the chloroplast large subunit ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcL*) gene (Hollingsworth *et al.*, 2009) and the nuclear ribosomal internal transcribed spacer (ITS) region (Schoch *et al.*, 2012) have been proposed as barcodes for the plant and fungi kingdoms, respectively.

The use of a single barcode region does not provide sufficient reliability for the identification of the majority of regulated pests. Therefore, several short standardized genetic markers have been identified as 'barcodes' for identification at the required taxonomic level in several pest groups. DNA barcoding protocols for eukaryotes and prokaryotes (a novelty in the DNA barcoding field) were developed and validated within the Quarantine Organisms Barcoding of Life (QBOL) Project financed by the 7th Framework Programme of the European Union. Within the DNA barcoding EUPHRESCO II project, test protocols for several quarantine pests and invasive plant species were added, and the use of polymerases with proofreading abilities was introduced to minimize the risk of polymerase chain reaction (PCR) errors. In addition, amplification primers were M13-tailed when possible to improve the user-friendliness of the protocols, allowing the generation of sequence data with a minimum number of sequencing primers. Regulated organisms are identified by finding the closest matching reference record, using a combination of Basic Local Alignment Search Tool (BLAST) hit identity, multi-locus sequence analysis (MLSA) and clustering in species-specific clades using multiple databases containing sequence data of regulated organisms and related species. Pest species in this Standard were selected on the basis of their pest status, economic impact, availability of material and pre-existing knowledge of loci with sufficient resolution.

This EPPO Standard describes the DNA barcoding protocols developed for the identification of a number of regulated arthropods, bacteria, fungi and oomycetes, invasive plant species, nematodes and phytoplasmas. Each organism group is covered in a separate Appendix. Protocols describe the extraction of nucleic acids and the amplification of short standardized marker(s). Since the identification of regulated pests is often based on several different markers, diagnostic schemes are provided to aid the selection of

[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.

appropriate protocols. When more than one marker is necessary, the markers are either used in parallel for species identification (e.g. invasive plant species and phytoplasmas) or a single marker is first used for genus identification (e.g. 16S for bacteria) and, depending on the genus, a second marker (sometimes in parallel with a third marker) is used for identification to species level. For some *Xanthomonas* bacteria a third marker is needed for identification at the pathovar or (sub)species level. For each identification based on several markers all consensus sequences produced need to be analysed in a MLSA which can be done in Q-bank. The generation of sequence data, assembly of raw sequence data and analysis of consensus sequences using BLAST and MLSA in online databases is discussed in Appendix 7. Appendix 8 provides an example of a sequencing analysis report that can be used to collate all relevant data, and Appendix 9 provides information on synthetic positive amplification controls (PACs).

It has to be noted that the outcome of DNA barcoding tests can be negatively affected by the incompleteness of databases, incorrectly identified species in databases, the amplification of pseudogenes or NUMTs and introgression or hybridization events. For that reason, the analysis of sequence data should be performed by proficient operators. DNA barcoding is consequently used in support of identification at a certain taxonomic level. Origin, host plant and other characteristics (e.g. morphological, biochemical, reactions on indicator plants) are typically needed to complete the diagnosis.

2. Reference material

A single synthetic PAC per organism group can be used to assess the efficiency of the PCR amplification. It can also be used as a standardized process control from amplification until sequence analysis and will give insight into the repeatability and reproducibility of each test (see also Appendix 7, Section 5.3 'Validation'). The synthetic PAC is designed in such a way that all tests in one Appendix can be monitored using a single control. When amplified, the synthetic PACs yield amplicons ranging from 560 to 720 base pairs, depending on the primers used. When sequenced, the synthetic PACs can easily be identified since, after translation of the nucleic acid sequence (reading frame 1, standard code), the following amino acid sequence is obtained twice: *KEEP*CALM*THIS*IS*MERELY*A*VERY*STRANGE*REFERENCE*PHRASE*WITH*EIGHTY*FIVE*CHARACTERS (stop codons are indicated as *). Synthetic PAC sequences are presented in Appendix 9, and are available from the NCBI: PAC arthropods v.1 (KT429638); PAC bacteria v.1 (KT429643); PAC fungi v.1 (KT429642); PAC invasive plant species v.1 (KT429639); PAC nematodes v.1 (KT429641); PAC phytoplasmas v.1 (KT429640), and can be ordered from commercial companies producing synthetic genes or gBlocks (e.g. ThermoFisher, IDT, Biomatik).

3. 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.

4. 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.

5. Acknowledgements

This protocol was originally drafted by: BTLH van de Vossenberg, M Westenberg, M Botermans, Dutch National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen, the Netherlands; J Hodgetts, Fera, Sand Hutton, York YO41 1LZ, UK; and B Cottyn, Institute for Agricultural and Fisheries Research, Plant Sciences Unit, Crop Protection, Burgemeester van Gansberghelaan 96, bus 2, 9820, Merelbeke, Belgium. It was reviewed by the Panel on Diagnostics and Quality Assurance as well as the Panels on Diagnostics in the different disciplines. The DNA barcoding protocols in this standard were developed, optimized and validated in an international test performance study within the QBOL Project financed by 7th Framework Program of the European Union, and the DNA Barcoding EUPHRESCO II Project.

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Appendix 1 – DNA barcoding of arthropods

1. General information

1.1 This appendix describes the protocols used to identify selected regulated arthropods by conventional PCR followed by Sanger sequencing analysis. Table 1 shows the regulated organisms that have successfully been tested with the protocols described in this section. It is very likely that other regulated arthropods can also successfully be identified using these protocols, but to date validation data has not been generated to support this.

Table 1. Regulated arthropods successfully identified with barcoding protocols

	Test			
Regulated organism	2.2 *COI*	2.3 *COI*[*]	2.4 *COI*[*]	Remarks
Anoplophora chinensis	x[†]			
Anoplophora glabripennis	x			
Anthonomus eugenii	x			
Helicoverpa zea	x			Listed as *Heliothis zea*
Liriomyza bryoniae	x			
Liriomyza sativae	x			
Spodoptera eridania	x			
Spodoptera frugiperda	x			
Spodoptera littoralis	x			
Spodoptera litura	x			
Tephritidae (non-European)[‡]	x			
Thrips palmi	x			

[*]In some cases the *COI* test using primers LCO1490 and HCO2198 (Section 2.2) fails to produce an amplicon. In those cases, the *COI* tests described in Sections 2.3 and 2.4 can be used alternatively.
[†]Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.
[‡]Several non-European *Tephritidae* sequences are available in Q-bank.

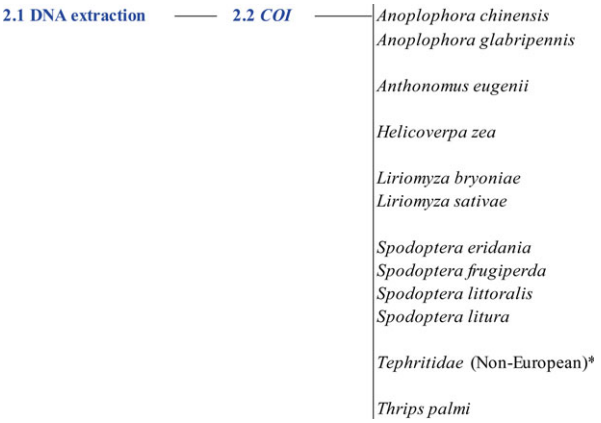


Fig. 1 Diagnostic testing scheme for identification of regulated arthropods using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used. *Several non-European *Tephritidae* sequences are available in Q-bank.

1.2 Protocols were developed by INRA (FR) as part of the QBOL Project financed by 7th Framework Programme of the European Union (2009–12). The protocols were further optimized by the Food and Environment Research Agency (Fera) (GB) as part of the EUPHRESCO II DNA Barcoding project (2013–14).

1.3 The mitochondrial *COI* gene test described in Section 1.2.2 is used for species identification of selected regulated arthropods (see Fig. 1, Table 1). If no amplicons are generated the COI tests described in Sections 1.2.3 and 1.2.4 can be used.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the BIO-X-ACT™ Short Mix (Bioline) reagents (cat. no. BIO-25026).

1.6 Molecular-grade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease free.

1.7 Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (Bio-Rad).

Note that validation data presented in Section 4 have been obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Tissue material (typically 10–50 mg) of all life stages of a single specimen is used as input for DNA extraction.

2.1.2 DNA is extracted using the Blood & Tissue kit (Qiagen) according to the animal tissue protocol.

2.1.3 When tissue material is stored in ethanol, all the ethanol should be removed prior to DNA extraction.

2.1.4 Grinding of the tissue material in a lysis buffer (provided) prior to DNA extraction can be performed but is not required in order to allow non-destructive DNA extraction.

2.1.5 After crushing, the sample should be incubated at 56°C for at least 1 h.

2.1.6 DNA is eluted in 200 µL of pre-heated (56°C) elution buffer (provided). When working with small amounts of tissue material, DNA is eluted in 50–100 µL of pre-heated elution buffer.

2.1.7 No DNA clean-up is required after DNA extraction.

2.1.8 The extracted DNA should either be used immediately or stored at −20°C until use.

2.2 PCR of the arthropod *COI* gene

2.2.1 PCR-sequencing of 709 bp (amplicon size including primers) of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene of arthropods is adapted from Folmer *et al.* (1994).

2.2.2 Primer sequences are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
LCO1490	GGTCAACAAATCA TAAAGATATTGG	X	X
HCO2198	TAAACTTCAGGGTG ACCAAAAAATCA	X	X

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
LCO1490	10 µM	0.5	0.2 µM
HCO2198	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proofreading activity.

2.2.4 Thermocycler profile: 3 min at 94°C, 5× (30 s at 94°C, 30 s at 45°C, 1 min at 72°C), 35× (30 s at 94°C, 1 min at 51°C, 1 min at 72°C), 10 min at 72°C.

2.2.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.2.6 The mitochondrial *COI* is a protein-coding region. Translation Table 5 (Invertebrate Mitochondrial Code) applies to the mitochondrial *COI* gene.

2.2.7 The primer pair LCO1490/HCO2198 results in a *COI* sequence with the codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.3 Alternative PCR of the arthropod *COI* gene – 1

2.3.1 PCR-sequencing of 745 bp (amplicon size including primers) of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene of arthropods (J. Y. Rasplus, unpublished[2]).

2.3.2 Primer sequences are described in the table below. The M13-tailed *COI* primer cocktail is prepared by pooling an equal volume of 10 µM of the five primers LCO1490puc-t1, LCO1490-Hym1-t1, HCO2198puc-t1, HCO2198Hym1-t1 and HCO2198Hym2-t1.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
LCO1490puc-t1	caggaaacagctatgacc TTTCAACWAATC ATAAAGATATTGG*	X	
LCO1490Hym1-t1	caggaaacagctatgacc TTTCWACAAATCA TAAADAYATTGG	X	
HCO2198puc-t1	tgtaaaacgacggccagt TAAACTTCWGGRT GWCCAAARAATCA	X	
HCO2198Hym1-t1	tgtaaaacgacggccagt TAAACTTCYGGAT GTCCRAAAAATCA	X	
HCO2198Hym2-t1	tgtaaaacgacggccagt TAAACTTCWGGRT GACCAAAAAATCA	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.3.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	10	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
Hymenoptera primer cocktail	10 µM total	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 Thermocycler profile: 3 min at 94°C, 5× (30 s at 94°C, 30 s at 45°C, 1 min at 72°C), 35× (30 s at 94°C, 1 min at 51°C, 1 min at 72°C), 10 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.3.6 The mitochondrial *COI* is a protein coding region. Translation Table 5 (Invertebrate Mitochondrial Code) applies to the mitochondrial *COI* gene.

2.3.7 The M13-tailed primer cocktail results in a *COI* sequence with the codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.4 Alternative PCR of the arthropod *COI* gene – 2

2.4.1 PCR-sequencing of 745 bp (amplicon size including primers) of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene of arthropods is adapted from Germain *et al.* (2013).

2.4.2 Primer sequences are described in the table below. The M13-tailed *COI* primer cocktail is prepared by pooling an equal volume of 10 µM of the five primers LCO1490-puc-t1, LCO1490Hym1-t1, HCO2198puc-t1, HCO2198Hym1-t1 and HCO2198Hym2-t1.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
LCO1490puc-t1	caggaaacagctatgacc TTTCAACWAATCA TAAAGATATTGG*	X	
LCO1490Hem1-t1	caggaaacagctatgacc TTTCAACTAAYCA TAARGATATYGG	X	

(continued)

[2]Developed in the framework of the QBOL project (http://www.qbol.org) in parallel to the test described under Section 2.4.

Table *(continued)*

Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
HCO2198puc-t1	tgtaaaacgacggccagt TAAACTTCWGGRT GWCCAAARAATCA	X	
HCO2198Hem1-t1	tgtaaaacgacggccagt TAAACYTCDGGAT GBCCAAARAATCA	X	
HCO2198Hem2-t1	tgtaaaacgacggccagt TAAACYTCAGGAT GACCAAAAAAYCA	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.4.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	10	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
Hemiptera primer cocktail	10 μM total	0.5	0.2 μM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.4.4 Thermocycler profile: 3 min at 94°C, 5× (30 s at 94°C, 30 s at 45°C, 1 min at 72°C), 35× (30 s at 94°C, 1 min at 51°C, 1 min at 72°C), 10 min at 72°C.

2.4.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.4.6 The mitochondrial *COI* is a protein-coding region. Translation Table 5 (Invertebrate Mitochondrial Code) applies to the mitochondrial *COI* gene.

2.4.7 The M13-tailed primer cocktail result in a COI sequence with the codon starting in reading frame 2 of the primer-trimmed consensus sequence.

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 DNA extraction: include an empty tube in the DNA extraction procedure as if it were a real sample.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: include a tube with no added template; instead add 2 μL 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 gBlock EPPO_PAC_Arthropods_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 1).

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size

When these conditions are met:

- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding Project in an international consortium of 11 participants. Additional data was generated by the Dutch NPPO laboratory.

4.1 Analytical sensitivity

Tissue material (typically 10–50 mg) of all life stages of a single specimen is used as input for DNA extraction. For all protocols a DNA concentration of 3.9 ng μL^{-1} is sufficient to generate an amplicon that can be sequenced, leading to a high-quality (HQ) consensus sequence (Phred score > 40) of at least 99%.

4.2 Analytical specificity

The locus indicated in Table 1 possesses sufficient interspecies variation to allow for identification to species level. In addition to the species listed in Table 1, species from several genera have successfully been amplified and sequenced by the Dutch NPPO using the protocols in this appendix (see the EPPO validation sheet for this appendix, http://dc.eppo.int/tps.php):

Test 1.2.2 *COI*: *Acanthocinus* (1), *Acleris* (1), *Adoxophyes* (1), *Anastrepha* (1), *Anoplophora* (8), *Apriona* (1), *Argyrogramma* (1), *Atherigona* (1), *Autographa* (1), *Bactrocera* (5), *Bombus* (1), *Cameraria* (1), *Carpomya* (1), *Ceratitis* (3), *Chloridea* (2), *Chromatomyia* (1), *Chrysodeixis* (1), *Chymomyza* (1), *Clepsis* (1), *Clytus* (1), *Conogethes* (1), *Contarinia* (1), *Copitarsia* (2), *Coremagnatha* (1), *Cydalima* (1), *Cydia* (1), *Dasineura* (3),

Deroceras (1), *Desmiphora* (1), *Deudorix* (1), *Diabrotica* (1), *Diaphania* (2), *Dorata* (1), *Drosophila* (2), *Dryocosmus* (1), *Earias* (1), *Elaphria* (2), *Enarmonia* (1), *Ephestia* (1), *Ephiphyas* (1), *Euclea* (1), *Euleia* (1), *Frankliniella* (1), *Grapholita* (1), *Helicoverpa* (2), *Heliothos* (1), *Helivocerpa* (1), *Hesperophanes* (1), *Himacerus* (1), *Hylotrupes* (1), *Hymenia* (1), *Hypena* (1), *Janetiella* (2), *Janus* (1), *Lasioptera* (2), *Liriomyza* (5), *Mamestra* (1), *Maruca* (1), *Mesopolobus* (1), *Monochamus* (7), *Muscina* (1), *Napomyza* (2), *Neoleucinodes* (1), *Orgyia* (1), *Ornidia* (1), *Ovachlamys* (1), *Ozodes* (1), *Palpita* (1), *Pemphredon* (1), *Placochela* (1), *Planoccoccus* (1), *Platynota* (2), *Pomacea* (1), *Prays* (1), *Psapharochrus* (1), *Pyrodereces* (1), *Rhagoletis* (1), *Rhectocraspeda* (1), *Rhinoncus* (1), *Sesia* (1), *Sinibotys* (1), *Spodoptera* (15), *Sternochetus* (1), *Strymon* (1), *Tetranychus* (1), *Thaumatotibia* (1), *Thecabius* (1), *Thrips* (3), *Torymus* (1), *Trichoferus* (2), *Tuta* (1), *Vittaplusia* (1), *Xylodiplosis* (1), *Xylotrechus* (1) and *Xystrocera* (1).

Test. 1.2.3 *COI* alternative 1: *Anoplophora* (4), *Apriona* (1), *Argyesthia* (1), *Bombus* (1), *Etiella* (1), *Grapholita* (1), *Leucinodes* (1), *Monochamus* (1), *Tretropium* (1) and *Trichoferus* (3).

Test 1.2.4 *COI* alternative 2: *Anoplophora* (4), *Apriona* (1) and *Argyesthia* (1).It has to be recognized that the potential for amplification and sequencing with the generic primers in this Appendix is much larger.

4.3 Selectivity

Selectivity does not apply as individual specimens are used.

4.4 Diagnostic sensitivity

Test performance study (TPS) partners in the EUPHRESCO II DNA Barcoding Project analysed five DNA samples of the following species: *Vespa crabo* (not regulated), *Bemisia tabaci*, *Liriomyza huidobrensis*, *Spodoptera eridania* and *Anoplophora glabripennis.* The overall diagnostic sensitivity obtained was 98%. All except one sample was correctly identified. One partner used conservative identification for the *Spodoptera eridania* sample (i.e. Lepidoptera sp.: order-level identification) which resulted in a diagnostic sensitivity of 91% for this sample. Re-analysis of data produced by this partner showed that species-level identification is possible and an overall diagnostic sensitivity of 100% could be obtained.

4.5 Reproducibility

The same DNA samples are analysed by different partners. Therefore in this situation the reproducibility is identical to diagnostic sensitivity.

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of the operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Appendix 2 – DNA barcoding of bacteria

1. General information

1.1 This appendix outlines protocols for the identification of selected regulated bacteria using conventional PCR followed by Sanger sequencing analysis. Table 2 shows the regulated organisms that have successfully been tested with the protocols described in this section. It is very likely that other regulated bacteria can successfully be identified using these protocols, but validation data has not been generated to support this.

1.2 The protocol was developed by the Institute for Agricultural and Fisheries Research (ILVO), University of Ghent, Belgium, and Agroscope, Switzerland, as part of the QBOL Project financed by 7th Framework Programme of the European Union (2009–12). As part of the EUPHRESCO II DNA Barcoding Project (2013–14), the protocols were further optimized by ILVO, Belgium.

1.3 A combination of two to three out of six tests is used to identify selected regulated bacteria; the *16S* ribosomal DNA (rDNA), *gyrB* (2×), *avrBs2* and *mutS*. After *16S* rDNA-based confirmation of the bacterial genus, the protocol follows the barcoding strategy as presented in the diagnostic testing scheme (see Fig. 2). Table 2 gives an overview of the loci needed for the selected regulated bacteria.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the Bio-X-Act Short Mix (Bioline) reagents (cat. no. BIO-25026).

1.6 Molecular-grade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease free.

1.7 Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (Bio-Rad).

The validation data presented in Section 4 were obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Cell pellets of pure cultures (maximum 2×10^9 cells) are used as starting material for the DNA extraction.

Table 2. Regulated bacteria successfully identified with barcoding protocols

Regulated organism	Test						Remarks
	2.2 *16S* rDNA	2.3 *gyrB* *Clavibacter*	2.4 *mutS* *Ralstonia*	2.5 *gyrB* *Xanthomonas*	2.6 *avrBs2* *Xanthomonas*	2. 7 mutS *Xylella*	
Clavibacter michiganensis spp.	x*	x					Gram +ve
Ralstonia solanacearum	x		x				Gram −ve
Xanthomonas alfalfae ssp. *citrumelonis*	x			x	x		Gram −ve
Xanthomonas axonopodis pv *dieffenbachiae*	x			x	x		Gram −ve
Xanthomonas citri subsp. *citri*	x			x	x		Gram −ve
Xanthomonas euvesicatoria	x			x	x		Gram −ve
Xanthomonas fragariae	x			x			Gram −ve
Xanthomonas fuscans subsp. *aurantifolii*	x			x	x		Gram −ve
Xanthomonas fuscans subsp. *fuscans*	x			x	x		Gram −ve
Xanthomonas gardneri	x			x			Gram −ve
Xanthomonas oryzae	x			x			Gram −ve
Xanthomonas perforans	x			x	x		Gram −ve
Xanthomonas translucens	x			x			Gram −ve
Xanthomonas vesicatoria	x			x			Gram −ve
Xylella fastidiosa	x					x	Gram −ve

*Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.

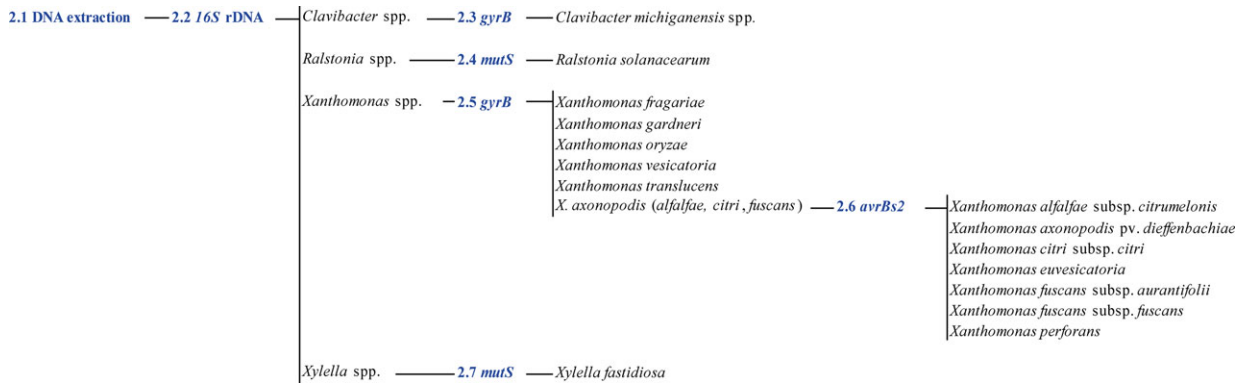


Fig. 2 Diagnostic testing scheme for identification of regulated bacteria using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used.

2.1.2 DNA is extracted using the Blood & Tissue kit (Qiagen) using the pre-treatment for Gram-negative or Gram-positive bacteria followed by the animal tissue protocol (starting at Step 2 or 4 for Gram-negative or Gram-positive bacteria, respectively). The pre-treatment for Gram-positive bacteria can also be used for the DNA extraction of Gram-negative bacteria.

2.1.3 DNA is eluted in 100 µL of elution buffer (provided). As the first elution fraction may still contain impurities, elution is performed twice using 50 µL of elution buffer and the two fractions are collected in a single micro-centrifuge tube.

2.1.4 No DNA clean-up is required after DNA extraction.

2.1.5 The extracted DNA should either be used immediately or stored until use at −20°C or below.

2.2 Conventional PCR *16S* rDNA bacteria

2.2.1 PCR of approx 1500 bp of the *16S* rDNA amplification is adapted from Edwards *et al.* (1989), followed by sequencing of a partial 309–350 bp fragment using the two reverse primers as adapted from Coenye *et al.* (1999).

2.2.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
pA (forward primer)	AGAGTTTGATCCT GGCTCAG	X	
pH (reverse primer)	AAGGAGGTGATCC AGCCGCA	X	
Reverse 358–339	ACTGCTGCCTCCCG TAGGAG		X
Reverse 536–519	GTATTACCGCGGCT GCTG		X

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
pA (forward primer)	10 μM	0.75	0.3 μM
pH (reverse primer)	10 μM	0.75	0.3 μM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.2.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (20 s at 98°C, 20 s at 60°C, 1 min at 72°C), 5 min at 72°C.

2.2.5 Cycle sequencing reactions of a small fragment from the amplified 1500 bp are performed using the primers reverse 358–339 and reverse 536–519 in separate reactions. The obtained dual coverage sequence (309–350 bp) fragment is used for genus identification.

2.2.6 *16S* rDNA is a non-coding but conserved locus that is transcribed in *16S* rRNA. Translation tables do not apply to *16S* rDNA.

2.3 Conventional PCR *gyrB Clavibacter michiganensis* spp.

2.3.1 PCR sequencing of 598 bp (amplicon size including primers) of the gyrase subunit B (*gyrB*) gene for *Clavibacter michiganensis* spp. is adapted from Richert *et al.* (2005).

2.3.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
GyrB 2F (M13-tagged)	caggaaacagctatgacc* ACCGTCGAGTTC GACTACGA	X	
GyrB 4R (M13-tagged)	tgtaaaacgacggccagt CCTCGGTGTTGC CSARCTT	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.3.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
GyrB 2F (M13-tagged)	10 μM	0.75	0.3 μM
GyrB 4R (M13-tagged)	10 μM	0.75	0.3 μM
Subtotal		23.0	
Genomic DNA extract	10 ng μL^{-1}	2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (10 s at 98°C, 10 s at 60°C, 30 s at 72°C), 5 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.3.6 The *gyrB* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the bacterial *gyrB* gene.

2.3.7 The M13-tailed primer pair GyrB 2F/GyrB 4R results in a *gyrB* sequence with a codon starting in reading frame 3 of the primer-trimmed consensus sequence.

2.4 Conventional PCR *mutS Ralstonia* spp.

2.4.1 PCR amplification of 803 bp (amplicon size including primers) of the DNA mismatch repair protein (*mutS*) gene for *Ralstonia* spp. identification is adapted from Wicker *et al.* (2007).

2.4.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
MutS-RsF (M13-tagged)	caggaaacagctatgacc* ACAGCGCCTTGA GCCGGTACA	X	
MutS-RsR (M13-tagged)	tgtaaaacgacggccagt GCTGATCACCGG CCCGAACAT	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.4.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
MutS-RsF (M13-tagged)	10 µM	0.75	0.3 µM
MutS-RsR (M13-tagged)	10 µM	0.75	0.3 µM
Subtotal		23.0	
Genomic DNA extract	10 ng μL^{-1}	2.0	
Total		25.0	

*Pr adequate PCR master mixes containing a polymerase with proof-reading activity.

2.4.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (10 s at 98°C, 10 s at 60°C, 30 s at 72°C), 5 min at 72°C.

2.4.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.4.6 The *mutS* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the bacterial *mutS* gene.

2.4.7 The M13-tailed primer pair MutS-RsF/MutS-RsR results in a *mutS* sequence with a codon starting in reading frame 2 of the complementary strand of the primer-trimmed consensus sequence.

2.5 Conventional PCR *gyrB Xanthomonas* spp.

2.5.1 PCR amplification 765 bp (amplicon size including primers) of the gyrase subunit B (g*yrB*) gene for *Xanthomonas* spp. identification is adapted from Parkinson *et al.* (2007).

2.5.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
XgyrPCR2F (M13-tagged)	caggaaacagctatgacc* AAGCAGGGCAAG AGCGAGCTGTA	X	
X.gyrrsp1 (M13-tagged)	tgtaaaacgacggccagt CAAGGTGCTGAA GATCTGGTC	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.5.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
XgyrPCR2F (M13-tagged)	10 µM	0.75	0.3 µM
X.gyrrsp1 (M13-tagged)	10 µM	0.75	0.3 µM
Subtotal		23.0	
Genomic DNA extract	10 ng μL^{-1}	2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.5.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (10 s at 98°C, 10 s at 60°C, 30 s at 72°C), 5 min at 72°C.

2.5.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.5.6 The *gyrB* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the bacterial *gyrB* gene.

2.5.7 The M13-tailed primer pair XgyrPCR2F/X.-gyrrsp1 results in a *gyrB* sequence with a codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.6 Conventional PCR *avrBs2 Xanthomonas* spp.

2.6.1 PCR amplification of approximately 905 bp (amplicon size including primers) of the avirulence protein (*avrBs2*) gene for *Xanthomonas* spp. identification is adapted from Hajri *et al.* (2009).

2.6.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
AvrBs2F (M13-tagged)	caggaaacagctatgacc* GGACTAGTCCTGCC GGTGTTGATGCACGA	X	
AvrBs2R (M13-tagged)	tgtaaaacgacggccagt CGCTCGAGCGGTGAT CGGTCAACAGGCTTTC	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.6.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
AvrBs2F (M13-tagged)	10 µM	0.75	0.3 µM
AvrBs2R (M13-tagged)	10 µM	0.75	0.3 µM
Subtotal		23.0	
Genomic DNA extract	10 ng μL^{-1}	2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.6.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (10 s at 98°C, 10 s at 60°C, 30 s at 72°C), 5 min at 72°C.

2.6.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.6.6 The *avrBs2* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the bacterial *avrBs2* gene.

2.6.7 The M13-tailed primer pair AvrBs2F/AvrBs2R results in an *avrBs2* sequence with a codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.7 Conventional PCR *mutS Xylella* spp.

2.7.1 PCR amplification of 851 bp (amplicon size including primers) of the DNA mismatch repair protein (*mutS*) gene for *Xylella* spp. identification (adapted from M. Maes, unpublished[3]).

[3]Developed in the framework of the QBOL project (http://www.qbol.org) in parallel to the test described under Section 2.4.

2.7.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
XFmutS-F (M13-tagged)	caggaaacagctatgacc* TTATAGCAGCGC TTTGAGTCGGT	X	
XFmutS-R (M13-tagged)	tgtaaaacgacggccagt GTGAACAGCGAT TCGAGCCG	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower-case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.7.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
XFmutS-F (M13-tagged)	10 µM	0.75	0.3 µM
XFmutS-R (M13-tagged)	10 µM	0.75	0.3 µM
Subtotal		23.0	
Genomic DNA extract	10 ng μL^{-1}	2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.7.4 Thermocycler profile: 1 min 30 s at 98°C, 30× (10 s at 98°C, 10 s at 60°C, 30 s at 72°C), 5 min at 72°C.

2.7.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.7.6 The *mutS* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the bacterial *mutS* gene.

2.7.7 The M13-tailed primer pair XFmutS-F/XFmutS-R results in a *mutS* sequence with a codon starting in reading frame 1 of the complementary strand of the primer-trimmed consensus sequence.

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 DNA extraction: include an empty tube in the DNA extraction procedure as if it were a real sample.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: include a tube with no added template, instead add 2 µL 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 gBlock EPPO_PAC_Bacteria_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 2).

3.2 Interpretation of results

Verification of the controls:
- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size

When these conditions are met:
- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding project in an international consortium of 11 participants. Additional data was generated by the Dutch NPPO laboratory.

4.1 Analytical sensitivity

Pellets of pure cultures are used for the DNA extraction. For all protocols a DNA concentration of 1.1 ng μL^{-1} is sufficient to generate an amplicon that can be sequenced, leading to a consensus sequence with a HQ (Phred score > 40) of at least 84%.

4.2 Analytical specificity

The combination of loci indicated in Table 2 possess sufficient interspecies variation to allow for identification to species level and, when relevant, also to the subspecies or pathovar level. Apart from the species listed in Table 1, species from several genera have successfully been amplified and sequenced using the protocols in this appendix by the Dutch NPPO (see the EPPO validation sheet for this Appendix, http://dc.eppo.int/tps.php):

Test 2.2.2 *16S* rDNA: *Acidovorax* (4), *Clavibacter* (1), *Curtobacterium* (1), *Dickeya* (7), *Pantoea* (1), *Pseudomonas* (2), *Ralstonia* (1), *Rhodococcus* (1) and *Xanthomonas* (4).

Test 2.2.3 *gyrB Clavibacter*: *Clavibacter* (1).

Test 2.2.5 *gyrB Xanthomonas*: *Xanthomonas* (10).

Test 2.2.6 *avrBs2 Xanthomonas*: *Xanthomonas* (7).

It has to be recognized that the potential of amplification and sequencing with the generic primers in this Appendix is much greater.

4.3 Selectivity

Selectivity does not apply as pure cultures are used.

4.4 Diagnostic sensitivity

TPS partners in the EUPHRESCO II DNA Barcoding Project analysed five DNA samples of the following species: *Clavibacter michiganensis* subsp. *michiganensis*, *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *begoniae* (not regulated), *Xanthomonas axonopodis* pv. *dieffenbachia* and *Xylella fastidiosa.* The overall diagnostic sensitivity obtained was 67% (*C. michiganensis* subsp. *michiganensis* 55%, *R. solanacearum* 91%, *X. a.* pv. *begoniae* 45%, *X. a* pv. *dieffenbachia* 45% and *X. fastidiosa* 100%). Identification at higher taxonomic levels was conservative due to a lack of confidence of the operators in making the identification at subspecies or pathovar level (i.e. *Ralstonia* sp. instead *R. solanacearum* ($n = 1$), *C. michiganensis* instead of *C. michiganensis* subsp. *michiganensis* ($n = 5$) and *X. axonopodis* instead of *X. a* pv. b*egoniae* ($n = 2$) or *X. a.* pv *dieffenbachiae* ($n = 3$)), and incorrect identifications led to relative low diagnostic sensitivity values for some samples. Re-analysis of the data provided by partners shows that identification at the required taxonomic level as listed in Table 2 is possible and an overall diagnostic sensitivity of 96% could be obtained

4.5 Reproducibility data

The same DNA samples are analysed by different partners. Therefore in this situation the reproducibility is identical to diagnostic sensitivity.

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Appendix 3 – DNA barcoding of fungi and oomycetes

1. General information

1.1 This Appendix describes the protocols for the identification of selected regulated fungi and oomycetes using conventional PCR followed by Sanger sequencing analysis. Table 3 shows the regulated organisms that have successfully been tested with the protocols described in this section. It is very likely that other regulated fungi and oomycetes can successfully be identified using these protocols, but

validation data has not been generated to support this.

1.2 Protocols were developed by the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (KNAW-CBS), Plant Research International, Business Unit Biointeractions and Plant Health, Wageningen, the Netherlands (PRI) and the Food and Environment Research Agency, York, United Kingdom (Fera), as part of the QBOL Project financed by the 7th Framework Programme of the European Union (2009–12). As part of the EUPHRESCO II DNA Barcoding Project (2013–14), the protocols were further optimized by the Dutch NPPO.

1.3 A combination of two out of six tests is used to identify selected regulated fungi and oomycete: *ITS*, *EF-1a*, *TUB2*, *CALM*, *ACT* and the mitochondrial *COI* gene (see Fig. 3). Table 3 gives an overview of the loci needed for the selected regulated fungi ond oomycetes.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the Bio-X-Act Short Mix (Bioline) reagents (cat. no. BIO-25026).

1.6 Molecular-grade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease free.

1.7 Amplification is performed in a Peltier-type thermocycler with a heated lid, e.g. C1000 (Bio-Rad).

Validation data presented in Section 4 have been obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction

2.1.1 Mycelium of pure cultures is removed from the agar surface (approximately 2 cm^2) using a sterile scalpel or micro-pestle and used as the starting material for the DNA extraction.

2.1.2 DNA is extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

2.1.3 Particular care should be given to ensure the sample is adequately homogenized. Micro-pestles can be used to grind fungal tissue but specialist equipment can be used when high-throughput is required (e.g. Retsch Mixer Mill MM301).

Table 3. Regulated fungi ond oomycetes successfully identified with barcoding protocols

	Tests						
Regulated organism	2.2 *ITS*	2.3 *EF-1a*	2.4 *TUB2*	2.5 *CALM*	2.6 *ACT*	2.7 *COI*	Remarks
Ceratocystis fagacearum	x*						
Ceratocystis fimbriata f. sp. *platani*	x	x					Listed as *Ceratocystis platani*
Ceratocystis virescens	x						
Lecanosticta acicola	x		x				Listed as *Scirrhia acicola*
Phytophthora ramorum	x					x	
Stagonosporopsis chrysanthemi	x				x		Listed as *Didymella ligulicola*
Verticillium alboatrum	x			x			Listed as *Verticillium albo-atrum*
Verticillium dahliae	x			x			

*Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.

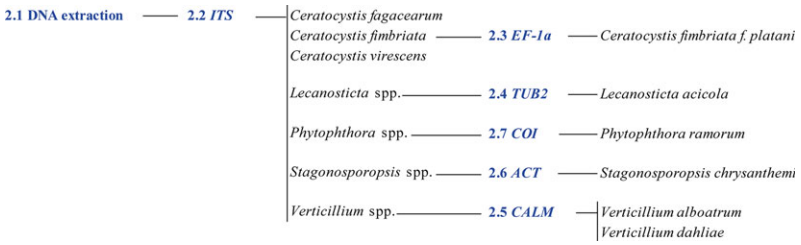


Fig. 3 Diagnostic testing scheme for identification of regulated fungi and oomycetes using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used.

2.1.4 DNA is eluted twice in 50 µL of elution buffer (provided in the extraction kit).

2.1.5 DNA extracts should be used immediately or stored at −20°C until use.

2.2 Conventional PCR *ITS* fungi and oomycetes

2.2.1 PCR-Sequencing of approximately 550–1700 bp (amplicon size including primers) of the nuclear ribosomal internal transcribed spacer (*ITS*) locus is adapted from White *et al.* (1990).

2.2.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
ITS5	GGAAGTAAAAGTCGTAACAAGG	X	X
ITS4	TCCTCCGCTTATTGATATGC	X	X

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
ITS5	10 µM	0.5	0.2 µM
ITS4	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.2.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 52°C, 1 min 40 s at 72°C), 10 min at 72°C.

2.2.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.2.6 *ITS* is a non-coding locus, containing a small conserved region that is transcribed in *5.8S* ribosomal RNA. Translation tables do not apply to *ITS*.

2.3 Conventional PCR *EF-1a* fungi

2.3.1 PCR sequencing of approximately 680 bp (amplicon size including primers) of the translation elongation factor 1 alpha (*EF-1a*) gene is adapted from Jones *et al.* (2011) and Oliveira *et al.* (2015).

2.3.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
EFCF1	AGTGCGGTGGTATCGACAAG	X	X
EFCF2	TGCTCACGGGTCTGGCCAT	X	X

2.3.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
EFCF1	10 µM	0.5	0.2 µM
EFCF2	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 52°C, 30 s at 72°C), 10 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.3.6 The nuclear *EF-1a* is a protein coding region. Translation Table 1 (Standard Code) applies to the nuclear *EF-1a* gene.

2.3.7 Primer pair EFCF1/EFCF2 results in an *EF-1a* sequence containing two introns, one of them starting in the primer-trimmed consensus sequence.

2.4 Conventional PCR *TUB2* fungi

2.4.1 PCR sequencing of approximately 450 bp (amplicon size including primers) of the nuclear beta-tubulin (*TUB2*) gene is adapted from Groenewald *et al.* (2013).

2.4.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
TUB2Fd	GTBCACCTYCARACC GGYCARTG	X	X
TUB4Rd	CCRGAYTGRCCRAAR ACRAAGTTGTC	X	X

2.4.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
TUB2Fd	10 µM	0.5	0.2 µM
TUB4Rd	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.4.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 52°C, 30 s at 72°C), 10 min at 72°C.

2.4.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.4.6 The nuclear *TUB2* is a protein-coding region. Translation Table 1 (Standard Code) applies to the nuclear *TUB2* gene.

2.4.7 Primer pair TUB2Fd/TUB4Rd results in a *TUB2* sequence containing three introns, one of them starting in the primer-trimmed consensus sequence.

2.5 Conventional PCR *CALM* fungi

2.5.1 PCR sequencing of approximately 520 bp (amplicon size including primers) of the nuclear calmodulin (*CALM*) gene is adapted from Carbone & Kohn (1999).

2.5.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	X	X
CAL-737R	CATCTTTCTGGCCATCATGG	X	X

2.5.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
CAL-228F	10 µM	0.5	0.2 µM
CAL-737R	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.5.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 50°C, 30 s at 72°C), 10 min at 72°C.

2.5.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.5.6 The nuclear *CALM* is a protein-coding region. Translation Table 1 (Standard Code) applies to the nuclear *CALM* gene.

2.5.7 Primer pair CAL-228F/CAL-737R results in a *CALM* sequence starting with an intron of the primer-trimmed consensus sequence.

2.6 Conventional PCR *ACT* fungi

2.6.1 PCR sequencing of approximately 290 bp (amplicon size including primers) of the nuclear actin (*ACT*) gene is adapted from Carbone & Kohn (1999).

2.6.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
ACT-512F	ATGTGCAAGGCC GGTTTCGC	X	X
ACT-783R	TACGAGTCCTTC TGGCCCAT	X	X

2.6.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
ACT-512F	10 µM	0.5	0.2 µM

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
ACT-783R	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.6.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 52°C, 30 s at 72°C), 10 min at 72°C

2.6.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.6.6 The nuclear *ACT* is a protein-coding region. Translation Table 1 (Standard Code) applies to the nuclear *ACT* gene.

2.6.7 Primer pair ACT-512F/ACT-783R results in an *ACT* sequence with a codon starting in reading frame 3 of the primer-trimmed consensus sequence and containing two introns.

2.7 Conventional PCR *COI* fungi

2.7.1 PCR sequencing of 727 bp (amplicon size including primers) of the mitochondrial cytochrome c oxidase I (*COI*) gene is adapted from Robideau *et al.* (2011).

2.7.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
OomCoxI-Levup	TCAWCWMGATGG CTTTTTTCAAC	X	X
OomCoxI-Levlo	CYTCHGGRTGWCC RAAAAACCAAA	X	X

2.7.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
OomCoxI-Levup	10 µM	0.5	0.2 µM
OomCoxI-Levlo	10 µM	0.5	0.2 µM

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity

2.7.4 Thermocycler profile: 5 min at 95°C, 40× (30 s at 94°C, 30 s at 52°C, 45 s at 72°C), 10 min at 72°C.

2.7.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.7.6 The mitochondrial *COI* is a protein coding region. Translation Table 5 (Invertebrate Mitochondrial Code) applies to the mitochondrial *COI* gene.

2.7.7 The primer pair OomCoxI-Levup/OomCoxI-Levlo results in a *COI* sequence with codon starting in reading frame 2 of the primer-trimmed consensus sequence.

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: DNA extraction of an Eppendorf tube containing 25 µL of molecular-grade water.

-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 gBlock EPPO_PAC_Fungi_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 3).

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size

When these conditions are met:

- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding Project in an international consortium of nine participants. Additional data was generated by the Dutch NPPO laboratory.

4.1 Analytical sensitivity

Pellets of pure cultures are used for the DNA extraction. For all protocols a DNA concentration of 0.05 ng μL^{-1} is sufficient to generate an amplicon that can be sequenced, leading to a consensus sequence with a HQ (Phred score > 40) of at least 83%.

4.2 Analytical specificity

The locus or combination of loci indicated in Table 3 possess sufficient interspecies variation to allow for identification to species level. Apart from the species listed in Table 1, species from several genera have successfully been amplified and sequenced by the Dutch NPPO laboratory using the protocols in this Appendix (see EPPO validation sheet for this appendix):

Test 3.2.2 *ITS*: *Atropellis* (1), *Boeremia* (1), *Ceratocystis* (2), *Chalara* (1), *Ciborinia* (1), *Colletotrichum* (1), *Diaporthe* (4), *Diplocarpon* (1), *Elsinoe* (3), *Epicoccum* (1), *Fusarium* (1), *Geosmithia* (1), *Gremmeniella* (1), *Heterobasidion* (1), *Melampsora* (2), *Ophiognomonia* (1), *Penicillium* (1), *Peyronellaea* (1), *Phialophora* (1), *Phoma* (2), *Phomopsis* (1), *Phytophthora* (8), *Phytopythium* (1), *Pseudocercospora* (1), *Pyrenochaeta* (1), *Stagonosporopsis* (1) and *Venturia* (1).

Test 3.2.4 *TUB2*: *Ciborinia* (1), *Colletotrichum* (1), *Fusarium* (1) and *Penicillium* (1).

Test 3.2.5 *CALM*: *Penicillium* (1).Test 3.2.6 *ACT*: *Colletotrichum* (1), *Entoleuca* (1), *Epicoccum* (1), *Phoma* (2) and *Stagonosporopsis* (1).

It has to be recognized that the potential of amplification and sequencing with the generic primers in this Appendix is much greater.

4.3 Selectivity

Selectivity does not apply as pure cultures are used.

4.4 Diagnostic sensitivity

TPS partners in the EUPHRESCO II DNA Barcoding Project analysed five DNA samples of the following species: *Ceratocystis fimbritia* f. sp. *platani*, *Lecanosticta acicola*, *Phytophthora ramorum*, *Stagonosporopsis chrysanthemi* and *Verticillium dahliae*. The overall diagnostic sensitivity obtained was 96% (*C. fimbritia* f. sp. *platani* 89%, *L. acicola* 100%, *P. ramorum* 100%, *S. chrysanthemi* 89% and *V. dahliae* 100%). One of the partners was not able to correctly identify the sample *S. chrysanthemi* as no amplicon was obtained for the *ACT* locus which is necessary for reliable species identification. Re-analysis of the data provided by partners show that identification at the required taxonomic level as listed in Table 3 is possible and an overall diagnostic sensitivity of 98% could be obtained.

4.5 Reproducibility

The same DNA samples are analysed by different partners. Therefore in this situation the reproducibility is identical to diagnostic sensitivity.

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Appendix 4 – DNA barcoding of invasive plant species

1. General information

1.1 This Appendix describes protocols for the identification of selected invasive plant species using conventional PCR followed by Sanger sequencing analysis. Table 4 shows the selected invasive plant species that have successfully been tested with the protocols described in this section. It is very likely that other invasive plant species can successfully be identified using these protocols, but validation data has not been generated to support this.

1.2 Protocols were developed by the Dutch NPPO.

1.3 Two tests in parallel are used to identify selected invasive plant species: targeting the chloroplast *trnH-psbA* intergenic spacer and the *rbcL* gene. *rbcL*, one of the standardized DNA barcodes for plants, does not give sufficient resolution for species demarcation for the selected invasive plant species, therefore *trnH-psbA* is added as an additional barcode region (see Fig. 4). Table 4 gives an overview of the selected invasive plant species.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the Bio-X-Act Short Mix (Bioline) reagents (cat.no. BIO-25026).

1.6 Molecular -rade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

1.7 Amplification is performed in a Peltier-type thermocycler with heated lid, e.g. C1000 (Bio-Rad).

Validation data presented in Section 4 have been obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction

2.1.1 About 1 g fresh or frozen (green) plant tissue is ground in 5 mL GH + grinding buffer (6 M guanidine hydrochloride, 0.2 M sodium acetate pH 5.2, 25 mM EDTA, 2.5% PVP-10), in a plastic grinding bag using Homex 6 (Bioreba AG) and used as starting material for the DNA extraction.

2.1.2 DNA is extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

2.1.3 DNA is eluted twice in 50 µL of elution buffer (provided in the isolation kit).

2.1.4 DNA extracts should be used immediately or stored at −20°C until use.

2.2 Conventional PCR *rbcL* invasive plants

2.2.1 PCR sequencing of 599 bp (amplicon size including primers) of the chloroplast large subunit ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcL*) gene is adapted from Kress & Erickson (2007) and Kress *et al.* (2009).

2.2.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
rbcL-a_f	ATGTCACCACAAAC AGAGACTAAAGC	X	X
rbcLa SI_Rev	GTAAAATCAAGTCC ACCRCG	X	X

Table 4. Regulated invasive plant species successfully identified with barcoding protocols

Regulated organism	Tests		Remarks
	2.2 *rbcL*[*]	2.3 *trnH-psbA*	
Ludwigia peploides	x	x	
Ludwigia grandiflora	x	x	
Hydrocotyle ranunculoides	x	x	
Myriophyllum aquaticum	x	x	
Myriophyllum heterophyllum	x	x	

[*]Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)[*]	2×	12.5	1×
rbcL-a_f	10 µM	0.5	0.2 µM
rbcLa SI_Rev	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.2.4 Thermocycler profile: 5 min at 95°C, 5× (30 s at 94°C, 30 s at 45°C, 30 s at 72°C), 35× (30 s at 94°C, 30 s at 50°C, 30 s at 72°C), 10 min at 72°C.

2.2.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.2.6 The chloroplast *rbcL* is a protein-coding region approximately 1430 bp in length. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the chloroplast *rbcL* gene.

2.2.7 Primer pair rbcL-a_f/rbcLa SI_Rev results in a sequence with codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.3 Conventional PCR *trnH-psbA* invasive plants

2.3.1 PCR sequencing of 300–900 bp (amplicon size including primers) of the chloroplast intergenic spacer between the histidine transfer tRNA (*trnH*) and the D1 protein of photosystem II (*psbA*) is adapted from Sang *et al.* (1997) and Tate (2002).

2.3.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
trnH2	CGCGCATGGTGGATTCACAATCC	X	X
psbAF	GTTATGCATGAACGTAATGCTC	X	X

2.3.3 Master mixes are prepared according to the table below.

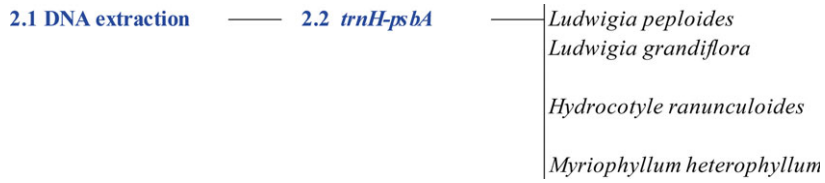


Fig. 4 Diagnostic testing scheme for identification of regulated invasive plant species using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
trnH2	10 µM	0.5	0.2 µM
psbAF	10 µM	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 Thermocycler profile: 5 min at 95°C, 5× (30 s at 94°C, 30 s at 45°C, 50 s at 72°C), 35× (30 s at 94°C, 30 s at 50°C, 50 s at 72°C), 10 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.3.6 The chloroplast *trnH-psbA* intergenic spacer is a non-coding region. Translation tables do not apply to *trnH-psbA*.

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: DNA extraction of an Eppendorf tube containing 25 µL of molecular-grade water.

-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 gBlock EPPO_PAC_Invasive_Plants_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 4).

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size

When these conditions are met:

- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding Project in an international consortium of eight participants. Additional data were generated by the Dutch NPPO laboratory.

4.1 Analytical sensitivity

Pellets of pure cultures are used for the DNA extraction. For all protocols a DNA concentration of 5 ng μL^{-1} is sufficient to generate an amplicon that can be sequenced, leading to a consensus sequence with a HQ (Phred score > 40) of at least 98%.

4.2 Analytical specificity

The combination of loci indicated in Table 4 possesses sufficient interspecies variation to allow for identification to species level. Apart from the species listed in Table 5, species from several genera have successfully been amplified and sequenced by the Dutch NPPO using the protocols in this Appendix (see the EPPO Validation Sheet for this Appendix, http://dc.eppo.int/tps.php):

Test 4.2.2 *rbcL*: *Carex* (1), *Centella* (1), *Cyperus* (3), *Hydrocotyle* (6), *Impatiens* (3), *Kyllinga* (1), *Lagarrosiphon* (1), *Ludwigia* (2), *Myriophyllum* (16), *Oxalis* (1), *Rotala* (1) and *Wolffia* (4).

Test 4.2.3 *trnH-psbA*: *Carex* (1), *Centella* (2), *Cyperus* (3), *Hydrocotyle* (6), *Impatiens* (3), *Kyllinga* (1), *Lagarrosiphon* (1), *Ludwigia* (2), *Myriophyllum* (17), *Oxalis* (1), *Rotala* (1) and *Wolffia* (4).

It has to be recognized that potential of amplification and sequencing with the generic primers in this Appendix is much greater.

4.3 Selectivity

Selectivity does not apply as individual specimens are used.

4.4 Diagnostic sensitivity

TPS partners in the EUPHRESCO II DNA Barcoding Project analysed five DNA samples from the following species: *Ludwigia peploides*, *Ludwigia grandiflora*, *Hydrocotyle ranunculoides*, *Hydrocoyile vulgaris* and *Myriophyllum hetrophyllum*. The overall diagnostic sensitivity obtained was 68% (*L. peploides* 50%, *L. grandiflora* 63%, *H. ranunculoide* 75%, *H. vulgaris* 63% and *M. hetrophyllum* 88%). Conservative identification at a higher taxonomic level (genus instead of species level) led to relative low diagnostic sensitivity values for some samples. Re-analysis of the data provided by partners shows that identification at the required taxonomic level as listed in Table 4 is possible and an overall diagnostic sensitivity of 100% could be obtained.

4.5 Reproducibility

The same DNA samples are analysed by different partners. Therefore in this situation the reproducibility is identical to diagnostic sensitivity.

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of the operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Table 5. Regulated nematodes successfully identified with barcoding protocols

	Tests			
Regulated organism	2.2 *18S* rDNA	2.3 *28S* rDNA	2.4 *COI*	Remarks
Aphelenchoides besseyi	x*	x	x	
Bursaphelenchus xylophilus	x	x		
Ditylenchus destructor	x	x		
Ditylenchus dipsaci	x	x		
Globodera pallida	x	x	x	
Globodera rostochiensis	x	x	x	
Meloidogyne chitwoodi	x		x	
Meloidogyne fallax	x		x	
Nacobbus aberrans	x			
Radopholus similis	x			

*Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.

Appendix 5 – DNA barcoding of nematodes

1. General information

1.1 This Appendix describes protocols for the identification of selected regulated nematodes using conventional PCR followed by Sanger sequencing analysis. Table 5 shows the selected regulated organisms that have successfully been tested with the protocols described in this Appendix. Other (regulated) nematode species can successfully be identified using these protocols, but validation data has not been generated to support this.

1.2 The protocols were developed by Agroscope, Switzerland, and the Laboratory of Nematology, Wageningen University, the Netherlands, as part of the QBOL Project financed by the 7th Framework Programme of the European Union (2009–12). As part of the EUPHRESCO II DNA Barcoding Project (2013–14), the protocols were further optimized by the Dutch NPPO.

1.3 A combination of three tests is used to identify selected regulated nematodes: the *18S* rDNA (small subunit, SSU), the *28S* rDNA (large subunit, LSU) and the mitochondrial *COI* gene (see Fig. 5). Table 5 gives an overview of the loci needed for the selected regulated nematodes.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the Phusion® High-Fidelity (New England Biolabs) reagents (cat. no. M0530).

1.6 Molecular-grade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease free.

1.7 Amplification is performed in a Peltier-type thermocycler with a heated lid, e.g. C1000 (Bio-Rad).

Validation data presented in Section 4 have been obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction

2.1.1 Single nematodes or cysts in 25 µL of molecular-grade water are used as input for DNA extraction.

2.1.2 DNA is extracted using the 'Single Worm Lysis' kit (ClearDetections) following the manufacturer's instructions.

2.1.3 Lysates should be used immediately or stored at −20°C until use.

2.2 Conventional PCR *18S* rDNA (SSU) – nematodes

2.2.1 PCR sequencing of approximately 1730 bp of the small subunit 18S ribosomal DNA (*18S* rDNA

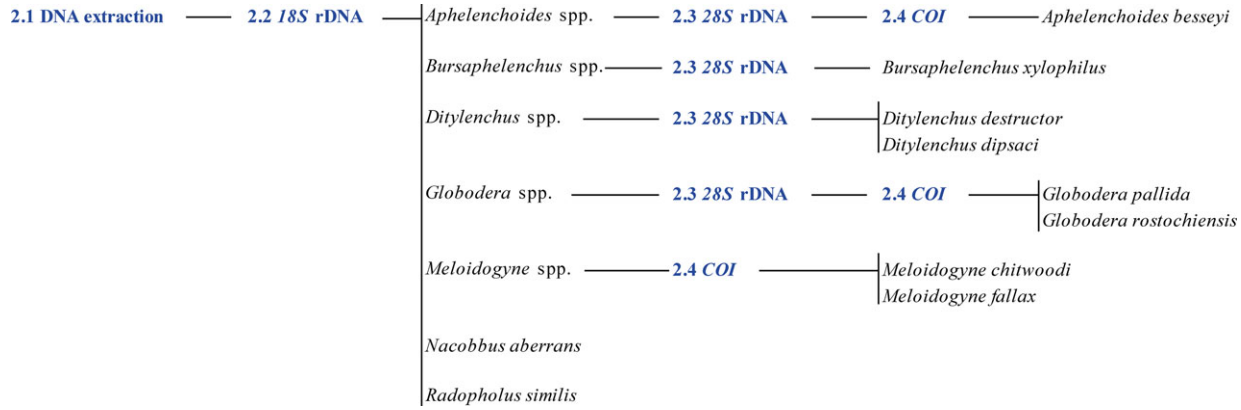


Fig. 5 Diagnostic testing scheme for identification of selected regulated nematodes using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used.

(SSU)) is adapted from Holterman *et al.* (2006) using two separate reactions: 988F/1912R (amplicon size including primers approximately 980 bp) and 1813F/2646R (amplicon size including primers approximately 880 bp).

2.2.2 Primer sequences and their application are described in the table below.

Reaction	Primer name	Primer sequence (5′–3′ orientation)	Primer used for PCR	Primer used for Sequencing
1	988F	CTCAAAGATTAAGCCATGC	X	X
	1912R	TTTACGGTCAGAACTAGGG	X	X
2	1813F	CTGCGTGAGAGGTGAAAT	X	X
	2646R	GCTACCTTGTTACGACTTTT	X	X

2.2.3 Master mixes are prepared according to the table below.

2.2.4 Thermocycler profile: 1 min at 98°C, 5× (10 s at 98°C, 20 s at 45°C, 60 s at 72°C), 35× (10 s at 98°C, 20 s at 54°C, 60 s at 72°C), 10 min at 72°C.

2.2.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.2.6 *18S* rDNA (SSU) is a non-coding but conserved locus that is transcribed in *18S* ribosomal RNA. Translation tables do not apply to *18S* rDNA (SSU).

2.3 Conventional PCR *28S* rDNA (LSU) – nematodes

2.3.1 PCR sequencing of approximately 1000 bp (amplicon size including primers) of the large subunit *28S* ribosomal DNA (*28S* rDNA (LSU)) is adapted from Holterman *et al.* (2008).

Reagent	Working concentration	Volume per reaction (µL) Reaction 1	Volume per reaction (µL) Reaction 2	Final concentration
Molecular-grade water	N.A.	16.05	16.05	N.A.
Phusion HF Buffer (NEB)*	5×	5.0	5.0	1×
dNTPs (NEB)	10 mM	0.5	0.5	200 µM
988F	10 µM	0.6	–	0.24 µM
1912R	10 µM	0.6	–	0.24 µM
1813F	10 µM	–	0.6	0.24 µM
2646R	10 µM	–	0.6	0.24 µM
Phusion DNA polymerase (NEB)	2 Units μL^{-1}	0.25	0.25	0.5 Unit
Subtotal		23.0	23.0	
Genomic DNA extract		2.0	2.0	
Total		25.0	25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
28–81for	TTAAGCATATCATTT AGC GGAGGAA	X	X
28–1006rev	GTTCGATTAGTCTTT CGCCCCT	X	X

2.3.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	16.05	N.A.
Phusion HF Buffer (NEB)*	5×	5.0	1×
dNTPs (NEB)	10 mM	0.5	200 µM
28–81for	10 µM	0.6	0.24 µM
28–1006rev	10 µM	0.6	0.24 µM
Phusion DNA polymerase (NEB)	2 Units μL^{-1}	0.25	0.5 Unit
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 Thermocycler profile: 1 min at 98°C, 5× (10 s at 98°C, 20 s at 45°C, 30 s at 72°C), 35× (10 s at 98°C, 20 s at 54°C, 30 s at 72°C), 10 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.3.6 *28S* rDNA (LSU) is a non-coding but conserved locus that is transcribed in *28S* ribosomal RNA. Translation tables do not apply to *28S* rDNA (LSU).

2.4 Conventional PCR *COI* – nematodes

2.4.1 PCR sequencing of 447 bp (amplicon size including primers) of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene is adapted from Hu *et al.* (2002).

2.4.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
JB3	TTTTTTGGGCATCCT GAGGTTTAT	X	X
JB5	AGCACCTAAACTTAAA ACATAATGAAAATG	X	X

2.4.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	16.05	N.A.
Phusion HF Buffer (NEB)*	5×	5.0	1×
dNTPs (NEB)	10 mM	0.5	200 µM
JB3	10 µM	0.6	0.24 µM
JB5	10 µM	0.6	0.24 µM
Phusion DNA polymerase (NEB)	2 Units μL^{-1}	0.25	0.5 Unit
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.4.4 Thermocycler profile: 1 min at 98°C, 40× (10 s at 98°C, 20 s at 41°C, 30 s at 72°C), 10 min at 72°C.

2.4.5 Cycle sequencing reactions are performed using the obtained PCR products with primers used for amplification in separate reactions.

2.4.6 Mitochondrial *COI* is a protein-coding region. Translation Table 5 (Invertebrate Mitochondrial Code) applies to the mitochondrial *COI* gene.

2.4.7 Primer pair JB3/JB5 results in a *COI* sequence with codon starting in reading frame 1 of the primer-trimmed consensus sequence.

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: DNA extraction of an

Eppendorf tube containing 25 µL of molecular-grade water.
-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 gBlock EPPO_PAC_Nematodes_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 5).

3.2 Interpretation of results

Verification of the controls:
- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size

When these conditions are met:
- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding Project in an international consortium of nine participants. Additional data was generated by the Dutch NPPO laboratory.

4.1 Analytical sensitivity

For all protocols DNA purified from a single nematode is sufficient to generate an amplicon that can be sequenced leading to a consensus sequence with a HQ (Phred score > 40) of at least 86%.

4.2 Analytical specificity

The locus or combination of loci indicated in Table 5 possess sufficient interspecies variation to allow for species-level identification. Apart from the species listed in Table 5, species from several genera have successfully been amplified and sequenced by the Dutch NPPO using the protocols in this Appendix (see EPPO Validation Sheet for this Appendix, http://dc.eppo.int/tps.php):

Test 5.2.2 *18S* rDNA: *Aphelenchoides* (5), *Bursaphelenchus* (3), *Cactodera* (1), *Ditylenchus* (2), *Globodera* (3), *Heterodera* (4), *Heterorhabditis* (1), *Longidorus* (1), *Meloidogyne* (7), *Nacobbus* (1), *Paratrichodorus* (3), *Pratylenchus* (6), *Radophilus* (1), *Steinernema* (2), *Subanguina* (1), *Trichodorus* (3) and *Xiphinema* (1).

Test 5.2.3 *28S* rDNA: *Aphelenchoides* (5), *Bursaphelenchus* (2), *Cactodera* (1), *Ditylenchus* (2), *Globodera* (2), *Heterodera* (4), *Heterorhabditis* (1), *Longidorus* (1), *Meloidogyne* (6), *Nacobbus* (1), *Paratrichodorus* (3), *Pratylenchus* (3), *Radophilus* (1), *Steinernema* (2), *Subanguina* (1), *Trichodorus* (1) and *Xiphinema* (1).

Test 5.2.4 *COI*: *Aphelenchoides* (5), *Bursaphelenchus* (3), *Cactodera* (1), *Globodera* (3), *Heterodera* (4), *Heterorhabditis* (1), *Laimaphelenchus* (1), *Longidorus* (1), *Meloidogyne* (8), *Nacobbus* (1), *Pratylenchus* (6), *Radophilus* (1), *Steinernema* (2) and *Xiphinema* (1).

It has to be recognized that the potential for amplification and sequencing with the generic primers in this Appendix is much greater.

4.3 Selectivity

Selectivity does not apply as individual specimens are used.

4.4 Diagnostic sensitivity

TPS partners In the EUPHRESCO II DNA Barcoding Project analysed five DNA samples of the following species: *Aphelenchoides besseyi*, *Aphelenchoides fragariae*, *Bursaphelenchus xylophilus*, *Ditylenchus dipsaci* and *Meloidogyne chitwoodi.* The overall diagnostic sensitivity obtained was 96% (*A. besseyi* 89%, *A. fragariae* 89%, *B. xylophilus* 100%, *D. dipsaci* 100% and *M. chitwoodi* 100%). One partner incorrectly analysed the sequence data for both *Aphelenchoides* species. Re-analysis of the data provided by partners shows that identification at the required taxonomic level as listed in Table 5 is possible and an overall diagnostic sensitivity of 100% could be obtained.

4.5 Reproducibility

The same DNA samples are analysed by different partners. Therefore, in this situation, the reproducibility is identical to diagnostic sensitivity.

One of the TPS participants reported that they also obtained non-specific amplicons during PCR. In such cases the PCR product of expected size should be excised from agarose gel (see also Appendix 7, Section 2.5).

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of the operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Appendix 6 – DNA barcoding of phytoplasmas

1. General information

1.1 This Appendix describes protocols for the identification of selected regulated phytoplasmas using conventional PCR followed by Sanger sequencing

Table 6. Regulated phytoplasmas successfully identified with barcoding protocols

Regulated organism	Tests*		Remarks
	2.2 *tuf*	2.3 *16S* rDNA	
Candidatus Phytoplasma mali	x	x	Listed as Apple proliferation mycoplasma
Candidatus Phytoplasma pruni	x	x	Listed as Peach rosette mycoplasma, Peach X-disease mycoplasma and Peach yellows mycoplasma
Candidatus Phytoplasma prunorum	x	x	Listed as Apricot chlorotic leafroll mycoplasma
Candidatus Phytoplasma pyri	x	x	Listed as Pear decline mycoplasma
Candidatus Phytoplasma solani	x	x	Listed as Potato stolbur mycoplasma
Grapevine flavescence dorée MLO	x	x	Listed as Grapevine flavescence dorée MLO

*Tests marked with 'x' need to be performed to reach reliable identification of the corresponding taxa. When multiple loci are indicated in the table, the MLSA tools in Q-bank should be used.

analysis. Table 6 shows the selected regulated organisms that have successfully been tested with the protocols described in this Appendix. It is very likely that other phytoplasmas can successfully be identified using these protocols, but validation data has not been generated to support this.

1.2 These protocols were developed by Institute of Integrated Pest Management, Aarhus University, Denmark and the University of Bologna, as part of the QBOL Project financed by the 7th Framework Programme of the European Union. As part of the EUPHRESCO II DNA Barcoding Project (2013–14), the protocols were further optimized by the Food and Environment Research Agency (Fera), United Kingdom.

1.3 Two tests in parallel are used to identify selected regulated phytoplasmas; elongation factor EF-Tu (*Tuf*) and *16S* rDNA (see Fig. 6). Table 6 gives an overview of the loci needed for the selected regulated phytoplasmas.

1.4 Primer sequences, amplicon sizes and thermocycler settings are provided in the test-specific sections. HPLC-purified primers should be ordered to avoid non-specific PCR amplification.

1.5 Reaction mixes are based on the Bio-X-Act Short Mix (Bioline) reagents (cat. no. BIO-25026).

1.6 Molecular-grade water is used to set up reaction mixes; this should be purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease free.

1.7 Amplification is performed in a Peltier-type thermocycler with a heated lid, e.g. C1000 (Bio-Rad).

Validation data presented in Section 4 have been obtained using the chemicals, equipment and methodology described in this Appendix and in combination with the guidance provided in Appendix 7.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Place 1 g of fresh or frozen plant tissue in a pre-cooled, sterile and dry mortar and add liquid nitrogen.

2.1.2 Homogenize the plant tissue using a sterile porcelain pestle.

2.1.3 Add 100 mg of the homogenized tissue to a pre-cooled microcentrifuge tube.

2.1.4 Alternatively, 100 µL of plant sap can be used for DNA extraction.

2.1.5 Proceed with DNA extraction using the DNeasy Plant Mini Kit (cat. no. 69104) according to the manufacturer's instructions (Qiagen).

2.1.6 No DNA clean-up is required after DNA extraction.

2.1.7 The extracted DNA should either be used immediately or stored at −20°C or below until use.

2.2 Conventional PCR *EF-Tu* – phytoplasmas

2.2.1 PCR sequencing of 480 bp (amplicon size nested-PCR including primers) of the Elongation factor Tu (*EF-Tu*) gene is adapted from Makarova *et al.* (2012).

2.2.2 Primer sequences are described in the table below. The Tuf340 PCR primer cocktail is prepared by pooling an equal volume of 10 µM of primers Tuf340a and Tuf 340b. The Tuf890 PCR primer cocktail is prepared by pooling an equal volume of 10 µM of primers Tuf890ra, Tuf890rb and Tuf 890rc. The Tuf400 PCR primer cocktail is prepared by pooling an equal volume of 10 µM of primers Tuf400a, Tuf400b, Tuf400c, Tuf400d and Tuf 400e. The Tuf835 primer cocktail is prepared by pooling an equal volume of 10 µM of primers Tuf835ra, Tuf835rb and Tuf 835rc.

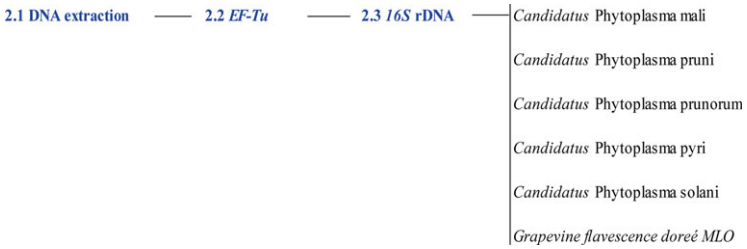


Fig. 6 Diagnostic testing scheme for identification of selected regulated phytoplasmas using DNA barcodes. The steps shown refer to the sections in this Appendix which should be followed to reach reliable identification of the corresponding taxa. When sequence data of multiple loci are generated, the MLSA tools in Q-bank need to be used.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for		
		PCR	nested-PCR	Sequencing
Tuf340a	GCTCCTGAAGAAA RAGAACGTGG	X		
Tuf340b	ACTAAAGAAGAAA AAGAACGTGG	X		
Tuf890ra	ACTTGDCCTCTTTC KACTCTACCAGT	X		
Tuf890rb	ATTTGTCCTCTTTC WACACGTCCTGT	X		
Tuf890rc	ACCATTCCTCTTTC AACACGTCCAGT	X		
Tuf400a (M13-tagged)	caggaaacagctatgacc GAAACAGAAAAAC GTCAYTATGCTCA*		X	
Tuf400b (M13-tagged)	Caggaaacagctatgacc GAAACTTCTAAAA GACATTACGCTCA		X	
Tuf400c (M13-tagged)	caggaaacagctatgacc GAAACATCAAAAA GACAYTATGCTCA		X	
Tuf400d (M13-tagged)	caggaaacagctatgacc GAAACAGAAAAAA GACAYTATGCTCA		X	
Tuf400e (M13-tagged)	caggaaacagctatgacc CAAACAGCTAAAA GACATTATYCTCA		X	
Tuf835ra (M13-tagged)	tgtaaaacgacggccagt AACATCTTCWACH GGCATTAAGAAAGG		X	
Tuf835rb (M13-tagged)	tgtaaaacgacggccagt AACACCTTCAATAG GCATTAAAAAWGG		X	
Tuf835rc (M13-tagged)	tgtaaaacgacggccagt AACATCTTCTATAG GTAATAAAAAAGG		X	
M13rev-29	caggaaacagctatgacc			X
M13uni-21	tgtaaaacgacggccagt			X

*Lower case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
Tuf340 primer cocktail	10 µM total	0.5	0.2 µM
Tuf890 primer cocktail	10 µM total	0.5	0.2 µM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.2.4 Thermocycler profile: 5 min at 95°C, 35× (30 s at 94°C, 30 s at 54°C, 60 s at 72°C), 10 min at 72°C.

2.2.5 The PCR test results in a 550-bp PCR product.

2.2.6 Two microliters of 1/30 diluted PCR product should be used as input for the nested PCR test.

2.2.7 Master mixes for the nested PCR are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
Tuf400 primer cocktail	10 µM	0.5	0.2 µM
Tuf835 primer cocktail	10 µM	0.5	0.2 µM
Subtotal		23.0	
1/30 diluted PCR product		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.2.8 Thermocycler profile: 5 min at 95°C, 35× (30 s at 94°C, 30 s at 54°C, 60 s at 72°C), 10 min at 72°C.

2.2.9 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.2.10 The *tuf* gene is a protein-coding region. Translation Table 11 (Bacterial, Archaeal and Plant Plastid Code) applies to the *tuf* gene.

2.2.11 The M13-tailed primer cocktail Tuf400/Tuf835 results in a *tuf* sequence with a codon starting in reading frame 2 of the primer-trimmed consensus sequence.

2.3 Conventional PCR *16S* rDNA – phytoplasmas

2.3.1 PCR sequencing of approximately 600 bp (amplicon size including primers) of the *16S* ribosomal DNA (*16S* rDNA) is adapted from Makarova *et al.* (2012).

2.3.2 Primer sequences are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
P1-ATT (M13-tagged)	caggaaacagctatgacc AAGAGTTTGATC CTGGCTCAGG*	X	
P625r (M13-tagged)	tgtaaaacgacggccagt ACTTAYTAAACC GCCTACRCACC	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower case characters indicate the universal M13 tails. These tails play no role in amplification of the target but are used for generating cycle sequence products.

2.3.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water	N.A.	9.5	N.A.
Bio-X-ACT Short mix (Bioline)*	2×	12.5	1×
P1-ATT (M13-tagged)	10 μM	0.5	0.2 μM
P625r (M13-tagged)	10 μM	0.5	0.2 μM
Subtotal		23.0	
Genomic DNA extract		2.0	
Total		25.0	

*Or adequate PCR master mixes containing a polymerase with proof-reading activity.

2.3.4 PCR cycling parameters: 5 min at 95°C, 35× (30 s at 94°C, 30 s at 54°C, 60 s at 72°C), 10 min at 72°C.

2.3.5 Cycle sequencing reactions are performed using the primers targeting the respective M13 tags in separate reactions.

2.3.6 *16S* rDNA is a non-coding but conserved locus that is transcribed in *16S* ribosomal RNA. Translation tables do not apply to *16S* rDNA.

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: DNA extraction of an Eppendorf tube containing 25 μL of molecular-grade water.

-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 gBlock EPPO_PAC_Phytoplasmas_1 (0.1 ng μL^{-1}; see Appendix 9) or genomic DNA of a relevant target organism (see Table 6).

3.2 Interpretation of results

Verification of the controls:

- NIC and NAC should produce no amplicons
- PAC should produce amplicons of the expected size
- All samples should produce amplicons of the expected size

When these conditions are met:

- Tests yielding amplicons of the expected size are used for cycle sequencing
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance criteria available

Performance criteria for the tests in this Appendix were determined under the EUPHRESCO DNA Barcoding Project in an international consortium of ten participants. Additional data was generated by the Dutch NPPO laboratory and Fera, UK.

4.1 Analytical sensitivity

For all protocols a DNA concentration of 30 ng μL^{-1} and a relative infection grade of 10% (i.e. 10× dilution) is sufficient to generate an amplicon that can be sequenced, leading to a consensus sequence with a HQ (Phred score > 40) of at least 98%.

4.2 Analytical specificity

The locus or combination of loci indicated in Table 6 possess sufficient interspecies variation to allow for identification to species level. In addition to the species listed in Table 6, the following species have successfully been amplified and sequenced using the protocols in this appendix by the Dutch NPPO: *Ca*. Phytoplasma asteris, *Ca*. Phytoplasma aurantifolia, *Ca.* Phytoplasma phoenicium and *Ca*. Phytoplasma trifolii.

4.3 Selectivity

Ca. Phytoplasma mali, *Ca.* Phytoplasma prunorum, *Ca.* Phytoplasma pyri and two isolates of *Ca.*Phytoplasma solani have been tested from *Malus*, *Prunus domestica* 'St Julien', *Pyrus* and *Catharanthus roseus*, respectively. Other matrices might apply and need to be verified by end-users before implementing the tests described in this Appendix.

4.4 Diagnostic sensitivity

TPS partners in the EUPHRESCO II DNA Barcoding Project analysed five DNA samples of the following species: *Ca.* Phytoplasma mali, *Ca.* Phytoplasma prunorum, *Ca.* Phytoplasma pyri and two isolates of *Ca.*Phytoplasma solani. The overall diagnostic sensitivity obtained was 96% (*Ca.* Phytoplasma mali 100%, *Ca.* Phytoplasma prunorum 90%, *Ca.* Phytoplasma pyri *1*00% and *Ca.* Phytoplasma solani 90% and 100%). Re-analysis of the data provided by partners shows that identification at the required taxonomic level as listed in Table 3 is possible and an overall diagnostic sensitivity of 98% could be obtained.

4.5 Reproducibility

The same DNA samples are analysed by different partners. Therefore, in this situation the reproducibility is identical to diagnostic sensitivity.

The outcome of data analysis is dependent on the databases used and relies on a combination of nucleotide similarity, specific clustering in tree views and the ability of end-users to recognize sequence data deposited in databases which is likely to be misidentified. The analysis of sequence data using online resources and the interpretation of BLAST and MLSA results heavily depends on the proficiency of the operators handling the data. All relevant (online) resources should be used to draw a final conclusion for the data-analysis. See Appendix 7 for guidance on data-analysis.

Appendix 7 – Sanger sequencing, consensus preparation and data-analysis

1. General information

1.1 This Appendix describes how to generate sequence data, how to create a consensus sequence and how to analyse data using online resources. This Appendix may also contain information that is useful for the analysis of sequences of viruses and viroids (although they do not have DNA barcodes).

1.2 Sequence data files containing chromatograms (also referred to as electropherograms or trace data, e.g. *.ab1, *.abi or *.scf) and quality scores (Phred scores) are used as input for consensus sequence preparation and data analysis. The sequence data files are sometimes referred to as reads.

1.3 The use of sequence data files without chromatograms (e.g. *.seq, *.fas or *.txt) might lead to unreliable results.

1.4 Sequencing analysis software that allows alignment and editing of sequence data containing chromatograms with Phred scores is essential for the creation of reliable consensus sequences (e.g. the Lasergene software package (DNAstar), CLC genomic workbench (CLC bio) or Geneious (Biomatters)).

1.5 Access to the Internet is needed to access online databases such as NCBI GenBank, BOLD and Q-bank.

2. Sanger sequencing

2.1 PCR products, together with the primers used for the sequencing reaction, can be sent to commercial companies for Sanger sequencing.

2.2 All of the indicated marker regions should be sequenced in forward and reverse directions as indicated under the specific test sections.

2.3 Sequencing primers indicated in the primer tables (Appendices 1–6) should be provided to the commercial company.

2.4 If multiple PCR products (>100 bp) are visible after amplification, the PCR product of expected size (see organism tables in Appendices 1–6) should be excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) before sending it for sequencing.

Below an example is provided of the steps that could be taken when PCR products are sequenced in-house:

2.5 Purify PCR products using a QIAquick PCR Purification Kit (Qiagen). Purified PCR product is eluted in 30–50 μL of elution buffer (provided). If multiple PCR products are visible on agarose gel after amplification, the PCR product of expected size (see organism tables in Appendices 1–6) should be excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen).

2.6 Separate cycle sequencing reactions are performed for each primer (see specific protocols) using Big-Dye Terminator v. 1.1 or v. 3.1 Cycle Sequencing Kits (Life Technologies) according to the manufacturer's instructions.

2.7 Cycle sequence products are purified using Sephadex G50 columns in 96-well multiscreen HV plates (Millipore) or the DyeEx 2.0 spin kit (Qiagen).
2.8 An equal volume of HiDi formamide (Life Technologies) should be added to the purified cycle sequence product.
2.9 Analyse the purified cycle sequence product: HiDi formamide on a Sanger sequence platform (e.g. 3500 Genetic Analyzer, Life Technologies).
2.10 Generated chromatograms are used to create a single consensus file.

3. Consensus sequence preparation

In general, overlapping sections are used to generate consensus sequences. When needed (e.g. when discriminatory sequences are located in overhangs with 1× coverage), sections that are covered only once can be included in the consensus sequence. Visual inspection of the assembly is an important part of the creation of a consensus sequence. Phred scores can be used to aid consensus sequence creation as they indicate the reliability of base-calling: a Phred score of 10 = 90%, 20 = 99%, 30 = 99.9%, 40 = 99.99% and 50 = 99.999% reliability for the selected base. Phred scores >40 are regarded as high-quality (HQ) data.

3.1 Upload the chromatograms in the sequencing analysis software.
3.2 Select the chromatograms (at least 2) needed for the preparation of consensus sequences. Chromatograms can be generated using, for instance, a forward and reverse primer (e.g. *COI* gene arthropods) or two reverse primers (e.g. *16S* rRNA gene, bacteria). In some cases, multiple PCR products are used to generate a single consensus sequence (e.g. *18S* rRNA gene, nematodes).
3.3 Assemble the chromatograms so that an alignment is obtained that shows the electropherograms of the individual reads.
3.4 Trim 3′ untemplated –dA from the consensus sequence.
3.5 Trim amplification primers from the consensus sequence. Internal sequence primer sequences can be retained. Appendix 8 shows a suggested form for preparation of consensus sequences and data analysis.
3.6 Assess the assembly visibly and edit where needed. Check the entire sequence in order to detect any errors in the assembly and consensus sequence. The following rules are used as a guide. Visual inspection of the assembly might lead to different decisions:
- Trim the low quality ends of the consensus sequence to prevent an unreliable consensus sequence because of low-quality bases: (i) for 1× coverage the Phred score should be at least 30 for the individual read, (ii) for 2× or more coverage it should be at least 20 for the individual reads.
- Bases in the consensus sequence with a Phred score < 20 should be noted as N.
- Make sure that the consensus sequence is shown in the right direction (5‘–3′ from the forward primer; see primer tables in Appendices 1–6). This is particularly important when using the BOLD database for data-analysis. When using a consensus sequence that has the wrong direction, BOLD will not be able to match the sequence to other sequences in the database.
- When polymorphisms (double peaks) are observed in good-quality data, IUPAC ambiguity codes should be used (see Table 7).
- When insertions or deletions (InDels) are present in coding sequences (the presence of InDels can be inferred by analysing the BLAST hit alignment), the consensus sequence can be converted to amino acids in order to check that there are no unexpected stop codons in the coding sequence (note that the correct reading frame should be used; see organism tables in Appendices 1–6).

3.7 Generate a consensus sequence from the assembly.

4. (Online) data analysis

Relevant resources should be used to draw a final conclusion for the data analysis. There are several online resources available that can be used for the analysis of the consensus sequence obtained. A detailed description of the different resources and the interpretation of BLAST results are shown in Section 5.

4.1 Document all (online) resources consulted, the settings used, results and conclusions per source. Appendix 8 shows a suggested form for preparation of consensus sequences and data-analysis.

Table 7. IUPAC ambiguity codes

Code	Represents	Complement
A	Adenine	T
G	Guanine	C
C	Cytosine	G
T	Thymine	A
Y	Pyrimidine (C or T)	R
R	Purine (A or G)	Y
W	weak (A or T)	W
S	strong (G or C)	S
K	keto (T or G)	M
M	amino (C or A)	K
D	A, G, T (not C)	H
V	A, C, G (not T)	B
H	A, C, T (not G)	D
B	C, G, T (not A)	V
N	any base	N
–	Gap	–

4.2 Document the results per resource used (e.g. by providing screenshots or pdf files of BLAST hits, MLSA results, tree views, alignments, etc.).
4.3 Draw a general conclusion from the conclusions per source, making use of conservative terms (e.g. Sample X possibly is/isn't taxon Z, or it is (very) likely/unlikely that Sample X is taxon Z) avoid using absolute terms (e.g. Sample X is taxon Z).
4.4 When a misidentification of an accession in the online databases is suspected, end-users can BLAST the sequence of the presumed misidentified organism against 'NCBI+organism' (see Section 5.3) to determine the reliability of the identification.
4.5 It has to be noted that PCR sequencing is used in support of species identification. Origin, host plant and other characteristics (e.g. morphological, biochemical, reactions on indicator plants) are typically needed to complete the diagnosis.

5. Essential procedural information

5.1 Controls

For a reliable test result to be obtained, the following external controls should be included for each sequencing run and derived consensus sequence(s) generation and sequence analysis:

Positive cycle sequence control (PCC), to monitor the efficiency of cycle sequence reactions, the generation of sequence data and consensus sequence preparation: amplicon of a sample with known identity and sequence analysis as a sequencing process control (e.g. amplicons obtained with synthetic PACs, or DNA with a known sequence). The percentage of high-quality bases and the sequence length obtained from this sample are indicative for cycle sequence reactions and the generation efficiency for sequence data. Alignment of the PCC consensus sequence with the known reference sequence (should be 100%) is indicative of the success of consensus sequence preparation.

Generating consensus sequences heavily depends on the proficiency of the operators handling raw data. The same applies to the interpretation of BLAST results. Synthetic PACs are standardized controls that can be used to unambiguously monitor success from cycle sequence reaction to sequence analysis. Between-run repeatability for individual operators and the overall reproducibility within a lab can be used to monitor trends in sequence analysis success. In addition, the proficiency of operators working with sequencing analysis can be monitored using blind samples with known sequences or by participation in proficiency tests.

When unclear results are obtained, sequence data is analysed by a second operator or the test is repeated.

5.2 Validation

Determining performance criteria for DNA barcoding is performed in two separate steps: (1) PCR reactions (all performance criteria described in PM7/98(2) apply unless stated otherwise in Appendices 1–6), and (2) creating consensus sequences and sequence analysis (only the performance criteria analytical specificity, diagnostic sensitivity and reproducibility are relevant).

The analytical specificity of the locus (or combination of loci) used can change over time because of the use of (online) databases with constantly changing content. Changes made to the content of (online) databases might influence the usability of generated sequence data for the identification on the required taxonomic level. Instead of determining performance criteria for the sequence data analysis step, the usability of generated data (i.e. analytical specificity) is evaluated each time an analysis is performed by determining if the generated data provides sufficient resolution between taxa (e.g. no overlap in inter- and intraspecific variation, or taxon-specific clustering). The validation status of a species–locus(loci) combination relies on the last time that combination was assessed. The protocols in this Standard have proven to be fit for purpose for the selected-regulated pests and pathogens. Only selected regulated pests that were previously tested by the authors of this Standard have been included in the Standard, but it should be noted that these protocols can be used for a much broader range of (non-regulated) organisms. Laboratories implementing these protocols have to verify each time that an analysis is performed that the resolution of the generated sequence(s) still allows species identification.

Synthetic PACs can be used to determine the repeatability and reproducibility of the sequence analysis steps (see Appendix 7, Section 5.1).

5.3 Background information on online resources

The most commonly used online databases and their application are described in the table below. Terms used in the table are explained in a glossary.

5.3.1 Glossary.

BLAST	In a BLAST search, a sequence is broken into small pieces (word size) that are matched with the data in the database (seeds). Rewards and penalties for matching and mismatching bases are awarded. Changing the scoring settings of the algorithm parameters can greatly influence the BLAST (especially the gap penalty) output which consists of hit names, accession numbers, max score, total score, E-value, coverage and similarity
Max score	Highest alignment score (bit score) between the query sequence and the database sequence segment. The scores of different alignments cannot be compared, nor can they be used to select the best alignment because their scale depends on the gap penalty

(continued)

Table *(continued)*

Total score	Sum of alignment scores of all segments from the same database sequence that match the query sequence (calculated over all segments). This score is different from the max score if several parts of the database sequence match different parts of the query sequence. The scores of different alignments cannot be compared, nor can they be used to select the best alignment because their scale depends on the gap penalty
E-value	The E-value (Expect value) indicates the reliability of the hit, and the closer it is to zero the more 'significant' a hit is (note: the hit, not the identity of the specimen!). BLAST hits are typically sorted on E-value (low to high). The first BLAST hit (lowest E-value) is not necessarily the most likely species identity. Particularly when sequence data with large changes in query coverage are present in the database the E-value can be unreliable to identify the best match. Because of this, tree views of the obtained BLAST hits are used to further determine the identity of the sequenced specimen
Consensus	A theoretical representative sequence in which each nucleotide is the one which occurs most frequently at that site in the different sequences. (e.g. sequences generated with the forward primer and reverse primer of a given amplicon in separate reactions). It is the results of multiple sequence alignments in which related sequences are compared to each other
Coverage	Percentage of the query length that is included in the aligned segments. This coverage is calculated over all segments
Similarity	Percentage of identical bases in the alignment. The percentage is calculated over all segments
MLSA	In multi-locus sequence analysis (MLSA), or multi-locus sequence typing (MLST), sequence data of more than one locus is analysed simultaneously
Gap penalty	If the gap penalty is too large, gaps are avoided and the sequences cannot be properly aligned. If the gap penalty is too low, gaps are inserted everywhere to prevent mismatches. This does not produce any informative alignment. The 'best' alignment is obtained for an intermediate gap penalty

	NCBI GenBank	BOLD	Q-bank
Hyperlink	http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome	http://www.boldsystems.org/index.php/IDS_OpenIdEngine	http://www.Q-bank.eu/
Database description	The NCBI GenBank sequence database is a publicly accessible database containing sequence data for more than 260 000 formally described species (Benson *et al.*, 2013). The sequence data in the NCBI database consists of a many loci from all organism groups that are relevant to the plant health field (bacteria, fungi, oomycetes, insects, invasive plant species, nematodes, phytoplasmas, viruses and viroids). Many quarantine and quality organisms, phylogenetically related species and look-alikes are represented in this database. Data in NCBI is checked for various technical aspects before publication. Through the taxonomy database (select 'Taxonomy' in the dropdown menu on the NCBI website), it is possible to see which organisms are present in the NCBI database	The BOLD database (Ratnasingham & Hebert, 2007) is the DNA BARCODE sequence database for the identification of animalia, fungi and plants. The database includes *COI* for animalia, *ITS* for fungi and *rbcL* and *matK* for plants. Sequence data in BOLD have to meet strict requirements to ensure species identity of the specimens in the database. Specimens and strains used to generate sequence data are vouchered. The *COI* database can be used for identification of arthropods and nematodes. Although the main focus of BOLD lies with *COI* sequences for animalia, the *ITS* and the *rbcL* and *matK* databases can be useful for fungi and invasive plants, respectively.	Q-bank is a scientifically curated database that focuses specifically on European Union-regulated plant pathogens, pests, invasive plants and related species. Sequence data of most pest 'barcodes' that are generated with the protocols described in this Standard are available. Specimens and strains used to generate the Q-bank sequence data are vouchered and can often be acquired via the curator of a database section
Database subsets	The NCBI database includes many subsets such as: Nucleotide collection (nr/nt) – 'nr' stands for 'non-redundant,' but it isn't Reference genomic sequences (refseq_genomic) – comprehensive, integrated, non-redundant, well-annotated set of sequences NCBI Genomes (chromosome) – complete genomes and chromosomes from reference sequences Typically the nr/nt database is used. End-	Within the COI database (animalia) several subsets of the database can be used: All records on BOLD barcode Barcode species-level records Public record barcode database Full-length record barcode database The first three options require a COI fragment of at least 500 bp for identification, while the 'Full length record barcode database' needs at least 640 bp. The first-mentioned	The Q-bank database has seven subsets: arthropods, bacteria, fungi, invasive plants, nematodes, phytoplasmas and viruses and viroids. The BLAST algorithm can be used to query all sequences in the entire database, while the MLSA tools are accessed through the organism-specific subset of the database

(continued)

Table *(continued)*

	NCBI GenBank	BOLD	Q-bank
Database subsets *(continued)*	users have to be aware that this database contains misidentified sequences. Additional analyses can be performed to determine if a sequence is derived from a misidentified specimen (e.g. analysis in other databases, BLAST of putative misidentified sequence to the nr/nt database restricted to species identity)	database ('All barcode records') also contains sequence data from specimens which are not identified to species level, and is less suitable for species identification. By default 'Barcode species level records' is selected The *ITS* database does not have subsets in the database and requires a fragment of at least 100 bp in order to perform a BLAST search. The database contains *ITS* sequence data from specimens which are not identified to species level and therefore does not have the same status as the 'Species-level barcode records' *COI* database The *rbcL* and *matK* database does not have subsets in the database, and requires a fragment of at least 500 bp to perform a BLAST search. The *rbcL* and *matK* database contains sequence data from specimens which are not identified to species level and therefore does not have the same status as the 'Species-level barcode records' *COI* database. There are very few *rbcL* and *matK* records on BOLD	
Frequently used analysis tools	Single-locus basic local alignment search tool (BLAST)	Single-locus BLAST	Single-locus BLAST Multi-locus BLAST
BLAST and MLSA parameters	In NCBI three BLAST pre-sets are available: megablast, discontinuous megablast and blastn Megablast is designed for the comparison of sequences with high similarity (>95%) and is in those cases very quick. Megablast utilizes a large word size (n = 28) Discontinuous megablast makes use of a smaller word size (n = 11) in which mismatches are allowed. GenBank indicates that this is particularly useful for comparison across species Blastn is the slowest algorithm, and also makes use of a word size n = 11, but if desired this can be adjusted to 7 Megablast is used by default, but if this does not yield useful hits other algorithms can be used. Under the heading 'Algorithm parameters' settings as (e.g.) number of hits to be shown, word size, match/mismatch scores, number of displayed results can be changed It is possible to restrict BLAST to a	It is not possible to adjust the BLAST settings in BOLD	BLAST: from the Q-bank homepage, the BLAST search can be accessed through: ID/Blast against all Q-bank sequences, but can also be accessed from the organism-related sections of the database. A disclaimer has to be checked before the BLAST search tool can be used. Under pairwise sequence alignment parameters, different BLAST settings such as word size, maximum hits to display and cut-off settings for minimum similarity and overlap can be adjusted. In general, the default settings are appropriate, but it is important to check which databases are selected for your search MLSA: MLSA is accessed under ID in the organism-specific part of the database. The disclaimer should be checked before the MLSA tool can be used. Under the DNA sequence data tab, sequences of different loci are submitted. Make sure that the

(continued)

Table *(continued)*

	NCBI GenBank	BOLD	Q-bank
BLAST and MLSA parameters *(continued)*	specific taxon or taxa (e.g. genus, species, subspecies), or to exclude certain taxa. To do so, type the name of the desired taxon or taxa in the 'Organism' field on the BLAST page. It should be noted that not all sequences in NCBI have a taxonomic name assigned to them and could be missed in the selection you make. Also, synonyms are not taken into account. It has to be noted that BLAST results restricted to a specific taxon sometimes show different similarity percentages in the hit table compared to the alignment. Usually the latter shows the correct percentage		number of loci used are correct (Minimum characters to be accounted, default = 1) under Polyphasic identification parameters. Other settings under Polyphasic identification parameters can be used as default
BLAST and MLSA output	BLAST results are by default displayed in three different ways: Graphic summary, a BLAST hit table (Descriptions) and a detailed overview per hit (Alignments). The Graphic summary shows the length of the query sequence (Sbjct) and the hit lengths and their position relative to the query sequence. The hit table shows, among others, the name of the hits, their accession number, the coverage with respect to the query sequence, the percentage similarity, and the E-value. The detailed overview per hit gives information about the percentage of agreement, overlap, an alignment between query and Sbjct and information relating to the accession number (e.g. locus). Simultaneous BLAST of multiple sequence items is possible to increase the sequence analysis throughput	Apart from the 'All barcode database records', the BLAST results of *COI* sequence data will be displayed as a hit table with similarity percentages, a graph showing the similarity scores and a probability that the sequence belongs to a particular taxonomic level (Identification summary). The 'All records barcode identification' database gives no identification summary. BOLD does not account for synonyms, so it is possible that the identification summary states that a certain sequence belongs to either species A or B, while A and B are synonyms The *ITS* and *rbcL* and *matK* databases show BLAST results largely in the same way as NCBI. Additionally, graphs with similarity scores and E-values are given Simultaneous search of multiple sequence items possible after registration	BLAST: BLAST results are displayed as a hit table showing, among others, the name of the hits, their accession number, the coverage with respect to the query sequence (% overlap) and the percentage similarity. Furthermore, the orientation of your sequence with respect to the hit is displayed under 'Direction' (+/+ or +/−). In Q-bank, the E-value is referred to as probability. A rating is assigned to the hit, the more stars are granted the more likely it is that a hit is correct (note: the hit, not the species identity!). Alignments can be accessed by expanding the hit results (click on the triangle next to the hit). Simultaneous BLAST of more than one sequence is not possible. MLSA: In the MLSA results, Q bank shows the number of loci that are included in the analysis ('Accounted') and the total weight assigned (usually 1 per locus). Also, the degree of similarity is displayed. Alignments of different loci can be accessed by expanding the hit (click the triangle next to the hit)
Tree views*	BLAST hit results can be displayed as a fast minimum evolution (FME) tree or neighbour-joining (NJ) tree view by selecting 'Distance tree of results' on the BLAST results page. Selecting 'show all' under 'collapse mode' will allow one to assess if a query sequence (highlighted in yellow) falls in a species-specific clade	COI BLAST hit results can be displayed as a NJ tree view by selecting 'Tree based identification' on the BLAST results page. Tree settings cannot be adjusted. The query sequence is highlighted in red. *ITS* and *rbcL* and *matK* BLAST hits cannot be shown in a tree view	BLAST hit and MLSA results can be displayed using different tree views by selecting 'Draw tree' on the BLAST or MLSA results page. Neighbour joining and UPGMA are the most commonly used algorithms. The query sequence is indicated with 'My data'. Apart from choosing the tree algorithm, tree settings cannot be changed. It has to be noted that the information displayed for the external nodes is dependent on the

(continued)

Table *(continued)*

	NCBI GenBank	BOLD	Q-bank
Tree views *(continued)*			subset of the database queried. Some subsets of the database provide more information than others for the external nodes. The full specimen record can be accessed by clicking on the external nodes in the tree view
Species included	Through the taxonomy database (select 'Taxonomy' in the dropdown menu on the NCBI homgepage), it is possible to see which organisms are represented in the NCBI database	Through the taxonomy database (select the 'Taxonomy' tab on the BOLD homepage) it is possible to see which species are present in the BOLD database	Overviews of species included in the Q-bank database are provided in the organism-related subsets of the database

*See also section 5.4 for the interpretation of tree views.

5.4 Interpretation of tree views

Tree views obtained from BLAST and MLSA results are used in addition to BLAST hits for reliable species identification. It should be noted that the usefulness of tree views is, similar to the interpretation of BLAST and MLSA hits, highly dependent on the availability of relevant loci and taxa in the database consulted. Furthermore, the implemented algorithms for multiple sequence alignments (ClustalW) and tree construction (fast minimum evolution, neighbour joining) do in some cases not show/optimally reflect the species position within the tree depending on the genetic variation of the chosen loci and the number of taxonomic differences from the reference sequences available in the database. In principle, an unknown sequence can be assigned to a particular taxon when it falls within a taxon-specific cluster.

It is important to realize that trees generated from (partial) gene sequences or sequence data from non-coding regions only show the relationship between these (partial) genes or regions and do not necessarily show a phylogenetic relationship among the taxa. To infer phylogentic relationships more in-depth analyses are necessary (for a practical handbook see Lemey *et al.*, 2009).

A tree consists of a root, branches, nodes and leaves (=external nodes) (see Fig. 7A). The external nodes show the taxa that are used. These taxa can be species, genera or families, but also subspecies or pathovars. The nodes of the tree represent the (hypothetical) ancestors, or better, represent sequences of the (hypothetical) ancestors. Groups of taxa with the same (hypothetical) ancestors form clades or clusters. When determining phylogenetic relationships, an outgroup is chosen to root the tree (=outgroup rooting) (Fig. 7A). However, when BLAST results are used to draw a tree, there is no outgroup and trees are typically midpoint rooted, which is indicated with a node on the branch

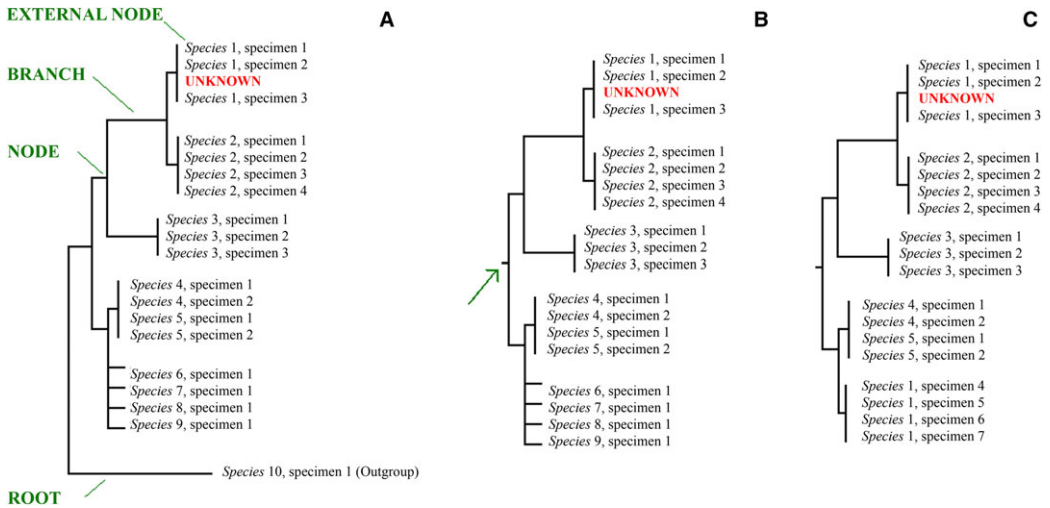


Fig. 7 (A) Outgroup rooted tree with species 1–10. Species 1, 2 and 3 form monophyletic groups, species 4 and 5 form a non-species-specific cluster and species 6–9 represent a polytomy. Species 10 is the outgroup in this cladogram. (B) Midpoint rooted tree. The same cladogram as in (A) but without an outgroup. This tree is rooted on the branch between the specimens with the lowest homology. (C) Midpoint rooted tree in which species 1 represents a polyphyletic group.

between the specimens with the lowest homology (Fig. 7B). In Fig. 7(A,B), all specimens of species 2 form a clade, but also all specimens of species 1 + the unknown sequence + species 2 and 3. Species 4 and 5 together form a non-species-specific clade. Based on the gene or region used to draw this tree, there is no resolution between species 4 and 5. If an unknown sequence would cluster in clade 4/5, identification on the basis of this tree is not possible. In this case, it can be said that the unknown sequence possibly belongs to species 4 or 5.

Different terms are used to indicate the relationship between external nodes. In Fig. 7A,B, species 2 is a sister group of species 1 + unknown sequence (and vice versa). Species 3 is again a sister group of species 1 + unknown sequence + species 2, and so on. In general, a branch splits into two branches after a node (=dichotomous). Specimens with a common (hypothetical) ancestor form a *monophyletic* group (e.g. all the specimens in species 2 in Fig. 7A–C). A *polyphyletic* group consists of specimens with different (hypothetical) ancestors (e.g. species 1 in Fig. 7C). The latter can sometimes occur in trees obtained from BLAST results. Specimens of the same species may be found at different places in the tree and form a polyphyletic group. Identification is then still possible, provided that the unknown sequence clusters with a species-specific clade. For instance: in Fig. 7(A–C) an unknown sequence is included in the analysis. In Fig. 7(A,B) the sequence clusters with a species-specific clade which contains all specimens of this species available in the database (no overlap with other species). In Fig. 7(C) the sequence falls in one of the species-specific clusters from the polyphyletic species 1. In both cases this provides a reasonably strong indication that the unknown sequence probably belongs to species 1. Sometimes it is not possible to determine the relationship between the different taxa (see species 6, 7, 8 and 9). This is called a *polytomy*. If a tree obtained from BLAST results shows a polytomy, this often indicates that the diagnostic resolution of the analysed locus or loci is not sufficient.

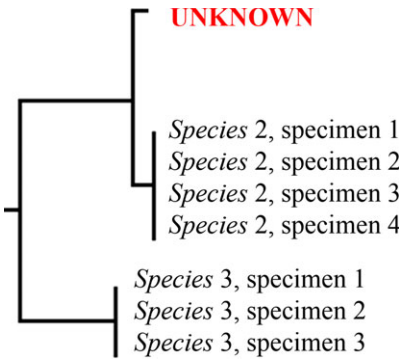


Fig. 8 The same midpoint rooted tree as in Fig. 7(B) without species 1 and 4–10.

The usefulness of tree views is highly dependent on the sampling of the relevant taxa. If some taxa are not represented it is difficult to interpret the tree. In Fig. 8, species 1 and species 4–10 (relative to Fig. 7B) are not included. It is impossible to see that the unknown sequence clusters with species 1 and might be misidentified as variation of species 2. When an unknown sequence clusters as sister to a species-specific cluster or as a single branch in a tree special caution is needed, since this could either be a result of variation within a species that has not been sequenced before or lack of sampling of other related species.

Appendix 8 – Suggested form for consensus sequence preparation and data analysis

This form can be used to document the locus/loci sequenced, sources and settings used, results obtained and conclusions drawn. It is important to document this information since databases with constantly changing content are used for identification. This Appendix may also contain useful information for the analysis of sequences of viruses and viroids (although they do not have DNA barcodes).

Date: Operator:

Table 1. Information concerning locus sequenced and consensus sequence preparation [copy this table for each locus used]

1	LIMS and/or collection number	
2	Name locus	(e.g. cytochrome *c* oxidase subunit I)
3	Characteristics locus	☐ coding ☐ non-coding ☐ mix coding and non-coding
4	Cycle sequence reactions and sequencing performed	☐ in-house ☐ external company (*)
5	BigDye terminator kit used	☐ version 1.1 ☐ version 3.1
6	*n* cycle sequence reactions performed: consensus based on *n* chromatograms	x: x (* when not 1:1)
7a	Assembly method	☐ *de novo* assembly ☐ reference assembly (go to 7b)
7b	Reference sequence used (collection or NCBI number)	
8	Untemplated –dA and amplification primers removed?	☐ yes ☐ no (*)
9	Are single-sequence reads used in the consensus sequence	☐ yes, how many bases 5′-end: … and 3′-end: … ☐ no
10	Orientation consensus sequence correct (5′–3′ from Fw primer)	☐ yes
11	Consensus length: expected consensus length (when available)	xxx bp: xxx bp (* when not 1:1)
12	% High-quality (HQ) bases (Phred score > 40)	xxx.x %

*Provide detailed explanation below.

Explanation and additional information on locus used and consensus sequence obtained:

Table 2. Sources used, analysis settings and analysis results

Source	Analysis information	Parameters	Explanation, reference to analysis results and conclusion per database[‡]
NCBI	Database used	☐ nucleotide collection (nr/nt) ☐ other (give details[†])	
	Selection algorithm	☐ megablast ☐ discont. megablast ☐ blastn	
	Parameters adjusted	☐ no ☐ yes (give details)	
	Tree method	☐ fast minimum evolution ☐ NJ	
	Restrict to organism(s) (optional)	☐ not used ☐ used (give details)	
	Exclude organism(s) (optional)	☐ not used ☐ used (give details)	
BOLD	Database used	☐ *COI* ☐ *ITS* ☐ *rbcL* & *matK*	
	Subset COI database (when used)	☐ all ☐ species level ☐ public record	
	Tree view used	☐ not used ☐ used (give details)	
Q-bank	Analysis method	☐ single locus[*] ☐ multi-locus (give details)	
	Parameters adjusted	☐ no ☐ yes (give details)	
	Tree method	When applicable (give details)	
Other	When applicable provide details		

[*]Turn non-redundant GenBank option off.
[†]Provide details in the last column of the table.
[‡]Number of nucleotides in analysis, % similarity with 1st or specific match, specific clustering/no specific clustering with taxon *Z*.

Data-analysis conclusion

[Draw a single conclusion from the results obtained using different resources. For instance: Based on the analysis of xxx nucleotides of locus A and xxx nucleotides of locus B in database 1, 2 and 3 we can conclude that sample xxx might be/presumably is/is not taxon *Z*.]

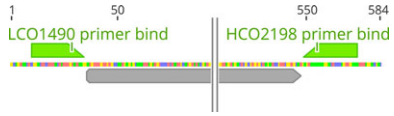
Analysis results and other supportive information

[For example, consensus sequence(s) and print screens of BLAST hit tables, tree views, alignment views, etc. with reference to Table 2 that lead to conclusions per database and to the general conclusion.]

Appendix 9 – gBlocks

The sections (Figs 9 to 14) below provide graphical representation and background information on the gBlocks that can be used as PAC for the DNA barcoding tests. gBlocks were designed by the Dutch NPPO in such a way that they can be used for all tests in a single organism group (or Appendix). Dark green annotated sequences indicate annealing sites for forward primers, whereas light green annotated sequences indicate annealing sites for reverse primers. The 513-nucleotide (nt) reference sequence phrase is indicated in yellow, and will result after translation (reading frame 1, standard code) in the following amino acid sequence twice: *KEEP*-CALM*THIS*IS*MERELY*A*VERY*STRANGE*RE-FERENCE*PHRASE*WITH*EIGHTY*FIVE*CHARAC-TERS (stop codons are indicated as *).

1. Arthropod tests



gBlock name: EPPO_PAC_Arthropods_1
version: 1
length: 584 nt
NCBI accession: KT429638
Sequence:
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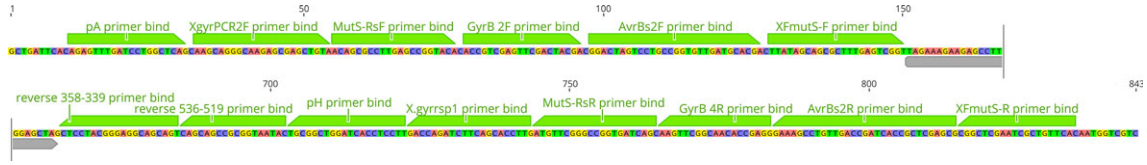
CGCGAATTATACTAGGCGTAGGTTGAACGCTATTAGTCAACTAGAGCGAATGGCGAATAGAGAGAATTTGAGCGGGAAAACTGTGAGTAGCCGCATA
GAGCTAGCGAGTAGTGGATTACTCATTAGGAAATCGGACATACCTACTAGTTCATCGTAGAGTAGTGCCATGCACGGGCTTGCACAGAGAGATCGTGA
AAGGAGGAACCATGATGCGCACTTATGTGAACACATATTAGTTGAATATCATGAATGGAAAGAGAGCTCTATTGAGCCTGAGTCGAGAGGTACTGAAG
TACGCGTGCAAACGGAGAGTGACGTGAGTTCGAAAGAGAGAATTGCGAATGACCTCACCGAGCATCCGAATGATGGATAACCCACTGAGAGATAGGG
CATACATATTGATTTATTGTGGAATGATGTCACGCGAGAGCATGTACCGAACGGAGCTAGTGATTTTTTGGTCACCCTGAAGTTTAAATGGTCGTC

2. Bacterial tests



gBlock name: EPPO_PAC_Bacteria_1
version: 1
length : 843
NCBI accession: KT429643
Sequence:
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GACTAGTCCTGCCGGTGTTGATGCACGACTTATAGCAGCGCTTTGAGTCGGTTAGAAAGAAGAGCCTTAGTGTGCTTTAATGTAGACCCACATAAGCTA
GATATCGTAGATGGAGCGCGAATTATACTAGGCGTAGGTTGAACGCTATTAGTCAACTAGAGCGAATGGCGAATAGAGAGAATTTGAGCGGGAAAAC
TGTGAGTAGCCGCATAGAGCTAGCGAGTAGTGGATTACTCATTAGGAAATCGGACATACCTACTAGTTCATCGTAGAGTAGTGCCATGCACGGGCTTG
CACAGAGAGATCGTGAAAGGAGGAACCATGATGCGCACTTATGTGAACACATATTAGTTGAATATCATGAATGGAAAGAGAGCTCTATTGAGCCTGAG
TCGAGAGGTACTGAAGTACGCGTGCAAACGGAGAGTGACGTGAGTTCGAAAGAGAGAATTGCGAATGACCTCACCGAGCATCCGAATGATGGATAAC
CCACTGAGAGATAGGGCATACATATTGATTTATTGTGGAATGATGTCACGCGAGAGCATGTACCGAACGGAGCTAGCTCCTACGGGAGGCAGCAGTCA
GCAGCCGCGGTAATACTGCGGCTGGATCACCTCCTTGACCAGATCTTCAGCACCTTGATGTTCGGGCCGGTGATCAGCAAGTTCGGCAACACCGAGGG
AAAGCCTGTTGACCGATCACCGCTCGAGCGCGGCTCGAATCGCTGTTCACAATGGTCGTC

3. Fungal and oomycete tests

gBlock name: EPPO_PAC_Fungi_1
version: 1
Length : 798
NCBI accession: KT429642
Sequence:
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CCCTATGTGCAAGGCCGGTTTCGCCTCATCACGATGGCTTTTTTCAACTAGAAAGAAGAGCCTTAGTGTGCTTTAATGTAGACCCACATAAGCTAGATA
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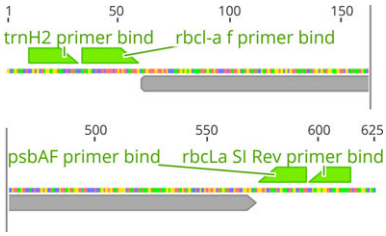
TGAGAGATAGGGCATACATATTGATTTATTGTGGAATGATGTCACGCGAGAGCATGTACCGAACGGAGCTAGGCATATCAATAAGCGGAGGAATGGCC
AGACCCGTGAGCAGACAACTTCGTCTTCGGCCAGTCTGGCCATGATGGCCAGAAAGATGATGGGCCAGAAGGACTCGTATTTGGTTTTTCGGACATCC
AGAGGAATGGTCGTC

4. Invasive plant species tests



gBlock name: EPPO_PAC_Invasive_Plants_1
version: 1
length : 625
NCBI accession: KT429639
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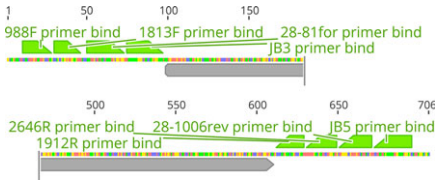
CATAAGCTAGATATCGTAGATGGAGCGCGAATTATACTAGGCGTAGGTTGAACGCTATTAGTCAACTAGAGCGAATGGCGAATAGAGAGAAATTTGAGC
GGGAAAACTGTGAGTAGCCGCATAGAGCTAGCGAGTAGTGGATTACTCATTAGGAAATCGGACATACCTACTAGTTCATCGTAGAGTAGTGCCATGCA
CGGGCTTGCACAGAGAGATCGTGAAAGGAGGAACCATGATGCGCACTTATGTGAACACATATTAGTTGAATATCATGAATGGAAAGAGAGCTCTATTG

5. Nematological tests



gBlock name: EPPO_PAC_Nematodes_1
version: 1
Length: 706
NCBI accession: KT429641
Sequence:
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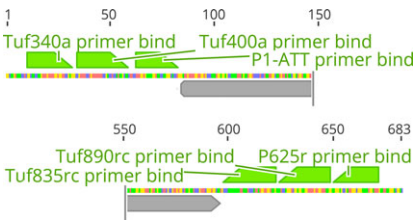
AGAAAGAAGAGCCTTAGTGTGCTTTAATGTAGACCCACATAAGCTAGATATCGTAGATGGAGCGCGAATTATACTAGGCGTAGGTTGAACGCTATTAG
TCAACTAGAGCGAATGGCGAATAGAGAGAATTTGAGCGGGAAAACTGTGAGTAGCCGCATAGAGCTAGCGAGTAGTGGATTACTCATTAGGAAATCG
GACATACCTACTAGTTCATCGTAGAGTAGTGCCATGCACGGGCTTGCACAGAGAGATCGTGAAAGGAGGAACCATGATGCGCACTTATGTGAACACAT
ATTAGTTGAATATCATGAATGGAAAGAGAGCTCTATTGAGCCTGAGTCGAGAGGTACTGAAGTACGCGTGCAAACGGAGAGTGACGTGAGTTCGAAA
GAGAGAATTGCGAATGACCTCACCGAGCATCCGAATGATGGATAACCCACTGAGAGATAGGGCATACATATTGATTTATTGTGGAATGATGTCACGCG
AGAGCATGTACCGAACGGAGCTAGCCCTAGTTCTGACCGTAAAAAAAGTCGTAACAAGGTAGCAGGGGCGAAAGACTAATCGAACCATTATGTTTTAA
GTTTAGGTGCTAATGGTCGTC

6. Phytoplasma tests



gBlock name: EPPO_PAC_Phytoplasmas_1
version: 1
Length: 683
NCBI accession: KT429640
Sequence:
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CTTAGTGTGCTTTAATGTAGACCCACATAAGCTAGATATCGTAGATGGAGCGCGAATTATACTAGGCGTAGGTTGAACGCTATTAGTCAACTAGAGCG
AATGGCGAATAGAGAGAATTTGAGCGGGAAAACTGTGAGTAGCCGCATAGAGCTAGCGAGTAGTGGATTACTCATTAGGAAATCGGACATACCTACT
AGTTCATCGTAGAGTAGTGCCATGCACGGGCTTGCACAGAGAGATCGTGAAAGGAGGAACCATGATGCGCACTTATGTGAACACATATTAGTTGAATA
TCATGAATGGAAAGAGAGCTCTATTGAGCCTGAGTCGAGAGGTACTGAAGTACGCGTGCAAACGGAGAGTGACGTGAGTTCGAAAGAGAGAATTGCG
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GAACGGAGCTAGCCTTTTTTATTACCTATAGAAGATGTTACTGGACGTGTTGAAAGAGGAATGGTGGTGCGTAGGCGGTTTAGTAAGTAATGGTCGTC