Bulletin OEPP/EPPO Bulletin (2017) **47** (2), 146–163 ISSN 0250-8052. DOI: 10.1111/epp.12380

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/62 (2)

Diagnostics
Diagnostic

PM 7/62 (2) '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum'[1]

Specific scope

This Standard describes a diagnostic protocol for '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum'.

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*

Specific approval and amendment

Approved as PM 7/62 *Candidatus* Phytoplasma mali and PM 7/63 *Ca.* P. pyri in 2006. Revised in 2017-02 as a single Standard as PM 7/62 (2) with the addition of '*Ca.* P. prunorum'.

1. Introduction

Fruit trees of the family Rosaceae may be seriously affected by phytoplasmas of the Apple Proliferation group (AP 16SrX group). The AP group includes '*Candidatus* Phytoplasma mali', which causes apple proliferation (AP), '*Ca.* P. prunorum', associated with European stone fruit yellows (ESFY) and '*Ca.* P. pyri', associated with pear decline (PD) (Seemüller & Schneider, 2004; Marcone *et al.*, 2010). Although '*Ca.* P. mali' infection occurs mainly in the genus *Malus*, it has also been occasionally identified in plants other than the typical host, for example stone fruits and both European pear (*Pyrus communis*) and Asian pear (*Pyrus pyrifolia*) (Lee *et al.*, 1995; Del Serrone *et al.*, 1998; Seemüller & Schneider, 2004; Mehle *et al.*, 2007). '*Ca.* P. pyri' is mainly associated with the genus *Pyrus* (Seemüller & Schneider, 2004). '*Ca.* P. prunorum' causes economically important disorders in apricot (*Prunus armeniaca*), Japanese plum (*Prunus salicina*) and peach (*Prunus persica*) (Carraro & Osler, 2003). European plums (*Prunus domestica*) as well as some other wild *Prunus* species (*Prunus spinosa*, *Prunus cerasifera*, *Prunus insititia*) are susceptible to infection but generally do not show symptoms. Such species represent a hidden source of infection (Carraro *et al.*, 1998a, 2004; Carraro & Osler, 2003). In contrast, *Prunus avium* has demonstrated a high level of resistance to '*Ca.* P. prunorum' (Jarausch *et al.*, 1999). Phytoplasmas from the AP group have also been detected in hazel (*Corylus avellana*), ash (*Fraxinus excelsior*), dog rose (*Rosa canina*), hackberry (*Celtis australis*), hawthorn (*Crataegus monogyna*), oak (*Quercus robur* and *Quercus rubra*), hornbeam (*Carpinus betulus*) and bindweed (*Convolvulus arvensis*) (Seemüller & Schneider, 2004).

Psyllids seem to play a crucial role in the transmission of phytoplasmas from the AP group (Tedeschi & Alma, 2004). '*Ca.* P. prunorum' is transmitted to host plants of *Prunus* species by the vector *Cacopsylla pruni* (Carraro *et al.*, 1998b). Additionally, the leafhopper *Asymmetrasca decedens* (synonym *Empoasca decedens*) has been suggested as a potential vector of this phytoplasma (Pastore *et al.*, 2004). '*Ca.* P. pyri' is transmitted to the host plants by two vectors, *Cacopsylla pyricola* (Davies *et al.*, 1992) and *Cacopsylla pyri* (Carraro *et al.*, 1998c). Known psyllid vectors of '*Ca.* P. mali' are *Cacopsylla picta* (synonym *Cacopsylla costalis*) (Frisinghelli *et al.*, 2000 and Jarausch *et al.*, 2003) and *Cacopsylla melanoneura* (Tedeschi & Alma, 2004). In addition to psyllids, some other insects have been reported as vectors of '*Ca.* P. mali', including the spittlebug *Philaenus spumarius*, the leafhopper *Artianus interstitialis* (Hegab & El-Zohairy, 1986) and possibly *Fieberiella florii* (Krczal *et al.*, 1988).

A flow diagram describing the procedures for detection and identification is presented in Fig. 1.

2. Identity

Name: '*Candidatus* Phytoplasma mali'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*

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

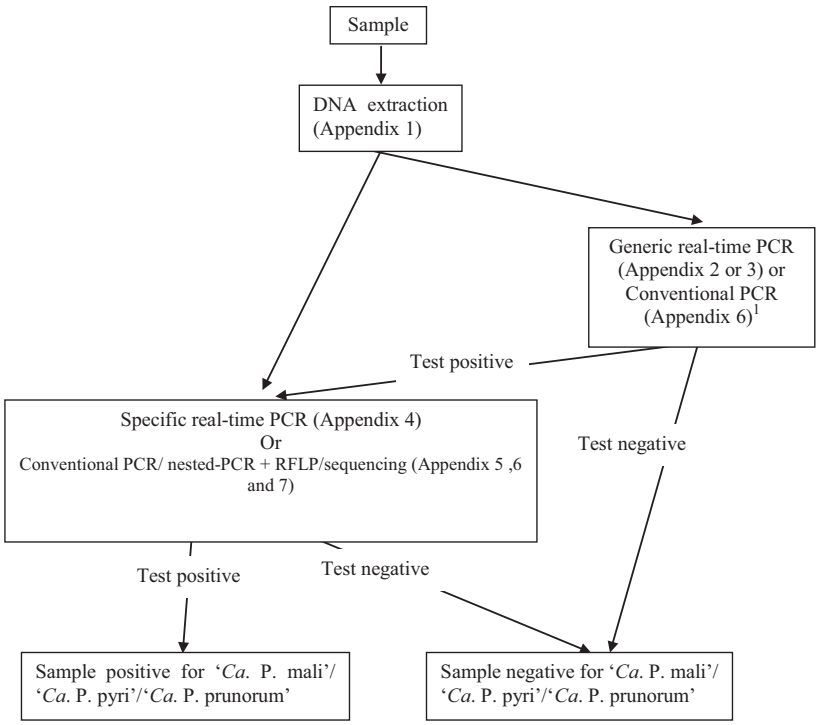

Fig. 1 Flow diagram for the detection and identification of '*Ca.* P. mali'/'*Ca.* P. pyri'/ '*Ca.* P. prunorum'. [1]Depending on the circumstances of use (e.g. imported plant material versus plant material tested for a specific phytoplasma survey) it may be useful to perform a generic test which would then identify presence of other phytoplasmas.

Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPMA
Phytosanitary categorization: EPPO A2 List no. 87; EU Annex designation I/A2

Name: '*Candidatus* Phytoplasma pyri'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPPY
Phytosanitary categorization: EPPO A2 List no. 95; EU Annex designation I/A2

Name: '*Candidatus* Phytoplasma prunorum'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPPR
Phytosanitary categorization: EU Annex designation I/A2

3. Detection

3.1 Disease symptoms

The severity of the disease depends on a number of factors including species, variety, rootstock and the age of the trees.

The distribution of phytoplasmas in the tree is uneven and is not constant over the year. It may vary from one year to the next, and in some years symptoms may not be observed. The distribution pattern in the tree is also dependent on temperature. In winter, the content of phytoplasmas in the aboveground part of the tree declines due to sieve-tube degeneration, and the phytoplasmas concentrate more in the roots. Phytoplasmas are detected in phloem tissues in shoots from midsummer to the end of sap flow. Detection on roots is possible throughout the year, although uneven distribution also applies here (Schaper & Seemüller, 1982; Seemüller *et al.*, 1984).

'Ca. P. mali'

The most typical symptom caused by '*Ca.* P. mali' is witches' broom at the end of shoots (Fig. 2). On diseased trees, leaves roll downward and become brittle, they are finely and irregularly serrated and are smaller than normal, with unusually enlarged stipules (Fig. 3). Fruits are smaller and flattened (Fig. 4), and peduncles longer. Early leaf reddening is a good indication of the presence of the disease. The presence of a fine hairy root system on nursery plants during winter may be another indication.

'Ca. P. pyri'

The most easily recognized symptoms occur in late summer with the development of premature autumn leaf colour on affected trees. Most cultivars develop a premature red colour (Figs 5 and 6), but some may develop a premature yellow colour. There may be some leaf cupping or curling and there is usually premature leaf drop. The following spring, affected trees suffer from weak growth and sparse pale foliage. The severity of the spring symptoms can vary from absence to death. There may be a line of necrotic tissue in the bark at the graft union between scion and rootstock.

Fig. 2 Witches' broom caused by apple proliferation (AP) is particularly evident in winter time. (Courtesy F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

Fig. 3 Leaves of AP infected trees (left) are smaller than normal ones (right) and have large stipules at the base of the stem. (Courtesy F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

'Ca. P. prunorum'

Typical symptoms are reddening and curling of leaves (Fig. 7), and sometimes lines of necrotic tissue in the bark (Fig. 8).

Possible confusion

The premature autumn leaf colour symptoms associated with apple proliferation, pear decline and European stone fruit yellows may also have several other causes. Water logging, root damage, ring barking caused by feeding animals, some bacterial cankers, rootstock and variety

Fig. 4 Apples cv. Jonagold from a healthy (left) and AP-infected tree (right). Infected fruits are undersized, misshapen and irregularly coloured. (Courtesy F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

Fig. 5 Reddening of the foliage with pear decline (left) and healthy tree (right) (EPPO Global Database).

incompatibility can all give rise to symptoms resembling those caused by phytoplasma infection.

3.2 Sampling and sample preparation

3.2.1 Sampling of asymptomatic plants

There is limited experience in the EPPO region with testing on asymptomatic plants. In Slovenia, testing in nurseries is performed on small roots sampled from at least three different root areas of the tree. Root parts should each be 10 cm long.

It should be noted that testing of roots for *Pyrus* species trees grafted on *Cydonia oblonga* is not recommended

Fig. 6 Reddening of the laminar tissue of tree with pear decline (EPPO Global Database).

Fig. 7 Characteristic leafroll symptoms on apricot. (Courtesy G. Morvan, INRA, Montfavet, FR.)

Fig. 8 Browning and necrosis of the middle layer of apricot bark (phloem) after a severe winter (Courtesy G. Morvan, INRA, Montfavet, FR.)

because this latter species is not sensitive to '*Ca.* P. pyri'. Testing of leaves as described in 3.2.2 can also be performed.

3.2.2 Sampling of symptomatic plants

Samples should be collected from shoots showing symptoms but in good condition (no necrotic areas) and not affected by other pests. Symptoms appear between June and October and the timing of appearance depends on the cultivar and the environment. The phytoplasmas may be unevenly distributed through the tree, requiring several different parts of the tree to be examined. It is advisable to examine the shoots from at least three different parts of the tree and collect a small branch from each part.

3.2.3 Sample preparation

Approximately 1–1.5 g of leaf mid-vein tissue and/or vascular tissue (phloem) from bark or roots should be randomly collected. For testing with real-time PCR, pooling of leaves, bark or roots collected from up to five plants is possible.

Material for testing should be used fresh or stored at −20°C (or lower depending on the storage time, e.g. −80°C for more than 2 years).

3.2.4 Vectors

Testing of vectors is only done for research purposes and is not described in this Standard.

3.3 Screening tests

3.3.1 Molecular methods

Different molecular methods for phytoplasma detection are available (Dickinson & Hodgetts, 2013); however, only two real-time PCR tests were included in the test performance study performed in 2011 in the framework of the Euphresco FruitPhytoInterlab project (EUPHRESCO FruitPhytoInterlab Group, 2011): real-time PCR tests developed by Christensen *et al.* (2004) and Hodgetts *et al.* (2009). DNA extraction is described in Appendix 1 and the two real-time PCR tests are described in Appendices 2 and 3, respectively. Positive samples should be further tested with one of the specific molecular tests (Fig. 1). A conventional PCR test with primers fU5/rU3, not evaluated during the Euphresco FruitPhytoInterlab project, is described in Appendix 6.

Phytoplasmas may occasionally be identified infecting plants other than their typical host; therefore depending on the circumstances of use (e.g. imported plant material versus plant material tested for a specific phytoplasma detection survey) it may be useful to perform a generic test (Appendices 2, 3 and 6) which would then detect other phytoplasmas.

3.3.2 Other tests

Testing on woody indicators and 4′,6-diamidino-2-phenylindole (DAPI) are methods recommended in PM 4/27 *Pathogen-tested material of* Malus, Pyrus (EPPO, 1999) *and* Cydonia and PM 4/30 *Certification scheme for almond, apricot, peach and plum* (EPPO, 2001). Such tests are mainly used in the framework of the production of certified material, not for routine testing.

4. Identification

4.1 Molecular methods

4.1.1 Molecular tests

In addition to the two generic real-time PCR tests mentioned in the previous section, a real-time PCR test for the specific detection of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (Appendix 4) and an AP group-specific nested PCR, with the primer pairs P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995), followed by group-specific PCR with f01/r01 (Lorenz *et al.*, 1995) (Appendix 5), were also included in the test performance study in 2011 (EUPHRESCO FruitPhytoInterlab Group, 2011). No relevant differences regarding sensitivity were observed between these tests. If the AP group-specific nested PCR test (Appendix 5) is used, the amplification product should be digested by the restriction enzymes *Ssp*I and *Bsa*AI/*Rsa*I for differentiation of '*Ca*. P. mali'/'*Ca*. P. pyri'/'*Ca*. P. prunorum' (Appendix 7). The primers f01/r01 may also be used in a conventional PCR test format, but analytical sensitivity is reduced.

A conventional PCR test with primers fU5/rU3 followed by restriction fragment length polymorphism (RFLP) is described in Appendices 6 and 7 respectively. This test was not evaluated during the EUPHRESCO FruitPhytoInterlab project. Its main advantage is to avoid the risks of cross contamination that may occur with a nested-PCR test, although it is known that analytical sensitivity with conventional PCR is reduced compared with nested-PCR.

Instead of RFLP analysis, the amplification product can also be sequenced and the resulting sequences can be compared to databases in order to identify the phytoplasma detected.

4.1.2 DNA barcoding

General procedures for DNA barcoding of phytoplasmas are described in an EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* including procedures for '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (EPPO, 2016).

5. Reference material

Institut national de la recherche agronomique, UMR GDPP Bordeaux, BP 81, 33883 Villenave D'Ornon Cedex, France.

http://www6.bordeaux-aquitaine.inra.fr/bfp_eng/Resources/Phytoplasmas-collection

Phytobacteriology Laboratory, Plant Pathology, DiSTA – *Alma Mater Studiorum* – University of Bologna, Italy (assunta.bertaccini@unibo.it). Phytoplasma Collection. International Phytoplasmologists Working Group. http://www.ipwgnet.org/index.php?option=com_content&view=article&id=29&Itemid=5

Sequences for different strains are available in Q-bank (http://www.q-bank.eu/Phytoplasmas/).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7. Performance criteria

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

8. Further information

Further information on these organisms can be obtained from:

Ms Bertacini, Phytobacteriology Laboratory, Plant Pathology, DiSTA – *Alma Mater Studiorum* – University of Bologna (UB) Italy, assunta.bertaccini@unibo.it

Mr Foissac UMR 1332 Biologie du Fruit et Pathologie, INRA–Bordeaux Aquitaine, 33882 Villenave d'Ornon Cedex, France, xavier.foissac@inra.fr

Mr Jarausch, Institut für Molekulare und Angewandte Pflanzenforschung Rheinland-Pfalz, RLP AgroScience GmbH, Breitenweg 71, 67435 Neustadt-an-der-Weinstrasse, Germany, wolfgang.jarausch@agroscience.rlp.de

9. Feedback on this Diagnostic Protocol

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

10. Protocol revision

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

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

11. Acknowledgements

The protocol for '*Ca*. P. mali' was originally drafted by: F. Costard, Ministère de l'Agriculture, Service de la Protection des Végétaux, Unité de virologie des ligneux, Villenave d'Ornon (France). The protocol for '*Ca*. P. pyri' was originally drafted by: D. L. Davies, Horticulture Research International, East Malling (UK). This revision (common protocol and addition of '*Ca*. P. prunorum') was prepared

by N. Mehle, National Institute of Biology, Večna pot 111, 1000 Ljubljana (Slovenia) (natasa.mehle@nib.si), and M. Loiseau, ANSES-LSV Plant Health Laboratory, 49044 Angers (France) (marianne.loiseau@anses.fr).

The revision was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

12. References

Carraro L, Ferrini F, Ermacora P & Loi N (2004) Transmission of European stone fruit yellows phytoplasma to *Prunus* species by using vector and graft transmission. *Acta Horticulturae* **657**, 449–453.

Carraro L, Loi N, Ermacora P, Gregoris A & Osler R (1998c) Transmission of pear decline by using naturally infected *Cacopsylla pyri* L. In: *Prociding of 17th International Symposium on Fruit Tree Virus Diseases* (Ed. Hadidi A). Acta Horticulturae, vol. 472, pp. 665–668.

Carraro L, Loi N, Ermacora P & Osler R (1998a) High tolerance of European plum varieties to plum leptonecrosis. *European Journal of Plant Pathology* **104**, 141–145.

Carraro L & Osler R (2003) European stone fruit yellows: a destructive disease in the mediterranean basin. In: *Virus and Virus-Like Diseases of Stone Fruits, with Particular Reference to the Mediterranean Region* (Ed. Myrta A, Di Terlizzi B & Savino V). *CIHEAM. Options Mediterraneennes Serie B*, Vol. 45, pp. 113–117.

Carraro L, Osler R, Loi N, Ermacora P & Refatti E (1998b) Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. *Journal of Plant Pathology* **80**, 233–239.

Christensen NM, Nicolaisen M, Hansen M & Schulz A (2004) Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions* **17**, 1175–1184.

Christensen NM, Nyskjold H & Nicolaisen M (2013) Real-time PCR for universal phytoplasma detection and quantification. In: *Phytoplasma: Methods and Protocols, Methods in Molecular Biology*, vol. 938 (Ed. Dickinson M & Hodgetts J), pp. 245–252. Springer Science+Business Media, LLC.

Davies DL, Guise CM, Clark MF & Adams AN (1992) Parry's disease of pears is similar to pear decline and is associated with mycoplasma-like organisms transmitted by *Cacopsylla pyricola*. *Plant Pathology* **41**, 195–203.

Del Serrone P, La Starza S, Krystai L, Kölber M & Barba M (1998) Occurrence of apple proliferation and pear decline phytoplasmas in diseased pear trees in Hungary. *Journal of Plant Pathology* **80**, 53–58.

Deng S & Hiruki C (1991) Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of Microbiological Methods* **14**, 53–61.

Dickinson M & Hodgetts J (Eds) (2013) *Phytoplasma: Methods and Protocols, Methods in Molecular Biology*, vol. 938, 421 p. Springer Science+Business Media, LLC.

Doyle JJ & Doyle JI (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**, 13–15.

EPPO (1999) Certfication scheme for Malus, Pyrus and Cydonia. *EPPO Bulletin*, **29**, 239–252 (title of the current published version is Pathogen-tested material of Malus, Pyrus and Cydonia).

EPPO (2001) Certification scheme for almond, apricot, peach and plum. *EPPO Bulletin* **31**, 463–478.

EPPO (2016) PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bulletin* **46**, 501–537.

EUPHRESCO FruitPhytoInterlab Group (2011) European interlaboratory comparison and validation of detection methods for '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma prunorum' and '*Candidatus* Phytoplasma pyri': Preliminary results. *Bulletin of Insectology* **64**(Supplement), S281–S284.

Fleiss JL, Levin B & Paik MC (2003) *Statistical Methods for Rates and Proportions*, 3rd edn. John Wiley and Son, New York, NY (US).

Frisinghelli C, Delaiti L, Grando MS, Forti D & Vindimian ME (2000) *Cacopsylla costalis* (Flor, 1861), as a vector of apple proliferation in Trentino. *Journal of Phytopathology* **148**, 425–431.

Hegab AM & El-Zohairy MM (1986) Retransmission of mycoplasma-like bodies associated with apple proliferation disease between herbaceous plants and apple seedlings. *Acta Horticulturae* **193**, 343.

Hodgetts J, Boonham N, Mumford R & Dickinson M (2009) Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas. *Applied Environmental Microbiology* **75**, 2945–2950.

Jarausch W, Eyquard JP, Mazy K, Lansac M & Dosba F (1999) High level of resistance of sweet cherry (*Prunus avium* L.) towards European stone fruit yellows phytoplasmas. *Advances in Horticultural Science* **13**, 108–112.

Jarausch B, Schwind N, Jarausch W, Krczal G, Dickler E & Seemüller E (2003) First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. *Plant Disease* **87**, 101.

Krczal G, Krczal H & Kunze L (1988) *Fieberiella florii* (Stål), a vector of apple proliferation agent. *Acta Horticulturae* **235**, 99–106.

Landis JR & Koch GG (1977) The measurement of observer agreement for categorical data. *Biometrics* **33**, 159–174.

Lee IM, Bertaccini A, Vibio M & Gundersen DE (1995) Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* **85**, 728–735.

Lorenz KH, Schneider B, Ahrens U & Seemüller E (1995) Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non-ribosomal DNA. *Phytopathology* **85**, 771–776.

Marcone C, Jarausch B & Jarausch W (2010) '*Candidatus* phytoplasma prunorum', the causal agent of European stone fruit yellows: an overview. *Journal of Plant Pathology* **92**, 19–34.

Mehle N, Brzin J, Boben J, Hren M, Frank J, Petrovič N *et al.* (2007) First report of '*Candidatus* Phytoplasma mali' in *Prunus avium*, *P. armeniaca* and *P. domestica*. *Plant Pathology* **56**, 721.

Mehle N, Nikolić P, Gruden K, Ravnikar M & Dermastia M (2013b) Real-time PCR for specific detection of three phytoplasmas from the apple proliferation group. In: *Phytoplasma: Methods and Protocols, Methods in Molecular Biology*, vol. 938 (Ed. Dickinson M & Hodgetts J), pp. 269–281. Springer Science+Business Media, LLC.

Mehle N, Nikolić P, Rupar M, Boben J, Ravnikar M & Dermastia M (2013a) Automated DNA extraction for large numbers of plant samples. In: *Phytoplasma: Methods and Protocols, Methods in Molecular Biology*, vol. 938 (Ed. Dickinson M & Hodgetts J), pp. 139–145. Springer Science+Business Media, LLC.

Nikolić P, Mehle N, Ravnikar M, Gruden K & Dermastia M (2010) A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. *Mollecular and Cellular Probes* **24**, 303–309.

Palmano S (2001) A comparison of different phytoplasma DNA extraction methods using competitive PCR. *Phytopathologia mediterranea* **40**, 99–107.

Pastore M, Raffone E, Santonastaso M, Priore R, Paltrinieri S, Bertaccini A *et al.* (2004) Phytoplasma detection in *Empoasca decedens* and *Empoasca* spp. and their possible role as vectors of European stone fruit yellows (16SrX-B) phytoplasma. *Acta Horticulturae* **657**, 507–511.

Papayiannis LC, Harkou IS, Markou YM, Demetriou CN & Katis NI (2011) Rapid discrimination of Tomato chlorosis virus, Tomato infectious chlorosis virus and co-amplification of plant internal control using real-time RT-PCR. *Journal of Virological Methods* **176**, 53–59.

Roberts RJ, Vincze T, Posfai J & Macelis D (2010) RESBASE-a data base for DNA restriction and modification: enzymes, genes and genomes. *Nucleic Acids Research* **38**, 234–236.

Schaper U & Seemüller E (1982) Condition of the phloem and the persistence of mycoplasma-like organisms associated with apple proliferation and pear decline. *Phytopathology* **72**, 736–742.

Schneider B, Seemüller E, Smart CD & Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology* (Ed. Razin S), pp. 369–380. Academic Press, San Diego, CA (US).

Seemüller E, Schaper U & Zimbelmann R (1984) Seasonal variation in the colonization patterns of mycoplasma-like organisms associated with apple proliferation and pear decline. *Zeitschrift für Pflanzenkrankenheiten und Pflanzenschutz* **91**, 371–382.

Seemüller E & Schneider B (2004) '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1217–1226.

Tedeschi R & Alma A (2004) Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura* (Homoptera: Psyllidae). *Journal of Economic Entomology* **97**, 8–13.

Weller SA, Elphinstone JG, Smith NC, Boonham N & Stead DE (2000) Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology* **66**, 2853–2858.

Appendix 1 – DNA extraction from plant material

CTAB procedure (modified from Doyle & Doyle, 1990)

Several methods have been developed and compared (Palmano, 2001). The method described below is an optimization of a method described by Doyle & Doyle (1990) for extraction of DNA from woody plants.

Nucleic acids can be extracted from fresh or frozen (−20 or −80°C) tissues [leaf veins, vascular tissue (phloem) from bark or roots].

Grind 1 g of tissue in 10 mL of 3% CTAB buffer [3% cetyl-trimethyl-ammonium bromide (CTAB) in 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl] at room temperature. Transfer 1 mL of the suspension to an Eppendorf tube, add 2 µL of 2-mercaptoethanol (for a final concentration of 0.2%). Vortex briefly and incubate for 20 min at 65°C. Then, add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10 000 *g* for 10 min. Recover the aqueous phase and precipitate the nucleic acids with an equal volume of cold isopropanol. Shake by inversion and centrifuge at 10 000 *g* for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, air dry and dissolve in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) or nuclease-free water.

Alternative method

Another DNA extraction method applicable to a large number of plant samples combines a simple and quick homogenization step of crude extracts with DNA extraction based on the binding of DNA to magnetic beads. This extraction method has been validated in combination with the PCR tests described in Appendices 2–4. It has also been used with other molecular tests (nested PCR and loop-mediated isothermal amplification, LAMP) and performed well; however validation data has not yet been published (Mehle N., pers. comm., 2016).

One gram of leaf mid-vein tissue or vascular tissue (phloem) from bark or roots is homogenized in 2 mL of extraction buffer (264 mM Tris, 236 mM Tris-HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05% Tween 20, pH 8.2) or lysis buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile) using tissue homogenizer (e.g. FastPrep®-24 with TN 12 × 15-TeenPrep™ Adapter, MP Biochemicals). Alternative grinding procedures include with liquid nitrogen using a mortar and pestle or homogenization in extraction bags using a Homex 6 homogenizer (BIOREBA).

Total DNA can be reliably extracted using a QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher® mL, Thermo Scientific) (Mehle *et al.*, 2013a).

Total DNA extract is eluted in 200 µL of elution buffer (QuickPick™ SML Plant DNA kit + KingFisher). For leaf mid-vein tissue and bark/root phloem tissue tenfold diluted DNA is suitable for testing.

Extracted total DNA can be kept at −20°C.

Other extraction methods may be used but should be validated in combination with the PCR test to be used.

Appendix 2 – Real-time PCR for the generic detection of phytoplasmas (Christensen *et al.*, 2004)

1. General information

1.1 The following real-time PCR protocol is performed for the detection of phytoplasmas.

1.2 The test was developed by Christensen *et al.* (2004) and the test description was published by Christensen *et al.* (2013).

1.3 Primers and probes were designed within the 16S rDNA. Probe and primers were based on alignments of 16S rDNA obtained from GenBank from a range of phytoplasma strains (one of each phytoplasma 16Sr group), bacteria and mycoplasmas. This test is considered as generic, although validation data is not available for all phytoplasmas.

1.4 Forward primer 5′ CGTACGCAAGTATGAAACTTAAAGGA 3′; reverse primer 5′ TCTTCGAATTAAACAACATGATCCA 3′; probe 5′ FAM-TGACGGGACTCCGCACAAGCG-TAMRA 3′.

1.5 The test performance study (Euphresco: FruitPhytoInterlab) was performed with a TaqMan Universal PCR Master Mix from Applied Biosystems.

1.6 Validation data has been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct). The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, vary between instrument models. The specific instrument manual should be consulted. When analysing the raw data it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. At the log view, this is the linear increase of fluorescence in the amplification plot.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 DNA extraction methods that are described in Appendix 1 may be used.

2.2 Real-time PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)*	Final concentration
Molecular-grade water†	N.A.	1.4	N.A.
TaqMan Universal PCR Master Mix (Applied Biosystems, containing UNG‡)	2×	5.0	1×
Forward primer	10 µM	0.3	0.3 µM
Reverse primer	10 µM	0.9	0.9 µM
Probe	2.5 µM	0.4	0.1 µM
Subtotal		8.0	
DNA		2.0	
Total		10.0	

*If a 25-µL reaction volume is used, multiply each component by 2.5.
†Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.
‡UNG or UDG (uracil-DNA glycosylase).

2.2.2 Real-time PCR conditions: UNG activation step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 cycles consisting of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[2]). The PAC should preferably be near the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example a plant cytochrome oxidase gene (Weller *et al.* 2000, Papayiannis *et al.* 2011).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: the same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Verification of the controls

- The PIC and PAC (as well as IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it produces no exponential amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

[2]Laboratories should take additional care to prevent risks of cross contamination when using cloned PCR products

4. Performance criteria available

Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab), in which the 10 participating laboratories analysed a total of 30 blind samples. This consisted of samples from 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca*. P. mali', 5 samples infected by '*Ca*. P. prunorum' and 5 samples infected by '*Ca*. P. pyri'. In five participating laboratories analytical sensitivity was also tested using a serial dilution of cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' at concentration of 10^7 to 10^1.

4.1 Sensitivity data

Diagnostic sensitivity: 100%
Analytical sensitivity for '*Ca*. P. mali': down to 10^1
Analytical sensitivity for '*Ca*. P. pyri': down to 10^1–10^2

4.2 Specificity data

Diagnostic specificity: 96%

4.3 Data on repeatability

Not available

4.4 Data on reproducibility

Agreement between laboratories – measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003): 0.926.[3]

Appendix 3 – Real-time PCR for the generic detection of phytoplasmas (Hodgetts *et al.*, 2009)

1. General information

1.1 The test was developed by Hodgetts *et al.* (2009).
1.2 Primers and probes were designed within 23S rDNA. This test is considered as generic, although validation data is not available for all phytoplasmas.
1.3 Forward primer JH-F1: 5′ GGTCTCCGAATGGGAAAACC 3′; forward primer JH-F all: 5′ ATTTCCGAATGGGGCAACC 3′; reverse primer JH-R: 5′ CTCGTCACTACTACCRGAATCGTTATTAC 3′; probe JH-P uni: 5′ FAM-AACTGAAATATCTAAGTAAC-MGB 3′.
1.4 The test performance study (Euphresco: FruitPhytoInterlab) was performed with a TaqMan Universal PCR Master Mix from Applied Biosystems.
1.5 Validation data has been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct). The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, vary between instrument models. The specific instrument manual should be consulted. When analysing the raw data it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. At the log view, this is the linear increase of fluorescence in the amplification plot.

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 DNA extraction methods that are described in Appendix 1 may be used.
2.2 Real-time PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)*	Final concentration
Molecular-grade water†	N.A.	1.7	N.A.
TaqMan Universal PCR Master Mix (Applied Biosystems), containing UNG‡	2×	5.0	1×
Forward primer (JH-F1)	10 μM	0.3	0.3 μM
Forward primer (JH-F all)	10 μM	0.3	0.3 μM
Reverse primer (JH-R)	10 μM	0.3	0.3 μM
Probe (JH-P uni)	2.5 μM	0.4	0.1 μM
Subtotal		8.0	
DNA		2.0	
Total		10.0	

*If a 25-μL reaction volume is used, multiply each component by 2.5.
†Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free.
‡UNG or UDG (uracil-DNA glycosylase).

2.2.2 Real-time PCR conditions: uracil *N*-glycosylase activation step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.

[3]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977)

- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[4]). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the external positive controls (PIC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example a plant cytochrome oxidase gene (e.g. Weller *et al.* 2000, Papayiannis *et al.* 2011) or eukaryotic 18S rDNA (AB Kit cat. no. 4319413E).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: the same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results:

Verification of the controls

- The PIC and PAC (as well as IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it produces no exponential amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab), where the 12 participating laboratories analysed a total of 30 blind samples This consisted of samples from 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca.* P. mali', 5 samples infected by '*Ca.* P. prunorum' and 5 samples infected by '*Ca.* P. pyri'.

4.1 Sensitivity data

Diagnostic sensitivity: 99.4%

4.2 Specificity data

Diagnostic specificity: 97.2%

4.3 Data on repeatability

Not available

4.4 Data on reproducibility

Agreement between laboratories measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003): 0.945.[5]

Appendix 4 – Real-time PCR for specific detection of '*Ca.* P. mali', '*Ca.* P. prunorum', and '*Ca.* P. pyri'

1. General information

1.1 The following real-time PCR protocol is performed for the detection and identification of '*Ca.* P. mali', '*Ca.* P. prunorum' and '*Ca.* P. pyri'.

1.2 The test was developed by Nikolić *et al.* (2010) and was published by Mehle *et al.* (2013b).

1.3 Primers and probes were designed within a variable region of the intergenic spacer region (IGS) between 16S and 23S rDNA. To design the specific sets of primers/probes the following nucleotide sequences with accession numbers were used: for '*Ca.* P. mali' (AF248958, AJ430067, AJ542541, AJ542542, APU54985, AY598319, CU469464, EF392654, EF392655, EF392656, EU168781, X68375), for '*Ca.* P. prunorum' (AJ542544, AJ542545, AJ575105, AJ575106, AJ575107, AM933142, AY029540, EF560638, EF560639, EF560640, EF560641, EF560642, EF560643, EF560644, EF560645, EF560646, ESU54988, EU168783, Y11933) and for '*Ca.* P. pyri' (AJ542543, AJ964959, DQ011588, PDU54989).

1.4 The AP amplicon covers a 147-bp region of the IGS of '*Ca.* P. mali', corresponding to nucleotides 1608–1754 in the isolate with accession number AJ542541. The ESFY amplicon covers a 147-bp region of the IGS of '*Ca.* P. prunorum', corresponding to nucleotides 1608–1754 in the isolate with accession number AJ542544.

[4]Laboratories should take additional care to prevent risks of cross contamination when using cloned PCR products

[5]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977)

The PD amplicon covers a 146-bp region of the IGS of '*Ca.* P. pyri', corresponding to nucleotides 1609–1754 in the isolate with accession number AJ542543.

1.5 Primer pairs are identical for all three species-specific assays: forward primer 5′ TGGTTAGAGCACACGCCTGAT 3′; reverse primer 5′ TCCACTGTGCGCCCTTAATT 3′. AP-specific probe: 5′ FAM-CAAAGTATTTATCTTAAGAAAACAAGC T-MGB 3′, ESFY-specific probe: 5′ FAM-CAAAATATTTATTTTAAAAAAACAAGCTC-MGB 3′, and PD-specific probe: 5′ FAM-AATATTTATTTTAAAAAAAGCTCTTTG-MGB 3′.

1.6 The test has been successfully performed using the Maxima Probe qPCR master mix (Fermentas) reagent and on a range of different real-time PCR systems including ABI (7900, 7900HT Fast, ViiA™7).

1.7 Validation data has been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (Ct). The transformation of the fluorescence signal into Ct data, as well as methods for baseline and threshold settings, vary between instrument models. The specific instrument manual should be consulted. When analysing the raw data it is important to adjust the cycle threshold (Ct) of the amplification plot to within the geometric (exponential) phase of amplification, preferably at the beginning of the geometric phase. At the log view, this is the linear increase of fluorescence in the amplification plot. It has been experimentally determined that an automatic baseline and threshold at 0.065 are usually suitable when using Maxima Probe qPCR master mix (Fermentas) and Applied Biosystems thermal cyclers 7900 or 7900HT Fast, while a threshold at 0.003 is usually suitable when using the thermal cycler ViiA™7.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 DNA extraction methods that are described in Appendix 1 may be used.

2.2 Real-time PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)*	Final concentration
Molecular-grade water†	N.A.	0.84	N.A.
Maxima™ qPCR master mix (Fermentas) containing UNG‡	2×	5.0	1×
Forward primer	10 µM	0.9	0.9 µM
Reverse primer	10 µM	0.9	0.9 µM
AP-, ESFY- or PD-specific probe	2.5 µM	0.36	0.09 µM
Subtotal		8.0	
DNA dilution		2.0	
Total		10.0	

*If a 25-µL reaction volume is used, multiply each component by 2.5.
†Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.
‡UNG or UDG (uracil-DNA glycosylase).

2.2.2 Real-time PCR conditions: UNG pre-treatment step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 cycles consisting of 15 s at 95°C and 1 min at 60°C.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[6]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

[6]Laboratories should take additional care to prevent risks of cross contamination when using cloned PCR products

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: the same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results:

Verification of the controls

- The PIC and PAC (as well as IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it produces no exponential amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

For amplicon names used in this section see point 1.4.

4.1 Analytical sensitivity data

- Validation data available from the National Institute of Biology (SI):

Determined using nonlinear modelling (programming environment R) based on the Ct value of the target dilutions in a positive sample.

The following dilutions of the sample DNA were tested (given the average Ct value at 10^1 given in brackets):

'*Ca.* P. mali' (22.5): 10^1 × diluted up to 10^7 × diluted

'*Ca.* P. prunorum' (25.7): 10^1 × diluted up to 10^7 × diluted

'*Ca.* P. pyri' (25.7): 10^1 × diluted up to 10^6 × diluted

Analytical sensitivity is represented as Ct values with 95% probability of detection (the dilution of sample DNA is given in parentheses):

AP amplicon: 34.1 (between $10^4$and 10^5)

ESFY amplicon: 33.2 (between 10^3 and 10^4)

PD amplicon: 37.8 (between 10^4 and 10^5)

- Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab):

The 12 (AP and PD amplicon)/13 (ESFY amplicon) participating laboratories analysed a total of 30 blind samples. The samples consisted of 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca.* P. mali', 5 samples infected by '*Ca.* P. prunorum' and 5 samples infected by '*Ca.* P. pyri'.

4.2 Diagnostic sensitivity

AP amplicon: 100%

ESFY amplicon: 100%

PD amplicon: 100%

In three (for '*Ca.* P. mali')/four (for '*Ca.* P. pyri') participating laboratories analytical sensitivity was also tested using a serial dilution of a cloned P1/P7 fragments from '*Ca.* P. mali' and '*Ca.* P. pyri' at concentration of 10^7 to 10^1.

Analytical sensitivity for '*Ca.* P. mali': 10^1

Analytical sensitivity for '*Ca.* P. pyri': 10^1

4.3 Analytical specificity data

- Validation data available from the National Institute of Biology (SI):

In silico analysis indicated no significant sequence homology with non-targets.

Number of strains of targets tested:

AP amplicon: 69

ESFY amplicon: 50

PD amplicon: 31

Number of non-targets tested:

AP amplicon: 154[7]

ESFY amplicon: 178[7]

PD amplicon: 164[7]

Percentage of accurate results:

AP amplicon: 100%

ESFY amplicon: 100%

PD amplicon: 100%

- Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab):

The 12 (AP and PD amplicon)/13 (ESFY amplicon) participating laboratories analysed a total of 30 blind samples. The samples consisted of 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca.* P. mali', 5 samples infected by '*Ca.* P. prunorum' and 5 samples infected by '*Ca.* P. pyri'.

4.4 Diagnostic specificity:

AP amplicon: 98.7%

ESFY amplicon: 93.8%

PD amplicon: 99.7%

4.5 Data on repeatability

- Validation data available from the National Institute of Biology (SI):

[7]Twenty-eight bacterial isolates that can be present as epiphytes or saprophytes on fruit trees, other phytoplasma strains cultivated in *Catharanthus roseus* and *Pennisetum purpureum*, DNA extracted from field samples of *Vitis vinifera* and from leaf veins or root phloem tissue of symptomatic and asymptomatic fruit trees, diagnostically predetermined to be infected with other phytoplasmas.

High target phytoplasma concentration:

AP amplicon (five samples with average Ct 22–28.1; no. of repeats: 5 × 5): 100% positive repeats

ESFY amplicon (five samples with average Ct 23–27.2; no. of repeats: 5 × 5): 100% positive repeats

PD amplicon (six samples with average Ct 25–31.8; no. of repeats: 6 × 5): 100% positive repeats

Medium target phytoplasma concentration:

AP amplicon (three samples with average Ct 28.1–31.1; no. of repeats: 3 × 5): 100% positive repeats

ESFY amplicon (two samples with average Ct 27.2–30.2; no. of repeats: 2 × 5): 100% positive repeats

PD amplicon (three samples with average Ct 31.8–34.8; no. of repeats: 3 × 5): 100% positive repeats

Low target phytoplasma concentration:

AP amplicon (three samples with average Ct 31.1–34.1; no. of repeats: 3 × 5): 100% positive repeats

ESFY amplicon (four samples with average Ct 30.2–33.2; no. of repeats: 4 × 5): 100% positive repeats

PD amplicon (four samples with average Ct 34.8–37.8; no. of repeats: 4 × 5): 95% positive repeats

4.6 Data on reproducibility

- Validation data available from the National Institute of Biology (SI):

Testing was done on 45 (AP amplicon)/37 (ESFY amplicon)/18 (PD amplicon) different days, with four (AP and PD amplicon)/five (ESFY amplicon) different operators and with two (ESFY and PD amplicon) different devices. Percentage of identical results:

Sample with medium target phytoplasma concentration:

AP amplicon (average Ct 28.4): 100%

ESFY amplicon (average Ct 27.3): 100%

PD amplicon (average Ct 31.8): 100%

Sample with low target phytoplasma concentration:

AP amplicon (average Ct 33.7): 100%

ESFY amplicon (average Ct 31.8): 100%

PD amplicon (average Ct 36.0): 89%

- Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab):

The 12 (AP and PD amplicon)/13 (ESFY amplicon) participating laboratories analysed a total of 30 blind samples. In particular: 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca*. P. mali', 5 samples infected by '*Ca*. P. prunorum' and 5 samples infected by '*Ca*. P. pyri'. Agreement between laboratories – measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003):

AP amplicon: 0.924[8]

ESFY amplicon: 0.84[8]

PD amplicon: 0.98[8]

[8]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977)

4.7 Other performance criteria available

The full validation data and report on the critical points in the diagnostic process and relating to uncertainty of measurement are available from the National Institute of Biology (SI) (see validation data deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php.)

Appendix 5 – AP group-specific nested PCR

1. General information

1.1 The following nested PCR protocol is performed for the detection and identification of AP group (16SrX) phytoplasmas.

1.2 Two sets of primers are used: P1/P7 primers (Deng & Hiruki, 1991; Schneider *et al.*, 1995) for first PCR and f01/r01 (Lorenz *et al.*, 1995) for second PCR (nested PCR). The first step confirms the presence of a phytoplasma while the second step (nested PCR) is specific for 16SrX group phytoplasmas.

1.3 The P1/P7 primers amplify the whole length of 16S and intergenic 16S–23S and a small part of 23S rRNA gene (1850 bp).

1.4 The amplicon size of nested PCR (f01/r01) is around 1100 bp.

1.5 Oligonucleotides:

P1: 5′-AAGAGTTTGATCCTGGCTCAGGATT-3′

P7: 5′-CGTCCTTCATCGGCTCTT-3′

f01: 5′- CGGAAACTTTTAGTTTCAGT-3′

r01: 5′- AAGTGCCCAACTAAATGAT-3′

1.6 The test performance study (Euphresco: FruitPhytoInterlab) was performed with a GoTaq DNA polymerase from Promega.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 DNA extraction methods that are described in Appendix 1 may be used.

2.2 Conventional PCR, followed by nested PCR

2.2.1 Master mix for PCR

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	16.375	N.A.
Green GoTaq Reaction buffer (Promega)	5×	5	1×
dNTPs (Promega)	10 mM	0.5	0.2 mM

(continued)

Table *(continued)*

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Forward primer (P1)	10 µM	1	0.4 µM
Reverse primer (P7)	10 µM	1	0.4 µM
GoTaq DNA polymerase (Promega)	5 U μL^{-1}	0.125	0.625 U
Subtotal		24	
Genomic DNA extract		1	
Total		25	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 PCR conditions: initial denaturation step at 94°C for 2 min; 36 cycles consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; final extension at 72°C for 8 min.

2.2.3 Master mix for nested PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	16.375	N.A.
Green GoTaq Reaction buffer (Promega)	5×	5	1×
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer (f01)	10 µM	1	0.4 µM
Reverse primer (r01)	10 µM	1	0.4 µM
GoTaq DNA polymerase (Promega)	5 U μL^{-1}	0.125	0.625 U
Subtotal		24	
1/30 diluted P1/P7 PCR product		1	
Total		25	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.4 Nested PCR conditions: initial denaturation step at 94°C for 2 min; 38 cycles consisting of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C; final extension at 72°C for 8 min.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[9]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive control (PIC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example a plant cytochrome oxidase gene (e.g. Weller *et al.* 2000, Papayiannis *et al.* 2011) or eukaryotic 18S rDNA(AB kit cat no. 4319413E)

Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract (the same matrix spiked with nucleic acid from the target organism).

3.2 Interpretation of results:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC, PAC (and if relevant IC) should produce amplicons of the expected size (depending on whether the target, endogenous or exogenous nucleic acid is used).

When these conditions are met:

- A test will be considered positive if amplicons of nested PCR around 1100 bp are produced.
- A test will be considered negative, if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

[9]Laboratories should take additional care to prevent risks of cross contamination when using cloned PCR products

In case of positive results, RFLP analysis (Appendix 7) or sequencing of PCR product should be performed to identify the phytoplasma.

4. Performance criteria available

Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab), where the 20 participating laboratories analysed a total of 30 blind samples. In particular: 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca*. P. mali', 5 samples infected by '*Ca*. P. prunorum' and 5 samples infected by '*Ca*. P. pyri'. In two participating laboratories analytical sensitivity was also tested using a standards of a cloned P1/P7 fragment from '*Ca*. P. mali' and '*Ca*. P. pyri' in concentrations from 10^7 to 10^1.

4.1 Sensitivity data

Diagnostic sensitivity – an estimation of the ability of the method to detect the target: 99.3%
Analytical sensitivity for '*Ca*. P. mali': 10^1–10^3
Analytical sensitivity for '*Ca*. P. pyri': 10^1

4.2 Specificity data

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target: 97.7%

4.3 Data on repeatability

Not available

4.4 Data on reproducibility

Agreement between laboratories – measured by calculation of the Kappa coefficient (Fleiss *et al.*, 2003): 0.94[10].

Appendix 6 – Conventional PCR for the generic detection of phytoplasmas (Lorenz *et al.*, 1995)

1. General information

1.1 The following PCR protocol is performed for the detection of phytoplasmas.
1.2 The test was developed by Lorenz *et al.* (1995).
1.3 The fU5/rU3 primers amplify a part of the 16S rRNA gene.
1.4 The amplicon size of PCR is around 862 bp.
1.5 Oligonucleotides:
fU5: 5′-CGGCAATGGAGGAAACT-3′
rU3: 5′-TTCAGCTACTCTTTGTAACA-3′
1.6 The performance study was performed with Amplitaq DNA polymerase from Applied Biosystems.

[10]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977)

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 DNA extraction methods that are described in Appendix 1 may be used.
2.2 Conventional PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	29.94	N.A.
Reaction buffer (Applied Biosystems)	10×	4	1×
dNTPs	20 mM	0.5	0.25 mM
BSA	50 mg mL^{-1}	0.04	0.05 mg mL^{-1}
Forward primer (fU5)	100 µM	0.2	0.5 µM
Reverse primer (rU3)	100 µM	0.2	0.5 µM
Amplitaq DNA polymerase (Applied Biosystems)	5 U μL^{-1}	0.12	0.6 U
Subtotal		35	
Genomic DNA extract		5	
Total†		40	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.
†Total reaction volume is recommended for proceeding with RFLP analysis or sequencing.

2.2.2 PCR conditions: initial denaturation step at 94°C for 2 min; 40 cycles consisting of 20 s at 94°C, 20 s at 55°C and 1 min at 72°C; final extension at 72°C for 4 min.

3. Essential procedural information

3.1 Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of

the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[11]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive control PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example a plant cytochrome oxidase gene (e.g. Weller *et al.* 2000, Papayiannis *et al.* 2011) or eukaryotic 18S rDNA(AB Kit cat. no. 4319413E)

Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls
- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract (the same matrix spiked with nucleic acid from the target organism).

3.2 Interpretation of results

Verification of the controls
- NIC and NAC should produce no amplicons.
- PIC, PAC (and, if relevant, IC) should produce amplicons of the expected size (depending on whether the target, endogenous or exogenous nucleic acid is used).

When these conditions are met
- A test will be considered positive if amplicons of PCR around 862 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

In case of positive results, RFLP analysis (Appendix 7) or sequencing of PCR product should be performed to identify the phytoplasma.

4. Performance criteria available

Validation data available from the Plant Heath Laboratory of ANSES (FR).

NB: This validation data was not obtained in the framework of the Euphresco project FruitPhytoInterlab, consequently the sensitivity data for this test cannot be compared with those presented for the other tests. For phytoplasmas relative quantification is not possible when samples are not analysed together.

4.1 Sensitivity data

Diagnostic sensitivity – an estimation of the ability of the method to detect the target: 98.55%

Last level at 100% positive results: 10^{-4} to 10^{-5} (levels tested between 10^{-1} and 10^{-8} for 3 different positive DNA extracts diluted in healthy DNA extract; one PD, one ESFY and one AP)

4.2 Specificity data

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target: 88.46%

4.3 Data on repeatability

97.56%

4.4 Data on reproducibility

Not available

Appendix 7 – RFLP

1. General information

1.1 The amplification products of AP group-specific nested PCR (Appendix 5) or of conventional PCR (Appendix 6) may be digested by the restriction enzymes for differentiation of '*Ca*. P. mali'/'*Ca*. P. pyri'/'*Ca*. P. prunorum'.

1.2 The protocol was published by Schneider *et al.* (1995).

1.3 For differentiation of AP group phytoplasmas the endonucleases *Ssp*I and *Bsa*AI or *Rsa*I proved to be useful.

1.4 Different profiles are obtained with each enzyme and allow the identification of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'.

2. Methods

2.1 PCR

2.1.1 Nested PCR and conventional PCR are described in Appendices 5 and 6

2.1.2 PCR/nested PCR product can be kept at −20°C

2.1.3 RFLP reaction

[11]Laboratories should take additional care to prevent risks of cross contamination when using cloned PCR products

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	10.7 (or 5.7)†	N.A.
Restriction enzyme buffer (Promega/ Fermentas)	10×	2.0	1×
BSA (Promega)	100×	0.2	1×
Restriction enzyme *Ssp*I (Promega/Fermentas)	10 U μL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)†	
Total		20	
Molecular-grade water*	N.A.	10.9 (or 5.9)†	N.A.
Restriction enzyme buffer (Biolabs/ Fermentas)	10×	2.0	1×
Restriction enzyme *Bsa*AI (Biolabs/Fermentas)	10 U μL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)†	
Total		20	
Molecular-grade water*	N.A.	10.9 (or 5.9)†	N.A.
Restriction enzyme buffer (Promega‡/ Fermentas)	10×	2.0	1×
Restriction enzyme *Rsa*I (Promega‡/Fermentas)	10 U μL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)†	
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.
†Depends on the nested PCR product: strong or weak.
‡Validation data for *Rsa*I obtained with restriction enzymes from Promega with amplicon of PCR using fU5/rU3 primers (Appendix 6).

2.1.3.1 Reaction incubation: 37°C for 4 h. Digested PCR products are subject to electrophoresis on 2% agarose gel along with a DNA ladder to size fragments.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, NAC and PAC controls from nested PCR (see Appendix 5) should be included. Three different PACs (PAC for '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum') are recommended.

3.2 Interpretation of results:

Verification of the controls

- NAC should produce no profiles.
- PACs should produce expected profiles (see below).

When these conditions are met:

- A test will be considered positive for '*Ca*. P. mali' if *Ssp*I digests the amplicons and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below. Generally, two fragments are visible on the electrophoresis gel for this phytoplasma.
- A test will be considered positive for '*Ca*. P. prunorum' if amplicons are not digested by *Ssp*I, and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below.
- A test will be considered positive for '*Ca*. P. pyri' if amplicons are not digested by *Ssp*I, and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below.
- Tests should be repeated if any contradictory or unclear results are obtained.

Expected RFLP electrophoretic profiles (size of the fragments) obtained with the two different PCRs followed by RFLP with *Rsa*I, *Bsa*AI and *SspI* (virtual RFLP analysis with http://tools.neb.com/REBsites/index.php (Roberts *et al.*, 2010):

Identified phytoplasma		'*Ca.* P. mali' 16SrX	'*Ca.* P. prunorum' 16SrX	'*Ca.* P. pyri' 16SrX
The fragment length expected from sequences obtained after nested PCR – Appendix 5 (bp)	*Rsa*I	744		744
			392	
			351	
		249	249	249
		44*	44*	44*
		16*	16*	16*
		4*	4*	4*
	*Bsa*AI	766		766
			452	
		251	251	251
		40*	40*	40*
	*Ssp*I		1056	1056
		713		
		344		
The fragment length expected from sequences obtained after amplification with primers fU5/rU3 – Appendix 6 (bp)	*Rsa*I	449		449
			392†	
		363	363†	363
			58*	
		44*	44*	44*
		16*	16*	16*
		4*	4*	4*
	*Bsa*AI	511		511
			452	
		365	365	365
			60	
	*Ssp*I		876	876
		827		
		49*		

*Bands are not always visible on electrophoresis gel at these lengths.
†See Fig 9.

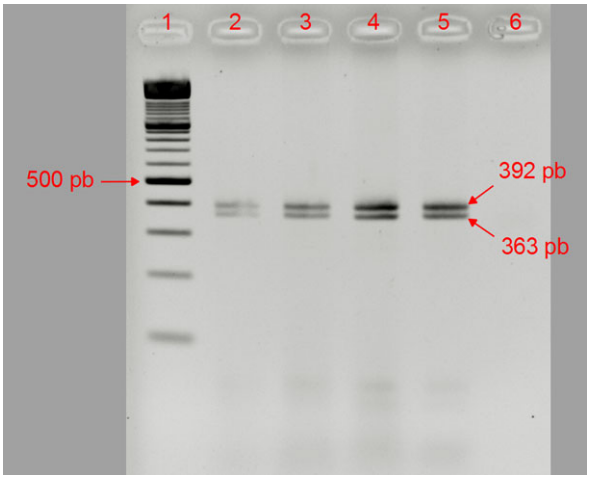

Fig. 9 Picture of a gel from a RFLP test with *Rsa*I after digestion of amplicons showing two distinct bands. 1, molecular marker 100 bp; 2 and 3, sample in duplicate; 4 and 5, positive amplification control in duplicate, DNA extract positive for '*Candidatus* Phytoplasma prunorum'; 6, negative amplification control, DNase-free water.

4. Performance criteria available

Not available for nested PCR followed by RFLP.

Validation data available from the Plant Heath Laboratory of ANSES (FR).Here, performance criteria are a result of generic PCR described in Appendix 6 followed RFLP described in this appendix.

4.1 Analytical sensitivity data

Last level at 100% positive results (levels tested between 1×10^{-1} and 1×10^{-8} for 3 different positive DNA extracts diluted in healthy DNA extract; one PD, one ESFY and one AP):

For '*Ca.* P. mali': 1×10^{-4}
For '*Ca.* P. prunorum': 1×10^{-4}
For '*Ca.* P. pyri': 1×10^{-5}

Diagnostic sensitivity – an estimation of the ability of the method to detect the target:

For '*Ca.* P. mali': 97.4%
For '*Ca.* P. prunorum': 100%
For '*Ca.* P. pyri': 96.7%

Last level at 100% positive results (levels tested between 1×10^{-1} and 1×10^{-8} for 3 different positive DNA extracts diluted in healthy DNA extract; one PD, one ESFY and one AP):

For '*Ca.* P. mali': 1×10^{-4}
For '*Ca.* P. prunorum': 1×10^{-4}
For '*Ca.* P. pyri': 1×10^{-5}

4.2 Analytical specificity data

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target

For '*Ca.* P. mali': 92.3%
For '*Ca.* P. prunorum': 91.7%
For '*Ca.* P. pyri': 100%

4.3 Data on repeatability

For '*Ca.* P. mali': 98.3%
For '*Ca.* P. prunorum': 100%
For '*Ca.* P. pyri': 97.8%

4.4 Data on reproducibility

Not available