



2004-018: Draft Annex to ISPM 27:2006 – *Phytoplasmas*

Comm no.	Para no.	Comment type	Comment	Explanation	Country
1.	G	Substantive	I support the document as it is and I have no comments		Lao People's Democratic Republic, Georgia, Thailand, New Zealand, Dominica, Mexico, Barbados, Canada, Ghana, OIRSA, Antigua and Barbuda, Malawi, Burundi, Belize, Gabon, Australia
2.	G	Substantive	<a href="#">Insert flow chart for detection of phytoplasma.</a>	This will be useful for understanding the procedure for detecting phytoplasma.	Japan
3.	G	Technical	<a href="#">The detection and diagnosis method using the DAPI stain method can be added as the first-step screening method after the symptom observation.</a>	Phytoplasma infections are characterized by an associated accumulation of DNA within the phloem, so infections can be demonstrated by treatment of plant tissues with the DNA-binding fluorochrome DAPI (4',6'-diamidino-2-phenylindole, 2HCl) on examination under UV light by fluorescence microscopy. Also, it is known that the DAPI stain method can be used for the large-scale diagnosis. In addition, this method does not need special expensive facilities and reagents, such as a PCR thermal cycler, DNA polymerase or related clean molecular test rooms It is suitable for the screening of suspected samples where it is not generally assumed that phytoplasma titre is extremely low (i.e., strongly suspected samples which show clear phytoplasma	Japan

Comm no.	Para no.	Comment type	Comment	Explanation	Country
				symptoms) or where there is a need to know quickly whether or not phytoplasma is present. Cited literature Seemüller E, 1976. Demonstration of mycoplasma-like organisms in the phloem of trees with pear decline or proliferation symptoms by fluorescence microscopy. <i>Phytopathologische Zeitschrift</i> , 85:368-372. Sinclair WA, Griffiths HM, Davis RE, Lee IM, 1992. Detection of ash yellows mycoplasma-like organisms in different tree organs and in chemically preserved specimens by a DNA probe vs. DAPI. <i>Plant Disease</i> , 76(2):154-158 Andrade N. M. & N. L. Arismendi, 2013, DAPI Staining and Fluorescence Microscopy Techniques for Phytoplasmas, <i>Phytoplasma Methods in Molecular Biology</i> , 938, pp.115-121.	
4.	8	Editorial	Phytoplasmas were first discovered by Doi <i>et al.</i> (1967) during their search for the agent of aster yellows. The unicellular organisms were called mycoplasma-like organisms because of their morphological similarity to animal mycoplasmas and their sensitivity to tetracycline antibiotics (Ishii <i>et al.</i> ,1967). Phytoplasmas are obligate prokaryotic plant pathogens that do not possess cell walls; are pleiomorphic in profile, with a mean diameter of 200–800 nm; and are non-motile. They inhabit the phloem sieve cells of their plant hosts. Phytoplasmas have genomes ranging in size from around 550 to 2 000 kb: they have a relatively small genome compared to other prokaryotes and lack several biosynthetic functions (Bai <i>et al.</i> , 2006; Marcone <i>et al.</i> , 1999; Oshima <i>et al.</i> , 2004).	spelling	EPPO, European Union, Georgia, Serbia
5.	8	Technical	Phytoplasmas were first discovered by Doi <i>et al.</i> (1967) during their search for the agent of aster yellows. The unicellular organisms were called mycoplasma-like organisms because of their morphological similarity to animal mycoplasmas and their sensitivity to tetracycline antibiotics (Ishii <i>et al.</i> ,1967). Phytoplasmas are obligate prokaryotic plant pathogens that do not possess cell walls; are pleiomorphic in profile, with a mean diameter of 200–800 nm; <del>and are non-motile.</del> They inhabit the phloem sieve cells of their plant hosts. Phytoplasmas have genomes ranging in size from around 550 to <del>2 000</del> <u>1 500</u> kb: they have a relatively small genome compared to other prokaryotes and lack several biosynthetic functions (Bai <i>et al.</i> , 2006; Marcone <i>et al.</i> , 1999; Oshima <i>et al.</i> , 2004).	1. Delete "and are non-motile", obvious. 2. Size of the genome is not more than 1500 kb. Namba <i>et al</i> 2005 (in <i>Mycoplasmas molecular biology pathogenicity and strategies for control</i> edited by Blanchard and Browning) refers to a genome ranging from 530 to 1350 Kbp.	EPPO, European Union, Georgia, Serbia
6.	8	Technical	Phytoplasmas were first discovered by Doi <i>et al.</i> (1967) during their search for the agent of aster yellows. The unicellular organisms were called mycoplasma-like organisms because of their morphological similarity to animal mycoplasmas and	This is an important reference for phytoplasma work.	United States of America

Comm . no.	Para . no.	Comment type	Comment	Explanation	Country
			their sensitivity to tetracycline antibiotics (Ishii <i>et al.</i> , 1967). Phytoplasmas are obligate prokaryotic plant pathogens that do not possess cell walls; are pleiomorphic in profile, with a mean diameter of 200–800 nm; and are non-motile. They inhabit the phloem sieve cells of their plant hosts. Phytoplasmas have genomes ranging in size from around 550 to 2 000 kb: they have a relatively small genome compared to other prokaryotes and lack several biosynthetic functions (Bai <i>et al.</i> , 2006; <a href="#">Davis et al. 2005</a> ; Marcone <i>et al.</i> , 1999; Oshima <i>et al.</i> , 2004).		
7.	9	Technical	Phytoplasmas are associated with a wide variety of symptoms in a diverse range of plant hosts (Lee <i>et al.</i> , 2000). Characteristic symptoms associated with of phytoplasma infection include virescence (the development of green flowers and the loss of normal flower pigments); phyllody (the development of floral parts into leafy structures); witches' broom (proliferation of auxiliary or axillary shoots) and other abnormal proliferation of shoots and roots; foliar yellowing, reddening and other discoloration; reduced leaf and fruit size; phloem necrosis; and overall decline and stunting ( <a href="#">Davis and Sinclair, 1998</a> ). Some plant species are tolerant or resistant to phytoplasma infections; these plants may be asymptomatic or exhibit mild symptoms (Lee <i>et al.</i> , 2000).	This is an important reference for phytoplasma symptoms.	United States of America
8.	11	Editorial	Geographically, phytoplasmas occur worldwide. The geographical distribution and impact of phytoplasma diseases depends on the host range of the phytoplasma as well as <a href="#">on the presence and</a> on the feeding behaviour of the insect vector. Some phytoplasmas have a broad range of plant hosts and polyphagous vectors and therefore have a wide distribution. <del>Conversely, many</del> <a href="#">Other</a> phytoplasmas have restricted host ranges and oligophagous or monophagous insect vectors, which restrict their geographical distribution. See Foissac and Wilson (2010) for a review of the geographical distribution of the main phytoplasma taxonomic groups.	Presence should be mentioned. Clearer wording.	EPPO, European Union, Georgia, Serbia
9.	12	Editorial	Phytoplasmas can be transmitted by insect vectors, dodder and grafting and can be spread by vegetative propagation of infected plant parts. Insect vectors of phytoplasmas, responsible for much of their natural spread, are restricted to phloem-feeding leafhoppers, plant hoppers and psyllids (Hemiptera, Auchenorrhyncha). They transmit the pathogen in a persistent manner. Weintraub and Beanland (2006) list more than 90 species that are known to be vectors, some of which are capable of vectoring more than one disease. Other methods of transmission of phytoplasmas include <del>via</del> dodder and <del>via grafting</del> <a href="#">transmission</a> . Dodders ( <i>Cuscuta</i> and <i>Cassytha</i> spp.) are parasitic vines that develop vascular connections with their hosts through haustoria. When a bridge is established between a healthy plant and a phytoplasma-infected plant, the phytoplasma will transfer to the healthy plant via the connecting phloem elements. Graft transmission and micropropagation of plants in tissue culture <del>can be used</del> <a href="#">are good ways</a> to maintain phytoplasmas for reference purposes.	Clearer with an additional comma in the last but one sentence. Other modifications for improved english.	EPPO, European Union, Georgia, Serbia

Comm . no.	Para . no.	Comment type	Comment	Explanation	Country
10.	12	Technical	Phytoplasmas can be transmitted by insect vectors, dodder and grafting and can be spread by vegetative propagation of infected plant parts. Insect vectors of phytoplasmas, responsible for much of their natural spread, are restricted to phloem-feeding leafhoppers, plant hoppers and psyllids (Hemiptera, Auchenorrhyncha). They transmit the pathogen in a persistent manner. Weintraub and Beanland (2006) list more than 90 species that are known to be vectors, some of which are capable of vectoring more than one <a href="#">phytoplasma disease</a> . Other methods of transmission of phytoplasmas include via dodder and via grafting. Dodders ( <i>Cuscuta</i> and <i>Cassytha</i> spp.) are parasitic vines that develop vascular connections with their hosts through haustoria. When a bridge is established between a healthy plant and a phytoplasma-infected plant the phytoplasma will transfer to the healthy plant via the connecting phloem elements. Graft transmission and micropropagation of plants in tissue culture are good ways to maintain phytoplasmas for reference purposes <a href="http://www.ipwgnnet.org/index.php?option=com_content&amp;view=article&amp;id=29&amp;Itemid=5">http://www.ipwgnnet.org/index.php?option=com_content&amp;view=article&amp;id=29&amp;Itemid=5</a> .	1. Vector do not transmit the disease but the phytoplasmas. 2. add a reference after the last sentence <a href="http://www.ipwgnnet.org/index.php?option=com_content&amp;view=article&amp;id=29&amp;Itemid=5">http://www.ipwgnnet.org/index.php?option=com_content&amp;view=article&amp;id=29&amp;Itemid=5</a>	EPPO, European Union, Georgia, Serbia
11.	12	Technical	Phytoplasmas can be transmitted by insect vectors, dodder and grafting and can be spread by vegetative propagation of infected plant parts. Insect vectors of phytoplasmas, responsible for much of their natural spread, are restricted to phloem-feeding leafhoppers, plant hoppers and psyllids (Hemiptera, Auchenorrhyncha). They transmit the pathogen in a persistent manner. Weintraub and Beanland (2006) list more than 90 species that are known to be vectors, some of which are capable of vectoring more than one disease. Other methods of transmission of phytoplasmas include via dodder and via grafting. Dodders ( <i>Cuscuta</i> and <i>Cassytha</i> spp.) are parasitic vines that develop vascular connections with their hosts through haustoria. When a bridge is established between a healthy plant and a phytoplasma-infected plant the phytoplasma will transfer to the healthy plant via the connecting phloem elements. Graft transmission and micropropagation of plants in tissue culture are good ways to maintain phytoplasmas for reference purposes.	In the second sentence of this paragraph, this list of known insect vectors should include these insects as well: 1. Mou, H.-Q., et al. 2013. Transcriptomic Analysis of Paulownia Infected by Paulownia Witches'-Broom Phytoplasma. PLoS ONE. Volume 8, Issue 10, 10 October 2013, Article number e77217 2. Hiruki, C. 1999. Paulownia witches'-broom disease important in East Asia. Acta Hort. 496: 63-68. 3. La, Y.J. 1968. Insect transmission of paulownia witches'-broom disease in Korea. Korea Observer 8:55-64.	United States of America
12.	14	Editorial	<a href="http://www.costphytoplasma.ipwgnnet.org/eu/index.htm">http://www.costphytoplasma.ipwgnnet.org/eu/index.htm</a>	The link to the website does not work any more. The COST program came to an end in September, 2013 and the website was transferred to the following address: <a href="http://www.costphytoplasma.ipwgnnet.org/">http://www.costphytoplasma.ipwgnnet.org/</a>	EPPO, European Union, Georgia, Serbia
13.	14	Substantive	<a href="http://www.costphytoplasma.eu/index.htm">http://www.costphytoplasma.eu/index.htm</a>	This link does not work.	Jamaica
14.	14	Substantive	<a href="http://www.costphytoplasma.eu/index.htm">http://www.costphytoplasma.eu/index.htm</a>	The page cannot be found.	Japan

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country
15.	14	Technical	<a href="http://www.costphytoplasma.eu/index.htm">http://www.costphytoplasma.eu/index.htm</a>	This link is no longer available. Suggest using: <a href="http://www.ipwgnnet.org/cost/index.htm">http://www.ipwgnnet.org/cost/index.htm</a>	United States of America
16.	20	Technical	Organisms that cannot be cultured <i>in vitro</i> are referred to as a ' <i>Candidatus</i> ' species (Murray and Stackebrandt, 1995). This has allowed the phytoplasmas to be assigned a provisional taxonomic status rather than be referred to by the name of the disease with which they are associated. Analysis of the sequence of 16S ribosomal (r)RNA genes provides the main basis for phytoplasma identification and classification.	Candidatus should be in simple quotes. This should be checked throughout the protocol.	EPPO, European Union, Georgia, Serbia
17.	20	Technical	Organisms that cannot be cultured <i>in vitro</i> are referred to as a <i>Candidatus</i> species (Murray and Stackebrandt, 1995). This has allowed the phytoplasmas to be assigned a provisional taxonomic status rather than be referred to by the name of the disease with which they are associated. Analysis of the sequence of 16S ribosomal (r)RNA genes provides the main basis for phytoplasma identification and classification.	Rewording of the first sentence: The convention of ' <i>Candidatus</i> ' species is used to refer to bacteria such as phytoplasmas that cannot be isolated and cultured <i>in vitro</i> (Murray and Stackebrandt, 1995).	United States of America
18.	21	Editorial	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a " <i>Candidatus</i> ( <i>Ca.</i> ) Phytoplasma" genus. Delineation of " <i>Ca.</i> Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $\geq 97.5\%$ similarity) in their 16S rRNA gene sequences. Descriptions of " <i>Ca.</i> Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 " <i>Ca.</i> Phytoplasma" species have been described.	"share a minimum of 97.5% similarity", so the symbol for "superior" should be replaced by the symbol for "superior or equal".	EPPO, European Union, Georgia, Serbia
19.	21	Editorial	The <a href="#">International Research Programme on Comparative Mycoplasmaology (IRPCM)</a> Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a " <i>Candidatus</i> ( <i>Ca.</i> ) Phytoplasma" genus. Delineation of " <i>Ca.</i> Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $>97.5\%$ similarity) in their 16S rRNA gene sequences. Descriptions of " <i>Ca.</i> Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 " <i>Ca.</i> Phytoplasma" species have been described.	Editrial correction	Japan
1.	21	Technical	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a " <i>Candidatus</i> ( <i>Ca.</i> ) Phytoplasma" genus. Delineation of " <i>Ca.</i> Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $\geq 97.5\%$	More complete description.	Georgia, Serbia

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			similarity, $\geq 1200$ nucleotides) in their 16S rRNA gene sequences. <a href="#">When a 'Candidatus' species includes phytoplasmas with different biological characteristics (vectors, host plants) they can be taxonomically distinguished following specific rules reported in IRPCM (2004).</a> Descriptions of "Ca. Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 "Ca. Phytoplasma" species have been described.		
21.	21	Technical	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a "Candidatus (Ca.) Phytoplasma" genus. Delineation of "Ca. Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $\geq 97.5\%$ similarity, $\geq 1200$ nucleotides) in their 16S rRNA gene sequences. <a href="#">When a 'Candidatus' species includes phytoplasmas with different biological characteristics (vectors, host plants) they can be taxonomically distinguished following specific rules reported in IRPCM (2004).</a> Descriptions of "Ca. Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 "Ca. Phytoplasma" species have been described.	More complete description.	EPPO
22.	21	Technical	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a "Candidatus (Ca.) Phytoplasma" genus. Delineation of "Ca. Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $>97.5\%$ similarity) in their 16S rRNA gene sequences. Descriptions of "Ca. Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 "Ca. Phytoplasma" species have been described.	Could the Acronym IRPCM be clarified.	Jamaica
23.	21	Technical	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a "Candidatus (Ca.) Phytoplasma" <del>genus</del> species. Delineation of "Ca. Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general, phytoplasmas within a species share a minimum of 97.5% similarity ( $>97.5\%$ similarity) in their 16S rRNA gene sequences. Descriptions of "Ca. Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 "Ca. Phytoplasma" species have been described.	Explanation for deletion of genus and substitution of species: The guidelines are for the description of species and not genus as in the original sentence.	United States of America
24.	21	Technical	The IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (2004) has published guidelines for the description of a "Candidatus (Ca.) Phytoplasma" genus. Delineation of "Ca. Phytoplasma" species is based on 16S rRNA gene sequences as well as on biological characteristics. In general,	More complete description.	European Union

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			phytoplasmas within a species share a minimum of 97.5% similarity (>97.5% similarity, <u>≥1200 nucleotides</u> ) in their 16S rRNA gene sequences. <u>When a 'Candidatus' species includes phytoplasmas with different biological characteristics (vectors, host plants) they can be taxonomically distinguished following specific rules reported in IRPCM (2004).</u> Descriptions of "Ca. Phytoplasma" species are published in the <i>International Journal of Systematic and Evolutionary Microbiology</i> and as of October 2013, 32 " <u>Ca. Phytoplasma</u> " species have been described.		
25.	22	Editorial	<b>3. Detection and Identification</b>  <u>Add a Flow chart after para.22..</u>	For more clarity.	China
26.	23	Technical	Polymerase chain reaction (PCR) techniques are the method of choice for phytoplasma detection. Successful molecular detection of phytoplasmas is dependent on appropriate sampling of plant tissue and reliable nucleic acid extraction methods (Firrao <i>et al.</i> , 2007; Palmano, 2001). Phytoplasmas can be unevenly distributed and in uneven titre throughout a plant, particularly in woody hosts, and symptomatic tissue is optimal for phytoplasma detection (Christensen <i>et al.</i> , 2004; Constable <i>et al.</i> , 2003; Garcia-Chapa <i>et al.</i> , 2003; Necas and Krska, 2006). Symptomless infection can occur in some plant hosts and if this is suspected it is important to thoroughly sample different tissues from the <del>one</del> plant for <u>DNA</u> phytoplasma isolation.	Phytoplasmas cannot be isolated as such; isolation of their DNA is more feasible.	EPPO, European Union, Georgia, Serbia
27.	25	Substantive	For most phytoplasma diseases, leaves with symptoms are the best sources of samples for diagnosis. Phytoplasmas reside almost exclusively in the phloem sieve elements of infected plants and therefore the cambium, petioles and midveins are used for DNA extraction. In some cases (e.g. X-disease phytoplasma), fruit peduncles contain the highest phytoplasma titre. Although phytoplasmas can be detected in roots and bark scrapings of dormant trees, generally it is best to test for phytoplasmas at the end of summer. Collected plant samples can be stored at -20 °C for up to 6 months prior to testing. Longer term storage is at -80 °C, or the plant material can be freeze-dried or dried over calcium chloride and stored at 4 °C.	Trunk should also be considered since one symptom of phytoplasma is the shedding of leaves in for example coconuts with Lethal Yellowing.	Jamaica
28.	25	Technical	For most phytoplasma diseases, leaves with symptoms are the best sources of samples for diagnosis. Phytoplasmas reside <del>almost exclusively</del> in the phloem sieve elements of infected plants and therefore the <del>cambium</del> , petioles and midveins are <u>often</u> used for DNA extraction. In some cases (e.g. X-disease phytoplasma), fruit peduncles contain the highest phytoplasma titre. Although phytoplasmas can be detected in roots and bark scrapings of dormant trees, generally it is best to test for phytoplasmas at the end of summer. Collected plant samples can be stored at -20 °C for up to 6 months prior to testing. Longer term storage is at -80 °C, or the plant material can be freeze-dried or dried over calcium	Second sentence: Note that the cambium consists of undifferentiated cells, and therefore does not contain any differentiated phloem cells of any kind. The cambium is a meristem, consisting of stem cells. Third sentence: This statement should be supported by a reference citation.	United States of America

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			chloride and stored at 4 °C.		
29.	26	Technical	<p>Various nucleic acid extraction methods have been reported for phytoplasma detection by PCR. A number of methods use an enrichment step to concentrate the phytoplasmas before nucleic acid extraction (Ahrens and Seemüller, 1992; Kirkpatrick <i>et al.</i>, 1987; Prince <i>et al.</i>, 1993). These techniques can be useful for hosts in which phytoplasmas are found in low titre, such as woody perennial plants, or for “difficult” hosts, from which high levels of compounds such as polysaccharides and polyphenols that can inhibit PCR are often co-extracted with the nucleic acid. In some simplified methods, plant tissue is ground directly in cetyltrimethyl ammonium bromide (CTAB)-based buffer or commercially available lysis buffers. Typically, a 2% CTAB buffer is used (it has been shown that a 3% solution is more reliable for grapevines) (Angelini <i>et al.</i>, 2001; Daire <i>et al.</i>, 1997). The DNA is then extracted directly from the lysate using commercially available silica spin columns (Green <i>et al.</i>, 1999; Palmano, 2001) or magnetic beads (Mehle <del>Boben</del> <i>et al.</i>, 2013<del>07</del>) or with organic solvents (Daire <i>et al.</i>, 1997; Zhang <i>et al.</i>, 1998). The method of using magnetic beads is generally performed on an automated nucleic acid extraction machine (e.g. KingFisher from Thermo Scientific). Most extraction methods are well validated for a variety of plant host species. The choice of method is dependent on the host being tested and the availability of facilities and equipment. It may be practical to use a method incorporating a phytoplasma enrichment step for woody perennial hosts and a simplified method for herbaceous hosts. For routine diagnostics it is important to validate an extraction method for a particular host to ensure reliability.</p>	<p>In Boben <i>et al.</i> (2007) no details about procedure are given. It would be better to include the following reference, because all details about procedure are given: Mehle N., Nikolić P., Rupar M., Boben J., Ravnikar M., Dermastia M. 2013. Automated DNA extraction for large numbers of plant samples. In: DICKINSON, Matthew (ed.), HODGETTS, Jennifer (ed.). Phytoplasma : methods and protocols, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press, 139-145</p>	EPPO, European Union, Georgia, Serbia
30.	27	Substantive	<p><a href="#">Review the reference of Christensen <i>et al.</i>, 2004 in line 5-6.</a></p> <p>A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used are the P1/P7 (Deng and Hiruki, 1991; Schneider <i>et al.</i>, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996; Lee <i>et al.</i>, 1993) primer pairs, which can be used as a nested-PCR assay. The P1/P7 primer pair amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. Real-time PCR has been reported to be as sensitive as nested-PCR (Christensen <i>et al.</i>, 2004) and is more amenable to high throughput analysis because post-amplification processing is not required. Real-time PCR using TaqMan probes is also more specific and there is less chance of cross-contamination than with conventional PCR, especially nested-PCR. False positives with closely related bacteria can occur with the PCR assays recommended in this protocol – a necessary compromise for a universal assay (L.W. Liefing, pers. comm.). Therefore, if the outcome is critical (e.g. post-entry quarantine samples, new host record, new distribution), the conventional PCR product should be sequenced.</p>	<p>Opinion of [27] was conflict with that of [51] in the sensitivity of nest-PCR and real time PCR. [27]'Real-time PCR has been reported to be as sensitive as nested-PCR' [51] '...and was found to be up to ten times more sensitive than conventional nested-PCR'</p>	China



Comm no.	Para no.	Comment type	Comment	Explanation	Country
31.	27	Substantive	A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used are the P1/P7 (Deng and Hiruki, 1991; Schneider <i>et al.</i> , 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996; Lee <i>et al.</i> , 1993) primer pairs, which can be used as a nested-PCR assay. The P1/P7 primer pair amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. Real-time PCR has been reported to be as sensitive as nested-PCR (Christensen <i>et al.</i> , 2004) and is more amenable to high throughput analysis because post-amplification processing is not required. Real-time PCR using TaqMan probes is also more specific and there is less chance of cross-contamination than with conventional PCR, especially nested-PCR. False positives with closely related bacteria can occur with the PCR assays recommended in this protocol – a necessary compromise for a universal assay (L.W. Liefing, pers. comm.). Therefore, if the outcome is critical (e.g. post-entry quarantine samples, new host record, new distribution), the conventional PCR product should be sequenced.	The sentences: "Real-time PCR using TaqMan probes is also more specific and there is less chance of cross-contamination than with conventional PCR, especially nested-PCR. False positives with closely related bacteria can occur with the PCR assays recommended in this protocol – a necessary compromise for a universal assay (L.W. Liefing, pers. comm.)" are not supported by any scientific paper. A personal communication should not be used for such statement.	EPPO, European Union, Georgia, Serbia
32.	27	Technical	A number of universal PCR primers have been designed that allow amplification of the 16S rRNA gene of any known phytoplasma. The most commonly used are the P1/P7 (Deng and Hiruki, 1991; Schneider <i>et al.</i> , 1995), <a href="#">SN910601/SN01119</a> (Namba <i>et al.</i> , 1993, Jung <i>et al.</i> , 2003) and R16F2n/R16R2 (Gundersen and Lee, 1996; Lee <i>et al.</i> , 1993) primer pairs, which can be used as a nested-PCR assay. The P1/P7 primer pair amplifies a PCR product that contains the entire 16S rRNA gene as well as the 16S/23S rRNA spacer region. Real-time PCR has been reported to be as sensitive as nested-PCR (Christensen <i>et al.</i> , 2004) and is more amenable to high throughput analysis because post-amplification processing is not required. Real-time PCR using TaqMan probes is also more specific and there is less chance of cross-contamination than with conventional PCR, especially nested-PCR. False positives with closely related bacteria can occur with the PCR assays recommended in this protocol – a necessary compromise for a universal assay (L.W. Liefing, pers. comm.). Therefore, if the outcome is critical (e.g. post-entry quarantine samples, new host record, new distribution), the conventional PCR product should be sequenced.	add primer and reference	Korea, Republic of, NEPPO
33.	28	Technical	As well as amplification of the 16S rRNA gene, PCR methods have also been used to amplify other regions of DNA for phytoplasma detection and classification, including ribosomal protein genes (Lee <i>et al.</i> , 1998; Lim and Sears, 1992; Martini <i>et al.</i> , 2007), the <i>tuf</i> gene (Schneider <i>et al.</i> , 1997; <a href="#">Makarova <i>et al.</i>, 2012</a> ), the 23S rRNA gene (Guo <i>et al.</i> , 2003) and the <i>secY</i> gene (Lee <i>et al.</i> , 2010). These primers may be useful when a second independent region of the phytoplasma genome is required.	The paper added is very relevant as universal primers are described that amplify a fragment of the <i>tuf</i> gene. This work was carried out as part of the Q-Bol project.	EPPO, European Union, Georgia, Serbia
34.	28	Technical	As well as amplification of the 16S rRNA gene, PCR methods have also been used to amplify other regions of DNA for phytoplasma detection and classification,	Suggest to include these important references	United States of

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			including ribosomal protein genes ( <a href="#">Jomantiene et al., 1998</a> ; Lee <i>et al.</i> , 1998; Lim and Sears, 1992; Martini <i>et al.</i> , 2007), the <i>tuf</i> gene (Schneider <i>et al.</i> , 1997), the 23S rRNA gene (Guo <i>et al.</i> , 2003) and the <i>secY</i> gene ( <a href="#">Davis et al., 2013</a> ; Lee <i>et al.</i> , 2010; <a href="#">Quaglino et al., 2014</a> ). These primers may be useful when a second independent region of the phytoplasma genome is required.		America
35.	<a href="#">32</a>	Technical	The PCR primers used in this assay are P1 (forward) (Deng and Hiruki, 1991), and P7 (reverse) (Schneider <i>et al.</i> , 1995) and <a href="#">SN910601/SN01119 (Namba et al., 1993, Jung et al., 2003</a> for the first-stage PCR:	add primer and reference	Korea, Republic of, NEPPO
36.	<a href="#">34</a>	Technical	<b>P7</b> (5'-CGT CCT TCA TCG GCT CTT-3').  <a href="#">SN910601(5'-GTTT GAT CCT GGC TCA GGA TT-3')</a>  <a href="#">SN011119(5'-TCG CCG TTA ATT GCG TCC TT-3')</a>	add primer	Korea, Republic of
37.	<a href="#">37</a>	Technical	<b>R16R2</b> (5'-TGA CGG GCG GTG TGT <u>ACGA</u> AAC CCC G-3').	In the original paper this is ACA and not AGA.	EPPO, European Union, Georgia, Serbia
38.	<a href="#">37</a>	Technical	<b>R16R2</b> (5'-TGA CGG GCG GTG TGT <u>ACGA</u> AAC CCC G-3').	This sequence is not correct.	United States of America
39.	<a href="#">38</a>	Technical	<del>The R16F2n primer has three nucleotides added to the 5' end of the R16F2 primer of Lee et al. (1995).</del>	This detail is not necessary and could generate confusion.	EPPO, European Union, Georgia, Serbia
40.	<a href="#">39</a>	Technical	The 20 µl reaction mixture is composed of the following reagent: 1× <i>Taq</i> polymerase buffer containing 1.5 mM MgCl <sub>2</sub> , 0.5 µM of each primer, 200 µM dNTPs, 1 U <i>Taq</i> DNA polymerase and 2 µl DNA template. The reaction is performed under the following thermocycling conditions: 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at either 53 °C (P1/P7 primers) or 50 °C (R16F2n/R16R2 primers), and 1 min at 72 °C; followed by a final extension for 10 min at 72 °C. For nested-PCR, 1 µl of the first-stage PCR products is used directly as template in the second-stage PCR. The PCR products are analysed by gel electrophoresis. The P1/P7 and R16F2n/R16R2 primers produce a 1 800 base pair (bp) and 1 250 bp amplicon, respectively. Conventional nested-PCR will determine if a sample is positive or negative for phytoplasma. To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced (see section 3.5).	1. it is surprising that it is suggested to use undiluted amplicons obtained by first stage PCR as target for the nested amplification. Generally a high dilution is recommended. 2. The sentence "To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced (see section 3.5)" should be moved to paragraph 77	EPPO, European Union, Georgia, Serbia

Comm no.	Para no.	Comment type	Comment	Explanation	Country
41.	39	Technical	The 20 µl reaction mixture is composed of the following reagent: 1× <i>Taq</i> polymerase buffer containing 1.5 mM MgCl <sub>2</sub> , 0.5 µM of each primer, 200 µM dNTPs, 1 U <i>Taq</i> DNA polymerase and 2 µl DNA template. The reaction is performed under the following thermocycling conditions: 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at either 53 °C (P1/P7 primers) or 50 °C (R16F2n/R16R2 primers), and 1 min at 72 °C; followed by a final extension for 10 min at 72 °C. For nested-PCR, 1 µl of the first-stage PCR products is used directly as template in the second-stage PCR. The PCR products are analysed by gel electrophoresis. The P1/P7 and R16F2n/R16R2 primers produce a 1 800 base pair (bp) and 1 250 bp amplicon, respectively. Conventional nested-PCR will determine if a sample is positive or negative for phytoplasma. To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced (see section 3.5).	Could the amount of each reagent be considered.	Jamaica
42.	39	Technical	The 20 µl reaction mixture is composed of the following reagent: 1× <i>Taq</i> polymerase buffer containing 1.5 mM MgCl <sub>2</sub> , 0.5 µM of each primer, 200 µM dNTPs, 1 U <i>Taq</i> DNA polymerase and 2 µl DNA template. The reaction is performed under the following thermocycling conditions: 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at either 53 °C (P1/P7 primers) or 50 °C (R16F2n/R16R2 primers), and 1 min at 72 °C; followed by a final extension for 10 min at 72 °C. For nested-PCR, 1 µl of the first-stage PCR products is used directly as template in the second-stage PCR. The PCR products are analysed by gel electrophoresis. The P1/P7 and R16F2n/R16R2 primers produce a 1 800 base pair (bp) and 1 250 bp amplicon, respectively. Conventional nested-PCR will determine if a sample is positive or negative for phytoplasma. To identify the phytoplasma present in positive samples, the amplicon will need to be sequenced (see section 3.5).	First sentence, suggest to provide the concentration of the reagent instead of volume. Concentration is more critical than volume. Third sentence, most commonly used is a 1:30 dilution, but 1 µl is also correct, so suggest incorporating this.	United States of America
43.	40	Editorial	The P1/P7 primer pair was used by Smart <i>et al.</i> (1996) to amplify the 16S/23S rRNA spacer region of ten different phytoplasma groups. Gundersen and Lee (1996) evaluated the R16F2n/R16R2 primers with representative phytoplasmas for ten distinct 16S rRNA groups. Since then, both primer pairs have been used to identify the phytoplasmas associated with numerous new diseases; for example, of <del>foxtail palm</del> <i>Wodyetia bifurcata</i> (Naderali <i>et al.</i> , 2013), <del>Chrysanthemum</del> <i>chrysanthemum</i> (Bayat <i>et al.</i> , 2013) and <del>oilseed rape</del> <i>Brassica napus</i> (Zwolińska <i>et al.</i> , 2011).	Latin names should always be given.	EPPO, European Union, Georgia, Serbia
44.	40	Technical	The P1/P7 primer pair was used by Smart <i>et al.</i> (1996) to amplify the 16S/23S rRNA spacer region of ten different phytoplasma groups. Gundersen and Lee (1996) evaluated the R16F2n/R16R2 primers with representative phytoplasmas for ten distinct 16S rRNA groups. Since then, both primer pairs have been used to identify the phytoplasmas associated with numerous new diseases; for example, of foxtail palm (Naderali <i>et al.</i> , 2013), chrysanthemum (Bayat <i>et al.</i> , 2013) and	This part is not necessary and includes some inaccuracies.	EPPO, European Union, Georgia, Serbia

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			oilseed rape (Zwolińska <i>et al.</i> , 2011).		
45.	44	Editorial	The reaction mixture for the 28S rRNA assay has the same components and is cycled under the same conditions as the phytoplasma assay, so that the two assays can be run simultaneously in separate tubes. The 28Sf/28Sr primer pair produces a 500–600 bp amplicon. However other primer pairs can also be used to check that the DNA is amplifiable.	1. Clearer with the addition of a comma in the first sentence. 2. Clearer (please see paragraph [41]).	EPPO, European Union, Georgia, Serbia
46.	48	Technical	Reverse primer (5'-TCT TCG AAT TAA ACA ACA TGA TCC A-3')	Sequence of primer needs to be verified with the original publication.	EPPO, European Union, Georgia, Serbia
47.	48	Technical	Reverse primer (5'-TCT TCG AAT TAA ACA ACA TGA TCC A-3')	This is incorrect.	United States of America
48.	50	Substantive	The 25 µl reaction mixture is composed of the following reagents: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer, 900 nM reverse primer, 200 nM FAM probe and 2 µl DNA template. The PCR is performed under the following thermocycling conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of: 15 s at 95 °C, 1 min at 60 °C. Real-time PCR results are analysed with the manufacturer's software.	The following footnote should be added: The use of the brand Applied Biosystems for the TaqMan PCR core reagent kit in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results	COSAVE, Uruguay, Chile, Brazil, Peru, Argentina
49.	50	Technical	The 25 µl reaction mixture is composed of the following reagents: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer, 900 nM reverse primer, 200 nM FAM probe and 2 µl DNA template. The PCR is performed under the following thermocycling conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of: 15 s at 95 °C, 1 min at 60 °C. Real-time PCR results are analysed with the manufacturer's software.	PCR based methods always involve the risk of false positive results due to the unintended reamplification of contaminating PCR products. The incubation of PCR reactions with Uracil-DNA Glycosylase (UNG) prior to the thermal cycling in combination with the use of dUTP in the PCR amplification is a commonly used technology to prevent such cross-contamination. This may also be suggested in the text. Additional modifications could be made: "300 nM forward primer, 900 nM reverse primer" is the original description in the paper, but later experiments have shown that 300 nM of each primer works fine. 2 µl DNA template: If undiluted this is quite a lot, the	EPPO, European Union, Georgia, Serbia

Comm no.	Para no.	Comment type	Comment	Explanation	Country
				original protocol is 1 µl in 25 µl	
50.	50	Technical	The 25 µl reaction mixture is composed of the following reagents: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer, 900 nM reverse primer, 200 nM FAM probe and 2 µl DNA template. The PCR is performed under the following thermocycling conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of: 15 s at 95 °C, 1 min at 60 °C. Real-time PCR results are analysed with the manufacturer's software.	Cosider putting the amount of each reagent to be used.	Jamaica
51.	50	Technical	The 25 µl reaction mixture is composed of the following reagents: 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer, 900 nM reverse primer, 200 nM FAM probe and 2 µl DNA template. The PCR is performed under the following thermocycling conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of: 15 s at 95 °C, 1 min at 60 °C. Real-time PCR results are analysed with the manufacturer's software.	First sentence, please consider this other reference, which is a newer publication: Methods Mol Biol. 2013;938:245-52. doi: 101007/978-1-62703-089-2_21. Christensen et al, reports 300nM forward primer, 300 nM reverse primer, 100 nM. First sentence: Christensen et al., 2004 reports 1 µl DNA template, this is probably a typo. Second sentence: This step may not be necessary if Uracil-DNA Glycosylase is not present in the Master Mix.	United States of America
52.	51	Technical	This method was evaluated by testing phytoplasmas from 18 different subgroups and was found to be up to ten times more sensitive than conventional nested-PCR (Christensen <i>et al.</i> , 2004).	This is not consistent with paragraph 27 which states that Real-time PCR has been reported to be as sensitive as nested-PCR (Christensen <i>et al.</i> , 2004).	EPPO, European Union, Georgia, Serbia
53.	52	Technical	Another universal real-time PCR assay is available that is designed for the 23S rRNA gene (Hodgetts <i>et al.</i> , 2009). A ring test programme between 22 laboratories determined that the Hodgetts <i>et al.</i> (2009) and Christensen <i>et al.</i> (2004) assays are similar in terms of sensitivity and specificity (EUPHRESO FruitPhytoInterlab Group, 2011). The Hodgetts <i>et al.</i> (2009) assay is based on a minor groove binder (MGB) TaqMan probe. <del>These probes are more expensive than non-MGB TaqMan probes.</del>	It is irrelevant with the methods of detection and identification of phytoplasmas.	China
54.	52	Technical	Another universal real-time PCR assay is available that is designed for the 23S rRNA gene (Hodgetts <i>et al.</i> , 2009). A ring test programme between 22 laboratories determined that the Hodgetts <i>et al.</i> (2009) and Christensen <i>et al.</i> (2004) assays are similar in terms of sensitivity and specificity (EUPHRESO FruitPhytoInterlab Group, 2011). The Hodgetts <i>et al.</i> (2009) assay is based on a minor groove binder (MGB) TaqMan probe. These probes are more expensive than non-MGB TaqMan probes.	It could be useful to include more details on primers and probes conditions because in the original paper only general concentrations are reported (also in the case of housekeeping control).	EPPO, European Union, Georgia, Serbia
55.	52	Technical	Another universal real-time PCR assay is available that is designed for the 23S rRNA gene (Hodgetts <i>et al.</i> , 2009). A ring test programme between 22 laboratories	This issue has not been brought up for other methods, so why bring it up here? Also true for	United States of

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			determined that the Hodgetts <i>et al.</i> (2009) and Christensen <i>et al.</i> (2004) assays are similar in terms of sensitivity and specificity (EUPHRESKO FruitPhytoInterlab Group, 2011). The Hodgetts <i>et al.</i> (2009) assay is based on a minor groove binder (MGB) TaqMan probe. <del>These probes are more expensive than non-MGB TaqMan probes.</del>	the suggested use of TaqMan Universal PCR Master Mix from Applied Biosystems (see [50])	America
56.	57	Technical	Alternatively, the 18S rRNA gene assay of Christensen <i>et al.</i> (2004) can be used to confirm that the DNA is amplifiable:	Recommended for monocot where the COX assay is less efficient.	United States of America
57.	61	Editorial	The reaction mixture for the COX and the 18S rRNA gene assays have the same components and are cycled under the same conditions as the phytoplasma real-time assay, so that the two assays can be run simultaneously in separate tubes.	Clearer with the addition of a comma.	EPPO, European Union, Georgia, Serbia
58.	61	Technical	The reaction mixture for the COX and the 18S rRNA gene assays have the same components and are cycled under the same conditions as the phytoplasma real-time assay so that the two assays can be run simultaneously in separate tubes.	Can also be used in a multiplexed assay in a single tube.	United States of America
59.	63	Editorial	For the test result obtained to be regarded as reliable, the following controls should be considered for each series of nucleic acid isolation and amplification of the target pest nucleic acid. Controls used will depend on the test used and the level of certainty required. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.	Clearer with the addition of a comma in the first sentence.	EPPO, European Union, Georgia, Serbia
60.	63	Technical	For the test result obtained to be regarded as reliable the following controls should be considered for each series of nucleic acid isolation and amplification of the target pest nucleic acid. Controls used will depend on the test used and the level of certainty required. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.	The minimum controls should include a control to monitor contamination during nucleic acid extraction. This should be included in PCR also.	EPPO, European Union, Georgia, Serbia
61.	65	Editorial	<b>Internal control.</b> For conventional and real-time PCR, plant internal controls ( <del>Housekeeping</del> <del>House-Keeper</del> Gene (HKG)) such as the general eukaryotic 28S rRNA gene (see section 3.1 for its use in the conventional nested-PCR) or the COX gene (see section 3.2 for its use in the <del>nested</del> real-time PCR) should be incorporated into the PCR protocols to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation, or the presence of PCR inhibitors.	1. Better wording 2. Section 3.1 is about "conventional nested-PCR" (please see paragraph [31]). 3. Section 3.2 is about "real-time PCR" (please see paragraph [45]). 4. Last sentence clearer with the addition of a comma.	EPPO, European Union, Georgia, Serbia
62.	68	Editorial	The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. If bulking of samples is done, then the quantity of positive control should be adjusted accordingly (e.g. ten lots of 20 mg	Clearer with the addition of a comma in the second sentence.	EPPO, European Union, Georgia,

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			sample bulked for DNA extraction, 2 mg infected leaf + 198 mg healthy plant tissue). If the positive control is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved.		Serbia
63.	68	Technical	The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. If bulking of samples is done then the quantity of positive control should be adjusted accordingly (e.g. ten lots of 20 mg sample bulked for DNA extraction, 2 mg infected leaf + 198 mg healthy plant tissue). If the positive control is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved.	Is it possible to have a scientific reference to support the recommendation on the amounts given?	EPPO, European Union, Georgia, Serbia
64.	76	Editorial	If internal control primers are also used, then the negative (healthy) control (if used), positive control and each of the test samples must produce the amplicon of the expected size. Failure of the samples to amplify <del>with</del> the internal control <del>primers</del> suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.	Edits	United States of America
65.	76	Technical	<del>For if</del> internal control <del>s primers are also used, then</del> the negative (healthy) control (if used), positive control and each of the test samples must produce the amplicon of the expected size. Failure of the samples to amplify with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.	"If internal primers are also used" is contradictory to the paragraph 63 ("...internal control...are the minimum controls that should be used").	EPPO, European Union, Georgia, Serbia
66.	76	Technical	If internal control primers <del>targeting plant DNA</del> are also used, then the <del>negative (healthy)</del> control (if used), positive control and each of the test samples must produce the amplicon of the expected size. Failure of the samples to amplify with the internal control primers suggests for example that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.	"Targeting plant DNA" - Added avoid confusion and help understand why healthy control will test positive with the internal control primers. Deletion of "negative" - Remove negative and leave healthy control only. Using "negative control" can cause confusion in this sentence.	United States of America
67.	77	Technical	<del>Change Para[77] into "The test on a sample should be confirmed by sequencing if it produces an amplicon of the correct size."The test on a sample will be considered positive if it produces an amplicon of the correct size.</del>	To identify the phytoplasmas present in positive samples, the amplicon will be need to be sequenced, according to the point of [39] and [81].	China
68.	81	Editorial	PCR products can be sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLASTN, available at the National Center for Biotechnology Information ( <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a> ). If the sequence shares less than 97.5% identity with its closest relative, the phytoplasma is considered to be a new " <i>Candidatus</i> Phytoplasma" species. In this case, the entire 16S rRNA gene should be sequenced and phylogenetic analysis performed. Sequencing a separate region of the genome such as the 16S/23S rRNA spacer region, <i>secY</i> gene,	Clearer with the addition of a comma in the third sentence.	EPPO, European Union, Georgia, Serbia

Comm no.	Para no.	Comment type	Comment	Explanation	Country
			ribosomal protein genes or the <i>tuf</i> gene is also desirable.		
69.	81	Technical	PCR products <del>should be</del> <del>can be</del> sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLASTN, available at the National Center for Biotechnology Information ( <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a> ). If the sequence shares less than 97.5% identity with its closest relative the phytoplasma is considered to be a new " <i>Candidatus</i> Phytoplasma" species. In this case, the entire 16S rRNA gene should be sequenced and phylogenetic analysis performed. Sequencing a separate region of the genome such as the 16S/23S rRNA spacer region, <i>secY</i> gene, ribosomal protein genes or the <i>tuf</i> gene is also desirable.	It's more logically.	China
70.	90	Editorial	Department of Environment and Primary Industries, AgriBio, 5 Ring Road, Bundoora Victoria, 3083 Australia, (Dr Fiona Constable; e-mail: <a href="mailto:fiona.constable@depi.vic.gov.au">fiona.constable@depi.vic.gov.au</a> ; tel.: +61 3 9032 7326; fax: +61 3 9032 7604).	1. Cf. paragraph [89]. 2. Cf. paragraph [89].	EPPO, European Union, Georgia, Serbia
71.	95	Editorial	This diagnostic protocol was drafted by Dr L.W. Liefing (Plant Health and Environment Laboratory, Ministry for Primary Industries, New Zealand), Professor P. Jones (Rothamsted Research, United Kingdom), Dr F. Constable (Department of Primary Industries, Victoria, Australia), Dr E. Torres (Laboratori de Sanitat Vegetal, Departament d'Agricultura, Alimentació i Acció Rural, Spain) <del>and</del> Dr W. Jelkmann (Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection and Fruit Crops, Germany) <del>and</del> Dr J. Verhoeven (Plant Protection Service, Departement Diagnostics, The Netherlands).	According to paragraph [2], "Consultation on technical level", Dr Verhoeven participated in the writing of the first draft of this DP, so why is he not acknowledged like the other authors?	EPPO, European Union, Georgia, Serbia
72.	105	Editorial	<b>Daire, X.D., Clair, D., Reinert, W. &amp; Boudon-Padieu, E.</b> 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of nonribosomal DNA. <i>European Journal of Plant Pathology</i> , 103: 507–514.	Suggest to add the following two references here: Davis, R.E., Sinclair, W.A. 1998. Phytoplasma identity and disease etiology. <i>Phytopathology</i> , 88:1372-1376 Davis R.E., Zhao Y., Dally E.L., Lee I.M., Jomantiene R., Douglas S.M. 'Candidatus Phytoplasma pruni', a novel taxon associated with X-disease of stone fruits, <i>Prunus</i> spp.: multilocus characterization based on 16S rRNA, <i>secY</i> , and ribosomal protein genes. <i>Int. J. Syst. Evol. Microbiol.</i> 63:766-776 (2013).	United States of America
73.	119	Editorial	<b>Jarausich, W., Lancas, M. &amp; Dosba, F.</b> 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different <i>Prunus</i> species detected by specific PCR. <i>Journal of Phytopathology</i> , 147: 47–54.	Suggest adding the following reference after this one: Jomantiene, R., Davis, R.E., Maas, J., Dally, E. Classification of new phytoplasmas associated with diseases of strawberry in Florida, based on analysis of 16S ribosomal-RNA and ribosomal-protein gene	United States of America



Comm no.	Para no.	Comment type	Comment	Explanation	Country
				operon sequences. International journal of systematic bacteriology, 48 (1), 1998, 269-277.	
74.	<a href="#">119</a>	Technical	<p><b>Jarausch, W., Lancas, M. &amp; Dosba, F.</b> 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different <i>Prunus</i> species detected by specific PCR. <i>Journal of Phytopathology</i>, 147: 47–54.</p> <p><a href="#">Jung HY, Sawayanagi T, Kakizawa S, Nishigawa H, Wei W, Oshima K, Miyata S, Ugaki M, Hibi T, Namba S (2003) "Candidatus Phytoplasma ziziphi", a novel phytoplasma taxon associated with jujube witches' broom disease. International Journal of Systematic and Evolutionary Microbiology 53:1037–1041.</a></p>	add reference for the primer added	Korea, Republic of, NEPPO
75.	<a href="#">127</a>	Technical	<p><b>Lim, P.-O. &amp; Sears, B.B.</b> 1992. Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and <i>Acholeplasma laidlawii</i> deduced from two ribosomal protein gene sequences. <i>Journal of Bacteriology</i>, 174: 2606–2611.</p> <p><a href="#">Makarova, O., Contaldo, N., Paltrinieri, S., Kawube, G., Bertaccini, A., Nicolai sen, M., 2012. DNA barcoding for identification of 'Candidatus phytoplasmas' using a fragment of the elongation factor Tuf gene. <i>PL OS One</i> 12 e52092</a></p>	New reference proposed see paragraph 38.	EPPO, European Union, Georgia, Serbia
76.	<a href="#">130</a>	Technical	<p><b>Marzachi, C.</b> 2004. Molecular diagnosis of phytoplasmas. <i>Phytopathologia Mediterranea</i>, 43: 228–231.</p> <p><a href="#">Mehle N., Nikolić P., Rupar M., Boben J., Ravnikar M., Dermastia M. 2013. Automated DNA extraction for large numbers of plant samples. In: DICKINSON, Matthew (ed.), HODGETTS, Jennifer (ed.). <i>Phytoplasma : methods and protocols</i>, (Methods in Molecular Biology, vol. 938), (Springer Protocols). New York: Humana Press, 139-145</a></p>	Additional reference (see paragraph 26).	EPPO, European Union, Georgia, Serbia
77.	<a href="#">132</a>	Technical	<p><b>Nadarali, N., Nejat, N., Tan, Y.H. &amp; Vadamalai, G.</b> 2013. First report of two distinct phytoplasma species, "<i>Candidatus Phytoplasma cynodontis</i>" and "<i>Candidatus Phytoplasma asteris</i>", simultaneously associated with yellow decline of <i>Wodyetia bifurcata</i> (Foxtail Palm) in Malaysia. <i>Plant Disease</i>, 97: 1504.</p> <p><a href="#">Namba S, Kato S, Iwanamis S, Oyaizu H, Shiozawa H, Tsuchizaki T (1993) Detection and differentiation of plant-pathogenic mycoplasma-like organism using polymerase chain reaction. <i>Phytopathology</i> 83:786–791</a></p>	add reference for the primer added	Korea, Republic of, NEPPO
78.	<a href="#">137</a>	Editorial	<b>Prince, J.P., Davis, R.E., Wolf, T.K., Lee, I.-M., Mogen, B.D., Dally, E.L., Bertaccini, A., Credi, R. &amp; Barba, M.</b> 1993. Molecular detection of diverse	Suggest adding the following reference: Quaglino, F., Zhao, Y., Casati, P., Bulgari, D.,	United States of

Comm . no.	Para . no.	Comment type	Comment	Explanation	Country
			mycoplasma-like organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. <i>Phytopathology</i> , 83: 1130–1137.	Bianco, P.A., Wei, W., Davis, R.E., 2013. Candidatus <i>Phytoplasma solani</i> , a novel taxon associated with stolbur- and bois noir-related diseases of plants. <i>Int j Syst Evol Microbiol</i> . 2013, Aug; 63 (Pt8):2879-94. Doi: 10.1099/ijs.0.044750-0. Epub 2013 Jan 18.	America
79.	141	Editorial	<b>Seemüller, E., Schaper, U. &amp; Zimbelmann, F.</b> 1984. Seasonal variation in the colonization pattern of mycoplasma-like organisms associated with apple proliferation and pear decline. <i>Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz</i> , 91: 371–382 <del>[in English with German summary]</del> .	This level of detail is not usually given.	EPPO, European Union, Georgia, Serbia
80.	146	Editorial	<b>Zhang, Y.P., Uyemoto, J.K. &amp; Kirkpatrick, B.C.</b> 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. <i>Journal of Virological Methods</i> , 71: 45–50.	Suggest adding the following reference: Zhao, Y., Davis, R.E., Wei, W., Shao, J., Jomantiene, R. <i>Phytoplasma Genomes: Evolution through mutually complementary mechanisms, gene loss and horizontal acquisition</i> . Chapter in <i>Genomics of plant-associated bacteria</i> . Hardcover – 3 Jul 2014 by Dennis C. Gross (editor), Ann Lichens-Park (editor), Chittaranjan Kole (editor).	United States of America