Molecular Diagnostic Tools for Detection and Differentiation of Phytoplasmas Based on Chaperonin-60 Reveal Differences in Host Plant Infection Patterns

Tim J. Dumonceaux1,2*, Margaret Green3, Christine Hammond1, Edel Perez4, Chrystel Olivier1

1. Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon, Saskatchewan, Canada, 2. Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 3. Canadian Food Inspection Agency, Centre for Plant Health, Sidney, British Columbia, Canada, 4. Instituto de Biotecnología y Ecología Aplicada (INBIOTECA), Universidad Veracruzana, Avenida de Las Culturas Veracruzanas, Xalapa, Veracruz, Mexico

*tim.dumonceaux@agr.gc.ca

Abstract

Phytoplasmas (’Candidatus Phytoplasma’ spp.) are insect-vectored bacteria that infect a wide variety of plants, including many agriculturally important species. The infections can cause devastating yield losses by inducing morphological changes that dramatically alter inflorescence development. Detection of phytoplasma infection typically utilizes sequences located within the 16S–23S rRNA-encoding locus, and these sequences are necessary for strain identification by currently accepted standards for phytoplasma classification. However, these methods can generate PCR products >1400 bp that are less divergent in sequence than protein-encoding genes, limiting strain resolution in certain cases. We describe a method for accessing the chaperonin-60 (cpn60) gene sequence from a diverse array of ‘Ca. Phytoplasma’ spp. Two degenerate primer sets were designed based on the known sequence diversity of cpn60 from ‘Ca. Phytoplasma’ spp. and used to amplify cpn60 gene fragments from various reference samples and infected plant tissues. Forty three cpn60 sequences were thereby determined. The cpn60 PCR-gel electrophoresis method was highly sensitive compared to 16S-23S-targeted PCR-gel electrophoresis. The topology of a phylogenetic tree generated using cpn60 sequences was congruent with that reported for 16S rRNA-encoding genes. The cpn60 sequences were used to design a hybridization array using oligonucleotide-coupled fluorescent microspheres, providing rapid diagnosis and
typing of phytoplasma infections. The oligonucleotide-coupled fluorescent microsphere assay revealed samples that were infected simultaneously with two subtypes of phytoplasma. These tools were applied to show that two host plants, *Brassica napus* and *Camelina sativa*, displayed different phytoplasma infection patterns.

**Introduction**

*’Candidatus Phytoplasma’* spp. are obligate intracellular Mollicutes that live and reproduce in the phloem tissue of plants and are transmitted by phloem-feeding leafhoppers, planthoppers, and psyllids [1]. Phytoplasmas infect a wide range of plants, including many species that are agriculturally significant [2]. These microorganisms induce developmental changes in infected plants, from virescence and phyllody to stunting and decline. In agronomically important crops, phytoplasma infection can alter inflorescence morphology and dramatically decrease seed set and/or fruit quality [3].

*’Ca. Phytoplasma’* spp. are generally difficult to culture in vitro and a recent report claiming to have cultured these bacteria [4] has been met with skepticism [5]. Diagnosis and differentiation of phytoplasma infection has therefore relied on molecular methods, principally PCR targeting a wide variety of regions within the 16S–23S rRNA genes [6]. The use of observed or virtual RFLP fingerprinting and DNA sequencing methods to differentiate *’Ca. Phytoplasma’* spp. allowed the grouping of phytoplasma strains that can be divergent in 16S rRNA-encoding sequences [3]. 16S–23S rRNA gene sequences have been effectively used to differentiate and classify phytoplasma strains [7–10], resulting in the identification of at least thirty groups of phytoplasma. Molecular diagnostic methods targeting 16S rRNA-encoding genes, including conventional PCR combined with direct sequencing and quantitative PCR (qPCR), have been employed to detect phytoplasma infection in plants and insects [6, 11, 12]. Limitations of rRNA-encoding gene targeted typing methods include the length of the amplicon generated (which can be >1500 bp) as well as the inability of ribosomal RNA-encoding gene sequences to differentiate certain subgroups of phytoplasma. [13–17].

These limitations have motivated the search for other molecular markers for the detection of phytoplasma infections. Protein-encoding genes are known to provide increased strain resolution compared to rRNA-encoding genes [18], but the use of protein-encoding genes requires tools such as PCR primers that are able to access the genes from genomes that may be highly divergent in sequence. The gene encoding the 60 kDa chaperonin (*cpn60*) has been shown to meet the criteria set out by the International Barcode of Life consortium for a barcode for the domain Bacteria [19]. Indeed it has been shown that *cpn60* sequences (often called *groEL* or *hsp60*) provide superior strain resolution for *’Ca. Phytoplasma’* spp.
compared to rRNA-encoding gene targets [14, 15]. However, the PCR amplification methods described for accessing cpn60 sequences from unknown and potentially divergent phytoplasma genomes [15] have been limited to specific subgroups (e.g. 16SrI, or ‘Ca. P. asteris’) and result in the generation of a sequence of ~1.4 kb, which is inconvenient for the rapid generation of novel sequences by direct sequencing of PCR products.

The cpn60 universal target (cpn60 UT) is a 549–567 bp (183–189 amino acid) region of the gene corresponding to nucleotides 247–828 of E. coli groEL [20]. The cpn60 UT can be amplified from nearly all bacteria using a set of degenerate universal primers [21, 22], and cpn60 UT sequences have been used to detect, identify, and differentiate a wide variety of bacterial groups [23–27]. Furthermore, the cpn60 UT has proven to be a useful target for the development of molecular diagnostic assays such as qPCR [28] and hybridization-based assays [24, 29] for other non-phytoplasma bacteria as well as specifically used to detect phytoplasma species using a loop-mediated isothermal DNA amplification assay [30].

The potential utility of previously described tools for accessing cpn60 is limited for ‘Ca. Phytoplasma’ spp. as samples will contain non-target bacterial and eukaryotic DNA, decreasing the specificity of the cpn60-based molecular diagnostic. However, the availability of full-length cpn60 gene sequences for certain ‘Ca. Phytoplasma’ spp., combined with the low G/C content observed in all ‘Ca. Phytoplasma’ spp. (approximately 21–28% G/C in 11 reported genomes), led us to investigate the possibility of designing a novel genus-level specific PCR-based assay for generating cpn60 UT sequences for additional ‘Ca. Phytoplasma’ spp. Our objective in the present study was to determine the feasibility of exploiting the cpn60 UT as a molecular diagnostic target for the detection and differentiation of ‘Ca. Phytoplasma’ spp. We describe two sets of PCR primers that successfully amplify the cpn60 UT from a wide variety of ‘Ca. Phytoplasma’ spp. that are highly divergent in sequence (61–98% identity). We used these sequences to develop molecular diagnostic assays that demonstrated differences in host plant infections between two related oilseed crops, Brassica napus and Camelina sativa.

**Materials and Methods**

**Provenance of plant samples and DNA extracts.**

In 2012, plants belonging to the species Linum usitatissimum, Brassica napus, Brassica napobrassica, Allium cepa, Daucus carota and Thapsi arvense that showed severe Aster yellows (AY) symptoms were sampled from fields located near Saskatoon, Saskatchewan, Canada (52.13° N, 106.68° W). DNA extracts of ‘Ca. P. phoenicium’ originated from infected plant tissue of Catharanthus roseus located in near Madruga, Mayabeque, Cuba (22.91° N, 81.85° W). These plants were sampled from areas that did not require specific permissions, and no endangered or protected plant species were sampled for this work. DNA extracts of Stolbur, Bois noir, (16SrXII) originated from 16SrXII-infected C. roseus sent by Dr. M. Maixner (JKI-Institute for Plant Protection in Fruit Crops and Viticulture,
Germany), DNA extracts of Aster yellows (16SrI and 16SrI-C) and DNA extracts of apple proliferation group or European stone fruit yellows and Pear decline (16SrX-B and 16SrX-C) were obtained from Dr. A. Bertaccini (University of Bologna, Italy) and Dr. X. Foissac (INRA-Bordeaux, France), respectively. Plants of *Ligustrum sinense* and *C. roseus*, infected with AY-16SrI-A were maintained in the laboratory and used as positive controls. Healthy *C. roseus* and *B. napus* grown in an insect-free growth chamber were used as negative controls. A comprehensive list of all source data obtained for strains of ‘Ca. Phytoplasma’ spp. analyzed in this study is shown in **S1 Table**.

**Plant sampling and DNA extraction.**
Approximately 0.1 g of finely cut leaf and stem tissues was placed in a well of a 96-well plate and lyophilized for 48 hours (−40°C, 0.120 mBar). Tissue was homogenized using a TissueLyser II (Qiagen) with one-3 mm glass bead at 30 Hz for 2 min. DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB)-based method [31]. Briefly, homogenized tissue was suspended in 250 μL of a solution of 55 mM CTAB, 100 mM Tris-Cl pH 8.0, 20 mM EDTA, and 1.4 M NaCl. 2-mercaptoethanol was added to this solution (4 μL/mL) immediately prior to DNA extraction and the samples were vortexed briefly and incubated at 65°C for 1 hour. Samples were extracted with 1.0 volume of chloroform, centrifuged, then the upper phase was transferred to a fresh tube and 1.0 volume of isopropanol was added. Samples were centrifuged, dried, and the DNA pellets were dissolved in 200 μL of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) containing RNase A (0.2 mg/mL). DNA extracts were stored at −20°C.

**Design of oligonucleotide-based tools targeting cpn60 of ‘Ca. Phytoplasma’ spp.**
Sequences for all amplification primers and hybridization probes along with optimized amplification conditions are shown in **S2 Table**. Primers for phytoplasma PCR amplification were based on the *cpn*60 UT primer annealing sites [22], but were adapted to phytoplasma sequences using full-length *cpn*60 genes from public databases (www.cpndb.ca and www.ncbi.nlm.nih.gov). One set of primers (H279p/H280p) was based on 18 full-length *cpn*60 sequences, which primarily represented the AY group. A second set of PCR primers (D0317/D0318) was designed based on the full-length *cpn*60 reported in the Peanut Witches’ Broom (PnWB) Phytoplasma genome sequence reported by Chung et al. [32]. Hybridization probes for the fluorescent microsphere detection assay were designed using PrimerPlex v2.62 (Premier Biosoft, Palo Alto, CA, USA).

**PCR amplification and sequencing.**
A dilution series of each DNA extract (neat, 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100) was routinely analyzed to compensate for the possibility of PCR inhibition.
Samples were analyzed using 16S rRNA gene-targeted universal bacterial PCR primers [33] to ensure that amplifiable bacterial DNA was present in the extract at the dilutions analyzed. *cpn60* UT amplicons were generated under the following conditions: 1 × PCR buffer (Life Technologies); 2.5 mM MgCl₂; 0.4 μM each primer (S1 Table); 0.5 mM each dNTP; and 1 U Taq DNA polymerase (Life Technologies). PCR primers were synthesized by IDT (Coralville, IA). PCR cycling conditions for *cpn60* were 95°C, 3 min (1x) followed by 30 cycles of 95°C, 30 sec; 42°C, 30 sec; 72°C, 30 sec. A final extension at 72°C (5 min) was performed. PCR products were either directly sequenced using the amplification primers, or were first ligated into the vector pGEM-T Easy (Promega, Madison, WI USA) according to the manufacturer’s recommendations, transformed into chemically competent *E. coli* JM109 (Promega), and sequenced using plasmid-targeted primers T7/SP6. For amplification of 16S–23S rRNA-encoding loci, primers P1 and Tint [6] were used with the recommended amplification conditions.

**Sensitivity, specificity, and limit of detection of *cpn60*-based molecular diagnostic assays.**

The diagnostic sensitivity and specificity, along with 95% confidence intervals, were calculated using 192 DNA extracts from field-collected plant tissues according to standard methods [34]. The results of 16S–23S rRNA locus-targeted PCR amplified with P1/Tint [6] were used as the gold standard to call positive and negative samples. The limit of detection (LOD) of each assay was determined by probit analysis of 8 replicates each of a dilution series of non-linearized plasmid DNA containing 10⁷–10¹ copies/reaction. Plasmids were diluted in 10 mM Tris-Cl, pH 8.0 containing 5 ng/ml yeast tRNA and stored in DNA low-bind tubes (Eppendorf). Probit analysis was conducted using SPSS (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 21.0) and the LOD was specified as the copy number that was predicted to yield a positive result in 95% of assays performed, as specified by Bustin et al. [35]. Plasmid templates for the LOD assays were as follows: for H279p/H280p, AY-0Y-M; for D0317/D0318, FD; for P1/Tint, PCR product from infected flax plants cloned into pGEM-T Easy.

**Phylogenetic analysis of phytoplasma *cpn60* sequences.**

Sequences were aligned using ClustalW [36] and analyzed using the Maximum Likelihood method based on the Tamura-Nei model [37]. Trees were bootstrapped 1000 times. Analysis was conducted in MEGA5 [38].

**Oligonucleotide-coupled fluorescent microsphere diagnostic assay.**

Phytoplasma *cpn60* was amplified using the phytoplasma PCR primer sets with the upstream primers modified with biotin and four phosphorothioate-modified
bases (S2 Table). Amplicons were rendered single-stranded by digestion with T7 exonuclease and hybridized to oligonucleotide-coupled fluorescent microspheres (Bio-Rad) as described previously [29, 39]. Hybridization probes had a 5-amino C12 modification at the 5’ end to facilitate bead coupling (IDT, Coralville, IA). The results were analyzed using a Bio-Plex instrument (Bio-Rad) and Bio-plex Manager software (v6.1.0.727). Duplicate aliquots of each amplification product were hybridized to the fluorescent microspheres, and in some cases duplicate amplification reactions were analyzed, for a total of 2 or 4 hybridization assays per sample. The median fluorescence intensity (MFI) of each bead in every sample was determined by measuring the fluorescence of 100 microspheres. The MFI of each microsphere in PCR-amplified samples was compared to that of a negative control (no template) using a Student’s t-test (one-tailed distribution). Positive results were scored as those with an MFI > 120 that were significantly greater than the negative control at \( P < 0.01 \).

### Determination of phytoplasma strains infecting oilseed crops at a single location.

Breeding lines and commercially available cultivars of *Camelina sativa* and *Brassica napus* plants were grown at the Agriculture and Agri-Food Canada research farm at Saskatoon, Saskatchewan, Canada, during the growing season of 2012. Plants were seeded in two rows in plots 3.0 m in length with 0.30 m between rows and 100 seeds per row, using a randomized complete block design. Rows of barley were used at the edge of each bloc. Tissue was harvested from plants showing signs of phytoplasma infection and DNA was extracted as described above. DNA was used as a template for nested PCR using primers P1/P6 [40] and R16F2/R16R2 [41] targeting the 16S–23S rRNA-encoding locus and products were directly sequenced using R16F2/R16R2. The same extracts were used as template for cpn60-targeted PCR using primers H279p/H280p and products were either directly sequenced using the amplification primers or were subjected to the fluorescent microsphere hybridization assay as described above.

### Results

**Design of oligonucleotide-based tools targeting *cpn60* of ‘Ca.Phytoplasma’ spp.**

Initial experiments aimed at developing a *cpn60*-targeted phytoplasma PCR assay used universal phytoplasma primer AY-groEL-F [15] and primer H280p (S2 Table). This primer set generated a PCR product of 826 bp that contained the entire *cpn60* UT along with 275 bp of upstream sequence. PCR products were generated using this primer set from DNA extracted from infected tissues of *Linum usitatissimum*. However, analysis of DNA extracted from infected *Brassica napus* plants revealed that this primer set had a very low sensitivity compared to
P1/Tint (0.489 with 90 positives analyzed), so a new primer was designed based on the sequence of the cpn60 UT universal primer annealing site.

This primer set, H279p/H280p (S2 Table), contained degenerate bases to capture the breadth of sequence heterogeneity observed in 18 full-length Phytoplasma cpn60 genes found in the cpnDB (www.cpnDB.ca). The sequences available for primer design were principally derived from the AY group, along with ‘Ca.P. mali’ (Apple Proliferation Phytoplasma). Selectivity of this primer set for ‘Ca. Phytoplasma’ spp. was increased by using a low Ta (42°C), exploiting the low G/C content observed in phytoplasma genes [14, 15, 32]. Primer set H279p/H280p successfully generated amplicons from a wide array of DNA extracted from infected plant tissues, including strains corresponding to ‘Ca. P. asteris’, ‘Ca. P. solani’, ‘Ca. P. mali’, ‘Ca. P. prunorum’, and ‘Ca. P. pyri’ (Fig. 1A). However, this primer set failed to amplify product from other ‘Ca. Phytoplasma’ species.

The availability of the complete genome sequence for Peanut Witches’ Broom Phytoplasma (PnWB; 16SrII group) [32] revealed the extreme sequence diversity to be expected at this locus: PnWB cpn60 was only 63–65% identical to any other phytoplasma cpn60 sequence reported, and would not have been expected to amplify with H279p/H280p. Therefore, a second set of primers was required to capture the diversity of phytoplasma cpn60 sequences. Primer set D0317/D0318 successfully generated cpn60 UT amplicon from ‘Ca. Phytoplasma’ spp., including ‘Ca.P. phoenicium’, ‘Ca.P. fraxini’, and ‘Ca. P. ulmi’. These templates did not amplify with H279p/H280p (Fig. 1B) and were highly divergent in cpn60 UT sequence compared to the other samples (Fig. 2). Based on these results, various ratios of H279p/H280p and D0317/D0318 were tested to determine if all of the observed sequence diversity of phytoplasma cpn60 could be captured in a single PCR primer cocktail. A ratio of 1:7 H279p/H280p:D0317/D0318 successfully generated cpn60 amplicon from all of the ‘Ca. Phytoplasma’ spp. tested in a single reaction (Fig. 1C).

In general, primer set H279p/H280p amplified cpn60 genes from ‘Ca.P. solani’ (Bois noir), ‘Ca.P. asteris’, and the 16SrX group and was not effective on other samples (Fig. 1). The only exception to this was ‘Ca.P. solani’ (Bois noir) isolate VL-06-1-20, Lebanon, which repeatedly did not amplify with primer set H279p/H280p (Fig. 1A, lane 11), yet had a sequence that was identical to those generated for other strains of ‘Ca.P. solani’ (Fig. 2), all of which amplified with that primer set. However, this sample was amplifiable with primer set D0317/D0318 as well as the optimized cocktail (Fig. 1 B, C-lane 11).

Diagnostic utility of cpn60-targeted amplification compared to 16S-23S-targeted PCR assay.

Using 192 DNA extracts from infected and uninfected B. napus as template, the sensitivity of the cpn60-targeted phytoplasma PCR (H279p/H280p) was high when compared to the 16S-23S-targeted PCR (P1/Tint), with 93% of 16S-23S-positive samples also testing positive with the cpn60-targeted assay (Table 1). However, the specificity observed was low: only 44% of negative samples tested...
negative using the cpn60 assay. This suggested that the cpn60 assay yielded a high rate of false positives, which may be expected due to the fact that the universal primer hybridization sites were used for this assay. We therefore determined the cpn60 amplicon DNA sequences from those 39 discordant samples that tested negative by 16S–23S PCR but positive by cpn60-targeted PCR. The DNA sequences obtained from 38 of the 39 samples (1 reaction failed) were identical or nearly identical to those of ‘Ca. P. asteris’ (16SrI group): AY-WB (GenBank NC_007716.1) and AY-OY-M (GenBank AB124807) (Fig. 2). This confirms that those DNA extracts, which had originally tested negative for phytoplasma using the 16S–23S assay, were indeed positive. The LOD of the cpn60-targeted assays was examined using serial dilutions of plasmid DNA (Table 2). Probit analysis revealed that the LOD of the cpn60-targeted assays was \( \sim 10^3 \) copies, which was about 10-fold lower than that observed for the 16S rRNA gene-targeted P1/Tint assay (Table 2).

**Molecular phylogeny of ‘Ca. Phytoplasma’ spp. based on cpn60 UT sequences.**

Sequences were determined for all of the phytoplasma cpn60 UT amplicons generated in this study. Overall, the sequence diversity at the cpn60 UT was high,
with the maximum divergence (61% sequence identity) observed between ‘Ca.P. fraxini’ (Ash Yellows) and ‘Ca.P. solani’ (Bois noir). The most similar cpn60 sequences were the sequences belonging to ‘Ca.P. asteris’ (AY), with 97–98% sequence identity observed. Phylogenetic analysis of all of the phytoplasma cpn60 UT sequences generated in this study, along with selected reference strains from cpnDB, is shown in Fig. 2. A total of 12 distinct groupings could be discerned, including 4 closely related groups of identical sequences (AY-OY-M; COL; CVB; and AY-WB). These sequences shared 97–98% identity and the reference sequences were all identified as ‘Ca.P. asteris’ (16SrI). Most of the infected plant samples we obtained from Canada yielded cpn60 sequences that clustered into these ‘Ca.P. asteris’-like (16SrI) groups.

Fluorescent microsphere assay for detection and identification of ‘Ca.Phytoplasma’ spp.

The cpn60 UT sequences generated using these primer sets were used to design a suite of hybridization probes for various ‘Ca.Phytoplasma’ spp. spanning the complete cpn60 UT sequence diversity that has been observed to date (S2 Table). The single exception to this was PnWB, for which a cpn60 sequence, but not template DNA, was available. Probes were coupled to fluorescent microspheres, mixed together, and hybridized to single-stranded PCR products generated using the cpn60-targeted phytoplasma primer sets (S2 Table). Results generated from a 4-plex bead mixture are shown in Fig. 3. The signal intensity generated using the

| Table 1. Sensitivity and specificity of cpn60-targeted PCR assay compared to 16S–23S PCR. |
|---------------------------------|------------------|------------------|---------|
|                                 | P1/Tint PCR results |                 |         |
| cpn60 PCR results*              | positive          | negative         | Total   |
| positive                        | 114               | 39               | 153     |
| negative                        | 9                 | 30               | 39      |
| total                           | 123               | 69               | 192     |
| cpn60 PCR sensitivity           | 0.927             | 0.046            | 0.95 CI |
| cpn60 PCR specificity           | 0.435             | 0.117            | 0.43 CI |

Positives and negatives were defined by the results of the P1/Tint assay and the corresponding numbers of positive and negative samples identified by the cpn60-targeted assay are indicated [34]. The sensitivity of the cpn60 PCR using P1/Tint as a gold standard was 0.927 (114 positives of 123), with a 95% confidence interval of 0.95. The specificity of the cpn60 PCR using P1/Tint as a gold standard was 0.435 (30 negatives of 69), with a 95% confidence interval of 0.117.

*assayed using primer set H279p/H280p.

CI, confidence interval.
plasmid DNA controls was highly variable, even though each contained the same copy number in the initial PCR \( (10^8 \text{ copies}) \). Since hybridization signal intensity can affect the definition of a positive result, we implemented a statistical definition of a positive result by specifying that each bead must generate a signal that is significantly greater than the background at \( p<0.01 \) using a Student’s t-test. Using this definition, we identified samples that were positive for various ‘Ca. Phytoplasma’ spp. from infected plant tissues, including AY-OY-M, AY-WB, ESFY, and Bois noir (Fig. 3). All of the 11 hybridization probes generated a signal only with the intended target (Fig. 4). Samples containing DNA from ‘Ca. P. asteris’ isolates AY-OY-M, AY-CVB, AY-COL, and AY-WB were readily distinguishable despite the fact that the \( cpn60 \) UT sequences of these strains were 97–98% identical (Figs. 3, 4).

The fluorescent microsphere assay (4-plex format) was implemented to examine 192 DNA extracts from \( B. \) napus. A summary comparing the results of

| gene target | primer set* | LOD (copy no. per assay) |
|-------------|-------------|--------------------------|
| \( cpn60 \) | H279p/H280p | 2355                     |
| \( cpn60 \) | D0317/D0318 | 4952                     |
| 16S–23S     | P1/Tint     | 34626                    |

*asee S1 Table.

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Fig. 3. \( cpn60 \)-targeted fluorescent microsphere hybridization assay to detect ‘Ca. Phytoplasma’ spp. Results are shown for a 4-plex assay (format used for analysis of 192 \( B. \) napus DNA extracts) on plasmid DNA controls \( (10^7 \text{ copies/PCR}) \) and on genomic DNA extracted from various infected plant tissues. Beads with a positive hybridization signal in each sample are identified (*). Samples from infected plant tissues are those described in S1 Table (onion, item #25; flax, item #41; vinca1, item #33; vinca2, item #35; carrot, item #15). Abbreviations: MFI, median fluorescence intensity; BN, Bois Noir; ESFY, European Stone Fruit Yellows; AY, Aster Yellows.

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cprn60 and 16S–23S PCR is shown in Table 3, and the complete results of all assays are shown in S3 Table. The fluorescent microsphere assay employed a PCR step that did not require agarose gel electrophoresis to determine a positive result and also returned strain-level information without the need for DNA sequencing. Overall the sensitivity of the PCR-fluorescent microsphere method was low, consistent with results we have observed with this assay using other samples (data not shown). However, the strain-level information returned for positive samples allowed the immediate determination of the strain of phytoplasma observed in each sample. In 29 of 30 cases in which both PCR product sequences and fluorescent microsphere hybridization results were obtained from the same templates, the results agreed. One sample (BN28T-94) appeared to be most closely related to AY-WB by direct sequencing of the PCR product but was positive for both AY-WB and AY-OY-M by the hybridization assay (S3 Table). Close examination of the sequence revealed that both sequences were present in the PCR product generated from this sample (S2 Fig. and S4 Table). This was subsequently confirmed by cloning this PCR product and sequencing several clones (data not shown). Similar double positive infections were noted in three other samples of B. napus (S3 Table).

Comparison of phytoplasma infection patterns in host plants.
The phytoplasma strains infecting two oilseed crops, Camelina sativa and Brassica napus, grown at the same geographic location at the same time were compared using 16S-23S rRNA and cprn60-encoding gene sequences. Within the infected B. napus plants examined, a mixture of two AY subtypes were identified using cprn60, with both AY-OY-M (65%) and AY-WB (35%) found in these samples (Table 4 and S5 Table). In contrast, only AY-OY-M was found in the C. sativa plants grown in the same location in the same year. The cprn60 UT sequences of these strains were 98% identical (S3 Fig.); however the 16S–23S rRNA-encoding sequences of the phytoplasma strains infecting the two oilseed crops were indistinguishable (S4 Fig.).

Discussion
Rapid, accurate diagnosis of phytoplasma infection and strain differentiation in host plants and insect vectors is essential to support disease surveillance, international trade in agricultural goods, and timing of interventions such as pesticide application to mitigate disease spread. Since ‘Ca. Phytoplasma’ spp. are obligate intracellular pathogens that are difficult or currently impossible to grow.
in culture, strain detection and differentiation has relied on methods based on the amplification of specific gene targets in the phytoplasma genome. Many different PCR methods have been developed targeting the 16S–23S rRNA-encoding gene region that can amplify targets from all known ‘Ca. Phytoplasma’ spp. [6, 40], and the phylogeny and classification of ‘Ca. Phytoplasma’ spp. has been described based on the sequences of this genomic region [7, 32].

While the 16S–23S rRNA-encoding region has proven to be very useful for the detection and differentiation of ‘Ca. Phytoplasma’ spp., certain disadvantages are associated with its use for routine screening. These limitations are highlighted by ongoing efforts to define alternative, protein-encoding molecular barcoding targets for phytoplasma detection and differentiation, which have resulted in an array of useful molecular tools becoming available for phytoplasma detection and differentiation, each with its own advantages and disadvantages. One such target is the elongation factor TU (tuf gene), which has been used to differentiate effectively strains of ‘Ca. P. asteris’ [42], and has been proposed as a useful molecular barcode for ‘Ca. Phytoplasma’ spp. [43]. Alternatively, a series of nine primer sets has been described that generate PCR products from a protein translocase subunit (secY) gene of a wide array of ‘Ca. Phytoplasma’ spp. [16]. The gene encoding RNA polymerase β-subunit (rpoB) has also been recently exploited as a molecular marker for phytoplasma [44]. All of these sequences are useful for differentiation of ‘Ca. Phytoplasma’ spp., and additional, non-ribosomal RNA-encoding molecular markers will provide further tools for the detection and differentiation of phytoplasma in plant and insect tissues.

Among the molecular markers proposed for DNA barcoding of phytoplasmas, only the gene encoding Cpn60 has been shown to be a molecular barcode for

Table 3. Summary of diagnostic assays conducted on 192 field-collected samples of *B. napus* from the 2012 growing season.

| test                                      | samples with typing results |
|-------------------------------------------|----------------------------|
|                                           | total pos | total neg | AY-OY-M | AY-WB | AY-OM and AY-WB | Total |
| cpn60 PCR-gel electrophoresis              | 153       | 39        | 27      | 11    | 0               | 38    |
| cpn60 PCR-fluorescent microsphere assay    | 125       | 67        | 53      | 68    | 4               | 125   |
| 16S–23S PCR-gel electrophoresis           | 123       | 69        | ND      | ND    | ND              | ND    |

Complete results for each sample are shown in S3 Table. 

a assayed using H279p/H280p. 

b only PCR products from samples that gave discordant results between P1/Tint (neg) and H279p/H280p (pos) were directly sequenced. One reaction failed so no typing information is available. For the fluorescent microsphere assays, all positive samples returned a typing result. 

c ND, not determined.

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Table 4. Determination of *Phytoplasma* subtypes from host plants grown in 2012 at a single location in Saskatoon, SK, Canada (52.13° N, 106.68° W).

| host          | Number of samples identified as AY subtype | total |
|---------------|-------------------------------------------|-------|
| *B. napus*    | AY-OY-M                                   | 42    |
|               | AY-WB                                     | 23    |
|               | total                                     | 65    |
| *C. sativa*   | AY-OY-M                                   | 70    |
|               | AY-WB                                     | 0     |
|               | total                                     | 70    |

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Bacteria that meets the criteria set forth by the International Barcode of Life in terms of barcode gap [19]. Furthermore, cpn60 has been shown to provide high resolution differentiation of *Ca. P. asteris* [14, 15]. However, the cpn60-targeted PCR assays that have been described are limited to certain subgroups, possibly due to the very high sequence divergence observed at this locus. The cpn60 UT has been exploited as a region of cpn60 that is accessible from nearly any bacterial genome (and many eukaryotic genomes) using a set of degenerate universal primers [22], and we sought to use this region as a basis for developing a PCR assay that can detect and differentiate *Ca.Phytoplasma* spp. in plant- or insect-derived DNA extracts. The primer sets that we have described yielded a PCR product with a size that is amenable to single-pass Sanger sequencing (552–555 bp when trimmed of primer sequences) and that possessed a very wide range of sequence diversity (61–98% identity). These primers were used to amplify the cpn60 UT from 43 phytoplasma-infected samples representing seven widely divergent 16Sr groupings. PCR based on cpn60 was more sensitive than 16S-23S-based PCR and had a lower LOD (Table 2).

One potential limitation of the use of the cpn60 UT for phytoplasma detection is the possibility of amplifying non-target bacterial and eukaryotic genes. Indeed, the specificity of the cpn60-based method we describe was low (43%), which suggests a high rate of false positives by this method. However, the sequences of these amplicons were identical or nearly identical to *Ca.Phytoplasma* spp., demonstrating that rather than being false positive by the cpn60-targeted assay, these samples were false negatives by the P1/Tint assay. In fact, the sensitivity of the 16S-23S-targeted PCR assay using the cpn60-targeted assay as a gold standard was only 0.745 (95% CI 0.069), suggesting that the cpn60-targeted PCR assay we have described is more sensitive than the 16S-23S-targeted assay.

The molecular phylogeny of *Ca.Phytoplasma* spp. that is described using cpn60 UT sequences is congruent with what has been described based on 16S rRNA-encoding sequences [7, 32], with three major clades identified. Group 1 is a basal clade that includes sequences derived from *‘Ca.P. asteris’* (AY; 16SrI) and *‘Ca. P. solani’* (Bois noir; 16SrXII). The second clade consists of strains identified as Apple Proliferation, Pear Decline, and European Stone Fruit Yellows (16SrX). The third clade, which is by far the most divergent both by 16S rRNA gene sequences and cpn60 UT sequences, consisted of a large number of 16Sr groupings. We amplified the cpn60 UT from three members of this clade (*‘Ca.P. fraxini’, ‘Ca.P. ulmi’, ‘Ca.P. phoenicium’*) and, despite the fact that we were unable to source DNA from an isolate of PnWB phytoplasma (16SrII), we would expect to be able to amplify its cpn60 UT since its sequence was used as the basis for the primer design in this study.

While these sequences represent a diverse sampling of the breadth of phytoplasma taxonomy, we were not able to access template DNA from all described *‘Ca.Phytoplasma’* spp. (or all representative 16Sr groups). For this reason it is not certain that the primer sets we describe will successfully amplify cpn60 from all known *‘Ca.Phytoplasma’* spp. This fact highlights one of the most important limitations of the phytoplasma cpn60 amplification strategy: genome
sequencing has revealed that certain ‘Ca. Phytoplasma’ spp. belonging to 16SrIII (including X-disease) lack the gene encoding Cpn60 [45], as has been noted in certain Mollicutes [46]. This does not necessarily mean, however that all phytoplasma in the 16SrIII group lack cpn60. Moreover, while a strength of the strategy we describe is the diversity of the sequences amplified, this very diversity may limit the ability to access novel cpn60 sequences from highly divergent ‘Ca. Phytoplasma’ spp. DNA extracts from samples of unknown status that test negative by the cpn60 PCR assay may therefore be negative, may contain PCR inhibitory compounds, may contain a phytoplasma cpn60 that is not recognized by the primers, or may contain DNA from a strain of phytoplasma that lacks cpn60. The first two possibilities can be countered using an internal amplification control [47], but the latter two possibilities mean that unknown samples that test negative by cpn60 PCR should be screened using 16S–23S or other amplification strategies. It is also likely that the cpn60-targeted PCR primers described here can be changed to improve their breadth of detection as new full-length phytoplasma cpn60 sequences accumulate from genomic sequencing efforts, which is quickly becoming the standard for bacterial species delineation [48–50]. Genomic sequencing of ‘Ca. Phytoplasma’ spp. will also provide a more comprehensive description of species that may lack the gene encoding Cpn60.

The use of cpn60 as a diagnostic target for ‘Ca. Phytoplasma’ spp. presents advantages regarding strain resolution compared to previously described 16S–23S-targeted PCR assays [14, 15]. The novel cpn60-based molecular diagnostic assays we have developed provide a set of tools to answer various questions regarding the presence of phytoplasma DNA in a sample. First, phytoplasma PCR followed by gel electrophoresis can sensitively determine if the sample contains DNA from a strain of phytoplasma that encodes Cpn60 in its genome, providing a binomial (positive/negative) result. Second, hybridization to oligonucleotide-coupled fluorescent microspheres provides similar binomial data but also rapidly returns information regarding the specific strain of phytoplasma that is present in the sample without the need for gel electrophoresis or amplicon sequencing. Application of these assays revealed a differential pattern of phytoplasma strain infection between Camelina sativa and Brassica napus that was difficult or impossible to identify using 16S–23S rRNA-encoding sequences. The identification of both AY-OY-M and AY-WB in B. napus plants but only the former strain in C. sativa plants from the same geographic location (and presumably the same potential pool of vectors) suggests that different vectors might have infected B. napus and C. sativa. In Saskatchewan, the aster leafhopper (Macrosteles quadrilineatus) is considered to be the main AY vector in oilseed crops [51], but other known AY vectors have been identified in and around those crops, sometimes in high numbers (Soroka et al, Can. Entomol., in press). Alternatively, C. sativa may have been infected with vectors carrying both subtypes but was only able to support the replication of one of the subtypes of phytoplasma. Multiple infections have been described in insect vectors [1] and in perennial and annual plants [51, 52]. Other explanations of this differential infection pattern such as vector feeding behavior or attraction/repellence from the host plants are also
possible, and the biological significance of this observation is presently unknown. However, the detection of multiple infections is of consequence regarding the development of tools (whether molecular or bioassays) to screen for phytoplasma resistance in oilseeds. The cpn60-based diagnostic tools we have described provide a means to address this and other questions related to phytoplasma strain distribution and plant and insect vector susceptibility. The availability of new cpn60 sequence data for phytoplasma also facilitates the development of other molecular diagnostic assays, such as loop-mediated isothermal DNA amplification [30] and quantitative PCR. These cpn60-based diagnostic tools will provide an important complement to existing 16S–23S, tuf, secY, rpoB, and other molecular diagnostic assays for phytoplasma.

Supporting Information

S1 Fig. Sequence alignment of cpn60-targeted PCR primer hybridization sites. Sequences are identified by their cpnDB ID numbers (cpndb.ca), or by their sample of origin; primer hybridization sites for plant samples were determined from sequences generated by primer pair AY-groELF-H280p (see text for details). These sites are immediately upstream (A) and downstream (B) of the universal target sequences described in this manuscript. The downstream sequences are reverse-complemented relative to the coding region of the cpn60 gene for clarity. The sequences of the cpn60-targeted amplification primers are shown below each alignment. Sequence logos demonstrating base distributions at each site were generated using the tool provided at http://weblogo.berkeley.edu/logo.cgi. doi:10.1371/journal.pone.0116039.s001 (PNG)

S2 Fig. Evidence of a mixed infection in B. napus sample BN28T-94. The PCR product generated from H279p/H280p was directly sequenced using the amplification primers. The indicated position was called as an ‘A’ but a clear ‘C’ signal can be seen on both strands. Similar results were observed at 11/11 sites of difference between these two sequences (S4 Table). doi:10.1371/journal.pone.0116039.s002 (PNG)

S3 Fig. cpn60 UT sequences identified in samples collected from infected B. napus and C. sativa plants. Two sequences were identified in the B. napus samples that were similar or identical to both AY-OY-M and AY-WB while the C. sativa samples displayed evidence of only a single strain of phytoplasma, AY-OY-M. The sequences of AY-OY-M and AY-WB were 98% identical. doi:10.1371/journal.pone.0116039.s003 (PNG)

S4 Fig. 16S-23S-encoding sequences identified in the same pool of infected B. napus and C. sativa samples. doi:10.1371/journal.pone.0116039.s004 (PNG)

S1 Table. Phytoplasma strain sources. doi:10.1371/journal.pone.0116039.s005 (XLSX)

S2 Table. Oligonucleotide sequences and amplification conditions.
S3 Table. **cpn60**-based PCR/sequencing and fluorescent microsphere assay results on infected and uninfected *B. napus* plants.
doi:10.1371/journal.pone.0116039.s007 (XLSX)

S4 Table. Evidence of mixed infection of AY-OY-M and AY-WB (Ca. *P. asteris*) in *B. napus* sample BN28T-94 revealed by fluorescent microsphere assay.
doi:10.1371/journal.pone.0116039.s008 (XLSX)

S5 Table. Determination of Phytoplasma strains infecting *B. napus* and *C. sativa* plants grown at the AAFC Experimental Research Farm in Saskatoon, Saskatchewan, Canada, in 2012.
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**Author Contributions**
Conceived and designed the experiments: TD CO MG. Performed the experiments: EP CH TD. Analyzed the data: TD EP CO MG. Contributed reagents/materials/analysis tools: EP MG. Wrote the paper: TD MG CO.

**References**

1. Weintraub PG, Beanland L (2006) Insect vectors of phytoplasmas. Annu Rev Entomol. pp.91–111.
2. Gasparich GE (2010) Spiroplasmas and phytoplasmas: microbes associated with plant hosts. Biologicals 38: 193–203.
3. Namba S (2011) Phytoplasmas: A century of pioneering research. J Gen Plant Pathol 77: 345–349.
4. Contaldo N, Bertaccini A, Paltrinieri S, Windsor HM, David Windsor G (2012) Axenic culture of plant pathogenic phytoplasmas. Phytopathol Mediterr 51: 607–617.
5. Zhao Y, Davis RE, Wei W, Shao J, Jomantiene R (2014) Phytoplasma Genomes: Evolution Through Mutually Complementary Mechanisms, Gene Loss and Horizontal Acquisition. In: Gross DC, editor. Genomics of Plant-Associated Bacteria. Berlin Heidelberg: Springer-Verlag. pp. 235–271.
6. Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, et al. (1996) Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. Appl Environ Microbiol 62: 2988–2993.
7. Maejima K, Oshima K, Namba S (2014) Exploring the phytoplasmas, plant pathogenic bacteria. J Gen Plant Pathol 80: 210–221.
8. Lee IM, Davis RE, Gundersen-Rindal DE (2000) Phytoplasma: Phytopathogenic mollicutes. Annu Rev Microbiol. pp.221–255.
9. Lee IM, Gundersen-Rindal DE, Davis RE, Bartoszyk IM (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int J Sys Bacteriol 48: 1153–1169.
10. Wei W, Davis RE, Lee IM, Zhao Y (2007) Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. Int J Syst Evol Microbiol 57: 1855–1867.
11. Angelini E, Luca Bianchi G, Filippini L, Morassutti C, Borgo M (2007) A new TaqMan method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. J Microbiol Meth 68: 613–622.

12. Nikolic P, Mele N, Gruden K, Ravnikar M, Dermastia M (2010) A panel of real-time PCR assays for specific detection of three phytoplasmas from the apple proliferation group. Mol Cell Probes 24: 303–309.

13. Lee IM, Zhao Y, Bottner KD (2006) SecY gene sequence analysis for finer differentiation of diverse strains in the aster yellows phytoplasma group. Molec Cell Probes 20: 87–91.

14. Mitrovic J, Contaldo N, Pattrinieri S, Mejia JF, Mori N, et al. (2011) The use of groEL gene for characterisation of aster yellows phytoplastmas in field collected samples. Bull Insectol 64: S17–S18.

15. Mitrovic J, Kakizawa S, Duduk B, Oshima K, Namba S, et al. (2011) The groEL gene as an additional marker for finer differentiation of ‘Candidatus Phytoplasma asteris’-related strains. Ann Appl Biol 159: 41–48.

16. Lee IM, Bottner-Parker KD, Zhao Y, Davis RE, Harrison NA (2010) Phylogenetic analysis and delineation of phytoplastmas based on secY gene sequences. Int J Syst Evol Microbiol 60: 2887–2897.

17. Martini M, Lee IM, Bottner KD, Zhao Y, Botti S, et al. (2007) Ribosomal protein gene-based phylogeny for finer differentiation and classification of phytoplasmas. Int J Syst Evol Microbiol 57: 2037–2051.

18. Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int J Syst Evol Microbiol 53: 1893–1900.

19. Links MG, Dumonceaux TJ, Hemmingsen SM, Hill JE (2012) The chaperonin-60 universal target is a barcode for bacteria that enables de novo assembly of metagenomic sequence data. PLoS One 7: e49755.

20. Hill JE, Penny SL, Crowell KG, Goh SH, Hemmingsen SM (2004) cpnDB: A Chaperonin Sequence Database. Genome Res 14: 1669–1675.

21. Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, et al. (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J Clin Microbiol 34: 818–823.

22. Hill JE, Town JR, Hemmingsen SM (2006) Improved template representation in cpn60 polymerase chain reaction (PCR) product libraries generated from complex templates by application of a specific mixture of PCR primers. Environ Microbiol 8: 741–746.

23. Chaban B, Musil KM, Himsworth CG, Hill JE (2009) Development of cpn60-based real-time quantitative PCR assays for the detection of 14 Campylobacter species and application to screening of canine fecal samples. Appl Environ Microbiol 75: 3055–3061.

24. Goh SH, Facklam RR, Chang M, Hill JE, Tyrrell GJ, et al. (2000) Identification of Enterococcus species and phenotypically similar Lactococcus and Vagococcus species by reverse checkerboard hybridization to chaperonin 60 gene sequences. J Clin Microbiol 38: 3953–3959.

25. Hill JE, Paccagnella A, Law K, Melito PL, Woodward DL, et al. (2006) Identification of Campylobacter spp. and discrimination from Helicobacter and Arcobacter spp. by direct sequencing of PCR-amplified cpn60 sequences and comparison to cpnDB, a chaperonin reference sequence database. J Med Microbiol 55: 393–399.

26. Paramel Jayaprakash T, Schellenberg JJ, Hill JE (2012) Resolution and Characterization of Distinct cpn60-Based Subgroups of Gardnerella vaginalis in the Vaginal Microbiota. PLoS ONE 7: e43009.

27. Schellenberg JJ, Dumonceaux TJ, Hill JE, Kimani J, Jaoko W, et al. (2012) Selection, Phenotyping and Identification of Acid and Hydrogen Peroxide Producing Bacteria from Vaginal Samples of Canadian and East African Women. PLoS One 7: e41217.

28. Dumonceaux TJ, Hill JE, Briggs SA, Amoako KK, Hemmingsen SM, et al. (2006) Enumeration of specific bacterial populations in complex intestinal communities using quantitative PCR based on the chaperonin-60 target. J Microbiol Meth 64: 46–62.

29. Dumonceaux TJ, Schellenberg J, Golekski V, Hill JE, Jaoko W, et al. (2009) Multiplex detection of bacteria associated with normal microbiota and with bacterial vaginosis in vaginal swabs by use of oligonucleotide-coupled fluorescent microspheres. J Clin Microbiol 47: 4067–4077.
30. Sugawara K, Himeno M, Keima T, Kitazawa Y, Maejima K, et al. (2012) Rapid and reliable detection of phytoplasma by loop-mediated isothermal amplification targeting a housekeeping gene. J Gen Plant Pathol 78: 389–397.

31. Daire X, Clair D, Reinitz W, Boudon-Padieu E (1997) Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. Eur J Plant Pathol 103: 517–514.

32. Chung W-C, Chen L-L, Lo W-S, Lin C-P, Kuo C-H (2013) Comparative Analysis of the Peanut Witches’-Broom Phytoplasma Genome Reveals Horizontal Transfer of Potential Mobile Units and Effectors. PLoS One 8: e62770.

33. Dorsch M, Stackebrandt E (1992) Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. J Microbiol Meth 16: 271–279.

34. Banoo S, Bell D, Bossuyt P, Herring A, Mabey D, et al. (2006) Evaluation of diagnostic tests for infectious diseases: general principles. NatRevMicrobiol 4: S21–S31.

35. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611–622.

36. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

37. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526.

38. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.

39. Dumonceaux TJ, Town JR, Hill JE, Chaban BL, Hemmingsen SM (2011) Multiplex detection of bacteria in complex clinical and environmental samples using oligonucleotide-coupled fluorescent microspheres. J Vis Exp: e3344.

40. Deng S, Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. J Microbiol Meth 14: 53–61.

41. Lee I-M, Hammond RW, Davis RE, Gundersen DE (1993) Universal Amplification and Analysis of Pathogen 16S rDNA for Classification and Identification of Mycoplasmalike Organisms. Phytopathol 83: 834–842.

42. Marcone C, Lee IM, Davis RE, Ragozzino A, Seemuller E (2000) Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and tuf gene sequences. Int J Syst Evol Microbiol 50 Pt 5: 1703–1713.

43. Makarova O, Contaldo N, Paltrinieri S, Kawube G, Bertaccini A, et al. (2012) DNA Barcoding for Identification of ‘Candidatus Phytoplasma’ Using a Fragment of the Elongation Factor Tu Gene. PLoS ONE 7: e52092.

44. Vailunas D, Jomantiene R, Davis RE (2013) Evaluation of the DNA-dependent RNA polymerase β-subunit gene (rpoB) for phytoplasma classification and phylogeny. Int J Syst Evol Microbiol 63: 3904–3914.

45. Saccardo F, Martini M, Palmano S, Ermacora P, Scortichini M, et al. (2012) Genome drafts of four phytoplasma strains of the ribosomal group 16SrIII. Microbiology 158: 2805–2814.

46. Clark GW, Tillier ERM (2010) Loss and gain of GroEL in the Mollicutes. Biochem Cell Biol 88: 185–194.

47. Hoofar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, et al. (2004) Practical Considerations in Design of Internal Amplification Controls for Diagnostic PCR Assays. J Clin Microbiol 42: 1863–1868.

48. Firrao G, Martini M, Ermacora P, Loi N, Torelli E, et al. (2013) Genome wide sequence analysis grants unbiased definition of species boundaries in “Candidatus Phytoplasma”. Syst Appl Microbiol 36: 539–548.

49. Richter M, Rossello-Mora R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106: 19126–19131.

50. Mende DR, Sunagawa S, Zeller G, Bork P (2013) Accurate and universal delineation of prokaryotic species. Nature Meth 10: 881–884.
51. Olivier C, Séguin-Swartz G, Galka B, Olfert O (2011) Aster yellows in leafhoppers and field crops in Saskatchewan, Canada, 2001–2008. Am J Plant Sci Biotechnol 141: 425–462.

52. Seemuller E, Kiss E, Sule S, Schneider B (2010) Multiple infection of apple trees by distinct strains of ‘Candidatus Phytoplasma mali’ and its pathological relevance. Phytopathol 100: 863–870.