

## Description of '*Candidatus Phytoplasma meliae*', a phytoplasma associated with Chinaberry (*Melia azedarach* L.) yellowing

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China tree yellows (ChTY) phytoplasma is associated with the yellowing disease of the China tree (*Melia azedarach*) in Argentina. According to partial 16S rRNA gene analysis, ChTY phytoplasma belongs to the 16Sr XIII group, subgroup G. Strains of species of ChTY have 98–99% 16S rDNA gene sequence similarity with 16SrXIII-group phytoplasmas, and less than 97.5% when compared to all '*Candidatus Phytoplasma*' described so far, except for the novel '*Candidatus Phytoplasma hispanicum*'. However, strains of species of ChTY are differentiated from the latter due to having additional molecular and biological attributes. The presence of unique features in the 16S rDNA sequence distinguishes ChTY from all species of '*Candidatus Phytoplasma*' currently described. The *in silico* RFLP profile of 16S rDNA (1.2 kb) and rplV-rpsC (1.3 kb) genes distinguished ChTY, as in the 16SrXIII-G subgroup within the 16SrXIII group. The phylogenetic analyses, based on 16S rDNA, rplV-rpsC and secA gene sequences, in addition to the restricted host range, characteristic symptoms and geographical distribution, confirm that the collective strains of the species ChTY represent a distinct lineage within the phytoplasma clade and support the description of a novel species of '*Candidatus Phytoplasma meliae*' with the reference strain being ChTY-Mo3 (Montecarlo, Argentina).

Phytoplasmas are pleomorphic bacteria without cell walls, which are derived from an acholeplasma-like ancestor. They are characterized by having small, AT-rich genomes encoding capabilities for a transkingdom parasitic lifestyle, on plants, insects and others (Lee *et al.*, 2000). These

pathogens cause disease on a broad scale and with a range of symptom intensities in more than 1000 species of plant (Bertaccini, 2007). Molecular analyses have provided considerable insight into the diversity and genetic interrelationships of phytoplasmas (Hogenhout *et al.*, 2008). RFLP analysis of 16S rRNA gene sequences has been widely used as a means to identify, differentiate and classify phytoplasmas into a series of groups and subgroups (Lee *et al.*, 1993, 1998). According to this classification scheme and data obtained from RFLP *in silico* analyses (Wei *et al.*, 2007, 2008; Zhao *et al.*, 2009a; Nejat *et al.*, 2013; Bertaccini *et al.*, 2014; Pérez-López *et al.*, 2016), 34 16Sr groups and more than 100 subgroups have been, so far, delineated. Due to difficulties in establishing axenic cultures, phytoplasmas have been assigned to the provisional genus '*Candidatus Phytoplasma*' (IRPCM, 2004). At the time of

**Abbreviations:** ChTY, China tree yellows; RFLP, Restriction Fragment Length **Polymerisation**; MPV, Mexican periwinkle virescence phytoplasma.

GenBank/EMBL/DDBJ accession numbers of gene sequence from representative strain [*Ca. Phytoplasma meliae*' ChTY-Mo3] are: KU850940, KU850944 and KU850948 (16S rDNA, secA and rplV-rpsC genes respectively).

Six supplementary figures and one supplementary table are available with the online Supplementary Material.

writing, 39 species of ‘*Candidatus Phytoplasma*’ have been described (Davis *et al.*, 2016) and another 12 additional species have been suggested on the basis of their mutually distinct 16S rRNA gene sequences (Wei *et al.*, 2007).

China-tree or, in Spanish, ‘paraíso’ (*Melia azedarach* L.) is a fast-growing species that is native to Southeast Asia (Ascher *et al.*, 1995), which has been widely introduced in South America as an ornamental shade tree and for commercial purposes. In addition to timber exploitation, this species is used for its active ingredients, which have diverse pharmacological properties (Rupa *et al.*, 2014; Sanna *et al.*, 2015). Two varieties of *M. azedarach* are grown in Argentina [(*gigantea*, which is commercially used for furniture manufacturing, and *umbraculifera*, which is mainly grown for gardening and landscaping (Ragonese, 1981)].

China-tree yellows disease has been associated with phytoplasmas in several South American and Asian countries, including Argentina (Vázquez *et al.*, 1983; Galdeano *et al.*, 2004; Arneodo *et al.*, 2007), Paraguay (Matsuoka *et al.*, 1986; Arneodo *et al.*, 2005), Brazil (Muñoz *et al.*, 1987; Duarte *et al.*, 2009), Bolivia (Harrison *et al.*, 2003), China (Qui *et al.*, 1998), Vietnam (Harrison *et al.*, 2006), and Korea (Han *et al.*, 2015). Disease symptoms include reduced leaf size and yellowing, and witches’-broom and dieback. According to the RFLP and sequence analyses of 16S rRNA genes, the China-tree yellowing-associated phytoplasmas reported in Asiatic countries are related to ‘*Candidatus Phytoplasma asteris*’. The phytoplasmas that occur in South America, rather, have been classified into three different subgroups: (i) China-tree decline phytoplasma (ChTDIII), attributed to 16SrIII group, subgroup B (Harrison *et al.*, 2003; Galdeano *et al.*, 2004; Arneodo *et al.*, 2005, 2007; Duarte *et al.*, 2009), (ii) Chinaberry yellows phytoplasma (CbY1), included in 16SrXIII group, subgroup C (Harrison *et al.*, 2003) and (iii) China tree yellows phytoplasma (ChTY) corresponding to the 16SrXIII group, subgroup G (Arneodo *et al.*, 2007, formerly 16SrXIII-C, reclassified by Pérez-López *et al.*, 2016). Studies on the geographical distribution of both phytoplasmas in Argentina revealed that while ChTDIII spreads over a wide latitudinal range, ChTY phytoplasma is restricted to subtropical regions of Northern Argentina, sharing vegetation and climatic characteristics with Bolivia and Paraguay (Arneodo *et al.*, 2007).

According to current taxonomy, the data we have up to now suggest that ChTDIII phytoplasma is related to ‘*Candidatus Phytoplasma pruni*’, a species within the 16SrIII-group. With respect to ChTY and related phytoplasmas in the 16SrXIII group, ‘*Candidatus Phytoplasma hispanicum*’ (Davis *et al.*, 2016) was recently described in association with the MPV (mexican periwinkle virescence phytoplasma, 16SrXIII-A). Here, we present genomic and biological characteristics of four strains of species of ChTY phytoplasma that support the description of the novel species ‘*Candidatus Phytoplasma meliae*’ (reference strain ChTY-Mo3). The key differences in ‘*Candidatus Phytoplasma hispanicum*’ are

discussed. Symptomatic leaf samples were collected in four locations situated in Northeastern Argentina: Montecarlo (Mo) and Cerro Azul (Ce) (Misiones Province), Presidencia Roque Saénz Peña (RS) (Chaco Province) and Yapeyú (Ya) (Corrientes Province). Leaf midrib and petiole portions were used for DNA extraction, according to Doyle & Doyle (1990). Healthy China tree plants maintained in greenhouses were grafted with infected branches to perpetuate isolates. Also, periwinkle ChTY-infected plants were obtained by dodder *Cuscuta subinclusa* transmission from China tree infected plants in order to compare the symptoms produced by ChTY with those of other phytoplasmas. Phytoplasma detection was conducted by PCR using the universal primers P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) and R16F2/R16R2 (Lee *et al.*, 1993), as previously described (Arneodo *et al.*, 2007). PCR-RFLP patterns of partial 16S rRNA gene (primers R16F2/R16R2) digested with *Mse*I, *Hpa*II, *Rsa*I and *Hae*III endonucleases (NEB) were evaluated first, in order to distinguish ChTY (16SrXIII-G) from ChTDIII (16SrIII-B) phytoplasmas (Arneodo *et al.*, 2005, 2007).

Four isolates of ChTY phytoplasma, representative of each location were selected for molecular characterization: ChTY-Mo3, ChTY-Ce3, ChTY-RS3 and ChTY-Ya4. 16S-23S rRNA (1.8 kb) and rplV-rpsC gene operons (1.3 kb) were amplified by PCR using primers P1/P7 and rpF1/rpR1 (Lim & Sears, 1992), respectively. A new pair of specific primers was designed, based on the ChTY *secA* gene sequence (KU950322). Primers (*secA*-ChTYFw: 5'-GCTTTAAGCG-GAAATCCCGTCCAT -3'/*secA*-ChTYRv: 5'-AACCC-CTTCCTTAGCTTCTAAGGC -3') were used to amplify a partial sequence of the *secA* gene (~680 pb) under the following cycling conditions: 35 cycles of 94 °C for 30 s (3 min initial denaturalization), 56 °C for 1 min and 72 °C for 1 min (5 min final extension). All PCR products were purified using Illustra MicroSpin S-400 HR Columns (GE) and cloned into a pGEM-T Easy vector (Promega). Three clones of each gene and ChTY-isolate were selected and sequenced in an automatic genetic analyzer (Unidad Genómica, Instituto de Biotecnología-INTA, Argentina) to obtain 3x coverage per base position. The consensus sequences were assembled using the Staden program package (Staden *et al.*, 2000), and deposited in the GenBank (NCBI/EMBL) database. Virtual RFLP analysis of 16S rDNA (1.2 kb) and phytoplasma classification into 16Sr groups and subgroups were carried out using *iPhyClassifier* (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>). In the case of ribosomal protein genes, rplV-rpsC (1.3 kb), *in silico* RFLP profiles were obtained using pDRAW32 software ([www.acaclone.com](http://www.acaclone.com)). Nucleotide sequence multiple alignments were conducted using MUSCLE (MULTIPLE Sequence Comparison by Log-Expectation). The best-fit model of nucleotide substitution was determined using the jModelTest ver 2.1.6 (Darriba *et al.*, 2012) and used for phylogenetic reconstructions. A phylogenetic tree was reconstructed with the maximum-likelihood method with phyML 3.0 software (Guindon *et al.*, 2010) and bootstrap (1000 repetitions) for statistical support.

**Table 1.** Analysis of 16S rRNA nucleotide identity between 'Candidatus Phytoplasma meliae' (ChTY-Mo3 reference strain) and species of 'Candidatus Phytoplasma', which have been accepted or proposed to date

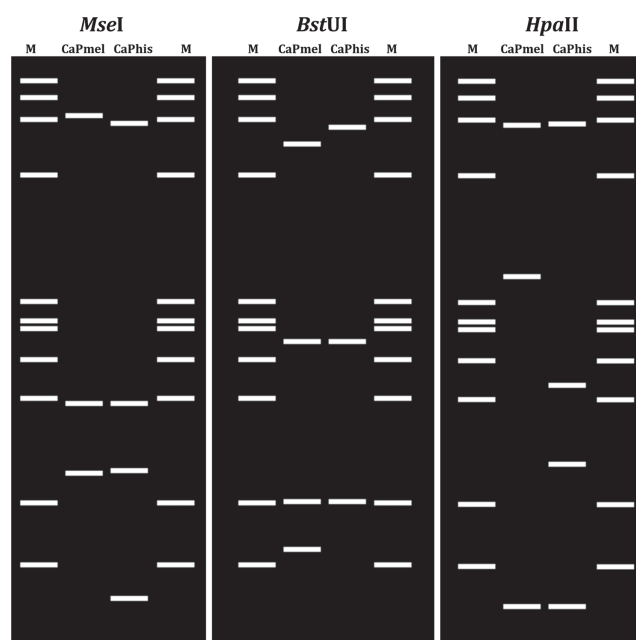
Formally described 'Ca. Phytoplasma species'	GenBank n°	16Sr group/ subgroup <sup>a</sup>	Id. 16S <sup>b</sup>	References
'Ca. Phytoplasma asteris'	M30790	16SrI-B	95, 73	Lee <i>et al.</i> (2004a)
'Ca. Phytoplasma aurantifolia'	U15442	16SrII-B	89, 69	Zreik <i>et al.</i> (1995)
'Ca. Phytoplasma australasia'	Y10097	16SrII-D	89, 79	White <i>et al.</i> (1998)
'Ca. Phytoplasmapruni'	JQ044393	16SrIII-A	90, 79	Davis <i>et al.</i> (2013)
'Ca. Phytoplasma phoenicium'	AF515636	16SrIX-D	89, 89	Verdin <i>et al.</i> (2003)
'Ca. Phytoplasma ulmi'	AY197655	16SrV-B	89, 80	Lee <i>et al.</i> (2004b)
'Ca. Phytoplasma ziziphi'	AB052876	16SrV-B	89, 88	Jung <i>et al.</i> (2003b)
'Ca. Phytoplasmarubi'	AY197648	16SrV-D	89, 88	Malembic-Maher <i>et al.</i> (2011)
'Ca. Phytoplasma trifolii'	AY390261	16SrVI-A	90, 54	Hiruki & Wang (2004)
'Ca. Phytoplasmamasudamericanum'	GU292081	16SrVI-I	90, 37	Davis <i>et al.</i> (2012)
'Ca. Phytoplasma fraxini'	AF092209	16SrVII-A	90, 30	Griffiths <i>et al.</i> (1999)
'Ca. Phytoplasma mali'	AJ542541	16SrX-A	92, 26	Seemüller & Schneider (2004)
'Ca. Phytoplasma pyri'	AJ542543	16SrX-C	92, 67	Seemüller & Schneider (2004)
'Ca. Phytoplasma spartii'	X92869	16SrX-D	92, 02	Marcone <i>et al.</i> (2004a)
'Ca. Phytoplasma prunorum'	AJ542544	16SrX-F	92, 09	Seemüller & Schneider (2004)
'Ca. Phytoplasma oryzae'	AB052873	16SrXI-A	90, 12	Jung <i>et al.</i> (2003a)
'Ca. Phytoplasma cirsiifolii'	KR869146	16SrXI-E	90, 37	Šafářová <i>et al.</i> (2016)
'Ca. Phytoplasma solani'	AF248959	16SrXII-A	95, 81	Quaglino <i>et al.</i> (2013)
'Ca. Phytoplasma australiense'	L76865	16SrXII-B	95, 89	Davis <i>et al.</i> (1997)
'Ca. Phytoplasma japonicum'	AB010425	16SrXII-D	95, 23	Sawayanagi <i>et al.</i> (1999)
'Ca. Phytoplasma fragariae'	DQ086423	16SrXII-E	96, 22	Valiunas <i>et al.</i> (2006)
'Ca. Phytoplasma hispanicum'	AF248960	16SrXIII-A	99, 82	Davis <i>et al.</i> (2016)
'Ca. Phytoplasma cynodontis'	AJ550984	16SrXIV-A	90, 79	Marcone <i>et al.</i> (2004b)
'Ca. Phytoplasma castaneae'	AB054986	16SrXIX-A	89, 06	Jung <i>et al.</i> (2002)
'Ca. Phytoplasma brasiliense'	AF147708	16SrXV-A	89, 14	Montano <i>et al.</i> (2001)
'Ca. Phytoplasma graminis'	AY725228	16SrXVI-A	94, 58	Arocha <i>et al.</i> (2005)
'Ca. Phytoplasma caricae'	AY725234	16SrXVII-A	93, 67	Arocha <i>et al.</i> (2005)
'Ca. Phytoplasma americanum'	DQ174122	16SrXVIII-A	95, 89	Lee <i>et al.</i> (2006)
'Ca. Phytoplasma rhamnii'	X76431	16SrXX-A	92, 00	Marcone <i>et al.</i> (2004a)
'Ca. Phytoplasma pini'	AJ632155	16SrXXI-A	90, 30	Schneider <i>et al.</i> (2005)
'Ca. Phytoplasma palmicola'	KF751387	16SrXXII-A	89, 89	Harrison <i>et al.</i> (2014)
'Ca. Phytoplasma omanense'	EF666051	16SrXXIX-A	89, 40	Al-Saady <i>et al.</i> (2008)
'Ca. Phytoplasma tamaricis'	FJ432664	16SrXXX-A	89, 40	Zhao <i>et al.</i> (2009b)
'Ca. Phytoplasma costaricanum'	HQ225630	16SrXXXI-A	94, 41	Lee <i>et al.</i> (2011)
'Ca. Phytoplasma malaysianum'	EU371934	16SrXXXII	91, 37	Nejat <i>et al.</i> (2013)
'Ca. Phytoplasma allosuarinae'	AY135523	ND	92, 41	Marcone <i>et al.</i> (2004a)
'Ca. Phytoplasma lycopersici'	EF199549	ND	93, 51	Arocha <i>et al.</i> (2007)
'Ca. Phytoplasma convolvuli'	JN833705	ND	95, 97	Martini <i>et al.</i> (2012)
'Ca. Phytoplasma balanitae'	AB689678	ND	89, 72	Win <i>et al.</i> (2013)
Provisional novel 'Ca. Phytoplasma' species and other incidentally cited strains:				
'Ca. Phytoplasma maluffae'	AF086621	16SrXII-A	90, 37	IRPCM (2004)
'Ca. Phytoplasma palmae'	AF498307	16SrIV-A	90, 95	IRPCM (2004)
'Ca. Phytoplasma vitis'	AF176319	16SrVIII-A	89, 80	IRPCM (2004)
'Ca. Phytoplasma cocostanzaniae'	X80117	16SrIV-C	90, 05	IRPCM (2004)

All the symptomatic China tree leaves analyzed ( $n=35$ ) were positive for phytoplasma infection. The presence of strains of species of ChTY was detected by 16S rDNA PCR-RFLP (Arneodo *et al.*, 2005, 2007) in 19 out of 35 samples tested, in every location surveyed. China tree plants experimentally infected in greenhouse conditions showed internode shortening, mainly in the apical region, leaf yellowing and size reduction (Fig. S1, available in the online Supplementary Material). Similar symptoms were observed in periwinkle plants infected with ChTY phytoplasma, which also showed internode shortening, leaf yellowing and size reduction (Fig. S2).

Sequence analysis showed 99.89–99.94% rDNA identity among ChTY-Mo3, ChTY-Ce3, ChTY-RS3 and ChTY-Ya4 strains, and less than 97.5% when compared to any ‘*Ca. Phytoplasma*’ species, except for ‘*Candidatus Phytoplasma hispanicum*’ MPV (AF248960) (Table 1), which had a 99.82 to 99.88% 16S rRNA gene sequence identity.

Virtual RFLP analysis of 16S rDNA showed that ChTY strains are members of the 16SrXIII-G subgroup (Pérez-López *et al.*, 2016) (Fig. S3, Table S1). Among the 17 enzymes considered for phytoplasmas classification (Lee *et al.*, 1998), *AluI*, *BstUI* and *HpaII* RFLP profiles clearly distinguished strains of species of ChTY from ‘*Candidatus Phytoplasma hispanicum*’ (Fig. 1), as demonstrated for *HpaII* by gel electrophoresis in previous works (Arneodo *et al.*, 2005, 2007).

The phylogenetic tree reconstructed by maximum-likelihood analysis, based on 16S rDNA sequences of ChTY



**Fig. 1.** *In silico* RFLP profile of the 16S rDNA partial gene (1.2 kb) obtained with *AluI*, *BstUI* and *HpaII* endonucleases. CaPmel: ‘*Candidatus Phytoplasma meliae*’ strain ChTY-Mo3 (reference sequence, KU850944); CaPhis: ‘*Candidatus Phytoplasma hispanicum*’ (AF248960); M:  $\phi$  X174-*HaeIII* digests.

strains, 16SrXIII group reference phytoplasmas, 39 ‘*Candidatus Phytoplasma* species’ and *Acholeplasma palmae* as the outgroup showed that ChTY was grouped within the 16SrXIII-group, but in a particular cluster with ChY phytoplasma. It was clearly separated from ‘*Candidatus Phytoplasma hispanicum*’, ‘*Candidatus Phytoplasma meliae*’ and ‘*Candidatus Phytoplasma hispanicum*’, which share a common ancestor within a major branch with ‘*Candidatus Phytoplasma asteris*’ and ‘*Candidatus Phytoplasma solani*’ (Fig. 2).

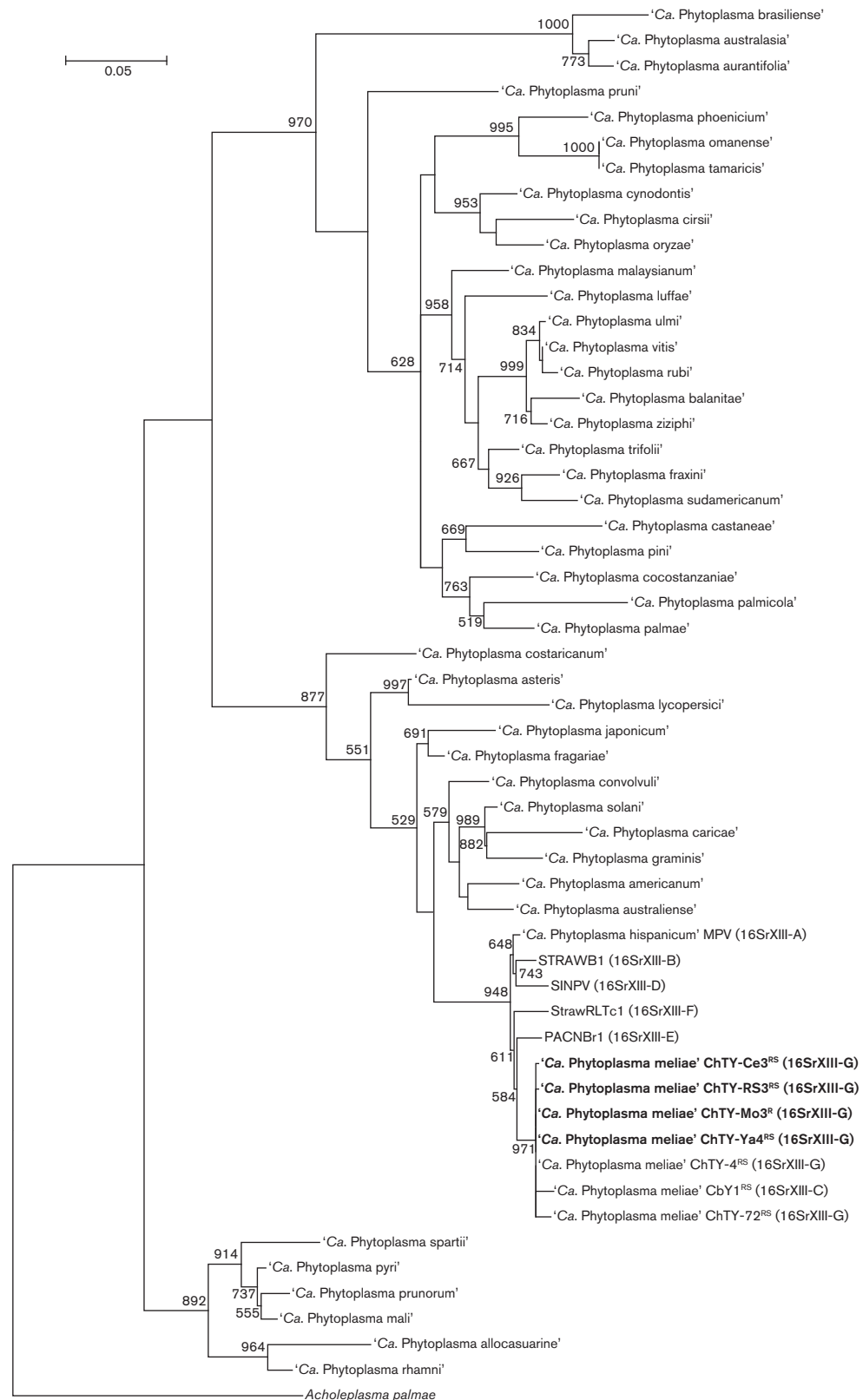
The 16S rDNA signature sequence characteristic to phytoplasmas 5′-CAAGAYBATKATGKTAGCYGGDCT-3′ (IRPCM, 2004), was found in position  $_{268}$ 5′-CAAGACGATGATGTG-TAGTCGGGCT-3′ $_{292}$  of the ChTY sequence with only one nucleotide change (position 286, T instead of C). The following sequences were identified as unique regions of the 16S rDNA gene of ChTY strains:  $_{296}$ 5′-GGCTGAACG-3′ $_{305}$ ;  $_{980}$ 5′-GCTTCTGCAAAGT-3′ $_{993}$  and  $_{1240}$ 5′-GAAGCGGAGCTT-3′ $_{1254}$ . These feature sequences were not present in the 16S rDNA gene of ‘*Candidatus Phytoplasma hispanicum*’.

Considering that ChTY phytoplasma has a higher 16S rDNA similarity with a ‘*Candidatus* species’ than the 97.5% cutoff value for designation of novel ‘*Candidatus Phytoplasma* species’, we analyzed other genes that would better reflect the differences between ChTY and ‘*Candidatus Phytoplasma hispanicum*’. The analysis of the rplV-rpsC operon from strains of species of ChTY-Mo3, ChTY-Ce3, ChTY-RS3 and ChTY-Ya4 (KU850944, KU850945, KU850946 and KU850947, respectively) revealed 100% identity among them and 95.14% with ‘*Candidatus Phytoplasma hispanicum*’ (EF193365). *BstUI*, *HaeIII*, *HhaI*, *MseI* and *TaqI* RFLP profiles (Fig. S4) clearly differentiated ChTY from ‘*Candidatus Phytoplasma hispanicum*’. For the *secA* gene, the sequences of strains of species of ChTY-Mo3, ChTY-Ce3, ChTY-RS3 and ChTY-Ya4 (KU850948, KU850949, KU850950 and KU850951, respectively) were compared with that of ‘*Candidatus Phytoplasma hispanicum*’ (EU168753). The analysis showed 100% identity among strains of species of ChTY and 94.10% with ‘*Candidatus Phytoplasma hispanicum*’.

Furthermore, phylogenetic trees reconstructed from the sequence of rpsC-rplV and *secA* genes were consistent with the 16S rDNA gene tree topology. In all cases, sequences of strains of species of ChTY formed a clade within the 16SrXIII-group cluster that separated it from ‘*Candidatus Phytoplasma hispanicum*’ (Figs S5 and S6).

On the basis of 16S rDNA sequence analysis, *in silico* RFLP patterns, phylogenetic trees and the detection of signature sequences, in addition to ribosomal protein and *secA* gene sequence analysis, symptoms, host range and geographical distribution, we propose the novel species ‘*Candidatus Phytoplasma meliae*’. The novel species is associated with China-tree yellowing disease in Argentina, Bolivia and Paraguay, with the following description.





**Fig. 2.** Phylogenetic tree inferred from an analysis of 16S rDNA sequences using the maximum-likelihood method. *Acholeplasma palmae* was used as the outgroup. The numbers on the branches are bootstrap (confidence) values (expressed as percentages of 1000 replicates). The GenBank accession number for each taxon is given in Table 1. The corresponding 16SrXIII

subgroups are also shown adjacent to each taxon and the strain of species of ChTY sequenced in this paper are in bold, <sup>RS</sup>: reference strain; <sup>RS</sup>: related strain.

## Description of ‘*Candidatus Phytoplasma meliae*’

Shares >97.5% 16S rDNA gene sequence identity with ‘*Candidatus Phytoplasma hispanicum*’ (Davis *et al.*, 2016), but additional genomic and biological features support a description as a separate species. For such cases, according to the IRPCM (2004), description of two different species is recommended only when all three of the following conditions apply: (i) the two phytoplasmas are transmitted by different vectors; (ii) the two phytoplasmas have a different natural plant host (or, at least, their behaviour is significantly different in the same plant host), and (iii) there is evidence of significant molecular diversity, achieved by either hybridization to cloned DNA probes, serological reactions or PCR-based assays. There is no evidence for the vectors that transmit these two phytoplasmas. However, their occurrence is limited to different geographic areas (‘*Candidatus Phytoplasma hispanicum*’ in Mexico - North America, ‘*Candidatus Phytoplasma meliae*’ in Argentina, Bolivia, and Paraguay - South America), so it could be assumed that the insect vectors involved are also different. With respect to the second condition, they have different natural plant hosts: ‘*Candidatus Phytoplasma hispanicum*’ has been described in association with *Catharathus roseus* (periwinkle), while ‘*Candidatus Phytoplasma meliae*’ has been detected only in *Melia azedarach* L. (China tree or paraíso). Moreover, the symptomatology observed in periwinkle plants experimentally infected with ‘*Candidatus Phytoplasma meliae*’ consisted of internode shortening and leaf yellowing/size reduction. These symptoms are different from those described for ‘*Candidatus Phytoplasma hispanicum*’ in the same host (virescence). With respect to the third condition, rpLV-rpsC and secA genes have significant sequence differences as well as *in silico* RFLP-profiles, SNPs and phylogeny, clearly distinguishing the species.

On the basis of these data, we propose the designation of the novel species ‘*Candidatus Phytoplasma meliae*’ as described below:

‘*Candidatus Phytoplasma meliae*’ (me’li.ae. N.L. gen. fem. n. meliae of Melia; of *Melia azedarach* referring to the plant host in which phytoplasma was described).

ChTY-Mo3 (Montecarlo, Argentina) is the reference strain (KU850940, KU850944 and KU850948 genbank accession for the 16S rDNA, rpLV-rpsC and secA, respectively). Related phytoplasma strains include ChTY-Ce3 (KU850941/KU850945/KU850949), ChTY-RS3 (KU850942/KU850946/KU850950) and ChTY-Ya4 (KU850943/KU850947/KU850951) (16S rDNA, rpLV-rpsC and secA, respectively) (present paper). Other related strains, based on 16S rDNA, are ChTY-4 and ChTY-72 (GenBank accession numbers

DQ444264 and DQ444265, respectively), and CbY1 (AF495882).

‘*Candidatus Phytoplasma meliae*’ [(Mollicutes) NC; NA; O, wall-less (GenBank accession number KU850940); oligonucleotide sequences of unique regions of the 16S rDNA gene are: <sup>296</sup>5’-GGCTGAACG-3<sub>305</sub>; <sup>980</sup>5’-GCTTCTGCAAAGT-3<sub>993</sub>; <sup>1240</sup>5’-GAAGCGCGAGCTTTT-3<sub>1254</sub>; P (*Melia azedarach*, phloem); M]

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## References

- Al-Saady, N. A., Khan, A. J., Calari, A., Al-Subhi, A. M. & Bertaccini, A. (2008). ‘*Candidatus Phytoplasma omanense*’, associated with witches’-broom of *Cassia italica* (Mill.) Spreng. in Oman. *Int J Syst Evol Microbiol* **58**, 461–466.
- Arneodo, J. D., Galdeano, E., Orrego, A., Stauffer, A., Nome, S. F. & Conci, L. R. (2005). Identification of two phytoplasmas detected in China-trees with decline symptoms in Paraguay. *Australas Plant Pathol* **34**, 583–585.
- Arneodo, J. D., Marini, D. C., Galdeano, E., Meneguzzi, N., Bacci, M., Domecq, C., Nome, S. F. & Conci, L. R. (2007). Diversity and geographical distribution of phytoplasmas infecting China-tree in Argentina. *J Phytopathol* **155**, 70–75.
- Arocha, Y., López, M., Piñol, B., Fernández, M., Picornell, B., Almeida, R., Palenzuela, I., Wilson, M. R. & Jones, P. (2005). ‘*Candidatus Phytoplasma graminis*’ and ‘*Candidatus Phytoplasma caricae*’, two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. *Int J Syst Evol Microbiol* **55**, 2451–2463.
- Arocha, Y., Antesana, O., Montellano, E., Franco, P., Plata, G. & Jones, P. (2007). ‘*Candidatus Phytoplasma lycopersici*’, a phytoplasma associated with ‘hoja de perejil’ disease in Bolivia. *Int J Syst Evol Microbiol* **57**, 1704–1710.
- Ascher, K. R. S., Schmutterer, H., Zebitz, C. P. W. & Naqvi, S. N. H. (1995). The Persian lilac or chinaberry tree: *Melia azedarach* L. In *The Neem Tree – Source of Unique Natural Products in Integrated Pest Management, Medicine, Industry and Other Purposes*, pp. 605–642. Edited by H. Schmutterer. Weinheim, Germany: VCH.
- Bertaccini, A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. *Front Biosci* **12**, 673–689.
- Bertaccini, A., Duduk, B., Paltrinieri, S. & Contaldo, N. (2014). Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *Am J Plant Sci* **5**, 1763–1788.
- Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* **9**, 772.
- Davis, R. E., Dally, E. L., Gundersen, D. E., Lee, I. M. & Habili, N. (1997). ‘*Candidatus phytoplasma australiense*’ a new phytoplasma taxon associated with Australian grapevine yellows. *Int J Syst Bacteriol* **47**, 262–269.
- Davis, R. E., Zhao, Y., Dally, E. L., Jomantiene, R., Lee, I. M., Wei, W. & Kitajima, E. W. (2012). ‘*Candidatus Phytoplasma sudamericanum*’, a novel taxon, and strain PassWB-Br4, a new subgroup 16SrIII-V phytoplasma,

from diseased passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.). *Int J Syst Evol Microbiol* 62, 984–989.

**Davis, R. E., Zhao, Y., Dally, E. L., Lee, I. M., Jomantiene, R. & Douglas, S. M. (2013).** 'Candidatus Phytoplasma pruni', a novel taxon associated with X-disease of stone fruits, *Prunus* spp.: multilocus characterization based on 16S rRNA, *secY*, and ribosomal protein genes. *Int J Syst Evol Microbiol* 63, 766–776.

**Davis, R. E., Harrison, N. A., Zhao, Y., Wei, W. & Dally, E. L. (2016).** 'Candidatus Phytoplasma hispanicum', a novel taxon associated with Mexican periwinkle virescence disease of *Catharanthus roseus*. *Int J Syst Evol Microbiol* 66, 3463–3467.

**Deng, S. & Hiruki, C. (1991).** Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *J Microbiol Methods* 14, 53–61.

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

**Duarte, V., Silva, E. G., Hass, I. C. R., Bedendo, I. P. & Kitajima, E. W. (2009).** First report of a group 16SrIII-B phytoplasma associated with decline of China tree in Brazil. *Plant Disease* 93, 666.

**Fernández, F. D., Meneguzzi, N. G., Guzmán, F. A., Kirschbaum, D. S., Conci, V. C., Nome, C. F. & Conci, L. R. Vilma, V. C. (2015).** Detection and identification of a novel 16SrXIII subgroup phytoplasma associated with strawberry red leaf disease in Argentina. *Int J Syst Evol Microbiol* 65, 2741–2747.

**Galdeano, E., Torres, L. E., Meneguzzi, N., Guzman, F., Gomez, G. G., Docampo, D. M. & Conci, L. R. (2004).** Molecular characterization of 16S ribosomal DNA and phylogenetic analysis of two X-disease group phytoplasmas affecting China-tree (*Melia azedarach* L.) and garlic (*Allium sativum* L.) in Argentina. *J Phytopathol* 152, 174–181.

**Griffiths, H. M., Sinclair, W. A., Smart, C. D. & Davis, R. E. (1999).** The phytoplasma associated with ash yellows and lilac witches'-broom: 'Candidatus phytoplasma fraxini'. *Int J Syst Bacteriol* 49, 1605–1614.

**Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010).** New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59, 307–321.

**Han, S.-S., Baek, S.-J., Lee, S.-H., Seo, S.-T. & Seralathan, K.-K. (2015).** First report of aster yellows phytoplasma (16SrI-B) associated with witches' broom disease of *Melia azedarach* var. *japonica* in Korea. *J Phytopathol* 163, 1055–1058.

**Harrison, N. A., Boa, E. & Carpio, M. L. (2003).** Characterization of phytoplasmas detected in Chinaberry trees with symptoms of leaf yellowing and decline in Bolivia. *Plant Pathol* 52, 147–157.

**Harrison, N. A., Carpio, M. L. & Boa, E. (2006).** First report of 'Candidatus phytoplasma asteris'-related strains infecting Chinaberry trees with leaf yellowing symptoms in Vietnam. *Plant Dis* 90, 527.

**Harrison, N. A., Davis, R. E., Oropeza, C., Helmick, E. E., Narváez, M., Eden-Green, S., Dollet, M. & Dickinson, M. (2014).** 'Candidatus Phytoplasma palmicola', associated with a lethal yellowing-type disease of coconut (*Cocos nucifera* L.) in Mozambique. *Int J Syst Evol Microbiol* 64, 1890–1899.

**Hiruki, C. & Wang, K. (2004).** Clover proliferation phytoplasma: 'Candidatus Phytoplasma trifolii'. *Int J Syst Evol Microbiol* 54, 1349–1353.

**Hogenhout, S. A., Oshima, K., Ammar, D., Kakizawa, S., Kingdom, H. N. & Namba, S. (2008).** Phytoplasmas: bacteria that manipulate plants and insects. *Mol Plant Pathol* 9, 403–423.

**IRPCM (2004).** 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int J Syst Evol Microbiol* 54, 1243–1255.

**Jomantiene, R., Davis, R. E., Maas, J. & Dally, E. L. (1998).** Classification of new phytoplasmas associated with diseases of strawberry in Florida, based on analysis of 16S rRNA and ribosomal protein gene operon sequences. *Int J Syst Bacteriol* 48, 269–277.

**Jung, H. Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Miyata, S., Oshima, K., Ugaki, M., Lee, J. T., Hibi, T. & other authors (2002).** 'Candidatus Phytoplasma castaneae', a novel phytoplasma taxon associated with chestnut witches' broom disease. *Int J Syst Evol Microbiol* 52, 1543–1549.

**Jung, H. Y., Sawayanagi, T., Wongkaew, P., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S., Ugaki, M. & other authors (2003a).** 'Candidatus Phytoplasma oryzae', a novel phytoplasma taxon associated with rice yellow dwarf disease. *Int J Syst Evol Microbiol* 53, 1925–1929.

**Jung, H. Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S., Ugaki, M., Hibi, T. & other authors (2003b).** 'Candidatus phytoplasma ziziphi', a novel phytoplasma taxon associated with jujube witches'-broom disease. *Int J Syst Evol Microbiol* 53, 1037–1041.

**Lee, I. M., Davis, R. E. & Gundersen-Rindal, D. E. (2000).** Phytoplasma: phytopathogenic mollicutes. *Annu Rev Microbiol* 54, 221–255.

**Lee, I. M., Bottner, K. D., Secor, G. & Rivera-Varas, V. (2006).** 'Candidatus Phytoplasma americanum', a phytoplasma associated with a potato purple top wilt disease complex. *Int J Syst Evol Microbiol* 56, 1593–1597.

**Lee, I. M., Bottner-Parker, K. D., Zhao, Y., Villalobos, W. & Moreira, L. (2011).** 'Candidatus Phytoplasma costaricanum' a novel phytoplasma associated with an emerging disease in soybean (*Glycine max*). *Int J Syst Evol Microbiol* 61, 2822–2826.

**Lee, I.-M., Hammond, R. W., Davis, R. E. & Gundersen, D. E. (1993).** Universal amplification and analysis of pathogen 16S rDNA for classification and identification of MLOs. *Phytopathology* 83, 834–842.

**Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E. & Bartoszyk, I. M. (1998).** Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int J Syst Bacteriol* 48, 1153–1169.

**Lee, I.-M., Gundersen-Rindal, D. E., Davis, R. E., Bottner, K. D., Marcone, C. & Seemüller, E. (2004a).** 'Candidatus Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *Int J Syst Bacteriol* 54, 1037–1048.

**Lee, I.-M., Martini, M., Marcone, C. & Zhu, S. F. (2004b).** Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of 'Candidatus Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *Int J Syst Bacteriol* 54, 337–347.

**Lim, P. O. & Sears, B. B. (1992).** Evolutionary relationships of a plant-pathogenic mycoplasma-like organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *J Bacteriol* 174, 2606–2611.

**Malembic-Maher, S., Salar, P., Filippin, L., Carle, P., Angelini, E. & Foissac, X. (2011).** Genetic diversity of European phytoplasmas of the 16SrV taxonomic group and proposal of 'Candidatus Phytoplasma rubi'. *Int J Syst Evol Microbiol* 61, 2129–2134.

**Marcone, C., Gibb, K. S., Streten, C. & Schneider, B. (2004a).** 'Candidatus Phytoplasma spartii', 'Candidatus Phytoplasma rhamnii' and 'Candidatus Phytoplasma allocasuarinae', respectively associated with spartium witches'-broom, buckthorn witches'-broom and allocasuarina yellows diseases. *Int J Syst Evol Microbiol* 54, 1025–1029.

**Marcone, C., Schneider, B. & Seemüller, E. (2004b).** 'Candidatus Phytoplasma cynodontis', the phytoplasma associated with bermuda grass white leaf disease. *Int J Syst Evol Microbiol* 54, 1077–1082.

**Martini, M., Marcone, C., Mitrović, J., Maixner, M., Delić, D., Myrta, A., Ermacora, P., Bertaccini, A. & Duduk, B. (2012).** 'Candidatus Phytoplasma convolvuli', a new phytoplasma taxon associated with bindweed yellows in four European countries. *Int J Syst Evol Microbiol* 62, 2910–2915.

**Matsuoka, K., Brune, A., Ansani, A. & Carvalho, M. G. (1986).** Association of a mycoplasma-like organism with *Melia azedarach* in Paraguay. *Revista Arvore* 10, 202–206.

- Melo, L., Silva, E., Flores, D., Ventura, J., Costa, H. & Bedendo, I. (2013). A phytoplasma representative of a new subgroup, 16SrXIII-E, associated with Papaya apical curl necrosis. *Eur J Plant Pathol* **137**, 445–450.
- Montano, H. G., Davis, R. E., Dally, E. L., Hogenhout, S., Pimentel, J. P. & Brioso, P. S. (2001). 'Candidatus Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease. *Int J Syst Evol Microbiol* **51**, 1109–1118.
- Muñoz, J. O., Nome, S. F. & Kitajima, E. W. (1987). A mycoplasma like organism associated with yellows of *Melia azedarach*. *Fitopatol Bras*:**12**, 95–97.
- Nejat, N., Vadamalai, G., Davis, R. E., Harrison, N. A., Sijam, K., Dickinson, M., Abdullah, S. N. & Zhao, Y. (2013). 'Candidatus Phytoplasma malaysianum', a novel taxon associated with virescence and phyllody of Madagascar periwinkle (*Catharanthus roseus*). *Int J Syst Evol Microbiol* **63**, 540–548.
- Pérez-López, E., Luna-Rodríguez, M., Olivier, C. Y. & Dumonceaux, T. J. (2016). The underestimated diversity of phytoplasmas in Latin America. *Int J Syst Evol Microbiol* **66**, 492–513.
- Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P. A., Wei, W. & Davis, R. E. (2013). 'Candidatus Phytoplasma solani', a novel taxon associated with stolbur- and bois noir-related diseases of plants. *Int J Syst Evol Microbiol* **63**, 2879–2894.
- Qui, B. S., Li, H. H., Sui, C. L. & Jiu, K. X. (1998). Amplification of phytoplasma 16SrDNA from 20 infected plants in China and their RFLP analysis. *Sci Silvae Sin* **34**, 67–74.
- Ragonese, A. E. (1981). Cultivo y mejoramiento del paraíso gigante (*Melia azedarach* var. gigantea cv. Garraasino), en la República Argentina. *Anales Academia Nacional De Agronomía y Veterinaria XXXV*, 5–10.
- Rupa, B. A., Azam, N. K., Mannan, A., Ahmed, N. & Hasan, N. (2014). Phytochemistry and pharmacological appraisals of persian lilac (*Melia azedarach* Linn): a quick comprehensive review. *Am J Ethnomed* **1**, 152–163.
- Šafářová, D., Zemánek, T., Válková, P. & Navrátil, M. (2016). 'Candidatus Phytoplasma cirsii', a novel taxon from creeping thistle [*Cirsium arvense* (L.) Scop]. *Int J Syst Evol Microbiol* **66**, 1745–1753.
- Sanna, G., Madeddu, S., Giliberti, G., Ntalli, N. G., Cottiglia, F., De Logu, A., Agus, E. & Caboni, P. (2015). Limonoids from *Melia azedarach* fruits as inhibitors of flaviviruses and *Mycobacterium tuberculosis*. *PLoS One* **10**, e0141272.
- Santos-Cervantes, M. E., Chávez-Medina, J. A., Acosta-Pardini, J., Flores-Zamora, G. L., Méndez-Lozano, J. & Leyva-López, N. E. (2010). Genetic diversity and geographical distribution of phytoplasmas associated with potato purple top disease in Mexico. *Plant Dis* **94**, 388–395.
- Sawayanagi, T., Horikoshi, N., Kanehira, T., Shinohara, M., Bertaccini, A., Cousin, M. T., Hiruki, C. & Namba, S. (1999). 'Candidatus phytoplasma japonicum', a new phytoplasma taxon associated with Japanese hydrangea phyllody. *Int J Syst Bacteriol* **49**, 1275–1285.
- Schneider, B., Seemüller, E., Smart, C. D. & Kirkpatrick, B. C. (1995). Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In *Molecular and Diagnostic Procedures in Mycoplasma*, vol. 1, pp. 369–380. Edited by S. Razin & J. G. Tully. San Diego: Academic Press.
- Schneider, B., Torres, E., Martín, M. P., Schröder, M., Behnke, H. D. & Seemüller, E. (2005). 'Candidatus Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. *Int J Syst Evol Microbiol* **55**, 303–307.
- Seemüller, E. & Schneider, B. (2004). 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and 'Candidatus Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *Int J Syst Evol Microbiol* **54**, 1217–1226.
- Staden, R., Beal, K. F. & Bonfield, J. K. (2000). The staden package, 1998. *Methods Mol Biol* **132**, 115–130.
- Valiunas, D., Staniulis, J. & Davis, R. E. (2006). 'Candidatus Phytoplasma fragariae', a novel phytoplasma taxon discovered in yellows diseased strawberry, *Fragaria x ananassa*. *Int J Syst Evol Microbiol* **56**, 277–281.
- Vázquez, A., Ducasse, D. A., Nome, S. F. & Muñoz, J. O. (1983). Declinamiento del paraíso (*Melia azedarach* L.), síntomas y estudios etiológicos de esta nueva enfermedad. *Revista de Investigaciones Agropecuarias – INTA* **18**, 309–320.
- Verdin, E., Salar, P., Danet, J.-L., Choueiri, E., Jreijiri, F., El Zammam, S., Gélie, B., Bové, J. M. & Garnier, M. (2003). 'Candidatus Phytoplasma phoenicium' sp. nov., a novel phytoplasma associated with an emerging lethal disease of almond trees in Lebanon and Iran. *Int J Syst Evol Microbiol* **53**, 833–838.
- Wei, W., Davis, R. E., Lee, I. M. & 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.
- Wei, W., Davis, R. E., Jomantiene, R. & Zhao, Y. (2008). Ancient, recurrent phage attacks and recombination shaped dynamic sequence-variable mosaics at the root of phytoplasma genome evolution. *Proc Natl Acad Sci USA* **105**, 11827–11832.
- White, D. T., Blackall, L. L., Scott, P. T. & Walsh, K. B. (1998). Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya, and their proposed inclusion in 'Candidatus Phytoplasma australiense' and a new taxon, 'Candidatus Phytoplasma australasia'. *Int J Syst Bacteriol* **48**, 941–951.
- Win, N. K., Lee, S. Y., Bertaccini, A., Namba, S. & Jung, H. Y. (2013). 'Candidatus Phytoplasma balanitae' associated with witches' broom disease of *Balanites triflora*. *Int J Syst Evol Microbiol* **63**, 636–640.
- Zhao, Y., Wei, W., Lee, I.-M., Shao, J., Suo, X. & Davis, R. E. (2009a). Construction of an interactive online phytoplasma classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *Int J Syst Evol Microbiol* **59**, 2582–2593.
- Zhao, Y., Sun, Q., Wei, W., Davis, R. E., Wu, W. & Liu, Q. (2009b). 'Candidatus Phytoplasma tamaricis', a novel taxon discovered in witches'-broom-diseased salt cedar (*Tamarix chinensis* Lour.). *Int J Syst Evol Microbiol* **59**, 2496–2504.
- Zreik, L., Carle, P., Bové, J. M. & Garnier, M. (1995). Characterization of the mycoplasma-like organism associated with witches'-broom disease of lime and proposition of a Candidatus taxon for the organism, 'Candidatus phytoplasma aurantifolia'. *Int J Syst Bacteriol* **45**, 449–453.