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

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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 i*n 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 16SrXIIII 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 (Restriction Fragment Length Polymorphism) 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

Abbreviations: ChTY, China tree yellows; MPV, Mexican periwinkle virescence phytoplasma; RFLP, Restriction Fragment Length Polymorphism; SNP, Single Nucleotide Polymorphism.

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.

'*Candidatus* Phytoplasma' (IRPCM, 2004). At the time of 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, 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* Pytoplasma 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). 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 '*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′-AACCCCTTCC TTAGCTTCTAAGGC-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 llustra 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 *i*Phyclassifier (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). 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	Id. 16S	References
'*Ca.* Phytoplasma asteris'	M30790	16SrI-B	95, 73	Lee *et al.* (2004a)
'*Ca.* Phytoplasma aurantifolia'	U15442	16SrII-B	89, 69	Zreik *et al.* (1995)
'*Ca.* Phytoplasma australasia'	Y10097	16SrII-D	89, 79	White *et al.* (1998)
'*Ca.* Phytoplasma pruni'	JQ044393	16SrIII-A	90, 79	Davis *et al.* (2013)
'*Ca.* Phytoplasma phoenicium'	AF515636	16SrIX-D	89, 89	Verdin *et al.* (2003)
'*Ca.* Phytoplasma ulmi'	AY197655	16SrV-B	89, 80	Lee *et al.* (2004b)
'*Ca.* Phytoplasma ziziphi'	AB052876	16SrV-B	89, 88	Jung *et al.* (2003b)
'*Ca.* Phytoplasma rubi'	AY197648	16SrV-D	89, 88	Malembic-Maher *et al.* (2011)
'*Ca.* Phytoplasma trifolii'	AY390261	16SrVI-A	90, 54	Hiruki & Wang (2004)
'*Ca.* Phytoplasma sudamericanum'	GU292081	16SrVI-I	90, 37	Davis *et al.* (2012)
'*Ca.* Phytoplasma fraxini'	AF092209	16SrVII-A	90, 30	Griffiths *et al.* (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 *et al.* (2004a)
'*Ca.* Phytoplasma prunorum'	AJ542544	16SrX-F	92, 09	Seemüller & Schneider (2004)
'*Ca.* Phytoplasma oryzae'	AB052873	16SrXI-A	90, 12	Jung *et al.* (2003a)
'*Ca.* Phytoplasma cirsii'	KR869146	16SrXI-E	90, 37	Šafářová *et al.* (2016)
'*Ca.* Phytoplasma solani'	AF248959	16SrXII-A	95, 81	Quaglino *et al.* (2013)
'*Ca.* Phytoplasma australiense'	L76865	16SrXII-B	95, 89	Davis *et al.* (1997)
'*Ca.* Phytoplasma japonicum'	AB010425	16SrXII-D	95, 23	Sawayanagi *et al.* (1999)
'*Ca.* Phytoplasma fragariae'	DQ086423	16SrXII-E	96, 22	Valiunas *et al.* (2006)
'*Ca.* Phytoplasma hispanicum'	AF248960	16SrXIII-A	99, 82	Davis *et al.* (2016)
'*Ca.* Phytoplasma cynodontis'	AJ550984	16SrXIV-A	90, 79	Marcone *et al.* (2004b)
'*Ca.* Phytoplasma castaneae'	AB054986	16SrXIX-A	89, 06	Jung *et al.* (2002)
'*Ca.* Phytoplasma brasiliense'	AF147708	16SrXV-A	89, 14	Montano *et al.* (2001)
'*Ca.* Phytoplasma graminis'	AY725228	16SrXVI-A	94, 58	Arocha *et al.* (2005)
'*Ca.* Phytoplasma caricae'	AY725234	16SrXVII-A	93, 67	Arocha *et al.* (2005)
'*Ca.* Phytoplasma americanum'	DQ174122	16SrXVIII-A	95, 89	Lee *et al.* (2006)
'*Ca.* Phytoplasma rhamni'	X76431	16SrXX-A	92, 00	Marcone *et al.* (2004a)
'*Ca.* Phytoplasma pini'	AJ632155	16SrXXI-A	90, 30	Schneider *et al.* (2005)
'*Ca.* Phytoplasma palmicola'	KF751387	16SrXXII-A	89, 89	Harrison *et al.* (2014)
'*Ca.* Phytoplasma omanense'	EF666051	16SrXXIX-A	89, 40	Al-Saady *et al.* (2008)
'*Ca.* Phytoplasma tamaricis'	FJ432664	16SrXXX-A	89, 40	Zhao *et al.* (2009b)
'*Ca.* Phytoplasma costaricanum'	HQ225630	16SrXXXI-A	94, 41	Lee *et al.* (2011)
'*Ca.* Phytoplasma malaysianum'	EU371934	16SrXXXII	91, 37	Nejat *et al.* (2013)
'*Ca.* Phytoplasma allocasuarine'	AY135523	ND	92, 41	Marcone *et al.* (2004a)
'*Ca.* Phytoplasma lycopersici'	EF199549	ND	93, 51	Arocha *et al.* (2007)
'*Ca.* Phytoplasma convolvuli'	JN833705	ND	95, 97	Martini *et al.* (2012)
'*Ca.* Phytoplasma balanitae'	AB689678	ND	89, 72	Win *et al.* (2013)
Provisional novel '*Ca.* Phytoplasma' species and other incidentally cited strains:				
'*Ca.* Phytoplasma luffae'	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 ChTY strains 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, BstU*I and *Hpa*II RFLP profiles clearly distinguished strains of species of ChTY from '*Candidatus* Phytoplasma hispanicum' (Fig. 1), as demonstrated for *Hpa*II 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 strains, 16SrXIII group reference phytoplasmas, 39 '*Candidatus* Phytoplasmas 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).

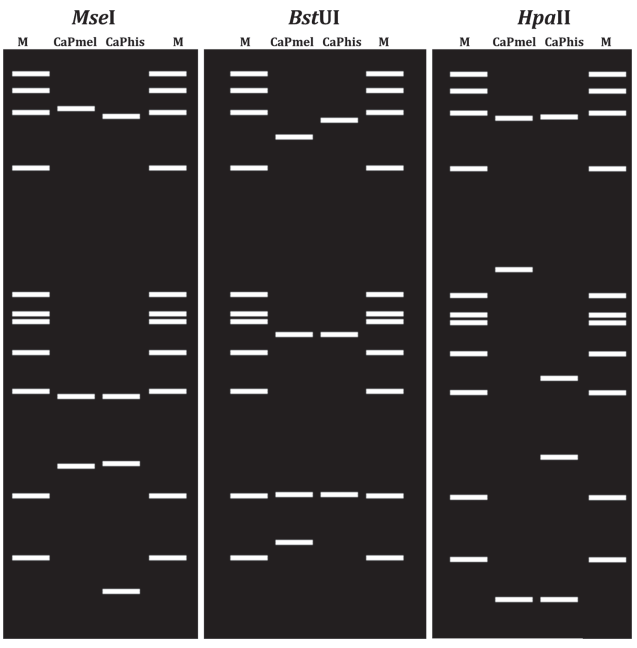

Fig. 1. *In silico* RFLP profile of the 16S rDNA partial gene (1.2 kb) obtained with *Alu*I, *BstU*I and *Hpa*II endonucleases. CaPmel: '*Candidatus* Phytoplasma meliae' strain ChTY-Mo3 (reference sequence, KU850944); CaPhis: '*Candidatus* Phytoplasma hispanicum' (AF248960); M: ϕ X174-*Hae*III digest.

The 16S rDNA signature sequence characteristic to phytoplasmas 5′–CAAGAYBATKATGTKTAGCYGGDCT-3′ (IRPCM, 2004), was found in position $_{268}$5′-CAAGACGATGATGTGTAGTCGGGCT-3′$_{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}$5′-GCTTCTGCAAAGT-$_{3993}$ and $_{1240}$5′-GAAGCGCGAGCTTTT-3$_{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). *BstU*I, *Hae*III, *Hha*I, *Mse*I and *Taq*I 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 constructed from the sequence of rpsC-rpLV and secA genes were consistent with the 16S rDNA gene tree topology. In all cases, sequences 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 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.

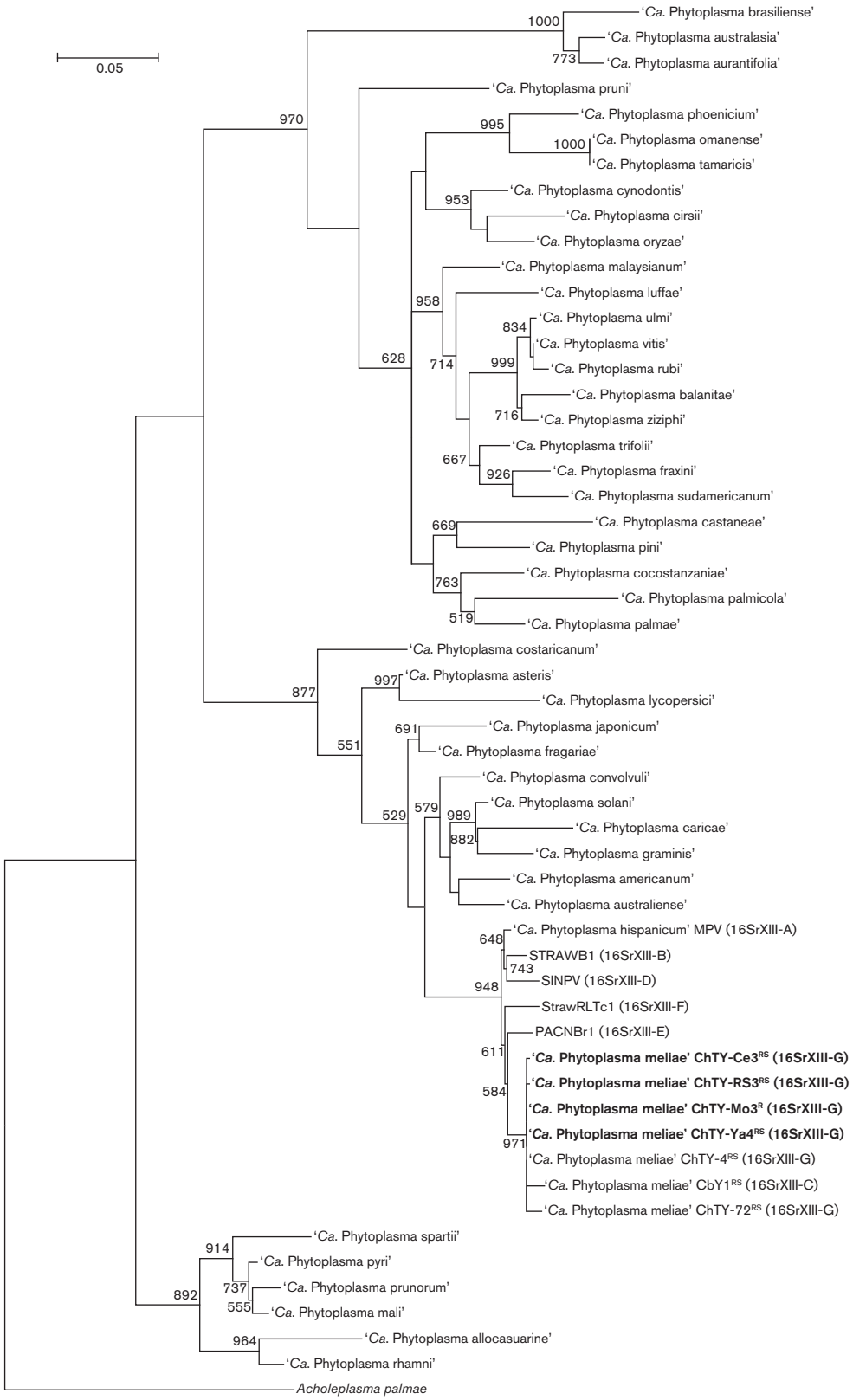

Fig. 2. Phylogenetic tree inferred from an analysis of 16S rDNA sequences using the maximum-likelihood method. *Acholeplasma palmae* was used as the outgroup. The numbers on the branches are bootstrap (confidence) values (expressed as percentages of 1000 replicates). The GenBank accession number for 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. [R]: reference strain; [RS]: related strain. Bar = number of nucleotide substitutions per site.

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 (Single Nucleotide Polymorphism) 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: $_{296}$5′-GGCTGAACG-3$_{305;}$ $_{980}$5′-GCTTCTGCAAAGT-3$_{993;}$ $_{1240}$5′-GAAGCGCGAGCTTTT-3$_{1254}$; P (*Melia azedarach*, phloem); M]

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