

Detection and identification of a novel 16SrXIII subgroup phytoplasma associated with strawberry red leaf disease in Argentina

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Strawberry red leaf phytoplasma was found in strawberry plants from production fields in Lules (Tucumán province) and Bella Vista (Corrientes province), Argentina. Characteristic strawberry red leaf symptoms were stunting, young leaves with yellowing at the edges, mature leaves which curled and were reddish at the abaxial face, flower and fruit deformation and death. The pathogen was detected with phytoplasma-universal primer pairs P1/P7 followed by R16F2n/R16R2 as nested primers in 13 diseased plants. Based on RFLP and sequence analysis of the amplified 16S rRNA gene, the phytoplasma was related to the 16SrXIII group (Mexican periwinkle virescence). *In silico* the RFLP profile of all the samples analysed revealed the presence of a unique pattern, showing that the novel phytoplasma is different from all the phytoplasmas currently composing the 16SrXIII group. The phylogenetic analysis was consistent with RFLP analysis as the strawberry red leaf phytoplasma was grouped within the 16SrXIII group, but formed a particular cluster. On this basis, the Strawberry red leaf phytoplasma associated with strawberry red leaf disease was assigned to a new subgroup, 16SrXIII-F.

Argentina is one of the few countries in the Southern Hemisphere with the climatic conditions for producing high-quality strawberry transplants. Around 100 million fresh and cold-stored transplants are produced annually in Argentinean nurseries for the domestic and foreign markets (Kirschbaum *et al.*, 2012). With respect to the strawberry fruit, Argentina exported 3493 tonnes in 2012 (SENASA, 2012). The fruit production areas are located at a wide range of latitudes (24° S–42° S), mainly in Tucumán, Santa Fé, Buenos Aires and Corrientes provinces (Kirschbaum *et al.*, 2000). Phloem-restricted pathogens,

such as phytoplasmas and bacteria-like organisms, have been reported globally to be associated with several diseases in strawberry plants (Maas, 1998). Phytoplasmas are wall-less, plant-pathogenic members of the class *Mollicutes* restricted to phloem tissue and are transmitted by sap-sucking insect vectors: mainly leafhoppers and psyllids (Davis & Lee, 2000). A classification scheme based on 16S rRNA gene (1.2 kb amplicon, F2nR2 primers) RFLP patterns derived from digestion by 17 restriction enzymes is often used to classify phytoplasmas into groups and subgroups (Wei *et al.*, 2008). Until now, 33 16Sr phytoplasma groups and more than 120 subgroups have been delineated (Zhao *et al.*, 2009; Lee *et al.*, 2011; Davis *et al.*, 2012; Nejat *et al.*, 2013; Bertaccini *et al.*, 2014). It has been established that strawberry plants serve as a natural host of divergent phytoplasmas belonging to at least six 16Sr groups (16SrI, 16SrIII, 16SrVI, 16SrVII, 16SrXII and 16SrXIII) (Harrison *et al.*, 1997; Jomantiene *et al.*, 1998, 1999, 2001; Padovan *et al.*, 2000; Fernández *et al.*, 2013). Phytoplasma-affected strawberry plants often display symptoms including

Abbreviations: StrawRL, strawberry red leaf phytoplasma.

The GenBank/EMBL/DDBJ accession numbers for the consensus 16S rRNA gene sequences of strains StrawRL-Tc1, StrawRL-Tc7, StrawRL-BV1 and StrawRL-BV2 are KJ921641, KJ921642, KJ921643 and KJ921644, respectively.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

virescence, flower and fruit phyllody, dwarfing, a multi-branching crown, leaf chlorosis, and flower and fruit abnormalities. In Argentina, strawberry red leaf disease was first observed in strawberry production fields in Lules (Tucumán province) and Bella Vista (Corrientes province) in 2009 and 2010. Affected plants present symptoms including stunting, young leaves with yellowing at the edges, mature leaves with curling and a reddish colouration at the abaxial face, flower and fruit deformation and death. These symptoms resemble those caused by phytoplasmas, and given the fact that there is no information available on the aetiology of this disease, we have carried out detection of the phytoplasma associated with strawberry red leaf disease and pathogen identification based on 16S rRNA gene sequence analysis.

Characterization of the 16S rRNA gene sequence from strawberry red leaf phytoplasma

Thirteen symptomatic strawberry (*Fragaria x ananassa* Duch. 'Camarosa') plants showing stunting, curled leaves and redness on the abaxial face were collected from production fields in Lules (Tucumán province) and Bella Vista (Corrientes province). Five symptomless strawberry plants were sampled as controls. DNA from MPV strain (Mexican periwinkle virescence, 16SrXIII-A) (supplied by Dr Ming Lee, e-mail:leem@ba.ars.usda.gov) and ChTYXIII strain (China tree yellows, 16SrXIII-C), collected from infected China-tree (*Melia azeradach* L.) and maintained in a greenhouse in Argentina, were used as the reference strains in this study. DNA was obtained according to the protocol of Doyle & Doyle (1990) with modifications in order to eliminate the presence of inhibitors in PCRs. Approximately 0.1 g of fresh petioles and midribs from each sample of strawberry plant were ground in sterile mortars with liquid N₂ and 10 mg polyvinylpyrrolidone (P-6755; Sigma). After incubation with 2 % CTAB (cetyl trimethylammonium bromide) buffer, the supernatant was washed twice with chloroform/isoamyl alcohol (24:1, v/v), DNA was precipitated using cold 2-propanol and pellets were washed with ethanol 70 % (v/v). Samples were tested by nested PCR using two pairs of universal primers, P1/P7 primers (Deng & Hiruki, 1991; Schneider *et al.*, 1995) to amplify a 1.8 kb fragment including the 16S rRNA gene, the 16S–23S spacer region and the 5' end of the 23S rRNA gene in the first round, and R16F2n/R16R2 as nested primers (Gundersen & Lee, 1996) to amplify a 1.2 kb fragment of the 16S rRNA gene. PCRs were performed in a thermal cycler (TRIO-Thermoblock, Biometra) in 40 µl reaction solution containing 40 ng of template DNA, 0.4 µM each primer, 200 mM dNTPs, 1 U GoTaq DNA Polymerase and 5 × polymerase buffer (Promega) as previously described (Deng & Hiruki, 1991; Schneider *et al.*, 1995; Lee *et al.*, 1998). DNA from asymptomatic strawberry plants and >reaction mixtures without DNA were used as negative controls. DNA from MPV and ChTYXIII strains were used as positive controls. PCR products were analysed by

electrophoresis on a 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

The 1.2 kb fragments amplified by nested PCR with the primers pairs R16F2n/R16R2 from StrawRL samples, ChTYXIII and MPV were digested with 14 restriction enzymes: *Alu*I, *Bam*HI, *Bst*UI, *Hha*I, *Hae*III, *Rsa*I, *Hinf*I, *Eco*RI, *Mse*I, *Kpn*I, *Sau*3AI, *Taq*I (New England Biolabs), *Hpa*I and *Hpa*II (Promega) according to manufacturers' instructions. Restriction fragments were resolved in 8 % (w/v) polyacrylamide or 1.5 % (w/v) agarose (Biodynamics SRL) + 0.5 % agarose Metaphor (BioWittaker Molecular Applications) gel buffered in Tris/Borate/EDTA (TBE), stained with ethidium bromide and visualized by UV transillumination. The resulting RFLP patterns were compared with those of the Mexican periwinkle reference strain (MPV, 16SrXIII-A) and China tree yellows (ChTYXIII, 16SrXIII-C) detected in Argentina. The 1.2 kb PCR product amplified with primers, R16F2n/R16R2, from four representative strains, StrawRL-Tc1 and StrawRL-Tc7 from Lules (Tucumán province) and StrawRL-BV1 and StrawRL-BV2 from Bella Vista (Corrientes province), were purified with MicroSpin™ S-400 HR Columns (Amersham Biosciences) and cloned in a pGEM-T Easy vector system (Promega). Competent cells of *Escherichia coli* DH5α were transformed with the recombinant plasmids following the method of Sambrook *et al.* (1989). Plasmid DNA extraction was performed using a Plasmid Extraction kit (Qiagen) according to the manufacturer's instructions. For each strain three clones were selected and sequenced from both extremes (4 × coverage per base position) using an automated DNA sequencer (Macrogen). The final sequences were assembled using the SeqMan program (Lasergene7.1; DNASTAR) with manual adjustment when necessary. The nucleotide sequences obtained were deposited in the GenBank database. The 16S rRNA gene sequences of the strawberry red leaf phytoplasma (StrawRL) strains were analysed with BLAST (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) in order to identify sequences with high similarity values. Sequence similarity was calculated using the CLUSTAL W option of the MEGALIGN program (Lasergene 7.1; DNASTAR). Collective RFLP patterns of StrawRL sequences and coefficients of similarity using 17 restriction enzymes were generated by virtual gel analysis using the *i*PhyClassifier program (Zhao *et al.*, 2009). The 16S rRNA gene sequences from four StrawRL strains (Tc1, Tc7, BV1 and BV2) were aligned with CLUSTAL W from the Molecular Evolutionary Genetics Analysis program (MEGA5) (Tamura *et al.*, 2011) along with eight sequences of 16SrXIII reference strains and nine sequences from 'Candidatus Phytoplasma' species that are closely related to the 16SrXIII group (IRPCM Phytoplasma/Spiroplasma Working Team—Phytoplasma Taxonomy Group, 2004; Arocha *et al.*, 2005; Lee *et al.*, 2006; Valiunas *et al.*, 2006; Lee *et al.*, 2011; Quaglino *et al.*, 2013). The phylogenetic tree was reconstructed by the neighbour-joining method using the MEGA5 program. *Acholeplasma palmae* (GenBank accession no. L33734) was used as an outgroup to root the tree. Bootstrapping was performed to estimate the stability and support for the inferred clades.

New phytoplasma subgroup 16SrXIII-F

A 1.2 kb rRNA gene product was amplified from the DNAs of all 13 diseased plants using a nested PCR assay. All strains of the StrawRL phytoplasma assayed had identical 16S rRNA gene RFLP patterns when *Mse*I, *Bam*HI, *Hha*I, *Hinf*I, *Eco*RI, *Sau*3AI, *Taq*I and *Hpa*I restriction enzymes were used and the profiles were indistinguishable from those of the ChTYXIII and MPV reference strains (data not shown). The *Bst*UI pattern of StrawRL strains was identical to that of the MPV strain, whereas restriction with *Hpa*II was identical to that of ChTYXIII (Fig. 1). RFLP patterns obtained with *Rsa*I, *Hae*III, *Alu*I and *Kpn*I were different from those of MPV and ChTYXIII (Figs 1 and 2), and also from the published patterns of strawberry green petal phytoplasma STRAWB2 (Jomantiene *et al.*, 1998), Mexican potato purple top phytoplasma SINPV (Santos-Cervantes *et al.*, 2010) and papaya apical curl necrosis PACN phytoplasmas (Melo *et al.*, 2013), and subgroups 16SrXIII-B, 16SrXIII-D and 16SrXIII-E.

The 16S rRNA gene sequence identity of StrawRL strains ranged from 99.45 % to 100 % among the strains, and from 97.8 % to 98.7 % with the 16SrXIII phytoplasma group. All the StrawRL strains analysed contained the sequence 5'-CAAGACGATGATGTGTAGCCGGGCT-3' (125–149nt) in the 16S rRNA gene portion of the *rrn* operon, matching with the signature sequence (5'-CAAGAYBATKATGKTAGCYGGDCT-3') that defines the taxa in the provisional genus 'Candidatus Phytoplasma' (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004). Collective RFLP patterns (Fig. S1, available in the online Supplementary Material) derived by *i*PhyClassifier from the 16S rRNA gene F2nR2 fragment of StrawRL were consistent with actual RFLP analysis and revealed that StrawRL phytoplasma differed from the reference patterns of all previously described

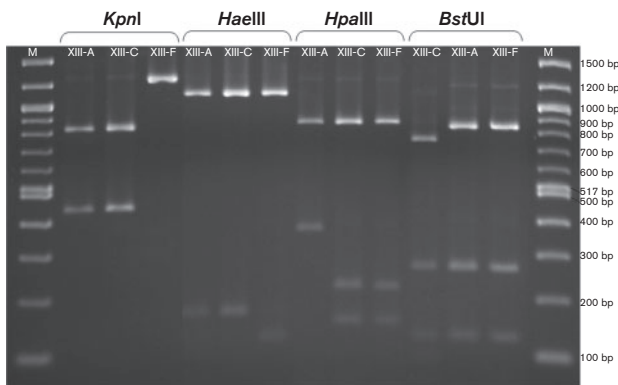


Fig. 1. RFLP analysis of 1.2 kb PCR products of the 16SrRNA gene (R16F2n/R16R2 primers) digested with *Kpn*I, *Hae*III, *Hpa*II and *Bst*UI restriction enzymes. Phytoplasma reference strains were MPV (16SrXIII-A) and ChTYXIII (16SrXIII-C) and StrawRL (16SrXIII-F). M, 100 bp DNA Ladder (New England Biolabs).

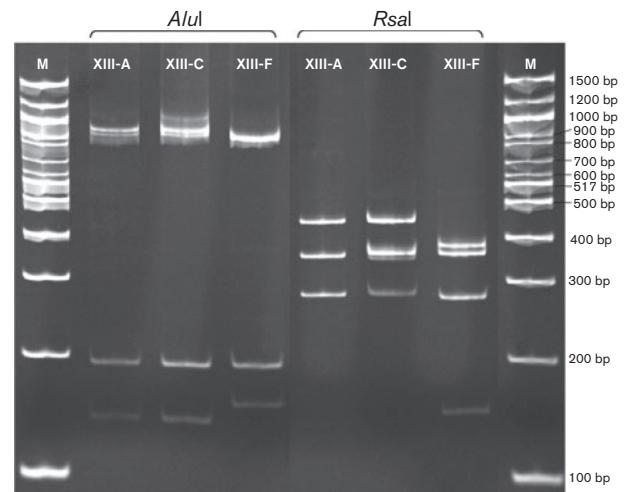


Fig. 2. RFLP analysis of 1.2 kb PCR products of the 16S rRNA gene (R16F2n/R16R2 primers) digested with *Alu*I and *Rsa*I restriction enzymes. Phytoplasma reference strains MPV (16SrXIII-A) and ChTYXIII (16SrXIII-C) and StrawRL (16SrXIII-F). M, 100 bp DNA Ladder (New England Biolabs).

16Sr groups/subgroups. The pattern of the reference group, 16SrXIII-A (GenBank accession no. AF248960), was most similar with a coefficient of 0.89. Five subgroups within the 16SrXIII group have been described: 16SrXIII subgroup A (Lee *et al.*, 1998, 2000); 16SrXIII subgroup B (Jomantiene *et al.*, 1998), 16SrXIII subgroup C (Harrison *et al.*, 2003), 16SrXIII subgroup D (Santos-Cervantes *et al.*, 2010) and 16SrXIII subgroup E (Melo *et al.*, 2013). Four key enzymes (*Alu*I, *Hae*III, *Kpn*I and *Rsa*I) were distinguished using *in silico* RFLP profiles of the strawberry red leaf phytoplasma (StrawRL-Tc1, KJ921641) from all the 16SrXIII subgroups (Fig. 3).

Phylogenetic analysis of near full-length 16S rRNA gene sequences from nine previously described 'Ca. Phytoplasma' species, eight 16SrXIII phytoplasma groups, four strains of StrawRL phytoplasma, with *A. palmae* as the out-group, yielded a consensus tree (Fig. 4) in which strains of StrawRL were grouped with the sequences from representative strains of the 16SrXIII group (bootstrap value, 100 %) and in the same clade, but they formed a discrete cluster (bootstrap value, 100 %). These results are consistent with those based on RFLP profiles and sequence analysis, supporting the assignment of StrawRL phytoplasma as a novel subgroup within group 16SrXIII.

Strawberry red leaf and diversity of phytoplasmas in South America

Phytoplasmas from the 16SrXIII group have been described to affect vinca (Lee *et al.*, 1998, 2000), strawberries (Jomantiene *et al.*, 1998), chinaberries (Harrison *et al.*, 2003; Arneodo *et al.*, 2005, 2007), potatoes (Santos-Cervantes

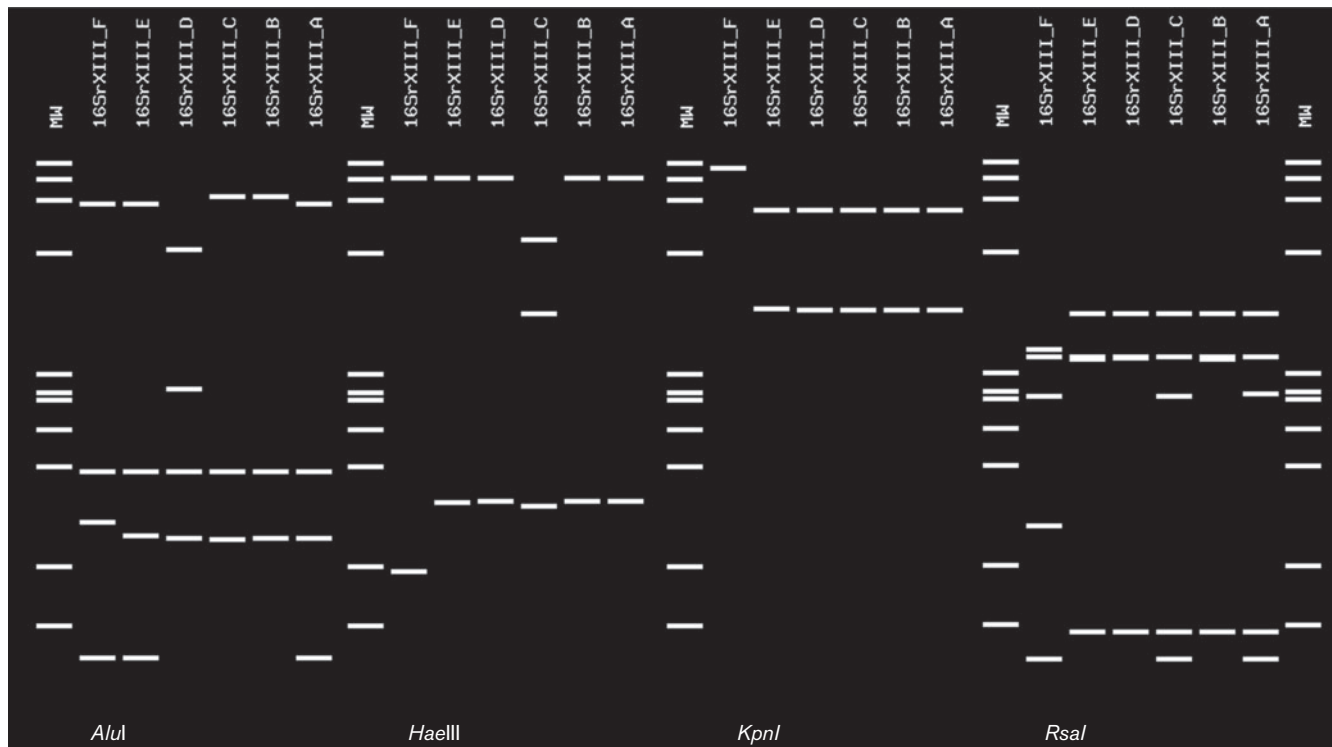


Fig. 3. *In silico* RFLP profile of partial 16S rRNA gene (1.2 kb) constructed using the *iPhyClassifier* program (Zhao *et al.*, 2009) from key enzymes (*AclI*, *HaeIII*, *KpnI* and *RsaI*) that distinguish StrawRL (16SrXIII-F) from subgroups 16SrXIII-A, -B, -C, -D and -E. Reference sequences used for each subgroup were: 16SrXIII-A (AF248960), 16SrXIII-B (U96614), 16SrXIII-C (AF495882), 16SrXIII-D (FJ914647), 16SrXIII-E (EU719111) and 16SrXIII-F (StrawRL-Tc1, KJ921641). MW, X174-*HaeIII* digests.

et al., 2010), broccoli (Eckstein *et al.*, 2013) and papaya (Melo *et al.*, 2013). In the present work, the StrawRL associated with Strawberry red leaf in Argentina production fields has been characterized based on 16S rRNA gene sequence analysis. Actual, *in silico* RFLP and sequence analysis classified StrawRL phytoplasma as a novel member of the 16SrXIII group. According to the classification scheme guidelines, a novel 16Sr subgroup can be proposed if the similarity coefficient value of the RFLP collective pattern of the F2nR2 fragment of a given phytoplasma is ≤ 0.97 (Lee *et al.*, 1998; Wei *et al.*, 2008). The highest value for StrawRL was 0.89 with 16SrXIII-A; therefore, we propose the assignment of StrawRL as a novel 16SrXIII subgroup F phytoplasma, with reference strains StrawRL-Tc1 (accession no. KJ921641) and StrawRL-Tc7 (KJ921642); StrawRL-BV1 (KJ921643) and StrawRL-BV2 (KJ921644) are related sequences. A BLAST search (<http://blast.ncbi.nlm.nih.gov/>) using the 16S rRNA gene (1244 bp) sequence of StrawRL (strain StrawRL-Tc1) as the query showed high similarity values with sequences of other 16SrXIII phytoplasma groups (Table S1), that have not been published yet. The associated strains include cauliflower stunt (JN818844), periwinkle witches' broom (AY204549), pepper purple top (JQ745314), potato purple top (JN087521) and tomato purple top (KC329499). Interestingly, two sequences

available in GenBank, associated with phyllody on strawberries in Brazil (SFP-Br02: EU719108 and SFP-Br03: EU719109), showed a high RFLP similarity coefficient (Table S1) with StrawRL. Moreover, SFP-Br03 has an identical *in silico* RFLP profile to that of StrawRL and is probably a StrawRL phytoplasma isolate, but more information is necessary to confirm this. In South America there are several lineages of phytoplasma that are not found on other continents (Montano *et al.*, 2000, 2001). For example, several phytoplasmas within group 16SrIII are associated with diverse host species that have been described as part of unique 16SrIII subgroups such 16SrIII-J (Montano *et al.*, 2000; Galdeano *et al.*, 2004; Amarel-Mello *et al.*, 2006, 2011; Fiore *et al.*, 2012), 16SrIII-V (Davis *et al.*, 2012), 16SrIII-U (Amarel-Mello *et al.*, 2011) or 16SrIII-W and 16SrIII-X (Galdeano *et al.*, 2013). Moreover, phytoplasmas belonging to subgroups 16SrVII-B (Barros *et al.*, 2002; Meneguzzi *et al.*, 2008) and 16SrVII-C (Conci *et al.*, 2005; Fernández *et al.*, 2013) of the ash yellows group (16SrVII) have been recorded only in South America. With respect to the 16SrXIII-group, phytoplasmas from subgroup 16SrXIII-C have been reported to affect chinaberry trees in Bolivia, Argentina and Paraguay (Harrison *et al.*, 2003; Arneodo *et al.*, 2005, 2007). RFLP and sequence analysis of the 16S rRNA gene clearly differentiates it from the rest of the

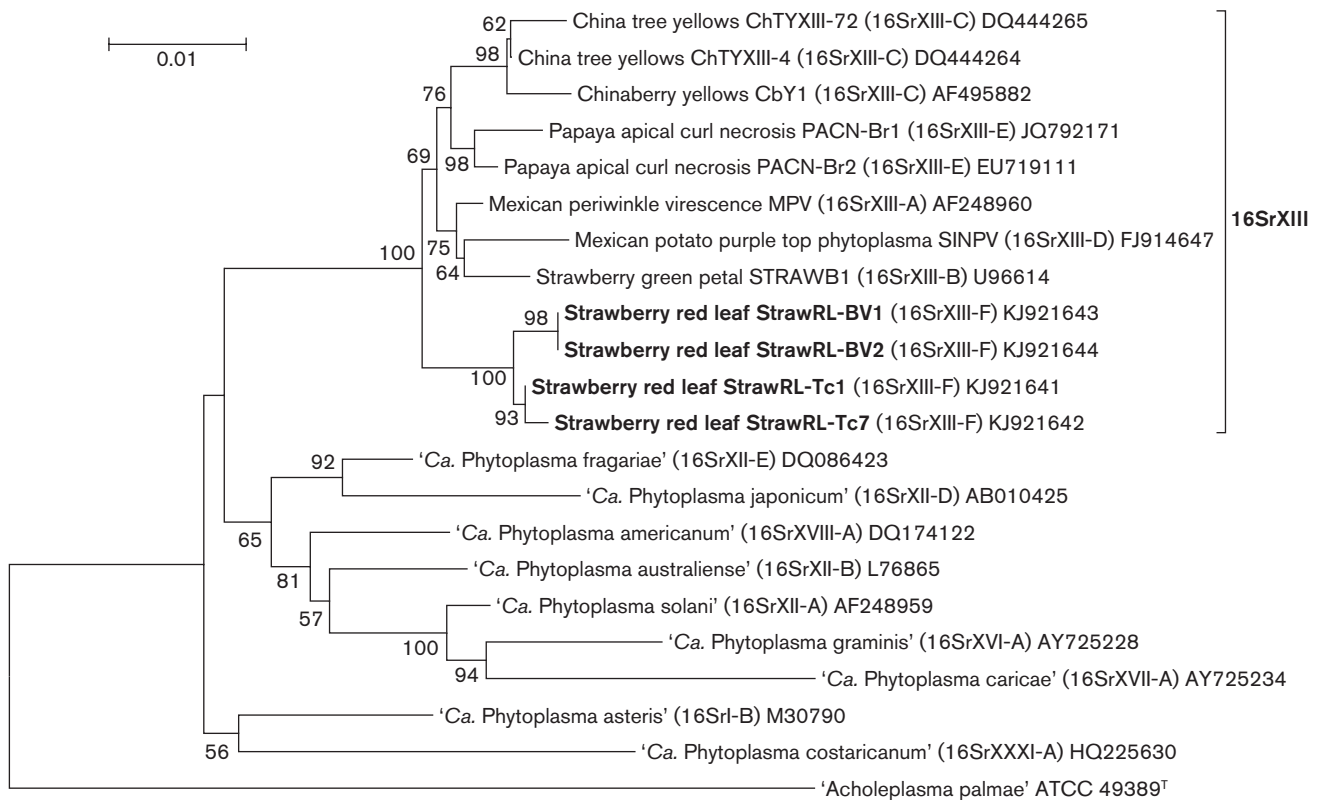


Fig. 4. Phylogenetic tree inferred from analysis of 16S rRNA gene sequences using the neighbour-joining method. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates. Numbers on the branches are bootstrap (confidence) values. The taxa used in the phylogenetical reconstruction included reference strains of nine previously identified species of '*Ca. Phytoplasma*', eight 16SrXIII reference strains, StrawRL strains (in bold type) (StrawRL-Tc1, -Tc7, -BV1 and -BV2) and *A. palmae* ATCC 49389^T as the outgroup.

16SrXIII group. These findings are consistent with the concept that a unique ecology and geographical separation provided favourable conditions for the divergence of phytoplasma lineages from those of other world regions (Montano *et al.*, 2001). In this study, the molecular characterization of strawberry red leaf phytoplasma extends knowledge of the diversity of the 16SrXIII group. Given the fact that the 16SrXIII-F subgroup has not yet been reported in another host, and considering the similarities found in sequences with isolates from strawberries in Brazil, StrawRL appears to prevail in strawberry plants; however, the assessment of weeds and/or other surrounding crop species is necessary to identify vector insects and confirm this. The study of the diversity of StrawRL phytoplasma isolates from different regions and the search for their vectors could uncover the behaviour of this pathosystem at a deeper level, thus improving the management strategies of the disease and preventing its spread to unaffected areas.

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