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.
virecence, flower and fruit phyllody, dwarfing, a multi-branaching 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 stuntng, 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 stuntng, 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: leeim@ba.ars.usda.gov) and ChTYXIII strain (China tree yellows, 16SrXIII-C), collected from infected China-tree (Melia azedarach 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 polyvinylpolypyrrolidone (P-6755; Sigma). After incubation with 2 % CTAB (cetyl trimethylammonium bromide) buffer, the supernatant was washed twice with chloroform/isoamylic 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 control 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, ChrTYXIII and MPV were digested with 14 restriction enzymes: AluI, BamHI, BstUI, HhaI, HaeIII, RsaI, HindI, EcoRI, MseI, KpnI, Sau3AI, TagI (New England Biolabs), HpaI and HpaII (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 MicroSpinTM S-400 HR Columns (Amersham Biosciences) and cloned in a pGEM-T Easy vector system (Promega). Competent cells of Escherichia coli DH5a 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 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 iPhyclasifier 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; Valunas 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. Bootstrap 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, *Hin* fl, *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 *Bsr*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 STAWB2 (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’-CAAGACGATGATGTTAGCCGGGC-3’ (125–149nt) in the 16S rRNA gene gen portion of the rrn operon, matching with the signature sequence (5’-CAAGAYBATKATGTKTAGCYGGDC-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 iPhyclassifier 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 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 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).

**Fig. 1.** RFLP analysis of 1.2 kb PCR products of the 16S rRNA gene (R16F2n/R16R2 primers) digested with *Kpn* I, *Hae* III, *Hpa* I 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).
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. Actually, 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 \( \leq 0.97 \) (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 phylody 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

![In silico RFLP profile of partial 16S rRNA gene (1.2 kb) constructed using the iPhyclassifier program (Zhao et al., 2009) from key enzymes (AII, 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.](http://www.microbiologyresearch.org/content/suppl/2018/11/03/ijsem.00032-2018.DC1.Fig3.jpg)
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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References


