Identification of three novel subgroups within the X-disease group phytoplasma associated with strawberry redness disease

Franco D. Fernández, Natalia G. Meneguzzi and Luis R. Conci*

Abstract

Strawberry plants showing symptoms of lethal redness disease were found in production fields located in Tucumán province, Argentina. The presence of phytoplasmas was confirmed by PCR of 16S rDNA gene using phytoplasma universal primers. According to the 16S rDNA gene sequence identity, the four isolates analysed are related to the X-disease group (16SrIII) (identity ~99%). These results were confirmed by in silico RFLP, actual RFLP and also by phylogenetic analyses of the 16S rDNA gene. This new phytoplasma was named as Strawberry X-Redness (StrawXR). The results from virtual and actual RFLP analyses of 16S rDNA gene revealed the presence of subgroup 16SrIII-J and three new 16SrIII subgroups. This is the first record of phytoplasmas from X-disease group associated strawberry in Argentina. These results confirm the prevalence of X-disease group and also contribute to the knowledge of diversity of phytoplasmas in this region.

Phytoplasmas are insect-transmitted plant phloem-inhabiting bacteria responsible for numerous diseases in diverse plant species worldwide [1]. Currently, despite numerous efforts, isolation and axenic cultivation of phytoplasmas remain a major challenge [2]. However, as in other prokaryotes, 16S rDNA gene is highly conserved in phytoplasmas [3] and useful for diversity analysis. Based on 16S rRNA gene sequence and RFLP profile analysis, a comprehensive scheme has been proposed to classify phytoplasmas into 16Sr groups/subgroups [4]. Currently, based on this classification system and the application of computer programs for virtual RFLP [5–9], 33 16Sr groups and more than 100 subgroups have been delineated. In Argentina, according to this classification system, phytoplasmas from four different 16Sr groups have been described affecting diverse plant species (16SrI, 16SrIII, 16SrVII and 16SrXIII) [10]. The X-disease (16SrIII) is one of the most diverse phytoplasma groups, not only for subgroup diversity but also for having a wide host range. Among the 25 16SrIII subgroups described [7, 11–13], only B, J, X and W have been reported in Argentina, and are known to affect numerous plant species [12, 14, 15]. Regarding strawberry (Fragaria x ananassa Duch.), several phytoplasmas from at least six 16Sr groups (16SrI, 16SrIII, 16SrVI, 16SrVII, 16SrXII and 16SrXIII) have been described that affect this species worldwide [16–22]. Two different phytoplasmas have been detected in strawberry in Argentina up to now. The ASP phytoplasma (Argentinean Strawberry Phyllody) has been reported to causing phyllody and virescence, while the StrawRL phytoplasma (Strawberry Red Leaf) has been associated with the lethal redness disease, characterized by symptoms such as stunting, yellowing at the edges of young leaves, curling and reddish colouration at the abaxial face of mature leaves, flower and fruit deformation and plant death. The molecular characterization of ASP 16S rDNA gene placed it within the 16SrVII-C subgroup [21], while StrawRL phytoplasma has been described as a new 16SrXIII-F subgroup [22]. Lethal redness disease is being recurrently detected in the Tucumán province (northwest), which is one of the main strawberry production regions in Argentina. It remains unknown, however, whether they are infected by previously reported StrawRL phytoplasma or different phytoplasmas. The goal of the present work was to detect, identify and characterize new phytoplasma isolates associated with lethal redness disease in strawberry based on 16S rDNA gene sequence analysis.

Sampling was conducted in Lules (Tucumán province), one of the most important strawberry production areas in Argentina. Midribs and petioles from 12 symptomatic and 5 asymptomatic strawberry plants were ground with liquid
nitrogen in sterile mortars. Total nucleic acid was extracted as described by Doyle [23] with some modifications [22]. Phytoplasma detection was performed by PCR using universal primers P1/P7 [24, 25] 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 [26] to amplify a 1.2 kb fragment of the 16S rRNA gene using the parameters previously reported [22]. For nested PCR, 1 µl of 1:25 dilution P1/P7 PCR product was used. DNA from periwinkle (*Catharanthus roseus* L. G. Don) infected with Argentinean Catharanthus Little Leaf (ACLL) phytoplasma [27] was used as a positive control, and DNA from healthy periwinkle and strawberry plants maintained in greenhouse, as negative controls. PCR-amplified DNA products were analysed through electrophoresis in agarose 1 % (w/v) gels, stained with ethidium bromide and visualized in UV transilluminator.

The 1.2 kb amplicons were purified using MicroSpin S-400HR Columns (Amersham Biosciences) and cloned in pGEM-T Easy vector system (Promega). Competent cells of *Escherichia coli* DH5α strain were transformed with the recombinant plasmids following Sambrook *et al.* [28]. Plasmid DNA extraction was performed using NucleoSpin (MACHEREY-NAGEL) according to the manufacturer’s

---

**Fig. 1.** *In silico* RFLP profile of partial 16S rRNA gene (1.2 kb) with enzymes *MseI*, *TaqI* and *HinfI* using the *iPhyclassifier* program [7] that distinguishes StrawXR-Arg2 (KU516388) and StrawXR-Arg4 (KU517704) isolates from 16SrIII-J reference pattern (AF147706). MW, *φ*X174-*HaeIII* digests.
instructions. Three clones for each isolate were selected and sequenced from both ends (3× coverage per base position) using an automated DNA sequencer (Unidad Genómica, Instituto de Biotecnología-Instituto Nacional de Tecnología Agropecuaria). The consensus sequence of each isolate was assembled using Staden program package [29] and deposited in GenBank (NCBI/EMBL). The consensus sequences were analysed using nucleotide–nucleotide basic local alignment search tool BLASTn (NCBI) in order to determine closely related phytoplasmas deposited in GenBank (NCBI/EMBL).

The complete 16S group/subgroup classification was performed using the online program iPhyClassifier [7]. The complete RFLP profiles were obtained using the 17 endonucleases proposed by Lee et al. [4] and compared to those of the iPhyClassifier database or with other phytoplasma sequences retrieved from GenBank. Those restriction enzymes showing pattern differences in silico among different isolates (BfAl, RsAl, TaqI and MsEl) were used to digest R16F2n/R16R2 PCR products (1.2 kb fragments) according to the manufacturer’s instructions. For phylogenetic analyses, multiple sequence alignments were performed using ClustalW2 including sequences from several ‘Candidatus Phytoplasma’ species, representative from 16Sr groups according to the previous classification established by the iPhyClassifier. Acholeplasma palmae was used as outgroup. The phylogenetic tree was reconstructed using the neighbour-joining method with MEGA software version 6 [30] and 1000 bootstrap replications in order to give statistical support to the inferred clades.

Phytoplasma infection was confirmed in four samples by nested PCR only. No amplification was observed in negative controls both from direct and nested PCRs. Consistent results were obtained after three-time repeated PCR. The PCR product (1.2 kb) obtained in the four phytoplasma positive samples showed an MsEl RFLP profile similar to the X-disease group (data not shown), which were therefore selected to be sequenced. In order to avoid name confusion with the strawberry red leaf phytoplasma (StrawRL, 16SrXIII-F), previously reported in association with the strawberry lethal redness [22], this phytoplasma has been named strawberry X-redness (StrawXR). The consensus sequences (1247 bp) of StrawXR-Arg4, StrawXR-Arg53 and StrawXR-Arg75 isolates from 16SrIII-X reference pattern (AF147706). MW, φX174-HaellI digests.

Fig. 2. In silico RFLP profile of partial 16S rRNA gene (1.2 kb) with enzymes BfaI and RsAl using the iPhyClassifier program [7] that distinguishes StrawXR-Arg53 (KU517705) and StrawXR-Arg75 (KU517706) isolates from 16SrIII-X reference pattern (AF147706). MW, φX174-HaeIII digests.

Phytoplasma infection was confirmed in four samples by nested PCR only. No amplification was observed in negative controls both from direct and nested PCRs. Consistent results were obtained after three-time repeated PCR. The PCR product (1.2 kb) obtained in the four phytoplasma positive samples showed an MsEl RFLP profile similar to the X-disease group (data not shown), which were therefore selected to be sequenced. In order to avoid name confusion with the strawberry red leaf phytoplasma (StrawRL, 16SrXIII-F), previously reported in association with the strawberry lethal redness [22], this phytoplasma has been named strawberry X-redness (StrawXR). The consensus sequences (1247 bp) of StrawXR-Arg4, StrawXR-Arg53 and StrawXR-Arg75 isolates have been deposited in the GenBank database (NCBI).

The BLASTn analysis of the 16S rDNA gene sequence of StrawXR isolates showed an identity ~99% against phytoplasmas from 16SrIII group (X-disease). Using CLUSTAL W2, the 16S rDNA gene sequence identity between four StrawXR isolates showed a higher homology between StrawXR-Arg53 and StrawXR-Arg75 (99, 28%) and between StrawXR-Arg2 and StrawXR-Arg4 (98, 72%).

According to the iPhyClassifier analysis, the StrawXR isolates belong to the 16SrIII group (X-disease); however, the similarity coefficient (F) showed affiliation with different subgroups (Table S1, available in the online Supplementary Material). The StrawXR-Arg4 is a variant of 16SrIII-J subgroup (AF147706) (F=0.98), differing only by HinfI profile (Fig. 1). StrawXR-Arg2 isolate also showed the highest similarity coefficient with the 16SrIII-J subgroup (F=0.95), with differences in MsEl and TaqI RFLP profiles (Figs 1 and S1). On the other hand, StrawXR-Arg53 and StrawXR-Arg75 isolates showed a similarity coefficient of 0.97 with the 16SrIII-X reference sequence (KC412026). The StrawXR-Arg53 isolate differed from 16SrIII-X reference sequence by the RsAl profile, while StrawXR-Arg75 changed in the BfaI profile (Figs 2 and S2). Also, StrawXR-Arg53 and StrawXR-Arg75 are differentiated among them by the pattern of the above-mentioned enzymes (Figs 2 and S2).

The phylogenetic tree was reconstructed using the 16S rDNA gene (1.2 kb) sequence from 25 representative
isolates from 16SrIII group, 14 ‘Candidatus Phytoplasma’ species and A. palmae as outgroup. All StrawXR isolates were grouped within the X-disease phytoplasma sequences (bootstrap, 100 %) (Fig. 3). Isolates StrawXR-Arg2 and StrawXR-Arg4 were clustered within the representative sequences from 16SrIII-J subgroup. On the other hand, StrawXR-Arg53 and StrawXR-Arg75 isolates were grouped in a particular cluster with sequences from 16SrIII-X and 16SrIII-W subgroups.

Phytoplasmas belonging to the X-disease (16SrIII) group have been cited affecting multiple plants species in South America. The X-disease phytoplasma diversity found in this region encompasses subgroups 16SrIII-J [11, 12, 14, 31–34], 16SrIII-B [12, 14, 15], 16SrIII-V [35], 16SrIII-U [11], 16SrIII-W, 16SrIII-X [12] and the recently renamed 16SrIII-Z [36; renamed by Pérez-López et al. [13]]. In the present work, we reported phytoplasmas from the X-disease group associated with the lethal redness disease of strawberry. According to the 16S rDNA gene molecular characterization, a phytoplasma could be assigned to a new subgroup if it shows 0.97 or lower similarity coefficient with those of all representative strains of a certain group derived from the 16S rDNA gene RFLP analysis [6]. The RFLP similarity coefficient (F) of StrawXR-Arg2 isolate was 0.95 with 16SrIII-J reference sequence (AF147706). Also, StrawXR-

---

**Fig. 3.** Phylogenetic tree inferred from analysis of 16S rDNA gene sequences using neighbour-joining method. A. palmae was used as the outgroup. The numbers on the branches are bootstrap (confidence) values (expressed as percentage of 1000 replicates). The GenBank accession number for each taxon is given within parentheses. The corresponding 16SrIII subgroups are also shown beside each taxon, and the StrawXR isolates sequenced in this paper are in boldface. Asterisks indicate new 16SrIII subgroups described in this work. Bar, 0.01 substitutions per nucleotide position.
Arg53 and StrawXR-Arg75 isolates showed F values of 0.97 with the reference sequence of 16SrIII-X subgroup (KC412026) and 0.95 between them. These isolates are clearly distinguished from 16SrIII-J and 16SrIII-X subgroups, respectively, as shown by the in silico RFLP analysis and must be assigned to three new subgroups within the X-disease group. Recently, a new 16SrIII subgroup, 16SrIII-Z, represented by broccoli stunt phytoplasma strain BSP-21 (JX626327) [36] was reclassified [13]. Currently, there is no official system for naming phytoplasmas when the letters of alphabet are exhausted for a particular 16Sr group. Because of this, no letters will be assigned to the three new subgroups described here until a proper nomenclature is officially proposed. Previous works showed that the strawberry lethal redness disease in Argentina is associated with the presence of a phytoplasma belonging to the 16SrXIII-F subgroup [22]. The presence of phytoplasmas belonging to the 16SrIII group associated to the same symptoms shows that strawberry plants could act as a natural reservoir of diverse phytoplasmas and are able to develop the same symptoms in the presence of different pathogens. This situation has also been reported in broccoli (Brassica oleracea) in Brazil, where three different phytoplasmas (16Sr1, 16SrIII and 16SrXIII groups) were associated to the broccoli stunt disease [36]. This is the first report of X-disease group phytoplasmas affecting strawberry crops in Argentina. This work contributes to increase the knowledge about the diversity of phytoplasmas present in South America and also supports the concept that a unique ecology and geographic separation provided favourable conditions for divergence of phytoplasma lineages from other regions of the world [37].

Funding information
This work was supported by INTA and FONCyT (PICT2010-604 and PICT2011-1172).

Acknowledgements
F. D. F. and N. M. are researchers at INTA, and L. R. C. is a researcher at INTA and a professor at UCC (Universidad Católica de Córdoba). We are very grateful to Dr Ernestina Galdeano and Dr Nacira Muñoz for English language corrections of the manuscript.

Conflicts of interest
The authors declare there are no conflicts of interest.

References


---

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.