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Delineation of a novel subgroup 16SrXIII-J phytoplasma, a '*Candidatus* Phytoplasma hispanicum'-related strain, based on computer-simulated RFLP and phylogenetic analysis

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Abstract

Symptoms of fruit phyllody and slow growth, which are suggestive of phytoplasma infection, were observed in strawberry plants cultivated in commercial fields. In order to provide evidence of association of phytoplasma with affected plants, assays for detecting and identifying were performed through computer-simulated restriction fragment length polymorphism (RFLP) and phylogenetic analysis. Total DNA was extracted from symptomatic and asymptomatic samples and used as template in nested PCR primed by the primers P1/Tint followed by R16F2n/16R2. Amplified DNA fragments of 1.2 kb from the 16S rRNA gene revealed the presence of phytoplasma in all symptomatic samples. Molecular detection was confirmed by electron transmission microscopy, which evidenced pleomorphic bodies in the phloem vessels. Nucleotide sequence representative of the strawberry phytoplasma shared 97.2 to 99 % similarity with phytoplasmas currently classified as members of the distinct subgroups within the 16SrXIII group. Similarity coefficient (F) values ranged from 0.70 to 0.92, indicating that strawberry phytoplasma delineates a new strain in addition to '*Candidatus* Phytoplasma hispanicum'-related strains. The evolutionary tree displayed that this strain emerges as a new branch in relation to those previously described. The novel strain, designated SFP (strawberry fruit phyllody) phytoplasma represents the new 16SrXIII-J subgroup and its sequence, denominated SFP-Br02, was deposited in the GenBank database (EU719108). These findings contribute for the knowledge of the genetic diversity existing among members of the group 16SrXIII and establishes strawberry as an additional host of representatives of this group in Brazil.

Phytoplasmas are wall-less bacteria associated with hundreds of diseases worldwide, which occur in cultivated and uncultivated plant species [1]. The principal classification scheme is based on the sequence variability of the 16S rRNA gene, which allows to allocate distinct phytoplasmas into groups and subgroups, according to their molecular and phylogenetic features [1].

Currently, on the basis of criteria for phytoplasma delineation are recognized more than thirty groups of phytoplasmas identified in numerous geographical regions of the world [2–5]. Specifically regarding 16SrXIII group, nine subgroups have been described [3–5]. The first representative of the 16SrXIII group was identified in periwinkle plants from Mexico that exhibited symptoms of virescence; this phytoplasma was delineated as reference strain of the subgroup 16SrXIII-A [6]. Today, the 16SrXIII group, previously denominated the Mexican periwinkle virescence (MPV) group, is classified as the putative species '*Candidatus* Phytoplasma hispanicum' [7]. A strain, genetically distinct from MPV phytoplasma, was found in association with diseases of strawberry in Florida (USA) and was indicated as representative of the 16SrXIII-B subgroup [8]. In Bolivia, the molecular characterization of phytoplasmas associated with yellowing and decline of chinaberry trees (*Melia azedarach*) revealed a new strain, which was described as representative of the 16SrXIII-C subgroup [9]. Later, a member of this subgroup were also described in diseased chinaberry trees that exhibited symptoms of decline, cultivated in Paraguay and Argentina [10, 11]. In Mexico, a MPV phytoplasma-related strain was identified in association with potato purple top disease [12]. Based on genetic variation in the 16S rRNA gene sequence, this strain was proposed as reference phytoplasma of the subgroup 16SrXIII-D. The subgroup 16SrXIII-E is represented by a phytoplasma detected in papaya harbouring apical curl necrosis disease that occurs in Brazilian orchards, causing chlorosis, defoliation and bunching of the crown leaves [13]. In Argentina,

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The GenBank accession number EU719108 corresponds to the 16SrRNA gene from strain SFP-Br02 (Strawberry Fruit Phyllody-Brazil 02).

the subgroup 16SrXIII-F was reported, whose representative is a phytoplasma associated with strawberry red leaf disease [3]. Representatives of the subgroups 16SrXIII-G and 16SrXIII-H were found in a study that reclassified phytoplasmas reported in chinatree and broccoli [4]. The reference phytoplasma of the subgroup 16SrXIII-I was characterized in strawberry plants from Mexico [5].

The strawberry cultivation has an economic and social role mainly in the Brazilian states located in the South and Southeast regions that present temperate and subtropical climates [14]. In Brazil, in a cultivated area of over 4000 ha, a total of approximately 105 000 tonnes has been produced and marketed for both fresh fruit and industry purposes [14]. In all producing areas of the world, the yield of the culture may be affected by diseases, including those associated with phytoplasmas, which induce a diversity of symptoms such as phyllody, virescence, dwarfing, chlorosis, green petal, witches' broom and flower and fruit deformations [3, 8]. In Brazil, in the state of Espírito Santo, strawberry plants were observed growing in commercial fields with symptoms suggestive of phytoplasma infection expressed by fruit phyllody (Fig. 1) and low growth. Analysis performed in the present study provides the molecular–phylogenetic characterization of a phytoplasma that delineates the novel 16SrXIII-J subgroup, which was found in association with diseased strawberry plants.

Ten symptomatic and three asymptomatic samples of strawberry plants were analysed by PCR assays. Total DNA was extracted from foliar tissues using the DNeasy Plant Mini Kit (Qiagen). Leaves from corn plants infected with the maize bushy stunt phytoplasma, a representative of 16SrI-B subgroup (GenBank: AY265208), and extracts of samples from asymptomatic strawberry were used as positive and negative controls, respectively.

Fig. 1. Fruits of strawberry exhibiting typical symptoms of phyllody associated with the phytoplasma representative of the novel 16SrXIII-J subgroup (left). Fruit asymptomatic (right).

In order to detect phytoplasmas, nested PCR assays were carried out with the universal primers pairs P1/Tint [15, 16] followed by R16F2n/R16R2 [17], according to protocols described previously by these authors. The DNA fragments of 1.2 kb generated by nested PCR were ligated into the pGEM T Easy Vector System I (Promega), transformed to the *Escherichia coli* DH5alpha strain, and subsequently sequenced. The sequences were aligned, compared among themselves, with the sequences of phytoplasmas of distinct groups, and with sequences of the representatives of subgroups belonging to 16SrXIII group available in GenBank. Nucleotide sequences were analysed using computer programs for reconstruction and sequence analysis (Bioedit, Phred phrap and multiple sequence alignment – CLUSTALW).

The detection of phytoplasmas was also conducted through transmission electron microscopy from segments of leaf veins appropriately prepared for visualization, following methodologies previously published [18, 19].

Computer-simulated restriction fragment length polymorphism (RFLP) analysis was performed with all distinct members of the subgroups into the 16SrXIII group (Table 1). The aligned and cut sequences were exported for restriction analysis with 17 restriction enzymes [20] onto virtual gel by using the pDRAW32 program, developed by AcaClone Software. The virtual RFLP patterns were compared and similarity coefficient (F) values were calculated between each pair of phytoplasma strains as previously described [6]. A phylogenetic tree was established from the sequence representative of the strawberry phytoplasma detected in this study and sequences from phytoplasmas belonging to the distinct subgroups classified into 16SrXIII group, using MEGA program [21] with the neighbour-joining method. Bootstrapping was repeated 1000 times and *Acholeplasma laidlawii* (M23932) was included as an outgroup.

Nested PCR yielded DNA fragments of 1.2 kb revealing the presence of phytoplasma in all diseased strawberry samples. Amplicons were also generated from positive control, but no amplification occurred from extracts of asymptomatic samples. Electron microscopy confirmed the molecular detection of phytoplasma in affected samples revealing the presence of typical pleomorphic bodies inside the phloem vessels (Fig. 2).

A majority consensus sequence was selected after comparison among the nucleotide sequences of the clones, which was chosen to represent the phytoplasma found in strawberry samples. This sequence designated SFP-Br02 (Strawberry Fruit Phyllody-Brazil 02), was deposited in GenBank database under accession number EU719108. The sequence identity of SFP phytoplasma varied from 97.2 to 99.0 % regarding strains that represent the current subgroups classified into the 16SrXIII group. Based on computer-simulated virtual RFLP patterns, the similarity coefficient (F) values for the phytoplasma found in strawberry in relation to the other representatives of 16SrXIII ranged from 0.70 to 0.92 (Table 1).

Table 1. Similarity coefficients (F) derived from strawberry fruit phyllody phytoplasma (SFP-Br02), a reference strain of the new 16SrXIII-J subgroup molecularly characterized in the present study, and phytoplasmas affiliated with subgroups previously described within the 16SrXIII group

Subgroups	16SrXIII-A	16SrXIII-B	16SrXIII-C	16SrXIII-D	16SrXIII-E	16SrXIII-F	16SrXIII-G	16SrXIII-H	16SrXIII-I	SFP-Br02
16SrXIII-A	1									
16SrXIII-B	0.95	1								
16SrXIII-C	0.86	0.82	1							
16SrXIII-D	0.92	0.89	0.82	1						
16SrXIII-E	0.92	0.87	0.79	0.70	1					
16SrXIII-F	0.89	0.80	0.74	0.83	0.81	1				
16SrXIII-G	0.91	0.90	0.95	0.87	0.83	0.80	1			
16SrXIII-H	0.95	0.94	0.91	0.91	0.91	0.84	0.92	1		
16SrXIII-I	0.87	0.92	0.84	0.87	0.85	0.78	0.86	0.88	1	
SFP-Br02	**0.83**	**0.76**	**0.74**	**0.75**	**0.77**	**0.92**	**0.72**	**0.76**	**0.70**	**1**

Virtual RFLP analysis of 16S rDNA sequences revealed distinct restriction patterns for the enzymes BfaI, DraI, HaeIII, HinfI, KpnI, MseI and RsaI when profiles from strawberry fruit phyllody phytoplasma were compared with profiles generated by the representative of the 16SrXIII-A subgroup. The profiles obtained for SFP phytoplasma were also different from those displayed by the subgroup XIII-B phytoplasma regarding the enzymes AluI, BfaI, HaeIII, HinfI, KpnI and RsaI. The SFP phytoplasma and chinatree decline phytoplasma (16SrXIII-C) showed restriction profiles that were distinguishable based on the endonucleases AluI, BstUI, DraI, HaeIII, HinfI, HpaII, KpnI, MseI and RsaI. Electrophoretic profiles generated by the SFP strain with AluI, DraI, HaeIII, HinfI, KpnI, MseI and RsaI were divergent from those produced by the reference phytoplasma belonging to the 6SrXIII-D subgroup. The enzymes DraI, HaeIII, HinfI, KpnI, MseI, RsaI and TaqI yielded RFLP patterns that allowed to distinguish SFP phytoplasma from the representative of the 16SrXIII-E subgroup. The restriction profiles derived from HaeIII, HinfI and MseI for strawberry fruit phyllody phytoplasma were differentiated from those found for strawberry leaf red phytoplasma affiliated with 16SrXIII-F subgroup. The restriction profiles generated by SFP phytoplasma were also distinct from those produced by the representative of the 16SrXIII-G subgroup for the enzymes AluI, BfaI, BstUI, DraI, HaeIII, HinfI, HpaII, KpnI, MseI, and RsaI. The SFP phytoplasma was distinguished from the 16SrXIII-H phytoplasma by the restriction enzymes AluI, BfaI, HaeIII, HinfI, KpnI, MseI, and RsaI. Finally, digest profiles showed by the reference phytoplasma of the 16SrXIII-I subgroup were different from those produced by SFP phytoplasma regarding the enzymes AluI, BfaI, DraI, HaeIII, HinfI, KpnI, MseI, and RsaI.

The pattern of branching of the phylogenetic tree displayed that the SFP phytoplasma emerges as a new branch, in addition to the nine other mutually distinct strains currently classified as representative of the subgroups of the 16SrXIII group (Fig. 3).

In Brazil, the occurrence of phytoplasmas in strawberry plants was firstly reported in the end of 1990s by transmission electron microscopy [22]. According to these authors, diseased plants showed fruit phyllody and reduced growth. The findings of the present study confirmed through molecular methodology the association of phytoplasma with strawberry plants that exhibited the same type of symptoms

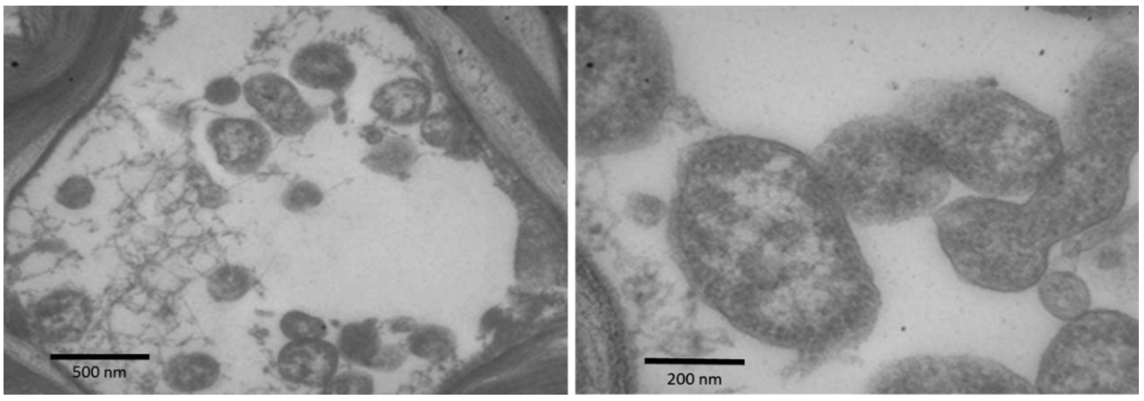

Fig. 2. Pleomorphic bodies of phytoplasma visualized in the phloem vessels of strawberry samples by transmission electron microscopy.

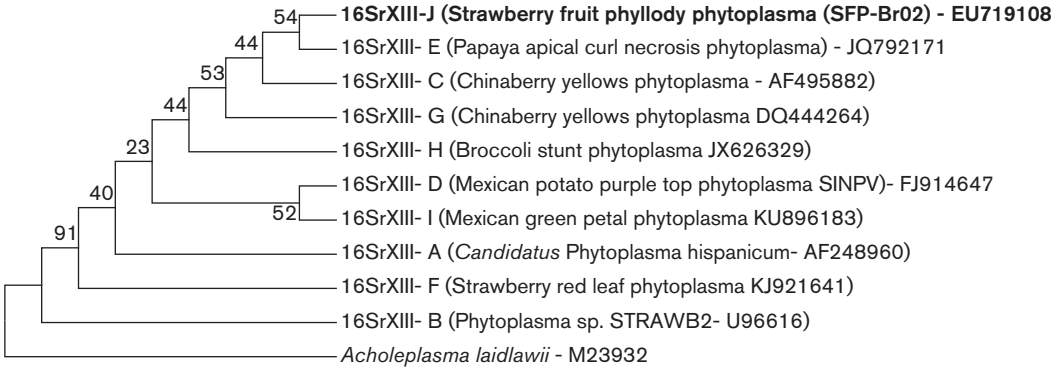

Fig. 3. Phylogenetic tree generated by sequences of 16S rRNA of the strawberry fruit phyllody phytoplasma/SFP-Br02 (this study) and reference phytoplasmas of the distinct subgroups affiliated with the 16SrXIII group, using the neighbour-joining method. *Acholeplasma laidlawii* was included as an outgroup. Numbers on the branches indicate bootstrap confidence values .

expressed by fruit phyllody and slow growth. The identification pointed this phytoplasma as more a member of the 16SrXIII group present in Brazil, in addition to the representatives of this group previously found in papaya [13] and broccoli [23].

Considering the current classification, few subgroups are recognized within the 16SrXIII group and few hosts have been reported for 16SrXIII phytoplasmas worldwide [3–5]. Thus, the representatives of the nine subgroups were found in a reduced and diversified number of herbaceous and woody species, including 16SrXIII-A in periwinkle (*Catharanthus roseus*) [6], 16SrXIII-B, 16SrXIII-F, and 16SrXIII-I in strawberry (*Fragaria x ananassa*) [3, 5, 8], 16SrXIII-C and 16SrXIII-G in chinatree (*Melia azedarach*) [4, 9, 10], 16SrXIII-D in potato (*Solanum tuberosum*) [12], 16SrXIII-E in papaya (*Carica papaya*) [13], 16SrXIII-H in broccoli (*Brassica oleracea var. italica*) [4]. Interestingly, these reports indicate that geographical distribution of these phytoplasmas is apparently restricted to countries of the American continent, including Argentina, Bolivia, Brazil, Mexico, Paraguay and the USA.

In the present study, virtual computer-simulated RFLP and values of similarity coefficients (F) provided support for delineation of a new subgroup 16SrXIII phytoplasma, following the current classification scheme [7, 20]. Thus, a new subgroup is recognized when a phytoplasma strain presents an F value equal or lower than 0.97 in comparison to those of all of the existing representative strains of a given group. The F values of the SFP phytoplasma ranged from 0.70 to 0.92 in relation to the representatives of the subgroups hitherto classified into the 16SrXIII group (Table 1). These values indicated that this strain is divergent from others 16SrXIII phytoplasmas and may delineate a new subgroup denominated 16SrXIII-J. The phylogenetic analysis also supports this delineation since the SFP phytoplasma represents a new emergent branch among those that comprise the evolutionary tree.

Our results confirmed the initial assumption that phytoplasmas are associated with the symptoms observed in diseased strawberry plants. Since this association was revealed, the phytoplasma molecularly characterized was identified as a representative of a novel subgroup 16SrXIII-J. However, assessment of the incidence and impact caused by the disease, as well as a survey to identify the host range and potential insect vectors are needed. This kind of information should certainly be useful for further understanding the epidemiological aspects and establishing management strategies to control the disease. In addition, our findings indicate strawberry as a new host for a member of the group 16SrXIII in Brazil and contribute to the increase of knowledge concerning the genetic diversity of phytoplasmas affiliated with the 16SrXIII group.

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

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