Original Paper

Occurrence of two new groups of *candidatus* phytoplasmas infecting soybean in Cuba

Ocurrencia de dos nuevos grupos de *candidatus* phytoplasmas infectando cultivos de soya en Cuba

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ABSTRACT: New symptoms similar to those induced by phytoplasmas, but different from those reported to date, were observed on soybean plants, so the objective of this research was to determine the possible presence of other groups of phytoplasmas infecting soybean crops in Cuba. Fifty-seven soybean plants showing symptoms of blistering and severe leaf mosaic were collected in several localities of the Cuban eastern region in 2014 and analysed by nested-PCR with primers targeting the 16S ribosomal DNA (16S rDNA). Phytoplasmas were detected in 31.58 % of symptomatic soybean plants. Conventional and in silico RFLP analyses of 16S rDNA sequences revealed the presence of '*Candidatus* Phytoplasma pruni' and '*Candidatus* Phytoplasma phoenicium' strains. Phytoplasmas belonging to the proposed new ribosomal subgroup 16SrIII-Z and the ribosomal subgroup 16SrIX-A were identified. Phylogenetic analysis corroborated the RFLP analyses, in which the Cuban 16SrIII-Z and 16SrIX-A subgroups formed a clade with representative sequences of the 16SrIII and 16SrIX groups, respectively. 16SrIII-Z was the most widespread subgroup (72.22 % of positive samples). This is the first report of phytoplasmas belonging to the 16SrIII and 16SrIX groups occurring in soybean in the country.

Key words: soybean, nested-PCR, phytoplasmas, RFLP, 16Sr groups.

RESUMEN: Nuevos síntomas similares a las inducidos por fitoplasmas, pero diferentes a los informados hasta la fecha, se observaron en plantas de soya, por lo que el objetivo de esta investigación fue determinar la posible presencia de otros grupos de fitoplasmas infectando los cultivos de soya en Cuba. Cincuenta y siete plantas de soya, que mostraban síntomas de ampollas y mosaico foliar severo, se recolectaron en varias localidades de la región oriental durante 2014 y se analizaron por PCR anidada con cebadores dirigidos al ADN ribosomal 16S (16S rDNA). Se detectaron fitoplasmas en el 31,58 % de las plantas de soya sintomáticas. Los análisis RFLP convencionales e *in silico* de las secuencias de ADNr 16S revelaron la presencia de las cepas '*Candidatus* Phytoplasma pruni' y '*Candidatus* Phytoplasma phoenicium'. Se identificaron fitoplasmas pertenecientes al nuevo subgrupo ribosomal propuesto 16SrIII-Z y al subgrupo ribosomal 16SrIX-A. El análisis filogenético corroboró los análisis RFLP, en los que los subgrupos cubanos 16SrIII-Z y 16SrIX-A formaron un clado con secuencias representativas de los grupos 16SrIII y 16SrIX, respectivamente. El 16SrIII-Z fue el subgrupo más extendido (72,22 % de muestras positivas). Este es el primer reporte de fitoplasmas pertenecientes a los grupos 16SrIII y 16SrIX que se presentan en soya en el país.

Palabras clave: soya, nested-PCR, fitoplasmas, RFLP, grupos 16Sr.

INTRODUCTION

Soybean (*Glycine max* L. Merr.) is one of the most cultivated legumes in the world, mainly because of its high oil and protein content (1). In Cuba, in order to replace the importation of grains, soybean production recently arose as an economic policy priority with a notable increase of the country area destined to soybean cultivation, for different uses (2).

A wide variety of pathogens can infect soybean and cause significant yield losses (3). The expansion of soybean cultivation in the world has caused the number and severity of the diseases affecting this crop to increase, and, among these diseases, phytoplasmas have acquired significant importance in the last years.

Phytoplasma diseases have had great impact on cropswith significant losses in production yield and quality (4, 5). According to Bertaccini and Lee (6), phytoplasmas infect more than 300 plant species worldwide, which limited the growth and photosynthesis of herbaceous and tree species (7).

Several soybean diseases have been associated with phytoplasma infections around the world: (i) a disease caused by 'Candidatus Phytoplasma asteris' was

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detected in soybean plants in Wisconsin (8); (ii) in Costa Rica, stunting, little leaf, shoot proliferation and aborted seed pods were associated with a putative new group related to group 16SrXII (9), which was later proposed as '*Candidatus* Phytoplasma costaricanum' and designated as 16SrXXXI group (10); (iii) phytoplasma-infected soybean has been reported in Taiwan (11); (iv) in Iran, phytoplasmas reported being transmitted by the seed (13) were found in soybean plants with symptoms of proliferation of sprouts and abortion of seeds and pods (12).

In Cuba, the presence of Aster yellows group phytoplasma and the evidence of two new subgroups 16SrI associated with stunting, chlorosis, crinkles, and aborted seed pods were reported in soybean (14). However, new soybean symptoms characterized by blistering and severe leaf mosaics were observed in the country. The objective of this research was to determine the possible presence of other phytoplasma groups infecting soybean in the country.

MATERIALS AND METHODS

Sample prospecting

From January to August 2014, surveys were conducted in soybean fields of Cuban Incasoy-27 cultivar in the localities of Holguin province in the eastern region of the country. Samples were randomly collected from 57 symptomatic plants displaying symptoms of blistering and severe leaf mosaic (Fig. 1) and from 10 symptomless plants.

Phytoplasma detection by nested PCR/

Total DNA was extracted from soybean plants according to Leiva *et al*. (15). For phytoplasma detection by nPCR, the combination of universal primers that target the phytoplasma 16S rRNA gene was used to amplify a 1.45 kb fragment from the first PCR reaction (primer pair R16mF2/R1) and a 1.25 kb fragment from the nested PCR (nPCR) reaction (primer pair R16F2n/R16R2) (15).

Phytoplasma identification by RFLP analysis

Each nPCR product was digested separately with the restriction endonucleases BstUI, HhaI, HpaII and KpnI (Promega) following the manufacturer's instructions. Digestion products were separated by electrophoresis on 3 % agarose gel and visualized by ethidium bromide staining in a transilluminator under UV light. RFLP patterns were compared with previously published patterns for different phytoplasmas (16).

Cloning and sequencing analysis of the 16SrDNAr region by BLASTn

Positive fragments of 1250 bp were purified using the Gel Band Purification Kit (GE Healthcare) and ligated into pGEM T-easy vector (Promega) according to supplier's instructions for subsequent transformation of competent cells of Escherichia coli DH5. Three inserts of each strain were sequenced in both directions using M13 primers (Macrogen, South Korea). The 16SrDNA sequences were compared with those available in GenBank at National Center of Biotechnology by BLASTn (17).

Insilico RFLP analysis and phylogenetic construction

Virtual RFLP analysis was performed for the 16S rDNA sequences (1250 bp) of the Cuban phytoplasma strains using the software program iPhyClassifier (18, 19). Each 16S rDNA sequences were digested in silico with AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI, and TaqI restriction enzymes. RFLP in silico patterns were compared with subgroup phytoplasmas reported to 16SrIII X-disease group (18, 20) and 16SrIX group 'Ca. phytoplasma phoenicium' (21).

The 16S rDNA gene sequences were aligned with MUSCLE algorithm (22) implemented in MEGA v. 6.06 (23). A phylogenetic tree for phytoplasma sequences was constructed using the maximum likelihood (ML) method with Tamura-Nei nucleotide substitution model and gamma-distributed. Bootstrap-

Figure 1. Soybean plants displaying symptoms of blistering (A) and severe leaf mosaic (B and C)./*Plantas de soya que muestran síntomas de ampollas (A) y mosaic severo en las hojas (B y C).*

ping (3,000 replications) was used to estimate the stability and support of the branches.

RESULTS AND DISCUSSION

Phytoplasma detection by nested PCR

Nested PCR fragments of phytoplasmas (1250 bp) were detected in 18 out of 57 symptomatic soybean plants (Table 1). No amplification was obtained from total DNA extracted from asymptomatic plants. These results indicate the presence of phytoplasmas in the 31.58 % of samples displaying blistering and severe leaf mosaic symptoms. Phytoplasmas were detected by nested PCR in a low percentage (31.58 %) of symptomatic soybean plants. The remaining symptomatic samples may have resulted negative because of the low phytoplasma concentration or the occurrence of any other pathogen in these soybean plants . In this sense, soybean plants with symptoms such as mosaic, blistering, leaf yellowing, and floral abortion were found positive to Begomovirus in Cuba (24). Further studies will focus on whether mixed infection of begomoviruses and phytoplasmas could be occurring in soybean plants in the country.

The incidence of phytoplasma was higher in Holguín, Uñas and La Rosa (6, 5 and 5 positive plants, respectively, in the samples tested) than in La División (2 positive plants in 5 tested).

Phytoplasma identification by RFLP analysis

The presence of 16SrIII (13 samples) and 16SrIX (5 samples) groups were identified when they were compared with patterns previously reported by (16) (Table 1).

The thirteen samples of the 16SrIII-Cu177 strain showed unique profiles with HpaII endonuclease and a similar pattern with HhaI endonuclease (Fig. 2 A) with other 16SrIII phytoplasma subgroups (X-disease group) when it was compared with previously reported patterns (16).

However, the two 16SrIX-Cu205 and three 16SrIX-Cu185 strains showed profiles identical to the 16SrIX-A subgroup 'Ca. Phytoplasma phoenicium' (Fig. 2B). These results suggest that the Cuban soybean is infected by phytoplasmas within to two distinct subgroups belonging to the 16SrIII and 16SrIX groups, a novel one (16SrIII-Cu177 strain), and these two subgroups occurring in the field.

Analysis of the 16SrDNA region sequencing by BLASTn

The sequence (1250 bp) of the 16SrIII-Cu177 strain (GenBank accession no KU749597) showed the highest nucleotide (nt) sequence identity (99 %) with several sequences of 16SrIII phytoplasma strains identified in different hosts. Also, this sequence showed high nt sequence identity (98.88 %, 1233 bp identical out of 1247 bp compared) with *Manihot esculenta* witches'-broom phytoplasma (GU193977). Additionally, the strain detected in soybean showed nt sequence identity of 98.79 % with a 16SrIII phytoplasma detected in clover in Lithuania (KC283217) and crotalaria in Brazil (KF941133), which are crop legumes.

The sequences of 16SrIX-Cu205 (KU749595) and 16SrIX-Cu185 (KU749596) showed nt sequence identity of 100 % with each other and the highest nt sequence identity (99.36 %, 1242 bp identical out of 1250 bp compared) with several sequences of 16SrIX phytoplasma strains including 'Crotalaria juncea' witches'-broom phytoplasma (KF941131) and Pigeon pea witches'-broom phytoplasma (KJ817871) detected in legumes.

In silico RFLP analysis and phylogenetic construction

In silico RFLP analysis indicated genetic variability in the 16SrIII phytoplasma associated with soybean (Fig. 3), and it confirmed the results obtained by conventional RFLP (Fig. 2). Unique *in silico* RFLP patterns were observed with HpaII (Fig. 3A)

Table 1. Detection of phytoplasma groups in soybean plants with blistering and severe leaf mosaic symptoms collected in the localities of Holguin province in the eastern region of Cuba./*Detección de grupos de fitoplasmas en plantas de soya que muestran síntomas de ampollas y mosaico severo en las hojas, colectadas en localidades de la provincia Holguín en la region oriental de Cuba*.

Locality	# of samples collected	Phytoplasma Positive[1]	16SrIII[2]	16SrIX[2]
Uñas	21	5	3	2
Holguín	11	6	4	2
La Rosa	19	5	4	1
La División	5	2	2	-
San Mateo	1	-	-	-
Total	**57**	**18**	**13**	**5**

[1]Phytoplasma detected by nested-PCR followed by RFLP with HaeIII

[2]Phytoplasma groups differentiation by RFLP analysis

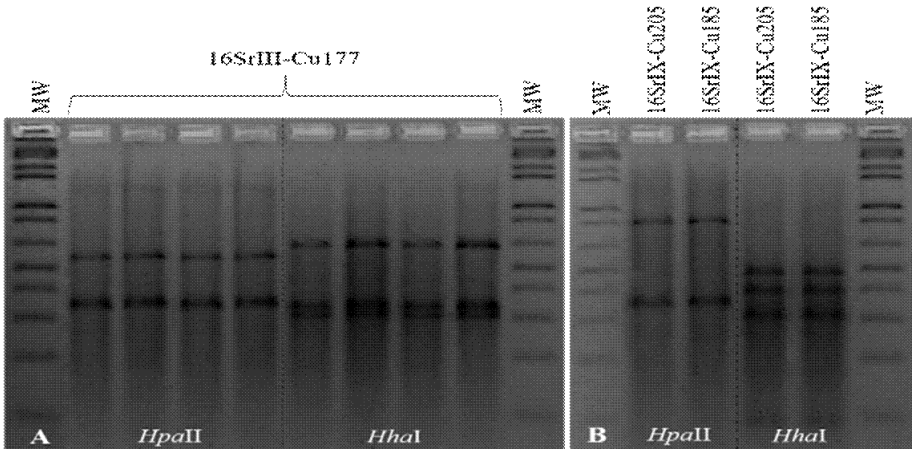

Figure 2. RFLP patterns of phytoplasma 16S rDNA gene (~1.2 kb) amplified with primer pairs R16mF2/R1 (in direct PCR) and R16F2n/R16R2 (nestedPCR) from the samples with infection of the Soybean X-Disease phytoplasma (A) and 'Ca. Phytoplasma phoenicium' (B) strains detected in soybean plants in Cuba. The PCR amplicon were digested using restriction enzymes HhaI and HpaII and subjected to 3% agarose gel electrophoresis. MW, molecular weight marker (1Kb Plus DNA Ladder); fragment sizes (bp) from top to bottom: 12000, 5000, 2000, 1650, 1000, 850, 650, 500, 400, 300, 200, 100./*RFLP del gen 16S ADNr de fitoplasmas (~1.2 kb) amplificado con los cebadores R16mF2/R16mR1 (primera reacción) y R16F2n/R16R2 (reacción anidada) de las muestras con infecciones de Soybean X-Disease phytoplasma (A) y 'Ca. Phytoplasma phoenicium' (B) detectadas en plantas de soya en Cuba. Los productos del nPCR fueron digeridos con las enzimas de restricción HhaI y HpaII, sujetos a electroforesis en gel de agarosa al 3 %. MW. Marcador de peso molecular (1Kb Plus DNA Ladder); tamaño de fragmento de arriba hacia abajo (pb): 12000, 5000, 2000, 1650, 1000, 850, 650, 500, 400, 300, 200, 100.*

for the 16SrIII-Cu177 strain. The 16SrIX-Cu205 and 16SrIX-Cu185 strains showed a profile similar to those from members of the 16SrIX-A subgroup for all enzymes analyzed (Fig. 3B). Together, the results of conventional and *in silico* RFLP indicate that the 16SrIII-Cu177 variant is a new subgroup within the 16SrIII group, and the 16SrIX-Cu205 and 16SrIX-Cu185 strains belong to the 16SrIX-A subgroup.

Phylogenetic analysis based on the 16S rDNA sequences of the 16SrIII soybean X-Disease phytoplasma and '*Ca.* Phytoplasma phoenicium'strains detected in this study and those of 53 other phytoplasmas yielded the consensus tree presented in Figure 4. The 16SrIII Soybean X-Disease phytoplasma strain grouped into a same branch of the '*Ca*. Phytoplasma pruni' phytoplasma (16SrIII-A), which in turn clustered closely to other phytoplasmas of the 16SrIII group, including Soybean veinal necrosis phytoplasma (AF177383) detected in Lithuania (Fig. 4).

The 16SrIII-Cu177 strain grouped into distinct branches, closely to Cirsium white leaf phytoplasma (AF373106) showing a greater genetic distance to other phytoplasma sequences (Fig. 4), thus providing additional evidence that these phytoplasmas represent a new subgroup. The 16SrIX-Cu205 and 16SrIX-Cu185 strains grouped ito a same branch of the '*Ca.* Phytoplasma phoenicium', which in turn clustered closely to other phytoplasmas of the 16SrIX group, including Pigeon pea witches-broom phytoplasma (AF248957), which is a member of the 16SrIX-A subgroup detected in the US. (Fig. 4)

Results presented here confirm the occurrence of phytoplasmas in soybean plants exhibiting blistering and severe leaf mosaic symptoms in Cuba. Based on RFLP patterns (HpaII), the strain 16SrIII-Cu177 is a member of a new ribosomal subgroup and is proposed to be named 16SrIII-Z, while the 16SrIX-Cu205 and 16SrIX-Cu185 strains are members of 16SrIX group '*Ca*. Phytoplasma phoenicium', which were classified within the 16SrIX-A subgroup.

Soybean X-Disease phytoplasma and '*Ca*. Phytoplasma phoenicium strains were characterized as members of the 16SrIII and 16SrIX groups, respectively, based on RFLP analysis of PCR-amplified 16S rDNA using the restriction endonucleases BstUI, HhaI HpaII and KpnI.

Other soybean diseases have been associated with phytoplasmas from other groups, including a disease causing symptoms of stunting, chlorosis, crinkle and aborted seed pods that was associated with a 16SrI group phytoplasma in Cuba (14). In Costa Rica, the 16SrXXXI group was proposed as the new '*Ca*. P. costaricanum' associated with soybean stunt symptoms (10). Additionally, a disease caused by a subgroup 16SrII-V phytoplasma was reported in Taiwan (11), and another in Iran, showing symptoms of bud proliferation and aborted seed pods was associated with phytoplasmas of the 16SrVI group (12).

These facts suggested that a high variability of phytoplasmas could be occurring in the soybean fields of Cuba. Accordingly, a high genetic variability of 16SrI phytoplasmas was reported in soybean in the same region by (14). Our results confirm this hypothe-

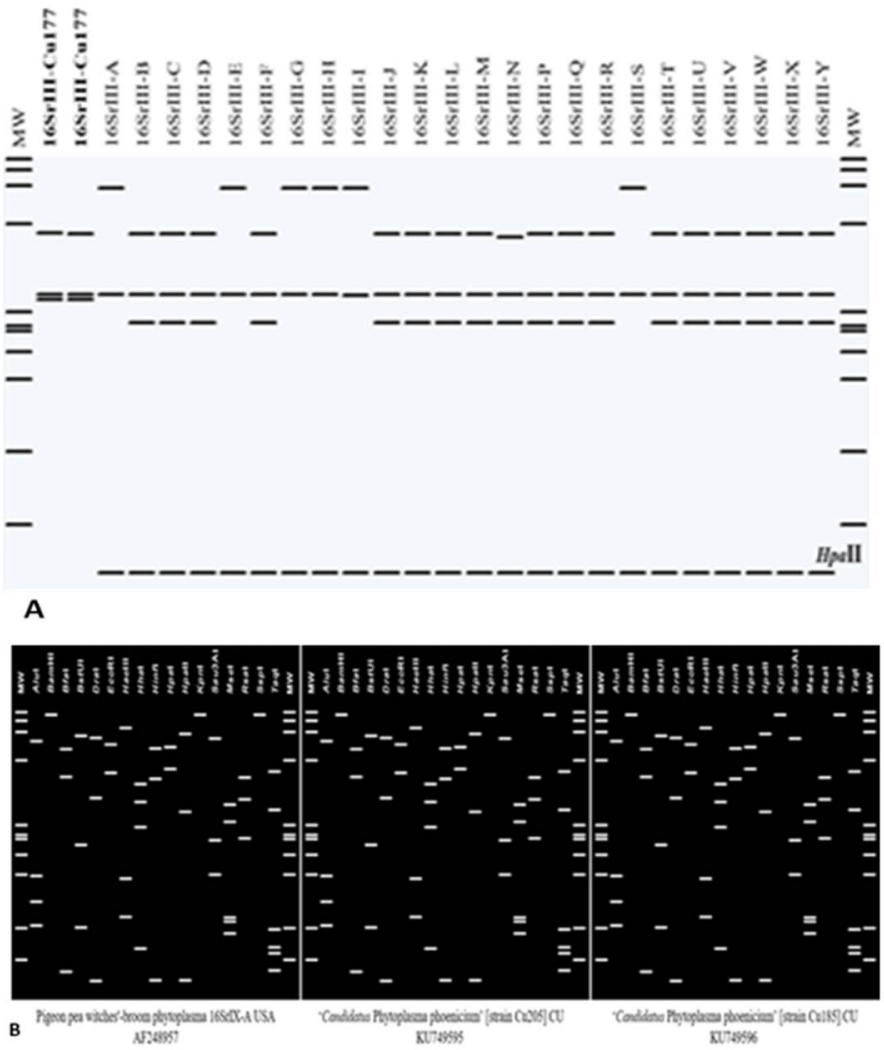

Figure 3. Computer-simulated virtual RFLP patterns derived from *in silico* digestions of phytoplasma 16S rDNA gene fragments amplified with R16F2n/R16R2 (~1.2 kb). (A) Strains of the 16SrIII group detected in soybean (this study) and representative subgroups were used with two enzymes: HhaI (top) and HpaII (bottom) and, (B) Strains of the 16SrIX group detected in soybean (this study) and representative 16SrIX-A subgroup were used with 17 enzymes recognised to phytoplasma classification. MW, molecular weight marker (øX174 DNA digested with HaeIII); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72./*RFLP virtual derivado de digestiones de fragmentos del gen 16S ADNr amplificados con los cebadores R16F2n/R16R2 (~1.2 kb). (A) Cepa del grupo 16SrIII detectado en soya (en este estudio), y (B) Cepa del grupo 16SrIX detectado en soya (en este estudio) y subgrupo 16SrIX-A. MW: marcador de peso molecular (øX174 DNA digerido con HaeIII); tamaños de fragmentos de arriba hacia abajo (pb): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.*

sis, with the detection of phytoplasmas belonging to two groups, including one novel subgroup.

Phylogenetic analysis was consistent with RFLP analyseswhen showed that Soybean X-Disease phytoplasma classified as 16SrIII-Z subgroup cluster within the same clade with Ca. Phytoplasma pruni (16SrIII group). In addition, the 16SrIII-Z subgroup was the most frequently detected one (72.22% of the infected samples).

The results presented at this point indicate that phytoplasmas identified in soybean plants in the country are genetically diverse. The identification of two phytoplasma groups, including a new subgroup in soybean plants from production fields, reinforce the urgency of developing a more effective crop management strategy. Further studies are required to identify their potential vectors, as well as a more reliable molecular characterization tool to analyze phytoplasma diversity in soybean varieties, which may have important epidemiological implications for the disease control.

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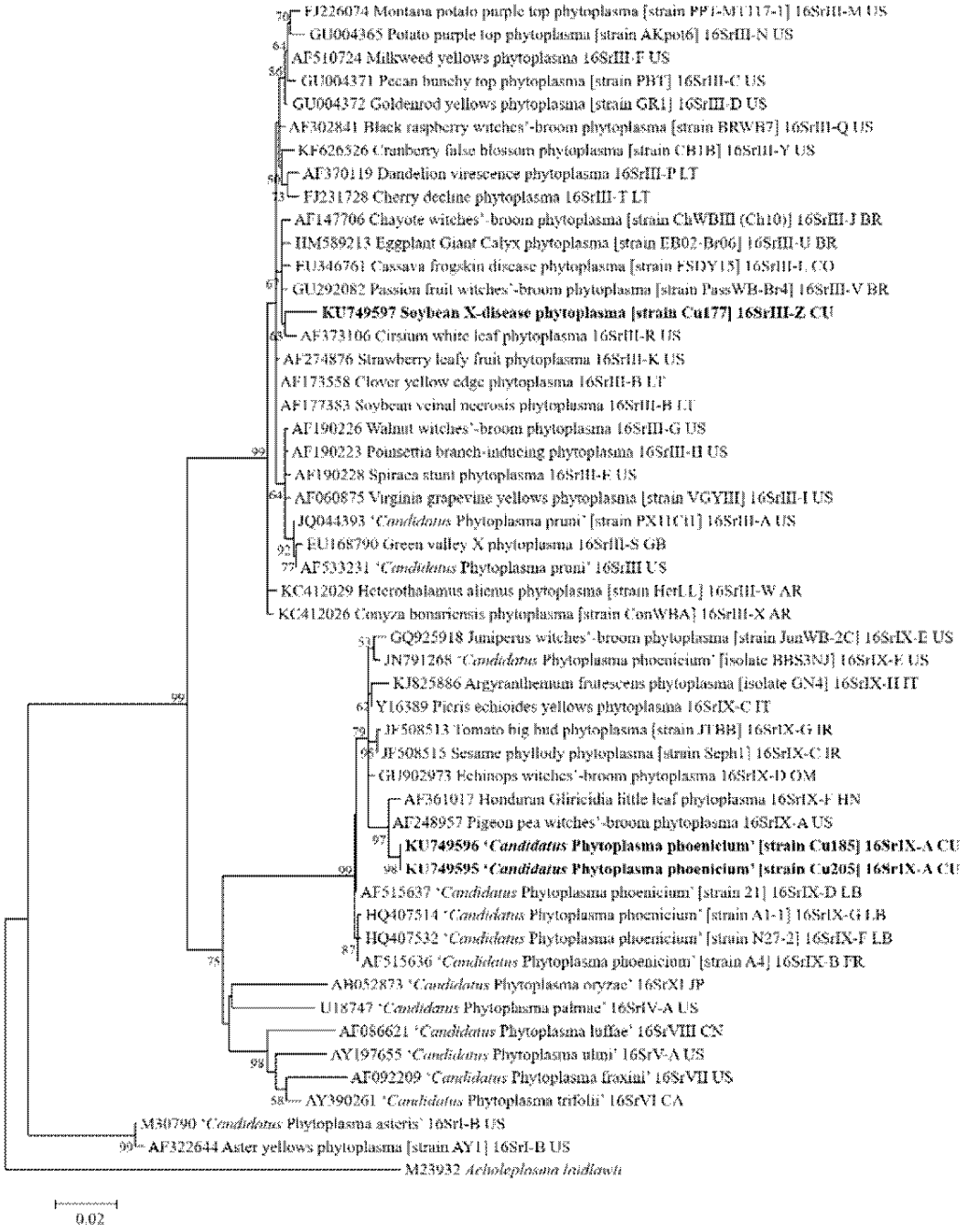

Figure 4. Phylogenetic relationships based on 16SrDNA sequences of phytoplasmas detected in soybean plants in Cuba (Soybean X-Disease phytoplasma and 'Ca. Phytoplasma phoenicium' strains) and representative phytoplasma subgroups characterized within 16SrIII and 16SrIX groups available in GenBank. Phylogenetic tree was inferred using the maximum likelihood (ML) method with Tamura-Nei nucleotide substitution model and gamma-distributed using 3,000 bootstrap replications. Bootstrap values are shown at the branch points. Also shown are GenBank accession numbers, phytoplasma disease, strains, subgroup and country abbreviations: Argentina (AR), Brazil (BR), Cuba (CU), Colombia (CO), France (FR), United Kingdom (GB), Honduras (HN), Iran (IR), Italy (IT), Lebanon (LB), Lithuania (LT), Oman (OM), and United States of America (US)./*Relación filogenética basada en secuencias del gen 16S ADNr de fitoplasmas identificados en cultivos de soya en la región oriental de Cuba (X-Disease SoybeanCu177), "Ca. Phytoplasma phoenicium" (CPP-SoybeanCu185 y CPP-SoybeanCu205) y Aster yellow Phytoplasmas (SoySTp-1 Cu, SoySTp-2 Cu, SoySTp-3 Cu) y fitoplasmas representativos de los subgrupos caracterizados como grupo 16SrI, 16SrIII y 16SrIX avalados en GenBank. El árbol filogenético fue inferido usando máxima verosimilitud (ML) con el método de distribución gamma modelo de sustitución de nucleótidos Tamura-Nei con remuestreo de 3000 repeticiones. Se muestran también los números de acceso al GenBank de las enfermades causadas por fitoplasmas, las cepas y abreviaturas de los países: Argentina (AR), Brasil (BR), Cuba (CU), Colombia (CO), Francia (FR), Reino Unido (GB), Honduras (HN), Irán (IR), Italy (IT), Líbano (LB), Lituania (LT), Oman (OM), y Estados Unidos de América (US)*.

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