Original Article

Molecular characterisation of '*Candidatus* Phytoplasma asteris' and a *Bean golden yellow mosaic virus* isolate present in mixed infection in common bean in Cuba

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Caracterización molecular de '*Candidatus* Phytoplasma asteris' y un aislado de *Bean golden yellow mosaic virus* presentes en infección mixta en frijol común en Cuba

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ABSTRACT: Common bean fields of Havana and Mayabeque provinces, Cuba, were surveyed from October to March in 2011 to 2013 to examine symptoms associated with the mixed infections by phytoplasmas and begomoviruses. Total DNA was extracted from ninety-nine symptomatic and ten symptomless plants and used as a template in phytoplasma detection by nested PCR using universal primers that target the 16S rRNA gene, and in the PCR for amplification of the begomovirus N-terminal region of the Rep protein, the intergenic region, and the N-terminal region of the coat protein. The sequences of phytoplasmas and begomoviruses obtained were compared with those of reference at the GenBank by BLAST*n*. Of the total samples collected, 72.72 % were positive to these infections. Of these, phytoplasmas were detected in 11.11 %, begomoviruses in 17.17 %, and 44 % showed a mixed infection by both pathogens. RFLP and phylogeny analyses confirmed the identification of the phytoplasma as a member of the group 16SrI '*Candidatus* Phytoplasma asteris', subgroup 16SrI-B. Sequence analysis revealed that the begomovirus coexisting with the 16SrI phytoplasma in common bean plants was the *Bean Golden Yellow Mosaic Virus* (BGYMV). The sequence of the A component of the present BGYMV showed 98 % similarity with that of a previously BGYMV isolated in Cuba (AJ544531), and its B component was closely related to that of the clade of the American isolate BGYMV-US (DQ119825).

Key words: *Phaseolus vulgaris* L., begomoviruses, phytoplasma, mixed infection.

RESUMEN: Se llevaron a cabo encuestas en Cuba durante octubre a marzo de 2011 a 2013, con observaciones de una mezcla de síntomas similares a los asociados con las infecciones por fitoplasmas y begomovirus en campos de frijol común (*Phaseolus vulgaris* L.) de las provincias La Habana y Mayabeque. El ADN total se extrajo de 99 plantas sintomáticas y diez asintomáticas; se utilizaron como moldes en un PCR anidado para detectar fitoplasmas, utilizando cebadores universales que flanquean al gen del ARNr16S, así como de un PCR para amplificar la región N-terminal de la proteína Rep de los begomovirus, la región intergénica y región N-terminal de la proteína de la cápsida. Tanto las secuencias de fitoplasma como del begomovirus obtenidas, se compararon con las de referencia en el GenBank por BLASTn. Del total de muestras colectadas, el 72,72 % fue positivo a estas infecciones. En el 11,11 % se detectó fitoplasma, en el 17,17 % begomovirus y 44 % presentó infecciones mixtas de ambos patógenos. Los análisis de RFLP y filogenia confirmaron la identificación del fitoplasma como miembro del grupo 16SrI '*Candidatus* Phytoplasma asteris', subgrupo 16SrI-B. El análisis de secuencia reveló que el begomovirus coexistiendo con el fitoplasma 16SrI, en plantas de frijol común, era el virus del mosaico dorado del frijol (BGYMV). El aislado BGYMV actual mostró 98 % de similitud en la secuencia de componentes A con la de un aislado anterior de BGYMV de Cuba (AJ544531), y su componente B estaba estrechamente relacionado con el del clado del aislado estadounidense BGYMV-US (DQ119825).

Palabras clave: *Phaseolus vulgaris* L., begomovirus, fitoplasma, infección mixta.

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INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) (Fabaceae) is native to the Americas. It is a legume with global importance as a nutritional source and its demand is expected to increase in the coming years because of the current trend of population to grow and increase its consumption (1). In Cuba, the edaphoclimatic conditions are favourable for growing common bean, and it is produced throughout the national territory. However, its national production does not cover the consumption demand. At present, the average national yield of the common bean production has been between 0.8 and 1 t/ha (2). The crop is susceptible to several diseases (3,4), and, among them, those caused by begomoviruses and phytoplasmas are considered important (5,6).

Begomoviruses (*Geminiviridae* family), characterized by circular ssDNA encapsidated in a twinned icosahedral capsid (7) are considered emergent global plant pathogens affecting different economical crops (8). Bipartite begomoviruses, such as *Bean dwarf mosaic virus* (BDMV), *Bean golden mosaic virus* (BGMV), *Bean golden yellow mosaic virus* (BGYMV) and *Bean calico mosaic virus* (BCaMV), have been previously reported affecting common bean, and BGYMV that causes very similar symptoms to BGMV has been the limiting factor for this crop production in the Mesoamerican and Caribbean areas (9).

In Cuba, in the last years, begomoviruses emerged as a main problem for solanaceous and fabaceous crops (8, 10). BGYMV caused 90 % losses of in common bean during the '90s (11). The BGYMV A component was identified and characterized, and it was classified as the Cuban strain BGYMV-[CU] by the International Committee of Viral Taxonomy (12). In 2018, Chang *et al.* (5) detected their presence in 89.5 % of the bean producing areas of Mayabeque province. Recently, *Tobacco leaf curl Cuba virus* was detected on common bean plants in the central region of the country (13), and two new begomoviruses have lately been identified, *Common bean mottle virus* (CBMoV) and *Common bean severe mosaic virus* (CBSMV) (5).

Phytoplasmas are wall-less prokaryotes that inhabit the phloem and are naturally transmitted mainly by leafhoppers (Cicadellidae) and planthoppers (Fulgoromorpha), and less frequently by psyllids (Psyllidae) (14). These pathogens are associated with diseases of more than 1000 plant species (15).

Phytoplasmas of the 16SrI group were identified on several crops in Cuba (16), including *Vicia faba* L., which was the first report of phytoplasmas affecting *Fabaceae* in our country (17), common bean (6), and soybean and jack bean (16).

On the other hand, phytoplasmas and begomoviruses can naturally occur in the same plant host (8, 18, 19). Natural mixed infections of phytoplasma and begomoviruses were reported in Mexico for 16SrIII phytoplasmas with the *tomato yellow leaf curl virus* (TYLCV) in tomato, and the *tomato chino La Paz virus* in pepper (20). Phytoplasmas of the groups 16SrI, 16SrXII '*Ca.* Phytoplasma solani', and 16SrV '*Ca*. Phytoplasma ulmi' were identified in tomato infected with TYLCV (21). In addition, a 16SrII phytoplasma and a *New Delhi tomato leaf curl* begomovirus was identified in India (22).

In Cuba, Leyva *et al*. (8) detected the coexistence of begomoviruses and phytoplasmas in soybean crops. Zamora (23) reported the presence of mixed infections of phytoplasmas and begomoviruses in common bean. However, the molecular characteristics of the isolates associated with mixed infections were not described.

The present study was aimed to characterize those pathogenic begomoviruses and phytoplasmas associated with mixed infections in common bean fields of Havana and Mayabeque provinces so that important tools can subsequently be provided to management of these diseases.

MATERIAL AND METHODS

Surveys, nucleic acid extraction, and reference controls

Surveys for phytoplasmas and begomoviruses were conducted in common bean fields of cv. ´Bat-304´ from October to March in 2011 to 2013 in the provinces of Havana and Mayabeque in south-western Cuba. The leave samples were taken from eight farms of three municipalities: Cotorro (23°01′34″N 82°14′51″O) in Havana province, and San José de Las Lajas (22°58′04″N 82°09′21″O) and Güines (50°52′04″N 1°52′25″E) in Mayabeque province. The midribs were removed from the collected leaves with a sterile scalpel and subjected to total DNA extraction (24).

For both PCR assays, specific for phytoplasmas and begomoviruses, ´Mexican potato purple top phytoplasma´, belonging to ´*Ca*. Phytoplasma asteris´ (16Sr-I group) and TYLCV were used, respectively, as the positive controls. They were from the Interdisciplinary Centre of Research for Integral Regional Development of National Polytechnic Institute (CIIDIR-IPN), Unit Sinaloa, Mexico.

Phytoplasmas and begomoviruses detection

To detect phytoplasma DNA, it was performed a nested PCR assay using universal primers that amplify the 16S rRNA gene of phytoplasmas: R16mF2/R1 for the first round and R16R2/F2n for the nested reaction (25). A dilution to 1/30 was made from the first round to the second. A PCR assay including the Rep-DGSAR (*Xba*I)/ and a pCP70-*BamH*1 primer pair

was used for the detection of begomoviruses (26). PCR products were electrophoresed in 1 % agarose gels, and visualized under UV transillumination, after staining with ethidium bromide as recommended by the manufacturer.

Restriction Fragment Length Polymorphism (RFLP) for phytoplasma

RFLP with restriction enzymes *Kpn*I and *Mbo*I was performed on the phytoplasma PCR products for the preliminary characterization of the phytoplasma detected in common bean plants (6). RFLP profiles were electrophoresed in 2 % agarose gels and visualized under UV transillumination, after staining with ethidium bromide as recommended by the manufacturer.

DNA sequencing of full-length genome of begomoviruses and PCR products of phytoplasma

Total DNA was used as a template for Rolling Circle Amplification (RCA) to obtain the complete begomoviruses genomes (TempliPhi ™ 100 Amplification kit, GE Healthcare, UK) (5). Genome concatemers generated during the begomovirus DNA amplification were digested with restriction enzymes: *Nco*I, *Puv*I and *Kpn*I (Promega, USA) to release the full-length genomes (13). PCR products were electrophoresed in 1 % agarose gels and visualized under UV transillumination, after staining with ethidium bromide as recommended by the manufacturer.

The complete genomic products from representative common bean samples of each province were purified on spin columns (Wizard Cat. No. A9281, Promega, Madison, USA) as recommended by the manufacturer and then cloned in pNEB193 vector (Stratagene, USA). The ligation products were transformed into *Escherichia coli* JM109 (Promega, USA), as recommended by the manufacturer. The recombinant plasmids selected were sequenced by the Sequencing Service CINVESTAV, (Irapuato, Mexico).

PCR products of phytoplasma from representative common bean samples of each province were purified on spin columns (Wizard Cat. No. A9281, Promega, Madison, USA) as recommended by the manufacturer. Purified PCR products were cloned in a pGEM-T Easy Vector System (Cat. Nº. A1360, Promega, Madison, USA) as recommended by the manufacturer and sequenced using primers M13F/R at the Sequencing Service, CINVESTAV, (Irapuato, Mexico).

Phylogenetic analyses

The obtained sequences of both phytoplasmas and begomoviruses were compared with those of reference at the GenBank by BLAST*n* (27) and aligned using the MUSCLE algorithm implemented in MEGA v. 6.0 (28). For 16S rDNA sequences a phylogenetic tree was constructed using the maximum likelihood (ML) method with the Hasegawa-Kishino-Yano (HKY) model and gamma distribution rate variation (+G). For the sequences of DNA-A and DNA- B begomoviruses, a phylogenetic tree was constructed using the Maximum Likelihood method with the Tamura-Nei model and gamma distribution rate variation (+G). Bootstrapping (3000 replications) was used to estimate the stability and support for the branches.

RESULTS

Surveys of phytoplasmas and begomoviruses symptoms

A total of 99 symptomatic common bean plants exhibited symptoms of mosaic, crinkle, leaf size reduction, leaf deformation, yellowing, chlorosis and dwarfism (Figure 1).

Detection of phytoplasmas and begomoviruses

Phytoplasmas were detected by nested PCR in 55 out of 99 leaf samples, while begomoviruses were detected by PCR in 63 out of 99 symptomatic common bean plants from all localities of Havana and Mayabeque provinces (Table 1). No phytoplasma amplification was obtained from those symptomless plants collected.

Based on the molecular analysis, the province with the lowest infection incidence was Havana, from which with only two leaf samples were positive to phytoplasma and four samples to begomovirus (Table 1).

Mixed infections were detected in 44 leaf samples from common bean plants expressing phytoplasma and begomovirus disease-like symptoms (Table 1). These results confirmed that the common bean plants exhibiting the above mentioned symptoms were associated with begomoviruses and phytoplasmas.

The detection of both pathogens in 44 samples established an occurrence probability of mixed infections of 0.44 in the total plants evaluated. The occurrence probability of mixed infections, P (AB), was calculated. The probability level of begomoviruses (B) occurring in first instance was 0.72, and of phytoplasmas (A) was 0.80. These results suggest that the occurrence probability of a mixed infection will be 1.11 times higher if phytoplasmas are first detected in the field samples.

Restriction Fragment Length Polymorphism (RFLP) for phytoplasma

Using *Kpn*I endonuclease, the fifty-five common bean samples positive to phytoplasma showed identical profiles to those of '*Ca*. Phytoplasma asteris' (16SrI-B subgroup) (Figure 2). Similar results were obtained with *Mbo*I endonuclease (data not showed).

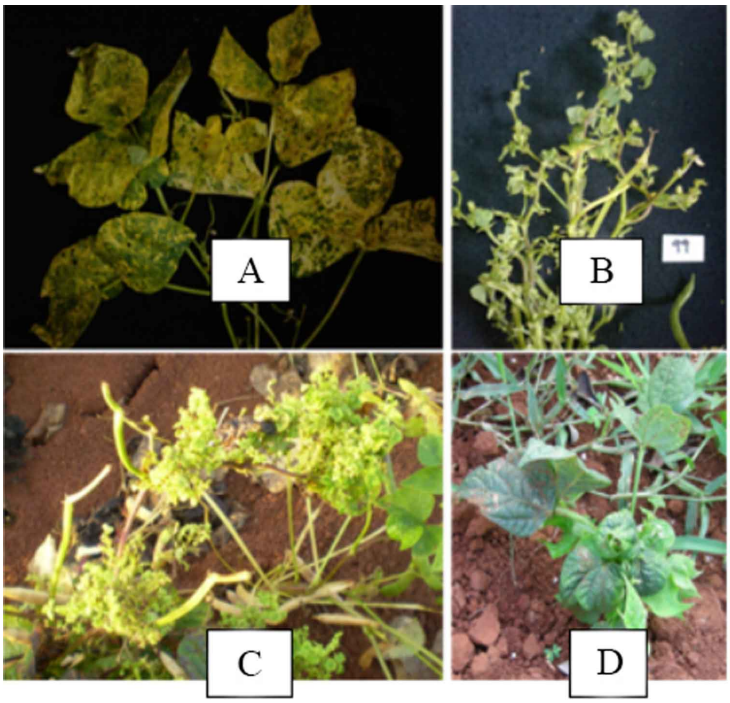

Fig. 1. *Phaseolus vulgaris* L. plants collected showing different symptoms: A, intense yellow mosaic; B, small leaves, stunting, dwarfism, phyllody and deformed pod; C, yellowing, short internodes, stunting and shoot proliferation; D, short internodes, stunting and dwarfism. /Plantas de *Phaseolus vulgaris* L. colectadas que muestran diferentes síntomas: A, mosaico amarillo intenso; B, hojas pequeñas, retraso del crecimiento, enanismo, vaina filodada y deformada; C, amarillamiento, entrenudos cortos, atrofia y proliferación de brotes; D, entrenudos cortos, retraso del crecimiento y enanismo.

Table 1. Positive common bean samples to phytoplasma, begomovirus or mixed infection of both/ Muestras de frijol común positivas a cada uno: fitoplasma, begomovirus o infección mixta de ambos.

Locality/ Province	Samples	nPCR*/RFLP phytoplasma	PCR Begomovirus	Mixed infection
Cotorro/ Havana	19	2	4	1
San José de Las Lajas/Mayabeque	52	34	38	28
Güines/Mayabeque	28	19	19	15
Total	**99**	**55**	**61**	**44**

*Nested-PCR

Table 2. Relationship and incidence probability of the detection of begomoviruses and phytoplasma in symptomatic common bean plants/ Relación y probabilidad de incidencia de la detección de begomovirus y fitoplasma en plantas sintomáticas de frijol común.

Mixed infections	Phytoplasma (A)		Begomoviruses (B)	
	+	-	+	-
Positive	44	0	44	0
Negative	11	44	17	38
Total	55	44	61	38
χ^2	63.36		16.42	
P	0.0001		0.0001	
PCO*	80a		72.13b	

*Percentage of conditional occurrence (PCO)

**Different letters (a/b) indicating a significant difference by using McNemar's proportion comparison test at $p<0.05$.

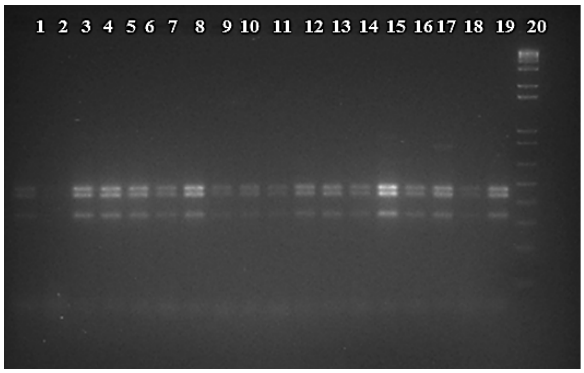

Fig. 2. *Kpn*I RFLP profiles of R16R2/F2n amplicon. Lanes 1-18: PCR Positive common bean samples. Lane 19: Mexican potato purple top phytoplasma 'Ca. Phytoplasma asteris' positive control. Lane 20: 1Kb molecular weight marker (Promega, USA). / Perfiles del RFLP con *Kpn*I del amplicón R16R2 / F2n. Carriles 1-18: Muestras de frijol común positivas para PCR. Carril 19: Control positivo: Fitoplasma de la punta morada de la papa mexicana ('Ca. Phytoplasma asteris'). Carril 20: marcador de peso molecular de 1 Kb (Promega, EE. UU.).

These results indicate that common bean is infected in Cuba by group 16SrI of phytoplasmas.

Sequence identity analysis of full-length genome of begomoviruses and PCR products of phytoplasmas

The amplified genomic DNA from representative symptomatic common bean samples infected by a begomovirus exhibited a *Kpn*I fragments of ~2,6 kbp. BLAST*n* analysis showed that the clones corresponded to begomoviral DNA-A (2644 nt) and DNA-B (2608 nt), respectively. DNA-A (KU160634) showed a nucleotide identity of 98 % with the begomovirus BGYMV isolates (AJ544531), (KX185517; KX185518), isolated previously in Cuba. In turn, DNA-B (KU145406) sequences obtained from 20 % of the clones showed a sequence identity of 95,71 %, 94,33 %, 93,84 %, and 90,75 % with the begomovirus strains BGYMV-US (DQ119825), BGYMV-MX (AF173556), BGYMV-DO (L01636), and BGYMV-GU (M91605).

The 16S rDNA sequences of three clones of Cuban *Phaseolus* phytoplasma (KR732321, KR732322, and KR732323) showed their highest sequence identity (99 %) with those of phytoplasma members of the group 16SrI, '*Ca*. Phytoplasma asteris', including the Chinese periwinkle phyllody phytoplasma (GU113145) and 'Canavalia ensiformis' yellowphytoplasma (KR232799).

Phylogenetic analyses

The phylogenetic analysis based on the 16S rDNA sequences of the 16SrI phytoplasma strains detected in this study and of those of 14 other phytoplasmas yielded the consensus tree shown in Figure 3.

The 16SrI Cuban *Phaseolus* phytoplasma strains grouped into a same branch of the AY phytoplasma, which in turn clustered closely to other phytoplasma strains of the 16SrI group previously detected in several crops in Cuba (Fig.3).

The phylogenetic analysis of the DNA-A of BGYMV (KU160634) detected in this study was placed within the monophyletic cluster of the Mesoamerican begomovirus group (Fig.4), more closely related to the strain BGYMV-CU (KX185517; KX185518) reported in Cuba. The analyses carried out with the B component sequence (data not show) confirmed that obtained for the A component, because BGYMV CU (KU145406) was placed closely to BGYMV-US (DQ119825) in the monophyletic cluster with Mesoamerican isolates.

DISCUSSION

The symptoms observed on the common bean plants in the fields of the provinces Havana and Mayabeque examined in the present study slightly differed from those observed by Zamora *et al*. (6) in production areas of San Jose de Las Lajas, Mayabeque, which these authors described as short internodes, dwarfism, leaf yellowing, shoot proliferation, and chlorosis of crown leaves in approximately 10 % of the plants. They also differed from those described by Moreira *et al*. (29), associated with "amachamiento" in Costa Rica.

The presence of phytoplasmas and begomoviruses was confirmed by PCR in 72,72 % of the common bean plants collected. Single infection of phytoplasmas and begomoviruses was detected in 11,11 % and 17,17 %, respectively, of the total plants tested. A mixed infection by both phytoplasma and begomovirus pathogens was obtained in 44,44 % of the plants surveyed. This is the first work that describes the molecular characterization of phytoplasmas and begomoviruses coinfecting plants in the common bean crop. Leyva *et al.* (8) detected a 15 % of coexistence of begomoviruses and phytoplasmas in the soybean crop in Cuba.

Based on the sequence of DNA-A component, the begomovirus was identified as BGYMV, confirming the previous results of Echemendía *et al*. (12) and Chang *et al.* (5). BGYMV has been reported affecting common bean in several countries of Central America, South America and the Caribbean (5, 9).

Other begomoviruses that have been reported in common bean in America are *Bean calico mosaic virus* (BCaMV); *Tomato golden mosaic virus* (TGMV), *Tomato yellow leaf curl virus* (TYLCV), *Rhynchosia golden mosaic virus* (RhGMV), *Sida mottle virus* (SiMoV), *Tobacco leaf curl Cuba virus (TobLCCuV)*, *Common bean mottle virus* (CBMoV) and *Common bean severe mosaic virus* (CBSMV), and BGMV (5,8,30,31,32,33,34). The latter virus caused losses in 100,000 ha in North Argentina.

The characterization of the full-length sequence of DNA-B component of BGYMV has a significant impact on BGYMV epidemiology in the country since

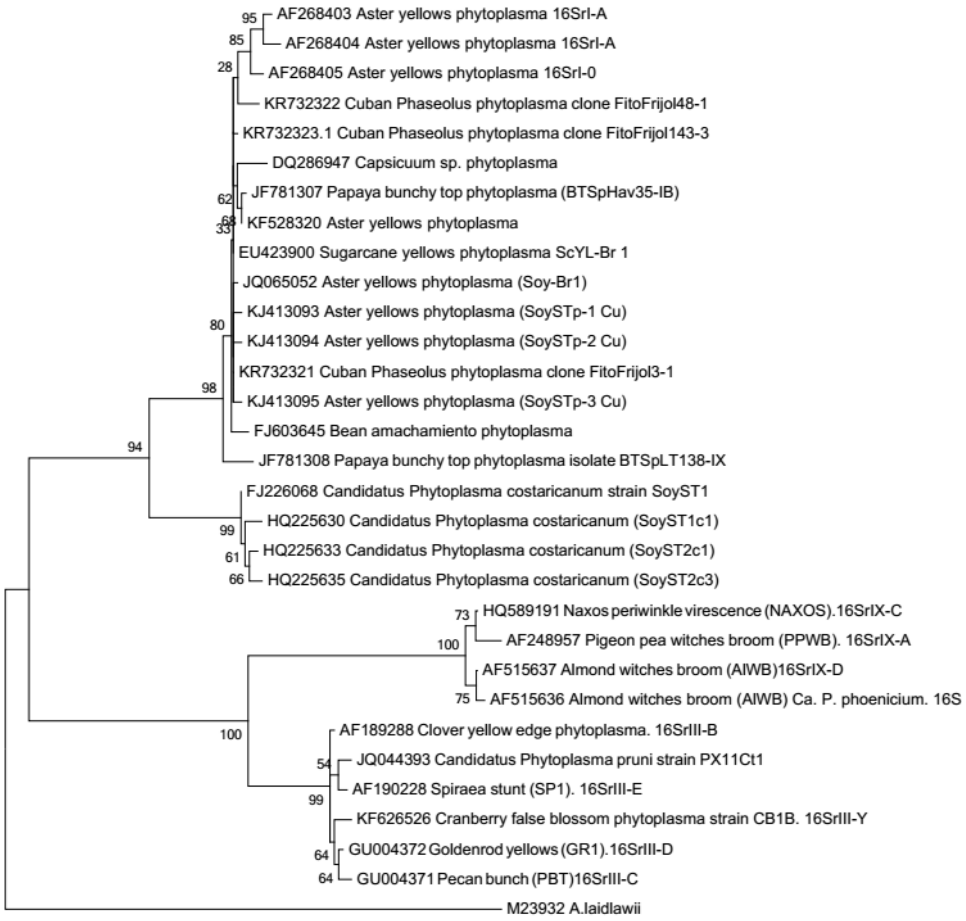

Fig. 3. Phylogenetic relationships based on 16SrDNA sequences of phytoplasmas causing common bean disease in Cuba and of representative phytoplasmas available in GenBank. Phylogenetic tree was inferred using the maximum likelihood (ML) method implemented in MEGA 6.06, with the HKY +G nucleotide substitution model and 3000 bootstrap replications. Bootstrap values are shown at the branching points/ Relaciones filogenéticas basadas en secuencias de 16SrDNA de fitoplasmas que causan la enfermedad del frijol común en Cuba y fitoplasmas representativos disponibles en GenBank. El árbol filogenético se infirió utilizando el método de máxima verosimilitud (ML) implementado en MEGA 6.06, con el modelo de sustitución de nucleótidos HKY + G y 3000 replicaciones de remuestreo. Los valores de remuestreo se expresan en los puntos de ramificación.

DNA-B is associated with the virus cell-to-cell movement through the plant plasmodesmata and the plant host range and symptoms (35).

The confirmation of the detection of the 16SrI group in common bean, particularly the subgroup 16SrI-B, is of a great epidemiological impact for phytoplasma infection in common bean in Cuba. This is the phytoplasma group with the widest plant and vector host range and the most complex epidemiology (36). It is a noteworthy fact that the symptoms described in common bean in 2012 differ from those observed in the present study.

The high incidence of both BGYMV and the 16SrI phytoplasma in common bean fields indicates that it is required a more effective disease management strategy to target both pathogens and their vectors simultaneously.

BGYMV is prevalent in common bean cultivated areas in Havana and Mayabeque provinces, suggesting that surveillance of this virus, which has devastated common bean fields in South America, required being reinforced.

In view of the capability of BGYMV and the 16SrI phytoplasma to coexist on a same common bean host plant, the Cuban policy for management of common bean diseases is required to be revised more in detail. This is a potential threat for the the common bean crop in Cuba. Additional studies on the epidemiology of both plant pathogens are also needed to develop more effective strategies for crop management and disease control.

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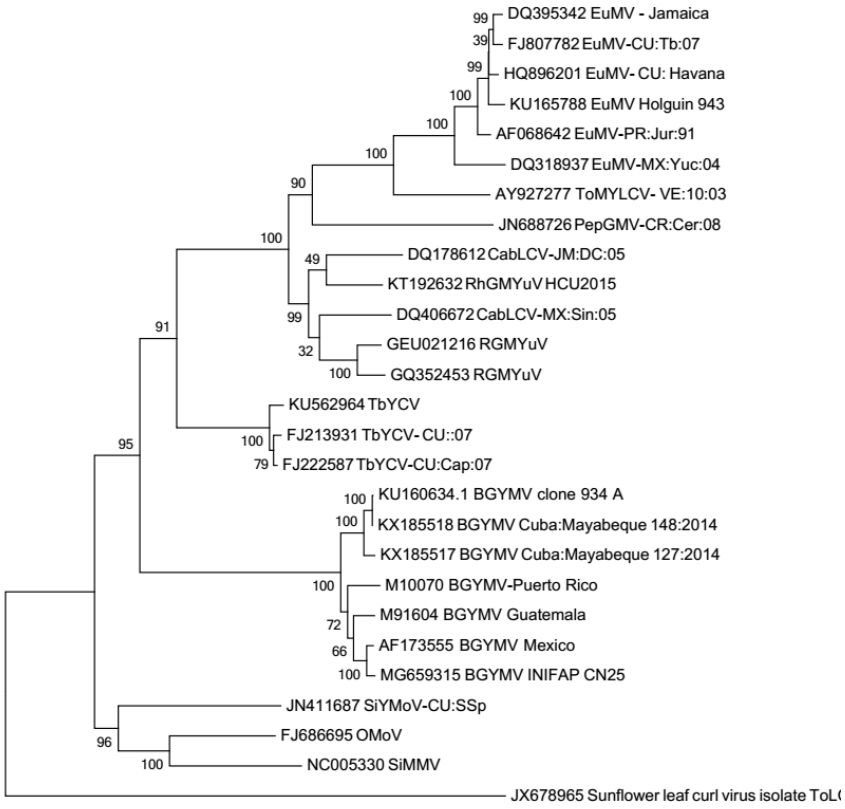

Figure 4. Phylogenetic relationships of DNA-A of *Bean golden yellow mosaic* virus detected in common bean plants with several GenBank reference sequences of begomoviruses affecting Fabaceae and species of other plant families. Phylogenetic tree was constructed using the Maximum Likelihood method with the Tamura-Nei model and gamma distribution rate variation (+G). Bootstrapping (3000 replications) was used to estimate the branch stability and support. / Relaciones filogenéticas del ADN-A del *virus del mosaico amarillo dorado del frijol* detectado en plantas de frijol común con diferentes secuencias de referencia del GenBank de begomovirus que afectan a Fabaceae y especies de otras familias de plantas. Los árboles filogenéticos se construyeron utilizando el método de máxima verosimilitud con el modelo de Tamura-Nei y la variación de la tasa de distribución gamma (+ G). Se realizó el remuestreo (3000 repeticiones) para estimar la estabilidad y el soporte de las ramas.

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