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Phytopathological Note

Bocconia frutescens little leaf, a new plant disease associated to a ´*Candidatus* Phytoplasma pruni´ related strain in Costa Rica

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ABSTRACT

Objective/Background. *Bocconia frutescens* (Papaveraceae) is a small tree distributed naturally from Mexico to Argentina and the Caribbean Bassin. *Bocconia* trees showing symptoms resembling phytoplasmas infection, such as little leaves and witches´-broom, were found in Cartago province, Costa Rica. Detection and identification of the potential phytoplasmas associated with *B. frutescens* little leaf symptoms was the objective out of this study.

Materials and Methods. Evaluation of leaves tissue using transmission electron microscopy (TEM), nested PCR using universal and specific primers to amplify phytoplasmas 16S rRNA and sec*A* genes. Nucleotidic sequences (Sanger method) were obtained from amplicons, and used for BLASTn, phylogenetic analyses, and *in silico* RFLP's.

Results. Presence of phytoplasmas into phloem tissue, only in symptomatic trees, was evidenced by TEM. Comparison of partial sequences (16Sr and *sec*A genes) by BLASTn, in silico RFLP´s and phylogenetic analyses, showed the occurrence of a ´*Candidatus* Phytoplasma pruni´ related strain in the samples evaluated.

Conclusion. Phytoplasmas were found only in the symptomatic *B. frutescens* trees evaluated. The phytoplasmas were identified as a ´*Ca.* Phytoplasma pruni´ related strain. This is the first report of *B. frutescens* as a natural host of ´*Ca.* Phytoplasma pruni´.

Keywords: Papaveraceae, semi-nested PCR, *in silico* RFLP's, 16SrIII-F, sec*A* gene

Introduction

Bocconia frutescens (Papaveraceae), a small tree (2 to 3 m in height), light-demanding and short-lived species, is found naturally from Mexico throughout Central America to Argentina and in the Caribbean islands. It was introduced to Hawaiian Islands and Reunion Island, where it is considered an invasive species (Sherley, 2000; Tassin *et al*., 2006). It is known by different names: "llora-sangre", "trompeto", "cacho-venado", plume-poppy, parrot weed, and bois codine (Francis, 2004). In Costa Rica, it is also named "guacamayo", "tabaquillo", "cola de gallo" and "tora" (Soto, 2007), and it is distributed between 100 and 3300 masl on both Pacific and Atlantic slopes, along roadsides, riverbanks, open fields, and forest light gaps (Boucher and Nishida, 2014). Various tissues of *B. frutescenses* are employed in ethnobotanical medicine, and *in vitro* evaluations of different extracts of this species have shown activity against different microorganisms. However, different alkaloids have been identified in this species (Lunagómez *et al*., 2020).

Several trees of *B. frutescens* showing symptoms reminiscent of phytoplasmas´ infection (Lee *et al*., 2000; Hogenhout *et al*., 2008; Maejima *et al*., 2014) were observed in roadsides, secondary succession areas and riversides, in some localities at Cartago province, Costa Rica.

Phytoplasmas (class Mollicutes) can induce different symptoms in plants, including witches'-broom, dwarfing and yellowing. They cause plant diseases worldwide and are responsible for devastating damage to many economically important crops and natural ecosystems. These phytopathogenic mollicutes are tiny cell wall-less prokaryotes living in the phloem of plant hosts or infecting different tissues of insect species responsible for their transmission among plants, including the families Cicadellidae, Cixiidae, Delphacidae, Derbidae, and Psyllidae (Lee *et al*., 2000, Weintraub and Beanland, 2006; Hogenhout *et al*., 2008; Maejima *et al*., 2014; Trivellone and Dietrich, 2021). The objective of this study was to detect and identify the potential phytoplasmas associated with little leaves symptoms observed in *B. frutescens.*

Samples from symptomatic *B, frutescens* trees observed in some localities at Cartago province, Costa Rica (Table 1) and from healthy trees were collected and evaluated by transmission electron microscopy (TEM) and molecular studies (nested and semi-nested PCR, cloning and sequencing, *in silico* RFLPs and phylogenetic analyses).

Foliar samples from 18 symptomatic *Bocconia* trees were collected and evaluated, in two different times during rainy season, in Capellades (*n*=9),

Table 1. Localities at Cartago province, Costa Rica, where trees of *Bocconia frutescens* were observed with little leaf symptoms associated with phytoplasmas.

Locality	Latitude (N)	Longitude (W)	Altitude (masl)	Symptomatic tree tested/ positive[z]
Capellades	9° 55' 19.35"	83° 47' 28.31"	1622	9/9
Cervantes	9° 52' 54.99"	83° 49' 13.35"	1502	4/4
Cervantes	9° 52' 22.30"	83° 49' 48.42"	1502	2/2
Pacayas	9° 54' 42.24"	83° 48' 47.86"	1751	3/3
Juan Viñas	9° 54' 25.65"	83° 46' 44.95"	1398	0
La Verbena	9° 56' 36.82"	83° 41' 41.39"	1116	0
Chirraca	9° 54' 05.26"	83° 42' 15.87"	937	0
Guayabo	9° 58' 12.20"	83° 41' 22.65"	1116	0

[z]Nested PCR analysed, a sample per tree evaluated in 2 different times.

Cervantes (*n*=6) and Pacayas (*n*=3) localities, and two healthy *Bocconia* trees were sampled at San Pedro de Montes de Oca (San Jose province, 9°56'27.11"N/ 84°02'34.73"W, 1236 masl). Pieces of midribs (1-2 mm long) from one of the symptomatic trees collected in Pacayas and from one healthy (San Pedro) were fixed and processed for transmission electron microscopy (TEM). These pieces were fixed (over-night, 15 °C) with Karnovsky solution (cacodylate 0.05 M buffered), after washing (cacodylate 0.05 M buffer) were post-fixed with osmium tetra-oxide (1%). The dehydration process was done using an ethanol/propylene oxide series, and embedded with propylene oxide: epoxy resin (Spurr´s medium-hard), to finally polymerized by heating. Ultra-thin sections were obtained of three different blocks per each tree samples. The sections were double stained to be observed at 100kV using a Hitachi TEM H-7100.

DNA was extracted from leaf midribs using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) from two *Bocconia* healthy trees, the 18 samples from *Bocconia* trees with little leaves, and from a positive control of *Sechium edule* infected with a 16SrI-B phytoplasma (Villalobos *et al*., 2002). Nested-PCR reactions were done with phytoplasmas universal primers: P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) in the first amplification, and specific primers R16F2n/ R16R2 (Gundersen and Lee, 1996) in the second round. PCR mix of 25 µL contained DreamTaq Green PCR Master Mix (2x) (Fermentas, St. Leon-Rot, Germany), 0.4 uM of each primer and 2 µL of DNA. The product of the first round was diluted 1/40 to be used in the second PCR round. The termocycle profiles used are shown in Table 2 and these were run in a PCR Gradient Palm Cycler (Corbett Research Model CG1-96, Australia). The products of the nested PCR were evaluated by electrophoresis (1% agarose and GelRed staining).

Table 2. Data about oligonucleotides (primers) and thermocycle profiles herein used for the detection of phytoplasmas genes 16S rRNA and *secA* translocase gene.

Primer name (location)	Oligonucleotide sequence (5'→3') (reference)	Primers pair (amplicon size expected), thermocycle profile (reference)
P1 (16S rRNA)	AAGAGTTTGATCCTGGCTCAGGATT (Deng and Hiruki, 1991)	**P1/P7** (1800 bp) and **R16F2n/R16R2** (1200 bp) 94 °C for 2 min, 30 cycles of (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min), 72 °C for 10 min (Villalobos *et al*., 2019).
P7 (23S rRNA)	CGTCCTTCATCGGCTCTT (Schneider *et al*., 1995)	
R16F2n (16S rRNA)	GAAACGACTGCTAAGACTGG (Gundersen and Lee, 1996)	
R16R2 (16S rRNA)	TGACGGGCGGTGTGTACAAACCCCG (Gundersen and Lee, 1996)	
P1A (16S rRNA)	AACGCTGGCGGCGCGCCTAATAC (Lee *et al*., 2004)	**P1/16S-SR** (1500 bp) and **P1A/16S-SR** (1200 bp) 94 °C for 2 min, 38 cycles of (94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min), 72°C for 10 min (Villalobos *et al*., 2011).
16S-SR (16S rRNA)	GGTCTGTCAAAACTGAAGATG (Lee *et al*., 2004)	
SecAfor1 (*secA* gene)	GARATGAAAACTGGRGAAGG Hodgets *et al*. (2008)	**SecAfor1/SecArev3** (840 bp) and **SecAfor2/SecArev3** (480 bp) 94 °C for 2 min, 35 cycles of (94 °C for 30 s, 53 °C for 60 s and 72 °C for 90 s), final extension 72 °C for 15 min (Hodgets *et al*., 2008).
SecArev3 (*secA* gene)	GTTTTRGCAGTTCCTGTCATNCC Hodgets *et al*. (2008)	
SecAfor2 (*secA* gene)	GAYGARGSWAGAACKCC Hodgets *et al*. (2008)	

Six amplicons from the nested-PCR assay, two amplicons randomly selected per each sampled locality, were directly sequenced in both directions using primers R16F2n /R16R2 after purification (Macrogen Inc., Korea). The contig sequence for each sample was obtained using BioEdit v7.2.5 (Hall, 1999) and these sequences were compared among them and identified by BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

One sample from Cervantes locality (code 16.1266) was selected for further characterization of the phytoplasma associated with symptoms of little leaves of *Bocconia*. A second, longer amplification product for the 16S rRNA gene was obtained with a semi-nested PCR using primer pairs P1/16S-SR and P1A/16S-SR (Deng & Hiruki, 1991; Lee *et al*., 2004). The reaction mix used were the mentioned above. The product of the first round PCR was diluted 1/40 previous to use in the semi-nested PCR. The thermocycle profiles used are also shown in Table 2. The amplicon was cloned (TA system) and three bacterial colonies were sequenced in both directions (Macrogen Inc., Korea). VecScreen tool (https://www.ncbi.nlm.nih.

gov/tools/vecscreen/) was used to identify and remove cloning vector sequences. A contig sequence per clone (total=3) was assembled, all sequences showed 100% identity to each other. The final sequence of 1.5 Kbp (available at GenBank PP353584) was used to search similarity (BLAST) and also was analysed by virtual RFLPs at the *i*PhyClassifier (Zhao *et al*., 2009) platform (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi). Additionally, this 16Sr sequence from symptomatic *Bocconia* was aligned (ClustalW) with 29 strains of 16SrIII group, seven ´*Ca.* Phytoplasma species´, and one *Acholeplasma laidlawi* (M23932); evolutionary analyses were conducted with MEGA X (Kumar *et al*., 2018), using the Maximum Parsimony method and a bootstrap of 1000 replicates. Also, the 13 unique oligonucleotide sequence regions (UOSRs) from the 16Sr sequence of ´*Ca*. Phytoplasma pruni´ (JQ044393) were compared using ClustalW (Thompson *et al*., 1994), with 16Sr partial sequence obtained herein (PP353584).

A partial sequence for the non-ribosomal *secA* gene was also obtained for sample 16.1266, as a second DNA barcode for identification of the phytoplasma associated to little leaves symptos. A semi-nested PCR was done using primers pairs SecAfor1/SecArev3 and SecAfor2/SecArev3, according to Hodgets *et al*. (2008). The reaction mix used were the mentioned above, but the product of the first round was diluted 1/20 before its use in the semi-nested PCR. The termocycle profiles used are shown in Table 2. The amplicon was directly sequenced in Macrogen Inc. (Korea) with the primers pair used in the semi-nested round. The partial sequence obtained (457 nt, GenBank Acc. No. PP375806) was compared to other phytoplasmas sequences using the BLASTn algorithm in GenBank. It was aligned (ClustalW) with sec*A* sequences of ten 16SrIII strains, seven different '*Ca.* Phytoplasma species´, and a *Bacillus subtilis* strain (D10279) to conduct evolutionary analyses in MEGA X, using the Maximum Parsimony method and a bootstrap of 1000 replicates.

Bocconia frutescens trees showing bunches of little leaves on branches (Figure 1A), proliferation of axillary shoots (witches'-broom) with little leaves on trunk (Figure 1C), and dieback (Figure 1B) symptoms, were observed in roadsides, secondary succession areas and riversides, in some localities at Cartago province (Costa Rica). Additionally, symptomatic isolated trees in road-sides were observed near to Turrialba town (Table 1). The symptoms were present in some branches of the diseased trees. No differences in symptomatology among trees from the different localities were detected. We propose the name *Bocconia frutescens* little leaf (BfLL) for this disease.

All the results confirmed the presence of phytoplasmas associated to the *Bocconia* trees displaying little leaves and witches'-broom. Pleomorphic wall-less structures similar to phytoplasmas were only observed by TEM in the phloem tissue from symptomatic *Bocconia* trees (Figure 2A). Phloem tissue from healthy *Bocconia* did not show any structure resembling phytoplasmas or other bacteria. The nested-PCR

Figure 1. *Bocconia frutescens* (Papaveraceae) trees showing symptoms reminiscent of phytoplasmas infection, observed in Cartago province, Costa Rica; A) witches'-broom and bunches of little leaves observed in some branches; B) Witches'-broom, defoliation, and die-back; C) Detail of axillar proliferation with leaves reduced in size.

(P1/P7, R16F2n/R2) in agarose gel electrophoresis showed fragments near to 1.2 Kb from all symptomatic samples (n=18) and positive control, but no amplification was obtained from healthy trees and a PCR-mix control (water rather than DNA). The 16S rRNA sequences (*ca*. 1200 nt) obtained for six amplicons shared 100% similarity between them. The resulting sequences indicated that the phytoplasma in *B. frutescens* is related to different strains of the 16SrIII group, and so it is a related strain of ´*Ca*. Phytoplasma pruni´ (JQ044393, Davis *et al.*, 2013). BfLL 16Sr sequence showed a high percentage of similarity in GenBank with Mexican Xalapa periwinkle virescence phytoplasma (99.67%, KY778008-09), ´*Ca*. Phytoplasma pruni´ (99.50%, MH428959), and Milkweed yellows phytoplasma (99.48%, AF510724).

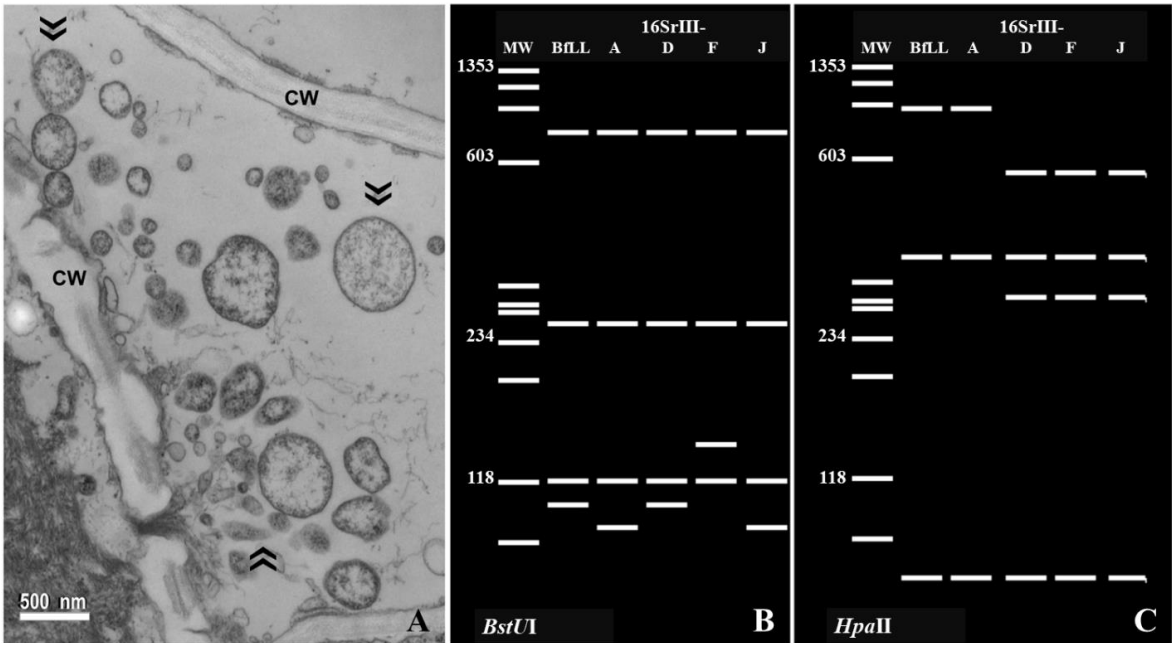

Figure 2. **(A)** Pleomorphic phytoplasmas bodies (») found inside phloem of *Bocconia frutescens* trees with little leaves and witches' broom symptoms observed in Cartago province, Costa Rica. CW = cell wall. **(B** and **C)** Profiles of virtual RFLPs obtained at *i*PhyClassifier to 16Sr F2/R2n fragment of phytoplasmas associated to *B. frutescens* little leaf (BfLL, GenBank Acc. No. PP353584) using *BstU*I (**C**) and *Hpa*II (**D**). Molecular weight (MW) fragment sizes (bp): 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.

According to *i*PhyClassifier, the 1.5 Kbp 16Sr partial sequence (sample 16.1266, PP353584) showed similarities of 98.87% with ´*Ca*. Phytoplasma pruni´ (JQ044393). Therefore, the phytoplasma infecting *B. frutescenses* in Cartago province (Costa Rica) is a member of ´*Ca*. Phytoplasma pruni´ (Davis *et al*., 2013) by the similarity threshold for the 16S rRNA gene as indicated in the new guidelines to '*Ca*. Phytoplasma' species delimitation (Bertaccini *et al.*, 2022, IRPCM 2004). Moreover, the results indicated that the sequence is different from the reference patterns of all previously established 16SrIII subgroups. The most similar was the reference pattern of the 16SrIII-F (AF510724) with a similarity coefficient of 0.95, suggesting that the phytoplasma associated to BfLL may represent a new 16SrIII subgroup. The computer simulated RFLPs displayed virtual patterns not corresponding to 16SrIII-F subgroup using *BstU*I and *Hpa*II enzymes. The virtual pattern obtained with *BstU*I matches to 16SrIII-D, meanwhile the profile displayed using *Hpa*II corresponds with 16SrIII- A, G, L and S subgroups (Figure 2B and 2C).

The phytoplasma signature sequence 5'CAAGACTATGATGT TAGCTG GACT3´ reported to ´*Ca*. Phytoplasma pruni´ (positions 258-282, JQ044393,

Davis *et al.,* 2013), is identical in the strain infecting *Bocconia* (positions 237-261, PP353584). The comparison of 13 UOSRs of ´*Ca*. Phytoplasma pruni´, reported by Davis *et al.* (2013), with BfLL´s 16Sr partial sequence (PP353584), showed that seven out of 13 UOSRs matched in BfLL´s. Additionally, single nucleotide polymorphisms (SNPs) were evidenced in five other UOSRs, and a dinucleotide polymorphism unmatched to UOSR between 817 to 828 positions (JQ044393) (Table 3).

Table 3. Unique oligonucleotide sequence regions (UOSR) in the 16S rRNA gene of '*Candidatus* Phytoplasma pruni' rrnA (JQ044393, Davis *et al*., 2013) compared to 16Sr sequence of BfLL associated phytoplasma (PP353584). Position in the respective sequence is shown in parenthesis, every nucleotide corresponding to a SNP is displayed in standard DNA nucleotides color, bold font and underlined.

`*Ca*. Phytoplasma pruni` rrnA (JQ044393), Davis *et al*. (2013)	**Bocconia little leaf associated phytoplasma (PP353584)**
5'-CACATTAGTTAGTTGG**T**AGGGTAAAGGCCTA CC-3' (226 to 258)	**C** (221)
5'-GTACCTCGGTATG-3' (402 to 414)	idem (381-393)
5'-TTATTAAGGAAGAAAAAAGAGTGGAAAAAC TCCCT-3' (425 to 459)	idem (404-438)
5'-ACGGTACTTAA-3' (462 to 472)	idem (441-451)
5'-TAATAAGTCTATAGTTTAATTTCAG**T**GCTTA ACGCT GTTGTGCTATAG-3' (571 to 618)	**C** (575)
5'-GTTTTACTAGAGTGAG-3' (624 to 639)	idem (603-618)
5'-TAAAA**CT**GGTAC-3' (817 to 828)	**AC** (801-802)
5'-TTTCTTGCGAAGTTA-3' (970 to 984)	idem (949-963)
5'-ATGGAGGT**C**ATCAGGAAAACAGGTG GTGC-3' (995 to 1023)	**T** (982)
5'-CTTGTCGTTA**G**TTGCCAGCATGTAAT-3' (1083 to 1108)	**A** (1072)
5'-GATGGGGACTTTAACGA-3' (1109 to 1125)	idem (1088-1104)
5'-GGTTGATACAAAG-3' (1211 to 1223)	idem (1190-1202)
5'-TCTCA**A**AAAATCAATC-3' (1252 to 1267)	**C** (1236)

The dendrogram resulting from the phylogenetic analyses of 37 phytoplasma 16S rRNA gene sequences (Figure 3) showed that the phytoplasma detected in *B. frutescens* clustered within the 16SrIII group (99% support). The BfLL phytoplasma is included in the same cluster with variant strains of 16SrIII-F, previously found infecting *C. roseus* in Costa Rica (MH428959, Villalobos *et al*., 2019) and Xalapa (Mexico) (KY778008-09, Pérez-López *et al.,* 2017). The nearest cluster contained representative strains of subgroups 16SrIII-F, C, D, M and N (AF510724, FJ376627, FJ376626, FJ376629, FJ226074, respectively).

The sec*A* partial sequence obtained from a phytoplasma infecting BfLL (PP375806) showed 94.97-97.81% (*e* value = 0) similarity with different X-disease strains (´*Ca*. Phytoplasma pruni´) available at GenBank. Additionally, phylogenetic analysis using partial sequence to *secA* gene (Figure 4) placed BfLL´s associated phytoplasma, more related to sec*A* sequences from representatives of 16SrIII subgroups D and F.

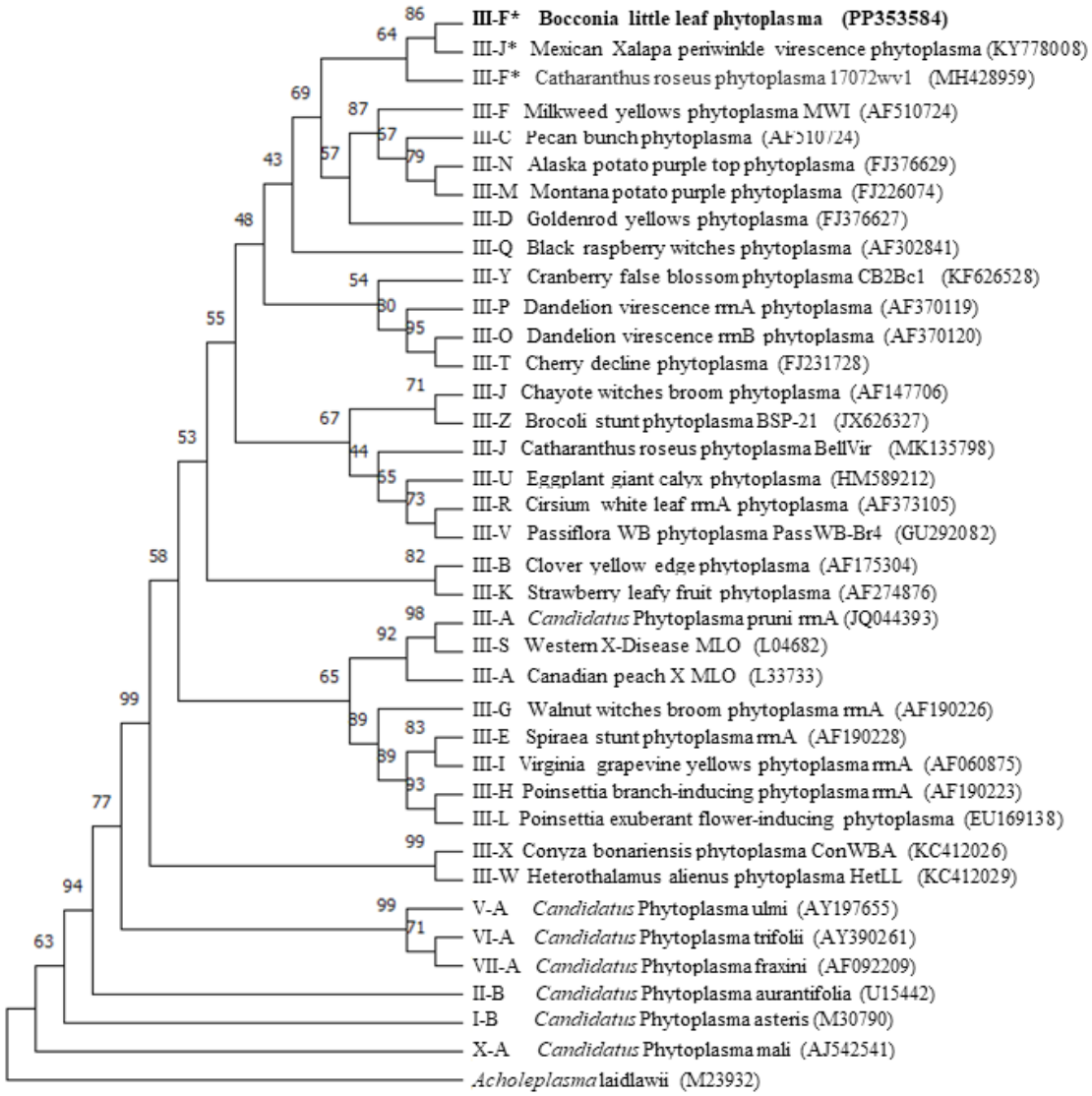

Figure 3. Dendrogram obtained from phylogenetic analysis using Maximum Parsimony method of partial 16S rRNA gene sequences from *Bocconia frutescens* little leaf (BfLL) phytoplasma, 29 sequences of 16SrIII group´s strains, seven '*Ca.* Phytoplasma species´ and *Acholeplasma laidlawii* as outgroup. The bootstrap test selected was 1000 replicates. GenBank accession number of every sequence is shown in parentheses. BfLL phytoplasma is displayed in bold. * = tentatively new subgroups.

Pérez-López *et al*. (2017) suggested that Xalapa strain (KY778008) is a North American lineage of the South American 16SrIII-J subgroup. However, according to phylogenetic relationships obtained herein (Figure 4), the strains found in Costa Rica (PP353584 and MH428959) and Xalapa (KY778008) may represent a new potential geographical lineage and putative new 16SrIII subgroups, currently

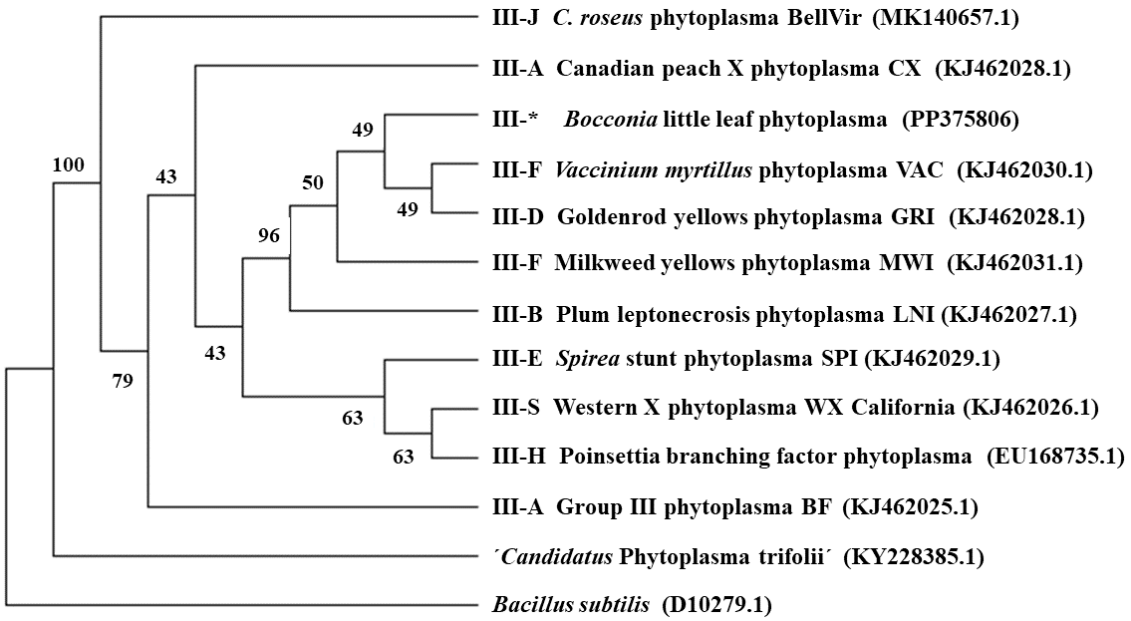

Figure 4. Dendrogram obtained from phylogenetic analysis with Maximum Parsimony using partial *secA* gene sequences from *Bocconia frutescens* little leaf associated phytoplasma, ten sequences of secA from different 16SrIII subgroups, '*Ca.* Phytoplasma trifolii´ and *Bacilus subtilis* (outgroup). The bootstrap test selected was 1000 replicates. GenBank accession number of every sequence is shown in parentheses. * = tentatively new subgroup.

reported in Central America and Mexico. Additionally, in Costa Rica was reported a strain of 16SrIII-L associated to cassava frog-skin disease (Pardo *et al.,* 2014). Also, a representative of 16SrIII group was reported infecting *Spondias purpurea* in El Salvador (Parada *et al*., 2006). These findings suggest that different strains of 16SrIII group may be disperse in the Central America region, representing a concern to agriculture and natural ecosystems.

The 16SrIII phytoplasma group is highly diverse, it has a wide geographic distribution and a great number of subgroups associated to a high diversity of host-plant species (Zhao *et al.*, 2009, Pérez-López *et al*., 2017). According to Bertaccini *et al.* (2022), 203 strains showing 98.80-100% nucleotide identities compared to the ´*Ca.* Phytoplasma pruni´ reference strain have been described. This is the highest number of strains reported for one of the 49 officially published ´*Ca.* Phytoplasma´ species. Diseases associated with this phytoplasma group have been reported in different countries in Europe, Asia, America and Africa (Foissac and Wilson 2009). Across the Americas, the number of reports from South America is particularly high (Fiore, 2023; Pérez-López *et al*., 2016).

To our knowledge, this is the first report of the natural occurrence of a '*Ca*. Phytoplasma pruni' related strain in *Bocconia frutescens* (Papaveraceae) in Costa Rica or worldwide.

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