Mexican Journal of Phytopathology
ISSN: 2007-8080

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*Phytopathological Note*

# Identification of phytoplasmas associated with Bunchy Top disease of papaya in Colima, Mexico

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***Section:***
*Periodical Issue*

***Received***:
05 March, 2024
***Accepted***:
19 May, 2024
***Published***:
18 June, 2024

**Citation:**
Valadez-Ramírez P, Ochoa-Martínez DL, Valdovinos-Ponce G, Blanco-Rodríguez E *et al*., 2024. Identification of phytoplasmas associated with Bunchy Top disease of papaya in Colima, Mexico.
Mexican Journal of Phytopathology 42(3): 24
https://doi.org/10.18781/R.MEX.FIT.2403-2

## ABSTRACT

**Background/Objective.** Phytoplasmas, rickettsiae and viruses have been detected in papaya plants with Bunchy Top disease (BT). In 2019, papaya plants with BT-like symptoms were observed in agroecosystems of Colima, Mexico. In order to determine the BT-associated phytoplasmas species or subgroups, asymptomatic and symptomatic plants were collected from papaya agroecosystems in four papaya producer municipalities, as well as papaya-associated weeds and insects.

**Materials and Methods.** Phytoplasma detection and identification was conducted by PCR, sequencing and phylogenetics of translocase subunit SecA (*secA*) and 16S ribosomal RNA (*16Sr*) genes, and PCR-RFLPs *in vitro* and *in silico* for *16Sr* gene.

**Results.** In papaya, phytoplasma groups 16SrI (subgroup AF), 16SrX, and 16SrXIII were identified in 2.08% (4 out of 192) symptomatic samples. The results of RFLPs *in silico* analysis showing the presence of 16SrX and 16SrXIII (sub)groups. In papaya-associated weeds and insects, phytoplasmas of group 16SrI (subgroups AF and B) were identified in 1.7% (3 out of 174) and 1.1% (2 out of 185) evaluated samples, respectively. Phytoplasma-carrying weeds were *Amaranthus palmeri* and *Echinochloa colona*; positive insects were *Micrutalis calva* and *Balclutha mexicana*.

**Conclusion.** It is the first time that phytoplasmas 16SrI-AF, 16SrX y 16SrXIII are associated with Bunchy Top disease of papaya in agroecosystems from Colima, Mexico. Phytoplasmas 16SrX y 16SrXIII are first reported in papaya plants at the

world level and in Mexico, respectively. Phytoplasma-carrying weeds and insects are new records as natural reservoirs and potential vectors.

**Key words:** *Carica papaya*, '*Candidatus* Phytoplasma', weeds, insects

## Introduction

Mexico is the third largest producer of papaya (*Carica papaya*) in the world and the main supplier of fresh fruit for the United States of America and Canada, where annual sales are greater than 350,000 tons, with a value of 335 million dollars (SIAP, 2020). In this country, the production of the caricaceae is located in the South-Southeast and Center-West regions, with the states of Oaxaca, Colima and Chiapas being the main producer states (SIAP, 2020). The state of Colima, located in the West on the coast of the Pacific Ocean, has a record of papaya production of 192,417 tons and is the main national exporter (Colima State Papaya Farmers Council [COEPAPAYA A.C.], 2017; SIAP, 2020).

In 2019, in 64 papaya agroecosystems visited in four municipalities of the state of Colima, plants showed stems with shortened internodes that caused the bunching of apical leaves. These plants also displayed leaves with chlorosis, yellowing, deformation and/or marginal necrosis. These symptoms are typical of the Bunchy Top (BT) disease (Cook, 1931; Story and Halliwell, 1969), and similar to those described in Cuba as "*papaya bunchy top*" (PBT) and "*bunchy top symptom*" (BTS) syndromes (Acosta *et al*., 2013).

BT is induced by phytoplasmas, rickettsiae and viruses, occasionally in mixed infections (phytoplasmas + rickettsiae or phytoplasmas + viruses), and it has been reported in the Dominican Republic, Cuba, Haiti, Jamaica, Puerto Rico, Costa Rica, Peru (Martorell and Adsuar, 1952; Story and Halliwell, 1969; Davis *et al*., 1998; Arocha *et al*., 2003; Acosta *et al*., 2013; Luis-Pantoja *et al*., 2015; Wei *et al*., 2017), and more recently, in Nigeria (Kazeem *et al*., 2021).

Considering that in Mexico phytoplasmas have been found in papaya plants with symptoms similar to BT (Pogoshyan *et al*., 2004; Navarrete-Yabur *et al*., 2005; Rojas-Martínez *et al*., 2011), and that in Colima this disease has become more economically important and its study has been scarcely covered (Colima papaya farmers, personal communication), the following goals have been set: a) to determine the presence of phytoplasmas in papaya plants with BT symptoms, as well as in weeds and insects related with the crop, and b) to establish their identity at the 16Sr group and/or subgroup level.

In November and December of 2019, 256 papaya samples were gathered from 64 commercial agroecosystems located in the municipalities of Tecomán, Colima,

Ixtlahuacán and Armería. Out of all the samples, 64 were asymptomatic and 192 displayed the symptoms described earlier, related to BT. A compound sample was taken from every plant, consisting of three leaves (blade and petiole), each one collected from the upper, middle and lower strata of the plant. From every papaya plant (symptomatic or asymptomatic sampled) up to five species of weeds present within a 2-meter diameter were gathered (depending on their availability and greater abundance). Insects were also gathered with a beating entomological net using three downward sweeps on the papaya plant and adjacent weeds. The insects were kept in 96% ethanol for later classification into morphospecies. The weeds and insects that tested positive for phytoplasmas were identified at the species level.

From the central veins and petioles of papaya leaves and weeds, as well as from the entire specimens of insects (one to five, depending on size and availability), the total DNA was extracted using a phytoplasma DNA enrichment protocol based on CTAB (Ahrens and Seemuller, 1992) and with the help of a homogenizer (Tissue Lyser, Qiagen, Germany) (Stillson and Szendrei, 2020). The DNA was evaluated by UV spectrophotometry (NadoDrop 2000, Fisher Scientific, USA) and by PCR amplification of the ribosomal S16 (*rps16*) gene of plants (Oxelman *et al*., 1997) or cytochrome oxidase subunit I (*COI*) of insects (Folmer *et al*., 1994), as internal controls in reaction volumes of 25 µL with 50 ng of DNA.

The detection of phytoplasmas was performed on 200 ng of DNA by nested endpoint PCR of the *16Sr* genes (first round: primers P1/P7 and nested: primers R16F2n/R16R2 and R16mF2/R16mR1) (Deng and Hiruki, 1991; Schneider *et al*., 1995; Gundersen and Lee, 1996; Lee *et al*., 1993) and *secA* (first round: primers secAFor1/secARev3 and nested: primers SecAFor5/SecARev2) (Dickinson and Hodgetts, 2013).

Identification was performed by sequencing, PCR-RFLPs *in vitro* and *in silico* of the gene *16Sr* and phylogenetic analysis of *secA* and *16Sr*. For sequencing, the nested PCR amplicons for *16Sr* (primers R16mF2/R16mR1) and *secA* of the positive samples were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) and sequenced in both directions with the primers R16mF2/R16mR1 and SecAFor5/SecARev2, using the dideoxy or chain termination method at Macrogen Inc. (Seoul, South Korea). The resulting sequences were analyzed with the MEGAX program (Kumar *et al*., 2018) and the BLASTn function of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), for deposition in the GenBank (https://submit.ncbi.nlm.nih.gov/).

For the *in vitro* PCR-RFLPs, approximately 1 µg of each purified product of the nested PCR for the *16Sr* gene (primers R16F2n/R16R2) of the positive samples, quantified by UV spectrophotometry (NanoDrop 2000, Fisher Scientific, USA) was conducted separately with 10 U of the restriction enzymes *Taq* I, *Hae* III, *Sau* 3AI, *Kpn* I, *Tru* I1 (*Mse* I), *Alu* I and *Rsa* I (Thermo Scientific, USA), following

the instructions by the manufacturer to confirm that the amplified fragment corresponded to the DNA of phytoplasmas (Lee *et al*., 1998). The products were separated by electrophoresis in 3% agarose gels (w/v) in a 1×TAE buffer and dyed with ethidium bromide (Sambrook and Russell, 2001).

For the *in silico* RFLPs, the sequences of the amplicons of the nested PCR (primers R16mF2/R16mR1), edited manually with MEGAX (Kumar *et al*., 2018) were analyzed using the software *i*PhyClassifier (https://acortar.link/AikrtQ) to assign and/or confirm species, 16Sr groups and subgroups.

The phylogenetic analyses were carried out independently with the *secA* and *16Sr* genes. To do this, the sequences of each one of the analyzed genes from different groups of phytoplasmas reported worldwide (49 for *secA* and 51 for *16Sr*) and from *Bacillus subtilis* (GenBank: X62035 for *secA*) or from *Acholeplasma laidlawii* (GenBank: U14905 for *16Sr*) as outgroups were downloaded from the GenBank and aligned with Clustal W. Phylogenies were built with the neighbor-joining method with 1000 bootstrap repetitions. All analyses were carried out in MEGA X (Kumar *et al*., 2018).

Phytoplasmas were detected in 2.08% (4 out of 192) papaya plants with symptoms related to BT (Table 1). No phytoplasmas were found in asymptomatic plants. The positive plants were gathered in the municipalities of Colima and Ixtlahuacán (Table 1) and they displayed stems with shortened internodes and yellow chlorotic leaves (Figure 1A). Positive samples were confirmed by sequencing the *secA* (GenBank: ON303285-ON303288) and *16Sr* (GenBank: PP348058-PP348061) genes. Sequence analysis of the *secA* and *16Sr* genes with BLASTn indicate that, in two of the four positive samples, the phytoplasmas belong to the 16SrI group (GenBank *secA*: ON303286 and ON303287; *16Sr*: PP348059 and PP348060), whereas the remaining two belong to the 16SrX (GenBank *secA*: ON303288; *16Sr*: PP348061) and 16SrXIII (GenBank *secA*: ON303285; *16Sr*: PP348058) groups, respectively.

**Table 1.** Detection of phytoplasmas in papaya (*Carica papaya*), weeds and insects associated with the crop in Colima, Mexico, in November and December of 2019.

| Municipality | Number of positive samples to phytoplasmas / Number of analyzed samples (%) | | |
|---|---|---|---|
| | Papaya | Weeds | Insects |
| Armería | 0/64 (0.0) | 1/74 (1.4) | 1/60 (1.7) |
| Colima | 2/64 (3.1) | 0/31 (0.0) | 0/32 (0.0) |
| Ixtlahuacán | 2/64 (3.1) | 1/35 (2.9) | 0/46 (0.0) |
| Tecomán | 0/64 (0.0) | 1/34 (2.9) | 1/47 (2.1) |
| Total | 4/256 (1.6) | 3/174 (1.7) | 2/185 (1.1) |

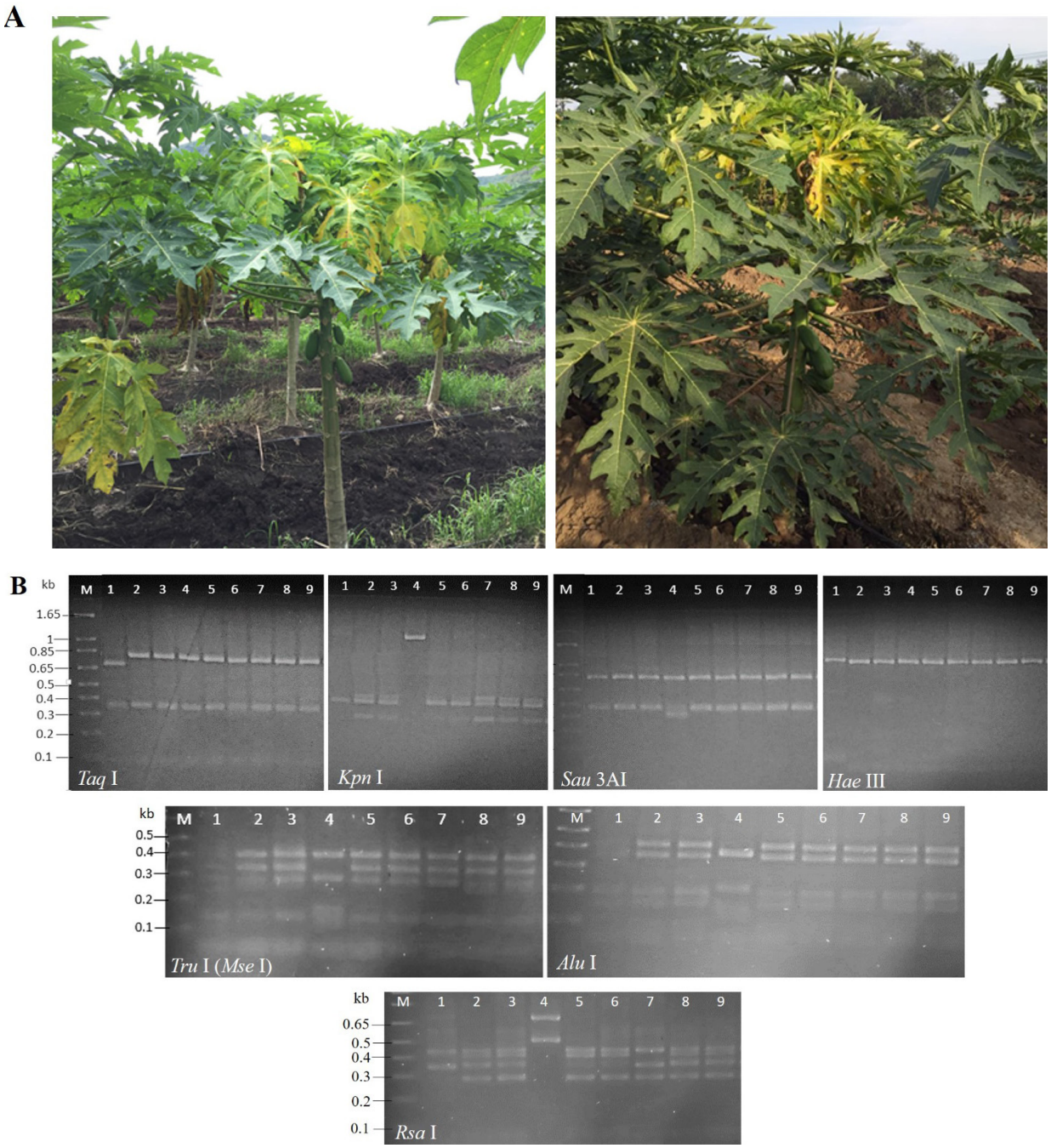


**Figure 1.** Detection of phytoplasmas in agroecosystems papaya with Bunchy Top disease in Colima, Mexico. **A)** Papaya plants showing stems with shortened internodes and yellow, chlorotic leaves, positive to phytoplasmas. **B)** *In vitro* RFLPs profiles of gene *16Sr* (primers R16F2n/R16R2) of the phytoplasmas under study. Lanes 1-4: samples 1S-4S-papaya, Lanes 5-6: samples 5S-6S-*Amaranthus palmeri*; Lane 7: sample 7S-*Echinochloa colona*; Lane 8: sample 8S-*Micrutalis calva*; and Lane 9: sample 9S-*Balclutha mexicana*. The DNA was digested with 10 U of every enzyme and the products were separated by electrophoresis in 3% (w/v) agarose gels in 1×TAE buffer. Lane M: 1 kb plus molecular weight marker (Thermo Scientific, EUA).

The profiles of the *in vitro* RFLPs of the *16Sr* gene (Figure 1B) were consistent with those reported by Lee *et al*. (1998) and with those obtained using *i*PhyClassifer (Figure 2) for the restriction enzymes used, which helped authenticate the DNA of the phytoplasmas found in this study. The *in silico* RFLPs analysis of the *16Sr* gene confirmed the presence of the '*Candidatus* Phytoplasma asteris' [group 16SrI (percentages of identity: 99.44 and 99.60) species and subgroup 16SrI-AF (coefficient of similarity: 1.00)], '*Ca*. Phytoplasma rhamni' [group 16SrX (percentage of identity: 93.31), undetermined subgroup] and '*Ca*. Phytoplasma hispanicum' [group 16SrXIII (percentage of identity: 99.37), subgroup 16SrXIII-D (coefficient of similarity: 0.95)] (Table 2). Regarding the percentages of identity

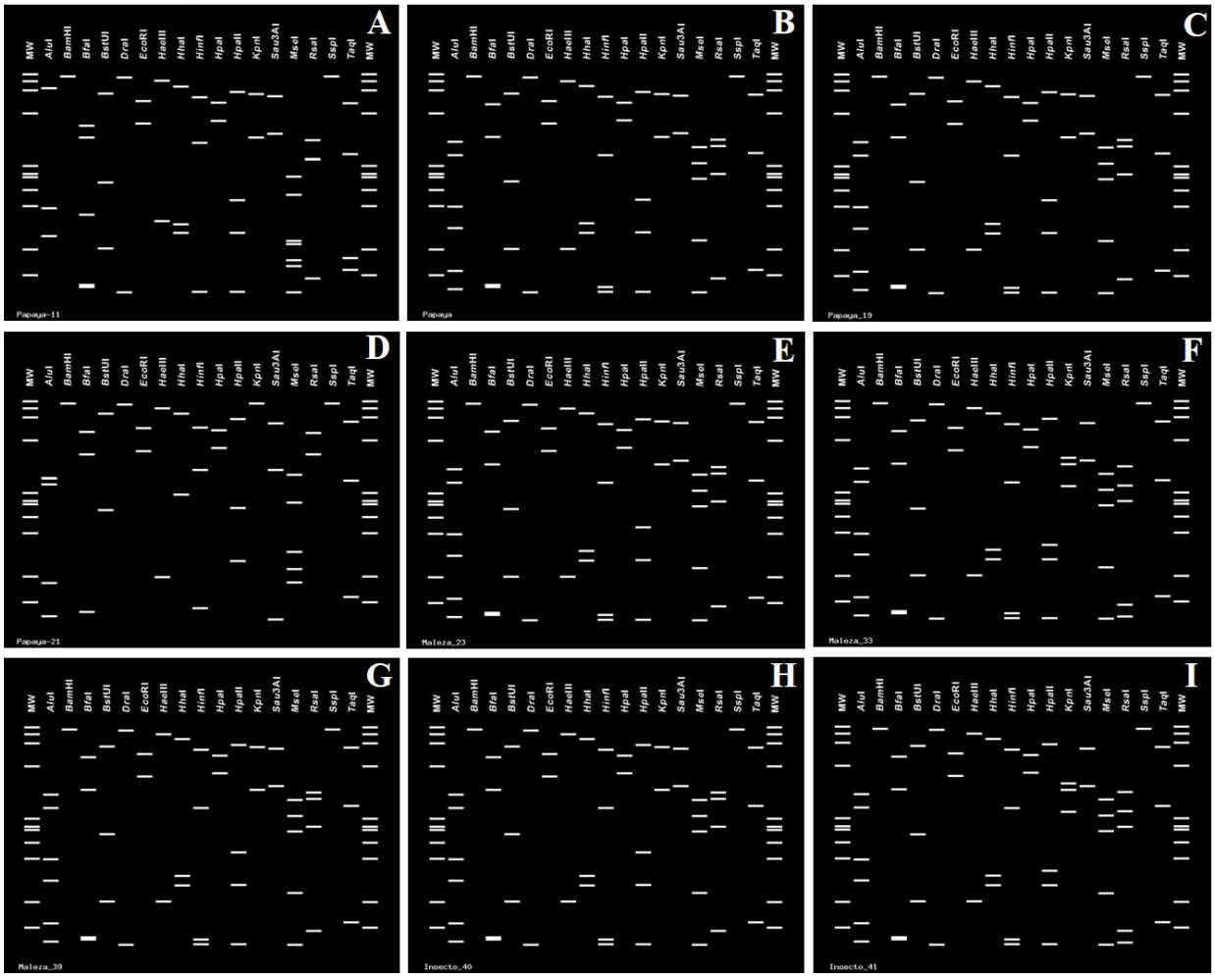


**Figure 2.** *In silico* RFLPs profiles of gene *16Sr* of the phytoplasmas under study, generated with *i*PhyClassifier with 17 restriction enzymes. A) sample 1S-papaya; B) sample 2S-papaya; C) sample 3S-papaya; D) sample 4S-papaya, E) sample 5S-*Amaranthus palmeri*; F) sample 6S-*Amaranthus palmeri*; G) sample 7S-*Echinochloa colona*; H) sample 8S-*Micrutalis calva*; I) sample 9S-*Balclutha mexicana*. Lanes MW: φX174 DNA digested with *Hae* III.

**Table 2.** Phytoplasmas identified in papaya, weeds and insects associated with the crop in Colima, Mexico, in November-December of 2019.

| Sample (GenBank *16Sr*) | '*Candidatus* Phytoplasma sp.' | Group 16Sr (% similarity) | Subgroup (coefficient of similarity) |
|---|---|---|---|
| 1S-papaya (PP348058) | '*Ca.* Phytoplasma hispanicum' | 16SrXIII (99.37) | D (0.95)[y] |
| 2S-papaya (PP348059) | '*Ca.* Phytoplasma asteris' | 16SrI (99.44) | AF (1.00) |
| 3S-papaya (PP348060) | '*Ca.* Phytoplasma asteris' | 16SrI (99.60) | AF (1.00) |
| 4S-papaya (PP348061) | '*Ca.* Phytoplasma rhamni' | 16SrX (93.31) | NA[z] |
| 5S-*Amaranthus palmeri* (PP348062) | '*Ca.* Phytoplasma asteris' | 16SrI (99.44) | AF (1.00) |
| 6S-*Amaranthus palmeri* (PP348063) | '*Ca.* Phytoplasma asteris' | 16SrI (99.68) | B (0.98) |
| 7S-*Echinochloa colona* (PP348064) | '*Ca.* Phytoplasma asteris' | 16SrI (99.60) | AF (1.00) |
| 8S-*Micrutalis calva* (PP348065) | '*Ca.* Phytoplasma asteris' | 16SrI (99.52) | AF (1.00) |
| 9S-*Balclutha mexicana* (PP348066) | '*Ca.* Phytoplasma asteris' | 16SrI (99.68) | B (0.98) |

[y]Potential new subgroup.

[z]NA: does not apply due to the low percentage of identity of the group assigned in *i*PhyClassifier.

and coefficients of similarity obtained for the phytoplasmas of groups 16SrX and 16SrXIII, this will have to be analyzed with other studies, if we are dealing with new (sub)groups.

The identification of the groups of phytoplasmas obtained here was also corroborated by phylogenetic inferences of the *secA* and *16Sr* genes, as the phylogenies obtained for both genes of the phytoplasmas detected in this study grouped with sequences corresponding to the same 16Sr groups of phytoplasmas previously identified in Mexico (GenBank *16Sr*: MN807429) or in other countries (Figure 3).

To our knowledge, this study is the first to document the presence of 16SrI-AF, 16SrX and 16SrXIII phytoplasmas in papaya plants with BT in Mexico and Colima. In addition, globally, it is the first time that the presence of the 16SrX has been reported in papaya. In Mexico, the first detection of phytoplasmas in papaya was made by scanning electron microscopy in Baja California Sur (Poghosyan *et al*., 2004). Subsequently, via molecular biology in Yucatan, Campeche, Quintana Roo (Navarrete-Yabur *et al*., 2005), Michoacan and Veracruz, where the positive samples were assigned to the subgroup 16SrI-C (Rojas-Martínez *et al*., 2011).

Along with its detection in Mexico, the group 16SrI has been reported in papaya in Cuba (Acosta *et al*., 2011; 2017; Acosta-Pérez *et al*., 2017), Peru (Hodgetts *et al*., 2009), Sri Lanka (Abeysinghe *et al*., 2014) and China (Yu *et al*., 2023), whereas 16SrXIII, only in Brazil (Melo *et al*., 2013). Other groups of economic importance in papaya are 16SrII (White *et al*., 1998; Panda *et al*., 2022), 16SrXII (Gibb *et al*., 1996; Barbieri *et al*., 2023), 16SrXV (Wei *et al*., 2017) and 16SrXVII (Arocha *et al*., 2005).

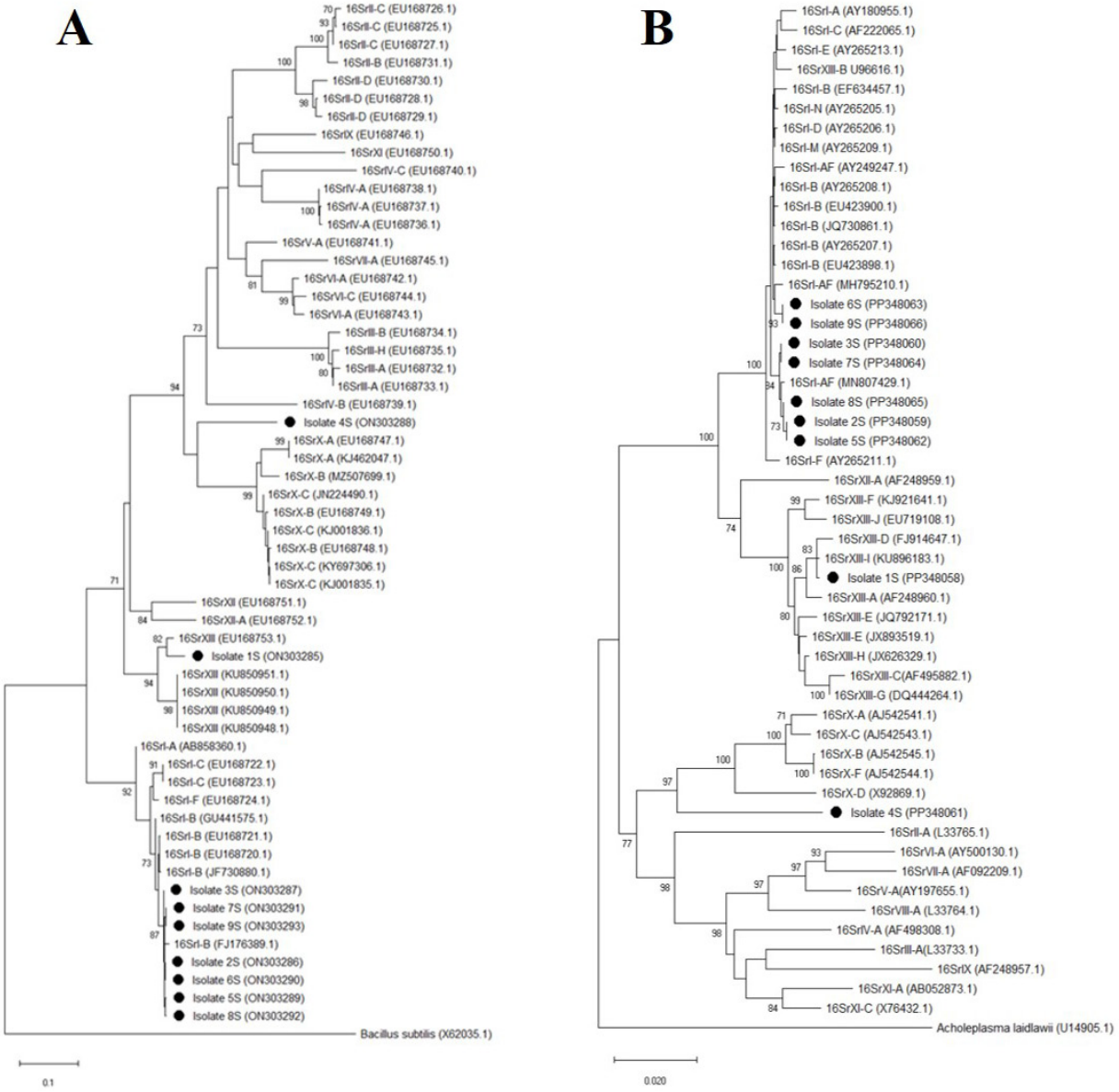


**Figure 3.** Phylogenetic trees of the *secA* (A) and *16Sr* (B) trees obtained using the neighbor-joining method of 16Sr groups of phytoplasmas identified in papaya agroecosystems in Colima, Mexico (black circles) and from phytoplasma groups from other parts of the world. Accession number are shown in parentheses. Samples 1S-4S: papaya, samples 5S-6S: *Amaranthus palmeri*, sample 7S: *Echinochloa colona*, sample 8S: *Micrutalis calva*, sample 9S: *Balclutha mexicana*. The gene sequence of *secA* of *Bacillus subtilis* and the *16Sr* gene sequence of *Acholeplasma laidlawii* were used as outgroups. In each phylogeny, bootstrap values (from 1000 replicates, greater than 70%) are shown on the branches. The scale bar indicates the number of nucleotide substitutions per site.

In Mexico, the presence of group 16SrX has never been reported (Pogoshyan-Melkonyan *et al*., 2019), whereas 16SrXIII has only been associated with potato and tomato plants in Sinaloa (Santos-Cervantes *et al*., 2010) and Baja California Sur (Holguín-Peña *et al*., 2007). In the future, whether the low percentage of the detection of phytoplasmas is due to the presence of other organisms such as rickettsiae or viruses that may be inducing the studied symptoms is something that must be determined (Davis *et al*., 1998; Arocha *et al*., 2003; Acosta *et al*., 2013; Luis-Pantoja *et al*., 2015).

In the case of weeds and insects, the detection of phytoplasmas was positive in three out of 174 (1.7%) and two out of 185 (1.1%) samples, respectively, and with the exception of Ixtlahuacán, the positive samples were obtained regardless of not having recorded phytoplasmas in papaya plants (Table 1). Colima was the only municipality in which there were no weeds nor insects that were positive to phytoplasmas (Table 1).

The weeds in which phytoplasmas were found were asymptomatic, two of which were identified as *Amaranthus palmeri* (Amaranthaceae) (GenBank *16Sr*: PP348062-PP348063, *secA*: ON303289-ON303290), and the other one, as *Echinochloa colona* (Poaceae) (GenBank *16Sr*: PP348064, *secA*; ON303291. The insects were identified as *Micrutalis calva* (Hemiptera: Membracidae) (GenBank *16Sr*: PP348065, *secA*: ON303292) and *Balclutha mexicana* (Hemiptera: Cicadellidae) (GenBank *16Sr*: PP348066, *secA*: ON303292) (Table 2).

The *in silico* RFLPs analyses of the *16Sr* gene helped assign the phytoplasmas detected to the species '*Ca*. Phytoplasma asteris' (group 16SrI, percentage of identity: 99.44-99.68), subgroups 16SrI-AF (coefficient of similarity: 1.00) in *A. palmeri* (sample 5S), *E. colona* (sample 7S) and *M. calva* (sample 8S), and 16SrI-B (coefficient of similarity: 0.98) in *A. palmeri* (sample 6S) and *B. mexicana* (sample 9S) (Table 2).

The detection of phytoplasmas in insects and weeds related with the papaya agroecosystems suggests that the arthropods spread bacteria between plants and that weeds can play an important part as a source of inoculum (Duduk *et al*., 2018). For Mexico, this investigation pioneers in questioning whether weeds and insects related to papaya are carriers/sources of phytoplasma inoculum, since it is the first time they are reported as carrying species of the group 16SrI phytoplasmas in papaya agroecosystems. Weeds add to the records of those that have been associated with papaya crops and have been reported as hosts of 16SrII phytoplasmas in Cuba and Ethiopia, such as *Anoda acerifolia* (Malvaceae), *Euphorbia heterophylla* (Euphorbiaceae), *Malvastrum coromandelianum* (Malvaceae) and *Rhynchosia minima* (Fabaceae) (Arocha *et al*., 2007; Bekele *et al*., 2011). Although *A. palmeri* is a common weed in different Mexican agroecosystems, it has only been reported as a host of phytoplasmas of 16SrIII group phytoplasmas as a weed associated with

the cultivation of luffa (*Luffa acutangula*) (Cucurbitaceae) (Santos-Cervantes *et al*., 2021).

Regarding insects *B. mexicana* and *M. calva*, it is the first time these are recorded as carriers of phytoplasmas in papaya agroecosystems. Previously, the positive detection of phytoplasmas was reported in specimens of the genus *Orosius* in Australia (Padovan and Gibb, 2001), *E. papayae* in Cuba (Acosta-Pérez *et al*., 2010; Acosta *et al*., 2017) and *E. stevensi* in Trinidad (Haque and Parasram, 1973). However, to date, only the vectorial capacity of *E. stevensi* in Trinidad (Haque and Parasram, 1973) and *E. papayae* in Puerto Rico (Adsuar, 1946) and Cuba (Acosta-Pérez *et al*., 2010; Acosta *et al*., 2017) has been proven.

Regarding *Balclutha*, in Mexico this genus is part of the entomofauna in *Vaccinium corymbosum* (Pérez-Mejía *et al*., 2020), *Vitis vinifera* (Almendra-Paxtlan *et al*., 2021), *Capsicum annuum* (Velásquez-Valle *et al*., 2018) and *Zea mays* (Pinedo-Escatel and Moya-Raygoza, 2018) agroecosystems, but neither the genus nor any species have been reported as carriers of phytoplasmas. In other countries, only in specimens of the *B. hebe* species have phytoplasmas of group 16SrXIII been found in *Brassica oleracea* (Canale and Bedendo, 2020), 16SrI in *Solanum tuberosum* (Girsova *et al*., 2016), 16SrII in *Trigonella foenum-graecum* (Malik *et al*., 2020) and 16SrIX in *Prunus dulcis* (Dakhil *et al*., 2011).

Regarding *Micrutalis*, its presence in Mexico has been recorded in amaranth (*Amaranthus hypocondriacus*) and *Prosopis* agroecosystems (Salas-Araiza and Boradonenko 2006), and the species *M. calva* in *Z. mays* (Pinedo-Escatel, 2014). *Micrutalis calva* has the vectorial capacity for the *Tomato pseudo-curly top virus* (TPCTV) in tomato in Florida (Simons and Coe, 1958).

Based on the importance of papaya in Mexico and other countries in which it is planted, it will soon be determined if *B. mexicana* and *M. calva* have the capacity to transmit phytoplasmas in papaya and in the weeds that make up its agroecosystem.

This study reports for the first time the presence of phytoplasmas 16SrI-AF, 16SrX and 16SrXIII in papaya plants with symptoms of Bunchy Top disease in agroecosystems in the state of Colima, Mexico. The phytoplasmas of groups 16SrX and 16SrXIII are reported for the first time worldwide and in Mexico in papaya, respectively. The weeds *Amaranthus palmeri* and *Echinochloa colona*, and the insects *Micrutalis calva* and *Balclutha mexicana*, associated with papaya agroecosystems, are carriers of phytoplasma subgroups 16SrI-AF and 16SrI-B, making them new records as natural reservoirs and potential vectors of the bacteria.

## Acknowledgments

To the National Agrifood Health, Safety and Quality Service (SENASICA Mexico) for funding this project. To the papaya farmers of the state of Colima for the collection of the samples. To the

anonymous reviewers of this manuscript for their valuable comments. Pedro Valadez-Ramírez thanks the Colegio de Postgraduados for the support provided to carry out his doctoral studies.

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