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A survey of auchenorrhynchan insects for identification of potential vectors of the 16SrIV-D phytoplasma in Florida

De-Fen Mou^{1,*}, Alessandra R. Humphries¹, Noemi Soto¹, Ericka E. Helmick¹, Marina S. Ascunce², Erica M. Goss², and Brian W. Bahder¹

Abstract

Lethal bronzing disease is caused by the 16SrIV-D phytoplasma and is fatal to many palm species. This disease has caused significant economic losses to nursery and landscaping industries in Florida. As is the phloem-limited nature of phytoplasmas, the lethal bronzing disease phytoplasma is transmitted only by phloem-feeding insect vectors. However, the vector remains unconfirmed and this impedes the development of Integrated Pest Management programs that target vector populations. The primary objective of this study was to identify potential vectors of the 16SrIV-D phytoplasma in Florida by surveying auchenorrhynchans using sticky traps at the area where the disease is actively spreading, and screening the specimens for the presence of the phytoplasma. Cixiidae, Cicadellidae, Derbidae, and Membracidae insects were collected consistently during the 1-yr survey. The total number of a cixiid planthopper, *Haplaxius crudus* (Van Duzee) (Hemiptera: Cixiidae), was significantly higher than other insects that were collected on the traps. Of 3,734 specimens tested by nested polymerase chain reaction, approximately 0.672% of the *H. crudus* specimens and 1 unidentified leafhopper (Cicadellidae sp.) tested positive for the 16SrIV-D phytoplasma. The 16S sequences amplified from the positive specimens were confirmed by sequencing and phylogenetic analysis. Based on the population survey and the polymerase chain reaction assay results, we suggest that *H. crudus* is the potential vector of the 16SrIV-D phytoplasma. However, the vector competency of *H. crudus* needs to be further investigated due to the possibility of feeding residue being detected. The study has important implication for current lethal bronzing disease management by monitoring and controlling vector candidates in disease areas.

Key Words: lethal bronzing disease; Texas Phoenix Palm Decline; phytoplasma vector; palm disease; Cixiidae; Cicadellidae

Resumen

La enfermedad del bronceado letal es causada por el fitoplasma 16SrIV-D y es fatal para muchas especies de palmeras. Esta enfermedad ha causado pérdidas económicas importantes a las industrias de viveros y paisajismo en Florida. Como la naturaleza de los fitoplasmas se limita al floema, el fitoplasma la enfermedad del bronceado letal solo puede ser transmitido por insectos vectores que se alimentan del floema. Sin embargo, el vector responsable permanece sin confirmar y esto impide el desarrollo de programas de manejo integrado de plagas enfocados a las poblaciones del vector o vectores. El objetivo principal de este estudio fue identificar los posibles vectores del fitoplasma 16SrIV-D en Florida mediante el muestreo de auchenorrhynchans utilizando trampas adhesivas en el área donde la enfermedad se está propagando activamente y examinando las muestras para detectar la presencia del fitoplasma. Los insectos pertenecientes a las familias Cixiidae, Cicadellidae, Derbidae y Membracidae fueron recolectados consistentemente durante el muestreo de un año. El número total de chicharritas cixiídeos, *Haplaxius crudus* (Van Duzee) (Hemiptera: Cixiidae), fue significativamente mayor que otros insectos que fueron recolectados en las trampas. De 3,734 muestras analizadas por reacción en cadena de la polimerasa anidada, aproximadamente el 0.672% de las muestras de *H. crudus* y 1 chicharrita no identificada (Cicadellidae sp.) dieron positivo para el fitoplasma 16SrIV-D. Las secuencias 16S amplificadas de las muestras positivas se confirmaron mediante secuenciación y análisis filogenético. Según el estudio de la población y los resultados de reacción en cadena de la polimerasa, sugerimos que *H. crudus* es el vector potencial del fitoplasma 16SrIV-D. Sin embargo, la habilidad de *H. crudus* como vector responsable debe de investigarse más a fondo debido a la posibilidad de detectar residuos de alimentación. Este estudio tiene implicaciones importantes en el manejo actual de la enfermedad del bronceado letal al monitorear y controlar los candidatos a vectores en áreas donde la enfermedad esté presente.

Palabras Claves: enfermedad del bronceado letal; vector del fitoplasma; enfermedad de las palmeras; Cixiidae; Cicadellidae

Lethal bronzing disease is a lethal decline caused by the 16SrIV-D phytoplasma. Lethal bronzing disease, also known as Texas Phoenix Palm Decline, was first discovered in Texas in 1980 in *Phoenix canariensis*

H. Wildpret (Arecaceae) and *Phoenix dactylifera* L. (Arecaceae) (McCoy et al. 1980) and properly characterized by Harrison et al. (2002b). Symptoms of lethal bronzing disease start with premature fruit drop

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and necrosis of flowers and emerging inflorescences followed by discoloration of older leaves which turn a reddish-brown color. These symptoms may be caused by blockage of vascular tissue that prevents nutrient movement in infected plants. The disease progression is then followed by the death of the spear leaf and the death of the entire tree (Harrison et al. 2002b; Bahder & Helmick 2019).

A recent study reported that lethal bronzing disease host range expanded from 12 to 16 palm species (Bahder et al. 2019a), and include economically important fruit crops like the edible date palm (*P. dactylifera*) and coconut palm (*Cocos nucifera* L.; *Arecaceae*), but also include several important ornamental and landscape palm species, such as the cabbage palm (*Sabal palmetto* (Walter) Lodd. ex Schult. & Schult.f.; *Arecaceae*), wild date palm (*Phoenix sylvestris* (L.) Roxb.; *Arecaceae*), Canary Island date palm (*P. canariensis*), and queen palm (*Syagrus romanzoffiana* (Cham.) Glassman; *Arecaceae*). The active spread of lethal bronzing disease is threatening the palm nursery and landscape industries, and has an economic impact of approximately \$404 million (Khachatryan 2012). Currently, lethal bronzing disease is reported to have spread into Mexico (Lara et al. 2017) and Florida (Bahder et al. 2018a). Since the introduction of lethal bronzing disease in Florida, there has been significant economic loss, and lethal bronzing disease is currently a significant threat to the sustainable production of palms for the nursery industry. The distribution of lethal bronzing disease was expanded to 31 counties in Florida, and a local palm nursery owner estimated the economic loss of \$4.5 million due to lethal bronzing disease infection in *P. sylvestris* (Bahder et al. 2019a).

As the incidence of lethal bronzing disease increases, the sustainability of palm production is increasingly at risk. However, an effective disease management program for lethal bronzing disease has not yet been developed. As phytoplasmas are restricted to phloem tissue in the vascular system (Doi et al. 1967), phytoplasma diseases, including lethal bronzing disease, are transmitted only by phloem-feeding insects (Weintraub & Beanland 2006). Identification of insect vectors of lethal bronzing disease is beneficial for developing disease management programs that use the vector(s) as the control target. However, the insect vector(s) of lethal bronzing disease has not been identified, impeding the development of a sustainable management program.

All known phytoplasma vectors belong to the order Hemiptera, the suborder Auchenorrhyncha, and superfamilies Cicadoidea and Fulgoroidea (Weintraub & Beanland 2006). There are 102 species of the known phytoplasma vectors, including the families Cicadellidae, Cixiidae, Delphacidae, Derbidae, Flatidae, and Psyllidae. Among them, leafhoppers (Hemiptera: Cicadoidea: Cicadellidae) is the largest family that consists of approximately 80% of vector species whereas the remaining are predominantly transmitted by fulgoroids (Cixiidae, Delphacidae, Derbidae, and Flatidae) (Weintraub & Beanland 2006; Wilson & Weintraub 2007). Previous efforts were made to identify the insect vector of lethal bronzing disease. Halbert et al. (2014) proposed 3 insect species, *Haplaxius crudus* Van Duzee (Hemiptera: Cixiidae), *Ormenaria rufifascia* Walker (Hemiptera: Flatidae), and *Omolocna joi* Wilson (Hemiptera: Derbidae), as potential vectors of lethal bronzing disease due to their abundance and observation of the insects feeding on palms in lethal bronzing disease affected areas. There was no testing whether these insects carry the 16SrIV-D phytoplasma. On the other hand, in other studies, there are a few insects that were tested by nested polymerase chain reaction assay to examine the presence of the phytoplasma in the insects. *Cedusa* species collected in Jamaica were tested and showed positive for 16SrIV phytoplasmas (Brown et al. 2006); *H. crudus* in Mexico also was reported to carry 16SrIV phytoplasmas, including the 16SrIV-D phytoplasma (Narváez et al. 2018). Despite these results, no thorough and systematic population survey of

all auchenorrhynchan insects found on diseased palms has been conducted or screened for the presence of the phytoplasma.

Lethal bronzing disease first appeared at the Fort Lauderdale Research and Education Center, Davie, Broward County, Florida, USA, in 2014 and began to spread (Bahder et al. 2018a). The existence of both lethal bronzing disease infected palms and healthy but susceptible palm hosts at the Fort Lauderdale Research and Education Center suggests the existence of an efficient vector in the area, and provides an excellent opportunity to survey for vector candidates. Population surveys allow monitoring of palm host-associated insects, assessment of local insect diversity, and simultaneously reveals which species are feeding on infected palms, shedding insight on which species could be the vector candidates. Additionally, detecting the phytoplasma from the insects provides further evidence of the potential vector of lethal bronzing disease.

The primary objective of this study was to identify potential vectors of the 16SrIV-D phytoplasma in Florida. Populations of auchenorrhynchan insects that are associated with palms were surveyed throughout the Fort Lauderdale Research and Education Center, and were screened for the presence of the phytoplasma to determine what species were carrying the phytoplasma. Identifying potential vectors of the phytoplasma will serve as the foundation for further studies to test vector competency of phytoplasma transmission by transmission assays. Ultimately, knowing the vector of the lethal bronzing disease phytoplasma and its transmission biology will aid the development of a sustainable management program.

Materials and Methods

STUDY SITE AND SAMPLE COLLECTION

The site used in this study was the Fort Lauderdale Research and Education Center (344 Km²) because of the active spread of lethal bronzing disease in various palm species and continual emergence of new disease outbreaks in different locations around the research center. Traps were set at various locations where there was active lethal bronzing disease spread, as well as areas with no visible disease (Fig. 1) to obtain comprehensive insect diversity and abundance for the population survey. One yellow sticky trap (Phercon Unbaited AM Yellow Sticky Traps, Gempler's, Janesville, Wisconsin, USA) was set up within a palm canopy of lethal bronzing disease infected palm (FLREC-1), and 9 more yellow sticky traps were set up within various palm canopies. Four of the traps (FLREC-1, FLREC-2, FLREC-3, FLREC-4) were hung on phytoplasma-infected palms, and more traps (FLREC-5, FLREC-6, FLREC-7, FLREC-8, FLREC-9, and FLREC-10) were placed in canopies of healthy palms in areas with no noticeable disease spread. Four sticky traps (FLREC-11, FLREC-12, FLREC-13, and FLREC-14) were set up due to the emergence of new lethal bronzing disease infected trees in the corresponding area. The initial date of trap set up for FLREC-1 was 11 Oct 2017; FLREC-2 to FLREC-10 was 18 Oct 2017; FLREC-11 was 15 Mar 2018; and FLREC-12 to FLREC-14 was 29 Mar 2018; and the last set up date for all traps was 26 Oct 2018.

The palms used for the traps set up included *S. palmetto* (FLREC-1, FLREC-2, FLREC-6, FLREC-7, FLREC-8, FLREC-9, FLREC-10, FLREC-11, FLREC-12, FLREC-13, FLREC-14), *Phoenix roebelenii* O'Brien (*Arecaceae*) (FLREC-3), *Sy. romanzoffiana* (FLREC-4), and *Sabal mauritiformis* (H.Karst.) Griseb. & H.Wendl. (*Arecaceae*) (FLREC-5). Traps were collected and replaced weekly until 2 Nov 2018. After collection of the traps, the number of auchenorrhynchan insects on the traps were counted, and the collected auchenorrhynchans were identified to species or family and placed individually in a microcentrifuge tubes (1.5 mL) and stored at -20 °C until it was processed for DNA extraction.

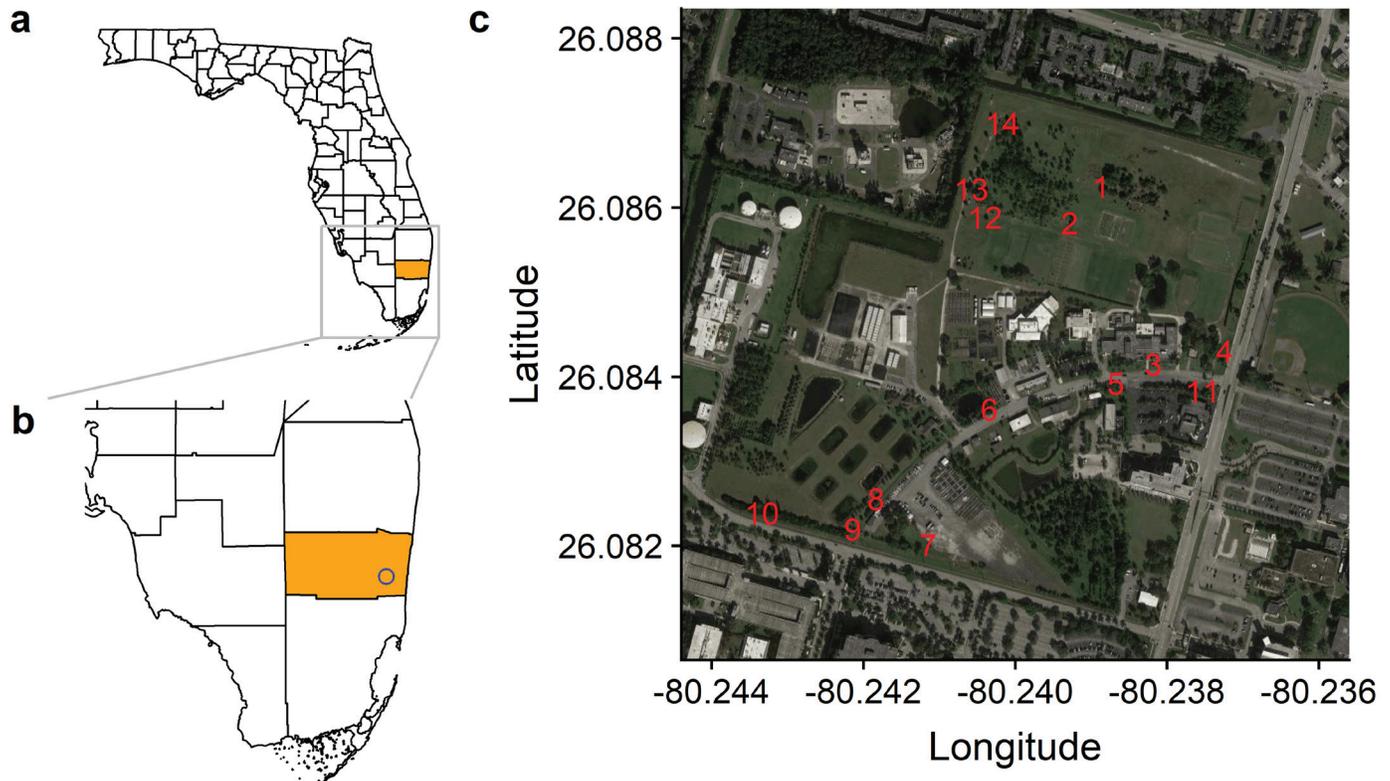


Fig. 1. (a) Map of Florida, USA, showing Broward County in yellow; (b) South Florida showing the location of the Fort Lauderdale Research and Education Center labeled in a circle; (c) Map of the Fort Lauderdale Research and Education Center study area showing the locations of the sticky traps according to the trap identification in red. The map was generated from Google Maps, Imagery@2019, DigitalGlobe, US Geological Survey, US Department of the Interior, Reston, Virginia, USA.

DNA EXTRACTION

The total DNA of collected auchenorrhynchs were extracted individually using a column DNA extraction kit (DNeasy Blood and Tissue kit, Qiagen, Hilden, Germany) following the manufacturer's instructions with modification of the lysis time to 24 h without grinding the specimens so that morphological identification of the specimens was possible (Bahder et al. 2015). The concentration and purity of the extracted DNA were measured using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

NESTED POLYMERASE CHAIN REACTION ASSAY AND SEQUENCING OF POLYMERASE CHAIN REACTION PRODUCTS

The extracted total DNA was screened for the presence of the 16SrIV-D phytoplasma by nested polymerase chain reaction assays. Polymerase chain reaction assays were examined by 16SrIV-D semi-specific primer sets, P1m (5'-TCCTGGCTCAGGATTAC-3') / LY16-23Sr (5'-TTGAGAATTTACGTTGTTTATCTAC-3') (Harrison et al. 2002a) for primary polymerase chain reaction with the following cycling conditions, initial denaturation at 95 °C for 120 s, then 34 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 120 s, followed by final extension at 72 °C for 8 min. For nested polymerase chain reaction, the primer set LY16-Sf2 (5'-AACGGGTGA GTAACACGTAAG-3') / LY16-23Sr2 (5'-TTAGACTGTGGGCTAAATG-3') (Harrison et al. 2008) was used with the initiation at 95 °C for 120 s, 34 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 120 s, extension at 72 °C for 180 s, followed by final extension at 72 °C for 8 min. All polymerase chain reaction reactions were conducted in 25 µL reactions containing 5X GoTaq Flexi Buffer (Promega, Madison, Wisconsin, USA), 25 mM MgCl₂, 200 µM

dNTPs, 0.5 µM of forward and reverse primer, 2% PVP-40, 1 U GoTaq Flexi DNA polymerase (Promega, Madison, Wisconsin, USA), and 2 µL of DNA template with sterile water to make the final reaction volume to 25 µL. Amplified polymerase chain reaction products of the 16S rRNA sequence of the phytoplasma were purified with ExoSAP-IT Express polymerase chain reaction product cleanup reagent (Applied Biosystems, Waltham, Massachusetts, USA) and sent for Sanger sequencing (Eurofins Genomics, Louisville, Kentucky, USA).

IDENTIFICATION OF INSECT SPECIMENS

According to the nested polymerase chain reaction assays, 2 specimens, *H. crudus*, belonging to Cixiidae, and 1 unidentified Cicadellidae tested positive with the phytoplasma. To verify and identify the taxon of the specimens, mitochondrial cytochrome c oxidase subunit I (COI) genes were amplified from 1 of the phytoplasma positive *H. crudus* specimens and the single phytoplasma positive Cicadellidae specimen.

Polymerase chain reaction assays were conducted with the primer set LCO1490 (5'-GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'-TCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) was used for the polymerase chain reaction with the initiation at 95 °C for 120 s, 34 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, followed by final extension at 72 °C for 5 min. Polymerase chain reactions were performed the same as in the 25 µL reactions using the same reagents as used for the nested polymerase chain reaction assay. Amplified polymerase chain reaction products of the COI gene were purified with ExoSAP-IT Express polymerase chain reaction product cleanup reagent and sent for Sanger sequencing (Eurofins Genomics, Louisville, Kentucky, USA).

DATA ANALYSIS

The DNA sequences of polymerase chain reaction products were assembled using DNA sequence assembler software DNA Baser v. 4.36 (Heracle BioSoft, Arges, Romania) for contig assembly. The COI gene amplified from *H. crudus* and the Cicadellidae specimen were aligned in BLASTn (<https://blast.ncbi.nlm.nih.gov/>) in the National Center for Biotechnology Information, US National Library of Medicine (NCBI) database and the Barcode of Life Database (BOLD). The contigs of the 16S rRNA gene sequences amplified from insects were aligned in BLASTn in the National Center for Biotechnology Information database. The 16S sequences were submitted to National Center for Biotechnology Information GenBank, and were used to build a phylogenetic relationship using maximum likelihood algorithm with bootstrap of 1,000 replications in MEGA X (Kumar et al. 2018) with the 16S sequences for all known subgroups of the 16SrIV phytoplasmas, the A subgroup of all known phytoplasma, and with *Acholeplasma palmarum* Tully et al. (Acholeplasmataceae) as an outgroup.

Population survey data were analyzed by the generalized linear model with quasi-poisson distribution. The number of specimens collected between families were analyzed, and multiple comparison Tukey tests was performed to further analyze the significant difference. All statistical tests were performed by using R vers. 3.6.0 (R Core Team 2018).

Results

POPULATION SURVEY OF AUCHENORRHYNCHANS

One-yr weekly surveys of auchenorrhynchs using yellow sticky traps were conducted at the Fort Lauderdale Research and Education Center where on-going lethal bronzing disease spread is well documented (Bahder et al. 2018a). During the survey, due to the different initial set up times, 55 wk of data were collected for FLREC-1, 54 wk for FLREC-2 to FLREC-10, 33 wk for FLREC-11, and 31 wk for FLREC-12 to FLREC-14. At least 11 species belonging to 9 families (Acanaloniidae, Cixiidae, Cicadellidae, Cercopidae, Clastopteridae, Derbidae, Flatidae, Membracidae, Tropiduchidae) were collected and identified. The species numbers presented here may be underestimated due to the unidentified species. The number of the collected insect specimens varied between species and collection dates.

The total number of specimens collected for the 4 major families of auchenorrhynchs (Cixiidae, Cicadellidae, Derbidae, and Membracidae) that were consistently found on the traps are listed in Table 1. Among the 4 auchenorrhyncha families, Cixiidae was the most abundant on the traps at 2,829 specimens in total, and 4.33 specimens on average per trap were captured per wk (Table 1). The number of specimens was analyzed by using the quasi-poisson regression model due to the overdispersion of the data, which is the variation of the data that is

Table 1. Number of major auchenorrhynchan insects collected by yellow sticky traps at the Fort Lauderdale Research and Education Center, Davie, Florida, USA.

Insect taxon	Total no. of specimens	Mean (per trap per week)	SE
Cixiidae	2,829 a	4.33	1.88
Cicadellidae	403 b	0.60	0.15
Derbidae	138 b	0.19	0.08
Membracidae	350 b	0.53	0.16

Different letters indicate significant difference of the total specimen number between species.

greater than the mean. The analysis results showed significant differences in the number of collected specimens between families ($df = 3$, $P < 0.0001$). Further post-hoc pairwise contrast analysis revealed that the number of Cixiidae specimens was significantly higher than the number of Cicadellidae and Membracidae specimens ($P = 0.036$ and 0.035 , respectively) and marginally significantly higher than Derbidae ($P = 0.0531$). Within the families of Cixiidae, Derbidae, and Membracidae, 4 species, *H. crudus* (Cixiidae), *Cedusa inflata* (Ball) (Hemiptera: Derbidae), *O. joi* (Derbidae), *Idioderma virescens* Van Duzee (Hemiptera: Membracidae) (Fig. 2) were collected. These 4 species are known palm feeders (Tsai & Fisher 1993; Howard et al. 2001).

Besides the major auchenorrhynchs, insect specimens that represent 5 different families including Acanaloniidae, Cercopidae, Clastopteridae, Flatidae, and Tropiduchidae were captured by the traps (Table 2). Because all specimens of these families were tested for the phytoplasma, the total numbers of the specimens for these families are the same as the number of specimens tested for the 16SrIV-D phytoplasma (Table 2). The survey data indicated the appearance of the hoppers of the 5 families in palm canopies are more likely a single event rather than regular events, implying a weak association between these insects and palms and, therefore, unlikely to be the vector candidates of the phytoplasma disease.

The number of specimens collected for the 5 major auchenorrhynchs species at Fort Lauderdale Research and Education Center is illustrated in Figure 3b. The dominant species throughout the study period was *H. crudus* and showed peak populations from Jul to Aug, with the highest peak consisting of 244 specimens on 20 Jul 2018, after which the population decreased sharply (Fig. 3b). *Idioderma virescens* also showed peak population numbers but from Sep to Oct 2017, with 50 specimens on 31 Aug 2018, the largest number on the traps. The populations of *C. inflata* collected throughout the study were less than *H. crudus* and *I. virescens*. *Omolicna joi* was collected only from 1 trap (FLREC-10) from Nov to Jan and from Aug to Nov; no specimen was found from Feb to Jul 2018. The number of specimens collected for the Cicadellidae family is the combination of several unidentified species and shows the abundance of the leafhoppers within this family.

SCREENING AUCHENORRHYNCHANS FOR THE 16SRIV-D PHYTOPLASMA

All auchenorrhynchs collected by the sticky traps were tested for the presence of the 16SrIV-D phytoplasma in the total DNA extracted from the insect specimens. Nine families of auchenorrhynchs, a total of 3,734 specimens, were tested using 16SrIV-D semi-specific nested polymerase chain reaction assays. Of the 3,734 specimens, 19 *H. crudus* specimens and 1 specimen of Cicadellidae tested positive for the phytoplasma, a positive rate of 0.672% and 0.248%, respectively (Table 2). No specimens in other families captured by the traps tested positive for the 16SrIV-D phytoplasma. These results indicate that *H. crudus* and the Cicadellidae carried the 16SrIV-D phytoplasma. The collection dates of the positive specimens showed 6 positive specimens on 22 Feb 2018, and 1 to 2 specimens at other times (Fig. 3a). The occurrence of the phytoplasma positive *H. crudus* is unlikely related to the number of specimens collected in the same wk. The 16SrIV-D phytoplasma positive Cicadellidae showed only 1 on 15 Feb 2018 (Fig. 3a), and the other 402 specimens in the family were tested negative for the phytoplasma (Table 2).

All 16SrIV-D phytoplasma positive *H. crudus* specimens were identified visually using the description by Kramer (1979). Additionally, the COI gene sequence was amplified from 1 identified *H. crudus* specimen and aligned in the National Center for Biotechnology Information database, the BLAST results showed > 99% identity with the *H. crudus* COI

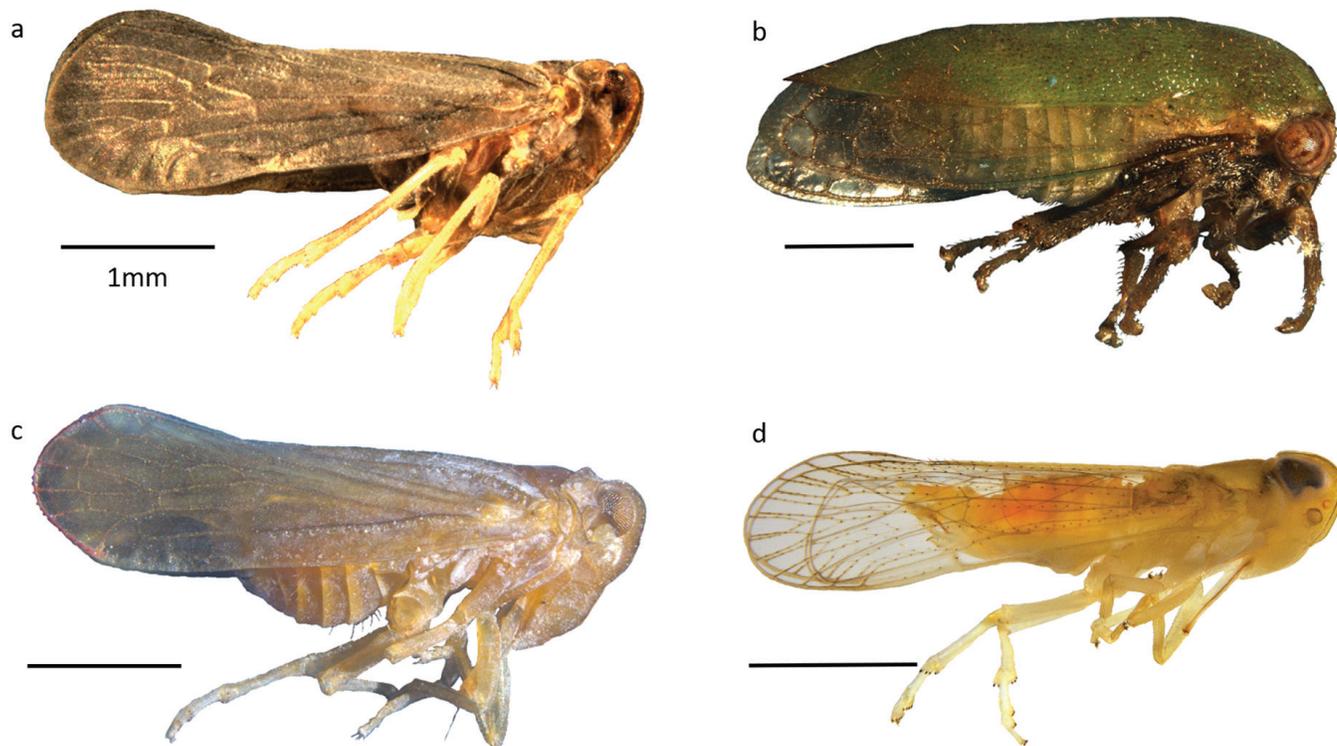


Fig. 2. Species of auchenorrhynchans that were consistently collected by sticky traps at the Fort Lauderdale Research and Education Center. (a) *Cedusa inflata* (Derbidae); (b) *Idioderma virescens* (Membracidae); (c) *Omolicna joi* (Derbidae); (d) *Haplaxius crudus* (Cixiidae).

gene sequence in the database (GenBank Accession no. EU183616.1). However, due to the damaged morphological characters of the phytoplasma positive leafhopper specimen on the sticky trap, the assignment of the leafhopper to a taxon beyond family level was not possible. There was no sequence in the National Center for Biotechnology Information database and Barcode of Life Database matches with the COI sequence that amplified from the phytoplasma positive leafhopper specimen. Therefore, the taxonomy of the phytoplasma positive Cicadellidae specimen remains unclear.

The sequences of 16S gene isolated from the positive *H. crudus* specimens and 1 Cicadellidae specimen were submitted to GenBank (Accession nos. MN053948-MN053964, MN180413-MN180415), and were used for the phylogenetic analysis (Fig. 4). The results of the

phylogenetic relationships showed the isolates of the phytoplasma obtained from *H. crudus* and the Cicadellidae specimen in this study resolved with the other 16SrIV-D sequences from GenBank, demonstrating the isolates amplified from *H. crudus* were 16SrIV-D phytoplasma.

Discussion

In this study, 19 *H. crudus* of over 2,829 specimens and 1 unidentified Cicadellidae specimen of over 403 specimens tested positive when all 3,734 collected specimens were tested for the 16SrIV-D phytoplasma. The phytoplasma positive rates were 0.672% for *H. crudus* and 0.248% for the Cicadellidae specimens. *Haplaxius crudus* and an

Table 2. Number of auchenorrhynchan insects collected by sticky traps and tested for 16SrIV-D phytoplasma by nested polymerase chain reaction assays and the number of positive specimens.

Family	Species	No. of specimens tested	No. of polymerase chain reaction positive	Phytoplasma positive rate
Acanaloniidae	Unidentified sp.	1	0	0
Cixiidae	<i>Haplaxius crudus</i>	2,829	19	0.672%
Cicadellidae	Unidentified spp.	403	1	0.248%
Cercopidae	Unidentified spp.	2	0	0
Clastopterae	<i>Clastoptera</i> spp.	2	0	0
Derbidae	<i>Cedusa inflata</i>	113	0	0
	<i>Omolicna joi</i>	26	0	0
Flatidae	<i>Melormenis basalis</i>	5	0	0
	<i>Ormenoides</i> sp.	1	0	0
Membracidae	<i>Idioderma virescens</i>	350	0	0
Tropiduchidae	<i>Pelitropis rotulata</i>	2	0	0
Total		3,734	20	

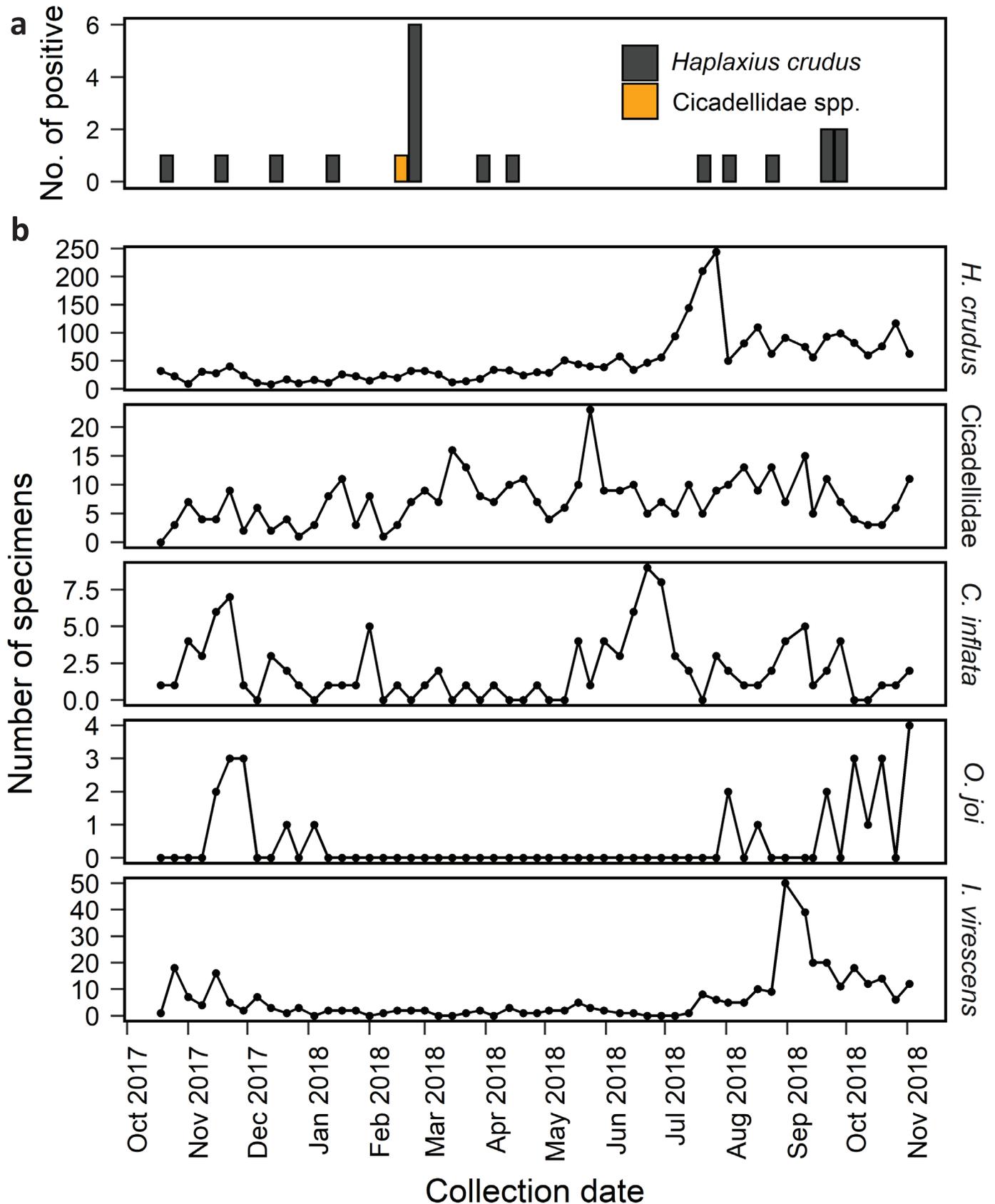


Fig. 3. (a) The number of insects tested positive for the 16SrIV-D phytoplasma by nested polymerase chain reaction assays; (b) Total number of major auchenorrhynchan insects collected by sticky traps at the Fort Lauderdale Research and Education Center from 11 Oct 2017 to 2 Nov 2018. Different scales for the number of specimens (y-axis) were used to fit the data ranges.

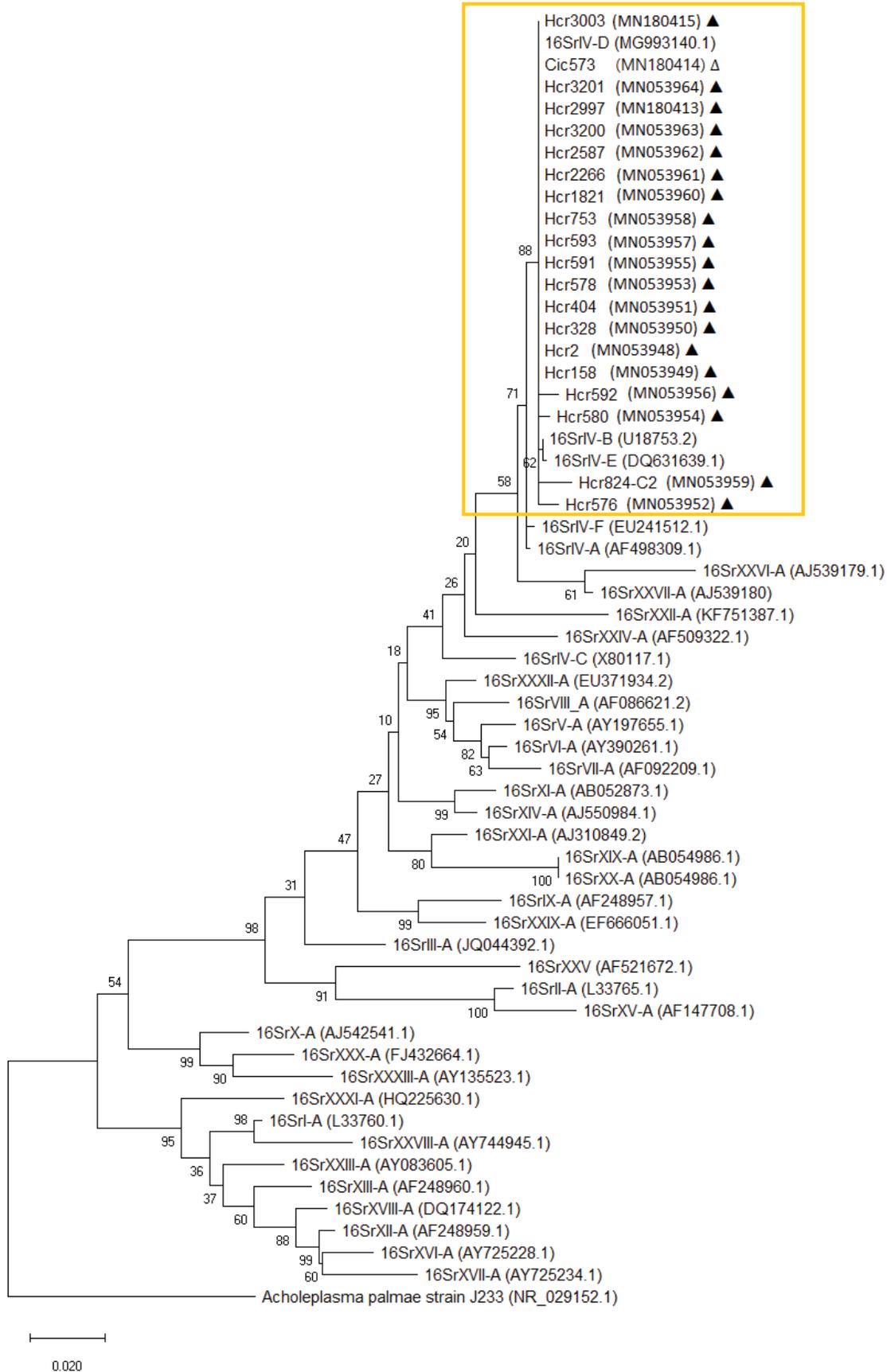


Fig. 4. Phylogenetic relationships generated from the 16S rRNA gene for 16SrIV phytoplasmas by using maximum likelihood (1,000 replicates) methods in MEGA. The 16S partial sequence amplified from *Haplaxius crudus* (indicated by the black triangle) and unidentified Cicadellidae specimen (indicated by the white triangle) from this study were included in the analysis.

unidentified Cicadellidae specimen tested positive for the 16SrIV-D phytoplasma, suggesting these 2 species are the vector candidates of the phytoplasma. However, multiple *H. crudus* specimens tested positive by nested polymerase chain reaction throughout the study, and population survey showed *H. crudus* was the most abundant species in the survey of auchenorrhynchan insects. These results, therefore, additionally support the theory that *H. crudus* is the potential vector of the 16SrIV-D phytoplasma.

The potential vector, *H. crudus*, formerly *Myndus crudus* Van Duzee (Hemiptera: Cixiidae), is a cixiid planthopper that has a close association with various palm species (Howard et al. 2001). The adult of *H. crudus* feeds on palms, whereas the nymphs feed on a wide range of weeds and grass hosts (Tsai & Kirsch 1977). Although the higher populations in Oct to Nov 2018 than the same mo in 2017 may be due to the increased number of traps set up in 2018; *H. crudus* showed a significantly higher population number than other species in the study. The abundance of *H. crudus* also was reported in other studies conducted in South Florida (Tsai & Mead 1982), North Florida (Halbert et al. 2014), and Brazil (Silva et al. 2019). There were numerous studies focusing on *H. crudus* because it was considered to be the vector of lethal yellowing phytoplasma disease which is caused by the 16SrIV-A phytoplasma (Tsai 1977; Eden-Green 1978). Howard et al. (1983) confirmed *H. crudus* as the vector of lethal yellowing in Florida by transmission assays (Howard & Thomas 1980; Howard et al. 1983). Furthermore, Narváez et al. (2018) reported 80 specimens (out of 2,726 specimens) of *H. crudus* tested positive for 16SrIV phytoplasmas in Mexico, and 6 out of 20 samples from the positive specimens confirmed was 16SrIV-D phytoplasma by sequencing. Results of both Narváez et al. (2018) and this study demonstrate the ability of *H. crudus* to carry the 16SrIV-D phytoplasma.

Other than these 19 *H. crudus* positive specimens, 3 more *H. crudus* specimens tested positive by the nested polymerase chain reaction, but the sequences were in poor quality and were unable to be assembled. Additionally, a leafhopper (*Texananus* sp.; Hemiptera: Cicadellidae) tested positive for the phytoplasma, and the 16S partial sequence isolated from the *Texananus* specimen was aligned with 16SrI-B, Aster yellow phytoplasma (GQ365729.1) with > 99% identity. The leafhopper, *Texananus* sp., was reported as the vector of Aster-yellow virus (Severin & Klostermeyer 1950) and has been considered to have association with other phytoplasmas (Crosslin et al. 2005; Munyaneza et al. 2008).

Some portion of the specimens collected by the sticky traps in our survey were unable to be identified due to morphological damage. Molecular identification methods will be needed to identify these specimens to species. However, the use of COI gene sequence data from the unidentified specimens is still unreliable for identifying the specimens to species because the 5' region of COI for most species of these taxa are not available in GenBank or the Barcode of Life Database. The 16SrIV-D phytoplasma positive rate for the unidentified Cicadellidae specimens may be higher than 0.248% if the Cicadellidae specimens captured by the traps can be identified and the total number of the same species can be counted. Moreover, Cicadellidae insects are commonly known as grass feeders and not palm feeders; the result of the Cicadellidae specimens that tested positive for the 16SrIV-D phytoplasma implies that there are potential phytoplasma reservoirs in weed or grass plant hosts. Knowing the taxonomy of the unidentified Cicadellidae specimens will be critical to further investigating the potential weed and grass hosts of the 16SrIV-D phytoplasma. Future study needs to be conducted to identify the Cicadellidae species that tested positive for the 16SrIV-D phytoplasma. The focus of future studies should be surveying leafhoppers (Cicadellidae) at the Fort Lauderdale Research and Education Center, morphologically identifying the

specimens, and then obtaining the COI gene sequences of the specimens to compare with the phytoplasma positive Cicadellidae specimen to confirm the species identification.

Omolocna joi was implicated as a potential vector of the 16SrIV-D phytoplasma in Florida (Halbert et al. 2014). We confirmed the presence of *O. joi* at the Fort Lauderdale Research and Education Center, located in Broward County, Florida, in this survey, which is the southernmost record in Florida to date. The population of *O. joi* was captured only during the fall and winter, which matches with the findings in northern Florida (Halbert et al. 2014). However, of a total of 26 tested specimens, no specimen tested positive for the phytoplasma. Additionally, *O. rufifascia* also was implicated as a potential vector (Halbert et al. 2014) in our study; however, no specimens were collected.

The specimens tested in this study were collected by sticky traps; the adhesive glue of the traps and the time period that the insects stayed on the trap may have caused minor DNA degradation of the specimens and the potential phytoplasma DNA in the specimens. Degradation of the DNA may affect the polymerase chain reaction results for testing for the phytoplasma. Additionally, there is a possibility that the limited detection threshold of nested polymerase chain reaction for the phytoplasma detection (Bahder et al. 2018b) was unable to detect a low titer of the phytoplasma that might be present in the insects. Furthermore, there was a study that tested lethal bronzing disease phytoplasma titer of the spear leaf and non-spear leaves from 3 lethal bronzing disease infected palm species; the phytoplasma was detected only from the spear leaf but not in the non-spear leaves (Bahder et al. 2019b). The unevenly distributed phytoplasma or extremely low titer of the phytoplasma in palm leaves may decrease the chance for *H. crudus* to acquire the phytoplasma.

Our study suggests *H. crudus* is the potential vector of the 16SrIV-D phytoplasma. Nineteen specimens tested positive for the 16SrIV-D phytoplasma by nested polymerase chain reaction and was confirmed as the phytoplasma by sequencing and phylogenetic analysis. However, the results do not demonstrate the vector competency of the insect. This is because phytoplasmas can be detected in non-vector insects due to individuals feeding on infected tissue, and residual pathogen may be detected from the insect gut (Vega 1993). Transmission assays will be essential for demonstrating the potential vectors' ability to transmit the 16SrIV-D phytoplasma and for confirming them as a true vector. Identification of the insect vector of the lethal bronzing disease phytoplasma will aid in the development of disease management methods to target the insect vector and contribute to management of the disease.

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