Presence of 16SrIV phytoplasmas of subgroups A, D and E in planthopper *Haplaxius crudus* Van Duzee insects in Yucatán, Mexico

María Narváez¹ · Roberto Vázquez-Euán² · Nigel A. Harrison³ · Germán Nic-Matos¹ · Jean Francois Julia⁴ · Jean Luc Dzido⁴ · Sandrine Fabre⁴ · Michel Dollet⁴ · Carlos Oropeza¹

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Abstract

The present study was carried out to determine if group 16SrIV phytoplasmas, causing lethal yellowing (LY) disease, are present in *Haplaxius crudus* Van Duzee (Hemiptera: Cixiidae) insects associated with palms in Yucatán, Mexico. *Haplaxius crudus* feral insects were captured from palm foliage at two locations (Chicxulub Puerto and CICY, Mérida, where LY-type diseases are active) and evaluated individually for the presence of phytoplasma DNA by a group 16SrIV-specific nested PCR assay. The results showed positive detection in *H. crudus* insects in a proportion of 2.7% (of the total 2726 analyzed) during a 3-year period of study. The percentage of detection was different for each site, 5.9% positive of 799 insects from Mérida and 1.7% of 1927 from Chicxulub Puerto. Positive detections were also obtained in extracts from 5.3 to 1.2% of males and females, respectively. Sequencing and in silico RFLP and phylogenetic analyses of PCR-amplified rDNA products indicated that *H. crudus* insects from Chicxulub Puerto harbored phytoplasma strains of subgroups 16SrIV-A or 16SrIV-D, whereas in insects from Mérida the strains found were 16SrIV-A, 16SrIV-D or 16SrIV-E. The diversity of subgroup strains detected in *H. crudus* coincided with strains previously identified in palms showing LY-type disease syndromes in Yucatán thereby implicating *H. crudus* as a candidate vector of 16SrIV phytoplasmas in this region of Mexico.

Keywords Lethal yellowing-type syndromes · *Haplaxius crudus* · Phytoplasmas · PCR detection

Introduction

Lethal yellowing (LY) is a devastating disease that affects at least 40 palm species, including coconut (*Cocos nucifera* L.), in different countries of the Caribbean basin (Ntushelo et al. 2013; Gurr et al. 2016). Occurrence of phytoplasmas (formerly mycoplasma-like organisms or MLO) in the phloem of coconut palms showing LY symptoms was first confirmed by transmission electron microscopy (TEM) (Beakbane et al. 1972; Heinze et al. 1972) and a cause–effect

relationship between phytoplasmas and LY was further supported by remission of early stage symptoms on palms in response to treatment with tetracycline but not penicillin antibiotics (McCoy 1972; Hunt et al. 1974). Genotypic characterization of coconut-associated phytoplasmas was made possible when pathogen-specific PCR, RFLP-typing and sequence analysis of PCR-amplified rDNA became available (Harrison et al. 1994a, b; Harrison et al. 2002a, c, 2008). As a result, phytoplasmas associated with different LY-type disease syndromes affecting numerous palm species have been classified within 16SrIV group (Lee et al. 1998) which is currently composed of at least 5 subgroups of strains (A, B, D, E, F) (Ntushelo et al. 2013). Of these subgroups, the most widely distributed is subgroup A (i.e., 16SrIV-A) that has been associated with extensive losses of the once prevalent Atlantic Tall (AT) coconut ecotype in Jamaica, Florida, Mexico and Honduras (Oropeza et al. 2005). Recently, subgroup D (16SrIV-D) strains have been associated with decline and mortality of several Phoenix palm species (Harrison et al. 2008), *Sabal palmetto* Walter (Harrison et al. 2009) and *Sabal mexicana* Mart (Vázquez-Euán et al. 2011).

✉ Carlos Oropeza
cos@cicy.mx

¹ Unidad de Biotecnología, Centro de Investigación Científica de Yucatán (CICY), A.C., Calle 43 No. 130 x 32 y 34, Chuburná de Hidalgo, 97205 Mérida, Yucatán, Mexico

² Departamento de Investigaciones Científicas y Tecnológicas, Universidad de Sonora, Hermosillo, Sonora, Mexico

³ University of Florida, Research and Education Center (UF-REC), Fort Lauderdale, FL 33314, USA

⁴ CIRAD, UMR IPME, 34398 Montpellier, France

Initial searches for a vector of LY disease were guided by the knowledge that phytoplasmas are phloem-limited, thus implicating phloem-feeding leafhoppers, planthoppers (*Auchenorrhyncha*; families *Cicadelloidea* and *Fulgoroidea*) and Psyllidae (Sternorrhyncha) (Weintraub and Beanland 2006), as the most plausible candidate vectors. Subsequent surveys of insect populations established that the cixiid *H. crudus* (formerly *Myndus crudus*) (Emeljanov 1989; Holzinger et al. 2002; Ceotto and Bourgoin 2008; Ceotto et al. 2008) was by far the most abundant potential vector consistently found on coconut palms and that populations of this species were as much as 40-fold higher in areas of high LY incidence than in disease-free areas (Howard 1980) and distribution of *H. crudus* also coincided with the known distribution of LY in the Americas (Howard et al. 1983). These findings led to transmission studies in Florida and Jamaica by a methodology relying upon the massive introduction of feral insects, collected in areas affected by the disease, into insect-proof cages containing healthy palms (Dollet 1992; Howard 1995). Using this approach, *H. crudus* was confirmed as a vector of LY in Florida after successful transmission of LY to several host palm species that included: *C. nucifera*, *Veitchia merrillii* Becc., *Pritchardia thurstonii* F. Muell and Drude., *Pritchardia pacifica* Seem and H. Wendl., *Trachycarpus fortunei* (Hook.) H. Wendl. and *Phoenix canariensis* Vasc. and Franco in three separate studies (Howard et al. 1983, 1984; Howard 1995). However, similar LY-controlled transmission attempts with this planthopper species in Jamaica failed to produce positive results (Schuiling and Johnson 1973; Eden-Green and Schuiling 1978; Eden-Green 1978, 1979). Thus, evidence implicating this species as a vector elsewhere in the Caribbean basin has remained elusive.

The phytoplasmas of the 16SrIV group causing the LY disease have been found to cause severe damage to several species of palms in Yucatán, Mexico (Narváez et al. 2006, 2016; Vázquez-Euán et al. 2011; Córdova Lara et al. 2017). Therefore, it is suspected that *H. crudus* might be involved as a vector of LY phytoplasmas in Yucatán as well. However, to date, there is no evidence reported supporting a role of this insect species, or any other, as a vector of LY phytoplasmas in Yucatán. Hence, in this study, we report the use of PCR amplification and analysis of phytoplasma rDNA sequences to determine if group 16SrIV phytoplasmas are present in *H. crudus* insects associated with palms in Yucatán.

Materials and methods

Nomenclature and identification of insects

For the nomenclature of cixiids of the genus previously known as *Myndus*, the term *Haplaxius* was used instead,

based on reviews published by Emeljanov (1989), Holzinger et al. (2002), Ceotto and Bourgoin (2008) and Ceotto et al. (2008).

Collection of insects

The Planthopper *H. crudus* adults were collected from palms (frequently from *Thrinax radiata* Lodd. ex Schult. and Schult. f and *C. nucifera*) at two sites in the state of Yucatán in Mexico, about 25 km away from each other, namely Chicxulub Puerto (21°17'38"N, 89°36'30"W) and at Centro de Investigación Científica de Yucatán (CICY) in Mérida (20°58'04"N, 89°37'18"W) (Fig. 1a), where other species of palms have been previously affected by LY-type disease syndromes (Table 1; Fig. 1b) and attributed to infection by 16SrIV phytoplasmas. The phytoplasmas detected in resident palms were identified as subgroup A strains at Chicxulub Puerto (Narváez et al. 2006) and as subgroups A or D at CICY (Vázquez-Euán et al. 2011). *Haplaxius crudus* (Fig. 1c) was collected from palm foliage using a handheld trap consisting of an outer 50 mL polypropylene tube fitted with a protruding inner 15 mL tube (Fig. 1d). Planthoppers were collected from the foliage of palms with LY symptoms and on nearby asymptomatic palms. Once in the laboratory, they were immediately observed with a dissecting microscope for species identification purposes and then stored in 70% ethanol at 4 °C until further analysis. A group of 346 insects was separated into males and females (Fig. 1e) for separate examination according to their sex. Insects collected were identified at CICY based on dichotomous keys of morphological characteristics (Kramer 1979; Triplehorn and Johnson 2005), and some of them were sent to: Thierry Bourgoin and Paola Ceotto (MNHN, Paris) for confirmation of identification.

DNA extraction and analysis of detection by PCR

Haplaxius crudus genomic DNA was extracted by the method of Paxton et al. (1996). For nested PCR, the first amplification was carried out using universal primers PI/P7 (Deng and Hiruki 1991), and the second amplification using the 16SrIV specific primers LY16Sf/LY16Sr (Harrison et al. 2002b, c). PCRs were carried out in a PCR Express Thermal Cycler (Thermohybrid) and reaction mixture for PCR amplifications consisted of 1 unit of Mango Taq Polymerase (Bioline), 50 ng of each primer, 125 µM of each dNTP (Invitrogen, Carlsbad, CA, USA) and standard PCR buffer containing 1.5 mM MgCl₂, all within a 25 µL total volume and the corresponding DNA template. In the case of the original DNA extracts from each planthopper, an aliquot was diluted 1:10 with sterile deionized water and 2 µL of each dilution was used as template for the first round of PCR. Thermal cycling

Fig. 1 Sites of capture of planthopper *H. crudus* insects in Yucatán, Mexico where lethal yellowing (LY)-type diseases are active (a). *T. radiata* palm with LY symptoms in Chicxulub Puerto (b). *H. crudus* before (c) and during capture from a palm leaf (d). Male and female *H. crudus* (e)

Table 1 16SrIV subgroup phytoplasma strains associated with other palm species in two sites in Yucatán, Mexico

Site	Palm species	<i>n</i> Palms analyzed	Phytoplasma strain	References
Chicxulub Puerto	<i>Coccothrinax readii</i>	8	16SrIV-A	Narvaez et al. (2006)
	<i>Thrinax radiata</i>	5	16SrIV-A	Narvaez et al. (2006)
	<i>Sabal mexicana</i>	1	16SrIV-A	Vázquez-Euán et al. (2011)
CICY-Mérida	<i>Gaussia maya</i>	3	16SrIV-A	CICY, present report
	<i>Thrinax radiata</i>	1	16SrIV-A	Vázquez-Euán et al. (2011)
	<i>Thrinax radiata</i>	1	16SrIV-D	Vázquez-Euán et al. (2011)
	<i>Sabal mexicana</i>	6	16SrIV-D	Vázquez-Euán et al. (2011)
	<i>Pseudophoenix sargentii</i>	1	16SrIV-D	Vázquez-Euán et al. (2011)

Strains were determined by in silico analysis of rDNA amplicon sequences obtained from the palms

parameters consisted of 92 °C for 2 min followed by 35 cycles of 94 °C for 60 s, 54 °C for 50 s and 72 °C for 80 s and a final cycle at 72 °C for 10 min before cooling to 4 °C. The product of first round PCR assay was diluted 1:40 with sterile water and 5 µL was used as template for the second amplification reaction with 16SrIV group-specific primers (LY16Sf and LY16Sr). The amplification conditions used were described previously in Harrison et al. (2002a). An aliquot (10 µL) of each final reaction mixture was electrophoresed through a 1% agarose gel using TAE (40 mM Tris-acetate, 1 mM EDTA), as running buffer. DNA in the gel was stained with ethidium bromide and visualized by UV transillumination.

Sequencing and BLAST analysis

The nested PCR products were purified with QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and cloned using a vector pGEM-T Easy kit (PROMEGA, Madison, WI, USA) and One Shot TOP 10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA). For recombinant plasmid purification, a Plasmid Miniprep kit (QIAGEN, Hilden, Germany) was used. Cloned inserts were sequenced in full on automated equipment (Davis Sequencing, Inc., Los Angeles, CA, USA). Purified PCR products were sequenced in both directions. The 16S DNA sequences were aligned with phytoplasma group/subgroup representatives available in

GenBank using ClustalW software (Hall et al. 2011) and the consensus sequences were submitted to GenBank. A search of the GenBank nucleotide database (NCBI) for homologous sequences was performed by BLAST analysis.

In silico RFLP's analysis

The phytoplasma sequence corresponding to the F2n/R2 region (1251 pb) was subjected to in silico RFLP analysis using iPhyClassifier software (Zhao et al. 2009), using 17 restriction endonucleases (*AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *Sau3AI*, *MseI*, *RsaI*, *SspI* and *TaqI*) and compared with corresponding profiles of strains of different 16SrIV subgroups provided by the software database.

Phylogenetic analysis

Phylogenetic analysis of partial 16S rRNA gene sequences representative of phytoplasmas detected in planthoppers *H. crudus* during this study, from group 16SrIV previously identified in palms and from other phytoplasma groups, was used to construct phylogeny by Neighbor-Joining method with 1000 replications for each bootstrap value using the phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.06 (Tamura et al. 2013). *Acholeplasma palmae* sp. nov., was used as an outgroup to root the phylogenetic tree.

Results

Occurrence of LY strains in palms in different sites in Yucatán

Palms of different species had already been associated with 16SrIV phytoplasma strains in the two sites used for planthopper collection: 16SrIV-A strains in Chicxulub Puerto and both 16SrIV-A and 16SrIV-D strains at CICY in Mérida (Fig. 1a, Table 1). Among the palm collection at CICY, native Maya palms [*Gaussia maya* (O. F. Cook) H. J. Quero] have developed LY symptoms and died. DNA extracts from this species yielded positive results by nested PCR assay

Table 3 Detection by PCR of 16SrIV phytoplasma DNA in female and male *Haplaxius crudus* captured in CICY, Yucatán, Mexico during 2007

Insect sex	Insects analyzed			
	Captured	% of all	PCR positive	% Positive
Female insects	98	28.3	2	2
Male insects	248	71.7	7	2.8
All insects	346	100	9	2.6

Insects included here are contained in Table 2

employing primer pairs P1/P7 followed by LY16Sf/LY16Sr. A sequence of the resulting rDNA products (KF716176) derived from *G. maya* shared 99.55% identity with phytoplasma subgroup 16SrIV-A strains associated with LY disease in Florida (AF498309, HQ613874 and EU241516).

Detection of 16SrIV phytoplasma group by PCR assay in *H. crudus* DNA extracts

Using 16SrIV group-specific nested PCR (LY16Sr/LY16Sf), positive detection was obtained for *H. crudus* from both collection sites over a 3-year period, 2006–2008 for CICY and 2007–2009 for Chicxulub Puerto (Table 2). The percentage of phytoplasma positive insects at CICY varied from 20.5% in 2006 to 2.6% in 2007 and no positive detections in 2008. Because of this trend, no further insect sampling or PCR analysis was carried out during 2009 at this site. At Chicxulub Puerto by comparison, percentages of phytoplasma positives remained at about 1% during the 4 years of the study period.

Detections of 16SrIV phytoplasma DNA in female and male *H. crudus*

In a batch of 346 *H. crudus* collected from palms during 2007, insects were separated by sex (Fig. 1d, e) and corresponding DNA extracts analyzed separately by nested PCR assay. The proportion of female insects/male insects found was 98 (28%)/248 (72%) (Table 3). Positive PCR amplification of 16SrIV phytoplasma DNA was obtained in 2% of the female insects, 2.8% of male insects and 2.6% of the 346 total insects captured (Table 3).

Table 2 Detection by PCR of 16SrIV phytoplasma DNA in *Haplaxius crudus* captured during the period 2006–2009 in two sites in Yucatán, Mexico

Year	CICY in Mérida			Chicxulub Puerto		
	Captured	PCR positive	% Positive	Captured	PCR positive	% Positive
2006	190	39	20.5	18	10	55
2007	299	08	2.60	620	6	0.96
2008	310	00	0	542	9	1.60
2009	ND	ND	ND	747	8	1.07
Total	799	47	5.9	1927	33	1.7

ND not determined

16SrIV phytoplasma strains in *Haplaxius crudus*

Nested PCR products from 20 planthoppers (8 captured at CICY and 12 captured at Chicxulub Puerto) were sequenced and subjected to Blast analysis. Twelve of these sequences (KJ922130, KJ922132, KJ922134, KJ922137, KJ922138, KJ922139, KJ922140, KJ922141, KJ922142, KJ922143, KJ922145, KJ922146) shared between 99.51 and 100% of

Table 4 16SrIV phytoplasma strains found in *Haplaxius crudus* insects captured in two locations in Yucatán, Mexico during 2007

Present study cases					Sequences compared with Acc. no.
Insect	Site	Acc. no.	Subgroup	% Identity	
1C	CICY	KJ922129	E	98.69	DQ631639
2C	CICY	KJ922130	A	99.51	HQ613874.1
3C	CICY	KJ922131	E	99.35	DQ631639
4C	CICY	KJ922132	A	99.91	DQ645632.1
5C	CICY	KJ922133	D	100	EF042899.1
6C	CICY	KJ922134	A	99.75	HQ613874.1
7C	CICY	KJ922135	D	100	EF042899.1
8C	CICY	KJ922136	D	99.68	EF042899.1
1Cx	Chicxulub	KJ922137	A	99.68	DQ645632.1
2Cx	Chicxulub	KJ922138	A	100	DQ645632.1
3Cx	Chicxulub	KJ922139	A	99.75	DQ645632.1
4Cx	Chicxulub	KJ922140	A	100	DQ645632.1
5Cx	Chicxulub	KJ922141	A	99.75	HQ613874.1
6Cx	Chicxulub	KJ922142	A	99.67	DQ645632.1
7Cx	Chicxulub	KJ922143	A	99.83	DQ645632.1
8Cx	Chicxulub	KJ922144	D	99.91	EF042899.1
9Cx	Chicxulub	KJ922145	A	100	HQ613874.1
10Cx	Chicxulub	KJ922146	A	100	DQ645632.1
11Cx	Chicxulub	KJ922147	D	99.43	EF042899.1
12Cx	Chicxulub	KJ922148	D	99.67	EF042899.1

Analyses carried out were based on the F2n/R2 portion (1251 bp) of the 16S rRNA gene. Insect cases included here are contained in Table 2

identity with comparable sequences of subgroup 16SrIV-A strains associated with LY in Florida (HQ613874), Cuba (DQ64563) or Mexico (GU47359) (Table 4). In six sequences (KJ922133, KJ922135, KJ922136, KJ922144, KJ922147, KJ922148), there was a 99.43–100% identity with a phytoplasma 16SrIV-D strain associated with Texas Phoenix palm decline (AF434989, HQ613874, HQ613875 and EF042899) (Table 4). In two sequences (KJ922129, KJ922131), there was a 98.69–99.35% of identity with a phytoplasma 16SrIV-E strain associated with coconut lethal decline in Dominican Republic (DQ631639) (Table 4).

The virtual RFLP analysis of the F2n/R2 region (1251 bp) of the 16S rRNA gene, of representative sequences of each of the 3 subgroups identified (Ac. No. KJ922138), (Ac. No. KJ922131) and (Ac. No. KJ922133), was produced from in silico digestion with *iPhyClassifier* software (Zhao et al. 2009) using 17 restriction endonuclease enzymes. Predicted digestion fragment found that differences in profiles between strains were evident for digestion with *AluI* and *MseI*, resolving the 3 subgroup strains A, D and E (Fig. 2).

Phylogenetic and molecular evolutionary analyses using MEGA 6.06 software (Tamura et al. 2013) were performed using the 16S rDNA sequences obtained from insect DNA extracts (Table 4). In the phylogenetic tree obtained (Fig. 3), the phytoplasma sequences clustered tightly together with strains 16SrIV-A (12 sequences), 16SrIV-D (6 sequences) and 16SrIV-E (2 sequences), forming three distinct subclades with previously reported phytoplasmas found in different palm species and in different locations in Mexico and other countries.

Discussion

The Cixiidae *H. crudus* is considered a vector of 16SrIV phytoplasmas, because it could transmit LY agent in insect-proof cage transmission experiments (Howard et al. 1983, 1984; Howard 1995), and positive detection of 16SrIV

Fig. 2 Virtual RFLP patterns derived from in silico digestions, using *iPhyClassifier* software, of F2n/R2 fragments of the 16S rRNA gene of phytoplasmas 2Cx Phytoplasma *Haplaxius crudus* (KJ922138),

3C Phytoplasma *Haplaxius crudus* (KJ922131) and 5C Phytoplasma *Haplaxius crudus* (KJ922133)

Fig. 3 Phylogenetic tree inferred from analysis of 16S rRNA gene sequences. The maximum-parsimony analysis was performed using the Close-Neighbor-Interchange (CNI) algorithm in MEGA 6.06. The initial tree for the CNI search was obtained with the random addition of sequences (10 replicates). The reliability of the analysis was subjected to a bootstrap test with 2000 replicates. The percentages (> 60)

of replicate trees in which the associated taxa clustered together in the bootstrap analysis are shown next to the branches. Sequences from *Haplaxius crudus* DNA used to construct the dendrogram are indicated by (filled triangle) and the GenBank accession numbers are shown in parentheses

phytoplasma that was obtained in insect DNA extracts (Harrison and Oropeza 1995) in studies carried out in Florida. Also, an association of 16SrIV phytoplasmas with Hemiptera insects has already been reported in Jamaica with the Derbidae *Cedusa* spp. (Brown et al. 2006) and in Cuba with the Cixiidae *Nymphocixia caribbea* Fennah (Dollet et al. 2010), but not in Mexico where LY-type syndromes have been affecting palms of different species since the late seventies in the last century. Therefore, the present study was carried out to find out if *H. crudus* insects were associated with 16SrIV phytoplasmas causing LY-type syndromes in Yucatán.

Positive detection was obtained for *H. crudus* insects captured in the two sites sampled (Chicxulub Puerto y CICY, Mérida), where palms of different species infected with 16SrIV phytoplasmas have been previously reported (Narváez et al. 2006; Vázquez-Euán et al. 2011). Considering all the 2726 insects captured and analyzed, results showed that 2.9% were positive. For CICY, there were 799 analyzed and 5.9% positive, and for Chicxulub Puerto, 1927 analyzed and 1.7% positive. But we found a fluctuation of the percentage of infected insects in the case of CICY, decreasing from 20 to 0% within 3 years. In the case of Chicxulub Puerto, the percentage changed little from around 1 to 1.7%. Such fluctuations are not uncommon. For instance, in the case of pear decline (PD), caused by phytoplasmas and transmitted by the psyllid *Cacopsylla pyricola* Foester, a study in California showed that the percentage of infected *C. pyricola* ranged from 0 to 45% depending on the orchard, the month and year of collection (Blomquist and Kirkpatrick 2002). In a more closely related case, searching for potential vectors of the phytoplasma-associated Lethal Disease in Tanzania affecting coconut, two homopteran species were found positive for phytoplasma detection with percentages ranging from 0.16% for *Diastrombus mkurangai* Wilson and 25% for *Meenoplus* spp. The relevance of these homopterans as LD vectors is still not defined; nevertheless, percentages of detection are highly contrasting.

In a parallel study (CICY, unpublished results), it has been observed that the presence of *H. crudus* insects infected with 16SrIV phytoplasmas both in CICY and Chicxulub has continued, which is consistent with occurrence of palms of different species developing LY-type symptoms in both sites, as in the case of *C. readii* H. J. Quero (Narváez et al. 2006), *T. radiata* and *S. mexicana* in Chicxulub Puerto, and *T. radiata*, *S. mexicana*, *P. sargentii* H. Wendl. ex Sarg. subsp. *Sargentii* in CICY, Mérida (Vázquez-Euán et al. 2011).

Also research has explored whether phytoplasmas can be present in females and male insects, and if there are differences in proportions and transmission. In a study phytoplasmas associated with Flavescence Dorée in *Vitis vinifera* L. were found in 27% of females and in 20% of males of the insect vector *Scaphoideus titanus* Ball., (Marzorati et al.

2006). In the case of *Macrosteles quadrilineatus* Forbes, the insect vector of aster yellows phytoplasma (AY), positive PCR detection was obtained for females and males, and infected females were significantly more likely than infected males to transmit aster yellows phytoplasma (Beanland et al. 1999). Although, in a 1 year study more male than female *M. quadrilineatus* leafhoppers were infected with AY phytoplasmas (Beanland et al. 2014). Similarly, in the present study female and male *H. crudus* insects were analyzed by PCR to determine if both could be infected with 16SrIV phytoplasmas that are related to LY. Captures showed that over two times more male than female insects were collected, and also positive detection was found for both female 2% and male 2.8%. Therefore, both female and male insects should be considered as putative vectors of 16SrIV phytoplasmas. Also, based on the association of the phytoplasmas with female insects, transovarial transmission should be considered as a subject for future studies, and also in view that there are studies already reporting the presence of the sugarcane white leaf phytoplasma in nymphs (at different developmental stages) of the putative insect vector, the leafhopper, *Matsumuratettix hiroglyphicus* Matsumura (Hanboonsong et al. 2002) and the presence of 'Ca. Phytoplasma prunorum' in eggs, nymphs and newly emerged adults of the insect vector, the psyllid *Cacopsylla pruni* Scopoli (Tedeschi et al. 2006).

The final part of the present study focused on identifying the 16SrIV phytoplasmas found in *H. crudus* insects at the subgroup level. The sequences obtained from the phytoplasma DNA from the two sites sourced were compared by BLAST analysis, and high identity percentages were found with phytoplasma strains 16SrIV-A (associated with LY phytoplasma in the USA, Cuba and Mexico) and 16SrIV-D (associated with Texas Phoenix palm decline in the USA), but also in the particular case of CICY high identity percentage was also found with a 16SrIV-E strain (associated with coconut lethal decline in Dominican Republic).

Virtual RFLP analysis showed differences in band profiles that were consistent with the occurrence of the three strains identified by BLAST analysis. In addition, phylogenetic analysis also confirmed this, showing that the three different phytoplasma sequences clustered tightly together with strains 16SrIV-A, 16SrIV-E and 16SrIV-D, respectively (Wei et al. 2007), forming three distinct subclades with reported phytoplasmas found in different palm species and in different locations in Mexico and other countries. Therefore, these three types of analyses strongly support the occurrence of strains 16SrIV-A, 16SrIV-D and 16SrIV-E in *H. crudus* insects in Yucatán, Mexico, with the first two strains appearing in a greater proportion. Similarly, the phytoplasma diversity found in palm and non-palm species in this region of Mexico includes strains 16SrIV-A and 16SrIV-D as the most commonly found.

In conclusion, the results of reported here confirm an association of the planthopper *H. crudus* (male and female) with 16SrIV phytoplasmas in a proportion of 2.7% of the total studied (2726) collected from two different sites (25 km away) where LY disease is present affecting different palm species. Also phytoplasma diversity was also found within the insects analyzed, and strains of three different subgroups A, D and E were identified. These data allow us to believe that *H. crudus* to be a candidate vector of 16SrIV phytoplasmas in Yucatán, as reported in the case of Florida. However, this hypothesis will have to be tested using techniques such as phytoplasma transmission within insect-proof cages as previously carried out in Florida (Howard et al. 1983, 1984; Howard 1995). In addition, for future studies, we should also look for other potential vector species, a possibility strengthened by the discovery of group 16SrIV phytoplasmas in *N. caribbea* (Dollet et al. 2010) and *Cedusa* spp. (Brown et al. 2006). The second one was found associated with understory vegetation on coconut farms affected by the disease in Jamaica (Brown et al. 2008). Some of these plant species were reported to harbor similar group 16SrIV phytoplasmas in symptomless individuals (Brown et al. 2008; Brown and McLaughlin 2011). Hence, in addition to extending studies on *H. crudus* as a potential vector of LY, we should also screen other insect species in Mexico for group 16SrIV phytoplasma detection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in publication of this paper.

Research involving human and animal participant This article does not contain any studies with human subjects or animal performed by any of the authors.

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