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Deliverable Title

Report on the phytoplasma detection and identification in *Haplaxius crudus* nymphs and other potential cixiid lethal yellowing (LY) vector identification.

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List of acronyms and abbreviations

16S rDNA	- 16S ribosomal deoxyribonucleic acid
16S rRNA	- 16S ribosomal ribonucleic acid
18S rDNA	- 18S ribosomal deoxyribonucleic acid
µM	- micromolar
bp	- base pair
BSA	- bovine serum albumin
CICY	- Centro de Investigación Científica de Yucatán
C. nucifera	- *Cocos nucifera*
COLPO	- Colegio de Postgraduados
CTAB	- cetyl trimethylammonium bromide
DNA	- deoxyribonucleic acid
dNTPs	- deoxynucleotides
EDTA	- ethylenediaminetetraacetic acid
E. petreae	- *Eustachys petreae*
H_2O	- water
H. caldwelli	- *Haplaxius caldwelli*
H. crudus	- *Haplaxius crudus*
H. skarphion	- *Haplaxius skarphion*
LY	- lethal yellowing
M	- molar
ml	- milliliter
mtCOI DNA	- mitochondrial cytochrome C oxidase I deoxyribonucleic acid
mtDNA	- mitochondrial deoxyribonucleic acid
NaCl	- sodium chloride
NJ	- Neighbor Joining
PCR	- polymerase chain reaction
P. laxum	- *Panicum laxum*

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PVP-40	- polyvinylpyrrolidone 40
RFLP	- restriction fragment length polymorphism
rDNA	- ribosomal deoxyribonucleic acid
RNA	- ribonucleic acid
rpm	- revolution per minute
Tris	- tris(hydroxymethyl) aminomethane
Tris-HCl	- tris(hydroxymethyl) aminomethane chloridrate
U	- unit
UCHIL	- Universidad de Chile
UNIBO	- *Alma Mater Studiorum* - University of Bologna
UV	- ultraviolet

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Executive summary

Nowadays the only species that has been confirmed as vector of the phytoplasmas 16SrIV-A and -D in coconut palms in Mexico, is *Haplaxius crudus*. However, other species of *Haplaxius*, such as *H. skarphion*, *H. caldwelli*, among others, captured in coconut palm orchards in Mexico, have been found positive for the presence of these phytoplasmas, but it has not yet been possible to confirm their role as insect vectors. To generate information in this regard, the focus of the present work was to develop the rearing technique for *Haplaxius* species, detect phytoplasmas in nymphs of cixiids and identify molecularly the *Haplaxius* species when the insects are still in the nymph stage. For the first time, the rearing of *H. crudus* in captivity was obtained and the first report about the detection of the phytoplasma 16SrIV-A in nymphs of the Cixiidae is presented here. Moreover, by using PCR and RFLP analyses, it was possible identify rapidly and specifically *H. crudus* and *H. caldwelli* in their nymph stages.

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1. Introduction

The genus *Haplaxius* Fowler has a distribution in the New World (Ferreira *et al*., 2010), which includes 34 species from North America and Northern Mexico; in addition, there are 31 species in the Neotropic (Bartlett *et al*., 2011). However, currently the only species that has been confirmed as vector of the phytoplasmas belonging to the ribosomal subgroups 16SrIV-A and -D, is *Haplaxius (Myndus) crudus* (Narvaez *et al*., 2018), however other species are suspected to play this role in areas with presence of lethal palms decay (Dollet *et al*., 2010; Halbert *et al*., 2014). In some coconut plantations, it is reasonable to think that other *Haplaxius* species, such as *H. skarphion*, *H. caldwelli*, among the others, may be participating in the transmission of phytoplasmas due to their phylogenetic proximity to the main vector (Bertin *et al*., 2010a), also having overlapping ecological niches in coconut plantations (Ramos *et al*., 2018). Certainly, *H. skarphion* and *H. caldwelli* have already been found to be positive for the phytoplasma of group 16SrIV (Ramos, 2018), but it is necessary to identify the ribosomal subgroup and which are the vectors involved in the transmission. Moreover, in some exploratory surveys in these plantations, phytoplasma-positive cixiids nymphs have been found which have not been identified in either genus or species.

Bertin *et al*. (2010b), mention that the modern identification of cixiids is based on morphological characteristics and is restricted to a small number of specialist entomologists with extensive experience about the families belonging to the Cixiidae genus. Even for experts, the morphological distinction of closely related species is difficult. The morphological identification of *H. crudus*, *H. skarphion* and *H. caldwelli* is possible for adults with dichotomous clues such as those of Kramer (1983), but not for their nymph stages. These nymph stages of cixiids species are difficult to observe in the field, because they feed on roots of their host plants (Sforza *et al*., 1999) and have a low mobility. The molecular identification of insects provides a fast and reliable key tool that can contribute to expand the knowledge about the identifications of cixiids. In addition, these markers can also be applied successfully to nymphs, whose morphological characteristic is limited to a few species of the Cixiidae family.

Ceotto *et al*. (2008) demonstrated that the phylogenetic trees obtained using the mitochondrial cytochrome C oxidase I (mtCOI DNA) and the 18S rDNA nucleotide sequences were congruent, and that the trees were consistent with the morphological classification. Nuclear and mitochondrial genomes have different modes of inheritance, and the effectiveness of nuclear loci and mitochondrial DNA for monitoring phylogeny may differ between phylogenetic levels (Song and Liang, 2013). Mitochondrial and ribosomal DNA, as well as some nuclear genes are more conserved and have repeatedly provided knowledge about the genetic evolution of species and genera that recently diverged (Behura, 2006). Therefore, the genes encoding mtDNA, rDNA and other nuclear DNAs are commonly used for the species identification (Bertin and Bosco, 2013). The focus of this work was to develop

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the rearing technique for *Haplaxius crudus*, detect phytoplasmas in nymphs of cixiids and identify molecularly the *Haplaxius* species when the insects are still in the nymph stage.

2. Reproduction in captivity of *Haplaxius crudus*

2.1. Material and methods

The main host species of *Haplaxius crudus* nymphs are *Brachiaria humidicola*, *B. mutica*, *Eustachis petraea* and *Paspalum maximum* (Ramos *et al*., 2018). Three localities in Mexico (Pailebot, Cárdenas, Tabasco) with coconut orchards in which LY disease is active, were monthly monitored with the objective to characterize the initial environmental conditions in which the immature stages of *H. crudus* reproduce and develop. In these orchards, in addition to the main *H. crudus* host plants indicated above, six other weeds were also present (Table 1).

The reproduction in captivity of *H. crudus*, was carried out in cages of own design, and with adult insects captured in the field.

Table 1: Alternative host families and species found in the coconut localities visited. Soil parameters measured

Host family	Host species	Coconut locality	Soil parameters measured
Poaceae	*Brachiaria decumbens* Stapf	-Pailebot -Cárdenas -Tabasco	-Humidity -Temperature -Texture -Freatic mantle -Depth -others
	**Brachiaria humidicola* (Rendle) Schweick		
	**Brachiaria mutica* (Forssk.) Stapf		
	Digitaria abyssinica (Hochst. Ex A. Rich.) Stapf		
	* *Eustachys petraea* (Sw.) Desv.		
	Leersia hexandra Sw.		
	Panicum laxum Sw.		
	**Paspalum maximun* Jacq.		
Portulacaceae	*Portulaca pilosa* L		
Cyperaceae	*Cyperus ligularis* L.		

*Main host species of *H. crudus* nymphs (Ramos *et al*., 2018).

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Preliminary assays for reproduction in captivity of *H. crudus*

To this purpose, the most favorable conditions for the development of the insect vector were adopted. These were the ones existing in Pailebot (Figure 1), in association to the grass species *Panicum laxum*. This site remains flooded from December to February. The water gradually withdraws during the following months, leaving favorable soil conditions for the development of nymphs (Figure 1). In order to confirm that the nymphs developed in this locality correspond to *H. crudus*, four soil samples with nymphs and waxy exudate were collected, two in April and two in May 2019, respectively. Each sample was introduced in a pot-cage and transferred to the laboratory. The cages were maintained at room temperature (25-30°C), and monitored every 15 days for two months, to record the emergence of adult insects (Figure 2).

Figure 1: Sampling during 2019 in the locality of Pailebot. Above: monthly sequence of the soil drying process. Below left and center: nymphs with waxy exudates. Below right: nymph of *H. crudus*

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Figure 2: Preliminary assays for reproduction in captivity of *H. crudus*. Left: inside of a pot-cage with waxy secretions of nymph. Right: pot-cages in the laboratory

Assays for reproduction in captivity of *H. crudus*

Four one-year-old palms *Pritchardia pacifica* 30-50 cm high, were planted in plastic pots (0.5 m diameter x 0.47 m high) whit sandy soil sterile from Pailebot site. *P. laxum* seeds were sown in each pot. Oviposition take place in the weed, simulating what happen in the field. For each pot a cylindrical cage with iron frame of 1 m height x 0.2 m radius was designed. The cage coat consists of transparent tulle. On the cover of the cage, openings were made to allow the introduction of insects (Figure 3). The insects released in each pot-cage have been captured in the center of the *Adonidia merrillii* palm orchards, in the localities of Pomoca, Huimanguillo and Tabasco. The capture occurred in the morning, with CICY´s modified dispositive of capture (Narvaez *et al*., 2018). The modification consists in the assembly of two falcon tubes: The first 15 ml tube, with an opening in the lower end, was inserted in the opening of another 50 ml tube. In each cage, 18 to 22 insects were released, using the same number of males and females. The insect collection was carried out weekly during one month, and for each capture one assay cage was used, being the cage number 1 the first used for the assay (Table 3). The cages were placed in a shaded greenhouse at 30-35°C, and the soil moisture was maintained by means of continuous irrigation.

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Figure 3: Assays for reproduction in captivity of *H. crudus* with pot-cages and *P. laxum* grass. A: general appearance of insect cages and their components; B: grass base of *P. laxum* with waxy exudate; C and D: nymphs in 4 and 5 instars; E: *H. crudus* adults born in captivity

2.2. Results and discussion

Preliminary assays for reproduction in captivity of *H. crudus*

Seventy-five adult insects emerged in the four pots-cages (Table 2). The emergence was discontinuous possibly due to the different egg laying times of the females. The survival average of the adults was nine days. Some insects born in captivity were introduced into Eppendorf tubes in 90% alcohol and conserved at -20ºC.

Table 2: Number of adult individuals of *H. crudus* emerged in the pot-cages used in the preliminary assay for reproduction in captivity

Pot-cage	Range of days for insect's emergence and number of insects emerged				Total number of insects emerged
	< 15 days	16-30 days	31-45 days	>45 days	
1	5	4	6	3	18
2	7	7	6	1	21
3	2	5	4	2	13
4	6	9	5	3	23

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Total	20	25	21	9	75

All the insects emerged in the pot-cages were identify as *H. crudus*, this means that *P. laxum* behaves as a suitable host for it use in the assays for the reproduction in captivity of this insect species. The number of adult insects that have emerged during this test could be considered low. However, it must be taken into account that the number of males and females present at the start of the assay was unknown. In addition, there was no information available about the amount of adult individuals that one insect pair is capable of generating. *P. laxum* is a weed native to America and can grow between 30 and 60 cm in height (Melendez, 1997). It is a very good host of *H. crudus* and grows very well in the pot-cages. In this work, the weed has been used for the first time to achieve, successfully, the reproduction in captivity of *H. crudus*.

Assays for reproduction in captivity of *H. crudus*

The 83 mature insects that were born in the four cages were all identified as *H. crudus*. The days for the emergence of insects ranged from 40 to 64 (Table 3). The days for the emergence in the pot-cages 1 and 2, coincide with the means reported by Tsai and Kirsch (1978), for nymphs produced in the laboratory in Saint Agustin grass, *Stenotaphrum secundatum*. On the other hand, for the pot-cages 3 and 4, the reproduction time was 40 days, due to the increase of the number of the hours with temperature higher than 30°C, during the assays weeks at the end of March and beginning of April.

During these trials no nymphs were captured, in order to maintain the habitat stability in the pot-cages. However, nymphs and exudates of the nymph waxes were observed in the field early, as described (Howard, 2012). In addition, it was not necessary to capture nymphs since the morphological descriptions of the five instants of nymphs of *H. crudus* are known (Wilson and Tsai, 1982).

Table 3: Adult insects of *H. crudus* born in captivity

Pot-cage	*H. crudus* introduced in pot-cage (1:1-female:male)	Days for the emergence	Number of *H. crudus* adults born
1	18	64	8
2	22	47	21
3	20	40	28
4	22	40	26

2.3. Conclusions

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The reproduction of *H. crudus* in captivity was possible, with pot-cages, sandy soils and an average daytime temperature of 30-35°C. This is the first reproduction in captivity of *H. crudus*.

3. Detection and identification of phytoplasmas in nymphs of cixiids

3.1. Material and methods

In previous studies it was found that the weed *E. petreae* was positive to the phytoplasma 16SrIV-A (Teran, 2014). Because of this, it was considered to determine if the nymphs of the cixiids associated with the rhizosphere of this weed were also positive to phytoplasma presence. In a coconut palm plantation where 12 coconut palm hybrids were established to assess their resistance to LY, 15 plant of *E. petreae* were surveyed for the presence of cixiids nymphs. The nymphs were captured using a mouth aspirator with collecting vial (pooter). This system allows storing the captured nymphs in a plastic container (collecting vial). Once the nymphs were collected they were transported to the phytopathology laboratory. The nymphs were separated, inserted in 2 ml tubes with 90% ethanol, and stored at -20°C until DNA extraction.

DNA extraction

The total DNA of each insect was extracted using the CTAB method described by Harrison *et al*. (1996) and Brown *et al*. (2006). Before starting the DNA extraction process, the ethanol was removed from the insect by evaporation. The insects were placed individually in 1.5 ml tubes containing 300 µl of CTAB extraction buffer (2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 3 M NaCl; 1% PVP-40 and 1% 2-mercaptoethanol) and macerated with micro pestles. Subsequently, extracts were incubated at 65°C for one hour in a water bath, then 300 µl of phenol-chloroform-isoamyl alcohol (25: 24: 1) was added and centrifuged (Eppendorf Centrifuge 5427R, Germany) at 14,000 rpm for 10 minutes. The supernatant was placed in a new 1.5 ml tube and the DNA was precipitated by adding 3 M sodium acetate and cold isopropanol volumes (30 µl and 180 µl, respectively), mixed by inversion and incubated at -20°C for 1 hour. In order to precipitate the DNA pellet, it was centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded. Two washes of the DNA pellet were performed with 100 µl of 70% ethanol and allowed to dry at room temperature for 15-20 minutes. The pellet was re-suspended in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The total DNA obtained from the insects was visualized by electrophoresis in 1% agarose gels in TAE 1 X buffer (Tris base, acetic acid and EDTA) stained with ethidium bromide.

Nested Polymerase Chain Reaction (nested PCR)

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The P1/P7 primers (Deng and Hiruki, 1991; Schneider *et al*., 1995) were used for the amplifications. Resulting products were diluted 1: 40 with ultrapure water, and 2 µl of the dilution was amplified for 35 cycles using LY-specific 16S rRNA gene primer pair 503F/LY16Sr (Harrison *et al*., 1999).

PCR amplifications were performed with a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using 1 U of the Mango Taq™ DNA polymerase (Bioline, UK) in a total volume reaction of 25 µl, that contained 2 µl of dNTPs (1.25 mM), 5 µl of 10 X PCR buffer, 5 units of Taq-polymerase, and 2 µl of each primer (25 ng/µl). The reaction was performed with an initial 2 minutes cycle at 94°C, followed by 35 cycles of 1 minute at 94°C, annealing for 50 seconds at 60°C, and extension for 80 seconds at 72°C. Reactions were terminated with 10 minutes at 72°C extension step and cooled to 4°C. The PCR products were analysed by electrophoresis with a 1% agarose gel stained with ethidium bromide and visualized examined by using a UV translluminator Molecular Image® Gel Doc™ XR System (BioRad, Hercules, CA). Negative controls were DNA extracted from non-symptomatic plants and sterile water. Coconut palm DNA (GenBank accession number GU473590) previously identified as infected by LY was used as a positive control (Vazquez-Euan *et al*., 2011).

PCR products purification and sequencing

PCR amplicons from the 503F/LY16Sr primers were purified in columns of the QIAquick GelExtraction kit (QIAGEN®, Hilden, DE, USA) according to the manufacturer's instructions. All purified products were quantified in a nanodrop spectrophotometer (Jenway-GenovaNano, UK). The sequencing was performed with the 3500xl Genetic Analyzer (Applied Biosystems, USA). The sequencing was performed in both directions.

3.2. Results and discussion

Six out of 33 nymphs (Figure 4), whose species was unknown, collected in the rhizosphere of *E. petreae*, were positive to the phytoplasma 16SrIV-A (Figure 5). *H. crudus*, in its nymph stages, may coexist with nymphs of *H. skarphion* and *H. caldwelli* in some species of poaceas and also with nymphs of another cixiid, *Oeclus snowi* (Ramos *et al*., 2018). This means that in the coconut palm there is a complex pathosystem enclosing multitrophic interactions that deserve further research.

Figure 4: Cixiid nymph in the 4th instar, associated to the rhizosphere of *Eustachys petreae*

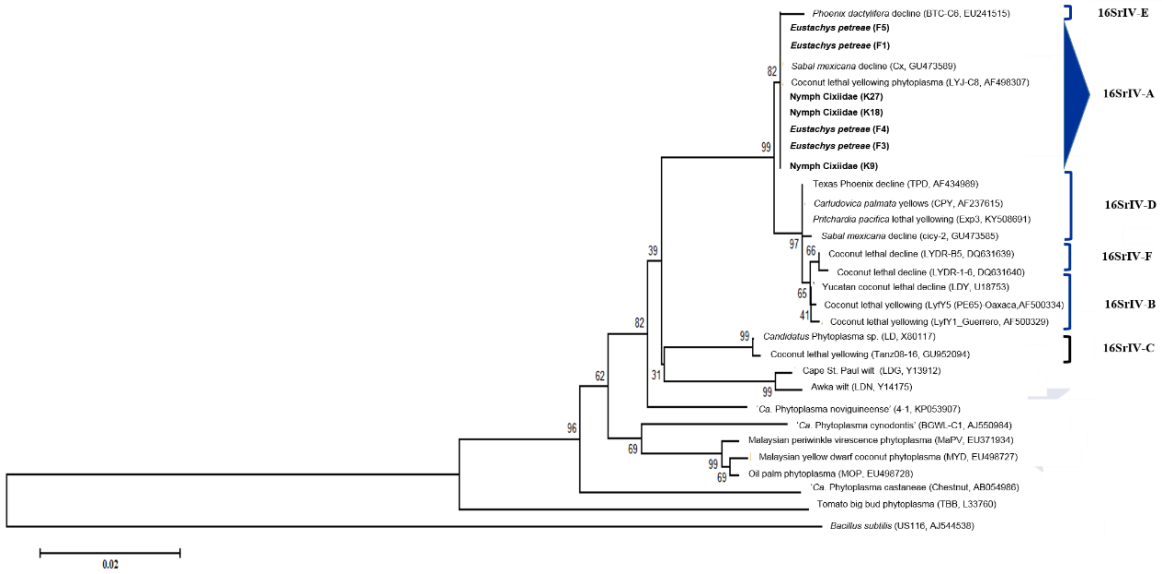

Figure 5: Phylogenetic tree derived by the analysis of the sequences obtained with primers 503F/LY16Sr. The tree was build using the Neighbor-Joining method. The model was Tajima-Nei (Tajima and Nei, 1984). *Bacillus subtilis* (GenBank accession number AJ544638) was used as an external group. The sequences of the phytoplasma strains in *Eustachys petraea* and nymph of Cixiidae are in bold. The length of the branches represents the distances between sequences. Bootstrap values for 2,000 replicas are shown in the branches. The GenBank accession number for each sequence is in parentheses, followed by phytoplasma ribosomal subgroup

3.3. Conclusions

This is the first report about the detection of the phytoplasma 16SrIV-A in nymphs of the Cixiidae family. It is possible that the nymphs have acquired the phytoplasma feeding on the roots of the *E. petreae*. It was necessary to identify the nymphs by a molecular tool as described below.

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4. Molecular identification of *H. crudus* and *H. caldwelli* nymphs.

4.1. Material and methods

Insect collection and DNA extraction

Adult individuals were collected with a hand-held leaf blower by vacuum under coconut (*C. nucifera*) and kerpi (*Adonidia merrillii*) palms leaflets, early in the morning or in the afternoon. The capture of *H. crudus* and *H. caldwelli* was carried out during 2017-2018 in palms with symptoms of lethal yellowing in several locations in the Chontalpa region, Tabasco, Mexico. All the specimens were preserved in 90% ethanol and stored at -20°C. Subsequently, the specimens were stored at -80°C in the genomics laboratory of the University Juárez Autónoma of Tabasco. Genomic DNA was extracted from adults using cetyltrimethylammonium bromide (CTAB) according to the Harrison *et al*. (1996) and Brown *et al*. (2006). The ethanol was removed from the insect before starting the extraction process. The DNA extraction was carried on as described in 3.1.

PCR amplification of the cytochrome C oxidase subunit I region in the mtDNA (mitochondrial DNA)

The nucleotide sequence data were generated using the *cytochrome C oxidase I (COI)* gene. The mtDNA genes have many advantages including a relatively rapid, easy to used mutation rate and known PCR primers (Bazrafkan *et al*., 2016). A fragment of the *COI* gene of approximately de 1 kb was amplified for the three species through a series of PCR assays (Simon *et al*., 1994; 2006; Lunt *et al*., 1996) whose amplification products overlap (Table 4). The primers HCCOI F/R (Table 4) were designed in this work from sequences of *H. crudus* genome (GenBank accession number KF472314).

The PCRs were carried out in 25 µl of reaction volume, with 5 X of PCR buffer, $MgCl_2$ 50 mM, 100 ng of each primer, 100 µM of dNTP (Invitrogen, USA), 0.5 U of Mango Taq™ (Bioline, United Kingdom) and 50 ng of template. The PCR cycle conditions for mtDNA were: initial denaturation at 95°C for 2 minutes, 35 cycles at 94°C for 1 minute, 48-60°C according to the first pair used for 1 minute, 72°C for 1 minute and a final extension at 72°C for 5 minutes. PCR products were visualized by electrophoresis on a 2.0% agarose gel stained with ethidium bromide.

PCR products purification and sequencing

PCR amplicons obtained were purified in columns of the QIAquick GelExtraction kit (QIAGEN®, Hilden, DE, USA) according to the manufacturer's instructions. All purified

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products were quantified in a nanodrop spectrophotometer (Jenway-GenovaNano, UK). The amplicons sequences were obtained with 3500xl Genetic Analyzer (Applied Biosystems, USA) at the Biotechnology Institute of the National Autonomous University of Mexico. The sequencing was performed in both directions.

Table 4: Sequences of primers used for the PCR amplification of a fragment of the *COI* gene of *H. crudus* and *H. caldwelli*

Primer	Sequence (5´ - 3´)	Reference
HCCOI (R)	AATGAAAATGGGCGACTA	This work
HCCOI (F)	ATTGCAGTACCGACAGGA	This work
UEA1 (F)	GAATAATTCCCATAAATAGATTTACA	Lunt *et al*., 1996
TY-N-1438d (F)	GAAWAATTCCYATAAWTARATTTACA	Zhang and Hewitt 1996
LCO1490-L (F)	GGTCWACWAATCATAAAGATATTGG	Nelson *et al*., 2007
LCO1490 (F)	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*., 1994
C1-J1709 (F)	AATTGGGGGGTTTGGAAATTG	Simon *et al*., 2006
C1-J-1718 (F)	GGGGGGTTTGGAAATTGATTAGTGCC	Simon *et al*., 1994
C1-N1738 (R)	TTTATTCGTGGGAATGCTATGTC	Simon *et al*., 2006
HCO2198 (R)	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*., 1994
HCO2198-L (R)	TAAACTTCWGGRTGWCCAAARAATCA	Nelson *et al*., 2007
C1-J-2183 (Jerry) (F)	CAACATTTATTTTGATTTTTTGG	Simon *et al*., 1994
C1-N-2659 (Mila1) (R)	GTCAATCCAGTAAATAATGG	Simon *et al*., 1994
UEA8 (R)	AAAAATGTTGAGGGAAAAATGTTA	Lunt *et al*., 1996
UEA9 (F)	GTAAACCTAACATTTTTTCCTCAACA	Lunt *et al*., 1996
C1-N2776 (R)	GGTAATCTGAATAACGTCGAGG	Simon *et al*., 2006
TL2-N-3014 (Pat) (R)	TCCATTGCACTAATCTGCCATATTA	Simon *et al*., 1994
UEA10 (R)	TCCAATGCACTAATCTGCCATATTA	Lunt *et al*., 1996

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Sequencing and restriction fragment length polymorphism (RFLP) analyses of amplified DNA obtained by the primers C1-J-2183 and UEA8

Using the sequences obtained from *H. crudus* and *H. caldwelli* with the primers C1-J-2183 and UEA8 (Ceotto *et al*., 2008; Halbert *et al*., 2014; Silva *et al*., 2019), a phylogenetic tree was constructed with the Neighbor Joining method (Saitou and Nei, 1987) using the MEGA7 program (Kumar *et al*., 2016). Further, the same sequences were analyzed with the NEBcutter V2.0 program (http://nc2.neb.com/NEBcutter2/) to define the restriction sites. Based on these, the restriction enzymes selected were: *Alu*I, *Ssp*I, *Cla*I, *Rsa*I and *Taq*I (Promega Corporation, Madison, USA). The digestion of the PCR products was carried out according to the manufacturer's instructions. A reaction volume of 20 µl contain: 13 µl of ultrapure H_2O, 2 µl of corresponding buffer for each enzyme, 2 µl of BSA, 2 µl of PCR product and 1 U of the enzyme. Ten µl of the digested product was mixed with 2 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). The RFLP profiles will be separated by electrophoresis on a 3% agarose gel at 80 V for 1 h and stained with ethidium bromide. The amplicons from *H. crudus* and *H. caldwelli* obtained using the primer pair C1-J-2183/UEA8, were analyzed in the NEBcutter V2.0 program.

4.2. Results and discussion

PCR amplification of the cytochrome C oxidase subunit I region in the mtDNA (mitochondrial DNA)

Among the primers listed in Table 4, only from 15 pairs was possible to obtain an amplification product from *H. crudus* and *H. caldwelli*. Five out 15 primer pairs were able to amplify the *COI* gene of both species of cixiids. Direct PCR amplicons were generated with the primer pairs UEA1/ C1-N1738 (TM: 55°C), UEA 9/UEA10 (TM: 55°C), LCO1490-L/HCO2198-L (TM: 55°C). Meanwhile, positive results were obtained by semi-nested PCR amplification using C1-J-1718/C1-N2776 (TM: 64°C) primers above on C1-J1709/C1-N2776 (TM: 49°C) amplicons.

Sequencing and restriction fragment length polymorphism (RFLP) analyses of amplified DNA obtained by the primers C1-J-2183 and UEA8

The sizes of the sequences amplified with these pairs of primer ranged from 550 to 560 bp. A phylogenetic tree was constructed (Figure 6). The individuals of *H. crudus* found in Tabasco grouped in the same cluster with those from USA (Halbert *et al*., 2014; Ceotto *et al*., 2008) and clearly separated from insects belonging to the species *H. caldwelli*. The virtual RFLP,

allows to infer that the restriction enzymes *Alu*I, *Ssp*I, *Cla*I, *Rsa*I and *Taq*I can specifically identify both species (Table 5).

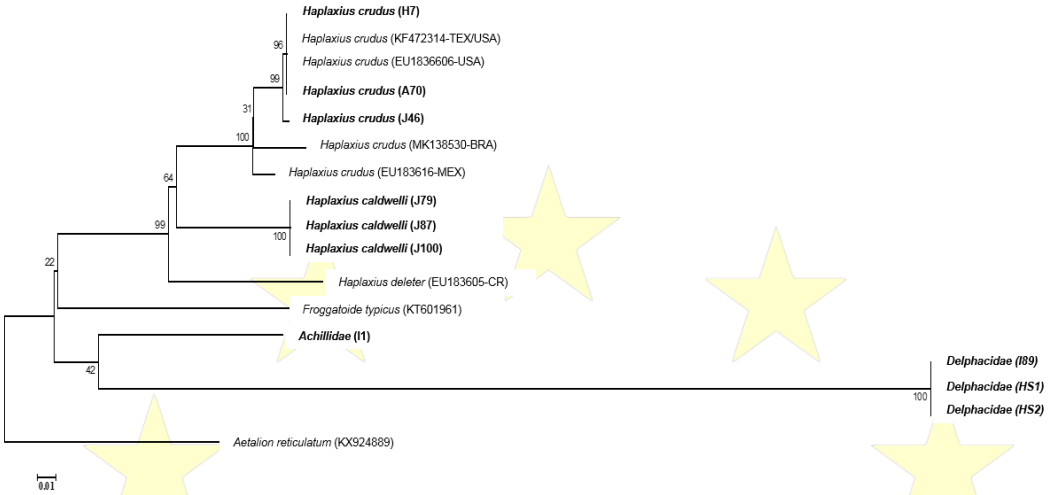

Figure 6: Phylogenetic tree derived by the sequence analysis of the sequences obtained with the primers C1-J-2183/UEA8. The tree was build using the NJ method. The model was Tajima-Nei (1984). *Aetalion reticulatum* (GenBank accession number KX924889) was used as an out group. The sequences of the insects obtained in this work are in bold. The length of the branches represents the distances between sequences. Bootstrap values for 2,000 replicas are shown in the branches. The GenBank accession number for each sequence is in parentheses

Table 5: Virtual RFLP analyses of DNA from *H. crudus* and *H. caldwelli* with *Alu*I, *Ssp*I, *Cla*I, *Rsa*I and *Taq*I on C1-J-2183/UEA8 amplicons

Restriction enzyme	*H. crudus* RFLP fragments (bp)	*H. caldwelli* RFLP fragments (bp)
*Alu*I	271, 215,114	270, 152, 99, 42, 21, 15
*Ssp*I	321, 279	321, 237, 41
*Cla*I	-	398, 201
*Rsa*I	351, 249	-
*Taq*I	-	398, 201

4.3. Conclusions

With these new molecular tools, it is possible detect and identify rapidly the *Haplaxius* species in their nymph stages.

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