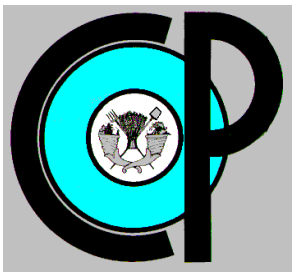

# COLEGIO DE POSTGRADUADOS

**INSTITUCIÓN DE ENSEÑANZA E INVESTIGACIÓN EN CIENCIAS AGRÍCOLAS**

**CAMPUS MONTECILLO**

**PROGRAMA DE POSTGRADO EN FITOSANIDAD**

**ENTOMOLOGÍA Y ACAROLOGÍA**

## PREVALENCIA DEL VIRUS DE LA LEPROSIS DE LOS CÍTRICOS TIPO CITOPLASMÁTICO Y NUCLEAR EN *Brevipalpus spp.* RECOLECTADO DE DIFERENTES ESPECIES DE CÍTRICOS

ANA KAREN BELTRÁN BELTRÁN

T E S I S

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**PREVALENCIA DEL VIRUS DE LA LEPROSIS DE LOS CÍTRICOS TIPO CITOPLASMÁTICO Y NUCLEAR EN *Brevipalpus spp*. RECOLECTADO DE DIFERENTES ESPECIES DE CÍTRICOS**

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## RESUMEN

El virus de la leprosis de los cítricos causa pérdidas importantes a la citricultura mundial; en los últimos meses éste se ha expandido en México, de ahí la importancia de conocer el vector, el complejo *Brevipalpus*, ubicarlo en cada zona productora y analizar si tiene alguna relación con el tipo de virus adquirido por estos ácaros. La prevalencia del virus de la leprosis de los cítricos C (CiLV-C) y OFV-citrus se determinó en poblaciones de campo en 15 estados productores de cítricos. Los ácaros se recolectaron de huertos de naranjas, toronjas, mandarinas, limas y limas dulces. Solo *Brevipalpus yothersi* y *B. californicus* fueron encontrados; *B. yothersi* fue el más abundante. Los virus CiLV-C y OFV-citrus se presentan en ambas especies de ácaros. Sin embargo, una mayor proporción de *B. yothersi* portaba CiLV-C comparado con *B. californicus*. La proporción de ácaros portadores de OFV-citrus y simultáneamente de ambos virus (CiLV-C y OFV-cítricos) fue muy baja pero similar para ambas especies de ácaros. Se discuten las implicaciones de nuestros resultados para la epidemiología de la leprosis de los cítricos.

**Palabras clave:** CiLV-C, OFV-citrus, *B. yothersi, B. californicus,* prevalencia, *Citrus*.

**PREVALENCE OF THE LEPROSIS VIRUS OF CYTOPLASMIC AND NUCLEAR CITRUS TYPES IN *Brevipalpus spp*. COLLECTED FROM DIFFERENT CITRUS SPECIES**

Ana Karen Beltrán Beltrán, MC.
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## ABSTRACT

The citrus leprosis virus causes important losses to the global citrus industry, in recent months in Mexico it has expanded; hence the importance of knowing the vector, the *Brevipalpus* complex, locate it in each production area and analyze if it has any relationship with the type of virus acquired by these mites. The prevalence of citrus leprosis virus C (CiLV-C) and OFV-citrus was determined in field populations in 15 citrus producing states. Mites were collected from orange, grapefruit, mandarin, lime and sweet lime orchards. Only *Brevipalpus yothersi* and *B. californicus* were found and *B. yothersi* was the most abundant. CiLV-C and OFV-citrus viruses occur in both mite species. However, a higher proportion of *B. yothersi* carried CiLV-C compared to *B. californicus*. The proportion of mites carrying OFV-citrus and simultaneously of both viruses (CiLV-C and OFV-citrus) was very low but similar for both mite species. The implications of our results for the epidemiology of Citrus leprosis are discussed.

**Keywords**: CiLV-C, OFV-citrus, *B. yothersi, B. californicus*, prevalence

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## INTRODUCCION GENERAL

La enfermedad conocida como leprosis de los cítricos se describió por primera vez en Florida en el año 1901 (Fawcett, 1909), pero desapareció en 1962 (Childers *et al.,* 2001), y posteriormente fue identificada en América del Sur, Paraguay, donde se conoció como "lepra explosiva" (Spegazzini, 1920), rápidamente se extendió a Argentina (Bitancourt, 1934), Brasil (Bitancourt, 1955), Costa Rica (Araya, 2000), Panamá (Domínguez *et al.,* 2001), Guatemala (Mejía *et al*., 2002), Bolivia (Gómez *et al.,* 2005), Colombia (León *et al*., 2006), Belice y México.

El agente causal de la enfermedad incluye dos tipos: el citoplasmático y el nuclear. Para el tipo citoplasmático se reconocen al Citrus leprosis virus C (CiLV-C) y el Citrus leprosis virus C2 (CiLV-C2), en el caso del nuclear se encuentran el Citrus leprosis virus nuclear (CiLVN) y el Orchid fleck dichorabdovirus strain citrus (OFV-citrus) (Kitajima *et al*., 1972; Ali *et al.,* 2014; Cruz-Jaramillo *et al.,* 2014; Roy *et al.,* 2015; Ramos-González *et al*., 2017).

Los síntomas de la enfermedad incluyen lesiones en las frutas, hojas y ramas pequeñas, causando la caída prematura de la fruta, defoliación y la muerte de ramas que conducen a un declive serio del árbol (Rodrigues *et al*., 2003).

La forma de transmisión del virus de una planta infectada a otra sana es mediante un ácaro fitófago del género *Brevipalpus*, el cual inocula el virus al perforar la pared celular de la planta (Kitajima *et al*., 2011; Hartung *et al*., 2015). El virus se desarrolla sólo en las células en que el ácaro vector introduce sus estiletes para alimentarse sin ingresar al sistema vascular de la planta, por lo que sólo se producen lesiones localizadas en hojas, frutos y ramas (Childers *et al.,* 2003). Debido a que el

proceso de transmisión de un virus mediante un vector es muy específico, a la fecha se considera que sólo las especies de *Brevipalpus* como *B. phoenicis sensu lato* y *B. californicus* están estrechamente relacionadas con la transmisión de los agentes causales de la leprosis (Rodrigues *et al.,* 1997).

A partir de 2015, se redescribió a *B. phoenicis* s.l., lo que resultó en un complejo de siete especies (Beard *et al*., 2015), de las cuales se ha encontrado a *B. yothersi* y *B. papayensis* en México (Sánchez-Velázquez *et al.,* 2015; Salinas-Vargas *et al.,* 2016), siendo la primera la especie predominante (Sánchez-Velázquez *et al*., 2015; Salinas-Vargas *et al*., 2016). Para *B. californicus* aún no se realiza la redescripción. Este último también se encuentra en cítricos en México, pero es menos frecuente que *B. yothersi*. Estos ácaros no se habían considerado de importancia económica, a pesar de tener alrededor de 500 plantas hospederas, tanto cultivadas como silvestres, debido a que el daño ocasionado no reportaba pérdidas económicas (Childers *et al.,* 2003a). Sin embargo, a partir de 2005, con la aparición de la leprosis de los cítricos y de los antecedentes que existían de ser la enfermedad viral más importante en la industria citrícola de Brasil, la Dirección General de Sanidad Vegetal implementó acciones para erradicar a la leprosis de los primeros estados afectados (Chiapas, Tabasco y Veracruz) (Izquierdo-Castillo *et al.,* 2011). A pesar de los esfuerzos realizados, en la actualidad la leprosis se encuentra prácticamente en todos los estados citrícolas del país, así como en los cítricos de traspatio.

## OBJETIVOS

Determinar la prevalencia del virus de la leprosis de los cítricos, tipo nuclear y citoplasmático, en ácaros del género *Brevipalpus* recolectados en árboles de *Citrus sinensis, C. paradisi, C. reticulata* y *C. x lemon*

Determinar las especies del género *Brevipalpus* en *C. sinensis, C. paradisi, C. reticulata* y *Citrus x lemon* de diferentes zonas citrícolas de México.

## HIPOTÉSIS

La prevalencia del virus de las leprosis nuclear y citoplasmático está relacionada con la especie del ácaro vector y la especie de cítrico.

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# CAPÍTULO 1. PREVALENCE OF CITOPLASMATIC (CILV-C) AND NUCLEAR (OFV-CITRUS) VIRUSES, THE CAUSATIVE AGENTS OF CITRUS LEPROSIS DISEASE, IN MITES OF THE GENUS *BREVIPALPUS*

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**RESUMEN**

La prevalencia del virus de la leprosis de los cítricos C (CiLV-C) y OFV-citrus se determinó en poblaciones de campo en ácaros *Brevipalpus* de 15 estados productores de cítricos en México. Los ácaros se recolectaron de huertos de naranjas, toronjas, mandarinas, limas y limas dulces. Solo *Brevipalpus yothersi* y *B. californicus* fueron encontrados y *B. yothersi* fue el más abundante. Los virus CiLV-C y OFV-citrus se encontraron en ambas especies de ácaros. Sin embargo, una mayor proporción de *B. yothersi* que *B. californicus* portaba CiLV-C. La proporción de ácaros que portaban OFV-citrus y ambos virus simultáneamente (CiLV-C y OFV-cítricos) fue muy baja pero similar para ambas especies de ácaros. Se discuten las implicaciones de nuestros resultados para la epidemiología de la leprosis de los cítricos.

**Palabras clave:** CiLV-C, OFV-citrus, *B. yothersi, B. californicus,* naranja, limon, toronja, mandarina, lima.

**ABSTRACT**

Prevalence of Citrus leprosis virus C (CiLV-C) and OFV-citrus were determined in field populations of *Brevipalpus* mites from 15 citrus-producing states in Mexico. Mites were collected from orange, grapefruit, mandarin, lime and sweet lime orchards. Only *Brevipalpus yothersi* and *B. californicus* were found and *B. yothersi* was the most abundant. The viruses CiLV-C and OFV-citrus were found in both mite species. However, a higher proportion of *B. yothersi* were carrying CiLV-C than *B. californicus*. The proportion of mites carrying OFV-citrus and both viruses simultaneously (CiLV-C and OFV-citrus) was very low but similar for both mite species. The implications of our results for the epidemiology of Citrus leprosis are discussed.

**Keywords**: CiLV-C, OFV-citrus, *B. yothersi, B. californicus,* orange, lime, grapefruit, mandarin, sweet lime.

## Introduction

Citrus leprosis is a viral disease of numerous citrus species; it is considered to be the most economically important viral disease of citrus in Brazil (Bastianel et al. 2010). The causative agents are citoplasmatic and nuclear type viruses. Two citoplasmatic type viruses (Citrus leprosis virus C [CiLV-C] and Citrus leprosis virus C2 [CiLV-C2] [*Cilevirus*]) and two nuclear type viruses (Citrus leprosis virus nuclear [CiLVN] and the Orchid fleck dichorabdovirus strain citrus [OFV-citrus] [*Dichorhavirus*]) have been recognized (Locali-Fabris et al. 2006; Roy et al. 2014; Ramos-González et al. 2016). Citrus leprosis was first reported in Mexico in 2005 and, since then, the Mexican government has made numerous attempts to minimize or eradicate the disease from localities with infected citrus trees (Izquierdo-Castillo et al. 2011). However, despite all these efforts, this disease is now found in the majority of the citrus-producing regions of Mexico (MaTeresa Santillan-Galicia, personal communication).

Virus transmission from infected to healthy plants is via mites from the genus *Brevipalpus* (Rodrigues et al. 2000; Rodrigues and Childers 2003; Kondo et al. 2003; Leon et al. 2017; Tassi et al. 2017). Mites acquire the virus (become viruliferous) when they pierce the cellular wall of infected plant cells during feeding. When viruliferous, mites feeding on healthy plants inoculate those plants with the virus (Kitajima et al. 2011; Hartung et al. 2015). The virus only develops within the cells into which it has been inoculated and does not enter the vascular system of the plant, thereby only producing very localized lesions in leaves, fruits and branches (Childers et al. 2003; Bastianel et al. 2010). The species *B. phoenicis sensu lato* (s.l.) (Geijskes), *B. californicus* (Banks) and *B. obovatus* (Donnadieu) have been described as vectors of viruses that cause Citrus leprosis (Childers et al. 2003; Rodrigues and Childers 2013). Recently, the existence of seven cryptic species within *B. phoenicis* s.l. has been reported (Beard et al. 2015), of which *B. yothersi*

(Baker) and *B. papayensis* Baker have been recorded in Mexico, in addition to *B. californicus. B. yothersi* is the most abundant and widely distributed mite species in Mexican citrus orchards (Sánchez-Velázquez et al. 2015; Salinas-Vargas et al. 2016). It is not known whether all the cryptic species within *B. phoenicis* s.l., are able to transmit viruses associated with Citrus leprosis. Recent publications have reported the ability of *B. yothersi* to transmit CiLV-C experimentally in bean plants (*Phaseolus vulgaris* L.) (Tassi et al. 2017). Garcia-Escamilla et al. (2018) reported the ability of *B. yothersi* and *B. californicus* to transmit CiLV-C and OFV-citrus to seven different citrus species. It remains important to confirm whether the most abundant *Brevipalpus* species are able to transmit viruses causing Citrus leprosis. We believe that detecting these viruses in field populations of mites is an important first step to identifying potential relationships between particular viruses and mite species. While finding a particular virus more frequently in one mite species than another does not guarantee that the mite will be able to transmit the virus, it does set the basis for future validation experiments and provide insight into the epidemiology of this disease. For this reason, we systematically sampled *Brevipalpus* mites from different citrus species in 16 Mexican states, and evaluated them for viruses associated with Citrus leprosis.

## Material and Methods

### *Mite collection*

Mites were collected on orange (*Citrus sinensis* (L.) Osbeck), grapefruit (*C. paradisi* Macfad.), mandarin (*C. reticulate* Blanco), persian lime (*C. latifolia* Yu. Tanaka), key lime (*C. aurantifolia* (Christm.) Swingle) and sweet lime (*Citrus limetta* Risso) in the states of Campeche, Chiapas, Hidalgo, Jalisco, Morelos, Nuevo León, Oaxaca, Puebla, Querétaro, San Luis Potosí, Sinaloa, Tamaulipas, Veracruz, Quintana Roo and Zacatecas. In general, five orchards were sampled from

each state except for Chiapas and Tamaulipas where seven orchards were sampled, and Sinaloa where two orchards were sampled. Five trees were sampled in each corner and the centre of every orchard (25 trees per orchard). From each tree, 20 leaves were sampled, five from each cardinal point and all 1.5 m above the ground. Samples were collected in plastic bags and each tree represented an experimental unit. Each plastic bag was labelled and transported to the laboratory inside a cool box, where all mites were collected.

Once in the laboratory, each leaf was visually inspected and all *Brevipalpus* mites were counted and collected. *Brevipalpus* mites were deposited individually inside 2 mL Eppendorf tubes containing one mL of RNA stabilizer (RNA Later®, Thermo Fisher Scientific Inc. Waltham, MA USA). All Eppendorf tubes were stored at -20 °C until required.

***Molecular identification of mites and detection of citrus leprosis viruses in Brevipalpus mites***

***RNA and DNA extraction from mites***

RNA and DNA were extracted from each individual mite. RNA was used for virus detection and identification while the DNA was used for molecular identification of the mite. RNA was extracted first from each mite using the Quick-RNA™ Tissue/Insect Microprep extraction kit (Zymo Research, Irvine, CA, USA). Mites were placed individually into ZR Bashing Bead Lysis Tubes each containing 800 µL of RNA lysis buffer. Lysis tubes were placed in a mixer mill (MM400, Retsc®, Haan, Germany) and the mite disrupted at 15 Hz for 10 minutes. Lysis tubes were then centrifuged at 12,000 g for one minute and 400 µL of the supernatant transferred into a Zymo-Spin IIIC column in a 2 mL collection tube and centrifuged at 8,000 g for 30 seconds. This step was repeated twice to process the 800 µL of the RNA lysis buffer. The Zymo-Spin IIIC column was retained to subsequently extract the DNA (see below), and RNA extraction was continued using

the RNA lysis buffer collected in the 2 mL collection tube following the manufacturer's instructions. RNA samples were stored at -20 °C. For DNA extraction, the Zymo-Spin IIIC column previously used for the RNA extraction was used again but with the buffers contained in the Quick-DNATissue/Insect Microprep Kit™ (ZYMO Research Corporation, Irving, Ca, USA). DNA Pre Wash Buffer (400 µL) was added to the Zymo-Spin IIIC column, and placed in a new collection tube, and the manufacturer's instructions were followed starting at this step. DNA was stored at -20 °C until required. Prior to storage of DNA and RNA samples at -20 °C, their concentrations were estimated using a NanoDrop™ (Thermo Fisher Scientific, Inc. Waltham, MA, USA).

*Molecular identification of mites*

For molecular identification of the mites, the extracted DNA was used as a template. A region of the mitochondrial gene Cytochrome Oxidase Subunit I (COI) was amplified using the primers DNF and DNR (Navajas et al. 1996). Amplifications were done by PCR in a 25 µL reaction volume containing 2.5 µL of buffer 10X (600 mM Tris-SO4 [pH 8.9]), 180 mM ammonium sulphate), 1 mM of $MgCl_2$, 0.2 µM of each primer, 0.2 mM of dNTP's, 0.5 µL of Taq DNA polymerase (Quiagen, GmbH, Hilden, Germany) and 5 µL (approx. 8 ng) of DNA. PCR amplifications were done using a MyCycler (BIO-RAD Laboratories Inc., Hercules, CA, USA), with the following thermal conditions: one cycle of 4 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 60 s at 54 °C and 60 s at 72 °C with a final extension at 72 °C for 5 min. PCR products were visualized on 1.5 % agarose gels in 1X TAE. The gels were stained with ethidium bromide (10 mg $mL^{-1}$) and photographed. PCR products were sent to Macrogen Inc. (Geumchen-gu, Seoul, Korea) for direct sequencing.

*Detection of virus by RT-PCR in mites*

Virus detection was achieved by reverse transcriptase and PCR. Complementary DNA (cDNA) was obtained for the extracted RNA by reverse transcriptase reactions using the Sensiscript® kit (QIAGEN®, Hilden, Germany). Reactions were made in final volumes of 20 µL, each containing 2 µL of 10X RT buffer (provided with the kit), 0.5 mM of each dNTP, 10 units of RNase inhibitors, 1 µL of reverse transcriptase, 7 µL of RNase-free distilled water, 1 µM of Oligo (dt) 18 primer (Thermo Fisher Scientific Inc. Waltham, MA, USA) and 5 µL of RNA (0.5-1.3 ng/µL). All tubes were incubated for 60 mins at 37 °C in a thermocycler model MyCycler™ (BIORAD Laboratories Inc., Hercules, CA, USA).

Detection of cytoplasmic (CiLV-C) virus and nuclear type (OFV-citrus) virus, was achieved using PCR with the complementary DNA (cDNA) obtained previously as a template. CiLV-C was detected using the primers MPF and MPR (Locali-Fabris et al. 2006). OFV-citrus was detected using three sets of primers, each designed to amplify a different strain of the virus. These primers were: OFVF and OFVR (Ali et al. 2014), NPF and NPR (Roy et al. 2015a), CNSV2F and CNSV2R (Cruz-Jaramillo et al. 2014). Concentrations of the PCR reagents were the same for all primers. Each reaction contained 2.5 µL of 10X PCR buffer (Tris-Cl, KCl, $(NH_4)2SO_4$, 15mM $MgCl_2$; pH 8.7), 0.18 µM of each primer, 0.2 mM of each dNTP, 0.2 µL of Taq polymerase (5 U/µL) (QIAGEN, GmbH, Hilden, Germany), 3 µL of cDNA and enough sterile distilled water to achieve a final volume of 25 µL per reaction. Thermal conditions for the CiLVC was one cycle of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 57 °C and 1 min a 72 °C, followed by a final extension at 72 °C for 10 min. For the OFVF/OFVR and NPF/NPR primer combinations: one cycle of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 61 °C and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. For the CNSV2F/CNSV2R primer combination: one

cycle of 5 min at 94 °C, 35 cycles of 35 s at 94 °C, 20 s at 62 °C and 45 s at 72 °C, followed by a final extension at 72 °C for 10 min. All PCRs were done in a MyCycler™ thermal cycler. PCR products were visualized as described previously. The expected band sizes were 339 bp for CiLV-C, 160 bp for the OFVF/OFVR primer combination, 681 bp for the NPF7NPR combination and 480 bp for the CNSV2F/CNSV2R combination. Although the identity of the viruses detected was based mainly on the size of the PCR products, some PCR products were also sent to Macrogen Inc. (Geumchen-gu, Seoul, Korea) for direct sequencing to confirm the identity of the viruses.

*Sequence handling and analysis*

Sequences from mites were edited using BioEdit v.7.1.9 (Hall 1999). Multiple alignments were made using Clustal W (Thompson et al. 1994) implemented in BioEdit. After alignment and trimming, the final length of the COI sequences for *Brevipalpus* were 358 bp (49 sequences). GenBank accession numbers are listed in Table 1.

**Table 1**: GenBank accession numbers of COI sequences used for the phylogenetic analysis. * = Sequences used as a reference for analyses.

| Species | Plant host | Sample ID | GenBank | Reference |
|---|---|---|---|---|
| *Brevipalpus yothersi* | *Citrus×latofolia* | 126E | MK424501 | This study |
| " | " | 184E | MK424508 | " |
| " | " | 217E | MK424520 | " |
| " | *Citrus×sinensis* | 119E | MK424500 | " |
| " | " | 155E | MK424502 | " |
| " | " | 167E | MK424503 | " |
| " | " | 175E | MK424504 | " |
| " | " | 187E | MK424511 | " |
| " | " | 200E | MK424515 | " |
| " | " | 203E | MK424517 | " |

| | | | | |
|---|---|---|---|---|
| " | " | 210E | MK424518 | " |
| " | " | 219E | MK424521 | " |
| " | " | 221E | MK424522 | " |
| " | " | 226E | MK424527 | " |
| " | " | 236E | MK424532 | " |
| " | " | 237E | MK424533 | " |
| " | " | 242E | MK424535 | " |
| " | " | 243E | MK424536 | " |
| " | " | 245E | MK424537 | " |
| *B. californicus* | *Citrus×latofolia* | 181E | MK424541 | " |
| " | " | 172E | MK424547 | " |
| " | " | 218E | MK424548 | " |
| " | " | 122E | MK424546 | " |
| " | *Citrus×sinensis* | 234E | MK424538 | " |
| " | " | 235E | MK424539 | " |
| " | " | 208E | MK424540 | " |
| " | " | 213E | MK424545 | " |
| " | " | 239E | MK424544 | " |
| " | " | 191E | MK424543 | " |
| " | " | 240E | MK424542 | " |
| *B. yothersi** | " | O2-2 | KF954990 | Sánchez-Velázquez et al. 2015 |
| *B. yothersi** | " | O4-2 | KF954991 | Sánchez-Velázquez et al. 2015 |
| *B. californicus** | ----- | Line 16 | DQ789591 | Groot and Breeuwer 2006 |
| *B. californicus** | ----- | Line 19 | DQ789594 | Groot and Breeuwer 2006 |
| *B. californicus** | ----- | HAP40 | KC291402 | Navia et al. 2013 |
| *B. obovatus** | ----- | US20 1 | KC291383 | Navia et al. 2013 |
| *B. obovatus** | ----- | Line 15 | KC291383 | Navia et al. 2013 |
| *Cenopalpus pulcher** | ----- | Cenopu45 | AY320029 | Rodrigues et al. 2004 |

COI sequences from mites were analysed using maximum likelihood in Molecular Evolutionary Genetic Analysis (MEGA) ver. 5 for Windows, with the Close-Neighbour-Interchange algorithm (Tamura et al. 2011). The robustness of branches was estimated by bootstrap analysis with 1000 repeated samplings of the data (Felsenstein 1985). For phylogenetic analysis, additional sequences were retrieved from GenBank and used for comparison (Table 1). For the virus sequences, all were edited as described previously. In total, nine sequences were obtained for the CiLV-C virus, and six for the OFV-citrus virus. To confirm virus identities, all sequences were used to query NCBI´s GenBank data base using the Basic Local Alignment Tool (BLASTN) implemented in Genbank. All searches resulted with E values between 0 and $3^{-165}$, 98-100% identity percentages, and query coverage of 100% for all the sequences used.

***Data analyses***

All the mites collected and evaluated in this study are shown in Table 2. This includes the citrus species and geographical origin from which they were collected, and whether each mite was carrying CiLV-C, OFV-citrus, both viruses or neither virus. The number of mites that were positive for the presence of OFV- citrus virus reflects the combined data from the three primers used. For statistical analysis, we used only data from orange and lime as they provided the most abundant samples, because these are the most frequent orchards in the field. First, we analysed the effect of citrus species (orange vs. lime) on the presence of *B. yothersi* and *B. californicus*. Then, the frequency of detection of the viruses, individually or in combination, was compared between the *Brevipalpus* mite species. Data were analysed using binomial tests to compare between specific pairs of treatments. All analyses were done using GenStat ver. 8.0 (Payne et al. 2005).

## Results

### Molecular identification of mites and the effect of citrus species on their presence

Phylogenetic analysis confirmed the presence of only two species within the genus *Brevipalpus*: *B. yothersi* and *B. californicus* (Fig. 1). The phylogenetic placement was supported by bootstrap values greater than 97%. On both orange and lime, *B. yothersi* mites were significantly more abundant than *B. californicus* ($P<0.001$) (Fig 1A lime; Fig 1B orange).

**Table 2**. *Brevipalpus californicus* and *B. yothersi* mites collected from different citrus species, location and the viruses detected in each individual.

| State | Citrus species | Total mites | CiLV-C | OFV-citrus | CiLV-C and OFV-citrus | Without virus |
|---|---|---|---|---|---|---|
| *Brevipalpus californicus* | | | | | | |
| Chiapas | orange | 2 | 1 | - | - | 1 |
| Hidalgo | orange | 1 | - | - | - | 1 |
| Jalisco | lime | 3 | 1 | 1 | - | 1 |
| | sweet lime | 2 | - | - | - | 2 |
| Morelos | lime | 1 | - | 1 | - | - |
| Oaxaca | lime | 3 | - | 2 | - | 1 |
| Quintana Roo | orange | 1 | - | - | - | 1 |
| S. L. Potosi | orange | 3 | - | - | - | 3 |
| Sinaloa | orange | 1 | - | - | - | 1 |
| Tamaulipas | orange | 1 | - | - | - | 1 |
| Zacatecas | lime | 6 | 1 | 1 | 1 | 3 |
| *Brevipalpus yothersi* | | | | | | |
| Campeche | lime | 6 | 1 | 1 | - | 4 |
| | orange | 1 | - | - | - | 1 |
| Chiapas | orange | 10 | 3 | 1 | 1 | 5 |
| Hidalgo | orange | 13 | 4 | 1 | 0 | 8 |
| Jalisco | lime | 1 | - | - | - | 1 |
| Nuevo Leon | orange | 2 | - | - | - | 2 |
| | grapefruit | 3 | 1 | - | - | 2 |
| Morelos | orange | 1 | - | - | - | 1 |
| | lime | 9 | - | - | - | 9 |
| Oaxaca | orange | 1 | - | 1 | - | - |
| | lime | 2 | 1 | - | - | 1 |
| Quintana Roo | orange | 10 | - | 2 | - | 8 |
| | lime | 5 | 1 | - | - | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Queretaro | orange | 14 | 2 | 2 | 1 | 9 |
| Puebla | lime | 12 | 2 | 2 | - | 8 |
| S.L. Potosi | orange | 10 | - | 2 | 2 | 6 |
| Sinaloa | orange | 1 | 1 | - | - | - |
| Tamaulipas | orange | 1 | 1 | - | - | - |
| | grapefruit | 2 | - | - | - | 2 |
| Veracruz | orange | 12 | 3 | - | - | 9 |

**Virus detection in mites and its relationship with mite species and citrus species**

CiLV-C and OFV-citrus viruses were detected in both species of mites collected from different citrus species and localities (Table 2). The proportion of *B. yothersi* mites carrying CiLV-C was greater than the proportion of *B. californicus* carrying CiLV-C ($P<0.001$) (Fig 3A). There was no significant difference in the proportion of *B. yothersi* mites and *B. californicus* mites carrying OFV-citrus virus ($P = 0.079$) (Fig 3B). Also, the proportion of *B. californicus* and *B. yothersi* mites carrying both viruses were not significantly different ($P= 0.176$) (Fig 3C).

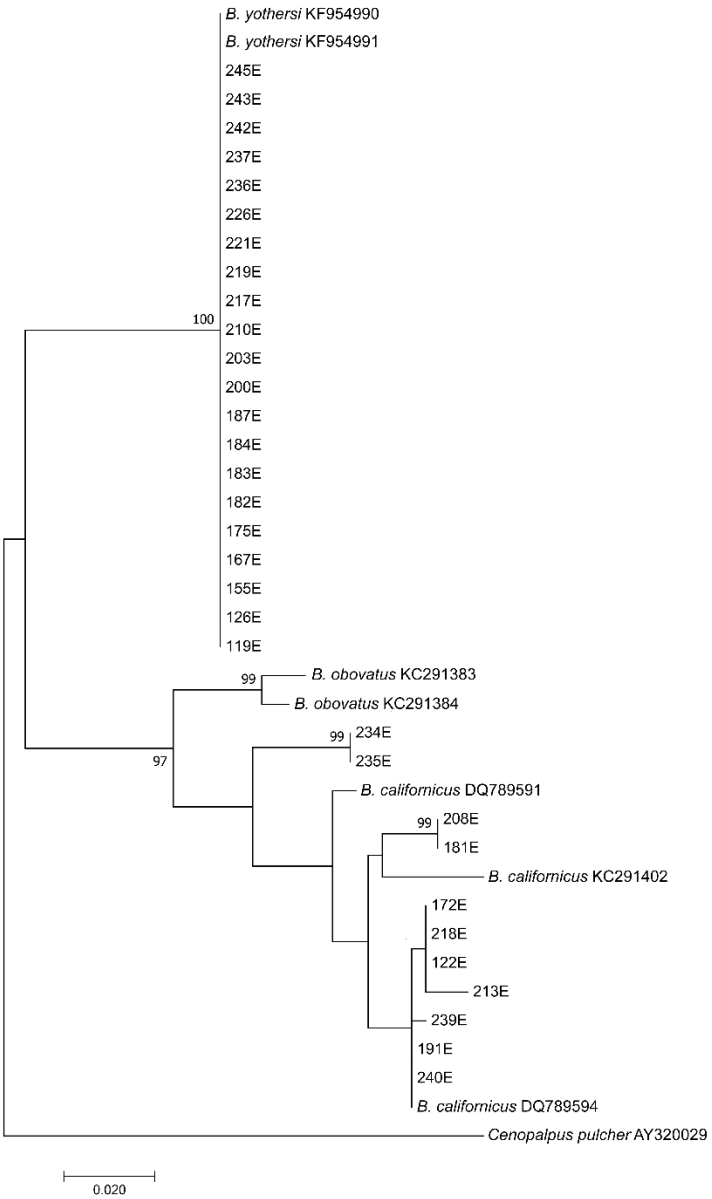


**Figure 1**. Dendrogram inferred from Maximum Likelihood analysis of subsamples of COI sequences. Analysis was done using a subsample of sequences as an example of the outcome. Other *Brevipalpus* species and *Cenopalpus pulcher* (Canestrini and Fanzago) (Acari: Tenuipalpidae) were used as the references and the outgroup respectively, and are labelled according to their GenBank accession numbers. Only bootstrap values above 80% are shown.

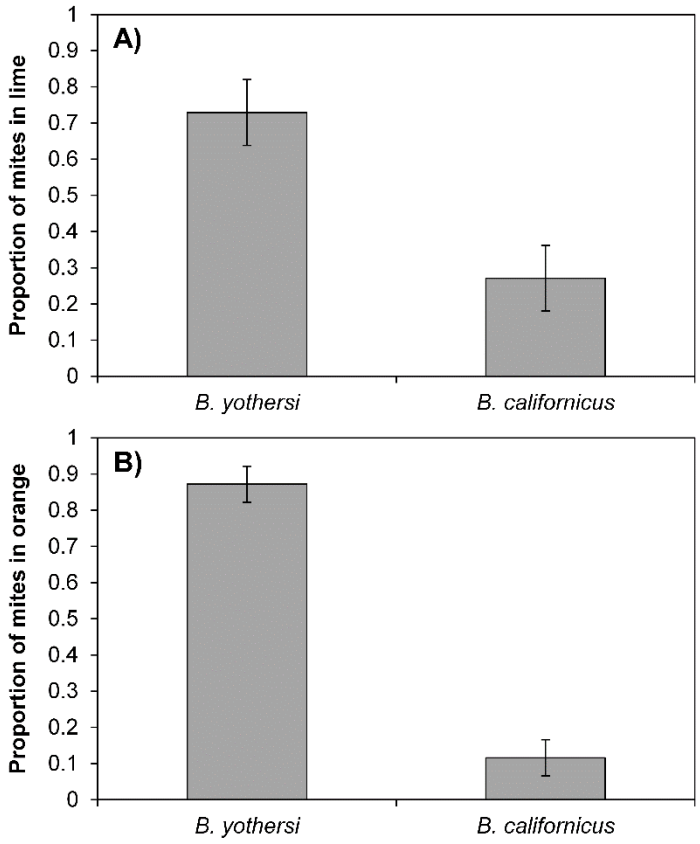


**Figure 2**. Proportion of *B. yothersi* and *B. californicus* mites found on A) lime and B) orange. Error bar represent standard error of the difference of the mean.

## Discussion

In line with previous reports (Sanchez-Velazquez et al. 2015; Salinas-Vargas et al. 2016), we only found *B. yothersi* and *B. californicus* in the citrus orchards we sampled. We can also confirm previous studies showing that *B. yothersi* is the most abundant and widely distributed species in Mexican citrus orchards (Fig. 2). Although *B. californicus* was found on both citrus species, it was always in very low densities, suggesting that it is less successful in establishing in citrus orchards than *B. yothersi*; it is possible that *B. californicus* may have other more suitable plant hosts but this remains to be confirmed experimentally. Recently, García-Escamilla et al. (2018) reported that both *B. yothersi* and *B. californicus* established on all the citrus species they studied. However, the

authors provided no population parameters, making it difficult to assess whether any of the species were more successful than others at establishing on the particular citrus species studied.

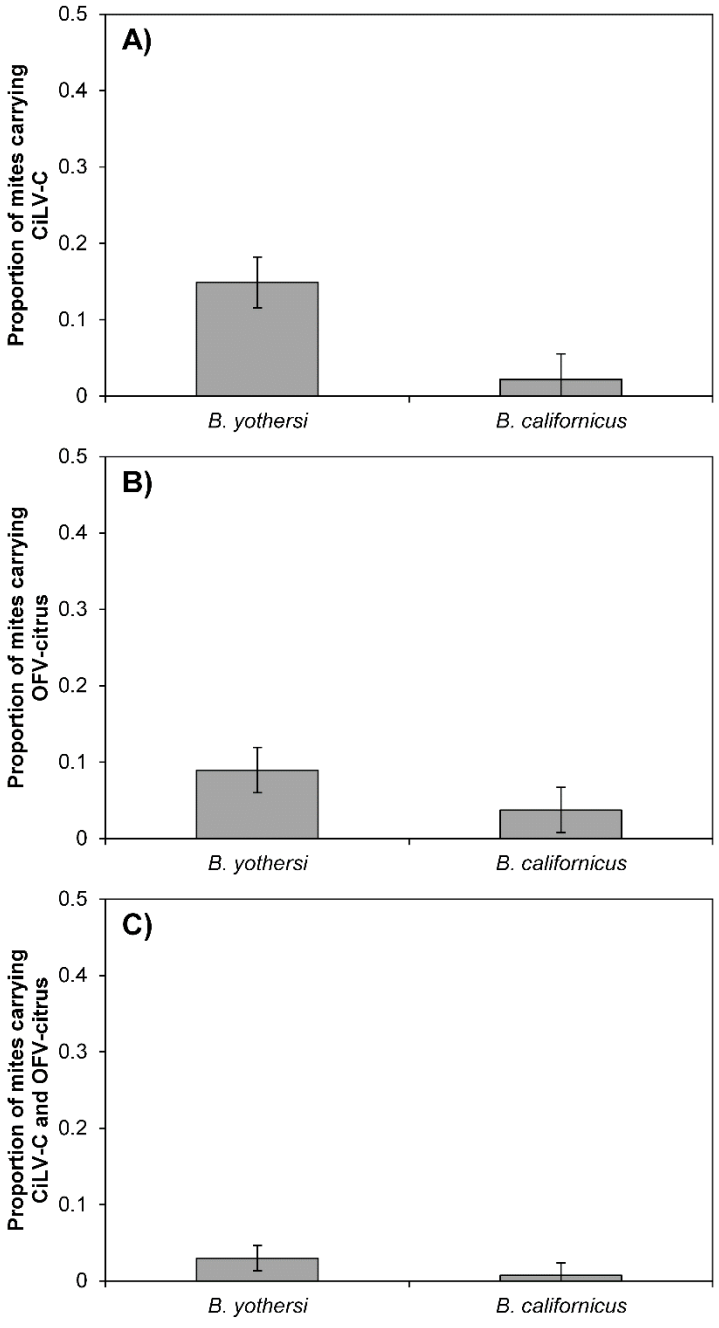


**Figure 3**. Proportion of mites of the species *B. yothersi* and *B. californicus* carrying: A) CiLV-C, B) OFV-citrus and C) both CiLV-C and OFV-citrus. Error bars represent standard error of the difference of the mean.

Our results indicate that *B. yothersi* and *B. californicus* can acquire both CiLV-C and OFV-citrus viruses, but that *B. yothersi* appears to be more efficient at acquiring CiLV-C than *B. californicus* (Fig 3A). It is therefore likely that *B. yothersi* may also be more efficient at transmitting CiLV-C than *B. californicus,* as suggested by García-Escamilla et al. (2018). The situation is different for OFV-citrus virus; despite being found in fewer mites than CiLV-C, its frequency of occurrence was similar in both *B. yothersi* and *B. californicus*. It has been reported that the transmission method for CiLV-C is of the persistent circulate type (Tassi et al. 2017), and that it does not replicate in the mite (Kitajima and Alberti 2014; Tassi et al. 2017). Whether OFV-citrus has the same transmission type remains to be demonstrated. However, the similarity in symptomatology between OFV-and CiLV-C (Roy et al. 2015b) suggests that this is possible. Therefore, it is likely that both *Brevipalpus* species have the capability to transmit the OFV-citrus. Previous studies have reported that, under controlled conditions, *B. californicus* can transmit OFV virus to New Zealand spinach (*Tetragonia expansa* Murray) and bean (*Phaseolus vulgaris* L.) (Kondo et al. 2003). Currently, no studies have confirming, experimentally, the ability of *B. yothersi* to transmit OFV-citrus to citrus trees and this should certainly be the subject of future experimentation. The fact that, although in very small proportions of mites, both viruses were found in the same individual mite regardless of species (Fig 3C), suggesting that a single individual mite could transmit both viruses, although it is also likely that, even if both viruses are inoculated by a single mite, only one will succeed and infect the plant.

In conclusion, *B. yothersi* and *B. californicus* are the most common species in citrus orchards, with *B. yothersi* being the most abundant in lime and orange trees. CiLV-C and OFV-citrus were found in both *Brevipalpus* species. Significantly more *B. yothersi* individuals contained

CiLV-C compared with *B. californicus*. No differences were found in the number of individuals of either *Brevipalpus* species carrying OFV-citrus or both viruses (CiLV-C + OFV-citrus) together.

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# DISCUSIÓN GENERAL Y CONCLUSIONES

Algunas especies de ácaros del género *Brevipalpus* (Acari: Tenuipalpidae) se han reconocido como vectores del CiLV (Arena *et al.*, 2016a; Bastianel *et al.*, 2010); encontramos a la especie *B. yothersi* como la más ampliamente distribuida en México (Figura 2), coincidiendo con los datos proporcionados por Salinas-Vargas *et al.* (2016) y Sánchez-Velázquez *et al.* (2015); y a el virus del tipo citoplasmático como el más común (Figura 3A). La prevalencia de CiLV-C en B. *yothersi* (Figura 3A) puede ser explicada analizando la relación epidemiológica en este patosistema vector-virus, ésta es el resultado de un proceso coevolutivo que ha generado ventajas a ambos, como sucede con otras interacciones virus-herbívoros explicadas por Casteel y Falk (2016)

El hecho de tener presencia de un tipo de virus en una especie de ácaro no indica que este tenga la capacidad para transmitirlo. Se conoce que *Brevipalpus* transmite al virus de la leprosis de forma persistente circulativa (Tassi *et al.*, 2017), y esto sugiere, que se determinó la presencia de alguno de los virus en cantidades suficientes para su reconocimiento por técnicas moleculares. Suponemos que algunos ácaros podrían tener cantidades bajas de inoculo y que, por tanto, aunque el virus podía estar presente no se expresó en los resultados.

Freitas *et al*. (2018) refieren que las hojas infectadas con CiLV-C parecen ser las preferidas por los ácaros para la colonización y oviposición, coincidiendo con lo observado por nosotros en campo, pues se identificó preferencia hacia hojas dañadas, no solo por el virus de la leprosis, si no por aquellas con presencia de lesiones generadas por insectos. Además, se debe considerar que el virus al debilitar las defensas de la planta, facilita la colonización de los ácaros de una manera más

eficiente (Arena *et al.,* 2016b), desencadenando así, claramente el sistema inmune de la planta (Arena *et al.,* 2016b).

Las bajas densidades de población detectadas en la toma de muestras, sugieren que incluso una baja población puede llegar a causar daños importantes a la producción, lo cual es también mencionado por Maldonado *et al.* (2016) quienes señalan que los ácaros vectores tienen baja capacidad ambulatoria y que, por tanto, su dispersión se lleva a cabo, principalmente, en árboles contiguos de una fila; esto es consecuencia de sus hábitos alimentarios (Rodrigues *et al.* 2001). Aunque también podría deberse a que el muestreo fue al azar y que simplemente se tomaron hojas poco infestadas.

Otro factor a considerar son las condiciones climáticas. Laranjeira *et al.* (2015) señalan que el aumento de insolación y el fotoperíodo promueven una mayor tasa de infestación por *B. phoenicis*, especie que ha sido subdividida en ocho especies, dentro de la cual se incluye a *B. yothersi* (Beard *et al.,* 2015).

Debido a que muy pocos individuos de *B. californicus* fueron recolectados, no se pudo hacer un análisis para determinar si la prevalencia de los virus causantes de la leprosis estaba relacionada con este vector, ni con el cítrico donde fue recolectado. Sin embargo, la alta prevalencia de *B. yothersi* en todas las especies de cítricos, indica que no influye el hospedero en el desarrollo de esta especie.

Con respecto a la prevalencia de los virus, estos sí pueden estar influenciados por la especie de cítrico, ya que CiLV-C se desarrolla mayormente en naranja que en limón y OFV-citrus es más común en limón que en naranja. No obstante, podemos encontrar a ambos virus tanto en naranja, mandarina, limón y toronja, de acuerdo con lo observado por González-García *et al.* (2019, en prensa).

Las dos especies de ácaros que se encontraron en los huertos muestreados fueron *B. yothersi* y *B. californicus*, siendo el primero el más predominante.

La prevalencia del virus citoplasmático (CiLV-C) fue mayor que el de tipo nuclear (OFV-citrus), el cual se detectó mayormente en *B. yothersi*, en comparación con el OFV-citrus que se encontró tanto en B. *yothersi* como en *B. californicus*.

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