El Colegio de la Frontera Sur

Variabilidad genética del nematodo *Metaparasitylenchus hypothenemi* parásito de la broca del café

TESIS
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Con orientación en Entomología Tropical

Por

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Variabilidad genética del nematodo *Metaparasitylenchus hypothenemi* parásito de la broca del café

Para obtener el grado de **Maestro en Ciencias en Recursos Naturales y Desarrollo Rural**.

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Dedicatoria

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RESUMEN

El nematodo *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) es un parásito de la broca del café *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae) en México y Guatemala. En esta investigación, nuestros objetivos fueron estimar la variabilidad genética de las poblaciones de *M. hypothenemi*, así como estimar la filogenia intra e inter-específica y determinar el potencial del gen COI para identificar al nematodo. Para ello, se colectaron adultos de la broca del café en 18 localidades de la región Soconusco, Chiapas; de los cuales, se obtuvieron los nematodos. Se utilizaron cinco hembras adultas de *M. hypothenemi* por cada localidad para realizar la extracción de ADN, amplificación y secuenciación del fragmento de 648 pb del gen COI. Se analizaron un total de 76 secuencias, dando como resultado 6 haplotipos, de los cuales, el haplotipo H1 fue el más frecuente y el probable ancestral. Los valores más altos de diversidad genética se registraron en las poblaciones de Salvador Urbina, La Alianza y Los Cacaos y se observó alta diferenciación genética y flujo genético restringido entre poblaciones (F_{ST}=0.66, Nm= 0.5, $p<0.001$). El análisis filogenético de máxima verosimilitud reveló dos linajes bien diferenciados (100 % bootstrap). Estos resultados sugieren que la variación y diferenciación genética entre poblaciones de *M. hypothenemi* son afectadas por el distanciamiento geográfico y probablemente con procesos adaptativos mediados por factores ambientales locales como la fragmentación del paisaje, el clima y la historia evolutiva. Además, se plantea la posibilidad de que *M. hypothenemi* esté constituida por dos linajes. Finalmente, se confirma la viabilidad de las secuencias del gen COI para identificar al nematodo *M. hypothenemi* y el potencial de esta herramienta para estudiar sus poblaciones desde una perspectiva molecular.

Palabras clave: diversidad genética, COI, árbol filogenético, gen, broca del café

CAPITULO 1

1.- Introducción

La broca del café (BC) *Hypothenemus hampei* (Ferrari) (Coleóptera: Curculionidae: Scolytinae), es la principal plaga que afecta a cultivo de café (Le Pelley 1968; Vega et al. 2009; Vega et al. 2015) considerado como uno de los productos agrícolas que más se comercializa en el mundo (Bongase 2017; Vegro y de Almeida 2020). La BC fue introducido al continente Americano a través de semillas de café infestadas procedentes de la República Democrática del Congo en 1913 (Berthet 1913), reconocida como sitio de origen de este insecto. Posteriormente, la BC se dispersó a todas las zonas productoras de café en América y el Caribe (Vega et al. 2009). Se infiere que este insecto se estableció en Brasil y consecutivamente invadió Guatemala y posteriormente México. En México, la BC fue detectado por primera vez en 1978 (Baker et al. 1989). Actualmente, se ha distribuido en los 13 estados cafetaleros más importantes de la República Mexicana: Chiapas, Veracruz, Oaxaca, Puebla, Guerrero, Hidalgo, San Luis Potosí, Nayarit, Jalisco, Tabasco, Colima, Querétaro y Morelos (SENASICA 2018). La BC es una plaga primaria, porque daña directamente el producto que se consume (fruto de café), principalmente por efecto de la alimentación de la hembra colonizadora y su progenie (Damon 2000; Gauthier 2010). Además, los ataques pueden propiciar la entrada de microrganismos que se desarrollan en el fruto, disminuyendo la calidad del producto y provocando considerables pérdidas económicas (Vega et al. 2009).

Hasta el momento, son muchos los enemigos naturales de la BC que han sido estudiados y usados como agentes de control biológico de este insecto plaga, entre los que destacan algunos parasitoides (Bustillo et al. 2002; Infante et al. 2005; Espinoza et al. 2009), depredadores (Henaut et al. 2001; Philpott y Armbrecht 2006; Armbrecht y Gallego 2007; Kellermann et al. 2008) y entomopatógenos (bacterias, hongos y nematodos) (Bustillo et al. 2002; Molina y López 2002; Sánchez y Rodríguez 2007). A pesar de la cantidad de enemigos naturales evaluados, la naturaleza criptica de este insecto limita el tiempo de acción de los agentes de control biológico. Sumado a esto, la dificultad en el establecimiento de las crías, problemas en la producción, reducidas tasas de parasitismo,

necesidad de contacto directo con el insecto, problemas de almacenamiento y altos costos de producción son factores que han sido determinantes en su uso (Ruales 1997; Infante et al. 2001; Vega et al. 2009).

Un campo relativamente inexplorado, son los parásitos de insectos, los cuales no matan a su hospedero, pero ocasionan un daño fisiológico reduciendo o evitando la fecundidad del insecto hembra y disminuyendo la longevidad (Vega et al. 2015). En ese sentido, se ha reportado la presencia de dos nematodos parásitos infectando a la BC de forma natural. El primero, *Panagrolaimus* sp. (Rhabditida: Panagrolamidae) en la India (Varaprasad et al. 1994) especie que no fue identificada y su naturaleza parasitaria no ha sido estudiada. El segundo, *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae), el cual se ha reportado parasitando a larvas, pupas y adultos de la BC en México (Castillo et al. 2002; Poinar et al. 2004). *M. hypothenemi* se considera el primer nematodo que parasita de manera natural a la BC en América (Vega et al. 2009).

1.1.- Generalidades de *Metaparasitylenchus hypothenemi*

1. 1. 1.- Características de la hembra fertilizada de vida libre (HFV)

La ovoviviparidad de *M. hypothenemi*, es una de las características que se ha utilizado para reconocer a la especie en campo debido a que es relativamente fácil observar los huevos en la HFV (Pérez et al. 2015). Las HFV poseen un cuerpo blanco, recto o ligeramente curvado (con el dorso hacia afuera); cono de la cabeza ausente, estilete presente, normalmente no retirado al cuerpo; poro excretor cerca del extremo de la cabeza; vulva subterminal; ano no visible (Poinar et al. 2004) (Fig. 1).

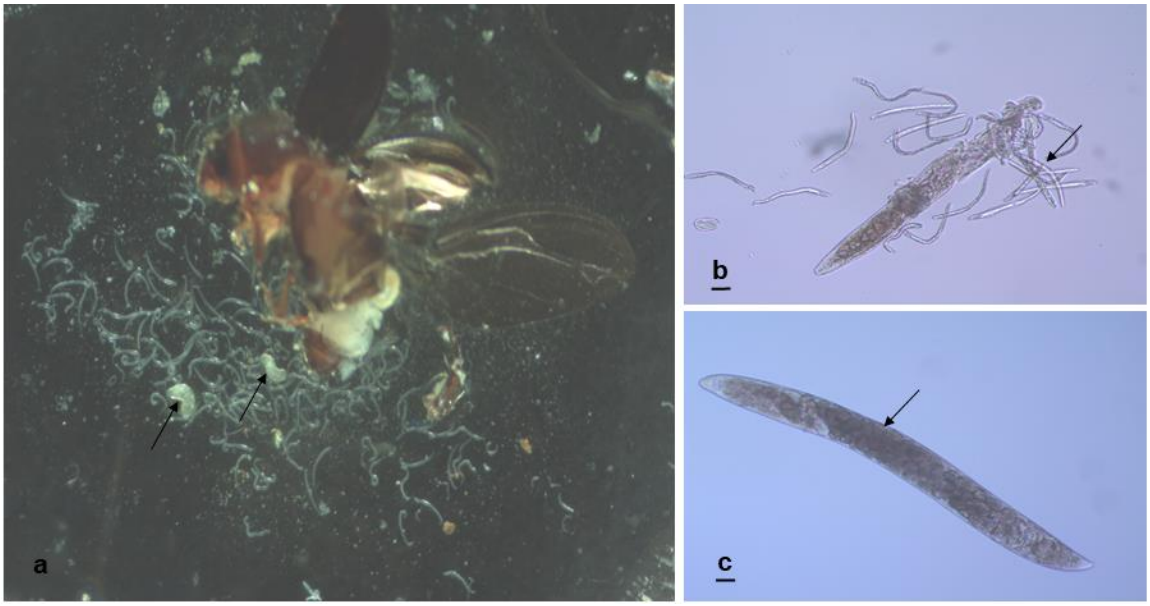

Fig.1. *Metaparasitylenchus hypothenemi.* a. Hembra fertilizada de vida libre (HFV) y juveniles extraídas de la cavidad corporal de *Hypothemus hampei*. Note la diferencia de tamaño en la HFV y los juveniles. Longitud de *H. hampei* = 1.7 mm. b. Juveniles emergiendo del interior de la HFV, Barra: 37 µm. c. Extremo posterior de la HFV que muestra los huevos en desarrollo, Barra: 30 µm; (Fotos tomadas en el presente trabajo utilizando el microscopio Olympus CX31 con el objetivo 10x).

1. 1 .2.- Ciclo de vida y modo de acción

Metaparasitylenchus hypothenemi posee un ciclo de vida simple de aproximadamente 16 días, incluye el huevo, cuatro estadios juveniles separados entre sí por mudas (J1-J4) y los adultos. Las hembras, en su etapa adulta, tienen la capacidad de infectar al hospedero luego de ser fertilizadas. A pesar de que no está claro el cómo la HFV infecta a la BC, autores como Castillo et al. (2002) sugirieron que la infección ocurre en el suelo, dentro de frutos de café infestados que han caído sobre la hojarasca. Asimismo, Poinar et al. (2004) sugirieron que la infección de la BC es iniciada por la HFV, quien activamente busca a un hospedero apropiado para introducirse a través de las aberturas naturales (boca, ano y espiráculos) y penetra la pared intestinal del insecto hasta llegar al hemocele. Una vez en el hemocele, la HFV aumenta progresivamente de tamaño al mismo tiempo que ocurre el desarrollo de los huevos. La primera muda (J1) se inicia

dentro del huevo con la cutícula fundida que queda en la cáscara del huevo o se saca del huevo y se deposita en el útero de la hembra. Después de que los huevos eclosionan y los juveniles de primera etapa han abandonado a la progenitora, la segunda muda (J2) ocurre dentro del hospedero. Las mudas subsiguientes (J3 y J4) se dan después de que los juveniles emergen del hospedero a través de los tractos alimentarios y aberturas genitales, este periodo es considerado de vida libre. Cuando adquieren la madurez sexual, los nematodos copulan y la HFV busca un nuevo hospedero. Las hembras suelen eliminar las cutículas de la tercera y cuarta muda por separado o simultáneamente, mientras que los machos las eliminan simultáneamente. Los mismos autores sugieren que las hembras de la BC parasitadas, son las responsables de transportar a los nematodos al fruto de café en planta, después de abandonar los frutos secos sobre la hojarasca. Cuando el insecto hembra parasitada oviposita dentro del fruto en la planta, simultáneamente los nematodos juveniles emergen del cuerpo del insecto y permanecen dentro del fruto. Al eclosionar los huevos del insecto hembra, los nematodos juveniles han alcanzado su madurez sexual. En este momento, las HFV ingresan a las larvas del insecto. Luego, los nematodos crecen lentamente y pasan a través de las larvas, pupas y luego a los adultos causando una reducción en la fecundidad o la esterilidad de las hembras adultas. La esterilidad de la BC causada por *M. hypothenemi*, ha sido estudiada por Castillo et al. (2019), demostrando que el nematodo provoca no solo la esterilidad parcial, sino en múltiples ocasiones la esterilidad completa de las hembras de la BC.

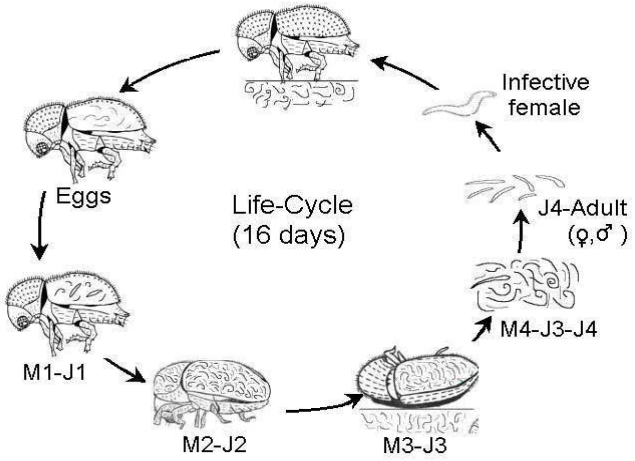

Fig. 2. Esquema del ciclo de vida de *Metaparasitylenchus hypothenemi* (Tomado de Poinar et al. 2004).

1. 1. 3.- Distribución

Metaparasitylenchus hypothenemi fue detectado por primera vez infectando a hembras adultas de la BC en una plantación comercial de café localizada en el municipio de Cacahoatán (Soconusco, Chiapas, México) (Castillo et al. 2002; Poinar et al. 2004). Este hallazgo, condujo a la realización de un muestreo intensivo en los cafetales de la región, registrando al nematodo en 12 de 20 localidades distribuidas en los municipios de Acapetahua, Tuzantán, Tapachula, Cacahoatán y Unión Juárez (Pérez et al. 2015). Los mismos atores sugirieron que *M. hypothenemi* podría estar distribuido ampliamente en todas las áreas cafetaleras del estado de Chiapas. Adicionalmente, se ha detectado a este nematodo infectando a la BC en Honduras y Guatemala (A. Castillo, comunicación personal).

En cuanto a la distribución geográfica de los nematodos asociados a insectos, autores como Millar y Barbercheck (2002) y Stuart et al. (2015), observaron que la distribución de los nematodos depende principalmente de la capacidad de dispersión de sus hospederos. Por lo que no sería extraño que la BC sea el responsable de dispersar a *M. hypothenemi* a través de largas distancias y consecuentemente ocasionar el aislamiento reproductivo entre sus poblaciones, ya que la BC puede desplazarse en un rango de 300 a 500 m desde su área de reproducción a través de corrientes de aire (Leroy 1936; Baker 1984). Además, Poinar et al. (2004), sugirieron la posibilidad de que la BC haya sido el responsable de transportar a *M. hypothenemi* durante su proceso de invasión a los países cafetaleros, aunque, actualmente no hay registros de este parásito en otras regiones del mundo.

Hasta el momento, sólo se conocen algunos aspectos de la biología y ecología de esta especie (Castillo et al. 2002; Poinar et al. 2004; Pérez et al. 2015; Castillo y Infante 2019; Castillo et al. 2019), pero no se han realizado estudios de genética de poblaciones de *M. hypothenemi*. La realización de este tipo de estudios es importante para proporcionar información sobre la diversidad genética de la especie, la estructura de sus poblaciones así como dilucidar los procesos evolutivos involucrados en su distribución actual.

1. 2.- ADN mitocondrial (ADNmt) y el gen Citocromo Oxidasa Subunidad I (COI)

El análisis de secuencias de ADNmt ha sido muy utilizado para estudios relacionados en el campo de la genética de poblaciones y la sistemática (Behura 2006; Arif y Khan 2009). Su aplicación ha logrado determinar el grado de variación y divergencia genética dentro y entre poblaciones de especies de nematos asociados parasíticamente a insectos (Grant 1994; Blouin et al. 1999; Blouin 2002; Oliveira et al. 2011; Valadas et al. 2014; Zieman et al. 2015). Existen múltiples ventajas que convierten al ADNmt como el marcador genético más apropiado para este tipo de estudios: a) está presente en casi todos los organismos eucariontes, en cada célula y en múltiples copias, lo que la hace factible para su amplificación de forma relativamente sencilla, b) se hereda estrictamente de forma materna por lo que carece de recombinación, c) presenta una alta tasa de evolución y d) carece de intrones (Avise 1987; Vázquez 2007; Gissi et al. 2008). El genoma mitocondrial está constituido por 37 genes que codifica dos ácidos ribonucleicos ribosomales (ARNr), 22 ARN de transferencia (ARNt) y 13 genes para proteínas: tres subunidades del Citocromo Oxidasa (COI, COII, COIII), una subunidad del citocromo b (cyt b) oxido reductasa, siete subunidades (ND-1, 2, 3, 4, 4L, 5 y 6) del complejo NADH deshidrogenasas y dos subunidades (6 y 8) del complejo ATP cintetasa (DiMauro 2007). Entre los genes que codifican para proteínas, el Citocromo Oxidasa Subunidad I (COI), es uno de los marcadores más eficaces que se ha utilizado para resolver con éxito filogenias en nematodos entopomatógenos del género Steinernema y Heterorhabditis (Szalanski et al. 2000; Nadler et al. 2006; Lis et al. 2018) y de la familia *Travassosinematidae* (Singh et al. 2015). Esto se debe a que es altamente variable a nivel intra-específico y presenta un alto nivel de conservación en su contenido entre diferentes organismos eucariontes (Gissi et al. 2008; Galtier et al. 2009). Lo que ha permitido la separación de especies estrechamente relacionadas y entre organismos de la misma especie (Hebert et al. 2003). Por ello este marcador ha resultado una herramienta útil en la identificación de organismos, comúnmente conocido como el código de barras de la vida (Francis et al. 2010).

1.3.- Código de Barras de la Vida (Barcode of Life Data Systems; BOLD)

El proyecto código de barras de la vida, se presenta como una iniciativa mundial, que tiene como objetivo inventariar toda la biodiversidad animal a través de un fragmento estándar de 648 pb del gen COI (Hebert et al. 2004). Esta herramienta permite realizar identificaciones taxonómicas de especies y declarar especies nuevas. El protocolo consiste en la extracción del ADN y secuenciación del fragmento estándar del espécimen de interés, una vez obtenidas estas secuencias, se realiza un alineamiento para comparar las secuencias de interés con las ya disponibles en la base de datos del sistema de código de barras de la vida (Barcode of Life Data Systems: BOLD). Cuando las secuencias de códigos de barras de los especímenes no coinciden con ninguna especie presente en la base de datos de referencia, se puede deducir que la base de datos está incompleta y que posiblemente se trata de una especie nueva (Casiraghi et al. 2010). Actualmente esta base de datos cuenta con 19,603 registros disponibles al público del filo Nematoda, representando 2,312 especies reportadas para 130 países. De los cuales solo 11 especies (0.47%) de la familia *Allantonematidae* han sido registradas (BOLD, www.boldsystems.org.). Estos datos demuestran el gran vacío de información para nematodos con asociación parasítica a insectos.

Esta tesis se ha dividido en tres capítulos, esta sección introductoria corresponde al capítulo I. En el capítulo II, se presentan los resultados de la investigación, a través de un manuscrito, en el cual analizó la variabilidad genética entre poblaciones de *M. hypothenemi*. Finalmente, en el capítulo III se presentan las conclusiones generales de este trabajo.

2.- Justificación

A pesar de la disponibilidad comercial de nematodos entomopatógenos y su uso como agentes de control biológico contra la BC, aún no está clara la efectividad de estos entomopatógenos en campo, teniendo en cuenta diversas limitaciones: costo, almacenamiento y aplicación adecuada del producto y costos de mano de obra. Es por esto que surge la necesidad de estudiar a *M. hypothenemi*, parásito natural de la BC, que ha demostrado tener potencial para causar un daño fisiológico a este insecto. Estudios encaminados al conocimiento de la bio-ecología y el origen geográfico de este nematodo son necesarios para determinar su potencial como agente de bio-control contra la BC.

Este trabajo representa el primer estudio de genética de poblaciones de *M. hypothenemi* en la única región en donde ha sido reportada. De esta manera, se espera contribuir con la generación del conocimiento científico base al informar por primera vez la diversidad genética de *M. hypothenemi*, la inferencia de múltiples hipótesis de su origen geográfico y la relación entre sus poblaciones.

3.- Pregunta de investigación

¿Estudiar la variabilidad genética de *M. hypothenemi* permitirá diferenciar sus poblaciones geográficas?

4.- Hipótesis

La especie *M. hypothenemi* presenta niveles de variabilidad genética que permiten diferenciar sus poblaciones geográficas.

5.- Objetivos

5.1- Objetivo general

Estimar la variabilidad genética y evaluar el gen COI como marcador molecular de la especie *M. hypothenemi.*

5.2- Objetivos específicos

- Identificar los haplotipos del gen COI en las poblaciones naturales del nematodo *M. hypothenemi.*
- Estimar las relaciones evolutivas de *M. hypothenemi* con otras especies de nematodos registradas en la base de datos GenBank (base de datos de secuencias genéticas) a partir de los datos obtenidos con el marcador COI.
- Determinar la utilidad del gen COI como marcador de la especie mediante la metodología del “Código de Barras”.

CAPÍTULO 2.- “Genetic Variability of the Coffee Berry Borer Parasitic Nematode *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) using COI Sequences”

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Abstract

The nematode Metaparasitylenchus hypothenemi (Tylenchida: Allantonematidae) is a parasite of the coffee berry borer (Hypothenemus hampei; Coleoptera: Curculionidae: Scolytinae) in Mexico and Guatemala. Our goals were to determinate the potential of the mitochondrial gene cytochrome oxidase subunit I (COI) to identify this parasitic nematode, as well as to analyse the phylogeny and genetic variation of its populations in Mexico. Five reproductive females of M. hypothenemi in 18 localities were obtained from parasitized wild coffee berry borer females for DNA extraction, amplification and COI sequencing. The COI marker has the potential to identify M. hypothenemi and to differentiate it from other parasitic nematodes registered in the COI gene bank. Our phylogenetic analyses showed two well differentiated lineages in the M. hypothenemi populations. Six haplotypes of M. hypothenemi were identified in 18 populations distributed along a 100 km long linear transect. No ubiquity of haplotypes was observed in these populations, with a haplotype most frecuent observed in 16 populations. Representative, endemic, and geographically isolated haplotypes were observed in some of these populations. M. hypothenemi populations showed a high genetic differentiation, with a restricted gene flow among the 18 sampled populations. These results confirm the viability of the COI gene sequences to identify this species and demonstrates the potential of this tool to study their populations from a molecular perspective.

Keywords	DNA barcode, phylogenetic tree, nematode, coffee berry borer

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Data will be made available on request

Genetic Variability of the Coffee Berry Borer Parasitic Nematode *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) using COI Sequences

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Declarations of interest: none

Abstract.

The nematode *Metaparasitylenchus hypothenemi* (Tylenchida: Allantonematidae) is a parasite of the coffee berry borer (*Hypothenemus hampei*; Coleoptera: Curculionidae: Scolytinae) in Mexico and Guatemala. Our goals were to determinate the potential of the mitochondrial gene cytochrome oxidase subunit I (COI) to identify this parasitic nematode, as well as to analyse the phylogeny and genetic variation of its populations in Mexico. Five reproductive females of *M. hypothenemi* in 18 localities were obtained from parasitized wild coffee berry borer females for DNA extraction, amplification and COI sequencing. The COI marker has the potential to identify *M. hypothenemi* and to differentiate it from other parasitic nematodes registered in the COI gene bank. Our phylogenetic analyses showed two well differentiated lineages in the *M. hypothenemi* populations. Six haplotypes of *M. hypothenemi* were identified in 18 populations distributed along a 100 km long linear transect. No ubiquity of haplotypes was observed in these populations, with a haplotype most frecuent observed in 16 populations. Representative, endemic, and geographically isolated haplotypes were observed in some of these populations. *M. hypothenemi* populations showed a high genetic differentiation, with a restricted gene flow among the 18 sampled populations. These results confirm the viability of the COI gene sequences to identify this species and demonstrates the potential of this tool to study their populations from a molecular perspective.

Key words: DNA barcode, phylogenetic tree, nematode, coffee berry borer

1. Introduction

The nematode *Metaparasitylenchus hypothenemi* Poinar (Tylenchida: Allantonematidae) is an obligate endoparasite of the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae), the most important insect pest of coffee worldwide (Le Pelley, 1968; Barrera, 1984; Vega et al., 2015). *Metaparasitylenchus hypothenemi* was discovered in a commercial coffee plantation in southeastern Mexico attacking *H. hampei* adults (Castillo et al., 2002), a pest detected for the first time in Mexico in 1978 (Baker, 1984). *Metaparasitylenchus hypothenemi* is ovoviviparous and its reproductive females and/or several hundred juveniles can be easily recognized inside the haemocoel from a parasitized coffee berry borer, while the eggs can only be observed inside reproductive females (Poinar et al., 2004). Although details about its biological cycle are known, ecological aspects related to its adaptation to the host and environment are still unknown. The geographical distribution of parasitic nematodes associated with insects depends of the dispersal capacity of their hosts (Millar and Barbercheck, 2002; Stuart et al., 2015). Poinar et al. (2004) suggests the possibility that *M. hypothenemi* was carried by this pest during its invasion to the coffee-growing countries of America, although there are no records of this parasite in other regions of the world.

The spread of *H. hampei* to coffee-growing countries around the world has been caused mainly by human activities (Damon, 2000), although it is known that this pest can use air currents to move 300 to 500 m from its breeding area (Leroy, 1936; Baker, 1984), which could influence long distance dispersal and gene flow patterns. Some aspects related to the biology and ecology of this nematode species have already been studied (Poinar et al., 2004; Castillo and Infante, 2019; Castillo et al., 2019), but there are no studies on the population genetics of *M. hypothenemi*. DNA barcoding based on the mitochondrial gene cytochrome oxidase subunit 1

(COI) can be useful in identifying individual specimens (Hebert et al., 2003). Mitochondrial DNA (mtDNA) has also been used to elucidate the evolutionary relationships between species and their population divergence because it is maternally inherited, lacks recombination or high mutation rates, and for its relative ease for DNA amplification from very small amounts of tissue (Marsjan and Oldenbroek, 2007). The COI gene has potential for the construction of phylogenies among closely related individuals (Saeb and David, 2014) and has been successfully used to infer phylogenetic relationships between nematode species (Saeb and David, 2014; Lis et al., 2018; Fitza et al., 2019), including the parasitic nematode *Deladenus proximus* (Tylenchida: Neotylenchidae) (Hartshorn et al., 2017), as well as the entomopathogenic nematodes *Heterorhabditis* (Blouin et al., 1999) and *Steinernema* (Kikuchi, 2016). The objective of this study was to analyse the genetic divergence, genetic structure and phylogeny of the natural populations of *M. hypothenemi* in the Soconusco, the only region in the world where this nematode has been systematically recorded.

2. Materials and Methods

2.1. Nematode collection

Five reproductive females of *M. hypothenemi* were collected each of 18 localities from the Soconusco region, Chiapas, Mexico (Table 1). Infective juveniles were excluded from this study, due to anecdotal records of another species of nematode parasitizing *H. hampei* in this region (Poinar et al., 2004). Nematodes were extracted from the abdominal cavity of *H. hampei* females, which were obtained from 100 coffee berries collected from coffee plants per locality. Each insect was placed on a single cavity microscope slide and immersed in saline solution for nematode dissection using entomological needles and forceps. Eggs can be observed within a

reproductive female of *M. hypothenemi*, whose body width is five times larger than a free-living female. The dissected nematode females were stored in 2.5 ml microtubes containing 96% alcohol at -20 °C, until molecular analysis. The samples obtained in each sampled locality were considered as a population.

2.2. Genomic DNA extraction, PCR amplification and sequencing.

Genomic DNA was individually extracted from the complete body of 90 adult female *M. hypothenemi*. DNA extraction and PCR amplification were performed using the standardized protocols of the Bar Code of Life project (Ivanova et al., 2006). PCR amplification was only achieved in 76 samples, from a 645 bp COI fragment using the universal primers: ZplankF1_t1: 5'-TGTAAAACGACGGCCAGTTCTASWAATCATAA RGATATTGG-3' (forward) y ZplankR1_t1: 5'-CAGGAAACAGCTATGACTTCAGGRTGRCCR AARAATCA-3' (reverse) (Prosser, 2013). The final volume of the PCR mix was 14 µl, containing 0.125 µl of each primer (10 µM), 0.625 µl de $MgCl_2$ (50 mM), 2 µl of ultrapure water, 0.0625 µl of each dNTP (10 mM), 1.25 µl of 10X PCR buffer, 6.25 µl of trehalose10%, 0.06 µl of Taq ADN polimerase 5U/µl (Platinum® Taq, Invitrogen) y 3.5 µl of DNA template. Amplification was carried out on 96-well acrylic plates using a thermal cycler (Master Cycler Pro Eppendorf) under the following conditions: denaturation to 94° C for 1 min, followed by 30-35 cycles of 94 °C for 30 sec, 51-54 °C for 40 sec, 72 ° C for 1 min and a final extension of 72 °C for 10 min. The PCR samples were separated on 2% agarose gels (E-Gel 96 Invitrogen, Carlsbad, CA), stained with ethidium bromide before being observed under UV light.

Positive PCR products were bidirectionally sequenced using M13F and M13R primers (Messing, 1983). The sequences were edited using Code Aligner v. 8.0.1 (Codon Code Corporation, Dedham, Massachusetts) and uploaded to the Barcode of Life Data System database (BOLD,

www.boldsystems.org), where they were labeled as Parasitic Nematode of Coffee Berry Borer (PNBC). COI sequences were deposited in GenBank under accession numbers MT520707 to MT520790.

2.3. Interspecific Phylogenetic Analysis

An interspecific phylogenetic analysis was carried out to know the potential of COI to molecularly identify *M. hypothenemi* and compare its genetic similarity with the sequences of other parasitic nematode species stored in GenBank database. Multiple sequence alignment (ClustalW) was done on 76 sequences obtained from the COI gene using MEGA X v. 7.0.26 (Tamura et al., 2007). Each obtained sequence (560 bp) was compared with the sequences deposited in the GenBank sequence database (http://www.ncbi.nlm.nih.gov/Genbank/) using Basic Logic Alignment Tool (BLAST).

Thirty-five sequences representing 29 parasitic nematode species, showing the highest percentage of genetic similarity, were obtained from the GenBank database. These sequences, together with 12 *M. hypothenemi* sequences were analysed using the maximum likelihood (ML) method with 10,000 start-up replicas, under the GTR + G (General Time Reversible + gamma distribution) evolution model in the MEGA X v software. 7.0.26 (Kumar et al., 2016). Previously, the evolution model was estimated using the JModeltest v. 0.1.1 (Posada, 2008), which allowed choosing the model with the smallest AIC (Akaike Information Criterion), to approximate the process of molecular evolution (Akaike, 1974).

2.4. Intraspecific Phylogenetic Analysis

An intraspecific phylogenetic analysis was performed to estimate evolutionary relationships between sampled populations. The intra-specific phylogeny was estimated using the TIM3 + I

evolution model (Tamura 3- parameter model + invariant sites), as suggested by JModeltest, applying the maximum likelihood method with 10,000 starting replicas, implemented in MEGA X v. 7.0.26 (Kumar et al., 2016). *Howardula aoronymphium* (Tylenchida: Allantonematidae, GenBank: AY589466) was used as an outgroup.

2.5. Genetic Structure and Differentiation

The COI sequences obtained from 18 localities were used to elucidate the structure and genetic diversity of *M. hypothenemi* in relation to its geographical distribution. We estimated the number of segregation sites (S), number of unique sites (Su), mean number of pairwise differences (k), number of haplotypes (h), diversity of COI haplotypes (Hd), nucleotide diversity (Π) and genetic structure using the software DnaSP v. 6.12.03 (Rozas and Librado, 2009).

Possible historical changes in population size were estimated using neutrality tests (D and F) using the software DnaSP v. 6.12.03 (Tajima, 1989; Fu and Li, 1993). The degree of genetic differentiation between *M. hypothenemi* populations was estimated using the fixation index (F_{ST}), and between populations using an analysis of molecular variance (AMOVA), also estimating the number of migrants per generation (Nm) using Arlequinv.3.5 (Excoffiera and Lischer, 2010).

The relationship between genetic differences [FST] and geographic distances between populations was analyzed by applying the Mantel test (Mantel, 1967). Two matrices (genetic differences between pairs of populations versus geographic distances) were compared. The geographical distances (km) were calculated with the QGIS v 3.10.3 software (https://qgis.org/es/site). Statistical analysis was based on 1000 simulations using R studio with the software "Vegan" (Oksanen et al., 2010).

2.6. Haplotype Network

158 To infer each indivicual´s genealogy based on the haplotypes observed in all populations and to
159 display potential alternative connections between them,a network analysis was carried out
160 between the haplotypes of each population, using the Median-Joining criterion with the software
161 NETWORK v. 5.0.
162
163 **3. Results**
164 3.1. Interspecific Phylogenetic Analysis
165 BLAST analysis shows that *M. hypothenemi* and 29 species of parasitic nematodes obtained from
166 the GenBank database have a genetic similarity within the range 77.8-84.8% (Table 2). The
167 phylogenetic relationship of 12 *M. hypothenemi* sequences in relation to 35 additional sequences
168 from 29 species of parasitic nematodes in GenBank resulted in a separate phylogenetic tree with
169 three well-defined clades (Fig. 1). The inter-specific phylogenetic analysis grouped the *M.*
170 *hypothenemi* specimens in clade III, separated and independent of the rest of the species included
171 in the analysis (99% bootstrap). At this last clade, two subgroups were formed with the *M.*
172 *hypothenemi* sequences, isolating the samples collected in the Los Cacaos locality (Fig. 1).
173 Furthermore, the specimens from the GenBank were grouped into two clades. Clade I, consisting
174 of 28 species of parasitic nematodes; and clade II, formed only by the species *Dracunculus*
175 *insignis* (Camallanida: Dracunculidae) (Fig. 1).
176 3.2. Intraspecific Phylogenetic Analysis
177 The tree topology revealed two independent clades with high support (100% bootstraps). Clade I
178 formed by two monophyletic groups, which corresponds to 96% of the specimens distributed in
179 the municipalities of Tapachula, Cacahoatán, Unión Juárez and San Pablo (Guatemala). Clade II
180 formed by three specimens belonging to the Los Cacaos population, located in the municipality

of Acacoyagua. A phylogenetic separation (16.5%) was observed between clade I and clade II specimens (Fig. 2).

3.3. Genetic Structure and Differentiation

The analysis of the 76 sequences revealed 93 polymorphic sites (16.6%), whose A-T content (67.6%) was higher than the G-C content (32.4%) in the 18 populations of *M. hypothenemi*. A total of six haplotypes were identified in a linear transect (approximately 100 km) in the Soconusco region (Fig. 3A). These haplotypes are distributed heterogeneously in the 18 populations with a frequency of 1-3 haplotypes/population (Table 3). The greatest diversity (three haplotypes) was observed in the SU, LC y LA (two haplotypes) populations, followed by SL, LU, DM, FO and SA with two haplotypes; while a single haplotype was registered in BR, EZ, RI, SD, SR, SJ, MP, RS, OA and BV populations (Table 3). The most frequent haplotype (H1) was registered in 50 specimens belonging to 16 populations (65.8%), followed by H2 present in 17 specimens belonging to eight populations (22.4%). In contrast, the H3-H4, H5, and H6 haplotypes were endemic to the LC, BR and SU populations, respectively (Fig. 3 B). Most of the populations shared two haplotypes (H1 and H2), without any ubiquitous haplotype. The H1 haplotype was registered in seven geographically adjacent populations, while H1-H2 was registered in seven geographically adjacent populations, indicating a low dispersal of the parasite and restricted gene flow in its populations (Fig. 3 B). H3-H4 were registered only in the LC population, closely related to each other and geographically separated from the rest of the haplotypes (Fig. 3 B). H5 was registered as a unique and endemic haplotype of the BR population, contrasting with H6 mixed with H1 and H2, only registered in the SU population. The geographic distribution patterns of the haplotypes indicate that the populations are genetically separated from each other, by a restricted genetic flow and a high degree of endemism. Likewise,

the geographical distribution of *M. hypothenemi* coincided with its intraspecific phylogeny, clearly separating populations that have different haplotypes (Fig. 3A and B).

The results of the neutrality tests (D for Tajima' and D, F for Fu and Li) are presented in Table 4. None of the populations studied presented statistically significant values (P> 0.05). However, negative values were observed for SU, LU, DM, SA and LC populations. These results indicate that the sequences have evolved in a neutral way and that the mutations produced do not exert selection pressure on them; that is, the null hypothesis of molecular evolution is not rejected and the populations do not show any demographic change (expansion or contraction of the population) (Duret, 2008).

The fixation index (F_{ST}) revealed a pronounced genetic differentiation between the 18 populations (Fst = 0.66, P <0.05), with a low number of migrants per generation (Nm = 0.50). The EZ and BR populations showed greater genetic differentiation in relation to the other populations. The range of genetic distances of the EZ population compared to the rest was 0.05-1.0, distant from each other by a range of 1.7-66.5 km. The range of the genetic distance between BR and the other populations was 0.7-1.0, distant from each other by a range of 9.5-50.1 km. Likewise, LC and BR were the most geographically distant populations. The geographical separation range of LC in relation to other populations was 50.1-87.1 km, with a genetic distance range of 0.5-0.7 between them. The BR population, geographically separated from the rest by 9.5-50.1 km, showed a genetic distance of 0.7-1.0 in relation to the others. The geographically closest populations (1.8 - 7.5 km) are in the municipality of Unión Juárez, whose genetic distance was 0 (Table 5). These results indicate that *M. hypothenemi* populations possess a genetic divergence apparently related to geographic isolation. The AMOVA results indicated that higher genetic variation occurred between populations (66.61%) than within populations (33.39%) (Table 5).

229 The Mantel test indicates a significant correlation between the genetic pairwise distances and the
230 geographic distances (km) of the *M. hypothenemi* populations ($r = 0.4728$, $p < 0.0001$), indicating
231 a pattern of isolation by distance (Fig. 4).
232
233 3.4. Haplotype Network
234 The results of the network analysis between the haplotypes identified for *M. hypothenemi*
235 describe two well-defined clades (Fig. 3B). Clade II grouped the H3 and H4 haplotypes, detected
236 only in the LC population, while the clade I grouped haplotypes H1, H2, H5 and H6, present in
237 rest of the populations studied. The number of mutational steps (88) between clades I and II,
238 indicate genetic isolation. The first clade specimens were collected in the municipalities of
239 Tapachula, Cacahoatán, Unión Juárez and San Pablo (Guatemala), while the second clade
240 specimens were collected in the municipality of Acacoyagua. The frequency of the H1 haplotype
241 in the analyzed populations and the number of its branches (Fig3A), indicate that it could be the
242 ancestral haplotype.
243
244 **4. Discussion**
245 This work demonstrates the applicability of COI for the identification of *M. hypothenemi* and to
246 genetically analyse the natural populations of this coffee berry borer endoparasite. It also shows
247 that the COI is a valuable tool to study the genetic variability of the parasitic nematode *M.*
248 *hypothenemi*, allowing us to know that its populations are genetically different from each other,
249 with representative, endemic and geographically isolated haplotypes in some of these
250 populations.

Nematodes have a wide diversity of habitats and life forms, nevertheless the genetic diversity of this group of organisms is poorly known (Olivera et al., 2011). The identification of *M. hypothenemi* using classical taxonomy has allowed to know distinctive morphological of this species, although its identification and detection remains complicated, due to its small size and the subtleness of its morphological characteristics. For this reason, a fast and reliable method for the identification of these organisms is required. It is feasible to identify nematode species using DNA barcoding. For example, it has been possible to identify eleven new species of marine nematodes using DNA barcoding (Martínez et al., 2020). However, COI is not yet widely used to identify parasitic nematode species. The results obtained by the BLAST analysis showed similarity percentages of less than 95%, demonstrating an information gap when *M. hypothenemi* was compared with evolutionarily close species. So far, only two species have been registered naturally parasitizing coffee berry borer adults: *Panagrolaimus* sp. in India (Varaprasad, 1998) and *M. hypothenemi* in Mexico (Castillo et al., 2002). However, the presence of *M. hypothenemi* has only been detected in this region of the world, although its identification using classical taxonomy is difficult, which would allow to expand our knowledge about the geographic range of this species.

Surprisingly, three specimens from LC population are phylogenetically separated from the other populations, isolated by 88 mutational steps in the haplotype network, which reveals a historical isolation pattern (Slatkin, 1987). In contrast, the rest of the haplotypes are only separated from each other by a mutational step, indicating a recent demographic expansion of the populations (Forster, 2004).

The intraspecific genetic divergence observed in the LC population (16.4%) was higher than the intraspecific limit proposed by the barcode (1-2%; Lanteri, 2007), which was perhaps caused by

the presence of a geographically isolated ancestral haplotype. Our phylogenetic analyses indicate that a sub-speciation process is occurring locally within populations of *M. hypothenemi*, perhaps as a result of mutation, gene drift or natural selection. This type of allopatric speciation is known as peripatric and occurs when few individuals in a population are isolated from the rest (Eichler, 1966). It could also be a different species or a cryptic species, *whith a morphology conserved, but genetically divergent.* However, genetic studies with greater representativeness of the isolated population and additional morphological studies would help to confirm the occurrence of this speciation process. Nuclear markers could be used to determinate if differentiation occurs in the genome or is restricted to mtDNA.

The genetic structure of *M. hypothenemi* populations is mainly influenced by restricted gene flow, conditioned by geographic isolation. Our results indicate that H1 was the most frequent, and perhaps the probable ancestral haplotype, distributed along the border line between Mexico and Guatemala. In addition, the presence of this parasite in coffee plantations from Honduras, previously been described by other authors (Poinar et al. 2004), suggesting the possibility that *M. hypothenemi* is widely distributed in Central America.

The general genetic diversity of *M. hypothenemi* (Hd =0.519, Π=0.0134) has higher Hd values than those reported in another study carried out with the *Heterorhabditis marelatus* ND4 mitochondrial gene, with values close to zero (Hd =0.16, Π= 0.002) (Blouin et al., 1999). Probably, the differences in these results were conditioned by the mobility of the infected host, since the impact of *M. hypothenemi* on the survival of its host is unknown, whereas *H. marelatus* kills its host in 24 ± 48 h (Del pino, 2005), limiting the dispersal of the nematode. This genetic diversity was higher compared to that observed in other studies that used 40-120 specimens per population (Klimpel et al., 2007; Powers et al., 2018), perhaps due to number of samples used per

297 population or because mtDNA presents a high rate of evolution (Brown et al., 1979; Avise et al.,
298 1987). Thus, our sample size may have influenced the estimation of population parameters and a
299 larger number of specimens per population in subsequent studies is recommended.

300 Although *H. hampei* is endemic to Africa (Le Pelley, 1968), Poinar et al. (2004) hypothesized
301 that *M. hypothenemi* infected the insect for the first time in the New World. Coincidentally, the
302 results of the neutrality analysis (D from Tajima and D, F from Fu and Li) show no historical
303 changes in the size of the populations, indicating that the populations studied are not under the
304 effects of natural selection. Thus, it is feasible that parasitism of *H. hampei* by *M. hypothenemi* is
305 a new ecological interaction, as occurs when a parasite is introduced to a new environment, and
306 not an evolutionary response (Bush et al., 2001). The determination of the origin of the parasite
307 and the age of a parasite-host interaction is complex, due to the variety of mechanisms involved
308 in the historical evolution of a parasitism, with the possibility of an initially accidental
309 association (Rico, 2011). However, the diversity of scolytids associated with the coffee
310 plantations of Soconusco is very high (Equihua et al., 1992) and *H. hampei* populations can reach
311 up to 11 million per ha in a mixed plot of robusta and arabica coffee (Baker and Barrera, 1993).
312 Under these conditions, it is probable that an endemic parasitic nematode from this region could
313 have formed one or more new parasite-host associations with the *H. hampei* (Bickford et al,
314 2007), which explain why some populations of *M. hypothenemi* showed negative values in the
315 neutrality tests (D for Tajima and F for Fu and Li), characteristic of a population with a recent
316 demographic expansion (Ramírez et al., 2008). The high values of genetic diversity and the small
317 values of nucleotide diversity also suggest the recent demographic expansion of *H. hypothenemi*
318 populations (Hamilton 2009), occurred probably when this pest invaded this region in 1978, 40
319 years ago (Baker, 1984).

Our results demonstrate that *M. hypothenemi* populations have wide genetic differentiation and few migrants per generation (Fst = 0.666, $p<0.05$; Nm = 0.50). The greatest genetic divergence of *M. hypothenemi* was observed between geographically most distant populations, with a restricted flow of genes probably conditioned by the limited dispersal capacity of the nematodes (Fonseca and Netto, 2006; Ali et al., 2016). Parasitic nematodes are very susceptible to UV radiation and dehydration, in any of their infective forms (Strong et al. 1996; Patel et al., 1997; Georgis et al., 2006). Also, restricted gene flow may be associated with adaptive processes mediated by local environmental factors such as landscape fragmentation and climate (McGaughran et al., 2014).

The results obtained in the Mantel test indicate that the genetic differentiation between *M. hypothenemi* populations is related with geographic distance, discarding an effect caused by the mobility of its host. Larose and Schwander (2016) used the 18S mitochondrial marker (18s rRNA) to study unidentified endoparasites, closely related to the Mermithidae family. Similarilty, they reported that the genetic differentiation between these species is mainly due to geographic separation and not to host-driven divergence. This work added six new *M. hypothenemi* geographic location sites, in addition to those previously known (Perez et al., 2015), although we believe that the location of new sites may be conditioned by the result of a random interaction caused by the transport of infested fruits with the pest and the adaptation of the parasite to local weather conditions.

The present work presents the first evidence on the American origin of this nematode. Our study also opened the possibility for future studies on the genetic and geographical diversity of this species, as well as its relationship with environmental variables and the search for new distribution areas in America, essential for the design of conservation strategies for this species and its management for control of this important pest.

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Figures captions

Fig. 1. Phylogenetic relationships (interespecific) of the nematode *M. hypothenemi* (13 secuences) with others 29 species of parasitic nematodes recorded in GenBank (35 secuences), using Maximum Likelihood.

Fig. 2. Phylogenetic relationships (intraspecífic) of *M. hypothenemi* (76 sequences) using Maximum Likelihood. A sequence of the parasitic nematode *Howardula aoronymphium* (Tylenchidae: Allantonematidae) was used as outgroup (GenBank AY589466). The scale bar represents the number of expected nucleotide substitutions per site. Municipalities: TAP, Tapachula; UJA, Unión Juárez; CAC, Cacahoatán; ACA, Acacoyagua y SPG, San Pablo Guatemala.

Fig. 3. (A). Distribution of haplotypes in the four municipalities from the Soconusco region, Chiapas, México and the municipality of San Pablo, Guatemala. (B) Haplotype network development using Network v. 5.0. Colors represent each haplotype. The size of the circles is proportional to the frequency of the haplotype inside the populations. The numbers on the lines connecting the haplotypes represent the mutational steps. The white rhombus (mv1) represents an extinct haplotype or an unsampled point.

Fig. 4. Relationship between genetic distance (FST) and geographic distance (km) of 18 populations of the nematode *M. hypothenemi* from Soconusco región, Chiapas, México.

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Fig. 1

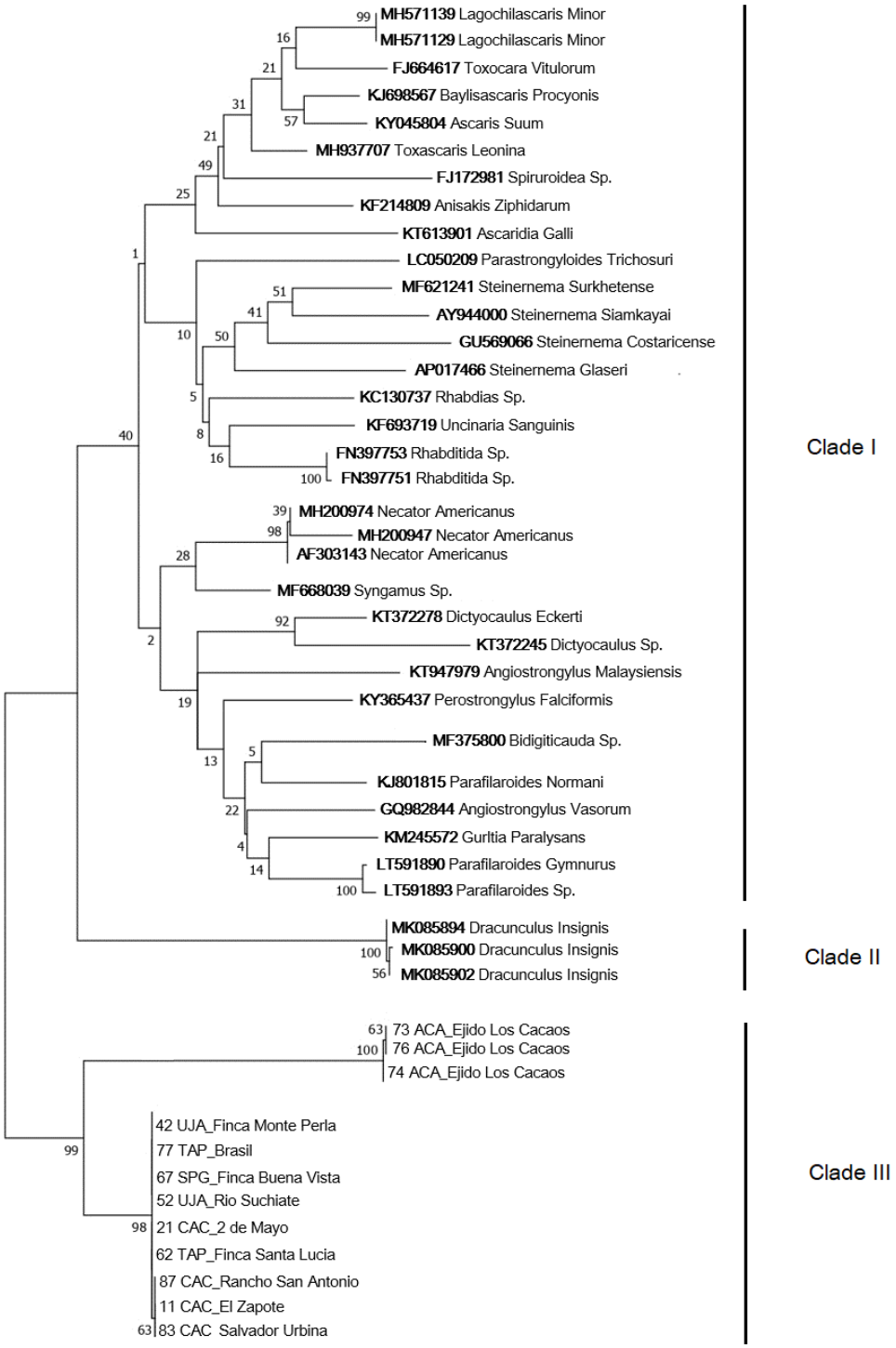

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Fig. 2

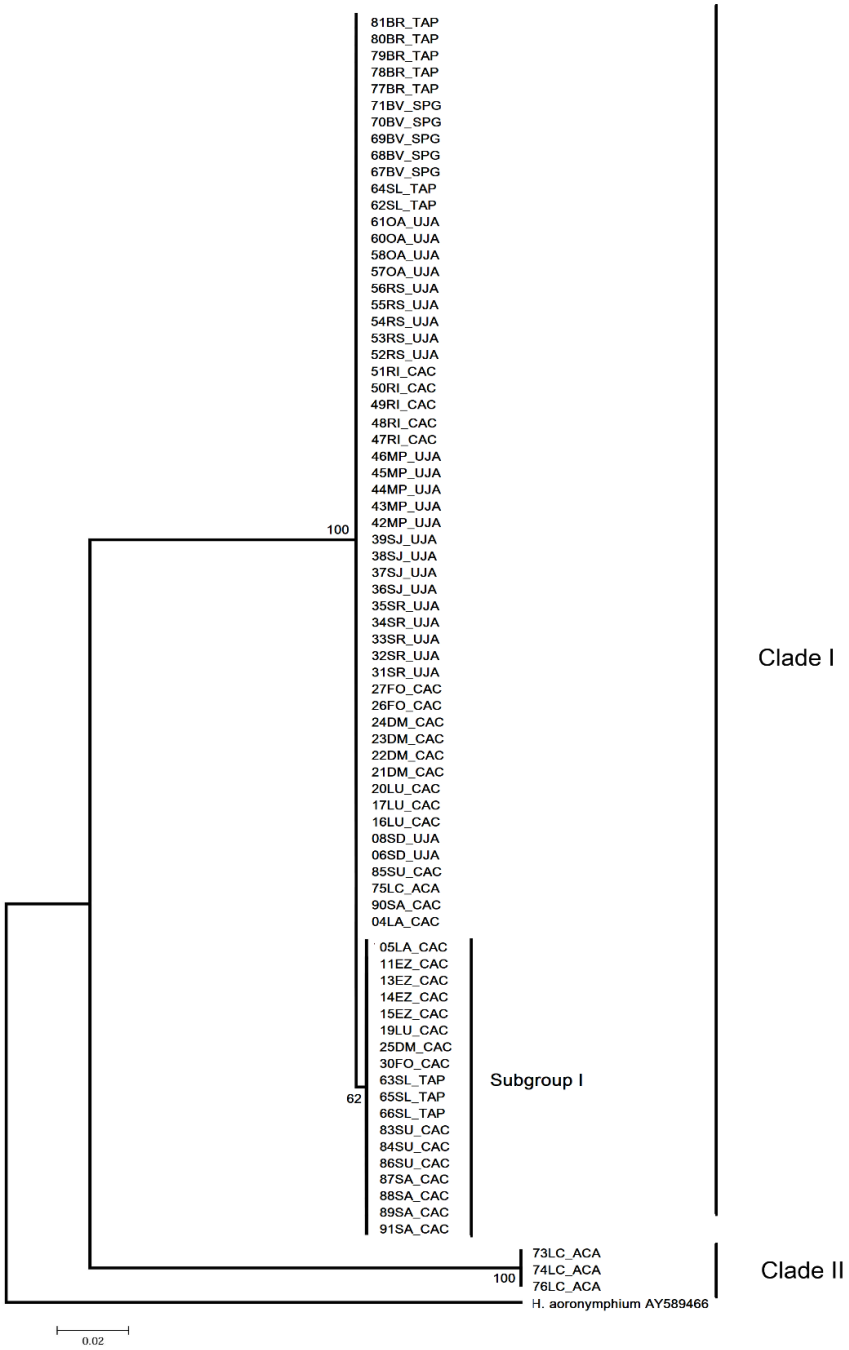

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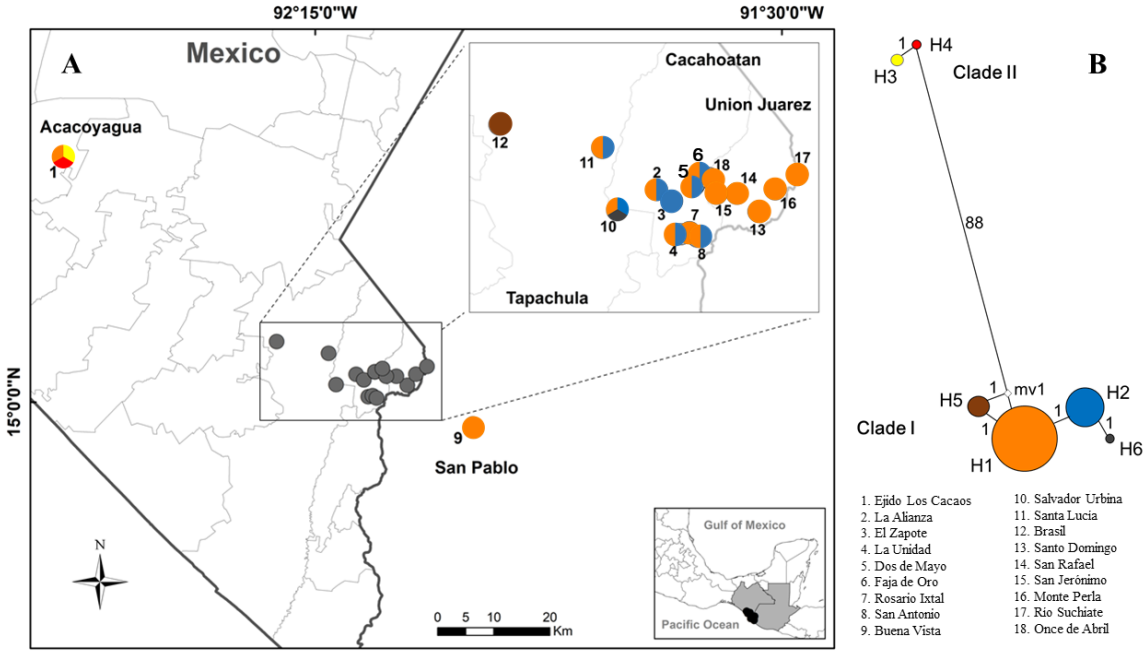
Fig. 3


92°15'0"W
91°30'0"W
15°0'0"N
A
Mexico
Acacoyagua
Cacahoatan
Union Juarez
Tapachula
San Pablo
Gulf of Mexico
Pacific Ocean
0 5 10 20
Km
N
B
H4
H3
Clade II
88
H5
mv1
H2
H6
Clade I
H1
1. Ejido Los Cacaos
2. La Alianza
3. El Zapote
4. La Unidad
5. Dos de Mayo
6. Faja de Oro
7. Rosario Ixtal
8. San Antonio
9. Buena Vista
10. Salvador Urbina
11. Santa Lucia
12. Brasil
13. Santo Domingo
14. San Rafael
15. San Jerónimo
16. Monte Perla
17. Rio Suchiate
18. Once de Abril

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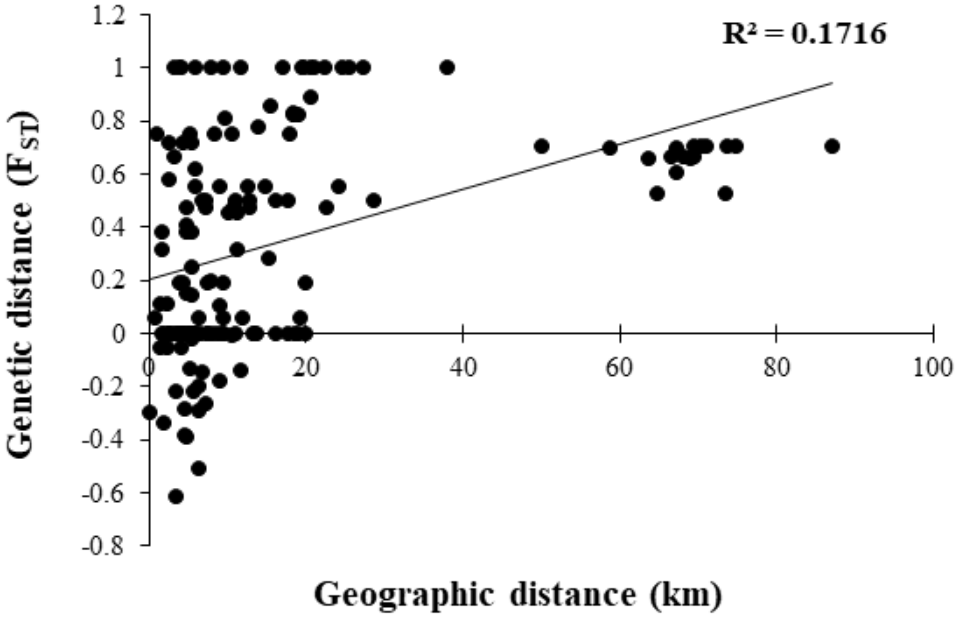
572 Fig. 4



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582 Table 1. Locality and geographical coordinates of the collected CBB samples infected with *M.*

583 *hypothenemi* in Mexico and Guatemala.

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Municipality	Locality	Country	Code	Geographic coordinates	
				Latitude	Longitude
Tapachula	Santa Lucía	Mexico	SL	15°04'42.5"	92°13'42.5
	Finca Brasil	Mexico	BR	15°05'50.3"	92°18'43.1"
	Salvador Urbina	Mexico	SU	15°02'26.9"	92°12'03.9
Cacahoatán	La Alianza	Mexico	LA	15°02'40.8"	92°11'03.2"
	El Zapote	Mexico	EZ	15°02'08.4"	92°10'18.7"
	La Unidad	Mexico	LU	15°00'31.4"	92°09'53.2
	Dos de Mayo	Mexico	DM	15°02'55.0"	92°09'14.5"
	Faja de Oro	Mexico	FO	15°02'56.4"	92°09'14.4"
	Rosario Ixtal	Mexico	RI	15°00'37.2"	92°09'28.0"
	San Antonio	Mexico	SA	15°00'24.2"	92°09'04.7"
Unión Juárez	Santo Domingo	Mexico	SD	15°01'37.5"	92°06'06.9"
	San Rafael	Mexico	SR	15°02'29.8"	92°07'10.3"
	San Jerónimo	Mexico	SJ	15°02'26.9"	92°08'08.9"
	Monte Perla	Mexico	MP	15°02'42.1"	92°05'17.8"
	Río Suchiate	Mexico	RS	15°03'26.1"	92°04'13.2"
	Once de Abril	Mexico	OA	15°03'14.8'	92°08'30.9"
Acacoyagua	Los Cacaos	Mexico	LC	15°23'23"	92°39'13.0"
San Pablo	BuenaVista	Guatemala	BV	14°57'53.1	91°59'48.7"

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605 Table 2. Species of parasitic nematodes recorded in the GenBank used to compare

606 phylogenetically with the nematode *M. hypothenemi*.

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Species	**Origin**	***N**	**BLAST result (% identity)**	****GenBank accession ID**
Perostrongylus falciformis	Germany	1	84.85	KY365437
Parafilaroides gymnurus	North Sea	1	84.77	LT591890
Parafilaroides sp.	USA	1	84.41	LT591893
Syngamus sp.	USA	1	84.09	MF668039
Anisakis ziphidarum	Japan	1	84.08	KF214809
Bidigiticauda sp.	Brazil	1	84.02	MF375800
Parafilaroides normani	Australia	1	83.93	KJ801815
Toxcara vitulorum	Japan	1	83.42	FJ664617
Necator americanus	Brazil	3	83.33	MH200974
			83.36	MH200947
			83.71	AF303143
Lagochilascaris minor	Mexico	2	83.30	MH571139
			83.30	MH571129
Dracunculus insignis	USA	3	83.30	MK085894
			83.30	MK085900
			83.13	MK085902
Parastrongyloides trichosuri	Japón	1	82.86	LC050209
Baylisascaris procyonis	China	1	82.80	KJ698567
Gurltia paralysans	Chile	1	82.68	KM245572
Ascaris suum	Denmark	1	82.62	KY045804
Angiostrongylus vasorum	Denmark	1	80.93	GQ982844
Steinernema surkhetense	India	1	80.11	MF621241
Toxascaris leonina	Poland	1	79.92	MH937707
Steinernema siamkayai	Thailand	1	79.73	AY944000
Rhabditida sp.	Chile	2	79.65	FN397753
			79.26	FN397751
Ascaridia galli	China	1	79.30	KT613901
Uncinaria sanguinis	Australia	1	79.17	KF693719
Steinernema Glaseri	Japan	1	78.97	AP017466
Rhabdias sp.	Mexico	1	78.89	KC130737
Dictycaulus sp.	Hungary	1	78.79	KT372245
Steinernema costaricense	Costa Rica	1	78.73	GU569066
Dictycaulus eckerti	Hungary	1	78.65	KT372248
Spiruroidea sp.	India	1	77.90	FJ172981
Angiostrongylus malaysiensis	Malaysia	1	77.84	KT947979

* Number of sequences used in the analysis

** GenBank accession number

608 Table 3. Frequency of female haplotypes of *Metaparasitylenchus hypothenemi* from the

609 Soconusco Region, Chiapas, Mexico.

Municipality	Population	*N	Haplotipe frequency
Tapachula	SL	5	H1(2), H2(3)
	BR	5	H5(5)
	SU	4	H1(1), H2(2), H6(1)
Cacahoatán	LA	2	H1(1), H2(1)
	EZ	4	H2(4)
	LU	4	H1(3), H2(1)
	DM	5	H1(4), H2(1)
	FO	3	H1(2), H2(1)
	RI	5	H1(5)
	SA	5	H1(1), H2(4)
Unión Juárez	SD	2	H1(2)
	SR	5	H1(5)
	SJ	4	H1(4)
	MP	5	H1(5)
	RS	5	H1(5)
	OA	4	H1(4)
Acacoyagua	LC	4	H1(1), H3(2), H4(1)
San Pablo	BV	5	H1(5)

* Number of sequences used in the analysis; the number in parenthesis is the female specimens observed for each haplotype.

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Tabla 4. Estimation of the genetic diversity in 18 populations of the nematode *M. hypothenemi* collected in the Soconusco region, Chiapas, Mexico.

Population	**N**	**S**	**H**	**Su**	**Hd**	**Π**	**K**	**Tajima**	**Fu and Li**	
								D	**D**	**F**
SL	5	1	2	0	0.600	0.00107	0.600	1.224	1.224	1.157
BR	5	0	1	0	0	0	0	0	0	0
SU	4	2	3	2	0.833	0.00179	1.000	-0.709	-0.709	-0.604
LA	2	1	2	1	1	0.00179	1	-	-	-
EZ	4	0	1	0	0	0	0	0	0	0
LU	4	1	2	1	0.500	0.00089	0.500	-0.612	-0.612	-0.478
DM	5	1	2	1	0.400	0.00072	0.400	-0.816	-0.816	-0.771
FO	3	1	2	1	0.666	0.00119	0.666	-	-	-
RI	5	0	1	0	0	0	0	0	0	0
SA	5	1	2	1	0.400	0.00071	0.400	-0.816	-0.816	-0.771
SD	2	0	1	0	0	0	0	-	-	-
SR	5	0	1	0	0	0	0	0	0	0
SJ	4	0	1	0	0	0	0	0	0	0
MP	5	0	1	0	0	0	0	0	0	0
RS	5	0	1	0	0	0	0	0	0	0
OA	4	0	1	0	0	0	0	0	0	0
LC	4	92	3	91	0.833	0.0824	46.166	-0.836	-0.836	-0.895
BV	5	0	1	0	0	0	0	0	0	0
Total	**76**	**93**	**6**	**0**	**0.519**	**0.0134**	**7.555**	**-2.06227***	**2.16746****	**0.55398**

N, number of sequences; S, number of segregating sites; H, number of haplotypes; Su, number of unique sites; Hd, haplotypic diversity; Π, nucleotidic diversity; K, average number of nucleotidic differences. The D índices of Tajima (Tajima, 1989), D and F de Fu and Li (Fu and Li, 1993). The hyphen represents an estimate not performed due to a limited number of sequences used. *P<0.05. **P<0.02.

Tabla 5. Genetic (F_{ST}) and geographic distances (km, above the diagonal) calculated for 18 populations of the nematode *M. hypothenemi* collected in the Soconusco region, Chiapas, México and San Pablo, Guatemala.

	LA	**SD**	**EZ**	**LU**	**DM**	**FO**	**SR**	**SJ**	**MP**	**RI**	**RS**	**OA**	**SL**	***BV**	**LC**	**BR**	**SU**	**SA**
LA	0	**9.3690**	**1.7021**	**4.5493**	**3.3898**	**3.3987**	**7.2098**	**5.4069**	**10.6806**	**4.8243**	**12.7553**	**4.8254**	**6.1989**	**22.6751**	**64.8518**	**15.3811**	**1.9256**	**5.5919**
SD	0	0	**7.8446**	**7.2901**	**6.2765**	**6.2903**	**2.5414**	**4.0700**	**2.5091**	**6.4920**	**4.8619**	**5.3740**	**15.2053**	**13.5984**	**73.4943**	**24.6554**	**11.1444**	**5.9469**
EZ	0.38462	1	**0**	**3.1014**	**2.4531**	**2.4812**	**5.8632**	**4.0543**	**9.3627**	**3.2266**	**11.5546**	**3.9150**	**7.9007**	**21.0198**	**66.5539**	**17.0398**	**3.3029**	**3.9520**
LU	-0.37931	-0.2632	0.66667	0	**4.5989**	**4.6415**	**6.2272**	**4.8122**	**9.4263**	**0.7996**	**11.8202**	**5.6574**	**10.5149**	**19.3228**	**68.9932**	**19.1241**	**5.3935**	**1.5162**
DM	-0.21495	-0.2903	0.72028	-0.28099	**0**	**0.0434**	**3.9188**	**2.2068**	**7.3302**	**4.2815**	**9.3664**	**1.4807**	**8.9290**	**19.8306**	**67.3408**	**18.3990**	**5.3098**	**4.6729**
FO	-0.61538	-0.2	0.57895	-0.38776	-0.29921	0	**3.9246**	**2.2213**	**7.3295**	**4.3249**	**9.3589**	**1.4605**	**8.9158**	**19.8483**	**67.3190**	**18.3893**	**5.3201**	**4.7159**
SR	0.47368	0	1	0.0625	0	0.18919	**0**	**1.8143**	**3.4995**	**5.5003**	**5.7464**	**2.8545**	**12.8031**	**16.1144**	**70.9683**	**22.3020**	**9.0792**	**5.2534**
SJ	0.38462	0	1	0	-0.05263	0.11111	0	0	**5.3116**	**4.1820**	**7.5147**	**1.6299**	**11.1353**	**17.6329**	**69.5073**	**20.5948**	**7.2667**	**4.1681**
MP	0.47368	0	1	0.0625	0	0.18919	0	0	**0**	**8.6472**	**2.4169**	**6.0561**	**16.0444**	**13.5434**	**73.7083**	**25.5726**	**12.5663**	**8.2104**
RI	0.47368	0	1	0.0625	0	0.18919	0	0	0	0	**11.0469**	**5.1833**	**10.9301**	**18.6181**	**69.5003**	**19.7071**	**5.8946**	**0.8250**
RS	0.47368	0	1	0.0625	0	0.18919	0	0	0	0	**0**	**7.9763**	**17.7618**	**13.1501**	**74.7380**	**27.2663**	**14.6698**	**10.6248**
OA	0.38462	0	1	0	-0.05263	0.11111	0	0	0	0	0	0	**10.0097**	**18.9658**	**68.1207**	**19.5317**	**6.7509**	**5.3779**
SL	-0.50442	0.28571	0.19463	-0.00515	0.10714	-0.17978	0.5	0.45205	0.5	0.5	0.5	0.45205	**0**	**28.7232**	**58.6541**	**9.5287**	**5.1844**	**11.7297**
***BV**	0.47368	0	1	0.0625	0	0.18919	0	0	0	0	0	0	0.5	0	**87.0806**	**38.0555**	**24.2594**	**17.8163**
LC	0.52541	0.52619	0.66906	0.66184	0.70123	0.60808	0.70368	0.66424	0.70368	0.70368	0.70368	0.66424	0.70134	0.70368	**0**	**50.0599**	**63.6108**	**70.3169**
BR	0.85507	1	1	0.82558	0.83333	0.82558	1	1	1	1	1	1	0.8125	1	0.70479	0	**13.8541**	**20.5322**
SU	-0.33333	0.31429	0	0.14286	0.24837	-0.01961	0.55224	0.5	0.55224	0.55224	0.55224	0.5	-0.12971	0.55224	0.66098	0.78102	**0**	**6.7157**
SA	-0.21495	0.62264	-0.05263	0.31416	0.41176	0.15167	0.75	0.72028	0.75	0.75	0.75	0.72028	-0.13636	0.75	0.70388	0.88889	-0.14428	0

* Site located in San Pablo, Guatemala.

CAPITULO 3.- Conclusiones

- La diversidad y diferenciación genética observada dentro y entre poblaciones de *M. hypothenemi* ocasionada por el flujo restringido de genes, está asociada al distanciamiento geográfico. Esto probablemente por procesos adaptativos mediados por factores ambientales locales como la fragmentación del paisaje y el clima.

- Nuestros resultados filogenéticos y de inferencia genealógica basada en los haplotipos de *M. hypothenemi*, indican una divergencia genética considerable entre la población de Los Cacaos, en Acapatahua y el resto de las poblaciones y de acuerdo a estos resultados, se sugiere que un proceso de sub-especiación está ocurriendo localmente dentro de las poblaciones de *M. hypothenemi*, tal vez como resultado de la heterogeneidad geográfica de la región, historia evolutiva, deriva genética, así como las presiones de selección natural. Sin embargo, los estudios genéticos con mayor representatividad de la población aislada, el uso de otros marcadores genéticos y estudios morfológicos adicionales de especímenes machos y hembras de *M. hypothenemi* ayudarían a confirmar la ocurrencia de este proceso de especiación.

- El presente trabajo muestra que el marcador COI es una herramienta valiosa para estudiar la genética poblacional del nematodo parásito *M. hypothenemi*, lo que permite comprender los aspectos ecológicos relacionados con su adaptación al huésped y al medio ambiente, que permanecieron desconocidos hasta antes del presente estudio. Además, se demuestra la aplicabilidad de las secuencias de COI para la identificación de esta especie.

- Este trabajo agregó seis nuevos sitios de ubicación geográfica de *M. hypothenemi*, además de los conocidos previamente (Pérez et al. 2015). No obstante, planteamos la hipótesis de que la ubicación de los nuevos sitios, puede estar condicionada por el flujo ocasional de la BC, causada por el transporte de frutas infestadas con la plaga y una consecuente adaptación del parásito a las condiciones climáticas locales.

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