UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
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INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

IDENTIFICACIÓN MORFOLÓGICA, MOLECULAR Y ANÁLISIS DE INFECCIÓN POR LEISHMANIA, BARTONELLA Y/O WOLBACHIA EN FLEBOTOMINOS (DIPTERA: PSYCHODIDAE: PHEBLOTOMINAE) DE LA ESTACIÓN DE BIOL. TROP., LOS TUX. VER.

TESIS
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INDICE

Identificación morfológica, molecular y análisis de infección por *Leishmania, Bartonella* y/o *Wolbachia* en flebotominos (Diptera: Psychodidae: Phlebotominae) de la Estación de Biología Tropical los Tuxtlas, Veracruz

RESUMEN

Los flebotominos, son dípteros de gran relevancia en salud publica debido a su capacidad de transmitir diversos patógenos a nivel mundial. No obstante, en México aun quedan áreas sin explorarse por lo que aún se desconoce mucho acerca de la biología, ecología, distirbución y taxonomía de este grupo. Es por eso que en este trabajo fue realizar un estudio enfocado en: 1) la identificación morfológica y molecular de flebotominos, 2) conocer la diversidad de estas especies y 3) su posible asociación con tres patógenos (*Leishmania*, *Bartonella* y *Wolbachia*), en la región de Los Tuxtlas, en el Estado de Veracruz. Adicionalmente se generaron datos complementarios sobre preferencias alimenticias, por lo que fue posible resaltar el posible papel de dos especies (*Psathyromyia aclydifera* y *Psychodopygus panamensis*) como vectores potenciales de *Leishmania infantum* en la zona de los Tuxtlas en Veracruz. En este proyecto se proporciona información básica acerca de las interacciones entre flebtominos, patógenos y hospederos, resaltando la relevancia de realizar estudios entomológicos y epidemiológicos en Méxcio, debido a que la dinámica de transmisión de patógenos, asi como la abundancia y distribución de especies de flebotominos difiere entre sitios geográficos. Este tipo de estudios permitirán enriquecer los programas de vigilancia epidemiológica para poder reducir el número de casos de leishmaniasis en Veracruz y otras partes de México

INTRODUCCIÓN

Las enfermedades transmitidas por vector son definidas como todas aquellas bacterias, parásitos o virus que son trasmitidos por artrópodos (flebotominos, mosquitos, moscas, chinches, piojos, pulgas y garrapatas, entre otros) a vertebrados terrestres incluyendo al ser humano. Estas enfermedades representan aproximadamente el 17% de las enfermedades infecciosas, y anualmente causan más de 700, 000 defunciones a nivel mundial (OMS, 2020a).

Dentro de este grupo de artrópodos considerados vectores, destacan por su gran relevancia en salud pública y veterinaria, los dípteros hematófagos comúnmente conocidos como flebotominos, ya que son capaces de transmitir diversos patógenos como: protozoarios del género *Leishmania* (Kinetoplastida: Trypanosomatidae), las bacterias *Bartonella bacilliformis* (Rhizobiales: Bartonellaceae) y *Rickettsia* spp. (Rickettsiales: Rickettsiaceae), así como algunos arbovirus (principalmente de las familias Bunyaviridae, Reoviridae and Rhabdoviridae) (Akhoundi et al., 2016; Ready, 2013; Young and Duncan, 1994).

No obstante, su mayor relevancia está relacionada con la transmisión del protozoario *Leishmania*, agente causal de la enfermedad conocida como leishmaniasis. Esta enfermedad es considerada un serio problema de salud a nivel mundial, ya que cerca de 12 millones de personas padecen de esta enfermedad y se reportan dos millones de casos humanos nuevos anualmente. Se estima que 350 millones de personas están en riesgo de contraer la enfermedad (Alvar et al., 2012; OMS, 2020b).

En México, los casos de leishmaniasis se han registrado al menos en 25 estados del país con 6,595 casos nuevos registrados durante el periodo de 2010-2019. Aunque se estima que cerca de 16 millones de personas están en riesgo de contraer la enfermedad, siendo el principal factor de riesgo las poblaciones que viven cerca de plantíos de café, cacao y chicle, ya que son suelos ricos en materia orgánica que favorecen la abundancia de flebotominos (Alvar et al., 2012; DGE, 2020; Mikery-Pacheco and Vera, 2018; Pech-May et al., 2016; Ready, 2013; Sanchez-Tejeda et al., 2001).

Aunque en los últimos 20 años el conocimiento sobre la biología, ecología y distirbución de los flebotominos ha incrementado exponencialmente, aún quedan muchas áreas geográficas sin explorarse de las cuales muchas de ellas son endémicas para la tranmision de la leishmaniasis. Es por eso que el objetivo de este trabajo fue realizar un estudio enfocado en conocer la diversidad de especies de flebotominos y su posible asociación con patógenos como: *Leishmania*, *Bartonella* y *Wolbachia* en la región de Los Tuxtlas, en Veracruz.

ANTECEDENTES

Los flebotominos (Diptera: Psychodidae: Phlebotominae) también son conocidos como: papalotillas, chitre, palomilla, manta blanca, quemador, pringador, jején, titira entre otros. Se caracterizan por ser dípteros nematóceros que se agrupan dentro de la subfamilia Phlebotominae, la cual incluye cerca de 1,026 especies a nivel mundial de las cuales 546 especies se distribuyen en América (Galati, 2019; Shimabukuro et al., 2017). Estos dípteros alcanzan tamaños entre 2-5 mm, sus cuerpos están cubiertos de sedas, tienen patas muy largas y la posición de sus alas formando un ángulo de 45° (forma de "V") en relación a su cuerpo. Tienen un ciclo de vida holometábolo (es decir que pasan por 4 etapas de desarrollo), existe dimorfismo sexual y tienen actividad crepuscular o nocturna con limitada capacidad de vuelo (Montes de Oca-Aguilar et al., 2013a; Pech-May et al., 2013). Ambos sexos se alimentan de azúcares, sin embargo en época reproductiva las hembras requieren de la ingesta de sangre de vertebrados para la maduración de huevos (Montes de Oca-Aguilar et al., 2013a). Dicha característica, está relacionada con su capacidad y competencia vectorial para poder transmitir *Leishmania*, *Bartonella bacilliformis*, *Rickettsia* spp., así como algunos arbovirus (Akhoundi et al., 2016; Ready, 2013; Young and Duncan, 1994).

En México, 52 especies válidas y dos especies fósiles de flebotominos han sido descritas hasta el momento, basándose principalmente en caracteres morfológicas ubicados principalmente en las genitalias de machos y hembras, asi como en estructuras de la cabeza (el cibario, antenas, palpos y ascoides). La taxónomia de estos dípteros es un trabajo que requiere de cierta expertiz lo que puede dificultar su identificación, debido a que durante el procesamiento algunas estructuras pueden dañarse, o incluso porque algunas algunas estructuras son muy parecidas entre ellas o que hay especies cripticas. No obstante, con el uso de herramientas moleculares se ha recurrido a métodos complementarios que faciliten la identificación de especies, siendo el gen Citocromo Oxidasa subunidad 1 (COI) el más utilizado. Este gen es comúnmente usado como un marcador genético para la identificación de especies, desde que se propuso como un gen adecuado para generar "Códigos de Barras de DNA" (Folmer et al., 1994; Hebert et

al., 2003). No obstante, son muy pocas las especies de flebotominos en México que cuentan con esta información para la identificación molecular (Adeniran et al., 2019; Florin and Rebollar-Téllez, 2013; Pech-May et al., 2013).

De las especies de flebotominos que se distribuyen en México, sólo cuatro son consideradas vectores principalmente de *Leishmania mexicana* agente causal de la leishmaniasis cutánea localizada (LCL), la forma clínica más común en el país. Aunque siete especies más, son consideradas vectores sospechosos de la transmisión de *Leishmania* sp. (González et al., 2011; Ibáñez-Bernal et al., 2017, 2011, 2010, 2006; Martins et al., 1978; Pech-May et al., 2010). Los casos de leishmaniasis se han registrado al menos en 25 estados del país y se estima que cerca de 16 millones de personas están en riesgo de transmisión,principalmente en áreas donde hay plantíos de café, cacao y chicle ya que son suelos ricos en materia orgánica que favorecen la abundancia de flebotominos (Alvar et al., 2012; DGE, 2020; Mikery-Pacheco and Vera, 2018; Pech-May et al., 2016; Ready, 2013; Sanchez-Tejeda et al., 2001).

En México, hasta el momento sólo se ha realizado un estudio acerca de las bacterias patógenas que pueden estar asociadas con estos dípteros. Reportando por primera vez la presencia de la bacteria *Wolbachia* en el flebotomino *Lutzomyia cruciata*, aunque con una baja prevalencia respecto a otros estudios realizados en América (Mikery-Pacheco et al., 2012; Ono et al., 2001). No obstante este hallazgo resulta interesante ya que es una bacteria que tiene un papel importante como parásito reproductivo y dependiendo su hospedero, puede conferir diversos beneficios, por lo que podría ser una alternativa como control biológico (Werren, 1997).

A pesar de que en los últimos años los estudios acerca de la biología y ecología de los flebotominos han incrementando principalmente en los estados de Chiapas, Nuevo León, Tamaulipas y la Península de Yucatán, el conocimiento acerca de este grupo está incompleto en otras partes del país (Berzunza-Cruz et al., 2015; Canto-Lara et al., 1999; Pech-May et al., 2010; Rebollar-Téllez et al., 1996a; Rodríguez-Rojas et al., 2017; Van Wynsberghe et al., 2000).

Tal es el caso del estado de Veracruz, el cual no sólo tiene una gran extensión territorial, sino que es considerado uno de los siete estados con mayor número de casos de leishmaniasis asociados principalmente a plantíos de cacao, en el cual se han reportado 209 casos de leishmaniasis cutánea y algunos casos esporádicos de leishmaniasis visceral en el periodo del 2010-2019. El número anual de casos oscila entre 14 y 27 casos con una incidencia que varía del 0.18 al 0.39 por cada 100, 000 habitantes (DGE, 2020). Adicionalmente, es un estado que alberga gran riqueza de especies de flebotominos, contando con 23 especies (Ibáñez-Bernal et al., 2011; Martins et al., 1978; Moo-Llanes et al., 2013). Y se estima que el número de especies podría ser aún mayor, si se llevarán a cabo estudios ecológicos en este estado (Moo-Llanes et al., 2013).

JUSTIFICACIÓN

A pesar de que Veracruz, es uno de los estados con mayor número de casos de leishmaniasis y cuenta con una gran diversidad de especies de flebotominos, pocos estudios se han realizado acerca de la biología, ecología y detección de patógenos en este grupo de dípteros. Es por eso que hacer un estudio de caso, nos permitirá conocer aspectos básicos acerca de la dinámica poblacional de los flebotominos, la diversidad de especies y su asociación con bacterias y protozoarios, con la finalidad de proporcionar información acerca de las interacciones entre estos dípteros, patógenos, hospederos y el ambiente. Esto permitirá enriquecer los programas de vigilancia epidemiológica para poder reducir el número de casos de leishmaniasis en Veracruz.

OBJETIVO GENERAL

Caracterizar la comunidad de flebotominos y analizar su infección por *Leishmania, Bartonella* y *Wolbachia* en la Estación de Biología Tropical, Los Tuxtlas, Veracruz.

OBJETIVOS PARTICULARES

- Identificación morfológica de flebotominos.
- Identificación molecular de los flebotominos con el gen Citocromo Oxidasa Subunidad 1 (COI)
- Evaluar la infección de los flebotominos por *Leishmania, Bartonella* y *Wolbachia.*
- Analizar las prevalencia de la infección por cada patógeno.
- Caracterización ecológica de las especies recolectadas (abundancia y prevalencia asociada a distintas épocas del año).

MATERIAL Y METODO

Área de estudio

El muestreo se realizó en la Estación de Biología Tropical Los Tuxtlas, localizada en el municipio de San Andrés Tuxtla en el Estado de Veracruz (N18° 34´ W95° 04´) (Fig. 1).

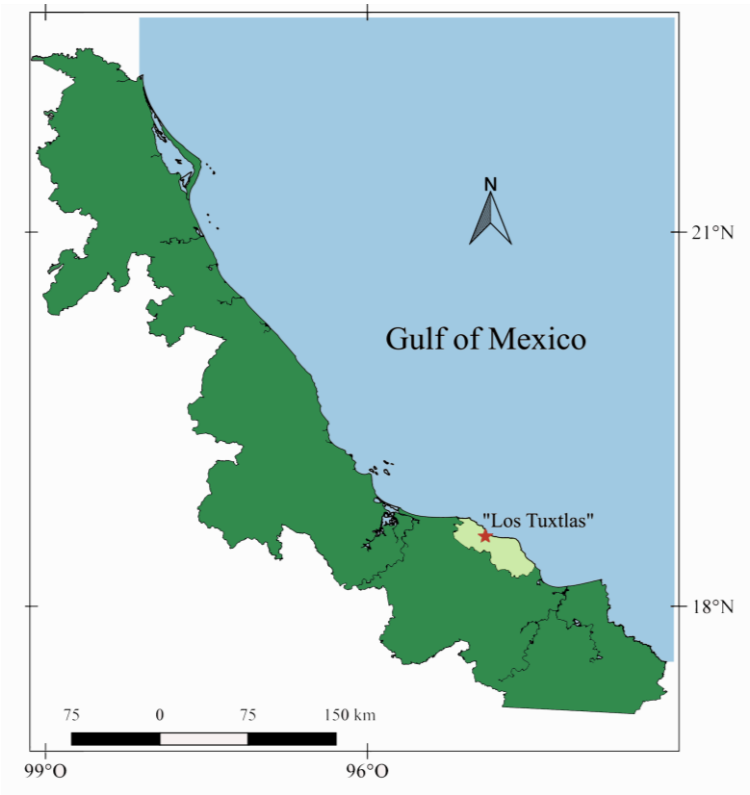

Fig 1. Mapa del área de estudio ubicada en la Estación de Biología Tropical Los Tuxtlas, Veracruz.

Esta zona está rodeada de una alta diversidad de ecosistemas, así como de gran complejidad geológica y ecológica que forma parte del último parche de bosque tropical húmedo. La estación se sitúa a una altitud de 150 a 700 metros sobre el nivel del mar y cubre un área de 644 hectáreas. Se caracteriza por tener un ambiente cálido y húmedo, con una temperatura promedio de 26°C. Este sitio, tiene una estacionalidad marcada, la época de lluvias se registra de Junio a Febrero siendo Agosto y Noviembre los meses que tienen los picos más altos, con un promedio anual de lluvia de 4500 mm. Por otro lado, la

época de secas comienza en Marzo y finaliza en Mayo, siendo este último mes el más caliente. La vegetación predominante es selva alta y mediana perennifolia, liquidámbar, bosque, manglares y potreros (Dirzo et al., 1997; Soto and Gama, 1997). Presenta una alta diversidad y riqueza de especies, incluyendo especies endémicas y en peligro de extinción tanto de flora como de fauna (Dirzo et al., 1997).

Los flebotominos fueron muestreados durante tres noches consecutivas durante 10 muestreos, en Marzo y Octubre 2011; Enero, Marzo, Abril, Junio, Agosto y Noviembre 2012; Abril y Mayo 2013. Fueron colectados usando 10 trampas de luz incandescente (modelo 512; John W. Hock Co., Gainesville, FL, USA), las cuales se activaron de las 18:00 a 11:00 h. Estas trampas se colocaron en un transecto de 100 m con al menos 10 m de distancia entre cada trampa (siempre en la misma posición durante todos los muestreos), colgadas de árboles o ramas (principalmente de *Astrocaryum mexicanum*) usando una cuerda a una distancia aproximada de un metro sobre el suelo, cerca de madrigueras o materia orgánica en descomposición. Una vez que se retiraron las trampas, se mantuvieron bajo refrigeración durante 12 h para bajar el metabolismo de los organismos colectados, posteriormente se separó y preservó el material en etanol al 70%, y finalmente se congeló a -20°C.

Identificación morfológica de flebotominos

Para realizar la identificación morfológica se realizaron preparaciones permanentes usando Euparal siguiendo el procedimiento propuesto por Ibáñez-Bernal (2005). Los especímenes colectados se separaron en sexos, los machos se montaron en laminillas completamente. En el caso de las hembras el 80% se montaron completas y del resto sólo la cabeza, alas y los últimos segmentos del abdomen, ya que el resto del cuerpo se utilizó para el procesamiento molecular. La selección de los ejemplares se realizó al azar.

Los especímenes colectados fueron clasificados de acuerdo con propuesta de Galati (2003), mientras que la identificación morfológica se basó en la propuesta de Young and Duncan (1994) y en las claves de Ibáñez-Bernal (Ibáñez-Bernal, 2005a, 2005b, 1999). Para las abreviaturas de los géneros y

subgéneros se utilizó el sistema propuesto por Marcondes (2007). Todo el material identificado morfológicamente está protegido bajo el permiso de colección SGPA/DGVS/09346/16 licencia FAUT-0317 de la Secretaria de Ambiente y Recursos Naturales.

Extracción de DNA

Para el análisis molecular, el 20% de las hembras colectadas fue procesado. El tórax, patas y abdomen de las hembras se separaron individualmente en tubos de 1.5 µL y se preservaron en etanol al 70%, excepto el tubo que tenía los primeros segmentos del abdomen. La extracción de DNA se estandarizó a partir de los segmentos del abdomen usando la resina Chelex-100. Para mejorar los resultados usando estructuras quitinosas se realizaron algunas modificaciones al protocolo de García-Gonzáles *et al.* (2004). Primero se preparó una solución al 10% de la resina Chelex-100 (BIO-RAD, California, USA), usando agua libre de nucleasas al 70°C. Al tubo de 1.5 µL que contenía los segmentos del abdomen se le añadieron 500 µL de la solución y 20 µl de proteinasa K (SIGMA). Después se mezcló con ayuda de un vórtex a una velocidad baja y se incubo a 56°C durante 12h. Luego se incrementó la temperatura a 100°C durante 15 min, con la finalidad de mejorar la desnaturalización de las proteínas. Las muestras fueron centrifugadas a 14, 000 revoluciones por minuto durante 10 min, y solo se usó el sobrenadante para la amplificación de DNA. La calidad y concentración del DNA fue cuantificado en relación a la absorbancia entre 260/280 nm usando un NanoDrop 2000 / 2000c (Thermo Fisher, EE. UU.).

Reacción en Cadena de la Polimerasa (PCR)

La PCR se realizó usando un termociclador C1000 Thermal Cycler (BioRad Laboratories, USA). En el caso particular de la identificación molecular de flebotominos y la detección de *Leishmania*, la mezcla de reacción fue preparada en un volumen final de 25 µL con 0.3 µL de ABM Hot Start DNA polimerasa (Applied Biological Materials Inc., Canada), 2.5 µL de 10X buffer, 1.5 µL de 25 mM $MgSO_4$, 0.6 µL de 2 mM dNTP, 1 µL de 10 pmol cada oligonucleótido, 3 µL de DNA y 15.1 µL de agua

ultrapura. Los amplificados fueron visualizados mediante electrophoresis en un gel de agarosa al 1.5% teñido con GelStar (Lonza, Switzerland).

Mientras que para la amplificación de Rickettsiales (*Wolbachia* y *Rickettsia*) y *Bartonella* la mezcla de reacción fue preparada en un volumen final de 25 µL con 12.5 µL de GoTaq Green Master Mix (2X Promega Corporation, Madison, WI, USA), 1 µL de cada oligo (100 ng each), 10 µL de DNA (~50 ng), y 0.5 µL de agua libre de nucleasas. Los amplificados fueron visualizados en un gel de agarosa al 2% teñidos con Smartglow.

Los genes, oligos y condiciones que se utilizaron se estandarizaron con base en literatura especializada (Tabla 1), y para todos los genes se incluyó como control negativo agua en vez de DNA. Exclusivamente para la detección de *Leishmania*, se incluyó como control positivo una cepa de *Le. amazonensis* (IFLA/BR/67/PH8, ATCC 50159) que circula en Colombia.

Todos los productos positivos, que se obtuvieron para cada gen amplificado se enviaron a secuenciar y purificar al Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, UNAM.

Finalmente, para calcular la prevalencia de bacterías y parásitos se tomaron en cuenta los parámetros de Bush *et al.* (1997), donde se evluó el número de especies infectadas por un linaje en específico de bacteria o parásito y el número de flebotominos analizados por especie.

Tabla 1. Datos moleculares para la identificación molecular y detección de patógenos en flebotominos

Objetivo	Gen	Oligos	Pares de Bases (bp)	Referencia
Identificación molecular de flebotominos	Citocromo Oxidasa Subunidad I (COI)	LCO1490 (5'-GGT CAA CAA ATC ATA AAG TATTG G−3') HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AATCA−3')	≈708	(Folmer et al., 1994; Lozano-Sardaneta et al., 2019b)
Detección de *Leishmania*	Espaciador transcrito interno subunidad 1 (ITS-1)	LITSR (5'-CTG GAT CAT TTT CCG ATG -3') L5.8S (5'-TGA TAC CAC TTA TCG CAC TT−3')	≈ 300–350	(El Tai et al., 2001; Lozano-Sardaneta et al., 2020)
Detección de *Bartonella*	Citrato sintasa (*glt*A)	BhCS871.p (5'-GGG GAC CAG CTC ATG GTG G−3') BhCS1137.n (5'-AAT GCA AAA AGA ACA GTA AAC A−3')	≈379	(Birtles and Raoult, 1996; Lozano-Sardaneta et al., 2019a; Norman et al., 1995)
Detección de *Rickettsia*	16S rDNA	EHR01F (5'-GCC TAA CAC ATG CAA GTC GAA CG−3') EHR02R (5'-GCC CAA TAA TTC CGA ACA ACG−3')	≈495	(Telford III et al., 2011)
Detección de *Rickettsia*	Citrato sintasa (*glt*A)	RpCS.877p (5'-GGG GGC CTG CTC ACG GCG G−3') RpCS.1258n (5'-ATT GCA AAA AGT ACA GTG AAC A−3')	≈390	(Wood et al., 1987)
Detección de *Wolbachia*	Proteína de superficie (*wsp*)	81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC 3') 691R (5'-AAA AAT TAA ACG CTA CTC CA 3')	≈600 bp	(Braig et al., 1998)

Análisis de datos y secuencias

Las secuencias obtenidas de todos los genes fueron visualizadas y editadas en el programa GeneStudio™ Professional Edition Version 2.2.0.0 (GeneStudio, Inc., USA). Cada secuencia fue comparada con secuencias disponibles en la base de datos de NCBI usando el algoritmo BLASTn (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), como una confirmación preliminar de la identidad de cada gen amplificado.

Posteriormente las secuencias fueron alineadas para cada gen, usando ClustalW en el programa MEGA v6.0 (Tamura et al., 2013) y MAFFT versión 7 (Katoh et al., 2017).

Identificación molecular de flebotominos

Para el alineamiento de secuencias de COI amplificadas, se calculó el número de haplotipos (H), sitios polimórficos (s) y diversidad nucleotídica por especie (π), usando el programa DnaSP v5.10 programa (Librado and Rozas, 2009). Mientras que el programa FINGERPRINT (http://evol.mcmaster.ca/fingerprint/), se utilizó para representar la heterogeneidad y diversidad nucleotídica obtenida (Lou and Golding, 2007).

Las distancias genéticas se calcularon utilizando el modelo de sustitución de Kimura-2 (K2P) y las secuencias fueron analizadas mediante un análisis de Neighbor-Joining (NJ), el cual se construyó en MEGA v6.0. Se analizó el barcode gap (gap entre las distancias intra e interespecífica K2P) para confirmar que la región COI era adecuada para la discriminación interespecífica. La representación gráfica del barcode gap fue calculada usando ggplot2 y EasyGgplot package (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016) en la plataforma R 3.5 (R Core Team, 2018).

Para determinar la limitación de la región COI en la retención de información filogenética, debido a la saturación de sustitución, se realizaron gráficos de saturación de transición/transversión sobre divergencia y se calculó el índice de saturación de sustitución (Iss) en el programa DAMBE versión 7.0 (Xia, 2018). Todas las secuencias obtenidas en el presente estudio se han depositado en GenBank con números de acceso MK744133-MK744156.

Detección de *Bartonella* en flebotominos

Las secuencias de *gltA* obtenidas para la detección de *Bartonella* se analizaron mediante un análisis de NJ usando como modelo de sustitución Tamura 3-parametros con 10,000 réplicas de

bootstraps. Las secuencias obtenidas fueron depositadas en GenBank bajo números de acceso MN325838 and MN325839.

Detección de *Rickettsia* y *Wolbachia* en flebotominos

Para el análisis de secuencias para la detección de *Rickettsia* sp. (16S rDNA, *gltA*) se realizó un análisis concatenado usando el método de Máxima Verosimilitud (ML) en MEGA 6.0. El modelo de sustitución que se ajustó mejor al modelo de sustitución de Kimura 2 parámetros (K2P) con una puntación de BIC de 1.466.686 y un valor de probabilidad de -668.202.

Mientras que para el análisis de secuencias del gen *wsp*, el alineamiento se realizó usando otras secuencias generadas previamente por Zhou *et al.* (1998) y añadiendo todas las secuencias de *wsp* asociadas con flebotominos. Debido al alto nivel de polimorfismos que tiene el gen *wsp*, y a que contiene varios singletons y gaps, el alineamiento se tradujo a las secuencias de aminoácidos para facilitar el alineamiento (Zhou et al., 1998). Para el análisis filogenético de estas secuencias, se realizó un análisis de Maxima Veroimilitud en MEGA 6.0 con 1, 000 bootstraps, usando el modelo de sustitución de Tamura 3-parameters (T92) + Gamma distribution (0.38). Todos los sitios alineados con menos de 95% de cobertura fueron eliminados del análisis. Las distancias genéticas entre las secuencias generadas en este trabajo y su similitud con otras cepas de *Wolbachia* se calcularon en MEGA 6.0. Las secuencias obtenidas se depositaron en GenBank con los siguientes números de acceso para *Rickettsia* 16S (MT158807) y *glt*A (MT1654485), y *Wolbachia wsp* (MT533592, MT533593).

Detección de *Leishmania* en flebotominos

Debido al alto nivel de polimorfismos, alto número de singlentons y gaps que tiene la región del ITS-1 presente en *Leishmania* se hizo un análisis de Maxima Parsimonia (MP) en MEGA 7.0 con 1,000 bootstraps, usando el algoritmo de Subtree-Pruning-Regrafting (SPR). Todos los sitios alineados con menos de 95% de cobertura fueron eliminados del análisis. Todas las secuencias obtenidas se depositaron en GenBank con los códigos de acceso MN422055-MN422063 y MN503524-MN503527.

Diversidad de especies de flebotominos

La diversidad de especies fue analizada en línea en el software R usando la librería SpadeR (Species Prediction and Diversity Estimation) (Chao et al., 2015). La diversidad de especies fue estimada usando la abundancia de especies de flebotominos identificados a nivel de especies. Se incorporaron tres medidas basadas en los números de Hill: la riqueza de especies ($q = 0$) con el estimador Chao1, diversidad Shannon ($q = 1$) con la entropía exponencial de Shannon y la diversidad de Simpson (q = 2) con la inversa de la concentración de Simpson (Gotelli and Chao, 2013). Estos índices fueron adoptados con el método de Chao y Jost (2015) para obtener mayor precisión de diversidad de especies continuos y de bajo sesgo. Además, obtuvimos las varianzas con un método de bootstrap para calcular los intervalos de confianza (Chao and Jost, 2015). Los intervalos de confianza no superpuestos (nivel de confianza del 95%) se consideraron estadísticamente significativos (p <0.05).

Para evaluar qué especies de flebotominos fueron más abundantes, se aplicó el índice de abundancia de especies (ISA), el cual fue normalizado a valores entre cero y uno, usando el "Índice estandarizado de abundancia de especies" del SISA (Roberts and Hsi 1979). Los valores del SISA cercanos a uno corresponden a las especies más abundantes. Este índice es útil para compensar abundancias y métodos de captura (Rodríguez-Rojas and Rebollar-Téllez 2017). Se calculó una prueba Z para probar si las proporciones sexos de los flebótomos muestreados eran similares y para evaluar diferencias significativas (p <0.05).

CAPÍTULO I. DNA BARCODING AND FAUNA OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE) FROM LOS TUXTLAS, VERACRUZ, MEXICO

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DNA barcoding and fauna of phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) from Los Tuxtlas, Veracruz, Mexico

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ABSTRACT

Mexico has great diversity of phlebotomine sand flies related to cases of leishmaniasis, yet few studies have dressed the molecular taxonomy of these sand fly species. The use of the cytochrome oxidase subunit 1 (COI) gene, as a DNA Barcode has facilitated the molecular identification of sand flies species worldwide. We use the DNA barcode as a useful tool for the identification of phlebotomine sand flies of the natural reserve Los Tuxtlas from Veracruz, México. A fragment of 536 bp of the COI gene was obtained from 36 individuals belonging to eight species of five genera (*Dampfomyia, Lutzomyia, Psathyromyia, Psychodopygus* and *Brumptomyia*) with coverage between 92–100%, and found similarities ranging from 93–98% with other New World phlebotomine sand flies. The NJ dendogram grouped sand flies into eight clusters according to identified species, supported by bootstrap of 97%–100%. In conclusion, all phlebotomine sand flies were correctly identified and agree with the morphological identification, also could separate genetics the isomorphic females of the genus *Brumptomyia.*

1. Introduction

Phlebotomine sand flies are dipterans of the family Psychodidae, which exhibit a great epidemiologic relevance as vectors of several human pathogens such as *Leishmania* parasites, the bacteria *Bartonella bacilliformis* and some arboviruses (Young and Duncan, 1994). More than 1000 sand fly species have been described worldwide (Shimabukuro et al., 2017), however only about 10% of these sand flies species serve as *Leishmania* vectors. Therefore, the accurate identification of sand fly species is very important for establishing control measures to reduce *Leishmania* transmission (Absavaran et al., 2009; Azpurua et al., 2010; Young and Duncan, 1994).

Although phlebotomine sand flies are a group of insects very studied worldwide, the taxonomic knowledge of this group is far from complete. This is mainly because the taxonomy of this group is based on morphological characters, requires considerable training and taxonomic expertise, due to the intraspecific variation and sometimes the lack of distinct morphological characters (Azpurua et al., 2010; Beati et al., 2004; Contreras-Gutiérrez et al., 2014; Terayama et al., 2008). In the recent years, DNA sequencing has become an important tool for studies of biological diversity and classification of American sand flies species. In particular, the mitochondrial gene cytochrome oxidase subunit 1 (COI) has been used for this purpose in many sand flies DNA barcode initiatives from Panama, Colombia, Brazil and Peru in order to produce species tags for taxonomic identification and in some cases, for resolving phylogenetic relationships at species level, due to their high evolutionary rate as well as for their low recombination (Absavaran et al., 2009; Arrivillaga et al., 2002; Azpurua et al., 2010; Beati et al., 2004; Cohnstaedt et al., 2011; Contreras-Gutiérrez et al., 2014; Depaquit, 2014; Pinto et al., 2015).

Currently, 54 phlebotomine sand flies species have been recorded in Mexico, of which only some are considered potential vectors of *Leishmania mexicana* and *Leishmania infantum,* the most important prevalent protozoa causing cutaneous or visceral leishmaniasis respectively (Alvar et al., 2012; Ibáñez-Bernal et al., 2004, 2017; Moo-Llanes et al., 2013). Despite the considerable diversity of sand fly species, few studies have addressed the molecular taxonomy of this subfamily. Research has focused mainly on the epidemiology of leishmaniasis and the detection of pathogens (Florin and Rebollar-Téllez, 2013; Pech-May et al., 2010, 2013; Sánchez-García et al., 2010; Sanchez-Tejeda et al., 2001). For this reason, we validated the use of COI DNA barcode as a useful tool for the identification of sand flies of the natural

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Table 1
List of species collected in Los Tuxtlas, Veracruz and their classification according to Galati (2003).

Phlebotomine sand fly species	♂	♀	Total	DNA available	COI-PCR
Psathyromyia (*Xiphopsathyromyia*) ***aclydifera****	266	322	588	89	5
Dampfomyia (*Coromyia*) ***beltrani***	2	1	3	1	1
Psathyromyia (*Forattiniella*) ***carpenteri***	9	52	61	38	5
Micropygomyia (*Micropygomyia*) ***cayennensis***	1	0	1	-	-
Lutzomyia (*Tricholateralis*) ***cruciata***▼	2	24	26	11	6
Bichromomyia ***olmeca olmeca***▼	10	12	22	4	2
Psychodopygus ***panamensis***▼	65	115	180	52	6
Pintomyia (*Pifanomyia*) ***serrana***	0	4	4	-	-
Psathyromyia (*Psathyromyia*) ***shannoni***▼	2	8	10	4	2
Psathyromyia (*Psathyromyia*) ***undulata***	2	4	6	2	1
Brumptomyia spp.	0	10	10	4	3
Brumptomyia ***hamata****	1	0	1	-	-
Brumptomyia ***mesai***	9	0	9	-	-
Doubtful specimens (mounting problems)	-	9	9	9	5
8 genera, 12 species	**369**	**561**	**930**	**214**	**36**

DNA available: number of specimens available for DNA extraction, COI-PCR: number specimens submitted for COI gene amplification
* New record for Veracruz State
▼ Species with relevance for *Leishmania* transmission in Mexico

reserve "Los Tuxtlas" from Veracruz, México.

2. Material and methods

2.1. Study area and sampling methods

Sampling was conducted at the Estación de Biología Tropical, Los Tuxtlas, located in the municipality of San Andrés Tuxtla in Veracruz, Mexico (N18° 34´ W95° 04´). All collection was done on public land. Sand flies were sampled during three consecutive nights in March and October 2011; January, March, April, June, August and November 2012; April and May 2013. Samplings were conducted from 18:00 to 11:00 h using automatic CDC miniature light traps (model 512; John W. Hock Co., Gainesville, FL, USA). Traps were hung from trees or branches using a rope approximately one meter above the ground. The collected material was maintained under refrigeration during 12h, after which the sand fly specimens were separated from other arthropods, preserved and stored in 70% ethanol.

2.2. Identification of sand flies

For morphological identification, sand flies were mounted on slides using "Euparal medium", following the procedures outlined by Ibáñez-Bernal (2005b). For molecular analysis, 20% of the collected female sand flies were selected randomly. The thorax, legs and abdomen were dissected and deposited in a microtube containing 70% ethanol and kept at −20 °C for this analysis. The remaining parts of the female body, as well as the collected male sand flies, were completely mounted on slides for morphological identification. Identification was carried out following the keys and classification proposed by Galati (2003), Young and Duncan (1994) and Ibáñez-Bernal (1999, 2005a, 2005b). The abbreviations of generic names were used according to Marcondes (2007).

2.3. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted only from the abdomen of individual female sand flies using the "Chelex-100 resin" (García-González et al., 2004). In order to amplify a ≈708 bp fragment of the mitochondrial COI gene, the primers LCO1490 (5´-GGT CAA CAA ATC ATA AAG ATA TTG G-3´) and HCO2198 (5´ TAA ACT TCA GGG TGA CCA AAA AAT CA-3´) were used (Folmer et al., 1994). The reactions were conducted in a C1000 Thermal Cycler (BioRad Laboratories, USA) under the following conditions: initial denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s; and a final extension at 72 °C for 5 min. The reaction mixture was prepared in a final volume of 25 μL reaction with 0.3 μL of ABM Hot Start DNA polymerase (Applied Biological Materials Inc., Canada), 2.5 μL of 10X buffer, 1.5 μL of 25 mM $MgSO_4$, 0.6 μL of 2 mM dNTP, 1 μL of 10 pmol of each oligonucleotide, 3 μL of the DNA template, and 15.1 μL of ultrapure H_2O. The negative control contained water instead of DNA, and in order to avoid cross contamination, no positive control were used. The amplification products were subjected to electrophoresis in 1.5% agarose gel stained with GelStar (Lonza, Switzerland). Positive PCR products (both strands) were purified and sequenced at the Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, UNAM.

2.4. Data analysis

The electropherograms were visualized and edited in GeneStudio™ Professional Edition Version 2.2.0.0 (GeneStudio, Inc., USA) in order to generate a consensus sequence for each specimen. Each consensus sequence was compared with all sequences available at NCBI database using BLASTn (Basic Local Alignment Search Tool) with megablast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as a preliminary confirmation of the identity of each specimen. The DNA consensus sequences were aligned using the ClustalW in MEGA v6.0 software (Tamura et al., 2013) and MAFFT version 7 server (Katoh et al., 2017).

The number of haplotypes (H), polymorphic sites (s), and nucleotide diversity per species (π), was calculated using DnaSP v5.10 program (Librado and Rozas, 2009). The web program FINGERPRINT (http://evol.mcmaster.ca/fingerprint/) was also used to depict the nucleotide heterogeneity and nucleotide diversity (Lou and Golding, 2007). Genetic pairwise distances were estimated using the Kimura-2-parameter substitution model (K2P) and Neighbor-Joining (NJ) tree was built in MEGA v6.0.

The barcode gap (gap between intra and interspecific genetic K2P distances) was analysed to confirm that COI region was suitable for interspecific discrimination. Graphical depiction of barcode gap was calculated using ggplot2 and EasyGgplot package (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016) in the platform R 3.5 (R Core Team, 2018).

In order to determine the limitation of COI region on the retention of phylogenetic information, due to substitution saturation, we created the transition/transversion saturation plots over divergence and calculated the index of substitution saturation (Iss) in the program DAMBE version 7.0.35 (Xia, 2018). All the sequences obtained in the present study have been deposited into GenBank under accessions numbers MK744133-MK744156.

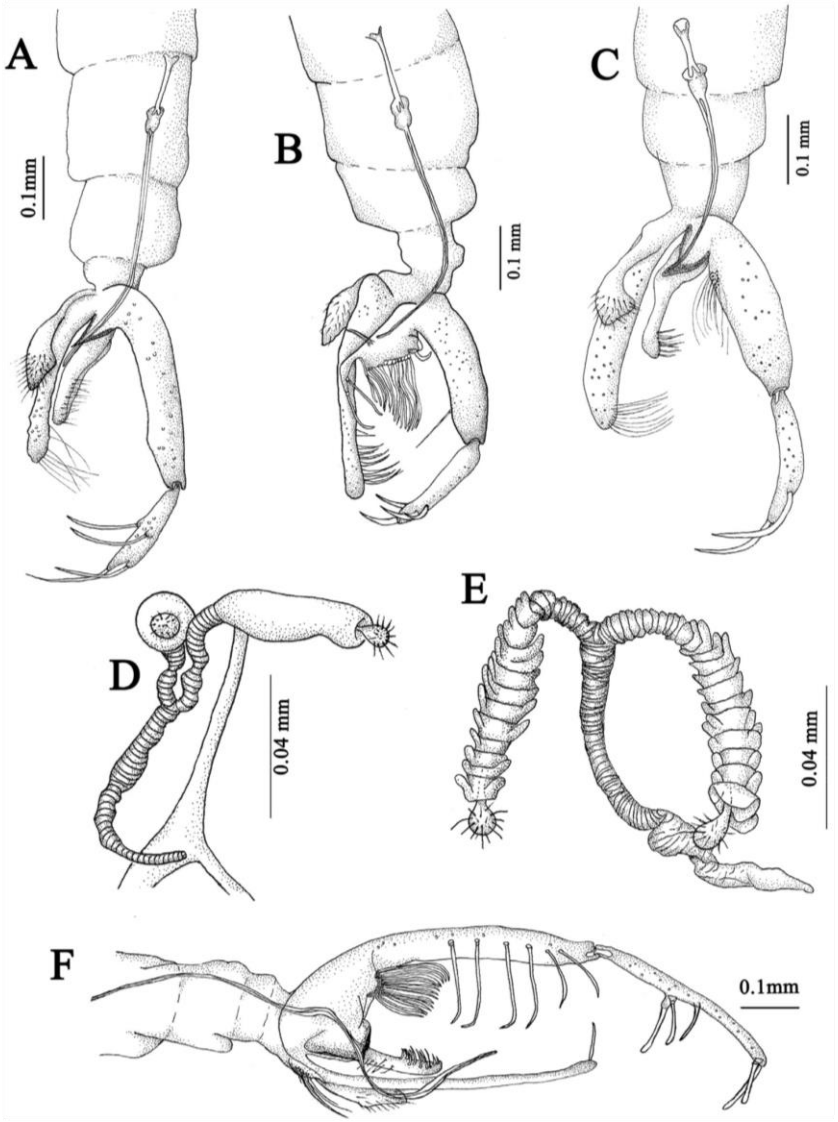

Fig. 1. Morphological characteristics of sand fly species from Los Tuxtlas, Veracruz. A. genitalia *Pa. shannoni* male; B. genitalia *Ps. panamensis* male; C. genitalia *Da. beltrani* male; D. Spermathecae *Pa. shannoni* female; E. Spermathecae *Ps. panamensis* female; F. genitalia *Br. mesai* male.

3. Results

3.1. Specimens collected

A total of 930 specimens were collected during this study, corresponding to twelve sand fly species belonging to eight genera *sensu* Galati (Table 1, Fig 1). The most abundant species were: *Psathyromyia* (*Xiphopsathyromyia*) *aclydifera* (Fairchild & Hertig), *Psychodopygus panamensis* (Shannon), *Psathyromyia (Forattiniella) carpenteri* (Fairchild & Hertig) and *Lutzomyia (Tricholateralis) cruciata* (Coquillett), representing 92.8% of the specimens collected. Overall, the proportion of females (60%) was a higher than that of males (40%). Four species of the sand fly fauna collected at Los Tuxtlas have epidemiological relevance for *Leishmania* transmission in Mexico (Table 1).

All collected species are new records for Los Tuxtlas except for *Br. mesai* and *Da. beltrani* that had previously been reported in this area (Ibáñez-Bernal, 1997). Furthermore, *Br. hamata* (Fig. 2) and *Pa. aclydifera* (Fig 3) are new records for State of Veracruz (Ibáñez-Bernal et al., 2011). The slide-mounted sand flies for morphological identification are sheltered under collection permission SGPA/DGVS/09346/16 license FAUT-0317 of the Secretaria de Medio Ambiente y Recursos Naturales.

3.2. Sequences analysis

We selected a female representative of each taxon from every date of collection for the COI gene amplification by PCR and DNA sequencing (Table 2). However, COI consensus sequences were obtained only for eight species, since sequences for *Pa. undulata, Bi. olmeca* and one sequence of *Brumptomyia* sp. either had poor quality or were too short for contig-assembly. All specimens used for barcode analyses were characterized morphologically. The set of obtained sequences had a

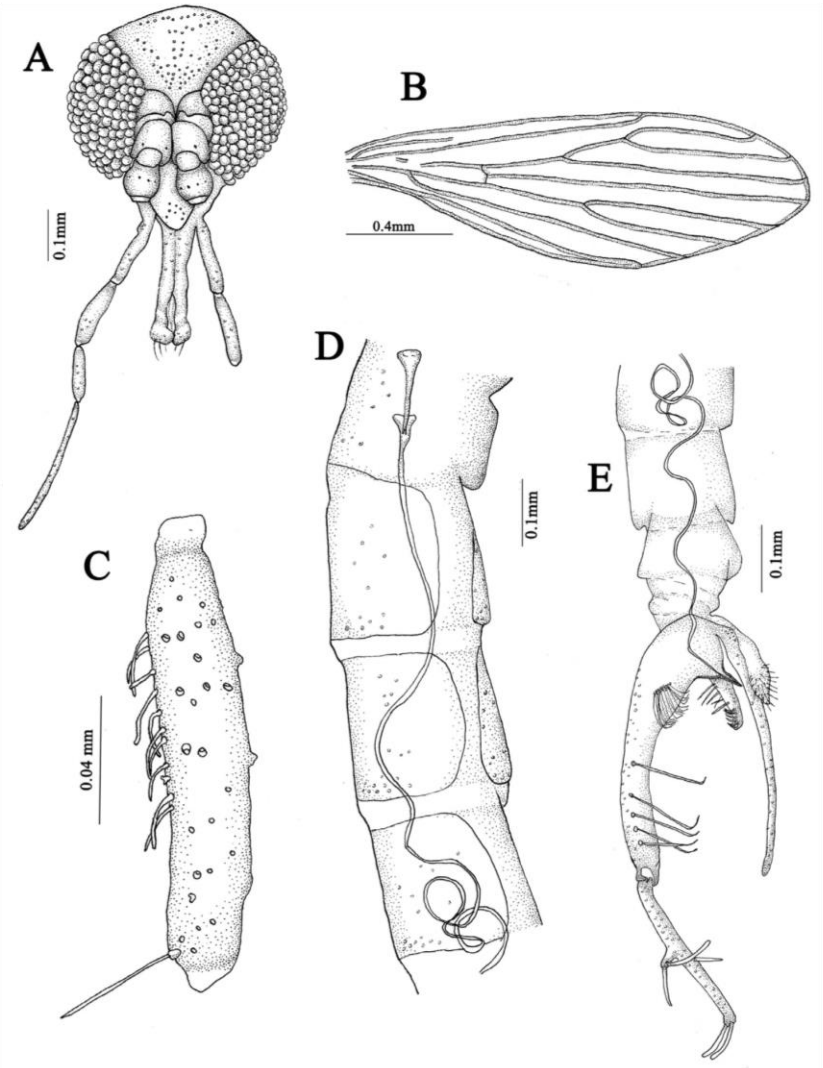

Fig. 2. Morphological characteristics of *Brumptomyia hamata.* A. head; B. wing; C. palp III with Newstead´s scales; D. sperm pump; E. genitalia.

coverage between 92–100%, and similarities ranging from 93–98% with other New World phlebotomine sand fly COI sequences (Azpurua et al., 2010; Contreras-Gutiérrez et al., 2014; Florin and Rebollar-Téllez, 2013; Romero-Ricardo et al., 2016; Vivero et al., 2017).

The multiple alignments of consensus and references sequences from GenBank containing 536 sites, no INDEL events or stop codons were observed inside the coding region, ruling out the presence of pseudogenes or nuclear copies of mitochondrial origin (NUMT). A total of 292 conserved sites, 244 variable sites and 211 parsimony-informative sites were observed. The total nucleotide diversity per site was $\pi = 0.14681$ and G + C = 0.367. The heterogeneity per nucleotide according to FINGERPRINT is shown in Fig. 4A and sites with highest nucleotide diversity are shown in Fig. 4B. Most of the sites with the highest diversity (dark grey) can be found at the initial and central region of the amplified COI gene. According to our sequences, the number of haplotypes per species varied between two and six (Table 2), being *Lu. cruciata* the sand fly taxon with the higher number of haplotypes (H = 6).

The NJ dendogram shows that the specimens of the same species were grouped in the same branch supported by high bootstrap values between 97%–100% (Fig. 5). Molecular taxonomy using COI barcoding allowed the identification of eight species belonging to five genera of Phlebotominae. The analysis of the specimens of genus *Brumptomyia* only permitted to identify the samples as *Br. mesai* and *Br. hamata* by using COI barcode, due to a lack of sufficient morphological characters to separate the females of this genus by traditional taxonomy. This was also the case for doubtful specimens that could not be accurately identified by morphological characters due to the loss of structures of morphological interest that occurred during the mounting procedure. However, we were able to differentiate two specimens of *Da. beltrani* and *Ps. carpenteri* as well as one specimen of *Lu. cruciata* (Table 1).

The intraspecies genetic distances calculated with the K2P model range from 0.5% to 2.4%. We observed the highest intraspecific divergence for *Pa. aclydifera,* followed by *Lu. cruciata* (Table 2). Otherwise, the interspecific distance ranged from 12.4% to 20.9%, where *Ps.*

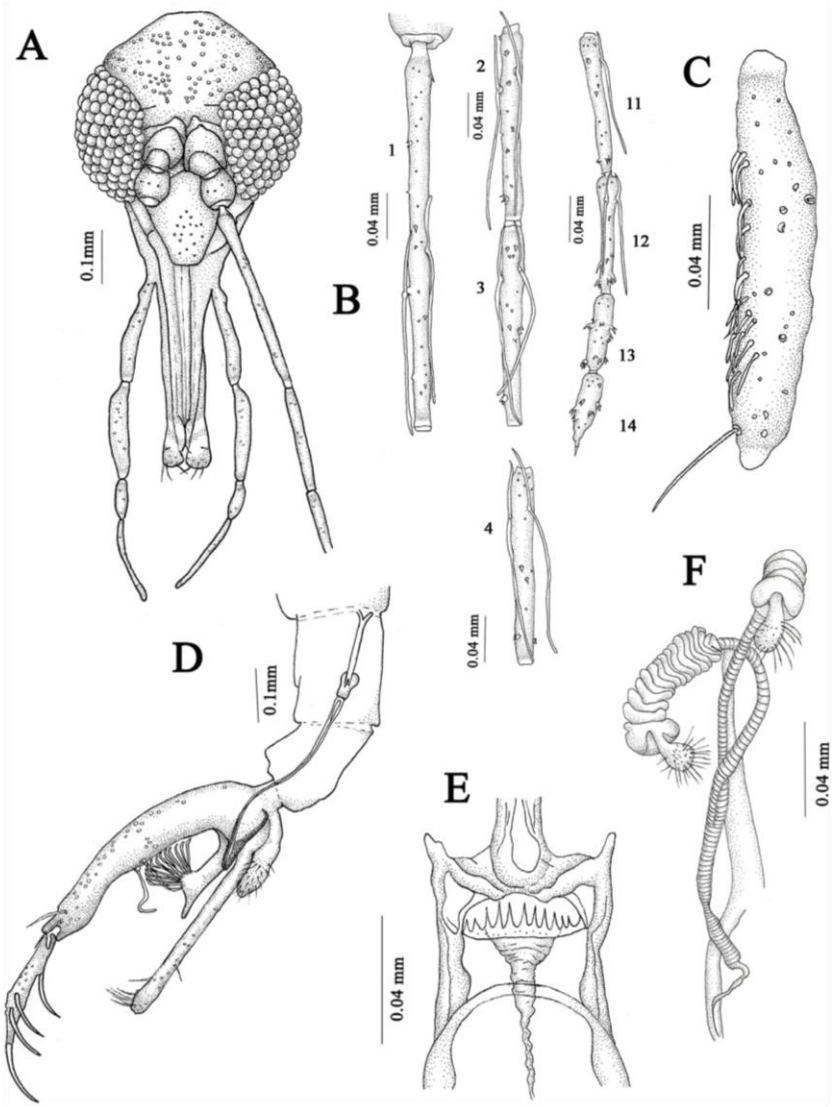

Fig. 3. Morphological characteristics of *Psathyromyia aclydifera.* A. head; B. flagellomeres (1–4; 11–14); C. palp III with Newstead´s scales; D. genitalia; E. cibarium; F. spermathecae.

panamensis and *Pa. carpenteri* showed the smallest interspecific distance, followed by *Br. mesai* and *Br. hamata* (Table 3).

To evaluated if the gene COI was a good indicator for species differentiation in spite of their observed genetic variability, we look for the "barcoding gap", by comparison of 1.817 pairwise distances of 63 American sand fly species (including our sequences and GenBank reference sequences). It can clearly be seen that interspecific and intraspecific distances have different means, although an overlap between them is observed (Fig. 6A). Despite this overlap, there a few interspecific comparisons that could be considered as small as intraspecific distance (Fig 6B). This has been reported for some species group in the genera *Trichopygomyia, Migonemyia, Dampfomyia, Psychodopygus, Pintomyia, Micropygomyia, Psathyromyia, Nyssomyia, Evandromyia, Bichromomyia, Lutzomyia, Brumptomyia* and *Warileya,* genera *sensu* Galati. Furthermore, the K2P distance distribution (density) suggests that ≈95% of the analysed sand fly species could be identified successfully. The barcoding gap was also calculated and compared using all codon positions and removing the third codon. In this assay, it appears that much of the genetic diversity and species differentiation capacity from this COI region lies in the variability of the third codon position, since the removal of this position reduced the observed distances (from maximum distance 0.2670, it declined to 0.086), which led to a greater overlap between intra- and interspecific distances (Fig. 6C and D).

The transitions/transversions versus genetic distance plot, clearly shows that the COI is saturated with transversions when genetic distances are >0.21 using all codon positions (Fig. 7A). Removal of the third of the codon resulted in the disappearance of the transversion saturation (Fig. 7B).

Additionally, the saturation test of Xia showed that by using only the 1st + 2nd codon position, the Iss = 0.261 when compared with all codon positions the Iss = 0.386. Although both values were significantly lower than Iss.c ($P < 0.0000$), it can be assumed that the

Table 2
Sand fly species identified by barcode from Los Tuxtlas, and COI intraspecific variability.

Species	N	Sex	H	Intraspecific distance	Accession numbers
Pa. aclydifera	3	F	3	0.0242	MK744147–MK744148
Ps. panamensis	2	F	2	0.0051	MK744136–MK744137
Pa. carpenteri	7	F	3	0.0083	MK744138–MK744144
Lu. cruciata	6	F	6	0.0120	MK744152–MK744156
Bi. olmeca olmeca	–	F	–	–	-
Pa. shannoni	2	F	2	0.0051	MK744145–MK744146
Pa. undulata	0	F	–	–	-
Da. beltrani	3	F	3	0.0085	MK744133–MK744135
Br. hamata	1	F	1	–	MK744149
Br. mesai	1	F	1	–	MK744150
10 species	**25**		**21**		**–**

n: number of sequences consensus obtained per specie, F = female, H: number of haplotypes per species.

gene COI has undergone some saturation on the third position of the codon.

4. Discussion

This study evaluated the use of DNA barcode as a reliable method to differentiate phlebotomine sand fly fauna of the Los Tuxtlas region in Veracruz, Mexico. The analysis NJ allowed the successful assignment of 25 COI sequences to eight Mexican species. The dendogram clearly showed that all sequences from the same species cluster together with strong branch support (bootstrap 97–100%), despite being sequences from different geographical locations (Azpurua et al., 2010; Contreras-Gutiérrez et al., 2014; Florin and Rebollar-Téllez, 2013; Pinto et al., 2015; Romero-Ricardo et al., 2016; Vivero et al., 2017). The barcode results agreed with the morphological identification for most of the specimens. Additionally, it allowed to identify, specimens with isomorphism problems, such as the two female specimens of the genus *Brumptomyia* that had high degree of morphological similarity (Absavaran et al., 2009; Ibáñez-Bernal, 1999). Even species that were damaged during the slide-mounting procedure could be identified, as was the case with some of *Pa. carpenteri, Da. beltrani* and *Lu. cruciata* females.

The COI gene allowed all the phlebotomine sand flies from Los Tuxtlas to be identified, the intraspecific genetic distance between these species ranged from 0.5% to 2.4%, the highest values corresponded to *Pa. aclydifera* (0.024) in spite of having only three haplotypes, followed by *Lu. cruciata* (0.018) that showed the higher number of haplotypes per species for this dataset (Table 2). However, those distances values fell within the expected range of intraspecific variability if we consider that the distances close to 2% are considered normal, according to barcode parameters and the distance values in documented Dipteran species (Scarpassa and Alencar, 2013). Interspecific variability for this species ranged between 12.4% to 20.9%, although the range of interspecific genetic divergence found in this study might be considered low, compared with previous reports from Colombia (Contreras-Gutiérrez et al., 2014; Romero-Ricardo et al., 2016), Peru (Nzelu et al., 2015), and Brazil (Rodrigues et al., 2018; Scarpassa and Alencar, 2013). This is probably due to composition of the fauna or the number of individuals used in this barcoding analysis. Nonetheless the range of distances observed allowed for the species identification process.

Regarding the high genetic variability of *Pa. aclydifera* (0.024), we did not expect high values due to very few specimens, yet it seems possible that these taxa may possess a higher genetic pool given their abundance in the area and their high intraspecific variability.

We expected a higher genetic variability for *Lu. cruciata* given the higher number of samples and haplotypes, although their intraspecific genetic distances can be considered as moderate and seems to be related to temporality effects, since the analysed specimens came from different collection dates. Despite that none of our species had a striking high intraspecific divergence; it would be interesting to explore the genetic structure for both *Pa. aclydifera* and *Lu. cruciata* nationwide or at a continental scale, since there is strong evidence that the genetic pool of *Lu. cruciata* may be sensitive to geographical barriers and could have adapted to local habitat characteristics (Pech-May et al., 2013). For that reason, it would be very interesting to explore the relationship between population isolation and *Leishmania* transmission cycles.

The barcoding gap analysis of 63 American species, showed little overlap between the minimum interspecific divergence and the maximum intraspecific distances. We found surprisingly low divergence between *Ty. triramula* (subtribe Lutzomyiina) and *Pa. aragaoi* (subtribe Psychodopygina) with a K2P divergence of 1.29%. Futhermore, the

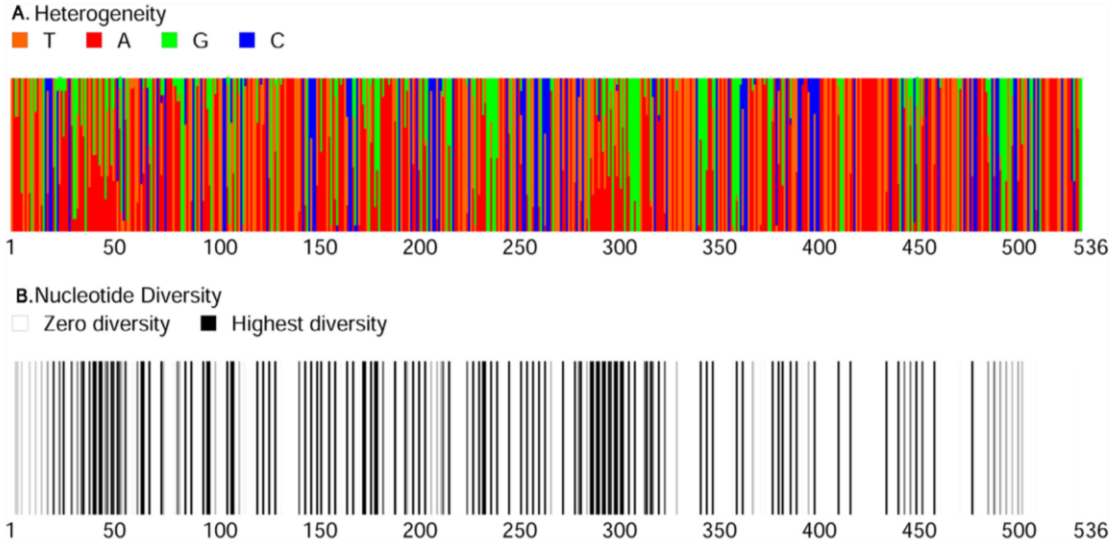

Fig. 4. Distribution of heterogeneity (A) and nucleotide diversity (B) for a region of 536 sites from COI gene of eight phlebotomine sand fly species from Los Tuxtlas, Veracruz, Mexico.

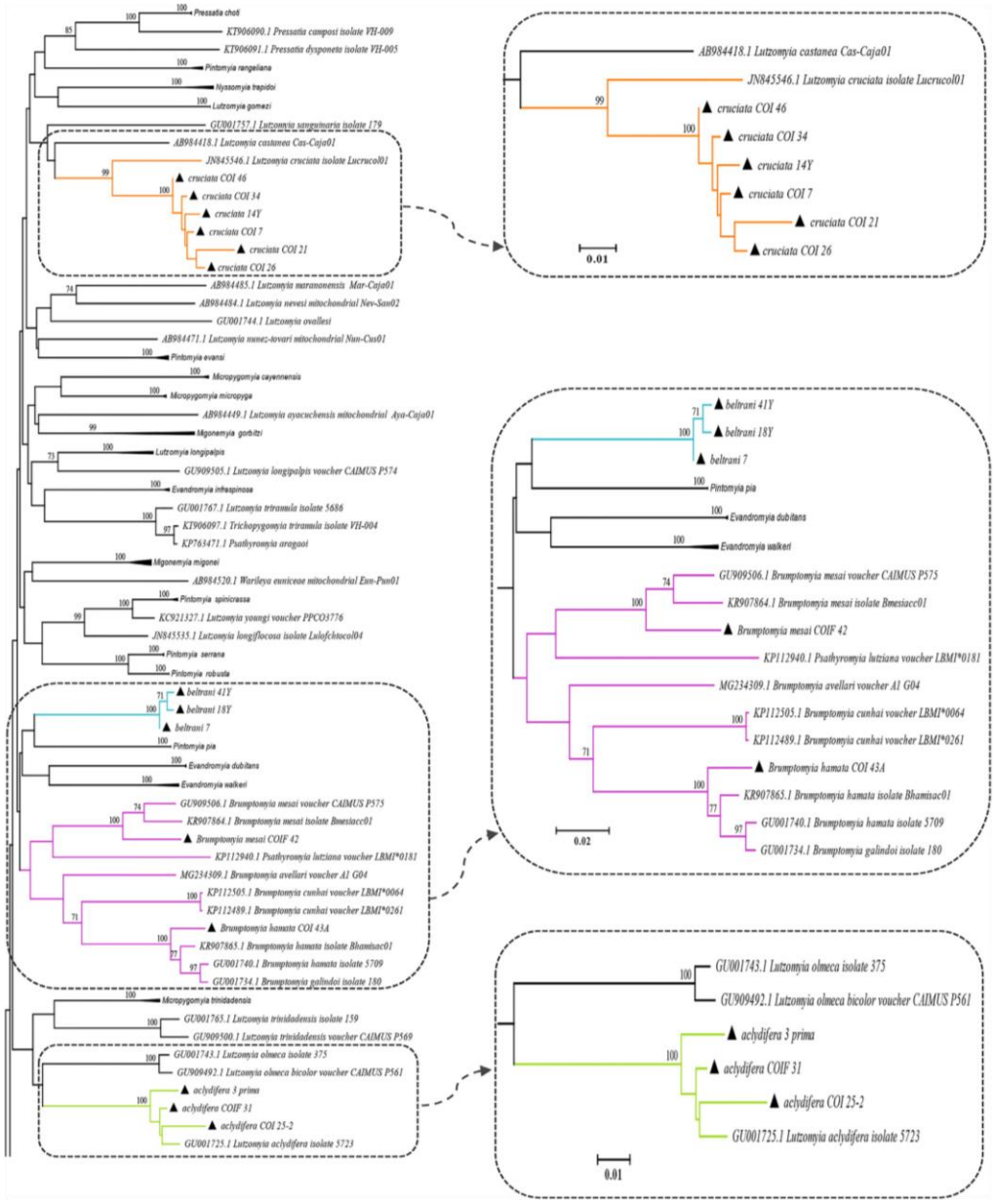

Fig. 5. Neighbor-joining tree of COI sequences of sand flies from Los Tuxtlas, Veracruz. Numbers indicate bootstrap values (only >70% are shown). The dotted square in the NJ tree was zoomed for the eight species identified in this study.

divergence between *Pintomyia* species from the series *serrana* (*Pi. serrana* and *Pi. robusta*) and *townsendi* (*Pi. youngi, Pi. spinicrassa* and *Pi. longiflocosa*) ranged between 3.24%–6.73% of divergence. This pattern was also evident between species of *Pressatia* (*Pressatia choti* vs *Pressatia camposi*, divergence 5.83%) and also remarkable among *Nyssomyia* species (especially *N. umbratilis, N. antunesi, N. yuilli, N. whitmani, N. intermedia and N. anduzei*), whose K2P divergences ranged from 2.1 to 6.8%. Those relative low values for interspecific divergences may be a result of the analysis of closely-related species that recently underwent a speciation process or this may be a genetic signal that some of those taxa are conspecifics. Contrarily, the genetic intraspecific variability analysis within the 63 American species showed high divergence for *Pa. shannoni* (6.9%), *Mi. trinidadensis* (8.23%), *Bi. flavicutellata* (8.29%), *Mg. gorbitzi* (8.5%) and *Pa. runoides* (9.7%). If we consider that recognized species complex as *Lu. longipalpis sensu lato* has K2P divergence values of around 7.6%, seems reasonable to speculate that some of these afore mentioned species with high intraspecific variability may represent population structuration or even some of them might represent cryptic species.

Besides the remarkable usefulness of COI barcode for species discrimination, it could also be a valuable tool for epidemiologic studies and vectorial control programs, where species identification is the basis

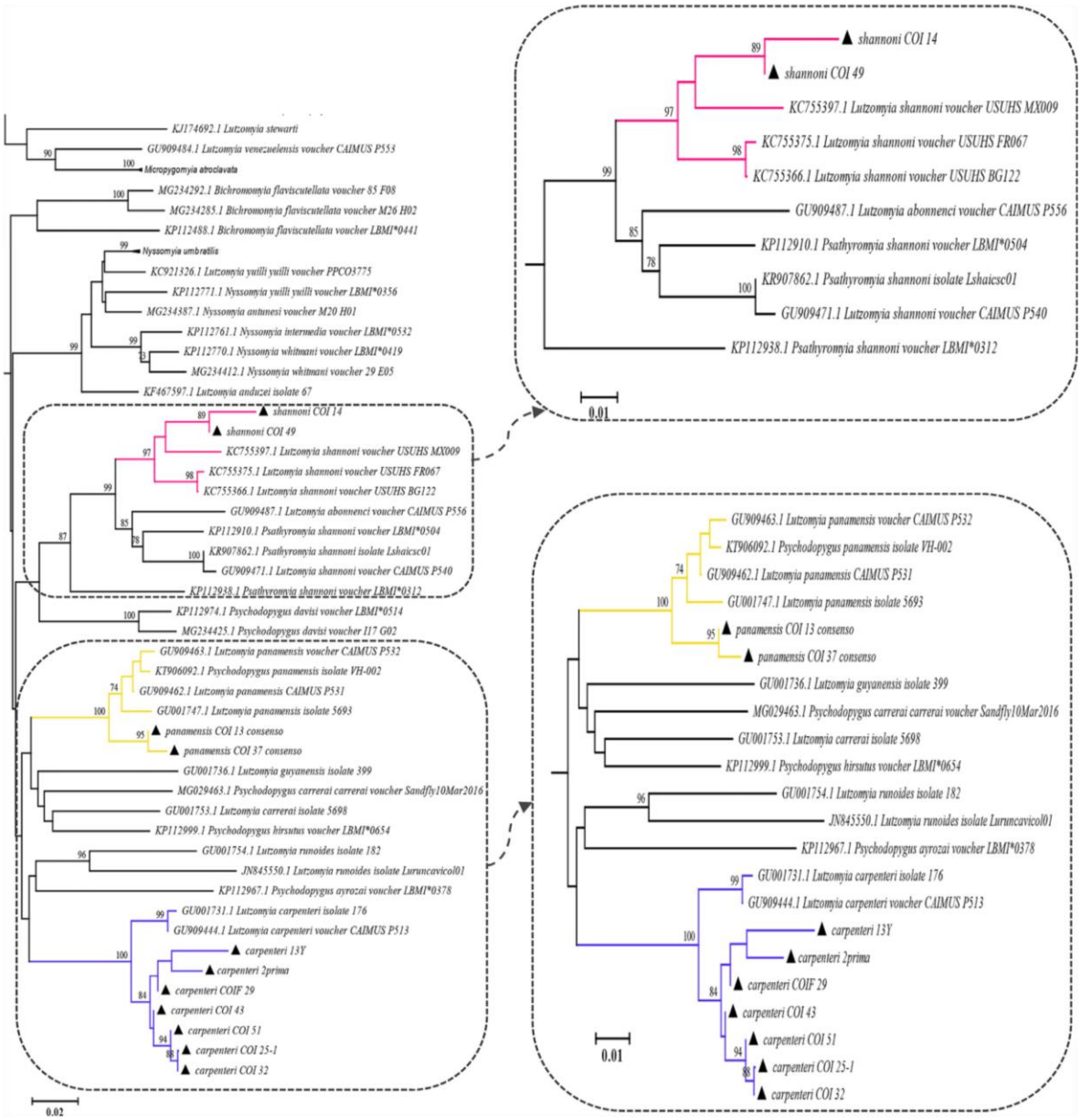

Fig. 5. (*continued*)

Table 3
Interspecific genetic distance (K2P) of COI among sand fly species from Los Tuxtlas.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
(1). *Pa. aclydifera*								
(2). *Br. hamata*	0.209							
(3). *Pa. carpenteri*	0.158	0.180						
(4). *Lu. cruciata*	0.160	0.139	0.167					
(5). *Ps. panamensis*	0.147	0.201	0.124	0.151				
(6). *Da. beltrani*	0.145	0.189	0.160	0.181	0.156			
(7). *Br. mesai*	0.163	0.128	0.142	0.172	0.150	0.128		
(8). *Pa. shannoni*	0.141	0.207	0.143	0.200	0.147	0.183	0.175	

of their effectiveness. However the capabilities of COI region must be used with caution when it comes to describing new species based only on genetic divergences, because species boundaries surely are not uniform among phlebotomine sand flies lineages (Cohnstaedt et al., 2011). We now describe the usefulness of the DNA barcode as a reliable method to differentiate the phlebotomine sand fly fauna of the natural reserve Los Tuxtlas in the State of Veracruz (Mexico) and provide the first sequences for seven Mexican sand flies species: *Da. beltrani, Pa. aclydifera, Pa. carpenteri, Ps. panamensis, Lu. cruciata, Br. hamata* and *Br. mesai*. The sequences of *Pa. shannoni* were already available at GenBank from previous studies from State of Quintana Roo (Florin and Rebollar-Téllez, 2013). Our study also contributed to the enrich the COI barcode libraries with an important set of sequences that could be useful for

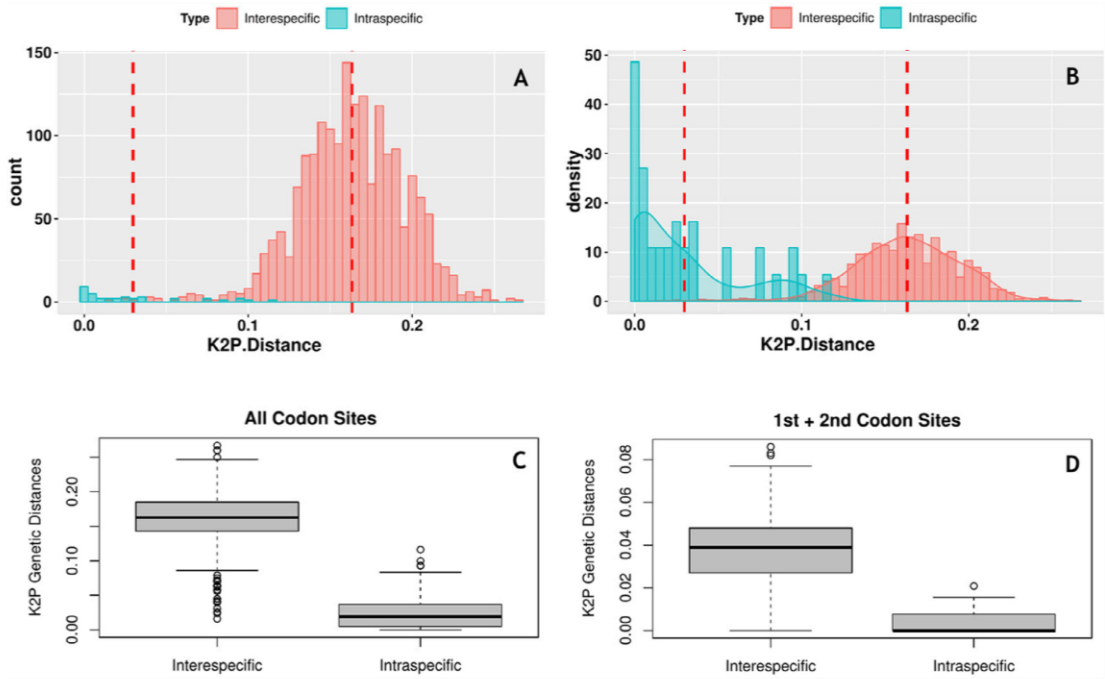

Fig. 6. Barcode gap of American phlebotomine sand flies using Kimura-2-parameter substitution model. The frequency (A) and density (B) of calculated intra- and interspecific genetic distances shows that their means (dashed red lines) are statistically different (Wilcoxon sum rank test W = 67355, $p = 2 \times 10^{-16}$). The boxplot shows the dropping of genetic distances from barcoding gap, including all codon positions (C) compared to a dataset without the 3rd codon site (D). Even after removal of the 3rd codon site, statistical differences were observed between intra- and interspecific means (Wilcoxon sum rank test W = 66926, $p = 2 \times 10^{-16}$).

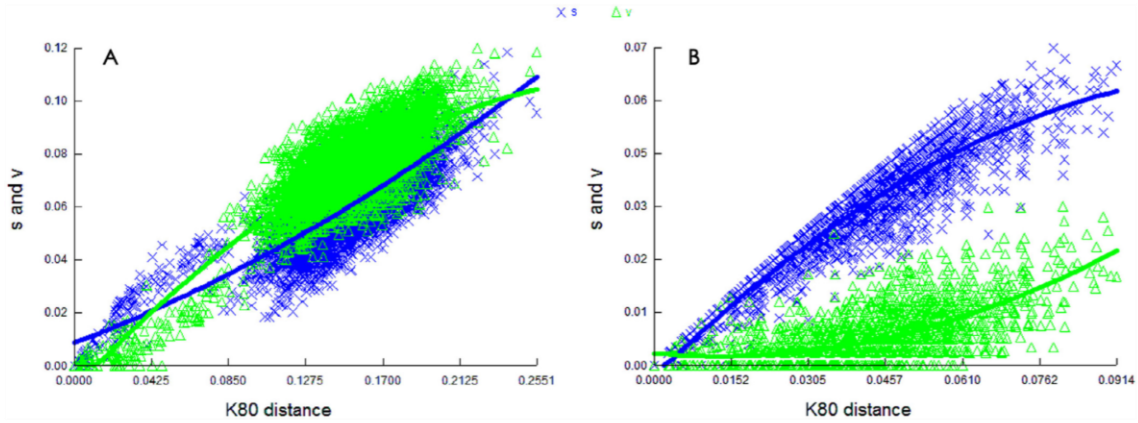

Fig. 7. Plot of transitions (blue) and transversions (green) over Kimura 2-parameters distances (K80) with all codon positions (A) and after removal of third codon position (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

future comparisons, as well as for genealogical studies of interesting taxa such as *Pa. shannoni, Ps. panamensis* and *Lu. cruciata* which are considered important vectors of *Leishmania* species in Latin America (Florin and Rebollar-Téllez, 2013; Nzelu et al., 2015; Pech-May et al., 2010).

In conclusion, this study provides for first time, COI barcode sequences for the identification of eight Mexican sand fly species. All the phlebotomine sand flies were separated in clusters with high confidence (97–100%) that agreed with their morphological identification. We additionally update the species richness for State of Veracruz in Mexico, with the new records of *Pa. aclydifera* and *Br. hamata*, that were confirmed with the DNA barcode analysis. Taken together the COI barcode not only allows the differentiation of sand fly species, but is also a powerful tool that enables to distinguish between isomorphic females, as occurs in the species of the genus *Brumptomyia* and furthermore permits to analyses damaged specimens.

Declaration of Competing Interest

None.

Acknowledgments

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CAPÍTULO II. DETECCIÓN MOLECULAR DE PATOGENOS ASOCIADOS CON FLEBOTOMINOS

- **First report of Bartonella sp. in sand flies (Diptera: Psychodidae: Phlebotominae) from southern Mexico**

- **Rickettsial agents detected in the genus Psathyromyia (Diptera: Phlebotominae) from a Biosphere Reserve of Veracruz, Mexico**

- **Molecular detection of Leishmania infantum in sand flies (Diptera: Psychodidae: Phlebotominae) from Veracruz, Mexico**

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SCIENTIFIC NOTE

FIRST REPORT OF *BARTONELLA* SP. IN SAND FLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE) FROM SOUTHERN MEXICO

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ABSTRACT. The genus *Bartonella* encompasses several zoonotic species that cause emerging infectious diseases in humans. These species are transmitted to humans by hematophagous arthropods. In South America, sand flies are the most important vectors of *Bartonella bacilliformis*, the etiological agent of Verruga peruana. Due to the importance of bartonellosis in public health, the aim of this study was to detect *Bartonella* in wild populations of phlebotomine sand flies in southern Mexico. In total, 29 sand flies belonging to 6 species were collected. Only 2 specimens were positive, representing a prevalence of 8.69% (2/23). This appears to be the first report of *Bartonella* sp. in phlebotomine sand flies outside the endemic area of Verruga peruana in South America. Further studies are needed to clarify the relation between this bacterium and sand flies in Mexico.

KEY WORDS Host, intracellular, *Lutzomyia*, vector, Veracruz

The genus *Bartonella* encompasses more than 33 species of Gram-negative, facultative intracellular and hemotropic bacteria, which can cause chronic intraerythrocytic infections in their mammalian hosts, especially rodents and humans (Chomel et al. 2009). Several blood-feeding arthropods are involved in the transmission of these bacteria, such as fleas, sand flies, sucking lice, Acari, and other arthropods (Kosoy et al. 2012, Kamani et al. 2013, Battisti et al. 2015, Regier et al. 2016).

Some *Bartonella* species are associated with an increasing number of emerging and re-emerging diseases in the human population (Chomel et al. 2003, Regier et al. 2016). In particular, *B. bacilliformis*, which causes an endemic anthropozoonosis in Peru, has also been reported in outbreaks in southeastern Ecuador and southern Colombia. Additionally, some unconfirmed cases have been reported in Thailand, Bolivia, Chile, and Guatemala (Patiño-Camargo 1939, Hambuch et al. 2004, Sanchez Clemente et al. 2012, Maroli et al. 2013). This pathogen is transmitted by sand fly species such as *Pintomyia verrucarum* (Townsend), *Lutzomyia noguchii* (Shannon), and *Lu. peruensis* (Shannon) in the localities of Huarochiri, Lima, and Urubamba, Cusco, Peru (Noguchi et al. 1929, Hertig 1942, Montoya et al. 1998, Ellis et al. 1999, Villaseca et al. 1999).

In Mexico, few studies have been carried out for the detection of *Bartonella* in arthropods (Sánchez-Montes et al. 2016, López-Pérez et al. 2017, Moskaluk et al. 2018). Despite 54 species of sand flies having been recorded in Mexico, no studies have been conducted to detect the presence of *Bartonella* in sand flies (Ibáñez-Bernal 2005, Ibáñez-Bernal et al. 2015). Thus, the aim of this study was to detect a possible natural infection of sand flies with *Bartonella* in a natural reserve in Los Tuxtlas, Veracruz, Mexico.

Sampling was conducted at the Estación de Biología Tropical, Los Tuxtlas, located in the municipality of San Andrés Tuxtla in Veracruz, Mexico (18°34′N, 95°04′W). Sand flies were sampled during 3 consecutive nights between April and May 2016. Sampling was carried out between 18:00 and 23:00 h, using 5 automatic light traps (model 512; John W. Hock Co., Gainesville, FL, USA). Traps were hung from trees or branches approximately 1 m above the ground. The collected material was separated, fixed, and stored in 70% ethanol. The head and the last segments of the abdomen of the female and male sand flies were mounted on slides using Canada balsam medium for morphological identification, following the procedures outlined by Ibáñez-Bernal (2005). For molecular analysis, we separated the thorax, legs, and abdomen individually in a microtube with 70% ethanol. Identification was carried out according to the classification proposed by Galati (2003).

Genomic deoxyribonucleic acid (DNA) was extracted from the abdomen of sand fly females using Chelex-100 (García-González et al. 2004). For

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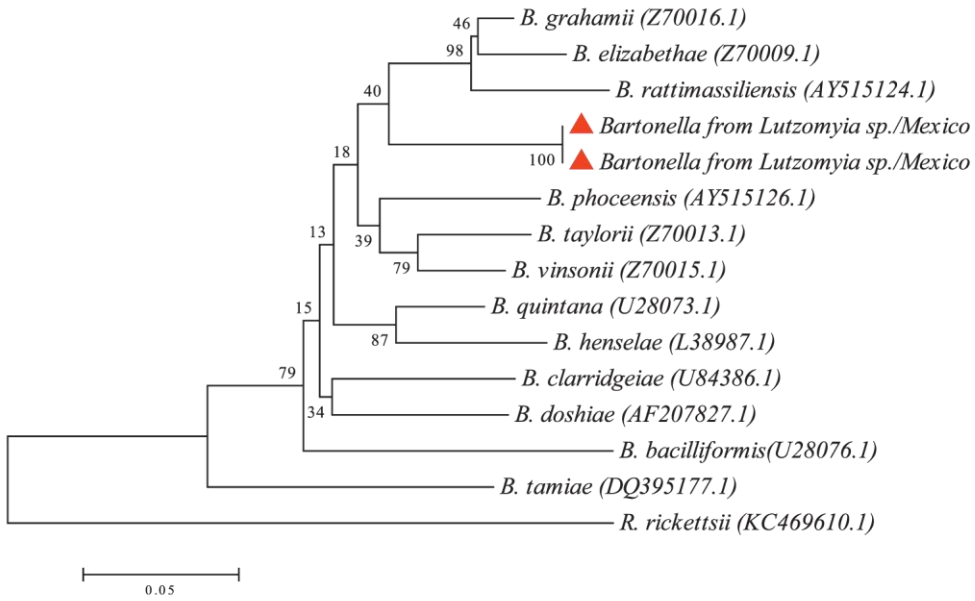

Fig. 1. Dendrogram for *Bartonella* species based on *gltA* sequences. A Tamura 3-parameter distance model and 10,000 iterations were used. Sequences obtained in this study are marked with a red triangle.

Bartonella detection, we amplified an ~379-base-pair (bp) fragment of the citrate synthase gene (*gltA*) using the primers BhCS871.p and BhCS1137.n (Norman et al. 1995). A reaction mixture was prepared in a final volume of 25 µl, with 12.5 µl of GoTaq® Green Master Mix (2X Promega Corporation, Madison, WI, USA), 1 µl of each primer (100 ng each), 10 µl of DNA (~50 ng), and 0.5 µl of nuclease-free water. We included *Bartonella* DNA taken from a flea as a positive control, whereas the negative control consisted of ultrapure water devoid of DNA. We followed the polymerase chain reaction (PCR) conditions specified by Kamani et al. (2013). The amplification products were subjected to electrophoresis in 2% agarose gel, and positive samples were sequenced at Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico.

Each sequence was compared with sequences available in the National Center for Biotechnology Information database using BLASTn (Basic Local Alignment Search Tool), as a preliminary confirmation of *Bartonella* amplification. The DNA sequences were aligned with other *Bartonella* sequences in GenBank using CLUSTAL W in MEGA 6.0. Furthermore, a neighbor joining analysis was carried out using the Tamura 3-parameter distance model with 10,000 iterations. The sequences obtained were deposited in GenBank under accessions numbers MN325838 and MN325839.

In total, 29 phlebotomine sand fly specimens, belonging to 6 species, were collected: *Bichromomyia olmeca olmeca* (Vargas and Díaz-Nájera), *Brumptomyia mesai* (Sherlock), *Lu. cruciata* (Coquillett), *Psathyromyia carpenteri* (Fairchild and Hertig), *Pa. undulata* (Fairchild and Hertig), and *Psychodopygus panamensis* (Shannon). Five specimens could not be identified due to the damage inflicted during the slide mounting process and were therefore recorded as *Lutzomyia* sp. The most abundant species were *Lu. cruciata*, with 7 specimens, and *Pa. carpenteri*, with 5 specimens, whereas the least abundant were *Pa. undulata*, with 2 specimens, and *Br. mesai* with 1 specimen. The proportion of females (75.86%) was higher than that of males (20.68%).

All female specimens were tested for *Bartonella* DNA, and 2 specimens of *Lutzomyia* sp. tested positive, representing a prevalence of 8.69% (2/23). Recovered sequences showed a 100% similarity between them. The obtained consensus sequences had 307 bp, 99% coverage, and 96% similarities with sequences of *Bartonella* sp. from Thailand and China (FJ668633 and FJ589054). Additionally, the neighbor joining analysis (Fig. 1) grouped our sequences close to *B. grahamii*, *B. elizabethae*, and *B. rattimassiliensis*. For more than 80 years, extensive research on the epidemiology of *B. bacilliformis* has been carried out in South America, yet only 1 study detected the presence of *B. grahamii* in *Lu. peruensis* from Peru (Ellis et al. 1999). In this study, we show, for the first time, the presence of *Bartonella* in phlebotomine sand flies outside the endemic area of Verruga peruana in South America. Our work also represents the 1st record of natural infection by *Bartonella* sp. in sand flies from Mexico. Surveillance studies of *Bartonella* in Mexico are scattered

and restricted to few localities in northern (Janos Biosphere Reserve), central (San Luis Potosi), and southeastern Mexico (Yucatan Peninsula), showing 14 new genetic linages (Rubio et al. 2014, Sánchez-Montes et al. 2016). The prevalence of *Bartonella* sp. in this study was 8.69% (2/23); this was higher compared to the other studies, which reported a prevalence less than 2% (Ellis et al. 1999, Villaseca et al. 1999).

The recovered sequences exhibited a close relationship with several *Bartonella* species previously detected in rodents and their ectoparasites, such as *B. grahamii* and *B. elizabethae*, which are emerging pathogens that can cause endocarditis in dogs and humans (Bitam et al. 2012, Regier et al. 2016). However, little is known about the potential vectors of this genus, their geographic distribution, or their genetic diversity (Kosoy et al. 2012). We cannot confirm whether sand flies are vectors of this bacterium in this area, since the arthropod could feed on blood meal from an infected host and not necessarily transmit the microorganism. It is possible that other lineages of *Bartonella* (pathogenic or nonpathogenic) can circulate in the fauna from Veracruz, yet the range of vectors involved in the transmission of *Bartonella* species remains to be established (Maroli et al. 2013, Battisti et al. 2015). Since an ample diversity of *Bartonella* species has been recorded in Mexico, further studies are needed to establish the role of sand fly species as potential vectors in order to understand the complex ecological networks in the life cycle of this bacterium.

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Research paper

Rickettsial agents detected in the genus *Psathyromyia* (Diptera: Phlebotominae) from a Biosphere Reserve of Veracruz, Mexico

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ABSTRACT

Phlebotomine sand flies are considered the main vectors of *Leishmania,* the causal agents of leishmaniasis, which is a serious emerging public health problem worldwide. The use of biological control alternatives, like endosymbiotic bacteria (*Wolbachia* and *Rickettsia*), have been proposed to decrease sand fly populations and reduce *Leishmania* transmissions, yet only few records on the detection of *Wolbachia* or *Rickettsia* in sand flies are available worldwide. The aim of this study was to perform the molecular detection of Rickettsial agents associated with sand flies from the last patch of a rainforest in south-eastern Mexico, where a high prevalence of *Leishmania infantum* has been reported. Sampling effort of sand flies covered 300 trap-nights between 2011 and 2013, and a total of 925 specimens from twelve species were morphologically identified. Using PCR techniques, we identified a new lineage of the endosymbionts *Rickettsia* in *Psathyromyia aclydifera* (prevalence of 19.54%), and *Wolbachia* in *Psathyromyia shannoni* and *Lutzomyia* sp. (prevalence of 25%). The detected *Wolbachia* lineage was similar to the *w*Whi strain found in *Pa. shannoni* from Colombia and *Nyssomyia whitmani* from Brazil; whereas the identified *Rickettsia* represents a new lineage worldwide. This is the first record of Rickettsial agents associated to sand flies from this region, yet it remains for analysed if these bacteria possibly play a role as vector control agents, capable of reducing the sand fly populations in Mexico.

1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are arthropods considered vectors of protozoans of the genus *Leishmania*, the causal agents of leishmaniasis worldwide [1]. Leishmaniasis transmission is a serious emerging and re-emerging public health problem of great relevance, with an estimated two million new human cases per year worldwide, 350 million people at risk of contracting the disease, and 12 million people already infected [2,3].

Vector control as a strategy of disease management has been done using insecticides (pyrethroids and chlorfluazuron as a growth inhibitor) to control the populations of sand flies [4]. The use of vertical fine-mesh nets impregnated with insecticide has shown to be a longer-lasting solution [5,6]. However, the constant use of some of these techniques causes resistance of sand fly species in endemic areas of leishmaniasis, and in some cases their implementation is inaccessible for some communities. Therefore, the biological control is an interesting strategy, and can be achieved by the use of endosymbiont bacteria (*e.g. Flavobacterium*, *Rickettsia*, *Spiroplasma* or *Wolbachia* among others). The use of "reproductive parasites" has the advantage that they facilitate their own propagation, manipulating the reproductive success of their arthropod host, even if this may affect the fitness of the host [7].

The α-proteobacteria *Wolbachia* (Rickettsiales: Anaplasmataceae) is an intracellular symbiont that is maternally or horizontally transmitted in a wide range of nematodes and arthropods [8,9]. This bacterium group induces reproductive abnormalities such as cytoplasmic incompatibility (CI), parthenogenesis, feminization of males, and male-killing in their host, which can cause reproductive isolation and speciation [9,10]. In some cases, the presence of this bacteria might confer protection by avoiding the establishment of some viruses (*e.g. Dengue*,

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Zika, Chikungunya in mosquitoes Culicidae), and filarial nematodes (e.g *Dirofilaria*) [11–13]. There are no studies on the use of *Wolbachia* as a control agent in sand flies [11]. Yet it is noteworthy that nineteen *Wolbachia* strains belonging to subgroups A and B have been naturally detected in sand fly species of medical importance from: Mexico, Panama, Colombia, Brazil, Europe, middle East, India and China [4,14].

Rickettsia (Rickettsiales: Rickettsiaceae) is another α-proteobacteria considered reproductive parasite, whose relevance has increased rapidly, since it was found in a wide range of insects. Some species of this genus have negative effects on their arthropod hosts by causing male embryonic lethality, thelytokous parthenogenesis, increasing their susceptibility to insecticides, reduce fecundity, longevity and body size of some arthropods [7,15–19]. Nevertheless, it is also beneficial in helping to resist heat shock and induce oogenesis in its hosts [16,18,19]. In the case of sand flies, little is known about this interaction, although there are three scattered records for species of sand flies that are distributed in the US, Brazil and China [20-22].

In Mexico, the state of Veracruz has great richness of sand fly species [23–25], and is one of the seven states with the highest number of human leishmaniasis cases associated with cacao plantations. During the period 2010 to 2019, 209 confirmed cases of cutaneous leishmaniasis and sporadic cases of visceral leishmaniasis have been recorded [2,26,27], and recently a high prevalence of *Leishmania infantum* was recorded in the tropical rainforest region of Los Tuxtlas [28]. However, knowledge on the Rickettsial endosymbionts associated with sand fly species from Mexico is very scarce, since only one record exists [4]. For that reason, the aim of this study was to perform the molecular detection of *Wolbachia* and *Rickettsia* in sand flies from the Los Tuxtlas, Veracruz, Mexico.

2. Material and method

2.1. Study area and sampling collections

The study site was located at Los Tuxtlas Tropical Biology Station (LT), which is part of the Biosphere Reserve of Los Tuxtlas, at the east coast of Mexico (N18° 34′ W95° 04′) (Fig. 1). The station is situated at an altitude between 150 and 700 m above sea level and covers an area of 644 ha. This region is characterized by a tropical environment with an average annual temperature of 26 °C. The rainy season spans from June to February with an average rainfall of 4, 500 mm per year, peaking between August and November. The dry season lasts from March to May, where May is the hottest month [29,30].

Sand flies were sampled for three consecutive nights during three consecutive years: 2011 (March and October), 2012 (January, March, April, June, August and November) and 2013 (April and May). Sampling was carried out between 18:00 and 23:00 h, using ten light traps (model 512; John W. Hock Co., Gainesville, FL, USA). Light traps were hung from trees or branches approximately one meter above ground in transects of 100 m in the same locations. The collected material was maintained under refrigeration during 12 h, and then the sand fly specimens were separated and stored individually in 70% ethanol.

2.2. Identification of sand flies

The sand flies were mounted on slides using Euparal medium for morphological identification, following the procedures outlined by Ibáñez-Bernal [31]. The collected female sand flies were dissected individually with sterile needles, in order to separate the head and the

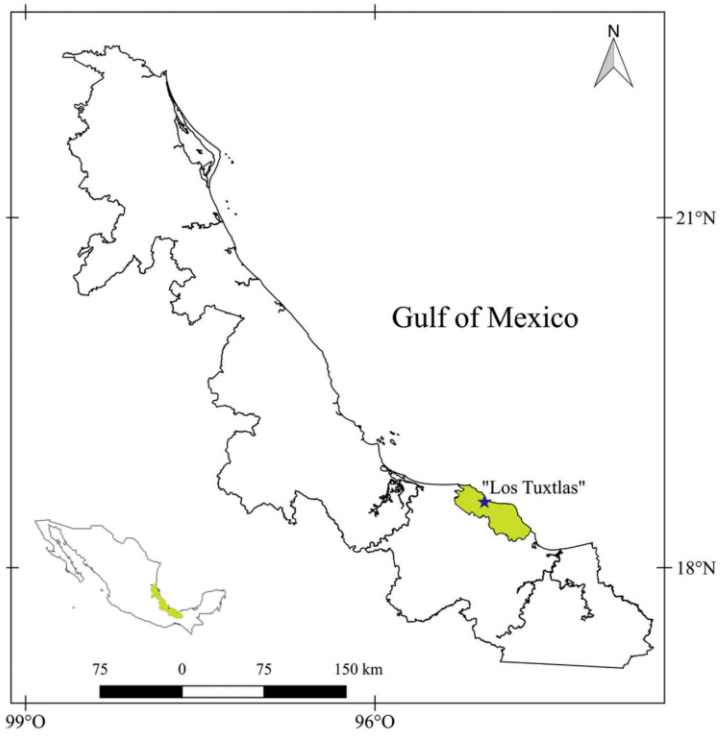

Fig. 1. Map depicting the location of Los Tuxtlas Tropical Biology Station, Veracruz, Mexico.

last segments of the abdomen for species identification, and the rest of the body was used for DNA extraction. Male sand flies were completely slide-mounted and used only for morphological identification. Phlebotomine sand fly species were classified according to the Galati [32] proposal, and the taxonomic identifications were based on Young and Duncan [33]. We use the abbreviation system proposed by Marcondes [34].

2.3. DNA extraction

DNA was extracted only from the first segments of the abdomen, using the Chelex-100 resin. To improve results for chitinous structures, some modifications were performed to the protocol of García-Gonzáles et al. [35]. First, a 10% solution of the resin Chelex-100 (BIO-RAD, California, USA) was prepared with heated nuclease-free water at 70 °C. The segments were placed in 1.5 μL tubes and 500 μL of the solution together with 20 μL of proteinase K (SIGMA) were added. After mixing, the samples were incubated at 56 °C during 12 h. After the incubation, the temperature was increased to 100 °C during 15 min, in order to enhance protein denaturation. The samples were centrifuged at 21, 952 G-force for 10 min, and only the supernatant was used for DNA amplification. DNA quality and concentration were quantified with the absorbance ratio between 260/280 nm using a NanoDrop 2000/2000c (ThermoFisher, USA).

2.4. Polymerase Chain Reaction (PCR) conditions

For detection of Rickettsial agents, several gene fragments were targeted: a ≈ 495 base pair (bp) fragment for the ribosomal gene 16S rDNA was amplified using the primers EHR01F (GCC TAA CAC ATG CAA GTC GAA CG) and EHR02R (GCC CAA TAA TTC CGA ACA ACG) [36], a ≈ 390 bp fragment for the citrate synthase (*gltA*) was amplified using the primers RpCS.877p (GGG GGC CTG CTC ACG GCG G) and RpCS.1258n (ATT GCA AAA AGT ACA GTG AAC A) [37] for *Rickettsia* detection, and a ≈ 600 bp fragment for the surface protein (*wsp*) for *Wolbachia* detection was amplified using the primers *wsp* 81F (5′ TGG TCC AAT AAG TGA TGA AGA AAC 3′) and *wsp* 691R (5′ AAA AAT TAA ACG CTA CTC CA 3′) [38]. The PCR reactions were performed in a C1000 Thermal Cycler (BioRad Laboratories, USA) under the previously reported conditions for each set of primers [36–38].

The reaction mixture was prepared in a final volume of 25 μL containing 12.5 μL GoTaq® Green Master Mix, 2× Promega Corporation (Madison, WI, USA), 1 μL of each primer (100 ng each), 10 μL DNA template (~50 ng), and 0.5 μL nuclease-free water. We did not include a positive control to avoid cross-contamination, and the negative control consisted of ultrapure water instead of DNA. The amplified products were analysed by electrophoresis in 2% agarose gels stained with Smartglow. Positive PCR products were sequenced at Laboratorio Nacional de Biodiversidad (Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, UNAM). The prevalence of Rickettsial agents was calculate according to Bush et al. [39], taking into account the number of specimens infected by a bacterial lineage and the number of sand fly specimens of each species that was analysed.

2.5. Data analysis

The 16S rDNA, *gltA* and *wsp* electropherograms were visualized and edited in the software Chromas. Each sequence was compared with all the sequences available at NCBI database using BLASTn (Basic Local Alignment Search Tool) as a preliminary confirmation of Rickettsial agents. Sequences retrieved from the 16S rDNA and *gltA* genes were aligned with other reference sequences deposited on GenBank using MEGA version 6.0. For the analysis of these sequences, a concatenated analysis was generated using the Maximum Likelihood (ML) method in MEGA 6.0. The Kimura-2-parameter substitution model (K2P) that fitted best with the multiple alignment showing a BIC score of 1,466,686 and a likelihood value of −668,202, was chosen.

The obtained sequences of the *wsp* gene were aligned with previous sequences generated by Zhou et al. (1998). Due to the high polymorphism of the *wsp* gene fragment, containing many singletons and gaps, we translated the sequences into amino acids, to facilitate the alignment [40]. For the phylogenetic analysis of these sequences, a Maximum Likelihood reconstruction was performed in MEGA 6.0 with 1000 bootstraps, using the Tamura 3-parameters (T92) + Gamma distribution (0.38) substitution model, considering complete deletion of gaps. All alignment sites with less than 95% site coverage were eliminated from the analysis. The genetic distances between the sequences generated in this work and their similarity with other *Wolbachia* strains were calculated in MEGA 6.0. The obtained sequences were deposited in GenBank under the following accessions numbers *Rickettsia* 16S rDNA (MT158807) and *gltA* (MT1654485), and *Wolbachia wsp* (MT533592, MT533593).

3. Results

3.1. Specimens collected

Sampling effort of sand flies included 300 trap-nights between March 2011 and May 2013. A total of 925 specimens from twelve species and eight genera *sensu* Galati [32] were morphologically identified (Table 1). *Psathyromyia aclydifera, Psychodopygus panamensis,* and *Psathyromyia carpenteri* accounted for 90% of the collected sand flies. DNA was extracted from 36.69% (204/556) of the collected females, belonging to ten species (Table 1). We obtained an average DNA concentration of 59.2 ng/μL (44 μL - 82.40 μL), and quality values of 260/280 showing 1.45 ± 0.06 (1.18–1.50) for each specimen.

3.2. Rickettsia DNA detection

A total of 17 specimens tested positive using the 16S rDNA-PCR, all positive specimens were identified as *Pa. aclydifera* (17/87 prevalence 19.54%). These 17 positive specimens were confirmed to contain *Rickettsia* DNA using the *gltA* gene, yet we were only able to obtain five complete sequences. The highest prevalence was observed in *Pa. aclydifera* specimens collected during August 2012, showing 44.44% (16/36), whereas the lowest prevalence was recorded in March 2012 showing 4% (1/25).

The 16S rDNA and *gltA* sequences obtained in this study were 100% identical between all the infected sand flies. Furthermore, the set of sequences of 16S rDNA exhibited a 99.48% similarity with sequences of a *Rickettsia* endosymbiont *Quadrastichus mendeli* from Italy (KX592503.1) and with a *Rickettsia limoniae* endosymbiont of *Limonia*

Table 1
List of sand fly species collected from Los Tuxtlas, Veracruz, 2011–2013.

Species	Males	Females	Total	DNA[a]
Psathyromyia aclydifera	266	322	588	87
Dampfomyia beltrani	2	4	6	4
Psathyromyia carpenteri	9	52	61	38
Micropygomyia cayennensis	1	0	1	0
Lutzomyia cruciata	2	24	26	9
Lutzomyia sp.	0	1	1	1
Bichromomyia olmeca olmeca	10	12	22	4
Psychodopygus panamensis	65	115	180	52
Pintomyia serrana	0	4	4	0
Psathyromyia shannoni	2	8	10	4
Psatrhyromyia undulata	2	4	6	1
Brumptomyyia hamata	1	1	2	1
Brumptomyia mesai	9	1	10	1
Brumptomyia spp.	0	8	8	2
12 species	**369**	**556**	**925**	**204**

[a] Female specimens available for DNA extraction.

chorea (AF322442.1), available in GenBank. The sequences of *gltA* had a 100% similarity with sequences of a *Rickettsia* endosymbiont of *Lutzomyia apache* from US (EU368001.1), and a *Rickettsia* endosymbiont of *Polythore lamecede* (LR780479).

The multiple alignments of the sequences contained 681 sites (384 pb for the 16S rDNA gene and 297 pb for the *gltA* gene). The concatenated analysis revealed the presence of a putative new lineage of a *Rickettsia* endosymbiont of *Pa. aclydifera* with a high bootstrap support value (99%). This linage is related with other *Rickettsia* endosymbiont species (Fig. 2).

3.3. Wolbachia DNA detection

Only two specimens tested positive, one *Pa. shannoni* (1/4 prevalence of 25%) and one *Lutzomyia* sp. (1/1 prevalence of 100%). The presence of the *Wolbachia* sp. was detected in *Pa. shannoni* collected in November 2012, and in *Lutzomyia* sp. collected in August 2012. The sequences obtained for the *wsp* gene were 100% identical, and exhibited similarities of 99.81% with sequences of a *Wolbachia* endosymbiont of *Drosophila sturtevanti* from Panama (AY620215.1) and *Wolbachia* sp. of *Nyssomyia whitmani* from Brazil (AF237885.1).

The multiple alignments of the sequences contained 554 sites, 306 conserved and 248 variable sites. The phylogenetic analysis with ML showed that the phlebotomine sand flies positive for *Wolbachia* DNA clustered with another *Wolbachia* endosymbiont of *Pa. shannoni* from Colombia and *Nyssomyia whitmani* from Brazil [9], with a high bootstrap value (99%) (Fig. 3). The sequences obtained were 0.002% different from the sequences of *Pa. shannoni* and *Ny. whitmani* from Colombia, according to the genetic distances.

4. Discussion

Symbiotic relationships between reproductive parasites and their hosts are very common in nature, and have been studied recently [41,42]. Nonetheless, this topic is still limited for phlebotomine sand fly species worldwide, and only 2% of the diversity of sand fly species has been related to reproductive parasites (mainly *Wolbachia* or *Rickettsia*), therefore the roles of these bacteria associated with sand fly species remain unclear. This study represents the first record of Rickettsial endosymbionts associated to sand flies from Veracruz.

The use of the *wsp* gene for the detection of *Wolbachia* has shown to be useful for phylogenetic analysis and identification of *Wolbachia* strains associated with different arthropod species, including sand flies [8,9,40]. We obtained a low prevalence (0.98%) for *Wolbachia* DNA as compared to other studies, where the prevalence ranged from 2.8 to 37.19% [9,43–46]. In Mexico, a single previous record showing *Wolbachia* associated to *Lu. cruciata* has been reported in the state of Chiapas, and also showing a low prevalence of 0.48% [4]. The low prevalence of *Wolbachia* in the country could be related to low densities of these bacteria, as well as to the behaviour of the sand flies and their distribution, since it has been reported that high temperatures and older sand flies are associated to lower infection rates [45,47].

In general, *Wolbachia* strains associated with sand flies are classified into the subgroups A or B. However, in this analysis the subgroup A is subdivided into two clades, one of which is represented mainly by Neotropical sand fly species, including the *Wolbachia* strain *w*Whi and the sequences generated in this study. The second group includes *Wolbachia* strains of Old World sand flies, such as *Ph. sergenti, Ph. mongolensis, Ph. duboscqi*, and *Ph. papatasi* (Fig. 3). The *Wolbachia* lineage found in this study clustered with a previous strain *w*Whi (with a bootstrap of 99% and a genetic difference of 0.002%) recorded in *Pa. shannoni* from Colombia and *Ny. whitmani* from Brazil. We observed that the lineage obtained in this study grouped in a clade with a 70% bootstrap value (Fig. 3). This could be attributed to a possible change in the position 263 of the nucleotide sequences, where the *w*Whi strain has an adenine and the sequences of our study show a guanine.

Wolbachia is usually vertically transmitted in arthropod populations [10]. However, in some sand fly species, *Wolbachia e.g.* strain *w*Whi is related to horizontal transmissions between closely related species [9,11,45]. The role of *Wolbachia* in the reproduction of phlebotomine sand fly species is not completely clear. Sand flies from the Old World have been shown to induce cytoplasmic incompatibility [45,48], with likely partial maternal transmission, and in some cases low variability in mitochondrial DNA haplotypes (*e.g.* in *Ph. papatasi*) [9,49,50]. In contrast, it has been speculated that in Neotropical sand flies, these bacteria may be a genetic barrier separating similar species, and generating intraspecific incompatibilities that drives to species diversification and speciation [43,45]. Even though the role of *Wolbachia* strains associated with the Mexican sand fly species is unclear, their presence in the *Lu. cruciata* and *Pa. shannoni* complexes possibly

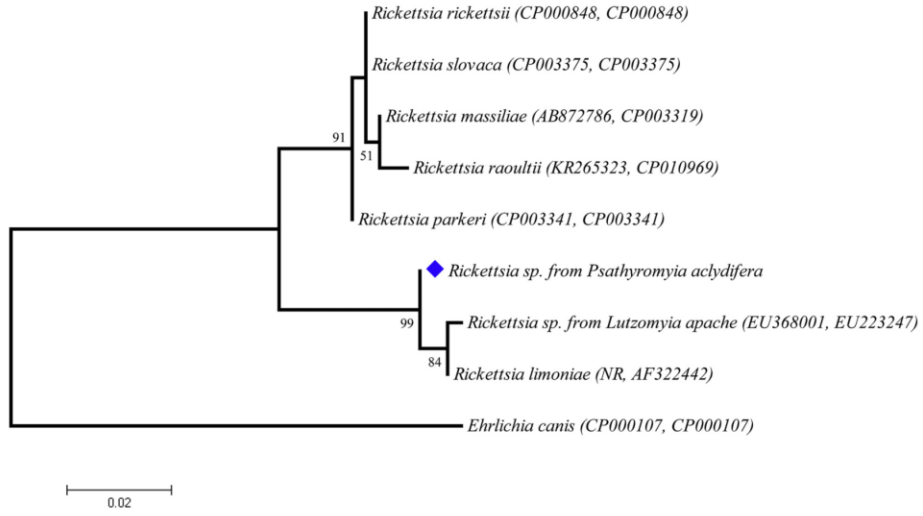

Fig. 2. Concatenated analysis of 16S rDNA and *glt*A genes of *Psathyromyia aclydifera* from Los Tuxtlas, Veracruz, Mexico. The obtained sequences are marked with a diamond.

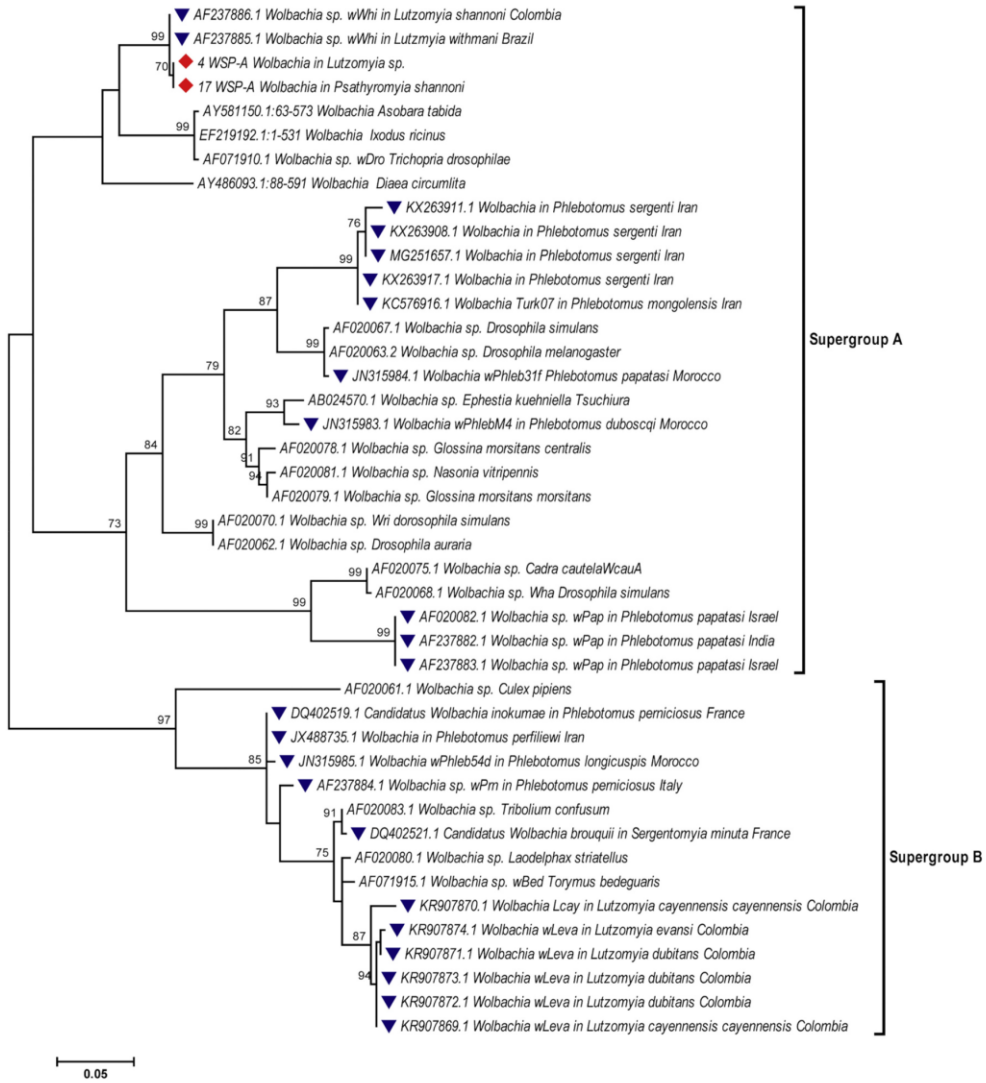

Fig. 3. Phylogenetic analysis using Maximum Likelihood for a fragment of 554 pb the *wsp* gene detected in sand flies from Los Tuxtlas, Veracruz, Mexico. The obtained sequences are marked with a diamond. The triangle shows other *Wolbachia* strains associated to phlebotomine sand flies.

facilitates species diversification and genetic isolation of populations of these sand fly species, which are important vectors of *Le. mexicana* widely distributed in the country [4,51–53]. Thus, it is necessary to sample sand fly populations from different locations to determine the diversity of *Wolbachia* lineages in Mexico [44].

The presence of a new *Rickettsia* associated with *Pa. aclydifera* showed a prevalence of 8.3%, yet little is known on the effect they exert on the fitness of their hosts [54]. A phylogenetic proposal has classified *Rickettsia* species associated with amoeba, booklice, crane flies, leeches and sand flies into a group called "Torix group" [55]. This group is characterized by *Rickettsia* species that are transmitted vertically, and this association could have a beneficial effect on the host. In some cases, their presence has an important role in oogenesis and removal these bacteria halts egg production and reproduction, and even host sizes, since infected organisms can be larger than uninfected organisms [18,56]. It is likely that *Rickettsia* associated with *Pa. aclydifera* was transmitted horizontally in this sand fly species, possibly during feeding of aphids, by parasitoids or mites, co-feeding or by sexual transmission [54,55,57,58]. In comparison to other studies showing a high prevalence (96–100%) of these bacteria, we recorded a low prevalence of this lineage (8.3%), which was only detected in two samplings [18,20,56,59]. This finding is interesting, since the presence of a non-pathogenic *Rickettsia* has been correlated with the decrease of transmission capacity other pathogens (*e.g.* another *Rickettsia* species). Furthermore, *Rickettsia* species initially described as non-pathogenic could have been reported to cause diseases in some vertebrates [54,58]. Taken together, these data clearly call for more studies to help elucidate the impact of this interaction.

Although the genus *Psathyromyia* includes nearly 47 sand fly species [60], few species are considered vectors or have been described to be naturally infected with *Leishmania* spp., and little is known on its endosymbionts in America. We record for the first time in Mexico the co-infection of *Le. infantum* and *Rickettsia* sp., in five specimens of *Pa. aclydifera* collected in August 2012 (13.88%) and of *Le. infantum* and

Wolbachia sp. in *Pa. shannoni* collected in November 2012 (50%), based on current data and those of our previous study [28]. This is interesting, since the coinfection of *Wolbachia-Leishmania* is not commonly reported, although a previous record of this coinfection in *Ni. tapidoi* from Panama has been documented [43]. More studies are warranted, since a random coinfection cannot be discarded.

Although the interactions between bacterial endosymbionts considered reproductive parasites are relevant as possible biological controls, *Wolbachia* associated with phlebotomine sand fly species does not seem an ideal strategy for controlling sand fly populations, since *Wolbachia* appears to induce different effects depending on their sand fly species and their distribution [8,50]. Yet, in cases where the horizontal transmission of *Wolbachia* strains occur, this bacterium could be beneficial, since it favours the drive of transgenes through wild sand fly populations, thereby possibly decreasing the transmission of parasites and viruses [44,45,47,48].

Further studies focused on the relationship between Rickettsial endosymbionts associated with sand fly species are needed to provide complementary information on the role of these bacteria as possible vector control strategies for sand fly populations, in order to decrease the transmission of *Leishmania* in Mexico.

Declaration of Competing Interest

None.

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Molecular detection of *Leishmania infantum* in sand flies (Diptera: Psychodidae: Phlebotominae) from Veracruz, Mexico

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ABSTRACT

Phlebotomine sand flies are vectors of *Leishmania* parasites causing different clinical forms of leishmaniases that represent a serious emerging public health problem worldwide. Mexico harbours a large diversity of sand flies, yet only six species have been considered suspected vectors of *Leishmania*. The disease has been recorded in 25 states, where the State of Veracruz has recorded the highest number of cases with leishmaniases, although no systematic or epidemiological surveillance studies of *Leishmania* vectors have been carried out in the region. For that reason, the aim of this study was to perform the molecular detection of *Leishmania* DNA in phlebotomine sand flies collected from a humid tropical region in Veracruz. We confirmed the presence of *Leishmania* DNA in eight sand fly species. Sand flies with the highest infection were *Psathyromyia aclydifera* and *Pychodopygus panamensis*. This is also the first report of *Leishmania* DNA in *Psathyromyia aclydifera, Psathyromyia carpenteri, Dampfomyia beltrani* and *Brumptomyia mesai*. Our findings highlight the importance of entomological surveys and epidemiological studies, since they enable to determine whether sand fly species may be potential *Leishmania* vectors in a given area of the Mexico, as transmission dynamics can vary in the different regions.

1. Introduction

The obligate protozoan parasites of the genus *Leishmania* (Trypanosomatida: Trypanosomatidae) cause several neglected vector-borne diseases known as leishmaniases (Akhoundi et al., 2016; Harris et al., 1998). These diseases are caused by 21 *Leishmania* species that are transmitted by more than 30 phlebotomine sand fly species in 98 countries (Aransay et al., 2000; Oryan and Akbari, 2016). Leishmaniases represents a serious emerging public health problem, since two million new cases are estimated per year, with 350 million people at risk of contracting the disease and 12 million people already infected (Akhoundi et al., 2016; Alvar et al., 2012). These diseases has a broad spectrum of clinical manifestations ranging from visceral (VL), local cutaneous (CL), diffuse cutaneous (DCL) and mucocutaneous (ML) leishmaniases (Akhoundi et al., 2016).

Given that leishmaniases encompass a group of diverse and complex vector-borne diseases, the detection of the parasite and the analysis of genetic diversity of *Leishmania* parasites, are useful for epidemiological studies in order to design and apply appropriate control measures (Aransay et al., 2000; El Tai et al., 2001; Freitas-Lidani et al., 2014; Harris et al., 1998). Some conventional techniques such as light microscopy and parasite isolation show low sensitivity to identifying the *Leishmania* species, and they are difficult to perform when processing large number of samples. However, the screening for *Leishmania* infections in large numbers of samples can be optimized by molecular techniques, such as the polymerase chain reaction (PCR), which are rapid, sensitive and specific (Aransay et al., 2000; Arrivillaga-Henríquez et al., 2017; Freitas-Lidani et al., 2014). Specifically, the use of nuclear ribosomal Internal Transcribed Sequence (ITS-1) was recently proposed for the detection and characterization of *Leishmania* parasites in the Neotropical region (Arrivillaga-Henríquez et al., 2017).

In Mexico, 54 phlebotomine sand fly species have been recorded, from which *Bichromomyia olmeca olmeca, Lutzomyia cruciata, Nyssomyia ylephiletor, Psathyromyia shannoni, Psychodopygus panamensis* and *Pintomyia ovallesi* are the species that have been incriminated or are suspected to be vectors of *Leishmania* (Biagi et al., 1965; Pech-May et al., 2010). Leishmaniases cases have been recorded in at least 25 states, predominantly in the states of Chiapas, Tabasco, Quintana Roo, Campeche, Yucatan, Veracruz and Nayarit where "chicle", coffee or cacao plantations represent a risk factor, since they harbour abundant

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phlebotomine sand flies (Alvar et al., 2012; Ibáñez-Bernal et al., 2015; Moo-Llanes et al., 2013; Pech-May et al., 2010; Rebollar-Téllez et al., 1996; Sánchez-García et al., 2010; Sanchez-Tejeda et al., 2001). An estimated 16 million people are at risk of becoming infected in 13 states of southeastern Mexico (CENAPRESE, 2014; Pech-May et al., 2016). The majority of the reported cases in Mexico are caused by *Leishmania* (*Leishmania*) *mexicana,* although *Leishmania* (*Viannia*) *braziliensis* has also been reported in Nayarit (Sanchez-Tejeda et al., 2001), *Leishmania* (*Leishmania*) *infantum* in Guerrero and Chiapas, and additionally *Leishmania* (*Viannia*) *panamensis* and *Leishmania* (*Viannia*) *guayanensis* have been reported in the southern states of Mexico (Alvar et al., 2012).

The State of Veracruz is one of the seven states with the highest number of cases with leishmaniases reported between 1990–2011 (Carrada-Figueroa et al., 2014). These cases were related to outbreaks of ML, VL, and CL mainly in cacao plantations (Alvar et al., 2012; Pérez et al., 2014; Sanchez-Tejeda et al., 2001). On the other hand, a total of 23 sand fly species have been recorded in this state (Ibáñez-Bernal et al., 2011; Moo-Llanes et al., 2013). However, no systematic or epidemiological surveillance study of *Leishmania* vectors has been carried out in the region. The aim of this study was to perform the molecular detection of *Leishmania* DNA in phlebotomine sand flies collected from a humid tropical region in Veracruz.

2. Material and methods

2.1. Study area and sampling collections

The study site is located at the Biosphere Reserve of Los Tuxtlas, the last parch of a humid tropical forest of Veracruz, is located on the east coast (N18° 34´ W95° 04´) (Fig. 1). The station is situated at an altitude between 150 and 700 meters above sea level and covers an area of 644 hectares. This place is characterized by a warm and humid environment and an average annual temperature of 26ºC. The rainy season spans from June to February with an average rainfall of 4500 mm per year. The dry season lasts from March to May, where May is the hottest month. The predominant ecosystem is high evergreen jungle, but there are other important ecosystems such as: evergreen forests, medium evergreen jungle, high jungle with liquidambar, mangroves and paddocks (Dirzo et al., 1997; Soto and Gama, 1997). Sand flies were sampled for three consecutive nights of the same week in March and October 2011, January, March, April, June, August and November 2012, April and May 2013. Sampling was carried out between 18:00 and 23:00 h, using ten light traps (model 512; John W. Hock Co., Gainesville, FL, USA). Light traps were hung from trees or branches (mainly of *Astrocaryum mexicanum*), approximately one metre above ground, near burrows or decomposing organic matter, in a transect with at least 10 m distance between each trap (always in the same locations). The collected material was maintained under refrigeration during 12 h. Later sand fly specimens were separated and stored in 70% ethanol.

2.2. Identification of sand flies

The sand flies were mounted on slides using Euparal medium for morphological identification, following the procedures outlined by Ibáñez-Bernal (Ibáñez-Bernal, 2005b). The collected female sand flies were dissected individually with sterile needles, in order to separate the head and the last segments of abdomen for species identification. The thorax and most of the abdomen was used for DNA extraction. Male sand flies were completely slide-mounted and used only for morphological identification. Phlebotomine sand fly species were identified according to Galati (2003) and also using the taxonomic keys of Young and Duncan (1994) and Ibáñez-Bernal, 2005, Ibáñez-Bernal, 2005, 1999). The abbreviation of generic names was performed according to Marcondes (2007).

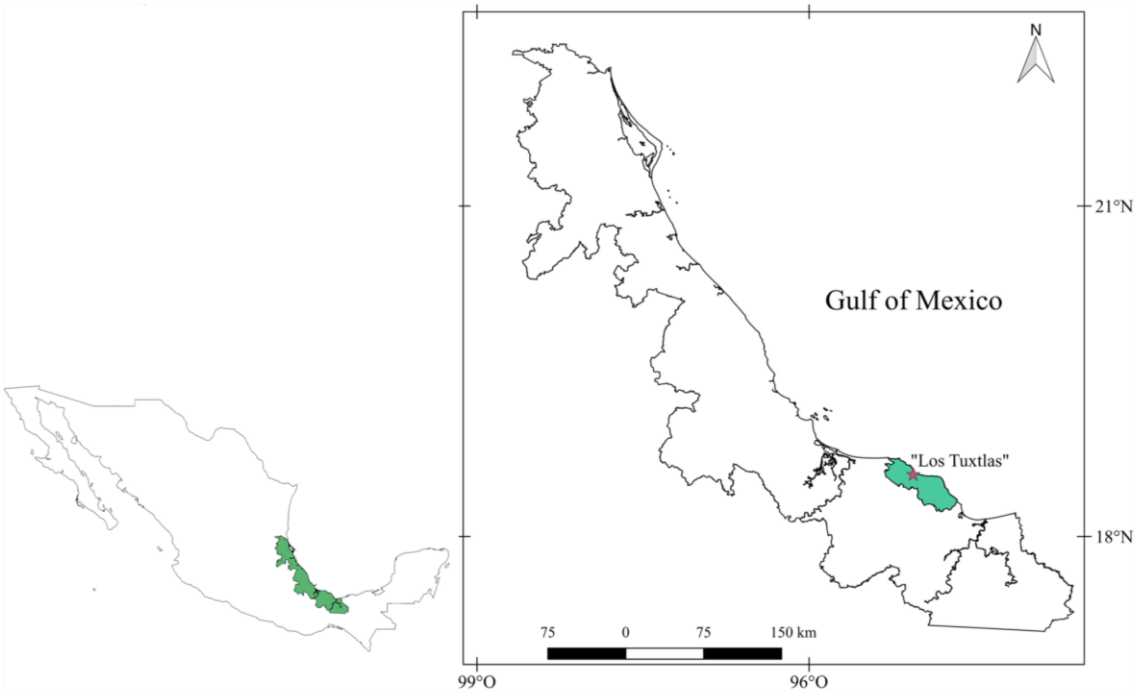

Fig. 1. Map showing the study area of the Biosphere Reserve of Los Tuxtlas, Veracruz, Mexico. The star pink shows the location "Los Tuxtlas".

2.3. DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted individually from the abdomen of the females using 500 μL of a 10%solution of the resin Chelex-100 (García-González et al., 2004). For *Leishmania* detection, pools of three specimens were made and a ≈ 300–350 bp fragment of the nuclear ribosomal ITS-1 region of *Leishmania* (ITS1-PCR) was amplified, using the primers LITSR (5′- CTG GAT CAT TTT CCG ATG - 3′) and L5.8S (5′- TGA TAC CAC TTA TCG CAC TT −3′) (El Tai et al., 2001). The PCR reactions were done in a C1000 Thermal Cycler (BioRad Laboratories, USA) under the following conditions: initial denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s; and final extension of 72 °C for 5 min. Reaction mixture was prepared in a final volume of 25 μL with 0.3 μL of ABM Hotstart DNA polymerase (Applied Biological Materials Inc., Canada), 2.5 μL of 10X buffer, 1.5 μL of 25 mM $MgSO_4$, 1.5 μL of 2 mM dNTP, 1 μL of 10 pmol of each oligonucleotide, 3 μL of the DNA template, and 15.1 μL of ultrapure H_2O. We included a WHO reference strain of *Leishmania amazonensis* (IFLA/BR/67/PH8, ATCC 50159) as a positive control, in order to exclude any cross-contamination. The negative control consisted of ultrapure water instead of DNA. The amplification products were analysed by electrophoresis in 1.5% agarose gels stained with GelStar (Lonza, Switzerland). If a pool tested positive, we repeated the PCR individually, using the same conditions as above. Positive PCR products were sequenced at Laboratorio Nacional de Biodiversidad (Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, UNAM). Prevalence of *Leishmania* was calculated according to Bush et al., 1997.

2.4. Data analysis

The ITS-1 electropherograms obtained were visualized, edited and assembled in GeneStudio ™ Professional Edition Version 2.2.0.0 (GeneStudio, Inc., USA) in order to generate a consensus sequence per sample. Each consensus sequence was compared with all sequences available at NCBI database using BLASTn (Basic Local Alignment Search Tool) with the megablast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), as a preliminary confirmation of *Leishmania* DNA identification. The DNA consensus sequences were aligned with other *Leishmania* reference sequences deposited on GenBank using MAFFT version 7 (Katoh et al., 2017). Due the high polymorphism of the region and the high number of singletons and gaps, *Leishmania* identification was performed using the Maximum Parsimony (MP) method in MEGA 7.0 with 1000 bootstraps, using the Subtree-Pruning-Regrafting (SPR) algorithm. All alignment sites with less than 95% site coverage were eliminated from analysis. All of the sequences obtained have been deposited in GenBank under the accession numbers MN422055-MN422063, and MN503524-MN503527.

3. Results

3.1. Specimens collected

Sampling effort of sand flies was done during 30 nights between March 2012 and May 2013. A total of 924 specimens from twelve species and eight genera *sensu* Galati were morphologically identified (Table 1). *Psathyromyia* (*Xiphopsathyromyia*) *aclydifera, Psychodopygus panamensis* and *Psathyromyia* (*Forattiniella*) *carpenteri* accounted for 90% of the collected phlebotomine sand flies. Less abundant species ranged from one to 26 individuals (Table 1). Overall, the proportion of females (60%) was a higher than that of males (40%). Species with epidemiological relevance for *Leishmania* transmission in Mexico were *Ps. panamensis, Lu. cruciata, Bi. olmeca* and *Pa. shannoni*. These four species accounted for 25.75% of the collected sand fly specimens.

Table 1
List of sand fly species collected from Los Tuxtlas, Veracruz.

Species	Males	Females	Total	DNA**	Females with eggs***
Psathyromyia aclydifera	266	322	588	87	6/87
Dampfomyia beltrani	2	4	6	4	1/4
Psathyromyia carpenteri	9	52	61	38	35/38
Micropygomyia cayennensis	1	–	1	–	–
*Lutzomyia cruciata**	2	24	26	9	8/9
*Bichromomyia olmeca olmeca**	10	12	22	4	2/4
*Psychodopygus panamensis**	65	115	180	52	23/52
Pintomyia serrana	0	4	4	–	–
*Psathyromyia shannoni**	2	8	10	4	3/4
Psatrhyromyia undulata	2	4	6	1	1/1
Brumptomyyia hamata	1	1	2	1	0/1
Brumptomyia mesai	9	1	10	1	1/1
Brumptomyia spp.	–	8	8	2	2/2
12 species	**369**	**555**	**924**	**203**	**82/203**

*Relevant species for *Leishmania* transmission in Mexico, **Female specimens available for DNA extraction, *** Females available for molecular analysis with eggs.

3.2. Leishmania detection by ITS1-PCR

For the detection of *Leishmania* DNA, we analysed 36.57% (203/555) of the collected females, belonging to ten species (Table 2). A total of 203 female specimens were organized in 63 pools, 30 of which were positive for molecular detection of *Leishmania* DNA (infection rate by pool 47.61%) using the ITS1-PCR (Fig. 2). Through individual PCR, we confirmed the presence of *Leishmania* DNA in 38 specimens (infection rate 18.71%) of eight sand fly species (Table 2). Higher infection rates were observed in specimens collected during November 56.25% (9/16), followed by those collected in August 25.53% (12/47). The lowest infection rates were recorded in March 2012 with 6.38% (3/47) and April 2013 with 9.67% (3/31) (Table 2). Sand flies with the highest infection numbers were *Pa. aclydifera* (12/87) and *Ps. panamensis* (10/52). In addition, we also detected the presence of *Leishmania* DNA in *Pa. aclydifera, Pa. carpenteri, Da. beltrani* and *Br. mesai.*

On the other hand, in seven species that tested positive for *Leishmania* DNA, we found that the 8.86% (18/203) had the presence of eggs in the abdomen. The sand flies species *Lu. cruciata* 44.4% (4/9), *Pa. carpenteri* 13.15% (5/35), and *Ps. panamensis* 7.69% (4/52) had the highest prevalence of female specimens with eggs in the abdomen together with *Leishmania* DNA. The other four species (*Pa. aclydifera, Bi. olmeca olmeca, Pa. shannoni* and *Br. mesai*) showed one or two specimens with both characteristics.

The sequencing of the ITS-1 region of *Leishmania* was successful in 30 of the 38 positive samples (78.94%), the remaining eight sequences were too short to be included in the analysis. The set of consensus sequences exhibited similarities, ranging from 98.74 to 100% with sequences of *Leishmania infantum* (MG778653.1, MN648760.1, MN412821.1, MF688836.1, MN648742.1, KX664449.1, MK510946.1, MF977313.1 and KU975158.1) available in GenBank.

The multiple alignment of consensus sequences contained 319 sites, 305 conserved sites and 14 variable sites. The phylogenetic analysis with the MP tree revealed that all phlebotomine sand flies that tested positive for *Leishmania* DNA, clustered with the *Le. infantum* reference sequences, showing a high confidence (97 bootstrap). However, there were some nucleotide differences which were not unexpected, given the high intra and interspecific variability of this non-coding region (Fig. 3).

4. Discussion

Leishmaniasis are a neglected tropical diseases causing an important public health problem worldwide, so it is important to improve our

Table 2
Number of females of sand fly species analysed by ITS-1 for detection of the*Leishmania*DNA.

Year	Collection month	Species	n	Specimens infected	Specimens infected with eggs	Infection (%)	Accession Number
2012	March	*Pa. aclydifera*	25	1/25	1/25	4%	**MN422060**
		Da. beltrani	1	0/1	0/1	0	
		Lu. cruciata	2	1/2	1/2	50%	
		Ps. panamensis	17	0/17	0/17	0	
		Pa. shannoni	1	1/1	1/1	100%	
		Pa. undulata	1	0/1	0/1	0	
	June	*Pa. aclydifera*	15	0/15	0/15	0	
		Pa. carpenteri	4	1/4	1/4	25%	**MN503526**
		Lu. cruciata	2	0/2	0/2	0	
		Ps. panamensis	15	5/15	2/15	33.33%	
		Pa. shannoni	1	1/1	1/1	100%	**MN422057**
		Br. hamata	1	0/1	0/1	0	
		Brumptomyia sp.	1	0/1	0/1	0	
	August	*Pa. aclydifera*	36	6/36	0/36	16.66%	**MN422055**
		Da. beltrani	2	1/2	0/2	50%	**MN503524**
		Pa. carpenteri	2	0/2	0/2	0	
		Lu. cruciata	2	2/2	2/2	100%	
		Bi. olmeca olmeca	2	1/2	0/2	50%	**MN422062**
		Ps. panamensis	3	2/3	1/3	66.66%	**MN422061**
	November	*Pa. aclydifera*	5	5/5	0/5	100%	**MN422059**
		Pa. carpenteri	3	1/3	1/1	33.33%	**MN503527**
		Ps. panamensis	6	2/6	2/6	33.33%	**MN422058**
		Pa. shannoni	2	1/2	0/2	50%	
2013	April	*Pa. aclydifera*	1	0/1	0/1	0	
		Pa. carpenteri	16	0/16	0/16	0	
		Lu. cruciata	1	0/1	0/1	0	
		Bi. olmeca	1	1/1	1/1	100%	**MN422056**
		Ps. panamensis	10	1/10	1/10	10%	**MN422063**
		Br. mesai	1	1/1	1/1	100%	**MN503525**
		Brumptomyia sp.	1	0/1	0/1	0	
	May	*Pa. aclydifera*	5	0/5	0/5	0	
		Da. beltrani	1	0/1	0/1	0	
		Pa. carpenteri	13	3/13	3/13	23.07%	
		Lu. cruciata	2	1/2	1/2	50%	
		Bi. olmeca olmeca	1	0/1	0/1	0	
		Ps. panamensis	1	0/1	0/1	0	
Total	**6 collections**	**10 species**	**203**	**38/203**	**18/203**	**18.71%**	

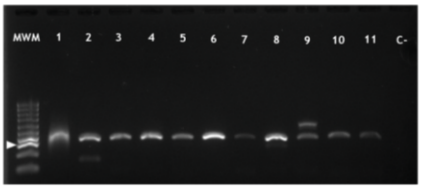

Fig 2. Electrophoresis in 1.5% agarose gel for*Leishmania*ITS1 amplicons (≈330pb) obtained from sand fly DNA.The numbers correspond to specimen samples 1-3= *Pa. aclydifera*, 4-6= *Pa. carpenteri*, 7-9= *Ps. panamensis*, 10= *Lu. cruciata*, 11= *Da. beltrani*, C-= negative control (ultrapure water), MWM: Hyperladder 100bp molecular weight marker (Bioline,UK).

knowledge on *Leishmania* species, as well as on their vectors and hosts, and gain better insight into the eco-epidemiologies of the diseases (Alvar et al., 2012; Pech-May et al., 2013, 2010). Finding infected phlebotomine sand flies provide crucial information on infection rates of sand flies in areas of high population abundances and species endemicity (Aransay et al., 2000). The State of Veracruz ranks third in diversity of species, reporting 23 phlebotomine sand fly records to date (Ibáñez-Bernal et al., 2006; Moo-Llanes et al., 2013). During the period of 2010-2018, 172 confirmed cases of cutaneous leishmaniasis and sporadic cases of VL have been reported in this state. The number of cases by year ranged from 14 to 27, with a median of 22 annual cases and the incidence rate varied from 0.18 to 0.39 per 100,000 inhabitants (Dirección General de Epidemiología DGE, 2020). However, this is the first study aimed at the molecular detection of *Leishmania* in sand fly species of this area.

The analysis of ITS-1 sequences obtained from phlebotomine sand flies showed that the DNA corresponded to *Le. infantum* with a remarkable branch support (97%) in the phylogenetic analysis. This *Leishmania* species is responsible of causing VL in the New World (Akhoundi et al., 2016; Mhaidi et al., 2018). To date, cases of VL in Mexico have been recorded mainly in the State of Chiapas (Alvar et al., 2012; Mikery-Pacheco et al., 2015; Pech-May et al., 2016, 2010), although sporadic cases have been recorded in the State of Veracruz (Alvar et al., 2012; Becker et al., 2005). Specifically, *Pintomyia evansi* and *Lutzomyia longipalpis* are sand flies suspected to be the vectors for this parasite species, but none of them were collected in our study area. It is possible that the infection by *Le. infantum* may be more widespread in phlebotomine sand fly species, as well as in more hosts and reservoirs than previously assumed in Mexico, since we were able to detect this parasite in eight sand fly species (Ibáñez-Bernal et al., 2006, Ibáñez-Bernal et al., 2004; Sanchez-Tejeda et al., 2001). Prior to our study, the only sand fly species to be incriminated or suspected as possible vectors for *Leishmania* (mainly *Le. mexicana*) in endemic areas of the country had been *Lu. cruciata, Bi. olmeca olmeca, Ps. panamensis* and *Pa. shannoni* (Biagi et al., 1965; Pech-May et al., 2010). We now show the first records of *Le. infantum* DNA in *Pa. alcydifera, Da. beltrani, Pa. carpenteri* and *Br. mesai*, although we do not know if these species are involved in the transmission of *Leishmania* in Mexico or if they can be considered as suspected vectors. Interestingly, in seven of these species, female specimens testing positive for *Leishmania* DNA also harboured eggs, which allows us to speculate that the parasites persist after digestion of the blood meal. Clearly, more studies on the relationships between *Leishmania* and local sand fly species are warranted in order to prove if are they competent vectors in this area or if they only feed on blood meals from hosts and reservoirs infected with *Leishmania*.

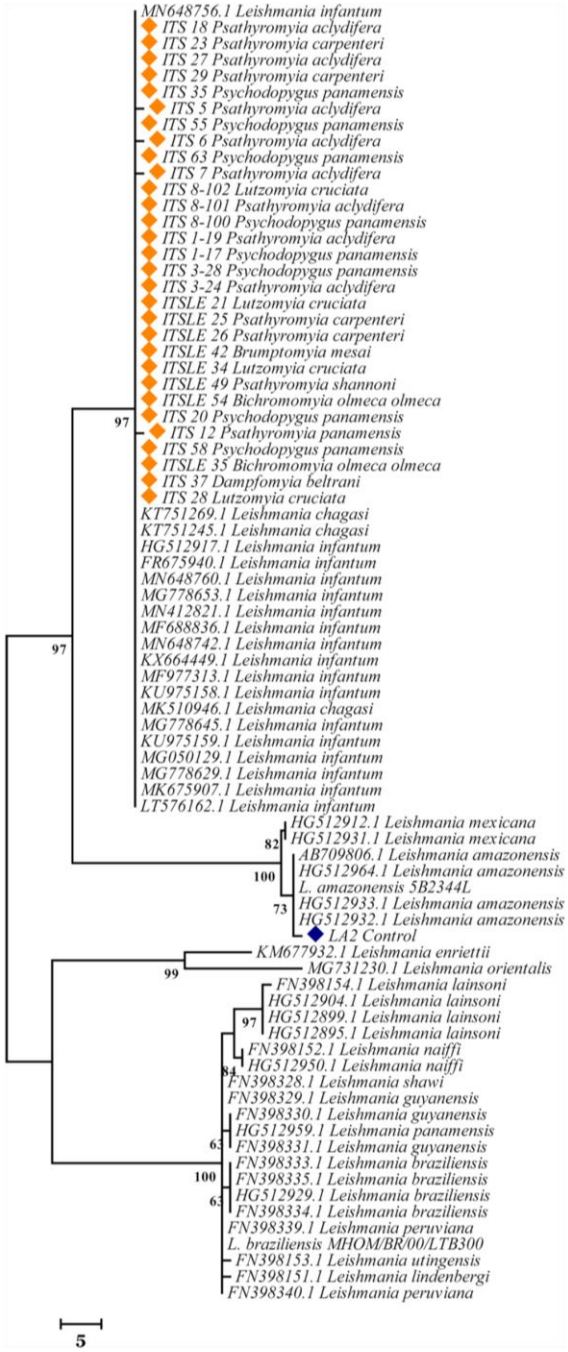

Fig 3. Phylogenetic analysis using Maximun Parsimony method (Tree length = 137 steps, Consistency Index = 0.8292, Retention Index = 0.9796). Eighty sequences were used and the bootstrap values are shown next to the branches. The sequences obtained are marked with an orange triangle, and the positive control with a blue triangle.e.

Our study now shows that a focus of *Le. infantum* infection is present at the Biosphere Reserve Los Tuxtlas. Its circulation is most likely enzootic, yet more studies are needed in order to define the risk of acquiring VL by the human population near the conserved area (Ajaoud et al., 2013).

It is noteworthy, that we found a high infection rate (18.71%) of sand flies, as compared to other studies (Andrade-Narvaez et al., 1990; Berzunza-Cruz et al., 2015; Pech-May et al., 2010; Sánchez-García et al., 2010; Sanchez-Tejeda et al., 2001). The sand fly species showing the highest infection values were *Pa. aclydifera* and *Ps. panamensis*. Most of the infected phlebotomine sand flies were detected during the dry season between August and November (21/38 infected females). This coincides with other studies suggesting that the climate modulates and/or influences sand fly populations, infection rates and biting behaviours (Rebollar-Téllez et al., 1996,b, Rebollar-Téllez et al., 1996; Baum et al., 2013; Pech-May et al., 2016; Rodríguez-Rojas et al., 2016). Records of *Lu. cruciata, Bi. olmeca olmeca* and *Pa. shannoni* in Campeche and Quintana Roo have shown that their abundance and biting rates decrease during the rainy season, but in general in our study these species were not abundant (Rebollar-Téllez et al., 1996; Rodríguez-Rojas et al., 2017; Sánchez-García et al., 2010).

The abundance of sand flies suspected as vectors in Mexico was low with exception of *Ps. panamensis*. This is probably related to exclusive use of light traps, since a high abundance of this species has previously been associated with other collecting techniques in tropical areas of Mexico (Pech-May et al., 2010; Pérez et al., 2014; Rebollar-Téllez et al., 2005, Rebollar-Téllez et al., 1996; Rodríguez-Rojas et al., 2017; Rodríguez-Rojas and Rebollar-Téllez, 2017).

Conversely, the high number of *Pa. aclydifera* specimens testing positive for *Leishmania* DNA is noteworthy, since this species had previously only been recorded only in Chiapas, Jalisco and Campeche, where it was neither abundant nor infected with *Leishmania* (Ibáñez-Bernal et al., 2015; Lozano-Sardaneta and Cañeda-Guzmán, 2015; Rebollar-Téllez et al., 1996). At this point, we are tempted to speculate on the possible participation of *Pa. aclydifera* in the transmission of *Leishmania* in this region, considering that it was the most abundant sand fly species and additionally showed a high infection rate. Yet more studies are needed to establish their biting rates of humans, seasonal variations of infection rates, blood meal preferences and the peaks of sand fly activities (Maia et al., 2015; Pech-May et al., 2016; Quiroga et al., 2017; Rêgo et al., 2015). Furthermore, it is essential to determine if wild and domestic animals are hosts of *Leishmania* parasites and establish the risk of human infections, given the transmission dynamics in our study site (Quiroga et al., 2017).

The detection of a female with eggs and *Leishmania* DNA in *Brumptomyia mesai* is another interesting finding, since the genus *Brumptomyia* does not include species with epidemiological importance in the transmission of *Leishmania* to humans, as some species have blood meal preferences for armadillos (Quiroga et al., 2017; Rebollar-Téllez et al., 1996; Young and Duncan, 1994). This finding is in accordance with the literature, where this phlebotomine genus has been reported to test positive *Leishmania* DNA (Quiroga et al., 2017). In addition, other phlebotomine sand flies without a vectorial background, such as *Warileya rotundippennis*, have been shown to harbour *Leishmania* parasites and to bite humans in an active leishmaniasis focus in Colombia (Moreno et al., 2015).

Our findings now highlight the importance of conducting entomological surveys and epidemiological studies to determine whether sand fly species may be potential vectors of *Leishmania* in a given area and if transmission dynamics differ between geographic sites.

Declaration of Competing Interest

None.

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CAPÍTULO III. SPECIES DIVERSITY AND BLOOD MEAL SOURCES OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) FROM LOS TUXTLAS, VERACRUZ, MEXICO

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Species diversity and blood meal sources of phlebotomine sand flies (Diptera: Psychodidae) from Los Tuxtlas, Veracruz, Mexico

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ABSTRACT

Phlebotomine sand flies can transmit several species of the genus *Leishmania*, that cause leishmaniasis, a serious neglected tropical disease worldwide. Although Mexico is an endemic country for the disease, studies on the biology, ecology, and the identification of blood meal sources of phlebotomine sand flies in some states remain unexplored. For this reason, this study aimed to evaluate the species diversity of sand flies, and identify their blood meal sources in the Biosphere Reserve Los Tuxtlas, Veracruz, an area with a high prevalence of *Leishmania infantum*. The cumulative sampling effort of sand flies covered 300 trap-nights between March 2011 and May 2013. For estimating species diversity, we calculated the species richness ($q = 0$), the diversity of the species ($q = 1$) and the dominant species ($q = 2$). To identify the blood meal sources, we amplified and sequenced a fragment of ≈400 bp of the vertebrate *Cytb* gene. A total of 951 specimens belonging to 15 species were collected. *Psathyromyia aclydifera* and *Psychodopygus panamensis* were the most abundant species. We were able to identify seven terrestrial vertebrate species, among which human beings were the most common source of the blood meal. In this study, relevant information on the structure of sand fly populations and their blood meal sources was obtained, providing basic and important information about the interactions between sand flies, hosts and *Leishmania* species.

1. Introduction

The phlebotomine sand flies (Diptera: Phlebotominae) are vectors of *Leishmania* (Kinetoplastida: Trypanosomatidae), *Bartonella bacilliformis* (Rhizobiales: Bartonellaceae) and some viruses (of the families Bunyaviridae, Reoviridae and Rhabdoviridae) (Ready, 2013). The main sand fly-borne disease is leishmaniasis, a serious emerging neglected public health problem worldwide, since one million new cases are estimated per year, 350 million people are at risk of contracting the disease, and 12 million people are already infected (Alvar et al., 2012; OMS, 2020).

The subfamily Phlebotominae includes about 1,026 species, of which almost 546 species are present in the New World (Galati, 2019). In Mexico, 52 extant valid and two fossil phlebotomine sand fly species have been recorded. However, only six of them are suspected of being vectors mainly for *Leishmania mexicana,* the etiological agent of cutaneous localized leishmaniasis, which is the main clinical form in the country (Ibáñez-Bernal et al., 2017, 2011, 2010, 2006; Martins et al., 1978; Pech-May et al., 2010). Leishmaniasis cases have been recorded in at least 25 States of Mexico, with 6595 new cases recorded between 2010 and 2019. It is estimated that around 16 million people are at risk for the transmission, mainly the people that live near chicle, coffee or cacao plantations, since these areas harbour abundant phlebotomine sand flies (DGE, 2020; Mikery-Pacheco and Vera, 2018; Pech-May et al., 2016; Ready, 2013). Although several studies have been carried out on

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the biology and ecology of sand flies, in the States of Chiapas, Nuevo León, Tamaulipas and the Yucatan Peninsula, the knowledge is far from complete in these states and elsewhere in Mexico (Berzunza-Cruz et al., 2015; Canto-Lara et al., 1999; Pech-May et al., 2010; Rebollar-Téllez et al., 1996a; Rodríguez-Rojas et al., 2017; Van Wynsberghe et al., 2000).

The State of Veracruz holds a high number of leishmaniasis cases associated with cacao plantations, and 209 confirmed cases of cutaneous leishmaniasis and sporadic cases of visceral leishmaniasis have been reported from 2010 to 2019. The number of cases by year ranged from 14 to 27, and the incidence rate varied from 0.18 to 0.39 per 100,000 inhabitants (DGE, 2020). Additionally, Veracruz is currently one of the Mexican states with the highest richness of sand fly species (Ibáñez-Bernal et al., 2011; Lozano-Sardaneta et al., 2019; Moo-Llanes et al., 2013), which could be potentially higher, if ecological studies were performed in this state (Moo-Llanes et al., 2013). Only few studies have focused on phlebotomine fauna and ecology in this area, yet none have addressed sand fly blood meal preferences (Mikery-Pacheco and Vera, 2018; Pérez et al., 2014). For this reason, this study aimed to determine the species diversity of sand flies, and to identify their blood meal sources in an area with a high prevalence of *Leishmania infantum* in Veracruz (Lozano-Sardaneta et al., 2020). We aimed to provide basic information to help understand the interaction between phlebotomine sand flies, hosts, and *Leishmania* species.

2. Materials and methods

2.1. Study area and sampling protocol

The study site was at the Los Tuxtlas Biological Station, within the Biosphere Reserve of Los Tuxtlas, located in the municipality of San Andrés Tuxtla (N18° 34′ W95° 04′). This Biosphere Reserve has an area of 155122 ha, harbouring a high ecosystem diversity, geological and ecological complexities. This area is one of the remaining areas of a humid tropical forest in Veracruz (Fig. 1), although in the last years, the demographic increase, and agricultural and livestock activities reduced their extension (Dirzo et al., 1997). Los Tuxtlas is situated at an altitude between 150 and 700 m above sea level and covers an area of 644 ha. It is characterized by a warm and humid climate with an average annual temperature of 26 °C. The rainy season lasts from June to February with an average rainfall of 4 500 mm per year. The dry season begins in March and lasts until May, where May is the hottest month. The predominant vegetation is tropical rainforest, although there are patches of mangroves and paddocks (Dirzo et al., 1997; Soto and Gama, 1997). It exhibits a high biodiversity and species richness, including endemic and endangered species of flora and fauna (Dirzo et al., 1997). The fauna include 565 species of birds, 139 of mammals, 166 of amphibians and reptiles, 109 of fish and 1 117 species of insects (SEMARNAT, 2016).

Sand flies were collected during three consecutive nights throughout three consecutive years (2011: March and October; 2012: January, March, April, June, August, and November; 2013: April and May). Sampling was carried out from 18:00 to 23:00 h, using only 10 incandescent light traps (model 512; John W. Hock Co., Gainesville, FL, USA) per night. A transect of 100 m with at least 10m distance between each trap (always in the same locations) were used. Traps were hung from trees or branches, approximately one meter above the ground, near burrows or decomposing organic matter. The specimens were collected nightly and maintained under refrigeration at 4°C for 12 h, to lower the metabolism of the collected sand fly specimens to be separated the next day and fixed in 70% ethanol.

2.2. Identification of sand fly species

The sand flies were mounted on slides using Euparal medium, following the procedures outlined by Ibáñez-Bernal (2005). The collected female sand flies were dissected, to separate the head and the last segments of the abdomen for species identification. Male sand flies were completely slide-mounted and used only for morphological identification. Phlebotomine sand fly species were classified according to Galati (2019), and the taxonomic identifications were based on Young

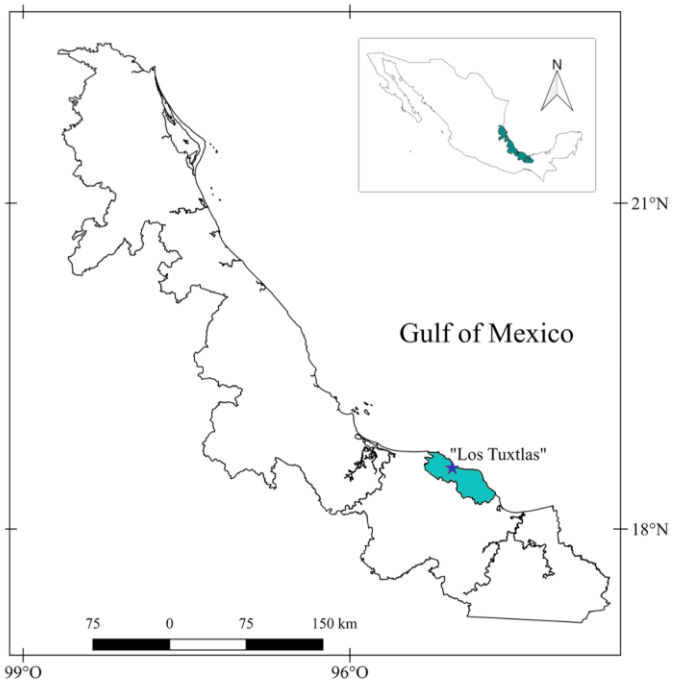

Fig. 1. Map showing the study area of Biosphere Reserve of Los Tuxtlas, Veracruz, Mexico. The star shows the location of Los Tuxtlas Biological Station.

and Duncan (1994). We used the abbreviation system proposed by Marcondes (2007).

2.3. Species diversity

The species diversity was estimated using phlebotomine sand fly abundance data identified to species level. We incorporated three measurements based on Hill numbers: the species richness ($q = 0$) with the Chao1 estimator, that is insensitive to the relative abundance of species, Shannon diversity ($q = 1$) with the exponential of Shannon entropy, which is sensitive to the relative abundance of the species, Simpson diversity ($q = 2$) with the inverse of Simpson concentration (Gotelli and Chao, 2013) that incorporates dominant species. The effective number of species is a measure of the number of species where each species is weighted by its abundance. These indices were adopted with the method of Chao and Jost (2015) to obtain precision, as well as continuous and low-bias species diversity profiles. Furthermore, we obtained the variances with a bootstrap method (number of bootstraps equal to 100) to calculate the confidence intervals (Chao and Jost, 2015). Non-overlapping confidence intervals (95% confidence level) were considered statistically significant, $p < 0.05$. All species diversity estimates were done in the online R software using the SpadeR library (Species Prediction and Diversity Estimation in R) (Chao et al., 2015).

To evaluate the most abundant sand flies species, we applied the index of species abundance (ISA), which was normalized to values between zero and one by the SISA 'Standardized Index of Species Abundance' (Roberts and Hsi 1979). SISA values close to one correspond to the most abundant species; this index is useful to compensate for abundances and trapping methods (Rodríguez-Rojas and Rebollar-Téllez 2017). A Z-test was calculated to test if the sexual proportions of the sampled sand flies were similar, and evaluate significant differences ($p < 0.05$).

2.4. DNA extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted only from the first segments of the abdomen, using the resin Chelex-100 (Lozano-Sardaneta et al., 2020). For blood meal source identification, we amplified a segment of the Cytochrome B (*Cyt*b) using the primers *cyt*b+ (5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3') and *cyt*b- (5'-TGT AGT TRT CWG GGT CHC CTA-3') (Cupp et al., 2004). The PCR reactions were performed in a C1000 Thermal Cycler (BioRad Laboratories, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 36 cycles at 90°C for 30 s, 56 °C for 30 s, and 72 °C for 50s, and a final extension at 72 °C for 3 min. The reaction mixture was prepared in a final volume of 25 μL containing 12.5 μL GoTaq® Green Master Mix 2X Promega Corporation (Madison, WI, USA), 1 μL of each primer (100 ng each), 10 μl DNA template (~50 ng), and 0.5 μL nuclease-free water. To standardize PCR procedures, we used chicken (*Gallus gallus*), bison (*Bison* bison) and gecko (*Lepidodactylus lugubris*) DNA as positive controls. To avoid cross-contamination, the DNA of these specimens was manipulated independently from phlebotomine sand fly samples. The negative control consisted of ultrapure water instead of DNA. The amplified products were analysed by electrophoresis in 2% agarose gels stained with Smartglow. Positive PCR products were sequenced at the Laboratorio Nacional de Biodiversidad (Laboratorio de Secuenciación Genómica de la Biodiversidad y de la Salud, Instituto de Biología, UNAM).

2.5. Molecular data

The electropherograms were visualized and edited in the Chromas software. Each sequence was compared with available sequences in the GenBank database using BLASTn tool. For the identification of the blood meal sources, we considered several criteria such as expected value, percentage of identity, query cover, and total score, to confirm the similarity of sequences. The DNA sequences were aligned with other reference sequences of vertebrates deposited in GenBank using MEGA version 6.0. We made a phylogenetic reconstruction based on the Maximum Likelihood (ML) inference method in MEGA 6.0 with 1 000 non-parametric bootstraps, using the General time Reversible model (GTR) + Gamma distribution with Invariant sites substitution model (G+I). All alignment sites with less than 95% site coverage were eliminated from the analysis. We used sequences of *Pintomyia nuneztovari* (AB761137.1) as outgroup. The obtained sequences were deposited in GenBank under the following accessions numbers MT946505-MT946524. A chord diagram was created in the R studio software using the circlize library (Gu et al., 2014) to show specific associations among sand flies and feeding sources, as well as sand flies, feeding sources, and *Leishmania infantum* infections (using previously published data) (Lozano-Sardaneta et al., 2020), indicating their respective frequencies.

3. Results

3.1. Phlebotomine sand fly species

The cumulative sampling effort of sand flies covered 300 trap-nights between March 2011 and May 2013. A total of 951 specimens belonging to 15 species and eight genera *sensu* Galati (2019) were identified (Table 1). The genus *Psathyromyia* recorded the highest number of species (four species). *Psathyromyia aclydifera* recorded the highest number of species (62.35%; SISA = 0.88), which was obtained mainly during samplings done in October 2011, April and August 2012. This was followed by *Psychodopygus panamensis* (19.66%; SISA = 0.86), *Psathyromyia carpenteri* (6.51%; SISA = 0.66), *Lutzomyia cruciata* (2.83%; SISA = 0.51), and *Bichromomyia olmeca olmeca* (2.31%; SISA = 0.46) (Table 1). The remaining species represented only 6.30% of the total abundance (Table 1). The proportion of females (62%) was significantly higher than that of males (38%) ($Z = -6.85$, $p < 0.05$).

3.2. Species diversity

The empirical species diversity was lower than the estimated species diversity in each of the 10 months sampled. The species diversity of the order ($q = 0$), showed that the highest species richness was found in April and June 2012, with nine species each month. This was followed by samplings done in March and October 2011, August 2012, and May 2013, during which eight species were obtained. Interestingly, the value of the effective number of species of order ($q = 0$) indicated that in March 2011, there was a 3.3 times higher estimated diversity than in November 2012. However, we expected to capture at least 19 species in March 2011 and only six species in November 2012. Thus, the expected species richness was higher than the values sampled each month. The estimated diversity shared some similarities with the empirical diversity, showing that the differences were not statistically significant ($p > 0.05$) (Fig. 2A).

Species diversity of the order ($q = 1$) (Shannon exponential) presented 1.5 to 3 times more species richness in April 2013 and March 2011, respectively, than the remaining months. The lowest effective number of species was observed in January 2012 and August 2012, with 1.72 and 1.99 effective species, respectively. There were statistically significant differences ($p < 0.05$) in Shannon diversity, mainly in October 2011, January and August 2012 (Fig. 2B). In March 2011, the confidence intervals were large (Fig. 2A, B), due to the little abundance obtained.

Regarding the empirical and estimated diversity, in March 2011 and April 2013, there were 3.2 times more dominant species than January and August 2012 for ($q = 2$) (Simpson diversity). In March 2011 and April 2013, there was a more effective number of dominant species (Fig. 2C).

3.3. Blood meal identification

For the blood meal identification, a total of 204 female specimens belonging to 10 species were analysed (Table 2). Despite that we were

Table 1
Phlebotomine sand fly species collected from the Biosphere Reserve of Los Tuxtlas, Veracruz, Mexico. The indices of diversity and SISA values are included.

Species	Mar 2011	Oct 2011	Jan 2012	Mar 2012	April 2012	June 2012	Aug 2012	Nov 2012	April 2013	May 2013	Total	%	SISA
	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂	♀|♂		
Psathyromyia aclydifera	0|0	65|51	29|27	32|39	66|39	31|34	62|39	8 |6	5|11	29|20	327|266	62.36	0.881
Dampfomyia beltrani	0|0	1|1	0|0	0|0	1|0	0|1	2|0	0|0	0|0	1|1	5|3	0.84	0.195
Psathyromyia carpenteri	1|0	6|0	0|0	0|5	1|1	4|0	2|0	3|0	23|3	13|0	53|9	6.52	0.657
Micropygomyia cayennensis	0|0	0|0	0|0	0|0	0|1	0|0	0|0	0|0	0|0	0|0	0|1	0.11	0.024
Lutzomyia cruciata	2|0	2|0	3|0	0|0	4|0	4|1	1|0	0|0	3|0	6|1	25|2	2.84	0.514
Dampfomyia deleoni	1|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	1|0	0.11	0.043
***Micropygomyia* sp.**	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	1|0	1|0	0.11	0.014
Bichromomyia olmeca olmeca	0|0	1|0	1|0	2|0	3|1	1|6	1|1	0|0	2|0	2|1	12|10	2.31	0.462
Micropygomyia oppidana	1|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	1|0	0.11	0.043
Pintomyia ovallesi	0|0	0|0	2|0	0|0	0|0	0|0	0|0	0|0	0|0	0|0	2|0	0.21	0.076
Psychodopygus panamensis	4|4	4|10	0|1	14|23	37|6	23|15	6|2	10|0	18|4	2|4	118|69	19.66	0.857
Pintomyia serrana	0|0	2|0	0|0	0|0	0|0	0|0	1|0	1|0	1|0	0|0	5|0	0.53	0.167
Psathyromyia shannoni	1|0	2|2	0|0	0|0	1|0	1|0	0|0	1|0	0|0	0|0	6|2	0.84	0.205
Psathyromyia undulata	4|2	0|0	0|0	1|0	2|0	1|1	0|0	0|0	0|0	0|1	8|4	1.26	0.224
***Brumptomyia* sp.**	0|0	2|0	0|0	0|0	0|0	3|0	0|0	0|0	3|0	2|0	10|0	1.05	0.19
Brumptomyia hamata	0|0	0|0	0|0	0|0	0|0	0|0	0|1	0|0	0|0	0|0	0|1	0.11	0.038
Brumptomyia mesai	0|1	0|0	0|0	0|1	0|0	0|2	0|0	0|0	0|5	0|1	0|10	1.05	0.2
Abundance	21	149	63	117	163	128	118	29	78	85	574|377	100%	
Species richness (S)	8	8	5	6	9	9	8	5	7	8	15		
Shannon diversity ($q = 1$)	5.34 (3.01-7.67)	2.34 (1.88-2.79)	1.63 (1.13-2.14)	2.59 (2.14-3.05)	2.77 (2.28-3.26)	3.71 (3.02-4.41)	1.91 (1.49-2.32)	3.27 (2.46-4.09)	4.57 (3.86-5.29)	3.74 (2.90-4.59)	3.44 (3.17-3.72)		
Simpson diversity ($q = 2$)	4.05 (2.36-5.74)	1.58 (1.36-1.79)	1.26 (1.02-1.50)	2.13 (1.82-2.43)	2.06 (1.76-2.35)	2.71 (2.25-3.16)	1.35 (1.18-1.53)	2.74 (2.03-3.45)	3.87 (3.25-4.48)	2.52 (1.92-3.11)	2.25 (2.10-2.41)		

The empirical diversity of Shannon and Simpson indices are shown with the confidence intervals

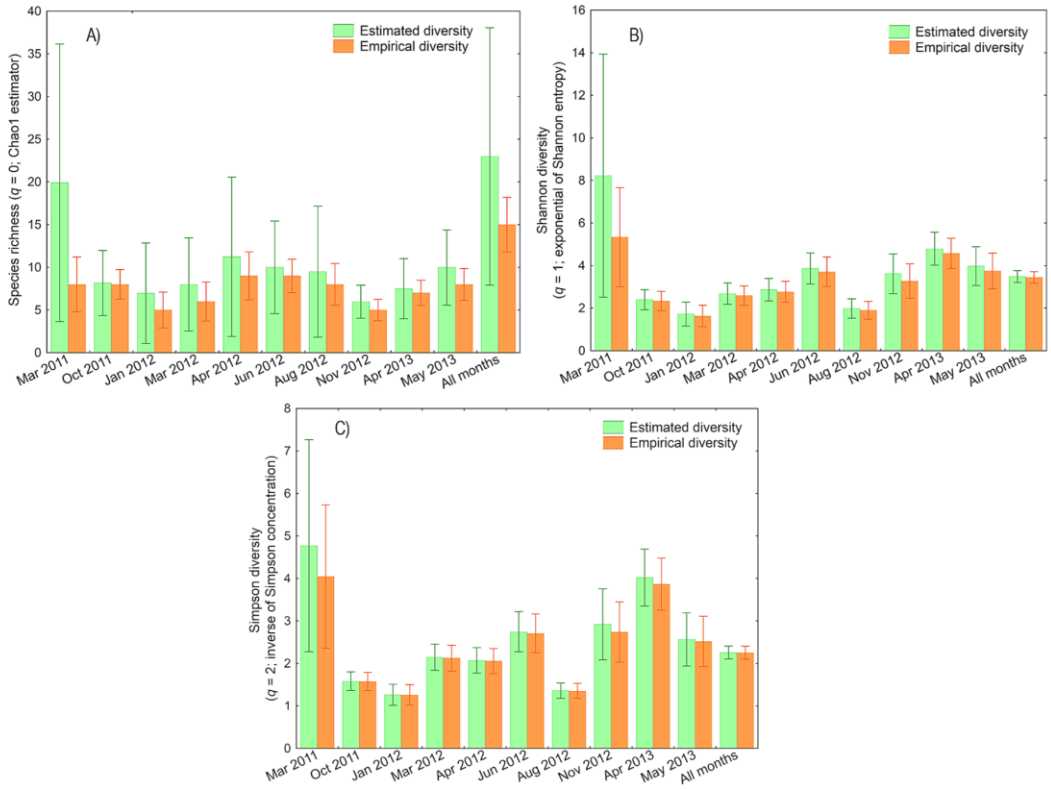

Fig. 2. Species diversity of phlebotomine sand flies collected from Biosphere Reserve of Los Tuxtlas, Veracruz, Mexico. A) Species richness (estimated and empirical diversity). B) Exponential of Shannon entropy (estimated and empirical diversity). C) Inverse of Simpson concentration (estimated and empirical diversity).

Table 2
Sand fly species used for blood meal identification.

Species	Females	DNA♠	Females with eggs*	Blood meal•
Pa. aclydifera	322	87	6/87	16/87
Da. beltrani	4	4	1/4	1/4
Pa. carpenteri	52	38	35/38	9/38
Lu. cruciata	24	9	8/9	-
Bi. olmeca olmeca	12	4	2/4	1/4
Ps. panamensis	115	52	23/52	10/52
Pa. shannoni	8	4	3/4	-
Pa. undulata	4	1	1/1	-
Br. hamata	1	1	0/1	-
Br. mesai	1	1	1/1	-
Br. sp.	8	2	2/2	-
10 species	551	203	82/203	37/203

♠Female specimens available for DNA extraction, * Females available for molecular analysis with eggs,
• Female positive for blood meal preferences

unable to collect information on the feeding status of female sand flies (full fed, half engorged or partially fed), we found that 40% (82/203) of them were female gravid, which suggests that they had previously ingested blood meal sources. To identify the blood meal sources, we amplified a fragment of ≈400 bp of the *cyt*b gene in 37/203 (18.22%) female specimens, belonging to five sand fly species collected in six sampling periods (Table 3). We were able to identify seven vertebrate species in the ML cluster analysis with high bootstrap values (Suppl. 1). Of these *Psathyromyia aclydifera, Pa. carpenteri* and *Ps. panamensis* were the species that showed a higher number of interactions with blood meal sources. It is noteworthy that although *Pa. aclydifera* had fed on 5/7 blood meal sources, the most frequent hosts detected was human beings (*Homo sapiens*) (Table 3, Fig. 3). Humans were the most common blood meal sources of four (4/5) sand fly species. In contrast, the river otter (*Lontra longicaudis*), the Pallas bat (*Glossophaga soricina*), and the Mediterranean house gecko (*Hemidactylus turcicus*) were the least frequent hosts (Fig. 3). In addition to detecting the blood meal sources in *Pa. aclydifera, Pa. carpenteri, Ps. panamensis* and *Bi. olmeca olmeca*, these sand flies were also shown to be infected with *Leishmania infantum* (Fig. 3, Table 3), as was previously reported (Lozano-Sardaneta et al., 2020).

4. Discussion

Estimating sand fly species diversity and identifying their natural blood meal sources are necessary to obtain reliable ecological and biological information for control and preventive programs against leishmaniasis (Baum et al., 2013; Rodríguez-Rojas et al., 2017). In Mexico, the knowledge on sand fly species has increased, although studies of the ecology, biology, distribution and identification of blood meal sources remain limited (Berzunza-Cruz et al., 2015; Rodríguez-Rojas et al., 2017). This study represents the first record on sand fly species diversity and identification of their blood meal sources from Los Tuxtlas, Veracruz, a region with a high incidence of *Leishmania infantum* (Lozano-Sardaneta et al., 2020).

We report a high sand fly species richness, including 15 of the 24 species that had previously been recorded in Veracruz (Ibáñez-Bernal et al., 2017, 2011, 2006; Lozano-Sardaneta et al., 2019; Martins et al., 1978). Although most of the collected sand fly species had been previously reported in Los Tuxtlas (Lozano-Sardaneta et al., 2019), we now show new records of *Da.*

Table 3
Blood meal sources identified from phlebotomine sand fly females collected in Los Tuxtlas, Veracruz, Mexico

Species	Sampling	Blood meal	Total score (n)	Query cover (%)	E-value	Identity (%)	Accession code
*Pa. aclydifera**	March 2012	*Canis lupus familiaris*	355	100%	8e-94	99.50%	MK937051.1
	March 2012	*Homo sapiens*	382	99%	6e-102	99.53%	LC088152.1
	June 2012	*Glossophaga soricina*	572	100%	3e-159	100%	AF382865.1
	August 2012	*Hemidactylus turcicus*	384	100%	2e-102	98.64%	HQ833732.1
	August 2012	*Canis lupus familiaris*	788	100%	1e-105	100%	KT447701.1
	August 2012	*Homo sapiens*♠	382	99%	6e-102	99.53%	KP126162.1
	May 2013	*Homo sapiens*	382	99%	6e-102	99.53%	LC088152.1
	May 2013	*Oryzomys couesi*	459	100%	4e-125	99.23%	KP778457.1
Da. beltrani	March 2012	*Homo sapiens*	382	99%	6e-102	99.53%	LC088152.1
*Pa. carpenteri**	May 2013	*Gallus gallus*♠	709	100%	0.0	99.75%	MN013407.1
	May 2013	*Homo sapiens*♠	342	100%	5e-90	97.06%	LC469901.1
	May 2013	*Lontra longicaudis*	395	100%	3e-112	99.55%	AF057123.1
*Bi. olmeca olmeca**	August 2012	*Canis lupus familiaris*♠	394	100%	1e-105	100%	KT447701.1
*Ps. panamensis**	March 2012	*Homo sapiens*	382	99%	6e-102	99.53%	LC088152.1
	March 2012	*Oryzomys couesi*	468	100%	7e-128	100%	JQ966248.1
	November 2012	*Homo sapiens*♠	360	99%	2e-95	100%	LC088152.1
	April 2013	*Gallus gallus*	365	100%	5e-97	100%	MK163563.1
	May 2013	*Homo sapiens*	382	99%	6e-102	99.53%	LC088152.1

Sand fly species (*) infected with *Leishmania infantum* and detection of blood meal preferences (♠).

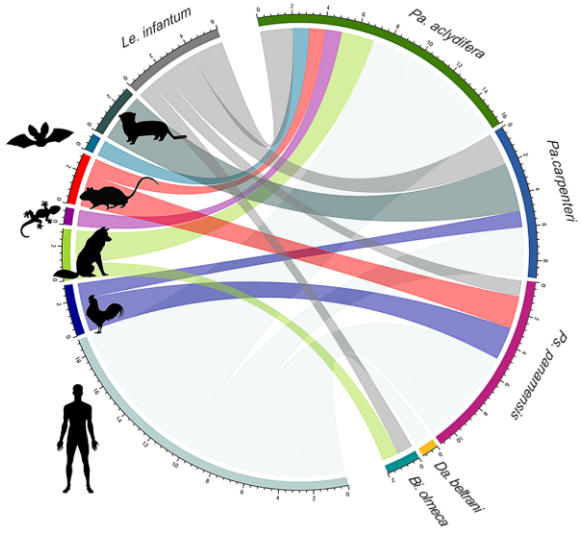

Fig. 3. Chord diagram for blood meal sources of phlebotomine sand fly species. Relationships and frequencies between sand fly species, their blood meal sources and infection with *Leishmania infantum* in Los Tuxtlas, Veracruz, Mexico.

deleoni, Mi. oppidana, and *Pi. ovallesi* for this area. *Pintomyia ovallesi* also represents a new record for the state of Veracruz, increasing the number of sand fly species from 24 to 25. This number of species represents almost half (48%) of the phlebotomine sand fly fauna of Mexico, which until now comprises 52 extant valid species.

The empirical diversity values obtained of orders ($q = 0$, 1, and 2) were lower than the estimated diversity. Sand fly species diversity varied monthly, and the highest species richness was recorded mainly in April and June 2012. Meanwhile, in March 2011 and April 2013, the most abundant species ($q = 1$) and dominant species ($q = 2$) were observed. The estimated richness ($q = 0$) were 23 effective numbers, while the obtained richness were 15 species, of which *Pa. aclydifera, Ps. panamensis* and *Pa. carpenteri* were the three most abundant and dominant species. Although *Ps. panamensis* had previously been reported to be highly abundant in other parts of Mexico, its presence in this area is relevant, since for more than 30 years it had not been reported in Veracruz with this high abundance (Lozano-Sardaneta et al., 2020, 2019; Martins et al., 1978; Pech-May et al., 2010). Both the high numbers of expected species and the faunal composition of the Los Tuxtlas, can be related to the characteristics of this area since the temperature, altitude, and the diverse vegetation types provide enough decomposing organic matter and a suitable climate that enable various sand fly species to successfully complete their life cycle (Ready, 2013; Soto and Gama, 1997). However, other factors could determine the peaks of activity and abundance of sand fly species include insufficient temporal and spatial sampling effort, environmental factors (including high temperature, rainfall, and altitude) or the use of only one type of trap. This could explain why some sand fly species were missing in the collected samples, and species such as *Lu. cruciata, Pa. shannoni*, and *Bi. olmeca olmeca*, which are considered anthropophilic species, were not as abundant as compared to studies done other parts of Mexico (Dantas-Torres et al., 2014; Moo-Llanes et al., 2013; Pech-May et al., 2010; Pérez et al., 2014; Rebollar-Téllez et al., 2005; Rodríguez-Rojas and Rebollar-Téllez, 2017).

The highest richness, abundance, and dominance of sand fly species was obtained during March and April, coinciding with the dry season (March to May). These findings are consistent with other studies conducted in Brazil and Mexico (mainly in the Chiapas and Yucatan Peninsula states), where greater abundances were recorded during the warm season, before to the rainy season, or after the decreasing rainfall (Pérez et al., 2014; Rebollar-Téllez et al., 1996a, 1996c; Sales et al., 2019). The high abundance of sand fly species frequently correlates with high temperatures and relative humidity, whereas a negative correlation is found during the rainy season for most sand fly species, although there are some exceptions (*e.g. Lutzomyia longipalpis*) (Pérez et al., 2014; Sales et al., 2019). The scarce collection of phlebotomine sand flies during the rainy season may also be related to their decreased flight activity. Thus, a reduced sampling success does not necessarily imply that sand flies are scarce or absent (Sales et al., 2019).

The blood preferences of sand fly species are not well known. However, several studies suggest that phlebotomine sand flies are opportunistic feeders and their host preference depends on the availability and abundance of the blood meal sources of each area, although the most frequently reported hosts are chickens, humans, rodents and cattle (Anaguano et al., 2015; Baum et al., 2013; Pereira Júnior et al., 2019). In this study, a high diversity of blood meal sources was obtained in May 2013 with four hosts (human, mouse, chicken, and river otter), followed by three hosts recorded in March 2012 (human, mouse, and dog) and August 2012 (human, gecko, and dog). Seven hosts were detected in five sand fly species, among which humans were the most common blood meal source (51.35%), followed by dogs (8.10%),

chickens (8.10%), and rodents (8.10%).

The sand fly species are frequently restricted to areas near blood meals sources. Therefore human activities can have an influence in the feeding habits, which could explain why it was the most common blood source. This behaviour may alter the natural cycle of *Leishmania* and increase the risk of transmission (Baum et al., 2013; Chaskopouloua et al., 2016; Sandoval-Ramírez et al., 2020). Canines and rodents (*e.g.* rice rats of the genus *Oryzomys*) are common blood meal sources for sand flies and also are considered reservoir hosts of *Leishmania*. Therefore they also have and important role in the cycle, given their proximity to humans (Baum et al., 2013; Roque and Jansen, 2014; Sandoval-Ramírez et al., 2020; Van Wynsberghe et al., 2000). The presence of blood meal from the Pallas bat (*G. soricina*) detected in a *Pa. aclydifera* specimen (not infected with *Le. infantum*) is interesting, since the Pallas bat is considered a reservoir host of *Le. mexicana* and *Le. infantum* in Mexico and Brazil, respectively (Berzunza-Cruz et al., 2015; Roque and Jansen, 2014). Yet, it remains to be established whether this bat species plays a role in the wild cycle of *Leishmania* spp. in Los Tuxtlas.

Our results also suggest that *Pa. aclydifera* and *Ps. panamensis* could be highly anthropophilic. In the case of *Ps. panamensis*, this is not unexpected, since this sand fly species has been anthropophilic and previously been incriminated as a vector of *Le. mexicana* in Mexico (Pech-May et al., 2010). On contrast, *Pa. aclydifera* appears to be an opportunistic feeder, showing preferences for diverse hosts, which in this area were human beings, dogs, rodents, geckos, and bats.

In a previous study in this region, a high infection rate with *Le. infantum* was detected in *Pa. aclydifera* and *Ps. panamensis*, mainly during August and November 2012 (Lozano-Sardaneta et al., 2020). Hence, these sand fly species possibly play a role in the transmission of *Leishmania* spp. in Los Tuxtlas. Yet more studies need to be done in this area, since the high infection rate registered in. August and November 2012 (rainy season) contrasts with our current results showing that the highest abundance, richness, and dominance of species are expected during the dry months. Furthermore, other studies have shown that the transmission peaks frequently occur during the months with a greater abundance of sand fly species (Pech-May et al., 2010; Rebollar-Téllez et al., 1996b). However, in some cases the abundance pattern of a sand fly species does not always correlate with their monthly infectious bite rate (Pech-May et al., 2010). It is therefore possible that these species (*Pa. aclydifera* and *Ps. panamensis*) may be less dependent on climatic fluctuations, which would reduce the impact on their abundance and dominance, favouring their possible role in the transmission of *Leishmania* spp. in Los Tuxtlas. Interestingly, in August 2012 the blood hosts identified in *Pa. aclydifera* were humans, dogs, and geckos, whereas for *Bi. olmeca olmeca* the only detected blood meal were dogs, and in November 2012 the blood meal detected were humans in *Ps. panamensis*. Meanwhile, the presence of *Le. infantum* was detected only in specimens of *Pa. aclydifera* and *Ps. panamensis* with human blood, and *Bi. olmeca olmeca* with dog blood.

Thus, the high prevalence of *Le. infantum* from Los Tuxtlas, is probably related to a greater abundance of infected hosts during the rainy season (from June to February), and judging from our results, it is highly likely that humans and dogs are the most abundantly infected host. Nonetheless, the high abundance of sand fly species and the presence of *Leishmania* DNA are not sufficient to incriminate a vector species, since we were not able to confirm how the infection occurs (Chaskopouloua et al., 2016; Sandoval-Ramírez et al., 2020). Therefore, complementary studies including hosts samplings are necessary.

We contribute novel data on the structure of sand fly populations and their blood meal sources, providing basic and important information on the interactions between sand flies, hosts, and *Leishmania infantum*. According to the data obtained, our study area seems to have completely different population dynamics, as compared to other areas in Mexico. For this reason, more detailed sampling of these sand fly species is necessary to understand their role in the cycle of leishmaniasis, enabling a more effective planning of prevention strategies and control of this disease in Veracruz, Mexico.

CRediT authorship contribution statement

Yokomi N. Lozano-Sardaneta: Conceptualization, Methodology, Writing - review & editing, Visualization, Formal analysis. **Eduardo I. Jiménez-Girón:** Methodology, Formal analysis, Writing - review & editing. **Jorge J. Rodríguez-Rojas:** Methodology, Formal analysis, Writing - review & editing. **Sokani Sánchez-Montes:** Methodology, Formal analysis, Writing - review & editing. **Lucía Álvarez-Castillo:** Methodology, Writing - review & editing. **Víctor Sánchez-Cordero:** Visualization, Resources, Writing - review & editing. **Ingeborg Becker:** Formal analysis, Writing - review & editing, Resources, Project administration.

Declaration of Competing Interest

The authors declare that have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.105831.

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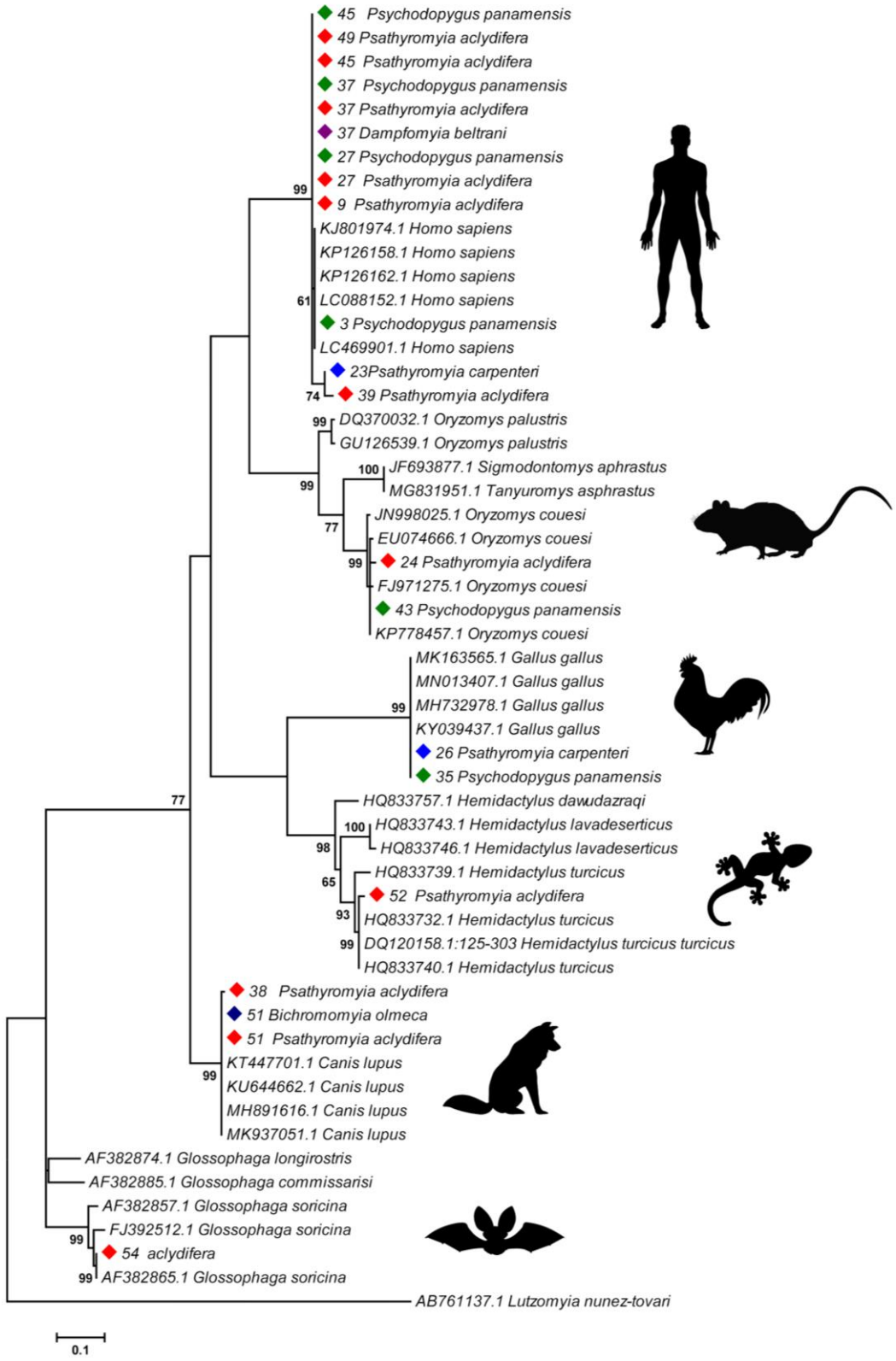

Suppl 1. Maximum Likelihood tree for blood meal preferences of sand fly species using Cyt *b* sequences. The obtained sequences are marked with a diamond.

DISCUSIÓN

Aunque en los últimos 20 años el estudio de los flebotominos en México se ha incrementado exponencialmente, aún existen muchas áreas geográficas sin explorarse, por lo que el conocimiento aún se considera incompleto. Estos dípteros tienen un papel importante en la transmisión de patógenos, por lo que la relevancia de los estudios acerca de la biología y ecología de los flebotominos cada vez es más importante, debido a su gran utilidad para mejorar los programas de vigilancia y control vectorial. Es por eso que en este trabajo se caracterizó por primera vez la comunidad de flebotominos que se distribuyen en la Estación de Biología Tropical, Los Tuxtlas, Veracruz, y se realizó la detección molecular de las bacterias *Bartonella* y *Wolbachia*, así como del protozoario *Leishmania.*

Durante los muestreos realizados se encontró una alta riqueza de especies, obteniendo 15 especies de flebotominos de las 23 que previamente habían sido registradas para el estado de Veracruz (Ibáñez-Bernal et al., 2017, 2011, 2006; Martins et al., 1978).Todas las especies colectadas en este estudio fueron identificadas morfológicamente y representan nuevos registros para la región de Los Tuxtlas, excepto *Brumptomyia mesai* y *Dampfomyia beltrani*, ya que dichas especies previamente estaban reportadas para esta zona (Ibañez-Bernal, 1997). Adicionalmente, *Psathyromyia aclydifera* y *Pintomyia ovallesi* también representan nuevos registros para el estado de Veracruz, aumentando el número de especies de flebotominos de 23 a 25 (Lozano-Sardaneta et al., 2019; 2020). Por lo que el estado de Veracruz cuenta con el 48% (25/52) de la fauna de flebotominos de México, la cual hasta el momento incluye 52 especies válidas existentes.

De forma complementaria a la identificación morfológica de las hembras, se recurrió al uso de la amplificación del gen COI (Folmer et al., 1994; Hebert et al., 2003), para evaluar si era un método confiable para diferenciar a las especies de flebotominos que se colectaron durante este trabajo. Se logró amplificar un fragmento del gen COI de 650 pares de bases aproximadamente en 25 ejemplares que

pertenecían a ocho especies diferentes de flebotominos y mediante un análisis de similitud, se corroboró su identidad. Con base en este análisis, tanto las secuencias generadas en este trabajo como otras secuencias disponibles en GenBank, se agruparon por especies con un soporte de rama alto (boostrap 97–100%) (Lozano-Sardaneta et al., 2019b), sin importar su distribución geográfica (Azpurua et al., 2010; Contreras-Gutiérrez et al., 2014; Florin and Rebollar-Téllez, 2013; Pinto et al., 2015; Romero-Ricardo et al., 2016; R. Vivero et al., 2017). Empleando este método, también fue posible separar a las especies que fueron clasificadas dentro del género *Brumptomyia* en *Br. mesai* y/o *Br. hamata*, que son las dos especies reportadas para México (Absavaran et al., 2009; Ibáñez-Bernal, 1999). En el caso particular de este género, los caracteres morfológicos para identificar a las hembras no son suficientes, debido a su gran similitud, lo cual dificulta su identificación. Por lo tanto, este método complementario es de gran utilidad ya que permitió separar a las especies de forma más eficiente.

La variabilidad intraespecífica observada en este estudio osciló entre 0.5% y 2.4%, siendo *Pa. aclydifera* (2.4%) y *Lutzomyia cruciata* (1.8%), las especies con los valores más altos de variación. La variación interespecífica observada estuvo en un rango de 12.4% a 20.9%, aunque en comparación con otros estudios realizados en Colombia (Contreras-Gutiérrez et al., 2014; Romero-Ricardo et al., 2016), Panamá (Azpurua et al., 2010), Perú (Nzelu et al., 2015) y Brasil (Rodrigues et al., 2018; Scarpassa and Alencar, 2013), la variación obtenida fue baja. Dentro de las muestras analizadas, algunas especies de flebotominos presentaron valores altos de variación, lo que apoya la hipótesis de que algunas de estas especies podrían incluir especies cripticas, como es el caso de *Psathyromyia shannoni* o *Lu. cruciata.* Esto último puede estar relacionado con que algunas especies de flebotominos pueden ser más sensibles a barreras geográficas o de temporalidad, favoreciendo que se adapten a las características del hábitat local, lo cual se reflejará en su variación intraespecífica e interespecífica (Florin and Rebollar-Téllez, 2013; Pech-May et al., 2013). Por lo que estudios complementarios son necesarios para dilucidar los factores (bióticos y abióticos) que influyen en la variabilidad genética de este grupo, principalmente aquellos que se distribuyen en las zonas endémicas de leishmaniasis.

La diversidad de especies de flebotominos fluctuó mensualmente, principalmente en abril y junio del 2012, cuando se registró la mayor riqueza de especies, mientras que en marzo del 2011 y abril del 2013 se observaron las especies más abundantes (q = 1) y las más dominantes (q = 2), lo que coincide principalmente con la época de secas. Este hallazgo coincide con lo que se ha reportado en otros estudios realizados en México (Chiapas y la Península de Yucatán principalmente) y Brasil, en donde también registraron que la mayor diversidad de especies se correlaciona principalmente con la temporada de secas (altas temperaturas y humedad relativa), mientras que hubo poca correlación con la época de lluvias (ya sea antes o después de esta) para la mayoría de las especies, aunque pueden existir algunas excepciones (Pérez et al., 2014; Rebollar-Téllez et al., 1996d, 1996b; Sales et al., 2019).

Aunque en este estudio se colectó el 60% (15/25) de las especies que se han reportado para Veracruz, la riqueza esperada para este estudio fue de 23 especies, siendo *Pa. aclydifera, Psychodopygus panamensis* y *Psatrhyromyia carpenteri* las tres especies más abundantes y dominantes. En el caso particular de *Pa. aclydifera*, resulta un hallazgo interesante ya que hasta el momento solo se ha reportado en Chiapas, Jalisco y Campeche, aunque nunca había sido reportada con gran abundancia, al igual que *Pa. carpenteri*. Mientras que *Ps. panamensis*, es una especie ampliamente distribuida en México, aunque para el estado de Veracruz no había sido reportada con esta abundancia por más de 30 años (Lozano-Sardaneta et al., 2020, 2019b; Martins et al., 1978; Pech-May et al., 2010).

La riqueza esperada de flebotominos para la región de Los Tuxtlas probablemente pueda estar relacionada con las características de esta zona (altitud, temperatura, vegetación, etc.) lo que favorece que diversas especies de flebotominos puedan establecerse y completar su ciclo de vida de forma exitosa (Ready, 2013; Soto and Gama, 1997). Esto posiblemente también favorece la capacidad y competencia vectorial de estos dipteros para transmitir diversos patógenos en esta zona.

Detección de *Bartonella* sp.

Aunque en los últimos años, los estudios sobre los linajes de *Bartonella* que circulan en México han incrementado, aún están dispersos y restringidos a algunas localidades de Chihuahua, San Luis Potosí y la Península de Yucatán. No obstante, sigue existiendo poca información respecto a los posibles vectores de esta bacteria, su distribución geográfica, y/o diversidad genética (Kosoy et al., 2012).

En este estudio se detectó *Bartonella* sp., en dos ejemplares de flebotominos del género *Lutzomyia* sp., las cuales fueron colectados durante el mes de mayo 2016, representando una prevalencia del 8.69% (2/23). Las secuencias recuperadas de estos amplificados tuvieron una cobertura del 99% y una similitud del 96% con secuencias de *Bartonella* sp., detectada en Tailandia y China (FJ668633 y FJ589054). Con base en el análisis realizado, estas secuencias son muy parecidas a las especies de *Bartonella* que están estrechamente relacionadas con roedores y sus ectoparásitos, que en algunos casos pueden ocasionar endocarditis en perros y humanos (Regier et al., 2016).

Aunque la especie detectada en este estudio no es *Bartonella bacilliformis*, la principal especie relacionada con flebotominos en América, este hallazgo representa un nuevo registro para esta bacteria fuera de la zona endémica de la Verruga Peruana, en donde solo se ha reportado la presencia de *B. bacilliformis* y *B. grahammi* asociada con estos dípteros (Ellis et al., 1999). Adicionalmente representa el primer registro de *Bartonella* sp. asociada con flebotominos en México.

Si bien no podemos confirmar que los flebotominos puedan tener un papel importante en la transmisión de esta bacteria en el país o en la zona de los Tuxtlas, indirectamente sabemos que otros linajes de esta bacteria (patógenos o no patógenos) están circulando en la fauna que se distribuye en Veracruz. Por lo tanto, debido a la gran diversidad de linajes de *Bartonella* registrados en México, sería conveniente seguir realizando estudios complementarios para poder confirmar si algunas especies de flebotominos están relacionadas con la trasmisión de estas bacterias en el país.

Detección de *Wolbachia* sp. y *Rickettsia* sp.

La detección de parásitos reproductivos y sus hospederos es muy común y estudiado ampliamente (Weinert et al., 2015). No obstante, en cuanto a su relacion con especies de flebotominos se sabe poco a nivel mundial, ya que hasta el momento sólo el 2% de la diversidad de especies de estos dípteros están relacionados con algún linaje de parásitos reproductivos (*Wolbachia* o *Rickettsia*). Este estudio representa el primer registro de rickettsiales endosimbiontes asociados a flebotominos del género *Psathyromyia* que se distribuyen en Los Tuxtlas.

Para la detección de *Wolbachia* se amplifico un fragmento del gen *wsp*, obteniendo un total de dos positivos (2/204), asociados con las especies de flebotominos *Pa. shannoni* y *Lutzomyia* sp., lo cual representa el segundo registro de cepas de *Wolbachia* asociadas a flebotominos de México. Su prevalencia (0.98%) fue baja en comparación con otros estudios realizados en flebotominos en donde reportan una prevalencia entre el 2.8% y el 37.19% (Azpurua et al., 2010; Bordbar et al., 2014; Ono et al., 2001; Parvizi et al., 2003; R. J. Vivero et al., 2017). Esto puede estar relacionado con bajas densidades de estas bacterias, así como con el comportamiento de los flebotominos y su distribución, ya que se ha reportado que en zonas con altas temperaturas y en flebotominos viejos su tasa de infección puede ser menor (Parvizi et al., 2013a, 2013b).

La cepa de *Wolbachia* detectada en este estudio fue muy parecida a la cepa *wWhi* (con un bootstrap del 99% y una diferencia genética de 0.002%) detectada en *Pa. shannoni* de Colombia y *Nyssomyia whitmani* de Brasil. Aunque se desconoce el rol que puede desempeñar *Wolbachia* en los flebotominos, algunos autores sugieren que en las especies de flebotominos que se distribuyen en América, esta asociación posiblemente sirva como una barrera genética que separa especies similares de flebotominos, generando incompatibilidad intraespecífica, ocasionando diversificación y especiación (Azpurua et al., 2010).

También, se detectóun nuevo linaje de *Rickettsia* sp., asociado con *Pa. aclydifera* con una prevalencia del 8. 3% (Perlman et al., 2006). Aunque poco se sabe acerca de la relevancia de esta asociación, con base en la propuesta filogenética de Weinert *et al.* (2009) las especies de *Rickettsia* asociadas con flebotominos pueden transmitirse verticalmente, y posiblemente esta asociación podría tener un efecto benéfico sobre el hospedero, tal como la inducción de la ovogénesis, el aumento del tamaño e incluso con la disminución de la capacidad de transmisión de otros patógenos (Kikuchi and Fukatsu, 2005; Nováková and Šmajs, 2019; Perlman et al., 2006; Perotti et al., 2006).

Sin embargo, más estudios enfocados en la detección de rickettsiales endosimbiontes (*Wolbachia* y *Rickettsia*) en flebotominos son necesarios, en pro de brindar información complementaria sobre su posible relevancia como control biológico y/o protección contra la transmisión de otros patógenos, con la finalidad de disminuir los casos de leishmaniasis en México.

Detección de *Leishmania infantum*

La leishmaniasis es una enfermedad tropical desatendida de gran relevancia en salud pública a nivel mundial. Por lo que, el saber si los flebotominos están infectados con este protozoario, proporciona información crucial sobre las tasas de infección de estos dípteros en áreas de alta abundancia de especies (Aransay et al., 2000). A pesar de que Veracruz registra una alta incidencia de casos de leishmaniasis, este es el primer estudio dirigido a la detección molecular del género *Leishmania* en especies de flebotominos de Los Tuxtlas. Con base en las secuencias analizadas, la identidad de estas corresponde a *Le. infantum,* agente causal del cuadro clínico visceral en América (Akhoundi et al., 2016). En este estudio, esta especie se detectó por primera vez en ocho especies de flebotominos, aunque no sabemos si estas especies están involucradas en la transmisión de *Le. infantum*. Previamente especies como *Lu. cruciata*, *Bi. olmeca olmeca*, *Ps. panamensis* y *Pa. shannoni* habían sido incriminadas como vectores de *Le. mexicana* en áreas endémicas de leishmaniasis de México (Biagi et al., 1965; Pech-May et al., 2010). Por lo que no podemos descartar su posible rol en la transmisión de otras especies de *Leishmania*, debido a la alta tasa de

infección (18.71%), detectada en este estudio en comparación con otros estudios realizados en el país (Andrade-Narvaez et al., 1990; Berzunza-Cruz et al., 2015; Pech-May et al., 2010; Sánchez-García et al., 2010; Sanchez-Tejeda et al., 2001). Por lo tanto, es probable que en esta zona exista un número elevado de poblaciones humanas y animales en riesgo de transmisión de leishmaniasis.

Las especies de flebotominos con mayor número de ejemplares infectados fueron *Pa. aclydifera* y *Ps. panamensis*, principalmente durante la época de secas. En el caso particular de *Pa. aclydifera* es un hallazgo interesante debido a su gran abundancia durante el muestreo y su limitada distribución, por lo que posiblemente puede estar desempeñando un papel importante en la transmisión de este parásito en Los Tuxtlas (Maia et al., 2015; Pech-May et al., 2016; Quiroga et al., 2017).

Preferencias alimenticias en flebotominos

Aunque este no fue un objetivo particular de este trabajo, de forma complementaria logramos establecer las preferencias alimenticias de los flebotominos colectados durante este estudio. Para muchas especies de flebotominos las fuentes alimenticias no son bien conocidas, aunque en general se sugiere que son organismos oportunistas que se alimentan de los hospederos que tengan al alcance. Por lo tanto, las preferencias alimenticias son dependientes de la disponibilidad y abundancia de los hospederos de las zonas de estudio, no obstante los organismos más comunes que se han reportado en la literatura son gallinas, ganado, roedores y humanos (Anaguano et al., 2015a; Baum et al., 2013; Pereira Júnior et al., 2019).

En este estudio se detectó la presencia de DNA de siete hospederos en cinco especies de flebotominos entre los cuales los humanos fueron la fuente de alimento más común (51,35%), seguida de perros (8,10%), pollos (8,10%) y roedores (8,10%). Aunque la zona de Los Tuxtlas es una zona conservada, en los últimos años las actividades humanas han perturbado la zona, por lo que no es extraño que se haya detectado la presencia de humanos en el estudio. Por otro lado, en el caso particular de los perros y roedores, se ha reportado que son fuentes de alimento comunes y también son considerados reservorios de *Leishmania*, desempeñando un papel importante en el ciclo de transmisión debido a su

proximidad con humanos (Baum et al., 2013; Roque and Jansen, 2014; Sandoval-Ramírez et al., 2020; Van Wynsberghe et al., 2000).

Con estos hallazgos complementarios, es posible que especies como *Pa. aclydifera* y *Ps. panamensis* sean especies antropofilicas de relevancia en la trasmisión de *Leishmania.* Debido a que estas dos especies fueron las que estuvieron infectadas con *Le. infantum* durante los meses de mayor prevalencia del parásito (Lozano-Sardaneta et al., 2020). De acuerdo con los datos de diversidad de especies, se observó que no son especies dependientes de las fluctuaciones climáticas, por lo que su abundancia y dominancia no cambió durante el muestreo, favoreciendo su posible papel en la trasmisión de *Le. infantum* en Los Tuxtlas. Los meses de mayor prevalencia de *Le. infantum* (agosto y noviembre 20121), coinciden con los meses donde se encontró que humanos y perros fueron las fuentes alimenticias más comunes, por lo tanto es probable que durante la época de lluvias la abundancia de hospederos infectados sea mayor.

Si bien no es posible incriminar a un vector basándose solamente en la abundancia y/o la presencia del DNA de algún parásito (por ejemplo, *Leishmania*), la evidencia generada en este proyecto acerca de la estructura poblacional de flebotominos, proporciona información básica y relevante sobre las interacciones entre flebotominos, sus parásitos y hospederos, abriendo la posibilidad de realizar estudios complementarios que nos permitan dilucidar el papel de estas especies como posibles vectores de *Leihsmania* y otras bacterias en México. Aparentemente la dinámica poblacional que observamos en esta zona, resultó ser complemente distinta a lo que previamente se había reportado para otras áreas de México. Es por eso que los estudios entomológicos en otras áreas del país son necesarios, ya que es la única forma de realizar una planificación más eficiente de las de las estrategias de prevención y control de enfermedades transmitidas por flebotominos en México.

RESUMEN DE RESULTADOS

1. Se logró identificar morfológicamente 15 especies de flebotominos, de las cuales 13 representan nuevos registros para la zona de Los Tuxtlas, y dos de ellas son nuevos registros para el Estado de Veracruz, aumentando la riqueza de especies de 23 a 25.

2. Se proporcionaron por primera vez, secuencias de códigos de barras usando el gen COI para siete especies de flebotominos mexicanos, lo cual representa una herramienta complementaria a la identificación morfológica. La identificación molecular de flebotominos no sólo facilita los estudios epidemiológicos, sino también permitirá distinguir entre especies isomorfas, como las especies del género *Brumptomyia*, o entre ejemplares que hayan sido dañados morfológicamente durante su procesamiento.

3. Se registró por primera vez la presencia de un linaje de *Bartonella* sp., asociado con flebotominos en México.

4. Se encontró por primera vez, la coinfección de *Leishmania infantum* y de *Rickettsia* sp., en ejemplares de *Pa. aclydifera*. Así mismo, se encontró coinfección entre *Le.infantum* con *Wolbachia* sp. en *Pa. shannoni*.

5. Se encontró la presencia de *Leishmania infantum* en ocho especies de flebotominos con una prevalencia del 18.7%.

6. Por primera vez, se identificaron las preferencias alimenticias de cinco especies de flebotominos, siendo humano el más común.

7. Con base en los resultados de diversidad de especies, la detección de *Leishmania* y las preferencias alimenticias, es posible que especies como *Pa. aclydifera* y *Ps. panamensis* sean de gran relevancia en la dinámica de transmisión de patógenos para la zona de Los Tuxtlas.

CONCLUSIONES

Los hallazgos obtenidos en este trabajo enriquecen la literatura sobre flebotominos y sus patógenos en México. Resaltan la importancia de realizar estudios entomológicos y epidemiológicos, para determinar las especies que pudieran ser relevantes en la transmisión de patógenos en diferentes áreas del país y si su dinámica de transmisión difiere entre sitios geográficos. Conocer la distribución geográfica y temporal de los flebotominos en todas las zonas del país es esencial para llevar acabo un manejo integral de las enfermedades transmitidas por vector, ya que por los resultados obtenidos, cada especie tiene su propia dinámica poblacional. Es posible que existan otras especies que puedan ser de relevancia en la transmisión de patógenos en áreas específicas de México, aunque se requieren estudios complementarios para dilucidar esta hipótesis.

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