Genetic Diversity and Prevalence of *Leishmania mexicana* in *Bichromomyia olmeca olmeca* in an Endemic Area of Mexico

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Genetic Diversity and Prevalence of *Leishmania mexicana* in *Bichromomyia olmeca olmeca*[1] in an Endemic Area of Mexico

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Abstract. Leishmaniases are endemic in southwestern Mexico, and different sand fly species are infected with *Leishmania mexicana* Biagi. One of the most abundant vectors and dominant species is *Bichromomyia olmeca olmeca* (Vargas and Díaz-Nájera). We analyzed the genetic variability of *Bichromomyia olmeca olmeca* and the prevalence of *Leishmania mexicana* infections with an endemic focus: "the Ejido 20 de Junio" (Mancolona), Campeche, Mexico, where patients with leishmaniases are reported throughout the year. Genetic diversity analysis of 102 sequences of a 270-bp fragment of the 3´ end of mitochondrial cytochrome *b* (cyt *b*) gene of *Bichromomyia olmeca olmeca* revealed 17 haplotypes. The nucleotide diversity and nucleotide polymorphism index were low. The neutrality test and Mismatch test showed population expansion. Prevalence of *Leishmania mexicana* was 24.5% in 102 females analyzed. This is the first study showing the genetic diversity of *Bichromomyia olmeca olmeca* sand flies in the Campeche region of Mexico, and also provides novel information on the high infection rate of *Bichromomyia olmeca olmeca* by *Leishmania mexicana.* Our finding of high sand fly infection rates during the end of the dry and hot weather of July enriches the literature because high infection rates had been reported only during the rainy season (November) in the region.

Introduction

Human leishmaniases are endemic in southwestern Mexico. The first scientific record for the parasite dates from 1912 (Seidelin 1912), beginning with chicle gum exploitation to produce chewing gum from the tree *Manilkara zapota* (L.) P. Royen in the Yucatan Peninsula (Beltrán and Bustamente 1942). *Leishmania mexicana* Biagi was identified as the etiological agent of "Chiclero´s Ulcer" (Biagi 1953, Garnham 1962). New World *Leishmania* vectors are small hematophagous Diptera of the subfamily Phlebtotominae of Psychodidae (Young and Arias 1991). In Mexico, the sand fly *Bichromomyia olmeca olmeca* (Vargas and Díaz-Nájera) is

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one of the main vectors of *Leishmania mexicana* (Biagi et al. 1965; Rebollar-Téllez et al. 1996; Sánchez-García et al. 2010; Pech-May et al. 2010; 2016). Studies in the Yucatan Peninsula showed that at least four other species are vectors of *Leishmania*: *Psychodopygus panamensis* Shannon, *Lutzomyia cruciata* Coquillet, *Psathyromyia shannoni* Dyar, and *Lutzomyia ylephiletor* Fairchild and Hertig (Rebollar-Téllez et al. 1996; Sánchez-García et al. 2010; Pech-May et al. 2010; 2016). *Bichromomyia olmeca olmeca* is one of the most abundant species at Campeche (Andrade-Narvaez et al. 2003; Pech-May et al. 2010; 2016), Quintana Roo (Cruz-Ruíz et al. 1994, Sánchez-García et al. 2010, May-Uc et al. 2011, Mendez-Perez and Rebollar Téllez 2012), and Yucatán (Gonzales et al. 2010, Mendez-Perez and Rebollar-Téllez 2012). In Mexico, the sand fly is also present in the states of Chiapas, Oaxaca, Tabasco, and Veracruz (Ibañez-Bernal 2002; Rebollar-Téllez et al. 2004; 2005). Several studies confirmed that in the wild, *Bichromomyia olmeca olmeca* harbors *Leishmania mexicana* (Rebollar-Téllez et al. 1996; Sánchez-Garcia et al. 2010; Pech et al. 2010; 2016). In their local sylvatic environment, sand flies are thought to be structured in local asynchronous populations that disperse to colonize new niches, thereby enabling the parasite to come into contact with, and infect, new host populations (Wilson and Benjamin 1980, Hanski and Gilpin 1997).

Use of molecular methodology, especially DNA sequencing, has profoundly influenced sand fly taxonomy (Harley 2009) and helped infer phylogenetic relationships and analyze genetic diversity (Hodgkinson et al. 2002, 2003; Torgerson et al. 2003). The molecular marker cytochrome *b* (cyt *b*) has a high nucleotide divergence rate in phlebotomine sand flies, and therefore has been used successfully for several phlebotomine species (Hodgkinson et al. 2002, 2003; Coutinho-Abreu et al. 2008; Scarpassa and Alencar 2012; Pech-May et al. 2013).

Bichromomyia olmeca olmeca in the Ejido 20 de Junio (Mancolona) co-exists in areas with high transmission of cutaneous leishmaniasis, and Pech-May et al. (2010) found it naturally very infected with *Leishmania mexicana*. Understanding the local genetic diversity of the vector, and the process responsible for it, are paramount for management of the vector and understanding dynamics of wildlife diseases. The main goal of the study was to analyze genetic diversity of the *Bichromomyia olmeca olmeca* vector and the prevalence of *Leishmania mexicana* infections in an endemic focus where patients with leishmaniasis were reported throughout the year (Hernández-Rivera et al. 2015). The study yielded novel information on the high infection rate of *Bichromomyia olmeca olmeca* by *Leishmania mexicana* during the end of the dry and hot weather of July, before the rainy season began, which enriches the literature because to date high infection rates had been reported only during the rainy season (November) in the region.

Materials and Methods

The Calakmul Biosphere is located at Calakmul 350 km south of the state capital. The region is Reserve characterized by undulating uplands with elevations 100 to 350 m above sea level. The main soil types are rendzinas (Pérez-Salicrup 2004). Total annual rainfall ranges from 1,400 to 900 mm, with high evapotranspiration and few permanent sources of surface water. Two main types of seasonal deciduous forests cover the area: upland forest (*selva mediana*) and low wetland forest (*selva baja indundable*). The area is endemic for cutaneous leishmaniasis, the highest

occurrence index of the disease in the state, with approximately 50 human cases reported every year (Hernández-Rivera et al. 2015).

The "Ejido 20 de Junio" (Mancolona) is in the municipality of Calakmul, Campeche, in southwestern Mexico (18°06'52'' S; 89°48'30.76'' W; 190 m above sea level) (Fig. 1). The land is used for cultivating crops, and farmers travel by foot, bicycle, or horseback on narrow footpaths from villages to their plots. Most transmission of *Leishmania* is thought to occur along the paths and in the cultivated plots (Pech-May et al. 2010).

Sand flies were collected during five consecutive nights from 16-20 July 2009. Each night, two Shannon traps (1.6 x 2.5 x 1.6 m) at least 100 m apart were randomly placed at the same time in the forest. The traps were tied to trees and stretched so the base was 30 cm above the ground. Every night two persons remained inside the traps attracting the sand flies. The humans were protected from bites by long-sleeved shirts and trousers and fine-mesh mosquito netting covering the head (Perez et al. 1987). To capture sand flies, glass aspirators were used during a 4-hour period (1800-2100 hours). Specimens caught were stored in a clear wide-mouthed jar and transported to a field-based laboratory where they were separated and washed in a Petri dish containing 2% detergent solution (Extran MA02, pH 7.2, EMD Chemicals, Gibbstown, NJ). After rinsing in clean water in another Petri dish, the flies were dried on filter paper and preserved in 500 μl of 80% ethanol. The head of each specimen was separated for taxonomic identification and the rest of the body (thorax and abdomen) were stored at -20°C for DNA extraction. The heads were mounted according to the protocol described by Ibáñez-Bernal (2005b). The taxonomic classification was based on Young and Duncan (1994), Ibáñez-Bernal (2000, 2005ab), and Galati (2003).

DNA was extracted individually from each female sand fly according to methods described in Pech-May et al. (2013). As an extraction check, a 450-bp fragment of the 18S rRNA gene was amplified using the oligonucleotides Lu.18SrRNA-1S and Lu.18S rRNA-1R (Kato et al. 2005) following the conditions reported by Sánchez-García et al. (2010). A 365-pb fragment from the 3´ end of mitochondrial cytochrome *b* (cyt *b*) was amplified using the oligonucleotides 11226 and 11587 following conditions reported by Hodgkinson et al. (2002, 2003).

To detect *Leishmania* DNA, each sample was evaluated using LMC-1S and LMC-1R oligonucleotides that specifically recognize all members of the *Leishmania* genus. Positive samples were tested additionally for *Leishmania mexicana* DNA, using specific oligonucleotides IR1 and LM17 and protocol reported by Berzunza-Cruz et al. (2009). PCR products were visualized on 1.5% agarose gels stained with ethidium bromide (0.5 μg/ml) under ultraviolet light. PCR products were purified using the QIAquick™ PCR purification kit (QIAGEN, Valencia, CA), and sequencing was on a Prisma 310 ABI (High-Throughput Genomics Center, Department of Genome Sciences, University of Washington).

DNA sequences were aligned and edited using Mega v.7.0 software (Kumar et al. 2015). The sequences obtained in the study were deposited in GenBank (accession numbers: KY947524, KY947525, KY947526, KY947527, KY947528, KY947529, KY947530, KY947531, KY947532, KY947533, KY947534, KY947535, KY947536, KY947537, KY947538, KY947539, and KY947540).

Genetic diversity was measured using the number of mutations (η), number of segregating sites (S), number of unique sites (Su), number of haplotypes (Nh), haplotype diversity (h), nucleotide diversity (π), and nucleotide polymorphism index (Θ). Tajima's D (Tajima 1989) as well as D and F indices (Fu and Li 1993) were

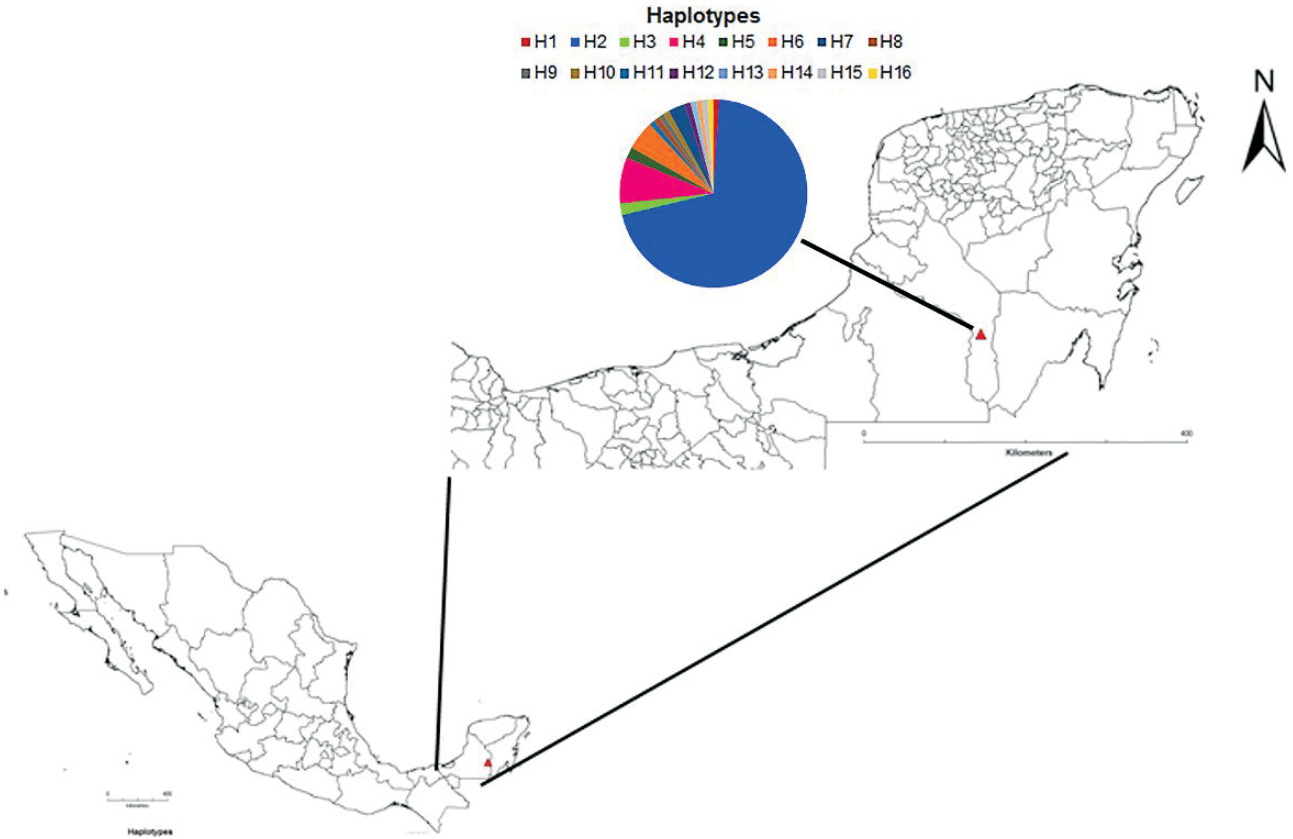

Fig. 1. Red arrowhead shows capture site of *Bichromomyia olmeca olmeca* sand flies in the border area of Campeche and Quintana Roo states in southwestern Mexico. Haplotype code and color are shown.

estimated to test for neutrality, considering segregating sites using DnaSP v.5.10 software (Rozas and Librado 2009). Significant negative values were expected in populations that had experienced an increase in effective population size (Tajima 1989, Fu 1997, Ramos-Onsins and Rozas 2002). A mismatch distribution test was computed with ARLEQUIN v. 3.5 (Excoffier and Lischer 2010), Harpending's raggedness index *R* (Harpending 1994) and the sum of squares deviations (Ssd) were calculated to validate population expansion signal using 10,000 replicates. In populations that underwent rapid demographic expansion, the mismatch distribution was expected to have a smooth unimodal curve (Rogers and Harpending 1992). Single haplotypes were used for intra-specific phylogenetic analysis. The best-fit model of evolution was estimated using the Akaike Information Criterion (Akaike 1974) as implemented in JModeltest v.0.1.1 software (Posada 2008). We used MrBayes: Bayesian Inference Phylogeny v.3.2. software (Ronquist et al. 2012), and four Markov Chain Monte Carlo chains were run for 10,000,000 generations (sampled every 1,000 generations) to allow adequate time for convergence (=0.003). The first 25% of sampled trees were considered burn in. The tree was visualized with FigTree V1.4 (http://tree.bio.ed. ac.uk/software/figtree/). *Lutzomyia cruciata* was used as an outgroup for the MrBayes analysis (GenBank accession number KC791221). Additionally, uncorrected pairwise '*p*' distances were calculated to establish intra- and interspecific variation (Sánchez-Montes et al. 2016).

The infection rate was calculated with the proportion of *Leishmania mexicana* infected by *Bichromomyia olmeca olmeca* divided by the total number of flies examined, using PCR. The mean bite rate of *Bichromomyia olmeca olmeca* for the month of July was estimated as the number of female sand flies per person per night. For estimating mean potential infectious bites, a mathematical model from Rabinovich and Feliciangeli (2004) modified by Pech-May et al. (2010) [(proportion of infected *Bichromomyia olmeca olmeca*) (mean human biting rate) (30 days in a month)] was used.

Results

In total, 102 *Bichromomyia olmeca olmeca* specimens were collected during the five-night period. The 3' end of the cytochrome *b* gene amplified approximately 270 nucleotides. The 102 sequences had 249 conserved sites, 21 variable sites, four parsimony informative sites, and 17 singletons and were A-T rich (78.5%). In total, 17 haplotypes were identified; the most frequent haplotype was H2 (n = 71) (Fig. 1). The haplotype diversity (Hd) was 0.5092 ± 0.6, whereas the nucleotide diversity (π = 0.00321 ± 0.0005) and the nucleotide polymorphism index (Θ = 0.01497 ± 0.003) had low values. Neutrality tests Tajima's D (-2.284), Fu and Li´s F (-4.543), and Fu and Li´s D (-4.720) were significant negatives ($p < 0.02$). The mismatch distribution was unimodal, which is consistent with the population expansion model (R = 0.07384, p = 0.8324; Ssd = 0.00088, p = 0.8656) (Fig. 2).

Bayesian phylogeny was constructed using the GTR model, being the most appropriate for the data (-INL = 655.1010, Delta AIC = 0, AIC = 1392.2019). Analysis provided maximum support (1.0 posterior probability, PP) for the major clade (Fig. 3). Intraspecific *p* distances ranged from 0-0.04 between sequences of *Bichromomyia olmeca olmeca.*

Of the 102 *Bichromomyia olmeca olmeca* specimens collected and analyzed, 25 were infected with *Leishmania mexicana*, 24.5% of the total number. The mean

biting rate by *Bichromomyia olmeca olmeca* during July was 5.1 female sand flies per person per night, while 1.44 was the mean number of potentially infectious bites that a person might receive in July.

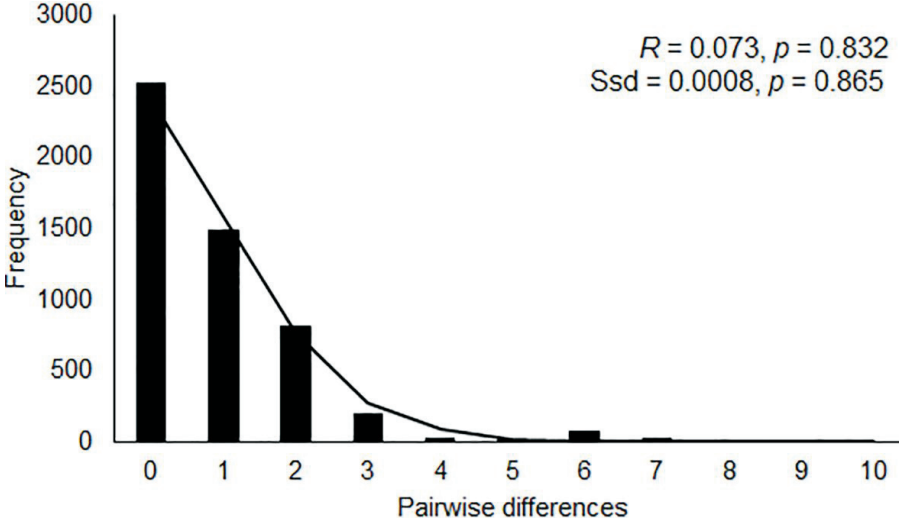

Fig. 2. Mismatch distribution among pairwise differences among haplotypes of the *Bichromomyia olmeca olmeca* population from Ejido 20 de Junio (Mancolona). Bars show the observed distribution and the line the distribution simulated under a sudden expansion model. The *p* value is from the sum of square deviations (Ssd), and Harpending's raggedness index *R* is the goodness-of-fit test for the sudden expansion model.

Discussion

Genetic variability of the *Bichromomyia olmeca olmeca* vector and prevalence of *Leishmania mexicana* infections were analyzed in an endemic focus where patients with leishmaniasis were reported throughout the year (Hernández-Rivera et al. 2015). The haplotype diversity was moderate, but the nucleotide diversity and nucleotide polymorphism index were low compared with another study using the same genetic marker with *Lutzomyia cruciata* in Mexico (Pech-May et al. 2013). The low nucleotide diversity estimate for *Bichromomyia olmeca olmeca* was consistent with a panmictic population (Mirabello and Conn 2006). The low genetic diversity might be caused by small effective dispersal rates and/or genetic drift and inbreeding because of fragmentation or change of landscape. The dispersion pattern for the small hematophagous sand flies depends on several factors including average flight distance, wind speed, and distance to resources. Dispersion capabilities vary according to sand fly species [for New World *Lutzomyia longipalpis* (Lutz and Neiva), the average flight distance varied between 0.5 km and

a minimum of 50 m] (Morrison et al. 1993, Casanova et al. 2005) and heterogeneity of the landscape, which influences dispersion of females looking for suitable blood sources and selecting sites for resting, mating, and laying eggs. Female sand flies seek shelter to avoid direct exposure to sunlight and desiccation (Montes de Oca-Aguilar et al. 2013).

Pérez-Mendez and Rebollar-Téllez (2012) analyzed morphometric variability of several *Bichromomyia olmeca olmeca* populations in the Yucatan Peninsula and concluded such variability was because of geographical distance among localities. Because our study was focalized in a specific area, the changes of landscape in "Ejido 20 de Junio" throughout time played an important role in low genetic variability of *Bichromomyia olmeca olmeca*.

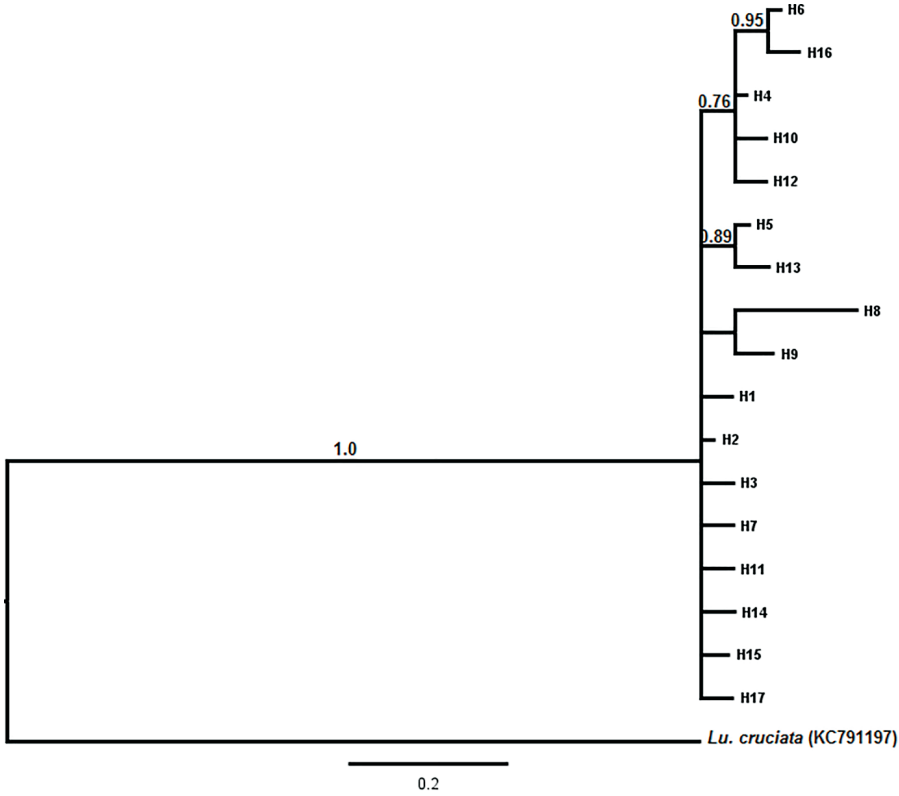

Fig. 3. Bayesian Inference (BI) topology tree for 270 nucleotides of the 3´ end of cytochrome *b* (cyt *b*) of *Bichromomyia olmeca olmeca* from Ejido 20 de Junio (Mancolona) inferred using the GTR model. Numbers on each branch (above branch) represent posterior probabilities obtained in the BI. *Lutzomyia cruciata* was used as the outgroup. The scale bar represents the expected number of nucleotide substitutions per site.

Landscape changes because of deforestation and anthropogenic land use (e.g., agriculture) cause loss of genetic diversity (reflected in high frequency of H2 and few unique haplotypes). *Lutzomyia cruciata* from Nuevo Montecristo and San Antonio Buenavista (Chiapas, Mexico) had low genetic diversity because of uncontrolled deforestation and land use for cattle-raising (Pech-May et al. 2013). According to historical records, long- and short-term impacts led to landscape heterogeneity. Long-term impacts occurred in the ecosystem of "Ejido 20 de Junio" after the Calkamul municipality began to be exploited for natural resources, with intensive deforestation for timber and intensified cultivation of *Capsicum annum* L., at the end of the 20th Century (Keys 2005). From 1954 to 1981, chicle exploitation and arrival and the establishment of different indigenous ethnic groups provoked a continuous change of the landscape because every ethnic group introduced different strategies to exploit the land. Short-term changes include recent activities such as programs for recovery of deforested areas and preservation of sylvatic areas after crop rotation. Cultivation of crops such as pepper (*Piper nigrum* L.), corn (*Zea mays* L.), and pineapple (*Ananas comosus* (L.) Merr.), and introduction of honey bees (*Apis mellifera* L.) led to fluctuations in the ecological niche and blood sources for *Bichromomyia olmeca olmeca* sand flies, affecting interactions between populations (Vester et al. 2007).

At Calakmul, Campeche, several villages especially in the northern part of Calakmul where the village "Ejido 20 de Junio" is located, showed prevalence of cutaneous leishmaniasis (Hernández-Rivera et al. 2015). In the area, four species of wild rodents are reservoirs for *Leishmania mexicana*: black-eared rice rat (*Oryzomys melanotis* Thomas), hispid cotton rat (*Sigmodon hispidus* Say and Ord), big-eared climbing rat (*Ototylomys phyllotis* Merriam), and Yucatan deer mouse (*Peromyscus yucatanicus* J. A. Allen and Chapman) (Canto-Lara et al. 1999). Studies on *Leishmania* vectors at the Ejido 20 de Junio (Mancolona) showed several Phlebtotominae species positive for *Leishmania mexicana* (Pech-May et al. 2010), indicating the *Leishmania* life cycle in the sylvatic area of Calakmul depends on the interaction of several vector sand fly species and different species of hosts. The distribution area of *Bichromomyia olmeca olmeca* at Campeche comprised 43.3% of the state, mainly rural areas. The number of people exposed to *Bichromomyia olmeca olmeca* in rural areas was 107,343 of a total of 328,009 persons, which contrasts with the lack of exposure of humans in urban areas (Pech-May et al. 2016). The sand fly species is one of the most important vectors in the area, based on its abundance and anthropophilic biting habit (Rebollar-Téllez et al. 1996, Pech-May et al. 2010). Abundance of *Bichromomyia olmeca olmeca* in July was greater than reported by Pech-May et al. (2010) that was done over a 4-month span. Yet, biting rate and inoculation rate (number of infectious bites to which a person living in the area would be exposed during the month) were similar to the reported (Pech-May et al. 2010, 2016).

Our data provide novel information on the high infection rate of *Bichromomyia olmeca olmeca* by *Leishmania mexicana* during the end of the dry and hot weather in July, before the rainy season starts in the region. The result enriches the current literature where high sand fly infection rates had been reported only during the rainy season (November) in the region (Pech-May et al. 2010).

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