UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

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ECOLOGÍA

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TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

BRENDA SOLÓRZANO GARCÍA

TUTOR PRINCIPAL: DR. GERARDO PÉREZ PONCE DE LEÓN, INSTITUTO DE BIOLOGÍA, UNAM.

COMITÉ TUTOR: DR. JOSÉ MARTÍN GARCÍA VARELA, INSTITUTO DE BIOLOGÍA, UNAM.
DR. GERARDO SUZAN AZPIRI, FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA, UNAM.

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Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión del Subcomité por Campo de Conocimiento Ecología y Manejo Integral de Ecosistemas del Posgrado en Ciencias Biológicas, celebrada el día 23 de enero de 2017, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **SOLORZANO GARCÍA BRENDA** con número de cuenta **513023675** con la tesis titulada: **"EFECTO DE LA FRAGMENTACIÓN Y AISLAMIENTO DE POBLACIONES DE *ALOUATTA PALLIATA* Y *ATELES GEOFFROYI* EN LA DIVERSIDAD GENÉTICA DEL NEMÁTODO *TRYPANOXYURIS* SP. (OXYUROIDEA)"**, realizada bajo la dirección del **DR. GERARDO PÉREZ PONCE DE LEÓN:**

Presidente:	DR. LUIS ENRIQUE EGUIARTE FRUNS
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Suplente	DR. GERARDO SUZAN AZPIRI

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a 21 de marzo de 2017.

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

c.c.p. Expediente del (la) interesado (a).

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RESUMEN

Los procesos microevolutivos en parásitos están moldeados por su historia de vida y demografía, así como por aspectos relacionados con la biología de sus hospederos y el ambiente en el que se desarrollan. La migración en parásitos está determinada en gran medida por la capacidad de dispersión de sus hospederos. Aquellos fenómenos que interfieran con la movilidad de este último, como por ejemplo la fragmentación del hábitat, se espera reduzcan también el flujo genético entre las poblaciones de sus parásitos. En México existen tres especies de primates, el mono aullador de manto (*Alouatta palliata*), el mono aullador negro (*A. pigra*) y el mono araña (*Ateles geoffroyi*). La fragmentación y pérdida de hábitat han aislado a diversas poblaciones de primates, ocupando remanentes de selva inmersos en una matriz de vegetación distinta a la original. Estos primates son hospederos de nematodos oxiuros pertenecientes al género *Trypanoxyuris*, los cuales presentan ciclo de vida directo, sin fase de vida libre y son altamente específicos, dependiendo de sus únicos hospederos, los primates, para dispersarse. En este trabajo determinan los efectos que la pérdida de hábitat y fragmentación del hábitat y el consecuente aislamiento de las poblaciones de primates pudieran tener en la genética de poblaciones de sus parásitos oxiuros. Entre marzo de 2013 y mayo de 2015 se colectaron 420 excretas de 68 tropas de primates en 52 localidades a lo largo de su rango de distribución en México. Se obtuvieron secuencias de tres marcadores moleculares: un gen mitocondrial (cox1: 293 secuencias), y dos genes nucleares (18S: 10 secuencias 28S: 38 secuencias). Para la determinación taxonómica de las especies de *Trypanoxyuris*. Se realizaron análisis morfológicos y filogenéticos de máxima verosimilitud e inferencia Bayesiana, para cada gen y concatenado. Diversos análisis fueron aplicados para evaluar la genética de poblaciones en *T. minutus* y *T. atelis*, empleando las secuencias de cox1. La historia poblacional y demográfica de ambas especies de oxiuros fue determinada mediante métodos de coalescencia. Se identificaron cinco especies de oxiuros, dos en cada especie de primate: *T. atelis* y *T. atelophora* en mono araña, *T. minutus* y *T. multilabiatus* en mono aullador de manto, y *T. minutus* y *T. pigrae* en mono aullador negro. *Trypanoxyuris multilabiatus* y *T. pigrae* se describen como especies nuevas en este trabajo. La información obtenida a través de la extracción de ADN de los huevos de otros helmintos

presentes en las excretas confirma la presencia de *Strongyloides* sp. y el trematodo *Controrchis biliophilus* como parte de la fauna parasitaria de los primates mexicanos. No se detectó diferenciación ni estructura genética en las poblaciones de *T. minutus* y *T. atelis*, a pesar de la alta fragmentación del hábitat y el aparente aislamiento de las poblaciones de primates. La dispersión pasiva de huevos, los grandes tamaños poblacionales de los parásitos, y un mayor movimiento de primates que lo esperado, podrían estar impidiendo la diferenciación genética de las poblaciones de oxiuros presentes en distintos fragmentos de selva.

ABSTRACT

Microevolution in parasites is molded by their life history and demography, as well as by the biology of their hosts, and environmental features. Migration is highly determined by the dispersal capability of their hosts. Those phenomena that interfere with host movement, such as habitat fragmentation, are expected to equally wane parasite gene flow. Three species of primates occur in México, the mantled howler monkey (*Alouatta palliata*), the black howler monkey (*A. pigra*), and the spider monkey (*Ateles geoffroyi*). Habitat loss and fragmentation have caused the isolation of many of these primate populations inhabiting forest remnants within a matrix of vegetation unlike the original. Mexican primates are hosts, among other parasites, of pinworms nematodes belonging to the genus *Trypanoxyuris*, which have a direct life cycle with no free-living stages, and are highly host-specific, depending on their only hosts, the primates, for dispersal. The aim of this study was to determine the effects that habitat loss and fragmentation, and the consequent isolation of host populations could have on the population genetics of their pinworms. A total of 420 fecal samples from 68 monkey troops were collected between March 2013 and May 2015 in 52 localities across primate distribution range in Mexico. Sequences of mitochondrial (cox1) and nuclear (18S and 28S) markers were obtained. Morphological revisions along with maximum likelihood and Bayesian phylogenetic analyses were used to identify the different *Trypanoxyuris* species. The cox1 sequences were employed to assess the population genetics of *T. minutus* and *T. atelis*. Coalescent methods were used to investigate the population history and demography of both pinworm species. Five pinworm species were identified, two in each species of primate: *T. atelis* and *T. atelophora* in spider monkey, *T. minutus* and *T. multilabiatus* in the mantled howler monkey, and *T. minutus* and *T. pigrae* in the black howler monkey. *Trypanoxyuris multilabiatus* and *T. pigrae* are described as new species. The information obtained through the DNA extraction from the eggs of other helminths found in the feces confirms *Strongyloides* sp. and the trematode *Controrchis biliophilus* as part of the parasitic fauna from Mexican primates. No genetic differentiation and population structure were detected neither in *T. minutus*, nor *T. atelis* despite habitat fragmentation and host isolation. Passive egg dispersion, the large

populations in the parasite, and a higher mobility of primates than expected, may be impeding the genetic differentiation of pinworm populations inhabiting forest fragments.

INTRODUCCIÓN GENERAL

INTRODUCCION GENERAL

Biología evolutiva del parasitismo

La biología evolutiva puede ser estudiada a dos niveles, el micro y macroevolutivo (Nadler 1995; Huyse et al. 2005). La diferencia entre estos niveles radica en la magnitud de las modificaciones genéticas; la macroevolución contempla el origen de adaptaciones complejas y los cambios ocurridos por arriba del nivel de especie, mientras que la microevolución comprende pequeños cambios evolutivos ocurridos en las poblaciones de una especie, siendo un proceso continuo y gradual (Reznick y Ricklefs 2009).

La microevolución en parásitos está mediada tanto por aspectos propios del parásito, como son la demografía, el ciclo de vida, el modo de transmisión y la especificidad hospedatoria, como por aspectos extrínsecos relacionados con la abundancia y movilidad de los hospederos, y por las características ambientales que intervienen en la sobrevivencia de ambos (Nadler 1995; Criscione et al. 2005; Barrett et al. 2008). La mayoría de estos factores influyen sobre el tamaño de las poblaciones de parásitos y su capacidad de dispersión, lo cual se ve reflejado en la diversidad genética y por ende en su capacidad de adaptación (Gandon y Michalakis 2002), y en la diferenciación genética poblacional, y por ello, en su potencial de especiación (Huyse et al., 2005).

Las poblaciones de parásitos, a diferencia de los organismos de vida libre, son fragmentadas, y dicha fragmentación se define en tres escalas principales (Combes, 2001). La más conspicua es la fragmentación a nivel hospedero individual; cada infrapoblación se encuentra habitando hospederos (“fragmentos”) distintos. Le siguen la fragmentación por especie de hospedero; y finalmente, la fragmentación espacial, la cual es similar a la fragmentación de las poblaciones en organismos de vida libre, en donde la discontinuidad en las poblaciones se da principalmente por la heterogeneidad ambiental. La cantidad de flujo genético entre poblaciones de una especie en los distintos niveles de fragmentación determinará si los procesos evolutivos suceden en conjunto o si se van formando subpoblaciones que evolucionan de manera independiente unas de otras (Nadler 1995; Huyse et al. 2005).

La intensidad de flujo genético en parásitos está íntimamente relacionada con su capacidad de sobrevivencia fuera del hospedero, con el número de hospederos y vectores

potenciales y con la movilidad de dichos hospederos y vectores. Aquellas especies de parásitos que logren permanecer viables en el ambiente por largos periodos de tiempo en cualquiera de los estadios de su ciclo de vida, con capacidad de infectar a varias especies de hospederos y con hospederos vágiles, tendrán mayores oportunidades de flujo genético que aquellos parásitos con periodos de sobrevivencia muy cortos fuera del hospedero, con una alta especificidad hospedatoria, y cuyos hospederos tiendan a ser sedentarios (Nadler 1995; Barrett et al. 2008; Blasco-Costa y Poulin 2013). No obstante, la movilidad del hospedero depende no sólo de su capacidad para dispersarse, sino también de propiedades del paisaje como la cantidad de hábitat y su distribución, conectividad y la configuración de la matriz (Tischendorf et al., 2003).

Efectos de la pérdida y fragmentación de hábitat en la estructura genética de las especies

La fragmentación del hábitat es un proceso mediante el cual un hábitat continuo es reducido y fraccionado, y cuyos remanentes se encuentran contenidos en una matriz de hábitats distintos al original (Fahrig, 2003). En conjunto, pérdida y fragmentación del hábitat son considerados entre las principales amenazas para la biodiversidad (Fischer y Lindenmayer 2007); aunque sus efectos sobre la supervivencia de un organismo, parásito o de vida libre, serán específicos para cada especie y dependerán de los requerimientos ecológicos y la biología de las mismas (Betts et al., 2014).

En México los procesos de fragmentación y pérdida masiva del hábitat comenzaron alrededor de 1940-1960, debido principalmente al desarrollo de políticas públicas que incentivaban la deforestación con propósitos agropecuarios y de colonización de los trópicos para la expansión de asentamientos humanos (Gonzalez-Montagut, 1999; Merino-Perez y Segura-Warnholtz, 2007). Dichos procesos de deforestación han continuado hasta nuestros días, aunque en menor intensidad en las últimas décadas (Mas et al., 2004; Masera et al., 1997), eliminando gran parte de la cobertura forestal del país. Se estima que las selvas mexicanas se han reducido hasta ocupar solamente el 10% de su área original (Masera et al., 1997). Por ejemplo, en la región de Los Tuxtlas, para 1986 más del 80% de las selvas originales habían desaparecido (Dirzo y Garcia, 1992). Para el año 2000, el paisaje de la zona norte de Los Tuxtlas estaba constituído por más de 1000 fragmentos de

selva remanentes separados unos de otros por más de 500m (Mendoza et al., 2005). Asimismo, de 1974 a 1991 se perdió cerca del 24% de la cobertura forestal de la selva Lacandona (Mendoza y Dirzo, 1999).

Procesos sinérgicos de pérdida y fragmentación del hábitat llevan a la disminución del hábitat disponible, a la reducción del tamaño de los fragmentos remanentes y al aumento en la distancia que separa dichos fragmentos (Bascompte y Solé, 1996; Fahrig, 2003). Esta modificación en la configuración del paisaje tiene importantes repercusiones, no solo en el tamaño de las poblaciones de flora y fauna silvestre, sino también en la movilidad de los organismos, parásitos o de vida libre, reduciendo el número de rutas de desplazamiento y aumentando los costos de dichos desplazamientos (Bender et al., 2003; Tischendorf et al., 2003). Las implicaciones genéticas en poblaciones de organismos en hábitats fragmentados y aislados incluyen una mayor deriva génica debido a un tamaño efectivo poblacional más pequeño, y una reducción en el flujo genético debido a un menor movimiento entre fragmentos (Keyghobadi, 2007; DiBattista, 2008).

En las últimas décadas, los avances tecnológicos han permitido evaluar las consecuencias genéticas de la pérdida y fragmentación del hábitat en distintos tipos de organismos. Las evidencias empíricas obtenidas a partir de estos estudios son contradictorias, mostrando que no siempre los procesos de pérdida y fragmentación del hábitat llevan a una erosión genética de las poblaciones (Aguilar et al., 2008). Los efectos inmediatos de la fragmentación dependerán del tamaño efectivo poblacional en cada fragmento y de la variabilidad genética de la población original previo a los sucesos de pérdida y fragmentación del hábitat (Aguilar et al., 2008).

En plantas se ha observado que los efectos de la fragmentación suceden más a nivel de diversidad genética que de diferenciación poblacional. Aquellas poblaciones que se encuentran en fragmentos tienden a ser genéticamente menos diversas que las poblaciones de hábitats continuos (Cuartas-Hernández y Núñez-Farfán, 2006; Aguilar et al., 2008; Chávez-Pesqueira et al., 2014). También se ha observado que el flujo genético dado por la dispersión del polen es menos afectado por la fragmentación del hábitat que el flujo genético dado por dispersión de semillas (Hamilton, 1999).

En animales, la mayoría de los estudios muestran un efecto negativo de la pérdida y fragmentación del hábitat en su estructura genética poblacional. En general, las poblaciones

en fragmentos presentan una pérdida de heterocigosis, una mayor endogamia y una mayor diferenciación genética que las poblaciones en hábitats continuos (Keyghobadi, 2007; Oliveiras de Ita et al., 2012). Aquellas especies más generalistas serán menos susceptibles a estos efectos genéticos que organismos más especialistas (Keyghobadi, 2007). Dado que el parasitismo es una interacción ecológica obligatoria para el parásito, los efectos de la fragmentación y pérdida del hábitat en la genética de poblaciones y evolución de una especie de parásito, dependerán no sólo de historia de vida del mismo parásito, sino también de la manera en que el hospedero definitivo, hospederos intermediarios y vectores sean afectados.

Biología de parásitos oxiuros del género *Trypanoxyuris*

Los oxiuros son nematodos parásitos que se caracterizan por tener un ciclo de vida directo, sin estadios de vida libre. Presentan un sistema sexual haplo-diplode, en donde los machos son haploides derivados de huevos sin fecundar, y las hembras son diploides derivadas de huevos fecundados (Adamson, 1989). Estos parásitos se transmiten a través de la ingestión de huevos, los cuales son expulsados al ambiente junto con las heces del hospedero. La autoinfección y retroinfección son comunes en estos nematodos (Adamson 1989; Felt y White 2005). Su ciclo de vida cuenta con dos mudas dentro del huevo, sin fase extra intestinal dentro del hospedero (Adamson, 1989).

Los huevos de oxiuros son muy sensibles a la escasez de humedad y sobreviven sólo unos pocos días fuera del hospedero (Adamson, 1989; Nadler, 1995), lo cual limita su capacidad de dispersión. Una vez ingeridos, los huevos eclosionan en el intestino delgado en donde las larvas continúan su desarrollo. Los adultos se establecen en el intestino grueso y las hembras grávidas migran a la zona perianal para depositar nuevos huevos y así concluir el ciclo (Adamson, 1989).

Los oxiuros de primates tienen una alta especificidad hospedatoria y presentan interesantes patrones coevolutivos con sus hospederos, llegándose a encontrar una especie de parásito por cada especie de hospedero (Hugot 1999). *Trypanoxyuris* es un género de parásitos perteneciente a la familia Oxyuridae, el cual se encuentra únicamente infectando primates Neotropicales (Platyrrhini) (Hugot et al. 1996).El género *Trypanoxyuris* fue descrito por Vevers (1923) a partir de individuos obtenidos de un mono saki (*Pithecia*

monachus) de la República Cooperativa de Guyana. Junto con el género *Enterobius*, encontrado en primates del viejo mundo (Catarrhini), y *Lemuricola* encontrado en lémures (Strepsirrhini), conforman los parásitos oxiuros de primates (Hugot et al. 1996). Previo a este trabajo existían 19 especies reconocidas de *Trypanoxyuris* descritas para las 165 especies de primates Neotropicales.

Primates no humanos en México

En México existen tres especies de primates habitando las selvas tropicales del sureste del país: el mono aullador de manto (*Alouatta palliata),* el mono aullador negro (*A. pigra)* y el mono araña (*Ateles geoffroyi*). El mono aullador de manto se distribuye desde el sureste de Veracruz, pasando por Centroamérica hasta los Andes en Colombia y Ecuador (Fig 1A); mientras que el mono aullador negro se encuentra solamente en la Península de Yucatán, Belice y el noreste de Guatemala (Fig 1A) (Cortés-Ortiz et al., 2015). El mono araña se distribuye desde el sureste de Veracruz, a través de Centroamérica hasta Panamá (Fig 1B) (Rylands et al., 2006). De esta manera, las poblaciones de primates en México constituyen las más norteñas de los primates Neotropicales.

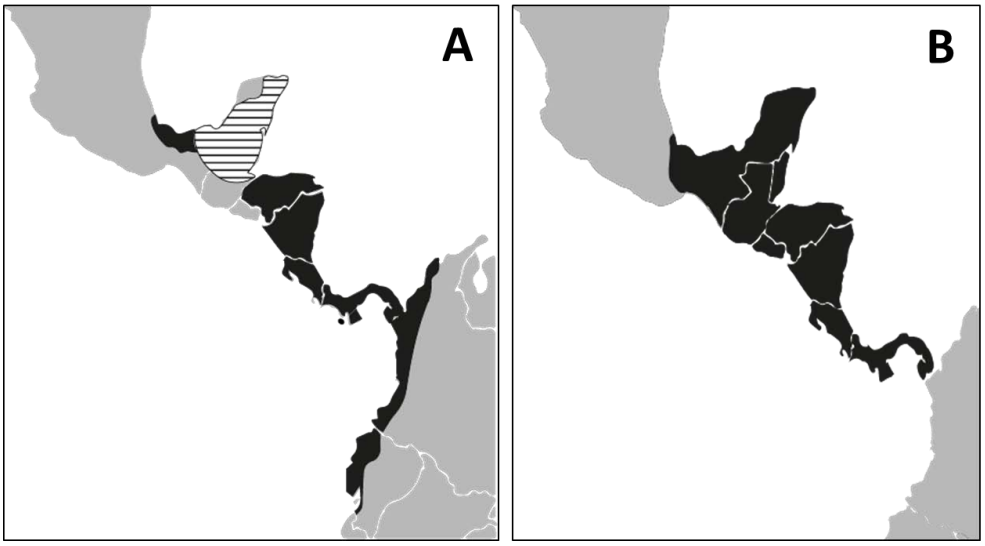

Figura 1. Rango de distribución de las tres especies de primates que se encuentran en México. A) distribución del mono aullador de manto, *Alouatta palliata* (negro), y distribución del mono aullador negro, *A. pigra* (rayas). B) distribución del mono araña, *Ateles geoffroyi* (negro). Modificado de Rylands et al., 2006.

Los monos aulladores se caracterizan por vivir en grupos sociales cohesivos; el tamaño promedio de grupo reportado para *A. palliata* en México va de 3 hasta más de 25 individuos, conformados por varios machos y hembras (Estrada y Coates-Estrada, 1996; Cristobal-Azkarate et al., 2005; Estrada et al., 2006). Aunque en monos aulladores individuos de ambos sexos dejan el grupo natal antes de su primera reproducción, las hembras son las más propensas a dispersarse, alejándose mayores distancias que los machos, en donde algunos pueden hasta permanecer en el grupo natal (Di Fiore y Campbell, 2007). El ámbito hogareño que se ha reportado para los monos aulladores de manto en México es de 10-75 ha dependiendo de la calidad del hábitat disponible (Estrada, 1982; Serio-Silva y Rico-Gray, 2002)

Por el contrario, los monos araña forman sociedades de fisión-fusión, en las que individuos de grupos grandes forman subgrupos más pequeños y cambiantes día a día en composición y tamaño (Aureli y Schaffner, 2008), con la intensión de ser más eficientes en el forrajeo y mitigar la competencia directa con individuos del mismo grupo. En mono araña los machos son filopátricos y solamente las hembras se dispersan del grupo natal (Di Fiore y Campbell, 2007). El ámbito hogareño reportado para mono araña en México es de 95-166 ha (Ramos-Fernández y Ayala-Orozco, 2002).

Estudios sobre la historia biogeográfica y evolutiva estiman que las especies de primates mexicanos divergieron de sus antepasados amazónicos hace ~ 2.2 – 3.7 millones de años (Lynch Alfaro et al., 2015). Se ha inferido que estos primates colonizaron Centroamérica al formase el Istmo de Panamá, el cual conectó Norte y Sudamérica; sin embargo, estudios moleculares recientes han llevado a proponer una nueva ruta de colonización a través de lo que pudo haber sido un continuo de hábitat que conectaba la costa norte de Sudamérica con Centroamérica pasando por las Guayanas (Lynch Alfaro et al., 2015; Morales-Jimenez et al., 2015).

Las tres especies de primates mexicanos están amenazadas (SEMARNAT, 2010) debido principalmente a los efectos conjuntos de la pérdida y la fragmentación de su hábitat, causadas en su mayoría por el cambio de uso de suelo derivado de las actividades humanas (Rodríguez-Luna et al., 2009; Estrada, 2015). De esta manera, lo que antes era un continuo de selvas, ahora constituye un paisaje conformado por remanentes de selva dentro de una matriz de vegetación antropizada constituida en su mayoría por campos agrícolas y

ganaderos (Gonzalez-Montagut 1999; Mas et al. 2004; Duran-Medina et al. 2009). Debido a que los primates mexicanos son principalmente arborícolas, su desplazamiento a través de la matriz para dispersarse entre fragmentos de selva es muy limitado (Mandujano et al. 2004; Mandujano y Estrada 2005), y por ende las poblaciones de primates que habitan estos remanentes de hábitat han quedado en su mayoría aisladas unas de otras, impidiendo el intercambio de individuos entre poblaciones de los distintos fragmentos.

Existen pocos estudios en los que se evalúe la estructura genética de las poblaciones de primates en México y su relación con la fragmentación y pérdida del hábitat; no obstante se ha reportado que poblaciones de mono aullador de manto en el estado de Veracruz presentan una baja diversidad genética y una diferenciación genética poblacional de baja a alta, dependiendo el marcador molecular empleado (Argüello-Sánchez, 2012; Dunn et al., 2014; Alcocer-Rodríguez, 2015; Jasso-del Toro et al., 2016). Asimismo, se ha reportado una diferenciación genética en poblaciones de mono aullador negro en fragmentos, como resultado del aislamiento poblacional (García del Valle et al., 2005). Desafortunadamente no existe un estudio que evalúe la genética de poblaciones de mono araña en México y la información disponible se deriva de análisis filogeográficos en donde se incluyen algunas poblaciones de varias especies de mono araña a lo largo de su distribución. Estos análisis muestran una alta variabilidad genética en poblaciones de *A. geoffroyi*, lo cual coincide con otras especies de monos araña (Ruiz-García et al., 2016). Por el contrario, al comparar la variabilidad genética entre especies de monos aulladores se observa que *A. palliata* es la especie menos diversa (Ruiz-Garcia et al., 2007).

Los primates mexicanos son hospederos de diversos parásitos, encontrando varios tipos de helmintos y protozoarios. La mayoría de los estudios parasitológicos en estos primates se han realizado mediante análisis coproparasitológicos a través de la identificación de los huevos de parásitos presentes en las excretas; no obstante en ocasiones la morfología de los huevos es muy similar impidiendo la distinción de especies. Entre las especies de parásitos más comunes se encuentran *Trypanoxyuris* sp., *Controrchis* sp., y *Strongyloides* sp (Bonilla-Moheno, 2002; Vitazkova y Wade, 2007; Cristobal-Azkarate et al., 2010; Trejo-Macias, 2010; Valdespino et al., 2010). De éstas, los nematodos oxiuros del género *Trypanoxyuris* son casi siempre los más prevalentes ((Bonilla-Moheno, 2002;

Vitazkova y Wade, 2007; Cristobal-Azkarate et al., 2010; Trejo-Macias, 2010; Valdespino et al., 2010).

Objetivos e hipótesis de la tesis

En este trabajo se buscó evaluar si la fragmentación del hábitat y el consecuente aislamiento de las poblaciones de primates han sido suficientes, en tiempo y en espacio, para ocasionar diferencias genéticas entre las poblaciones de sus parásitos oxiuros. Asimismo, se buscó conocer la diversidad de helmintos que parasitan a los primates en México, especialmente de oxiuros dado que hasta ahora sólo *Trypanoxyuris minutus* había sido reportado para ambas especies de monos aulladores (Trejo-Macias et al., 2007), y en el caso del mono araña sólo se había mencionado la presencia de nematodos del género *Trypanoxyuris* sp. (Bonilla-Moheno, 2002). Por tanto, se plantearon las siguientes hipótesis y objetivos:

Hipótesis

1. Dada la alta especificad hospedatoria de los oxiuros y que las especies de primates en México pertenecen a dos géneros distintos (*Alouatta* y *Ateles*), estarán parasitados por distintas especies de *Trypanoxyuris.*

2. Dadas las características biológicas de los oxiuros, como alta especificidad hospedatoria, ciclo de vida directo, y baja sobrevivencia fuera del hospedero, se encontrarán diferencias en la estructura genética entre las distintas poblaciones de parásitos para cada sitio muestreado, como consecuencia de los procesos de pérdida y fragmentación del hábitat que han impedido la migración y dispersión del hospedero.

3. Dado que los ciclos reproductivos del parásito son más cortos que los del hospedero, se encontrarán mayores diferencias genéticas en los oxiuros como resultado de la fragmentación y aislamiento, que las reportadas para las poblaciones de los hospederos.

Objetivos generales

1. Determinar si la fragmentación y aislamiento de poblaciones del hospedero (*Alouatta palliata* y *Ateles geoffroyi)* en el sureste de México tiene algún efecto en la diversidad y estructura genética del nematodo *Trypanoxyuris* sp.

2. Determinar la diversidad de especies de oxiuros que parasitan a las tres especies de primates en México.

Objetivos Particulares

1. Determinar el nivel de variación genética de *Trypanoxyuris* sp. en *Alouatta* y *Ateles* utilizando marcadores nucleares (18S, 28S) y mitocondriales (cox1).

2. Determinar la estructura genética intra e interpoblacional de *Trypanoxyuris* spp. en las dos especies de hospederos (*Alouatta palliata* y *Ateles geoffroyi)*.

3. Conocer si los patrones genético poblacionales de *Trypanoxyuris* spp. resultantes de la fragmentación y aislamiento poblacional, son congruentes con los reportados para sus hospederos. Establecer si estos patrones pueden constituir un indicador de la historia de fragmentación de las selvas y aislamiento de las poblaciones de primates en México.

Para lograr lo anterior se examinó la estructura genética de individuos pertenecientes a diferentes poblaciones de *Trypanoxyuris,* colectando oxiuros de poblaciones de mono aullador y mono araña, aisladas a lo largo de su rango de distribución en México. Se emplearon métodos de colecta no invasivos, utilizando los parásitos adultos y los huevos presentes en las excretas de estos primates. Asimismo, se compararon los resultados encontrados en este trabajo con lo reportado acerca de la estructura y variación genética de las poblaciones de primates mexicanos, con la intención de determinar si los

procesos evolutivos resultantes de la fragmentación y el aislamiento poblacional, son congruentes entre el parásito y el hospedero.

Estructura de la tesis

Los resultados son presentados en cuatro capítulos. En el primero se describen las especies de *Trypanoxyuris* que parasitan a los monos araña en México, utilizando tanto caracteres moleculares como morfológicos para diferenciar las distintas especies.

En el segundo capítulo se presentan las especies de *Trypanoxyuris* que se encuentran parasitando a las dos especies de monos aulladores en México (*Alouatta* spp). Allí se describen dos especies nuevas de *Trypanoxyuris,* cuyas características son detalladas en dos apartados: un artículo extenso y una nota.

El análisis de los efectos de la fragmentación del hábitat en la estructura genética de *Trypanoxyuris* es presentado en el tercer capítulo. En el último y cuarto capítulo se presenta el registro de los helmintos parásitos de las tres especies de primates en México encontrados a partir de combinar los análisis parasitológicos de las muestras colectadas a lo largo de este trabajo, con técnicas moleculares y análisis filogenéticos; además, el listado generado se complementa con una revisión de los helmintos que han sido reportados en la literatura para los primates mexicanos.

Finalmente, se incorpora una discusión general y las conclusiones de este trabajo. Como datos complementarios y a manera de apéndice se presentan los registros del muestreo realizando (Apéndice I) y los datos de porcentaje de infección estimados para cada parásito en cada hospedero y en cada localidad (Apéndice II).

CAPÍTULO 1

CAPÍTULO 1. PARÁSITOS OXYURIDOS DE MONO ARAÑA (*Ateles geoffroyi*) EN MÉXICO

En este capítulo se describen las especies de *Trypanoxyuris* que parasitan a los monos araña de vida libre en México. Se presenta una descripción morfológica de los oxiuros encontrados y se realiza un análisis de Inferencia Bayesiana a partir del gen mitocondrial cox1, para determinar las relaciones filogenéticas de estos *Trypanoxyuris* y otras especies de oxiuros de primates. Este capítulo constituye el primer registro de *T. atelis* y *T. atelophora* en monos araña en México. La información se presenta en un artículo publicado en la revista *Parasitology International* con el título "***Trypanoxyuris atelis* and *T. atelophora* (Nematoda: Oxyuridae) in wild spider monkeys (*Ateles geoffroyi*) in tropical rain forest in Mexico: Morphological and molecular evidence**".

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Trypanoxyuris atelis and *T. atelophora* (Nematoda: Oxyuridae) in wild spider monkeys (*Ateles geoffroyi*) in tropical rain forest in Mexico: Morphological and molecular evidence

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ABSTRACT

Two species of pinworms, *Trypanoxyuris atelis* and *Trypanoxyuris atelophora* were collected from the black-handed spider monkey (*Ateles geoffroyi*) in several localities across southeastern Mexico, representing the first record for both species in Mexican primates. Identification of pinworm species was based on morphological and molecular data. These pinworms are distinguished from other congeners, and from each other, by the buccal structure, the lateral alae, and the morphology of the oesophagus. Phylogenetic analyses based on sequences of the mitochondrial cytochrome *c* oxidase subunit 1 gene placed *T. atelis* as the sister species of *Trypanoxyuris minutus*, a parasite of the howler monkey *Alouatta palliata*, and *T. atelophora* as the sister species of *T. microon*, a parasite of the night monkey, *Aotus azarae*. These relationships were supported with high posterior probability values by Bayesian inference. Comparisons of additional pinworm taxa from Neotropical primates are needed to assess oxyurid diversity, and to better understand the evolutionary relationships among these nematodes and their primate hosts.

1. Introduction

The black-handed spider monkey (*Ateles geoffroyi*) is one of the three primate species that occur in Mexico [1]; its distribution range extends from southern Mexico to Panama [2]. Although numerous studies have been conducted on this primate species in Mexico [3–7] their helminth parasite fauna has barely been studied [8,9], and their oxyurid nematodes have not been identified.

Spider monkeys are susceptible to parasitism with pinworms of the genus *Trypanoxyuris* Vevers, 1923 [10]. The taxonomic history of the species of *Trypanoxyuris* that parasitises *Ateles* spp. is controversial. The first pinworm reported from spider monkeys was *T. atelis*, which was originally described from spider monkeys in South America by Cameron [11] as *Enterobius atelis*, and later changed to *Buckleyenterobius atelis* by Sandosham [12]. Meanwhile, Kreis [13] reported that a pinworm of spider monkeys from Panama was a different species from *E. atelis*, and named it *Oxyuronema atelophora*. However, the identity of this oxyurid was in doubt, as it was considered a synonym of *T. atelis*, and was later included within the same subgenus as *Trypanoxyuris* (*Paraoxyuronema*) *atelis* by Hugot et al. [14]. More recently, Hasegawa et al. [15] examined pinworm specimens from spider monkeys, reporting the presence of two species that could be distinguished by conspicuous morphological differences, proposing the reestablishment of *Buckleyenterobius* and *Oxyuronema* as subgenera; *Trypanoxyuris (Buckleyenterobius) atelis*, and *Trypanoxyuris (Oxyuronema) atelophora*. In any case, in the absence of a phylogenetic framework for *Trypanoxyuris*, hypotheses such as these subgeneric categories remain untested.

Here, we present morphological and molecular evidence from pinworms collected from free-ranging spider monkeys over most of their geographic range in Mexico. These data are used to test the validity of *T. atelis* and *T. atelophora* based on descriptions by Hasegawa et al. [15]. The morphological comparisons are supplemented by a molecular phylogenetic analysis of cytochrome *c* oxidase subunit 1 gene (COI) sequences, which assesses their evolutionary relationships among other members of *Trypanoxyuris*, and provides additional evidence concerning species status.

2. Materials and methods

2.1. Specimen collection

Five geographic areas of southeastern Mexico were sampled, across the states of Veracruz, Tabasco and Campeche (Table 1). In three of the five areas (Fig. 1) more than one forest fragment was sampled (see Table 1 for geographic coordinates in each area). All samples were collected from free-ranging populations, except those in Villahermosa,

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1383-5769/

Table 1
Collecting sites, number of adult nematodes recovered (N), prevalence (positive samples/total samples), and GenBank and Colección Nacional de Helmintos (CNHE) accession numbers for each *Trypanoxyuris* species recovered from spider monkey faeces in Mexico.

Map ID	Geographic area	Locality coordinates		Species	N	Prevalence	GenBank	CNHE
		North	West					
1	Los Tuxtlas, Veracruz	18°22′40″	94°46′10″	*T. atelis*	1	2/22		9375
		18°23′19″	94°45′13″		2		KP266344	
		18°22′40″	94°46′10″	*T. atelophora*	1	2/22	KP266369 KP266368	9381
		18°23′19″	94°45′13″		3			
2	Uxpanapa, Veracruz	17°15′30″	94°38′54″	*T. atelis*	8	7/21	KP266347–48 KP266345–46	9377
		17°24′50″	94°19′51″		3			
		17°15′30″	94°38′54″	*T. atelophora*	7	4/21	KP266374–79	
3	Las Choapas, Veracruz	17°19′26″	93°58′53″	*T. atelis*	13	2/16	KP266353–56 KP266349–51 KP266352	9376 9378
		17°11′58″	94°02′16″		5			
		17°12′21″	94°01′16″		6			
		17°19′26″	93°58′53″	*T. atelophora*	4	3/16	KP266380–82 KP266370–73	9383 9382
		17°12′21″	94°01′16″		9			
4	Villahermosa, Tabasco	17°59′58″	92°56′06″	*T. atelis*	44	2/10	KP266357–61	9380
5	La Libertad, Campeche	18°30′45″	90°29′24″	*T. atelis*	39	10/13	KP266362–67	9379
		18°30′45″	90°29′24″	*T. atelophora*	12	6/13		9384
	Total			*T. atelis*	121	23/82		
				T. atelophora	36	15/82		

Tabasco which come from spider monkeys reared in the Parque Museo La Venta Zoo. Adult pinworms were recovered from faeces of spider monkeys in situ and fixed either in 100% ethanol for DNA extraction, or 4% formalin for morphological analyses. Faecal samples were also collected, keeping them on ice until transported to the laboratory, where they were preserved at −20 °C. In order to establish a correlation between morphological features and DNA sequences, most specimens were cut in half; the anterior portion was used for morphological study, and the remainder was used for DNA extraction.

2.2. Morphological analyses

For morphological examination worms were cleared with alcohol–glycerol solution, and observed using an Olympus BX51 light microscope equipped with differential interference contrast (DIC). Measurements are presented in micrometres (μm) unless otherwise stated, with the range followed by the mean (in parentheses). Specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM) (Table 1). Also, some specimens were preserved and processed for scanning electron microscopy (SEM). Five pinworms were dehydrated through a graded series of ethyl alcohol and then critical point dried with carbon dioxide. The specimens were mounted on metal stubs with silver paste, coated with gold and examined in a Hitachi Stereoscan Model S-2469N at 10 kV.

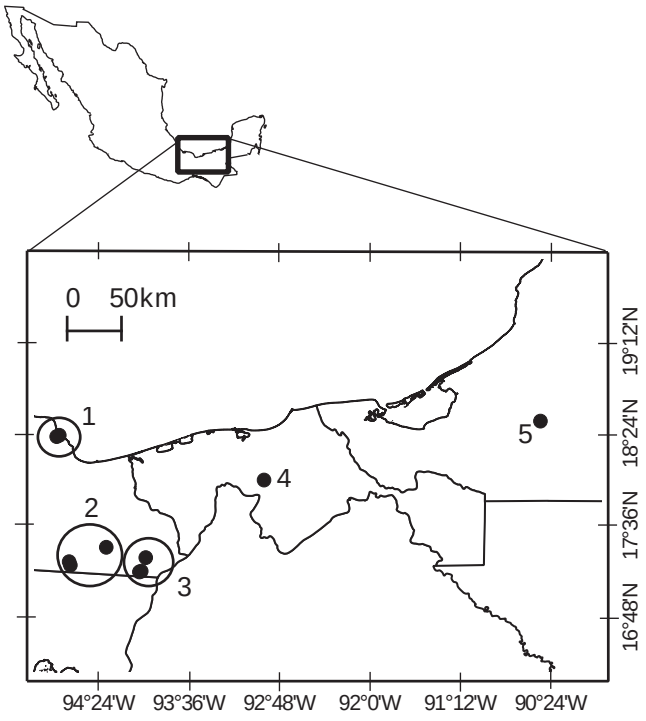

Fig. 1. Collecting sites of *Trypanoxyuris atelis* and *T. atelophora* from free-ranging spider monkeys in Mexico (excepting those from Villahermosa, Tabasco). Numbers refer to map ID in Table 1.

2.3. Amplification and sequencing of DNA

Pinworms fixed in ethanol were digested overnight at 56 °C in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using the DNAzol® reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. A fragment of the mitochondrial COI gene was amplified using the primers TrycoxF, 5′-TGGTTGGCAGGTCTTTATC-3′ (forward) and TryCoxR, 5′-AACCAACTAAAAACCTTAATMC-3′ (reverse) that were designed for this study. The PCR conditions were: initial denaturation at 94 °C for 1 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, and a post-amplification extension for 7 min at 72 °C. PCR products were treated with Exo-SAP (Thermo scientific), according to the manufacturer's instructions. PCR products were sent to the High Throughput Genomics Unit of the University of Washington, US, for sequencing. Contigs were assembled and base-calling differences were resolved using Geneious v.5.1.7 (Biomatters). Sequences were checked for accuracy using the translated amino acid sequences based on the invertebrate mitochondrial genetic code in MESQUITE v.2.75 [16]. Sequences were deposited in GenBank (Table 1).

2.4. Phylogenetic analysis

DNA sequences were aligned using CLUSTAL W and MESQUITE v.2.75, and no gaps were required to align the nucleotide sequences. In addition to the DNA sequences obtained in this study, we used sequences for each of the following species of *Trypanoxyuris* deposited in GenBank: *Trypanoxyuris microon* Linstow, 1907 from night monkeys (*Aotus azarae*) (AB626878–79), 3 sequences of *T. atelis* from a woolly monkey (*Lagothrix lagotricha*), hairy spider monkey (*Ateles belzebuth*), and black-handed spider monkey (*A. geoffroyi*) (AB626875–77), and 2

sequences of *Enterobius vermicularis* Linnaeus, 1785, and 1 sequence of *Enterobius anthropopitheci* Gedoelst, 1916 from chimpanzees (*Pan troglodytes*) (AB626873, 80, 60), all captive in a zoo in Japan; 2 sequences of *E. vermicularis* from humans (AB626865, 68), and 2 sequences of *Enterobius macaci* Yen, 1973 from wild Japanese macaques (*Macaca fuscata*) (AB626858–59). Additional COI sequences are available in GenBank [17], but they do not correspond to the COI region that was used in this study. To complement the analysis, we also generated 3 sequences of *Trypanoxyuris minutus* Schneider, 1866 from free-ranging Mexican howler monkeys (*Alouatta palliata*) (KP266341–43). Phylogenetic analysis was conducted by Bayesian Inference (BI) employing Monte Carlo Markov Chain analysis in the program BEAST v.1.7.5 [18]. The settings were the GTR substitution model, 10 million generations, sampling every 1000 generations, a heating parameter value of 0.3, and a "burn-in" of 10% estimated with Tracer v.1.5 [19]. The Bayesian consensus tree was rooted using the *Syphacia frederici* Roman, 1945 sequence (AB282593) as the outgroup, a decision based on the phylogeny constructed by Hasegawa et al. [20]. Genetic divergence (p-distance) was calculated using MEGA v.6 [21].

3. Results

One-hundred and fifty seven pinworms were recovered from 138 spider monkey's faeces in eight localities corresponding to five geographic areas of southeastern Mexico. Two species of *Trypanoxyuris* were identified, *T. atelis* and *T. atelophora*, and these represent new records for hosts from Mexico. *Trypanoxyuris atelis* had a higher prevalence than *T. atelophora* (Table 1). Both parasites occurred in all localities, although samples from Villahermosa, Tabasco (from a population of captive spider monkeys), were only infected with *T. atelis*. Mixed infections with these two pinworms were common, with 50% of the positive samples containing both parasite species.

3.1. Morphology

The two pinworm species, *T. atelis* and *T. atelophora*, exhibit conspicuous morphological differences that are in agreement with the redescription published by Hasegawa et al. [15]. *T. atelophora* possess two lips, one dorsal lip, and one deeply concave ventral lip, making the oral aperture resemble a reverse triangle (Fig. 2B); the lateral alae show a double crest in females (Fig. 2C), and a single crest in males (Fig. 3B); the oesophagus includes pronounced median and posterior bulbs, separated from each other by an isthmus (Figs. 2D and 3A). On the other hand, *T. atelis* is characterised by having two lips, one dorsal and one ventral, both laterally elongated (Fig. 4B); lateral alae are single crested in both males and females; oesophagus lacking a pronounced median bulb (Fig. 4A). Both species are similar in size (Table 2 for measurements), although the oesophagus is shorter in *T. atelophora*. Unfortunately, no males of *T. atelis* were collected in our samples. Eggs

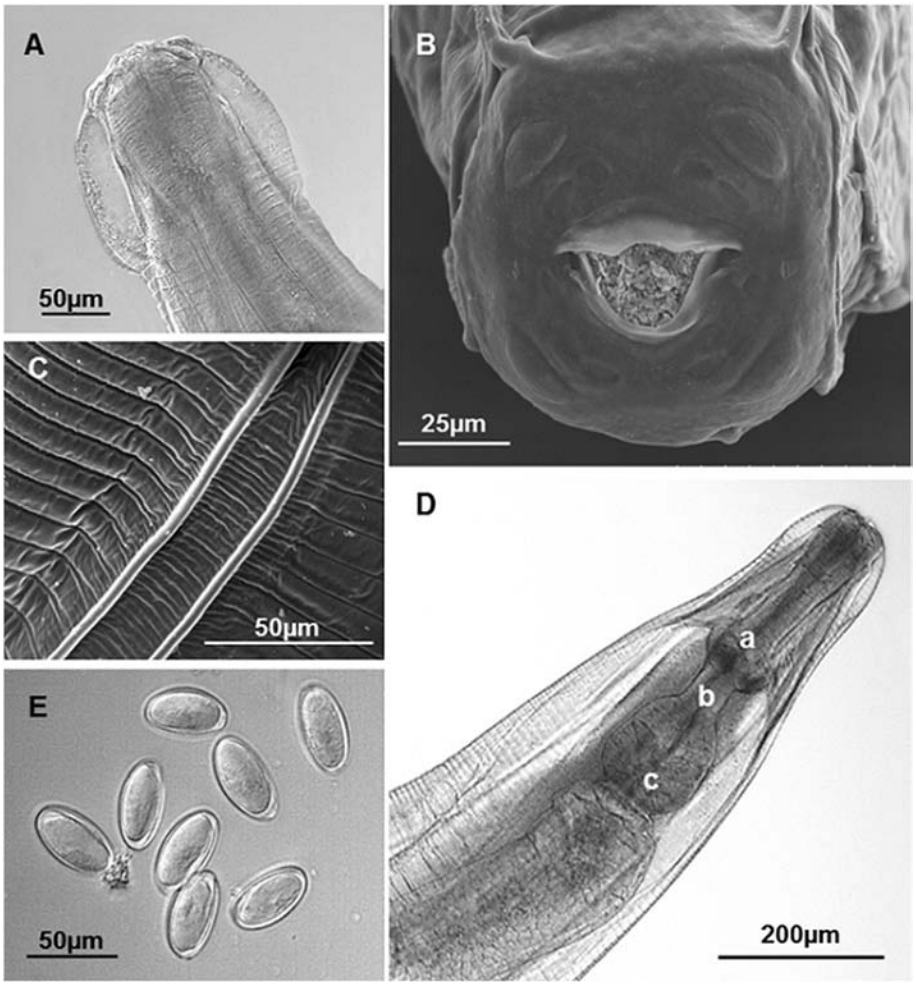

Fig. 2. Female specimens of *Trypanoxyuris atelophora*. (A) anterior region; (B) SEM en face view showing buccal structure, ventral lip concave making the oral aperture resemble a reverse triangle; (C) lateral alae double crested; (D) oesophagus: a = median bulb, b = isthmus, c = posterior bulb; (E) eggs.

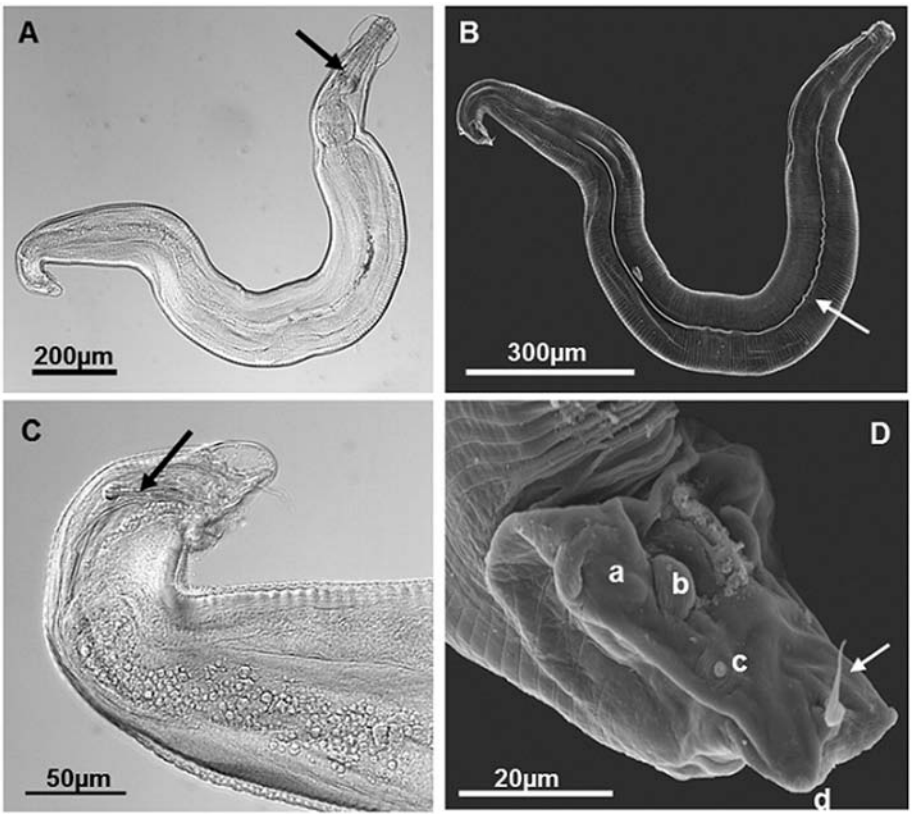

Fig. 3. Male specimens of *Trypanoxyuris atelophora* (A) full body, arrow pointing to median bulb; (B) SEM of full body, arrow pointing to single crested lateral alae; (C) posterior region, arrow pointing to spicule; (D) SEM of posterior region, a,b,c,d = caudal papillae, arrow pointing to the tail spike.

are similar in shape and size, making this character not useful in distinguishing these species, although *T. atelis* eggs possess more conspicuous longitudinal ridges than *T. atelophora*. In general, measurements of the specimens collected in Mexico overlap with those presented by Hasegawa et al. [15].

3.2. Phylogenetic analysis

Forty-two sequences of the COI mitochondrial gene, each with a length of 835 bp, were obtained in this study, including 24 of *T. atelis*, 15 of *T. atelophora*, and 3 of *T. minutus*. The final alignment consisted of 54 sequences, including those obtained from Genbank, and was used to conduct a phylogenetic analysis and to estimate levels of genetic divergence. The alignment was trimmed to 599 bp to avoid missing data, because the additional GenBank COI sequences were shorter. The phylogenetic tree obtained through BI shows that both *Enterobius* and *Trypanoxyuris* are monophyletic groups, with high posterior probabilities (Fig. 5). Within *Trypanoxyuris*, two well-supported clades are recovered, one including *T. microon* as the sister species of *T. atelophora*, and *T. minutus* as the sister species of *T. atelis* (Fig. 5). Genetic divergence ranged from 0 to 9% within species, from 9.2 to 13.2% among species of the same genus, and from 12.5 to 17.5% among genera (Table 3).

The *T. atelis* clade is further subdivided into three subclades, one containing the specimen recovered from a captive woolly monkey (*L. lagotricha*) in Japan by Hasegawa et al. [15], as the sister taxon of those obtained by the same authors from captive spider monkeys (*A. geoffroyi* and *A. belzebuth*) in Japan plus those sequenced in the present study from black-handed spider monkeys (Fig. 5). The genetic divergence within the 24 specimens of *T. atelis* from spider monkeys in Mexico was very low (0 – 1.5%). In contrast the average genetic divergence among the three subclades of *T. atelis* was 8% (7 – 8.9%). The most divergent sequence was that of the woolly monkey (8 – 8.9 %).

4. Discussion

This is the first record of *T. atelis* and *T. atelophora* from spider monkeys in Mexico. The most conspicuous morphological diagnostic traits for these oxyurids are the buccal structures, the lateral alae, and the structure of the oesophagus [15,22]. The morphological data presented here are consistent with the redescription of *T.* (*Buckleyenterobius*) *atelis* and *T.* (*Oxyuronema*) *atelophora* presented by Hasegawa et al. [15]. The molecular phylogenetic analysis strongly supported grouping each morphotype (corresponding to *T. atelophora* or *T. atelis*) into separate clades. Nevertheless, we elected not to use the subgeneric category, since we believe that information is lacking concerning the morphological characters that define them. We trust that further studies on pinworms from Neotropical primates will shed light on the taxonomic significance of the subgenus category.

In general COI haplotypes are highly variable and rapidly evolving among nematodes, readily distinguishing closely related species [23]; however, the sequence divergence among *T. atelis* subclades (8%) is greater than the average divergence within species obtained in this, and previous studies. Blouin [24] reported a maximum COI sequence difference within nematode species of about 6%. Nakano et al. [25] reported a pairwise divergence among COI sequences of *E. vermicularis* ranging from 0.3 to 6.5% (average 3.5%). The divergence level between *T. atelis* from spider monkeys in Mexico, and those from captive spider monkeys in Japan suggest the potential existence of a complex of cryptic species within *Trypanoxyuris* i.e., species that are genetically different

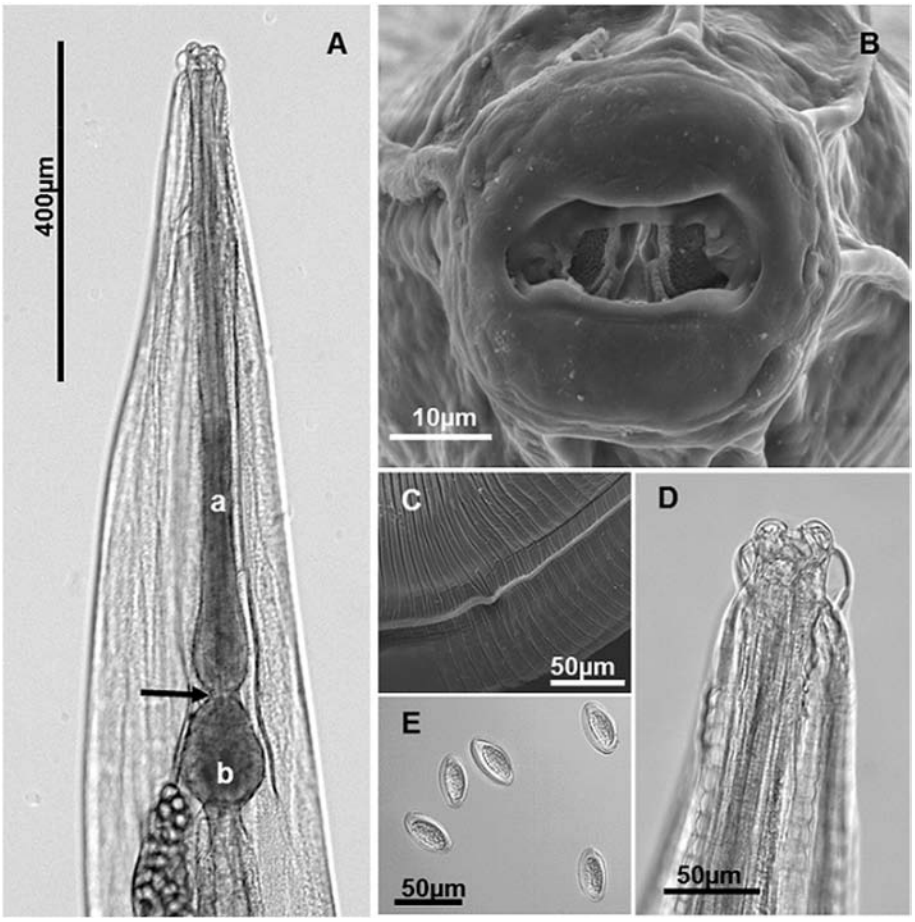

Fig. 4. Females of *Trypanoxyuris atelis* (A) oesophagus: a = corpus, b = posterior bulb, arrow pointing to isthmus; (B) SEM of buccal structure; (C) lateral alae single crested; (D) anterior region; (E) eggs.

but morphologically indistinguishable [26,27]. An alternative explanation could be related to the history of captivity of these spider monkeys and their descendants kept in Japanese zoos. For example, perhaps the zoo-kept spider monkeys have acquired a genetically distinct species of *Trypanoxyuris* during captivity. It seems unlikely that the relatively short history of isolation in zoos has been sufficient to cause the observed level of sequence differentiation between pinworms of captive and free-ranging spider monkeys. More studies of pinworms from different spider monkey species are needed to understand their biodiversity, phylogenetic history and genetic divergence.

Table 2
Measurements of *Trypanoxyuris atelis* and *T. atelophora* recovered from spider monkey faeces in Mexico. Mean values in parentheses, in comparison to those by Hasegawa et al. [15].

	T. atelis		*T. atelophora*			
	♀		♀		♂	
	This study $n = 25$	Ref $n = 11$	This study $n = 17$	Ref $n = 1$	This study $n = 1$	Ref $n = 2$
Length (mm)	3.1 – 6.5 (4.6)	4.3 – 5.3 (4.7)	2.7 – 7.7 (4.8)	3.88	1.66	1.7 – 1.9 (1.8)
Width in midbody	213.7 – 491.4 (325.8)	267 – 351 (323.8)	284.1 – 634 (426.7)	280	135.4	120 – 128 (124)
Nerve ring	164.4 – 273 (203.9)	205 – 176 (188.9)	137.8 – 244.7 (187.2)	163	120.1	132 – 134 (133)
Oesophagus length	776.3 – 946.4 (863.1)	743 – 864 (804)	361.4 – 522.9 (420.7)	422	266.1	317 – 318 (317.5)
Oesophagus corpus length	652.8 – 806.4 (734.6)	605 – 718 (661.9)	80.7 – 161.6 (119.8)	157	82.9	119 – 132 (126)
Oesophagus width at middle	30.5 – 54.6 (40.7)	37 – 45 (41.6)	35.9 – 66.3 (47.6)	48	31.1	32 – 38 (35)
Median bulb length	–	–	47.6 – 93.4 (69.1)	61	48.5	43 – 45 (44)
Median bulb width	–	–	51.4 – 107.2 (85.8)	74	44.2	51 – 54 (52.5)
Isthmus length	–	–	37.6 – 87.2 (66.6)	48	33.5	42 – 45 (43.5)
Isthmus width	–	–	29.5 – 68.2 (43.7)	35	30.8	–
Bulb length	105.3 – 154.7 (131.1)	122 – 128 (125.2)	112.2 – 201.3 (158.9)	138	99.8	94 – 96 (95)
Bulb width	88.8 – 156 (121.3)	112 – 128 (119.8)	83.2 – 154.1 (120.6)	99	81.9	70 –72 (71)
Excretory pore	609.2 – 1372 (974.4)	784 – 1050 (925.6)	696.2 – 1395 (951.4)	582	480	416 – 448 (432)
Vulva (mm)	1.04 – 1.93 (1.58)	1.3 – 1.7 (1.4)	1.14 – 2.71 (1.64)	1.08	–	–
Spicule length	–	–	–	–	37.7	45 – 50 (47.5)
Spike	–	–	–	–	17.4	15–19 (17)
Tail length	420.3 – 1010 (718.3)	950 – 1240 (1090)	540.3 – 1175 (936.4)	740	71	45 – 50 (47.5)
Egg length	32.5 – 43.8 (39.2)	40 – 46 (42.1)	34.1 – 50.5 (45.5)	–	–	–
Egg width	16.3 – 26.8 (20.3)	21 – 24 (22.3)	22.2 – 28.7 (24.8)	–	–	–

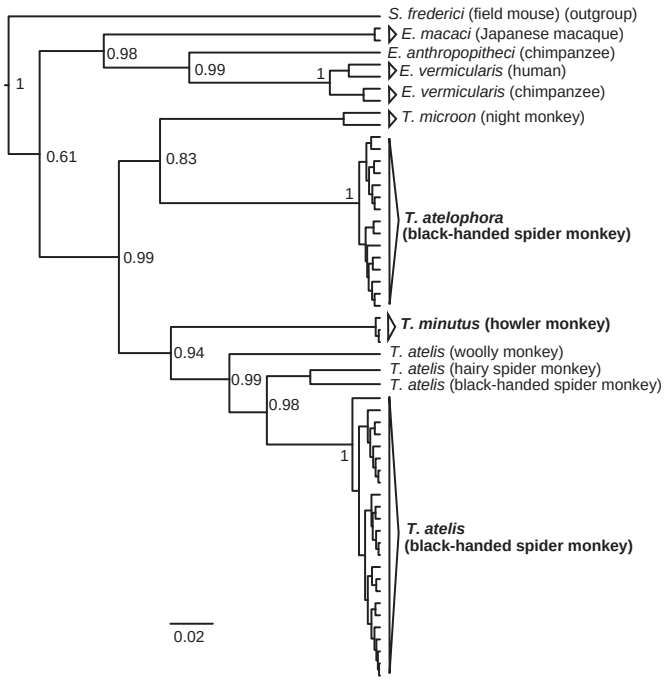

Fig. 5. Phylogenetic tree (Bayesian consensus tree) based on COI gene sequences of *Trypanoxyuris atelis* and *T. atelophora* from spider monkeys in Mexico. The values at the nodes represent the posterior probabilities. Bold type indicates sequences obtained in this study.

The divergence level found between the sequence of *T. atelis* from a captive woolly monkey (*L. lagotricha*) in Japan and those from spider monkeys suggests that these individuals may not be conspecific. It is notable that investigators have reported a different species of *Trypanoxyuris* as a parasite of *L. lagotricha*, i.e., *T. lagothricis* Buckley, 1931 [22,28], and that host-specificity has been argued to be a general feature for oxyurids infecting primates, where a pinworm species is often specific to one genus of primates [10]. For these reasons, more morphological and molecular examinations of pinworms from woolly monkeys are necessary to confirm the taxonomic status and phylogenetic relationships of this species with other members of the genus *Trypanoxyuris*.

The placement of *T. atelis* as the sister species of *T. minutus* is in agreement with previous phylogenetic analyses using morphological data [10]. These two species of pinworms parasitise *A. geoffroyi* and *A. palliata*, respectively, and these primate species both belong to the family Atelidae [2], and are distributed sympatrically over most of their ranges [2]. This pattern of association between parasites and their hosts suggests a codivergence scenario [29]; however this needs to be determined after conducting the proper cophylogenetic analyses including a more exhaustive taxon sampling for species of *Trypanoxyuris* that have not yet been sequenced. In contrast, the phylogenetic analysis showed that *T. atelophora* is the sister species of *T. microon*, which is a pinworm from a night monkey (*Aotus* spp.). Spider monkeys and night monkeys belong to different families (night monkeys to Aotidae); *Aotus* spp. are widely distributed in South America, and at least one species is sympatric with *A. geoffroyi* in Panama [2]. In this case, the host association between *T. atelophora* and *T. microon* suggests, instead, a host-switching event (see Charleston and Robertson [30]) that resulted from sympatry of their primate hosts; however, this also must be tested with a cophylogenetic analysis involving more pinworm species.

In this research, we found that two pinworm species are parasites of a single primate species, and that these nematodes can be found in the same individual host simultaneously. This pattern of co-occurrence is not commonly reported for the oxyurid fauna of primates of new world monkeys, although it has been observed in primates such as *Colobus* spp., *Semnopithecus entellus*, and *Eulemur fulvus* [10]. The results presented here suggest that the diversity of pinworms in Neotropical primates may be higher than expected. More data, both morphological and molecular, are needed, first, to uncover patterns of species diversity, with the potential finding of cryptic species, and second, to better understand the evolutionary history among pinworm species, thereby improving pinworm taxonomy and understanding of cophylogeny.

Future survey work where pinworms are identified based on a combination of molecular and morphological data will render more accurate identification of the specimens. Moreover, studying pinworm diversity through the integration of ecological and evolutionary frameworks will provide context for the distribution patterns of the Oxyuridae among primate orders, as well as increase our understanding of host and parasite dynamics, such as ecological fitting, co-adaptation and diversification, and the impacts of environmental perturbation and transformation [31].

5. Conclusions

This is the first record of *T. atelis* and *T. atelophora* from spider monkeys in Mexico. These two pinworm species exhibit conspicuous morphological differences that are in agreement with the redescription published by Hasegawa et al. [15]. The molecular phylogenetic analysis strongly supported two clades, one including *T. microon* as the sister species of *T. atelophora*, and *T. minutus* as the sister species of *T. atelis*. The high genetic divergence within *T. atelis* suggests that the diversity of pinworms in Neotropical primates may be higher than expected. More studies of pinworms from different spider monkey species are needed to understand their biodiversity, phylogenetic history and genetic divergence.

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Table 3
Genetic divergence within (bold) and among pinworm species using COI gene. P-distances are expressed in percentage. Range values inside ().

	T. minutus	*T. atelis*	*T. atelophora*	*T. microon*	*E. macaci*	*E. vermicularis*
T. minutus	**0.1 (0 – 0.2)**					
T. atelis	9.6 (9.2 – 11.4)	**2.2 (0 – 9)**				
T. atelophora	12.3 (11.7 – 12.9)	11.6 (10.5 – 13.5)	**1 (0.2 – 2.3)**			
T. microon	12.3 (11.9 – 12.9)	11.9 (10.9 – 14)	12.2 (11.4 – 13.2)	**3**		
E. macaci	13.7 (13.5 – 13.9)	13.8 (13.2 – 15)	13.1 (12.5 – 14)	14 (13.5 – 14.5)	**0.3**	
E. vermicularis	14.4 (14 – 14.9)	15.1 (14 – 17.5)	15.2 (14.5 – 16)	15.9 (15.2 – 16.5)	12.9 (12.4 – 13.4)	**3.5 (1.3 – 4.7)**
E. anthropopitheci	15.2 (15.2 – 15.4)	14.8 (14.4 – 15.7)	16.1 (15.5 – 16.9)	15.6 (15 – 16.2)	12.7	11.9 (11.4 – 12.4)

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CAPÍTULO 2

CAPÍTULO 2. PARÁSITOS OXYURIDOS DE MONOS AULLADORES (*Alouatta* spp.) EN MÉXICO

Se presentan las especies de *Trypanoxyuris* que parasitan a los monos aulladores de vida libre en México: el mono aullador de manto (*Alouatta palliata*) y el mono aullador negro (*Alouatta pigra*). La información se presenta en dos apartados; el primero es un artículo publicado en la revista *Parasitology International* titulado "**Pinworm diversity in free-ranging howler monkeys (*Alouatta* spp.) in Mexico: Morphological and molecular evidence for two new *Trypanoxyuris* species (Nematoda: Oxyuridae)**" en donde se describen dos especies nuevas de *Trypanoxyuris* con base en caracteres moleculares y morfológicos; una de estas especies se encuentra en *A. palliata* y la otra en *A. pigra*. Asimismo se analizan las relaciones filogenéticas de este grupo mediante métodos filogenéticos de Máxima Verosimilitud e Inferencia Bayesiana, empleando marcadores mitocondriales y nucleares.

El segundo apartado es una nota aceptada en la revista *The Journal of Parasitology* con el título "**The missing fellow: first description of the *Trypanoxyuris pigrae* male (Nematoda:Oxyuridae), a parasite of the black howler monkey (*Alouatta pigra*) in Mexico**", en la cual se hace únicamente la descripción del macho de *T. pigrae* y se corrobora su identidad empleando información molecular.

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Pinworm diversity in free-ranging howler monkeys (*Alouatta* spp.) in Mexico: Morphological and molecular evidence for two new *Trypanoxyuris* species (Nematoda: Oxyuridae)

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ABSTRACT

Two new species of *Trypanoxyuris* are described from the intestine of free-ranging howler monkeys in Mexico, *Trypanoxyuris multilabiatus* n. sp. from the mantled howler *Alouatta palliata*, and *Trypanoxyuris pigrae* n. sp. from the black howler *Alouatta pigra*. An integrative taxonomic approach is followed, where conspicuous morphological traits and phylogenetic trees based on DNA sequences are used to test the validity of the two new species. The mitochondrial cytochrome oxidase subunit 1 gene, and the nuclear ribosomal 18S and 28S rRNA genes were used for evolutionary analyses, with the concatenated dataset of all three genes used for maximum likelihood and Bayesian phylogenetic analyses. The two new species of pinworms from howler monkeys were morphologically distinct and formed reciprocally monophyletic lineages in molecular phylogenetic trees. The three species from howler monkeys, *T. multilabiatus* n. sp., *T. pigrae* n. sp., and *Trypanoxyuris minutus*, formed a monophyletic group with high bootstrap and posterior probability support values. Phylogenetic patterns inferred from sequence data support the hypothesis of a close evolutionary association between these primate hosts and their pinworm parasites. The results suggest that the diversity of pinworm parasites from Neotropical primates might be underestimated.

1. Introduction

Trypanoxyuris Vevers, 1923 is a genus of oxyurid nematodes that belong to the subfamily Enterobiinae; this genus inhabits the large intestine of Neotropical primates and some rodents [1,2], and has a direct life cycle, with infection occurring by ingestion of eggs via heteroinfection and autoinfection [3,4]. Nineteen species of *Trypanoxyuris* have been described as parasites of primates [5–7]. In Mexico, previous parasitological surveys have reported three species occurring in free-ranging primates: *Trypanoxyuris atelis* Cameron, 1929, and *Trypanoxyuris atelophora* Kreis, 1932, both in spider monkeys (*Ateles geoffroyi*) [8], and *Trypanoxyuris minutus* Schneider, 1866 in howler monkeys (*Alouatta* spp.) [9].

Howler monkeys are the most widespread non-human primates in the Neotropics, with a distribution that ranges from southeastern Mexico to northern Argentina [10]. Two of the nine currently recognized species of howler monkeys occur in Mexico, the mantled howler monkey (*Alouatta palliata*) and the black howler monkey (*Alouatta pigra*). These two species are mainly allopatric, except for a small area in Mexico where they are sympatric [11]. Whereas parasitological studies are relatively common in howler monkeys [4,12–17], little specific attention has been given to their pinworm diversity or more generally to assessments of their helminth diversity using DNA data.

In this study we describe two new species of *Trypanoxyuris* from free-ranging howler monkeys, one in *A. palliata*, and one in *A. pigra*. Morphological and molecular evidence for comparison and description of these species comes from pinworms collected from across howler monkey distribution ranges in Mexico. The morphological comparisons are supplemented by a molecular phylogenetic analysis employing both mitochondrial protein-coding and nuclear ribosomal genes, which provide additional evidence for the distinction between the new species, and permit assessment of their evolutionary relationships among other members of *Trypanoxyuris* for which DNA sequences are available.

2. Materials and methods

2.1. Specimen collection

A total of 22 free-ranging howler monkey troops were surveyed, inhabiting 10 localities across south-eastern Mexico (Fig. 1) in the states

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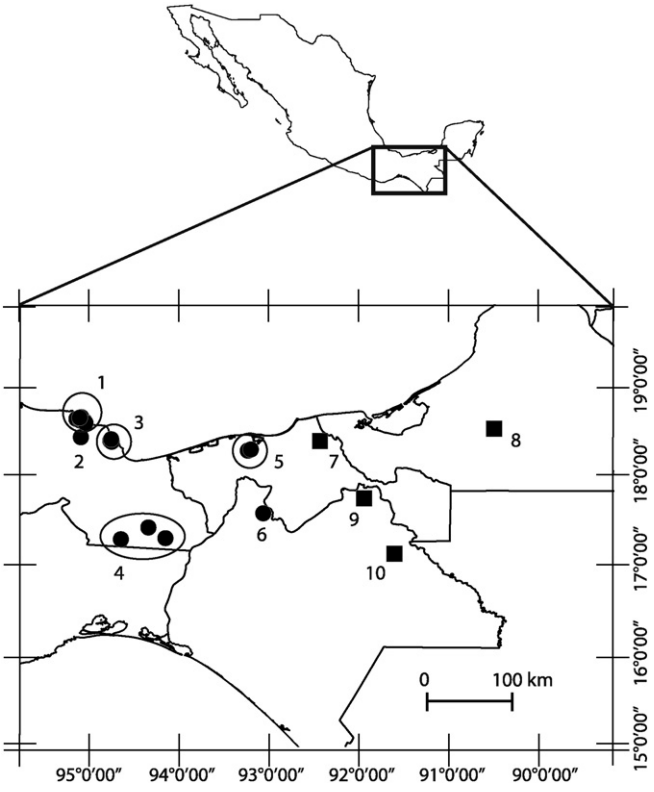

Fig. 1. Collecting sites of *Trypanoxyuris minutus*, *T. multilabiatus* n. sp. and *T. pigrae* n. sp. from free-ranging howler monkeys in Mexico. Circles: samples from *Alouatta palliata*; squares: samples from *A. pigra.* Numbers refer to map ID in Table 1.

of Veracruz, Tabasco, Campeche, and Chiapas (Table 1). Non-invasive sampling techniques were employed, recovering adult pinworms solely from fresh faeces of howler monkeys in situ. Adult pinworms were fixed either in 100% ethanol for DNA extraction, or 4% formalin for morphological analyses. Faecal samples were also collected and immediately placed on ice until transported to the laboratory, where they were preserved at −20 °C. Due to the small size of *Trypanoxyuris* males, recovery of males from the preserved samples was made following the procedure suggested by Hasegawa [18]. In order to establish a linkage between morphological features and DNA sequences of individuals, most specimens were cut in half with the anterior portion used for the morphological study, and the remainder used for DNA extraction.

2.2. Morphological analyses

Worms were cleared with alcohol-glycerol solution, and observed using an Olympus BX51 light microscope equipped with differential interference contrast (DIC). En face observations were made following the technique proposed by Hasegawa et al. [6]. Measurements are presented in micrometres (μm) unless otherwise noted, with the range followed by the mean (in parentheses). Specimens were also preserved and processed for scanning electron microscopy (SEM). Twelve pinworms were dehydrated through a graded series of ethanol and then critical point dried with carbon dioxide. The specimens were mounted on metal stubs with carbon adhesive, and then gold coated and examined in a 15 kV Hitachi Stereoscan Model SU1510 scanning electron microscope. Specimens of the two new *Trypanoxyuris* species were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM) (Table 1).

Table 1
Collecting localities, number of adult nematodes recovered (N), host species (*Alouatta* spp.), and GenBank and Colección Nacional de Helmintos (CNHE) accession numbers for each *Trypanoxyuris* species recovered from howler monkey faeces in Mexico. Map ID corresponds with numbers in Fig. 1.

Map ID	Species	N	Host	Locality	Coordinates		GenBank			CNHE
					North	West	Cox1	18S	28S	
1	*T. minutus*	90	*A. palliata*	Los Tuxtlas, Veracruz	18°35′29.4″	95°03′10.9″	KU285479			
					18°37′04.4″	95°08′30.6″	KU285480		KU285467	
					18°36′20.8″	95°′05′44.4″				
					18°38′15.8″	95°05′13.4″			KU285466	9920
					18°34′23″	95°02′26.8″				
					18°37′51″	95°06′20″			KU285465	
2	*T. minutus*	9	*A. palliata*	Agaltepec Island, Veracruz	18°24′50.7″	95°05′33.5″	KU285484			9921
3	*T. minutus*	51	*A. palliata*	Sierra Santa Marta, Veracruz	18°22′34.6″	94°45′55.8″	KU285481	KU285455		9919
					18°21′49″	94°45′0.9″				
					18°23′28.3″	94°45′20.3″				
4	*T. minutus*	15	*A. palliata*	Uxpanapa, Veracruz	17°16′43.2″	94°38′51.5″				9918
					17°17′27.9″	94°09′7.4″			KU285464	
					17°24′35.7″	94°20′30.2″	KU285483			
5	*T. minutus*	62	*A. palliata*	Comalcalco, Tabasco	18°15′50.5″	93°14′1.6″	KU285482			
					18°16′40.2″	93°12′9.1″				9917
6	*T. minutus*	62	*A. palliata*	Pichucalco, Chiapas	17°34′3.4″	93°03′52.5″	KU285485	KU285457		9916
10	*T. minutus*	1	*A. pigra*	Metzabok, Chiapas	17°07′6.7″	91°36′13.1″			KU285463	
2	*T. multilabiatus* n. sp.	24	*A. palliata*	Agaltepec Island, Veracruz	18°24′50.7″	95°05′33.5″	KU285486		KU285470	9914
							KU285488		KU285472	
3	*T. multilabiatus* n. sp.	5	*A. palliata*	Sierra Santa Marta, Veracruz	18°22′44.8″	94°46′7.6″				9915
4	*T. multilabiatus* n. sp.	6	*A. palliata*	Uxpanapa, Veracruz	17°16′43.2″	94°38′51.5″			KU285471	
					17°24′35.7″	94°20′30.2″	KU285490		KU285473	
5	*T. multilabiatus* n. sp.	2	*A. palliata*	Comalcalco, Tabasco	18°16′40.2″	93°12′9.1″	KU285487	KU285453		
6	*T. multilabiatus* n. sp.	8	*A. palliata*	Pichucalco, Chiapas	17°34′3.4″	93°03′52.5″	KU285489	KU285454		9912
										9913
7	*T. pigrae* n. sp.	13	*A. pigra*	Pantanos de Centla, Tabasco	18°22′13.3″	92°26′0.3″	KU285494			9911
							KU285495			
8	*T. pigrae* n. sp.	2	*A. pigra*	La Libertad, Campeche	18°30′34″	90°29′47.6″				
9	*T. pigrae* n. sp.	15	*A. pigra*	Catazajá, Chiapas	17°43′57.6″	91°56′36.3″	KU285492	KU285458	KU285469	9910
10	*T. pigrae* n. sp.	14	*A. pigra*	Metzabok, Chiapas	17°07′6.7″	91°36′13.1″	KU285491	KU285456	KU285468	9908
							KU285493			9909

2.3. Amplification and sequencing of DNA

Individual pinworms fixed in ethanol were digested overnight at 56 °C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using the DNAzol® reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. A fragment of the mitochondrial cytochrome c oxidase subunit 1 gene (cox1), and regions of the small (18S) and large (28S) subunits of the nuclear ribosomal genes were amplified by PCR, using the primers in Table 2.

Given the regions of the cox1 gene available in GenBank for some *Trypanoxyuris* species, and due to difficulties with amplification of this gene in certain *Trypanoxyuris* species, two sets of primers (TryCox and Pr) amplifying overlapping cox1 regions (nucleotides spanning positions 119–960, and 722–1113 respectively, numbered with reference to the *Enterobius vermicularis* Linnaeus, 1785 complete mitochondrial genome – GenBank EU281143) were used in this study (Table 2). The PCR conditions for cox1 were: initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, 1 min at 54 °C for TryCox primers (1 min at 40 °C for Pr primers), 72 °C for 2 min, and a post-amplification extension for 7 min at 72 °C. For the 18S and 28S the PCR conditions were: initial denaturation at 94 °C for 4 min, followed by 33 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and a post-amplification extension for 7 min at 72 °C. PCR products were treated with Exo-SAP-IT (Thermo Scientific), according to the manufacturer's instructions. Cox1 amplification products were sequenced by the High Throughput Genomics Unit of the University of Washington, U.S.A., whereas nuclear ribosomal PCR products were sequenced at the University of California, Davis, College of Biological Sciences Sequencing Facility. Contigs were assembled and base-calling differences resolved using Geneious v.5.1.7 (Biomatters). As an additional check on accuracy, cox1 nucleotide sequences were translated using MESQUITE v.2.75 [19] and the invertebrate mitochondrial genetic code. All sequences obtained in this study were deposited in GenBank (Table 1).

2.4. Phylogenetic analyses

Cox1 sequences were aligned using CLUSTAL W and MESQUITE v.2.75; no gaps were required for alignment. In addition to the cox1 sequences obtained in this study, we used sequences from GenBank for the following *Trypanoxyuris* species: *T. microon* Linstow, 1907 from night monkeys (*Aotus azarae*) (AB626878-79); *T. atelis* from a woolly monkey (*Lagothrix lagotricha*), hairy spider monkey (*Ateles belzebuth*), and black-handed spider monkey (*Ateles geoffroyi*) captive in Japan (AB626875-77); *T. atelis* (KP266344, KP266347) and *T. atelophora* (KP266370, KP266380) from Mexican spider monkeys (*A. geoffroyi*); *E. vermicularis* and *E. anthropopitheci* Gedoelst, 1916 from chimpanzees (*Pan troglodytes*) (AB626880, AB626860); *E. vermicularis* from humans (AB626865, AB626868); and *Enterobius macaci* Yen, 1973 from wild Japanese macaques (*Macaca fuscata*) (AB626858-59).

Alignments of the 18S and 28S sequences were performed separately using ProAlign v0.5a0 [20]. To add phylogenetic context for these analyses we included the following 18S sequences from GenBank: *T. atelis* (AB626596-97) from spider monkeys; *T. atelis* (AB626595) from a woolly monkey; *E. vermicularis* from humans (JF934731, HQ646164), *E. vermicularis* and *E. anthropopitheci* (AB626601-02) from chimpanzees, and *E. macaci* from a wild Japanese macaque (AB626604). We also generated two sequences of *T. atelis* and two sequences of *T. atelophora* for the 18S, and three sequences of *T. atelis*, two sequences of *T. atelophora*, and one sequence of *E. vermicularis* for the 28S region. Sequences of *Oxyuris equi* Schrank, 1788 were used as outgroups for the three genes (KP404095 for cox1, EF180062 for 18S, and the present study for 28S).

Maximum likelihood (ML) and Bayesian Inference (BI) were performed separately for each gene, and for the concatenated dataset (rDNA + cox1) partitioned by gene. Since sequence data for each gene was not complete for all taxa (e.g., additional 28S sequences for *Trypanoxyuris* sp. were not available in GenBank, and cox1 sequences were shorter in *T. pigrae* n. sp.), missing data ("?") was used in the concatenated data set and in the cox1 data set, in order to expand the number of taxa compared. For the cox1 dataset two individuals had 50.6% missing data. In the concatenated data set, ten of the 20 analyzed terminals had from 13.8% to 43.7% missing data. Overall mean data completeness was 82% of characters. It is established that failing to add taxa because of missing data can lead to reduced phylogenetic accuracy [21], and our analyses assume that the increase in accuracy gained by adding taxa outweighs loss of resolution caused by inclusion of missing data.

MrModeltest v. 2.3 [22] was used to select the best model of evolution for each gene using the Akaike information criterion. The GTR + I + G substitution model was the best model for all three genes. The ML tree was inferred using the program RAxML v.8 [23] as implemented in the CIPRES Science Gateway [24]. ML clade support was assessed using bootstrap resampling with 1000 replications. BI analyses were performed using MrBayes v.3.2.2 [25] and the CIPRES Science Gateway [24]. Bayesian analyses included two simultaneous runs of Markov chain Monte Carlo, each for four million generations, sampling trees every 4000 generations, a heating parameter value of 0.2, and a "burn-in" of 25%. A 50% majority-rule consensus tree was constructed from the post burn-in trees. Genetic divergence (p-distance) was calculated using MEGA v.6 [26]; standard error of the distances was estimated by bootstrap resampling with 100 replications.

Table 2
Primer sequence information. PCR = amplification; SEQ = sequencing. REF = reference.

Locus	Primer name	Sequence (5′-3′)	Tm (°C)	Use	Ref
Cox1	TryCoxF	TGGTTGGCAGGTCTTTATC	56	PCR & SEQ	(1)
	TryCoxR	AACCAACTAAAAACCTTAATMC	52	PCR & SEQ	(1)
	Pr-a	TGGTTTTTTGTGCATCCTGAGGTTTA	67	PCR & SEQ	(2)
	Pr-b	AGAAAGAACGTAATGAAAATGAGCAAC	62	PCR & SEQ	(2)
18s	G18s4	GCTTGTCTCAAAGATTAAGCC	58.6	PCR & SEQ	(3)
	136	TGATCCTTCTGCAGGTTCACCTAC	64.5	PCR & SEQ	(4)
	651	GCGACGGGCGGTGTGTAC	66.7	SEQ	(5)
	135	CGGAGAGGGAGCCTGAGAAACGGC	71.4	SEQ	(5)
28s	391	AGCGGAGGAAAAGAAACTAA	56.3	PCR & SEQ	(4)
	501	TCGGAAGGAACCAGCTACTA	60	PCR & SEQ	(6)
	503	CCTTGGTCCGTGTTTCAAGACG	65	SEQ	(6)
	504	CAAGTACCGTGAGGGAAAGTTG	63	SEQ	(6)

(1) Solórzano et al. [8]; (2) Nakano et al. [28]; (3) Blaxter et al. [41]; (4) Nadler and Hudspeth [42]; (5) Nadler et al. [43]; (6) Smythe and Nadler [44].

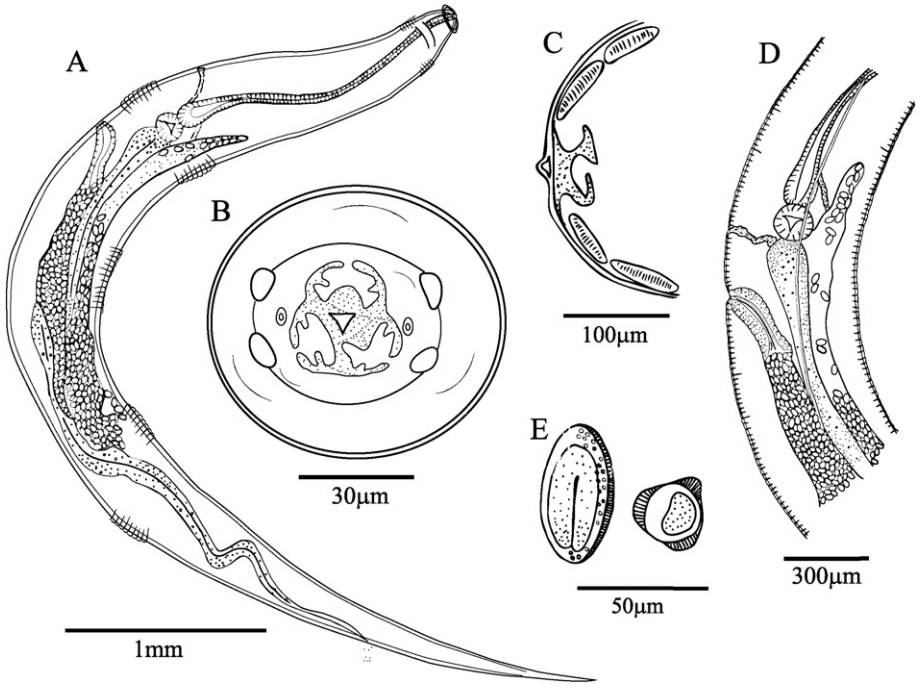

Fig. 2. *Trypanoxyuris pigrae* n. sp. (A) Full body female, lateral view. (B) Cephalic end, apical view. (C) Cross section showing lateral ala. (D) Middle portion of the body. (E) Eggs.

3. Results

3.1. Morphological description

3.1.1. Trypanoxyuris pigrae n. sp. (Figs. 2A–E, 3A, 4G–I)

Description based on 22 specimens, all of them females. No male specimens were found in the howler monkey faeces. Cuticle with transverse striations. Cephalic vesicle present. Cephalic tray quadrangular; buccal aperture triangular, delimited by 3 notched lips, one dorsal and two subventral; dorsal and left ventral lips are bilobulated (Fig. 2B). Labial structures surrounded by a circular furrow (Fig. 3A). Cephalic papillae readily visible, located in ventral and dorsal extremes of the cephalic tray with ventral papillae closest to the amphids (Fig. 3A). Two amphids, one on each side of the cephalic tray. Lateral alae single crested (Fig. 3A), beginning at nerve ring and terminating close to the caudal extremity. Oesophagus long with posterior spherical oesophageal bulb (Fig. 4G). Vulva located in the anterior 3rd of the body; muscular vagina longitudinally oriented, with distal vagina approximately perpendicular to longitudinal body axis (Fig. 2A, D). Tail long conical. Eggs ellipsoidal, symmetric, finely granulated, with 3 longitudinal ridges forming a triangular contour in cross section (Fig. 4H, I). Measurements are given in Table 3.

3.1.1.1. Taxonomic summary. Type-host: *A. pigra* (Lawrence, 1933), black howler monkey.

Site of infection: Not determined (samples were obtained from faeces).

Type-locality: Metzabok, Chiapas state, Mexico (17°07′6.7″ N, 91°36′ 13.1″ W).

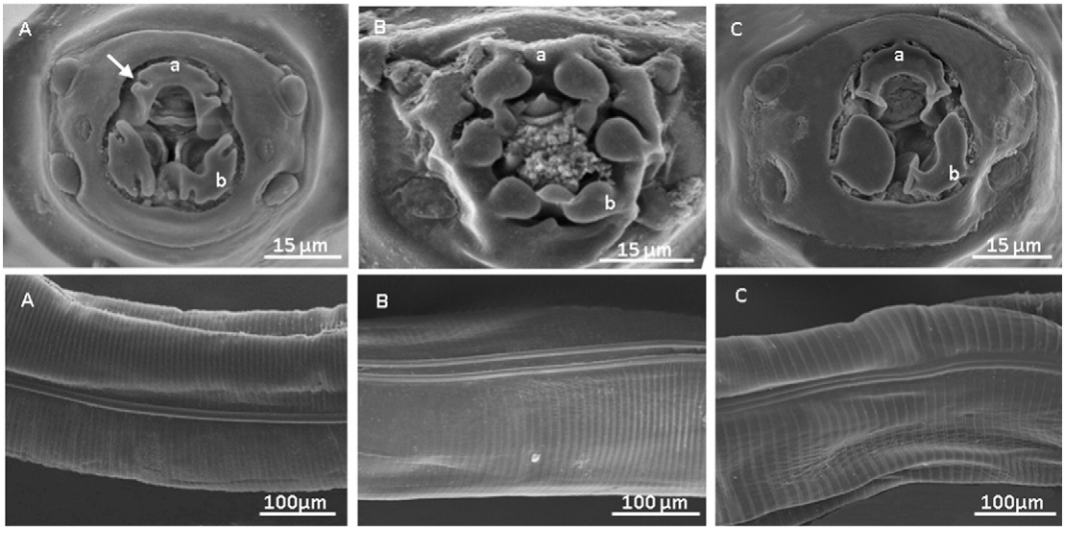

Fig. 3. SEM of females of the three species of pinworms found in howler monkeys. (A) *Trypanoxyuris pigrae* n. sp. (B) *T. multilabiatus* n. sp. (C) *T. minutus.* Buccal structures are shown at the upper row; a) dorsal lobulated lips; b) subventral lobulated lips; white arrow indicates notches found in the lips of *T. pigrae* n. sp. Lateral alae are shown in the lower row.

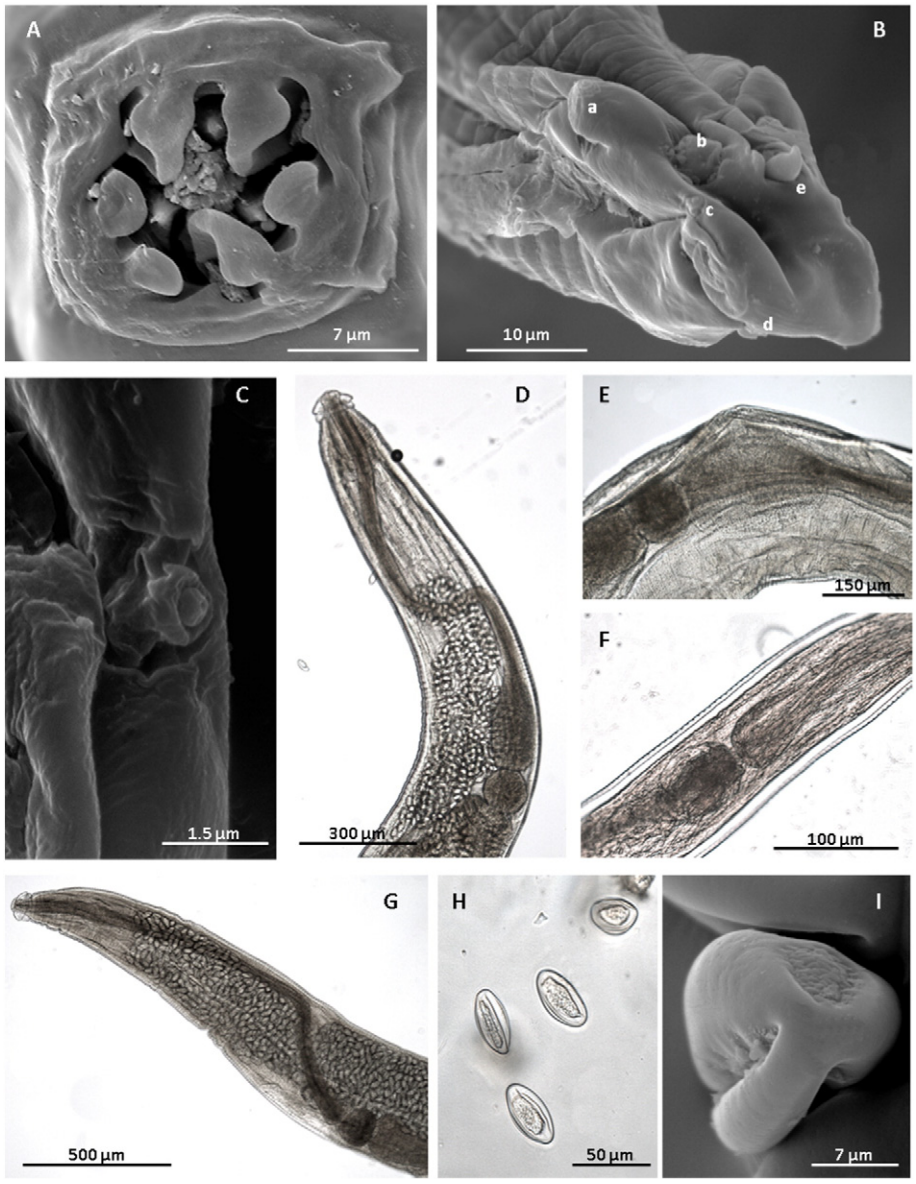

Fig. 4. New *Trypanoxyuris* species from howler monkeys. (A) *T. multilabiatus* male apical view. (B) *T. multilabiatus* male posterior extremity: a, b, c, d = caudal papillae, e = spicule. (C) *T. multilabiatus* male caudal papilla surrounded by ring-shaped thickening. (D) *T. multilabiatus* female anterior portion of the body. (E & F) *T. multilabiatus* oesophagus posterior pear-shaped widening; female and male respectively. (G) *T. pigrae* female anterior portion of the body. (H & I) *T. pigrae* eggs.

Additional localities: Pantanos de Centla, Tabasco, Mexico (18°22′ 13.3″ N, 92°26′0.3″ W); La Libertad, Campeche, Mexico (18°30′34″ N, 90°29′47.6″ W); Catazaja, Chiapas, Mexico (17°43′57.6″ N, 91°56′36.3″ W).

Type-material: Holotype CNHE: No. 9908; paratypes CNHE: No. 9909–9911.

Prevalence of infection: Metzabok 4/7 hosts (57%); Pantanos de Centla 4/7 (57%); La Libertad 2/6 (33%); Catazaja, Chiapas 8/11 (73%).

Etymology: The species name refers to the type-host of the oxyurid, *A. pigra*.

3.1.1.2. Remarks. In the present study we found mixed infections with *T. pigrae* and *T. minutus* occurring in the same individual host. These two pinworm species are morphologically very similar. The buccal structures are alike between these two species, as well as the morphology of the oesophagus, although on average the new species has a shorter oesophagus length than *T. minutus*. The most reliable distinctive characters are the lateral alae, being single crested in *T. pigrae* n. sp. and double crested in *T. minutus*, and the notched lips which are characteristic of *T. pigrae* n. sp. (Fig. 3). Careful enface observations can corroborate species identity. Egg size and shape are not useful features for discriminating *T. pigrae* n. sp. from *T. minutus*.

3.1.2. T. multilabiatus n. sp. (Figs. 5A–F, 3B, 6A–D, 4A–F)

Description based on 41 specimens. Cuticle with transverse striations. Cephalic vesicle present. Cephalic tray quadrangular; buccal aperture roughly round, delimited by 6 lips arranged in 3 pairs, one dorsal and two subventral; both dorsal lips lobulated, in left subventral pair only the most ventral lip lobulated, in right subventral pair lips not lobulated. Cephalic papillae readily visible, located in ventral and dorsal extremes of the cephalic tray, with ventral papillae closest to the amphids. Two amphids, one on each side of the cephalic tray. Lateral alae present in both sexes, extending from nerve ring level to the caudal extremity. Oesophagus with a conspicuous posterior pear-shaped widening,

Table 3
Measurements of adults of *Trypanoxyuris minutus*, *T. multilabiatus* n. sp., and *T. pigrae* n. sp. recovered from howler monkey faeces in Mexico. Measurements are presented in micrometres (μm) unless otherwise noted. Mean values in parentheses.

	T. minutus		*T. multilabiatus* n. sp.		*T. pigrae* n. sp.
	n = 27 ♀	n = 16 ♂	n = 31 ♀	n = 10 ♂	n = 22 ♀
Length (mm)	4.4–7.7 (5.6)	2.1–3.0 (2.6)	4.3–6.1 (4.9)	2.0–2.5 (2.3)	4.4–7.2 (5.8)
Width in midbody	297.3–543.7 (431.7)	117.3–173.3 (142.2)	365–341.8 (368.1)	79.9–141.9 (105.3)	143.9–560.6 (405.7)
Nerve ring	156–243.2 (203.5)	85.1–135.8 (108.3)	161.8–211.5 (185.6)	78.4–117.5 (90.2)	178.7–262.7 (204.6)
Oesophagus length	1618–2051.5 (1836.2)	659.6–834.3 (723.4)	1268.9–1439.2 (1371.6)	527.5–685.3 (614.7)	1212.5–1922.6 (1627.2)
Oesophagus corpus length	1483.8–1911.9 (1708.1)	555.0–735.4 (629.3)	948.3–1069.2 (1004.6)	450.4–591.5 (500.7)	1083.4–1784.6 (1497.5)
Oesophagus width at middle	37–54.5 (44.1)	17.0–34.3 (28.6)	31.6–41.3 (42.3)	22–32.4 (27.5)	31.5–49.3 (43.8)
Oesophagus posterior widening length	–	–	231.2–258.8 (256.7)	59.2–93.8 (74.5)	–
Oesophagus posterior widening width	–	–	125.5–191.3 (169.8)	41.5–67.4 (50.1)	–
Bulb length	113.7–148.2 (129)	70.7–93.6 (84.8)	101.6–113.5 (114.9)	58.9–85.6 (69.2)	109.4–166.5 (131.4)
Bulb width	109.6–155.8 (137.2)	66.3–93.6 (76.3)	102.6–115.9 (124.3)	51.3–70.1 (59.7)	100–162.6 (133.8)
Excretory pore	870.2–2210.1 (1292.4)	689.9–939.7 (814.3)	932.4–1342.1 (1161.6)	636.3–766.9 (714.4)	832.8–1910.9 (1317.2)
Vulva – anterior end (mm)	1.3–2.9 (1.7)	–	1.6–2.2 (1.5)	–	1.2–2.2 (1.7)
Vulva – posterior end (mm)	2.6–6.5 (4.0)	–	2.6–3.9 (3.1)	–	2.5–5.2 (4.1)
Spicule length	–	27.3–79.9 (58.44)	–	42.8–52.5 (47.3)	–
Tail appendage	–	11.6–15.6 (13.4)	–	–	–
Tail length	1294–2444.8 (1517.7)	–	1431.8–1507.9 (1416.4)	–	1268.2–1656.3 (1434.8)
Egg length	39.2–52.9 (47)	–	29.3–45.6 (38.8)	–	37.17–53.6 (45.7)
Egg width	20.8–29.9 (23.7)	–	13.2–25.4 (20.2)	–	16.4–27.3 (23.5)

anterior to oesophageal bulb; widening varies notably in size among individuals and it appears to be formed by muscular tissue and possesses a thin wall (Fig. 4D–F).

Females (n = 31): Dorsal lips with lobes in the most extreme opposite sides; subventral lip with lobe present towards the right end (Fig. 3B). Lateral alae composed of two parallel crests separated from each other by a wide transversely striated channel (Fig. 3B). Excretory pore located anterior to oesophageal bulb. Vulva located in the anterior 3rd of the body; muscular vagina longitudinally oriented, with distal vagina approximately perpendicular to longitudinal body axis (Fig. 5E, F), cellular diaphragm between vagina and uterine opening. Tail long, conical. Eggs ellipsoidal, symmetric, finely granulated, with 3 longitudinal ridges forming a triangular contour in cross section. Measurements are given in Table 3.

Males (n = 10): Lobulated lips less conspicuous than in females; lobes present in the opposite side of the lips comparing to females, with dorsal lips lobulated in the closets opposite sides, and subventral lip lobulated towards the left end (Fig. 4A). Lateral ala single crested. Excretory pore located after oesophageal bulb. Posterior body bent ventrally. Spicule long, slightly wider in the middle (Fig. 6D). Caudal alae present. Four pairs of caudal papillae present, all surrounded by ring-shaped thickenings (Fig. 4C); first pair large, directed laterally; second

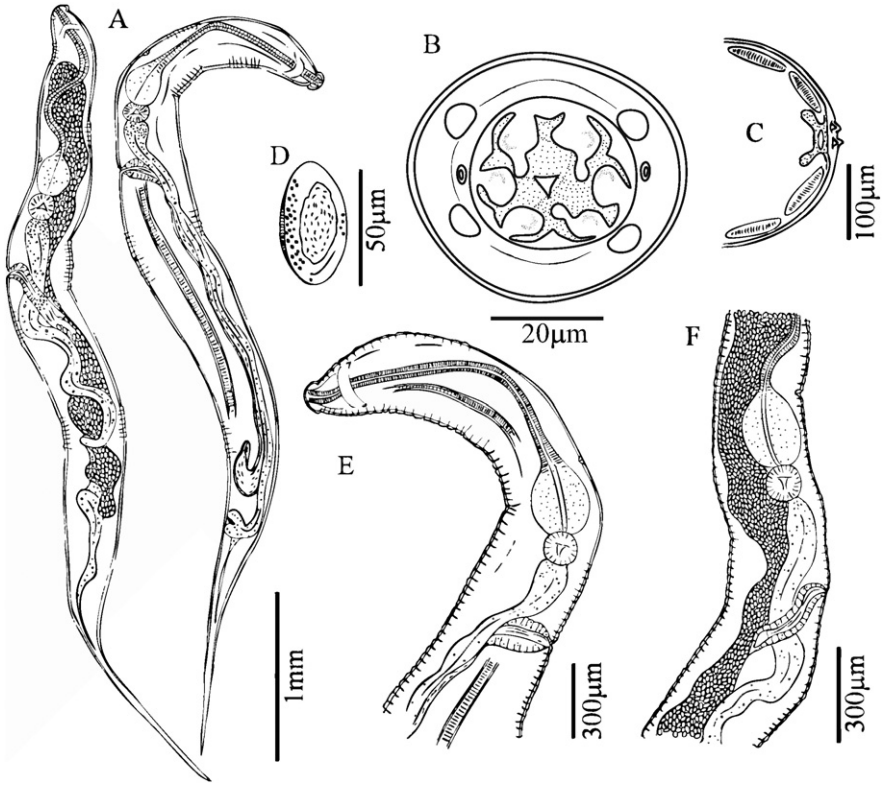

Fig. 5. *Trypanoxyuris multilabiatus* n. sp. (A) Females, full body, lateral view. (B) Cephalic end, apical view. (C) Cross section showing lateral alae. (D) Egg. (E) Anterior portion of the body, showing the shape of the oesophagus. (F) Middle portion of the body.

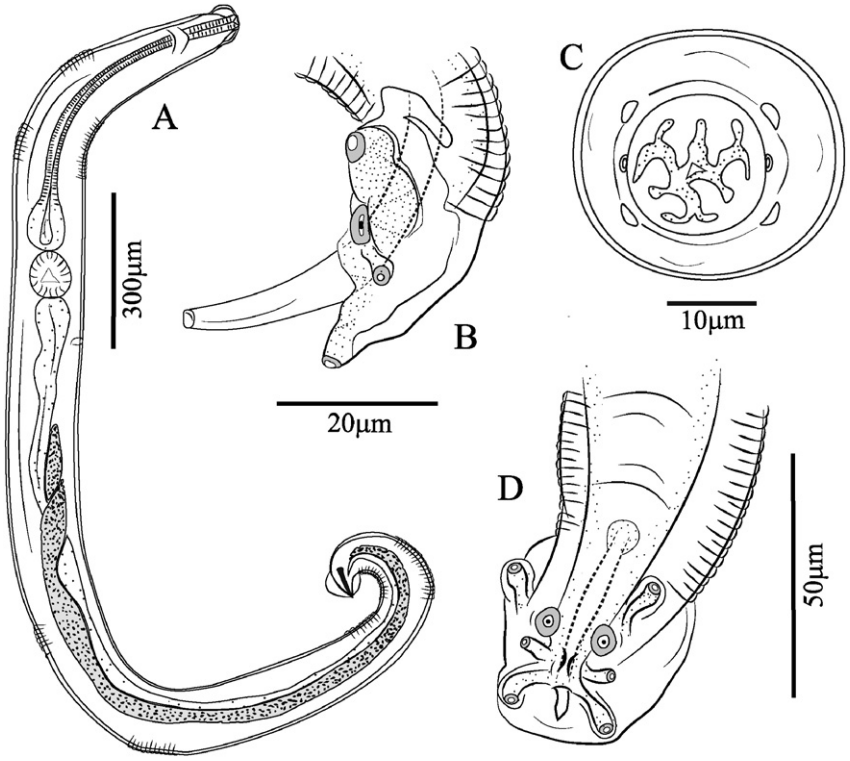

Fig. 6. *Trypanoxyuris multilabiatus* n. sp. (A) Male, full body, lateral view. (B) Posterior extremity, lateral view. (C) Cephalic apical view. (D) Posterior extremity, ventral view.

and third pairs minute, directed ventrally; fourth pair minute, directed posterolaterally (Fig. 6B, D). Tail appendage absent (Fig. 4B). Measurements are given in Table 3.

3.1.2.1. Taxonomic summary. Type-host: *A. palliata* (Gray, 1849), mantled howler monkey.

Site of infection: Not determined (samples were obtained from faeces).

Type-locality: Finca Santa Ana, Pichucalco, Chiapas state, Mexico (17° 34′3.4″ N, 93°03′52.5″ W).

Additional localities: Agaltepec Island, Catemaco, Veracruz, Mexico (18°24′50.7″ N, 95°05′33.5″ W), Sierra Santa Marta, Veracruz, Mexico (18°22′44.8″ N, 94°46′7.6″ W); Uxpanapa, Veracruz, Mexico (17°16′ 43.2″ N, 94°38′51.5″ W, and 17°24′35.7″ N, 94°20′30.2″ W); Comalcalco, Tabasco, Mexico (18°16′40.2″ N, 93°12″9.1″ W).

Type-material: Holotype CNHE: No 9912; allotype CNHE: No 9982; paratypes CNHE: No. 9913–9915.

Prevalence of infection: Agaltepec Island 3/7 hosts (43%); Sierra Santa Marta 1/5 hosts (20%); Uxpanapa 2/12 hosts (17%); Comalcalco 2/13 hosts (15%); Pichucalco 4/20 hosts (20%).

Etymology: The species name derives from the latin noun multi and the adjective labiatus, referring to the large number of lips, *multilabiatus.*

3.1.2.2. Remarks. Another species of pinworm, *T. minutus*, also parasitizes mantled howler monkeys in Mexico. In the present study we found mixed infections, with *T. minutus* and *T. multilabiatus* n. sp. occurring in the same individual host. The two pinworm species exhibit conspicuous morphological differences that make identification straightforward (Fig. 3). The distinctive buccal structures, oesophagus, and lateral alae, makes the new species easily distinguishable from *T. minutus.* Moreover, *T. multilabiatus* possess several features that distinguish it from all other described *Trypanoxyuris* species. For instance, *T. minutus* and *T. pigrae* n. sp. exhibit a circular furrow surrounding the lips; this furrow is not evident in *T. multilabiatus* (Fig 3); however, the arrangement of the lips, and the location of the lobulated lips are similar in the three species (Fig 3). Likewise, the shape of the oesophagus in the new species is unique within the genus *Trypanoxyuris*. The lacking of the tail appendage in males is another distinctive character of this species; the presence of this tail spike in the other species of *Trypanoxyuris* is a trait that distinguishes them from *Enterobius*, which lack such appendage [2]. Egg morphology is practically identical to *T. minutus*, and *T. pigrae* n. sp. which makes this character not useful for species identification, although the eggs of the new species tend to be somewhat smaller (Table 3).

3.2. Phylogenetic analysis

3.2.1. Concatenated data set

This data set consisted of both nuclear rDNA regions (28S and 18S) plus the cox1 gene sequences. The final alignment included 30 sequences and 2629 characters. Phylogenetic trees reconstructed by ML and BI methods yielded similar topologies, with *Enterobius* and *Trypanoxyuris* as monophyletic sister-groups (Fig. 7), and with very strong support for the *Trypanoxyuris* clade. Within *Trypanoxyuris* three major lineages were recovered. The first major lineage includes all sampled species (and individuals) from howler monkeys, *T. multilabiatus* n. sp., *T. pigrae* n. sp. and *T. minutus*. All the individuals sequenced from each of these three *Trypanoxyuris* species, sampled from across their distributional ranges in Mexico, are reciprocally monophyletic (Fig. 7), with *T. pigrae* n. sp. and *T. minutus* grouped as sister species. The clade of three species from howler monkeys is characterized by high bootstrap and posterior probability values, as is the sister-species relationship between *T. pigrae* n. sp. and *T. minutus*. The second major lineage is the sister group to the three species from howler monkeys, and consists of *T. atelis* individuals obtained from different primates of the subfamily Atelinae. This lineage is also supported by moderate ML bootstrap and high posterior probability values. Finally, a third lineage containing two species that parasitize primates from different host families was recovered (*T. atelophora* from Atelidae and *T. microon* from Aotidae) but with weak support.

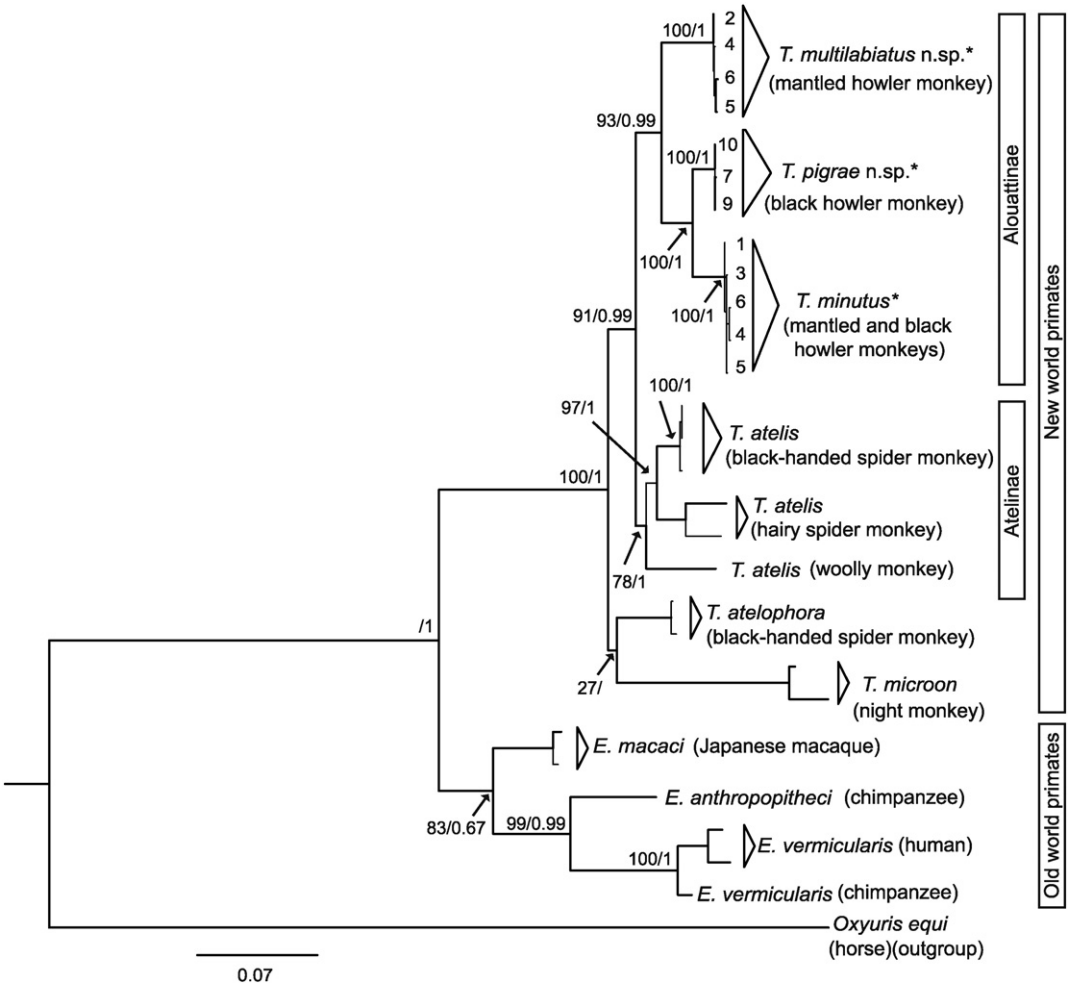

Fig. 7. Maximun likelihood phylogenetic tree of *Trypanoxyuris* species inferred with the concatenated data set (cox1 + 18S + 28S). Numbers at the nodes represent ML bootstrap percentages followed by posterior probabilities from Bayesian inference. Sequences obtained in this study are indicated with *. Numbers at the branch tips refer to map ID in Table 1 and Fig. 1. Bars indicate host family.

3.2.2. Nuclear ribosomal DNA data set

To evaluate the relative contribution of each data set to the delimitation of *Trypanoxyuris* species, analyses of each data set (locus) were conducted separately. Sequences of 1749 bp of 18S, and 1148 bp of 28S, were obtained in this study for each of the five species of *Trypanoxyuris* parasitizing primates in Mexico (*T. atelis*, *T. atelophora*, *T. minutus*, *T. pigrae* n. sp., and *T. multilabiatus* n. sp.). The 18S sequences were trimmed to 765 bp because the sequences obtained from GenBank and used for comparative analysis were shorter. Alignments of sequences from both ribosomal genes (18S and 28S) were combined in a partitioned data set consisting of 26 sequences and 1914 characters. Phylogenetic trees reconstructed by ML and BI yielded the same topology (see Fig. S1 in supplementary material), and were almost identical in topology to the one recovered with the concatenated dataset (Fig. 7), excepting the placement of *T. atelis* from a woolly monkey (*L. lagotricha*) as the sister taxon of the clade occurring in howler monkeys; nevertheless, this relationship was not strongly supported by ML bootstrap values or posterior probabilities. The isolates of the two new species were reciprocally monophyletic in these separate analyses of nuclear ribosomal sequences.

3.2.3. Cox1 data set

Twenty-one sequences of the cox1 gene were obtained in this study, including seven of *T. minutus* (1026 bp), five of *T. multilabiatus* n. sp. (1026 bp), five of *T. pigrae* n. sp. (437 bp), two of *T. atelis* (439 bp), and two of *T. atelophora* (439 bp). The final alignment including sequences from GenBank consisted of 33 sequences. This alignment was trimmed to 715 bp to ensure comparison of homologous regions of the cox1 gene. Each of the three species of *Trypanoxyuris* from howler monkeys is strongly supported as reciprocally monophyletic (see Fig. S2 in supplementary material). Overall the Bayesian and ML trees agree with the tree topology for the concatenated data set (Fig. 7), but the sister-group relationships of *Trypanoxyuris* species hosted by howler monkeys are quite different. However, these different results are supported by low bootstrap and posterior probability values.

3.3. Genetic divergence

The genetic divergence within species ranged from 0.5%–5.9% in cox1, from 0%–0.2% in 18S, and it was null for 28S sequences. Genetic divergence among pinworm species ranged from 8.5%–13.8% in cox1, from 0.2%–2% in 18S, and from 0.8%–5.8% in 28S (28S and cox1 shown in Table 4). Among pinworm genera, the genetic divergence ranged from 13%–17.8% in cox1, from 4.1%–5.3% in 18S, and from 17.2%–18.4% in 28S.

4. Discussion

T. multilabiatus n. sp. and *T. pigrae* n. sp. represent the 20th and 21st described species of *Trypanoxyuris* from primates. Both new species are readily distinguishable by morphological traits, such as their unique

Table 4
Mean genetic divergence among pinworm species. P-distances are expressed as percentages (±SE). 28S rDNA p-distances are above the diagonal; Cox1 p-distances below the diagonal. Bold numbers on diagonal indicate cox1 genetic divergence within species. 18S rDNA distances are not shown. *E. anthropo* = *E. anthropopitheci*.

	T. minutus	*T. multilabiatus* n. sp.	*T. pigrae* n. sp.	*T. atelis*	*T. atelophora*	*T. microon*	*E. macaci*	*E. anthropo*	*E. vermicularis*
T. minutus	**0.6 (0.2)**	3.8 (0.4)	0.08 (0.3)	3.7 (0.5)	5.7 (0.6)	–	–	–	18.3 (1.1)
T. multilabiatus n.sp.	9.7 (1.1)	**0.5 (0.2)**	3.8 (0.5)	3.5 (0.5)	5.0 (0.7)	–	–	–	18.1 (1.1)
T. pigrae n. sp.	10.5 (1.3)	9.1 (1.4)	**0.5 (0.2)**	4.0 (0.5)	5.8 (0.7)	–	–	–	18.4 (1.2)
T. atelis	10.7 (0.8)	10.3 (0.9)	8.5 (1.2)	**5.9 (0.6)**	3.2 (0.5)	–	–	–	17.2 (1.1)
T. atelophora	12.8 (1.1)	12.1 (1.2)	9.5 (1.6)	11.9 (1.0)	**1.1 (0.4)**	–	–	–	17.2 (1.1)
T. microon	13.6 (1.2)	12.6 (1.3)	12.1 (1.7)	13.0 (1.2)	12.5 (1.2)	**2.8 (0.6)**	–	–	–
E. macaci	14.6 (1.1)	13.1 (1.2)	14.1 (1.7)	14.6 (1.1)	14.3 (1.3)	16.1 (1.3)	**1.0 (0.4)**	–	–
E. anthropo	16.6 (1.2)	15.2 (1.3)	15.5 (1.5)	16.3 (1.1)	17.5 (1.2)	17.8 (1.3)	13.4 (1.0)	–	–
E. vermicularis	15.7 (1.2)	15.9 (1.2)	16.1 (1.5)	16.5 (1.1)	16.6 (1.2)	17.5 (1.3)	13.8 (1.1)	12.8 (1.1)	**4.2 (0.6)**

buccal structures, the shape of the lateral alae, and the structure of the oesophagus. In addition, molecular phylogenetic analyses strongly support the distinction of these species. The three genes used in this study (cox1, 18S and 28S) analyzed either separately or concatenated, unequivocally diagnosed and delineated *T. multilabiatus* n. sp. and *T. pigrae* n. sp. from the other *Trypanoxyuris* species for which sequences are available, placing each of them in separate monophyletic lineages.

The intraspecific genetic divergence estimated in this study for cox1 was up to 5.9%, which coincides with the 6% maximum cox1 sequence divergence previously reported within nematode species [27], and the 6.5% pairwise divergence among cox1 sequences reported for *E. vermicularis* [28]. The interspecific cox1 divergence in comparisons of *T. multilabiatus* n. sp. to congeners ranged from 9% to 12%; similar congeneric comparisons for *T. pigrae* n. sp. ranged from 8.5%–12%. These values exceed the 6% intraspecific threshold, and are characteristic of the range of genetic divergence between some pinworm species. Although genetic distances are useful for molecular prospecting, we caution against using a genetic yardstick as the only evidence to delimit parasite species [29,30]. Assumptions critical for using a genetic yardstick to delimit species can easily be violated, such as substitution rate constancy among different evolutionary lineages.

The limited divergence found in 18S rDNA limits its utility for assessing *Trypanoxyuris* relationships between closely related species. In contrast, the 28S rDNA provides enough resolution to discriminate between closely related species of *Trypanoxyuris.* The resolving power of both the 18S and 28S rDNA genes has been noted in studies conducted for other oxyurid nematodes [31]. Unfortunately, prior to the present study 28S sequences from *Trypanoxyuris* were unavailable in GenBank.

4.1. Parasite-host associations

It has been proposed that each species of pinworm parasitizes one genus of primates [5]. In this research, we found that the three species of *Trypanoxyuris* are parasites of *Alouatta* spp. Two species of *Trypanoxyuris* are found in a single *Alouatta* species, with some individual hosts parasitized by both species. *T. minutus* is found in the mantled howler monkey (*A. palliata*) and in the black howler monkey (*A. pigra*), whereas *T. multilabiatus* n. sp. was only found in the mantled howler, and *T. pigrae* n. sp. was exclusively found in the black howler. This observation suggests that, at least for howler monkeys, there is one common species of pinworm, as well as one additional species of pinworm for each howler species. Nevertheless, more intensive pinworm sampling from howler monkey species is needed in order to test this hypothesis.

Morphological differences among these pinworms are primary related to the alimentary system. We speculate that they have specialized in different diets as previously suggested in other studies [32], and that they occupy different habitats in the host intestine to partition the gut and minimize interspecific competition, facilitating coexistence of different *Trypanoxyuris* species within an individual host; although the non-invasive sampling we used in this study does not allow us to corroborate this possibility. Moreover, competition has been proposed as a mechanism promoting parasite diversity [33]; thus, the diversification of *Trypanoxyuris* within *Alouatta* could be a consequence of intra-host competition among *Trypanoxyuris* populations.

The phylogenetic relationships of *Trypanoxyuris* species discovered in this study can be explained in reference to the phylogeny of Neotropical primates. Our results are mainly consistent with the coevolution hypothesis between primates and their parasitic pinworms [5,34]. The clade formed by *T. multilabiatus* n. sp., *T. pigrae* n. sp., and *T. minutus* exclusively parasitizes howler monkeys (subfamily Alouattinae [35]), whereas *T. atelis* parasitizes monkeys from the subfamily Atelinae [35]; both primate hosts belong to the family Atelidae [35]. Also, the *T. atelis* clade shows three well supported groups, and this has previously been proposed to represent the existence of more than one species (perhaps cryptic species) within this clade [8]. In contrast, the relationship between *T. atelophora* and *T. microon* does not match the host phylogeny, and although support for this clade is weak, it could be explained as a result of a host switching event [8]. However, testing these hypotheses requires incorporating a more complete set of taxa including *Trypanoxyuris* species that have not yet been sequenced, and conducting detailed phylogenetic analyses [8].

The three species of pinworms occurring in howler monkeys in Mexico form a monophyletic clade with high support values in ML and BI analyses. These howler monkeys (*A. palliata* and *A. pigra*) are sister species and share a common phylogeographic history of colonization of Middle-America [36,37]. Parasite-host associations uncovered in this study, along with host evolutionary history, allow us to speculate about the origin of these pinworms. *T. minutus* has been reported across the Neotropics in several howler monkey species [38–40], some of them with older origins than *A. palliata* and *A. pigra*, suggesting a longer history of parasitism between this nematode and howler monkeys. Thus it seems likely that *T. minutus* was already present in the common ancestor of *A. palliata* and *A. pigra*, and was retained throughout the diversification process. One explanation for the host association pattern is that the two new species we describe here (*T. multilabiatus* n. sp., and *T. pigrae* n. sp.), are each the result of a peripheral isolate speciation event with the persistency in each case of the ancestral species (*T. minutus*). However, the phylogenetic hypothesis shows strong support for a sister-species relationship between *T. pigrae* n. sp. and *T. minutus* (Fig. 5), which is inconsistent with the expectation that *T. pigrae* n. sp. and *T. multilabiatus* n. sp. both have *T. minutus* as a close relative. An alternative possibility is that both *T. multilabiatus* n. sp. and *T. minutus* were present in the common ancestor of these two howler monkey species. In this scenario both pinworm species were retained in *A. palliata*, whereas *T. multilabiatus* n. sp. was lost in *A. pigra*, and *T. pigrae* n. sp. originated via speciation from the *T. minutus* ancestor. The phylogenetic results would appear to make this a plausible explanation. Finding *T. multilabiatus* n. sp. associated with other species of *Alouatta* would provide additional support for this hypothesis.

Although the exact scenario for the origin of the hosts *A. palliata* and *A. pigra* remains unclear [36], these ideas are consistent with the observation that since speciation occurred in these primates, both howler monkey species have been living in different types of habitats, adapting to distinct environmental conditions and food resources, which could

facilitate the speciation and diversification of their pinworms. In this respect, the results of our study cast doubts about the possible existence of more than one species in what has been considered *T. minutus* across the Americas. Most of the parasitological research in Neotropical primates is conducted through the identification of eggs found in faeces. Since *Trypanoxyuris* eggs belonging to different species seem to be morphologically indistinguishable, the implementation of molecular techniques to determine species identity is critical. The evolutionary history of these nematodes, and the processes underlying parasite-host associations will became clearer as more information is generated from different howler monkey species and populations across their distribution in the Neotropics.

Currently, *A. palliata* and *A. pigra* have an allopatric distribution, with a small contact zone where hybridization occurs [11]. It would be interesting to determine how their *Trypanoxyuris* species behave in this hybrid zone, and to what extent the host specificity pattern is maintained by *T. multilabiatus* n. sp. and *T. pigrae* n. sp., including the *Trypanoxyuris* diversity within hybrid howler monkeys.

The results presented here, together with previous studies [8], reveal five *Trypanoxyuris* species parasitizing the three species of Mexican primates. In addition, two new species of *Trypanoxyuris* have been recently discovered in a red uakari monkey (*Cacajao calvus*) from the Peruvian Amazon [7], although no sequence data were generated for these species. Empirical evidence suggests that pinworm diversity in Neotropical primates is higher than previously thought. The use of combined morphological and molecular methods makes the identification of pinworm species more accurate. However, two additional sampling strategies are essential for a more accurate estimation of *Trypanoxyuris* diversity and phylogeny. The first sampling strategy is to examine a broader range of host species, including those Neotropical primates whose parasite fauna has been poorly investigated. Second, survey efforts must be increased to cover representative areas throughout host distributions. Since pinworms and primates appear to have a close evolutionary relationship, increasing phylogenetic understanding of these parasites may provide insights to clarify some remaining controversies in Neotropical primate biogeography [37]. In that respect, further research in genetic, phylogenetic, and phylogeographic contexts are needed to uncover the evolutionary and ecological process underlying pinworm distribution and diversification.

5. Conclusion

T. multilabiatus n. sp. and *T. pigrae* n. sp. are two new species of pinworms parasitizing howler monkeys in Mexico. Conspicuous morphological traits distinguish these new species from their congeners, such as the buccal structures, the lateral alae, the structure of the oesophagus, and the tail appendage in males. In addition, reciprocal monophyly evidenced from molecular phylogenetic analyses corroborate the delimitation of the new species. The three species of pinworms occurring in howler monkeys in Mexico form a monophyletic clade with high support values. More exhaustive pinworm sampling in Neotropical primate species is needed to elucidate the evolutionary and ecological process underlying pinworm distribution and diversification.

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The Missing Fellow: First Description of the Trypanoxyuris pigrae Male (Nematoda:Oxyuridae), a Parasite of the Black Howler Monkey (Alouatta pigra) in Mexico.

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Abstract:	The first morphological description of the male of Trypanoxyuris pigrae Solórzano-García, Nadler, and Pérez-Ponce de León, 2016 is presented in this study. Morphological data are supported by molecular data. Specimens of T. pigrae were recovered after the necropsy of a roadkill black howler monkey (Alouatta pigra) in southeastern Mexico. Males of T. pigrae are characterized by having 3 notched lips, and a long esophagus with a posterior bulb; they also show a single crested lateral alae, , a single spicule, and 4 caudal papillae. Morphological features coincide with those of the previously described T. pigrae females, and molecular profiles confirmed species identification. Males of T. pigrae are very similar to those of T. minutus, another species of pinworm that also parasitizes the black howler monkey A. pigra; however, the shape of the lips represents a very reliable diagnostic feature. Because of this, detailed en face observations are recommended to discriminate between these pinworm species.

The Missing Fellow: First Description of the *Trypanoxyuris pigrae* Male (Nematoda: Oxyuridae), a Parasite of the Black Howler Monkey (*Alouatta pigra*) in Mexico

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ABSTRACT: The first morphological description of the male of *Trypanoxyuris pigrae* Solórzano-García, Nadler, and Pérez-Ponce de León, 2016 is presented in this study. Morphological data are supported by molecular data. Specimens of *T. pigrae* were recovered after the necropsy of a roadkill black howler monkey (*Alouatta pigra*) in southeastern Mexico. Males of *T. pigrae* are characterized by having 3 notched lips, and a long esophagus with a posterior bulb; they also show a single crested lateral alae, , a single spicule, and 4 caudal papillae. Morphological features coincide with those of the previously described *T. pigrae* females, and molecular profiles confirmed species identification. Males of *T. pigrae* are very similar to those of *T. minutus*, another species of pinworm that also parasitizes the black howler monkey *A. pigra;* however, the shape of the lips represents a very reliable diagnostic feature. Because of this, detailed *en face* observations are recommended to discriminate between these pinworm species.

Trypanoxyuris is a genus of oxyurid nematodes belonging to the subfamily Enterobiinae that are only found in Neotropical primates (Hugot et al., 1996). Currently there are 21 described species of *Trypanoxyuris*, of which 3 species, *Trypanoxyuris minutus*, *Trypanoxyuris multilabiatus,* and *Trypanoxyuris pigrae*, have been reported to parasitize howler monkeys (*Alouatta* spp.) (Solórzano-García et al., 2016). *Trypanoxyuris*

25 *pigrae* was recently described from adult pinworms recovered from the feces of black
26 howler monkeys (*Alouatta pigra*) in Mexico (Solórzano-García et al., 2016). Unfortunately,
27 no male specimens were found at that time, and only the description of the female was
28 provided. Here, we present the description of *T. pigrae* males based on morphological and
29 molecular data. Specimens were recovered from the intestine of a black howler monkey
30 found as a roadkill in the Catazajá-Palenque highway, Chiapas state, southeastern Mexico.
31 Pinworms found during necropsy were separated by sex and stored in 70% alcohol.
32 For species identification, worms were cleared with alcohol-glycerol solution, and observed
33 under an Olympus BX51 light microscope equipped with differential interference contrast
34 (DIC) for morphological analyses. Buccal structures are among the main diagnostic
35 features used to discriminate among *Trypanoxyuris* species (Hugot, 1985), thus *en face*
36 observations were made following the technique proposed by Hasegawa et al. (2004). Four
37 specimens were also preserved and processed for scanning electron microscopy (SEM)
38 following procedures described in Solórzano-García et al. (2016). Male specimens of *T.*
39 *pigrae* were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de
40 Biología, Universidad Nacional Autónoma de México (UNAM) (allotype CNHE: No
41 10300) (paratypes CNHE: No 10301), and in the Colección Biológica de la Universidad
42 Autónoma de Chiapas (UCHMVHN-S 0015). To confirm the morphological identification
43 using molecular data, a fragment of the cytochrome oxidase subunit I gene (cox1) was
44 sequenced from 3 *T. pigrae* male specimens. Genomic DNA was extracted using the
45 procedure followed by Solórzano-García et al. (2016), and cox1 was amplified following
46 Nakano et al. (2006). PCR products were treated with Exo-SAP (Thermo Scientific,
47 Waltham, MA) according to the manufacturer's instructions, and sequenced at the
48 sequencing facility of the Instituto de Biología, Universidad Nacional Autónoma de

México. Sequences were compared with those available in the GenBank nucleotide database, using the BLAST tool (https://blast.ncbi.nlm.nih.gov). Sequences obtained in this study were deposited in GenBank, accession number: KY200973-75.

Description of *Trypanoxyuris pigrae* males (Figs. 1A-E, 2A). Based on 28 specimens, with minimum and maximum values followed by the mean; measurements are expressed in µm unless otherwise stated: Body length 1.7–2.5 (2.2) mm, width in midbody 74.7–130 (109.8); cuticle with transverse striations. Lateral alae single crested, beginning at level of nerve ring and terminating at precloacal level. Cephalic tray quadrangular, with 2 amphids, 1 on each side; cephalic papillae readily visible, located in ventral and dorsal extremes of the cephalic tray . Buccal aperture triangular, delimited by 3 notched lips, 1 dorsal and 2 subventral; dorsal and left ventral lips bilobulated, with separation between lobes significantly smaller in the left ventral lip compared to dorsal lip. Nerve ring 112.8–168.7 (145.5) from anterior extremity. Excretory pore 556.5–777.6 (667.5) from anterior extremity. Esophagus 485.3–750.2 (610.4) long, with a posterior spherical esophageal bulb; corpus of the esophagus 420.3–662.3 (532) long excluding median bulb, and 14.9–35.1 (28.4) width at the middle; posterior esophageal bulb 58.6–88.3 (75.4) long, 48.1–83.5 (70.2) width. Posterior body vent ventrally. Spicule 27.4–57.5 (45.8) long. Caudal alae present, with 4 pairs of caudal papillae surrounded by ring-shaped thickenings; first pair large, directed laterally; second and third pairs minute, directed ventrally; fourth pair minute, directed posterolateraly. Tail appendage 6.7–18.5 (11.3) long.

The cox1 sequences obtained for *T. pigrae* males were 427 bp long and showed 100% and 99% identity with *T. pigrae* sequences available in GenBank (e.g., accession numbers: KU285491-95). Even though buccal structures in *T. pigrae* males mirror those of *T. pigrae* females, the notches present in the lips of the males are less conspicuous than in

73 females (Fig. 2A, B). Mixed pinworm infections have been reported for black howler
74 monkeys in Mexico, with *T. pigrae* and *T. minutus* occurring even in the same individual
75 host (Solórzano-García et al., 2016). In addition to being sister taxa (Solórzano-García et
76 al., 2016), these two pinworm species are also morphologically very similar (Trejo-Macias
77 et al., 2011; Solórzano-García et al., 2016), with no variation in the shape of spicule or
78 caudal papillae in males from either species. The most reliable character to distinguish
79 between males of these 2 species is the presence of notched lips of *T. pigrae* (Fig. 2);
80 hence, careful *en face* observations are highly recommended in order to discriminate
81 between these 2 *Trypanoxyuris* species.

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Figure 1. Males of *Trypanoxyuris pigrae.* (**A**) Full body; (**B**) SEM of midbody showing lateral ala single crested; (**C**)SEM of posterior region, a, b, c, d = caudal papillae, arrow pointing to tail appendage ; (**D**) posterior end, arrow pointing at the spicule; (**E**) Esophageal bulb.

Figure 2. SEM *en face* views showing buccal structures of *Trypanoxyuris* sp. from black howler monkeys. (**A**) *Trypanoxyuris pigrae* male; (**B**) *Trypanoxyuris pigrae* female; (**C**) *Trypanoxyuris minutus* male; (**D**) *T. minutus* female; a = dorsal lip; b= left subventral lip; arrow pointing to lip notches in *T. pigrae.*

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Figure 1

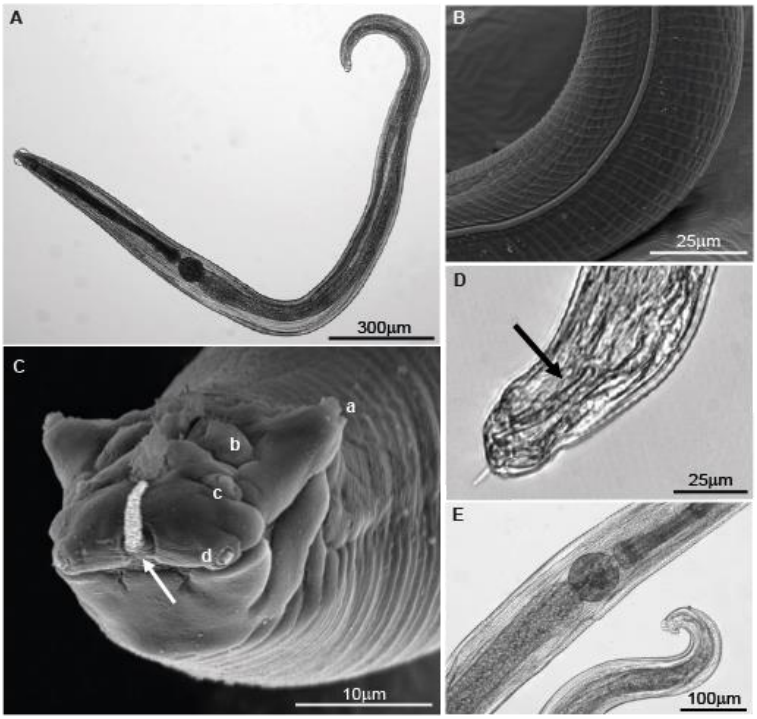
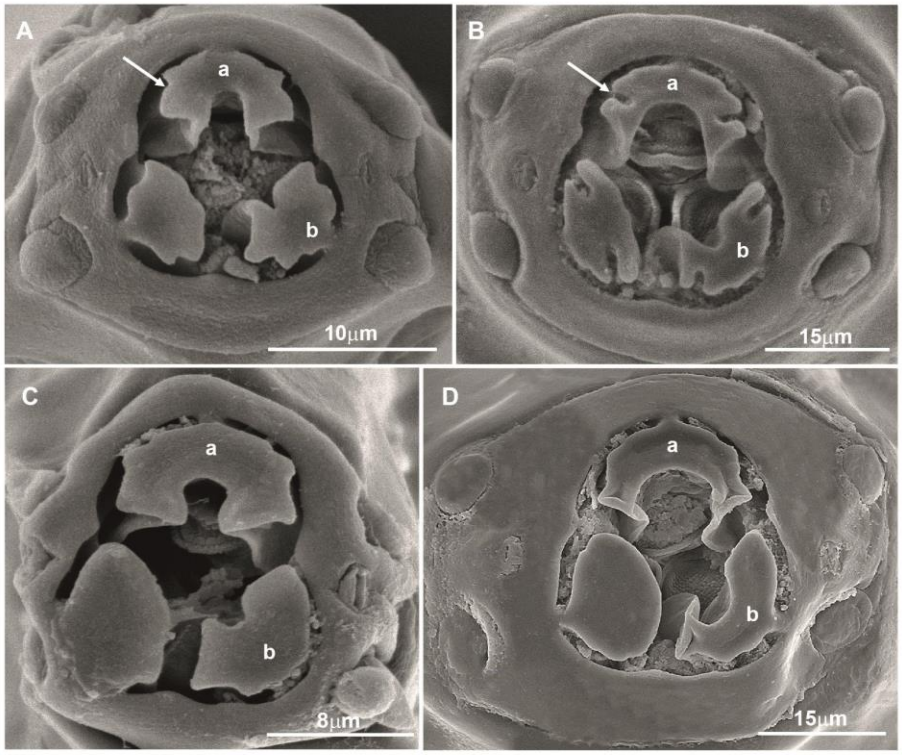


Figure 2



CAPÍTULO 3

CAPÍTULO 3. ESTRUCTURA GENÉTICA DE *Trypanoxyuris minutus* Y *T. atelis* Y SU RELACIÓN CON LA FRAGMENTACIÓN DEL HABITAT.

En este capítulo se aborda uno de los objetivos principales de este trabajo, el determinar el efecto del aislamiento de las poblaciones de primates, consecuencia de procesos de pérdida y fragmentación del hábitat, sobre la estructura genética de sus parásitos oxiuros. Para evaluar lo anterior se eligió la especie de *Trypanoxyuris* más abundante en dos especies de primates: *T. minutus* en mono aullador (*A. palliata*) y *T. atelis* en mono araña (*A. geoffroyi*).

Se aplicaron diversos análisis para determinar la diversidad genética, diferenciación poblacional y estructura genética, así como la historia poblacional y demográfica en las dos especies de oxiuros. Con base en los patrones genéticos encontrados, se proponen tres escenarios para explicar el flujo genético en parásitos son propuestos. La información se presenta en un manuscrito sometido a la revista *International Journal for Parasitology*, con el título "**Habitat fragmentation does not result in genetic structuring of pinworm populations in New World primates across tropical rainforests".**

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Abstract: Microevolution processes in parasites are driven by factors related to parasite biology, host abundance and dispersal, and environmental conditions. Here, we test the prediction that isolation of host populations results in reduced genetic diversity and high differentiation among parasite populations. We conducted a population genetic analysis of two pinworms, Trypanoxyuris minutus and T. atelis, commonly found parasitizing howler and spider monkeys in tropical rainforests across south-eastern Mexico, whose populations are currently isolated due to anthropogenic habitat loss and fragmentation. Mitochondrial DNA was employed to assess parasite genetic patterns, as well as to analyse their demography and population history. Both pinworm species showed high haplotype diversity but, unexpectedly, lower nucleotide diversity than that reported for other parasites. No genetic differentiation or population structure was detected in either pinworm species despite habitat loss and fragmentation and host isolation. The results suggest that primate inter-fragment dispersal movements might be higher than expected, and that passive dispersal might be facilitating gene flow between parasite populations, irrespective of fragment isolation. Also, large population sizes in parasites could be helping them to cope with the isolation and fragmentation of populations, blurring the effects of genetic drift. The present study highlights the complexity of the drivers that intervene in the evolutionary processes of parasites. Detailed genetic studies are needed, both in host and parasite populations, to assess the effects that habitat perturbation and environmental changes could have in the evolutionary dynamics of pinworms and primates.

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Habitat fragmentation does not result in genetic structuring of pinworm populations in New World primates across tropical rainforests

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Abstract

Microevolution processes in parasites are driven by factors related to parasite biology, host abundance and dispersal, and environmental conditions. Here, we test the prediction that isolation of host populations results in reduced genetic diversity and high differentiation among parasite populations. We conducted a population genetic analysis of two pinworms, *Trypanoxyuris minutus* and *T. atelis*, commonly found parasitizing howler and spider monkeys in tropical rainforests across south-eastern Mexico, whose populations are currently isolated due to anthropogenic habitat loss and fragmentation. Mitochondrial DNA was employed to assess parasite genetic patterns, as well as to analyse their demography and population history. Both pinworm species showed high haplotype diversity but, unexpectedly, lower nucleotide diversity than that reported for other parasites. No genetic differentiation or population structure was detected in either pinworm species despite habitat loss and fragmentation and host isolation. The results suggest that primate inter-fragment dispersal movements might be higher than expected, and that passive dispersal might be facilitating gene flow between parasite populations, irrespective of fragment isolation. Also, large population sizes in parasites could be helping them to cope with the isolation and fragmentation of populations, blurring the effects of genetic drift. The present study highlights the complexity of the drivers that intervene in the evolutionary processes of parasites. Detailed genetic studies are needed, both in host and parasite populations, to assess the effects that habitat perturbation and environmental changes could have in the evolutionary dynamics of pinworms and primates.

Keywords: population size, genetic structure, forest fragments, Nematoda, Oxyuridae.

1. Introduction

Microevolution in parasites is mediated by many factors related to the biology of the parasite and that of its host, as well as to environmental conditions intervening in their subsistence.

Parasite life history traits such as life cycle complexity, reproductive mode, and population sizes, along with host abundance and dispersal regulate some of the most important aspects of parasite population genetics (Nadler, 1995; Criscione et al., 2005; Blasco-Costa and Poulin, 2013; Lagrue et al., 2016). General patterns of parasite genetic structure have not been investigated in great detail (Blasco-Costa and Poulin, 2013), however, parasites with sexual reproduction capable of infecting multiple host species, and with high dispersal capabilities and long-lived definitive hosts, are expected to be highly diverse and poorly differentiated. In contrast, strong host specificity, autogenic life cycles, and an aggregated distribution of host populations (either through behavioural, environmental or geographical factors) will most likely promote genetic structure among parasite populations (Nadler, 1995; Criscione and Blouin, 2004; Barrett et al., 2008; Blasco-Costa et al., 2012).

In addition, processes of parasite local adaptation, speciation and coevolutionary dynamics will be affected by host and parasite gene flow (Criscione et al., 2005; Lagrue et al., 2016). Genetic interchange among parasite populations is believed to be strongly correlated with host dispersal ability (Blouin et al., 1995; Prugnolle et al., 2005; Louhi et al., 2010); however, host vagility is mediated not only by its dispersal capability but also by landscape properties such as habitat extent and arrangement, connectivity and matrix configuration (Tischendorf et al., 2003). Habitat loss and fragmentation are considered among the most important threats for biodiversity (Fischer and Lindenmayer, 2007). The expansion of human settlements and the associated changes in landscape configuration have reduced native vegetation to patches within a matrix of anthropogenic vegetation, harbouring isolated wildlife populations. The severity of the effects that these processes could have on the persistence of any organism are species-specific and depend on the species' ecology and life history requirements (Betts et al., 2014).

Oxyurid nematodes of the genus *Trypanoxyuris* are commonly found in New World primates (Hugot et al., 1996). Pinworms of primates are highly host-specific and show interesting patterns of host-parasite coevolution (Hugot, 1999). These nematodes are characterized by having a

direct life cycle with no free-living stages. They present a haplodiploid reproduction mode where males are haploid and derived from unfertilized eggs, while females are diploid and derived from fertilized eggs (Adamson, 1989). Transmission occurs by the ingestion of eggs which are passed to the external environment with host faeces and deposited in clusters; autoinfection and retroinfection are thus common transmission modes (Felt and White, 2005).

Trypanoxyuris minutus and *T. atelis* parasitize howler monkeys (*Alouatta* spp.) and spider monkeys (*Ateles geoffroyi*), respectively (Solórzano-García et al., 2015, 2016). In Mexico, these primates are considered endangered mainly due to habitat loss and fragmentation (Rodríguez-Luna et al., 2009; SEMARNAT, 2010), leaving isolated primate populations in what used to be a continuous tropical rainforest (Rodríguez-Luna et al., 2009; Solórzano-García et al., 2012). Both primate species can be considered as specialist dispersers since the probability of dispersal between forest fragments declines with increasing habitat loss; specifically by imposing higher risk of mortality while crossing the matrix (Estrada and Coates-Estrada, 1996; Mandujano et al., 2004; Pozo-Montuy and Serio-Silva, 2007).

In this study, we evaluate the population genetic patterns of *T. minutus* and *T. atelis* occurring in isolated howler and spider monkey populations in south-eastern Mexico. First, we assessed the amount and geographic distribution of genetic diversity among parasitic pinworms in fragmented tropical forests. Second, we tested whether the processes of habitat loss and fragmentation, and the consequent isolation of host populations have resulted in the genetic isolation and genetic structure of pinworm populations. Third, we investigated the demographic history of parasites based on the genetic data to reveal the genetic consequences of habitat fragmentation for the pinworm populations. In order to make regional and local inferences for both pinworm species, several primate populations were sampled across their distribution range in Mexico. Mitochondrial DNA was used to assess parasite genetic patterns among and within fragments and geographic regions, as well as to analyse their demographic and population history. The life history properties of pinworms, the fragmented condition of the habitat, the limited ability

of primates to cross the matrix and move across forest patches (Mandujano and Estrada, 2005), and the tight parasite-host association between pinworms and primates, fit the conditions for low diversity and strong genetic structure in parasite populations. Our unexpected results are discussed in light of their implications for primate ecology and conservation, in addition to contributing to our growing understanding of parasite evolution.

2. Methods

2.1. Collection of pinworm specimens

Trypanoxyuris minutus specimens were collected from free-ranging howler monkey troops inhabiting 16 isolated forest fragments assigned to four geographic regions across their distribution range in south-eastern Mexico (Fig 1). Distance between centroids of forest fragments in each geographic region ranged from 2 km – 77 km, while distance between centroids of regions ranged from 79 km – 226 km. Likewise, *T. atelis* specimens were collected from spider monkey troops inhabiting 15 forest fragments distributed in 6 regions (Fig 1). Distance between centroids of forest fragments in each geographic region ranged from 2 km – 142 km, while that between centroids of regions ranged from 130 km- 870 km. All spider monkey samples were collected from free-ranging populations, except those in Villahermosa, Tabasco which came from spider monkeys reared in a Zoo.

Non-invasive sampling techniques were employed to obtain adult pinworms from primate faeces. Faecal samples were collected right after defecation and immediately placed on ice until transported to the laboratory where they were preserved at −20°C. Whenever possible, adult pinworms were recovered *in situ* before storing the faecal sample. Searching for additional adult pinworms was done following the procedure proposed by Hasegawa (2009) using the frozen faecal samples. All recovered specimens were fixed in 100% ethanol for DNA extraction. An average of ten pinworms per host species per forest fragment was sampled, preferably from distinct host individuals.

2.2. DNA extraction, amplification and sequencing

Individual pinworms were digested overnight at 56 °C in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using the DNAzol® reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Mitochondrial DNA (mtDNA) has been shown to be an excellent molecular marker for population genetic studies in nematodes, given its high substitution rate (Blouin et al., 1998); thus a section of the cytochrome c oxidase subunit 1 gene (cox1) was amplified using the primers TrycoxF, 5′-TGGTTGGCAGGTCTTTATC-3′ (forward) and TryCoxR, 5′-AACCAACTAAAAACCTTAATMC-3′ (reverse). The PCR conditions were: initial denaturation at 94 °C for 1 min, followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, and a post-amplification extension for 7 min at 72 °C. PCR products were treated with Exo-SAP (Thermo Scientific), according to the manufacturer's instructions. Sequences were assembled and base-calling differences were resolved using Geneious v.5.1.7 (Biomatters). Sequences were aligned using Clustal W and MESQUITE v.2.75 (Maddison and Maddison, 2011), and checked for accuracy using the translated amino acid sequences based on the invertebrate mitochondrial genetic code. A total of 140 sequences of 841 bp were obtained for *T. minutus* (GenBank: **XXXX**), while 98 sequences of 844 bp were obtained for *T. atelis* (GenBank: **XXXX**).

2.3 Analysis of genetic variation and population differentiation

Molecular diversity indices including number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide differences (k) were derived using DnaSP v.5 (Rozas et al., 2003) for each forest fragment, and each geographic region, for each pinworm species. Genetic diversity parameters were not estimated for populations with less than 5 sampled parasite individuals, but these populations were included in

157 the regional and total estimations. To assess the amount of genetic differentiation among fragments
158 and regions, pairwise Fst were estimated using Arlequin v.3.5 (Excoffier et al., 2005). To assign the
159 genetic variation among or within populations we performed a hierarchical analysis of molecular
160 variance (AMOVA) as implemented in Arlequin using pairwise differences, and 1000 bootstrap
161 replicates to evaluate significance. Also, a median-joining haplotype network was constructed using
162 Network v.5.000 (Bandelt et al., 1999) to show the evolutionary relationships between haplotypes
163 from different fragments and regions. Because the resulting networks were too complex involving
164 multiple alternative linkages, we used the MP calculation post-processing option to visualize the
165 most parsimonious tree (Polzin and Dabeschmand, 2003).
166
167 *2.4 Analysis of population structure*
168 We used two methods to evaluate population structure of both pinworm species. A spatially
169 explicit Bayesian clustering method implemented in the program BAPS v.6 (Cheng et al., 2013).
170 BAPS defines the neighbourhood of each individual based on Voronoi tessellation, with
171 neighbouring individuals more likely to be co-assigned to a cluster than individuals far apart; also
172 the correlation between clusters decreases with the distance between sites (Corander et al., 2008).
173 We tested a maximum number of clusters (K) of K=5, K=10, K=15, running 10 replicates for each
174 value of K, using the spatial clustering of groups in the population mixture analysis.
175 Also, a discriminant analysis of principal components (DAPC) (Jombart et al., 2010) was
176 performed using the adegenet v.3.1.9. package (Jombart, 2008) for R (R Development Core Team
177 2010) to attempt to differentiate pinworm populations based on fragments and regions. DAPC does
178 not make assumptions regarding population genetic models, and the optimal number of population
179 clusters is established through the Bayesian Information criterion (BIC) using the find.clusters
180 function in adegenet v.3.1.9.
181 Finally, because primate movements between forest fragments decreases with the distance,
182 we evaluated if pinworm populations presented a pattern of isolation by distance (IBD) by

183 performing a Mantel test (Mantel, 1967) to correlate Edward's genetic distance (Edwards, 1971)
184 with geographic distance, using the R console. Geographical distances between forest fragments
185 were calculated using the raster v.2.5.8. package (Hijmans et al., 2016). Edward distance was
186 estimated using the adegenet v.3.1.9 package. Mantel tests were run for each pinworm species with
187 the ade4 v.1.7.4 (Dray and Dufour, 2007) package, with 999 repetitions.
188
189 *2.5 Demographic and population history*
190 To investigate the population history and demography of *T. minutus* and *T. atelis*, three
191 methods were used. First we calculated Fu's Fs neutrality test using DnaSp v.5. This test evaluates
192 if populations are evolving at equilibrium between mutation and genetic drift or if some non-
193 random process is happening such as natural selection or population expansion or decline. A
194 negative value of Fs indicates a larger number of alleles than expected given the observed level of
195 genetic diversity, consistent with population expansion; while a positive value suggests deficiency
196 of alleles as would be expected in a declining population. Second, we constructed a Bayesian
197 Skyline Plot (BSP) using the software BEAST v.1.7.5 (Drummond et al., 2005) to infer changes in
198 the population size of both pinworms through time. The BSP is a coalescent method that uses a
199 Markov chain Monte Carlo (MCMC) procedure to estimate a posterior distribution of effective
200 population sizes through time directly from a sample of gene sequences, given a specified
201 nucleotide substitution model (Drummond et al., 2005). For both pinworm species the appropriate
202 model of nucleotide evolution was HKY+I+G determined using the AIC criterion in MrModeltest
203 v.2.3 (Nylander, 2004). BSP analyses were run with the strict molecular clock option and a
204 mutation rate of 1.57×10^{-7} substitutions per site per generation, which was estimated from
205 *Caenorhabditis elegans* mtDNA (Denver et al., 2000). One hundred million iterations were
206 performed, sampling model parameters every 20,000 iterations and a 10% burn-in. Plots and the
207 performance of the MCMC process were visualized in Tracer v 1.5. (Rambaut et al., 2013). Third,
208 we used Lamarc v.2.1.3 software (Kuhner, 2006) to estimate the demographic parameters Θ_0 and

the population growth rate (g) of the expression $\Theta t = \Theta_0 \exp(-gt\mu)$. For mtDNA, Θ_0 equals $2\mu N_e$, where μ is the mutation rate of 1.57×10^{-7} substitutions per site per generation, and N_e is the effective population size of females. Positive values of g indicate that the population has been growing, and negative values indicate that it has been shrinking, while a value of zero indicates no change in population size. The analyses used the F84 model with the substitution rates corrected in order to adjust for the previously determined HKY+G+I model. We used the Bayesian search using 5 independent runs, 10 initial chains and 5 final chains each using 10,000,000 steps with a burn-in period of 10,000 steps. For both species of pinworms the growth priors ranged from -500 to 10,000 in order to insure that the search included growth, decline and no population change.

3. Results

3.1 Genetic diversity and population differentiation

Values of average molecular diversity within fragments and regions are shown in Tables 1 and 2. Populations of *T. minutus* and *T. atelis* were highly genetically diverse for mtDNA, showing a haplotype diversity per fragment or region ranging from 0.778 to 1.0 and a nucleotide diversity ranging from 0.0023 to 0.0126. Both haplotype and nucleotide diversity were higher in *T. atelis* than in *T. minutus*.

The low values of pairwise Fst in most pinworm populations revealed little genetic differentiation between forest fragments (Table 3). Notably for *T. minutus*, the southernmost populations showed the highest population differentiation compared to the rest of the populations; however, it was not a uniform pattern (Supplementary data Table S1). Also, the farthest regions (Tabasco and Chiapas) showed significant Fst values (Table 3), revealing limited gene flow between *T. minutus* in these regions and the rest of the populations. Pairwise Fst values in *T. atelis* indicated moderate differentiation between some populations (Fst = 0.085 – 0.731, Table S2); however, no geographical pattern was evident. Significant Fst values were observed between populations in forest fragments belonging to the same region, such as F13 in Los Tuxtlas and F16 in

Uxpanapa. At the regional level, Campeche and Quintana Roo showed the highest Fst values (Table 3), suggesting a moderate genetic differentiation between *T. atelis* populations in these regions and the rest.

AMOVA's Fst values also indicated low to moderate differentiation between populations of both pinworm species, with most of the variation distributed within populations rather than among populations or among regions (Table 4). These findings were supported by the haplotype networks. In both pinworm species, median-joining networks showed no clear distribution of haplotypes according to geographical location (Fig 2). Besides, an ancestral haplotype was not evident in either network. The most common haplotype in *T. minutus* was recovered from 17 specimens (15 from Los Tuxtlas and 2 from Uxpanapa); nine haplotypes were shared between regions, with only one present in the four regions. For *T. atelis*, the most common haplotype was recovered from seven specimens (four from Los Tuxtlas, one from Uxpanapa and two from Chiapas); five haplotypes were shared among regions; nevertheless no haplotype was present in all six regions.

3.2 Population structure

The optimal number of groups obtained from BAPS analysis was K=1 for *T. minutus* and K=2 for *T. atelis* regardless of the maximum value of K tested. Likewise, the DAPC was not able to discriminate population clusters in either pinworm species (Fig S1). These analyses in conjunction with the AMOVA and Fst results indicate a lack of correlation between genetic structure and the geographical distribution of the different fragments and regions. Patterns of IBD were tested for both pinworm species. Mantel tests show a modest relationship between genetic distances and geographical distances ($R^2 = 0.24$, $p = 0.028$) for *T. minutus.* In contrast, no IBD was observed in *T. atelis* populations ($R^2 = 0.16$, $p= 0.113$) (Fig S2).

3.3 Demography and population history

Overall, Fs values indicate a population expansion in both *T. minutus* and *T. atelis;* at local level, Fu's Fs were high and negative in most of the fragments, especially in *T. minutus* populations (Tables 1 and 2). The demographic expansion scenario was also supported by the other two methods employed to evaluate population history. Bayesian skyline plot (BSP) for *T. minutus* shows a strong and continuous population growth, while BSP for *T. atelis* shows a more drastic and population expansion (Fig 3). Lamarc analyses also indicate a rapid population growth with g= 3,111 (2,040-4,924, 95% PPD) for *T. minutus* and g= 811 (492-972, 95% PPD) for *T. atelis*. A value of g = 200 has been suggested as indicative of fast population growth, i.e. that the population has grown hugely in a relatively short period of time (Kuhner, 2006). Thus the values obtained for *T. minutus* and *T. atelis* support the idea of large population sizes in both species of pinworms.

4. Discussion

Here, we present a population genetic analysis of two highly host-specific parasite species, with haplodiploid reproduction, direct life cycle, and a transmission mode that commonly involves autoinfection and retroinfection; both pinworms occur in host populations living in forest fragments. The life history traits of these parasites along with the current isolation of their host populations due to habitat loss and fragmentation, lead to predictions of reduced genetic diversity and high differentiation between parasite populations (Criscione et al., 2005; Huyse et al., 2005; Barrett et al., 2008). Nevertheless, our results show genetic patterns that are inconsistent with these predictions, giving new insights into parasite population history and host ecology. In both parasites we found high haplotype diversity, low nucleotide diversity, a null population structure and signals of a large population sizes characteristic of population expansion. Nadler (1995) postulated that both parasite and host traits are responsible for the genetic make-up of parasite populations. The interplay of these factors in shaping the observed genetic patterns of *Trypanoxyuris* species in conjunction with landscape features is further discussed, along with the possible scenarios that could yield the lack of population structure.

286

287 *4.1 Genetic diversity*

288 A transmission mode where offspring constantly reinfect their natal individual host, and a
289 haplodiploid sex determination system may both increase inbreeding and limit gene flow between
290 populations, reducing parasite genetic diversity (Adamson, 1989; Nadler, 1995). However, both
291 pinworm species showed high haplotype diversity in all sampled populations, and, interestingly,
292 nucleotide diversity similar to that found in free-living animals (Goodall-Copestake et al., 2012).
293 Notably, the levels of nucleotide diversity found in these nematodes are considerably lower than
294 those reported for cox1 in other parasites with direct life cycles [$\pi = 0.012 – 0.021$ (Miranda et al.,
295 2008; Archie and Ezenwa, 2011; Haynes et al., 2014; Ács et al., 2016)], and instead resemble those
296 of parasites with asexual reproduction [$\pi = 0.006$ (Keeney et al., 2009; Marigo et al., 2015)]. The
297 level of nucleotide diversity found in the two species of *Trypanoxyuris* could be explained by their
298 haplodiploid condition and transmission mode; however, other pinworm species and even
299 *Enterobius vermicularis*, the sister genus of *Trypanoxyuris* and also a pinworm that parasitizes
300 primates, show higher levels of nucleotide diversity [$\pi = 0.014 – 0.049$ (Falk and Perkins, 2013;
301 Rodriguez-Ferrero et al., 2013)]. We believe that the relationship found between haplotype and
302 nucleotide diversity in the two pinworm species herein studied is probably a consequence of the
303 tight co-evolutionary associations between these parasites and their hosts, and the parasites' large
304 population sizes.

305 Little is known about the genetic diversity of primates occurring across Mexico;
306 nevertheless, a few local studies with howler monkeys (*A. palliata*) have found lower genetic
307 diversity in these populations compared with others along the species distributional range (Baiz,
308 2013; Dunn et al., 2014; Alcocer-Rodríguez, 2015; Jasso-del Toro et al., 2016). Howler and spider
309 monkeys occurring in Mexico represent the northernmost populations in these species' geographical
310 range (Rylands et al., 2006). The colonization of Mexican tropical rainforests is relatively recent,
311 with the primates moving from South to North America, apparently after the emergence of the

Panama land bridge (Cortes-Ortiz et al., 2003; Lynch Alfaro et al., 2015). The low genetic diversity in Mexican primates is thought to be a consequence of population bottlenecks suffered by these species during their expansion through Central America (Dunn et al., 2014). A reduction in host populations could have resulted also in a reduction in their pinworm populations, with current levels of nucleotide diversity reflecting the host-parasite biogeographical histories.

4.2 Population differentiation and genetic structure

High levels of host specificity have been proposed to increase parasite genetic structure since parasites with narrow host ranges are more likely to experience processes of local extinction (Barrett et al., 2008). Also, parasites with direct life cycles and no free living stages (such as pinworms) are expected to strongly rely on their host movements to disperse (Nadler, 1995; Criscione et al., 2005). In these cases, parasite gene flow mainly depends upon the potential of their hosts to disperse parasites between geographically isolated populations (Blasco-Costa et al., 2012). In spite of the high host specificity of pinworms and the isolation of host populations between forest fragments and regions, we found no genetic structure in either *Trypanoxyuris* species. At a local scale, pairwise Fst values show no differentiation between pinworm populations inhabiting forest fragments belonging to the same region, with the exception of only a few populations where moderate genetic differentiation was detected; however, this differentiation seems random and did not obey any geographical or spatial pattern. Moreover, since no differentiation was observed between other forest fragments of the same region separated by greater distances, we believe that factors other than the geographical distance are hindering the mobility of primates, causing the differentiation between parasite populations in these particular fragments. At a broader scale, Fst values suggest the emergence of population differentiation patterns, yet not enough to induce a clear genetic population structure. In *T. minutus*, we observed a tendency of the southernmost regions to differentiate from the rest. This is supported not only by the AMOVA results showing a slightly but significant variation among regions, but also by the existence of a rather week IBD pattern.

Contrary to *T. minutus*, no IBD was observed in *T. atelis* populations; instead the appearance of genetic differentiation between regions may be associated with environmental factors such as habitat type. Primate habitat in the regions of Campeche and Quintana Roo consists mainly of semi-deciduous low canopy tropical forest, while Los Tuxtlas, Uxpanapa and Chiapas are dominated by tall evergreen tropical rainforest. Environmental and climatic regimes characteristic of each forest type, along with differences in the feeding ecology of spider monkeys in each habitat could be promoting the observed Fst values between regions in *T. atelis* populations.

4.3 Habitat fragmentation and lack of population structure

Regardless of the limited gene flow between certain forest fragments and regions, neither of the different clustering methods applied could discriminate between pinworm populations, preventing us from detecting any genetic structure in these parasites from host populations in Mexico. This apparent panmixia suggests at least three possible scenarios. First, given the fragmented condition of the study sites and the low probability of primates crossing the matrix (Mandujano et al., 2004), pinworms may be using other sources of dispersal between forest fragments in addition to host movement. *Trypanoxyuris* eggs could be transported by wind or water to different forest fragments; however, the chances of these eggs being ingested by their hosts seem to be somewhat low, since monkeys rarely come down the trees to drink water from rivers (Campbell et al., 2005). In addition, pinworm eggs are really sensitive to low humidity and survive only a few days outside the host (Adamson, 1989; Nadler, 1995); this is particularly true for *Trypanoxyuris* in forest fragments, where edge effects impose hostile conditions for egg survival (Escorcia-Quintana, 2014), making gene flow through wind rather unlikely.

Second, movement of primates between forest fragments may be more frequent than expected. Even though the few available population genetic analyses of howler monkeys show limited gene flow between forest fragments in Los Tuxtlas and Uxpanapa (Alcocer-Rodriguez, 2015; Dunn et al., 2014), gene flow among parasite populations does not require reproductive

364 success of the host, only the dispersal of individuals. Thus, monkeys could be using different
365 fragments for feeding or just as transient visitors carrying their parasites with them. Little is known
366 about the capacity of monkeys to move between forest fragments; a study of howler monkeys in
367 Mexico reports a threshold distance between 60m-200m depending on landscape connectivity
368 (Mandujano and Estrada, 2005). However, hazardous movements have been observed in both
369 howler and spider monkeys (Chaves and Stoner, 2010; Herrera et al., 2015) to reach forest
370 fragments. The genetic structure of the two species of parasites suggests that monkeys overcome the
371 adversities imposed by the non-suitable matrix more often than previously thought. The relatively
372 frequent movement of primates between forest fragments has important implications for tropical
373 forest regeneration (Link and Di Fiore, 2006; Chaves et al., 2011; Arroyo-Rodríguez et al., 2015),
374 enhancing the conservation value of these organisms.

375 Finally, the third scenario assumes the actual absence of gene flow between pinworm
376 populations, with the relatively recent tropical forest fragmentation and the large population sizes of
377 the parasites blurring the effects of genetic drift, delaying the appearance of genetic structure.
378 Massive tropical forest fragmentation in Mexico only began around 1940-1960, primary motivated
379 by policies encouraging deforestation for farming purposes and human settlement expansions
380 (Gonzalez-Montagut, 1999; Merino-Perez and Segura-Warnholtz, 2007). In terms of evolutionary
381 time, the isolation of primate populations as a consequence of habitat loss and fragmentation is
382 quite recent. Variations in the genetic structure of populations due to landscape changes are not
383 instantly evident; there is a time lag between the occurrence of the landscape change and the genetic
384 response (Anderson et al., 2010). Moreover, the different demographic tests applied in the present
385 study indicate large population sizes of both *Trypanoxyuris* species, along with population
386 expansions and high population growth rate through time. Longer times are needed for genetic drift
387 to be reflected in the allelic frequencies of larger parasite populations (Nadler, 1995). All these
388 factors could be preventing us from detecting population structure in the parasites in spite of the
389 absence of gene flow.

Other studies using mtDNA to assess population genetic patterns in parasites with direct life cycles, have also not found a genetic structure (e.g., Braisher et al., 2004; Haynes et al., 2014; Archie and Ezenwa, 2011; Ács et al., 2016). Furthermore, a tendency of parasite populations to be less genetically differentiated than their hosts has been observed, with host dispersal being a poor predictor of parasite genetic patterns (Mazé-Guilmo et al., 2016). Our results concur with these previous observations, indicating that large population sizes in parasites can help them cope with the isolation and fragmentation of populations. They are also consistent with passive dispersion of parasites (through wind, water or other sources), as unlikely as it seems, occurring at higher rates than expected, facilitating gene flow between parasite populations regardless of host vagility; this phenomenon requires confirmation, however.

4.4 Implications for primate health and conservation

Forest loss and fragmentation, rather than decreasing population sizes, could be facilitating population growth in these parasites. Some studies have shown higher parasite prevalence in primates living in forest fragments due to an increase in host density and immune-suppression caused by the stress related to habitat perturbation and competition for resources (Gillespie and Chapman, 2006; Arroyo-Rodríguez and Dias, 2010). Furthermore, spider monkey latrines are found be closer to each other in forest fragments compared to continuous forest (González-Zamora et al., 2012). Thus, habitat loss and fragmentation could be promoting the transmission of pinworms by favouring contact between host individuals, but also by intensifying exposure to contaminated areas. This could explain why pinworm populations in these isolated host populations continue to grow despite habitat loss and fragmentation.

Howler and spider monkeys inhabiting the forest fragments sampled during the present study showed high prevalences of *Trypanoxyuris* infections (Solórzano-García and Pérez-Ponce de León, submitted.). Even though pinworms of primates are nor highly injurious parasites (Adamson, 1989), a howler monkey death caused by a severe *T. minutus* infection has been reported in Brazil

(Amato et al., 2002). Parasites are important components of any ecosystem, and should not be neglected from efforts toward biodiversity conservation (Gómez and Nichols, 2013); however, the health hazards imposed by dense parasite populations, along with the threats that habitat loss and fragmentation impose on monkey populations (Estrada et al., 2017), are jeopardizing not only host subsistence but also parasite survival.

4.5 Final comments and future directions

The results presented here provide a snapshot of pinworm population genetics from isolated host populations, and highlight the complexity of the factors that intervene in the evolutionary processes of parasites. Further studies expanding sampling efforts to include populations across the complete distribution range of these parasites, incorporating different molecular markers and tackling the role of environmental factors such as climatic features, topology and landscape connectivity, are essential to determine which of the proposed scenarios is more likely. Population structure in parasites with a direct life cycle and high host specificity is expected to parallel that of their hosts (Huyse et al., 2005). Detailed genetic assessment on non-human primate populations in Mexico remains essential to make the proper comparison between pinworm and primates, in order to determine if the genetic patterns that we found in parasites are also shown by their hosts, and also to understand how habitat perturbation and environmental changes affect the evolutionary dynamics between these parasites and their hosts.

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Figure 1. Collecting sites of pinworms across mantled howler monkeys distributional range (dashed area) and spider monkeys distributional range (grey area) in Mexico. Circles and ellipses correspond to geographic regions: A) Los Tuxtlas, B) Uxpanapa, C) Tabasco, D) Chiapas for mantled howler monkeys, E) Chiapas for spider monkeys, F) Campeche, G) Quintana Roo. Black polygons are the tropical forest fragments where sampling was performed; numbers correspond to forest fragment ID in Tables 1 and 2.

Figure 2. Median-Joining haplotype network for *T. minutus* (A) and *T. atelis* (B) based on cox1. Haplotype frequency is represented by the diameter of the circle.

Figure 3. Bayesian skyline plots of the change in effective population size (N_e) of *T. minutus* (A) and *T. atelis* (B). The black line is the median estimate of population size, and the grey lines indicate the 95% highest and lowest confidence intervals. A continuous but high population growth is observed for *T. minutus,* while a more sudden expansion is observed in *T. atelis.*

Figure S1. DAPC plots showing no population structure in *T. minutus* (A) and *T. atelis* (B).Histograms show eigenvalues from principal component analysis (PCA) and discriminant analysis (DA) respectively. In PCA histogram, shadowed bars indicate the number of PCs retained. DA eigenvalues correspond to the ratio of the variance between and within groups for each discriminant function.

Figure S2. Plots showing the relationship between genetic distances (Edward distance) and geographic distances among populations for *T. minutus* (left) and *T. atelis* (right).

Table 1. Genetic diversity and neutrality test for *T. minutus* from howler monkeys in Mexico. n = number of sequences, S = segregating sites, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, k = average number of nucleotide differences. Significant values are indicated by an asterisk, * $p \leq 0.05$, ** $p \leq 0.001$. Fragment ID number correspond to those in Figure 1.

Region	**Fragment**	**ID**	**n**	**S**	**h**	**Hd *(SD)***	**π *(SD)***	**k**	**Fu's Fs**
Los Tuxtlas	R.Huber	F1	12	13	9	0.955(0.047)	0.00467(0.00071)	3.92	-3.01
North	Montepío	F2	11	14	10	0.982(0.046)	0.00493(0.00061)	4.15	-5.30*
	P. escondida	F3	10	12	6	0.778(0.137)	0.00357(0.00098)	3.00	-0.82
	Organos II	F4	10	12	9	0.978(0.054)	0.00380(0.00054)	3.20	-5.26**
	Jicacal	F5	3						
	2 Abril	F6	7	7	5	0.857(0.137)	0.00328(0.00074)	2.76	-0.87
	Subtotal		**53**	**33**	**34**	**0.943(0.024)**	**0.00421(0.00034)**	**3.54**	**-32.8****
Los Tuxtlas	Agaltepec	F7	**10**	**15**	**8**	**0.933(0.077)**	**0.00510(0.00082)**	**4.29**	**-2.42**
Centre									
Los Tuxtlas	Magallanes	F9	11	12	10	0.982(0.046)	0.00454(0.00071)	3.82	-5.66*
South	M. Pilapa	F11	10	10	7	0.867(0.107)	0.00402(0.00055)	3.38	-1.73
	Playa	F12	8	10	8	1 (0.063)	0.00539(0.00079)	4.54	-4.21*
	Subtotal		**29**	**19**	**22**	**0.973(0.018)**	**0.00477(0.00043)**	**4.02**	**-16.7****
	Total		**92**	**42**	**53**	**0.957(0.012)**	**0.00450(0.00026)**	**3.79**	**-61.9****
Uxpanapa	El Fortuño	F14	4						
	M.Vidal	F15	5	9	5	1 (0.126)	0.00428(0.00087)	3.6	-1.90*
	Liberales	F17	5	8	4	0.900(0.161)	0.00428(0.00108)	3.6	-0.04
	Total		**14**	**17**	**11**	**0.967(0.037)**	**0.00512(0.00058)**	**4.31**	**-4.52***
Tabasco	H. la Luz	F20	10	12	9	0.978(0.054)	0.00407(0.00053)	3.42	-4.99**
	Comalcalco	F21	10	15	9	0.978(0.054)	0.00526(0.00068)	4.42	-4.01*
	Total		**20**	**20**	**17**	**0.979(0.024)**	**0.00469(0.00044)**	**3.94**	**-12.58****
Chiapas	Pichucalco	F25	**14**	**16**	**13**	**0.989(0.031)**	**0.00431(0.00076)**	**3.63**	**-9.80****
Total			**140**	**62**	**83**	**0.971(0.007)**	**0.00471(0.00020)**	**3.96**	**-124.72****

Table 2. Genetic diversity and neutrality test for *T. atelis* from spider monkeys in Mexico. n = number of sequences, S = segregating sites, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, k = average number of nucleotide differences. Significant values are indicated with an asterisk, * p≤ 0.05, ** p≤ 0.001. Fragment ID number correspond to those in Figure 1.

Region	**Fragment**	**ID**	**n**	**S**	**h**	**Hd *(SD)***	***π* (*SD*)**	**k**	**Fu's Fs**
Los	Guadalupe	F8	7	14	7	1(0.076)	0.00609(0.00088)	5.143	-2.94*
Tuxtlas	Magallanes	F10	2						
	Playa	F13	10	8	5	0.8(0.1)	0.00232(0.00073)	1.956	-0.65
	subtotal		**19**	**19**	**12**	**0.924(0.042)**	**0.00405(0.00068)**	**3.415**	**-4.83***
Uxpanapa	El Fortuño	F14	7	13	7	1(0.076)	0.00621(0.00092)	5.238	-2.89*
	Murillo Vidal	F16	3						
	Liberales	F17	1						
	El Jaguar	F18	10	14	9	0.978(0.054)	0.00529(0.00064)	4.467	-3.98*
	El Desengaño	F19	10	16	10	1(0.045)	0.00670(0.00064)	4.444	-6.42**
	subtotal		**31**	**32**	**28**	**0.994(0.010)**	**0.00625(0.00047)**	**5.277**	**-26.4****
Tabasco	**Villahermosa**	**F22**	**10**	**12**	**10**	**1(0.045)**	**0.00469(0.00066)**	**3.956**	**-6.94****
Campeche	La Libertad	F23	11	30	10	0.982(0.046)	0.01267(0.00198)	10.691	-2.05
	El Zapote	F24	3						
	Subtotal		**14**	**33**	**13**	**0.989(0.031)**	**0.01178(0.00183)**	**9.945**	**-4.26***
Chiapas	R. Agraria	F26	4	12	4	1(0.177)	0.0077(0.00186)	6.5	
	Guacamayas	F27	8	18	8	1(0.063)	0.00711(0.00246)	6	-3.40*
	Subtotal		**12**	**21**	**11**	**0.985(0.040)**	**0.00707(0.00180)**	**5.970**	**-4.74***
Quintana	P. Morelos	F28	10	17	10	1(0.045)	0.00685(0.00078)	5.778	-5.33*
Roo	San Joaquín	F29	2						
	Subtotal		**12**	**12**	**11**	**0.985(0.040)**	**0.00632(0.00072)**	**5.33**	**-5.23***
Total			**98**	**66**	**73**	**0.988(0.005)**	**0.00724(0.00059)**	**6.108**	**-91.9****

Table 3. Pairwise Fst between regions. *T. minutus* data is shown under the diagonal, *T. atelis* data is shown above the diagonal. Bold shadowed values are significantly different from zero. NA = not applicable, dashes indicate unsampled regions.

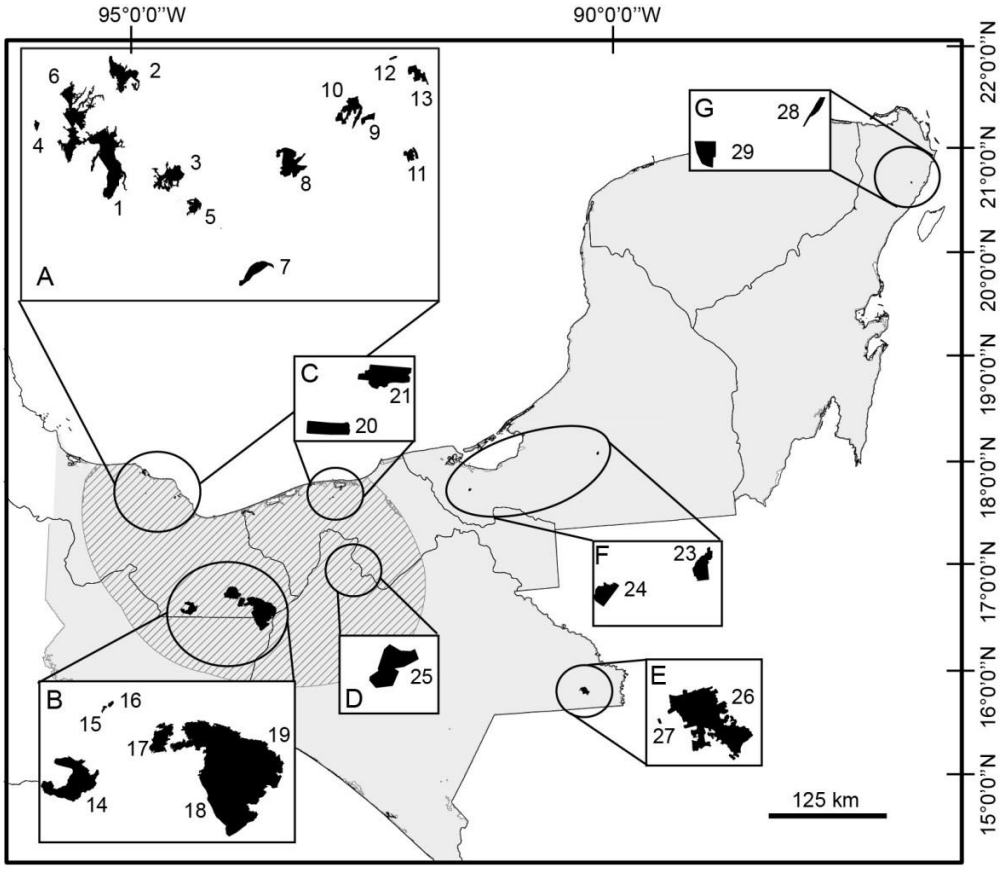
	TN	TS	Tuxtlas	UXP	TAB	CHI	CMP	QRoo
TS	0.002		----	----	----	----	----	----
Tuxtlas	NA	NA		0.012	**0.056**	0.034	**0.226**	**0.201**
UXP	-0.015	-0.019	-0.019		0.047	0.029	**0.175**	**0.101**
TAB	**0.103**	**0.094**	**0.089**	**0.055**		0.049	**0.191**	**0.239**
CHI	**0.106**	**0.088**	**0.087**	0.056	0.009		**0.133**	**0.143**
CMP	----	----	----	----	----	----		**0.092**
QRoo	----	----	----	----	----	----	----	

TN = Tuxtlas North, TS = Tuxtlas South, UXP = Uxpanapa, TAB = Tabasco, CHI = Chiapas, CMP = Campeche, QRoo = Quintana Roo.

Table 4. Analysis of molecular variance (AMOVA) of *T. minutus* and *T. atelis* populations based on cox1.

Source of Variation	d.f	Sum of squares	Variance components	Percentage of variation	Statistics	P value
Trypanoxyuris minutus						
Among regions	3	14.17	0.0867	4.28	Fct = 0.043	0.023
Among fragments within regions	12	29.99	0.0751	3.71	Fsc = 0.039	0.019
Within fragments	124	231.23	1.8648	92.01	Fst = 0.079	P< 0.0001
Trypanoxyuris atelis						
Among regions	5	41.70	0.2242	7.19	Fct = 0.088	0.089
Among fragments within regions	8	33.04	0.2555	8.20	Fsc = 0.072	0.014
Within fragments	84	221.46	2.6365	84.61	Fst = 0.154	P< 0.0001

Figure 1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90°0'0"W
22°0'0"N
21°0'0"N
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A
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125 km

Figure 2

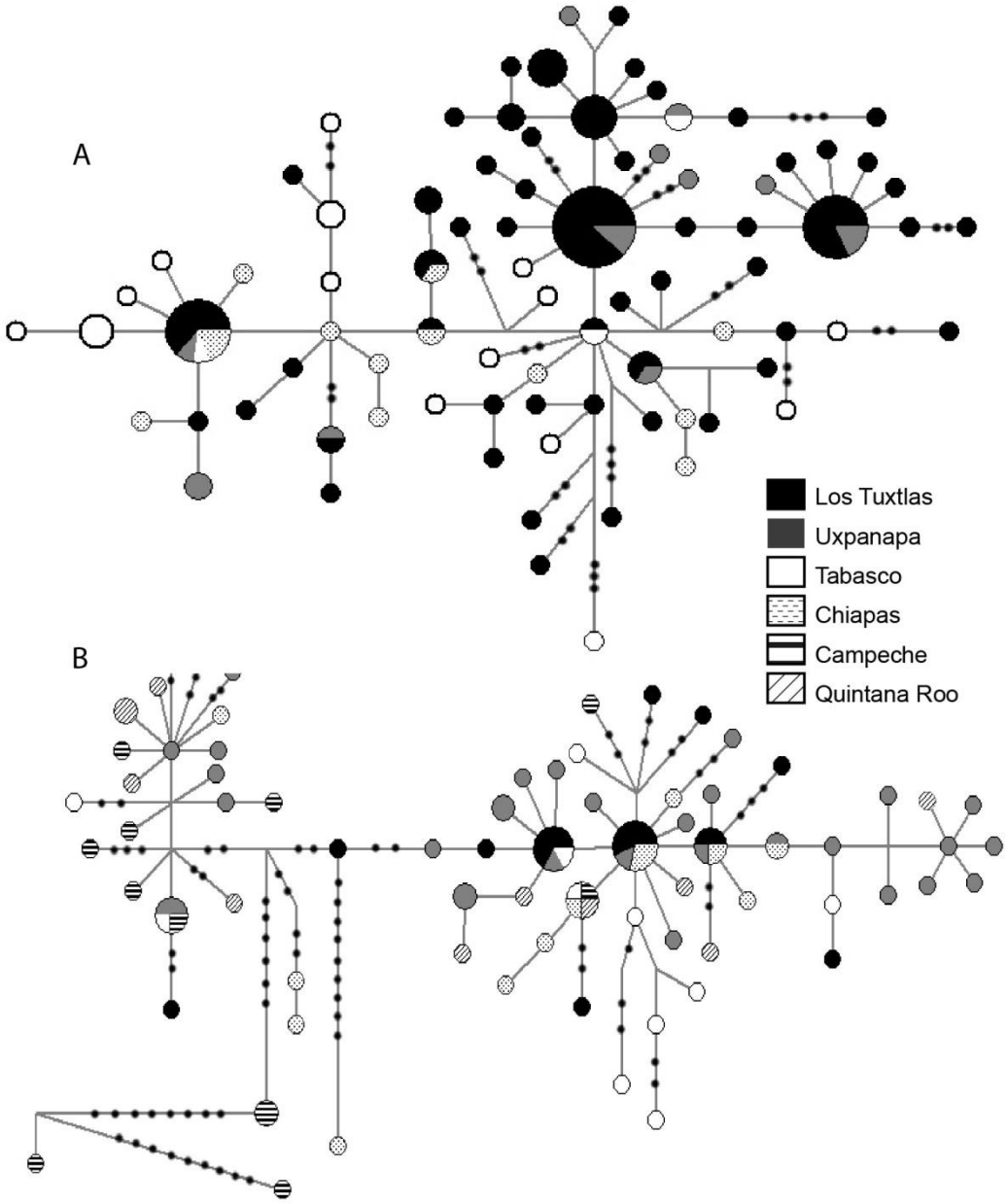
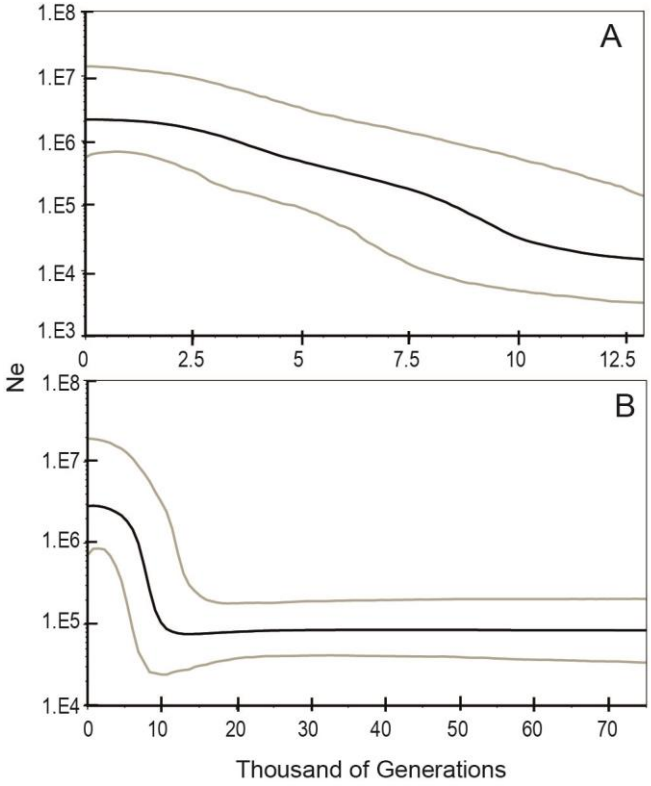

A
B
Los Tuxtlas
Uxpanapa
Tabasco
Chiapas
Campeche
Quintana Roo

Figure 3


A
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1.E8
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1.E5
1.E4
1.E3
0
2.5
5
7.5
10
12.5
0
10
20
30
40
50
60
70
Thousand of Generations

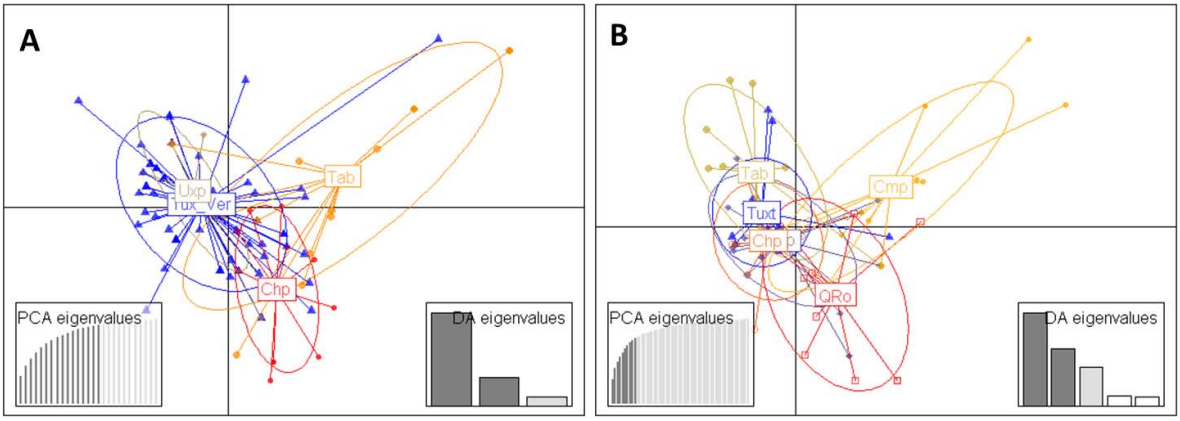
Table S1. Pairwise Fst values between *T. minutus* populations sampled at each forest fragment. Bold shadowed values are significantly different from zero. Fragment ID number correspond to those in Figure 1.

	F1	F2	F3	F4	F5	F6	F7	F9	F11	F12	F14	F15	F17	F20	F21
F2	-0.005														
F3	0.066	-0.002													
F4	0.084	0.023	-0.040												
F5	-0.009	-0.031	-0.069	-0.052											
F6	0.017	-0.033	-0.025	0.039	-0.019										
F7	-0.055	0.007	0.051	0.082	-0.053	0.014									
F9	0.048	0.011	0.004	0.004	-0.010	0.045	0.060								
F11	-0.034	-0.035	0.022	0.033	-0.022	0.036	-0.036	0.025							
F12	0.068	0.030	0.052	0.079	0.063	0.053	0.086	0.054	0.095						
F14	-0.043	-0.027	-0.022	-0.026	-0.200	0.015	-0.061	-0.008	-0.089	0.080					
F15	0.102	0.010	-0.056	-0.057	-0.110	0.049	0.085	-0.003	0.045	0.039	-0.053				
F17	0.058	**0.128**	**0.245**	**0.288**	0.248	0.140	0.051	**0.180**	0.165	0.119	0.211	**0.297**			
F20	0.049	0.078	**0.155**	**0.212**	0.165	0.054	0.031	**0.159**	**0.101**	**0.116**	**0.141**	**0.220**	0.038		
F21	0.038	0.080	**0.121**	**0.179**	0.051	0.074	0.008	**0.138**	**0.093**	0.062	0.095	**0.162**	0.047	0.010	
F25	0.013	0.062	**0.158**	**0.208**	**0.181**	0.068	0.020	**0.148**	**0.084**	**0.090**	**0.143**	**0.224**	-0.004	0.004	0.022

Table S2. Pairwise Fst values between *T. atelis* populations sampled at each forest fragment. Bold shadowed values are significantly different from zero. Fragment ID number correspond to those in Figure 1.

	F8	F10	F13	F14	F16	F18	F19	F22	F23	F24	F26	F27	F28
F10	-0.002												
F13	**0.105**	0.146											
F14	-0.034	0.041	0.107										
F16	**0.300**	0.521	**0.585**	0.132									
F18	0.082	0.074	0.033	0.099	**0.459**								
F19	0.034	0.014	0.042	0.073	**0.447**	-0.027							
F22	0.068	0.028	**0.085**	0.091	**0.465**	0.068	0.032						
F23	**0.126**	0.101	**0.285**	0.067	0.087	**0.261**	**0.241**	**0.227**					
F24	0.045	0.045	**0.366**	-0.052	0.094	**0.257**	0.198	**0.198**	-0.021				
F26	-0.112	-0.056	**0.172**	-0.091	0.200	0.097	0.038	0.067	0.038	-0.144			
F27	-0.038	-0.048	**0.113**	0.024	**0.345**	0.066	0.028	0.047	**0.173**	0.103	-0.064		
F28	0.065	0.083	**0.163**	-0.085	0.076	**0.174**	**0.165**	**0.172**	**0.117**	-0.030	-0.014	**0.118**	
F29	**0.401**	0.800	**0.731**	0.207	0.147	**0.527**	**0.524**	0.561	0.053	0.221	0.289	**0.407**	0.078

Figure S1


A
Uxp
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PCA eigenvalues
DA eigenvalues
B
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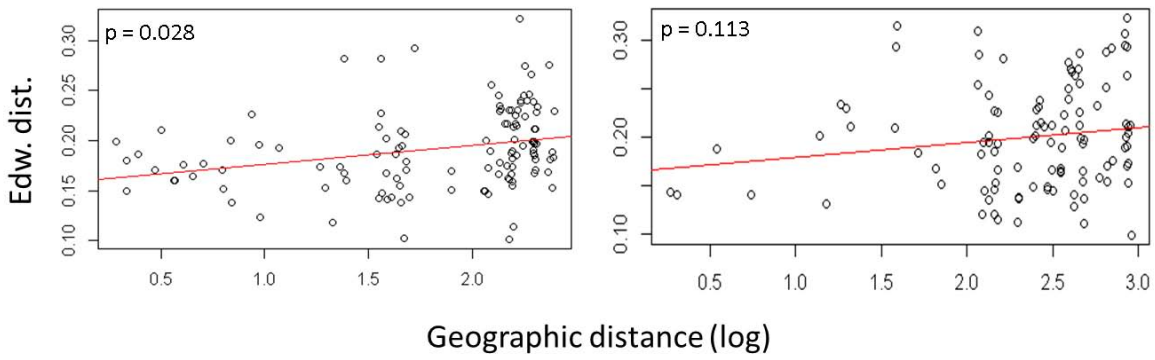
Cmp
QRo
PCA eigenvalues
DA eigenvalues

Figure S2



CAPÍTULO 4

CAPÍTULO 4. HELMINTOS PARÁSITOS DE PRIMATES MEXICANOS Y LA IMPLEMENTACIÓN DE TÉCNICAS MOLECULARES PARA SU DIAGNÓSTICO.

En este último capítulo se presentan los resultados del muestreo parasitológico realizado en las tres especies de primates mexicanos, combinando métodos de muestreo no invasivos con técnicas moleculares mediante la extracción de ADN a partir de los huevos de parásitos encontrados en las excretas de estos primates. Con la intención de corroborar la identidad taxonómica se realizaron análisis filogenéticos de Inferencia Bayesiana son realizados para cada morfotipo de huevo.. El diagnóstico parasitológico aquí presentado es complementado con una revisión de los helmintos parásitos reportados en la literatura para los primates mexicanos. Las ventajas del uso de métodos no invasivos en el diagnóstico molecular de parásitos de especies de hospederos en peligro son discutidas, junto con las implicaciones para su conservación. La información es presentada como un artículo titulado "**Helminth parasites of howler and spider monkeys in Mexico: insights into molecular diagnostic methods and their importance for zoonotic diseases and host conservation**", el cual ha sido sometido a revisión en la revista *International Journal for Parasitology: Parasites and Wildlife*.

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Abstract

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Keywords	DNA sequence; parasite egg; phylogenetic analysis; diagnosis
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Helminth parasites of howler and spider monkeys in Mexico: insights into molecular diagnostic methods and their importance for zoonotic diseases and host conservation

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Note: Supplementary data associated with this article

Abstract

The majority of the parasite assessments of New World primates have been conducted through the identification of the eggs found in faeces, though many species of parasites have very similar eggs, leaving uncertainty in the diagnosis. Here, we present the results of a parasite survey of the three species of primates distributed in Mexico, combining non-invasive sampling with molecular techniques via DNA extraction of the eggs found in the faeces. Mitochondrial and ribosomal DNA were employed for species identification and Bayesian phylogenetic analysis. Nine parasite taxa were found in the three primate species: the nematodes *Trypanoxyuris minutus, T. multilabiatus, T. pigrae, T. atelis, T. atelophora, Strongyloides* sp., unidentified Ancylostomatid, unidentified Ascarid, and the trematode *Controrchis biliophilus*. We were able to extract and amplify DNA from the eggs of the five species of *Trypanoxyuris* reported for Mexican primates, two morphologically different trematode eggs, and *Strongyloides* sp. Phylogenetic analysis confirmed that the two types of trematode eggs belong to *Controrchis biliophilus,* a member of the family Dicrocoeliidae. For *Strongyloides* sp., phylogenetic analysis and genetic divergence showed an association between our samples and *S. fuelleborni;* however, no species could be established due to the lack of more DNA sequences from *Strongyloides* sp. occurring in Neotropical primates. The use of molecular and phylogenetic methods could help to overcome the limitations imposed by traditional non-invasive sampling because eggs are primarily obtained from the faeces; however, its utility relies on the extant genetic library and the contributions that expand such library. The information presented here could serve as a basis for future research on primate parasitology, allowing a more accurate parasite diagnosis and a more precise evaluation of their zoonotic potential.

Keywords: DNA sequence, parasite egg, phylogenetic analysis, diagnosis.

1. Introduction

Parasites are important natural components of ecosystems because they actively intervene in the ecological, demographic and life history processes of their hosts, influencing the structure and organization of free-living organism communities (Poulin, 1999; Gómez and Nichols, 2013). The study of parasites provides information not only on host health but also on the evolutionary history and historical biogeography of the host-parasite associations (Brooks and McLennan, 1993), as well as the health of the ecosystem (Lafferty, 1997; Overstreet, 1997; Pérez-Ponce de León, 2014).

Parasites in wildlife vertebrates are challenging to study, and in most occasions the death of the host is required to obtain and identify its parasitic fauna. This has been a major limitation in studying rare and endangered species, such as many Neotropical primates, where sacrifice is unethical or even illegal. For this reason, the majority of the parasitic assessments of New World primates have been conducted via non-invasive sampling techniques. Non-invasive parasitic evaluations rely mostly on egg identification, though many species of parasites have very similar egg morphotypes, making it practically impossible to distinguish species, which results in uncertainty in the diagnosis. Furthermore, while information on human parasites and parasites of veterinary importance is available with detailed guides on parasite species and egg descriptions (Zajac and Conboy, 2006; Ash and Orihel, 2007; Taylor et al., 2015), only a few references are available regarding the parasitic diseases of wildlife mammals (see Samuel et al., 2001). No guides for the diagnosis of parasites in free-ranging primates are currently available, except for the references and diagnostic images compiled by Hasegawa et al. (2009) and the photographs of eggs and larvae presented in different papers on primate parasitology.

Molecular techniques have been mentioned as promising tools for parasitological studies, not only by facilitating species identification regardless of the parasite developmental stage but also by allowing the gathering of data on transmission modes, geographical spreads, ecological

dynamics, and evolutionary processes, thus widening the scope of parasitological research (Monis et al., 2002; Gasser, 2006).

In Mexico, there are three native species of primates: the mantled howler monkey (*Alouatta palliata*), the black howler monkey (*Alouatta pigra*), and the spider monkey (*Ateles geoffroyi*). These primates are all considered to be endangered species by Mexican law (SEMARNAT, 2010) and are threatened mainly by habitat loss and the illegal pet trade (Duarte-Quiroga and Estrada, 2003; Rodríguez-Luna et al., 2009). As habitat fragmentation and landscape anthropogenization increases, encounters between primates and domestic fauna and humans have become more common, and a clear parasitological diagnosis is critical to evaluate the possibilities of cross-infections and the risks that this could have for primate conservation and human health. The proper identification of parasite species is essential to addressing this issue.

We present the results of a parasite survey of these three Mexican primates along their distribution range in Mexico. Non-invasive sampling methods were combined with molecular techniques to enhance parasite species identification via DNA extraction of the eggs found in the primate faeces and by inferring their phylogenetic position. In addition, a list of all the helminths parasitizing primates in Mexico was summarized from available bibliographical sources with the aim of generating a checklist of the helminths in this group of mammals. This information could serve as a basis for future research on primate parasitology, assisting with a more accurate identification of parasite species. This could provide a more precise evaluation of their zoonotic potential, the implications for primate conservation and management and for public health.

2. Methods

2.1 Sample collection and parasitological examinations

The study area comprises the tropical rainforests of southeastern Mexico, including fragmented and continuous forests, protected and unprotected areas, and agroforestry lands across the primates distribution range in Mexico. A total of 420 samples were collected between 2013 and

2015 from 68 primate troops inhabiting 52 localities (Fig. 1). All samples correspond to free-ranging populations, except those in Villahermosa and Palenque, which correspond to captive populations in zoos. In most localities, more than one forest location was surveyed.

Non-invasive sampling techniques were employed, collecting faecal samples immediately after defecation to avoid contamination. In general, a single monkey troop was surveyed in one day, starting the collection at dawn and moving along with the troop to gather as many samples as possible, avoiding repeatedly sampling the same individual. On occasions where the monkey troop was too small (< 10 individuals) or there were many troops nearby, more than one troop was surveyed in a day. Faecal samples were placed in 50 ml falcon tubes, and stored at 4°C until transported to the laboratory, where they were preserved at -20°C. Preserved samples were examined for parasite eggs under direct light microscopy (10xm 40x, 100x) using flotation in saturated sodium chloride solution and simple sedimentation techniques (Greiner and McIntosh, 2009). Both methods were performed for each collected sample. The initial identification of the parasites was based on egg morphology, shape, size and colour. The percentage of infected hosts was estimated for each parasite taxa in each host species; in addition, we also quantified the number of hosts that were infected by at least one helminth species.

When a positive drop for any type of parasite was observed, the entire drop was transferred to a new slide and observed under the stereoscope, where eggs with different appearances were individually separated with the aid of a 0.5-10 µl micropipette and sited in a drop of distilled water (5 µl) on a new slide. The eggs were rinsed several times in fresh drops of distilled water to remove the concentrated solution and then placed in 0.5-ml Eppendorf tubes with 7 µl of distilled water and kept at -20°C until DNA extraction. Each egg morphotype was measured (length and width) and photographed to characterize its shape.

DNA was successfully extracted from a pool of 5 eggs of the same general appearance using the SIGMA REDExtract-N-Amp Tissue PCR Kit (St. Louis, MO, USA) and the Chelex® 100 (Bio-Rad, Richmond, CA, USA) chelating resin method. Whenever possible, two molecular

markers were used for species identification: the mitochondrial cytochrome c oxidase subunit 1 gene (cox1) and a region of the large subunit of the nuclear ribosomal gene (28S). For cox1, two sets of primers amplifying adjacent regions were used: pr-a: 5'-TGGTTTTTTGTGCATCCTGAGGTTTA-3', pr-b: 5'-AGAAAGAACGTAATGAAAATGAGCAAC-3' (Nakano et al., 2006), and LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3', HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al., 1994). PCR conditions for cox1 were as follows: initial denaturation at 94°C for 1 min, followed by 30 cycles at 94°C for 1 min, 40°C for 1 min, 72°C for 2 min, and post-amplification extension for 7 min at 72°C.

The 28S primers included 502: 5'-CAAGTACCGTGAGGGAAAGTTGC-3', and 536: 5'-CAGCTATCCTGAGGGAAAC-3' (García-Varela and Nadler, 2005). PCR conditions for 28S were as follows: 94°C for 4 min, followed by 34 cycles at 94°C for 0:30 min, 54°C for 0:50 min, 72°C for 1:30 min, and a post-amplification extension for 7 min at 72°C. PCR products were treated with Exo-SAP (Thermo scientific) according to the manufacturer's instructions and were sequenced at the Instituto de Biología, Universidad Nacional Autónoma de México. Sequences obtained in this study were deposited in GenBank (Supplementary material S1).

2.2 Phylogenetic analyses

To accomplish species identification, at least one molecular marker was used for each parasite taxa. The 28S sequences were used for all egg morphotypes, since it has been mentioned that ribosomal DNA performs better for diagnostic proposes than mitochondrial DNA (Blouin, 2002). In few cases, two molecular markers were used for phylogenetic analyses, as in the case of *Strongyloides* spp.

DNA sequences were aligned using CLUSTAL W and MESQUITE v. 2.75. For cox1, no gaps were required to align the nucleotide sequences. To infer the phylogenetic position of the different eggs within the phylogeny of the major helminth group they belong to (usually at the level

of order or family), we used a set of DNA sequences available in GenBank, using the closest identifiable egg species as a proxy by conducting a nucleotide blast (BLASTN) (Supplementary material S1). Phylogenetic analyses were conducted by Bayesian Inference (BI) employing Monte Carlo Markov Chain analysis in the program MrBayes v. 3.2.2 (Ronquist and Huelsenbeck, 2003) as implemented in the CIPRES Science Gateway (Miller et al., 2010). MrModeltest v. 2.3 (Nylander, 2004) was used to select the best model of evolution for each gene for each egg species using the Akaike information criterion. The Bayesian analyses included two simultaneous runs of Markov chain Monte Carlo, each for four million generations, sampling trees every 4000 generations, with a heating parameter value of 0.2 and a "burn-in" of 25%. A 50% majority-rule consensus tree was constructed from the post burn-in trees. Genetic divergence (p-distance) was calculated using MEGA v. 6 (Tamura et al., 2013); standard error of the distances was estimated by bootstrap resampling with 100 replications.

3. Results

3.1 Parasite diversity and percentage of infected hosts

Alouatta palliata contained the highest number of samples infected with at least one parasite species (97/126), followed by *Ateles geoffroyi* (124/248) and *Alouatta pigra* (19/46). Nine parasite taxa were found in the three primate species, the majority of which were nematodes, along with one species of trematode (Table 1, Fig. 2). Parasite species richness was similar in the two species of *Alouatta*, with three taxa per howler monkey species, while seven taxa of parasites were found in *A. geoffroyi.*

In general, nematodes of the genus *Trypanoxyuris* reached the highest percentage of infection in all primates. The eggs of these pinworms are morphologically undistinguishable among species (Solórzano-García et al., 2015, 2016); fortunately, adult pinworms were present in most of the faeces, making it possible to identify them at the species level. However, this was not the case

for the other nematodes, i.e., *Strongyloides* sp., the ancylostomid, and the ascarid, for which egg morphology is not a reliable method to establish species identification.

The helminth parasite fauna of the three species of primates is composed of 23 species, based on the information available in different bibliographical sources and the information provided by our field survey of the last two years. Of the 23 species, there are 3 platyhelminthes (2 trematodes and 1 cestode), 1 acanthocephalan, and 19 nematodes (Supplementary material S2). Parasite species richness is higher in *Alouatta palliata,* with 14 taxa reported, followed by *A. pigra* with 13 taxa, and *Ateles geoffroyi* with 11 taxa. *Alouatta palliata* is the most studied primate, since 17 of the 39 available parasitological reports address that species, while *A. pigra* and *A. geoffroyi* have been the focus of 11 studies each. Most of the parasitological research has been conducted with free-ranging primate populations (60%), with an equal number of studies in semi-captivity and captivity conditions (20% each).

3.2 Molecular identification of the eggs and the phylogenetic analysis

We were able to extract and amplify DNA from four of the six different egg morphotypes found in the faeces. The ancylostomatid and the ascarid eggs could not be sequenced because only two eggs for each of these taxa were found in the faeces. We successfully amplified the 28S for all the egg morphotypes. The mitochondrial gene, cox1, was more difficult to amplify, and we were only able to obtain a sequence for *Strongyloides* sp. and only 3 species of *Trypanoxyuris*.

Trypanoxyuris eggs

We were able to sequence both the cox1 and 28S genes for *Trypanoxyuris* eggs obtained from the three species of Mexican primates. We obtained sequences 700 bp long for the 28S gene from the eggs of five *Trypanoxyuris* species. The final alignment, including the sequences from GenBank, consisted of 19 terminals, including both adult and egg sequences. This alignment was trimmed to the 700 bp obtained to ensure comparison of the homologous regions. For cox1 gene,

we were able to obtain sequences 673 bp long from the eggs of *T. minutus, T. atelis* and *T. multilabiatus.* The final alignment was trimmed to 605 bp and consisted of 18 taxa including sequences from Genbank. Phylogenetic analysis on both genes placed each egg with its corresponding pinworm species with high nodal support through posterior probabilities (Fig. 3).

Trematodes eggs

Two different trematode egg morphotypes were found in the faeces of *A. palliata* and *A. geoffroyi*: one corresponding to *Controrchis biliophilus* and the other differing from this in the appearance of the content material inside the egg (see Fig. 2). Sequences of 786 bp for 28S were obtained for a sample of each trematode egg from each of the two host species. The final alignment, including the sequences from GenBank for the family Dicrocoeliidae and other species included in the order Plagiorchiida, consisted of 17 sequences. This alignment was trimmed to the 786 bp obtained to ensure a comparison of homologous regions. The phylogenetic tree shows that all egg sequences belong to the same clade regardless of differences in the egg shape and host species (Fig. 4), indicating that both egg morphotypes correspond to *C. biliophilus*. These relationships are supported by high posterior probability values. The clade containing the *C. biliophilus* sequences is placed as a sister taxon of the *Dicrocoelium* species within the family Dicrocoeliidae.

Strongyloides eggs

We were able to sequence both the cox1 (1079 bp) and 28S (686 pb) genes for *Strongyloides* eggs obtained from *Ateles geoffroyi*. Final alignment of cox1 included 9 species of *Strongyloides* from different host species; it was trimmed to 721 bp because some of the GenBank sequences were shorter. For 28S, the alignment consisted of 19 terminals including 10 species of *Strongyloides* from different host species. This alignment was trimmed to 686 bp to ensure a comparison of homologous regions; nevertheless, missing data (“?”) was allowed to expand the number of taxa

compared, specifically to include *S. cebus*, which in conjunction with *S. venezuelensis* had 52% and 55% of missing data, respectively.

Phylogenetic analyses were carried on separately for each molecular marker to assess its utility in species identification. Molecular analysis confirmed that these eggs belonged to the genus *Strongyloides*; however, its position within each phylogenetic tree varied between genes. For the 28S the two sequences obtained were identical to each other. The tree shows that our sequences are nested in an unresolved clade along with *S. fuelleborni* and a group containing 3 species, i.e., *S. cebus, S. venezuelensis and S. callosciureus* (Fig. 5). Genetic divergence between our samples and the other 4 species included in the clade varied from 4.7 to 5.1%. The cox1 tree shows that the only sample we were able to sequence for this marker for *Strongyloides* is nested as a sister species of a clade formed by *S. papillosus*, *S. fuelleborni* and *S. venezuelensis* (Fig. 5), with a sequence divergence of 18.9%, 14.4% and 16.4%, respectively. Needless to say, not all the same species of the family Strongyloididae are represented in both trees because sequences of both molecular markers are not yet available.

5. Discussion

Though primates are a relatively well studied group in Mexico (Estrada and Mandujano, 2003), only a few studies have focused on assessing their parasite diversity, and only free-ranging primate populations of six regions have been previously surveyed (Stroner and Gonzalez-Di Pierro, 2006; Trejo-Macias et al., 2007; Vitazkova and Wade, 2007; Cristobal-Azkarate et al., 2010). The information presented here increases the number of localities where parasites of these primates have been studied, contributing to a more complete parasitological evaluation across the distribution range of these primates in Mexico. The parasites found in the present study correspond with the previously reported taxa for the three Mexican primates; however, despite the more extensive sampling, the parasite species richness found was lower. Eggs of *Raillietina* sp., Strongylidae and *Necator* sp. have been previously found in the faeces of Mexican primates (Supplementary material

S2), and species such as *Ascaris lumbricoides, Calodium hepaticum, Dipetalonema gracile* and *Parabronema bonnei*, have been reported from necropsies of *A. palliata* and *A. geoffroyi* (Caballero, 1948; Caballero and Grocott, 1952; Villanueva-Jimenez, 1988); however, we did not find eggs that looked such as these parasites in any of the revised samples.

Given that the majority of parasitological studies on Mexican primates are based on egg identification, we cannot discard the possibility that some of those reported parasite taxa are a result of sample contamination or even a possible misidentification. Another limiting factor in accomplishing proper taxonomic identification is that egg samples are not deposited in parasite collections, and thus the identification cannot be independently verified and relies on the photographs provided by the authors. For example, *Enterobius* sp. have been reported for the three Mexican primates (García-Serrano, 1995; Rodríguez-Velázquez, 1996; Stroner and Gonzalez-Di Pierro, 2006; Supplementary material S2); nevertheless, co-evolutionary studies have shown that pinworms of the genus *Enterobius* only parasitize Old World primates and that *Trypanoxyuris* is the genus of pinworms found in New World monkeys (Brooks and Glen, 1982; Hugot et al., 1996; Hugot, 1999, 1998). Oxyurid eggs are very similar among members of the Enterobiinae subfamily, making it possible to mistake species. Moreover, molecular studies on pinworm diversity in Mexican primates have shown that five *Trypanoxyuris* species are found in these hosts (Solórzano-García et al., 2015, 2016). For these reasons, we believe that the records of *Enterobius* previously mentioned in the literature are in fact *Trypanoxyuris*.

Similarly, ancylostomatid eggs have been reported for *A. palliata* only in one location (Cristobal-Azkarate et al., 2010). According to the photographs of the two eggs presented in that study, the eggs lack the characteristic features of ancylostomatid eggs, such as a thin, smooth and colourless shells containing embryonic blastomeres (Rai et al., 1996) and instead resemble the eggs of *Parabronema bonnei* and a trematode, respectively. Two other nematodes, *Necator* sp. and *Trichostrongylus* sp., have been reported as parasites of spider monkeys in captivity (González Hernández, 2004; Villa-Espinoza, 2011; Suplementary information S2). Since these parasites have

not been reported in a free-ranging population, their presence could be the result of enclosure conditions, close contact with humans and other animals in captivity, and the health status of other animals in the zoos; thus, these parasites do not necessarily belong to the natural parasitic fauna of this primate.

The results presented here show that the application of molecular and phylogenetic methods could help overcome the limitations imposed by traditional non-invasive sampling. As suggested by Criscione et al. (2005), one of the three key uses of molecular markers is to link morphologically indistinguishable life stages to adult stages of known species. However, the utility of this approach relies in the availability of molecular information from previous parasitological studies, as exemplified by the three types of parasite eggs that we were able to sequence. *Trypanoxyuris* is undoubtedly the taxa with the most available information. Molecular data from the adults of the five pinworm species parasitizing Mexican primates have been published (Solórzano-García et al., 2015, 2016), which made egg identification via DNA analysis straightforward. This nematode genus contains 21 species that parasitize primates across the neotropics. The identification of the different *Trypanoxyuris* species can be easily obtained by sequencing samples from different host species and areas, increasing the extant genetic library.

Controrchis biliophilus is the only reported trematode species in Mexican primates (Supplementary material S2). The eggs are characterized by its brown colour, a thick shell and the presence of two readily visible eyespot remnants (Jiménez-Quiros and Brenes, 1957). Even though no molecular information is available for the trematode *C. biliophilus*, adult worms are held in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, which allowed us to confirm the identity of these eggs. Samples of *C. biliophilus* were collected from a troop of howler monkeys translocated to Agaltepec Island in Catemaco, Veracruz (Villanueva-Jimenez, 1988). We obtained sequences of *C. biliophilus* eggs from the same locality, enabling a greater confidence in the identification of the parasite. Trematode eggs with a slightly different morphology, specifically lacking the two eyespot remnants, has been previously reported

by Trejo-Macias et al. (2007), and this morphology was also observed in the present study for samples from *A. palliata* and *A. geoffroyi*. The molecular and phylogenetic analysis showed no differences in the 28S DNA sequences between *C. biliophilus* and the trematode egg with a slightly different morphology, confirming that this particular egg morphotype also corresponds with *C. biliophilus*.

Finally, *Strongyloides* sp. has been reported in Mexican primates, but species determination has not been established because the eggs of *Strongyloides* lack morphological features that allow for discrimination among a wide diversity of species. There are over 40 species of *Strongyloides* that parasitize vertebrates (Dorris et al., 2002). *Strongyloides stercoralis* and *S. fuelleborni* have been found in primates (Gillespie and Chapman, 2006; Chapman et al., 2009; Dupain et al., 2009), and *S. cebus* has been mentioned as the only species that naturally infects Neotropical primates (Mati et al., 2013). The phylogenetic analysis presented here confirmed that the eggs belonged to *Strongyloides*; however, they appeared to have a closer phylogenetic association with *S. fuelleborni* than with *S. cebus.* Furthermore, the genetic divergence between the eggs found and the species of *Strongyloides* for which sequences are available suggests that these might represent a new species, although this cannot be established at the moment due to the lack of additional DNA sequences from *Strongyloides* eggs occurring in Mexican primates. Unfortunately, we were not able to find any larvae or adults in the faeces that would allow us to take this inquiry any further.

Another important parasite is *Ascaris lumbricoides*. Adults of this species were found in a necropsy of an *A. palliata* specimen that died from natural causes in Los Tuxtlas, Mexico (see García-Prieto et al., 2012). This record adds to those made by several authors based on eggs found in the faeces, described as *Ascaris* sp., in the three Mexican primates (González Hernández, 2004; Cristobal-Azkarate et al., 2010; Trejo-Macias, 2010). *Ascaris lumbricoides* is a human parasite commonly found in other primates such as chimpanzees and gorillas (Lilly et al., 2002; Dupain et al., 2009). It has also been reported in several howler monkey species (Stuart et al., 1990,1998) and other Neotropical primates (Michaud et al., 2003) but is unusual, and in most cases these primates

were in close contact with humans. In the present study, we found two eggs of an ascarid in samples from *A. geoffroyi*. Unfortunately, we were not able to extract DNA from these eggs, but we believe that a more intensive sampling, where a large number of eggs could be gathered, would provide the molecular data needed to clarify the taxonomic identity of the ascarid and to elucidate its zoonotic potential.

The presence of parasites such as *Strongyloides fuelleborni* and *Ascaris lumbricoides*, both common human parasites and with the capability of causing severe illness (Crompton, 2001; Olsen et al., 2009), is of major concern for the conservation of free-ranging primate populations. A precise confirmation that these parasite species are occurring in Neotropical primates remains essential to determine possible transmission routes and the potential effects that habitat fragmentation and the increase of human encroachment into wildlife territory could have on the spread of these parasites. For example, by applying molecular techniques, Gasser et al., (2009) were able to show that *Oesophagostomum bifurcum* in humans was genetically distinct from those harboured by non-human primates, concluding that non-human primates were not reservoir hosts for human oesophagostomiasis and that the genetic variants had different transmission patterns.

There is no doubt that more research is needed to properly characterize the parasites of Neotropical primates. The combination of molecular techniques with non-invasive sampling methods has proved to be effective for a better understanding of parasite diversity, transmission modes, and evolutionary history. Even though parasite eggs found in faeces are vast sources of information, the isolation of eggs along with DNA extraction and amplification are highly laborious tasks. A wide variety of methods have been described to obtain DNA from parasite eggs found in faeces (Štefanić et al., 2004; Harmon et al., 2006; Trachsel et al., 2007; Demeler et al., 2013; Federer et al., 2016). Nevertheless the standardization of the molecular diagnostic procedures still remains a critical issue in order for such techniques to be widely applied in the parasitological study of endangered species. As new molecular methods emerge, such as new generation sequencing and meta-genomic analysis, and the costs for their application become more accessible, the surveillance

of the parasitic fauna of endangered species through non-invasive sampling will be easily accomplished and more accurate (Srivathsan et al., 2016). Nevertheless, this surveillance will rely on current efforts to molecularly typify the parasites found in these hosts.

The principal aim of this study was to present molecular data from the parasite eggs found in the faeces that could serve as a basis for future parasitological assessments on New World primates. The results presented here support the contention that ribosomal genes are more suitable than mitochondrial DNA for species diagnosis (Blouin, 2002). Since the divergence levels found for the amplified region of the 28S gene (within the same parasite species) was really low, we pose this should be the marker used for parasite species diagnosis. A more accurate parasite diagnosis would enable us to understand the ecological and evolutionary background of parasite-host associations, possibilities for cross-transmissions and their implications for primate conservation. Likewise, the proper identification of parasites when managing primate populations in captivity or for conservation proposes is essential. This is particularly important when moving individuals among zoos around the world, or when they are subjected to reintroduction and translocation programmes (Nunn and Altizer, 2006), to avoid disease outbreaks by the introduction of novel parasites that could threat the resident populations, including non-primates.

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390
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578
579
580 **Fig. 1.** Surveyed sites for parasites in Mexican primates. Dots indicate sampling sites, black:
581 *Alouatta palliata*; white: *A. pigra;* and grey: *Atetes geoffroyi*. Polygons indicate the primate
582 distribution range in Mexico, diagonal lines: *A. palliata;* dashes: *A. pigra;* and grey: *A. geoffroyi.*
583
584 **Fig. 2.** Egg morphotypes found in the faeces of Mexican primates. A) *Trypanoxyuris* sp., B)
585 *Controrchis biliophilus,* arrow pointing to the two eyespot remnants; C) trematode, diagnosed as *C.*
586 *biliophilus* by molecular data; D) unidentified ancylostomatid; E) *Strongyloides* sp.; F) unidentified
587 ascarid. Scale bar is equal to 15 µm.
588
589 **Fig. 3.** Phylogenetic trees based on 28S (left) and cox 1 (right) sequences of *Trypanoxyuris* sp.
590 Sequences obtained from the eggs are bold type and indicated with an *. Numbers at the nodes
591 represent posterior probabilities from Bayesian inference.
592
593 **Fig. 4.** Phylogenetic tree based on 28S sequences of *Controrchis biliophilus*. Sequences obtained
594 from the eggs are bold type and indicated with an *. Numbers at the nodes represent posterior
595 probabilities from Bayesian inference. Host species are indicated within parenthesis.

596

597 **Fig. 5.** Phylogenetic trees based on 28S (left) and cox 1 (right) sequences of *Strongyloides* eggs.

598 Sequences obtained from the eggs are bold type and indicated with an *. Numbers at the nodes

599 represent posterior probabilities from Bayesian inference.

600

601

Figure 1

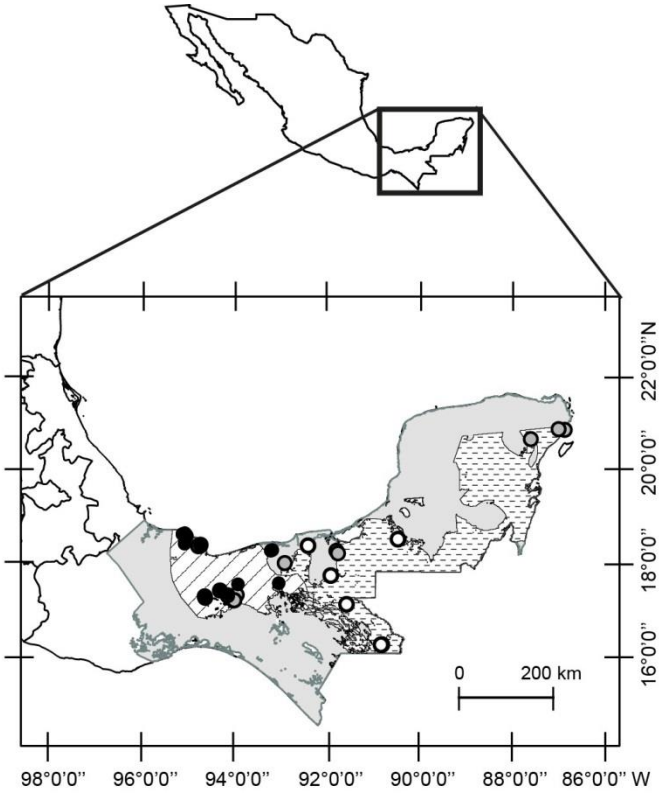
22°0'0"N
20°0'0"
18°0'0"
16°0'0"
0
200 km
98°0'0"
96°0'0"
94°0'0"
92°0'0"
90°0'0"
88°0'0"
86°0'0" W

Figure 2

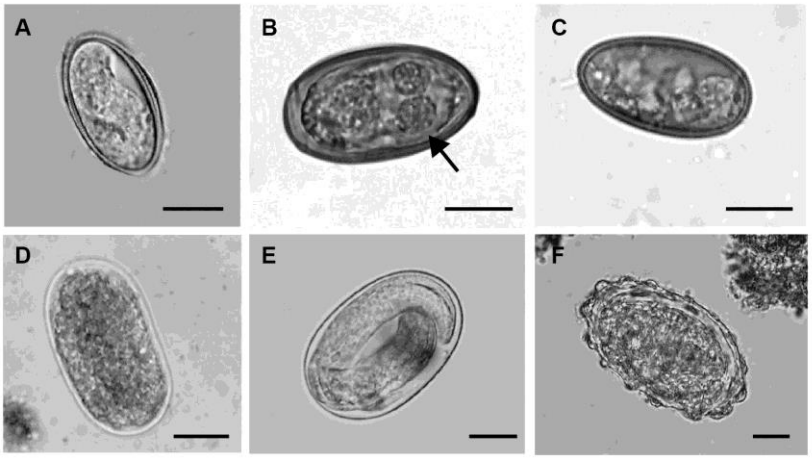

Figure 3

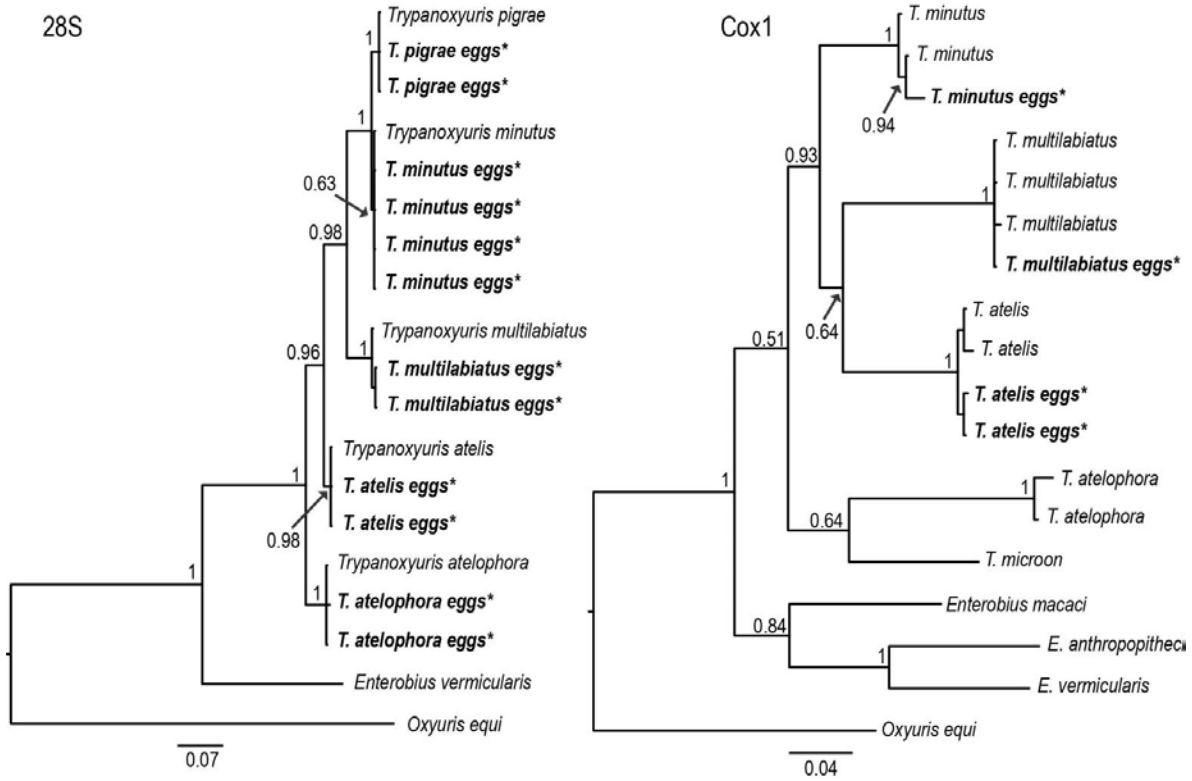
28S
Trypanoxyuris pigrae
T. pigrae eggs*
T. pigrae eggs*
Trypanoxyuris minutus
T. minutus eggs*
T. minutus eggs*
T. minutus eggs*
T. minutus eggs*
Trypanoxyuris multilabiatus
T. multilabiatus eggs*
T. multilabiatus eggs*
Trypanoxyuris atelis
T. atelis eggs*
T. atelis eggs*
Trypanoxyuris atelophora
T. atelophora eggs*
T. atelophora eggs*
Enterobius vermicularis
Oxyuris equi
0.07
Cox1
T. minutus
T. minutus
T. minutus eggs*
T. multilabiatus
T. multilabiatus
T. multilabiatus
T. multilabiatus eggs*
T. atelis
T. atelis
T. atelis eggs*
T. atelis eggs*
T. atelophora
T. atelophora
T. microon
Enterobius macaci
E. anthropopitheci
E. vermicularis
Oxyuris equi
0.04

Figure 4

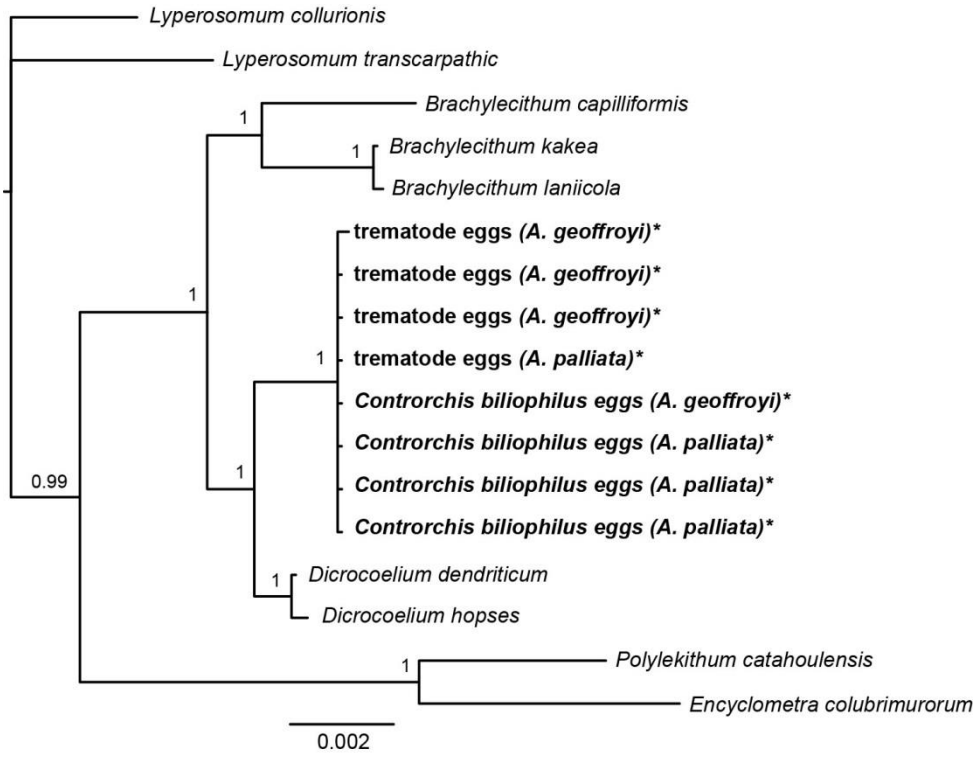
Lyperosomum collurionis
Lyperosomum transcarpathic
Brachylecithum capilliformis
Brachylecithum kakea
Brachylecithum laniicola
trematode eggs (A. geoffroyi)*
trematode eggs (A. geoffroyi)*
trematode eggs (A. geoffroyi)*
trematode eggs (A. palliata)*
Controrchis biliophilus eggs (A. geoffroyi)*
Controrchis biliophilus eggs (A. palliata)*
Controrchis biliophilus eggs (A. palliata)*
Controrchis biliophilus eggs (A. palliata)*
Dicrocoelium dendriticum
Dicrocoelium hopses
Polylekithum catahoulensis
Encyclometra colubrimurorum
1
1
1
1
1
1
1
1
0.99
0.002

Figure 5

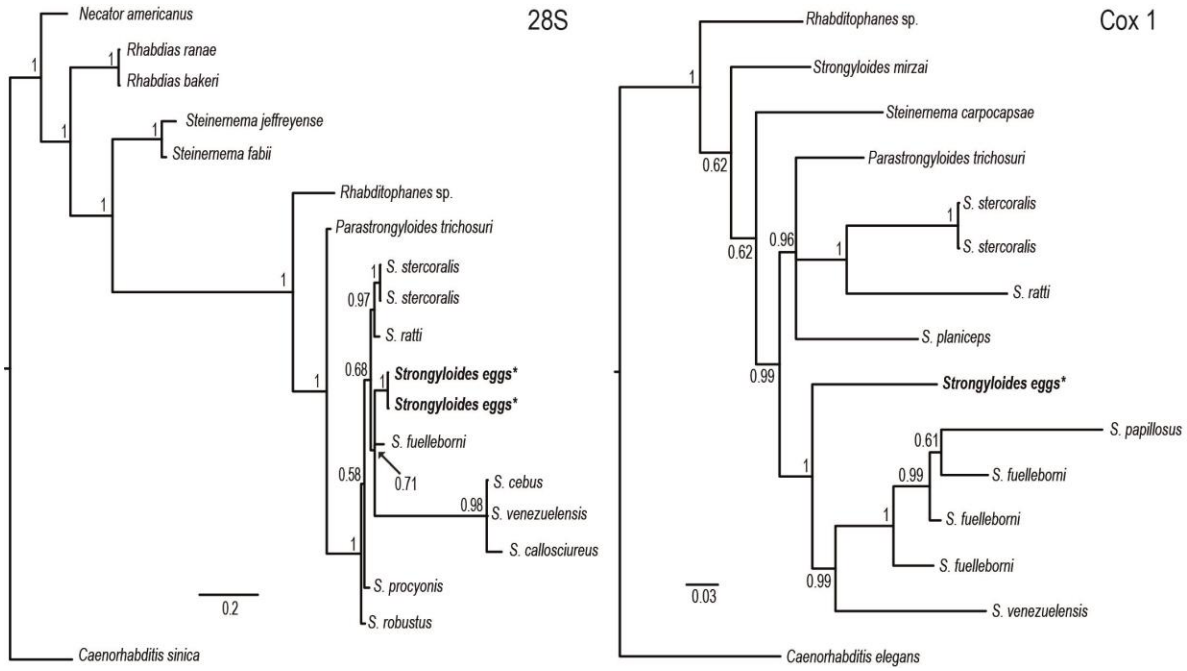
28S
Necator americanus
Rhabdias ranae
Rhabdias bakeri
Steinernema jeffreyense
Steinernema fabii
Rhabditophanes sp.
Parastrongyloides trichosuri
S. stercoralis
S. stercoralis
S. ratti
Strongyloides eggs*
Strongyloides eggs*
S. fuelleborni
S. cebus
S. venezuelensis
S. callosciureus
S. procyonis
S. robustus
0.2
Caenorhabditis sinica
Cox 1
Rhabditophanes sp.
Strongyloides mirzai
Steinernema carpocapsae
Parastrongyloides trichosuri
S. stercoralis
S. stercoralis
S. ratti
S. planiceps
Strongyloides eggs*
S. papillosus
S. fuelleborni
S. fuelleborni
S. fuelleborni
S. venezuelensis
0.03
Caenorhabditis elegans

Table 1. Percentage of infection of parasites and sampling effort for each Mexican primate species.

Parasite Phylum	Parasite taxa	*A. palliata*	*A. pigra*	*A. geoffroyi*
Platyhelminthes	*Controrchis biliophilus*	10.3%		2.0%
Nematoda	Ancylostomatid			1.6%
	Ascarid			0.8%
	Strongyloides sp.	2.2%	13.3%	
	Trypanoxyuris sp.	7.1%	15.2%	14.5%
	T. atelis			17.7%
	T. atelophora			9.7%
	T. minutus	57.9%	2.2%	
	T. multilabiatus	10.3%		
	T. pigrae		23.9%	
Sample size		126	46	248
Localities sampled		9	6	15
Forest fragments sampled		17	9	26
Troops sampled		22	10	36

Supplementary material S1. Taxa used in phylogenetic analysis and GenBank accession numbers

Species	Host	Source	Gene	GenBank
Brachylecithum capilliformis	*Locustella fluviatilis*	Genbank	28S	KU212184
Brachylecithum kakea	*Acrocephalus arundinaceus*	Genbank	28S	KU212180
Brachylecithum laniicola	*Lanius collurio*	Genbank	28S	KU212183
Caenorhabditis elegans	free living	Genbank	Cox 1	NC001328
Caenorhabditis sinica	free living	Genbank	28S	KF732844
Controrchis biliophilus	*Alouatta palliata*	eggs	28S	KY200964 KY200966-67 KY200969
Controrchis biliophilus	*Ateles geoffroyi*	eggs	28S	KY200963 KY200965 KY200968 KY200970
Dicrocoelium dendriticum	cattle	Genbank	28S	AF151939
Dicrocoelium hospes	cattle	Genbank	28S	AY251233
Encyclometra colubrimurorum	*Natrix natrix*	Genbank	28S	AF184254
Enterobius anthropopitheci	Chimpanzee	Genbank	Cox 1	AB626860
Enterobius macaci	*Macaca fuscata*	Genbank	Cox 1	AB626858
Enterobius vermicularis	human	specimen	28S	
		Genbank	Cox 1	AP017684
Lyperosomum collurionis	*Sylvia atricapilla*	Genbank	28S	AY222259
Lyperosomum transcarpathic	*Sorex minutus*	Genbank	28S	AF151943
Necator americanus	Mammals	Genbank	28S	KU180694
Oxyuris equi	horse	Genbank	28S	KU180675
		Genbank	Cox 1	KP404095
Parastrongyloides trichosuri	*Trichosurus vulpecula*	Genbank	28S	AB923880
		Genbank	Cox 1	LC050209
Polylekithum catahoulensis	*Ictalurus furcatus*	Genbank	28S	EF032698
Rhabdias bakeri	*Lithobates sylvaticus*	Genbank	28S	EU360840
Rhabdias ranae	*Lithobates pipiens*	Genbank	28S	EU360844
Rhabditophanes sp.	fungus gnat	Genbank	28S	JX674036
	free living	Genbank	Cox 1	LC050214
Steinernema carpocapsae	*Galleria mellonella*	Genbank	Cox 1	AY591323

Steinernema fabii	*Galleria mellonella*	Genbank	28S	KR527217
Steinernema jeffreyense	*Galleria mellonella*	Genbank	28S	KP164866
Strongyloides cebus	*Saimiri sciureus*	Genbank	28S	AB272236
Strongyloides callosciureus	*Callosciurus erythraeus*	Genbank	28S	AB272230
Strongyloides fuelleborni	*Macaca fuscata*	Genbank	28S	AB272235
		Genbank	Cox 1	AB526292
Strongyloides fuelleborni	human	Genbank	Cox 1	AB526282
Strongyloides fuelleborni	*Gorilla gorilla*	Genbank	Cox 1	AB526289
Strongyloides mirzai	*Trimeresurus flavoviridis*	Genbank	Cox 1	AB526307
Strongyloides papillosus	sheep	Genbank	Cox 1	LC050210
Strongyloides planiceps	*Nyctereutes procyonoides*	Genbank	Cox 1	AB526296
Strongyloides procyonis	*Procyon lotor*	Genbank	28S	AB205054
Strongyloides ratti	wild rat	Genbank	28S	LN609412
		Genbank	Cox 1	LC050211
Strongyloides robustus	*Glaucomys volans*	Genbank	28S	AB272232
Strongyloides sp.	*Ateles geoffroyi*	eggs	28S	KY200971-72
		eggs	Cox 1	submitted
Strongyloides stercoralis	human	Genbank	28S	DQ145661 KU180693
		Genbank	Cox 1	LC050212 NC028624
Strongyloides venezuelensis	wild rat	Genbank	28S	AB923884
		Genbank	Cox 1	LC050213
Trypanoxyuris atelis	*Ateles geoffroyi*	eggs	28S	KY200959-60
		eggs	Cox 1	submitted
		Genbank	28S	KU285474
		Genbank	Cox1	KP266344 KP266347
Trypanoxyuris atelophora	*Ateles geoffroyi*	eggs	28S	KY200961-62
		eggs	Cox 1	submitted
		Genbank	28S	KU285477
		Genbank	Cox1	KP266376 KP266371
Trypanoxyuris microon	*Aotus azarae*	Genbank	Cox 1	AB626878
Trypanoxyuris minutus	*Alouatta palliata*	eggs	28S	KY200951-54

		eggs	Cox 1	submitted
		Genbank	28S	KU285464
		Genbank	Cox 1	KU285479-80
Trypanoxyuris multilabiatus	*Alouatta palliata*	eggs	28S	KY200957-58
		eggs	Cox 1	submitted
		Genbank	28S	KU285473
		Genbank	Cox 1	KU285487-89
Trypanoxyuris pigrae	*Alouatta pigra*	eggs	28S	KY200955 -56
		eggs	Cox 1	submitted
		Genbank	28S	KU285469

Supplementary data S2. List of helminth species in three species of primates in Mexico including our field data and information from various bibliographical sources.

Primate host	Phylum	Parasite taxa	Locality	References
Mono aullador de manto (*Alouatta palliata*)	Platyhelminthes	*Controrchis biliophilus**$^{O\,\Delta}$	Los Tuxtlas, Ver.	[1–5]
			Agaltepec, Ver.	[5,6]
			Sierra de Santa Marta, Ver.	[5,7,8]
			Uxpanapa, Ver.	[5]
			Villahermosa, Tab.	[9]
			Comalcalco, Tab.	[5]
			Pichucalco, Chp.	[5]
		Raillietina sp*	Los Tuxtlas, Ver.	[4]
		Trematode*	Los Tuxtlas, Ver.	[2]
	Acanthocephala	*Prosthenorchis elegans* *	Los Tuxtlas, Ver.	[4]
	Nematoda	Ancylostomatidae*	Los Tuxtlas, Ver.	[3]
		Ascaris sp.*	Los Tuxtlas, Ver.	[3]
		Enterobius sp.$^{\Delta}$	Los Tuxtlas, Ver.	[1]
		Nematode	Los Tuxtlas, Ver.	[4]
		Parabromena sp.*	Los Tuxtlas, Ver.	[2–4]
		*P. bonnei**^{O}	Sierra de Santa Marta, Ver.	[7]
			Agaltepec, Ver.	[6]
		Trypanoxyuris sp.*	Los Tuxtlas, Ver.	[2,10]
		*T. minutus**$^{O\,\Delta}$	Los Tuxtlas, Ver.	[3,4,11,12]
			Sierra de Santa Marta, Ver.	[7,8,12]
			Agaltepec, Ver.	[6,12]
			Uxpanapa, Ver.	[12]
			Villahermosa, Tab.	[9]
			Comalcalco, Tab.	[12]
			Pichucalco, Chp.	[12]
		*T. multilabiatus**	Agaltepec, Ver.	[12]
			Sierra de Santa Marta, Ver.	[12]
			Uxpanapa, Ver.	[12]
			Comalcalco, Tab.	[12]
			Pichucalco, Chp.	[12]

		Strongylidae*	Los Tuxtlas, Ver.	[4]
		Strongyloides sp.*$^{\Delta}$	Los Tuxtlas, Ver.	[1,3,10]
Mono aullador negro	Platyhelminthes	*Controrchis* sp.*	Montes Azules, Chp.	[13]
(*Alouatta pigra*)		*C. biliophilus**	Montes Azules, Chp.	[2]
			Palenque, Chp.	[2,4,14]
			Calakmul, Cmp.	[2,14]
		Raillietina sp.*	Montes Azules, Chp.	[2]
			Palenque, Chp.	[2,4]
			Calakmul, Cmp.	[2]
		Trematode*	Montes Azules, Chp.	[2]
			Palenque, Chp.	[2,4]
			Calakmul, Cmp.	[2]
	Acanthocephala	*Prosthenorchis elegans**	Palenque, Chp.	[4]
	Nematoda	Ascarididae*	Palenque, Chp.	[4]
		Nematode*	Palenque, Chp.	[4]
		Enterobius sp.*	Montes Azules	[13]
		Parabronema sp.*	Palenque, Chp.	[4]
		Trypanoxyuris sp.*	Reforma Agraria, Chp.	[5]
			Montes Azules, Chp.	[2]
			Palenque, Chp.	[2]
			Calakmul, Cmp.	[2]
		*T. minutus**	Metzabok, Chp.	[12]
			Palenque, Chp.	[4,11,14]
			Calakmul, Chp.	[14]
		*T. pigrae**	Metzabok, Chp.	[12]
			Catazajá, Chp.	[12]
			Pantanos de Centla, Tab.	[12]
			La Libertad, Cmp.	[12]
		Strongylid*	Montes Azules, Chp.	[2]
			Palenque, Chp.	[2,4]
			Calakmul, Cmp	[2]
		Strongyloides sp.*	Montes Azules, Chp.	[13]
			Catazajá, Chp.	[5]
		Trichostrongylid*	Montes Azules, Chp.	[13]
Mono araña	Platyhelminthes	*Controrchis* sp.*	Sierra Santa Marta, Ver.	[5]
(*Ateles geoffroyi*)			Reforma Agraria, Chp.	[5]

Nematoda	Ancylostomatidae*	Sierra Santa Marta, Ver.	[5]
		Uxpanapa, Ver.	[5]
	Ascaris sp.$^{\Delta}$	Veracruz, Ver.	[15]
	Ascarid*	Uxpanapa, Ver.	[5]
	*Calodium hepaticum**	Palenque, Chp.	[16]
	*Dipetalonema gracile**	Mapastepec, Chp.	[17]
		Campeche	[17]
	Enterobius sp.$^{\Delta}$	Los Tuxtlas, Ver.	[18]
	Necator sp. $^{\Delta}$	Veracruz, Ver.	[15]
	Trypanoxyuris sp.*$^{\Delta}$	Quintana Roo	[19]
		Morelia, Mich.	[20]
		Punta Laguna, QR.	[5]
	*T. atelis**$^{\Delta\,\circ}$	Sierra Santa Marta, Ver.	[21]
		Uxpanapa, Ver.	[21]
		Villahermosa, Tab.	[21]
		La Libertad, Cmp.	[21]
		El Zapote, Cmp.	[5]
		Reforma Agraria, Chp.	[5]
		Puerto Morelos, QR.	[5]
	*T. atelophora**$^{\circ}$	Sierra Santa Marta, Ver.	[21]
		Uxpanapa, Ver.	[21]
		La Libertad, Cmp.	[21]
		El Zapote, Cmp.	[5]
		Reforma Agraria, Chp.	[5]
		Puerto Morelos, QR:	[5]
	Strongyloides sp.$^{\Delta}$*	Veracruz, Ver.	[15]
		Los Tuxtlas, Ver.	[18]
		Sierra Santa Marta, Ver.	[5]
		Uxpanapa, Ver.	[5]
		Villahermosa, Tab.	[5]
		El Zapote, Cmp.	[5]
		Morelia, Mich.	[20]
		Palenque, Chp.	[5]
		Reforma Agraria, Chp.	[5]
	Trichostrongylus sp.$^{\Delta}$	Morelia, Mich.	[20]

Chp: Chiapas; Cmp: Campeche; Mich: Michoacán; QR: Quintana Roo; Tab: Tabasco; Ver: Veracruz

* Free-ranging hosts, $^{\circ}$host in semi-captivity, $^{\Delta}$ host in captivity.

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DISCUSIÓN GENERAL

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En este proyecto se buscó determinar el efecto del aislamiento de las poblaciones de primates, consecuencia de la fragmentación y pérdida del hábitat, sobre la genética de poblaciones de sus oxiuros parásitos. Paralelamente se aplicaron técnicas moleculares que nos permitieran emplear métodos de colecta no invasivos para estudiar a profundidad tanto la diversidad como los procesos evolutivos en parásitos de primates.

A pesar de que los primates mexicanos son un grupo relativamente bien estudiado (Estrada y Mandujano, 2003; Estrada et al., 2006a), la información existente en relación a su fauna parasitológica es escasa (Stroner y Gonzalez-Di Pierro 2006; Trejo-Macias et al. 2007; Vitazkova y Wade 2007; Cristobal-Azkarate et al. 2010; Valdespino et al. 2010). Este estudio incluyó diversas localidades para las que no existían registros parasitológicos, además de confirmar, con análisis morfológicos, moleculares y filogenéticos, la identidad de los helmintos parásitos presentes en las muestras colectadas, contribuyendo de manera importante al conocimiento de la parasitología de los primates en México.

Durante este estudio se identificaron ocho taxa de nematodos y una especie de trematodo parasitando a las tres especies de primates mexicanos, cuya identidad fue corroborada con datos moleculares. Estos resultados, junto con los reportes previamente publicados muestran que la helminto fauna de los primates mexicanos está compuesta por 17 especies de parásitos: 3 platelmintos, 1 acantocefalo y 13 nematodos.

Cinco especies de oxiuros fueron identificadas en base a caracteres morfológicos y moleculares: *T. atelis* y *T. atelophora* que se encuentran en el mono araña, *T. minutus* y *T. multilabiatus* parasitan al mono aullador de manto, mientras que *T. minutus* y *T. pigrae* se encuentran en el mono aullador negro. Es importante mencionar que antes de este estudio sólo *T. minutus* había sido reportado como parásito de estos primates en México y Centroamérica (Stuart et al., 1998; Vitazkova, 2009; Trejo-Macias et al., 2011) y en la mayoría de los trabajos publicados la especie se refería únicamente como *Trypanoxyuris* sp. Por tanto, este trabajo constituye no sólo el primer registro de *T. atelis* y *T. atelophora* en mono araña en México, sino la descripción de dos especies nuevas, *T. multilabiatus* y *T. pigrae.*

Las principales diferencias morfológicas entre los distintos *Trypanoxyuris* observados en este estudio coinciden con lo publicado anteriormente para otras especies de

Trypanoxyuris (Hugot 1985), indicando que los caracteres más conspicuos para distinguir entre las distintas especies de *Trypanoxyuris* son el número y forma de los labios, la forma del ala lateral y la estructura del esófago. Dichos rasgos son más fácilmente observables en hembras que en machos, ya que éstas son significativamente de mayor tamaño. Dado que la forma y el tamaño de los huevos son prácticamente iguales en todas las especies de *Trypanoxyuris,* la presencia de individuos adultos o el diagnóstico molecular a partir de los huevos presentes en las excretas son necesarios para hacer una identificación a nivel de especie.

A pesar de que monos aulladores y monos araña son simpátricos en gran parte de su distribución en México (Rylands et al., 2006), estos primates no comparten especies de *Trypanoxyuris,* evidenciando la alta especificidad hospedatoria que presentan estos nematodos. Se ha mencionado que oxiuros y primates presentan importantes asociaciones co-evolutivas, existiendo una especie de oxiuro por cada especie de primate (Hugot 1999); sin embargo, en este trabajo encontramos dos especies de *Trypanoxyuris* en cada especie de hospedero. Este patrón de co-ocurrencia no había sido reportado en los oxiuros de primates Neotropicales (Hugot 1999), a excepción del mono uakari (*Cacajao calvus*) el cual también es parasitado por dos especies de *Trypanoxyuris* (*T. cacajo* y *T. ucayaliik*) (Conga et al., 2015). Lo anterior parece indicar que la diversidad de oxiuros en primates Neotropicales es mayor que lo esperado. Más estudios parasitológicos en las distintas especies de primates Neotropicales son necesarios para determinar con mayor certeza la diversidad de *Trypanoxyuris* y para poder identificar los eventos coevolutivos que pudieron dar origen a las asociaciones entre oxiuros y primates.

Uno de los objetivos principales de este trabajo fue poner a prueba la hipótesis de que la fragmentación y pérdida del hábitat promueven la diferenciación y estructura genética en las poblaciones de parásitos a través del aislamiento espacial de las poblaciones del hospedero. Los nematodos del género *Trypanoxyuris* constituyen un buen modelo para estudiar dicho fenómeno por tres motivos principales: 1) son parásitos de ciclo de vida directo y que sobreviven muy poco tiempo fuera del hospedero (Adamson, 1989); 2) las poblaciones de sus hospederos se encuentran aisladas debido a los procesos de fragmentación y pérdida de hábitat (Rodríguez-Luna et al., 2009); 3) la estrecha relación evolutiva entre oxiuros y primates (Hugot 1999). Lo anterior fue evaluado en las dos

especies más abundantes de *Trypanoxyuris* y sus dos especies de hospederos: *Trypanoxyuris minutus* en el mono aullador de manto, y *T. atelis* en el mono araña.

Los distintos análisis realizados en este estudio muestran que no existe una estructura genética en las poblaciones de ambas especies de *Trypanoxyuris*, a pesar de la alta fragmentación del hábitat y el aparente aislamiento de las poblaciones de primates. Estudios en los que se ha buscado evaluar el efecto de la fragmentación del hábitat en la genética de poblaciones de distintas especies de flora y fauna silvestres en México no han encontrado diferencias significativas en comparación con poblaciones en hábitat continuo (González-Astorga y Núñez-Farfán, 2001; Figueroa-Asquivel et al., 2010), a excepción de algunos casos en donde se ha registrado que las poblaciones en fragmentos presentan una menor diversidad genética y una mayor diferenciación y estructura poblacional (Cuartas-Hernández y Núñez-Farfán, 2006; Oliveiras de Ita et al., 2012; Chávez-Pesqueira et al., 2014). Estos patrones observados sugieren que la erosión genética causada por una reducción, tanto en el tamaño poblacional como en el flujo genético, en aquellas poblaciones que habitan fragmentos no es tan intensa como se esperaría, o que los efectos completos resultantes de la fragmentación y pérdida del hábitat tardan muchas generaciones en poder detectarse.

La aparente panmixia entre las distintas poblaciones de *Trypanoxyuris* sugiere dos escenarios opuestos: 1) el aislamiento de los fragmentos de hábitat no constituye una barrera para estos oxiuros ya que algunos mecanismos de dispersión pasiva de huevos (por aire o agua), o bien un mayor movimiento de primates entre los fragmentos, han mantenido el flujo genético entre poblaciones de estos parásitos; 2) si existe un aislamiento causado por la fragmentación del hábitat, pero los grandes tamaños poblacionales en parásitos han diluido los efectos de la deriva génica y se necesita mucho más tiempo para que la estructura genética entre las poblaciones de parásitos presentes en distintos fragmentos de selva sea evidente.

También se observó que las poblaciones de oxiuros son genéticamente mucho más diversas que las poblaciones de sus hospederos. Este patrón de poca diferenciación y alta diversidad ha sido reportado para otras especies de parásitos de ciclo directo (Braisher et al. 2004; Archie y Ezenwa 2011; Haynes et al. 2014) y puede ser consecuencia de los tamaños poblacionales mucho más grandes en parásitos que en hospederos, pero también

puede indicar que otros factores, aparte de la capacidad de dispersión del hospedero, están facilitando el flujo genético entre distintas poblaciones de parásitos (Mazé-Guilmo et al., 2016).

Los patrones observados en este estudio evidencian la complejidad de los procesos genético-evolutivos en parásitos. Dada la completa dependencia del parásito con su hospedero, la inclusión de información genética del hospedero es necesaria para lograr una comprensión más completa acerca de la microevolución en parásitos (Gandon y Michalakis 2002; Criscione 2008). Desafortunadamente, los estudios en donde se analizan la genética de poblaciones y los posibles efectos de la perturbación ambiental en primates a lo largo de su rango de distribución en México e incluso en Centroamérica, son muy escasos. Los pocos estudios existentes han reportado una baja diversidad genética y diferenciación poblacional en monos aulladores en fragmentos de selva en el estado de Veracruz (Argüello-Sánchez, 2012; Baiz, 2013; Dunn et al., 2014; Alcocer-Rodríguez, 2015; Jasso-del Toro et al., 2016). No obstante, los patrones poblacionales observados en dichos estudios varían según el marcador molecular empleado y en ocasiones son contradictorios. Esta falta de información impidió que en este estudio se realizara una comparación detallada entre lo ocurrido en oxiuros y primates, proponiendo solamente posibles escenarios en lugar de explicaciones más certeras a los patrones genéticos observados en *Trypanoxyuris.*

Con la finalidad de comprender mejor los procesos genético evolutivos en poblaciones de oxiuros de primates, es recomendable complementar la información obtenida en el presente trabajo de tesis con los siguientes cinco aspectos: a) añadiendo información derivada de marcadores moleculares distintos al ADN mitocondrial, como por ejemplo microsatélites o SNP's, los cuales al ser más variables pueden revelar estructuras genéticas no observadas con sólo la mitocondria (Vázquez-Domínguez et al., 2009); b) ampliando el muestreo a todo el rango de distribución de estas especies de hospederos; d) obteniendo información acerca de la genética de poblaciones de los hospederos y así poder comparar el paralelismo entre los procesos microevolutivos entre parásitos y hospederos; e) incorporando variables de tipo ambiental o de paisaje (características climáticas, topología, conectividad) que pudieran explicarnos con mayor precisión los patrones genéticos observados en las poblaciones de estos parásitos.

Este trabajo constituye un primer esfuerzo para implementar la parasitología molecular en primates en México. La mayoría de los estudios parasitológicos en primates se han realizado mediante la identificación de los huevos presentes en las excretas, los cuales en muchas ocasiones son prácticamente indistinguibles unos de otros, dejando ciertas imprecisiones en el diagnóstico. Los protocolos aquí empleados mostraron ser adecuados para el aislamiento y extracción de ADN de huevos de parásitos presentes en las excretas de primates, aunque aún es necesario estandarizarlos y en ciertos casos mejorarlos para aumentar el éxito de amplificación y secuenciación del material genético (por ejemplo con *Strongyloides*). La información molecular de las distintas especies de helmintos encontrados en este trabajo servirá de base para futuros estudios que busquen determinar la diversidad de parásitos en primates Neotropicales y conocer sus relaciones evolutivas.

En este trabajo se demostró que la combinación de técnicas moleculares con métodos de muestreo no invasivos es una herramienta de gran utilidad para realizar estudios taxonómicos y evolutivos en parásitos, especialmente en especies de hospederos amenazadas, como es el caso de los primates mexicanos. Con el desarrollo de nuevas técnicas moleculares, como la secuenciación masiva y análisis metagenómicos, la determinación de la fauna parasitológica de especies en riesgo podrá ser realizada de manera más eficaz y precisa.

Estudios de esta naturaleza pueden llegar a tener importantes implicaciones en la conservación tanto de los parásitos, pues forman parte importante de la biodiversidad (Gómez y Nichols 2013), como de sus hospederos, ayudando a la identificación y caracterización genética de parásitos, a determinar la riqueza parasitaria en hospederos y localidades, a realizar monitoreos de salud más certeros en organismos tanto cautivos como de vida libre, a determinar rutas y posibles mecanismos de transmisión y riesgo de zoonosis (Gasser, 2006), así como a evaluar los efectos que los cambios ambientales y modificaciones en el paisaje puedan estar teniendo en las dinámicas ecológicas y evolutivas entre parásito y hospedero. Además, a través del análisis de las estructuras genético-poblacionales en parásitos podemos inferir ciertos aspectos relacionados con la ecología y evolución de sus hospederos como mecanismos e intensidad de dispersión, patrones de distribución, demografía, historia biogeográfica, entre otros (Whiteman y Parker 2005; Gómez y Nichols 2013).

CONCLUSIONES GENERALES

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→ Se registraron en total cinco especies de oxiuros en los primates mexicanos. *Trypanoxyuris atelis* y *T. atelophora* se encuentran parasitando al mono araña. Las dos especies de monos aulladores comparten a *T. minutus*, mientras que *T. multilabiatus* se encuentra sólo en mono aullador de manto y *T. pigrae* sólo parasita al mono aullador negro.

→ Se describen dos especies nuevas de nemátodos del género *Trypanoxyuris*, *T. multilabiatus* y *T. pigrae*.

→ La forma de las estructuras bucales, la forma del ala lateral y la estructura del esófago constituyen los principales caracteres diagnósticos para las especies del género *Trypanoxyuris*.

→ Cada especie de *Trypanoxyuris* constituye un grupo monofilético, los cuales a su vez, presentan una estructura genealógica que comprende tres grupos con evolución independiente asociados a la subfamilia de hospederos a la que parasitan. Grupo 1: parásitos de monos aulladores (Alouattinae); Grupo 2: parásitos de atelinos (Atelinae); Grupo 3: parásitos de Atelinae y Aotidae.

→ Los oxiuros de primates mexicanos son altamente específicos encontrando dos especies de *Trypanoxyuris* por cada especie de hospedero, lo cual indica que la diversidad de oxiuros en primates Neotropicales está subestimada.

→ La fragmentación del hábitat no ha ocasionado una estructura genética entre las poblaciones de *T. minutus* y *T. atelis*. Esta panmixia indica que la dispersión pasiva de huevos, los grandes tamaños poblaciones, y un mayor movimiento de primates que lo esperado, podrían estar impidiendo la diferenciación genética de las poblaciones de oxiuros presentes en distintos fragmentos de selva.

→ Son necesarios más estudios en oxiuros y en primates, incorporando distintos marcadores moleculares y colectando información de distintas poblaciones a lo largo de su rango de distribución completo (México y Centroamérica), para determinar con mayor certeza sus procesos genético-evolutivos y para entender los efectos que las perturbaciones ambientales pudieran tener en la dinámica parásito-hospederos.

→ Los protocolos presentados son adecuados para el aislamiento y extracción de ADN de huevos de parásitos presentes en las excretas, facilitando el desarrollo de estudios taxonómicos y evolutivos de parásitos en especies de hospedero amenazadas al emplear técnicas de muestreo no invasivas.

→ El gen 28S constituye un marcador útil para realizar diagnosis molecular de parásitos, especialmente aquellos que infectan especies de hospederos amenazadas.

→ La información de las distintas especies de helmintos encontrados en este trabajo servirá de base para futuros estudios que busquen determinar la diversidad de parásitos en primates Neotropicales y conocer sus relaciones evolutivas

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APÉNDICE I

APÉNDICE I

Tabla de localidades de colecta de muestras para las tres especies de primates en México. Coordenadas son dadas en UTM, Zona 15 y Zona 16 Norte.

Hospedero	Región	Localidad	Fragmento	Coordenadas	Tamaño de tropa
Alouatta palliata	Los Tuxtlas	Montepio	Playa Escondida	Z15 283155 2056495	16
			Organos II	Z15 274021 2059896	7
			Rancho Huber	Z15 278877 2058500	7
			Montepío 1	Z15 279829 2062025	22
			Jicacal	Z15 284629 2054811	13
			Dos de Abril	Z15 277866 2061284	16
		Catemaco	Agaltepec	Z15 278950 2037274	54
		Sierra Santa Marta	Magallanes	Z15 313475 2032721	6
			Mirador Pilapa	Z15 315074 2031304	13
			Playa	Z15 314534 2034362	3
	Uxpanapa	Poblado 3	El fortuño	Z15 324862 1911131	5
		Liberales	Macizo	Z15 377555 1912123	1
		Murillo Vidal	M. vidal	Z15 357486 1925402	5
		San Miguel Allende	DG	Z15 399851 1940219	NI
	Tabasco	Comalcalco	Hacienda la Luz	Z15 475289 2019412	14
			Zona Arqueológica	Z15 478593 2020935	18

	Chiapas	Pichucalco	Finca Santa Ana	Z15 493146 1942349	18
Alouatta pigra	Tabasco	Pantanos de Centla		Z15 559855 2031254	4
	Campeche	La libertad	DT	Z15 764310 2048385	5
	Chiapas	Metzabok	Laguna Tzibaná	Z15 648553 1893200	6
		Catazajá	Carretera	Z15 612024 1960924	6
		Palenque	Aluxes		10
		Reforma Agraria	Guacamayas	Z15 728520 1798288	5
			Reserva Ejidal	Z15 730478 1798761	3
Ateles geoffroyi	Los Tuxtlas	Sierra Santa Marta	Magallanes I	Z15 313038 20329217	7
			Magallanes II	Z15 314028 2031845	5
			Playa I	Z15 314295 2034198	4
			Playa II	Z15 314718 2034076	8
			Guadalupe A	Z15 309901 2031460	9
			Guadalupe B	Z15 309919 2031083	5
	Uxpanapa	Poblado 3	El Fortuño	Z15 323614 1912228	4
		Liberales	Macizo	Z15 377555 1912123	15
			DL	Z15 374818 1916015	12
			DTL	Z15 375993 1914250	NI
		Primitivo R Valencia	San Felipe	Z 15 388109 1909108	15
		Poblado 15	El Jaguar	Z15 391396 1902634	8

	Murillo Vidal	DJ	Z 15 358641 1925858	2
	Desengaño	Macizo	Z 15 396978 1915187	10
Tabasco	Villahermosa	Parque La Venta	Z15 506864 1990129	13
Campeche	La libertad	DT	Z15 764981 2048736	3
	El Zapote	Las piñas	Z15 624392 2019312	4
Chiapas	Metzabok	Laguna Tzibaná	Z15 648553 1893200	3
	Palenque	Aluxes		3
	Reforma Agraria	Guacamayas	Z15 728520 1798288	6
		Reserva ejidal	Z15 731310 1798199	3
Quintana Roo	Puerto Morelos	Jardín Botánico	Z16 510073 2305134	20
		San Joaquín	Z16 496378 2306759	8
	Punta Laguna		Z16 434030 2283328	25

APÉNDICE II

APENDICE II

Tabla de porcentajes de infección para cada tipo de parásito en cada especie de hospedero por localidad, por región y en el muestreo total. N= tamaño de muestra, n = número de muestras positivas, % = porcentaje de infección

Hospedero	**Región**	**Localidad**	**N**	**Parásito**	**n**	**%**
Alouatta palliata	Tuxtlas Norte	Montepio	12	*Trypanoxyuris minutus*	11	92%
		Organos 2	8	*T. minutus*	5	63%
		Playa Escondida	19	*T. minutus*	11	58%
		Rancho Huber	8	*Controrchis biliophilus*	2	25%
				T. minutus	3	38%
		Sub total	47	*C. biliophilus*	2	4%
				T. minutus	30	64%
		Agaltepec	10	*C. biliophilus*	2	20%
				T. minutus	5	50%
				T. multilabiatus	5	50%
	Tuxtlas Sur	Magallanes	5	*C. biliophilus*	2	40%
				T. minutus	4	80%
		Mirador	3	*C. biliophilus*	1	33%
				T. minutus	3	100%
				T. multilabiatus	1	33%
		Playa	3	*C. biliophilus*	1	33%
				T. minutus	2	67%
		Subtotal	11	*C. biliophilus*	4	36%
				T. minutus	9	82%
				T. multilabiatus	1	9%
	Tuxtlas	Total	68	*C. biliophilus*	8	12%
				T. minutus	44	65%
				T. multilabiatus	6	9%
	Uxpanapa	Fortuño	5	*T. minutus*	3	60%
		Liberales	3	*T. minutus*	3	100%
		M. vidal	7	*T. minutus*	3	43%

				T. multilabiatus	1	14%
		San Miguel Allende	9	*C. biliophilus*	1	11%
				Trypanoxyuris sp.	1	11%
				T. minutus	2	22%
		Total	24	*C. biliophilus*	1	4%
				Trypanoxyuris sp.	1	4%
				T. minutus	11	46%
				T. multilabiatus	1	4%
	Tabasco	Hacienda la Luz	10	*T. minutus*	6	60%
		Comalcalco	13	*C. biliophilus*	3	23%
				Trypanoxyuris sp.	2	15%
				T. minutus	7	54%
				T. multilabiatus	2	15%
		Total	23	*C. biliophilus*	3	13%
				Trypanoxyuris sp.	2	9%
				T. minutus	13	57%
				T. multilabiatus	2	9%
	Chiapas	Pichucalco	20	*C. biliophilus*	2	10%
				Trypanoxyuris sp.	7	35%
				T. minutus	7	35%
				T. multilabiatus	4	20%
	Muestreo total		**135**	***C. biliophilus***	**14**	**10%**
				***Trypanoxyuris* sp.**	**10**	**7%**
				T. minutus	**75**	**56%**
				T. multilabiatus	**13**	**10%**
Alouatta pigra	Campeche	La Libertad	6	*T. pigrae*	2	33%
	Tabasco	P. Centla	7	*Trypanoxyuris* sp.	1	14%
				T. pigrae	3	43%
	Chiapas	Catazajá	11	*Strongyloides* sp.	1	9%
				Trypanoxyuris sp.	4	36%
				T. pigrae	4	36%
		Metzabok	7	*Trypanoxyuris* sp.	1	14%
				T. minutus	1	14%
				T. pigrae	2	29%
		Palenque	8	negativo	0	0%
		Reforma Agraria	7	*Trypanoxyuris* sp.	1	14%

		total	33	*Strongyloides* sp.	1	3%
			33	*Trypanoxyuris* sp.	6	18%
			33	*T. minutus*	1	3%
			33	*T. pigrae*	6	18%
	Muestreo Total		**46**	***Strongyloides* sp.**	**1**	**2%**
				***Trypanoxyuris* sp.**	**7**	**15%**
				T. minutus	**1**	**2%**
				T. pigrae	**11**	**24%**
Ateles geoffroyi	Los Tuxtlas	Magallanes	17	*Strongyloides* sp.	1	6%
				Trypanoxyuris sp.	4	24%
				T. atelis	1	6%
				T. atelophora	1	6%
		Magallanes DS	13	Ancylostomatido	1	8%
				Trypanoxyuris sp.	2	15%
				T. atelophora	1	8%
		Playa	15	*C. biliophilus*	2	13%
				Trypanoxyuris sp.	1	7%
				T. atelis	3	20%
				T. atelophora	5	33%
		Guadalupe	19	*C. biliophilus*	1	5%
				Ancylostomatido	2	11%
				Strongyloides sp.	2	11%
				Trypanoxyuris sp.	1	5%
				T. atelis	6	32%
				T. atelophora	1	5%
		total	64	*C. biliophilus*	3	5%
				Ancylostomatido	3	5%
				Trypanoxyuris sp.	8	13%
				Strongyloides sp.	3	5%
				T. atelis	10	16%
				T. atelophora	8	13%
	Uxpanapa	Fortuño	7	*T. atelis*	2	29%
				T. atelophora	1	14%
		Liberales	14	Ascarido	1	7%
				Strongyloides sp.	10	71%
				T. atelis	1	7%
		Murillo Vidal	12	*Strongyloides* sp.	7	58%

			T. atelis	2	17%
	Primitivo	6	*Strongyloides* sp.	2	33%
	El Jaguar	8	*Strongyloides* sp.	1	13%
			Trypanoxyuris sp.	1	13%
			T. atelis	3	38%
			T. atelophora	3	38%
	Desengaño	16	Ancylostomatido	1	6%
			Ascarido	1	6%
			Strongyloides sp.	4	25%
			T. atelis	2	13%
			T. atelophora	3	19%
	total	63	Ancylostomatido	1	2%
		63	Ascarido	2	3%
		63	*Strongyloides* sp.	24	38%
		63	*Trypanoxyuris* sp.	1	2%
		63	*T. atelis*	10	16%
		63	*T. atelophora*	7	11%
Tabasco	Villa Hermosa	10	*Strongyloides* sp.	1	10%
			T. atelis	2	20%
Campeche	La libertad	14	*T. atelis*	9	64%
			T. atelophora	3	21%
	El Zapote	9	*Strongyloides* sp.	1	11%
			Trypanoxyuris sp.	2	22%
			T. atelis	1	11%
			T. atelophora	2	22%
	total	23	*Strongyloides* sp.	1	4%
			Trypanoxyuris sp.	2	9%
			T. atelis	10	43%
			T. atelophora	5	22%
Chiapas	Metzabok	4	negativo	0	0%
	Palenque	6	*Strongyloides* sp.	2	33%
	Reserva ejidal	10	*C. biliiophilus*	2	20%
			Strongyloides	1	10%
			Trypanoxyuris sp.	2	20%
			T. atelis	3	30%
			T. atelophora	2	20%
	Guacamayas	9	*Strongyloides* sp.	1	11%

			Trypanoxyuris sp.	3	33%
			T. atelis	3	33%
			T. atelophora	1	11%
	total	29	*C. biliophilus*	2	7%
		29	*Strongyloides* sp.	4	14%
		29	*Trypanoxyuris* sp.	5	17%
		29	*T. atelis*	6	21%
		29	*T. atelophora*	3	10%
Quintana Roo	Puerto Morelos	27	*Trypanoxyuris* sp.	9	33%
			T. atelis	4	15%
			T. atelophora	1	4%
	San Joaquín	13	*Trypanoxyuris* sp.	4	31%
			T. atelis	2	15%
	Punta Laguna	19	*Trypanoxyuris* sp.	7	37%
	total	59	*Trypanoxyuris* sp.	20	34%
		59	*T. atelis*	6	10%
		59	*T. atelophora*	1	2%
Muestreo Total		**248**	***C. biliophilus***	**5**	**2%**
			Ancylostomatido	**4**	**2%**
			Ascarido	**2**	**1%**
			***Strongyloides* sp.**	**33**	**13%**
			***Trypanoxyuris* sp.**	**36**	**15%**
			T. atelis	**44**	**18%**
			T. atelophora	**24**	**10%**