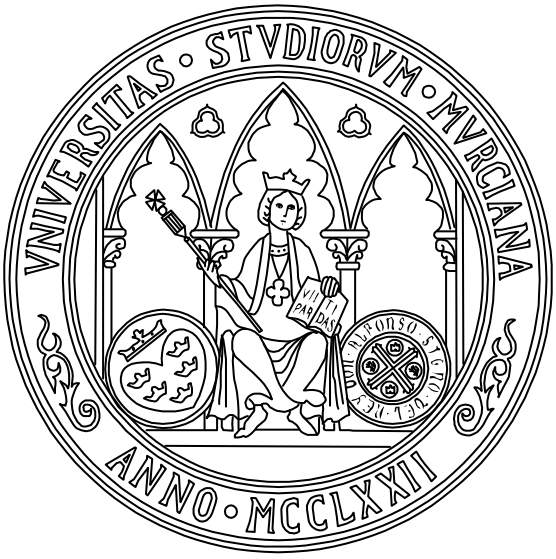

UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Molecular ecology and parasitic diversity of
wild populations in the Mesoamerican howler monkeys
Alouatta palliata and *A. pigra*

Ecología molecular y diversidad parasitaria de poblaciones
silvestres de las especies de mono aullador
Alouatta palliata y *A. pigra* en Mesoamérica

Dña. Claudia Villanueva García

2017

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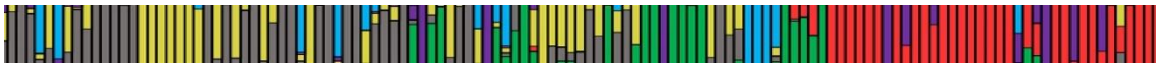

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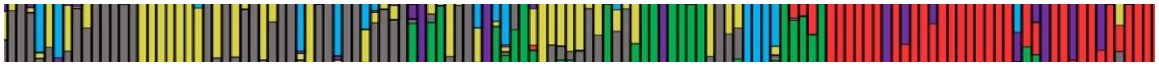
RESUMEN



Resumen

La región Mesoamericana constituye uno de los 25 *hotspots* o puntos calientes de biodiversidad en todo el mundo. Ocupa el segundo lugar en cuanto a número de especies de vertebrados endémicos y alberga alrededor del 7% de la biodiversidad mundial. Representa una conexión entre dos continentes principales, que habrían sido testigos de importantes intercambios bióticos hace 3 millones de años cuando ambos continentes se unieron. Representa el límite norte de la distribución geográfica de los primates silvestres en los Neotrópicos. Tres especies de estos primates co-ocurren en sus bosques: *Alouatta pigra* (conocido como mono aullador negro), *A. palliata* (mono aullador de manto dorado) y *Ateles geoffroyi* (mono araña).

México, clasificado como un país megadiverso, forma parte de los 12 países que poseen alrededor del 70% de la biodiversidad mundial. Incluye tres de las 25 regiones ecológicas prioritarias o *hotspots* del planeta, siendo Mesoamérica la mayor superficie, cubriendo todo el sureste de México, las costas del Atlántico y Pacífico y la Cuenca del Balsas. En México, esta región abarca nueve estados, en los cuales los procesos de fragmentación y cambios en el uso de la tierra han impactado seriamente la biodiversidad de la zona. Tabasco, es uno de los estados del sureste de México, incluido en el *hotspot* de Mesoamérica. Se caracteriza por una inmensa planicie aluvial costera plana con drenaje deficiente y grandes áreas de terrenos permanentemente o estacionalmente inundados. El área de estudio cubre un área de aproximadamente 118 km de ancho y 358 km de longitud a través de los estados de Tabasco y Chiapas, en el sureste de México.

Los monos aulladores pertenecen al género *Alouatta* que comprende 14 especies: *A. palliata, A. pigra, A. seniculus, A. belzebul, A. caraya, A. sara, A. discolor, A. arctoidea, A. guariba, A. juara, A. macconnelli, A. nigérrima, A. puruensis* y *A. ululata* (Crockett, 1998). Este género es uno de los más estudiados entre los primates americanos, desde el punto de vista de los patrones de alimentación, el uso de los recursos, su abundancia y otros aspectos poblacionales, como la distribución, uso del espacio y su conservación.

En México, el género *Alouatta* está representado por dos especies *A. palliata* (mono aullador de manto dorado) y *A. pigra* (mono aullador negro). Ambas especies se pueden distinguir basándose en caracteres morfológicos, genéticos y citogenéticos.

La hibridación natural es un fenómeno relativamente común entre los primates, tanto del Viejo como del Nuevo Mundo, y ha desempeñado un papel importante en su proceso evolutivo reticulado. En el género *Alouatta* hay muchos casos de pequeñas áreas de solapamiento entre especies, aunque la hibridación entre ellas sólo ha sido corroborada genéticamente en dos pares de especies: *A. palliata* y *A. pigra* en Tabasco, México y entre *A. caraya* y *A. guariba* en Brasil. Aunque *A. palliata* y *A. pigra* son alopátricas en la mayor parte de su rango, ambas especies tienen dos zonas de contacto: i) un área muy amplia de solapamiento en los estados de Tabasco y Campeche; ambas especies ocurrieron naturalmente en simpatría; ii) un área estrecha de márgenes contiguos, pero no superpuestos, en el este de Guatemala. En la zona de contacto de Tabasco y Campeche, muchos estudios han detectado eventos de hibridación natural.

Los monos aulladores tienen una gran capacidad de adaptación debido a su plasticidad ecológica y conductual, lo que les permite tolerar cambios en la estructura de su ecosistema, como la fragmentación y la reducción del hábitat. A pesar de la capacidad de las especies de *Alouatta* de tolerar la perturbación del hábitat, son sensibles a los altos niveles de pérdida de hábitat y fragmentación. Como consecuencia de la pérdida y alteración de sus hábitats, la mayoría de las especies y subespecies de *Alouatta* están incluídas en algunas de las categorías de la Lista Roja de especies amenazadas de la UICN y sus poblaciones mostraron una tendencia decreciente. En particular, ambas especies presentes en México están clasificadas como amenazadas por la UICN (*A. pigra* en peligro de extinción y *A. palliata* ssp. *mexicana* en peligro crítico). Los estudios realizados en Tabasco mostraron que *A. palliata* se ve más afectada por la disminución del tamaño de los fragmentos que *A. pigra.*

El parasitismo ocurre en primates como un proceso ecológico natural. La relación entre el parásito y el hospedador es muy importante porque deben cumplirse condiciones especiales para que este proceso ocurra. En el género *Alouatta* se ha descrito la presencia de endoparásitos gastrointestinales en siete de las catorce especies del género.

En esta tesis se analizaron dos especies de mono aullador pertenecientes al género *Alouatta* (*A. palliata* y *A. pigra*), con diferentes preferencias de hábitat y diferencias en la composición de grupos, en escenarios de cambio climático con el objetivo principal de determinar el estado de conservación y salud de sus poblaciones silvestres en los

remanentes de hábitat. Este estudio debe servir como punto inicial para proponer a corto plazo planes de manejo para la conservación del hábitat para estas dos especies.

Para lograr estos objetivos, se colectaron un total de 653 muestras fecales (215 de *A. palliata* y 438 de *A. pigra*) en 45 localidades, utilizando técnicas no invasivas. Adicionalmente, se colectaron muestras de tejido (n = 7 individuos) y de hueso (n = 5 individuos) procedentes de cadáveres de ambas especies. Las muestras fueron colectadas con el permiso de colecta científica del Ministerio de Ambiente y Recursos Naturales y el Subsecretario de Gestión para la Protección del Medio Ambiente NOM-059-SEMARNAT-2010; Referencia SGPA / DGVS / 04725/13. Las muestras fueron analizadas utilizando herramientas moleculares (ADN mitocondrial y nuclear) y análisis de parásitos. Los resultados de esta tesis se encuentran estructurados en 4 capítulos.

En el **capítulo 1** se analiza la estructura poblacional, la diversidad genética y la conectividad de las poblaciones de las especies parapátricas de monos aulladores, *Alouatta palliata* y *A. pigra* en el sureste de México. La pérdida de hábitat, la fragmentación y la hibridación son factores conocidos que afectan la estructura genética de las poblaciones de monos aulladores, lo que reduce la aptitud física y su capacidad para soportar cambios ambientales y enfermedades. Se recolectaron un total de 393 muestras fecales de *A. palliata* (125) y *A. pigra* (268) de las 45 localidades, así como se describieron varias características del paisaje, como son el uso de la tierra, el tamaño y el número de parches y varios índices de perturbación del hábitat. Se generaron modelos de nicho ecológico para ambas especies en varios escenarios climáticos (Último Máximo Glacial, Holoceno Medio, Presente y Futuro). Se utilizó una submuestra de heces (*A. palliata*: 67 y *A. pigra*: 217) para el análisis de ADN. Se amplificaron y secuenciaron dos fragmentos mitocondriales, citocromo b (*cytb*) y ATP-sintasa 6 y 8 (*ATPasa*) y uno nuclear (*Sry*), así como 10 loci (microsatélites). Se analizaron la diversidad genética, la estructura de la población, el flujo génico y la conectividad espacial entre ambas especies. También se exploraron los efectos de las variables del paisaje sobre la diferenciación genética. La distribución potencial de *A. palliata* y *A. pigra* indica una respuesta dispar en escenarios pasados, presentes y futuros y una zona de hibridación mantenida a través del tiempo. Los modelos obtenidos predicen que *A. palliata* tendrá un nicho potencial reducido en comparación con *A. pigra*. Los resultados de este estudio sugieren que los grupos de *A. palliata* y *A. pigra* estudiados en el sureste de México presentan una baja diversidad genética, pero similar a la publicada en

otros estudios con microsatélites para las mismas especies. La mayoría de los alelos mostraron desviaciones para el equilibrio de Hardy-Weinberg, probablemente como consecuencia de la endogamia. Los datos genéticos recuperaron tres grupos principales, tanto las dos especies parentales como individuos híbridos distribuídos en una extensa área de hibridación en el sur del estado de Tabasco. Los análisis de conectividad mostraron patrones diferentes, mientras que las poblaciones de las tierras altas alejadas entre sí presentan una alta conectividad, las poblaciones más cercanas de las tierras bajas se diferenciaron genéticamente, posiblemente debido a la pérdida y fragmentación del hábitat. Por lo tanto, es crucial preservar los fragmentos restantes y promover esfuerzos de conservación para regenerar el restablecimiento de la conectividad de las poblaciones de estos monos aulladores en peligro de extinción.

En el **capítulo 2** se analizaron los efectos de la pérdida y fragmentación del hábitat en la prevalencia y riqueza de parásitos gastrointestinales entre las especies parapátridas *Alouatta palliata* y *A. pigra* en el sureste de México. Se sabe que varios factores como la pérdida de hábitat y la fragmentación o hibridación producen cambios en el hospedador que influyen directamente en los procesos de parasitación e infección. Se analizaron muestras fecales de 498 individuos (147 de *A. palliata* y 351 de *A. pigra*) de cinco regiones del estado de Tabasco mediante un estudio coprológico. Se registraron características del paisaje como el tamaño del parche, el uso de la tierra y varios índices de perturbación del hábitat en cada lugar de muestreo. Una submuestra de 72 individuos se analizó mediante marcadores moleculares, utilizando 10 microsatélites (loci) y se asignó el genotipo a cada individuo. Se encontraron diferencias en las características del paisaje y en los índices de perturbación del hábitat entre las cinco regiones. Del mismo modo, se encontraron diferencias en la riqueza y prevalencia parasitarias entre las regiones, entre las especies hospedadoras y entre los orígenes genéticos. Se detectaron correlaciones entre las variables ambientales y la prevalencia de parásito-específica. Varios parámetros de alteraciones del hábitat, como el tamaño del parche o el índice de presión de uso circundante están relacionadas con la prevalencia y la riqueza del endoparásito, sin que se haya encontrado una relación clara con la fragmentación.

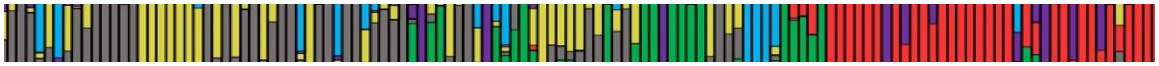
En el **capítulo 3** se pretende aclarar la especificidad del hospedador de *Blastocystis* spp. aíslado a partir de muestras fecales en los monos aulladores *Alouatta palliata* y *A. pigra*. Aunque se ha documentado en *Blastocystis* la presencia de la especificidad críptica del

hospedador, las diferencias en las tasas de infección y el alto polimorfismo genético dentro y entre las poblaciones de algunos subtipos han impedido aclarar su carácter de generalista o especialista de este parásito. En este capítulo se evaluó la variabilidad genética y la especificidad de acogida de *Blastocystis* spp. en monos aulladores silvestres en dos áreas tropicales en la región suroeste de México. Se analizaron muestras fecales para detectar la infección con varios subtipos de *Blastocystis* en 225 monos, de los cuales 59 correspondían a *Alouatta palliata* y 166 a *A. pigra*, pertenecientes a 16 sitios de muestreo. Se utilizó como marcador una región del gen de la subunidad pequeña del rDNA (SSUrDNA). Se realizaron análisis filogenéticos y de diversidad genética de acuerdo con las áreas geográficas donde se encontraron los monos. *Blastocystis* ST2 (subtipo 2) fue el más abundante (91,9%), seguido por ST1 y ST8 con 4.6% y 3.5%, respectivamente. No se observó asociación entre los subtipos de *Blastocystis* y las especies de *Alouatta*. En los análisis se usaron secuencias de SSUrDNA del GenBank de primates humanos y no humanos (NHP) como referencia. Los árboles de la red de haplotipos exhibieron diferentes distribuciones: ST1 mostró un perfil generalista ya que varios haplotipos de diferentes animales se distribuyeron homogéneamente con pocos cambios mutacionales. Para ST2, un centro mayor de dispersión agrupó a las muestras mexicanas, y se observaron altas diferencias mutacionales entre los NHP. Además, los valores de diversidad de nucleótidos y haplotipos, así como los índices de migración y diferenciación genética, mostraron valores diferentes para ST1 y ST2. Estos datos sugieren que las poblaciones de ST1 están sólo mínimamente diferenciadas, mientras que las poblaciones de ST2 en humanos son altamente diferenciadas de las de NHP. Las especificidades de generalista y especialista en cuanto a hospedador mostradas por las poblaciones de *Blastocystis* ST1 y ST2 indican procesos de adaptación distintos. Debido a que ST1 exhibe un perfil generalista, este haplotipo puede ser considerado una metapoblación. Por el contrario, ST2 se presenta como un conjunto de poblaciones locales con preferencias tanto para los seres humanos como para los NHP.

Finalmente, en el **capítulo 4** se describe un nuevo grupo de *Entamoeba* en monos aulladores (*Alouatta* spp.) que está asociado con parásitos de reptiles. Nuestro conocimiento de las especies de parásitos presentes en los hospedadores silvestres es incompleto, especialmente en primates no humanos (NHP). Protozoos como las amebas del género *Entamoeba* infectan una gran variedad de especies de vertebrados, incluídos los NHP. Sin embargo, tradicionalmente su identificación se ha hecho mediante la evaluación microscópica, por lo que las especies de amebas no siempre han sido identificadas

correctamente. En este capítulo se ha buscado *Entamoeba* spp. utilizando enfoques moleculares en monos aulladores de vida silvestre (*Alouatta palliata* y *A. pigra*) del sureste de México. En total se colectaron 155 muestras, de las cuales 46 eran de *A. palliata* y 109 de *A. pigra*. Se detectó un nuevo clado de *Entamoeba*, que se separó de otras especies descritas, aunque tenía una posición más cercana a *E. insolita*, así como también una secuencia que ha sido típicamente encontrada en iguanas con bajos valores de identidad compartida (<90%). Hemos designado este nuevo clado como linaje ribosómico 8 (RL8) y hemos demostrado que los miembros de este grupo no son exclusivos de los reptiles.

INTRODUCTION



Study area

The region of Mesoamerica, one of the 25 hotspots worldwide, occupies the second place in endemic vertebrates and hosts about 7% of the world's biodiversity (Myers *et al.*, 2000). It represents a land bridge between two major continents, which witnessed major biotic exchanges 3 million years ago when both continents joined. It represents the northern limit of the geographic distribution of wild primates in the Neotropics. Three species co-occur in these rainforests*: Alouatta pigra* (known as black howler monkey), *A. palliata* (known as golden mantled howler) and *Ateles geoffroyi* (spider monkey).

Despite this, human population growth combined with its continuous dependence on agriculture and high levels of poverty, have led to the disappearance of 90% of the forests in this region (Bryant *et al.*, 1997). This has placed the populations of some species at risk, as the quetzal (*Pharomachrus euptilotis*), the harpy eagle (*Harpia harpyja*), the jaguar (*Panthera onca*), the tapir (*Tapirus bairdii*) and also several species of howler monkeys (*Alouatta* spp.) among others.

Mexico, classified as a megadiverse country, is part of the 12 countries that hold about 70% of the world's biodiversity. It includes three of the 25 priority ecological regions or hotspots of the planet, being Mesoamerica the greater surface, covering all the south-eastern Mexico and the Atlantic and Pacific Coasts and the Balsas Basin. In Mexico, this region covers nine states, in which the processes of fragmentation and land use change have seriously impacted the biodiversity of the area. Tabasco, is one of these states of the Mexican southeast, included in the Mesoamerica hotspot. It is characterized by a huge flat coastal alluvial plain with poor drainage and large areas of permanently or seasonally inundated terrains. The Grijalva and lower Usumacinta rivers water the eastern and central parts of the plain. The weather is warm and moist, with an average temperature of ~26°C and an annual range of rainfall of 2,000 to 4,000 mm of precipitation. Chiapas, the neighbouring state, has areas with different climates, abundant rainfall and diverse topology.

The study site covers an area of about 118 km width and 358 km length across Tabasco and Chiapas states in south-eastern Mexico. The sampling in Tabasco State was carried out mainly in the lowlands (ranging from 1 to 213 masl), except for the southern units in which sampling took place in a mountain range region (with an altitude range of 50 to 900 m). The sampling sites of Chiapas and Guatemala also did not exceed 250 meters of elevation. The study region is characterized by a mosaic formed by native vegetation patches and

extensive urban areas, grasslands, crops, shrubs, flood areas, and riparian forests. The native vegetation comprises patches of evergreen forests and riparian forests, mangrove and marsh areas.

The genus *Alouatta*

Howler monkeys belong to the genus *Alouatta*, which comprises the largest species of primates distributed in the Neotropics, being found from the southeast of Mexico to the north of Argentina (Wolfheim, 1983). This genus includes 14 species: *A. palliata, A. pigra, A. seniculus, A. belzebul, A. caraya A. sara, A. discolor, A. arctoidea, A. guariba, A. juara, A. macconnelli, A. nigérrima, A. puruensis, A. ululata* (Crockett, 1998). This genus is one of the most studied among American primates. Studies on feeding patterns, resource use, abundance and other population aspects, distribution, space use and conservation have been developed (Estrada *et al.*, 2002; Stoner, 1994). One of the characteristic features of the species of the genus is the vocalization made by males that can be heard at great distances and give the monkey its common name of "howler" (Dunn *et al.*, 2015).

Howler monkeys inhabit Neotropical rainforest and they are characterized by their ecological and behavioural plasticity, what let them to tolerate changes in the structure of their habitat, such as the fragmentation and habitat loss. Howlers are largely folivorous, with a flexible diet consisting in more than 181 plant species (54 families, Cristóbal-Azkarate and Arroyo-Rodríguez, 2007), also combined with fruits and flowers. Although these primates are considered strictly arboreal, there are events reported them walking on the ground (Pozo-Montuy and Serio-Silva 2007), and occasionally swimming in the rivers or water source. They can memorize travel routes, that allow them to cope with changing ecological landscapes, whether natural or anthropogenic (Silver and Marsh, 2003). This behavioural plasticity facilitates them to find new areas of foraging or even executing short trips at the ground level (Bicca-Marques and Calegano-Márquez, 1998, Serio-Silva and Rico-Gray, 2000). This ecological and behavioural plasticity has allowed them to stablish in perturbed areas occupied by livestock, agriculture or even human infrastructure (Chinchilla *et al.,* 2005; Pozo-Montuy and Serio-Silva, 2007).

In Mexico, the genus *Alouatta* is represented by two species *A. palliata* (known as golden mantled howler) and *A. pigra* (known as a black howler monkey). Both species can be

distinguished based on genetic (Cortés-Ortiz *et al.,* 2003), cytogenetic (Steinberg *et al.,* 2008), and morphological characters (Smith, 1970). *A. palliata* is black with yellowish fur on the sides and some individuals present strands of blond hair in different parts of the body like the tail, while *A. pigra* is totally black and more corpulent (Fig. 1).

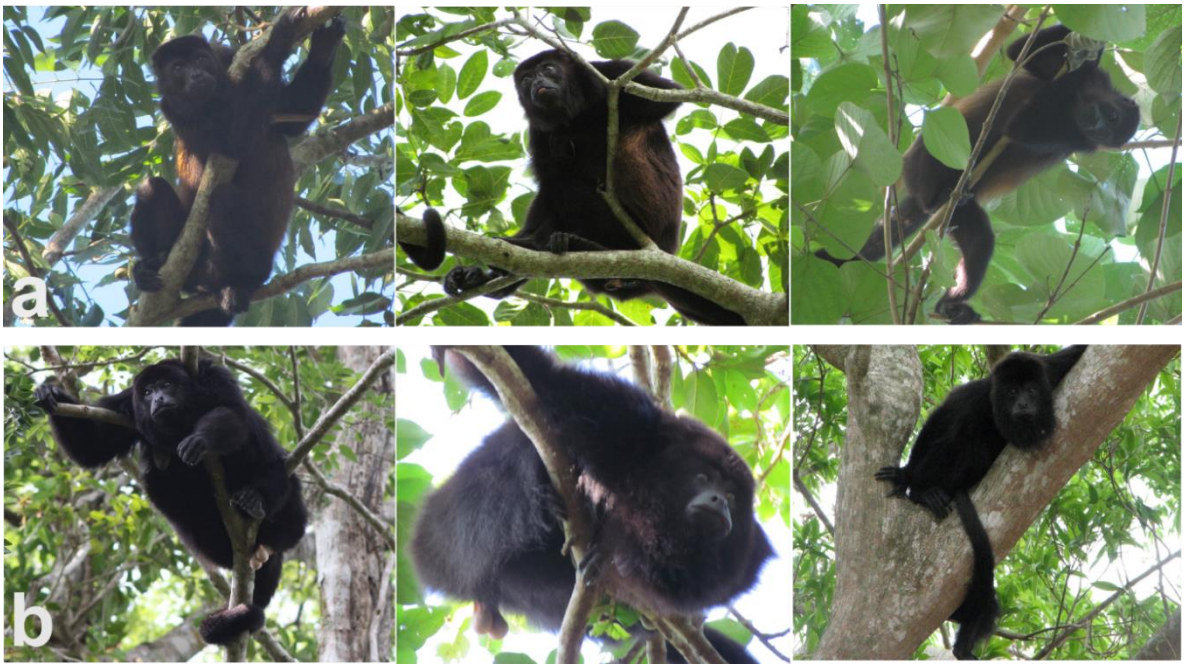


Figure 1. Howler monkeys. a) *A. palliatta* individuals, b) *A. pigra* individuals. Photos: Claudia Villanueva.

Regarding to its distribution ranges, *A. palliata* has a wider geographic distribution, occurring from southern Mexico, through all Central America to the coastal forests of Colombia and Ecuador. In Mexico, they have its largest representation in the region of the Tuxtlas, south of Veracruz. Across its distribution, five subspecies have been described: *A. p. mexicana*, *A. p. palliata*, *A. p. coibensis*, *A. p. trabeata*, and *A. p. aequatorialis* (Cortés-Ortiz *et al.,* 2015). The subspecies *A. p. mexicana* is distributed in Guatemala and in the Mexican states of Tabasco, Chiapas and Veracruz, but remain isolated for the remaining subspecies (Fig. 2). In contrast, *A. pigra* is an endemic species of the Mesoamerican region (Estrada, 2003) with a restricted geographic distribution in Guatemala, Belize and Southern Mexico, occurring only in the states of Tabasco, Chiapas, and the Yucatan Peninsula (Fig. 2). Both *A. palliata mexicana* and *A. pigra* found a natural barrier in their southern highland massif of Mexico and Guatemala (Sierra Madre de Chiapas and central highlands of Guatemala).

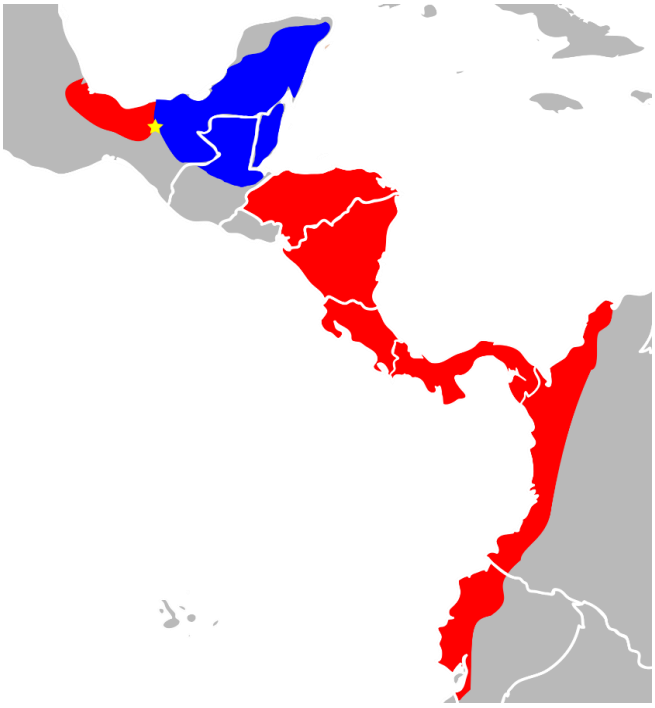


Figure 2. Geographic distribution of howler monkeys: *A. palliata* (red) and *A. pigra* (blue). Hybrid zone (yellow star). Distributions modified from IUCN 2017. The IUCN Red List of Threatened Species. Version 2016-3. www.iucnredlist.org. Downloaded on 13 January 2017.

Conservation status

Despite the ability of *Alouatta* species to tolerate habitat perturbation, they are sensitive to high levels of habitat loss and fragmentation that has taken place in the Mesoamerican hotspot. As a consequence, most of the *Alouatta* species and subspecies are included in some of the categories of the IUCN Red List of threatened and their populations showed a decreasing trend (IUCN, 2017). The two species present in Mexico are classified as threatened by the IUCN (endangered: *A. pigra*, and critically endangered: *A. palliata* ssp. *mexicana*). Studies conducted in Tabasco showed that *A. palliata* is more affected by decreases in fragment size than *A. pigra* (Dias *et al.,* 2013).

A. pigra populations are at risk due to the restricted distribution in Mesoamerica of the species, the rapid fragmentation and conversion of its natural habitat in agricultural fields and pastures lands and the continued hunting for food and capture for pets (Marsh *et al.,* 2008). On the other hand, *A. palliata mexicana* is classified as critically endangered (Cuarón *et al.,* 2008) because it is estimated that it will suffer a severely decline over 3 generations (36 years) due to past and ongoing rates of habitat loss (estimated at between 4.3 and 6.2% per year; Cuarón, 1997).

Analysis of Genetic Diversity in howler monkeys

The molecular genetic studies in howler monkeys across the last two decades has shed light to the evolutionary history and status of their populations of these threatened primates as well as their coevolution with parasites (Cortés-Ortiz *et al.,* 2003; 2007; Meireles *et al.,* 1999; Solórzano-García, 2016; Solórzano-García and de León, 2017). Different molecular markers have been used to answer diverse evolutionary, ecological and behavioural questions.

Mitochondrial DNA analysis

The introduction of the maternally inherited mitochondrial DNA (mtDNA) markers (Avise, 2000) has provided a privileged insight into the differentiation of populations and the causes of genetic diversity present below the species level, between and within populations. Among the mitochondrial DNA markers *cytochrome b* (*cytb)* and ATP-synth*ase 6 and 8* (*ATPase*) *genes* have been widely used in molecular studies on howler monkeys (Cortés-Ortiz *et al.,* 2003). These mitochondrial protein coding genes generally exhibit 5 to 10 times greater variability than protein coding nuclear genes, and have been used as molecular tools for estimating phylogenetic relations and phylogeographic structure in various groups of species. The information obtained provides a clear insight into i) the ecological and adaptive characteristics of species and ii) the historical and genetic variation among populations, which is necessary for understanding the evolution of taxa and to guide the conservation policies of evolutionary lineages.

Nuclear analysis

Sry: Analyzing genetic variations of the mammalian Y chromosome provides the genetic structure of the patrilineal lineages. The Y chromosome-linked *Sry* gene constitute the molecular switch for sex determination (Koopman *et al.,* 2001; Sinclair *et al.,* 1990). *Sry* may be useful to phylogeographic studies (Petit *et al.,* 2002) and may facilitate our understanding of population history and population isolations of species. A phylogeographic study has been used to explore the patrilineal origin and understand the direction of crosses between sympatric howler monkeys populations (Cortés-Ortiz *et al.,* 2007; Kelaita and Cortés-Ortiz, 2013).

Microsatellites (SSTRs): Simple sequence tandem repeats of two to six nucleotides are found in most nuclear genomes (Chambers and MacAvoy, 2000; Tautz, 1989), and have become a valuable tool for detecting population genetic structure, evaluating genetic diversity, testing parentage relationships and interpreting recent population history (Zhang

and Hewitt, 2003). The characteristics that have contributed to their popularity are their ubiquity across most organisms, their high variability resulting from high mutation rates and their single-locus co-dominant inheritance (Selkoe and Toonen, 2006; Sunnucks, 2000; Tautz, 1989). Microsatellite markers have become a current molecular marker in vertebrates and particularly in mammals and primates for population genetic studies. In particular, in howler monkeys there have been several studies that have analyzed the genetic structure (Jasso-del Toro *et al.,* 2016; Ruiz-García *et al.,* 2007), the hybridization process (Cortés-Ortiz *et al.*, 2007; Kelaita and Cortés-Ortiz, 2013) as well as other behavioral aspects (Ho *et al.,* 2014; Van Belle *et al.,* 2012).

Effects of hybridization and fragmentation on the genetic and parasitic diversity

Due to the presence of a highly-fragmented landscape and a unique hybrid zone across the Tabasco state, it represents an excellent opportunity to evaluate the effects of hybridization and fragmentation on the genetic and parasitic diversity of *Alouatta palliata* and *A. pigra*.

Hybridization in Howler Monkeys

Natural hybridization is a relatively common phenomenon among primates from both the Old and New Worlds, and have played an important role in their reticulate evolutionary process (Arnold and Meyer, 2006). In the genus *Alouatta* there are many instances of small areas of overlap between species (Cortés-Ortiz *et al.*, 2015), although hybridization between them has only been corroborated in two species pairs: *A. palliata* and *A. pigra* in Tabasco, Mexico and between *A. caraya* and *A. guariba* in Brazil.

Although *A. palliata* and *A. pigra* are allopatric in most of their range, both species have two contact zones (Fig. 3): a) a very broad area of broad overlap in Tabasco and Campeche states were both species naturally occurred in sympatry, and b) a narrow area of contiguous, but not overlapping ranges in eastern Guatemala (Baumgarten and Williamson, 2007).

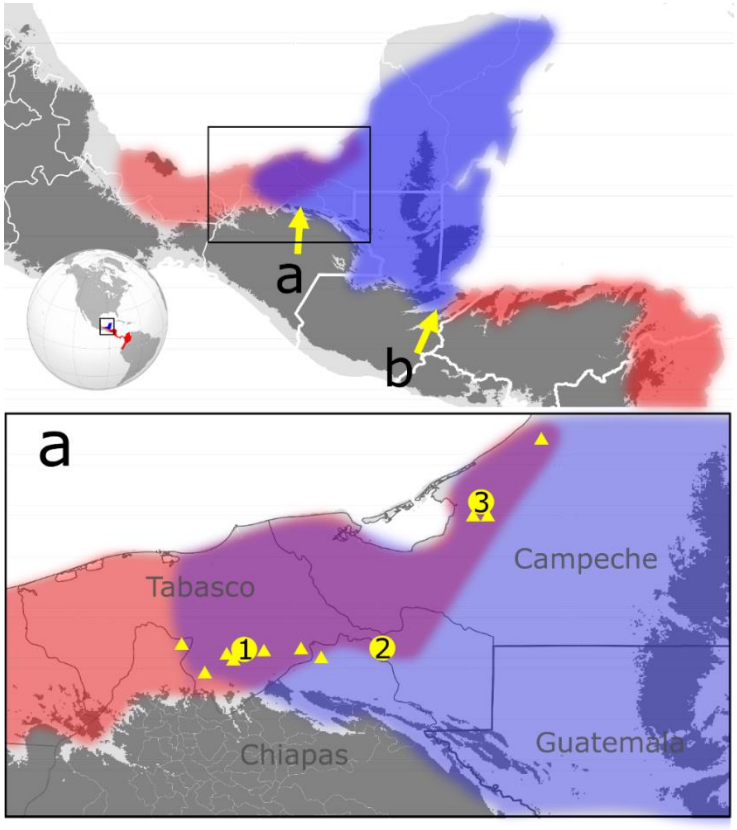


Figure 3. Geographic distribution of the contact zone (a and b, yellow arrows) between *A. palliata* (red) and *A. pigra* (blue). Altitude over 250 m are represented in dark grey. a) Detail of the hybrid zone and hybridization events (yellow circles and triangles) detected in the Tabasco and Campeche states. 1. Macuspana, Tabasco; 2. Zapata, Tabasco; 3. Laguna de Términos, Campeche. Yellow triangles from Kelaita and Cortés-Ortiz (2013).

In the contact zone of Tabasco and Campeche, many studies have reported events of natural hybridization (Fig. 3a). In Macuspana, Tabasco, several studies have shown hybridization along an area of 67 km2, generating a hybrid zone of 20 km wide (Cortés-Ortiz *et al.*, 2007; Horwich and Johnson, 1986; Smith, 1970). Other parapatric areas detected are around Zapata, Tabasco (Horwich and Johnson, 1986) and in the northern end of Laguna de Términos in Campeche (Serio-Silva *et al.,* 2005). Morphometric and genetic analyses (Kelaita and Cortés-Ortiz, 2013) revealed many instances of hybridization (Fig. 3a).

Hybrid individuals present mixed morphological features distinctive of each species (mainly subtle facial features, as well as pelage coloration, (see Fig 5.2 of Cortés-Ortiz *et al.*, 2015), although several studies have shown that backcrossed individuals are not morphologically

distinct based on overall appearance suggesting that molecular studies are needed (Cortés-Ortiz *et al.,* 2015).

Genetic confirmation of hybridization was obtained studying mtDNA and nuclear SRY and microsatellites in Macuspana hybrid area, Tabasco (Cortés-Ortiz *et al.,* 2007). This study suggested a postzygotic isolation mechanism with a unidirectional hybridization, in which only female F1 hybrids were viable and fertile in crosses of *A. palliata* males with *A. pigra* females. In contrast, crosses between *A. pigra* males and *A. palliata* females fail to produce fertile offspring (Fig. 4).

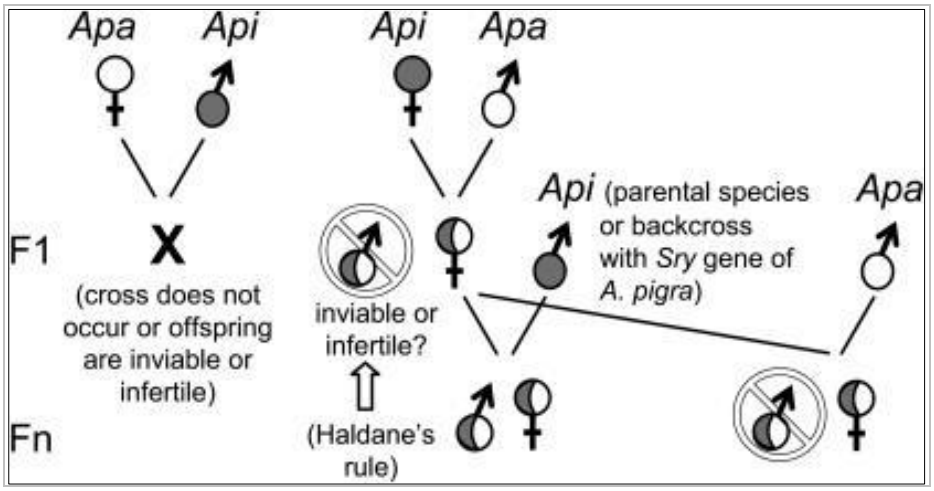


Figure 4. Possible outcomes of crosses between *A. palliata*, *A. pigra* and hybrid individuals based on genotypic data of individuals from the Mexican hybrid zone. From Figure 3 of Cortés-Ortiz *et al.*, (2007).

This asymmetric hybridization resulted in a genetic pattern with a high frequency of *A. pigra* mtDNA haplotypes (maternal heritage) introgressed in individuals with *A. palliata* nuclear background, and a low number of hybrid individuals with *A. pigra* nuclear background introgressed in individuals with *A. palliata* mtDNA haplotype. In the same way, all male hybrids have the *Sry* gene type (paternal heritage) coincident with the majority of their nuclear background. These observations agree with Haldane's rule, about hybrid sterility affecting the heterogametic sex (males) more than the homogametic sex (females), producing therefore, only females in the first generation of crossing (F1). Only viable or fertile males appear in the population after extensive backcrossing among multigenerational

hybrids (Fn) or between hybrids and purebred individuals (Fig. 3, Cortés-Ortiz *et al.*, 2007; 2015).

This hybrid zone between *A. palliata* and *A. pigra*, consist in a continuum of intraspecific variation from purebred individuals and first generation (F1), to backcrossed and multigenerational hybrids (Fn). In the contact zone, morphometric and genetic analysis revealed low levels of hybrid individuals (12%) and none of them were first generation hybrids (Kelaita and Cortés-Ortiz, 2013).

Habitat loss and fragmentation

Habitat loss and fragmentation are the primary drivers of the loss of biodiversity in the tropical region (Fahrig, 2003; Haddad *et al.,* 2015). In the last half century deforestation in tropical areas has rapidly increased leading to a loss of more than a third of all forest cover worldwide (Hansen *et al.,* 2013). Mesoamerica, represent one of the five hotspots more threatened due to forest loss by high deforestation rates (Brooks *et al.*, 2002). As a result, it has been pointed as a priority area for conservation (Myers *et al.*, 2000; Sarkar *et al.*, 2009). In particular, in Tabasco State the forest area has been severely reduced from 49.1% to 13.6 % of land surface due to deforestation and changes in land use to grasslands (Díaz-Gallegos *et al.*, 2010; Palma-López and Triano, 2002).

The effects of habitat loss and fragmentation have been largely studied in primates showing disparate responses depending on the specific ability of the species to deal with habitat fragmentation and disturbance (Irwin, 2016; Marsh, 2003; Marsh and Chapman, 2013). In particular, the genus *Alouatta* is known by their ability to tolerate habitat disturbance and their capacity to move among forest patches (Mandujano and Escobedo-Morales, 2008). Many studies have been conducted on howler monkeys to analyse the effects of forest fragmentation on different biological and ecological aspects (Fig. 4). They have shown that habitat loss and the consequent habitat fragmentation and reduction in patch size resulted in higher population densities (i.e. Clarke *et al.*, 2002; Cristóbal-Azkarate *et al.*, 2005; Estrada *et al.,* 2002), less food resources (i.e Arroyo-Rodríguez and Mandujano, 2006; López *et al.,* 2005; Marsh and Loiselle, 2003), higher physiological stress (Dunn, 2009; Martínez-Mota *et al.*, 2007), higher parasite prevalence and diversity (Gillespie and Chapman, 2006; Kowalewski and Gillespie, 2009) as well as changes in social organization and behaviour (Fig. 5, reviewed in Arroyo-Rodríguez and Dias, 2010).

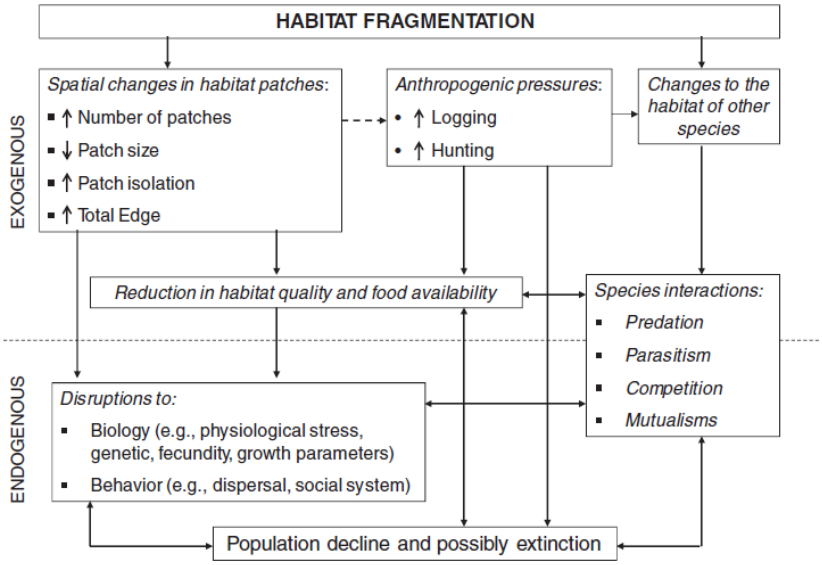


Figure 5. Flow diagram of two general threatening processes (exogenous and endogenous) arising from habitat fragmentation as experienced by a declining animal species (reviewed in Arroyo-Rodríguez and Dias, 2010).

Endoparasites in the genus *Alouatta*

Parasitism occurs in primates as a natural ecological process. The relationship between the parasite and the host is very important because special conditions must be fulfilled for this process to occur. The parasite needs to have adaptations to be able to carry out the parasitism to a specific host, as well as appropriate conditions for its survival. It is important to mention that in the parasite-host relationship, only the former benefits, while the latter may present tolerance (Campillo *et al.*, 1999). The specificity of a host varies among parasite species and it is influenced by the behaviour and ecology of both host and parasite, as well as their ecological and coevolution relationships. Some parasites are strictly host-specific, others show specificity for phylogenetically related or non-congenic species of hosts, while others are non-specific (generalist). Generalist parasites present ecological and behaviour adaptations that allow them to move and to switch between different hosts, increasing the potential contact with many potential hosts (Dick and Patterson, 2007; Simková *et al.*, 2013).

In the genus *Alouatta* the presence of gastrointestinal endoparasites has been reported in seven of the fourteen species of the genus (Balsells-Hernández, 2012; Barrios, 2005; Beltrán-Saavedra *et al.*, 2009; Chinchilla *et al.*, 2005; Eckert *et al.*, 2006; Guerrero *et al.*,

2012; Maldonado-López *et al.*, 2014; Martins, 2002; Milozzi *et al.*, 2012; Montoya, 2013; Roncancio-Duque and Benavides, 2013; Serur, 2008).

A relevant fact is that different genera of primates that inhabit sympatric zones have been found to share the same parasite richness, being the transmission of infections among different species more common than expected, such as the case of *A. palliata* and *Ateles geoffroyi* in a region of Costa Rica (Maldonado-López *et al.*, 2014) and *Alouatta seniculus* and *Ateles hybridus* in an area of Colombia (Roncancio-Duque and Benavides, 2013).

Regarding gastrointestinal parasites of zoonotic importance for the health of the populations of the genus *Alouatta,* the presence of *Giardia sp*. and *Entamoeba sp*. in free living populations of *A. belzebul* in Brazil, although there was no relationship between the presence of these parasites and the fragmentation of the area (Martins, 2002). It is relevant to mention that these species are reported as pathogenic and cause zoonosis in humans.

Several studies have shown that habitat loss and fragmentation produces changes in the host (such as population density, physiological stress, ranging patterns, intraspecific and interspecific contacts, and diet) that directly influence on the parasitation and infection process (Nunn and Altizer, 2006). In particular, in *Alouatta* species, several studies have pointed to higher endoparasite loads in fragmented patches (Eckert *et al.,* 2006; Stoner and Gonzalez Di Pierro, 2006; Trejo-Macias *et al.,* 2007; Trejo-Macias and Estrada, 2012; Vitazkova and Wade 2007). Not only fragmentation leads to changes in the presence of endoparasites, for example, studies carried out in *A. pigra* suggest that habitat characteristics and seasonality, as well as gender, age and diet of individuals, influence the load and prevalence of endoparasites. (Balsells-Hernández 2012; De Paiva *et al.*, 2010, Eckert *et al.;* 2006, Milozzi *et al.*, 2012). Additionally, hybridization might also affect parasitic prevalence and richness. Hybrids might present higher susceptibility to parasite infection than their parental species due to intrinsic factor such as genomic incompatibilities or extrinsic factors such as being exposed to parasite communities infecting both parental species. A recent study has showed that hybrids had much higher gastrointestinal parasitic prevalence than either of the parent primate species (Sommer *et al.*, 2014).

In Mexico, several studies have been conducted to evaluate the parasite richness and parasite load in *A. palliata* and *A. pigra*. Also, it has been evaluated the effects of fragmentation, seasonality and captivity in the parasite richness and prevalence. The parasitological records that have been reported is shown in Table 1.

Table 1. Gatrointestinal endoparasites of *Alouatta pigra* and *A. palliata* in Mexico.

SPECIES	STUDY SITE	AIM OF THE STUDY	REFERENCE	PARASITES FOUND
A. palliata *A. pigra*	Tacotalpa, Tabasco.	Compare and describe endoparasite diversity during rainy season vs. dry season.	Baños-Ojeda, 2016	*Trypanoxyuris* sp., *Cyclospora* sp., *Eimeriidae* sp., *Strongyloides* sp., Strongylid and *Blastocystis* sp.
A. palliata *A. pigra*	Macuspana, Tabasco.	Determine the transmission degree of *Trypanoxyuris sp.* in both species.	González-Hernández, 2014	*Trypanoxyuris* sp.
A. palliata *A. pigra*	Tuxtlas, Veracruz.	Characterize the parasites in howler and evaluate the effect of fragmentation.	Trejo-Macías *et. al,* 2007	*Trypanoxyuris minutus, Controrchis biliophilus* and Eimeriiidae oocyst.
A. palliata *A. pigra*	Tuxtlas, Veracruz, Palenque Chiapas.	Evaluate the habitat features that affect to prevalence and parasitic load.	Trejo-Macías *et. al*, 2012	*Controrchis biliophilus* and *Trypanoxyuris* sp.
A. palliata	Parque Museo La Venta, Tabasco.	Study the effect seassonal changes in the parasitic load of howler in semi-captivity.	Abogado-Reyes, 2005	*Trypanoxyuris minutus, Controrchis billiophilus* and Eimeriiidae oocyst.
A. palliata	Sierra of Santa Marta, Veracruz.	Test the effect of fragmentation on the parasite of howlers.	Valdespino, 2010	*Trypanoxyuris minutus, Controrchis biliophilus* and *Isospora arctopitheci.*
A. palliata	Sierra of Santa Marta, RegiónTuxtla.	Evaluate effect of fragmentation on the parasite richness and parasite load.	Aguilar, 2007	*Trypanoxyuris minutus, Controrchis biliophilus* and *Strongyloides* sp.
A. pigra	Catazajá rainforest, Chiapas.	Evaluate effect of seasonality on the parasite load.	Alvarado-Villalobos, 2010	*Controrchis biliophilus, Eimeria* sp. and *Trypanoxyuris* sp.
A. pigra	Tuxtlas, Veracruz.	Study the parasite load in two population on fragmented habitats.	García-Hernández, 2009	*Trypanoxyuris* sp., *Strongyloides sp. Isospora belli, Cyclospora* sp. and *Eimeria* sp.
A. pigra	Lacandona rainforest, Chiapas.	Evaluate effect of seasonality and fragmentation on the parasite load.	Stoner and Gonzales-Di Pierro, 2006	*Blastocystis* sp., *Entamoeba* sp. *Isospora* sp., *Enterobius* sp. *Strongyloides* sp. *Trichostrongyloide* sp.
A. pigra	Park of Palenque, Chiapas.	Characterize the parasites richness.	Vitazkova *and Wade*, 2006	*Controrchis biliophilus,Trypanoxyuris minutus,Giardia* sp. *Entamoeba* sp.
A. pigra	Tenosique mountain, Tabasco.	Characterize the parasites in natural populatios of howlers.	González-Hernández, 2014	*Strongyloides* sp., *Trypanoxyuris* sp. *Controrchis biliophilus, Cryptosporidium* sp.

Planning, objectives and hypothesis

In this thesis, two species of howler monkeys belonging to the genus *Alouatta*, with different habitat preferences and differences in group's composition were analysed in climate change scenarios to determine the state of conservation and health of wild populations in habitat remnants through molecular (mitochondrial and nuclear DNA) and parasitic approaches. This study should serve as an initial point for proposing, management plans for habitat conservation for these two threatened species.

To achieve these objectives, non-invasive techniques were implemented to collect a total of 653 scat samples (215 from *A. palliata* and 438 from *A. pigra)*, collected from 45 localities, gathering information on identification of species, sex, age, coordinates, and troop. Additional tissue (n=7 individuals) and bone (n=5 individuals) samples from both species were collected by recovery of carcasses of howler monkeys, or by chemical handling, for collection of biopsy samples. All the procedures were performed in accordance with the provisions of the Regulations of the Environment and Natural Resources Ministry and the Under-Secretary of Management for Environmental Protection NOM-059-SEMARNAT-2010; reference SGPA/DGVS/04725/13.

In the **first chapter**, population structure, genetic diversity and connectivity of populations of the species, *Alouatta palliata* and *A. pigra* from in South-eastern Mexico were analysed in a total of 393 fecal samples, 125 from *A. palliata* and 268 from *A. pigra*, with the aim to (1) provide a phylogeography pattern, genetic diversity and structure of the samples under study; (2) identify zones of hybridization in Southeast of Mexico and patterns of introgression in both species using genetic approaches and a MAXENT tool for construct a potential niche for hybrids model; (3) clarify the gene flow and genetic connectivity in the hybridization zone and (4) compare genetic diversity in zones with different landscape´s characteristics.

In **chapter two**, the effects of habitat loss and hybridization in gastrointestinal parasites prevalence and richness between the two parapatric species, *Alouatta palliata* and *A. pigra* were analysed. Tabasco represents an area with high habitat loss and fragmentation as well as a well-known area of hybridization between *A. palliata* and *A. pigra*. Fecal samples of 498 individuals (147 of *A. palliata* and 351 of *A. pigra)* from five regions across Tabaco state were analyzed by a coprologic study. Landscape features, habitat disturbance indexes as well as a genetic individual's assignment of a 72 individuals subsample was carried out in order to evaluate differential response of richness and

prevalence of endoparasites a) depending on host specificity b) depending of the degree of hybridization, c) depending on habitat features and d) on fragmentation level.

In **chapter three,** the genetic variability and host specificity of the parasite *Blastocystis* spp. in wild howler monkeys from two rainforest areas in the south-eastern region of Mexico were analysed for infection with *Blastocystis*. For achieving that, 225 fecal samples from *Alouatta palliata* (59) and *A. pigra* (166) monkeys were surveyed using a region of the small subunit rDNA (SSUrDNA) gene as a marker, comparing the sequences obtained with those in GenBank from human and non-human primates (NHP) to be used as references. The identification of a generalist or specialist profile of a parasite for its host is relevant for understanding its prevalence, transmission and other biological features. Therefore, the aim of the study was to assess the genetic variability and host specificity of *Blastocystis* spp. populations in the two howler monkey species *A. palliata* and *A. pigra*.

In **chapter four,** the presence of *Entamoeba* spp. in scats of the howler monkeys *A. palliata* and *A. pigra* from Mexico was analysed using molecular approaches. In total, 155 samples were collected, 46 from *A. palliata* and 109 from *A. pigra*. Universal forward and reverse oligonucleotides designed for *Entamoeba* species based on the consensus sequences from the 18S small subunit ribosomal RNA gene according to the highly-conserved regions reported in GenBank were tested and the PCR conditions were optimized. In *A. palliata* and *A. pigra,* the genus *Entamoeba* has traditionally been described and identified based on microscopic analyses of faeces but have not yet been identified at the species level using molecular methods. Therefore, the aim of the study was to clarify the genetic relationships of *Entamoeba* spp detected in *A. palliata* and *A. pigra,* with other species of *Entamoeba*, that parasite other vertebrates.

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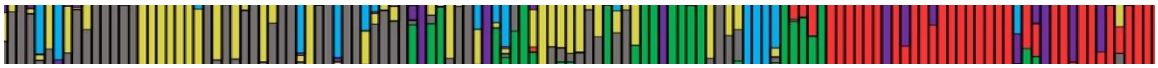
CHAPTER 1



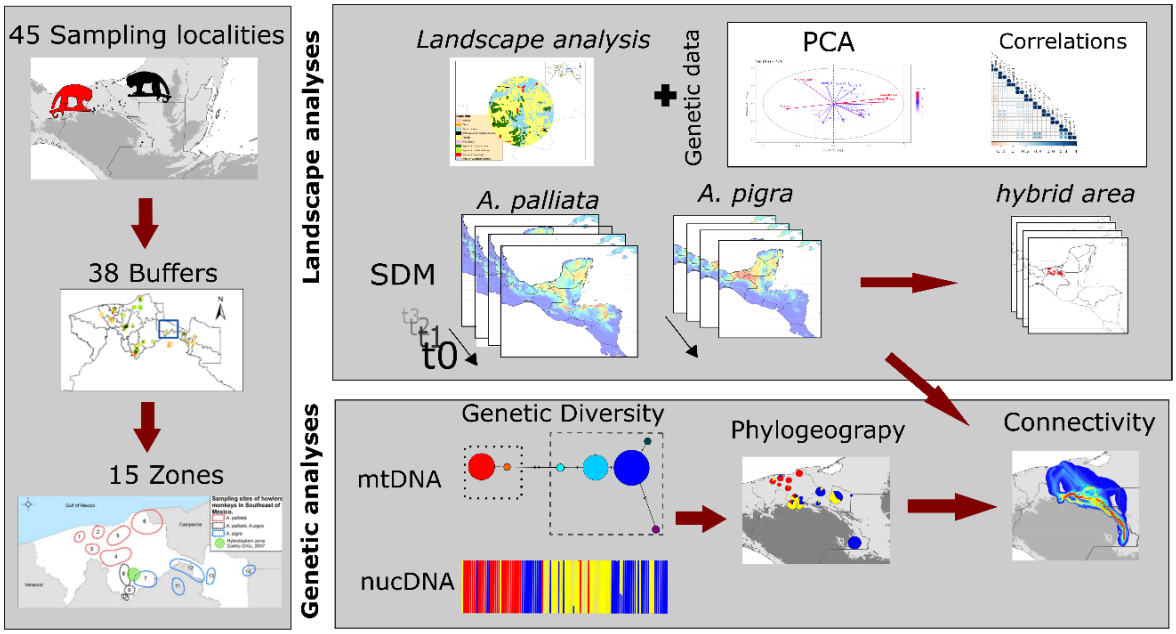
Population structure, genetic diversity and connectivity of populations in parapatric species, *Alouatta palliata* and *A. pigra* in Southeast of Mexico.

Population structure, genetic diversity and connectivity of populations in parapatric species, *Alouatta palliata* and *Alouatta pigra* in Southeast of Mexico.

Abstract

Habitat loss, fragmentation and hybridization are known drivers affecting the genetic structure of howler monkey populations reducing the fitness and their ability to withstand environmental changes and diseases. We have conducted a study to test the effect of these drivers on the genetic diversity of two parapatric species of howler monkeys *Alouata palliata* (gold mantled howler monkey) and *A. pigra* (black howler monkey) in Tabasco State, Mexico. A total of 393 faecal samples from *A. palliata* (125) and *A. pigra* (268) were collected from the 45 localities, as well as several landscape features such as land-use, size and number of patches, and several habitat perturbation indexes. Ecological niche models were generated for both species in several climatic scenarios (Last Glacial Maximum, Mid Holocene, Present and Future). A faecal subsample (*A. palliata:* 67 and *A. pigra*: 217) was used for DNA analyses. Two mitochondrial fragments, *cytochrome b* (*cytb)* and *ATP-synthase 6 and 8 genes* (*ATPase*) and one nuclear (*Sry*) were amplified and sequenced as well as 10 microsatellite loci were amplified and detected. Genetic diversity, population structure, gene flow and special connectivity among both species were analysed. The effects of landscape variables on the genetic differentiation were also explored. The potential distribution for *A. palliata* and *A. pigra* indicate disparate response to past, present and future scenarios and a maintained hybridization zone through time. Models predicts that *A. palliata* will have its potential niche reduced in comparison to *A. pigra*. The results of this study suggest that the groups of *A. palliata* and *A. pigra* studied from South-eastern Mexico, present a low genetic diversity, but similar to that reported in other studies with microsatellites for the same species. Most alleles showed deviations for the Hardy-Weinberg equilibrium, probably as a consequence of inbreeding. Genetic data recovered three main clusters, both parental species and hybrid individuals distributed across an extensive area of hybridization in southern Tabasco State. Connectivity analyses showed different patterns, whereas distant highland populations where highly connected, close lowlands population were genetically differentiated, possibly due to habitat loss and fragmentation. Therefore, it is crucial to preserve the remaining fragments and promote conservation efforts to regenerate reestablishing connectivity of populations of these endangered howler monkeys.

Keywords: hybridization, speciation, fragmentation, parapatric, hybrid zone.


45 Sampling localities
38 Buffers
15 Zones
Landscape analyses
Landscape analysis
Genetic data
PCA
Correlations
A. palliata
A. pigra
hybrid area
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Genetic analyses
Genetic Diversity
mtDNA
nucDNA
Phylogeograpy
Connectivity

Introduction

Habitat loss and fragmentation are one of the main threats in primates (reviewed in Estrada *et al.*, 2017). Howler monkeys (genus *Alouatta*) are one of the genera known for their ability to tolerate habitat disturbance and also for their capacity to move among forest patches in relation to other co-occurring primate species. Despite their capacity, they are sensitive to high levels of habitat loss and fragmentation, being the patch size of remnant forest the main factor constraining populations (reviewed in Arroyo-Rodríguez and Dias, 2010). As consequence of the anthropogenic pressures, there are several species and subspecies classified as threatened by the IUCN (i.e. Vulnerable: *A. palliata* ssp. *aequatorialis* and ssp. *coibensis*; Endangered: *A. pigra*, and Critically endangered: *A. palliata* ssp. *mexicana* and ssp. *trabeata*, IUCN, 2017).

An important consequence of habitat loss and fragmentation is the isolation of populations that produce inbreeding and genetic drift, resulting in lower genetic diversity and reduce the ability to withstand environmental changes and diseases (Whitehorn *et al.*, 2011). This pattern has been observed in several studies (i.e. Do Nascimento *et al.*, 2007; James *et al.*, 1997; Malgrem and Brush, 1978; Oklander *et al.,* 2007, 2010). However, recent studies on *A. pigra* showed higher heterozygosity in fragmented populations (del Valle, *et al.*, 2005; Winkler *et al.,* 2004). These contrasting results highlight the need of further studies to assess the genetic effects of the fragmentation on howler monkey's populations.

Natural hybridization is another driver that has played an important a role shaping the reticulate evolutionary process of primates (Arnold and Meyer, 2006). Natural hybridization of differentiated parapatric populations of subspecies, or closely related species commonly occurs among primates from both the Old and New Worlds, and frequently a persistent hybrid zone is formed in the contact areas (Detwiler *et al.*, 2005). Among New World primates, several cases of natural hybridization and hybrid between subspecies and species of *Samiri*, *Callithrix*, *Saguinus* and *Alouatta* have been reported (Aguiar *et al.*, 2008; Cortés-Ortiz *et al.,* 2007; Mendes, 1997; Peres *et al.,* 1996; Silva *et al.,* 1992). Concretely, there are numerous studies reporting natural hybridization between species of the genus *Alouatta* along its whole distribution range (reviewed in Cortés-Ortiz *et al.*, 2015).

The genetic effects of hybridization vary among species influencing different fitness-related traits. Various studies have shown that hybridization might have neutral effects (Bergman *et al.*, 2008), suppose a fitness advantage to the hybrid individuals (heterosis;

Charpentier *et al.*, 2008) or a disadvantage (outbreeding depression). Also, hybrid vigour and hybrid breakdown can be expressed simultaneously in hybrid populations because of the simultaneous masking of recessive deleterious alleles, dilution of genes that confer local adaptation, and disruption of coadapted gene interactions (Fenster and Galloway, 2000); decreasing of reproductive rates and increasing susceptibility to disease, parasites, predators, competitors and climatic changes (i.e. chapter 2). Extinctions of inbred populations may be triggered by demographic or environmental causes, yet ultimately result from the cumulative effects of inbreeding, especially in changing environments (Tocher and Decline, 1998). Different anthropogenic drivers of ecosystem change can interact with each other, generating additive or non-additive effects. Synergistic effects are expected as the result of different drivers' combination (Didham *et al.*, 2007). Several studies on primates have pointed out the indirect effects of multiple factors (Chapman *et al.,* 2006; 2007; Milton, 1996). For example, habitat loss and fragmentation have been suggested as potential factors that promote anthropogenic hybridization (Detwiler *et al.*, 2005).

In order to improve our understanding of how fragmentation and hybridization affect howler monkey genetic diversity and connectivity, we have conducted a study on two parapatric species of howler monkeys *Alouatta palliata* (gold mantled howler monkey) and *A. pigra* (black howler monkey) in Tabasco State, Mexico. This region represents an excellent opportunity to evaluate the individual and combined effects of several drivers of genetic diversity such as fragmentation and hybridization of these two parapatric species.

In the last 60 years, Tabasco forest area has been severely reduced from 49.1% to 13.6% of land surface due to deforestation and changes in land use to grasslands (Díaz-Gallegos *et al.,* 2010; Palma-López and Triano, 2002). This highly fragmented landscape has left the populations generally relegated to small fragments and many of them with a high level of isolation, what might generate genetic effects as inbreeding depression (Milton, *et al.*, 2009). Therefore, it is important to shed light on the effects of fragmentation in Mexican howler monkeys.

Additionally, this area is known as a zone of hybridization between *A. palliata* and *A. pigra*, concentrated on a relative small area of 20 km wide in Macuspana, Tabasco (Ho *et al.*, 2014; Kelaita and Cortés-Ortiz, 2013). Genetic studies have shown that hybridization and subsequent backcrosses are directionally biased, only female hybrids are viable and fertile, suggesting a postzygotic isolation mechanism and a subsequent outbreeding depression (Cortés-Ortíz *et al.*, 2007).

The combined effect of both mentioned drivers can also be studied. In Macuspana, hybrid individuals of two *Alouatta* species were observed in small and more isolated fragments, suggesting that deforestation and habitat fragmentation affects in some degree the natural hybridization process (Dias *et al.*, 2013). However further studies are needed to determine the influence on the anthropogenic hybridization on the natural hybrid zones.

The aims of this study include to (1) provide a phylogeography pattern, genetic diversity and structure of wild populations of *A. palliata* and *A. pigra* in South of Mexico; (2) identify zones of hybridization in Southeast of Mexico and patterns of introgression in both species; (3) clarify the gene flow and genetic connectivity across *Alouatta* population and with the hybridization zone (4) evaluate the effect of different landscape´s characteristics on the genetic diversity of the species.

Materials and methods

Study area

The study site covers an area of about 118 km width and 358 km length across Tabasco and Chiapas states in south-eastern Mexico (Fig. 1). The sampling in Tabasco State was carried out mainly in the lowlands (ranging from 1 to 213 masl), except for the southern units in which sampling took place in a mountain range region (with a maximum altitude of 406 masl). The sampling sites of Chiapas and Guatemala also did not exceed 250 meters of elevation. Forty-five localities were sampled grouped in 15 zones, covering mainly Tabasco state and some Chiapas and Guatemala localities (Table 1, Fig. 1).

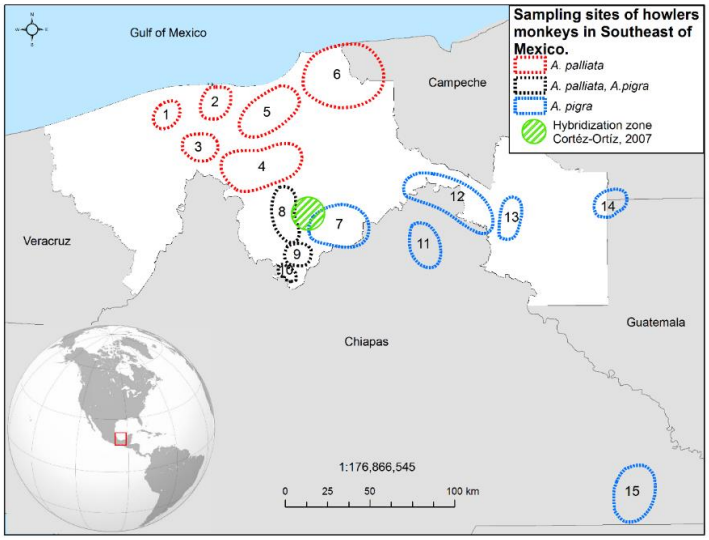

Figure 1. Sampling locations of mantled howler monkeys (*Alouatta palliata*, black circles) and black howler monkeys (*Alouatta pigra*, grey circles) or both (half black-half grey circles) from this study. Number identification of localities are explained in Table 1.

Table 1. Fifteen sampling zones (including 45 localities) for *Alouatta palliata* and *Alouatta pigra* in Southeast of Mexico. Tab: Tabasco. Chia: Chiapas. Number of zone which indicates the corresponding localities as represented in Fig. 1.

Zone	Id	Localities	Phenotype	Latitude	Longitude	masl
1	1.1	Carlos Greene, Comalcalco, Tab.	*A. palliata*	18.23894	-93.45537	9
2	2.1	Palestina, Paraiso, Tab.	*A. palliata*	18.34539	-93.18365	-1
	2.2	Mayan archaeological site, Comalcalco, Tab.	*A. palliata*	18.2799979	-93.2047695	5
	2.3	Cholula, Comalcalco, Tab.	*A. palliata*	18.284141	-93.209893	12
	2.4	Norte, Comalcalco, Tab.	*A. palliata*	18.3003616	-93.204453	4
3	3.1	Piedra, Cunduacan, Tab.	*A. palliata*	18.10359	-93.22632	8
	3.2	Cacao cultive, Cunduacan, Tab.	*A. palliata*	18.14256	-93.26161	11
4	4.1	Samaria, Cunduacan, Tab.	*A. palliata*	18.0136877	-93.1255827	0
	4.2	Mazaltepec, Centro, Tab.	*A. palliata*	18.0119484	-92.9991234	7
	4.3	La Venta, Centro, Tab.	*A. palliata*	18.000421	-92.936306	23
	4.4	Boqueron, Centro, Tab.	*A. palliata*	17.926953	-92.9829158	12
	4.5	Yumka, Centro, Tab.	*A. palliata*	18.000836	-92.806608	27
	4.6	Maluco, Centro, Tab.	*A. palliata*	18.0695557	-92.8142176	0
5	5.1	Mazateupa, Nacajuca, Tab.	*A. palliata*	18.216351	-93.000619	0
	5.2	Tabasquillo, Centla, Tab.	*A. palliata*	18.37737	-92.77884	0
6	6.1	Victoria, Centla, Tab.	*A. pigra*	18.5774706	-92.6241517	2
	6.2	Nueva Alianza, Centla, Tab.	*A. pigra*	18.525545	-92.640229	1
	6.3	Chochal, Jonuta, Tab.	*A. pigra*	18.423631	-92.255413	8
	6.4	San Juanito, Centla, Tab.	*A. pigra*	18.381522	-92.656127	0
	6.5	Tres Brazos, Centla, Tab.	*A. pigra*	18.36573	-92.63938	-3
7	7.1	Agua Blanca, Macuspana, Tab.	*A. pigra*	17.6797	-92.57551	213
	7.2	Celia Gonzalez de Rovirossa, Macuspana, Tab.	*A. pigra*	17.6854714	-92.385641	47
	7.3	Escondida, Jalapa, Tab.	*A. pigra*	17.6267046	-92.6530613	17
8	8.1	Jalapa, Jalapa, Tab.	*A. pigra*	17.7191561	-92.8069964	0
	8.2	Victor Fernandez Manero, Jalapa, Tab.	*A. palliata*	17.845057	-92.804545	24
	8.3	Poana, Tacotalpa, Tab.	*A. pigra*	17.5877706	-92.748522	19
9	9.1	Xicotencatl, Tacotalpa, Tab.	*A. palliata, A. pigra*	17.522577	-92.734639	53
10	10.1	Tapijulapa, Tacotalpa, Tab.	*A. pigra*	17.46178	-92.78136	79
	10.2	Florida, Tacotalpa,Tab	*A. pigra*	17.46687	-92.76248	222
	10.3	Villa Luz, Tacotalpa,Tab	*A. palliata, A. pigra*	17.44604	-92.76825	61
	10.4	Kolenchen, Tacotalpa,Tab	*A. pigra*	17.4434606	-92.757574	179
11	11.1	Acahual, Palenque, Chia.	*A. pigra*	17.6006514	-92.0372667	47
	11.2	Airport, Palenque, Chia.	*A. pigra*	17.5334418	-91.9940176	0
	11.3	Forest, Palenque, Chia.	*A. pigra*	17.6412313	-92.0314849	47
12	12.1	Pajaros, Jonuta, Tab.	*A. pigra*	17.896954	-92.093926	6
	12.2	Seja, Emiliano Zapata, Tab.	*A. pigra*	17.846483	-91.748345	9
	12.3	Chaschoc, Emiliano Zapata, Tab.	*A. pigra*	17.7967125	-91.7412737	0
	12.4	Jose Alfredo, Emiliano Zapata,	*A. pigra*	17.6613624	-91.6506949	26

		Tab.				
	12.5	Chacamax, Emiliano Zapata, Tab.	*A. pigra*	17.7153835	-91.7190358	9
13	13.1	Arenal, Balancan, Tab.	*A. pigra*	17.6577509	-91.5487348	51
	13.2	Caracolillo, Balancan, Tab.	*A. pigra*	17.6645761	-91.5414078	33
	13.3	Josefa Ortiz de Dominguez, Balancan, Tab.	*A. pigra*	17.77755	-91.50594	12
14	14.1	Guatemala	*A. pigra*	17.7855184	-90.9701725	64
15	15.1	Guacamayas, Reforma Agraria, Chia.	*A. pigra*	16.2566192	-90.8624539	142
	15.2	Roquera, Playon de la Gloria, Chia.	*A. pigra*	16.137996	-90.891848	212

Sampling of howler monkey troops

A total of 393 scat samples from *A. palliata* (n=125) and *A. pigra* (n=268) were collected from the 45 localities. In order to obtain reliable estimates of group size and composition, each of the sampling sites were surveyed staying with each troop more than 12 h and waited until all the members of the group defecated and collected one sample per animal. All the scats were carefully collected with gloves to avoid contamination and only the top of each sample was recovered. A portion was stored in absolute ethanol until processing in the laboratory. In accordance with howler monkey morphological traits, it was assigned the identification of species, sex, age, coordinates, and troop. Additional tissue (n=7 individuals) and bone (n=5 individuals) samples from both species were collected by recovery of carcasses of howler monkeys, or by chemical handling, for collection of biopsy samples. These samples were also stored in absolute ethanol.

Non-invasive techniques were implemented for sampling collection. All the procedures were performed in accordance with the provisions of the Regulations of the Environment and Natural Resources Ministry and the Under-Secretary of Management for Environmental Protection NOM-059-SEMARNAT-2010; reference SGPA/DGVS/04725/13. Academic authorities of the Universidad Juarez Autonoma de Tabasco authorized the present study (reference number UJAT-2013-43).

Landscape analyses

Analysis of the landscape characteristics

To study the effect of fragmentation, fifteen zones were obtained grouping sampling sites by proximity and similarity of landscape features, then these sites were analysed (Table 1, Fig. 2). To analyze how the influence of landscape characteristics on the genetic diversity of *A. palliata* and *A. pigra*, a landscape analysis was carried out. The geographic coordinate was registered at each site with a Garmin eTrex 30x GPS. In

each sampling point a buffer area of 3 km in diameter was realized using ARCGis 10.1. In each of the obtained buffers, the different types of vegetation and land-use were digitized from Spot 6 and 7 images of the year 2015 (1.5 m of resolution). The minimum mappable area was 0.5 ha and digitized at a scale of 1:3,000. The identification of the different types of vegetation and land use were made following the interpretation criteria of Chuvieco (2010) and through field checks.

In every buffer, all the different patches were classified in 10 different types of land-use. The size and number of all the patches of the same land-use was measured with fragstat software for ARCGIS 10.1 to calculate the average size (Ha) of the patches every land-use. Additionally, in the patch where the howler monkeys were sampled, it was measured the land-use as well as its percentage of natural vegetation surrounding the patch that was transformed into a surrounding use pressure index (SPI) of the sampled patch. This index ranges from 1 to 11.5, a value of 1 indicates minimal pressure, and as the value increases the pressure is higher (Pladeyra, 2001).

At buffer level, it was calculated a fragmentation index (ranging from 0 to 1, 0 in highly fragmented buffers and 1 in not fragmented ones) following the proposal of Díaz-Lacava (2003). Besides, vegetation type and land uses were grouped into two classes, natural and non-natural vegetation, following the classification of Galindo-Alcántara *et al.*, (2006) and INEGI, (2012). Finally, it was calculated for the latter, the percentage of the natural vegetation in the buffer.

The calculated metrics to evaluate the fragmentation characteristics of the areas where the howler monkey samples were collected were: percentage coverage, mean natural coverage, patch size, and maximum and minimum fragmentation index. In order to measure the pressure exerted on the forests and natural forests by neighboring polygons according to the use of the soil or vegetation they present (Pladeyra, 2001), the surrounding use pressure index (SPI) was calculated. The following are the formulas used to calculate each of the metrics mentioned above:

Percentage coverage = (coverage / total coverage of the buffer) * 100

Mean natural coverage = total natural coverage between buffer number

Mean patch size = the area of each patch / number of patches

Maximum and minimum index = Minimum and maximum value of fragmentation recorded in each zone.

$SPI = \Sigma (Pp * vp) / 100$

Where Pp, is the percentage of perimeter that is shared with the neighboring polygon, Vp, is the weighting value of the neighboring polygon.

Species distribution modelling (SDM), present, past and future scenarios

Besides the landscape analysis, bioclimatic variables at regional scale were considered for modelling their ecological niche. First, ecological-niche models were constructed to evaluate the suitable habitat for *A. palliata* and *A. pigra* using MAXENT version 3.3.3k. (Phillips *et al.*, 2006), a maximum entropy machine learning algorithm that estimates the probability distribution based on environmental information in an area associated only to presence-data. The model generates a probability distribution inferred by a set of environmental variables, derived from the occurrence data (Phillips *et al.*, 2006, 2008). Presence data were obtained by field registers of the present study and searching records of the presence of each species in databases and published scientific literature. Maxent was used with default settings while partitioning the geographical records between training and test samples (75% and 25%, respectively). This technique has been proven to achieve high predictive accuracy (Phillips *et al.*, 2006, 2008). The analyses were carried out for each species, considering their natural geographic distribution. In the case of *A. palliata*, it was modelled using a background that covers Mexico, Central America and pacific coast of South America (135 presence records used for training, 44 for testing and 10,102 background points), and for the model of *A. pigra* (174 presence records used for training, 57 for testing and 10,169 background points) the distribution covered Mexico, Guatemala and Belize countries as background. Initially, a total of 19 bioclimatic variables based on the model MIROC-ESM, Watanabe, *et al.*, 2011 were downloaded from the Worldly database version 1.4 (Humans *et al.*, 2005) at four different periods: the present, Mid-Holocene (MH; ~6,000 years BP), Last Glacial Maximum (LGM; ~ 21,000 years BP) and Future (2050) (Humans *et al.*, 2005), and; at a scale of 30 arc seconds (approximately 1km). (Except LGM at 2.5 min ≈ 4.5 km). Future climate scenario was modelled in the representative concentration pathway 4.5 (RCP4.5) scenario. Correlation among the initial 19 bioclimatic variables plus the altitude was measured with Pearson's correlation coefficient in *R package version*, 3.1 in order to avoid multi-collineality among variables (Pinheiro, 2011).

SDM were used to predict areas of temporal stability (regions in which species are predicted to occupy under several extreme climatic scenarios) and unstable areas (regions that disappear with climatic oscillations). Additionally, the variation in size of the overlapping area between both species distribution models across different periods was explored. Only grids with a probability of occurrence greater than 70% using ESRI ArcGIS v10.1 software were considered. The percentage of increase or decrease of areas of potential niches in each time scenario was calculated to evaluate the temporal variation of the area of sympatry.

Genetic analyses

DNA extraction

A scat subsample was used for DNA extraction from *A. palliata* (n=67) and *A. pigra* (n=217). Approximately 100 mg of each faecal sample was extracted using FavorPrep Stool DNA Isolation Mini Kits (Favorgen Biotech Corporation, Pingtung County, Taiwan) with an extended proteinase K digestion step, to maximize DNA yield. On the other hand, DNA of tissue samples was extracted using Invisorb Spin Tissue Mini Kit (Stratec, Berlin, Germany) with an extended proteinase K digestion step.

PCR amplification and sequencing

Three DNA fragments, corresponding to two mitochondrial fragments, *cytochrome b* (*cytb)* and *ATP-synthase 6 and 8 genes* (*ATPase*) and one nuclear (*Sry*) were amplified and sequenced (Table 3), using the primers designed by Cortés-Ortiz *et al.* (2003; 2007). PCR amplification was performed in a volume of 15 µL, which contained 2 µL of DNA (20-150 ng), 1× PCR buffer (1 mM KAPA Taq Buffer), 0.01 mg/mL of bovine serum albumin, 1.5 mM $MgCl_2$, 0.25 U polymerase (KapaTaq; KAPA Biosystems, Boston, MA, USA), and 0.25 mM of each primer. The PCR conditions were as follows: denaturalization at 94°C for 2 min, followed by 50 cycles (40 for *ATPase*) of denaturation for 30 s at 96°C, 30 s annealing at 55°C (60º for *ATPase*) and 1 min extension (90 s for *Sry*) at 72°C, followed by a final extension at 72°C for 10 min (15 min for *Sry*). Information of primers-used are described in Table 2.

Table 2. PCR primers for two mitochondrial fragments and one nuclear fragment for *Alouatta palliata* and *Alouatta pigra*.

Type DNA	Gene/ fragment	Primer	S	Primer sequence	Described in
Mitochondrial protein coding	*Cytb*	CB1	F	5´-CCATCCAACATCTCAGCATGATGAAA-3´ (26)	Cortés-Ortíz *et al.,* 2003
		CB2	R	3´-CCCTCAGAATGATATTTGTCCTCA-5´ (24)	
Mitochondrial protein coding	*ATPase*	LCO-CO2-L	F	5´-TARGCRTGTGWTTGGTGGGTCATTA-3´ (25)	Cortés-Ortíz *et al., 2003*
		LCO-CO3-H	R	3´-AGCATTAACCTTTTAAGTTAAAGATT-5´ (26)	
Nuclear protein coding	*Sry*	SW2	F	5´-CTTGAGAATGAATACATTGTCAGGG-3´ (25)	Moreira, 2002; Whitfield *et al.*, 1993
		SRY	R	3´-CGGTAAAAAGGAGAGTCTGCGTAG-5´ (24)	

All the amplicons were separated by electrophoresis on 1.5% agarose gel and *RedSafe* Nucleic Acid Staining Solution and sequenced on both strands by Secugen (ABI3730xl DNA Analyzer, Sanger DNA sequencing, Secugen, S. L., Madrid, Spain). Sequences were subjected to BLAST analysis via GenBank. Multiple sequence alignments, editing, assembly of strains were performed using MUSCLE (Edgar, 2004) program with the default parameters, and manually adjusted in GENEIOUS software version 7.1.3 trial (Kearse *et al.*, 2012).

Microsatellite genotyping

Initially, sixteen microsatellite markers were tested individually for the two *Alouatta* species; however, six loci were not used because of inconsistent amplification success. Finally, ten microsatellites were amplified in three multiplex PCR reactions (Table 3) following the method of Cortés-Ortíz *et al.* (2010). PCR amplification of loci was carried out with 50-100 mg of stool DNA in a total reaction volume of 13 µL with a particular PCR condition for each multiplex and specific fluorescent-dye primer combination (Table 3).

Table 3. Multiplex design of ten microsatellites in Mexican howler monkeys, including annealing temperatures for PCR reactions. Fluor means fluorochrome.

Locus	*Primer direction*	*Primer sequence (5´-3´)*	*Fluor*	*Temperature annealing (°C)*	*Multiplex*
Api07	F	TGCTTTCATGCCAACTCAAG	FAM	55°C	M1
	R	CTCAAAACCCTCACAGTGACAA			
Api08	F	GCTTCCTCTTCCCTTCTGCT	FAM		
	R	GGAGCCCTGAATTCTTTTGC			
Apm09	F	CAGGGTTCCTCTTTCACTGG	HEX		
	R	TTGGGATCACAAGTGCTTCA			
Api09	F	ACTTGCTGTGTGACCTTCAG	FAM	60°C	M2
	R	AATGTCTATCCAGCAGCCTCT			
D17S804	F	GCCTGTGCTGCTGATAACC	FAM		
	R	CACTGTGATGAGATGTCATTCC			
Ab17	F	GGAAACAGTGGAAGACAAAAGGAG	HEX		
	R	AGATGGCCAAAGATAAAGACATGTAAAA			
D5S111	F	GGCATCATTTTAGAAGGAAAT	NED		
	R	ACATTTGTTCAGGACCAAAG			
Apm04	F	TGAGAGTGAGCACCTGCCTA	NED	64°C	M3
	R	CAGCCCTGATCACAAAGTGT			
Apm01	F	CACGTGTGTCCAGCTTGTCT	PET		
	R	ATTCTGCTGCCCTTGAGTTC			
Ab04	F	AGCGCCTCTCCTGGTTTTTAC	VIC		
	R	AAAAATTCCCAAACCCCACC			

Initial denaturation for 5 min at 95°C, followed by 35 cycles (40 cycles for M2) of denaturation of 30 s at 95°C, 45 s (30 s for M3) of annealing temperature (64°C for M1, 60°C for M2 and 55°C for M3), and 90 s of extension (30 s for M3) at 72°C, with a final extension step of 30 min at 72°C. Resulting profiles (Secugen, S. L., Madrid, Spain) were analysed using GENEMAPPER 4.0 version (Chatterji and Pachter, 2006).

Genetic diversity indexes

To assess genetic variability, the average number of haplotypes (*Nh*), number of effective haplotypes (*Neh*), the information index (*I*), the mitochondrial (*cytb* and ATPase) and nuclear (*Sry*) diversity (*D*) and the unbiased diversity by population (*uD*) were calculated for both species using GENALEX v6 (Peakall and Smouse, 2006). Also GENALEX was used to calculate the genetic diversity based on ten microsatellite (Table 3) variation within species, that was evaluated by computing the number of alleles (Na), size range of alleles, observed (Ho) and expected heterozygosity (He), and Hardy-Weinberg equilibrium with GENEPOP 4.2 (Raymond and Rousset, 1995), conducted using Fisher's Method, as implemented with the default parameters for the Markov chain protocol (1000 dememorizations, 100 batches and 1000 iterations per batch). The presence of null alleles was tested using PopGenReport library (Adamack and Gruber, 2014) in R version 3.3.1 (R Development Core, Team, 2014).

Population structure and hybridization analyses

Networks were constructed for both haplotypes and genotypes. Network analyses based on haplotypes were conducted by Median Joining Network implemented in POPART (Leigh and Bryant, 2015). Additionally, a method called "minimum spanning network" (MSN) was used to calculate microsatellite genotype distances irrespective of ploidy level (Bruvo´s genetic distance, Bruvo *et al.*, 2004). MSN was calculated based on dissimilarity distance *diss.dit* and plotted with poppr.msn in *poppr* package using R software version 3.3.1 (R Development Core, Team, 2014).

Population structure based on microsatellite data of both species was explored using a Bayesian algorithm implemented in the software STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). An admixture model that assumed correlated allele frequencies was used. The results were based on simulations with 80,000 burn-in steps and 1,000,000 MCMC (Markov Chain Monte Carlo algorithm) iterations. Five runs were used for each K-value (K=1-10) to estimate the most likely value of K. STRUCTURE results were processed by the on-line software STRUCTURE HARVESTER (Earl and vonHoldt, 2012), for implementing the Evanno method, a Bayesian algorithm that allows the correct detection of the number of clusters (Evanno *et al.*, 2005). To explore the hybridization between

both species, an initial analysis was carried out to define both pure and hybrid individuals. Subsequently an intraspecific analysis was performed to estimate population structure. Because one of the assumptions of STRUCTURE analyses is Hardy–Weinberg equilibrium (HWE) for the populations and our data did not show EHW conditions (Masuda *et al.*, 2009), an alternative analysis, free of underlying assumptions of HWE, was carried out to explore population structure. A Discriminant Analysis of Principal Components (DAPC) was realized implemented in Adegenet library (Jombart and Ahmed, 2011) in R (version 3.3.1; R Development Core Team, 2014). The function "find clusters" was applied to determine the numbers of genetic clusters potentially present in the overall multilocus genotype dataset, estimating a probability of assignment of individuals to each cluster, while avoiding assignment of geographic information *a priori*.

Analyses of genetic differentiation and gene flow

Genetic differentiation was assessed using F_{ST} analysis (Balloux and Goudet, 2002).The total genetic differentiation between previously obtained clusters was assessed using analysis of molecular variance (AMOVA, Meirmans, 2006), in addition to pairwise F_{ST} estimates (Weir and Cockerham, 1984) implemented in ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). The significance of these estimators was assessed using a non-parametric permutation approach (10,000 permutations) and a Bonferroni correction was applied to significance values. Genetic variation between species were inferred with Shannon index with GENEPOP 4.2 (Raymond and Rousset, 1995).

In order to explore the relationship between genetic and geographic distance, a Mantel test analysis was calculated for both species and for every resulting DAPC cluster using GENALEX v6 (Peakall and Smouse, 2006).

To explore the spatial connectivity of *Alouatta* populations we calculated species' dispersal networks among shared haplotypes (*cytb* and *ATPase*) and genotypes (*Sry and microsatellites*) using SDMtoolbox v1 (Brown, 2014) in ArcGIS (ESRI, 2014). Genotypes of microsatellite were obtained with the assigned cluster of DAPC. Least-cost corridors (LCC) were calculated among all individuals using the inverse of species distribution modelling (SDM) as friction layer. The LCC were only carried out between *A. pigra* populations because of the low distance between sampling points in *A. palliata*.

Quantification of environmental variation and its association with genetic differentiation

In order to analyze the potential role of environment as a driving factor for genetic differentiation, we characterized the environmental space of each population using a principal component analysis (PCA) using FactoMine R package (Husson *et al.*, 2015) implemented in R. This procedure allowed us to discriminate and identify the main components of the environmental variables that best explained the distribution of the species and which of them contribute most to the model. After identifying the most important environmental variables, an AMOVA test was done to determine if there was a significant effect of those variables on the genetic data. The AMOVA was performed in R with the library poppr (Kamvar, *et. al*, 2014).

The most important environmental variables explaining the variance of principal components were further explored by correlation analyses. They were carried out by the function *cor()* in Library Stats, R (version 3.3.1; R Development Core Team, 2014) with Pearson correlations coefficients (significance level <0.05) were calculated to measure the linear dependence between two variables. Correlograms results were drawn with corrplot library.

Results

Landscape analyses

Landscape characteristics

A general estimation of every land-use type average size in all the buffer areas was done. This classification included natural tree vegetation, herbaceous natural vegetation, secondary vegetation and agroecosystems, these last two, despite of being of anthropic origin, are coverages that the monkeys are using as habitat and for that reason they were also included in this group. The unnatural class included agriculture, pasture, plantations, areas without vegetation, human infrastructures and settlements (Table 4).

Table 4. Classification of different types of vegetation and land-use in the sampling locations of wild populations of *Alouatta palliata* and *Alouatta pigra*.

Vegetation types and land-use	Description
Secondary vegetation	Massifs of vegetation in process of natural regeneration after having been modified the original vegetation. The arboreal species dominate.
Infrastructures and human settlements	Include human infrastructures and human settlements.
Agroecosystem	Include crops such as cacao *Theobroma cacao*.
Agriculture	This class includes temporary and perennial crops, mainly of herbaceous type such as banana and sugarcane.
Grassland	Herbaceous communities in which grass or graminoid species predominate, these communities are determined by natural conditions of climate and soil.
Natural vegetation arborea	All types of natural vegetation in which the dominant biological form is arborea, i.e. medium and high perennial forest, mangrove and common name “tintal” (*Haematoxylium campechianum*).
Herbaceous natural vegetation	In this category are grouped herbaceous plant covers, typical of marsh environments, whose root is attached to the bottom of the water bodies. In this class were grouped the following vegetation types (common names, following by scientific names): Popal (Thalia geniculata), Tular (*Typha latifolia)*, Chintul (*Cyperus articulatus)*, Carrizal (*Phragmites australis)*, Cibal or Popal (*Cladium* genus), (Magaña-Alejandro, 2010).
Areas without vegetation	Areas devoid of vegetation.
Plantations	Forest plantations such as eucalyptus, teak, and melina are included. Also plantations of oil palm and rubber were included.
Hydrology	Characterize all hydrology of sampling area.

Landscape description by zones

The largest number of "natural vegetation" samples was obtained in zones 10 and 12. Zone 10 is part of a protected natural area and 12 is a wetland area located in the lower basin of the Usumacinta River. Although these records come from patches of natural vegetation, which on average have more than 100 ha, both zones are areas with a high degree of fragmentation and the patches where the samples were collected are subject to a strong pressure by the matrix that surrounds them. This according to the surrounding use pressure index (SPI), (Table 5).

Zones 5, 9, 10, 14 and 15 are the only areas with a moderate degree of fragmentation according to the fragmentation index used. These areas are characterized by more than 50% of their surface covered by natural vegetation. In spite of this, the fragments where

the howler monkeys were recorded are subject to a low to medium degree of pressure by the surrounding matrix, according to the index of surrounding use pressure. Only in 8 sites (Tabasquillo, Nueva alianza and Tres Brazos in the municipality of Centla, Poana in Tacotalpa, Palenque in Chiapas, Chacamax in Emiliano Zapata, Arenal in Balancan and Guacamayas in Chiapas) belonging to zones 5, 6, 8, 11, 12 and 15, the patches where the howler monkeys were sampled have a low surrounding use pressure. However, although they are highly fragmented areas, they still maintain important fragments of natural vegetation with an average size of 59.46 ha (min = 1 ha, max = 2,108.6 ha).

Table 5. Metrics of habitat disturbance in the fifteen studied zones for *A. palliata* and *A. pigra*. The numbers in parentheses indicate the minimum and maximum values recorded in each zone.

Zone	Species	Average Natural cover (%)	Average patch size (ha)	Fragmentation index (Minimum-maximum)	Pressure index of surrounding use SPI
1	*A. palliata*	22.7	26.2	0.22	Intermediate
2		53.28	379.2 (87.5-886.6)	0.3-0.7	High
3		40.1	499.47 (7.6-991.2)	0.3-0.5	Intermediate, high
4		24.3	1110.2 (28.2-2092.8)	0.002-0.7	Intermediate, high
5		61.1	685.5 (366-1005)	0.6-0.7	Low, intermediate
6	*A. pigra*	26.1	694.7 (3.3-2005.3)	0.2-0.5	Low, intermediate
7		26.7	1243.42 (622-1864)	0.2-0.4	Intermediate,
8	*A. palliata*	27.4	974.6 (4.5-967.14)	0.1-0.4	Low, intermediate
9	*A. pigra*	57.7	1297.8 (1509.1-1086.6)	0.6	Low
10	*A. palliata*	73.3	446.3 (35.8-1074.2)	0.6-0.9	Intermediate, high
11	*A. pigra*	8.47	898.06 (85.3-1710.8)	0.08	High
12		39.3	910.3 (162.3-2108.6)	0.1-0.8	Low, intermediate
13		28.9	788.3 (33.1-2296.2)	0.1-0.6	Intermediate
14		73.6	1997.7	0.7	Intermediate
15		56.1	1048.1 (1145-1165.8)	0.5-0.6	Intermediate

Species distribution and climate niche modelling for *A. palliata* and *A. pigra* in four scenarios (LGM, MH, present and Future-2050)

We constructed past, present-day and future (2050 year) climate niche models using nine bioclimatic variables, all of which had a correlation degree lower than 0.80 (Pearson coefficient). The Jackknife procedure was implemented in Maxent (Maximum entropy modelling) to find the best set of predictor variables following a parsimony approach based on the average area under the receiving operator characteristics curve (AUC) test of ten replicates. The final set of bioclimatic predictor variables for the SDMs were BIO1, BIO4, BIO5, BIO8, BIO9, BIO10, BIO11, BIO16 and BIO17. This model had high value of AUC, for *A. palliata* (0.88) and *A. pigra* (0.93) indicating overall good performance. The environmental variable with highest information content for both species was BIO4 Temperature Seasonality Additionally BIO5 (Max Temperature of Warmest Month) was important for *A. palliata*, and BIO11 (Mean Temperature of Coldest Quarter) for *A. pigra.*

Last Glacial Maxima SDM projection showed very restricted potential species distribution with respect to current SDM for both species, although it was significantly lower for *A. pigra* (11 times smaller) than for *A. palliata* (two times) (Table 6). *A. pigra* showed several small glacial refugia scattered across southern highland massif of Mexico (Chiapas, Oaxaca, Puebla and Veracruz) and Belize. In the same way, *A. palliata* showed a fragmented distribution with several glacial refugia: in the current hybridization zone of Mexico, and in several refugia across Costa Rica, Colombia and Ecuador. Although glacial refugia for both species were inferred close to the actual hybrid zone, no overlap was detected in this period (Table 6, Supplementary material, Fig. S2).

MH SDM projection showed an expansion of the potential distribution range for both species from the inferred glacial refugia (9 times for *A. pigra* and 5 times for *A. palliata*). *A. pigra* SDM expand into Central America, what predicts a hybridization area that covers the actual hybrid area plus several areas in Guatemala and the Atlantic coast of Nicaragua.

The actual predicted habitat suitability area was consistent with the current distribution of both species (Fig. 2). *A. pigra* continued its range expansion from MC potential distribution, although at lower rates while a significant loss of potential distribution area in *A. palliata* is observed (almost to one third of its MH SDM distribution). The predicted overlap of potential climatic niches between both species across the Tabasco state is corroborated by hybridization observed in this area (Cortés-Ortiz *et al.*, 2015), (Fig. 3, Table 6).

Future projection (2050) showed also a dissimilar response to climate change for both species. *A pigra* showed an eastward and westward range expansion (76%) of its potential distribution range while *A. palliata* did not show substantial changes in its distribution range (Supp. Mat. Fig. S2, Table 6). The area of sympatry between both species is inferred to be reduced in an 80% (Table 6).

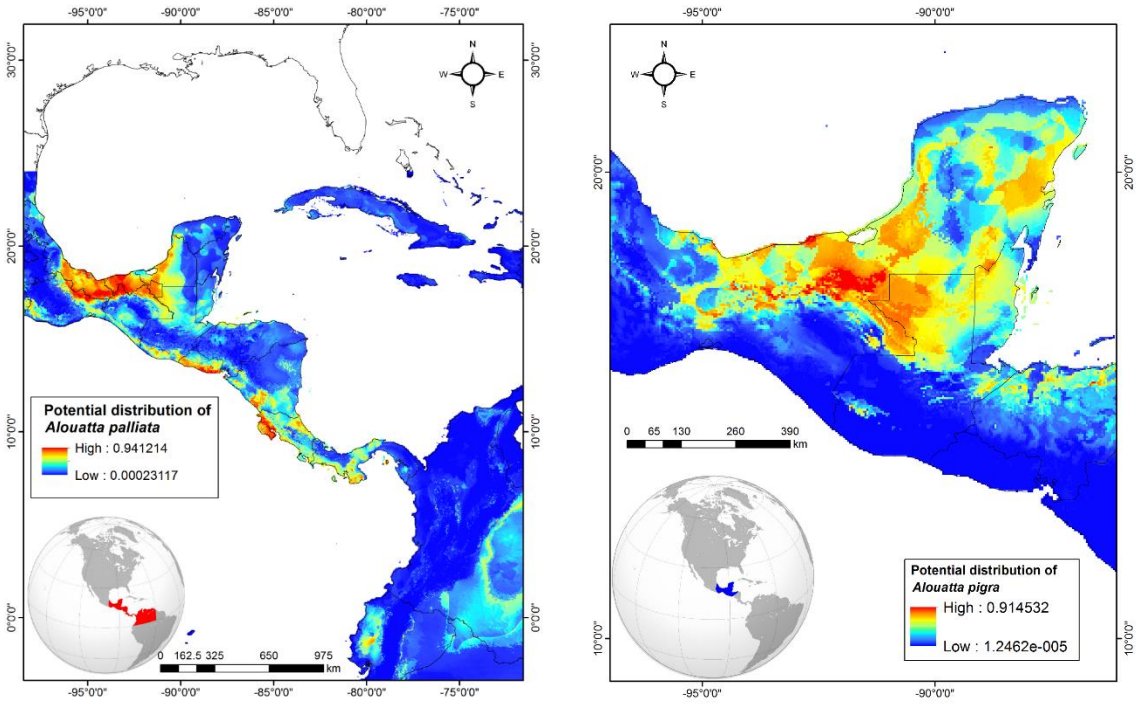

Figure 2. MAXENT actual niche model constructed for *A. palliata* and *A. pigra*. Areas with high probability of species occurrence in warm colors, areas enclosing low probability values in cool colors. Color graphic scale represents the percentage of probability of presence.

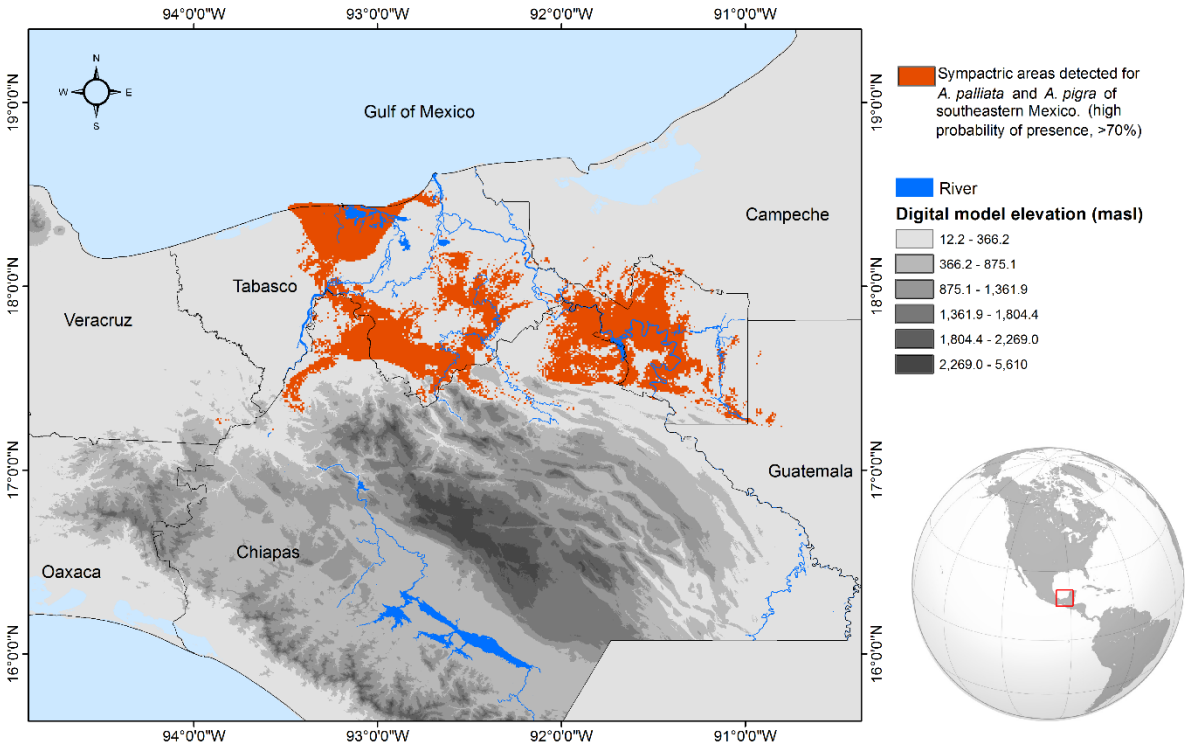

Figure 3. High probability of overlapping (>70%) of *Alouatta palliata* and *A. pigra* inferred from MAXENT niche models. MASL means meters above sea level.

Table 6. MAXENT scenarios for *Alouatta palliata*, *Alouatta pigra* and *A. palliata* x *A. pigra* crosses areas estimated (highly probability of presence, >60%).

Time	*A. palliata* area (ha x 10^6)	Size variation	*A. pigra* area (ha x 10^6)	Size variation	*A. palliata* x *A. pigra* area (ha x 10^6)	Size variation
Last Glacial Maximum (LGM)	5.08		0.53			
Mid Holocene (MH)	27.03	+ 432%	4.89	+ 821%	1.45	+ 43%
Present (PR)	10.22	- 62%	6.15	+ 26%	2.07	
Future Miroc, 2050 (MIR)	10.10	-1%	10.63	+ 73%	0.40	- 80%

Genetic analyses

Genetic diversity

The diversity indices for mtDNA and nuclear genes demonstrated low levels of genetic variation within howler monkey species, *A. palliata* and *A. pigra* (Table 7). The mitochondrial *cytochrome b* fragment was amplified in 162 howler monkeys and the resulting the length of the aligned sequences was 242 bp. Mitochondrial *ATPase* was

amplified in 243 individuals resulting in a 719 bp fragment. Nuclear *Sry* was sequenced in 24 individuals generating a 592 bp fragment. For *cytochrome b*, ATP*ase* and *Sry* fragments additional sequences were obtained from GenBank for individuals from Mexico of both species (Table 7). Only seven haplotypes using the *cytb* were determined, two haplotypes for *A. palliata* and five for *A. pigra* (Fig. 4a). *ATPase* fragments included ten haplotypes (H), six haplotypes for *A. palliata* and four haplotypes for *A. pigra* (Fig. 4b). Five mutational steps separated both species with *cytochrome b* and 34 mutational steps for ATP*ase*. For Sry only one haplotype (H1) was determined for *A. palliata* whereas three haplotypes were registered for *A. pigra*, and only one mutational step separated both species. Fourteen of the 24 haplotypes detected with the three fragments were known from previous studies (Cortés-Ortiz *et al.*, 2003 and Cortés-Ortiz *et al.*, 2007) whereas ten were novel (*cytb*: H2 for *A. palliata*, H3 and H7 for *A. pigra*; ATP*ase*: H4 and H7 for *A. palliata*; *Sry*: H6 for *A. palliata,* H1, H3, H4 and H5 for *A. pigra*; Fig. 4).

Table 7. Estimates of genetic variation in *Alouatta palliata* and *Alouatta pigra* inferred from two mitochondrial and one nuclear marker. +gb: including sequences of GenBank; *N*: sample size; *Nh*: number of haplotypes; *Neh:* number of effective haplotypes; *I*: Information Index; *D*: diversity and *uD*: unbiased diversity by population.

Specie	Locus	*N*	*Nh*	*Neh*	*I*	*D*	*uD*
A. palliata	*Cytb*	21	3	1.639	0.709	0.39	0.41
A. palliata (+gb)		59	2	1.034	0.086	0.033	0.034
A. pigra		141	6	2.072	0.942	0.517	0.521
A. pigra (+gb)		161	5	1.873	0.739	0.466	0.469
A. palliata	*ATPase*	20	4	2.985	1.192	0.665	0.7
A. palliata(+gb)		49	4	3.043	1.222	0.671	0.685
A. pigra		223	2	1.009	0.029	0.009	0.009
A. pigra (+gb)		303	3	1.032	0.090	0.031	0.031
A. palliata	*Sry*	1	NA	NA	NA	NA	NA
A. palliata (+gb)		5	2	1.471	0.5	0.32	0.4
A. pigra		23	5	2.507	1.116	0.601	0.628
A. pigra (+gb)		31	5	2.058	0.965	0.514	0.531

The microsatellites, as an insight into the genetic diversity of Tabasco and Chiapas howler monkeys, were firstly analyzed for each species. Genetic parameters based on microsatellite data for *A. palliata* and *A. pigra* individuals were estimated (Table 8). All microsatellite loci were highly polymorphic and the overall number of alleles ranged from five (*D17S804*) to twenty (*Ab17*). The number of alleles per locus ranged from 5 to 16 alleles (mean 3.6), and from 6 to 20 alleles (mean 3.4) across *A. palliata* and *A. pigra*

species respectively. All loci (except the *Apm*04 in *A. palliata*) exhibited deviation from Hardy–Weinberg equilibrium in both species. None of the loci showed occurrence of null alleles. *A. palliata* and *A. pigra* exhibited mean observed heterozygosity of 0.26 and 0.36 respectively, and the results showed an excess of homozygotes, except for the locus Apm01 in *A. pigra (*Table 8*).*

Additionally, an analysis was performed for each species considering the geographic location of the samples. In this case, the population parameters were inferred by grouping the samples into sets based on the geographic site and similarities of features habitat, grouping in five and eight sampling zones for *A. palliata* and *A. pigra* respectively (Table 9). The average genetic diversity, measured as unbiased expected heterozygosity (*uHe*) were similar for both species, 0.61 ± 0.03 (*A. palliata*) in the west to 0.638 ± 0.025 (*A. pigra*) in the east of Tabasco State (Table 9).

Table 8. Summary of microsatellite variability and estimates of genetic variation in *A. palliata* (n=41 individuals) and *A. pigra* (n=85 individuals) inferred from ten microsatellite loci (mean ± SE). Grand mean and standard error over populations and loci. All populations. Na: number of alleles; Ne: number of effective alleles; Ho: observed heterozygosity; He: expected heterozygosity; uHe: unbiased expected heterozygosity and H-W=Global Hardy-Weinberg test, heterozygote deficit.

	Alouatta palliata					***Alouatta pigra***				
Locus	***N_a***	***Size range (bp)***	***Ho***	***He***	***P-value (H-W)***	**N_a**	**Size range (bp)**	**Ho**	**He**	**P-value (H-W)**
Api07	7	108-124	0.379	0.605	**0.0117**	16	102-170	0.388	0.571	**0**
Api08	6	272-294	0.179	0.524	**0.0282**	6	272-288	0.286	0.289	**0.0298**
Apm09	8	157-178	0.300	0.615	**0.0009**	12	137-205	0.269	0.545	**0**
Ab04	12	158-202	0.651	0.738	**0.0417**	14	120-202	0.159	0.373	**0**
Ab17	16	190-262	0.233	0.589	**0.0003**	20	177-256	0.532	0.662	**0**
Api09	13	428-482	0.097	0.611	**0.0007**	15	405-482	0.246	0.460	**0**
Apm01	12	184-218	0.423	0.621	**0.0004**	9	183-213	0.586	0.548	**0.0253**
Apm04	8	238-252	0.253	0.458	0.5843	8	237-279	0.312	0.474	**0**
D17S804	5	156-178	0.135	0.386	**0.0032**	12	122-168	0.321	0.522	**0**
D5S111	6	156-178	0.090	0.287	**0.0006**	11	138-181	0.220	0.396	**0**
Mean±SE	3.55 ± 0.231		0.259 ± 0.031	0.528 ± 0.033		3.417 ± 0.16		0.356 ± 0.027	0.526 ± 0.021	

Comparing groups by area, the number of alleles per locus in *A. palliata* ranged from two to five alleles (mean 6.14), and showed a mean observed heterozygosity of 0.28. The number of alleles for *A. pigra* ranged from two to seven alleles (mean 4.34) and showed a mean observed heterozygosity of 0.39. The average genetic diversity (*uHe*) was bigger for *A. pigra* in comparison with *A. palliata* (Table 9). Of the 15 zones, only four zones are in Hardy-Weinberg equilibrium expectations. Two of these zones corresponding for *A.*

palliata, to Z1 (Carlos Greene, Comalcalco) and Z8-Z10 (Jalapa, and Villa Luz, Tacotalpa), while for *A. pigra*, Z11 (Palenque, Chiapas) and Z13 (Arenal, Caracolillo and Josefa Ortiz de Dominguez, Balancan) are in HWE.

Table 9. Summary of microsatellite variability and estimates of genetic variation in 5 sampling zones of *A. palliata* (n=40 individuals) and *A. pigra* (n=83 individuals) inferred from ten microsatellite loci (mean ± SE). Grand mean and standard error over populations and loci. All populations. N: sample size; Na: number of alleles; Ne: number of effective alleles; I=Shannon's Information Index; Ho: observed heterozygosity; He: expected heterozygosity; uHe: unbiased expected heterozygosity; H-W=Global Hardy-Weinberg test, heterozygote deficit, numbers in bold means disequilibrium of HW; F = Fixation Index = (He - Ho) / He = 1 - (Ho / He) and Fst = (Ht - Mean He) / Ht.

Sp	Zone	N	Na	Ne	I	Ho	He	uHe	P-value (H-W)	F	Fst
A. palliata	**Z1**	4.8 ± 0.44	3 ± 0.33	2.26 ± 0.29	0.87 ± 0.11	0.31 ± 0.08	0.51 ± 0.05	0.58 ± 0.05	0.0014	0.43 ± 0.16	
	Z2	8 ± 0.77	5 ± 0.68	3.5 ± 0.55	1.31 ± 0.13	0.2 ± 0.05	0.66 ± 0.04	0.71 ± 0.05	**0**	0.71 ± 0.08	
	Z3 Z4	8.5 ± 0.72	5 ± 0.54	3.43 ± 0.49	1.32 ± 0.12	0.28 ± 0.07	0.65 ± 0.04	0.70 ± 0.05	**0**	0.59 ± 0.10	
	Z5	5.9 ± 0.97	2.7 ± 0.60	1.89 ± 0.43	0.64 ± 0.20	0.18 ± 0.08	0.33 ± 0.10	0.36 ± 0.11	**0**	0.42 ± 0.15	
	Z8 Z10	3.5 ± 0.22	3.2 ± 0.20	2.64 ± 0.26	1.01 ± 0.08	0.44 ± 0.10	0.58 ± 0.04	0.69 ± 0.05	0.0675	0.20 ± 0.17	
	Mean ± SE	6.14 ± 0.40	3.78 ± 0.26	2.74 ± 0.20	1.03 ± 0.07	0.28 ± 0.04	0.55 ± 0.03	0.61 ± 0.03	**0**	0.47 ± 0.06	0.20 ± 0.03
A. pigra	**Z6**	6.6 ± 1.10	3.40 ± 0.70	2.57 ± 0.49	0.96 ± 0.18	0.18 ± 0.07	0.52 ± 0.08	0.57 ± 0.09	**0**	0.64 ± 0.15	
	Z7 Z8	7.10 ± 0.41	4.90 ± 0.48	3.34 ± 0.45	1.31 ± 0.11	0.44 ± 0.10	0.66 ± 0.04	0.71 ± 0.04	**0**	0.33 ± 0.16	
	Z9	10.60 ± 0.50	5.40 ± 0.83	3.64 ± 0.50	1.36 ± 0.15	0.52 ± 0.05	0.67 ± 0.04	0.71 ± 0.05	**0**	0.20 ± 0.10	
	Z10	18.10 ± 0.66	7.60 ± 0.85	4.63 ± 0.66	1.66 ± 0.13	0.30 ± 0.05	0.74 ± 0.04	0.76 ± 0.04	**0**	0.59 ± 0.07	
	Z11	4.10 ± 0.41	2.70 ± 0.33	2.21 ± 0.29	0.79 ± 0.13	0.64 ± 0.11	0.47 ± 0.07	0.58 ± 0.10	0.9483	-0.35 ± 0.09	
	Z12	10.90 ± 1.29	4.40 ± 0.64	3.01 ± 0.47	1.15 ± 0.13	0.44 ± 0.05	0.61 ± 0.04	0.65 ± 0.04	**0**	0.23 ± 0.11	
	Z13	5.30 ± 0.54	2.70 ± 0.33	2.05 ± 0.24	0.76 ± 0.13	0.29 ± 0.11	0.44 ± 0.07	0.50 ± 0.08	**0.0119**	0.47 ± 0.17	
	Z15	5.40 ± 0.76	3.60 ± 0.54	2.72 ± 0.44	1.05 ± 0.15	0.32 ± 0.10	0.57 ± 0.07	0.63 ± 0.08	**0**	0.43 ± 0.17	
	Mean ± SE	8.513 ± 0.548	4.338 ± 0.271	3.021 ± 0.178	1.130 ± 0.058	0.390 ± 0.032	0.586 ± 0.023	0.638 ± 0.025	**0**	0.319 ± 0.055	0.20 ± 0.04

Population structure and hybridization analyses

For *cytochrome b* the most common haplotype for *A. palliata* is H1 (98.3%), followed by H2 (1.7%); in the case of *A. pigra* the most frequent is H5 (65.2%), followed by H4 (32.9%). In *A. palliata* H1 is widely distributed in Veracruz and Tabasco States. In *A. pigra* H4 and H5 is present in Tabasco State and in the Southeast of Mexico. In *A. pigra* H2 and H3 are restricted to Balancan municipality in Tabasco (Fig. 4a).

For *ATPase* the most common haplotype for *A. palliata* is H10 (44.1%), followed by H8 (26.5%), H5 (17.6%) and H7 (11.8%); in the case of *A. pigra* the most frequent haplotype

is H1 (99.6%), followed by H3 (0.4%) (Fig. 4b). In *A. palliata* H5 and H10 is widely distributed in Veracruz and Tabasco States. In *A. pigra* H1 is present in Tabasco and Chiapas States, also in the Southeast of Mexico. In *A. pigra* H8 are restricted to southeast of Tabasco State and Central American region.

The most common occurrence of *Sry* haplotype for *A. palliata* is H7 (80%), followed by H6 (20%). For *A. pigra* the most common occurrence is H2 (64.5%), followed by H5 (25.8%), H3 and H4 with the same frequency (3.2% respectively). Significant differences in the haplotype distribution were observed at species and geographical levels (Fig. 4c). In *A. palliata* H4 is distributed in Veracruz and Tabasco States. In *A. pigra* H1 is exclusive of South of Chiapas state, and H2 is widely distributed in Tabasco and Campeche States.

STRUCTURE analysis based on microsatellite data showed the highest Δ*K* for *K*= 2 dividing both species populations. Finally, STRUCTURE for *K*= 3 found admixture between both species with a third cluster including hybrids of *A. palliata* and *A. pigra* (with different percentage of introgression) mainly in highlands of Tabasco State. However, STRUCTURE results were not taken into account because most of the loci of both species were not satisfy HWE conditions within populations (Masuda *et al.*, 2009).

Therefore, a DAPC was conducted to obtain further insights into the level of hybridization by species. Principal component analysis scatter plot for ten microsatellites for 126 howler monkeys from 15 sampling zones reveals population substructure and diversity. The cluster analysis performed by K-means and DAPC revealed the presence of three genetic groups (K = 3) in the Southeast of Mexico (Fig. 4d and Fig. 5), showing association with the geographical origin, as it can be observed in the minimum spanning network (MSN; Fig. 6), based on the presence of the same microsatellite genotype in different zones.

Three cluster populations are well differentiated (Fig. 4d). In this analysis, the species *A. palliata* and *A. pigra* appeared to be separated geographically in an East and West region of Tabasco State. In DAPC, hybrid individuals appeared to be a west-east distribution. The crosses of *A. palliata* and *A. pigra* occurred more frequently in *A. pigra* distribution area. F1 Individuals of howler monkey are present in Zone 12, at southeast of Tabasco State. Main contact area and hybridization area are occurring from zone 8 to zone 11. Although zones 4, 13 and 14 also present individuals allowing to cluster 3, recognized as hybrids (Fig. 5).

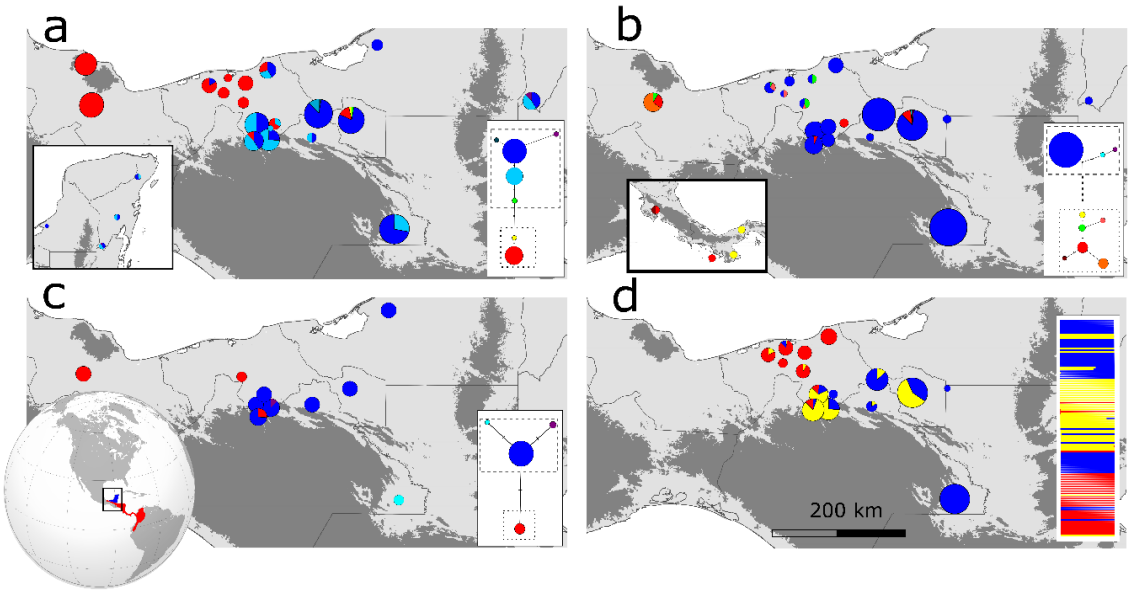

Figure 4. Geographic distribution of the genetic diversity of *A. palliata* and *A. pigra* populations. Pie charts show frequency of haplotypes/genotypes in each geographical sampling zone. (a) haplotypes of *Cytochrome b*, (b) haplotypes of *ATPase*, (c) haplotypes of *Sry,* (d) Three clusters obtained by discriminant analysis of PCs (DAPCs) of ten microsatellites. A median joining network is shown besides each marker. Sampling sites names are shown in Table 1. Dashed lines and cool colours represent *A. pigra* populations. Dotted lines and warm colours represent *A. palliata* populations. Yellow colour in (d) represents *A. palliata x A. pigra* crosses of putative hybrid individuals. The size of the circles is proportional to the number of individuals with a particular haplotype/genotype. In the haplotype network (a, b and c) each transversal bar represents a mutational step.

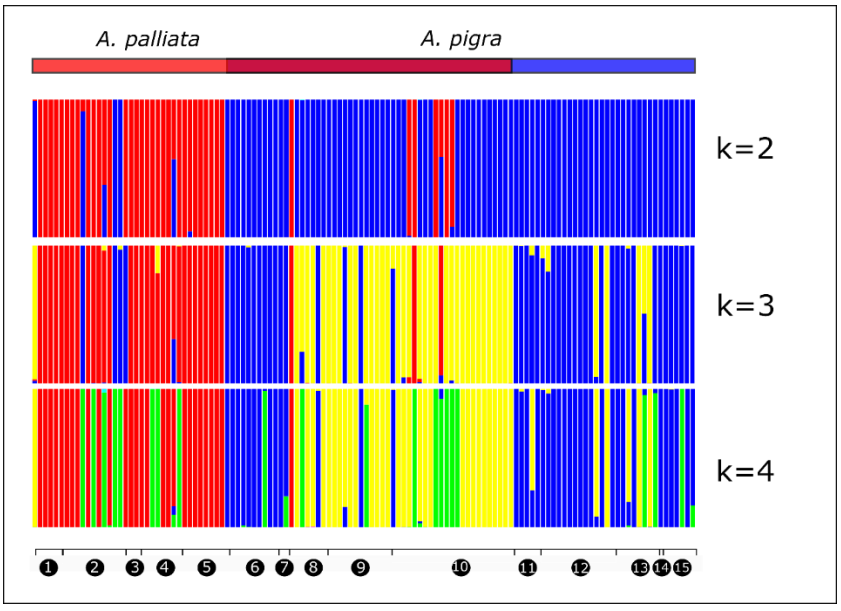

Figure 5. Population structure of *A. palliata* and *A. pigra* populations. DAPC often nuclear loci for 126 individuals. Vertical bars show the membership in a cluster for each individual. Separate colours represent separate clusters. K=2 shows well-resolved groups: red (cluster 1): *A. palliata* cluster; blue (cluster 2): *A. pigra* cluster; in K=3, yellow (cluster 3): putative hybrids of *A. palliata* and *A. pigra* (with different percentage of introgression) and shows mixed groups. Numbers below the graphic represent to zone identification in Table 1 and Fig. 1.

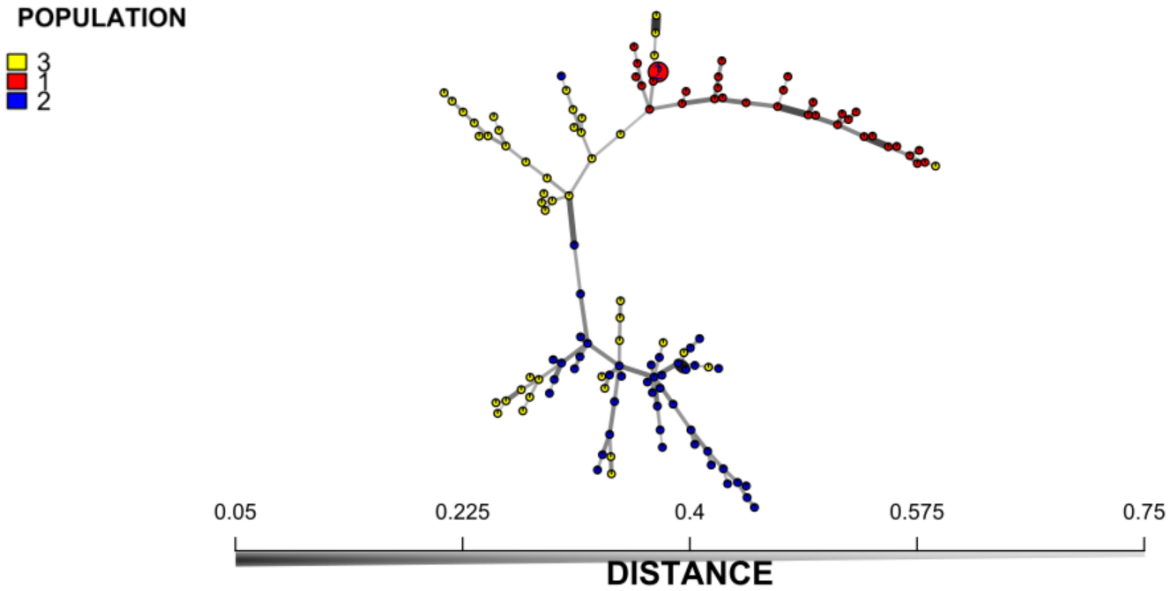

Figure 6. Minimum spanning network of howler monkey nuclear DNA region microsatellite genotype distances. Red dots represent individuals of *A. palliata* (cluster 1), blue dots represent individuals of *A. pigra* (cluster 2) and hybrid individuals are represented with yellow dots (cluster 3).

A similar general genetic pattern can be noted on the microsatellite minimum spanning network reconstruction (Fig. 6), as well as DACP also suggests the presence of three different clusters, and supports the hybridization process.

Analyses of genetic differentiation and gene flow

Interpopulation analysis of *Fst* values between the *A. palliata* and *A. pigra* populations are significantly different from 0 (*Fst*=0.117; p<0.001), indicating significant genetic differentiation. When pairwise *Fst* was calculated among DAPC clusters, it showed lower genetic differentiation between hybrids and purebread *A. pigra* (*Fst* = 0.073) than between hybrids and purebred *A. palliata* (*Fst*=0.114) or between purebred individuals of both species (cluster 1 and 2, *Fst*=0.128).

Analysis of molecular variance (AMOVA) showed that 15.78% for *A. palliata* and 2% for *A. pigra* of the total variance in data set was partitioned among populations (Global *Fst* = 0.42, *Fst*=0.29, $p < 0.001$, respectively, Table 10). Pairwise *Fst* values were significant for all comparisons. A significant positive correlation between geographic and genetic distance was obtained only for *A. pigra* (p= 0.0009).

Dispersal network results for *A. pigra* revealed high connectivity between populations of the highlands and low connectivity between lowland populations. MtDNA showed higher connectivity between southern Chiapas populations and Tabasco and northern Chiapas populations than nuclear dispersal networks (Fig. 7).

Table 10. AMOVA for *A. palliata* and *A. pigra* populations. Asterisks indicate significant contributions (p<0.01).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Alouatta palliata				
Among groups	7	4.689	0.028	**15.78**
Among individuals, within groups	7	2.006	0.046	**25.86***
Within individuals	68	7.040	0.104	**58.37***
Total	82	13.735	0.177	
Alouatta pigra				
Among groups	14	12.297	0.005	**2**
Among individuals within groups	12	6.354	0.066	**27.25***
Within individuals	193	33.162	0.172	**70.76***
Total	219	51.814	0.243	

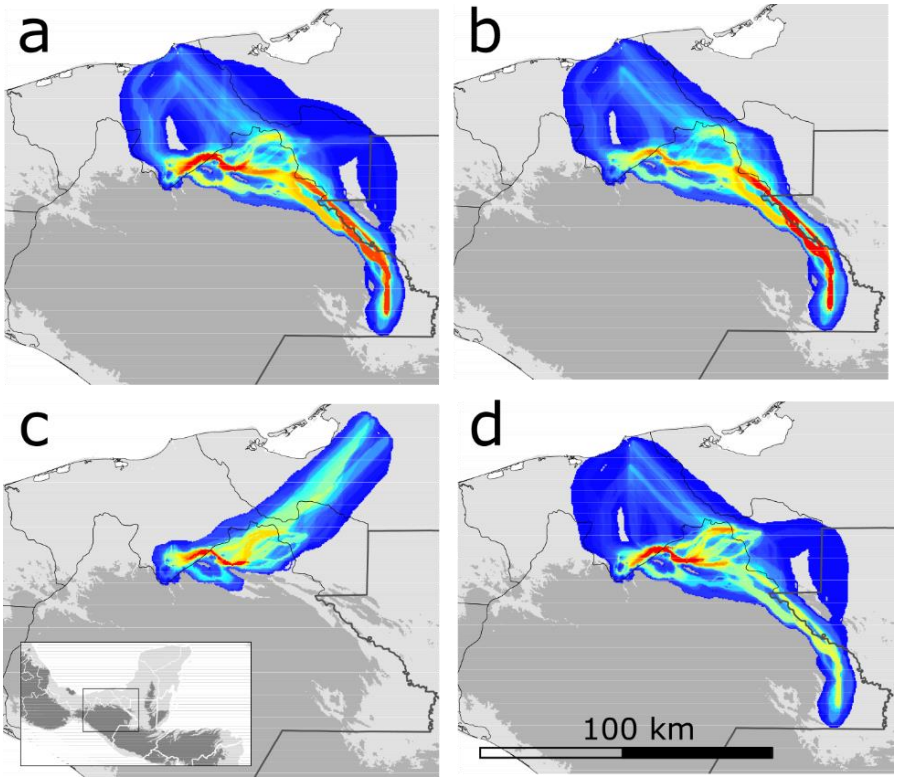

Figure 7. Dispersal networks of shared haplotypes or genotypes for *A. pigra* populations across southern Mexico. (a) ATPase, (b) cytB, c) SRY and d) microsatellites locus.

Effects of landscape variables on genetic diversity

PCA analysis showed low level of variance explained by the two first dimensions (13.2 and 11.3 %) There were several landscape variables with a high contribution to explain

the first two axes. Natural vegetation, fragmentation, arboreal vegetation and grassland contribute to explain dimension 1 (Fig.8). On the other hand, the surrounding natural vegetation, infrastructure and surrounding use pressure index (SPI) contribute to dimension 2. Additionally, some microsatellite loci, contribute to explain the model: Ab17 and Api08, in dimension 1 and Ab04 and Apm04 and Api09 on dimension 2. This could be explained because of the high level of polymorphism in these loci (Fig. 8).

Surrounding natural vegetation showed the highest correlation with molecular data (5 loci) followed by type of vegetation (4 loci) (Fig. 9). Loci Ab17 was positively correlated with high number of landscape features such as vegetation type, arboreal natural vegetation, or surrounding natural vegetation (Fig. 9). It was found a significant correlation ($p<0.05$) between D5s111 and Ab04 loci with several environmental variables. There was a positive correlation with herbaceous natural vegetation and negative with secondary vegetation. Both loci showed a distinct response to type of vegetation and SPI habitat disturbance index. Locus Api07 showed positive correlations with natural vegetation and low fragmentation, and a negative correlation with grassland and total area.

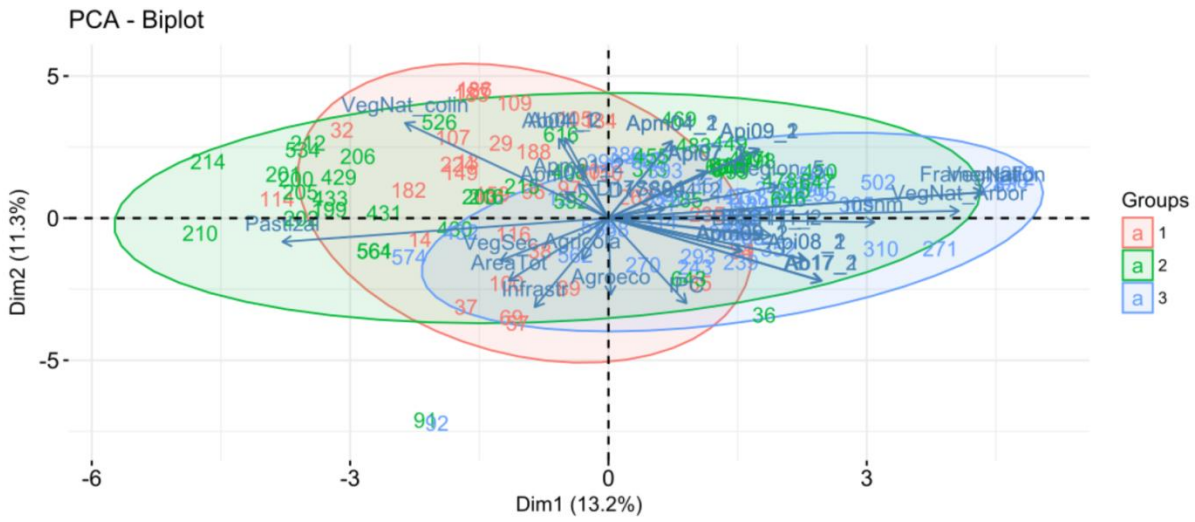

Figure 8. A bi-plot PCA of environmental and genetic (microsatellite) variables. Site clustering based on minimized centroid distances of environmental variables comprising the reduced data set (a). Cluster groups of DAPC K=3 are labelled with a red circle (*A. palliata*, cluster 1), green circle (*A. pigra*, cluster 2) denote sites within whole range of landscape and blue circle, while blue circles (*A. palliata* x *A. pigra*, cluster 3). Ellipses encompass the sites clustered by minimized centroid distances.

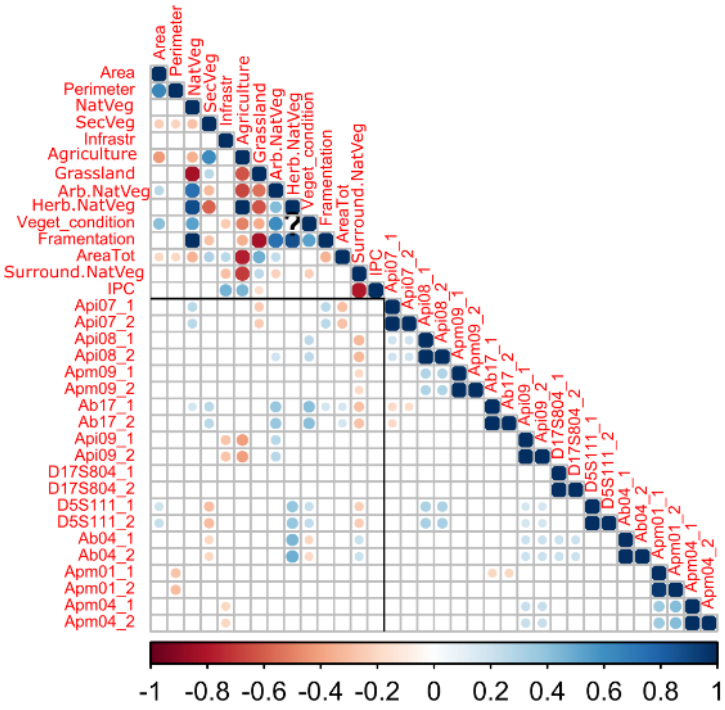

Figure 9. Correlations between genetic diversity and landscape variables ($p<0.05$). Blue indicates a positive relationship, red a negative one; colour intensity is proportional to Pearson's correlation coefficient.

Discussion

Our study presents a potential species distribution and its phylogenetic diversity for *A. palliata* and *A. pigra* in their hybridization zone in southern Mexico. We also explore the temporal dynamic of these areas across several climatic scenarios and the effects of landscape variables.

The results of the current distribution model of *A. palliata* and *A. pigra* were in general congruent in both species, and with previously reported studies for them (Vidal-García and Serio-Silva, 2011).

The tropical glacial refugia model suggest that Neotropical lowland forests become divided into a number of isolated smaller glacial refugia for the forest fauna, and from which they expanded during interglacial periods (Bennett and Provan, 2008; Haffer, 1969). Here we have explored the SDM of *A. palliata* and *A. pigra* during both extreme climatic scenarios, using the Last Glacial maxima (LGM), Mid Holocene (MH) and Present as models to understand their temporal dynamic across the Pleistocene climatic

oscillations. Our results support the tropical glacial refugia model, showing several small and isolate areas of potential habitat distribution scattered along Mesoamerican highlands during LGM and a range expansion during the interglacial periods (MH and present). These interglacial expansions agree with previous studies on tropical primates (Anthony *et al.*, 2007, Arora *et al.*, 2010) and do not support the hypothesis of tropical stability found in other tropical mammals (Lessa *et al.*, 2008)

SDM revealed an independent response to climatic oscillations between both species. First it has been identified a clear sign of expansion from refuge areas in both species after LGM, but both species expanded differently with *A. pigra* expansion been proportionally larger than *A. palliata. W*hile *A. palliata* reached its maximum distribution in MH, *A. pigra* continues to expand steadily (Supp. Mat. Fig. S3). Actual prediction of potential niche calculated for both species indicated that at present *A. palliata* distribution had been reduced with a decrease of almost 62% with respect the MH distribution. However, this is not the case for *A. pigra* that show an increase of 26%. Within the future climatic projection, *A. pigra* shows an expansion of the potential niche. This result was unexpected as at present it is a species with a more local or regional distribution with respect to its congeneric *A. palliata*. On the contrary, *A. palliata* is widely distributed from South-eastern Mexico to northern of Peru, thus, it was expected that this species would show a wider climatic niche and therefore a better future scenario in comparison with *A. pigra*. However, the results obtained indicate that its potential niche would become even lower than *A. pigra*. Therefore, a disparate response to climatic variations is obtained in both sister species. Previous studies have also shown a similar species-specific response to climate change in phylogenetically related taxa (i.e. Anadón *et al.*, 2015). Consequently, studies of landscape modelling and future scenarios are required to prioritize conservation strategies for the most threatened species.

Despite the potential species distribution has suffered contraction and expansions for both *Alouatta* species across climatic oscillations; the observed present-day hybrid zone has remained stable through all modelled climatic scenarios, but LGM. Although no sympatry area was detected in the LGM models, their respective glacial refugia were located 30 km apart in the Sierra Madre of Chiapas. Given the speciation process between both *A. palliata* and *A. pigra* species was 3 mya (Cortés-Ortiz *et al.*, 2003), it is likely that they have been in contact in previous climatic oscillations through the Pleistocene. These results support previous evidence of temporal stability in hybridization zones through climatic oscillations (Hewitt, 2011).

A. pigra was expected to present lower genetic variability than *A. palliata*. In addition, by its restricted distribution, both Mexican howler monkeys were expected to exhibit a northward decline of genetic variability in comparison with Central America howler monkeys (Ellsworth y Hoelzer, 2006). The results of this study suggest that the groups of *A. palliata* and *A. pigra* studied from South-eastern Mexico, present a low genetic diversity, but within the range of previously reported values for the same species. The mean number of alleles found (3.6 alleles per locus for *A. palliata* and 3.4 for *A. pigra*) show similar values than previous studies (2.88 - 6.3; Winkler *et al.*, 2004; Ruiz-García *et al.*, 2007; Milton *et al.*, 2009; Ellsworth and Hoelzer, 2006; Jasso-del Toro, *et al.*, 2016). In the same way, the observed heterozygosity values (Ho) were similar to previously reported. The results for *A palliata* (Ho = 0.259) were within the range of other studies (0.14 -0.56; Zaldivar *et al.*, 2003; Winkler *et al.*, 2004; Ruiz-García *et al.*, 2007; Milton *et al.*, 2009; Dunn *et al.*, 2014; Jasso-del Toro, *et al.*, 2016). *A.pigra* also showed similar values (Ho = 0.356) to previous studies (0.3-0.59; James *et al.*, 1997; del Valle *et al.*, 2005 ; Van Belle *et al.*, 2012). However, no differences have been found between *A. palliata* and *A. pigra*, despite differences in the mating system and number of individuals per group. Whereas *A. palliata* shows a multi-male composition with 2–45 individuals per group and *A. pigra* shows a uni-male composition and has smaller groups with 2–10 individuals per group (Pavelka and Chapman, 2006). Genetic differentiation (Fst=0.2) for both species in 5 and 8 zones.

Most alleles showed deviations for the Hardy-Weinberg equilibrium. One of the reasons for this is inbreeding, as it changes genotype proportions. Since the inbreeders are related, it is likely that for one characteristic they have matching alleles. Therefore, the frequency of homozygotes increases (as the genes the offspring receives from both related parents are more likely to be the same) and the frequency of heterozygotes decreases. The inbreeding in the case of *A. paliatta* and *A. pigra* could be the result of habitat fragmentation as the gene flow is decreased and populations become isolated. In all loci an excess of homozygotes is observed in *A. palliata* whereas in *A. pigra*, only locus *Apm01* shows similar values of expected and observed heterozygosity. The excess of homozygotes corresponds to that previously described in other studies for both species in the region (Ellsworth and Hoelzer, 2006; James *et al.*, 1997; Jasso-del Toro, *et al.*, 2016; Kelaita and Cortés-Ortiz, 2013).

Apart from inbreeding, the excess of homozygotes could be explained by a selection processes on adjacent genes of some loci. However, the excess of homozygotes has been observed in all loci in *A. palliata* and in all but one in *A. pigra*. If selection

processes can produce excess of heterozygotes in some loci it is not very likely that it affects to all loci analysed.

Contrary to our expectations, the present study for *A. palliata* carried out in non protected areas (Z2, Z3 and Z4), reported a greater genetic diversity than in areas with a reserve status (Z8 and Z10), a larger area and a lower habitat fragmentation, with an average of 2.88 alleles per locus, an observed heterozygosity of 0.14 and an expected heterozygosity of 0.23 considering 8 polymorphic loci (Jasso-del Toro *et al.*, 2016). However, our results demonstrate a low heterozygosity (0.26) when compared it with the research by Ellsworth and Hoelzer (2006), which report for both *A. palliata* (n = 8 Veracruz howler monkeys) and *A. pigra* (n = 28 Belizean howler monkeys) a heterozygosity of 0.36. Heterozygosity differences could be explained by the small sample of Mexican howler monkeys analyzed by Ellsworth and Hoelzer (2006).

At the outset of our study, we expected to find decreased interspecific heterozygosity in fragmented landscapes and in *A. pigra* populations in comparison with *A. palliata* populations. This hypothesis is confirmed, as a homozygosity excess is reported in the present study. Many loci displayed increase of homozygosity in recombinant hybrids in the species of the genus *Alouatta*, despite striking genetic differentiation between the two species, as shown by the PC analysis and minimum spanning network. These results are congruent with previously research reported in *A. palliata* and *A. pigra* populations in reduced and fragmented habitats, and a reduced level of heterozygosity was expected in northern howler monkey populations (Cortés-Ortiz, *et al.*, 2003; 2007; Jasso del Toro *et al.*, 2016).

Natural introgressive hybridization between species after secondary contacts have been described in all major groups of organisms and described it in a narrow zone. The present study provides clear evidence of an extensive area of hybridization between *Alouatta palliata* and *A. pigra*, being Tabasco State the main zone of hybridization. Analyses based on genetic data and model based on geographic information system have revealed a width zone of hybridization (estimated area of 2.07 ha x 10^6, >270 Km in lineal distance) in comparison with the previously reported hybrid zone (20 Km, Cortés-Ortíz *et al.*, 2007). Our analyses of a contact zone of the two divergent evolutionary species of the genus *Alouatta* show that a strong genetic structure is also maintained in parapatry. Hybrids were relatively few and mostly results of backcrosses. These results suggest that at least some post-mating isolation mechanisms are already in place between *A. palliata* and *A. pigra* species. Despite the high level of differentiation between

the lineages, some introgression still occurs, and displays clear signals of asymmetry, regarding both the mode of inheritance of markers and the species considered. First, nuclear DNA as inferred from microsatellites (biparentally inherited) included the most introgressed markers, followed by mtDNA (maternally inherited) and also the few number of samples that provided sequence data from the Y chromosome (paternally inherited) showed some sign of introgression. These differences between markers could be explained because mtDNA is more stable over time/conditions than nuclear DNA, and evolves slower than nuclear DNA, because maternal inheritance is maintained without recombination. Second, important biogeographic aspects of distribution of the hybrid zone were found, such as consistent altitudinal segregation.

The introgression of *A. palliata* and *A. pigra* occurred preferentially in the Southern part of the State, mainly in the highland zone, where probably both species remained in the barrier. We found many instances of introgression of a Northern *A. palliata* mtDNA haplotype (*Cytb* and *ATPase*) within a Southern *A. pigra* nuclear background (even in populations relatively far apart from the hybrid zone previously reported in Macuspana municipality by Cortés-Ortíz *et al.*, 2007), but there are few instances showing introgression on the other direction. This asymmetric introgression regarding species was expected from previous research (Cortés-Ortíz *et al.*, 2007), and it was explained by Haldane´s rule. It is also important to highlight that in the study of Cortés-Ortíz (2015) all male hybrids had the SRY gene type (reflecting the paternal lineage) coinciding with most of their nuclear background, which supports this theory. This differential introgression depending on the mode of inheritance, although exist evidence of other small mammals which present asymmetrical and differential gene introgression in a width contact zones (Berthier *et al.*, 2006; Gligor *et al.*, 2009; Macholan *et al.*, 2007).

When analyzing the genetic composition of hybrids, our results coincide with that reported by Cortés-Ortíz *et al.* (2015), in which the DNA haplotypes of the *A. pigra* matriline are more present than those of *A. palliata*. Although individuals with *A. palliata* matriarchal DNA were also found, this crossing could have been carried out with backcross males or multigenerational hybrids or between hybrids and pure individuals. Chromosomal incompatibilities could be the reason of the low number of hybrids. The patterns of genetic variation of hybrid / backcross individuals suggest that directionality in hybridization may be due to chromosomal, cyto-nuclear or genomic incompatibilities (Cortés-Ortíz *et al.*, 2015). In studies of the chromosomal arrangements of the howler monkeys of Mesoamerica Steinberg *et al.*, (2008) reported that *A. palliata* and *A. pigra* have different numbers of modal chromosomes (2n = 58 for *A. pigra* and 2n = 53 and 54

for *A. palliata* males and females, respectively), and males have different sex determination systems ($X_1X_2Y_1Y2$ quadrivalent in *A. pigra* and X_1X_2Y trivalent in *A. palliata*). These differences in karyotypes could act as a prezygotic isolation mechanism, as these disparity in autosomes and sex chromosomes may compromise the fertility of hybrids.

Further and long-term investigations in wild populations, including following descendant of crosses and backcrosses, should be performed in order to clarify reproduction, inbreeding and speciation mechanisms, and also to predict the viability of both species in the future.

SDMTools results revealed a genetic differentiation per species and between intraspecies analyses of populations of *A. pigra.* Several types of barriers can limit dispersal and gene flow among wild populations in the genus *Alouatta*. The results showed the prevalence of different elevational bands in the area. Landscape resistance could represent some kind of isolation effect, for example, i) natural barriers such as southern mountains and/or hydrological barriers of the State, and ii) anthropogenic barriers, such as infrastructure of the main cities. Our results agree with studies, in which they develop landscape connectivity models and evaluate gene flow in anthropic (urban) environments (butterfly, Leidner and Haddad, 2010; Munshi-South, 2012) or highly fragmented habitats (roe deer, Coulon *et al.*, 2004; lemur, Quéméré *et al.*, 2010; giant pandas, Zhu *et al.*, 2010). In these cases, gene flow is better explained by environmental variables than by geographic distances themselves. With the model of connectivity landscape and gene flow that was developed in the present study can be inferred the connectivity routes between populations of howler monkeys and identify the zones that offer a "low resistance" of mobility or corridor between populations. Connectivity models presented as in the present study will allow to identify and propose potential biological corridors that offer a low resistance for the migration of the current populations of the genus *Alouatta* in Mexico, and also for threatened or endangered species.

According to the dispersion network based on the MtDNA analysis, the result show that there is still connectivity between the *A. pigra* populations found in the mountains south of the State of Tabasco and the populations at the south of the State of Chiapas. Although the linear distances that exist within the *A. palliata* and *A. pigra* populations of the State of Tabasco are small (the closest ones less than 30 km), with respect to the more than 230 linear kilometres that exist between the Sierra de Tacotalpa and Playón de la Gloria in Chiapas, the MtDNA reveals the still existing gene flow and / or genetic connection between these geographically remote populations. It is important to note that

the inaccessibility of the physiographic barriers, in this sense is favouring *A. pigra*, allowing their populations to have as potential niche the slope of the mountains belonging to the Sierra Madre of Chiapas, where this exchange is taking place (Fig. 7). These results might be closely linked to the biological characteristics and behaviour of howler monkeys. *A. palliata* and *A. pigra* are mainly folivorous, spends most of their life eating plants or moving within the forest for foraging, and they will spend more time looking for food and will have to travel and visit more patches to feed.

Despite the fact that Mexican primates have a category of endangered species, in the case of *A. pigra* and as critically endangered, *A. palliata*, the State of Tabasco, because of its accelerated process of deforestation and loss of habitat, currently only contains small remnants of fragments with natural vegetation for howler monkeys. However, it is vital to preserve these fragments and promote conservation efforts to regenerate and recover areas of natural vegetation that allow re-establishing connectivity inside and outside the State. Especially for populations of howler monkeys that were located within urban landscapes and without a connection of vegetation that allows to preserve the genetic information of those individuals.

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Supplementary material

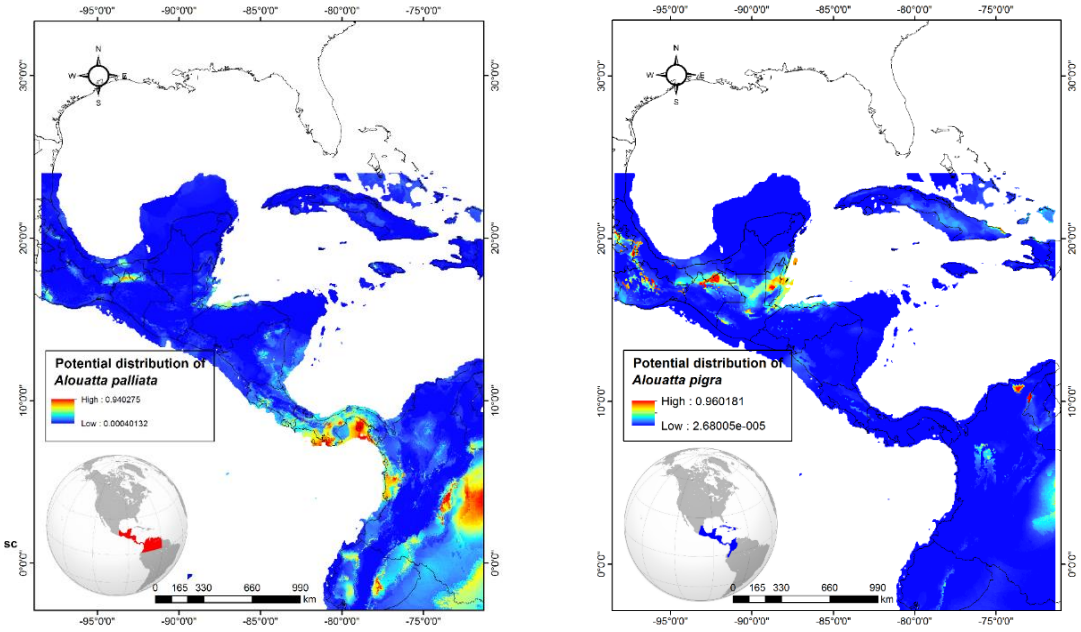


Figure 1S. MAXENT **Last glacial maximum** for *A. palliata* and *A. pigra* niche model constructed. Areas with high probability of species occurrence in warm colors, areas enclosing low probability values in cool colors. Color graphic scale represents the percentage of probability of presence.

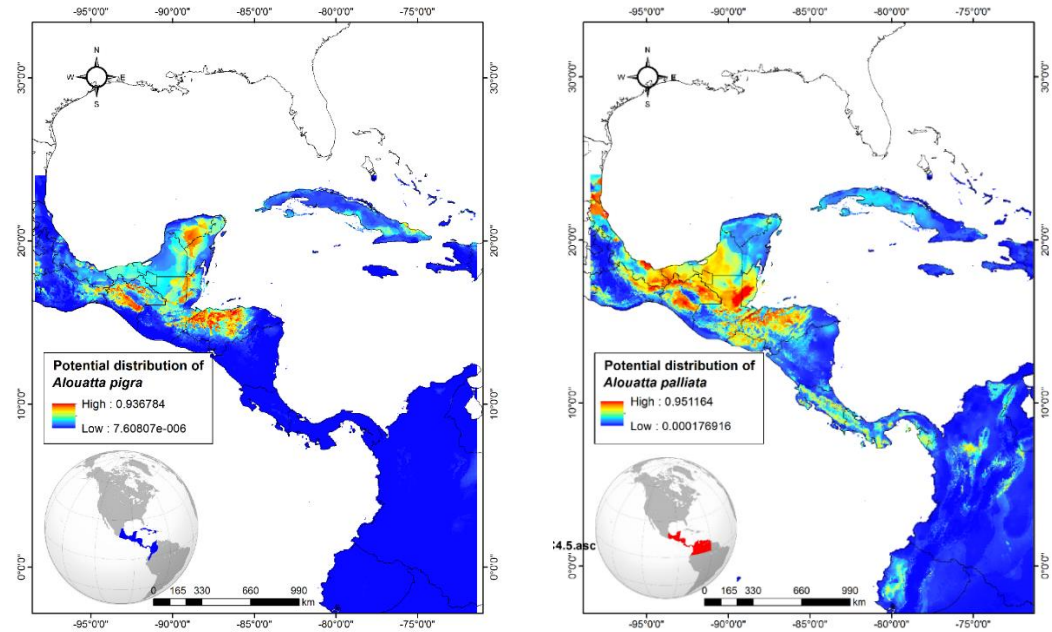


Figure S2. MAXENT **Future-2050 (Miroc 4.5)** niche model constructed for *A. palliata*. Areas with high probability of species occurrence in warm colors, areas enclosing low probability values in cool colors. Color graphic scale represents the percentage of probability of presence.

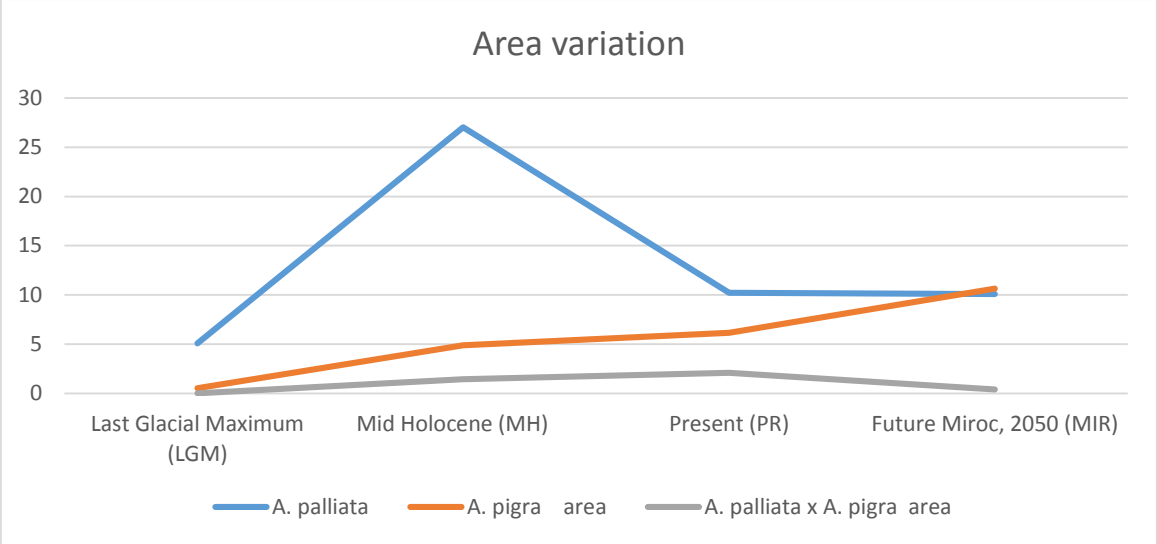
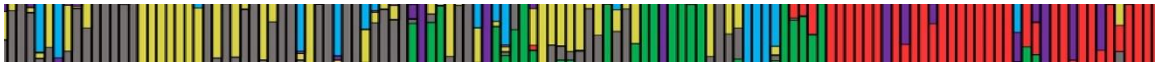


Figure S3. Estimated areas (ha x 10^6) of species distribution modelling for *Alouatta palliata*, *Alouatta pigra* and *A. palliata* x *A. pigra* crosses across several climatic scenarios (highly probability of presence, >60%).

CHAPTER 2



Effects of habitat loss and fragmentation in gastrointestinal parasites prevalence and richness between parapatric species, *Alouatta palliata* and *A. pigra* in Southeast of Mexico.

Effects of habitat loss and fragmentation in gastrointestinal parasites prevalence and richness between parapatric species, *Alouatta palliata* and *A. pigra* in Southeast of Mexico.

Abstract

Several factors like habitat loss and fragmentation or hybridization are known to produce changes in the host that directly influence on the parasitation and infection processes. We have analysed the effects of habitat disturbance and hybridization on parasitic richness and prevalence in the parapatric species *Alouatta palliata* and *A. pigra.* Faecal samples of 498 individuals (147 of *A. palliata* and 351 of *A. pigra)* from five regions across Tabaco state were analyzed by a coprologic study. Landscape features such as patch size, land use, and several habitat disturbance indexes were recorded in every sampling location. A subsample of 72 individuals were genotyped using 10 microsatellites loci, and a genetic individual's assignment was carried out. Differences in landscape features and habitat disturbance indexes were found between the five regions. In the same way, differences in parasitic richness and prevalence were found among regions, among host species and among genetic origins. Correlations between several environmental variables and specific parasites prevalence were detected. Several metrics of habitat disturbances as patch size or surrounding use pressure index are related with endoparasite prevalence and richness, with no clear relationship was found with fragmentation.

Keywords: endoparasite prevalence, fragmentation, howler monkeys, hybridization, landscape.

Introduction

The impact of habitat loss and fragmentation have been largely studied on how it affects parasite richness and prevalence on primates and in particular on *Alouatta* species (reviewed in Nunn and Altizer, 2006; Huffman and Chapman, 2009). Despite of a large number of studies analysing the relationships between perturbation and parasite abundance and richness on primates in general and in *Alouatta* species in particular, these interactions have not yet been well understood (Nunn and Gillespie, 2016). Several studies have shown that habitat loss and fragmentation produces changes in the host (such as population density, physiological stress, ranging patterns, intraspecific and interspecific contacts, and diet) that directly influence on the parasitation and infection processes (Arroyo-Rodríguez and Dias, 2010; Nunn and Altizer, 2006). However, about the impact of disturbance on disease there are two contrasting hypotheses, the dilution and the amplification effects, that predicts opposite effects. (Young *et al.* 2013). The first hypotheses, the dilution effect, postulate that higher biodiversity contributes to decrease parasitism, reducing the possibility of disease transmission, indeed it may modulate human disease risk. This implies several mechanisms, such as reduction of fitness, regulation of populations of susceptible hosts and interference with parasite transmission. For example, it is expected that fragmentation generates inbreeding depression in isolated population, what reduced the ability to withstand environmental changes and diseases, increasing parasite prevalence (Arroyo-Rodríguez and Dias, 2010). Therefore, biodiversity losses could aggravate epidemics that harm humans and wildlife, generating higher parasite prevalence (proportion of infected hosts) and richness (number of different parasite species per host), (Civitello *et al.*, 2015). In particular, in *Alouatta* species, several studies have pointed to higher endoparasite loads in fragmented patches (Eckert *et al.*, 2006; Trejo-Macias and Estrada, 2012; Vitazkova and Wade, 2007). On the other hand, the amplification effect predicts that increased biodiversity increases disease risk. The increment of host richness and abundance in undisturbed ecosystems can facilitate higher parasite richness and abundance (Hechinger and Lafferty 2005; Jones *et al.* 2008; Dunn *et al.* 2010; Keesing *et al.* 2006). These effects can also be present when species are introduced and they may act as alternative hosts for the parasite, with a subsequent spillback of the infection to native species of the host (Daszak, Cunningham and Hyatt 2000; Kelly *et. al*., 2009).

Other important factor that could influence parasitation is hybridization. Parasites are known to reduce substantially the fitness of infected host (Poulin, 2007), therefore differences between hybrid and parental infection levels could affect the relative

evolutionary success of hybrids. Four alternatives scenarios for infection level in hybrid versus parental taxa have been proposed: additive, dominance, hybrid resistance and hybrid susceptibility (Fritz *et al.,* 1994). Previous studies on primates has pointed to a hybrid susceptibility scenario where hybrids present higher susceptibility to parasite infection than their parental species due to intrinsic factor such as genomic incompatibilities or extrinsic factors such as being exposed to parasite communities infecting both parental species (Sommer *et al.*, 2014).

Different natural or anthropogenic factors can interact with each other, generating additive or non-additive effects on complex biological phenomena. Many indirect effects are expected as the result of a combination of different factors (Didham *et al.* 2007). Several studies on primate species have highlighted the importance of multifactor explanations (Chapman *et al.,* 2006; 2007; Milton, 1996).

In the last 60 years, Tabasco forest area in Mexico has been severely reduced from 49.1% to 13.6 % of land surface due to deforestation and changes in land use to grasslands (Díaz-Gallegos *et al.* 2010; Palma-López and Triano, 2002). This highly fragmented landscape has left the populations generally relegated to small fragments and many of them with a high level of isolation, what might generate a genetic footprint (Chapter 1) as well as a higher parasitic prevalence and richness. Therefore, it is important to shed light on the effects of habitat loss and fragmentation in Mexican howler monkeys. Additionally, this area is known as a region of hybridization between *A. palliata* and *A. pigra*, concentrated on a relative small area of 20 km wide in Macuspana (Tabasco), (Ho *et al.*, 2014; Kelaita and Cortés-Ortiz, 2013). The combined effect of both mentioned driver can also be studied. In Macuspana, hybrid individuals of two *Alouatta* species were observed in small and more isolated fragments, suggesting that deforestation and habitat fragmentation affects in some degree the natural hybridization process (Dias *et al.*, 2013). However further studies are needed to determine the influence on the anthropogenic hybridization on the natural hybrid regions.

In order to improve our understanding of the effects of fragmentation and hybridization on parasitation, we have conducted a study in the hybridization zone of *A. palliata* and *A. pigra*, with the objective to evaluate the effect of both natural and anthropogenic factors on gastrointestinal parasitic prevalence and diversity. This region represents an excellent opportunity to evaluate the effect of habitat loss, and fragmentation as well as hybridization on the parasitic diversity of these two parapatric species. Specifically, our hypotheses are that there is a differential response of richness and prevalence of endoparasites a) depending on host specificity b) depending of the degree of

hybridization, c) depending on habitat features and d) on fragmentation level. We expect a higher parasitation on *A. palliata* than on *A. pigra*, due to *A. palliata* populations sampling were in fragments of non-natural vegetation and closer to human populations in comparison with *A. pigra* populations. Additionally, we expect higher parasitation in hybrid areas and a lower parasitic diversity in grasslands, and in urbanized areas as well as in more fragmented landscapes.

Material and methods

Study area and landscape characteristics

Thirty-six sites in Southeastern Mexico grouped in twelve municipalities belonging to Tabasco and three of Chiapas were sampled in 2014 and 2015 (Figure 1, Table 1). In order to analyse a broad spatial scale, these sites were grouped into five major regions: (1) low lands eastern to Grijalva river defined by cacao cultivars, (2) low lands western of Grijalva river defined by a mangrove area, (3) highlands of Southern Central Tabasco dominated with fragmented rainforest, (4) lowlands of southwest Tabasco State dominated by grasslands, wetlands and oil palm cultivars, and (5) lowlands of western Chiapas state, a conserved mountain cloud forest area (Fig. 1, Table 1).

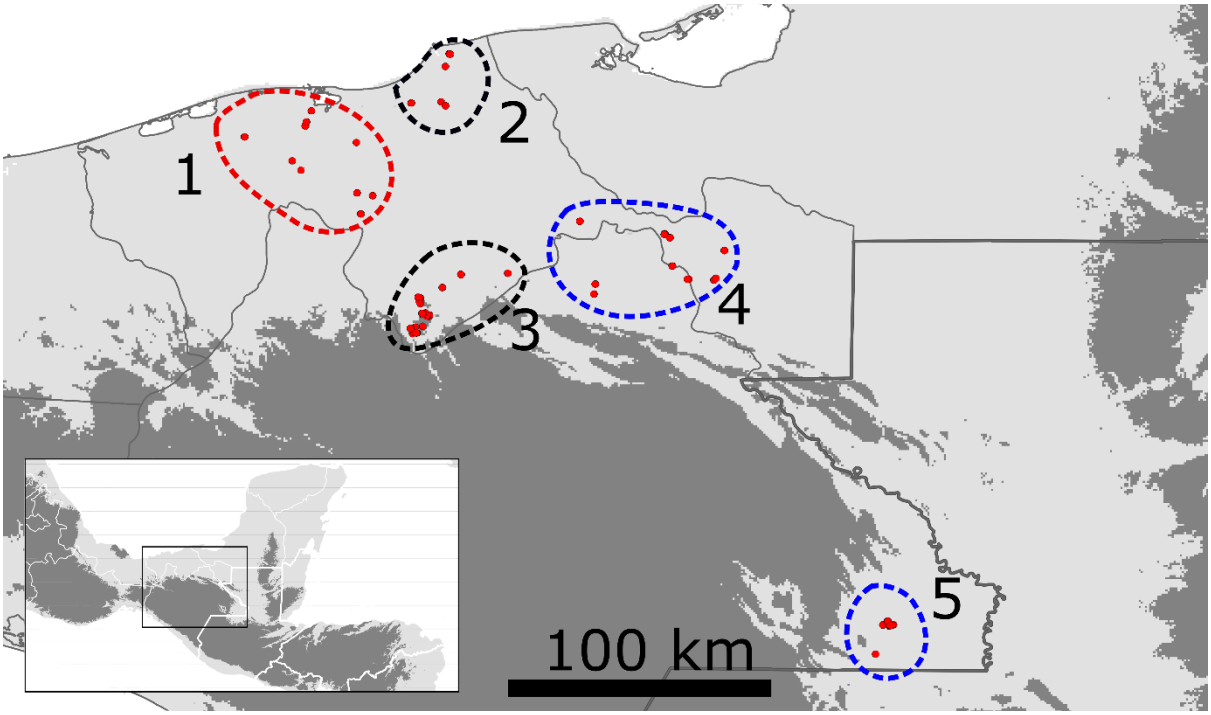


Figure 1. Sampling locations of mantled howler monkeys (*Alouatta palliata*, red circles) and black howler monkeys (*Alouatta pigra*, blue circles) or both (black circles) from this study. Numbers correspond to regions sampled as explained in Table 1.

Table 1. Sampling localities for *Alouatta palliata* and *Alouatta pigra* in Southeast of Mexico. N sample = number of samples.

Region	Specie	Site	Municipality	No. of samples	Latitude	Longitude
1	*A. palliata*	Carlos Green	Comalcalco	8	18.23894	-93.45537
		Norte		22	18.300362	-93.204453
		Cholula		28	18.284141	-93.209893
		Palestina	Paraiso	2	18.34539	-93.18365
		La Piedra	Cunduacan	1	18.10359	-93.22632
		Cacao plantation		3	18.14256	-93.26161
		Boqueron	Centro	8	17.926953	-92.982916
		Parque Museo La Venta		22	18.0003032	-92.935525
		C.I.C.N. Yumka		20	18.0003032	-92.935525
		Mazaltepec		4	18.01195	-92.99912
		Mazateupa	Nacajuca	1	18.21635	-93.00062
2		Tabasquillo	Centla	7	18.37722	-92.77867
	A. pigra	Tres Brazos		9	18.36573	-92.63938
		San Juanito		1	18.381522	-92.656127
		Nueva Alianza		1	18.525545	-92.640229
		La Victoria		10	18.5774706	-92.624152
3		Celia Gonzalez de Rovirossa	Macuspana	4	17.6854714	-92.385641
		Parque Estatal Agua Blanca		3	17.68119	-92.57778
		La Escondida Ranch	Jalapa	5	17.6267046	-92.653061
		Poana	Tacotalpa	42	17.5827706	-92.745202
		Xicotencatl		51	17.5095626	-92.706605
	A. palliata	Villa Luz		21	17.4455557	-92.763876
	A. pigra	Villa Luz		20	17.4461676	-92.768048
		Florida		11	17.46687	-92.76248
		Kolenchen		22	17.44263	-92.75666
		Tapijulapa		6	17.46045	-92.78121
4		Los Pajaros	Jonuta	4	17.896954	-92.093926
		Bertollini Ranch	Emiliano Zapata	14	17.830062	-91.728366
		Chacamax		6	17.71538	-91.71904
		Island 2		11	17.846483	-91.748345
		Island 3		21	17.843018	-91.750769
		Jose Alfredo Ranch		22	17.6613624	-91.650695
		Arenal	Balancan	12	17.6645761	-91.541408
		Primatology Center		19	17.77755	-91.50594
		Palenque	Chiapas	18	17.6412313	-92.031485
5		Las Guacamayas		36	16.25281	-90.83759
		Roquera		3	16.13788	-90.89208

In order to analyze the effects of landscape characteristics, a buffer area of 3 km in diameter was realized in each sampling locality using ARCGis 10.1. In each buffer, it was

measured the size and number of patches of different types of vegetation and land-use following protocol described in chapter 1.

Additionally, several indexes of disturbance such as the fragmentation level or surrounding use pressure index (SPI) were calculated. At patch level, in the patch where the monkey was sampled, it was measured the patch area, as well as the percentage of natural vegetation surrounding the patch that was transform into a surrounding use pressure index (SPI). This index ranges from 1 to 11.5, a value of 1 indicates minimal pressure. At buffer level, it was calculated a fragmentation index (FI) (ranging from 0 to 1, 0 in highly fragmented buffers and 1 in not fragmented ones) following the proposal of Díaz-Lacava (2003). Besides, vegetation type and land uses were grouped into two classes, natural and non-natural vegetation, following the classification of Galindo-Alcántara *et al.* (2006) and INEGI (2012).

Gastrointestinal endoparasite detection/identification/ analysis

498 individual samples of wild howler monkey troops were collected non-invasively and were fixed with 4% formalin and stored at room temperature until analyzed at the laboratory. 147 scats of *A. palliata* and 351 of *A. pigra.* Fecal samples were processed using the passive fecal flotation technique with saturated salt solution NaCl (sp.gr. 1.20) to recover eggs cysts and oocysts (Acevedo y Romero, 1987). Eggs, cysts and oocysts were identified according to taxonomic keys (Nunn and Altizer, 2005; Sloss *et al.*, 1994).

Parasite infections were described in terms of parasitic richness, prevalence and total parasitic prevalence. Parasite richness was estimated as the number of every parasite taxa recovered within every individual and in every region (Altizer *et al.*, 2007). Due to the low number of parasites detected, the prevalence was calculated as number of howler monkeys infected by the total number of individuals sampled in the considered region. Total parasitic prevalence was estimated as the sum of every species parasitic prevalence in every region.

Eight different gastrointestinal endoparasites were analysed in both *A. palliata* and *A. pigra* populations: *Blastocystis* sp., Strongylid egg, *Parabronema sp., Strongyloides sp., Trypanoxyuris sp., Controrchis sp., Cyclospora sp and* oocysts of Eimeriidae. Some of their biological and pathological features are described in Table 2.

Non-invasive techniques were implemented for sampling collection. All the procedures were performed in accordance with the provisions of the Regulations of the Environment and Natural Resources Ministry and the Under-Secretary of Management for Environmental Protection NOM-059-SEMARNAT-2010; reference

SGPA/DGVS/04725/13. Academic authorities of the Universidad Juarez Autonoma de Tabasco authorized the present study (reference number UJAT-2013-43).

Table 2. Description of main features of gastrointestinal endoparasites found in this study. 1) Barrios, 2005; 2) Montoya 2013; 3) Roncancio-Duque and Benavides, 2013; 4) Villanueva-García *et al.*, 2017; 5) Baños-Ojeda, 2016; 6) Helembrook, 2014; 7)García-Hernández, 2009; 8) Alvarado-Villalobos, 2010; 9) Mollericona *et al.*, 2013; 10) Chinchilla *et al.*, 2005; 11) Beltrán-Saaedra *et al.*, 2009; 12) Cristóbal-Azkarate *et al.*, 2010; 13) Stoner and Gonzales-Di Pierro, 2006; 14) Guerrero, 2012; 15) Balsells, 2012; 16) Martins, 2002; 17) Serur, 2008; 18) Valdespino, 2010; 19) Trejo-Macias, 2010; 20) Kowalzik *et al.*, 2010.

Name	Type	Described in *Alouatta*	Infection route	Pathogenicity	Limitant factor	Seaso_ nality	Frag_ menta_ tion effect	Zoonotic importance
Blastocystis sp.	Protozoan	yes (1,2,3)	fecal-oral	controversial (4)	water availability			
Strongylid egg	Nematode		Fecal – oral or maybe percuta_ neous	severe health effects (9)	Coinfection with Strongylodes sp,low rainfall	dry seasson (5)		
Cyclospora sp.	Protozoan		Fecal-oral	epidemics, severe health effects	water availability, host size	rainy sesson (5)		yes
Eimeriidae	Protozoan		fecal-oral		water availability (4)	rainy sesson (5,7)	Yes (8)	
Parabronema sp.	Nematode		Fecal-oral		water availability	rainy seasson		
Strongyloides sp.	Nematode	Yes (10-13)	Percuta_ neous and fecal-oral	adverse health effects (3,6,9)	Coinfection with strongylid, low rainfall	dry seasson (5,7)	yes (8)	yes (1,2,11,14)
Trypanoxyuris sp	Nematode	yes (10, 15-17)	fecal-oral			both (7,8,18)	yes (5, 8, 19)	
Controrchis sp.	Platyhelminth	yes (20)	Intermediate host (20)				yes (8,20)	

Effects of fragmentation and hybridization on parasitation

To test whether environmental variables and landscape characteristics may explain or correlate with the presence of parasitic species, a correlation analyses was performed using the function cor() in R (version 3.1.2; R Development Core Team, 2014).

To explore the effect of hybridization 72 individuals (14 *A. palliata* and 58 *A. pigra)* were genotyped from DNA of extracted from 100 mg faecal samples using FavorPrep Stool DNA Isolation Mini Kits (Favorgen Biotech Corporation, Pingtung County, Taiwan). Ten microsatellites were amplified in three multiplex PCR reactions following the procedure described in chapter 1. A Discriminant Analysis of Principal Components (DAPC) was realized using Adegenet library (Jombart and Ahmed, 2011) in R (version 3.1.2; R Development Core Team, 2014) to assignate individuals to genetic clusters in order to identify hybrid individuals (see chapter 1) Additionally, it was compared the parasitic richness and prevalence in the region 3 with other regions. This region is where most of

hybrids individual between *A. palliata* and *A. pigra* have been found (see Chapter 1 and Kelaita and Cortés-Ortiz, 2013).

Results

Analysis of landscape characteristic

Distinct land-uses were detected in the five regions (1-5; Fig. 2). Low lands eastern to Grijalva river (region 1) were the most anthropogenized one, dominated with agroecosystems, infrastructures and pastureland, also with a high number of patches (119). Low lands western of Grijalva river (region 2) was dominated by pastureland (60%) and natural tree vegetation (35%), it represents the lowest number of patches (28). Region 3 is dominated with natural tree vegetation (60%) and also secondary vegetation (37%). However, it is a highly-fragmented region, with the higher number of patches (185). Region 4 was a mixture of natural vegetation (36%), pastureland (23%), and secondary vegetation (19%), also with a high number of patches (127). Finally, lowlands of western Chiapas state (region 5) represents the region with lower anthropogenic impact, being all the patches of natural tree vegetation (100%) and low number of patches (39).

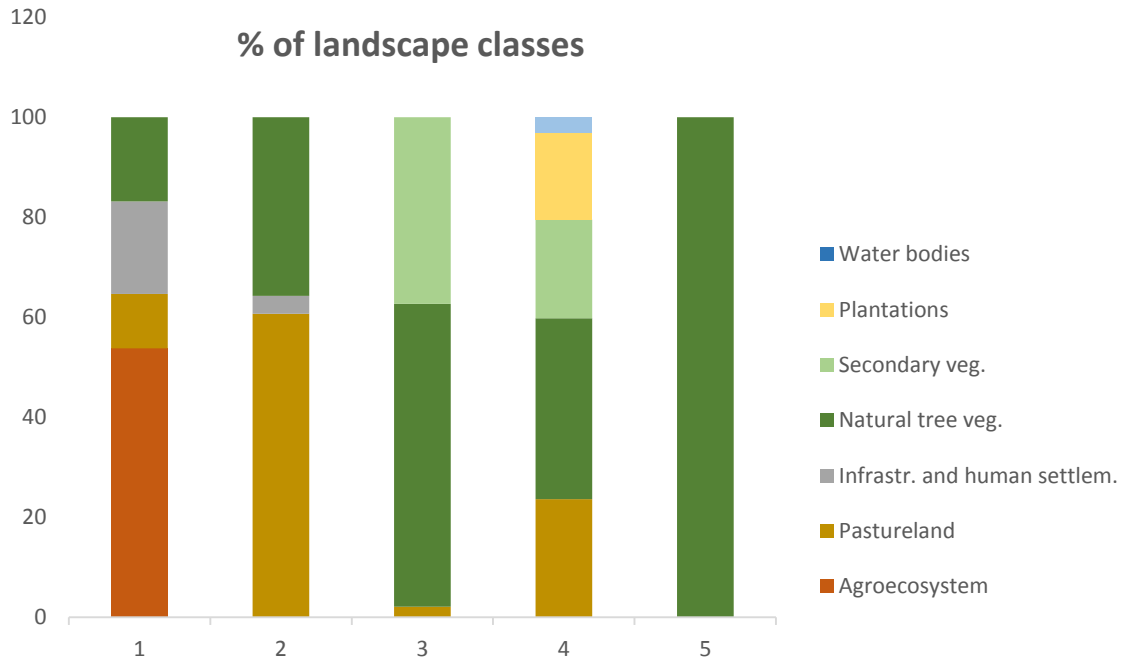


Figure 2. Average of predominant landscape classes in five Tabasco regions (1-5).

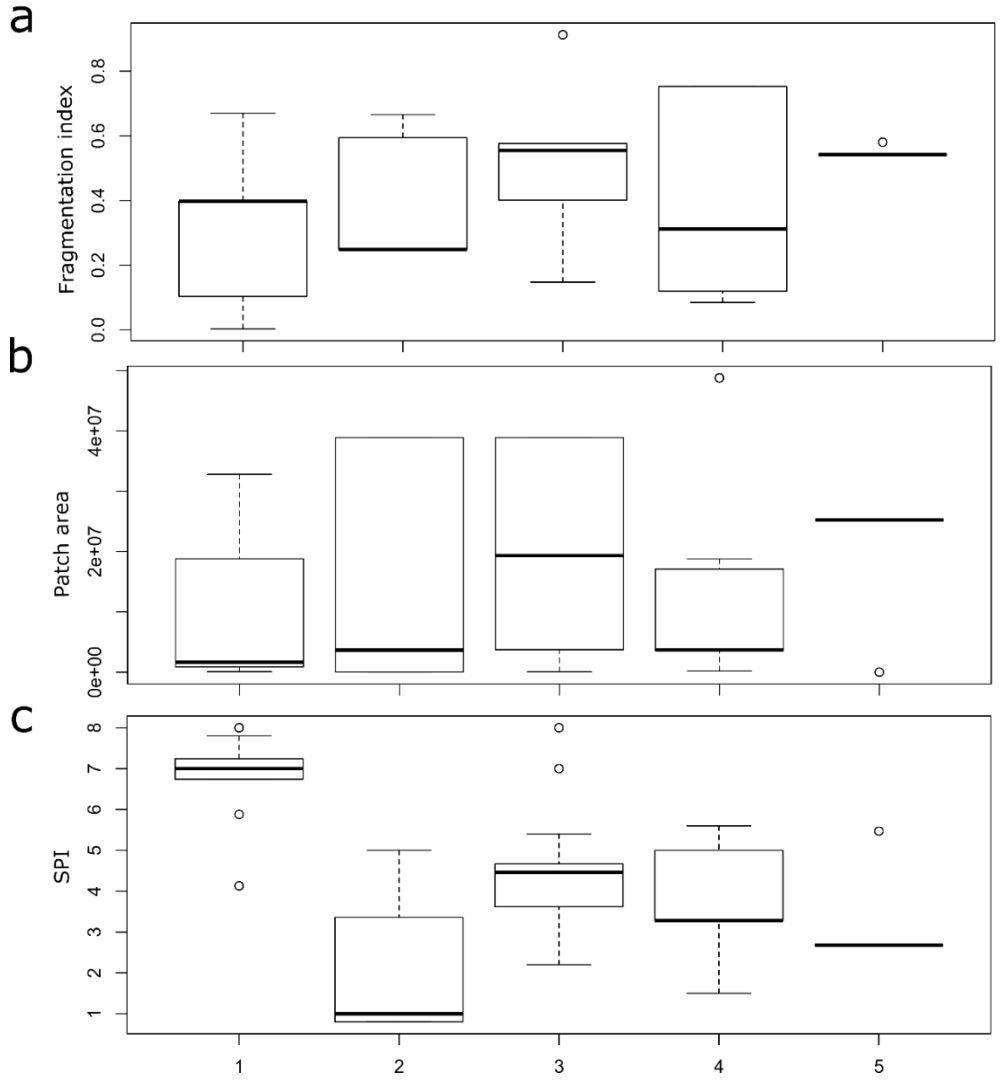


Figure 3. Boxplot of disturbance pressures for the five regions studied (1-5) in Southern Mexico: a) Fragmentation index (FI) at 3 km buffer level. b) Patch area (Ha) and c) surrounding use pressure index (SPI) at patch level.

Prevalence and richness of endoparasites in *A. palliata* and *A. pigra*

Trypanoxyuris sp was the most common parasite in both species. The proportion of individuals that present at least one parasite was higher for *A. palliata* (n=38, 25%) than for *A. pigra* (n=69, 19%). However, the coinfection with at least two parasites was more common in *A. pigra* (n= 28, 40%) than in *A. palliata* (n=3, 8%). The individuals with 3 parasites detected (n=6) were all *A. pigra*. All included *Trypanoxyuris* and four of them were also coinfected with *Controrchis* and *Strongyloides*. Individuals coinfected with two parasites (n=22) were mainly *A. pigra* (n=19) and all included *Trypanoxyuris,* and 40% of them also has *Eimeriidae* oocysts.

Host specificity was observed in 5 of the 8 species of parasites detected. *Parabronema* was found only in *A. palliata* whereas *Strongyloides, Cyclospora, Eimeriidae* oocyst and *Strongylid* egg were only found in *A. pigra.*

Region 1 only presented *A. palliata* individuals, whereas region 4 and 5 only showed *A. pigra*. Regions 2 and 3 presented both species although *A. palliata* in lower proportion (30 and 12% respectively). Individual parasite richness was very low varying from 0 to 3 parasites per individual, been regions 3 and 4 the ones with higher individual parasite richness. Parasitic richness and prevalence and total prevalence was high in regions 1, 3 and 4 (Table 3). Region 3 harbour the highest parasite richness with six species detected followed by region 1 and 4 with four species. On the other hand, only two species were detected in region 2 (from *A. pigra* individuals) and no species in region 5.

Table 3. Gastrointestinal parasitic richness of and prevalence of *A. palliata* and *A. pigra* in each of the Southern Mexico regions analysed (1 to 5).

		A. palliata				***A. pigra***				
	Zone	1	2	3	Tot	2	3	4	5	Tot
	n	119	7	21	147	21	164	127	39	351
	Richness	4	0	1	4	2	6	4	0	7
Prevalence	*Blastocystis* (cyst)	0	0	0	0.00	0	0.02	0	0	0.01
	Strongylid	0	0	0	0.00	0	0.02	0	0	0.01
	Cyclospora	0	0	0	0.00	0	0.03	0	0	0.01
	Parabronema	0.03	0	0	0.03	0	0	0	0	0.00
	Strongyloides	0	0	0	0.00	0	0.01	0.09	0	0.03
	Trypanoxyuris	0.25	0	0.05	0.21	0.10	0.24	0.13	0	0.17
	Controrchis	0.03	0	0	0.02	0.10	0	0.05	0	0.02
	Coccidia(Eimeriidae)	0.03	0	0	0.02	0	0.06	0.01	0	0.03
	Total Prevalence	0.34	0	0.05	0.28	0.19	0.37	0.28	0	0.28

Prevalence varies for each species, *Trypanoxyuris* is the species with higher prevalence (*A. palliata* 21% and *A. pigra* 17%). The remaining species presents a prevalence ranging between 1 and 3%.

Total prevalence was high in region 1, 3 and 4 (34, 37 and 28 % respectively) and low in region number 2 (19%) and 5 (0%).

Effect of fragmentation on parasitation

Only weak but significant ($p<0.05$) correlations were found between environmental variables and the degree of parasitation (Fig. 4). *Trypanoxyuris* appeared negatively correlated with infrastructure. *Controrchis* and *Strongyloides* were both negatively correlated with both FI (positively with fragmentation) and percentage of natural

vegetation, and additionally, the former was also positively correlated with grassland. On the contrary Eimeriidae was negatively correlated with fragmentation (negatively with FI), percentage of natural vegetation and patch area, what indicates that is a parasite indicator of low perturbation. Eimeriidae was also correlated with patch area. *Cyclospora* sp. and Strongylid do not appeared correlated with any of the environmental variables studied. Parasitic richness was correlated with all the gastrointestinal parasites found, and especially with *Trypanoxyuris.*

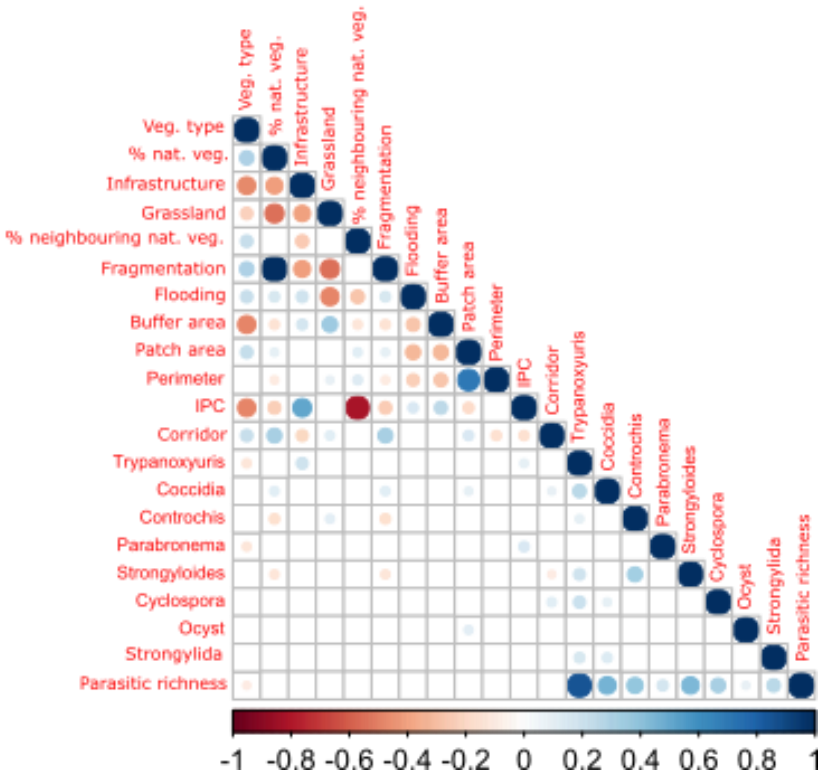


Figure 4. Correlations between parasite diversity and landscape variables ($p<0.05$). Blue indicates a positive relationship, red a negative one; colour intensity is proportional to Pearson's correlation coefficient.

Effects of hybridization on parasitation

The cluster analysis performed by DAPC revealed the presence of three genetic clusters (K = 3), belonging one of them to individuals considered as hybrid *A. palliata* x *A. pigra* (see chapter 1). Differences in genetic origin were obtained for the three clusters. Although parasitic richness was the same (3 spp) for cluster 2 (*A. pigra*) and cluster 3 (hybrids), the total prevalence was significatively higher for hybrid cluster (46%) than for the purebred cluster 1 (18%) and 2 (15%). Additionally, the prevalence of the parasites was significantly different for *Trypanoxyuris* and Eimeriidae. In the same way, the number of individuals coinfected with two parasites was significantly higher in hybrid cluster 3 (17%) than in cluster 2 (4%).

Table 3. Gastrointestinal parasitic richness of and prevalence in Southern Mexico of three genetic clusters obtained in *A. palliata* and *A. pigra.* Cluster 1 and 2 represent purebred individuals of *A. palliata* and *A. pigra,* respectively. Cluster 3 shows hybrid individuals.

Genetic cluster	1	2	3
n	11	26	35
Parasitic richness	1	3	3
Blastocystis	0.00	0.00	0.00
Strongylid	0.00	0.00	0.00
Cyclospora	0.00	0.00	0.03
Parabronema	0.00	0.00	0.00
Strongyloides	0.00	0.00	0.00
Trypanoxyuris	0.18	0.08	0.29
Controrchis	0.00	0.04	0.00
Eimeriidae	0.00	0.04	0.14
Total prevalence	0.18	0.15	0.46
n coinfected	0	1	6
% coinfectation	0.00	3.85	17.14

Discussion

In this study, we have found evidences that support that both habitat perturbation and hybridization promotes parasites richness and prevalence. This data support the dilution effect hypothesis that postulates that higher biodiversity contributes to decrease parasitism.

The parasites found in this study for both species coincide with those reported for Mexico in previous studies (García, 2009; Trejo, 2007; Vitaskova, 2006; Gonzales, 2014; Stoner and Gonzales-Di Pierro, 2006). *Trypanoxyuris sp.* had the highest prevalence, being the parasite most common reported for howlers (Valdespino *et al.*, 2010). However, our data of *Trypanoxyuris* prevalence (21% for *A. palliata* and 17% for *A. pigra*) are within the range of previously reported (González-Hernández, *et al.*, 2014).

When comparing parasite richness by region, highlands of Tabasco State (region 3) had one of the highest reported richness in Mexico for *A. pigra* (n = 6), similar to the richness reported for *A. pigra* by Stoner *et al.* (2006) in the Montes Azules Biosphere Reserve (n = 6). Contrary to expectations, in our study the most preserved region (region 5) had the lowest richness (n=0) despite a sufficient sampling effort (n=39).

Our results show that several metrics of habitat disturbance such as patch size or SPI are related with endoparasite prevalence and richness. It is well known that population density usually increases in the smallest patches, what might increase intraspecific competition, as well as parasite transmission (Arroyo-Rodriguez and Dias, 2010; González-Hernández *et al.* 2011).

These results agree with previous studies on *Alouatta* parasites (Santa Cruz *et al.* 2000, Trejo-Macías *et al.* 2007, Valdespino *et al.* 2010). However, fragmentation index showed direct correlation with some parasite species, like *Trypanoxyuris* sp. and *Parabronema* sp. The regions with lowest parasite prevalence and richness were lowlands of western Chiapas (region 5) followed by the low lands western of Grijalva river (region 2). However, although fragmentation in region 5 was the lowest (0.54), it was not the case in region 2, where the fragmentation at buffer level was the highest (the lowest fragmentation index=0.36). Additionally, correlations analyses showed not significant correlation between fragmentation and parasitic richness (Figure 4). These contrasting results could be related with methodological problems of the fragmentation metrics (Arroyo-Rodríguez and Mandujano, 2009). However, this result could also reflect that habitat loss rather than habitat fragmentation has larger effects on howler's parasitic diversity and prevalence. It has been shown that habitat fragmentation has not a strong effect on howler populations. On the contrary, habitat loss and vegetation attributes produced significant effects on howler population (reviewed in Arroyo-Rodriguez and Dias, 2010). It could be hypothesized that habitat loss is directly influencing over parasites richness and prevalence through the effects on its host.

On the other hand, our results showed significant individual correlations of fragmentation index with specific parasites such as Eimeriidae (-), *Controrchis* (+) and *Strongyloides* (+), what points to differential species-specific response to fragmentation. These results are in accordance with previous studies that also found individualistic responses of parasites to habitat features (Valdespino *et al.* 2010).

Trejo-Macías *et al.* (2007) studying parasitic richness and prevalence in *A. palliata* and *A. pigra,* showed that some parasites such as Strongylid and Eimeriidae only occurred in fragmented habitats. Our results do not support these conclusions, because we have only found Strongylid in low fragmented areas. In the same way, Eimeriidae showed a significant negative correlation with fragmentation (positive fragmentation index).

Our data clearly show higher parasitic prevalence in hybrid individuals (46%) than in their parental species (18% in *A. palliata* and 15% in *A. pigra*). Additionally, our data

showed that the probability of coinfection was more than four times higher in hybrid individuals (Table 3). There were no differences in parasitic diversity between hybrid (cluster 3) and *A. pigra* (cluster 2), however region 3, where the hybridization area takes place, was the one with highest parasitic richness. Further studies are needed with broader sampling to confirm the influence of hybridization on parasitic richness. In our study *Trypanoxyuris* sp. is higher in hybrid cluster 3 (29%) although lower than previously reported in a hybrid area (82%, in Macuspana, within region 3, González-Hernández *et al.*, 2014).

This is the first study to genetically test the combined effects of fragmentation and hybridization on parasite richness and prevalence between *A. palliata* and *A. pigra*. Detailed studies are needed in order to discern the synergistic effect of both factors.

Acknowledgments

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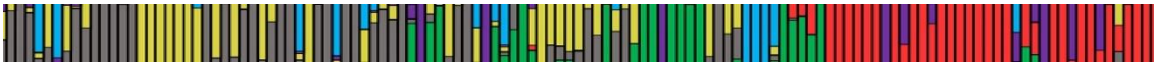
CHAPTER 3



Clarifying the cryptic host specificity of *Blastocystis* spp. isolates from *Alouatta palliata* and *A. pigra* howler monkeys

Clarifying the cryptic host specificity of *Blastocystis* spp. isolates from *Alouatta palliata* and *A. pigra* howler monkeys

Abstract

Although the presence of cryptic host specificity has been documented in *Blastocystis*, differences in infection rates and high genetic polymorphism within and between populations of some subtypes (ST) have impeded the clarification of the generalist or specialist specificity of this parasite. We assessed the 33 genetic variability and host specificity of *Blastocystis* spp. in wild howler monkeys from two rainforest areas in the southeastern region of Mexico. Fecal samples of 225 *Alouatta palliata* (59) and *A. pigra* (166) monkeys, belonging to 16 sylvatic sites, were analyzed for infection with *Blastocystis* ST using a region of the small subunit rDNA (SSUrDNA) gene as a marker. Phylogenetic and genetic diversity analyses were performed according to the geographic areas where the monkeys were found. *Blastocystis* ST2 was the most abundant (91.9%), followed by ST1 and ST8 with 4.6% and 3.5%, respectively; no association between *Blastocystis* ST and *Alouatta* species was observed. SSUrDNA sequences in GenBank from human and non-human primates (NHP) were used as ST references and included in population analyses. The haplotype network trees exhibited different distributions: ST1 showed a generalist profile since several haplotypes from different animals were homogeneously distributed with few mutational changes. For ST2, a major dispersion center grouped the Mexican samples, and high mutational differences were observed between NHP.

Furthermore, nucleotide and haplotype diversity values, as well as migration and genetic differentiation indexes, showed contrasting values for ST1 and ST2. These data suggest that ST1 populations are only minimally differentiated, while ST2 populations in humans are highly differentiated from those of NHP. The host generalist and specialist specificities exhibited by ST1 and ST2 *Blastocystis* populations indicate distinct adaptation processes. Because ST1 exhibits a generalist profile, this haplotype can be considered a metapopulation; in contrast, ST2 exists as a set of local populations with preferences for either humans or NHP.

Keywords: *Alouatta palliata, Alouatta pigra*, *Blastocystis*, haplotype diversity, host specificity, parasites.

Introduction

Blastocystis spp. is a common parasite that colonizes the intestines of many animals, including mammals, reptiles and birds. Although this microorganism is the most common eukaryotic parasite identified in human feces, its pathogenic role remains controversial. While some studies have reported that this parasite is associated with the development of cutaneous and intestinal disorders, other studies report no clinical manifestations (Poirier *et al.,* 2012; Scanlan and Stensvold, 2013; Yakoob *et al.,* 2010, Tan *et al.,* 2008, Scanlan *et al.,* 2014). In addition, *Blastocystis* exhibits high genetic polymorphism, presenting at least 17 subtypes (ST). The ST1-ST4 are frequently observed in humans but can also occur in birds, pigs, cows, dogs, rats and non-human primates (NHP). ST5 is common in pigs, and ST6 and ST7 are common in birds, while ST8 is documented in NHP and ST9 is only detected in humans. The ST10-ST17 have never been identified in people (Scanlan and Stensvold, 2013; Yoshikawa *et al.,* 2004; Stensvold *et al.,* 2009) although, a recent study showed that ST12 may occur in humans (Ramirez *et al.,* 2016). The potential zoonotic transmission of *Blastocystis* has been under debate, as studies have reported dissimilar results (Abe 2015; Parkar *et al.,* 2007; 2010; Yoshikawa *et al.,* 2009; Petrašova *et al.,* 2011; Alfellani *et al.,* 2013; Helenbrook *et al.,* 2015a). In Kathmandu, Nepal, the potential transmission of *Blastocystis* between local rhesus monkeys (*Macaca mulatta*) and children was assessed; the authors detected three subgroups of ST2 shared between the children and the monkeys, suggesting that the local rhesus monkeys might serve as a potential source for human ST2 infections (Yoshikawa *et al.,* 2009). Studies primarily concentrating on the genetic variability of *Blastocystis* recovered from humans and several primate genera, including Old and New World monkeys and prosimians, have confirmed the cryptic host specificity of ST1 and ST3. These parasites exhibit only minimal overlap between the sources, indicating that some ST3 isolates have adapted to NHP, while others have adapted to humans. Furthermore, reflecting considerable overlap in ST2, haplotypes exists among humans and NHP, and transmission among these species may occur in zoological parks and animal sanctuaries (Parkar *et al.,* 2007; Alfellani *et al.,* 2013; Stenvold *et al.,* 2012). In contrast, a study performed in the Rubondo Island National Park, Tanzania, where different autochthonous and introduced NHP live together, ST1-ST3 and ST5 were detected. Interestingly, the chimpanzees (*Pan troglodytes*) were parasitized only by ST1, which formed a unique phylogenetic clade. This finding suggested that Rubondo chimpanzees were colonized by a single, host-specific *Blastocystis* strain that circulates among the members of the group and that transmission of this genotype does not occur between primate populations and thus does not constitute a reservoir for human

infections (Petrašova *et al.,* 2011) Recently, Helenbrook *et al.* (2015a) studied fecal samples from humans and howler monkeys (*Alouatta palliata aequatorialis*) from Ecuador living in allopatric areas at close proximity. *Blastocystis* ST1-ST3 were detected in the human samples, while all monkeys had ST8, thus questioning the zoonotic potential of ST8. Furthermore, howlers are particularly interesting because these animals appear to be more sensitive to infectious diseases, such as yellow fever and gastrointestinal parasites. Different howler species live in forest fragments, disturbed habitats, and in close proximity to human populations (Crockett 1998; Sallis *et al.,* 2003; Helenbrook *et al.,* 2015b).

For many pathogens, the presence of multiple host species has important epidemiological and evolutionary implications, i.e., alternative host species might be reservoirs of a disease extinct in one host. In addition, parasites that interact with multiple host species may be less locally adapted, and consequently, these organisms are expected to be less specific. In addition, the assessment of host ranges can be hindered by the presence of cryptic species: even if a parasite is able to infect different host species, differences in infection rates among the alternative hosts might be interpreted as a consequence of a local adaptation process, leading to the preference for certain host species over others (Westram *et al.,* 2011). Hence, the identification of a generalist or specialist profile of a parasite for its host is relevant for understanding its prevalence, transmission and other biological features. Therefore, the aim of the present study was to assess the genetic variability and host specificity of *Blastocystis* spp. populations in howler monkey species *A*. *palliata* and *A*. *pigra* from two sylvatic areas of Mexico.

Materials and Methods

Study population

The genus *Alouatta* comprises 6 species widely distributed between Mexico and Argentina in allopatric or sympatric patterns. *A*. *palliata* and *A*. *pigra* howler monkeys are important inhabitants of some tropical rainforest areas in the southeastern region from Mexico, and both species are critically endangered and primarily distributed in states of Chiapas, Campeche, Quintana Roo, Tabasco, Veracruz and Yucatan. These arboreal monkeys live in troops, and their population densities vary considerably, ranging from <10 to approximately 100 individuals per km^2 (Crockett 1998). Although these monkeys are largely folivorous, their indiscriminate diet is likely one of the main reasons that these animals are adaptable to changing ecological landscapes, whether natural or anthropogenic (Helenbrook *et al.,* 2015b).

Sample strategy and study area

The present study was conducted in Tabasco and Chiapas states in the southeastern region of Mexico. Tabasco is a large flat coastal alluvial plain characterized by poor drainage and large areas of permanently or seasonally inundated terrains. The Grijalva and lower Usumacinta Rivers water the eastern and central parts of the plain. The weather is warm and moist, with a high constant temperature of ~26°C and an annual range of rainfall of 2000 to 4000 mm H_2O. Chiapas, the neighboring state, has areas with different climates, abundant rainfall and diverse topology. Furthermore, the study region is a mosaic formed by native vegetation patches and extensive urban areas, grasslands, crops, shrubs, flood areas, and riparian forests. The native vegetation comprises patches of evergreen forest and riparian forest, mangle and marsh areas (INEGI 2010). Field collection occurred in 2014 and 2015. The sampling sites were selected among vegetation areas used by *Alouatta* species for feeding, and there is information available on the general landscape, hydrography and flood vulnerability recorded from Tabasco's Ecological Planning Program (Gama, 2013). An arc-tool called “neighbor joining” in the Arcview 10.0 program was used to discriminate overlapping areas. Initially, we established a random minimum separation of 5 km between every population to avoid resampling the same population. This distance was established after considering the mobility reported for a group of *A*. *palliata*, which traveled less than 50 m on 12 of the 34 days during that these animals were followed. The *A*. *pigra* group traveled the same distance but on 15 of the 34 days (Amato and Estrada 2010). After interviews with local people, subdivisions and fieldwork, the presence of these monkeys was corroborated when howler troops were visually located using binoculars or based on their vocalizations. Sampling sites were selected considering 16 buffer areas, ranging from 7,852 m as minimum to 13,230 m as maximum, with distances of 10 to 357 km between each buffer. These sites included 14 sites in Tabasco and two sites in Chiapas (Table 1). The sites where *A*. *palliata* and *A*. *pigra* populations showed allopatric distribution were separated by the Grijalva River, living on the west and east sides of the river, respectively, except for one site in Tabasco (site 3, corresponding to the Tapijulapa, Cascadas de Villa Luz and Kolenchen localities) where both monkey species live in a sympatric area. The municipality Emiliano Zapata in Tabasco (site 8a) was of particular interest because, at this site, there is a group of small islands in the middle of the Chaschoc-Seja Lagoon (with some howlers living on two of the islands), with only boat access from the Bertollini Ranch (site 8b). Although some areas showed different grades of anthropogenic disturbance, among the 16 sites, 9 sites are considered as conserved zones, including forest and wetlands, while the other sites are meadows without human interactions, except for site 10 (Parque La Venta/ Tabasco), in which there is a proximity to humans and their homes.

Table 1. Infection rates of *Blastocystis* subtype (ST) for howler monkey populations. [1]N, number of analyzed howlers, [2]% *Blastocystis* infection, obtained as all positive samples for *Blastocystis*x100/N [3]% *Blastocystis* ST, obtained as positive samples for each STx100/all positive samples for *Blastocystis* in a specific site.[4]ST1 vs ST2+ST8 for *A. palliata* or *A. pigra*; *p*=0.9471, 95% Confidence interval (95%IC)=0.09-28.5.[5]ST2 vs ST1+ST8 for *A. palliata* or *A. pigra*; *p*=0.4981, 95%IC=0.29-9.17.[6]ST8 vs ST1+ST2 for *A. palliata* or *A. pigra*; *p*=0.7840, 95% .IC=0.71-20.85

Site ID	Localities/State	Species	N[1]	% *Blastocystis* infection[2]	% *Blastocystis* ST[3]			Accession number of GenBank
					ST1	ST2	ST8	
1	Playon de la Gloria/CHIAPAS	*A. pigra*	9	11.1 (1/9)	-	100 (1/1)	-	KT591833
2	Reforma Agraria/CHIAPAS	*A. pigra*	40	60 (24/40)	-	100 (24/24)	-	KT591789-94, KT591804-05, KT591814-17, KT591799-803, KT591806-09, KT591811-13
3	Tapijulapa, Cascadas de Villa Luz, Kolenchen/ TABASCO	*A. pigra,* *A. palliata*	5 8	20 (1/5) 37.5 (3/8)	-	100 (1/1) 100 (3/3)	-	KT591831, KT591839, KT591769
4	Poana, Xicotencatl/ TABASCO	*A. pigra*	22	18.2 (4/22)	-	50 (2/4)	50 (2/4)	KT591786-87, KT591852-53
5	Pochitocal/ TABASCO	*A. pigra*	5	0	-	-	-	-
6	Cascadas de Agua Blanca/ TABASCO	*A. pigra*	3	66.6 (2/3)	50 (1/2)	50 (1/2)	-	KT591851
7	Rancheria Josefa Ortiz de Dominguez/ TABASCO	*A. pigra*	5	80 (4/5)	-	100 (4/4)	-	KT591795-98
8a	Islands, Xeha Lagoon, Emiliano Zapata/ TABASCO	*A. pigra*	34	47(16/34)	6.3 (1/16)	93.7 (15/16)	-	KT591850, KT591770-75, KT591777-85
8b	Ranch Bertollini, Emiliano Zapata/ TABASCO	*A. pigra*	16	37.5 (6/16)	16.6 (1/6)	83.3 (5/6)	-	KT591848, KT591820-23,KT591838
9	Los Pajaros/ TABASCO	*A. pigra*	4	25 (1/4)	-	100 (1/1)	-	KT591788
10	Parque La Venta/ TABASCO	*A. palliata*	8	100 (8/8)	-	100 (8/8)	-	KT591840-47
11	Cocoa plantation and Ranch Cali/ TABASCO	*A. palliata*	3	33.3 (1/3)	-	100 (1/1)	-	KT591835
12	Carlos Greene/ TABASCO	*A. palliata*	30	26.6 (8/30)	12.5 (1/8)	87.5 (7/8)	-	KT591849, KT591824-30
13	Palestina/ TABASCO	*A. palliata*	4	25 (1/4)	-	100 (1/1)	-	KT591768
14	Tabasquillo/ TABASCO	*A. palliata*	7	28.6 (2/7)	-	50 (1/2)	50 (1/2)	KT591837, KT591854
15	San Juanito and Tres Brazos/ TABASCO	A. *pigra*	11	18.2 (2/11)	-	100 (2/2)	-	KT591818-19
16	Nueva Alianza and La Victoria /TABASCO	*A. pigra*	11	27.3 (3/11)	-	100 (3/3)	-	KT591834, KT591836, KT591832
		A. pigra	166	38.5 (64/166)	4.6 (3/64)	92.1 (59/64)	3.1 (2/64)	
		A. palliata	59	38.9 (23/59)	4.3 (1/23)	91.3 (21/23)	4.3 (1/23)	
	Total		**225**	**38.7 (87/225)**	**4.6 (4/87)[4]**	**91.9 (80/87)[5]**	**3.5 (3/87)[6]**	

Species identification and fecal sample collection

Species identification was performed using morphological characteristics: *A*. *pigra* is larger, and their hair color is completely black. Additionally, testes are evident in male *A*. *pigra* infants, unlike *A*. *palliata* males, in which the testicles do not descend until sexual maturity. The fur of *A*. *palliata* is not uniform in color and is typically dense, with golden flecks in the underarm region and areas with no pigment on the hands, feet and tail (Oropeza-Hernandez *et al.,* 2011). Fecal samples were collected using a non-invasive technique: waiting until the monkeys defecated. Only one sample per animal was collected. To this end, at least two persons monitored the howler monkey troops: one person recovered the fecal sample and the other person followed the howlers to obtain their approximate age, sex and some identification features of each individual, thereby guaranteeing no resampling. Additionally, we verified the species identification using mitochondrial and nuclear markers (Cortés-Ortiz *et al.,* 2009] and determined whether each sample and its corresponding monkey matched. All samples were fresh and carefully collected using gloves to avoid contamination from the soil, arthropods and vegetal detritus; only the top of each sample was saved and stored in 70% ethanol for further molecular analysis.

DNA extraction and PCR assays

DNA was extracted from approximately 100 mg of each fecal sample using the ZR Fecal DNA MiniPrep Kit (Zymo Research, Irvine, USA). The primers used for end-point PCR assays amplified an ~500 bp region of the small subunit rDNA (SSUrDNA) gene (Santín *et al.,* 2011; Sanchez- Aguillón *et al.,* 2013). PCR amplifications were performed in a final volume of 25 µL, containing 6.25 pmol of each primer, 1X PCR buffer (8 mM Tris-HCl, pH 8, and 20 mM KCl), 2.4 mM $MgCl_2$, 0.5 mM dNTPs, 0.01 mg BSA, and 1 U Taq DNA Polymerase (Promega). Up to 50 ng of DNA (~2 µL) was used as a template to amplify the genomic sequences. The following amplification conditions were used: 94°C for 5 min, followed by 36 cycles at 94°C-30 s, 54°C-30 s and 72°C-30 s, with a final extension step at 72°C for 10 min. The amplicons were assessed using electrophoresis on 1.2% agarose gels, after which the bands were purified using the AxyPrep PCR Clean-up Kit (Axigen Biosciences, CA, USA) and sequenced on both strands by a commercial supplier. Chromatograms were evaluated with Mesquite software using the Chromaseq package (Maddison and Maddison 2016;], with phred and phrap algorithms for base calling, assigning quality values to each one and assembling contigs (Ewing *et al.,* 1998a b; Gordon *et al,* 1998).

Phylogenetic reconstruction and genetic variation analysis

All sequences were subjected to BLAST search in the GenBank database, and the sequences obtained in this work were accessed with the numbers KT591768-KT591854. Multiple alignments were performed using the CLUSTAL W (Thompson *et al.,* 1994) and Muscle (Edgar 2004) programs, with manual adjustment in MEGA 5.05 software (Tamura *et al.,* 2001). The best-fit model of nucleotide substitution was determined using the Akaike Information Criterion in Modeltest software, version 3.7 (Posada and Crandall 1998) and the Hasegawa Kishino Yano model with gamma distribution and invariable sites. Phylogenetic reconstruction using Bayesian inference was performed using Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck 2003). The analysis was performed over 10 million generations, with sampling trees every 100 generations. Trees with scores lower than those at the stationary phase (burn-in) were discarded. The trees that reached the stationary phase were collected and used to build consensus trees. Other sequences were collected from GenBank and used as subtype references and for population genetics analysis (S1 Table).

A Median Joining Network analysis (Bandelt *et al.*, 1999] was performed using NETWORK 4.611(fluxus-engineering.com) with default settings and assumptions. Genetic diversity analyses within and between populations were performed using DnaSPv4 (Rozas *et al.,* 2003), and indices, such as nucleotide diversity (π), haplotype polymorphism (θ), gene flow (*Nm*),genetic differentiation index (F_{ST}) and Tajima's D test were obtained. These indices have been previously applied for population genetics studies in *Blastocystis* (Vargas-Sanchez *et al.,* 2015; Villalobos *et al.,* 2014). They denote the average proportion of nucleotide differences between all possible pairs of sequences in the sample (π); the proportion of nucleotide sites expected to be polymorphic in any suitable sample from this region of the genome (θ); the movement of organisms among subpopulations (*Nm*); and the differentiation between or among populations (F_{ST}). To support the data interpretation, populations strongly differentiated have an $Nm < 1$, whereas those with an $Nm > 4$ behave as a single panmictic unit. For F_{ST}, the following commonly used values for genetic differentiation were considered: 0 to 0.049, small; 0.05 to 0.149, moderate; 0.150 to 0.25, great; and values above 0.26 indicate enormous genetic differentiation. Negative values for Tajima's D test suggest a recent expansion process or an effect of purifying selection (Hartl and Clark 1997).

Statistical analysis

Descriptive statistics are expressed as mean and standard deviation (SD). Analysis by Student's t test for unrelated samples and Mantel–Haenszel test were applied; 95%

confidence intervals (95%CI) were also obtained. Data analysis was performed with SPSS software Version 15.0 (SPSS Institute, Chicago, IL).

Ethics statement

All fecal samples were collected with a non-invasive technique after monkeys defecated. Sampling and procedures were in accordance with the provisions of the Regulations of the Environment and Natural Resources Ministry and the Under-Secretary of Management for Environmental Protection NOM-059-SEMARNAT-2010; reference SGPA/DGVS/04725/13. Academic authorities of the Universidad Juarez Autonoma de Tabasco authorized our study (reference number UJAT-2013-IB-43).

Results

Frequency of *Blastocystis* spp. in *A. palliata* and *A. pigra*

In 14 sites from Tabasco and two from Chiapas, fecal samples from 225 howler monkeys, *A. palliata* (59) and *A. pigra* (166) were collected and analyzed. Table 1 summarizes the positive Blastocystis ST results for animals from each site; only at site 5 (Pochitocal, Tabasco) were the monkeys not parasitized. The frequency of Blastocystis spp. was 38.7% (n=87), 39% for *A. palliata* and 38% for *A. pigra;* being ST2 the most abundant (91.9%), followed by ST1 and ST8 with 4.6% and 3.5%, respectively. No association between Blastocystis ST and *Alouatta* species was found. In addition, we identified infected howlers with similar frequencies and ST distributions in flooded and non-flood areas (Fig 1).

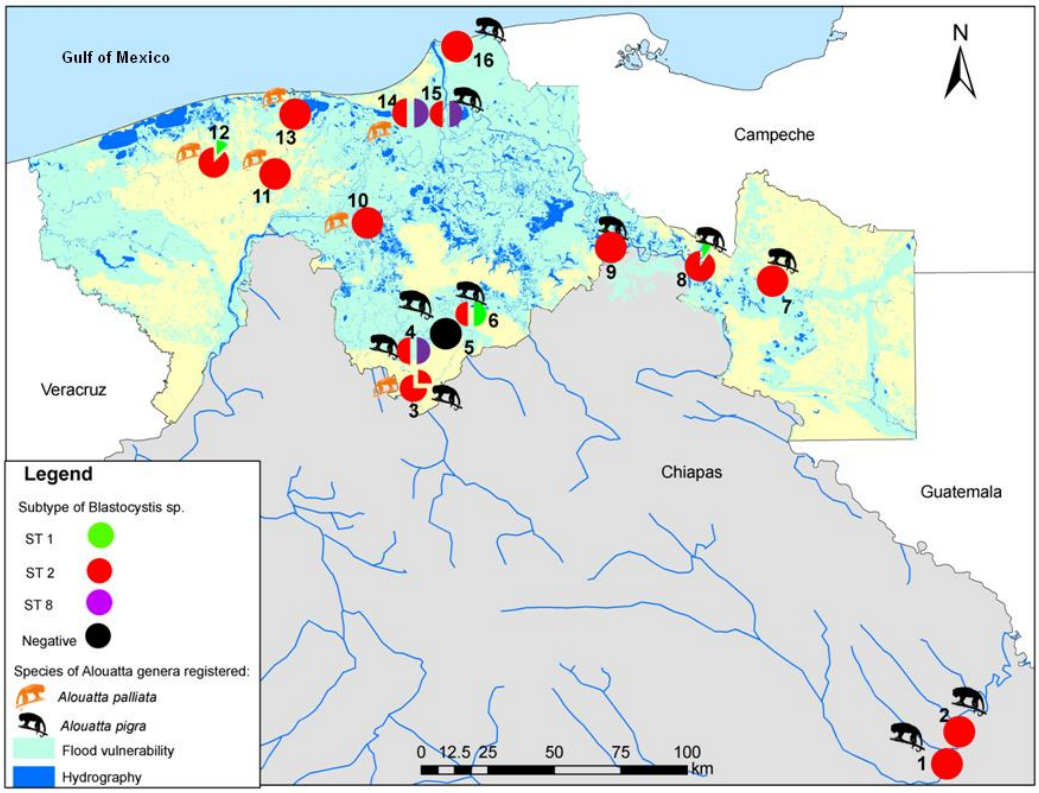


Figure 1. Field sampling sites, hydrography and frequency of *Blastocystis* ST in *Alouatta palliata* and *A. pigra* from Tabasco and Chiapas. The pie charts indicate those localities with positive samples and each colour represents a different ST; proportions of colours are according to ST frequencies.

Phylogenetic reconstruction and genetic variation analysis

The contigs were assembled using both forward and reverse chromatograms from each sample, and analysis using Mesquite/Chromaseq software showed well-defined peaks without significant background noise. A Bayesian phylogenetic tree was built for SSUrDNA using all worldwide available sequences recorded in GenBank (S1 Fig). The sequences were grouped into the ST1, 2, and 8 clusters. The haplotype networks for ST1 and ST2 showed contrasting distributions: for ST1 (Fig 2A), several haplotypes from different mammals (humans, NHP, and pigs) and birds are homogeneously distributed in different countries, and in general, few mutational differences are present among them. Conversely, for ST2 (Fig 2B), a principal dispersion center grouped most of the Mexican howlers together, with the remaining sequences also closely distributed. Interestingly, some haplotypes showed high mutational differences, particularly in the NHP vs. human haplotypes. For example, for the Mexican sequences, three howler haplotypes diverged from the principal dispersion center by over 40 mutational changes, with the most

divergent being a sample from one of the islands (site 8a). A similar phenomenon was observed for the ST2 sequences previously reported for *Cercopithecus hamlyni* from Spain (Santín *et al.,* 2011), with one haplotype close to the human haplotypes, and another haplotype markedly distant, with up to 76 mutational changes.

Figure 3 shows the ranges of F_{ST} and *Nm* among the different sampling sites in Tabasco and Chiapas. Despite the great geographic distances between some of the populations, minimal differentiation and high gene flow were observed. As expected, the population with the lowest differentiation and gene flow was in the islands (site 8a), in contrast with site 8b, which showed F_{ST} and *Nm* values similar to those of the other sampling sites. The populations at sites 2, 8a, 8b and 12 yielded negative Tajima's D values, but only for site 2, and this result was statistically significant (p<0.01).

Table 2 shows the comparisons between the indexes obtained for ST1 and ST2 of humans and NHP. The ST8 haplotype was not analyzed because of an insufficient number of sequences. These indexes support the topologies of the contrasting haplotype networks (Fig 2A and 2B). In the intra-ST comparison between humans and NHP, human *Blastocystis* ST1 populations had lower π values than those of NHP, whereas for ST2, human and NHP parasites had similar variability; despite the disparity between the number of available sequences in the GenBank of humans and NHP for ST1, the genetic diversity values were statistically significant. The *Nm* and F_{ST} values for ST1 and ST2 were inverted, suggesting that *Blastocystis* ST2 populations from humans and NHP are strongly differentiated with occasional gene flow, while scarcely any differentiation between ST1 parasite populations from humans and NHP was detected.

Table 2. Values of nucleotide diversity (π), haplotype polymorphism (θ), gene flow (*Nm*) and genetic differentiation index (F_{ST}) for sequences of humans and HNP populations of *Blastocystis* ST1 and ST2.

	N	**π±SD**	***p****	**θ±SD**	***p****	**F_{ST}**	***Nm***
ST1							
Humans	147	0.0241±0.0030	0.0001	0.0298±0.0080	0.0001	0.071	3.25
NHP	14	0.061±0.0169		0.0694±0.00026			
ST2							
Humans	94	0.0211±0.0022	0.247	0.0238±0.0070	0.0001	0.644	0.14
NHP	83	0.0204±0.0059		0.0774±0.0204			

*Student's t-test, for independent samples

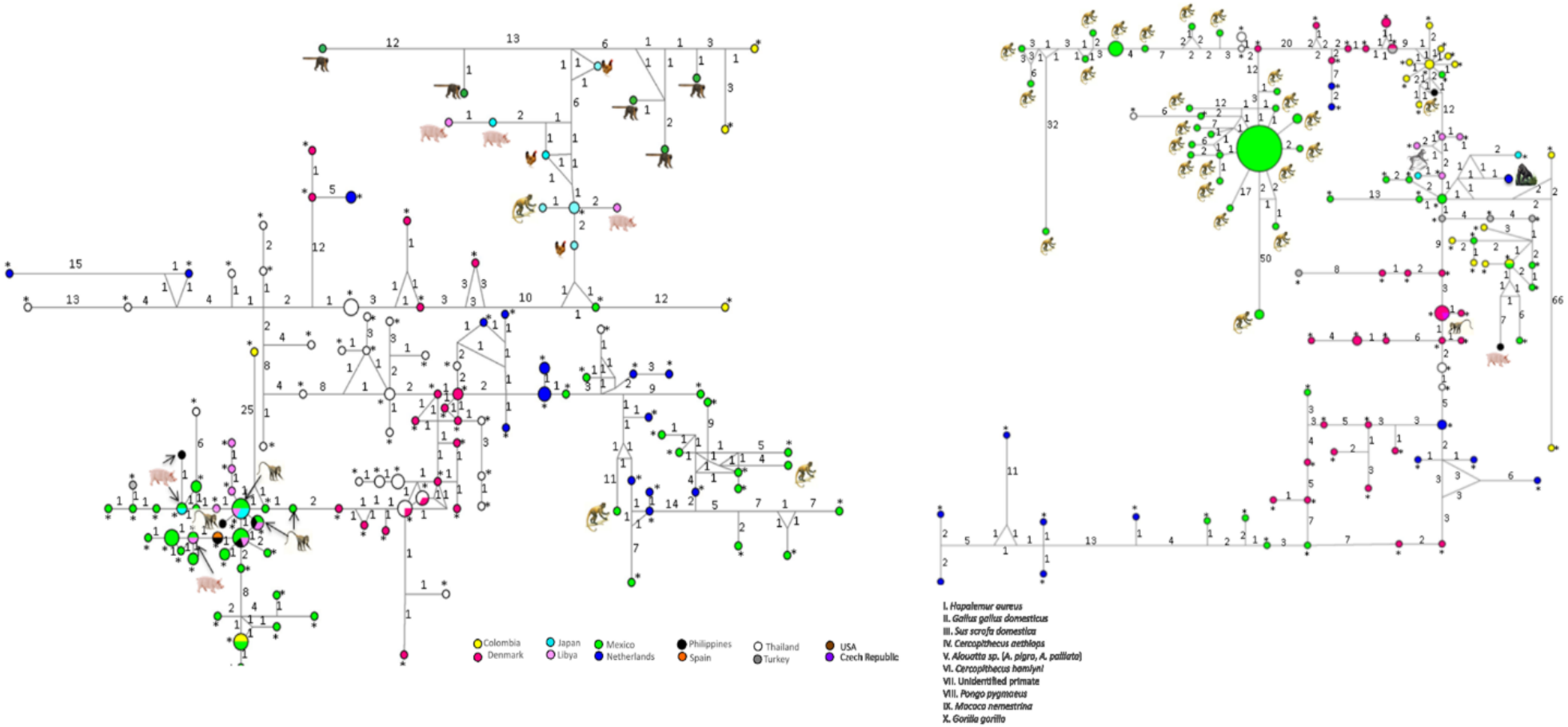


Figure 2. Haplotype networks for *Blastocystis.* Haplotype network trees using SSUrDNA sequences from different countries and hosts for ST1 (a) and ST2 (b). Numbers in branches refer to mutational changes; sizes of circles and colors are proportional to haplotype frequencies. For those animal haplotypes, an image and Roman reference numbers were included, while for human haplotypes, asterisks were added.

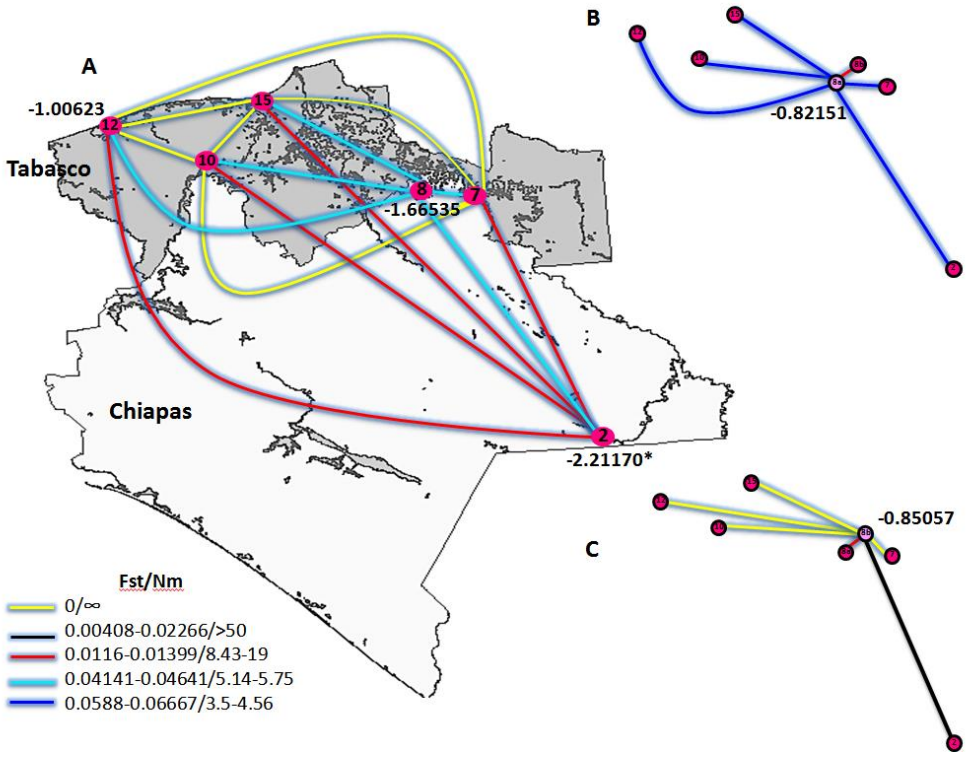


Figure 3. Schematic representation of interactions among population indexes. The gene flow (Nm), genetic differentiation index (F_{ST}), and Tajima's D values of *Blastocystis* ST by SSUrDNA analysis, according to different sampling sites. The number together the sampling size circle, mean the Tajima's D value. * p<0.01

Discussion

The few reports on the molecular identification of *Blastocystis* ST in *Alouatta* monkeys (Alfellani *et al.,* 2013; Helenbrook *et al.,* 2015; Ramírez *et al.,* 2014) have shown dissimilar results for this NHP genus. The results obtained in the present study not only strengthen the existence of cryptic host specificity for ST1 and ST2 but also show that these ST have different population structures, with certain haplotypes of ST2 showing a preference for either humans or NHP. Generalist parasites infecting several host species can actually be cryptic parasite species, each characterized by a different degree of host specificity, particularlyinfluenced by local adaptations that have led to a preference for certain host species over others and have zoonotic implications. Therefore, the generalist profile of some parasites may be a key process for the maintenance of their genetic diversity and population diversification (Stensvold *et al.,* 2012a b; Kankare *et al.,* 2005; Smith *et al.,* 2016; Westram *et al.,* 2011).

In the present study, we observed that ST1 exhibits a generalist profile because there was minimal differentiation between parasite populations from humans and NHP, as well

as substantial gene flow between them, suggesting that ST1 landscapes resemble a metapopulation (ensemble of interacting populations with a finite lifetime) (Le Gac *et al.,* 2007; Roulin *et al.,* 2015; Levins *et al.,* 1969; Hanski *et al.,* 1991). In contrast, some *Blastocystis* ST2 haplotypes have diverged similar to a set of local populations with preferences towards certain host species (humans or NHP). Both population structures have relevance for understanding the prevalence and transmission of *Blastocystis* ST. According to these findings, the putative metapopulation structure of ST1 implies a link with the processes of population turnover, extinction and establishment of new populations (Roulin *et al.,* 2015), i.e., *Blastocystis* ST1 may have the capacity to recolonize vacant niches, such as new hosts, even of different species, supporting the zoonotic transmission of this parasite (Yoshikawa *et al.,* 2004; Stensvold *et al.*, 2009, Ramirez *et al.,* 2016, Abe 2015; Parkar *et al.,* ;2010; Yoshikawa *et al.,* 2009; Petrašova *et al.*, 2011; Alfellani *et al.,* 2013). In addition, (Sanchez-Aguillon *et al.,* 2013) documented reinfection by *Blastocystis* ST1 in an asymptomatic patient who received anti-parasitic treatment and follow-up three months earlier.

However, the existence of ST2 as a set of resident populations locally adapted by each parasite population would support the preference for infecting either humans or NHP. Such a process of local adaptation is supported by Helenbrook *et al.* (2015c) who observed that *Blastocystis* was the unicellular parasite most frequent (60%) in samples of *A*. *palliata aequatorialis* howlers from Ecuador. Subsequently, in another study performed in similar populations of these howler monkeys, 68% of these animals were infected with *Blastocystis* ST8, while humans living in close proximity were infected with ST1, ST2 and ST3 Helenbrook *et al.* (2015a)]. Interestingly, although these subtypes (ST1-ST3) are distributed worldwide, they are common in America (Ramirez *et al.,* 2016; Vargas-Sanchez *et al.,* 2015; Villalobos*et al.,* 2014). In the present study, 39% and 38% of *A*. *palliata* and *A*. *pigra* howlers, respectively, were primarily infected with *Blastocystis* spp., and of these, 91.3% of *A*. *palliata* and 92.1% of *A*. *pigra* howler monkeys were harboring *Blastocystis* ST2. Other relevant findings regarding the host specificity of ST2 were derived from the phylogenetic tree of parasites from Rubondo Island, Tanzania (Petrašova *et al.,* 2011), in which the cluster for ST2 showed two clades for *Blastocystis*, those from humans and those from NHP.

In addition, as cited above, local adaptations are relevant for the maintenance of genetic diversity and population diversification. The high degree of intra-ST genetic polymorphism has been reliably documented, particularly for ST1 and ST3 (Yoshikawa *et al.*, 2009; Alfellani *et al.*, 2013; Stenvold *et al.*, 2012a b; Villalobos *et al.*, 2014; Ramirez

et al., 2014), suggesting that populations of recent origin have undergone a radiation process (Tomasini *et al.,* 2004). However, the great divergence of some haplotypes for ST1 and ST2 observed in the present study deserves special attention. The presence of divergent haplotypes with more than 40 mutational differences between sequences from NHP of Spain and Mexico, particularly in a monkey from an island (site 8a), suggests the island-continent model described by Wright (1940), in which many finite subpopulations (equivalent to the continent) are present, including the source of migrants to the island. When the amount of gene flow and the population size on the islands are both large, the allele frequency on the islands will soon become similar to that of the continent. However, if the population size on the island is small or if the rate of gene flow is low (as in the present study), then genetic drift could lead to random changes in allele frequency. As a result, the allele frequency on the islands may differ significantly from that on the continent and that of the migrants (Hedrick 2011). Alternatively, the population size could correspond to an epidemic population structure such as that depicted by Maynard-Smith *et al.* (1993), in which there is frequent recombination within all members of the population, such that the structure is a net rather than a tree.

It has been observed that the use of more than one genetic marker has facilitated the clarification of the differences between organism populations. However, some studies of genetic variability focused only on the SSUrDNA gene analysis have shown that this marker is sensitive enough to resolve phylogenetic relationships, population differentiation events and cryptic infections in both *Blastocystis* and other parasites (Chambouvet *et al.,* 2015; Aurahs *et al.,* 2009; Pillet *et al.,* 2012; Ramirez *et al.,* 2016; Abe 2004)

Since mixed ST infections are common (Ramirez *et al.,* 2014; Scanlan *et al.,* 2015; Jimenez-Gonzalez *et al.,* 2012), we used Mesquite/Chromaseq software to identify co-infections in the howler monkeys as this technique has been used to reduce bias and misinterpretations during sequence analysis (Maddison and Maddison 2016; Ewing *et al.,* 1998a b, Gordon *et al.,* 1998); in this way, this analysis could distinguish a double profile in the chromatogram sequences, suggesting mixed infections based on different ST. However, future use of ST-specific primers, cloning, and SSCP analysis should be performed to confirm the presence or absence of ST co-infections.

Although a definitive mode of transmission for *Blastocystis* has not been identified, it has been suggested that transmission occurs orally through water, food or direct contact with the parasite; here, *Blastocystis* transmission apparently occurs through water (Moe *et al.,* 1996), but wild howler monkeys have rarely been observed drinking river water. Instead,

these animals use arboreal water cisterns (Glander 1978), suggesting that transmission may occur when monkeys are in contact with contaminated leaves or other non-arboreal elements (i.e., lianas, vines and epiphytes) (Vitone *et al.,* 2004; Gonzalez-Hernandez *et al.,* 2014), wet ground or sewage during their movement between trees. Nevertheless, transmission may occur when monkeys drink water directly from the ground; Serio-Silva and Rico-Gray observed this behavior for *A*. *palliata* and *A*. *pigra* feeding and traveling on the ground, in small patches in locations with scarce trees, and during the dry and wet seasons in Balancan, Tabasco (Serio-Silva and Rico-Gray 2003).

The results of the present study demonstrate that howler monkey populations of *A*. *palliata* and *A*. *pigra* with locally adapted *Blastocystis* ST1 and ST2 populations exhibit distinct generalist and specialist types of host specificity. In addition, these data suggest that the *Blastocystis* ST2 populations in humans are highly differentiated from those of NHP, while the ST1 populations are only minimally differentiated. The host generalist and specialist specificities exhibited by ST1 and ST2 *Blastocystis* populations are thus distinct processes. Because ST1 exhibits a generalist profile, this haplotype can be considered a metapopulation; in contrast, *Blastocystis* ST2 exists as a set of local populations with preferences for either humans or NHP.

Furthermore, it is important to highlight the use of population genetics studies in the epidemiology of this eukaryote because these studies may clarity some aspects of host-parasite relations; for example, how genetic variability within a subtype is reflected in phenotypic and functional variability and its potential role on the symptoms of the parasite carriers (Stensvold and Clark 2016).

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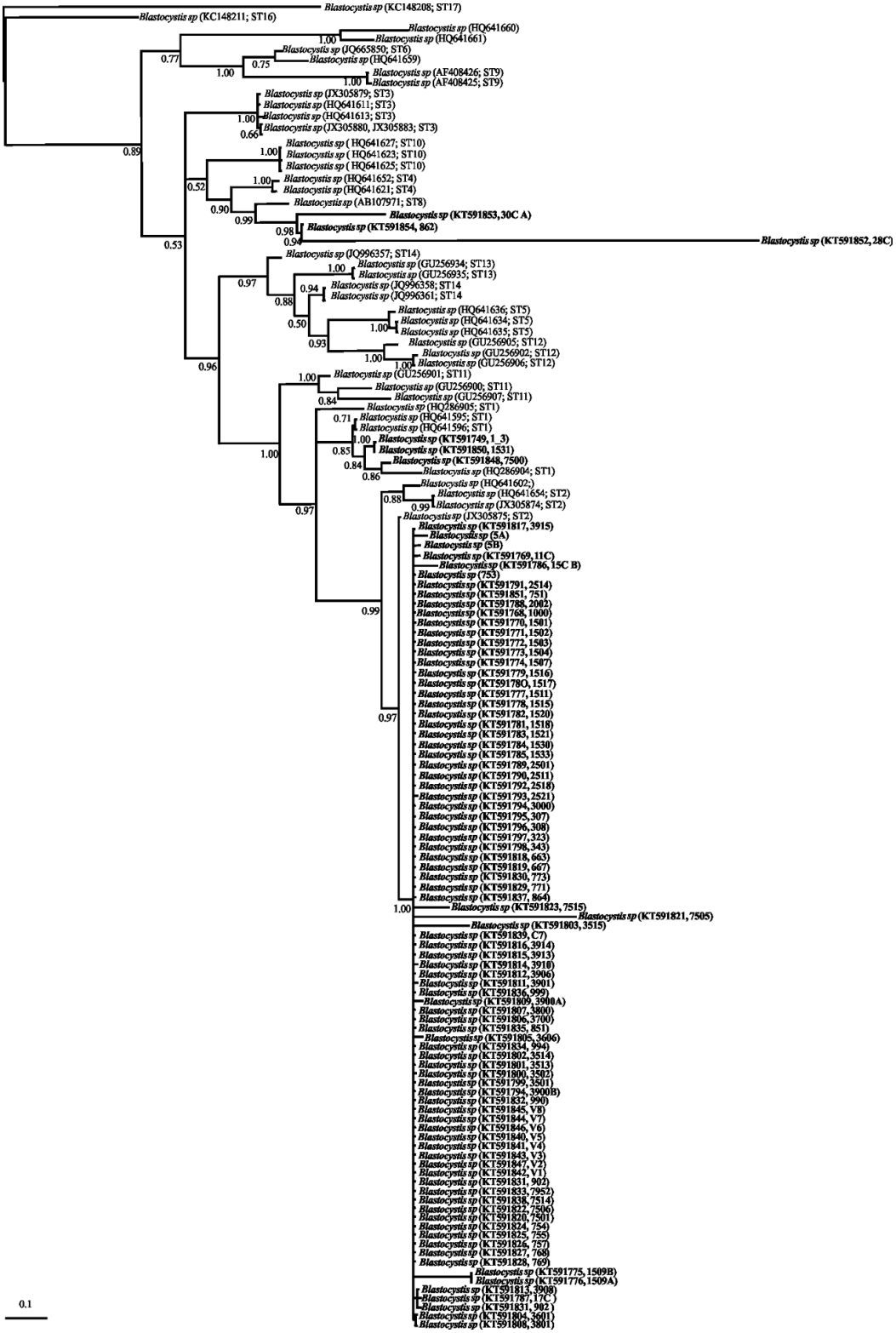
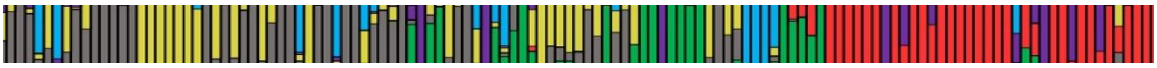
Supplementary material



Supplementary Figure 1. Phylogenetic inference of *Blastocystis* spp. Bayesian phylogenetic tree using a fragment of SSUrDNA sequences; the values of the nodes indicate posterior probabilities values using 10 million generations. ST and GenBank accession numbers are shown, as well as identification of each sample.

CHAPTER 4



New *Entamoeba* group in howler monkeys (*Alouatta* spp.) associated with parasites of reptiles

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Abstract

Our knowledge of the parasite species present in wildlife hosts is incomplete, especially in non-human primates (NHPs). Protozoans such as amoebae of the genus *Entamoeba* infect a large variety of vertebrate species, including NHPs. However, traditionally, their identification has been made through microscopic evaluation; therefore, amoeba species have not always been identified correctly. We searched for *Entamoeba* spp. using molecular approaches in free-ranging howler monkeys (*Alouatta palliata* and *A. pigra*) from southeast Mexico. In total, 155 samples were collected, with 46 from *A. palliata* and 109 from *A. pigra*. We detected a new clade of *Entamoeba*, which was separated from other described species but closer to *E. insolita*, as well as an unnamed sequence typically found in iguana species with low shared identity values (<90%). We designated this new clade as ribosomal lineage 8 (RL8) and we have shown that members of this group are not exclusive to reptiles.

Keywords: *Alouatta palliata*, *Alouatta pigra*, *Entamoeba* spp., ribosomal lineage.

Introduction

Parasites are essential components of biological communities and an important source of biodiversity (Poulin and Morand 2005). However, it is not surprising that most ecological, epidemiological, and evolutionary studies have focused mainly on parasites that are important for public health (Martínez-Hernández *et al.* 2014). The impact of parasitic infections in wild animal populations is recognized as an important factor that influences the distribution and density of species (Anderson 1979). This may be particularly important for parasites in endangered wildlife populations because their study can provide useful information about a host's health, infection risk, and the development of management programs (Gillespie *et al.* 2005). Even non-pathogenic parasites may become important when animal populations are malnourished or stressed, and their combined effects may predispose animals to other disadvantageous conditions (Scott 1988).

Several studies have shown that a fairly high diversity of parasites are harboured by non-human primates (NHPs), particularly gastrointestinal parasites, including protozoans and helminths (Vitazkova and Wade, 2006; Helenbrook *et al.* 2015; Villanueva-Garcia *et al.* 2017). Protozoans such as amoebae of the genus *Entamoeba* infect numerous types of vertebrates, including NHPs. However, at the molecular level, *Entamoeba* parasites have only been described in Old World primates: *E. chattonni*, *E. coli*, *E. dispar*, *E. hartmanni*, *E. histolytica*, *E. nutalli*, and *E. polecki* (Berrilli *et al.* 2011; Feng *et al.* 2011; Regan *et al.* 2014; Jirků-Pomajbíková *et al.* 2016). In addition, a high rate of infection by *E. coli*, *E. dispar*, and *E. hartmanni* (up to 80% of the total faeces analysed) has been observed (Jirků-Pomajbíková *et al.* 2016).

Mexican howler monkeys, *Alouatta palliata* and *Alouatta pigra*, are New World NHPs with extremely restricted ranges in the fragmented forests of Mexico. According to the International Union for Conservation of Nature, both species are critically endangered given the dramatic declines in their populations over the last 30 years due to habitat fragmentation and loss (Dunn *et al.* 2014). *A. pigra* is endemic to the southeast of Mexico, Guatemala, and Belize, and its populations may have been affected more severely by these events (Crockett 1998). Mostly helminths and protozoans have been identified in these monkeys based on microscopic evaluations. However, most of these previous studies were performed using animals kept in captivity, such as zoos, and with close human contact (Regan *et al.* 2014).

In *A. palliata* and *A. pigra*, the genus *Entamoeba* has traditionally been described and identified based on microscopic analyses of faeces (Stuart *et al.* 1998; Eckert *et al.* 2006; Vitazkova and Wade 2006; Trejo-Macías *et al.* 2007). However, to the best of our knowledge, amoebic parasites have not yet been identified at the species level using molecular methods. Molecular tools (such as PCR and sequencing) have helped to clarify the identification of species and to establish taxonomic relationships, particularly in hosts with restricted distributions, and have the potential to discover new parasite species (Clark *et al.* 2006).

In order to provide information regarding the presence of parasites in wildlife hosts and to help clarify their genetic relationships with other species, we studied *Entamoeba* spp. in the howler monkeys *A. palliata* and *A. pigra* from Mexico using molecular approaches.

Methods

Faecal samples from *A. palliata* and *A. pigra* distributed in forest habitats in Tabasco and Chiapas states, Mexico, were collected during 2014 to 2015. The study areas were selected using a maximum entropy distribution model (Maxent software, version 3.3.3k http://www.cs.princeton.edu/~schapire/maxent/). This program estimates the probability distribution for species based on environmental constraints (Phillips *et al.* 2006) using data regarding the presence of species and environmental variable layers for the area of study. Finally, an arc-tool called “neighbour joining” was used to discriminate overlapping areas within 5 km in the Arcview 10.0 program. The presence of howler monkeys was confirmed when troops were visually detected by binoculars or based on their vocalizations. Samples were collected from 27 localities (25 in eight municipalities from Tabasco state and two municipalities from Chiapas state), where the distances between each of the sites ranged from 10 to 357 km.

Faecal samples were collected using a non-invasive technique by waiting until the monkeys defecated and only one sample was obtained per animal. All of the samples were carefully collected with gloves to avoid contamination and only the top of each sample was recovered. A portion was then stored in 70% ethanol until processing in the laboratory and another portion was fixed in 4% formalin. The sampling procedures were performed in accordance with the provisions of the Regulations of the Environment and Natural Resources Ministry and the Under-Secretary of Management for Environmental Protection (NOM-059-SEMARNAT-2010; reference SGPA/DGVS/04725/13). The

academic authorities of the Universidad Juarez Autonoma de Tabasco authorized our study (reference number UJAT-2013-IB-43).

Entamoeba cysts were separated using the passive faecal flotation technique in saturated salt (NaCl) solution with a specific gravity of 1.20 (Acevedo and Romero 1987). Subsequently, DNA was extracted from approximately 100 mg of each faecal sample using a FavorPrep Stool DNA Isolation Mini Kit, according to the manufacturer's instructions (Favorgen Biotech Corporation, Pingtung County, Taiwan). PCR amplification was performed in a volume of 13 µL, which contained 2 µL of DNA (200 ng), 1× PCR buffer (1 mM KAPA Taq Buffer), 0.01 mg/mL of bovine serum albumin, 1.5 mM $MgCl_2$, 0.25 U polymerase (KapaTaq; KAPA Biosystems, Boston, MA, USA), and 0.25 mM of each primer. Universal forward and reverse oligonucleotides were designed for *Entamoeba* species based on the consensus sequences from the 18S small subunit ribosomal RNA gene according to the highly conserved regions reported in GenBank.

The designed forward primer set comprised: UniverF 5′-ATA ACG GRT AAC GAG GAA TTR GGG-3′ and the reverse primer, UniverRev 5′-GTT GAG TCA AAT TAA GCC GCA GGC-3′, which amplified a region of approximately 724 to 1100 bp depending on the amoeba species. The amplification conditions were as follows: a denaturing step at 95°C for 2 min, 35 cycles at 95°C for 30 s, 64°C for 25 s, and 72°C for 90 s, with a final extension step at 72°C for 30 min. The amplicons were separated by electrophoresis on 1.5% agarose gel and *RedSafe* Nucleic Acid Staining Solution, where the bands were excised under ultraviolet illumination and sequenced on both strands by a commercial supplier.

All of the sequences obtained in this study were subjected to BLAST analysis via GenBank. Multiple alignments were performed using CLUSTALW (Thompson *et al.* 1994) and MUSCLE (Edgar 2004) programs, and manually adjusted with MEGA 7 software (Tamura *et al.* 2011). Phylogenetic reconstruction using Bayesian inference was performed with the Mr. Bayes 3.1.2 program (Ronquist and Huelsenbeck 2003). The analysis was performed over 3 million generations with sampling trees every 100 generations. Trees with scores lower than those in the stationary phase (burn-in) were discarded. The trees that reached the stationary phase were collected and used to build consensus trees. Other *Entamoeba* sequences were obtained from GenBank for phylogenetic reconstruction: *E. coli* AF149914, AF149915, FR686364, AB444953; *E. chattoni* AF149912; *E. polecki* FR686392, FR686357, AF149913, LC082305; *E. suis* DQ286372; *E. gingivalis* D28490, KX027297-8; *E. kamaktli* KX027294-6; *E. equi* DQ286371; *E. invadens* AY769863; *E. ranarum* AF149908; *E. hartmanni* FR686378,

FR686381; *E. insolita* AF149909; *E. terrapinae* AF149910; *E. bovis* FN666252; *E. sp* FN666253, FR686358-65, KR025406, KR025409, AF149911; *E. moshkovskii*, AF149906, KP722604; *E. ecuadorensis* DQ286373; *E. dispar* Z49256, AB282661, KP722596, KP722599; *E. histolytica* X56991, KP233837, AB197936; *E. nuttalli* LC041205; *E. bangladeshi* KR025411; *E. muris* AB445018; and *E. struthionis* AJ566411.

Results

In total, 155 samples were obtained from howler monkeys, with 46 from *A. palliata* and 109 from *A. pigra*. No *Entamoeba* cysts were found in any of the faecal samples analysed by microscopy. However, PCR using faecal DNA detected eight positive samples for *Entamoeba* species: seven from *A. pigra* (6.42%) and one from *A. palliata* (2.17%), where the size of the amplified fragment was 900 bp. The positives samples were obtained from four sites in three municipalities of Tabasco State.

BLAST searches found regions of similarity in the sequences obtained from howler monkeys (ID 33, 22, 23, 24, 47, 57, and 60f) with shared identity values of 90% relative to *Entamoeba insolita* and only one (ID 3bal) sequence had a shared identity value of 99% with *Entamoeba* spp. (AF149911), a sister species of *E. insolita*. Lower similarity values were obtained with other *Entamoeba* species.

A Bayesian phylogenetic tree was built using small subunit rDNA (SSU rDNA) sequences from different *Entamoeba* species in the GenBank database. Our sequences were grouped into two clades with high posterior probability values: 3bal sequences (from *A. pigra*) were grouped with *Entamoeba* sp. NIH:1091:1 sequence (AF149911) and the others (one from *A. palliata* and six from *A. pigra*) were grouped in a new clade, which was clearly separated from the *E. insolita* (AF149909), *Entamoeba* sp. (AF149911), and Oedla (FR686365) sequences (Fig. 1). Similar results were observed in the haplotype network trees where a high number of mutations separated the three clades (Fig. 2).

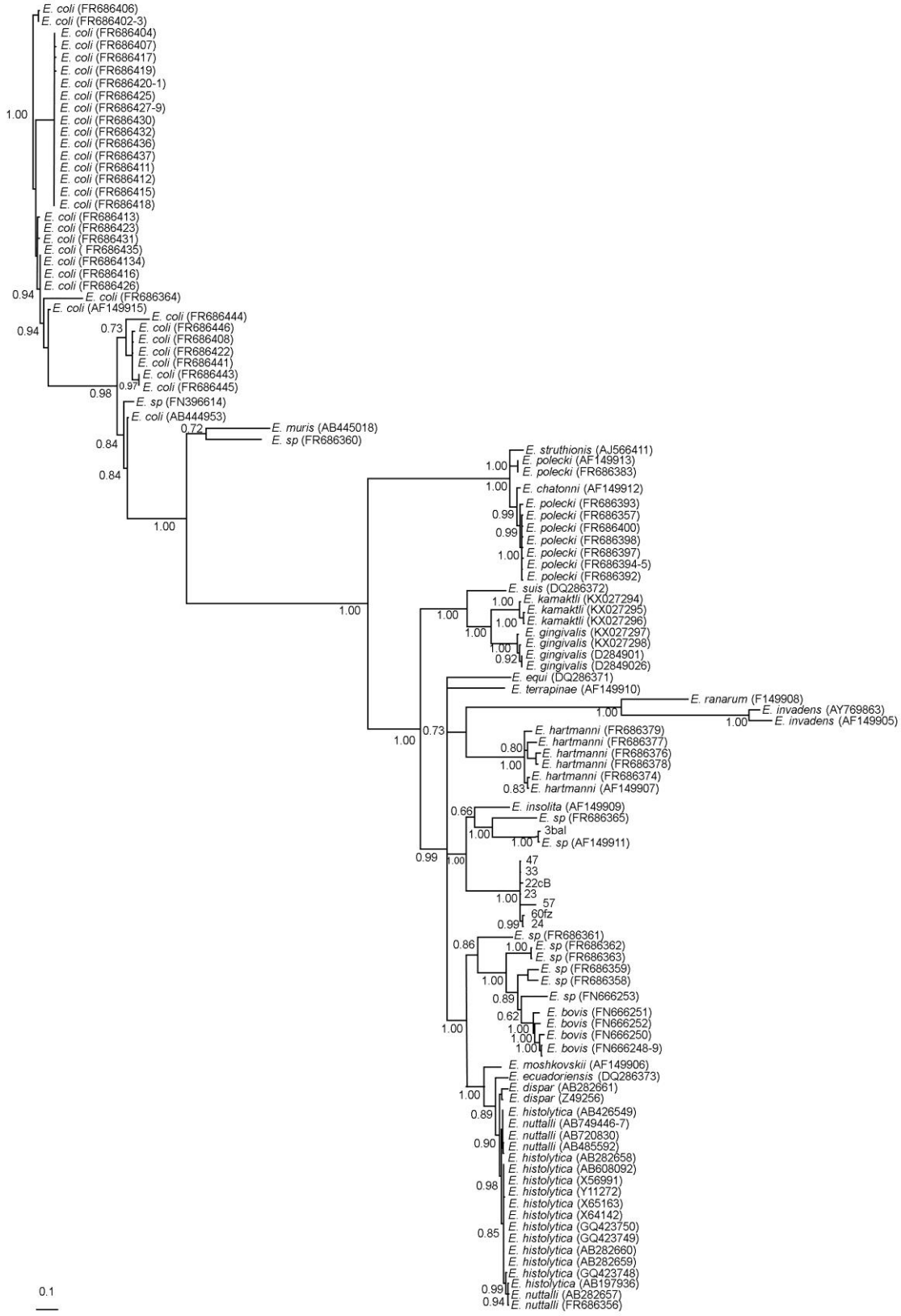

Figure 1. Bayesian phylogenetic tree based on sequences of *Entamoeba* species using a fragment in the region of the small subunit rDNA gene. The values on the nodes indicate posterior probabilities. The phylogenetic tree was sampled for over 3 million generations.

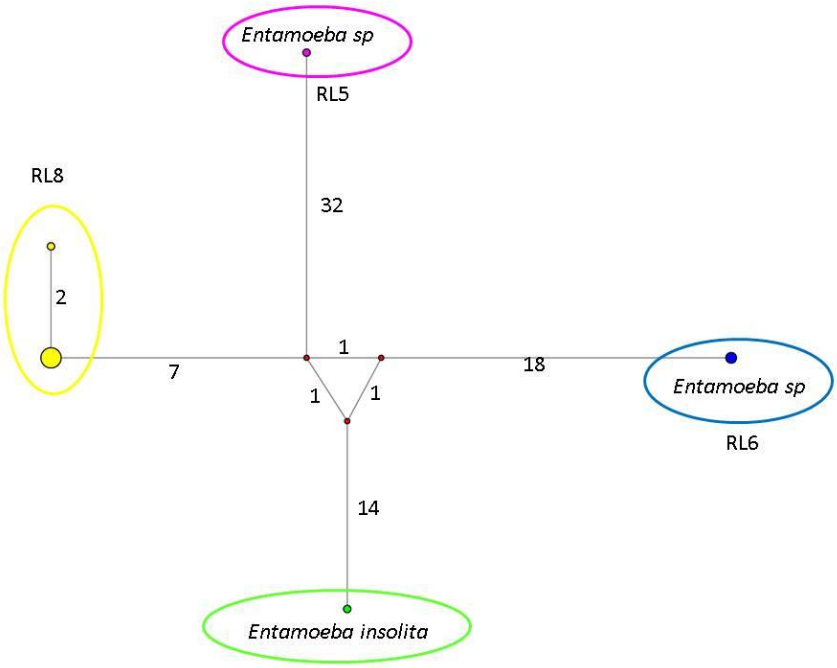

Figure 2. Haplotype network obtained for *Entamoeba* species using small subunit rDNA sequences. Numbers in branches refer to mutational changes. The sizes of circles are proportional to the haplotype frequencies. Each colour indicates a distinct ribosomal lineage (RL).

Discussion

It is fairly common to find reports of the presence of *Entamoeba* spp. in faecal samples from NHPs. However, most of these reports were based on morphological identification and the cysts were identified only to the genus level. In the present study, we identified a new clade of *Entamoeba* in howler monkey *Alouatta* spp. This clade was clearly separated from *E. insolita*, which was isolated from a tortoise, and an unnamed sequence recovered from an iguana (*Entamoeba sp*. NIH:1091:1), as well as the Oedla sequence isolated from *Geochelone pardalis* (tortoise), where these three groups of amoebae were isolated from reptilian species. The new clade identified in this study shared low identity values compared with the sequences reported previously for other amoebae (<90%), where high posterior probability values were obtained for the phylogenetic reconstructions with high numbers of mutations among the three clades. Silberman *et al.* (1999) clustered *E. insolita* with *Entamoeba* sp. NIH: 1091:1 after Stensvold *et al.* (2011) included the Oedla sequence in the same cluster, although the latter had a relatively low bootstrap value and thus the result was dependent on the type of analysis. Therefore, the Oedla sequence was assigned the name of *Entamoeba* RL5,

whereas *Entamoeba* sp. NIH: 1091:1 was designated as *Entamoeba* RL6. These assignments were suggested until the establishment of valid taxonomic names for the isolates. The ribosomal lineages (RLs) designated for these branches within phylogenetic trees lack strong affinities with previously described species, whereas the term subtype refers to well-supported phylogenetic clusters within a defined species. It was also suggested that subtypes should be defined using partial gene sequences, whereas ribosomal lineages should be assigned using complete SSU rDNA sequences (Stensvold *et al.* 2011). We used partial SSU rDNA sequences, but the phylogenetic tree had posterior probability and bootstrap support values of 1.00 and 100, respectively, and the sequences were not included in previously described species, thereby supporting the RL8 clade as a new group of *Entamoeba*. Only one sequence from *A. pigra* was not included in RL7, where it was located in the RL6 clade (*Entamoeba* sp. NIH:1091:1). Our results also suggest that the group comprising *E. insolita*, RL5, and RL6 is not exclusive to reptiles, as previously suggested. Therefore, exhaustive studies of *Entamoeba* host diversity are necessary before considering host specificity.

The lowest prevalence of *Entamoeba* and the limited geographic region where they were found in the present study may indicate that the new *Entamoeba* clade is located only in the southern region of Tabasco State. Therefore, more studies should search for this group in other hosts throughout the region. Specialized parasites are important because they can carry phylogenetic and population genetic information regarding the evolutionary history of their hosts (Rózsa and Vas 2014).

The two primate species considered in this study, *A. palliata* and *A. pigra*, are examples of species that have experienced habitat disruption, which could have affected their gastrointestinal parasites. Anthropogenic fragmentation and habitat destruction are the main causes of local extinction among NHP populations, thereby decreasing and isolating populations, as well as favouring parasitic infections (Stoner 1996; Shalk and Forbes 1997; Estrada and Mandujano 2003; Estrada *et al.* 2006). Due to the destruction of their habitat, new species of parasites can occupy new niches, which may affect other organisms, including humans, so more studies should analyse the parasite diversity in wild hosts and the relationships among these *Entamoeba* species, including the impacts on their hosts and other parasites.

Acknowledgments

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CONCLUSIONS

Chapter 1. Population structure, genetic diversity and connectivity of populations in parapatric species, *Alouatta palliata* and *Alouatta pigra* in Southeast of Mexico.

1. The potential distribution for *A. palliata* and *A. pigra* indicates disparate response to past, present and future scenarios and a maintained hybridization zone through time.
2. Last Glacial Maxima SDM proyection showed very restricted potential *Alouatta* distribution with respect to current SDM for both species, although it was significantly lower for *A. pigra* than for *A. palliata*.
3. *A. pigra* showed several small glacial refugia scattered across southern highland massif of Mexico (Chiapas, Oaxaca, Puebla and Veracruz) and Belize.
4. *A. palliata* showed a fragmented distribution with several glacial refugia: in the currect hybridization zone of Mexico, and in several refugia across Costa Rica, Colombia and Ecuador. Although glacial refugia for both species were inferred close to the actual hybrid zone, no overlap was detected in this period.
5. Models predicts that *A. palliata* will have its potential niche reduced in comparison to *A. pigra*.
6. The results of this study suggest that the groups of *A. palliata* and *A. pigra* studied from South-eastern Mexico, present a low genetic diversity, but similar to that reported in other studies with microsatellites for the same species.
7. Most alleles showed deviations for the Hardy-Weinberg equilibrium, probably as a consequence of inbreeding.
8. Genetic data recovered three main clusters, both parental species and hybrid individuals distributed across an extensive area of hybridization in southern Tabasco State.
9. Connectivity analyses showed different patterns, whereas distant highland populations where highly connected, close lowlands population were genetically differentiated, possibly due to habitat loss and fragmentation.
10. Therefore, it is crucial to preserve the remaining fragments and promote conservation efforts to regenerate reestablishing connectivity of populations of these endangered howler monkeys.

Chapter 2. Effects of habitat loss and fragmentation in gastrointestinal parasites prevalence and richness between parapatric species, *Alouatta palliata* and *A. pigra* in Southeast of Mexico.

1. Habitat perturbation and hybridization promotes parasites richness and prevalence. This data support the dilution effect hypothesis that postulates that higher biodiversity contributes to decrease parasitism.
2. Several metrics of habitat disturbance such as patch size or SPI are related with endoparasite prevalence and richness.
3. Population density usually increases in the smallest patches, what might increase intraspecific competition, as well as parasite transmission.
4. Our results agree with previous studies on *Alouatta* parasites.
5. Fragmentation index did not show direct correlation with parasite richness and prevalence.
6. No significant correlation between fragmentation and parasitic richness was found.
7. These results could also reflect that habitat loss rather than habitat fragmentation has larger effects on howler's parasitic diversity and prevalence.
8. It could be hypothesized that habitat loss is directly influencing over parasites richness and prevalence through the effects on its host.
9. These results found individualistic responses of parasites to habitat features. Significant individual correlations of fragmentation index with specific parasites such as Eimeriidae (-), *Controrchis* (+) and *Strongyloides* (+), what points to differential species-specific response to fragmentation.
10. A higher parasitic prevalence in hybrid individuals (46%) than in their parental species (18% in *A. palliata* and 15% in *A. pigra*). The probability of coinfection was more than four times higher in hybrid individuals.
11. The presence of *Trypanoxyuris* sp. is higher in the hybrid individuals.

Chapter 3. Clarifying the cryptic host specificity of *Blastocystis* spp. isolates from *Alouatta palliata* and *A. pigra* howler monkeys.

1. The results of the present study demonstrate that howler monkey populations of *A. palliata* and *A. pigra* with locally adapted *Blastocystis* ST1 and ST2 populations exhibit distinct generalist and specialist types of host specificity.
2. *Blastocystis* ST2 populations in humans are highly differentiated from those of NHP.

3. *Blastocystis* ST1 populations are only minimally differentiated.
4. The host generalist and specialist specificities exhibited by ST1 and ST2 *Blastocystis* populations are thus distinct processes.
5. ST1 exhibits a generalist profile, this haplotype can be considered a metapopulation.
6. *Blastocystis* ST2 exists as a set of local populations with preferences for either humans or NHP.

Chapter 4. New *Entamoeba* group in howler monkeys (*Alouatta* spp.) associated with parasites of reptiles.

1. A new clade of *Entamoeba* spp. in howler monkey *Alouatta* spp was reported in Tabasco State using molecular techniques.
2. *Entamoeba* clade was separated from *E. insolita*, and has homology with other sequences of *Entamoeba sp*. isolated from reptilian species.
3. The new clade identified in this study shared low identity values compared with the sequences reported previously for other amoebae (<90%), where high posterior probability values were obtained for the phylogenetic reconstructions with high numbers of mutations among the three clades.
4. RL8 clade, a new group *of Entamoeba* was reported. Only one sequence from A. *pigra* was not included in RL7, where it was located in the RL6 clade (*Entamoeba* sp. NIH:1091:1).
5. Our results also suggest that the group comprising *E. insolita*, RL5, and RL6 is not exclusive to reptiles, as previously suggested. Therefore, exhaustive studies of *Entamoeba* host diversity are necessary before considering host specificity.
6. The lowest prevalence of *Entamoeba* and the limited geographic region where they were found in the present study may indicate that the new *Entamoeba* clade is located only in the southern region of Tabasco State.