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GENETIC APPROACH OF THE COIBA ISLAND HOWLER MONKEY *Alouatta coibensis coibensis* FROM PANAMA, AND ITS CONSERVATION IMPLICATIONS

APROXIMACIÓN GENÉTICA DEL MONO AULLADOR DE LA ISLA DE COIBA *Alouatta coibensis coibensis* DE PANAMÁ Y SUS IMPLICACIONES PARA LA CONSERVACIÓN

Edgardo Díaz-Ferguson
Estación Científica Coiba (Coiba AIP), Panamá.
ediaz@coiba.org.pa https://orcid.org/0000-0002-2314-5021

Carlos W. Ramos
Universidad de Panamá, Facultad de Ciencias Naturales, Exactas y Tecnología, Departamento de Genética y Biología Molecular, Coiba Scientific Station (Coiba AIP), Panamá.
laito52@yahoo.com https://orcid.org/0000-0003-2344-9241

Yohanny Pineda
Universidad de Panamá, Facultad de Ciencias Naturales, Exactas y Tecnología, Departamento de Genética y Biología Molecular, Panamá.
yypr29@gmail.com https://orcid.org/0009-0002-7939-1659

Karol M. Gutiérrez-Pineda
Universidad de Panamá, Facultad de Ciencias Naturales, Exactas y Tecnología, Panamá.
madelinekarol@gmail.com https://orcid.org/0000-0001-9129-7716

Edgardo Castro-Pérez
Universidad de Panamá, Facultad de Ciencias Naturales, Exactas y Tecnología, Departamento de Genética y Biología Molecular, INDICASAT-AIP, Clayton, Panamá.
hdnacastro@gmail.com https://orcid.org/0000-0003-4884-9479

Pedro G. Méndez-Carvajal
Universidad de Panamá, Facultad de Ciencias Naturales, Exactas y Tecnología, Departamento de Fisiología y Comportamiento Animal, Panamá.
mendez55.pm@gmail.com https://orcid.org/0000-0003-1306-5869

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ABSTRACT

Studies on genetic diversity are key to conservation management because they may determine the resilience of primate populations under environmental and anthropogenic changes. Herein, we studied genetic diversity and connectivity in a population sample of the Coiba island howler monkey *Alouatta coibensis coibensis* from Coiba island in Panama. We collected 14 faecal samples from individuals at two sites on the northern and north-eastern sides of the island at the Gambute and Los Pozos trails, respectively. DNA was extracted from these samples, and partial sequences of cytochrome oxidase I (COI) were obtained and used as molecular markers to determine genetic diversity. Haplotypic diversity estimates ranged from 0.68 to 0.80 and from 0.0026 to 0.0041 for nucleotide diversity. Genetic connectivity based on reduced genetic distances and nonsignificant Hudson Snn test values suggests elevated connectivity between sites and the existence of a single population with good variability. Demographic history data identified positive Tajimas D as an indication of population contraction and reduced population size in this species. These results will be the baseline for establishing a better understanding of population structure, genetic health, connectivity patterns and reproductive behaviour along the largest island of the Central American Pacific. This information will allow scientists to understand and better evaluate the conservation status of this primate, confirming good availability of genetic flow even for this population living in isolation.

KEYWORDS

Genetic variability, genetic connectivity, haplotype diversity, nucleotide diversity, primates.

RESUMEN

Los estudios sobre diversidad genética son clave en el manejo de la conservación porque pueden detectar la resiliencia de la población de primates ante cambios ambientales y antropogénicos. En este trabajo estudiamos la diversidad genética y la conectividad en una muestra de la población de mono aullador *Alouatta coibensis coibensis* de isla Coiba en Panamá. Colectamos catorce muestras de heces de individuos de dos sitios del lado norte y noreste de la isla (Sendero Gambute y Los Pozos, respectivamente). Secuencias parciales del gen citocromo oxidasa I (COI) generadas a partir del DNA obtenido de esas muestras fueron utilizadas como marcador molecular para determinar la diversidad genética. Los valores de diversidad haplotípica presentaron un rango entre 0.68 y 0.80 y entre 0.0026 y 0.0041 para la diversidad de nucleótidos. La conectividad genética basada en distancias genéticas reducidas y valores estadísticos de Snn no significativos sugieren una conectividad elevada entre sitios y la existencia de una sola población con buena variabilidad. Los datos de la historia demográfica mostraron un Tajimas D positivo como un indicativo de la contracción y tamaño reducido de la población de esta especie. Estos resultados serán la base para una mejor comprensión de la estructura poblacional, salud genética, patrones de conectividad y comportamiento reproductivo a lo largo de la isla más grande del Pacífico Centroamericano. Esta información permitirá a los científicos, comprender y evaluar mejor el estado de conservación de este primate, confirmando una buena disponibilidad de flujo genético incluso para esta población que vive aislada.

PALABRAS CLAVES

Variabilidad genética. conectividad genética. diversidad haplotípica. diversidad nucleotídica. Primates.

INTRODUCTION

Studies on genetic diversity are key to conservation management because they may determine the resilience of primate populations under environmental and anthropogenic changes (Melo-Carrillo *et al*. 2020). Genetic diversity studies are needed to understand population fitness, population changes, connectivity, and effective population size to detect inbreeding, as isolation leads to changes at the evolutive and behavioural levels (Hughes *et al*. 2008; Milton *et al*. 2009; Reed *et al*. 2002). Few genetic studies have been performed on different species from the *Alouatta* genus facing isolation conditions. However, populations of *Alouatta palliata mexicana* at Selva Zoque in Mexico exhibited low genetic diversity when sequencing of mitochondrial genes from 45 individuals was conducted (Dunn *et al*. 2014). Low genetic variation has also been observed in *A. palliata palliata* from Costa Rica and Panama and in *A. pigra* in Guatemala, whereas studies in *A. seniculus*, *A. belzebuth* and *A. fusca* from Brazil have shown that the Amazonian-located species exhibit higher genetic variability than Mesoamerican species (Cortés-Ortiz *et al*. 2003; Zaldivar *et al*. 2003). These results may be related to biogeographical scenarios, founder effects or bottle neck effects related to dispersion capability (Milton *et al*. 2009).

In Panama, the Coiba Island howler monkey has been described as an endemic species and includes two subspecies: *Alouatta coibensis coibensis* and *Alouatta coibensis trabeata*. *Alouatta coibensis coibensis* is restricted to Coiba and Jicaron Islands, concentrating the only two populations for this taxon as a continental island species (Froehlich & Froehlich 1987; Méndez-Carvajal 2019a; Milton & Mittermeier 1977; Rylands *et al*. 2008; Thomas 1902). The Azuero howler monkey, *A. c. trabeata*, is located at the Azuero peninsula, including areas of Herrera, Los Santos, and Veraguas provinces (Méndez-Carvajal 2013). Both subspecies have been declared endangered by the International Union for Conservation of Nature (IUCN) (Cuarón *et al*. 2020; Méndez-Carvajal *et al*. 2021). Each subspecies lives under different habitat conditions; *A. c. coibensis* is restricted to islands, and *A. c. trabeata* is found on the mainland and lives in forest patches with incremental habitat fragmentation due to agricultural and cattle activities, compromising their population gene flow. Previous studies from Fundación Pro-Conservación de los Primates Panameños (FCPP) have been monitoring the Coiba Island howler´s populations as a long-term project since 2009 to the present, calculating 472 individuals and a group size average of 4.3 ind/groups, with 109 groups for the island comprising a habitat size of 504 km^2 (Méndez-Carvajal 2012; Méndez-Carvajal 2019). Additionally, the group structure for this species tends to have only one or two males per group (unimale-multifemale), which is different from *A. palliata*, which has 4 to 6 males and larger groups (multimale-multifemale) (Méndez-Carvajal 2012; Méndez-Carvajal 2019). The average group size observed represents the smaller reported for an *Alouatta* species, and genetic diversity studies are required to better understand their populations and any possible inbreeding effect due to isolation. As social structure has a significant effect on genetic variation (Smouse *et al*. 1981), our aim was to describe the genetic diversity of *A. c. coibensis* and to evaluate the implications for its conservation. Due to geographic isolation from the mainland of this species, low genetic diversity is expected (Jensen *et al*. 2013). In this study, the genetic diversity, connectivity, and demographic history of the species were briefly analysed. Understanding genetic diversity and connectivity is essential for evaluating isolation effects and other factors that have been suggested to affect population structure, such as low reproductive success, low food quality, and inbreeding (Méndez-Carvajal 2012; Oklander *et al*. 2017). These data will provide useful information for future studies of the connectivity and phylogeography of *A. coibensis*, together with demographic studies, will add relevant information concerning the variability of haplotype composition and phylogenetic relationships of Mesoamerican howler monkeys, providing information that could be comparable to other primate species living under similar habitat conditions and for conservation purposes.

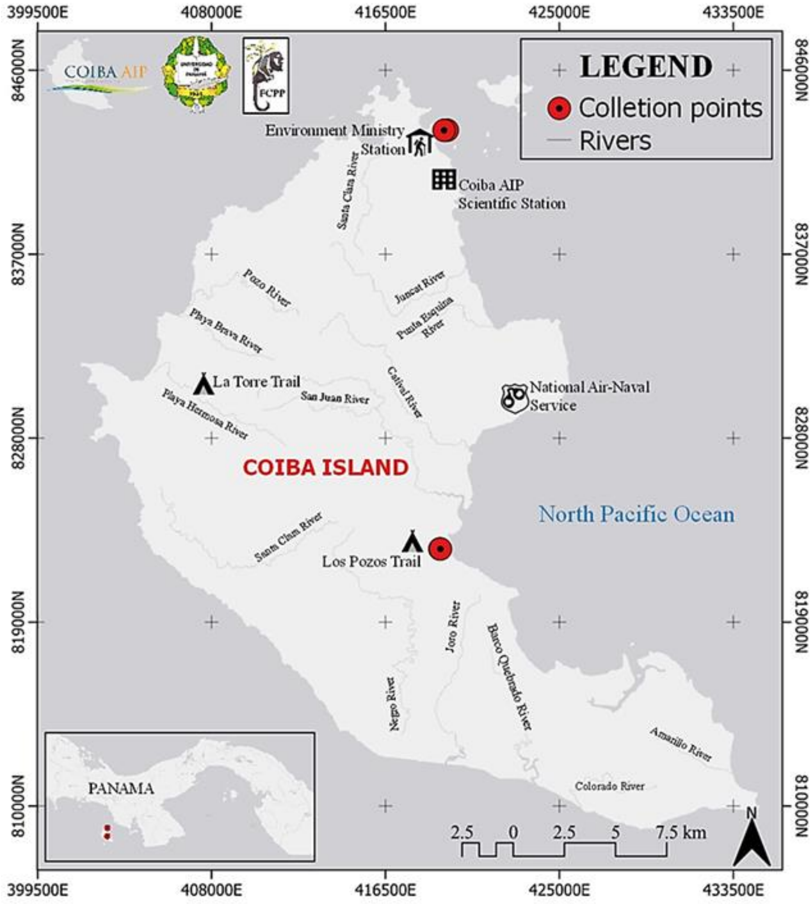
METHODS

Study site

Coiba Island is located on the Pacific side of Panama 24 km off the southwest coast of Veraguas Province, Panama. The island was a penal colony from 1919 to 2004, with 85% of land covered by Humid Tropical Forest (Ibañez 2011; Méndez-Carvajal 2019b). The canopy height reaches 50 m, mostly with tree species reported as suitable for the only two species of primates that inhabit this island (howler and capuchin monkeys *Cebus imitator*) (Méndez-Carvajal 2012).

The maximum elevation is 200 m.a.s.l., with 24 to 26 °C as a rank for temperature and annual precipitation of 3,403 mm (Ibañez 2011). To identify the groups of interest and collect the faecal samples, we used groups previously monitored from Gambute at the Environmental Ministry Station (N 07°37′ 34 7”; W 81° 43′ 46 9”) and Los Pozos (N 07°26′ 25 8”; W 81° 43′ 55 5”) (Figure 1).

Figure 1.
Study area, Coiba Island, Republic of Panama. Red dots show the sampling areas. Gambute location is marked as Environment Ministry Station.



Sample collection

Faecal samples from *A. c. coibensis* were collected immediately after defecation, deposited into plastic bags or vials containing 75% ethanol and stored in an icebox cooler. We recorded the date, hour, sex and, if possible, approximate age of the animal as recommended by Goossens *et al*. (2011) (Table I). Samples were subsequently transported for DNA. extraction from Coiba Island to the Laboratory of Genetics and Molecular Biology of the University of Panama.

Table 1.

Study animals, locations, and time of feacal collection.

No. Sample	Species code	Sex	Coordinates	Study site	Date	Time
1	A.c.c-oo1	AM	N 07°37′34 7” W 81°43′46 9”	G	July 17, 2019	15:00
2	A.c.c-002	AM	N 07°37′34 7” W 81°43′46 9”	G	July 17, 2019	15:18
3	A.c.c-003	AF	N 07°37′34 7” W 81°43′46 9”	G	July 17, 2019	15:19
4	A.c.c-004	AF	N 07°37′34 7” W 81°43′46 9”	G	July 17, 2019	11:10
5	A.c.c-005	AF	N 07°37′34.7” W 81°43′46 9”	G	July 17, 2019	15:00
6	A.c.c-006	AF	N 07°37′34.7” W 81°43′46.9”	G	July 17, 2019	15:00
7	A.c.c-007	AM	N 07°26′22 7” W 81°43′55 7”	LP	August 19, 2019	09:26
8	A.c.c-008	AM	N 07°26′25.8” W 81°43′55.5”	LP	August 19, 2019	09:26
9	A.c.c-009	JF	N 07°26′25 8” W 81°43′55.5”	LP	August 19, 2019	14:46
10	A.c.c-010	JF	N 07°26′25.8” W 81°43′55.5”	LP	August 19, 2019	14:46
11	A.c.c-011	AF	N 07°26′25.8” W 81°43′55.5”	LP	August 19, 2019	15:00
12	A.c.c-012	AF	N 07°26′25” W 81° 43′ 55 6”	LP	August 19, 2019	16:00
13	A.c.c-013	AF	N 07°26′25” W 81°43′55.6”	LP	August 19, 2019	16:00
14	A.c.c-014	AM	N 07°26′25” W 81°43′55.6”	LP	August 19, 2019	16:00

Note: A.c.c.=*Alouatta coibensis coibensis*; AF=Adult female; AM=Adult male; JF=Juvenile female; G=Gambute; LP=Los Pozos.

DNA extraction, COI amplification, sequencing, and alignment

DNA was extracted using the E.Z.N.A.® Stool DNA kit (Omega Bio-Tek Inc., Georgia USA). The concentration of DNA was evaluated spectrophotometrically using a Nanodrop, and the quality was verified electrophoretically. A 658 bp fragment of the COI region was amplified by PCR using the universal primers LCO 1490 (5’-GGT CAA CAAATC ATA AAG ATA TTG G-3’) and HCO 2198 (5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Folmer *et al*. 1994). The DNA amplification reaction was performed in 30 µL reaction volumes containing 15 µL 2X PCR Master mix (Omega Bio-Tek), 2 µL of DNA template (50 ng), 1 µL (0.33 µM) of each primer and 11 µL of nuclease-free ddH2O. PCR thermal cycler conditions consisted of initial denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 52 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 3 min. The PCR products of expected size were evaluated by electrophoresis in 1% agarose gels in 1X TAE buffer and stained with GelRed. PCR products were shipped to Psomagen (USA) for purification and bidirectional sequencing using the same universal primers with a BigDye Terminator 3.1 kit (Applied Biosystems, USA). Sequences were assembled, edited, aligned, and trimmed using SEQUENCHER 5.4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA). The final obtained sequences were compared to the GenBank (BLAST) and Barcode of Life Data System (BOLD) databases to determine the identity of each sequence.

Ethical approval and declaration of conflicts

Samples were collected in accordance with the ethical approval of the Environmental Ministry of Panama, based on scientific permit SE/APH-1-2019.

Data availability

The DNA sequence datasets generated during and/or analysed during the current study are available in the GenBank repository, [https://www.ncbi.nlm.nih.gov/genbank/] with the following accession numbers: MZ636576-MZ636589

Genetic diversity

We calculated haplotype number (h), haplotype diversity (Hd), polymorphic sites (S) and nucleotide diversity (π) using DnaSP v.5 (Librado & Rozas 2009). The haplotype network was constructed with the POPART software package (Leigh & Bryant 2015) using the TCS method (Clement *et al*. 2002). We applied the Tajimas D test to examine equilibrium between mutations (Tajimas 1989), and for demographic history and distances, we used Hudson Snn tests (Hudson 2000).

RESULTS

Sequence identity

Searches for sequence identity in GenBank revealed that all sequences generated in this study exhibited the highest percentages of identity (94%) with COI sequences from individuals identified as *Alouatta guariba*. Similar results were obtained using BOLD with the closest similarity of 93.26 with *Alouatta guariba* reference sequences, indicating the lack of COI sequences of the *Alouatta* species studied here in both databases. However, although there are no COI sequences for *A. coibensis* or *A. palliata* deposited in GenBank or BOLD, the highest percentages of identity or similarity for the sequences generated from our specimens matched sequences of the genus *Alouatta*.

Genetic diversity

A total of nine variable sites ($S = 9$) and six haplotypes were identified from 14 sequences. Haplotype diversity (Hd) was 0.73, and nucleotide diversity (π) was 0.0038 (Table II). Regarding genetic connectivity and demographic history, the results yielded a negative Tajimas D value of -1.125 ($p > 0.053$) for Los Pozos. Meanwhile, positive D values were observed for Gambute with D=0.83. The results from these two locations were not significantly different ($p>0.05$). The genetic distance between the two locations was reduced (GST=0.0086) with no significant genetic differentiation (Table II; Figure 2). The genetic distance using the Hudson test (Da) was 0.0094, which was not significant, revealing no evidence of genetic differentiation (Table 2).

Table 2.

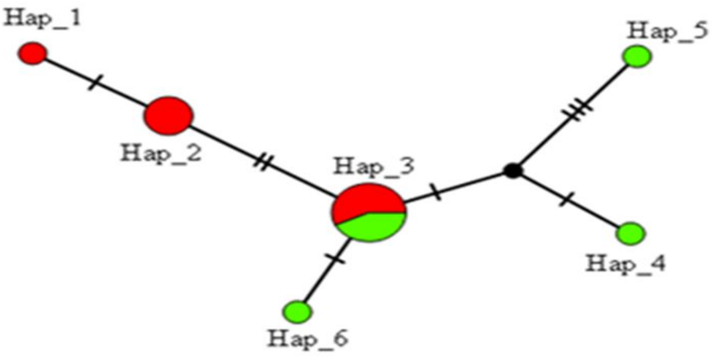
Genetic diversity parameters and accession numbers for the two analyzed populations.

Site	N	H	Hd	π	Snn	Da	AN
G	8	3	0.68	0.0026	p>0.05	0.0094	MZ636576-80 MZ636587-89
LP	6	4	0.80	0.0041			MZ636581-86

Note: G= Gambute; N=number of sequences; H= Haplotype number; Hd= Haplotype diversity; π = nucleotide diversity; Snn=Hudson (2000) genetic differentiation statistic between locations; Da= genetic distance; AN= GenBank accession number.

Figure 2.

TCS haplotype network. The size of the circles is proportional to the haplotype frequency. Black dots represent unsampled or missing haplotypes. Red and green colours correspond to sites where the sequences were obtained. Red: Gambute. Green: Los Pozos. Short bars represent mutational steps between haplotypes.



DISCUSSION

Sequence identity

The taxonomic status of *Alouatta coibensis* is controversial and has been poorly studied at the genetic level (Groves, 2001; Rylands *et al*., 1997). Regardless of the taxonomic status identity of specimens from Coiba, there were no previous COI sequences to compare in GenBank; therefore, our data for *A. c. coibensis* were deposited, contributing new information on the genetics of this species. Genetic characterization of this species is beneficial for unravelling gene flow patterns, understanding taxonomy and establishing better conservation practices (Rylands *et al*. 2006). In addition, genetic similarities between our *A. c. coibensis* sequences and *A. guariba* sequences in GenBank (94% similarity) support previous inferences regarding social and group structure similarities between the two species (Méndez-Carvajal 2012; Milton & Mittermier 1977). Although the IUCN considers this subspecies with synonymous names, some authors suggests that genetic data base for *A.coibensis* should be improved with more genetic studies (Cortés-Ortiz *et al*. 2003; Ruíz-García *et al*. 2017).

Genetic diversity

The TCS haplotype network revealed that individuals from the same locality were separated by fewer mutational steps than haplotypes from different localities (Fig. 2). Most of the haplotypes were singletons, except for haplotypes 2 and 3. Haplotypes 1 and 2 were present only in Gambute, while haplotypes 4, 5 and 6 were only present in Los Pozos. Haplotypes 4 and 5 are connected by a black node representing a missing intermediate haplotype. Gambute (Environment Ministry Station) exhibited the highest number of unique haplotypes compared to Los Pozos. Haplotype 3 was the most frequent haplotype and was directly connected to two of the remaining five haplotypes. This haplotype is present in 7 of the fourteen individuals sampled and is the only haplotype shared by individuals from both localities. The number of mutational steps among haplotypes of Los Pozos was higher (1-7) than among haplotypes from Gambute (1-4).

Genetic diversity in members of the Alouatta genus has been estimated using different molecular markers, including isozymes, restriction fragment profiles of mtDNA, microsatellites and nuclear and mitochondrial gene sequences (Barroso *et al*. 1988, Goncalves *et al*. 2004, James *et al*. 1997, 2008, Nascimento *et al*. 2005). Isozyme analysis of 14 loci in *Alouatta palliata* from Costa Rica displayed no variation and low genetic diversity compared to *A. seniculus* and *A. belzebul* from Brazil (Zaldivar *et al*. 2003). Populations of *Alouatta palliata* located in northern latitudes exhibit less genetic diversity than populations located in South America (Cortés-Ortíz *et al*. 2003; Ford

2006). In the case of *A. c. coibensis*, the data reported here are similar to the diversity patterns observed in South American species of the genus *Alouatta*, with a higher diversity compared to the data obtained from *A. palliata* and other species of the genus in Mesoamerica. However, the overall haplotypic and nucleotide diversity values for *A. c. coibensis* were consistent with the results obtained from COII in 124 individuals of *A. palliata* from different Latin American countries, with Hd values of 0.674 and π of 0.0038 (Ruíz-García *et al*. 2017), but lower than those observed in *A. belzebul* from Amazonian and Atlantic forests (Nascimento *et al*. 2008) and *A. caraya* from the Argentina and Paraguay populations (Ascunce 2007). The diversity values exhibited by individuals from *A. c. coibensis* were considerably higher than values obtained from mitochondrial control region sequences of *A. p. mexicana*, an endangered speciesinhabiting Selva Zoque, one of the largest tropical rainforests in northern Mexico (Cuarón *et al*. 2020; Dunn *et al*. 2014). Previous reports indicate that in general, island populations generally exhibit lower levels of genetic diversity than their mainland counterparts due to isolation, small size and increased inbreeding isolation, small size and increased inbreeding (Frankham 1997; De la Rúa *et al*. 2001; Jensen *et al*. 2013; Miller 2010). Compared to other species, *A. c. coibensis*, although with a small population size, exhibits increased genetic diversity even with other species that show similar habitat conditions, such as the Japanese macaque *Macaca fuscata*, which inhabits a Japanese island with a similar surface to Coiba´s (504.9 km^2) but with a higher population density (Hayaishi & Kawamoto 2006; Méndez-Carvajal 2012). Comparing the genetic diversity values of *A. c. coibensis* populations to the endangered proboscis monkey *Nasalis larvatus* from Southeast Asia, our data revealed higher levels of genetic diversity for Coiba´s populations. Coiba is the largest island on the Pacific coast of Mesoamerica, and it is uninhabited (except for small-size scientific research and national security stations) with a mature and little-altered rainforest (Méndez-Carvajal, 2019b; Milton & Mittermier, 1977). The island is considered a natural sanctuary with few natural predators (and hunting is not allowed) and without geographic barriers that reduce the free movement of individuals among groups within the island (Méndez-Carvajal 2002; Mittermier & Milton 1978). These habitat conditions may explain the relatively higher values of genetic diversity for *A. c. coibensis* compared to other limited habitats for island populations. Additionally, the levels of genetic diversity observed in Coiba island howler monkeys could be the result of the early dispersal of males to other groups or the result of several early migratory waves that populated the island causing a relatively highly diverse founder effect; however, there is currently no evidence on this type of dispersal pattern. The differences in genetic diversity observed between collection sites with greater genetic diversity in Los Pozos than Gambute may represent differences among two putative demes in which exchange of individuals occurs.

Genetic connectivity and demographic history

Genetic connectivity data are essential for understanding dispersal behaviour and other historical and contemporary processes that determine population structure (Oklander *et al*. 2017). Therefore, the lack of genetic differentiation among *A. c. coibensis* populations evidenced connectivity and no isolation within the northern section of the island. Based on demographic history and overall negative D values, we infer a population expansion for *A. c. coibensis* in the northern section of Coiba Island.

The genetic diversity of *A. c. coibensis* was moderate to high, even in an isolated environment. These findings and the observed connectivity indicate that *A.c. coibensis* is not inbreeding and demonstrates the viability of the species by gene flow. Studies of sex-biased dispersal are necessary to evaluate the possibility that the observed genetic diversity in small populations, such as Coiba Howler monkeys, is related to female phylopatry. As a part of the FCPP´s long-term research, we are expanding knowledge using different aspects of the *A. c. coibensis* ecology, including studying parasitology and chemistry ecology in their diet from other sites of Coiba, to better understand this primate in a long term and to apply conservation practices before tourism and other anthropogenic effects evolve in the future.

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