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Initial characterization of mitochondrial DNA control region haplotypes of the Antillean manatee (*Trichechus manatus manatus*, Sirenia:Trichechidae) in Guatemala

Grecia Mendez*[1]; https://orcid.org/0000-0002-8559-6557
Susan Carney[2]; https://orcid.org/0000-0003-3047-7435
Heidy Amelia Garcia[3]; https://orcid.org/0000-0002-2392-6768
Ester Quintana-Rizzo[4]; https://orcid.org/0000-0002-8957-0506

1. Department of Biology, Universidad del Valle, Guatemala; grecia.m.mendez@gmail.com (Correspondence*)
2. Department of Biology, Hood College, Frederick MD, USA; slc239@gmail.com
3. Fundación Defensores de la Naturaleza, Guatemala, Guatemala; hgarcia@defensores.org.gt
4. Department of Biology, Emmanuel College, Boston MA, USA; tetequintana@comcast.net

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ABSTRACT

Introduction: Small populations are at risk of losing genetic variability much faster than large populations; this subsequently decreases their ability to adapt when facing environmental changes. A small population of the endangered Antillean manatee (*Trichechus manatus manatus*) has been identified in Guatemala.

Objective: This study explored the genetic diversity of the Antillean manatee in Guatemala by analysing mitochondrial DNA control region haplotypes in the two most important habitats for the species, Bahía La Graciosa, a coastal bay and Bocas del Polochic, a coastal wetland, both located in the Izabal State.

Methods: Genetic samples were collected using non or minimally invasive sampling techniques: scraping of epidermal tissue, collection of floating feces, and collection of tissue from carcasses. DNA extractions, DNA amplification using polymerase chain reaction (PCR), and sequencing of the control D-loop region were used to process and analyse the samples.

Results: Seven mitochondrial DNA sequences were obtained from 36 samples collected (minimum of four and maximum of seven individuals). Four haplotypes were identified, A01, A03, A04, and J01. No other Central American country has reported this number of haplotypes in a manatee population, and it is the first time that haplotype A01 has been reported for the region. The Guatemalan manatee population comprises at least two genetic lineages, the Florida/Greater Antilles lineage (haplotypes A01, A03, and A04) and the Mesoamerican lineage (J01).

Conclusion: Further studies, with the use of nuclear markers, are necessary to understand the population dynamics between Bahia La Graciosa and Bocas del Polochic to identify the number of management units present in the country; also, the degree of relatedness with the Belizean population needs to be established to better coordinate conservation efforts.

Key words: Non-invasive genetic sampling; endangered species; control D-loop region; Lago de Izabal; Atlantic coast of Guatemala; conservation management plans.

RESUMEN

Caracterización inicial de haplotipos de la región control de ADN mitocondrial del Manatí Antillano (*Trichechus manatus manatus* Sirenia:Trichechidae) en Guatemala

Introducción: Las poblaciones pequeñas corren el riesgo de perder variabilidad genética mucho más rápido que una población de mayor tamaño; disminuyendo, así mismo, su capacidad de adaptarse ante cambios ambientales.

Una pequeña población de la especie en peligro de extinción el manatí antillano (*Trichechus manatus manatus*) ha sido identificada en Guatemala.

Objetivo: Este estudio explora la diversidad genética del manatí antillano en Guatemala mediante el análisis de haplotipos de la región de control del ADN mitocondrial en los dos hábitats más importantes identificados para la especie, Bahía La Graciosa, un bahía costera y Bocas del Polochic, un humedal costero, ambos localizados en el departamento de Izabal.

Métodos: Las muestras genéticas se colectaron utilizando técnicas de muestreo no invasivas o mínimamente invasivas: raspado de tejido epidérmico, recolección de heces y recolección de tejido extraído de cadáveres. Se usaron extracciones de ADN, amplificación de ADN médiate la reacción en cadena de la polimerasa (PCR) y secuenciación de la región control D-loop.

Resultados: Se obtuvieron un total de siete secuencias de ADN mitocondrial de 36 muestras recolectadas. Cuatro haplotipos fueron identificados, A01, A03, A04 y J01. Ningún otro país centroamericano ha reportado esta cantidad de haplotipos en una población de manatíes y es la primera vez que se reporta el haplotipo A01 para la región. La población de manatíes guatemaltecos comprende al menos dos linajes genéticos, el linaje Florida/Antillas Mayores (haplotipos A01, A03 y A04) y el linaje mesoamericano (J01).

Conclusión: Son necesarios más estudios, con el uso de marcadores nucleares, para comprender la dinámica poblacional entre Bahía La Graciosa y Bocas del Polochic y para poder identificar el número de unidades de manejo presentes en el país: además, se debe establecer el grado de relación con la población de Belice para coordinar mejor los esfuerzos de conservación.

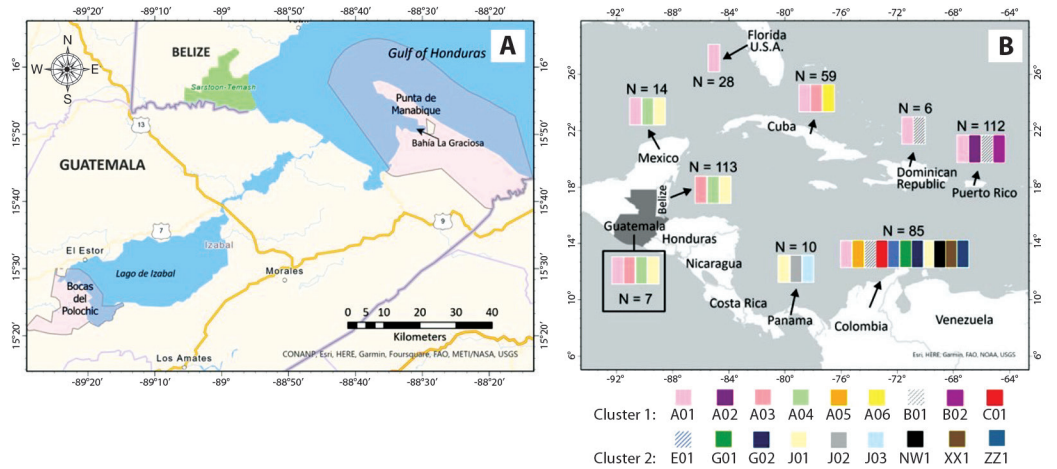
Palabras clave: Muestro genético no invasivo; especie en peligro de extinción; región control D-loop; Lago de Izabal; Costa Atlántica de Guatemala; planes de manejo de conservación.

INTRODUCTION

Genetic studies on the West Indian manatee, *Trichechus manatus* Linnaeus, 1758 have shown low haplotype and nucleotide diversity, with 29 haplotypes identified to date, distributed in three distinct lineages: Cluster I, Cluster II, and Cluster III (Alvarez-Aleman et al., 2022; Caballero et al., 2021; Díaz-Ferguson et al., 2017; Garcia-Rodriguez et al., 1998; Satizabal et al., 2012; Vianna et al., 2006). Cluster I includes haplotypes found in Florida and the Greater Antilles, Cluster II is distributed from the Gulf of Mexico to the Caribbean coast of South America, and Cluster III is exclusive to Brazil and Guyana (Vianna et al., 2006). The Antillean manatee (*T. m. manatus*), classified as endangered on the IUCN Red List (Self-Sullivan & Mignucci-Giannoni, 2008), is one of the two subspecies of the West Indian manatee and is found in small population pockets throughout most of its range.

In Central America, the genetic variability of the Antillean manatee has been studied in Belize (Hunter et al., 2010) and Panama (Díaz-Ferguson et al., 2017); these countries share the Cluster II lineage (Díaz-Ferguson et al., 2017). Belize has the largest manatee population in the western hemisphere with around 1 000 animals (Hunter et al., 2010) and, through conservation efforts, could assist in the recovery of the adjacent Guatemalan population (Quintana-Rizzo & Reynolds III, 2010). The most recent estimate is 150 individuals in Guatemala (Quintana-Rizzo & Reynolds III, 2010), and threats like illegal hunting limit the survival of the population (Machuca-Coronado & Corona, 2019). The objective of this study was to explore the genetic diversity of the Antillean manatee in Guatemala in two areas that are ecologically distinct but important for the species, Bocas del Polochic, an inshore wetland and Bahia La Graciosa, a coastal bay (Fig. 1A).

Manatee epidermal tissue, feces, and carcass skin cuts were collected at the two study sites between July 2012 and February 2011. To obtain epidermal tissue, a modified method, initially developed by Carney et al. (2007) was employed. A scraper with increased perforations (from 2 mm to 5 mm wide) and a lighter pole (4 m of aluminum instead of 2 m of PVC) were used to scrape the dorsal part of the manatee without hurting the animal and subsequently storing the tissue with 75 % ethanol and



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Location	N	HT	List of haplotypes	Reference
Belize	113	3	A03, A04, J01	Hunter et al., 2010
Guatemala	4≤N≥7	4	A01, A03, A04, J01	This study
Panama	10	3	J01, J02, J03	Díaz-Ferguson et al., 2017

Fig. 1. (A) Location of the two sampling sites and protected areas: Bocas del Polochic Wildlife Refuge in Lago de Izabal and Bahía La Graciosa, part of the Punta de Manabique Wildlife Refuge, both located on the Atlantic coast of Guatemala. (B) Manatee mitochondrial control region D-loop haplotypes found in Guatemala and other parts of the Americas and the number of samples analyzed per country. Each haplotype is depicted in distinct colors and grouped into its corresponding cluster, previously defined by Vianna et al. (2006). Data sources: Mexico, Florida and the Dominican Republic (Vianna et al., 2006), Cuba (Alvarez-Alemán et al., 2022), Puerto Rico (Hunter et al., 2012), Colombia (Caballero et al., 2021), Panama (Díaz-Ferguson et al., 2017), and Belize (Hunter et al., 2010). (C) Number of genetic haplotypes (HT) and sample size (N) for Antillean manatee in Guatemala and other Central American countries (Belize and Panama). Since floating fecal samples cannot be assigned to different manatees, the sample size of Guatemalan manatees in this study could be less than seven, which is the number of sequences reported but not less than four because four different haplotypes were identified.

1 X TE (pH 8). After a manatee sighting, fecal samples were collected within a 100 m radius of the sighting. These were collected using new disposable plastic bags and latex gloves (Muschett et al., 2009) and were stored at room temperature in sterile containers with 95 % ethanol using a proportion of 1:3 (Muschett et al., 2009). Lastly, skin cuts from dead carcasses were collected opportunistically and stored at -20 °C. Two extraction protocols were used to treat tissue samples with different degradation states: the phenol-chloroform method (Muschett et al., 2009) was used for samples with high DNA concentration and a silica method (Höss & Pääbo, 1993) was used for low DNA concentrations. Two extraction protocols were also used to assay fecal samples: Zhang et al. (2006) and Marrero et al. (2009). In the Zhang et al. (2006) protocol, instead of binding the DNA to a spin column with guanidine thiocyanate, the samples were resuspended in 500 µl of isopropanol at -20 °C. A modification was used to increase the purity index of the extractions to 1.6–2.0. This modification consisted of applying the silica method (Marrero, 2009) to the resuspended samples.

Mitochondrial control region D-loop DNA was amplified by PCR using 1X Buffer (Promega), 4 mM of $MgCl_2$, 150 µM of each dNTPs, 0.3 µM of each primer (CR-4 and CR-5), 1.5 U of Taq DNA polymerase (Promega) and 1 µl of extracted DNA with a concentration of 100 ng/µl in a total reaction volume of 25 µl. The PCR cycling conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, and a final extension

of 72 °C for 1 hr (García-Rodríguez et al., 1998). The optimum annealing temperature for fecal samples was 54 °C instead of 47 °C. PCR products were run on a 1 % agarose gel and visualized by electrophoresis to confirm amplification of the expected ~ 410 bp size. The dNTPs and primers were removed using the Novagen purification kit. The PCR products were bi-directionally sequenced (Macrogen Inc., Korea) to ensure a high confidence level in each nucleotide.

ChromasPro (2.1.10.1) was used to assemble the forward and reverse strands into contigs. A BLAST search of each contig was used to identify the top match haplotype, previously registered and defined by Vianna et al. (2006). A Clustal Omega alignment was generated between the contig and the haplotype, where shared polymorphisms and percentage alignment were used to define each sample as its respective haplotype.

A total of 36 manatee samples were collected. Of these, 29 samples (27 fecal samples and two tissue samples from carcasses) were obtained in Bahía La Graciosa, and seven samples (two fecal samples and five tissue samples from carcasses) were obtained in Bocas del Polochic. No epidermal tissue samples were collected during the study. Noise reduction was a primary challenge for approaching manatees since another research was simultaneously being carried out in the same boat. This field experience limited the application of this specific method in the region. However, approaching manatees is delicate due to their elusive behavior.

DNA was amplified from 22 of the samples collected; all came from Bahía La Graciosa. Seven samples were successfully sequenced; one came from tissue samples and six from fecal samples. The main reason for a lack of sequence results from tissue samples was contamination; the preservation, storage, and management of samples was not optimal. On the other hand, the limiting factor for processing fecal samples was degradation; fecal samples degrade very quickly when exposed to UV radiation.

Double coverage sequences ranged in length from 390-444 bp. Two sequences had read lengths of less than 410 bp, the length of characterized haplotypes in Genbank. However, these sequences of 390 and 392 bp in length were 100 % matches to the J01 haplotype, the most variable of the haplotype sequences identified here. To confirm each haplotype, forward and reverse chromatograms for each manatee sequence were aligned with the reference haplotype from Genbank (Supplementary Material 1). In all cases, there was a 100 % match between the reference sequence and the chromatograms. Only one sequence can be conclusively traced back to an individual since it consisted of a tissue sample from a carcass. The remaining samples consisted of feces collected exclusively in the bay area; therefore, there is a risk that a single individual was sampled multiple times and/or that these sequences could only be representative of a few bay area resident individuals.

Four haplotypes were identified, A01, A04, A03 and J01 (Fig. 1B and Fig. 1C), with respective frequencies of 43 %, 14 %, 14 %, and 29 %. These haplotypes have all been found in Central America, except for haplotype A01 (Fig. 1B). All manatee sequences have been registered in GenBank (OQ587957-OQ587965). Genetic diversity parameters like haplotype frequency, haplotype diversity, and nucleotide diversity could not be determined due to the aforementioned risk.

This study reports four manatee mitochondrial control region D-loop haplotypes for Guatemala, in a minimum of four individuals and a maximum of seven manatees. This is the highest number of haplotypes reported for Mesoamerica; all other genetically studied populations of the region, namely Belize, Panama and the Caribbean coast of Mexico, have reported three haplotypes (Díaz-Ferguson et al., 2017; Hunter et al., 2010; Nourisson et al., 2011) (Fig. 1B). This significant finding in a relatively small sample size strongly indicates that a more comprehensive population genetic analysis of the Guatemalan manatees is warranted. Belize, the adjacent manatee population to Guatemala, has

reported the haplotypes A03, A04, and J01 in 113 individuals; (Fig. 1C) (Hunter et al., 2010). These three haplotypes have all been identified in Guatemala, along with the A01 haplotype that was reported in three out of seven sequences. Panama has also reported a different set of three manatee haplotypes: J01, J02, and J03 (Díaz-Ferguson et al., 2017).

Each haplotype identified in Guatemala was matched with the corresponding cluster previously named and classified by Vianna et al. (2006) (Supplementary Material 2). These clusters are lineages inferred using nucleotides and sequential divergence parameters to construct phylogenetic relationships. Haplotypes A01, A03, and A04 belong to Cluster I (Florida and Greater Antilles) and haplotype J01 belongs to Cluster II (Mesoamerica) (Vianna et al., 2006). The presence of Cluster I and Cluster II gives a bimodal character to the manatee population of Guatemala. This pattern has also been detected in manatees sampled from Mexico, Belize, Colombia, and Venezuela (Vianna et al., 2006), indicating that the manatees in Guatemala likely share a common ancestry with the rest of the Mesoamerican manatee population.

The presence of the A01 and the A03 haplotypes in the study suggests that the Guatemalan manatees have a genetic relationship with the North American and/or Greater Antilles population (Fig. 1B). Haplotype A03 has been reported in Cuba (Alvarez-Alemán et al., 2022) and Belize (Hunter et al., 2010). A01 is the only haplotype reported for the Florida population and has a broad distribution across the wider Antilles (Hunter et al., 2012; Vianna et al., 2006). Florida manatees are known to travel outside their normal range [e.g., from Florida to Cuba, Alvarez-Aleman et al. (2010); from Florida to Mexico, Castelblanco et al. (2021)]; interestingly, the haplotype A01 has not been reported in the Belizean manatee population, where 16 times as many samples have been analyzed to date (Hunter et al., 2010).

A complete mitochondrial diversity study and an analysis of nuclear DNA markers would provide a further understanding of the origin and genetic status of the Guatemalan manatee population. Nuclear DNA markers would be fundamental in determining the number of management units present in the country. They would aid in delineating population genetic relationships with neighboring countries, like Belize, and across the region. Conservation efforts should focus on determining the distribution of each genetic lineage within the country as well as the degree of interbreeding and, prioritize the preservation of each ancestral lineage and promote habitat/population connectivity where possible.

Ethical statement: the authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

See supplementary material
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