Mitochondrial Reconstruction of Ecuadorian Snakes and the Isolation of COX1 for their DNA Barcode Identification

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URBAN BARCODE RESEARCH PROGRAM

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Abstract

In this study, we aim to sequence messenger RNA from four unknown snake samples to recover their full mitogenomes, in order to address gaps in understanding genetic diversity and the evolutionary history of Neotropical snakes. We used RNAseq and bioinformatics as an alternative approach to isolate mitochondrial sequences, allowing us to reconstruct the mitogenomes of these samples. Our primary objective is to offer insights into these unknown snakes and their phylogenetic relationships. After isolating the COX1 barcode gene from the mitochondrial sequences, we utilized DNA barcoding to compare it with sequences in public databases using the BLAST tool. This was followed by sequence alignment and phylogenetic tree inference, including bootstrap analysis, which allowed us to identify the closest relatives to our samples as *Chironius fuscus* (brown whipsnake), *Leptodeira ornata septentrionalis* (northern cat-eyed snake), and *Coniophanes longinquus* (lonely spot belly snake). Our observations bridge gaps in the understanding of Neotropical snakes and demonstrates the success of RNAseq in recovering mitochondrial genomes.

Introduction

- Reconstructing the mitochondrial genome of animals can provide many insights on evolutionary history and by demonstrating this data in a phylogenetic tree, applications of this can also entail information regarding taxonomy and identification.
- Historically, it has been theorized that snakes have originated from lizards, yet there still remain conflicting ideas on *how* that happened; whether snakes adapted for life on land, aquatic or burrowing. These opinions are largely due to a lack of fossil records of snakes because of their small size and fragility (Da Silva et. al 2018) However, with the application of phylogenetic trees, which show certain historical evolutionary traits over time, evidence can be provided to support one of these theories. Ex: snakes experiencing limb loss while being derived from lizards.
- By analyzing the mitochondrial genome of these unknown snake samples (we only are aware of the genus), we can identify the most closely related ancestor and provide new information on these snakes' evolutionary history.
- We chose to isolate the COX1 gene because it is a widely used marker for taxonomic identification and no other genetic sequence has such diversity and availability for biodiversity identification as COX1 (Rodrigues et al 2017).
- Moreover, based on current research literature, there seems to be a lack of studies focusing on reconstructing the mitochondrial genome of our target Ecuadorian Snakes (*Leptodeira*, *Chironius*, and *Coniophanes*). Therefore, our research proposal would be one of the first few attempts to utilize NGS & RNA Sequencing to reconstruct such mitochondrial genomes.

Methodology

Step 1: Sample Collection

A collection of four samples were taken from the Andes mountains in Ecuador for their mitochondrial genome isolation. We were only aware of identification at a generic level (genus only) and their species were not identified prior to extraction.

Fig 1: Andes mountains, Ecuador

Step 2: RNA Extraction and Sequencing

- We performed an Trizol RNA extraction on our samples in which we burst the tissue, then cells, organelles, nucleus and the nucleolus to isolate and purify the RNA.
- Our total RNA was quantified with an nanodrop and was sent to a sequencing company (Azenta). Following the processing of our samples, we received our sequences as FASTQ text files, and we began sequence realignment and mitochondrial COX1 isolation.

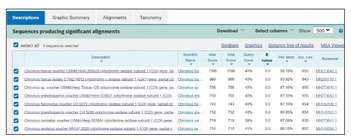

Fig 2: NCBI BLAST results for *Chironius fuscus*

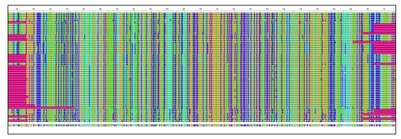

Fig 3: Alignment of nucleotide sequences

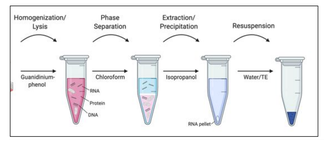

Fig 4: Steps of a basic Trizol RNA extraction

Step 3: Reconstruction of Mitogenome

- We employed two transcriptome assembly tools to reconstruct the mitochondrial genome: *R* and *Sublime*
- Using the Sublime text editor we were able to annotate the transcriptome and isolate the COX1 gene of about ~1,500 bp. We isolated transcript sequences that matched a reference mt-genome from related taxonomic groups to identify the COX1 gene.
- After getting the reference sequence, we put these files into the NCBI, and received a data output of the most closely related species.
- Following this, we used phylogenetic methods such as maximum likelihood using IQ-TREE and performing analysis using bootstrap identities. This software also allowed us to construct a phylogenetic tree and allowed us to see the evolutionary relationship between our samples and known species of snakes.

Fig 5: Phylogenetic tree for *Coniophanes longinquus*

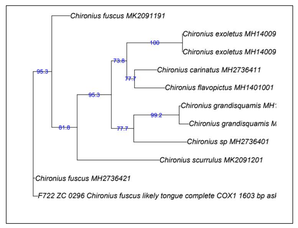

Fig 6: Phylogenetic tree for *Chironius fuscus*

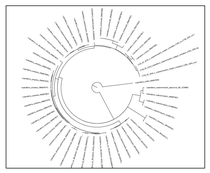

Fig 7: Phylogenetic tree for *Leptodeira Septentrionalis*

Fig 8: Mitogenome plot for *Coniophanes longinquus*

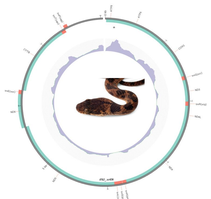

Fig 9: Mitogenome plot for *Leptodeira septentrionalis*

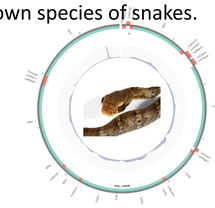

Fig 10: Mitogenome plot for *Chironius fuscus*

ID#	Species Identification	%GenBank Match	Brief Description
F726	*Chironius fuscus*	98.78%	Chironius fuscus or more commonly known as the brown sipo is found in parts of Colombia, Venezuela, Guyana, French Guiana, Brazil, Peru, Bolivia, Ecuador and Brazil's Atlantic forest, at elevation ranges from 189 to 1372m. Brown whipsnakes are active hunters, mainly consuming anurans (Boana boans, Leptodactylus petersii, L.mystaceus, Osteocephalus taurinus) whilst also, consuming salamanders, lizards, geckos, birds and mice. (
F745	*Leptodeira Septentrionalis*	98.44%	The Leptodeira septentrionalis, northern cat-eyed snake, is widely accepted as a docile creature, however it does have rear-fangs and is mildly venomous. Typically found in areas ranging from Mexico and Central America to Colombia, Venezuela, Ecuador and northwestern South America. These snakes medium sized snakes span anywhere from 15 inches to 24 inches long (as adults) and can blend into their surroundings with their slender bodies.(Brown Whipsnake
F752	*Coniophanes longinquus*	86.24%	This species natural region include coastal deciduous forests in countries like Peru and Ecuador, it is protected in the La Ceiba protective forest whilst living at altitudes of 1200 to 1430 meters above sea level. Males measure a length of 351 mm and females measure an average length of 422mm. Its diet consists of anurans and lizards. Males have medium brown backs and black spots that become continuous with black stripes. Females have more intense dorsal colors with yellowish brown and dark brown.(Brown Whipsnake

Results and Discussion

- We were able to successfully reconstruct the mitochondrial genomes of all four samples using phylogenetic analysis and bootstrap. However, when attempting to extract RNA from our samples, we ran into some difficulties due to the nature of our snake tongue samples (tongue tissue is harder to work with). Despite this initial challenge, we were able to follow up with new samples and successfully extract RNA, and continue reconstruction of the mitogenome.
- Something that we found interesting was how in previous studies, it has been documented that the *Chironius fuscus* has a lot more genetic variation than what is available (Breno et. Al 2018). Our documentation of this species can further contribute to current research regarding the environmental and genetic patterns of the evolution of the *Chironius species.*
- The *Leptodeira septentrionalis* has evolutionary history that has not been fully understood (Daza et al 2009). Through the use of our phylogenetic tree, which displays the genetic relationships among various species, we can understand many important facts such as ancestry, and evolutionary traits, which can help uncover more about this species.
- Currently, there is limited information regarding *Coniophanes Longinquus* population trends, habitat requirements and threats (NatureServe Explorer 2.0). This information is vital for conservationists to make informed decisions regarding habitat conservation. Our research can provide insights on the genetic diversity and population structure of this species, allowing researchers to infer key relationships that are crucial for conservation efforts.
- Moving forward, further scientific research will be needed to be employed to continue to understand more regarding the sector of neotropical snakes, aid conservation efforts and discover more about their evolutionary history.

References

Rodrigues, Marina Silva, et al. "Cytochrome c Oxidase Subunit 1 Gene as a DNA Barcode for Discriminating Trypanosoma Cruzi DTUs and Closely Related Species." Parasites & Vectors, vol. 10, no. 1, 16 Oct. 2017, https://doi.org/10.1186/s13071-017-2457-1.

Da Silva FO;Fabre AC;Savriama Y;Ollonen J;Mahlow K;Herrel A;Müller J;Di-Poï N; "The Ecological Origins of Snakes as Revealed by Skull Evolution." *Nature Communications*, U.S. National Library of Medicine, 22 May 2018, pubmed.ncbi.nlm.nih.gov/29371624/.

Daza, Juan M et al. "Complex evolution in the Neotropics: the origin and diversification of the widespread genus Leptodeira (Serpentes: Colubridae)." *Molecular phylogenetics and evolution* vol. 53,3 (2009)

Breno Hamdan, Anieli G. Pereira, Letícia Loss-Oliveira, Dennis Rödder, Carlos G. Schrago, Evolutionary analysis of Chironius snakes unveils cryptic diversity and provides clues to diversification in the Neotropics, Molecular Phylogenetics and Evolution, Volume 116, 2017, Pages 108-119, ISSN 1055-7903, https://doi.org/10.1016/j.ympev.2017.08.004.

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