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ANIMAL SCIENCE

The Brazilian Atlantic Bushmaster *Lachesis* (Linnaeus, 1766) Mitogenome With Insights On Snake Evolution And Divergence (Serpentes: Viperidae: Crotalinae)

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Abstract: This study presents the first complete mitogenome of the Brazilian Atlantic bushmaster *Lachesis* with insights into snake evolution. The total length was 17,177 bp, consisting of 13 PCGs, 22 tRNAs, two rRNAs and a duplicate control region (CRs). Almost all genes were encoded by the heavy-strand, except for the ND6 gene and eight tRNAs (tRNA-Gln, Ala, Asn, Cys, Tyr, Ser[TGA anticodon], Glu, Pro). Only ATG, ATA, and ATC were starting codons for protein-coding sequences. Stop codons mainly were TAA, AGA, AGG, and TAG; whereas ND1, ND3, and CYTB terminated with incomplete stop codons. Phylogeny retrieved *Lachesis* within the Crotalinae as the sister group of *Agkistrodon*; and the *Lachesis*+*Agkistrodon* clade as the sister group of (*Sistrurus*+*Crotalus*)+*Bothrops*. The tree supports Crotalinae, Viperinae, and Azemiopinae in the Viperidae family, being sister taxa of Colubridae+(Elapidae+Psammophiidae). The mean genetic distance across 15 snake families and 57 nucleotide sequences was 0.37. The overall mean value of genetic distance across the Crotalinae was 0.23, with *Lachesis muta* exhibiting the shortest distance of 0.2 with *Agkistrodon piscivorus*, *Protobothrops dabieshanensis* and *P. flavoviridis* and the greatest 0.25 with *Gloydius blomhoffii*, *Trimeresurus albolabris*, *S. miliarius*, and *Deinagkistrodon acutus*. The complete Atlantic *L. muta* mitogenome presented herein is only the third annotated mitogenome from more than 430 described Brazilian snake species.

Key words: Comparative analysis, genetic distance, mitochondrial genome, next generation sequencing, phylogenetic tree, snake.

INTRODUCTION

Snakes are a species-rich squamate clade with more than 4,038 extant species (Uetz et al. 2023). The monophyletic alethinophidians snakes are broadly distributed, morphologically diverse, and comprise most extant snakes, including the venomous viperid family. The bushmaster *Lachesis muta* (Linnaeus 1766) is a medically important Viperidae snake, occurring in South America in east and south of Venezuela, Trinidad and Tobago, Guyana, French Guiana, Suriname, Northeast of Bolivia, east of Peru, Ecuador, Colombia, and Brazil (Campbell & Lamar 2004, Nogueira et al. 2019). Due to the bushmaster specificity for inhabiting preserved rainforest, the complex venom-composition for bioprospection, and aggressive envenomation, *Lachesis* has been used as research-model for venomics (e.g. Pla et al. 2013), natural history and ecology (e.g. Diniz-Sousa et al. 2020), biogeography (e.g. Lira-da-Silva et al. 2009, Citeli et al. 2020), taxonomy (e.g. Fernandes et al. 2004), and snakebite epidemiology (e.g. Mise

et al. 2018). However, aspects of its genomic composition remain underexplored (see exceptions in Zamudio & Greene 1997).

Snakes' mitogenomes contain several unusual characteristics for vertebrates and represent an ideal model for exploring potential links between mitogenomic structure, its function, and evolution (Jiang et al. 2007). Some unique characteristics include single or duplicate control regions (CRs) that can act as an additional origin of heavy strand replication (Jiang et al. 2007); gene order rearrangements including gene loss, translocation, and duplication (Qian et al. 2018); shorter tRNA genes, translocation of the tRNALeu gene; and large non-coding regions (e.g. Zhou et al. 2022). An example of gene arrangement inferred to be ancestral to snakes is the mitogenome of the Typhlopidae *Indotyphlops braminus* (Yan et al. 2008). It has also been reported that a small non-coding region is highly homologous to the start of control regions I (CR I) and II (CR II) across Colubridae and Homalopsidae (He et al. 2010).

In general, tRNAs in snakes exhibit cloverleaf structures with some exceptions that may lack a dihydrouridine (DHU) arm/stem and TΨC loop (e.g. Zhou et al. 2022). The WANCY tRNA gene cluster and the control regions and their adjacent segments are hotspots for mitogenomes gene arrangement (Qian et al. 2018). There is so much variation in snake mitogenomes that three major types and 11 subtypes have been proposed to better understand its composition and evolution (Qian et al. 2018).

Despite the gene order rearrangements mentioned above, the mitogenome content may also show phylogenetic signal, valuable data for helping with species delimitation hypotheses and access to the evolution of taxa (e.g. Pan et al. 2019). The description and characterization of mitogenomes of South American snakes are scarce, and only the complete mitochondrial genomes of the pit viper *B. jararaca* (Almeida et al. 2016) and *M. surinamensis* (Pessoa et al. 2019) were published, despite the 430 species known to occur in Brazil (Costa et al. 2021). Publications on molecular-based research of *L. muta* are limited to fragments of mitochondrial DNA and nuclear genes (Zamudio & Greene 1997, Mason et al. 2019). No complete mitogenome has ever been reported. In this study, we produced the first complete mitogenome sequence of the Brazilian Atlantic bushmaster *Lachesis* by next generation sequencing and characterized its gene content, genome size, gene order, and repetitive sequences. We predict that the mitogenome arrangement of the Atlantic *L. muta* belongs to the Type III pattern proposed by Qian et al. (2018), marked by the duplication of the control regions and translocation of the tRNALeu gene. In addition, we used the mitogenome to infer the phylogenetic placement and relationships of the species and the genus *Lachesis*, but also the Crotalinae subfamily and the Viperidae family including all snake lineages with available complete mitogenomes. Furthermore, we explore the genetic distance of related taxa by exploring the power of mitogenomic sequences to provide insights into taxa limits and evolution.

MATERIALS AND METHODS

Sampling, sequencing and annotation

Mitogenome sequencing

Genomic DNA of one specimen of the Atlantic bushmaster *Lachesis* from Quebrangulo, Alagoas State, Brazil (Instituto Vital Brazil HPLANT 15,893) was extracted from ventral scale tissue using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer' protocol. Paired-end libraries were built with 50 ng of

genomic DNA through random fragmentation. The DNA is simultaneously fragmented and bound to specific adapters using the Nextera DNA Flex Library Prep kit (Illumina), according to the manufacturer's instructions. Subsequently, the libraries were diluted in a solution of Tris-HCl and 0.1% Tween, added to the flowcell, then to a clustering step using the NextSeq 500/550 kit (300 cycles) and sequenced in the Illumina NextSeq 500 sequencer.

Assembly and annotation

A total of 9,487,510 paired-end reads were generated by the Illumina platform (~1,4X genome coverage size) and used as input for the NOVOPlasty 3.5 (Dierckxsens et al. 2016) assembly. Reference genes of the mitogenome of the snake *Bothrops jararaca* were used as seeds to assemble and recover the complete mitogenome. We used the MITOS2 (Bernt et al. 2013) webservers (http://mitos2.bioinf.uni-leipzig.de/index.py) to annotate the mitogenome, considering genetic code 2 (vertebrate mitochondria) and RefSeq89 Metazoa database as parameters. The MITOS annotation was manually adjusted in GENEIOUS Prime 2022.2.2 (https://www.geneious.com) and the Artemis annotation tool (Rutherford et al. 2000). The tRNAs were searched for potential cloverleaf structures on tRNAscan-SE Search. The size of the mtDNA control region (CR) was compared by using the boundaries of tRNAs and sequence comparison with previously reported snake mitogenomes. A circular mitogenome map was generated using OGDRAW v1.3.1 (Greiner et al. 2019). To check annotation and gene order, we aligned the complete mitogenome sequence of the Atlantic *L. muta* with three Viperidae species (*Gloydius rubromaculatus*, *Agkistrodon piscivorus* and *Bothrops jararaca*), three Colubridae (*Ovophis okinavensis*, *Lycodon semicarinatus* and *Sibon nebulatus*), one Elapidae (*Sinomicrurus peinani*) and one Typhlopidae (*Indotyphlops braminus*). Nucleotide sequence alignment was performed in MAFFT v7.263 (Katoh & Standley 2013) using '-auto' strategy and normal alignment mode. We included all former species to represent a dataset with higher confidence in the alignment.

Evolutionary analyses

Phylogeny and genetic distance

To estimate the phylogenetic position of the Atlantic *L. muta*, we used representative species of all snake families with available complete mitogenomes in the GenBank database. A total of 57 species were included belonging to 15 families: Acrochordidae (n=2), Aniliidae (n=1), Viperidae (n=34) with subfamilies Azemiopinae, Crotalinae which holds *L. muta* species and Viperinae, Boidae (n=2), Colubridae (n=2), Cylindrophiidae (n=1), Elapidae (n=2), Leptotyphlopidae (n=1), Pareidae (n=2), Psammophiidae (n=1), Pythonidae (n=2), Tropidophiidae (n=1), Typhlopidae (n=2), Uropeltidae (n=1) and Xenopeltidae (n=2) (Supplementary Material - Table SI). Each nucleotide sequence of the 13 protein-coding genes (PCGs) was extracted from the 57 species and used as input by SPLACE (G.L Nunes et al. unpublished data: https://github.com/reinator/splace), which automatically splits the same genes from the different species into fasta files, individually aligns with MAFFT v7.263 using default parameters, and then concatenates the aligned genes from the same organism in a single fasta file, generating a supermatrix.

We performed a maximum likelihood analysis using the supermatrix generated by the 13 concatenated protein-coding regions (PCGs: 11,525 bp). Maximum likelihood analyses to test our mitogenome annotation and help explore the placement of *L. muta* within the snake's clade were conducted in IQ-TREE v1.6.12 (Nguyen et al. 2015). The best-fit substitution models

and partitioning schemes for each gene were estimated with ModelFinder (Kalyaanamoorthy et al. 2017), implemented in IQ-TREE (Matrix SI). We assessed nodal support using 5,000 bootstrap pseudoreplicates via the ultrafast-bootstrap (UFBoot) (Minh et al. 2013). The trees were visualized and edited in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree). The mitogenome data supporting this study are available in GenBank (OP773877). Reference number for other taxa is available in Table SI.

The genetic distances for the concatenated 13 PCGs regions between families and within the Crotalinae subfamily were calculated using MEGA 7 (Kumar et al. 2016) with 5,000 bootstrap replicates, the complete-deletion option, the Kimura 2-parameter model (Kimura 1980) and gamma distribution (shape parameter = 1).

RESULTS AND DISCUSSION

Atlantic Bushmaster mitochondrial genome annotation

Mitogenome composition and gene order

The complete mitogenome of the Atlantic bushmaster *Lachesis* (Instituto Vital Brazil HPLANT 15893; NCBI accession number submission OP773877) from the Atlantic Forest in Quebrangulo municipality, Alagoas State of Brazil, is a 17,177 bp length (Table I and Figure 1), circular, double-stranded DNA molecule (Figure 1). It includes 37 genes with 13 PCGs, two rRNA genes, 22 tRNA genes and two control regions (CR I, CR II) (Figure 1). The nucleotide composition is 33% of A, 12% of G, 26% of T, and 29% of C.

The gene order agrees with the Type III-B pattern, observed in alethinophidians and specifically Viperidae marked by the duplication of the control region and translocation of the tRNALeu gene (Figure 2) (Qian et al. 2018). The tRNA-Leu (TAA anticodon) gene is found between CRII and tRNA-Gln (Figure 1), not between 16S rRNA and ND1, as in most vertebrates. The majority of the 37 genes were encoded by the heavy-strand, except the ND6 gene and eight tRNAs (tRNA-Gln, Ala, Asn, Cys, Tyr, Ser[TGA anticodon], Glu, Pro).

The intergenic spacers totalize 75 bp, ranging from 1 to 45 bp in size; the longest was located between 16S rRNA and ND1. Overlapping nucleotides were found between six pairs of genes, with the length range from 1 to 22 bp; the largest was between COX3 and tRNA-Gly.

Protein-coding genes and codon usage.

The length of protein-coding genes varied from 165 bp in ATP8 to 1,788 bp in ND5. Herein, only the three starting codons for PCGs were adopted ATG (COX2, ATP8, ATP6, COX3, ND4L, ND4, ND5, ND6, CYTB), ATA (ND1, COX1, ND3) and ATC (ND2). Most stop codons were TAA, except for COX1, COX2 and ND4 with AGA; ND6 with AGG, ND2 with TAG; whereas ND1, ND3 and CYTB terminated with incomplete T, presumably transformed into complete stop codons through post-transcriptional polyadenylation (Ojala et al. 1981). As found in most snakes, most of these genes are coded on the heavy strand (H-strand) except for ND6.

Transfer and ribosomal RNAs

The tRNAs ranged from 55 bp in tRNA-Ser (GCT anticodon) to 73 bp in tRNA-Leu (TAA anticodon). Almost all the tRNAs could fold into a typical cloverleaf structure, except for tRNA-Ser (ATA anticodon), which lacked a D-arm or dihydrouridine arm. The complete loss of the D-arm is also observed in the tRNAs of mt-genomes of several vertebrates (Salinas-Giege et al. 2015). The function of tRNA is to help translate mRNA into protein and collaborates with various proteins from post-transcription to decoding in ribosomes. The 12S rRNA and 16S rRNA genes are 912 bp and 1,471 bp in length, respectively, separated by tRNA-Val.

Table I. Complete mitochondrial genome annotation of the Brazilian Atlantic bushmaster *Lachesis* and traits of codon and anticodon. *=tRNA with no D-arm. ITG= Intergenic space (no sign) or gene overlapping (-).

Locus	Strand	From	To	Length (pb)	ITG (bp)	Start	Stop	Anti-codon
tRNA-Phe	+	1	66	66	0			GAA
12S rRNA	+	66	977	912	-2			
tRNA-Val	+	975	1038	64	-9			TAC
16S rRNA	+	1029	2499	1471	45			
ND1	+	2544	3480	937	1	ATA	T- -	
tRNA-Ile	+	3481	3548	68	3			GAT
tRNA-Pro	-	3551	3614	64	1			TGG
CR II	+	3615	4628	1014	1			
tRNA-Leu	+	4629	4701	73	1			TAA
tRNA-Gln	-	4702	4771	70	1			TTG
tRNA-Met	+	4772	4835	64	1			CAT
ND2	+	4836	5867	1032	-1	ATC	TAG	
tRNA-Trp	+	5866	5930	65	1			TCA
tRNA-Ala	-	5931	5995	65	1			TGC
tRNA-Asn	-	5996	6068	73	3			GTT
OL	+	6071	6104	34	-1			
tRNA-Cys	-	6103	6161	59	1			GCA
tRNA-Tyr	-	6162	6222	61	-7			GTA
COX1	+	6215	7825	1611	-9	ATA	AGA	
tRNA-Ser	-	7816	7883	68	1			TGA
tRNA-Asp	+	7884	7946	63	1			GTC
COX2	+	7947	8639	693	-7	ATG	AGA	
tRNA-Lys	+	8632	8693	62	1			TTT
ATP8	+	8694	8858	165	-9	ATG	TAA	
ATP6	+	8849	9529	681	0	ATG	TAA	
COX3	+	9529	10335	807	-22	ATG	TAA	
tRNA-Gly	+	10313	10372	60	-2			TCC
ND3	+	10370	10715	346	1	ATA	T- -	
tRNA-Arg	+	10716	10779	64	1			TCG
ND4L	+	10780	11070	291	0	ATG	TAA	
ND4	+	11070	12407	1338	1	ATG	AGA	
tRNA-His	+	12408	12468	61	1			GTG
tRNA-Ser*	+	12469	12523	55	1			ATA
tRNA-Leu	+	12524	12595	72	3			TAG
ND5	+	12598	14385	1788	-4	ATG	TAA	

Table I. Continuation.

ND6	-	14381	14902	522	1	ATG	AGG	
tRNA-Glu	-	14903	14964	62	1			TTC
CYTB	+	14965	16078	1114	1	ATG	T- -	
tRNA-Thr	+	16079	16142	64	1			TGT
CR I	+	16143	17177	1035	0			

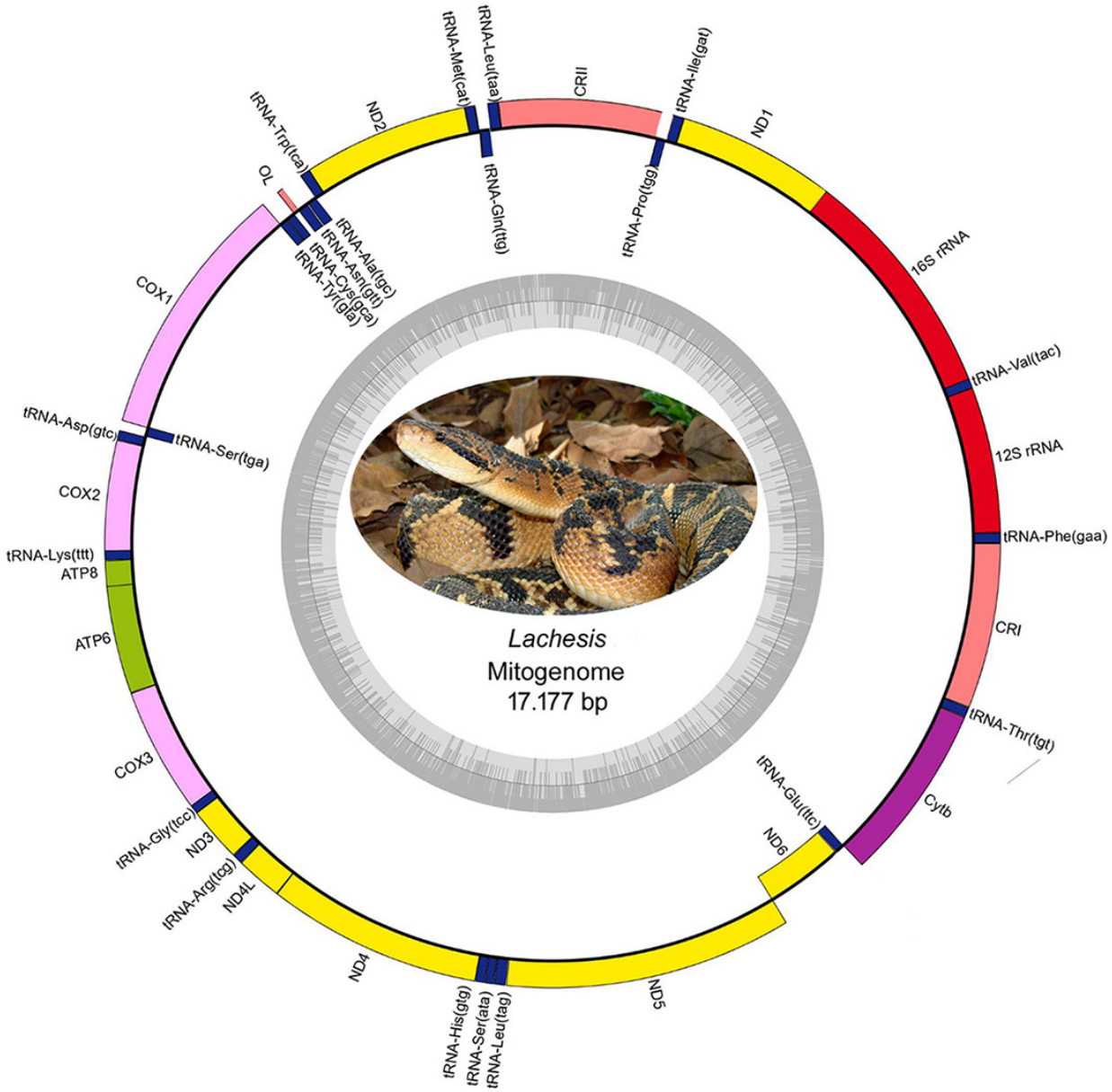

Figure 1. Mitochondrial genome map of the Brazilian Atlantic bushmaster *Lachesis*. Genes encoded on the heavy or light strand are respectively indicated on the outside or inside of the circular mitogenome map. The tRNAs are denoted by blue color and labelled according to the three-letter amino acid codes. Photo: Marco Freitas.

Figure 2. Comparison of mitochondrial gene organizations of the Brazilian Atlantic bushmaster *Lachesis*. Genes, control regions (CRs), non-coding regions (NCD), and light-strand replication origins are shown in boxes. Genes relevant to discussions on gene rearrangements are highlighted in different colors. Dotted arrows indicate the rearranged genes and the inferred evolutionary directions of the rearrangements.

Control region (CR)

Control region I is 1,035 bp in length and located between tRNA-Thr and tRNA-Phe, different from other snake families, where it is flanked by tRNA-Pro and tRNA-Phe, but similar to other Viperidae snakes (Figure 2). The CR II is 1,014 bp located between tRNA-Pro and tRNA-Leu (TAA anticodon), different from other snake families (tRNA-Ile-CRII-tRNA-Leu), but similar to other Viperidae (Figure 2). The CR is the main non-coding area of the mitochondrial genome and is known to contain controlling elements for replication and transcription (Formenti et al. 2021). The origin of the L-strand replication (OL) is 34 bp in length and is located between tRNA-Asn and tRNA-Cys in the WANCY region (Qian et al. 2018) (Table I).

MITOS annotation note

The program annotated a 53bp control region between tRNA-Gln and tRNA-Met not only for *Lachesis muta* but also for *Gloydius rubromaculatus*, *Agkistrodon piscivorus*, *Bothrops jararaca*, *Lycodon semicarinatus*, *Ovophis okinavensis*, *Indotyphlops braminus* and *Sinomicrurus peinani*. Such CR was not found in any other published mitogenome. MITOS also retrieved tRNA-Tyr between tRNA-His and tRNA-Leu (TAA), not found by tRNAscan-SE Search Server v1.21 or literature. These finds were disregarded in the final annotation.

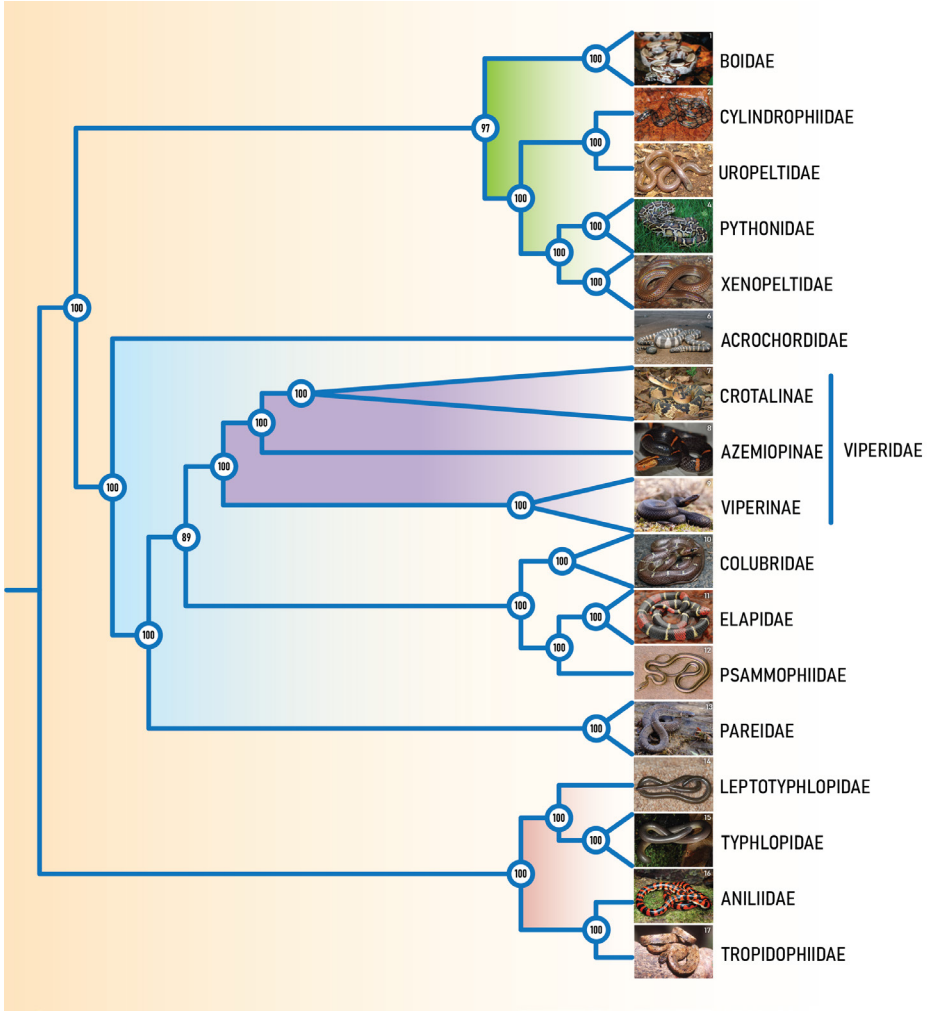

Figure 3. Maximum likelihood tree of 13 concatenated protein-coding genes. Phylogeny considered 57 species from 15 snake families, including the described mitogenome of the Brazilian Atlantic bushmaster *Lachesis*. Species terminal collapsed into their families. Numbers at the nodes indicate Ultrafast bootstrap support. Photos: Breno Hamdan (1,11,14,15,16), Sanoj Wijayasekara (2), S.R. Ganesh (3), Marcelo Duarte (4,17), Hari Krishnan (5), Aadit Patel (6, 10), Marco Freitas (7), Kaja Chocza (8), Halvard Midtun (9), Vivek Sharm (12), Matthieu Berroneau (13).

Mitogenome-based evolutionary analyses

Genetic distance

The estimate of average evolutionary divergence over all sequence pairs across the 15 snake families and 57 nucleotide sequences was 0.37. Considering the Atlantic *L. muta*, the shortest distance observed is 0.2 when compared with *A. contortrix* and *A. piscivorus*, whereas the greatest is 0.68 when compared with *I. braminus*. The overall mean value of distance across the Crotalinae subfamily is 0.23 (Table

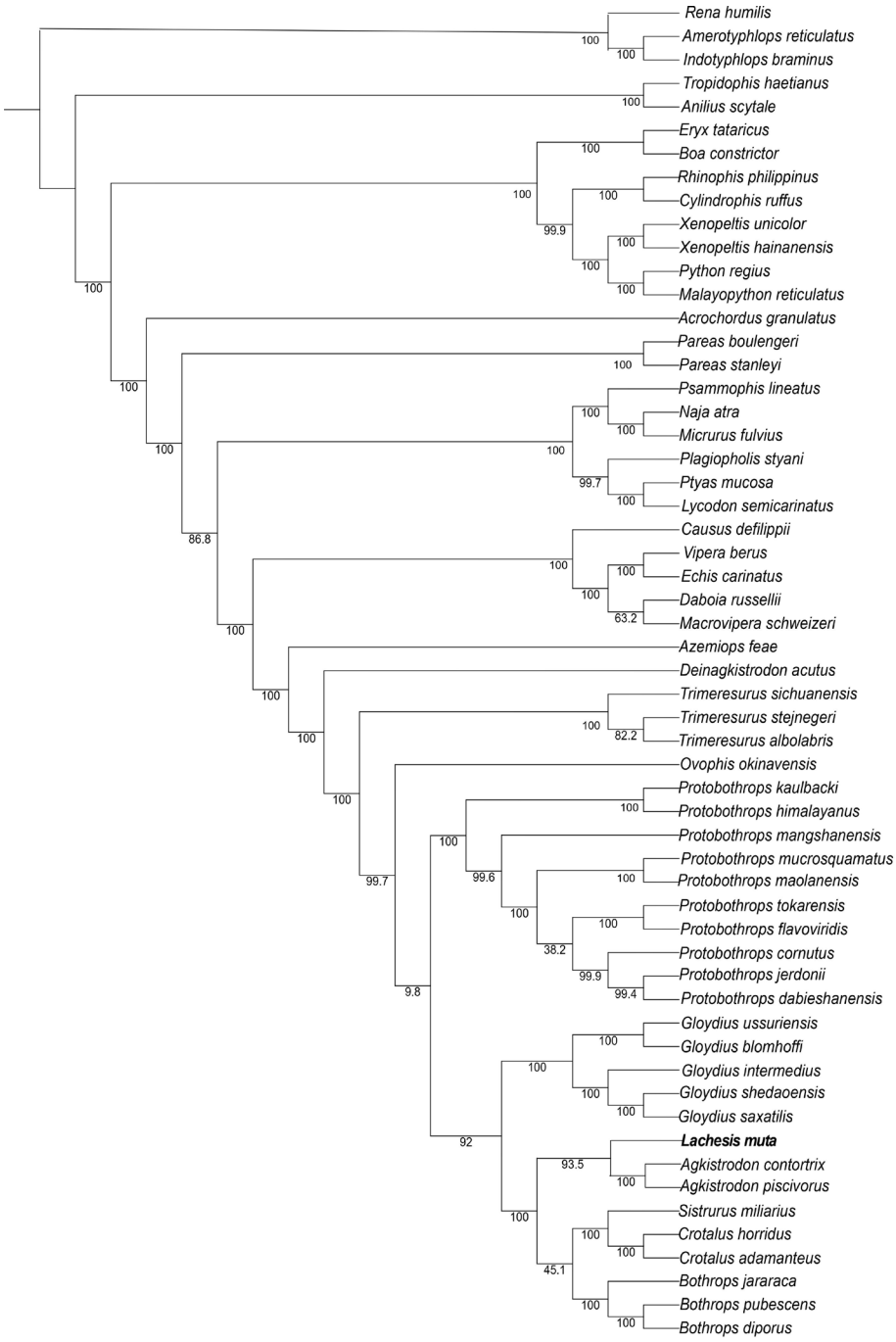

Figure 4. Maximum likelihood tree of 13 concatenated protein-coding genes. Phylogeny considered 57 species from 15 families, including the described mitogenome of the Brazilian Atlantic bushmaster *Lachesis* (bold). Numbers indicate Ultrafast bootstrap support.

SII). At the inter-family level, the highest value of the genetic distance was between Typhlopidae and Acrochordidae with 0.64. In contrast, the lowest value was between Xenopeltidae and Pythonidae at 0.26 (Table SIII). The scarcity of scientific papers that use multiple PCGs to estimate genetic distances limits the scope of the discussion for exploring mitogenome data to help infer taxa border. Genetic distance using the entire PCG may provide more powerful clues than using genes alone to explore cryptic diversity within snakes.

Phylogenetic analyses

The ML phylogenetic tree using the 13 PCGs showed well-supported clades retrieving all snakes' families with high support (>95). The lowest support at the inter-family level was Viperidae + (Colubridae + Elapidae), with a bootstrap value of 89. Monophyly of the Crotalinae subfamily was recovered with node support of 100 bootstraps (Figure 3), the same value observed for the clade Crotalinae+Azemiopinae. The clade was recently recovered (e.g. Zaher et al. 2019), and the overall relationships among the snake families sampled herein are primarily consistent with the results of recent studies (e.g. Burbrink et al. 2020).

The phylogenetic tree showed that the Atlantic *L. muta* was the sister species of *Agkistrodon* (*A. contortrix + A. piscivorus*). The *Agkistrodon + L. muta* clade formed a sister group with *(Sistrurus miliarius + (Crotalus horridus + Crotalus adamanteus)) + (Bothrops jararaca + (Bothrops pubescens + Bothrops diporus)*, with absolute statistical support (Figure 4). The phylogenetic relationship differs slightly from the literature, which shows the unresolved relationships between *Lachesis* and others and that *Agkistrodon* is sister to (*Sistrurus + Crotalus*) (Alencar et al. 2016) and not to *Lachesis*, as shown here. The differences between the two phylogenies may be attributed to (1) the use of 13 protein-coding mitochondrial genes in our study, compared to four PCGs and two rRNAs in Alencar et al. (2016), (2) the use of five independent nuclear markers in Alencar et al. (2016), and (3) the limited taxon sampling here, due to the limited availability of complete mitogenomes.

The phylogenetic analysis result was consistent with previous research. The first determined mitogenome sequence of the Atlantic *L. muta* provides fundamental data for further exploring mitogenome evolution in snakes.

The mitochondria have delighted science because of its unique bacterial origin, strategy in encoding subunits of the respiratory complexes and particular inheritance (Cavalier-Smith et al. 2006). The complete mitogenome is increasingly becoming a marker of choice. It has been widely used in the analysis of population structure, genetic diversity, species identification, phylogenetic analysis, origin, evolution, and conservation studies. In the last few years, high-throughput sequencing techniques have accelerated the sequencing of mitochondrial genomes and uncovered the great diversity of organizations, gene contents, and modes of replication and transcription found in eukaryotes (Briscoe et al. 2016). More than 430 snake species occur in Brazil (Costa et al. 2021); however, only two had their mitogenomes assembled and annotated, with no contributions for genetic distance nor phylogeny at the snake family level. In this study, we sequenced, assembled and annotated for the first time the mitogenome of the Atlantic threatened bushmaster *Lachesis muta* providing comparative mitogenome analyses, phylogeny at species and family level, and genetic distance data. We expect more efforts in exploring others Brazilian snakes' mitogenomes to use them in

integrative research, from the vertebrate gene order evolution to the species conservation approach.

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SUPPLEMENTARY MATERIAL

Table SI-SIII

Matrix S1.

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