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Article

Analysis of the Entire Mitogenome of the Threatened Freshwater Stingray Potamotrygon leopoldi (Myliobatiformes: Potamotrygonidae) and Comprehensive Phylogenetic Assessment in the Xingu River, Brazilian Amazon.

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Abstract: The present study characterized the complete mitochondrial genome of Potamotrygon leopoldi, commonly referred to as the "white blotched stingray", being is a member of the Potamotrygonidae family that are an extant group of stingrays that live exclusively in freshwater environments. P. leopoldi, endemic in the Xingu River from the Amazon region, are exploited by the commercial fisheries for food and specially to the ornamental industry, and this causes a big impact in the populations. There are used newly assembled PacBio long-read sequencing to assess the complete mitogenome of P. leopoldi and perform a comparative investigation in molecular evolution. The mitogenome has 17,504bp, containing 13 protein-coding, 22 tRNA, and 2 rRNA genes. The average of the composition in the mitogenome was A: 32.32%, T: 24.41%, C: 12.84%, G: 30.42% with an AT content of 56.73%. The values of AT and GC skewness were 0.13 and -0.40, respectively. Our phylogenetic analyses with mitogenome sequences of 40 elasmobranch species supports the monophyly for Potamotrygonidae family and indicates a close relationship to the Dasyatidae family and a sister relationship with P. orbignyi and P. falkneri. Also detected various amino acid sites under positive selection exclusively in P. leopoldi. This extensive comparative mitogenomic investigation offers novel and significant insights into the evolutionary lineage of neotropical freshwater stingrays and their closely related taxa. It stands as an indispensable resource for facilitating ongoing and prospective investigations into the molecular evolution of elasmobranchs.

Keywords: Mitogenome; Potamotrygoniadae; positive selection

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1. Introduction

Mitochondria are important organelles that are present in eukaryotic cells, being inherently related to the cellular energy production, while also having many contributions to homeostasis, such as nucleotide biosynthesis, apoptosis and other activities. These organelles typically have a circular genome and, over the generations, mutations might set a precedent for selective pressure[1].

The mitochondrial DNA (mtDNA) has about a ten to twenty times the nuclear DNA (nDNA) mutation rate, partially because of the absence of efficient mtDNA repair systems

[2]. Due to this mutation rate, the mitochondrial genome is an excellent tool to compare with other species and to help understand the recent evolutionary events that the animals suffered [3].

Stingray species have a fundamental ecological role in aquatic ecosystems, but many are usually fished for human consumption and to supply animals for the ornamental fish market. Data displayed by the Ministry of Development, Trade and Services of Brazil shows that the trade of ornamental freshwater fishes would moved 2.3 millions of dollars in 2023 [4]. Among these species, Potamotrygonidae stingrays are sought after for the domestic and international market due to its conspicuous coloration patterns. Potamotrygonidae is a monophyletic family of stingrays that evolved to live exclusively in freshwater environments, including four genera: Potamotrygon, Paratrygon, Heliotrygon and Plesiotrygon [5-7].

The genus Potamotrygon occurs in South America, with 39 species described, including species with wide distribution (e.g., P. motoro, P. orbignyi) and endemic species restricted to specific rivers (e.g., P. leopoldi, P. henlei) [8]. Notably, P. leopoldi inhabits the downstream waters of the Xingu River, from the Amazon region and is largely used as ornamental fishes, so it is a species particularly vulnerable to extinction [9]. Although there has been an increase in the knowledge about the biogeography and evolution of freshwater stingrays [10], there are still few studies that focus on the molecular changes associated with the emergence of adaptive characteristics that allowed adaptation of the stingrays from a marine environment to a freshwater ecosystem. In this sense, a comprehensive analysis of the mitochondrial genome may be a valuable strategy to expand our understanding of the evolutionary history of these species [11]. Currently, only five mitogenomes are described for the Potamotrygon genus, and reinforcements in the use of genomics are necessary to study the freshwater stingrays.

Therefore, we report the assembly and annotation of the complete mitochondrial genome of P. leopoldi and use the public available mitochondrial mitogenome of the myliobatiformes to investigate the evolutionary dynamics comparing with the marine group. Our investigation focused on understanding the evolutionary dynamics of Potamotrygon mitogenomes by comparing them with other stingrays and elasmobranchs, to understand the differences and the molecular adaptation of the freshwater stingrays. Additionally, we conducted an analysis of gene synteny to identify potential rearrangements within the Potamotrygonidae family, and we tested for amino acid sites under positive selection in the mitochondrial genes to search insights of the molecular adaptation of P. leopoldi and Potamotrygon genus. A mitogenome phylogeny was generated using data from mitogenomes of rays and sharks, enabling us to infer the phylogenetic relationship with the Potamotrygon genus, and the evolutionary time of speciation.

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on references.

2. Result

2.1. Mitochondrial genome structure

The complete circular mitogenome of P. leopoldi was sequenced using a Sequel I platform by the PacBio Systems, generating a total of 6,325,249,036 reads. We also used a set of sequences generated by Zhou et al., 2023 [12], with the Illumina Hiseq platform. It should be noted that Zhou et al., 2022 [12] assembled the total nuclear genome of such

specimens but the mitochondrial genome remained unannotated and with a large amount of redundancy. After these analyses, the total length of the mitogenome was 17,504 bp containing 13 PCGs, 22 tRNAs and 2 rRNAs genes (Figure 1). The P. leopoldi mitogenome was deposited in Genbank, under accession number OR896919.

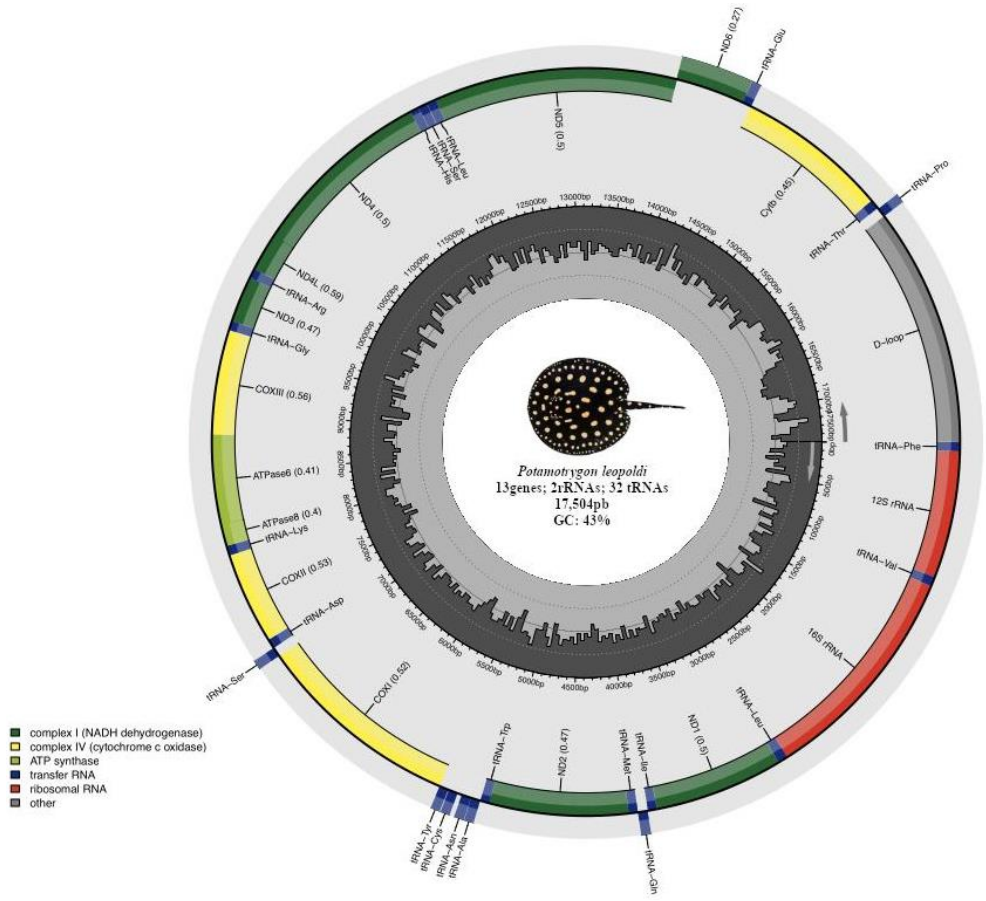


Figure 1. Circular genome map of P. leopoldi mitochondrial genome, showing 13 PCGs (in green and yellow), 2 rRNA (in red) genes, 22 tRNA genes (in blue), and the control region D-loop (in gray).

The mitogenome of the white blotched stingray has a nucleotide composition as follows: A: 32.32%, T: 24.41%, C: 12.84%, G: 30.42%. The A+T content is higher than C+G content, showing that the mitogenome has bias towards AT. The AT and GC skewness of the mitogenome sequence was 0.13 and -0.40, showing the A-skew and C-skew. Regarding the genes, 28 are encoded in the H-Strand (Heavy strand, +) and the other nine are encoded in the L-strand (Light strand, -), as seen in Table 1. The arrangement of genes was typical for vertebrates except for the variation in the length and the intergenic spacer region.

Table 1. Mitochondrial genome organization of Potamotrygon leopoldi.

Name	Type	Strand	Start	Stop	Intergenic nucleotide	Length	Anticodon & Start codon/Stop codon
tRNA-Phe	tRNA	+	1	69	0	69	-
12S rRNA	rRNA	+	70	1,030	0	961	-
tRNA- Val	tRNA	+	1,031	1,102	19	72	-

16S rRNA	rRNA	+	1,122	2,788	3	1667	-
tRNA-Leu	tRNA	+	2,792	2,866	0	75	-
ND1	PCG	+	2,867	3,841	1	975	ATG/TAA
tRNA-Ile	tRNA	+	3,843	3,910	4	68	-
tRNA-Gln	tRNA	-	3,915	3,986	0	72	-
tRNA-Met	tRNA	+	3,987	4,056	0	70	-
ND2	PCG	+	4,057	5,103	-1	1047	ATG/TAA
tRNA-Trp	tRNA	+	5,103	5,172	1	70	-
tRNA-Ala	tRNA	-	5,174	5,243	1	70	-
tRNA-Asn	tRNA	-	5,245	5,317	4	73	-
tRNA-Cys	tRNA	-	5,351	5,419	5	69	-
tRNA-Tyr	tRNA	-	5,425	5,493	1	69	-
COX1	PCG	+	5,495	7,048	6	1554	GTG/TAG
tRNA-Ser 2	tRNA	-	7,055	7,125	0	71	-
tRNA-Asp	tRNA	+	7,126	7,196	3	71	-
COX2	PCG	+	7,200	7,890	0	691	ATG/T(AA)
tRNA-Lys	tRNA	+	7,891	7,964	1	74	-
ATP8	PCG	+	7,966	8,133	-10	168	ATG/TAA
ATP6	PCG	+	8,124	8,807	-1	683	ATG/TAA
COX3	PCG	+	8,807	9,592	5	786	ATG/TAA
tRNA-Gly	tRNA	+	9,598	9,668	0	71	-
ND3	PCG	+	9,669	10,019	-1	351	ATG/TAA
tRNA-Arg	tRNA	+	10,019	10,089	0	71	-
ND4L	PCG	+	10,090	10,386	-7	297	ATG/TAA
ND4	PCG	+	10,380	11,760	3	1381	ATG/T(AA)
tRNA-His	tRNA	+	11,764	11,832	1	69	-
tRNA-Ser	tRNA	+	11,834	11,900	0	72	-
tRNA-Leu	tRNA	+	11,903	11,974	0	72	-
ND5	PCG	+	11,975	13,810	-4	1836	ATG/TAA
ND6	PCG	-	13,807	14,328	0	522	ATG/TAG

tRNA-Glu	tRNA	-	14,319	14,397	1	69	-
CYTB	PCG	+	14,399	15,541	4	1143	ATG/TAA
tRNA-Thr	tRNA	+	15,546	15,616	8	71	-
tRNA-Pro	tRNA	-	15,652	15,694	97	70	-
D-loop	CR		15,792	17,500		1708	-

The length of tRNA genes varied from 67 bp (tRNASer) to 75 bp (tRNALeu). All tRNA presented a typical cloverleaf secondary structure with a few base pair mismatches (Figure 2). The nucleotide frequencies of rRNA are A: 35.63%, T: 21.12%, G: 17.27%, C: 25.98%, with an AT content of 56.75%. Ten tRNAs showed secondary structure with mismatches: G-G base pair (instead of G-C), T-T (instead of T-A), A-A (instead of A-T), and G-T (instead of G-C) (Figure 2).

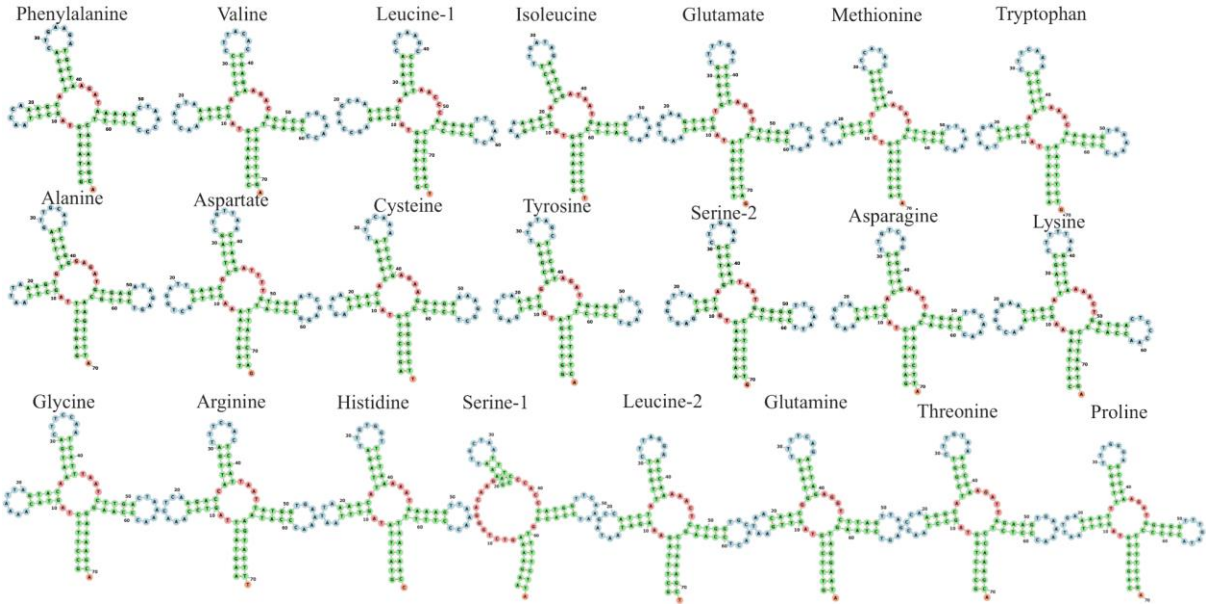


Figure 2. Secondary structure of tRNAs in the mitogenome of P. leopoldi visualized in the Forna web Server.

The gene order and orientation, along with the distribution of genes on both the heavy and light strands, exhibited complete congruence with the mitogenomes of other representatives within the Potamotrygon genus, including P. falkneri, P. magadlenae, P. motoro, P. Orbignyi, and the outgroup species, C. milii. We illustrate this notable similarity among the species in Figure 3. These findings underscore the remarkable conservation of gene organization and strand distribution within the Potamotrygon genus and contribute to our understanding of their evolutionary relationships.

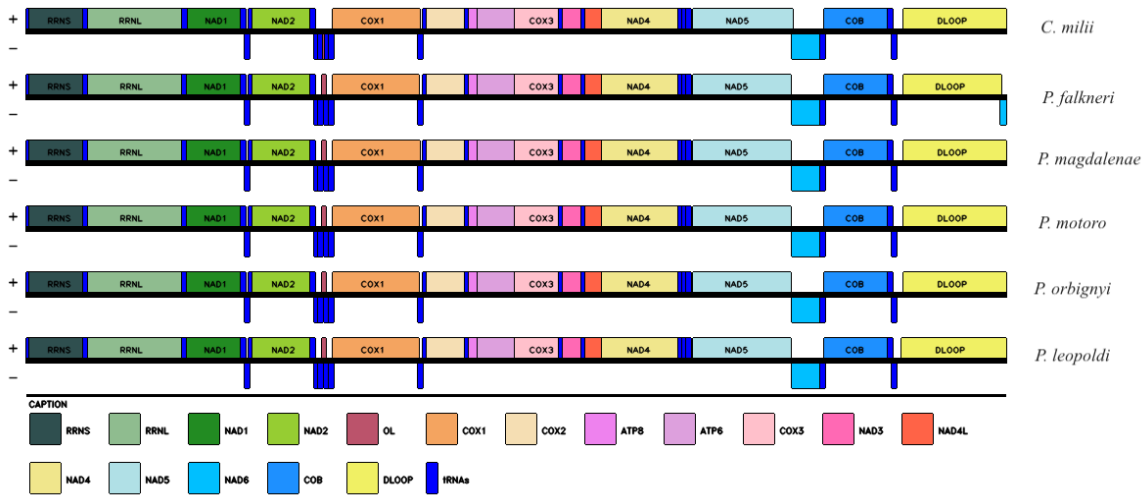


Figure 3. Arrangement of genes encoding rRNAs and proteins of P. leopoldi and other species of Potamotrygon genus. Heavy (+) and light (-) strands.

2.2. Protein-Coding Genes (PCGs)

The length of the protein-coding genes within the mitogenome of P. leopoldi was determined to be 11,444 base pairs (bp), with individual gene lengths ranging from 168 bp (ATP8) to 1,836 bp (ND5). The overall average A+T content was calculated to be 57.0%.

Comparative analysis with other Potamotrygonidae species revealed a significant similarity in the usage of conventional start and stop codons within most of the PCGs. Specifically, the majority of PCGs in P. leopoldi start the translation with the start codon ATG, and they commonly terminate with the stop codon TAA. However, notable exceptions were observed in two genes: the COXI gene utilized the start codon GTG, diverging from the typical ATG start codon pattern, and the ND6 gene employed the stop codon TAG instead of the more common TAA stop codon.

The PCGs comprised approximately 64.70% of the total mitochondrial genome in P. leopoldi. The average effective number of codons, an indicator of codon usage bias, was determined to be 57.65. Our analysis predicted a total of 3,811 codons from the 13 PCGs found. Figure 4 displays the Relative Synonymous Codon Usage (RSCU) for the amino acids, revealing a significant bias in codon usage for most of them. Comparative analysis of RSCU across mitogenomes from various elasmobranch families demonstrated interesting similarities. When comparing the five representatives of the Potamotrygonidae family with the outgroup *C. milii*, the codon usage patterns showed consistency, further supporting the close phylogenetic relationships among these taxa. These comprehensive results provide valuable insights into the genomic features and codon preferences within the mitogenome of P. leopoldi, and also contribute to improving our understanding of codon usage patterns and evolutionary relationships among elasmobranch families.

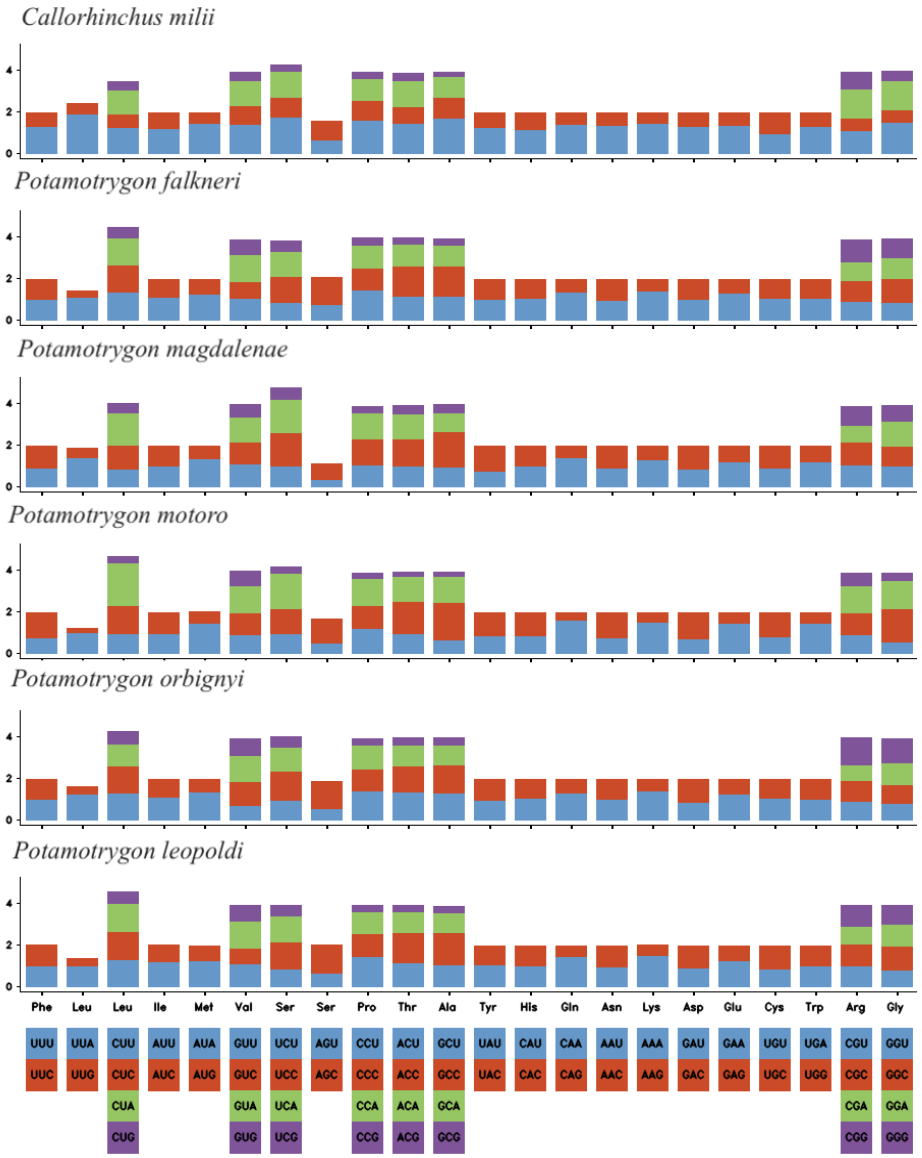


Figure 4. Relative Synonymous Codon Usage (RSCU) of the protein-coding genes of P. leopoldi and species from Potamotrygonidae family and the C. milii as outgroup.

2.3. Control region (CR)

The mitochondrial genome of P. leopoldi shares a conserved arrangement observed in other Potamotrygon mitogenomes, characterized by the positioning of the control region (CR) between the tRNAPhe and the tRNAPro (as illustrated in Figure 1). Notably, in P. leopoldi, this CR exhibits a high abundance of adenine (A) and thymine (T), resulting in a base composition of A=33.55%, T=29.92%, C=24.30%, and G=12.24%.

Within the CR of P. leopoldi, our investigation revealed the presence of two types of tandem repeats, the first consisting of a larger 128 bp-long repeat, and a smaller with 20 bp (Figure 5). These findings showed the genomic organization and sequence characteristics of the P. leopoldi mitogenome, offering valuable insights into the genetic diversity and evolutionary aspects within this species.

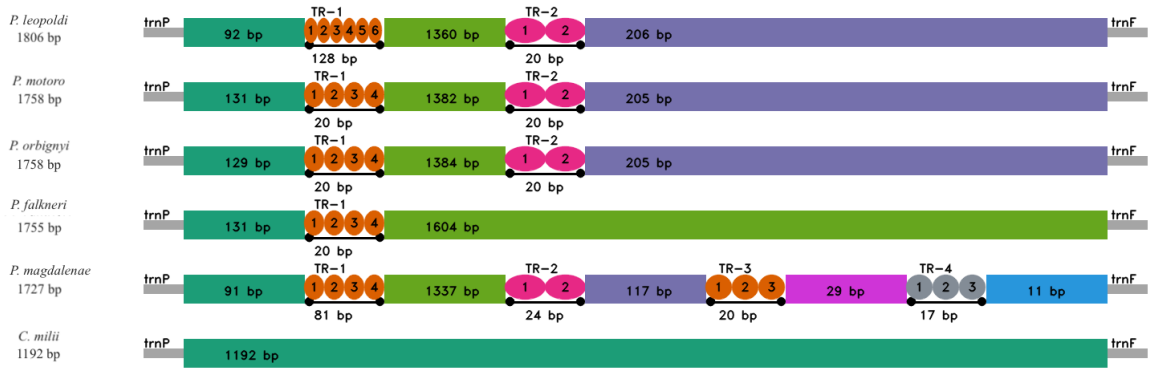


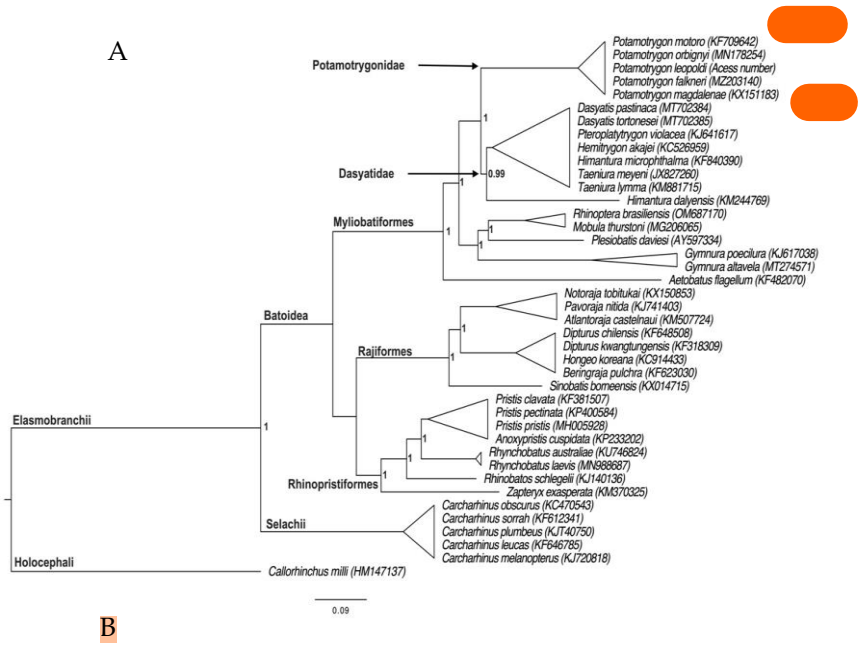
Figure 5. Organization of the CR in the complete mitogenomes of P. leopoldi, other four species from Potamotrygon genus and outgroup C. milii.

2.4. Adaptive molecular evolution

In the analysis of the mitogenome we found sites in positive selection when compared with myliobatiformes that have mitogenomes annotated in the GenBank. ND1-1 sites under positive selection, ND4-1 site under positive selection, ND5-3 sites under positive selection, COX I-6 site under positive selection, COX II-3 sites under positive selection. With this analysis, we observe that the positive selection may be related with the functional adaptive adjustment to the freshwater stingrays that have the adaptation to survive and colonize the freshwater on Amazon Basin.

2.5. Phylogenetic analyses

In the phylogenetic tree created through the BI method, the species display strong nodal support, forming separate clades for different families. Particularly, the family Potamotrygonidae is observed to be a monophyletic group and shares a sister relationship with the Dasyatidae family. Additionally, the Myliobatidae family is positioned as a basal group. Further examination of the tree reveals a sister relationship between P. leopoldi, P. falkneri and P. orbignyi (Figure 6).



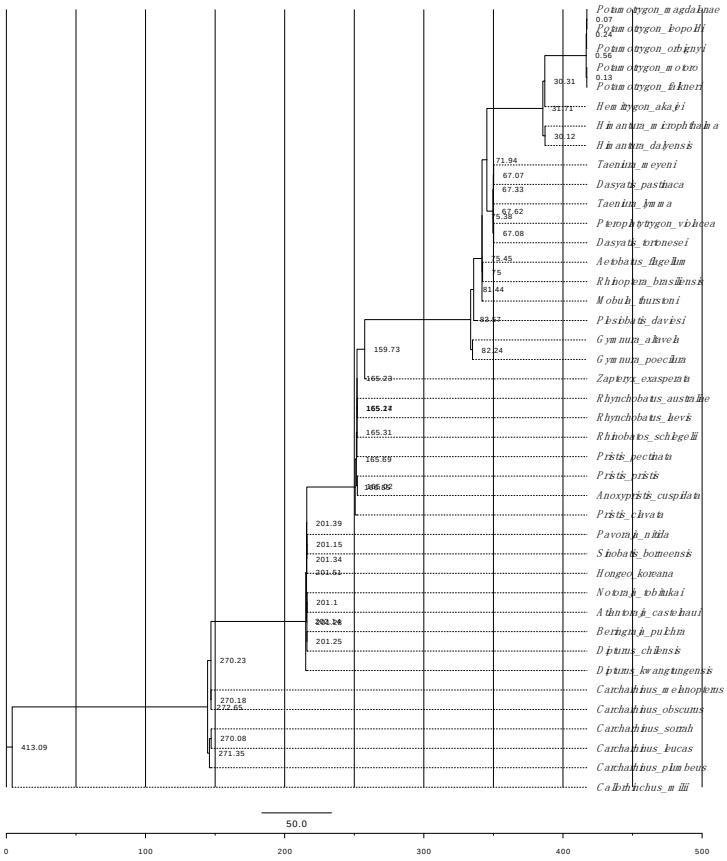


Figure 6. - A- Phylogenetic tree using the complete mitochondrial genome of species of order Myliobatiformes. GenBank Accession IDs are in brackets. The nodal support indicates the posterior probability in BI. B- Divergence time estimates in the class of chondrichthyes. The value above the node represents time to present, in My.

3. Discussion

3.1. Mitochondrial genome characterization

This article are performed a comprehensive sequencing of the complete mitochondrial genome of P. leopoldi. The sequencing approach yielded a coverage of 11x, and we complemented our data with publicly available sequences previously published by Zhou et al. in 2022 [12]. The utilization of next-generation sequencing (NGS) technology in this study presents multiple advantages, notably the generation of long reads, which helps compensate for the relatively lower coverage achieved. Furthermore, the incorporation of short-read sequences contributes to enhancing the assembly process, leading to a more precise description of the mitochondrial genome [13].

Within the Potamotrygonidae family, the length of mitogenomes varies, traditionally ranging from 17,429 bp (P. magdalenae) to 17,449 bp (P. orbignyi) [14]. Our analysis revealed that the mitogenome of P. leopoldi presents a length of 17,504 bp, making it larger than that of P. orbignyi, which previously held the record as the largest mitogenome described within the Potamotrygonidae family, with 17,449 bp [14].

This variation in mitogenome size can be attributed to differences in the length of the control region and intergenic regions. These regions are known to experience less influence from selection and exhibit a higher mutation rate, contributing to substantial variation among species [15]. Our study provides important insights into the genomic diversity within the Potamotrygonidae family and highlights the significance in shaping mitogenome size variation.

In the mitochondrial genome, nucleotides exhibit differential distribution between the two DNA strands, giving rise to DNA asymmetry [16]. To assess this asymmetry in

the studied mitogenome, AT/GC skewness values were calculated. In the mitogenome of P. leopoldi, there are observed positive values for AT skewness, indicating an abundance of adenine (A) over thymine (T). Conversely, negative values were noted for GC skewness, implying an excess of cytosine (C) relative to guanine (G). The observed asymmetry can be attributed to the outcomes of mitochondrial replication and transcription, as these molecular processes result in the unwinding of the DNA duplex, thereby facilitating the transient exposure of single-stranded DNA regions that exhibit an elevated susceptibility to mutational events [17-19]. The AT and GC skew values observed in the mitogenome of P. leopoldi were similar to those detected in other Potamotrygon species, such as P. falkneri [20]. These contribute to a better understanding of the genomic characteristics and evolutionary aspects within the Potamotrygon genus.

3.2. Protein-Coding Genes

The gene arrangement within the mitochondrial genome of P. leopoldi exhibited notable similarity to the commonly reported arrangement observed in most vertebrates [21]. Through a comprehensive comparative analysis involving four mitogenomes of species from the Potamotrygon genus, there are found a remarkably conserved gene arrangement, even in comparison to the outgroup species C. milii (Figure 3). Collectively, these findings strongly indicate a highly conserved mitogenome organization within the Potamotrygonidae. Nachtigall, Loboda and Pinhal [20] suggested that rearrangements in the mitochondrial genome of Batoidea species may be uncommon, which is in line with our findings that Potamotrygonidae mitogenomes have a conserved arrangement compared to other vertebrates. These observations contribute with valuable insights into the genomic organization and evolutionary patterns within the Potamotrygonidae family and highlight the significance of conserved gene arrangements in these species.

In many species, synonymous codons are not used at an equal frequency for specific genes, resulting in codon usage bias. Previous studies have reported the existence of such bias in the mitochondrial genomes of elasmobranchs and other fishes [22]. There are observed codon usage bias for several genes among the species within the Potamotrygonidae family. This occurrence can be influenced by multiple factors, such as gene length, translational selection, tRNA abundance, mutation rates, GC composition, and the amino acid composition of proteins.

To quantify the degree of codon bias within and among species, there are employed the effective number of codons (ENc). This metric ranges from 20 to 61, where a value close to 20 indicates that only one codon from each codon family is used, while a value close to 61 indicates that all synonymous codons are utilized equally [23]. In our investigation, P. leopoldi exhibited an elevated ENc value of 57.65, meaning that synonymous codons are equally used within the Potamotrygonidae family. This observation aligns with the findings depicted in Figure 4. These results provide valuable insights into the codon usage patterns within the Potamotrygonidae family and enhance our comprehension of the factors influencing codon bias in mitochondrial genomes of freshwater stingrays.

Control region (CR)

As in other Myliobatiformes mitogenomes, the CR found here has tandem repeat regions with variable copy numbers and lengths of repeat units (Figure 5). Our analysis showed that the CR of P. leopoldi has a similar CR pattern (Figure 5) to other representants in the Potamotrygon genus that have information of complete mitogenome in the public databases. Variability in the tandem repeat regions is common in elasmobranchs, and the high content of tandem repeats is responsible for the similar mtDNA genome size among the elasmobranchs [24]. Generally, the published mitogenomes of Potamotrygonidae lack discussion regarding such sequences within the control region. These features could

potentially play a role in the regulation of replication in the mitochondrial genome, as suggested in other vertebrates (e.g., [25], [26]).

Adaptive molecular evolution

Based on the results obtained in the study, the positive selection was found in ND1, ND4, ND5, COX I, and COX II. It demonstrates that the P. leopoldi, when compared with other Myliobatiformes and Potamotrygon species, has supported the hypothesis of the migration of the marine stingrays millions of years ago with a big adaptation, in this way, can seen the regions in positive selection that characterize the signature in the mitogenome. Supporting this idea, several studies have shown that, in ND genes, there is a functionally relation to proton piping process in the oxidative metabolism, are under strong positive selection in vertebrate species [27-32].

Noll et al., 2022 [32], found positive selection in penguins and the complex ND1 and ATP complex, play a central role in cellular energy production and are the genes that are present in the greatest evidence in selection in marine organisms. A study by Sebastian et al., 2022 [40] with clupeoid fish that colonized marine habitats over millions years presented positive selection in PCGs of animals lineages that migrate from marine to the freshwater in genes ND2, ND4, ND5, and ND6 and also has a relatively high number of amino acid changes. In the freshwater stingrays, the selective pressures are very similar to those that occurred in the clupeoid fish and would result from the selective pressures coming from niche changes, such as the transition from the saltwater to freshwater environment. Indeed, when conducting molecular evolutionary analysis on the clupeoid, certain mitochondrial genes have been identified with sites subject to positive selection in freshwater species, as opposed to their closely related saltwater counterparts [28].

3.3. Phylogenetic analyses

The phylogenetic analyses in this study illustrate the evolutionary positioning of Potamotrygon species (Figure 6). Our results reveal a close relationship between P. leopoldi and other species from the Potamotrygonidae family. The sequence of the five representants have a high similarity. The tree demonstrates monophyly, supporting the findings of Nachtigall, Loboda and Pinhal [20], who inferred the phylogeny of the Potamotrygonidae family and proposed that the genus Potamotrygon is also monophyletic. Additionally, gene similarity comparisons indicate a strong resemblance among species within the Potamotrygon genus, particularly in the mitochondrial sequence. This similarity is likely due to the well-established evolutionary biogeographic history of the genus.

4. Materials and Methods

4.1. Sample collection and DNA extraction

Tissue samples were collected from a captive population of stingrays (P. leopoldi), two of male and two of female at a farm located in Salinópolis, Pará, Brazil. The necessary permissions and access to the samples were obtained through the Secretary of Environment and Sustainability from Pará State (SEMAS-PA) under the Cooperation Agreement Nº05/2018.

Total genomic DNA was extracted from small fragments of the pectoral fin of adult P. leopoldi specimens using the Wizard Genomic DNA Purification Kit extraction method (Promega, Madison, WI, USA) in strict accordance with the supplier's instructions. Subsequently, the concentration and quality of the extracted DNA were assessed using both NanoDrop 1000 spectrophotometer and Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). These procedures ensure the reliability and validity of the genomic DNA samples obtained for further analysis.

4.2. Assembly, annotation and bioinformatic analysis of the complete mitogenome

Two genomic libraries were prepared (one consisting of two samples from a male and, the other, of two samples from a female) utilizing the SMRTbell Template kit 1.0 (Pacific Biosystems, Menlo Park, CA, USA) following the manufacturer's recommended protocols. The subsequent sequencing of these libraries was conducted on a PacBio Sequel I system (Pacific Biosystems) utilizing a SMRT Cell 1M v3 (Pacific Biosystems). In addition to our generated data, there are incorporated a set of complementary sequences of P. leopoldi (CRR191193, CRR191177, CRR191191, CRR191189, and CRR191192), recently generated by Zhou et al. 2023 [12] employing the Illumina HiSeq platform. This integration of complementary data sources contributes to a more comprehensive and robust analysis in our study.

Raw sequencing data was filtered to trim adapters and low-quality sequences using CANU software [33]. The resulting reads were then assembled using Unicycler [34]. Protein-coding genes (PCGs), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs) were predicted by using MitoAnnotator [35] and annotated by alignment with other mitogenomes of Potamotrygon. The secondary structure of tRNAs was visualized in the Forna web server (hhtp://rna.tbi.univie.ac.at/forna) [36]. The tandem repeat units of the Control Region (CR) were identified with the Tandem Repeats Finder Server (http://tandem.bu.edu/trf/trf.advanced.submit.html; with default settings, except for "Minimum Alignment Score To Report Repeat"=30) [37]. The AT and GC skews were calculated according to the following formulas: AT skew=(A – T)/(A + T) and GC skew=(G – C)/(C + G) [38]. Arrangement of genes encoding rRNA proteins, comparative relative synonymous codon usage (RSCU), and CR organization analysis were performed using in-house Python scripts. For these analyses are used the following mitogenomes deposited in Genbank: Potamotrygon falkneri (MZ203140), Potamotrygon orbignyi (MN178254), Potamotrygon motoro (KF709642), Potamotrygon magdalenae (KX151183) and Callorhinchus milii (HM137147).

4.3. Phylogenetic analysis

We reconstructed the phylogenetic tree using the complete mitogenome currently available for 40 species of elasmobranch. The species C. milii (HM137147) was used as an outgroup. To reconstruct the phylogenetic tree, we performed the multiple sequence alignment using the ClustalW alignment program with the default parameters in MEGA11 [39]. Bayesian Inferences (BI) were run using Markov-Chain Monte Carlo searches on two simultaneous runs of chains of 5,000,000 generations in Mr. Bayes version 3.1.2, with a sample frequency of every 1000th [40]. The first thousand trees were discarded as burn-in and the posterior probability of each node was calculated from the remaining trees, and examined in Figtree v1.4.4 [41].

The divergence time was estimated with BEAST v2.6.6 [42], and the reference in million years (MYA) between genera Pristis and Rhynchobatus (67.8 - 159.0 MYA) [43] was used as prior (www.timetree.org) for calibration [44].

To measure the effective number of codons (ENc) are estimated it through DAMBE 6.4.67 software. ENc designates the degree of codon bias for genes by computing deviation from uniform codon usage without any prior dependency over the sequence length or specific information of preferred codons [45].

5. Conclusions

In this study are presented the first complete mitochondrial genome of P. leopoldi, endemic in the Xingu River from the Amazon region, by integrating publicly available mitogenomes from other Potamotrygonidae species with our high-throughput sequencing. The P. leopoldi mitogenome comprises 13 PCGs, 22 tRNAs, and two rRNAs, with a structure similar to other Potamotrygon species. Phylogenetic analysis shows a sister relationship with P. orbignyi and P. falkneri, increasing the understanding of Potamotrygonidae evolution. The analysis of mitogenome created clues to understood the migration

of marine stingray and the adaptation of the actual freshwater stingray, in observation of the positive selection in the genes when compared with the marine stingray mitochondrial genome. The presented mitogenome data is valuable for future phylogenetic, DNA barcoding, and metabarcoding studies, aiding research in genetic diversity and evolutionary history for these important stingrays.

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Data Availability Statement: The mitogenome sequence data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov (accessed on 16 August 2024), under accession number OR896919.

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