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First description of the mitogenome of the endangered turtle *Erymnochelys madagascariensis* (Testudines: Podocnemididae) and its implications for conservation

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Abstract

First description of the mitogenome of the endangered turtle *Erymnochelys madagascariensis* (Testudines: Podocnemididae) and its implications for conservation.

Erymnochelys madagascariensis is ranked first on the EDGE of Existence program's list of priority reptiles. This species is the only living member of the family Podocnemididae found outside of South America. It has a unique evolutionary, as it is endemic to Madagascar. We present the first description of the complete mitogenome of the species. The assembled mitogenome is the third and the smallest described for the Podocnemididae. It has a length of 16,421bp, CG content of 38%, and presents 22 tRNAs, two rRNAs, 13 protein-coding genes (PCGs) and one non-coding region. The gene order and CG content were similar to the mitogenome of the *Podocnemis* species. Selective pressure analysis indicated the PCGs were under purifying selection except for *ATPase 8*. The phylogenetic analysis of PCGs of Pleurodira revealed that *Myuchelys* is a polyphyletic group. Our study demonstrates that the complete mitogenome can be a useful tool to assess genetic diversity via the identification of haplotypes among natural populations and detection of introgression events. This information may have important implications for conservation, especially in designing and implementing protected breeding areas and contributing to programs with restocking purposes.

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Keywords: Madagascar Big-headed Turtle, Mitochondrial DNA, Pleurodira, Purifying selection.

Resumo

Primeira descrição do mitogenoma da espécie ameaçada *Erymnochelys madagascariensis* (Testudines: Podocnemididae) e suas implicações para a conservação. *Erymnochelys madagascariensis* ocupa a primeira posição na lista de prioridades de répteis do programa EDGE of Existence. Esta espécie é a única descrita na família Podocnemididae encontrada fora da América do Sul e possui uma história evolutiva única para a herpetofauna de Madagáscar. Neste trabalho, apresentamos a primeira descrição do mitogenoma completo de *E. madagascariensis*. O mitogenoma montado é o terceiro e o menor mitogenoma descrito para Podocnemididae, com um comprimento de 16.421 pb, conteúdo de CG de 38% e apresenta 22 tRNAs, dois rRNAs, 13 genes codificadores de proteínas e uma região não codificadora. A ordem dos genes e o conteúdo de CG foram semelhantes ao mitogenoma das espécies de *Podocnemis.* A análise de pressão seletiva indicou que os genes codificadores de proteínas estavam sob seleção purificadora, com exceção de *ATPase 8*. A análise filogenética dos genes codificadores de proteínas de Pleurodira revelou que *Myuchelys* é grupo polifilético. Nossos dados demonstram que o mitogenoma pode ser uma ferramenta útil para avaliar a diversidade genética, uma vez que pode permitir a determinação de haplótipos entre populações naturais e detectar eventos de introgressão. Essas informações podem ter importantes implicações para a conservação, especialmente na concepção e implementação de áreas de reprodução protegidas e contribuir para programas com objetivos de repovoamento.

Palavras-chave: DNA mitocondrial, Pleurodira, Seleção purificadora, Tartaruga-de-cabeça-grande-de-madagascar.

Introduction

Madagascar is world-renowned for its unique fauna and flora (Wilmé *et al.* 2006). *Erymnochelys madagascariensis* (Grandidier, 1867), commonly known as the Madagascar Big-headed Turtle or Side-necked Turtle, is one of the most endangered reptile species. It is ranked first on the EDGE of Existence program's list of priority reptiles (EDGE 2023). The IUCN considers it critically endangered (Leuteritz *et al*. 2008).

This endemic freshwater turtle is native to slow-moving rivers, lakes, and swamps in western Madagascar, being the only living species of the family Podocnemididae found outside of South America (Leuteritz *et al.* 2008, Vargas-Ramirez *et al.* 2008), making it a true testament to Madagascar's biological uniqueness. *Erymnochelys* diverged from its closest living relative, *Podocnemis,* around 78 million years ago during the Late Cretaceous period (Vargas-Ramirez *et al.* 2008). Fossils of podocnemidid turtles, closely related to *Erymnochelys* and belonging to the same tribe (Erymnochelyini), have been discovered in Cenozoic deposits in Africa and Europe (Pérez-Garcia 2023). Madagascar is estimated to have separated from Africa around 150 million years ago and from India about 90 million years ago. This long period of isolation has allowed the local fauna and flora to evolve uniquely, leading to a high number of endemic species (Storey 1995), such as *E. madagascariensis.*

With fewer than 10,000 individuals, this species is threatened by human activities such as deforestation, agriculture, and the conversion of lakes to rice fields, resulting in habitat loss (Rakotomanana *et al.* 2013). *Erymnochelys madagascariensis* reaches sexual maturity at a late age (more than 15 years), so extraction of animals for food and medicine frequently results in the removal of individuals before they can contribute to the next generation (Leuteritz *et al.* 2008).

Efforts to conserve and protect the Madagascar big-headed turtle involve various strategies, including habitat preservation, anti-poaching measures, and captive breeding programs. Conservation organizations and researchers are working diligently to ensure the survival of this species and maintain the biodiversity of Madagascar's unique ecosystems.

A captive breeding program was implemented in 1999 to provide stock to be released in locations with depleted populations (Velosoa *et al.* 2013). At that time, no genetic data were available. Recent molecular studies have shown that captive populations have lower genetic diversity compared to native ones, suggesting that new strategies must be implemented to improve the success of the reproductive programs of *E. madagascariensis* (White *et al.* 2022).

According to White *et al.* (2022), the success of the breeding program of *E. madagascariensis* is directly associated with the knowledge of the genetic variation across the wild populations. Velosoa *et al.* (2013) pointed out that it is important to consider collection sites, molecular markers, and samples involved in this assessment. Until now, only a few microsatellite loci and fewer mitochondrial genes were used to estimate the genetic composition of natural and captive populations of Madagascar`s big-head turtle (Rafeliarisoa *et al.* 2006, White *et al.* 2022)

Herein, we present the first description of the complete mitogenome of *E. madagascariensis*. The use of the complete mitogenome offers a more comprehensive and detailed view of genetic variability compared to analyses based on a few mitochondrial genes. For applications in species management and conservation, mitogenomes are more advantageous than other nuclear markers because they allow us to trace the demographic and migratory history of populations. This information may have important implications for conservation, especially in designing and implementing protected areas for unique genetic groups that could contribute to breeding programs for restocking purposes.

Materials and Methods

In this study, we assembled and annotated the mitogenome of *E. madagascariensis* using paired-end sequencing reads (accession: SRR13244422) obtained from the Sequence Read Archive (SRA) of The National Center for Biotechnology Information (NCBI). The assemblage was conducted into the Galaxy Europe platform using the NovoPlasty v4.3.1. tool (Dierckxsens *et al.* 2017). A partial *CytB* sequence for *E. madagascariensis* (OL804198.1) was used as seed. The assembly was conducted by applying options for the mitochondrial genome with the following parameters: read length = 151, insert size = 300, and k-mer = 39.

Subsequently, annotation was performed using MITOS2 (Donath *et al.* 2019) in a module designed to annotate vertebrate mitochondrial genomes. Protein-coding regions (PCGs), Control Region (CR or D-Loop), and rRNA genes were also annotated manually and confirmed by comparison to the mitogenome of *Podocnemis unifilis* Troschel, 1848 (accession: JF802204) and *Podocnemis expansa* (Schweigger, 1812) (accession: MF359933).

The verification and correction of stop codons, nucleotide composition, and the Relative Synonymous Codon Usage (RSCU) were done using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura *et al.* 2021). Codon usage for each PCG was estimated using the Codon Usage web server (Stothard 2000) with the vertebrate mitochondrial code option.

We analyzed the selective constraints of PCGs using the Codon-based Z-Test of Selection implemented in MEGA11 (Tamura *et al.* 2021), considering the number of nonsynonymous substitutions per nonsynonymous site (dN) and the number of synonymous substitutions per synonymous site (dS). The mitogenome of *P. unifilis* (accession: JF802204) was used for comparisons. Hypothesis tests were performed considering the null hypothesis indicating neutrality (dN = dS) and alternative hypotheses

corresponding to Positive Selection (dN > dS) and Purifying Selection (dN < dS). The substitution model of Nei-Gojobori was applied, and gaps and missing data were treated as a pairwise deletion. P-values less than 0.05 were considered significant.

Relying on the propositions of Xiong *et al.* (2010) and Bernacki and Kilpatrick (2020), we sought to identify the primary structure of the CR by examining the presence of termination association sequences (TAS), conserved sequence blocks (CSBs) and the variable number of tandem repeats (VNTR). The number of repeats in the CR was investigated with the Microsatellite Repeats Finder web server (http://insilico.ehu.es/mini_tools/microsatellites/).

The Mfold web server (Zuker 2003), set in default mode with a temperature range of 33 to 40°C, was used to predict the secondary structure of this region. For every sequence analyzed using Mfold, we examined the first and most stable folding patterns generated by the program. We then compared these folding patterns, identifying similarities and differences in their secondary structures.

We conducted an additional step for annotation of tRNAs using ARWEN v1.2 (Laslett and Canbäck 2008) implemented in GeSeq (Tillich *et al.* 2017) with search mode: Metazoan Mitochondrial tRNAs and genetic code: Vertebrate Mitochondrial. The mitogenome of *Chelodina expansa* Gray, 1857 (accession: KY705230) was used as the reference genome. The secondary structures of the tRNAs were visualized using Forna (Kerpedjiev *et al.* 2015) implemented at the ViennaRNA Web Services site.

The graphic map of the circularized mitogenome was drawn using the online software OrganellarGenomeDRAW (Lohse *et al.* 2007).

A phylogenetic analysis was constructed considering the 13 PCGs from mitogenomes recovered from GenBank/RefSeq. For the evolutionary relationships, 40 species of Pleurodira (ingroup) and four species of Cryptodira (outgroup) were used in the analysis. A list of their respective accession numbers is available in Appendix I.

The PCGs were separated into individual datasets and aligned using MAFFT v.7.508 (Katoh and Standley 2013) under the L-INS-i method. We used the Concatenator v0.2.1 (Vences *et al.* 2022) tool for partitioning and concatenating the aligned sequences. Finally, we used IQ-TREE v2.2.2.6 (Minh *et al.* 2020) to infer the best-fit evolutionary models with ModelFinder and to conduct the phylogenetic analysis under the Maximum Likelihood (ML) method. Branch support was accessed with 10,000 replicates of ultrafast bootstrap approximation (UFBoot) (Minh *et al.* 2013). We visualized the resulting tree with iTOL v6 (Letunic and Bork 2021). The best evolutionary models according to the Akaike information criterion (AIC) were TIM+F+I+G4 for *ATPase 6*, HKY+F+I+G4 for *ATPase 8*, GTR+F+I+G4 for *COX I, CytB, ND2, ND4,* and *ND5*, GTR+F+R6 for *COX II*, TIM2+F+I+G4 for *COX III*, GTR+F+R8 for *ND1*, TVM+F+I+G4 for *ND3* and *ND4L*, and TN+F+I+G4 for *ND6*.

Results

The assembled mitogenome of *E. madagascariensis* (Genbank ID BK065183) is the third complete mitogenome described for the turtle family Podocnemididae. It is a closed-circular molecule of 16,421bp in length, CG content of 38%, and presents 22 tRNAs, two rRNAs, 13 PCGs, and one CR (Figure 1). The nucleotide composition is: A = 34.6%, T = 27.12%, C = 25.63%, and G = 12.65%.

One PCG (ND6) and eight tRNAs (tRNA-Glu, tRNA-Pro, tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, and tRNA-Ser_2) were encoded in the light strand. All other genes were encoded in the heavy strand.

Most PCGs used the standard start codon ATN (ATA, ATG, or ATT) except *ATPase8* (TTG). As for the stop codons, TAA (*COXII, COXIII, ATPase6, ND4, ND4L, ND5, CytB* and *ND1*) and TAG (*ND2, ND3*) were predominant. *COXI, ATPase8,* and *ND6* were the only PCGs with AGA, TGA, and AGG as stop codons, respectively.

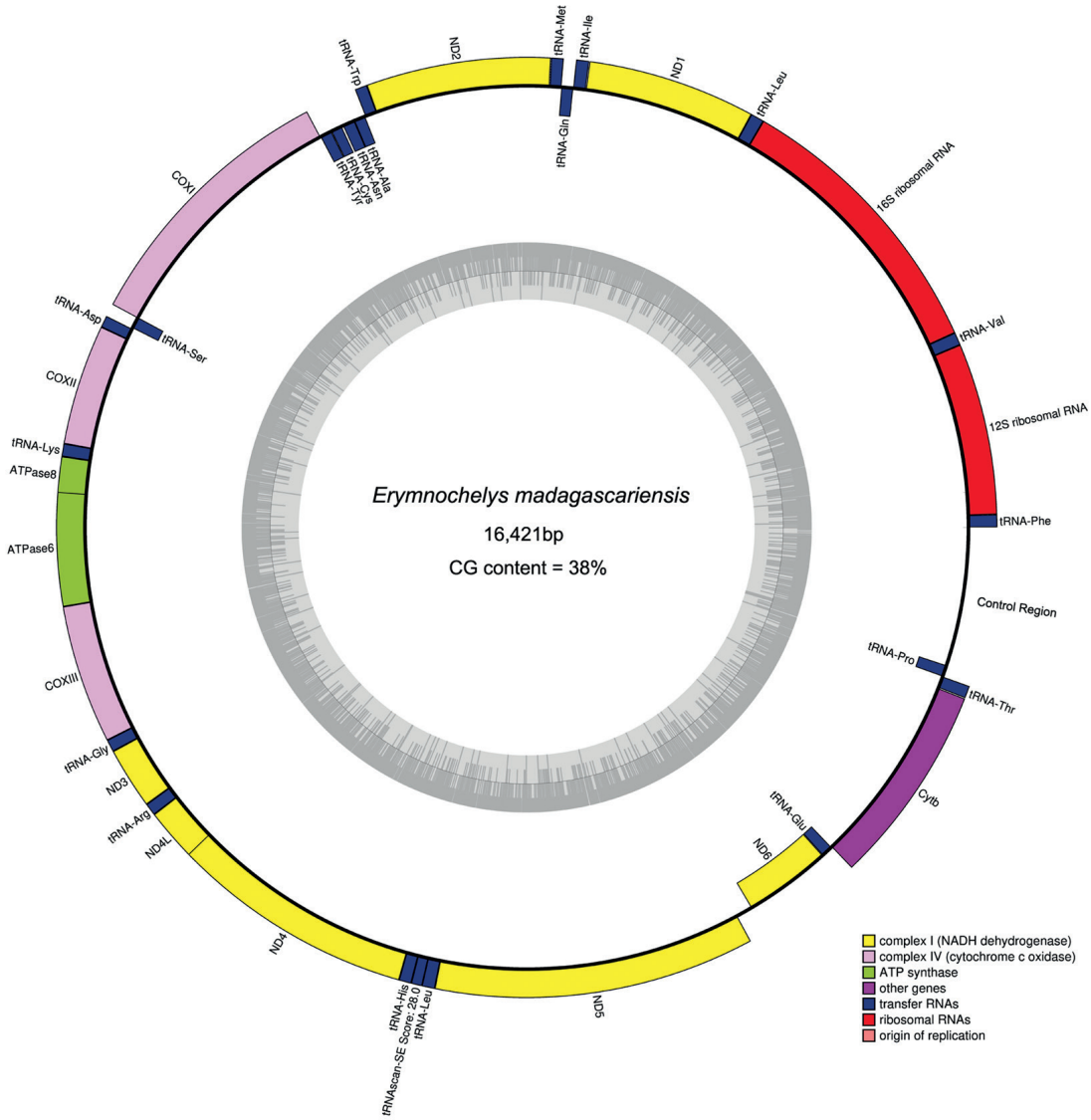

Figure 1. Organization of the mitogenome of *Erymnochelys madagascariensis*.

The RSCU (Relative Synonymous Codon Usage) of PCG codons is summarized in Figure 2.

In the mitochondrial DNA of *E. madagascariensis*, 3703 amino acids were encoded by PCGs. The most frequently used amino acids were Leucine (16.35%), Threonine (8.73%), and Isoleucine (8,51%). Cysteine was the least frequently used amino acid, with less than 0.67%. The analysis of selective constraints of PCGs indicated that most PCGs are evolving under purifying selection, except for *ATPase 8,* which appears to be under no selection (Table 1).

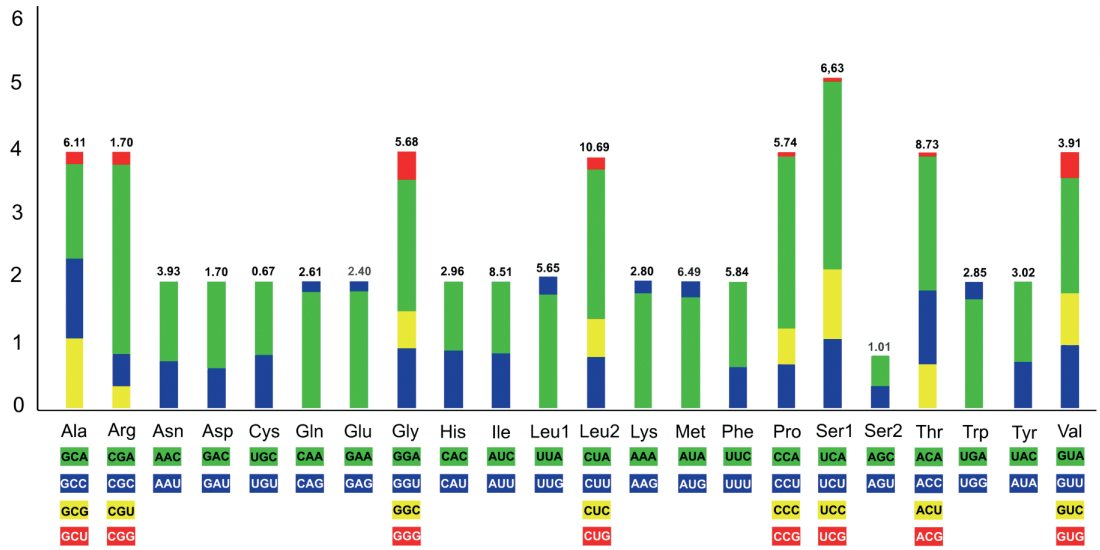

Figure 2. Codon usage of the 13 mitochondrial protein-coding genes of *Erymnochelys madagascariensis*. Codon families are indicated on the X-axis and frequency of RSCU on the Y-axis. The percentage of amino acid usage is indicated above each bar.

Table 1. Codon-based Test of Positive and Purifying Selection for analysis between PCGs sequences. Values of $p < 0.05$ are considered significant and are highlighted.

PCG	Positive selection	Purifying selection
ATP6	1	0.000000016
ATP8	0.0868	1
CytB	1	0
COX1	1	0
COX2	1	0.00000064
COX3	1	0
ND1	1	0.0000000003
ND2	1	0.00000000296
ND3	1	0.000231
ND4	1	0
ND4L	1	0.0000123
ND5	1	0
ND6	1	0.000000173

The lengths of tRNA genes ranged from 62bp (tRNA-Ser_1) to 74bp (tRNA-Ile). The tRNA-Leu and tRNA-Ser were duplicated as expected for vertebrate mitochondrial DNA (mtDNA), and tRNA-Ser1 was the only tRNA missing the dihydorouridinearm (D-arm) (Appendix II). Concerning the rRNAs, 12S rRNA presented 970bp, and 16S rRNA was 1,583bp in length.

The CR was positioned in the heavy strand, between tRNA-Phe and tRNA-Pro, with 830bp in length and GC content of 38.92% (A = 31.2%, C = 23,86%, G = 15.06%, and T = 29.88%). It was possible to identify three distinct regions within CR: (1) the left domain, spanning from the 3′end of tRNA-Pro to the 5′end of CSB-F; (2) the central domain, extending from the 5′end of CSB-F to the 5′end of CSB-1; and (3) the right domain, ranging from the 5′end of CSB-1 to the 5′end of tRNA-Phe. We were able to position five TASs, with two located in the left domain, two in the central domain, and one in the right domain (Figure 3).

Although the search for microsatellite repeats recovered 15 simple sequence repeats for the CR of *E. madagascariensis* (Table 2), we could only differentiate the VNTR2, which was characterized as an AT-rich region at the end of the right domain (Figure 3, Table 2). The most stable secondary structures of CR are presented in Figure 4. Every Mfold output generated comparable models featuring folds in all the domains except for two small loops, that were absent at temperatures of 39 and 40°C. In all models, a conserved stem-loop structure was observed at the 5′end of the left domain and the 3′end of the right domain. No folding loop was formed due to TASs binding.

The relationships among the living families of Pleurodira (Figure 5) received strong bootstrap support, recovering those groups as monophyletic. *Erymnochelys* was recovered as a sister group of *Podocnemis*. However, analysis of our assembled data set suggests the possibility that the genus *Myuchelys* is polyphyletic, as it appears within four separate clades within the Chelidae.

Table 2. Simple sequence repeats found in the Control Region of *Erymnochelys madagascariensis'* mitogenome.

Position	Length of repeats	Number of repeats	Repeats
3	3	2	TTCTTC
57	2	3	CCCCCC
64	9	2	GTGGGGGGGGTGGGGGGG
143	3	2	AACAAC
227	4	2	ATCCATCC
268	4	2	GCATGCAT
475	3	2	CATCAT
488	3	2	TAATAA
536	4	2	ACATACAT
623	3	2	ACCACC
648	4	2	ACATACAT
750	2	3	ATATAT
765	2	7	TATATATATATATA
780	2	8	ATATATATATATATAT
814	3	2	TCCTCC

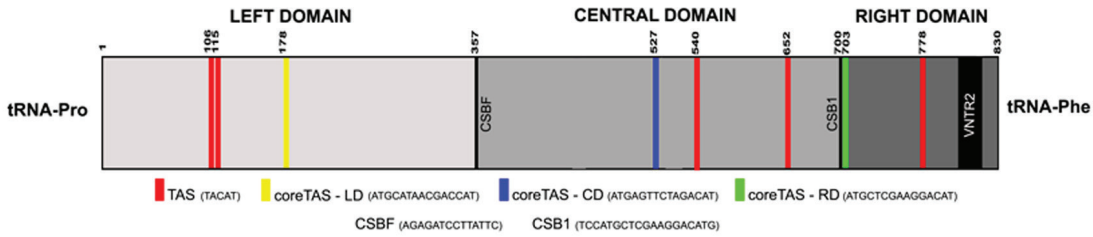

Figure 3. Representation of the primary structure of the Control Region of the mitogenome of *Erymnochelys madagascariensis.*

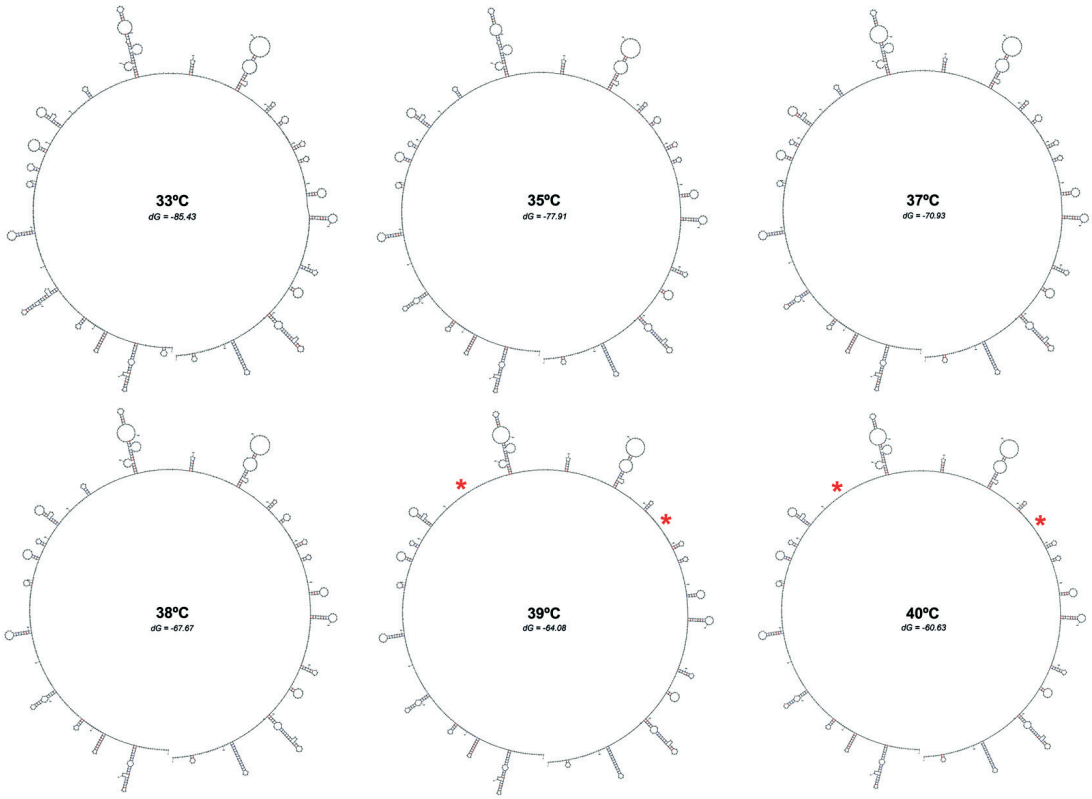

Figure 4. Mitochondrial Control Region possible folding models of *Erymnochelys madagascariensis* under different temperature conditions. The red signs indicate the absence of loops.

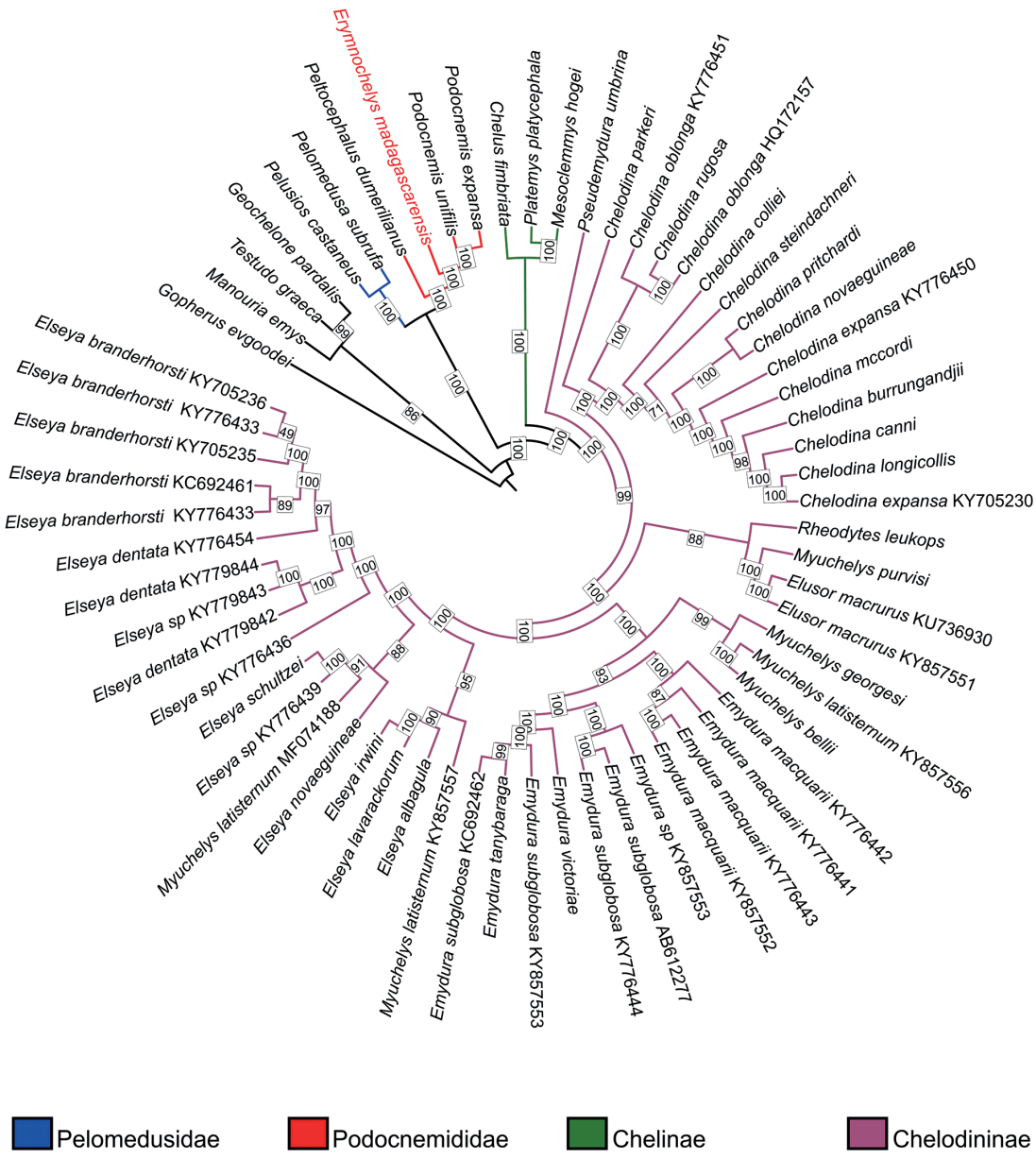

Figure 5. ML phylogenetic tree of Pleurodira inferred in the IQ-TREE employing nucleotide sequences of 13 PCGs. The tree was rooted using *Gopherus evgoodei* (Cryptodira) as outgroup. Ultrafast bootstrap values are indicated at the nodes.

Discussion

Mitochondrial DNA has proven to be a valuable tool for studying various animal groups. This marker allows for the analysis of genetic diversity, the determination of evolutionary relationships, and species identification (Trivedi *et al.* 2016, Georges *et al.* 2018, Carvalho *et al.* 2022).

The unique characteristics of this molecule make it essential for population diversity studies. Due to its non-recombining maternal inheritance pattern, it is possible to identify maternal lineages and introgression events (McDowall 2008, Vogel and Johnson 2008). The high rate of mtDNA substitution enables the identification of single nucleotide polymorphisms (SNPs) and rearrangements (McGuire *et al.* 2023), allowing for the determination of priority haplotypes that can be useful in breeding and conservation programs for threatened groups.

The analysis of *E. madagascariensis'* mitogenome revealed the smallest mitogenome documented among Podocnemididae species. When comparing gene order, CG content, and nucleotide composition, it was found to closely resemble those previously described in the mitogenomes of *P. unifilis* (Zhou *et al.* 2016) and *P. expansa* (Wang *et al.* 2018), reinforcing the evolutionary relationship between *Erymnochelys* and *Podocnemis*.

Within the family Podocnemididae, the CR region spans from 692bp in *P. expansa* (Wang *et al.* 2018) to 985bp in *P. unifilis* (Zhou *et al.* 2016), and this variation is likely attributed to the presence of VNTR. In general, the CR structure and composition in pleurodiran turtles differs considerably from those in Cryptodira. However limited information is available for pleurodiran families, limiting a comprehensive understanding of the CR evolution within this suborder (Bernacki and Kilpatrick 2020).

As the CR is a non-coding region that appears to undergo concerted evolution in Metazoa (Zhang and Hewitt 1997), it is known for its variability in size and composition, even among closely related taxa. Concerning the primary structure of the CR of *E. madagascariensis,* it was possible to identify the presence of TAS regions and the conserved blocks CSB-F and CSB1, which delineate the subdivision of the CR into three domains. These findings corroborate the proposal of Wang *et al.* (2011) and differ from those obtained by Bernacki and Kilpatrick (2020) for the Podocnemididae. While the presence of multiple TASs blocks and the terminal AT-rich VNTR2 appears to be a shared characteristic among different turtle families, our data and that of Bernacki and Kilpatrick (2020) seem to indicate that the absence of CSB2 and CSB3 blocks is a characteristic of pleurodires.

In evolutionary biology, a persisting question centers on the influence of natural selection and environmental factors on the differentiation of mitochondrial genes. Mitochondria play a pivotal role in cellular respiration and aerobic metabolism, imposing significant functional constraints on their genetic material, particularly concerning energy-related processes. A thorough examination of the selective pressures acting upon mitochondrial genes can offer valuable insights into the functional and ecological dimensions of how organisms evolve (Ding *et al.* 2023).

As expected, our analysis demonstrated that most PCGs of *E. madagascariensis* are under purifying selection, except for *ATPase 8*, similar to the results of Escalona *et al.* (2017) for the Cryptodira. Most mitochondrial genes appear to be subject to purifying selection (Shtolz and Mishmar 2019, Ding *et al.* 2023), eliminating harmful mutations and preserving mitochondrial functions. The limited instances of positive selection are often linked to cases of adaptation to new environmental or physiological conditions and are associated with sites of the OXPHOS complex I proteins (Escalona *et al.* 2017, Sahoo *et al.* 2023).

The phylogenetic reconstruction based on the mitochondrial PCGs recovered a sister group relationship between *Erymnochelys* and *Podocnemis*. This differs from the one proposed

by Ferreira *et al.* (2018) based on morphological traits, which placed *Peltocephalus* as a sister group of *Erymnochelys*. However, our findings are supported by the studies of Vargas-Ramirez *et al.* (2008) and Thomson *et al.* (2021), which are based on nuclear and/or mitochondrial data.

The coverage of the pleurodiran genera in our analysis is more comprehensive than that of Vargas-Ramirez *et al.* (2008). While our study includes fewer genera than Ferreira *et al.* (2018), it boasts a more robust species sampling. Evolutionary analysis involving numerous taxa using all PCGs relies on a substantial investment in constructing DNA datasets or utilizing available information from databases. This limitation restricted our ability to include all pleurodiran genera in our study.

Polyphyly observed in the genus *Myuchelys* in our study of the mitogenome was also found by other researchers who used both mitochondrial and nuclear genes in their phylogenetic reconstructions (Fielder *et al.* 2012, Le *et al.* 2013, Georges *et al*. 2018). However, *Myuchelys* was considered monophyletic in the study of Thomson *et al*. (2021), based on 15 nuclear genes. Although Thomson *et al.* (2021) included the most extensive coverage of genera used in evolutionary studies of turtles, they only included two samples each of *M. purvisi* (Wells and Wellington, 1985) and *M. latisternun* (Gray, 1867). Therefore, this limited sampling within these species may not have been sufficient to confirm or refute non-monophyly reported by other researchers.

The identification of polyphyly in evolutionary relationships based on mitochondrial data can be attributed to three possible explanations: (1) misidentification of species; (2) cryptic diversity, as suggested by Le *et al.* (2013), Fielder *et al.* (2012), and Kehlmaier *et al.* (2019); or (3) introgression (Kehlmaier *et al.* 2019). Our results indicate that some individuals of *Myuchelys* are genetically related to *Emydura* and others to *Elseya,* Australian genera found in sympatry with *Myuchelys*. Georges *et al.* (2018) have raised the possibility of hybridization between *M. georgesi* (Cann, 1997) and *Emydura macquarii* (Gray, 1830).

The decline of species can result from anthropogenic actions, such as habitat loss, overexploitation, and climate change. In light of this, many species and genetic groups could face extinction even before they are recognized by science (Kehlmair *et al.* 2019). *E. madagascariensis* is currently at risk of extinction due to anthropogenic activities, but we must also consider another risk to maintaining species' integrity: hybridization.

Considering the detrimental aspects of hybridization that can lead a species toward extinction, two noteworthy consequences are outbreeding depression and genetic swamping (Todesco *et al.* 2016). Our phylogenetic analysis has highlighted the possibility of hybridization among sympatric species in the Chelodininae. As instances of ancient introgression have been identified in the evolutionary history of Australasian turtles (Kehlmaier *et al.* 2019), monitoring the mtDNA these turtles enable us to detect potential introgression events.

White *et al.* (2022) employed microsatellites (SSR) and mitochondrial genes (*CytB* and *COXI*) to assess the genetic variability of *E. madagascariensis*. Their data indicate that *COXI* reveals a division of genetic groups based on north-western and south-western distributions, a pattern not recovered by SSR analysis. The mtDNA analysis also facilitated the identification of haplotypes associated with the watershed where these animals were collected, given that females exhibit fidelity to a specific river for breeding. This finding helps explain the genetic structure observed within the species.

This underscores the utility of mitogenomes in evaluating the genetic diversity of *E. madagascariensis* and other endangered Pleurodira. Utilizing the entire mtDNA molecule proves invaluable for identifying introgression events and cryptic diversity, which have significant implications for conservation efforts, especially in establishing and managing protected breeding areas. Determining haplotypes among natural and captive populations is fundamental to the success of programs that restock and preserve these species.

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Appendix I. *Taxa included in the phylogenetic analysis of the present study.*

Species	Genbank accession	Family/Subfamily
Chelodina burrungandjii	KY776447.1	Chelidae/Chelodininae
Chelodina canni	KY776448.1	
Chelodina colliei	KY776449.1	
Chelodina expansa	KY705230.1	
Chelodina expansa	KY776450.1	
Chelodina longicollis	KJ713173.1	
Chelodina mccordi	KY705231.1	
Chelodina novaeguineae	KY776446.1	
Chelodina oblonga	KY705234.1	
Chelodina oblonga	KY776451.1	
Chelodina parkeri	KY705232.1	
Chelodina pritchardi	KY705233.1	
Chelodina rugosa	HQ172157.1	
Chelodina steindachneri	KY776452.1	
Elseya albagula	KY776453.1	
Elseya banderhorsti	KC692461.1	
Elseya banderhorsti	KY705235.1	
Elseya banderhorsti	KY705236.1	
Elseya banderhorsti	KY776433.1	
Elseya banderhorsti	KY776434.1	
Elseya dentata	KY776454.1	
Elseya dentata	KY779842.1	
Elseya dentata	KY779844.1	
Elseya irwini	KY776435.1	
Elseya lavarackorum	KY776437.1	
Elseya novaeguineae	KY776438.1	
Elseya schultzei	KY776440.1	
Elseya sp.	KY779843.1	
Elseya sp.	KY776436.1	
Elseya sp.	KY776439.1	
Elusur macrurus	KU736930.1	
Elusur macrurus	KY857551.1	
Emydura sp.	KY857553.1	
Emydura subglobosa	KC692462.1	
Emydura subglobosa	KY776444.1	
Emydura subglobosa	KY776445.1	
Emydura subglobosa	AB612277.1	
Emydura tanybaraga	KY857559.1	

Appendix I. *Continued.*

Species	Genbank accession	Family/Subfamily
Emydura victoriae	KY857554.1	Chelidae/Chelodininae
Emydura macquarii	KY776441.1	
Emydura macquarii	KY776442.1	
Emydura macquarii	KY776443.1	
Emydura macquarii	KY857552.1	
Myuchelys bellii	KY924930.1	
Myuchelys georgesi	KY857555.1	
Myuchelys latisternum	KY857556.1	
Myuchelys latisternum	KY857557.1	
Myuchelys latisternum	MF074188.1	
Myuchelys purvisi	KY883378.1	
Peltocephalus dumerilianus	AB970731.1	
Pseudemydura umbrina	KY486272.1	
Rheodytes leukops	KY857558.1	
Chelus fimbriata	HQ172156.1	Chelidae/Chelinae
Mesoclemmys hogei	MF615513.1	
Platemys platycephala	KC692464.1	
Pelomedusa subrufa	AF039066.1	Pelomedusidae
Pelusios castaneus	KC692463.1	
Podocnemis expansa	MF359933.1	Podocnemididae
Podocnemis unifilis	JF802204.1	
Geochelone pardalis	DQ080041.1	Cryptodira (outgroup)
Gopherus evgoodei	CM017320.1	
Manouria emys	DQ080040.1	
Testudo graeca	DQ080049.1	

Appendix II. *The secondary structure of tRNA genes inferred for the mitogenome of* Erymnochelys madagascariensis.