Journal of Tropical Biodiversity and Biotechnology
Volume 10, Issue 01 (2025): jtbb13529
DOI: 10.22146/jtbb.13529

Research Article

The Complete Mitochondrial Genome of Critically Endangered Painted Terrapin, *Batagur borneoensis* (Testudines: Geoemydidae)

Nor Ainsyafikah Madiran[1], Han Ming Gan[2], Shahreza Md Sheriff[3], Aliyu Garba Khaleel[4], Muhammad Zaid Nasir[5], Chik Maslinda Omar[5], Mohamad Zulkarnain Mohd Dali[1], Muhammad Syafiq Aiman Mohd Nasir[1], Norshida Ismail[1], Ahmad Syazni Kamarudin[1]*

1)School of Animal Science, Aquatic Science and Environment, Faculty of Bioresources and Food Industry, UniSZA Besut Campus, 22200 Besut, Terengganu, Malaysia
2)Patriot Biotech Sdn Bhd, Bandar Sunway, 47500, Subang Jaya, Selangor, Malaysia
3)Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu, Kuala Nerus, Terengganu, Malaysia
4)Department of Animal Science, Faculty of Agriculture and Agricultural Technology, Kano University of Science and Technology, Wudil, P.M.B. 3244 Kano State, Nigeria
5)WWF Setiu Field Office, Lot 10575, Tanah Lot, Kg. Pagar Besi, 21020 Kuala Terengganu, Terengganu, Malaysia
* Corresponding author, email: ahmadsyazni@unisza.edu.my

Keywords:
Batagur borneoensis
Conservation
Genetic diversity
Mitogenome
Next-generation sequencing
Submitted:
26 May 2024
Accepted:
06 August 2024
Published:
24 February 2025
Editors:
Miftahul Ilmi
Liya Audinah

ABSTRACT

Characterising mitochondrial genomes is a key to studying evolution in vertebrates including turtles. This study employed Next-Generation Sequencing (NGS) to characterise mitochondrial DNA sequences in *Batagur borneoensis* (Schlegel & Muller, 1844). We reported the nearly complete mitogenome to clearly characterise the gene sequence of *B. borneoensis* which has been deposited in GenBank under the accession number PP228865. Phylogenetic analyses using Maximum Likelihood (ML) on the 13 protein-coding genes were conducted with MEGA X Version 11 software. This study presents the second in-depth analysis of the *B. borneoensis* mitochondrial genome, spanning 16,397 base pairs and containing 13 protein-coding genes, 22 transfer RNAs (tRNAs), two ribosomal RNAs (rRNAs), and a major non-coding region, two non-coding regions: L-strand origin replication (OL) and control region (OH). The sequence length and organisation of this species' mitochondrial genome fall within the typical range and gene arrangement found in vertebrate species. Most genes, except for seven tRNAs and *nad6*, were encoded on the primary DNA strand. All protein-coding genes (PCGs) began with an ATG initiation codon, except for *cox1* and *trnF* which started with GTG codon, and *nad3_0*, started with a TTA codon. These findings enhanced our understanding of nucleotide composition and molecular evolution in the genus *Batagur*. Phylogenetic analyses identified vulnerable and ecologically important species, aiding biodiversity and ecosystem protection. They also expanded the dataset for comparative studies within the Geoemydidae family. Additionally, this research may help develop primers and conservation strategies for future studies.

How to cite:
Madiran, N.A. et al., 2025. The Complete Mitochondrial Genome of Critically Endangered Painted Terrapin, Batagur *borneoensis* (Testudines: Geoemydidae). *Journal of Tropical Biodiversity and Biotechnology*, 10(1), jtbb13529. doi: 10.22146/jtbb.13529

INTRODUCTION

The Painted Terrapin *(Batagur borneoensis)* is an aquatic turtle characterised by its hard shell. Other than Malaysia (Peninsular and Borneo), the species also can be found inhabiting Indonesia in Sumatra and southern Thailand. Notably, significant populations of this species are identified in a few rivers in Terengganu, Melaka and Negeri Sembilan (Kolandaiveloo et al. 2020). As a result of live trade, painted terrapins are now distributed worldwide. The Turtle Survival Coalition lists the painted terrapin (*B. borneoensis*) as Critically Endangered and among the 25 most imperilled tortoises and freshwater turtles globally. Population in Indonesia and Malaysia are uncertain, with Malaysia seeing a severe decline, justifying its status on the IUCN Red List and CITES Appendix II. The species faces rapid decline due to habitat degradation, coastal development, local consumption, and international trade (Toomey 2016; Kolandaiveloo et al. 2020). In Indonesia, it is also categorised as a Critically Endangered under IUCN 2010 and CITES Appendix II according to Hernawan et al. (2019). Previous studies produced and analysed molecular data to clarify the identity and evolutionary relationship of Geomydidae groupings (Kundu et al. 2020). The ongoing evolution of living organisms defies quantification through a singular speciation theory. Multiple biological and environmental factors intricately contribute to genetic modifications across generations, resulting in the transformation of genes in descendant species from those of their ancestral populations (Kundu et al. 2020). Aside from natural selection, genetic traits within a population undergo frequent alterations at random in response to a number of biotic and abiotic events, resulting in a species' evolutionary dynamics. The utilisation of gene sequences has been pivotal in elucidating the phylogenetic relationships and evolutionary mechanisms inherent within earth's biota, with a particular focus on reptiles. Among these taxa, Testudines, comprising turtles, tortoises, and terrapins, stand out as among the most ancient extant lineages, characterised by an extensive evolutionary chronicle (Lourenço et al. 2012). Meanwhile, a next-generation sequencing (NGS) is a powerful tool in genomics research that can simultaneously sequence millions of DNA fragments, offering detailed insights into genome structure, genetic variations, gene activity, and changes in gene behaviour (Satam et al. 2023). Mitochondrial DNA (mtDNA) barcoding has significantly simplified species identification and assessment of phylogenetic relationships, especially for morphologically similar, closely related taxa. This technique uses a specific portion of the mitochondrial genome to distinguish species with wide-ranging distributions (Sokefun 2022). As a result, a complete mitogenome from this study will be helpful in understanding the genus *Batagur's* deep evolutionary branching and primer design in the future. To date, despite the numerous species in the *Batagur* genus, only mitogenomes of six species in which; *B. borneoensis*, *Batagur affinis affinis* (Cantor, 1847), *Batagur affinis edwardmolli* Praschag, Holloway, Georges, Päckert, Hundsdörfer & Fritz 2009, *Batagur kachuga* (Gray, 1831), *Batagur dhongoka* (Gray, 1834) and *Batagur trivittata* (Duméril & Bibron, 1835) have been reported in GenBank (OQ808844, OQ409915, OQ645446, NC 069558, NC069559, NC032300 and KX817298).

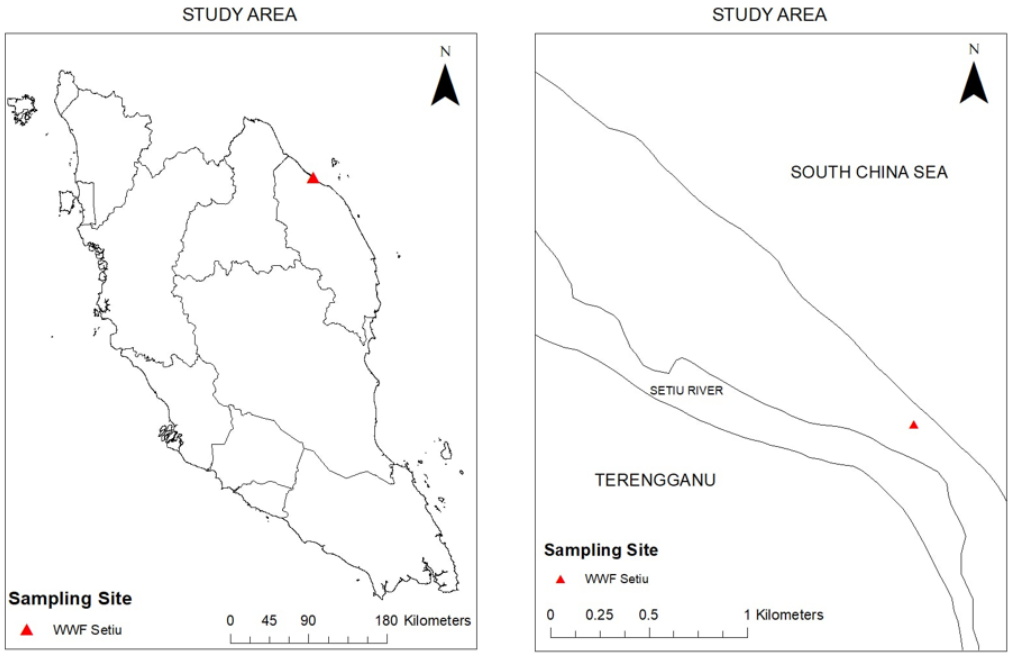
Despite a previous mitochondrial genome sequence of *B. borneoensis* available in GenBank (OQ808844), the sequence reported in this study originates from a distinct geographic location and different life stage, providing new insights into mitochondrial variability and adaptation within the species. Therefore, the objective of this study is to characterise the complete mitochondrial genome of critically endangered *B. borneoensis* specifically from the Setiu River, Terengganu. Thus, this study is the first publication on the complete mitochondrial genome of the critically endangered Painted Terrapin, *B. borneoensis*.

MATERIALS AND METHODS

Sample collection and mitochondrial DNA Extraction

In this study, we employed the genome skimming method to obtain the near-complete mitochondrial genome (Figure 1). The tissue sample was acquired from the World Wide Fund for Nature (WWF-Malaysia) Hatchery in Kampung Penarik Setiu, Terengganu (Figure 2a and 2b). On November 15th 2020, the deceased sample was taken from a recently hatched *Batagur's* egg reared at the WWF Hatchery (5.641482°N, 102.75159°E) and deposited at the Universiti Sultan Zainal Abidin (UniSZA) repository under the voucher number UniSZA/Reptile/01/2022. The survey was carried out with the previous approval of the Wildlife Authority granted to the World Wide Fund for Nature (WWF-Malaysia) and Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Research Ethics Committee, UAPREC (Letter No: UAPREC/06/009). All experiments were carried out in conformity with the relevant standards and legislation. A tissue sample weighing 20-50 mg was obtained from the carcass of a dead body juvenile terrapin and utilised for DNA extraction using the WizPrep gDNA Mini Kit (WizBio, Korea), following the manufacturer's guidelines.

Figure 1. A juvenile Painted Terrapin (*B. borneoensis*) from Setiu River, Kg. Penarik Setiu, Terengganu (own photo).



(a) (b)

Figure 2. (a) Map of Peninsular Malaysia, red triangle represents the location of Setiu, Terengganu on the east coast of Malaysia. (b) Map showing the sampling site where a juvenile terrapin carcass was collected by WWF Setiu, located at the Setiu River in Kg. Penarik, Setiu, Terengganu.

RNAse treatment and DNA Measurement

Approximately 2 mL of the DNA blood samples underwent treatment with 1 µL of 10 mg mL^{-1} RNAse at room temperature for 30 minutes, then purified with a 1x volume of SPIR bead (Oberacker et al. 2019). Then, 2 µL of pure DNA was examined with a Denovix high-sensitivity kit (Denovix, Wilmington, Delaware).

Illumina library preparation and partial genome sequencing

About 100 mg of DNA were fragmented into 350 bp fragments using a Bioruptor, followed by NEB Ultra II library preparation (NEB, Ipswich, MA) as per the manufacturer's instructions. Sequencing was conducted on a NovaSEQ6000 (Illumina, San Diego, CA) with a run configuration of 2 x 150 bp, resulting in approximately 1 GB of data per sample.

Assembly and Annotation

The total 16,397 base pairs of raw reads underwent trimming to remove low-quality bases and Illumina adaptor sequences using FASTP v0.21 (Chen et al. 2018). Trimmed readings were used for de novo assembly in MegaHIT-default settings (Li et al. 2015). MitoZ was used to identify and circularise the contigs originating from mitochondria. The circularised fish mitogenome was uploaded to MitoAnnotator (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html) for mitogenome re-orientation and annotation (Iwasaki et al. 2013). The circularised mitogenome of non-fish was re-oriented using a reference genome and annotated with MITOS (Bernt et al. 2013).

Phylogenetic Reconstruction

The complete mitogenome of *B. borneoensis* specimens obtained in this present study was analysed for a phylogenetic relationship with six complete mitogenomes of turtle species under the *Batagur* genus available from GenBank with three out group from other genera namely *Pangshura sylhetensis* Jerdon, 1870, *Pangshura tentoria* (Gray, 1834) *and Mauremys mutica* (Cantor, 1842). The

ClustalW tool in MEGA X version 11 was used to compare and align voucher sequences from GenBank with consensus sequences derived from this investigation for each species (Kumar et al. 2018). A Maximum Likehood (ML) trees were constructed using IQ-TREE multicore version 1.6.12 for Linux 64-bit with the TIM2+F+G4 as the best-fit substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017) chosen according to Bayesian Information Criterion (BIC) and visualized using software FigTree v1.4.4 (Rambaut 2018)

RESULTS AND DISCUSSION

Sequence Variation

The secondary intact mitochondrial genome of *B. borneoensis* (16,397 bp) was subjected to analysis and annotation. The assembly encompassed 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, and two non-coding regions: L-strand origin replication (OL) and control region (OH). Aside from NADH dehydrogenase subunit 6 (nad6) and seven tRNA, the majority of genes were found to be encoded on the sense (majority) strand.

The mitochondrial genome (16,397 bp) of the endangered painted terrapin, *B. borneoensis* was submitted to GenBank and assigned an accession number PP228865 in the current research. The mitogenome contains 37 genes, comprising 13 mitochondrial PCGs *in B. borneoensis; the* standard start codon (ATG) and six stop codons (AGG, TAA, AGC, GAA, TAG, and AGA) are utilised for translation initiation and termination (Figure 3). The start codon ATG is employed in all PCGs except for cox1 (GTG) and *nad3_0* (TTA), while 22 PCGs use anticodons, including *trnS2*, *trnD*, *TmK*, *trnG*, *trnR*, *trnH*, *trnS1*, *trnL1*, *trnE*, *trnT*, *trnP*, *trnV*, *trnL2*, *trnL*, *trnI*, *trnQ*, *trnM*, *trnW*, *trnA*, *trnN*, *trnC*, and *trnY*. Six PCGs (cox2, atp8, atp6, cox3, nad4, and cob) use the stop codon TAA, while cox1 employs AGG, *nad3_1* uses AGC, *nad3_0* utilises GAA, *nad6* employs AGA, and TAG is only present in *nad5*, *nad1*, and *nad2*.

Control region (CR)

Non-coding regions in the metazoan mitogenome consist of the origin of replication (OR), intergenic spacers, and the control region (CR). The mitochondrial OL of *B. borneoensis* is 27 base pairs located between 5145-5306 regions, which consist of a cluster of trnN (73bp) and trnC (66bp) genes with the CR comprising 883 base pairs situated between 15515-16397 (Figure 3).

The overall A + T content of the mitogenome was higher (58.0 %) than the G + C content (42.0 %) which is typical for a mitogenome sequence. The protein-coding genes *COX1*, *trnF*, and *nad3* start with codons other than ATG. Most protein-coding genes end with the stop codon TAA. Twelve protein-coding genes terminate with complete stop codons (AGG, AGC, AGA, TAA, and TAG), while the remaining three end with TA as partial stop codons, which are assumed to be completed to TAA by post-transcriptional polyadenylation (Anderson et al. 1981). Most mitochondrial genes in *B. borneoensis* are encoded on the H-strand, except for the ND6 gene and eight tRNA genes. The trnC(gca) gene is the shortest among the mitochondrial protein-coding genes, while the ND5 gene is the longest. The 12S and 16S ribosomal RNAs are 965 and 1601 base pairs long, respectively.

The mitochondrial replication origin (OR) plays a crucial role in mitogenome replication. Non-coding regions within the metazoan mitogenome are indispensable for the processes of DNA replication and preservation (Fernandez-Silva et al. 2003), encompassing the origin of replication (OR), diverse intergenic spacers, and the control region (CR). The process of polyadenylation at the 3'-end of mRNA, occurring after transcription, typically

serves to complete incomplete stop codons (Boore 1999). However, our study did not uncover evidence of the polyadenylation process, suggesting that the absence of stop codons or overlaps between protein-coding genes (PCGs) may have arisen due to selective pressures aimed at reducing the size of the mitochondrial genome.

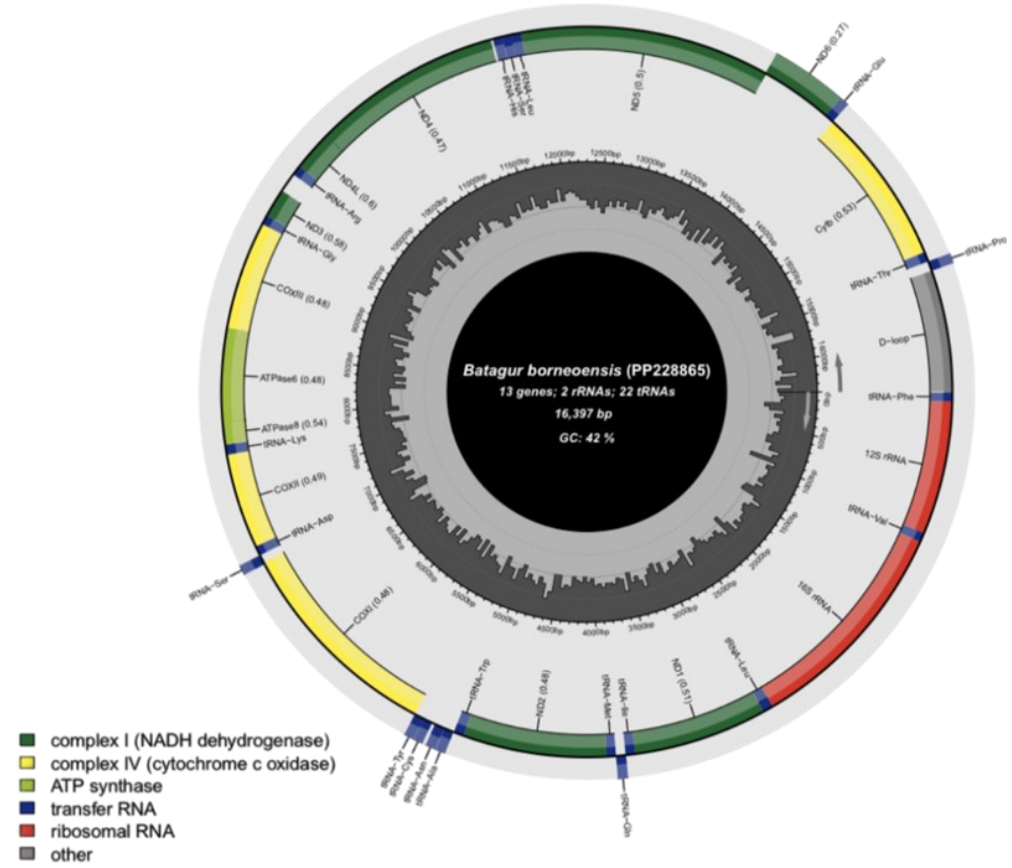


Figure 3. A novel genome map of the Malaysian *B. borneoensis* mitochondrial genome.

Phylogenetic Tree Analysis

There was a total of 22381 positions in the final dataset. The sequences used in this study including seven ingroups species, *B. borneoensis*, *B. trivittata*, *B. dhongoka*, *B. kachuga*, *B. affinis* and three outgroups, *P. sylhetensis*, *P. tentoria* and *M. mutica*.

The ML phylogenetic tree (Figure 4) offers insights into the evolutionary relationships among *Batagur* species, resulting in a well-resolved tree with all species supported with high bootstrap values. Figure 4 illustrates the construction of a TIM2+F+G4 as the best-fit substitution model selected by ModelFinder (Kalyaanamoorthy et al. 2017) using the dataset and IQ-TREE (Nguyen et al. 2015). According to the clustering in the phylogenetic tree, *B. borneoensis* forms a distinct group, with *B. trivittata* is the sister species, suggesting a close evolutionary relationship between these two species but relatively distant from other *Batagur* species in terms of evolutionary distance. Meanwhile, *B. affinis affinis* and *B. kachuga* share the closest evolutionary relationship. Unlike the conventional CO1 or CytB partial gene sequences, the full mitogenome provides a significantly higher number of variable sites, which will be valuable for delineating the evolutionary relationships of *Batagur* species in the future.

This research utilises comprehensive mitochondrial DNA data and bioinformatic analysis to investigate the evolutionary position and taxonomic classification of *B. borneoensis*. Mitochondrial genome has proven to be a valu-

able avenue for conducting phylogenetic and evolutionary investigations in vertebrates, including turtles (Kundu et al. 2019). Several studies have shown that phylogenies derived from mitogenome provide more robust insight than to those obtained from a single gene (Zhang et al. 2021). In addition, public Whole-Genome Sequencing (WGS) datasets which include numerous mitogenome sequences, are highly valuable across various field, including population research, disease association studies, and conservation genetics. Furthermore, the data supplied by WGS may also be relevant in other mitochondrial DNA (mtDNA) studies (Sturk-Andreaggi et al. 2022).

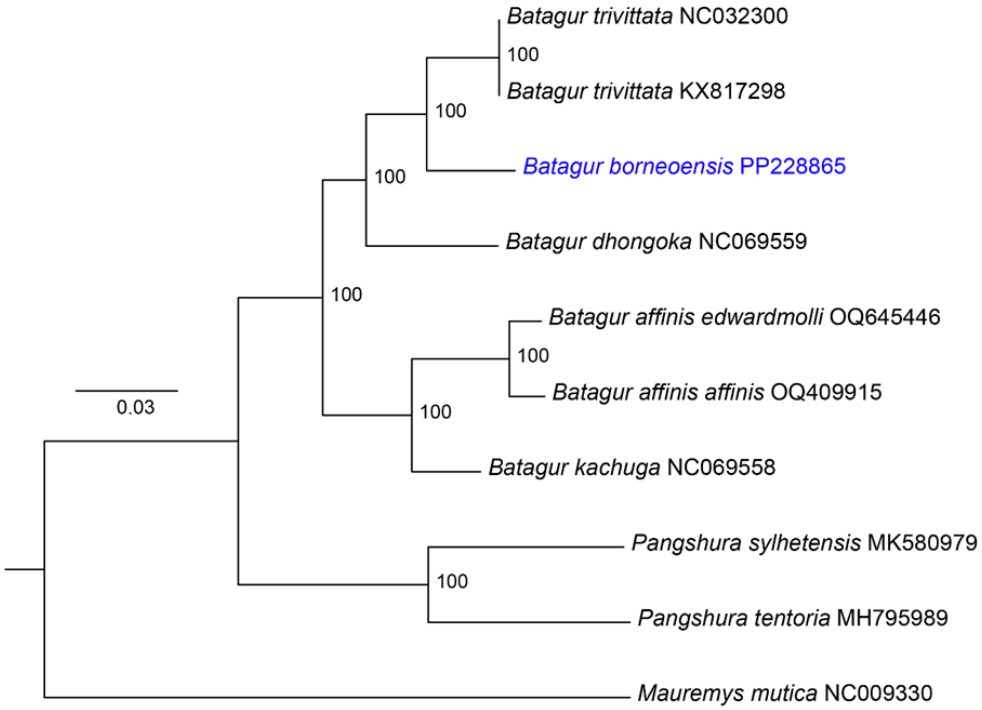


Figure 4. A Maximum Likehood phylogenetic tree from ten complete mitogenome of species in the Geomydidae family.

CONCLUSIONS

In conclusion, our study unveiled the gene organisation and features of the complete mitochondrial genome of *B. borneoensis*, marking the second characterisation of this species' entire mitogenome. Spanning 16,397 bp, the *B. borneoensis* mitogenome forms a circular molecule encompassing a regulatory region and the standard complement of 37 vertebrate mitochondrial genes. Its genetic arrangement and structural organisation closely resemble standard configurations found in other turtles mitogenome. These insights significantly contributed to our understanding of nucleotide composition and molecular evolution within *B. borneoensis* mitogenomes, providing essential data for comparative mitogenomics and advancing phylogenetic analyses within the Geomydidae family.

AUTHOR CONTRIBUTION

Conceptualisation, H.M.G, A.S.K, N.A.M; writing and laboratory work, N.A.M and M.S.A.M.N and A.G.K.; sampling and providing sample collection, M.Z.N and C.M.O; supervised and drafting, A.S.K and N.I; project administration, M.Z.M.D and S.M.S. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

We would like to express our biggest gratitude to Ministry of Higher Education (MOHE) for partially providing funding support for this research

through Fundamental Research Grant Scheme project (FRGS/1/2024/SSI03/UNISZA/02/3). We would also like to thank Nagao Natural Environment Foundation (UniSZA/2023/PPL/NEF (021)) and World Wide Fund for Nature (WWF-Malaysia) for the endless opportunity and support towards us through this research.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Anderson, S. et al., 1981b. Sequence and organization of the human mitochondrial genome. *Nature*, 290(5806), pp.457–465. doi.org/10.1038/290457a0

Bernt, M. et al., 2013. MITOS: Improved de novo metazoan mitochondrial genome annotation. *Molecular Phylogenetics and Evolution*, 69(2), pp.313–319. doi: 10.1016/j.ympev.2012.08.023

Boore, J.L., 1999. Animal mitochondrial genomes. *Nucleic Acids Research*, 27 (8), pp.1767–1780. doi: 10.1093/nar/27.8.1767

Chen, Y. et al., 2018. SOAPnuke: a Map Reduce acceleration-supported software for integrated quality control and preprocessing of high-throughput sequencing data. *GigaScience*, 7(1), pp.1-6. doi: 10.1093/gigascience/gix120.

Fernández-Silva, P., Enriquez, J.A. & Montoya, J., 2003. Replication and transcription of mammalian mitochondrial DNA. *Experimental physiology*, 88 (1), pp.41-56. doi: 10.1113/eph8802514.

Hernawan, E. et al., 2018. Literature Review: Distribution, Ecology, History and Conservation of Painted Terrapin (Batagur borneoensis Schlegel and Muller 1845) in Indonesia. *International Journal of Sciences: Basic and Applied Research*, 42(5), pp.199-209.

Iwasaki, W. et al., 2013. MitoFish and MitoAnnotator: A mitochondrial genome database of fish with an accurate and automatic annotation pipeline. *Molecular Biology and Evolution*, 30(11), pp.2531–2540. doi: 10.1093/molbev/mst141

Kalyaanamoorthy, S. et al., 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14, pp.587–589. doi: 10.1038/nmeth.4285

Kolandaiveloo, V. et al., 2020. Endoscopy gender determination and reproductive hormone profiles of Painted Terrapins (*Batagur borneoensis*) subjected to *ex situ* incubation. *Journal of Veterinary Medical Science*, 82(4), pp.497–502. doi: 10.1292/jvms.19-0477

Kumar, S. et al., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35(6), pp.1547–1549. doi: 10.1093/molbev/msy096

Kumar, S., Stecher, G. & Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), pp.1870–1874. doi: 10.1093/molbev/msw054

Kundu, S. et al., 2019. The first complete mitochondrial genome of the Indian Tent Turtle, Pangshura tentoria (Testudines: Geoemydidae): Characterization and comparative analysis. *Ecology and Evolution*, 9(18), pp.10854–10868. doi: 10.1002/ece3.5606

Kundu, S. et al., 2020. The complete mitochondrial genome of the endangered Assam Roofed Turtle, Pangshura sylhetensis (Testudines: Geoemydidae): Genomic features and phylogeny. *PLoS ONE*, 15(4), e0225233. doi: 10.1371/journal.pone.0225233

Li, D. et al., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics*, 31(10), pp.1674–1676. doi: 10.1093/bioinformatics/btv033

Lourenço, J.M. et al., 2012. Dating cryptodiran nodes: Origin and diversification of the turtle superfamily Testudinoidea. *Molecular Phylogenetics and Evolution*, 62(1), pp.496–507. doi: 10.1016/j.ympev.2011.10.022

Nei, M. & Kumar, S., 2000. *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Nguyen, L.T. et al., 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology and Evolution*, 32(1), pp.268-274. doi: 10.1093/molbev/msu300

Oberacker, P. et al., 2019. Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation. *PLoS Biology*, 17(1), e3000107. doi: 10.1371/journal.pbio.3000107

Rambaut, A., 2018. *FigTree v1.4.4*. Institute of Evolutionary Biology, University of Edinburgh.

Satam, H. et al., 2023. Next-Generation Sequencing Technology: Current Trends and Advancements. *Biology (Basel)*, 12(7), 997. doi: 10.3390/biology12070997. Correction published in *Biology (Basel)*, 13(5), pp.286. doi: 10.3390/biology13050286.

Sokefun, O., 2022. DNA barcode analysis of the endangered green turtle (Chelonia mydas) in the breeding grounds of Badagry, Lagos, Nigeria. *Acta Entomology and Zoology*, 3(2), pp.40–43. doi: 10.33545/27080013.2022.v3.i2a.75

Sturk-Andreaggi, K. et al., 2022. The Value of Whole-Genome Sequencing for Mitochondrial DNA Population Studies: Strategies and Criteria for Extracting High-Quality Mitogenome Haplotypes. *International Journal of Molecular Sciences*, 23(4), 2244. doi: 10.3390/ijms23042244

Tamura, K., Stecher, G. & Kumar, S., 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), pp.3022-3027. doi: 10.1093/molbev/msab120.

Toomey, D., 2016, 'Pulling the stunningly unique painted terrapin back from the brink', in *Mongabay News & Inspiration from Nature's Frontline*, viewed from *https://news.mongabay.com/2016/08/pulling-the-stunningly-beautiful-painted-terrapin-back-from-the-brink/*

Zhang, L. et al., 2021. Complete mitochondrial genomes reveal robust phylogenetic signals and evidence of positive selection in horseshoe bats. *BMC Ecology and Evolution*, 21(1), 199. doi: 10.1186/s12862-021-01926-2