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Research Article

Mitochondrial Genetic Diversity in a Captive Malaysian Giant Turtle (*Orlitia borneensis*) Population: Implications for Conservation Breeding

Andreas Bandang Hardian[1,2*], Irhamna Putri Rahmawati[1], Josephine Vanda Tirtayani[3], Nathan Rusli[4], Berta Alviyanto[3], Randy Kusuma[3], Adwitiya Irene Kumaralalita[3], Danuja Widigdaya[3], Safira Kinanti Pribadi[5], Tania Salsabila Utari[5], Fitri Ekasari[5], Anastasia Cahyakusuma Wahyuningtyas[5], Rini Widayanti[6], Herdhanu Jayanto[7]

[1]*Yayasan Konservasi Alam Yogyakarta-Wildlife Rescue Centre Jogja, Yogyakarta 55167, Indonesia*
[2]*Laboratory of Veterinary Anatomic Pathology, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang 65151, Indonesia*
[3]*Gembira Loka Zoo, Jalan Kebun Raya No. 2, Yogyakarta 55171, Indonesia*
[4]*Indonesia Herpetofauna Foundation, Bogor 16923, Indonesia*
[5]*Bachelor of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang 65151, Indonesia*
[6]*Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[7]*KONKLUSI - Kolaborasi Inklusi Konservasi (Yayasan), Sleman, Yogyakarta 55284, Indonesia*

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ABSTRACT

Generating genetically sustainable assurance colonies of critically endangered Malaysian giant turtles (*Orlitia borneensis*) for in situ genetic rescue and population reinforcement initiatives requires intensive exploration of genetic profiles, beyond merely providing reproductive viable individuals. When a pedigree and history report are unavailable, a conservation breeding programme requires additional genetic information to assess the individual's relatedness and prevent inbreeding and crossbreeding. We proposed a genetic-distance-based parental candidate determination using sequence analyses of mitochondrial ATP synthase subunits 8 and 6 (ATP86), cytochrome c oxidase subunit 1 (CO1), cytochrome B (CytB), and NADH dehydrogenase subunit 4 (ND4). Our study aimed to explore population structure, haplotypes, presumptive genetic localities, and potential genetic markers to identify individuals as potential founder and parental stockfor breeding. Our primers successfully amplified partial sequences of the aforementioned encoding genes. Sequence analyses and median-joining haplotype computation revealed potential geographic localities and haplotypes, indicating the presence of conserved populations of the Malaysian giant turtles in Malaysia and Indonesia. Moreover, there was evidence of the establishment of island-based localities in the Sumatra-Belitung cluster population. Our study suggested that more haplotypes and localities, based on Malaysian giant turtle genetics, need to be considered in the captive breeding programme. These genetic data provide a critical basis for optimizing pairing strategies among breeding individuals, reducing the risk of inbreeding and supporting the retention of genetic diversity within the Malaysian giant turtle (*Orlitia borneensis*) population.

1. Introduction

Malaysian giant turtles (*Orlitia borneensis*), known regionally as the Malayan giant turtle, are among the most threatened turtles in the world. This species is distributed throughout western Peninsular Malaysia, with the vast majority of its range in eastern Sumatra and Kalimantan, Indonesia. It has faced multiple anthropogenic threats, including excessive hunting and consumption, and habitat fragmentation from land-use

*Corresponding Author
E-mail Address: andreasbandangh@ub.ac.id

conversion, for more than two decades. This resulted in it being listed as a protected species in Indonesia since 1999 and categorized as Endangered (EN) on the IUCN Red List in 2003, before being upgraded to Critically Endangered (CR) in 2020 (Horne *et al.* 2020). Despite its critical status, this species remains poorly studied, further hindering the effectiveness of existing conservation efforts. Since its first description by Gray (1873), only about eight studies have examined the genetics of this species, with an additional twelve studies using it merely as an outgroup comparator. Ecological and veterinary research on this species is also remarkably scarce. Although this species has been listed as an Evolutionarily Distinct and Globally Endangered (EDGE) species and serves as a complementary indicator for Target 4 of the Kunming-Montreal Global Biodiversity Framework, the current level of conservation attention suggests that its recovery potential remains critically low.

Freshwater turtles or terrapins play an important role as scavengers in aquatic environments, removing decomposing organic matter and thereby maintaining water quality for natural fish hatching (Santori *et al.* 2020; Piczak *et al.* 2025). They also help reduce nitrogenous compound deposits resulting from decomposition, thereby mitigating the risk of algal blooms (Santori *et al.* 2020). Several studies also reported that terrapins contribute to seed dispersal in aquatic ecosystems, propagating aquatic plants that provide shelters for numerous fish and other organisms (Calviño-Cancela *et al.* 2007; Tulipani & Lipcius 2014; Falcón *et al.* 2020). Considering the potential ecological role of the Malaysian giant turtle in providing such ecosystem services, there is an urgent need to prioritize its conservation.

Although *Orlitia borneensis* is legally protected throughout most of its range and occurs within several protected areas, its population continues to decline (Horne *et al.* 2020). Ex situ conservation efforts have been initiated both within and outside its native range. Successful captive breeding through egg incubation and hatching has been reported in a zoo (Brockman *et al.* 2013), and additional unreported breeding successes are known to have occurred in Indonesia. These achievements highlight the potential for establishing assurance colonies to support future population restocking as part of a One-Plan conservation approach. However, many existing captive populations lack basic information on the geographic origins of their individuals, raising concerns about appropriate genetic pairing within assurance colonies. Moreover, decisions regarding suitable translocation sites remain unaddressed. Given the species' broad distribution along the eastern coast of Sumatra, the Bangka Belitung Islands, and Borneo (Horne *et al.* 2020), it is highly likely that prolonged geographic isolation has led to the development of distinct genetic localities among *O. borneensis* populations in Indonesia.

Genetic quality is a crucial pillar in maintaining genetic diversity and lineage purity in conservation breeding. Genetic analyses provide valuable references for assessing relatedness among individuals, identifying potential parental stocks based on their geographical origins, and preventing both inbreeding and unintended crossbreeding (Taylor 2015; Wellmann & Bennewitz 2019). For example, inbreeding has been widely reported to induce morphological and physiological defects that greatly affect the fitness of species in nature (Crnokrak & Roff 1999; Keller & Waller 2002; Adavoudi & Pilot 2021; Moran *et al.* 2021). Genetic rescue by introducing new individuals with a relatively distant genetic profile to a genetically saturated population might be an option to enrich the genetic richness pool, preventing inbreeding and defective traits from being expressed in the offspring (Frankham 2015; Supple & Shapiro 2018; Kyriazis *et al.* 2024; Onorato *et al.* 2024). In these cases, specific genetic markers generated by targeted gene amplification primers are pivotal for determining the parental candidates. Salleh and Esa (2024) reported a complete mitochondrial DNA (mtDNA) sequence of MGT from Malaysia, which is valuable for the Malaysian giant turtle's ancestral study. The other Malaysian giant turtle's encoding gene ever studied are including cytochrome B (CytB), CO1, NADH dehydrogenase subunit 4 (ND4), R35, Cmos, Rag1, Rag2, 12S rRNA, 16S rRNA, and control region (CR) (Wu *et al.* 1998; Honda *et al.* 2002; Feldman *et al.* 2004; Le *et al.* 2007; Hsieh *et al.* 2008; Reid *et al.* 2011; Palupcikova *et al.* 2012; Salleh *et al.* 2023).

Our study aimed to explore the population genetic diversity in small representative sample size from captive population and find any single nucleotide polymorphism as potential genetic marker based on selected mitochondrial encoding genes. We developed primers for targeted mitochondrial DNA (mtDNA) encoding gene amplification to profile the genetics of the Malaysian giant turtle (*Orlitia borneensis*) using conventional PCR and Sanger sequencing. Although numerous protein-coding genes exist within the mtDNA, not all contain genetic markers suitable for identifying individuals based on their localities (Harrison 1989; Mitra *et al.* 2018). While Sanger sequencing has lower

throughput compared to whole-genome sequencing, it provides excellent accuracy and resolution for shorter PCR products (Harrison 1989; Mitra *et al.* 2018). Thus, performing targeted amplification followed by Sanger sequencing offers a cost-effective and practical approach for conservation applications, particularly in species identification, translocation planning, and captive breeding programmes.

2. Materials and Methods

2.1. Permit and Ethics

This study was performed under the research permit document No. 3 Tahun 2025 and supervision by the Ministry of Forestry Republic Indonesia. All procedures conducted in this study was approved by Research Ethics Commission Faculty of Veterinary Medicine Universitas Gadjah Mada according to ethical clearance document No. 33/EC-FKH/int./2025.

2.2. Sampling Design and Collection Protocol

Our turtle inventory included approximately 57 individuals, which were grouped by estimated age, sex, and institutional origins. However, no locality origin information was available for any of the institutions. Of the 20 individuals classified as adults according to the body size and available health records, nine individuals–including one female–were randomly selected for sample collection. These nine individuals were considered physically healthy and designated as potential parental stock for future captive breeding following comprehensive health examinations. An additional sample was obtained from a Belitung Island individual with confirmed locality data and collection records.

For each individual, at least 0.5 mL blood was collected using a 23-gauge needle and 3-mL-syringe and preserved in 70% ethanol for subsequent genomic DNA extraction. Blood samples were primarily collected from the hind limb via the femoral vein, located between the gastrocnemius and fibularis longus muscles or between the fibularis longus and fibularis brevis muscles (Figure 1). Because vessel positioning can vary from the lateral to the medial aspect of the hind limb, adjusting the turtle's position—either in dorsal or ventral recumbency—was sometimes necessary to optimise access to the collection site. Blood samples preserved in 70% ethanol were stored in 1.5 mL microcentrifuge tubes sealed with elastic tape to prevent ethanol evaporation, labelled, and kept at room temperature. All samples were subsequently recorded and annotated for further analyses (Table 1).

2.3. Primer Design

We targeted four encoding genes from mitochondrial DNA (mtDNA) to be amplified through conventional polymerase chain reaction (PCR) followed by Sanger sequencing: mitochondrial ATP synthase subunit 8 and 6 encoding gene (ATP86), cytochrome c oxidase subunit 1 (CO1) encoding gene, cytochrome B (CytB) encoding gene, and NADH dehydrogenase subunit 4 (ND4) encoding gene. Reference genomes of *Orlitia borneensis* were obtained from the National Center for Biotechnology Information (NCBI) database with accession number NC_082188.1 and OQ808845.1 as provided by Salleh and Esa (2024).

All primers were designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3Plus (https://www.primer3plus.com/index.html) with the following settings: minimum product size at 1000 base pairs (bp), primer length between 20-24 bp, melting temperature (Tm) between 45-55°C, guanine-cytosine (GC%) content between 40-60°C; and at least 100 bp of flanking sequence on both sides of targeted gene region. Primer pairs with the lowest self-complementarity and self-3′-complementarity scores were selected. The final primer pairs and corresponding oligonucleotide sequences are presented in Table 2.

We performed an in-silico testing to see the specificity and priming site of our primer pairs using NCBI Nucleotide BLAST® platform. The pictorial attachment sites and predicted amplicon coverage were presented in Figure 2. The predicted amplicon size of ATP86, ND4, CO1, and CytB encoding gene primers are consecutively 968 bp, 1800 bp, 1001 bp, and 1061 bp.

2.4. DNA Extraction, Amplification, and Sequencing

The collected blood samples preserved in 70% ethanol underwent coagulation; therefore, the samples were gently resuspended through soft grinding, and approximately 50 µL of each was used for DNA extraction. Genomic DNA was isolated using the Zymo Research Quick-DNA™ Miniprep Plus Kit following the manufacturer's protocol, with a modified overnight incubation at 56°C during the lysis phase to enhance yield.

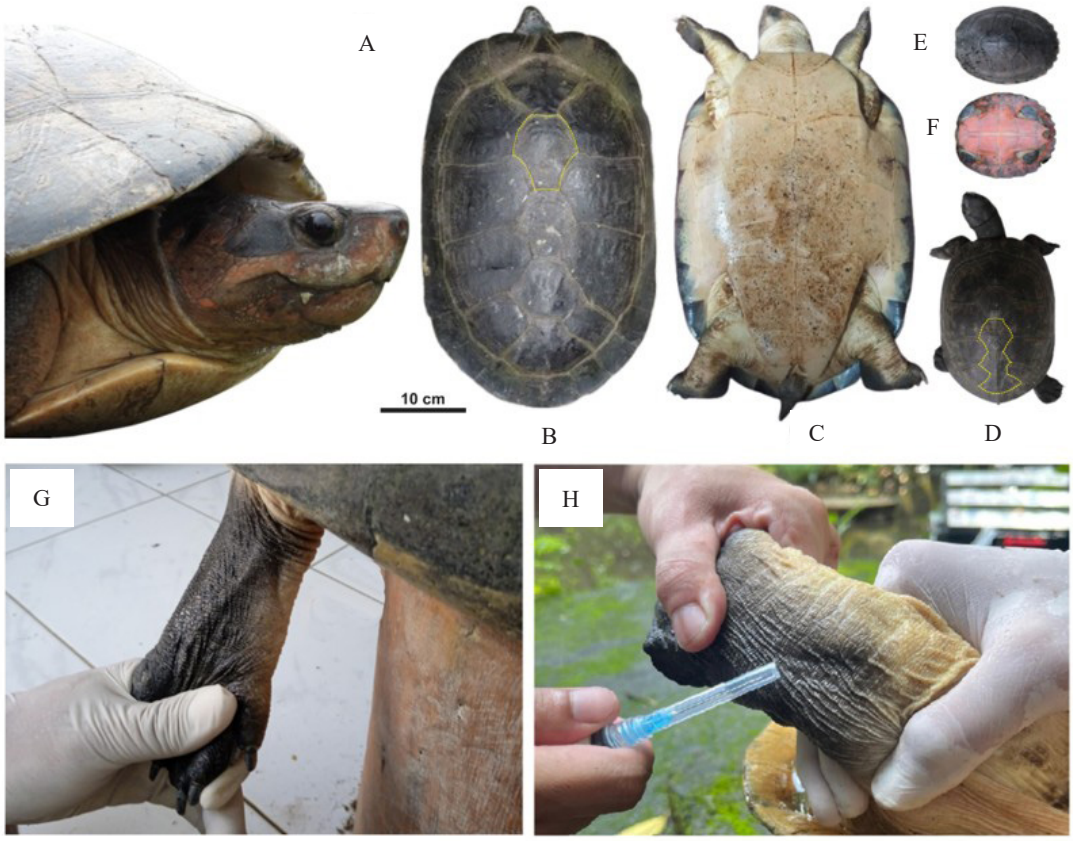

Figure 1. Phenotypic characteristic features of *O. borneensis* (A-F). Note that pebble-like granular scales around the eyes (A) and mushroom-like dorsal scute (yellow dashed line polygon) of dorsal carapace view (B). Plastron and ventral part are apparently yellow to light brown with no hinge (C). Juveniles usually appear with dorsal keel along dorsal scutes (yellow dashed line polygon – D). Newborns possess distinct serrated posterior marginal scutes and pale red plastron. Blood sampling site in ventral (G) and dorsal (H) recumbency of *O. borneensis*. Note that the accessible blood vessels appear as elongated bulging in a groove between gastrocnemius muscle and presumptive fibularis muscle group at the middle lateral aspect of the turtle's hind legs

Table 1. Information of Malaysian giant turtle individuals involved in this study

Individual name	Origin	Sampling site
M1	Malaysia (NCBI Genebank)	Depository Museum of Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia
M2	Malaysia (NCBI Genebank)	Depository Museum of Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia
OB 1	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 2	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 3	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 8	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 12	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 13	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB 14	Unknown	Wildlife Rescue Centre (WRC) Jogja, Yogyakarta, Java
OB G1	Unknown	Gembira Loka Zoo, Yogyakarta, Java
OB G5	Unknown	Gembira Loka Zoo, Yogyakarta, Java
OB BA	Belitung Island	Lenggang River, Eastern Belitung, Bangka Belitung

Table 2. Primer pairs sequences and predicted PCR product size

Encoding gene	Primer name	Oligonucleotide sequence	Tm (°C)	Predicted product size (bp)
ATP86	OBATPF	5' TGAGCAATCCCATCATTAGG 3'	52.1	968
	OBATPR	5' TGCGGTAGAAATGAGTTGAA 3'	51.8	
ND4	OBND4F	5' TCTGACCAACTGCACTAATC 3'	52.1	1800
	OBND4R	5' GGTACGAATGTGATGGAGAA 3'	51.9	
CO1	OBCOF	5' ATCACAATACCAAACACCCC 3'	52.8	1001
	OBCOR	5' GGCTGGTTCTTCATAGGTG 3'	52.9	
CytB	OBCYBF	5' ACGAAAAACTCACCCCATAA 3'	51.8	1061
	OBCYBR	5' ATAGAATGGAGGCTGTTTGG 3'	52.3	

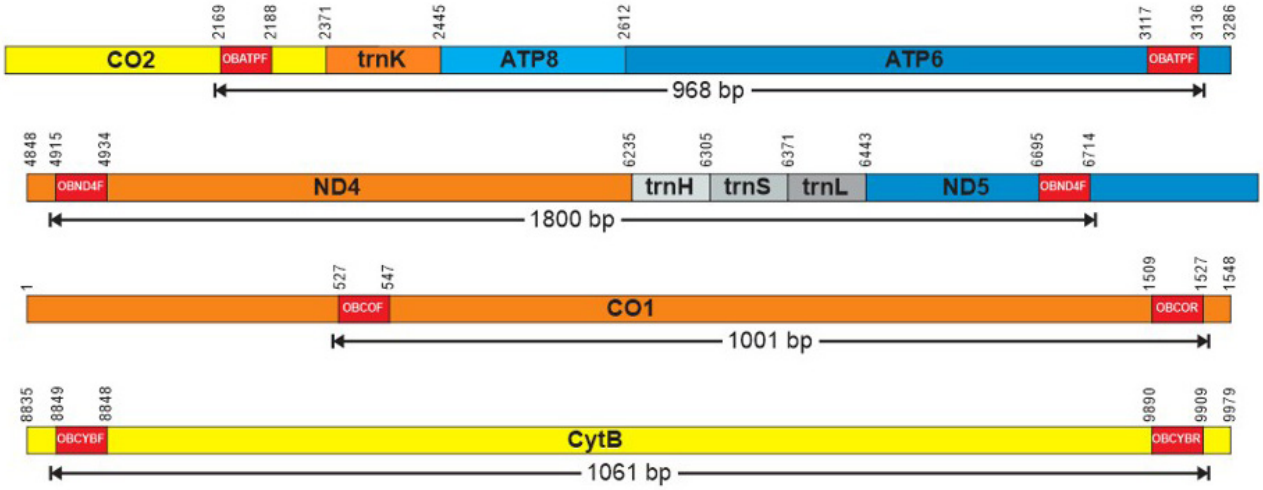

Figure 2. The bar charts illustrate the attachment site of each primer pair in targeted reference genomes. Both forward and reverse primers (red bars) flank several encoding genes except in CO1 and CytB encoding genes which apparently are within the targeted genes. The vertical ascending numbers above the bars indicate the nucleotide residual site number of reference genomes where the primers and encoding genes are located in ranges. The black lines under the bars indicate the primer coverage length with predicted PCR product size. Abbreviations: cytochrome c oxidase subunit 2 (CO2), tRNA-Lys (trnK), ATP synthase subunit 8 (ATP8), ATP synthase subunit 6 (ATP6), NADH dehydrogenase subunit 4 (ND4), tRNA-His (trnH), tRNA-Ser (trnS), tRNA-Leu (trnL), NADH dehydrogenase subunit 5 (ND5), cytochrome c oxidase subunit 1 (CO1), cytochrome B (CytB)

We employed all primer pairs for targeted mtDNA encoding gene amplification with single annealing temperature after several primer optimization attempts. Each PCR reaction contained 3 µL of extracted DNA as a template, mixed with Meridian Bioscience® 2× MyTaq HS Red Mix, following the manufacturer's recommended composition. The PCRs were run in thermal cycler with following conditions: pre denaturation at 94°C for 5 minutes, 35 cycles of denaturation (94°C for 30 seconds), annealing temperature at 51°C, elongation (72°C for 90 seconds), and ended with post elongation 72°C for 5 minutes. PCR products were visualised through agarose gel electrophoresis to confirm amplicon length against predicted product sizes. Amplicons displaying multiple bands were purified using gel extraction prior to Sanger sequencing, which was conducted on an Applied Biosystems™ 3730×l DNA Analyzer.

2.5. Sequence Analysis

All sequences were analysed in MEGA 11.0.13 for multiple sequence alignment, editing, searching potential single nucleotide polymorphism (SNP) pattern, and determining substitution model for nucleotide substitution type. We included several freshwater turtle species from NCBI Genbank for phylogenetic reconstruction using Maximum Likelihood (ML) analysis and bootstrap method phylogeny test with 1000 replications: *Orlitia borneensis* NC_082188.1 and OQ808845.1, *Batagur affinis* OQ645446.1, *Batagur borneoensis* NC_082187.1, *Carettochelys insculpta* NC_014048.1, *Cuora amboinensis* NC_014769.1,

Cyclemys dentata NC_018793.1, *Siebenrockiella crassicollis* AY434571.1, and *Trachemys scripta elegans* NC_011573.1. The phylogenetic analysis for ATP86 and ND4 amplicons used HKY (Hasegawa-Kishino-Yano) + I for substitution model whereas CO1 and CytB amplicons used HKY+G+ I and GTR (General Time Reversible) + G respectively. The phylograms were converted in Newick format and further edited in FigTree v1.4.4 for clade grouping by colour. We constructed a median joining haplotype network using DnaSP v6.12.03 and NETWORK 10.2.0.0 to see the putative haplotype number within the sample pool. Genetic pairwise distances were computed in MEGA 11.0.13 with 1000-time bootstrap replication for variance estimation and Kimura-2 parameter for substitution model.

2.6. Population Genetic Diversity

We analysed the genetic diversity of this small population based on available sequences from our study. The computations were performed in DnaSP v6.12.03 to conclude polymorphic sites (S), total number of mutations (η), haplotype (h), haplotype diversity (Hd), nucleotide diversity (π), theta estimator comparison of polymorphic sites (Watterson's theta) and nucleotide diversity (θW, θπ) according to each encoding genes. We estimated the mutation neutrality with Tajima's D test in DnaSP v6.12.03 (α=0.05). Haplotype diversity (Hd) levels were classified as described by Nei & Kumar (2000): low (0-0.5) and high (0.5-1). Nucleotide diversity levels were categorized as low (0.01-0.04), medium (0.05-0.07), and high (0.08-0.1).

3. Results

3.1. Primer Specificity and PCR Product Validation

We validated the specificity of our designed primers according to the produced amplicon length through PCR and similarity coverage of the sequenced amplicon. All primer pairs partially flanked the targeted regions and successfully produced amplicon length exactly identical to our predicted PCR products according to the DNA ladder (Figure 3). Amplicons of ATP86 and CytB appeared boldest, thick, and distinct than other two amplified encoding genes, indicating the optimum PCR condition and excellent amount of PCR products (Figure 3, A and C). The lowest amount of amplicon was present in CO1 encoding gene amplification with relatively faint thinner bands (Figure 3, D). All amplicons presented very fade smears along the lanes and several multiple very faint bands were observed in CytB and CO1 amplicon lanes (Figure 3, C and D).

3.2. Potential Genetic Markers based on SNP

Our findings revealed several nucleotide residue sites that may serve as promising genetic markers for distinguishing Malaysian giant turtle (*Orlitia borneensis*) populations by locality. Not all amplicons from the targeted coding genes were fully sequenced; therefore, only sequences with relatively intact and complete forward-strand reads, as determined from chromatogram quality, were included in the analysis. After multiple rounds of resequencing and optimization, we successfully obtained partial sequences for the ATP86, ND4, CytB, and CO1 genes, with lengths of 261 bp, 573 bp, 998 bp, and 416 bp, respectively. These sequences represent approximately one-third of the total targeted gene lengths, except for the CytB amplicon, which covered nearly 90% of the gene. Although the amplicon bands corresponded to the expected target sizes, sequencing results yielded shorter coverage than anticipated. This discrepancy was likely due to incomplete sequencing reads or potential degradation of amplicons following gel electrophoresis validation. Nevertheless, the obtained sequences revealed several potential genetic markers and single nucleotide polymorphisms (SNPs) after multiple sequence alignment. Notably, several sequence sites from the Indonesian *O. borneensis* samples exhibited consistent, identical nucleotide residues that were distinct from those of the two Malaysian-origin individuals. (Table 3 to 6, pink highlights) from Genebank as reference genomes (ATP86 at 560 (C→T); CytB at 253 (A→G), 681 (T→C); CO1 720 (T→C), 804 (T→C); ND4 201 (C→T), 246 (C→T), 579 (T→C)). Several sequences also displayed consistent nucleotide substitutions reflecting putative Malaysia-Sumatra-Belitung cluster (Table 3 and 4, blue highlights) including sequences from Malaysia and Bangka (ATP86 at 403 (C→T), 419 (A→G), 443 (C→T), 500 (A→G), 554 (A→G), 584 (C→T); CytB at 149 (T→C), 201 (T→C), 597 (G→A)). Additionally, OB G5 and OB 1 apparently became the closest relative to OB BA (Belitung origin) with several consistent residual sites (Table 4, yellow highlights) of partial CytB encoding gene (at 130 (C→T), 207 (C→T), 1029 (G→A)) and ATP86 encoding gene sequences (at 564 (A→G)). These findings of potential SNPs can be considered as markers for clustered locality with current validated origins which are private to certain groups.

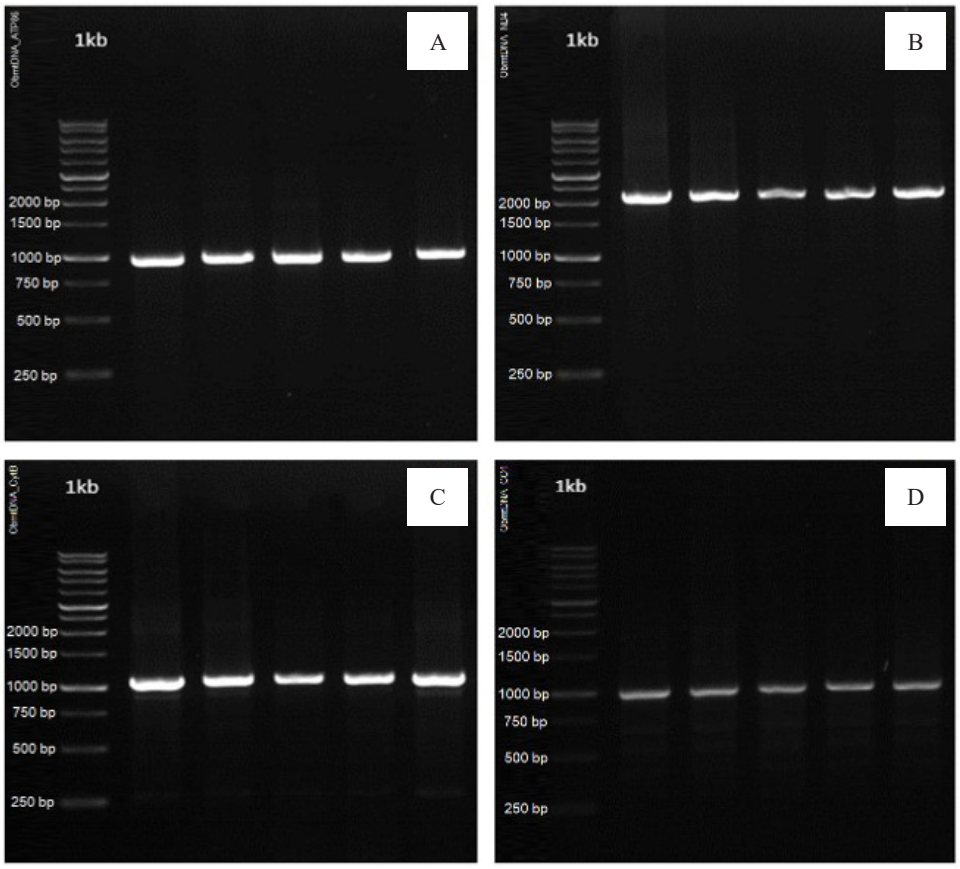

Figure 3. Amplicon bands of ATP86 (A), ND4 (B), CytB (C), CO1 (D) encoding genes amplifications were visualised in gel electrophoresis. At the same PCR condition, the best yield was presented consecutively in ATP86, CytB, ND4, and CO1 amplicons. The marker was 1 kb DNA ladder with lower bold band at 1000 bp and upper bold band at 3000 bp

Table 3. Putative locality-based genetic markers in partial ATP86 encoding gene sequence

	393	403	419	443	500	554	560	564	584
M1	A	C	A	C	A	A	C	A	C
M2	.	.	.	.	.	.	.	.	.
OB BA	.	.	.	.	.	.	T	G	.
OB 1	.	.	.	.	.	.	T	G	.
OB G5	.	.	.	.	.	.	T	G	.
OB G1	.	T	G	T	G	G	T	.	T
OB 14	.	T	G	T	G	G	T	.	T
OB 13	G	T	G	T	G	G	T	.	T
OB 3	G	T	G	T	G	G	T	.	T

Table 4. Putative locality-based genetic markers in partial CytB encoding gene sequence

	130	149	201	207	240	253	255	493	582	583	597	681	816	1029
M1	C	T	T	C	C	A	C	A	G	A	G	T	G	G
M2	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OB 3	.	C	C	.	T	G	.	.	A	G	A	C	A	.
OB 13	.	C	C	.	T	G	.	.	A	G	A	C	A	.
OB 14	.	C	C	.	.	G	T	G	A	.	A	C	.	.
OB G1	.	C	C	.	.	G	T	G	A	.	A	C	.	.
OB G5	T	.	.	T	.	G	.	.	A	.	.	C	.	A
OB BA	T	.	.	T	.	G	.	.	.	.	.	C	A	A
OB 1	T	.	.	T	.	G	.	.	.	.	.	C	A	A

Table 5. Putative locality-based genetic markers in partial CO1 encoding gene sequence

	606	630	675	720	804	892	898	956	957	969
M1	G	T	G	T	T	G	G	A	A	A
M2	.	.	.	.	.	.	.	.	.	.
OB 14	A	C	.	C	C	.	.	.	.	.
OB 1	.	.	.	C	C	C	C	T	G	T
OB 2	A	C	A	C	C	C	C	T	G	T
OB 3	A	C	A	C	C	.	.	.	.	.
OB 8	A	C	A	C	C	.	.	.	.	.
OB 12	A	C	A	C	C	.	.	.	.	.
OB 13	A	C	A	C	C	.	.	.	.	.
OB G1	A	C	A	C	C	.	.	.	.	.

Table 6. Putative locality-based genetic markers in partial ND4 encoding gene sequence

	201	228	246	262	411	579	658	675	676
M1	C	A	C	T	G	T	G	A	A
M2	.	.	.	.	.	.	.	.	.
OB G1	T	G	T	C	A	C	.	C	C
OB 14	T	G	T	C	A	C	C	C	.
OB 13	T	.	T	.	.	C	C	.	C
OB 3	T	.	T	.	.	C	.	.	.
OB G5	T	.	T	.	.	C	.	C	.

3.3. Phylogenetic Tree and Haplotype Network

The phylogenetic reconstruction of each partial encoding gene amplicon supported the presence of distinct localities for Malaysian giant turtles in Indonesia. We observed several monophyletic groups, particularly those that are closely related to Malaysia and Belitung origin individuals (Figure 4, respectively in orange and blue highlights) in ATP86 and CytB phylogram topology. These phylogenetic analyses also corroborated the distinctness between Malaysian and Indonesian populations, as individuals from Malaysia consistently formed separate monophyletic clades across all tree topologies. In the CO1 tree topology, the inclusion of two individuals (OB2) within the Belitung cluster further reinforced the presence of this lineage. Other individuals were dispersed across clusters, forming polyphyletic and paraphyletic groups (Figure 4; pink highlights).

There was no distinct pattern of haplotype grouping observed in the networks constructed from the available sequences, particularly among the group of Malaysian giant turtle individuals from Indonesia. Distinct haplotype groups were identified for ATP86 (four haplotypes), CO1 (five haplotypes), CytB (five haplotypes), and ND4 (six haplotypes) genes. However, the topologies of these haplotype networks did not exhibit consistent trends. The haplotype analyses nonetheless indicated that Malaysia-origin individuals (Figure 5; orange dots) were genetically distinct from those of Indonesian origin. Several individuals presumed to be from Belitung clustered within a single or closely related haplotype network (Figure 5; blue dots). In contrast, other Indonesian-origin individuals exhibited greater haplotype diversity (Figure 5; yellow dots), suggesting a richer genetic pool within the current captive population.

3.4. Population Genetic Distance and Diversity

The intraspecies genetic distances based on selected mtDNA encoding genes showed low differences (≤ 0.018) among MGT individuals (Supplementary Table 7-10). The average genetic distance according to partial ATP86, CytB, CO1, and ND4 encoding genes consecutively were 0.018, 0.007, 0.010, and 0.008. The CytB and ND4 encoding genes apparently became the most conserved regions in this study compared to CO1 and ATP86 those were relatively diverse. In the one hand, *Batagur borneensis* became genetically the closest relative to *Orlitia borneensis* according to partial sequence of ATP86 and CytB encoding genes (respectively at average 0.164 and 0.162). On the other hands, analyses in partial CO1 and ND4 encoding gene sequences showed that *Batagur affinis* possessed the closest relatedness to *Orlitia borneensis* (distance average at 0.139 and 0.205 consecutively).

We computed several genetic diversity parameters based on selected partial mtDNA encoding genes sequences. Since almost all individuals came from a same cohort, the fixation index was not estimated in this

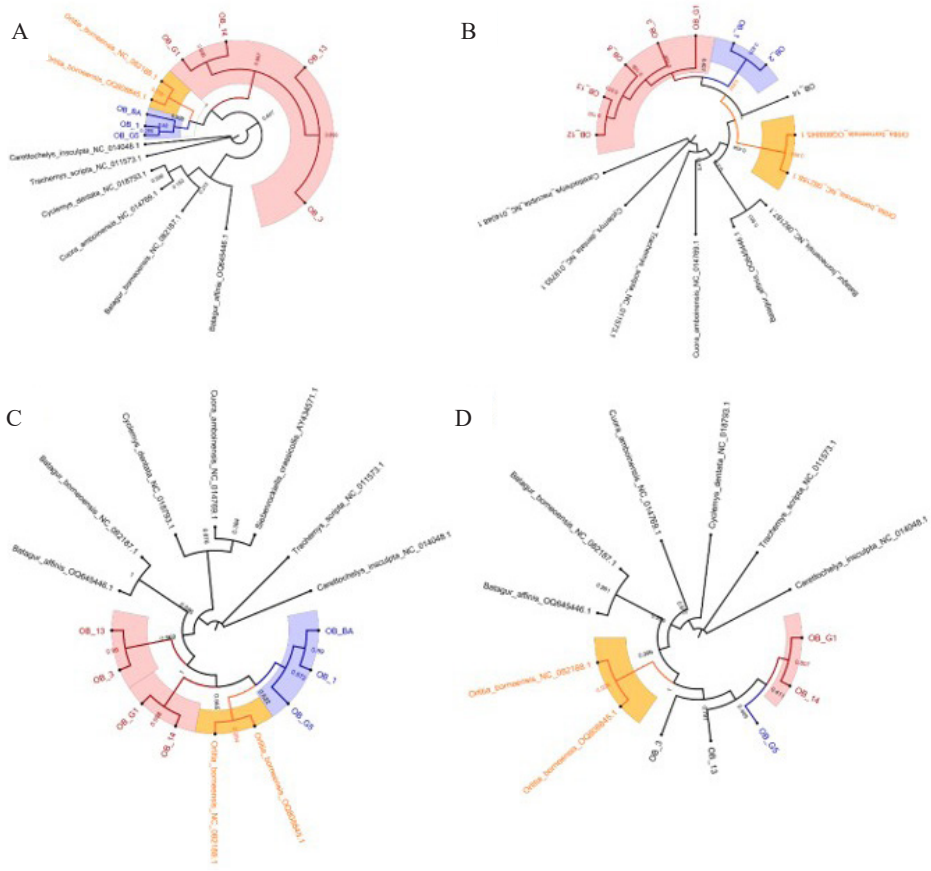

Figure 4. Rooted phylogram topologies of partial ATP86 (A), CO1 (B), CytB (C), and ND4 (D) encoding gene amplicons. All trees consistently presented distinct monophyletic branches of Malaysia-origin (orange highlights). A putative Belitung-origin (blue highlights) individuals stood consistently as a single separate branch in three phylograms. The other Indonesia-origin individuals remained spreading across the trees (pink highlight). The number in the branch nodes indicated consensus bootstrap number

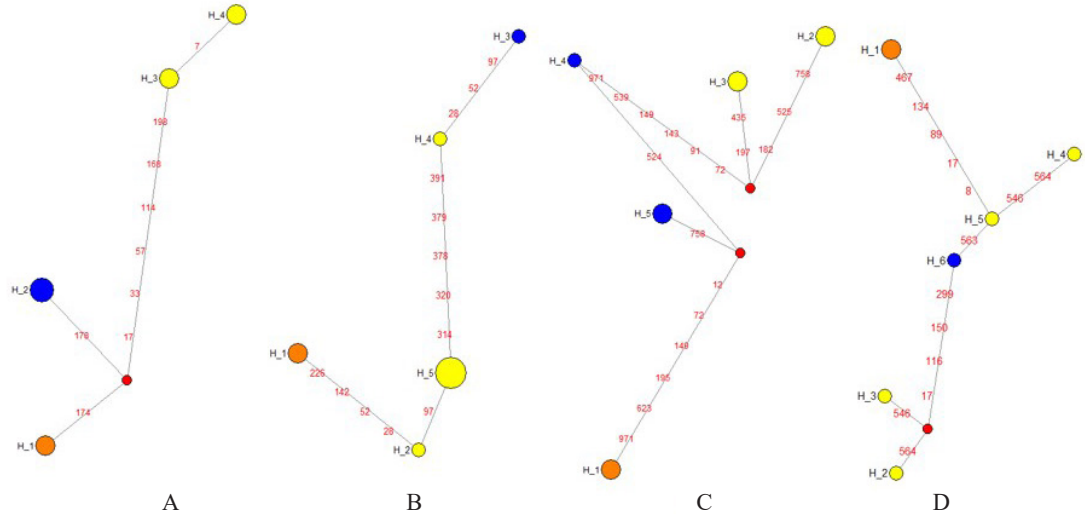

Figure 5. Median-joining haplotype networks of partial ATP86 (A), CO1 (B), CytB (C), and ND4 (D) encoding gene sequences. Malaysia-origin individuals (orange dots) were diverged from the other haplotype groups whereas previously predicted Belitung-origin individuals were clustered and or closely related (blue dots). The rest Indonesia-origin individuals remained scattered across the networks (yellow dots). The red numbers indicate the mutation and/or nucleotide substitution sites. The red dots indicate hypothetical ancestral nodes

study. The results of genetic diversity parameters through DNA polymorphism and Tajima's D test computation are presented in Table 7. Haplotype diversities of all partial mtDNA encoding sequences indicated high variation (Hd > 0.05). Nucleotide diversities were apparently at low level ($0.01 < \pi < 0.04$). The ATP86 encoding gene displayed highest nucleotide diversity supported by the largest number of mutation relatively compared to other sequenced encoding genes. The CytB encoding gene apparently presented the lowest nucleotide diversity and number of mutation relative to its sequenced sites. Tajima's D values calculated based on nucleotide diversity (π) and Watterson's theta (θW) resulted in diverse values, however all of partial sequenced encoding genes showed no statistical significant difference ($p > 0.05$).

4. Discussion

4.1. Amplification and Sequencing Evaluation

The incomplete sequence lengths obtained after amplicon validation by gel electrophoresis were likely associated with less suboptimal sequencing conditions or sample handling errors following electrophoresis. The correctly predicted PCR product sizes observed in all targeted gene bands confirmed that the primer pairs annealed to the intended sites and that amplification proceeded accurately. The presence of single, distinct bands with minimal nonspecific amplification—such as multiple bands, primer dimers, or smears—further indicated high primer specificity and optimized PCR conditions. Therefore, it is highly probable that reduced sequencing performance led to incomplete read lengths and limited nucleotide coverage. The need for multiple resequencing attempts to achieve analysable sequences also supports the likelihood of suboptimal sequencing conditions. Additionally, short-read sequencing constraints and small-truncated DNA fragments generated by unspecific primer annealing and polymerization pause in PCR or sequencing reactions may have affected the chromatogram quality and sequence completeness (Westberg *et al.* 1999; Hebert *et al.* 2018). Our procedure that involved gel extraction to isolate amplicons may have further inhibited sequencing reactions due to the presence of gel debris or DNase contamination (Gaastra & Jørgensen 1984). Moreover, UV (~300 nm) exposure during visualisation on transilluminators could have caused minor damage to double-stranded DNA, including the amplified fragments, although this effect is generally considered minimal (Gründemann & Schömig 1996).

Performing sequencing with higher throughput may capture more nucleotide residue sites, thereby providing additional genetic markers and improving the accuracy of sequence alignment analyses. Despite the relatively low coverage and shorter sequence yields compared to the predicted targets, all partial sequences presented high similarity (greater than 99%) and phylogenetic clustering to Malaysian giant turtle (*Orlitia borneensis*) reference genomes from NCBI GenBank database, revealing several single nucleotide polymorphisms (SNPs) that may serve as potential locality markers. Sanger sequencing has been reported to have lower fidelity and efficiency for targets exceeding 1 kb in length (Hebert *et al.* 2018). Because this method requires bidirectional reads and analysis to achieve complete amplicon coverage—often with truncated regions at the 5′ and 3′ ends due to dye blobs—its resolution is lower than that of more advanced sequencing platforms (Kieleczawa 2005). Long-read next-generation sequencing technologies (e.g., PacBio, Oxford Nanopore) are therefore recommended to achieve greater sequencing depth and higher-quality amplicon coverage (Combrink *et al.* 2023).

4.2. Importance of Genetic Diversity and Potential Localities to Conservation Breeding

The distinction between founder representation and parental stock selection is fundamental in conservation breeding programmes. Founder individuals determine the initial genetic composition of the captive population

Table 7. Genetic diversity parameters of a captive Malaysian giant turtle population based on selected mtDNA encoding genes

Parameters	ATP86	CO1	CytB	ND4
Individuals (N)	9	10	9	7
Number of sites (nt)	261	416	998	573
Polymorphic sites (S)	9	10	14	9
Total number of mutations (η)	9	10	14	9
Haplotype (h)	4	5	5	6
Haplotype diversity (Hd)	0.833±0.080	0.756±0.130	0.889±0.071	0.952±0.096
Nucleotide diversity (π)	0.018±0.002	0.009±0.002	0.007±0.001	0.008±0.001
Watterson's theta (θW)	0.0127	0.0085	0.0052	0.0064
Tajima's D	1.806 (p>0.05)	0.528 (p>0.05)	1.360 (p>0.05)	1.024 (p>0.05)

and should ideally represent diverse genetic lineages and geographic origins. While the parental stock comprises of a subset of these founders selected for active breeding, with the aim of translating genetic diversity into viable offspring whilst minimizing relatedness among mating pairs. Based on established principles of genetic management (Frankham *et al*. 2002; Frankham 2008), a clear distinction between these roles is critical to avoid suboptimal breeding strategies, particularly in populations with unknown provenance.

In this case, the high haplotype diversity (Hd) supported that the captive population we manage remained in high variety of genetic pool that indirectly indicated low potential of inbreeding. Because most individuals procured from confiscation, it is possible that nonrelated individuals from random, undetected origins may have contributed to and enriched the genetic diversity within closed population. Nevertheless, low nucleotide diversity (π) might be related to relatively short sequence yields resulted in our study which may not comprehensively represent the whole sequences of each encoding genes. Tajima's D value estimates the mutation neutrality in population indicating whether the population undergoes neutral condition, selection or demographic changes (expansion/contraction) (Stajich & Hahn 2005). Though the Tajima's D values in our study showed tendency of balancing selection (population contraction) that declined the heterozygosity due to allele loss (increasing genetic drift), the statistical analysis indicated that this captive population remained neutral ($p>0.05$).

Aside from relatively short recovered sequences, our study suggested that the mtDNA ATP encoding gene became the most hypervariable region among other three encoding genes which perhaps reflected the highest mutation rate (ATP>CO1>ND4>CytB). It agreed with the finding reported in previous studies (da Fonseca *et al.* 2008; Eo & DeWoody 2010) that supported mitochondrial ATP encoding gene mutating fastest than other three major encoding genes. We believed that there is positive association between ATP encoding gene mutation rate with geographical isolation inducing adaptive energy harvesting in striving isolated environment (Noll *et al.* 2022). This notion apparently is consistent to the phylogram pattern and haplogroup diagram in this study. Belitung origin individual was present as a single branch in phylogram and possessed specific genetic marker, leading to assumption that isolated population of *Orlitia borneensis* in Belitung might form a genetic locality. However, this finding should be supported by another sequence analyses with longer coverage of targeted encoding genes. Though inconsistencies were present in relatedness distance among *Orlitia borneensis*, *Batagur affinis*, and *Batagur borneensis* according to our analysis, genus *Batagur* was still confirmed as closest relative to *Orlitia borneensis*.

The trends of islands-based genetic differentiation among wild animals in Indonesia has been well documented in several studies, particularly for species inhabiting Sumatra, Java, and Borneo. Genetic marker analyses have demonstrated significant genetic distances among island populations in various Indonesian endemic species, including pangolins, orangutans, tarsiers, and binturongs (Goossens *et al.* 2009; Widayanti & Susmiati 2012; Sitam *et al.* 2023; Hardian *et al.* 2024), with comparatively fewer studies focusing on freshwater turtles in Asia (Fritz *et al.* 2014; Protiva *et al.* 2016; Salleh *et al.* 2023). To date, there have been no reports reclassifying the Malaysian giant turtle (*Orlitia borneensis*) based on island distribution or genetic divergence. Considering the historical dispersal of Indochinese fauna into the Sundaland archipelago, it is plausible that *O. borneensis* populations have undergone geographical isolation, potentially leading to processes of speciation or subspeciation through long-term genetic differentiation.

Our pilot study revealed clear genetic distinctness between Malaysian giant turtles (*Orlitia borneensis*) from Peninsular Malaysia and those from Indonesia, suggesting a historical divergence event that separated these populations and allowed them to evolve within localized genetic pools. The relatively high haplotype variation observed among Indonesian individuals also indicates the existence of small, isolated populations that have remained separated over extended periods, forming multiple familial clusters with limited gene flow. Although no new species or subspecies classifications have yet been proposed for *O. borneensis*, the presence of high haplotype diversity provides valuable options for enhancing genetic variability in captive breeding programmes—particularly to prevent inbreeding within genetically saturated populations where geographic origin is unknown. However, if future taxonomic revisions recognize distinct subspecies, conservation breeding strategies must prioritize maintaining genetic purity to prevent the loss of unique genetic diversity within the broader population.

Our results also identified a potential Belitung lineage that appears to be geographically restricted to Belitung Island. Distinct single nucleotide polymorphisms

(SNPs) consistently differentiated Malaysian-origin from Indonesian-origin individuals, providing molecular evidence for this divergence. It is widely recognized that each wildlife species—although sometimes elusive in behavior—plays a unique ecological role shaped by thousands of years of evolutionary adaptation to its habitat (Malhi *et al.* 2022; Ruikar *et al.* 2024). Further research on the ecological importance of the Malaysian giant turtle, particularly its role in food webs and ecosystem processes, is therefore essential.

The geological and paleoecological characteristics of Belitung Island—such as its ancient river networks and position within the Sundaland shelf (Kamiludin 2019)—highlight its ecological uniqueness, potentially distinct from that of Sumatra and Borneo. These paleo-river systems may have facilitated the historical dispersal of Malaysian giant turtles among islands before the onset of modern insular isolation. Fossil records indicate that *O. borneensis* extended as far as Central Java during the Middle Pleistocene (Setiyabudi *et al.* 2016; Claude *et al.* 2024), suggesting a wider prehistoric range prior to the separation of Belitung Island during the Late Pleistocene, approximately 13,000 years ago (Solihuddin 2014). Nevertheless, the presence of the Belitung-specific lineage, together with its potential ecological significance, provides compelling evidence to support stronger conservation measures for the Belitung landscape. Protecting this island from excessive mining and land-use conversion is therefore essential to preserving both the unique evolutionary heritage and the ecological integrity of the Malaysian giant turtle population.

Increasing the number of specimens representing each island or prospective locality would help to reduce inaccuracies resulting from low sample size and limited sequence coverage across the targeted genes, especially to substantiate specific Belitung-origin population. We acknowledge that our sample size was small, as this study was intended as an initial investigation. However, obtaining confirmed individuals with verified origins remains challenging, particularly given that misidentification of this species is still common in the field. The Malaysian giant turtle (*Orlitia borneensis*) is frequently mistaken for *Batagur* spp., *Siebenrockiella crassicolis* or *Cuora amboinensis* due to morphological similarities and overlapping local names. Considering their preferences for the riparian-aquatic and estuarine area with nearly their entire life cycles occuring underwater, the dispersal and radiation of Malaysian giant turtles might be strictly following river bodies, branches or other aquatic networks, potentially leading to sympatric distributions with the aforementioned species. We predict that their geographical localities might be separated by non-aquatic barriers such as mountainous areas or plateaus and apparently more restricted to lowlands rich in aquatic networks. Thus, to enhance the representation of genetic diversity and identify potential parental candidates for breeding, future sampling should prioritize areas along major aquatic networks to capture a broader range of localities. Developing a species distribution model (SDM) that integrates updated occurrence data of Malaysian giant turtles, the distribution of sympatric species, and natural food availability could further assist in identifying priority sampling sites and monitoring hotspots.

4.3. Pairing Proposal Considering the Genetic Distance

In the absence of pedigree or origin data, we propose a genetic-distance-based framework that explicitly separates founder identification from parental stock selection in the captive breeding of Malaysian giant turtles (*Orlitia borneensis*) (Figure 6). Individuals confiscated from illegal trade often lack traceable provenance; therefore, genetic analyses should first be used to characterise the diversity within the population and identify a representative founder pool.

From this genetically characterized founder population, parental stock can then be selected based on a combination of health status, reproductive maturity, and genetic distance. Compared to nuclear DNA, which is a product of biparental inheritance, mtDNA offers greater stability due to its strictly maternal transmission and limited recombination (Galtier *et al*. 2009; Hagström *et al.* 2013; Ladoukakis & Zouros 2017). Consequently, greater genetic distance in mtDNA indicates more distinct maternal lineages, reducing the likelihood of inadvertent inbreeding between closely related individuals, thereby can be used as a practical proxy to minimize the risk of inbreeding when selecting parental pairs.

In addition to genetic assessment, all prospective breeding individuals must be in good physical health. Comprehensive physical and laboratory evaluations—including hematology, blood biochemistry, and parasitological screening—should be conducted to identify potential diseases transmissible to offspring. Screening for turtle ranavirus, herpesvirus, and adenovirus is also strongly recommended (Johnson *et al*. 2008; Winter *et al.* 2022; Okoh *et al.* 2023). Following health assessment, candidate individuals from the founder

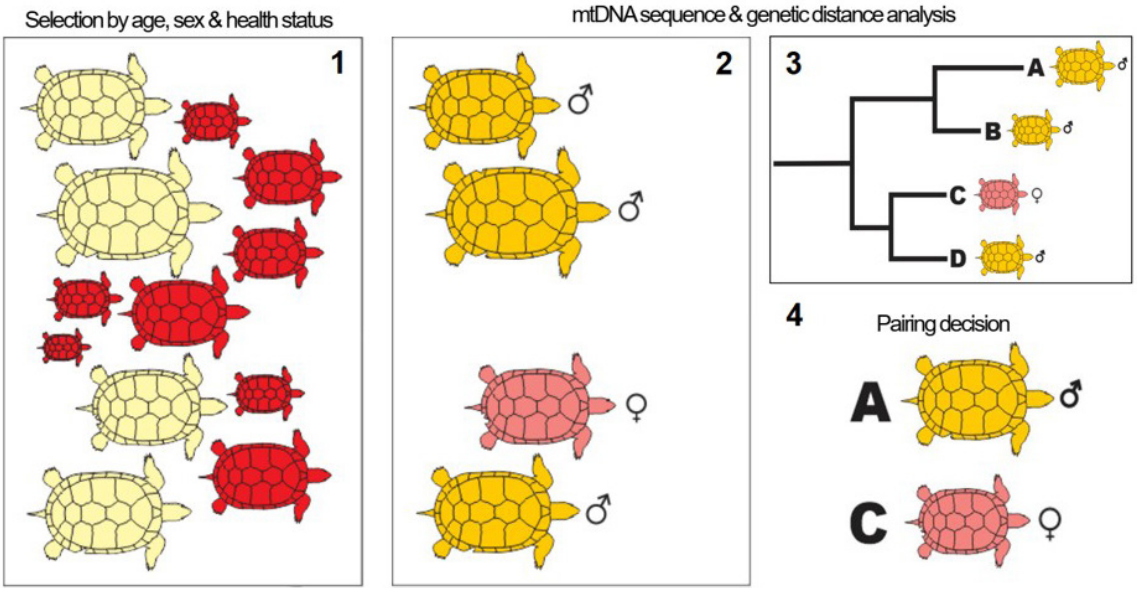

Figure 6. Proposed scenario considering genetic distance for parental selection and pairing process for Malaysian giant turtles with no information of history or pedigree. The turtles are grouped based on age, size, sex, and health status (1). Unsuitable individuals are coloured red. Selected individuals with male-female composition (pink and orange individuals) are tested for genetic distance based on mtDNA sequences (2). Reconstruction of phylogram to visualize the comparative genetic distance among selected individuals (3). The male-female pairs with farthest genetic distance (A and C individuals) are selected for breeding programmes (4)

pool should be grouped according to age, size, and sex. Only individuals meeting health and reproductive criteria should be advanced as parental stock candidates. Genetic analyses can then be used to estimate pairwise genetic distances among candidates. Final parental pairs should subsequently be selected by matching the male and female with the greatest genetic distance within the candidate group. This two-step framework—first ensuring adequate founder representation and subsequently optimizing parental stock selection—provides a structured approach to maintaining genetic diversity in captive population with limited background information.

Although this approach seems practical and applicable in such conditions, several challenges must be addressed. Accurate sex determination through either physical and/or genetic methods is critical; however, no reliable technique currently exists for this species. Phallus palpation through intracloacal examination may be used as an option, although inexperienced breeders often yield inaccurate results. Furthermore, performing palpation without sufficient anatomical knowledge can cause cloacal injury, potentially leading to ascending infections of the reproductive organs. Additionally, incompatibility between male and female individuals may result in aggressive interactions, sometimes leading to physical injury. For species exhibiting temperature-dependent sex determination (TSD), maintaining an appropriate sex ratio among offspring can be managed through temperature-controlled incubation. However, this method cannot be validated until individuals reach sexual maturity, which may take several years. Assurance colonies serve as genetic reservoirs for eventual reintroduction or other forms of conservation translocation as part of genetic rescue programmes. Therefore, before any release, it is essential to evaluate both the genetic saturation of the source population and the ecological carrying capacity of the recipient habitat to ensure the sustainability and effectiveness of the translocation effort.

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Supplementary Materials

Supplementary Table 7. Genetic distance matrix of several terrapin species based on partial ATP86 encoding gene sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
*Orlitia borneensis*_OQ808845.1															
*Orlitia borneensis*_NC_082188.1	0.000														
OB_BA	0.008	0.008													
OB_G5	0.008	0.008	0.000												
OB_G1	0.028	0.028	0.028	0.028											
OB_14	0.028	0.028	0.028	0.028	0.000										
OB_13	0.032	0.032	0.032	0.032	0.004	0.004									
OB_3	0.032	0.032	0.032	0.032	0.004	0.004	0.000								
OB_1	0.008	0.008	0.000	0.000	0.028	0.028	0.032	0.032							
*Batagur affinis*_OQ645446.1	0.184	0.184	0.184	0.184	0.189	0.189	0.195	0.195	0.184						
*Batagur borneoensis*_NC_082187.1	0.152	0.152	0.163	0.163	0.168	0.168	0.173	0.173	0.163	0.169					
*Carettochelys insculpta*_NC_014048.1	0.248	0.248	0.254	0.254	0.265	0.265	0.265	0.265	0.254	0.277	0.254				
*Cuora amboinensis*_NC_014769.1	0.170	0.170	0.181	0.181	0.197	0.197	0.197	0.197	0.181	0.198	0.156	0.283			
*Cyclemys dentata*_NC_018793.1	0.251	0.251	0.251	0.251	0.233	0.233	0.233	0.233	0.251	0.241	0.224	0.326	0.189		
*Trachemys scripta*_NC_011573.1	0.257	0.257	0.257	0.257	0.269	0.269	0.275	0.275	0.257	0.267	0.266	0.299	0.287	0.278	

Supplementary Table 8. Genetic distance matrix of several terrapin species based on partial CytB encoding gene sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
*Orlitia borneensis*_OQ808845.1																
*Orlitia borneensis*_NC_082188.1	0.000															
OB_3	0.009	0.009														
OB_13	0.009	0.009	0.000													
OB_14	0.008	0.008	0.005	0.005												
OB_G1	0.008	0.008	0.005	0.005	0.000											
OB_G5	0.006	0.006	0.009	0.009	0.008	0.008										
OB_BA	0.006	0.006	0.009	0.009	0.010	0.010	0.002									
OB_1	0.006	0.006	0.009	0.009	0.010	0.010	0.002	0.000								
*Batagur affinis*_OQ645446.1	0.169	0.169	0.165	0.165	0.169	0.169	0.164	0.164	0.164							
*Carettochelys insculpta*_ NC_014048.1	0.255	0.255	0.249	0.249	0.251	0.251	0.252	0.252	0.252	0.234						
*Cyclemys dentata*_NC_018793.1	0.175	0.175	0.172	0.172	0.177	0.177	0.172	0.172	0.172	0.177	0.245					
*Cuora amboinensis*_NC_014769.1	0.182	0.182	0.178	0.178	0.182	0.182	0.177	0.177	0.177	0.177	0.237	0.142				
*Batagur borneoensis*_ NC_082187.1	0.163	0.163	0.160	0.160	0.166	0.166	0.161	0.161	0.161	0.100	0.225	0.168	0.164			
*Siebenrockiella crassicollis*_ AY434571.1	0.204	0.204	0.205	0.205	0.203	0.203	0.205	0.205	0.205	0.209	0.250	0.190	0.184	0.195		
*Trachemys scripta*_NC_011573.1	0.200	0.200	0.194	0.194	0.197	0.197	0.199	0.199	0.199	0.193	0.238	0.183	0.195	0.195	0.232	

Supplementary Table 9. Genetic distance matrix of several terrapin species based on partial CO1 encoding gene sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
*Orlitia borneensis*_OQ808845.1																
*Orlitia borneensis*_NC_082188.1	0.000															
OB_14	0.010	0.010														
OB_1	0.017	0.017	0.017													
OB_2	0.024	0.024	0.015	0.007												
OB_3	0.012	0.012	0.002	0.019	0.012											
OB_8	0.012	0.012	0.002	0.019	0.012	0.000										
OB_12	0.012	0.012	0.002	0.019	0.012	0.000	0.000									
OB_13	0.012	0.012	0.002	0.019	0.012	0.000	0.000	0.000								
OB_G1	0.012	0.012	0.002	0.019	0.012	0.000	0.000	0.000	0.000							
*Trachemys scripta*_NC_011573.1	0.155	0.155	0.158	0.176	0.176	0.161	0.161	0.161	0.161	0.161						
*Cyclemys dentata*_NC_018793.1	0.173	0.173	0.176	0.188	0.188	0.173	0.173	0.173	0.173	0.173	0.172					
*Cuora amboinensis*_NC_014769.1	0.179	0.179	0.176	0.184	0.184	0.172	0.172	0.172	0.172	0.172	0.175	0.167				
*Carettochelys insculpta*_ NC_014048.1	0.196	0.196	0.186	0.205	0.198	0.183	0.183	0.183	0.183	0.183	0.181	0.175	0.220			
*Batagur borneoensis*_ NC_082187.1	0.170	0.170	0.167	0.178	0.178	0.163	0.163	0.163	0.163	0.163	0.157	0.188	0.156	0.189		
*Batagur affinis*_OQ645446.1	0.142	0.142	0.132	0.150	0.150	0.135	0.135	0.135	0.135	0.135	0.167	0.153	0.166	0.202	0.092	

Supplementary Table 10. Genetic distance matrix of several terrapin species based on partial ND4 encoding gene sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13
*Orlitia_borneensis*_OQ808845.1													
*Orlitia borneensis*_NC_082188.1	0.000												
OB_G1	0.014	0.014											
OB_14	0.014	0.014	0.004										
OB_13	0.009	0.009	0.009	0.009									
OB_3	0.005	0.005	0.009	0.009	0.004								
OB_G5	0.007	0.007	0.007	0.007	0.005	0.002							
*Batagur affinis*_OQ645446.1	0.203	0.203	0.205	0.205	0.208	0.204	0.206						
*Batagur borneoensis*_NC_082187.1	0.213	0.213	0.221	0.221	0.224	0.219	0.222	0.122					
*Carettochelys insculpta*_NC_014048.1	0.407	0.407	0.421	0.421	0.418	0.412	0.415	0.385	0.382				
*Cuora amboinensis*_NC_014769.1	0.222	0.222	0.235	0.235	0.235	0.231	0.233	0.220	0.225	0.350			
*Cyclemys dentata*_NC_018793.1	0.294	0.294	0.302	0.302	0.297	0.292	0.295	0.271	0.249	0.373	0.207		
*Trachemys scripta*_NC_011573.1	0.327	0.327	0.342	0.342	0.343	0.337	0.340	0.305	0.334	0.336	0.273	0.299	