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Mitochondrial D-Loop Analysis Reveals High Haplotype Diversity in the Belitung Tarsier (*Cephalopachus bancanus saltator*)

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ABSTRACT

Habitat fragmentation from ongoing land clearing on Belitung Island poses a critical threat to *Cephalopachus bancanus saltator*, an endangered primate species endemic to the region. Comprehensive genetic data for this subspecies remain scarce, and this study aimed to assess its genetic diversity by analyzing mitochondrial Displacement Loop (D-loop) sequence variation. Ear tissue samples from six individuals were collected at two geographically distinct sites, Bukit Peramun and Batu Mentas. DNA extraction, amplification using DLTARPROF and DLTARBFR primers, and sequencing produced fragments ranging from 418 to 424 base pairs. Comparative BLAST analysis revealed sequence similarity of 88.12% to 89.76% with *Tarsius bancanus* (GenBank accession NC_002811.1). Haplotype diversity (Hd) reached 1.0 in both populations, indicating exceptionally high intraspecific variation. Pairwise genetic distances ranged from 0.0074 to 0.0370, while divergence from the outgroup species *Carlito syrichta* reached 0.1777. Phylogenetic reconstruction using the neighbor-joining method identified two distinct clades, with bootstrap support values ranging from 68% to 93%. The results indicate high haplotype diversity among the sampled individuals, likely attributable to the small sample size (n = 6) and the use of D-loop markers. These findings are preliminary and provide a baseline for future genetic studies. Safeguarding forest connectivity and reducing habitat loss are essential to preserve this high level of genetic diversity, which is critical for maintaining the long-term survival, adaptability, and evolutionary potential of this vulnerable primate.

1. Introduction

Tarsiers are small primates with a geographically restricted distribution in Southeast Asia. Although broadly sympatric across the region, most populations occur allopatrically or parapatrically, with only limited zones of overlap. This distribution pattern provides important insights into taxonomy and evolutionary divergence, as island populations frequently exhibit distinctive morphological and genetic characteristics resulting from geographic isolation (Shekelle *et al.* 2017).

Three tarsier genera are recognized: *Tarsius* (Sulawesi), *Carlito* (southern Philippines), and *Cephalopachus* (Sundaland). Within *Cephalopachus bancanus*, four subspecies have been identified: *C. b. bancanus* (Bangka and southern Sumatra), *C. b. borneanus* (Borneo), *C. b. saltator* (Belitung Island), and

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C. b. natunensis (Serasan Island) (Groves and Shekelle 2010). All subspecies face increasing anthropogenic pressure, but *C. b. saltator* is particularly vulnerable due to its insular isolation, small population size, and restricted range, with an estimated density of only 19–46 individuals/km² (Yustian *et al*. 2009; Shekelle and Yustian 2020). The remaining populations persist in a few spatially isolated forest fragments, including Bukit Peramun and Batu Mentas, separated by anthropogenic landscapes that may limit connectivity. Despite legal protection and Endangered status under the IUCN, ongoing habitat degradation from tin mining, oil palm expansion, and illegal logging continues to fragment forests and increase extinction risk (Syafutra *et al*. 2017).

Genetic studies of *C. bancanus* in Sumatra and Borneo have revealed substantial intraspecific variation and clear phylogeographic structure (Widayanti and Solihin 2007). Similarly, studies of Sulawesi tarsiers demonstrate that geographic isolation has been a key driver of genetic differentiation and speciation (Shekelle *et al*. 2017). These findings suggest that the Belitung population may harbor unique genetic variants, but molecular data for *C. b. saltator* remains almost entirely lacking. This knowledge gap hinders both taxonomic clarification and the development of evidence-based conservation strategies.

Mitochondrial DNA (mtDNA) markers are widely used in primate phylogeography because of their high copy number, maternal inheritance, and relatively rapid mutation rates (Wirdateti *et al*. 2015). Among these, the non-coding Displacement Loop (D-loop) is particularly informative, as its hypervariable regions provide high-resolution detection of intraspecific diversity and population structure (Kowalczyk *et al*. 2021). Previous studies have shown that the D-loop is effective for distinguishing tarsier populations across geographic regions (Widayanti and Solihin 2007; Widayanti 2008).

The mitochondrial D-loop has the advantage of containing hypervariable regions that are highly polymorphic and evolve faster than mtDNA coding segments. This high diversity is also evident between individuals who do not have direct maternal relationships (Satiyarti and Anggita 2018). This allows identification at the individual or varietal level, making it ideal for diversity analysis within species (Kowalczyk *et al*. 2021). The D-loop also serves as the main regulatory site for mtDNA replication and transcription (Gustafsson *et al*. 2016).

Molecular information on *Cephalopachus bancanus saltator*, an endemic subspecies of Belitung Island, remains extremely limited. Existing genetic references are largely restricted to studies of the mitochondrial D-loop region in *Tarsius bancanus* and *T. spectrum* (Widayanti and Solihin 2007). Given the importance of molecular data for taxonomy, population differentiation, and conservation, the lack of genetic information for the Belitung tarsier represents a significant knowledge gap, particularly in the context of ongoing deforestation and land-use change.

This study aimed to assess genetic diversity in the Belitung Tarsier using mitochondrial D-loop sequences in response to knowledge gaps and the urgent conservation status of *C. b. saltator*. Belitung Island has undergone extensive habitat fragmentation due to tin mining and conversion of lowland forests to oil palm and other land uses, resulting in increasingly isolated forest patches that may constrain tarsier movement (Kusuma *et al*. 2024). This landscape context supports the hypothesis that prolonged insular isolation and fragmentation may be associated with high haplotype diversity and limited gene flow. The findings provide baseline molecular data to support future taxonomic clarification and conservation planning for this geographically restricted subspecies.

2. Materials and Methods

2.1. Study Area

The research samples consisted of ear tissue specimens collected from Bukit Peramun, Air Selumar Village, Batu Mentas, Kelekak Datuk Village, Belitung. The sampling locations are shown in Figure 1. This study was conducted from August to December 2024 at the Genetics and Biotechnology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, and the Biotechnology Laboratory, Specialist Medical Education Program, Faculty of Medicine, Universitas Sriwijaya, Palembang.

2.2. Determination of Cell Density

The materials used in this study were 2× MyTaq HS Red Mix Bioline, agarose gel (1.5% and 2%), 100 bp plus ladder, DNA primers (DLTARPROF and DLTARBFR), Qiagen DNeasy Blood and Tissue Kit, and tarsier ear tissue samples from Belitung Island, collected specifically from (Bukit Peramun: n = 4; Batu Mentas: n = 2). Detailed information about the analyzed samples is presented in Table 1.

2.3. Procedures

2.3.1. Sample Collection and Preservation

The ear tissue sample was collected from six tarsier individuals on Belitung Island: four from Bukit Peramun

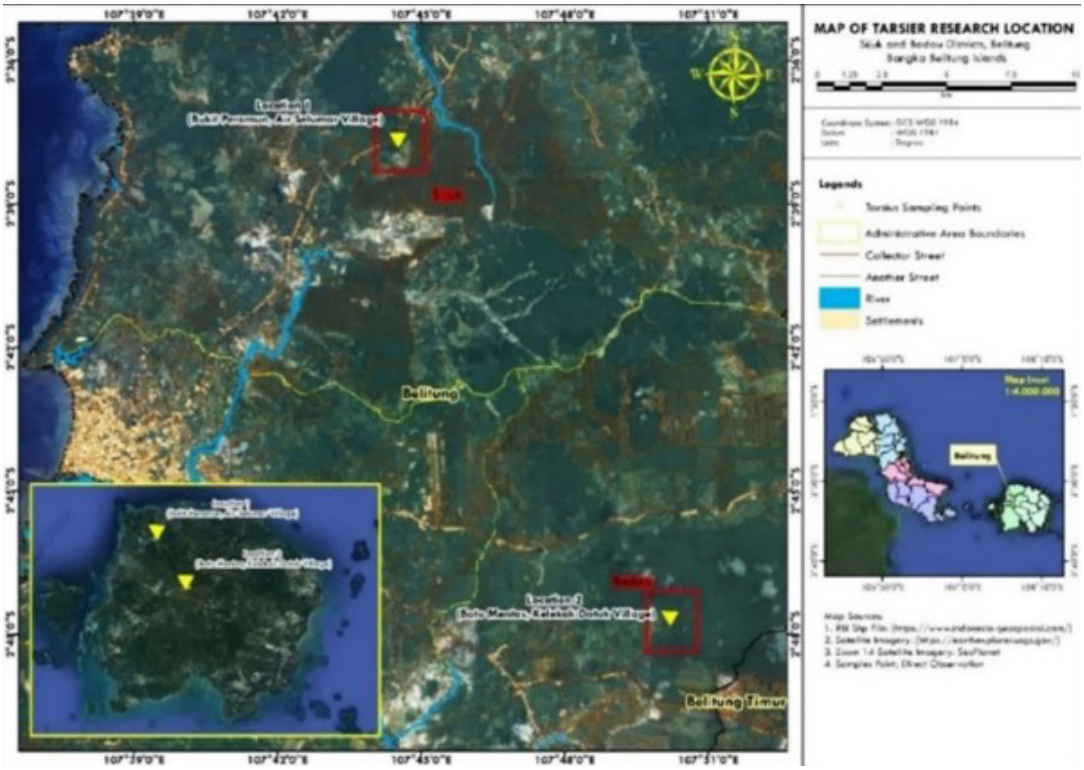

Figure 1. Sampling locations for Belitung Island tarsiers: Location 1: Bukit Peramun, Air Selumar Village; Location 2: Batu Mentas, Kelekak Datuk Village

Table 1. Sample data of Tarsier individuals from Belitung Island

Sample name and code	Sample type	Sample origins
Tarsier Belitung 1 (BEL 1)	Ear Tissue	Bukit Peramun, Air Selumar Village, Belitung
Tarsier Belitung 2 (BEL 2)	Ear Tissue	
Tarsier Belitung 3 (BEL 3)	Ear Tissue	
Tarsier Belitung 4 (BEL 4)	Ear Tissue	
Tarsier Belitung 5 (BEL 5)	Ear Tissue	Batu Mentas, Kelekak Datuk Village, Belitung
Tarsier Belitung 6 (BEL 6)	Ear Tissue	

in Air Selumar Village and two from Batu Mentas in Kelekak Datuk Village. The sample collection process was conducted in accordance with the protocol. All sampling procedures were conducted using non-lethal, minimally invasive methods to minimize stress and potential harm to the animals. Individuals were briefly restrained by trained personnel, and a small ear tissue biopsy was collected using sterile instruments under field conditions. Tissue sampling was limited in size and conducted in accordance with established wildlife ethical guidelines for primates, after which each individual was immediately released at the site of capture. Genetic resource access permits for this research were obtained from the Directorate General of Nature Resources and Ecosystem Conservation (Ditjen KSDAE), Ministry of Environment and Forestry of the Republic of Indonesia (Number SK85/KSDAE/SET.3/KSA.2/5/2023). The six ear tissue samples were preserved in absolute ethanol and subsequently processed for total DNA isolation.

2.3.2. Total DNA Isolation

Total DNA isolation from ear tissue samples was performed following the manufacturer's protocol for the Qiagen DNeasy Blood and Tissue Kit. The DNA isolation process consists of three main stages: lysis, extraction or separation of DNA from solid materials such as proteins and cellulose, and DNA purification.

2.3.3. DNA Quantity Test

The quantification test for pure DNA from ear tissue samples of Tarsier from Belitung Island was conducted using a NanoDrop Thermo Scientific spectrophotometer.

Calibration, as an initial step in NanoDrop usage, was performed by blanking the instrument with 1 µL of NFW (nuclease-free water) applied using a foam-tipped swab. The sample was then placed onto the NanoDrop surface in a volume of 1-2 µL for DNA concentration measurement.

2.3.4. DNA Quality Test

The DNA quality test was performed using agarose gel electrophoresis with an agarose concentration of 1.5%.

2.3.5. DNA Amplification

Extracted samples with sufficient purity and concentration will then undergo amplification. Amplification is performed using Polymerase Chain Reaction (PCR), which begins with instrument calibration or preheating for 30 minutes before use. The specific D-loop primers for Tarsier used in this study are presented in Table 2.

DNA amplification was performed with 2x MyTaq HS Red Mix (Applied Biosystems) in a T100 Thermal Cycler. DNA amplification was performed in a 25 µL PCR reaction, with the required components detailed in Table 3.

After all reagents were added to the microtube master mix, the mixture was homogenized using a micropipette. The tubes containing the reagents were homogenized to prevent evaporation during PCR. The prepared tubes were then placed into the wells of the Thermal Cycler and configured according to the protocol in Table 4.

Table 2. D-loop primer base pair sequence

Primer	Sequence (5'-3')	Amplified D-Loop Gene Size (bp)
DLTARPROF (Forward)	5'-CTG GCA TTC TCC ATA AACT-3.'	417
DLTARBFR (Reverse)	5'-GTT GCT GAT TTC ACG GAG GAAG-3'	417

Table 3. Mastermix compositions

Component	Volume (µL)
2x MyTaq HS Red Mix Bioline	13
Primer DLTARPROF (Forward)	1
Primer DLTARBFR (Reverse)	1
DNA Template	2
Nuclease-Free Water	8
Total	25

Table 4. PCR condition protocol

Steps	Times	Temperature (°C)
Pre-denaturation	2 minutes	94
Denaturation	30 second	94
Annealing	45 second	48.4
Elongation	1 minute	72
Extension	5 minutes	72
Number of cycles	35 cycles	

(source: Widayanti and Solihin 2007). Note: except for annealing based on the optimization trial

2.3.6. Electrophoresis Gel Agarose

PCR products were separated by 2% agarose gel electrophoresis in 1× TBE buffer. Gels were prepared by dissolving 2 g of agarose in 100 mL buffer, staining with GelRed, and casting with a comb. A DNA size marker (5 µL with 1 µL loading dye) and 3 µL of PCR products were loaded into the wells. Electrophoresis was conducted at 80 V and 400 mA for 40 min, and DNA bands were visualized under UV illumination using a GelDoc system.

2.3.7. Sequencing DNA

PCR products that passed quality verification by agarose gel electrophoresis were sequenced. To ensure reproducibility, each sample was amplified in at least two independent PCR reactions. Amplicons showing a single band of the expected size were purified to remove residual primers, nucleotides, enzymes, and other contaminants, and subsequently sequenced bidirectionally (forward and reverse) using the same primers. Sequencing was performed by Apical Scientific (1st BASE, Malaysia) via PT. Genetika Science using the Sanger method with capillary electrophoresis on an ABI PRISM® 3730xl Genetic Analyzer and BigDye™ Terminator v3.1 chemistry. Resulting chromatograms (.ab1 files) were used for downstream sequence editing and alignment.

2.3.8. Data Analysis

Genetic data obtained from laboratory analyses were processed and presented in tables and figures. DNA sequences were assembled into contigs using GeneStudio and edited prior to homology searches using BLAST (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related sequences. Two mitochondrial genome sequences retrieved from GenBank (*Tarsius bancanus*, NC_002811.1; *Carlito syrichta*, NC_012774.1) were used as reference

sequences in BLAST searches, sequence alignment, and phylogenetic analyses, with *C. syrichta* designated as an outgroup. Subsequent analyses, including multiple sequence alignment, assessment of nucleotide variation, calculation of pairwise genetic distances, and phylogenetic reconstruction using the Neighbor-Joining method, were conducted in MEGA version 11. Node support was evaluated using 1,000 bootstrap replicates. A median-joining haplotype network was constructed in PopART to visualize relationships among haplotypes, while haplotype diversity and other genetic diversity indices were estimated using DnaSP (latest version).

3. Results

3.1. Isolation of Total DNA

The sample with the highest DNA concentration was BEL 6, with a value of 139.3 ng/µl, while the lowest concentration was observed in BEL 4, measuring 5.8 ng/µl. The DNA purity value of BEL 4 was 1.79, which is still considered relatively pure. Meanwhile, BEL 2 had a purity value of 2.12 (Table 5). DNA concentration is considered adequate when the isolated DNA concentration is above 20 ng/µl, and the DNA extract purity ranges from 1.8 to 2.0. If the purity value is below 1.8, the isolated DNA may be contaminated with protein; conversely, if the purity value is above 2.0, the DNA isolation may be contaminated with RNA.

The electrophoresis result for sample BEL 4 showed a faint, barely visible DNA band (Figure 2). The condition observed in sample BEL 4 was due to DNA degradation. Degraded DNA appears very faint because its molecules have been fragmented into smaller pieces. DNA degradation is a dynamic process influenced by environmental factors, including temperature, humidity, and ultraviolet radiation. The postmortem interval affects organisms in various ways, with mechanisms such as hydrolysis, oxidation, and depurination compromising the structural integrity of DNA.

Table 5. Total DNA quantity test

Sample name and code	Sample weight (mg)	Concentration (ng/µl)	Purity (A260/A280)	Sample origins
Tarsier Belitung 1 (BEL 1)	19.6	92.5	1.93	
Tarsier Belitung 2 (BEL 2)	15.2	50.9	2.12	Bukit Peramun, Air
Tarsier Belitung 3 (BEL 3)	27.5	109.6	1.92	Selumar Village, Belitung
Tarsier Belitung 4 (BEL 4)	10.9	5.8	1.79	
Tarsier Belitung 5 (BEL 5)	29.4	105.1	1.86	Batu Mentas, Kelekak
Tarsier Belitung 6 (BEL 6)	43.9	139.3	1.87	Datuk Village, Belitung

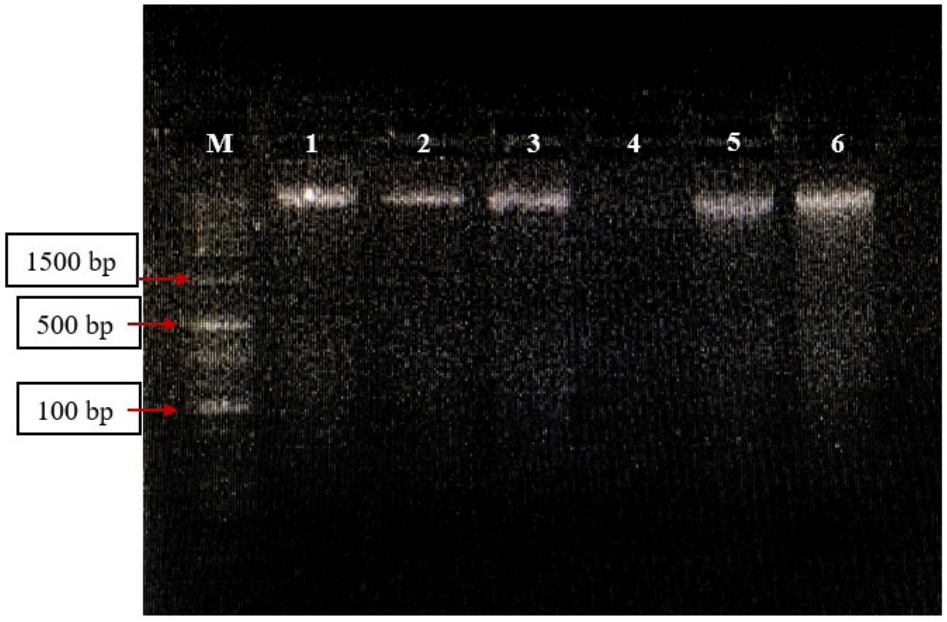

Figure 2. Agarose gel electrophoresis of DNA concentration was consistently observed across all six individuals (BEL 1 – BEL 6), indicating a successful DNA isolation process and suitability of the products for subsequent amplification

3.2. Polymerase Chain Reaction (PCR) Product

The primers DLTARPROF and DLTARBFR successfully amplified all six test samples (Figure 3). The PCR products were visualized at an annealing temperature of 48.4°C. PCR product band intensities varied among samples. The clearest and thickest band was observed in sample BEL 3, while the faintest band was seen in sample BEL 4. The faint band in BEL 4 was due to its very low DNA concentration and purity below 1.8, as indicated in Table 5.

3.3. Total Base Pairs of the D-Loop Gene

The DNA sequences from the contigs in each sample, BEL 1 to BEL 6, have a total of different base pairs, as shown in Table 6.

The six Tarsier test samples from Belitung Island exhibited varying D-loop gene sizes. The DNA amplification results for these samples ranged from 418 bp to 424 bp, as indicated in Table 6. A partial D-loop DNA fragment measuring 417 bp was successfully obtained through amplification using the primers DLTARPROF and DLTARBFR. Subsequently, DNA sequencing was performed using the same primers, and the resulting sequences were aligned with the *Tarsius bancanus* genome sequence in GenBank.

3.4. Homology Search Basic Local Alignment Search Tool (BLAST) NCBI

The results of the homology search carried out using BLAST NCBI on the six samples are shown in Table 7.

The observed D-loop sequence lengths ranged from 418 to 424 bp, with multiple nucleotide variations including substitutions, insertions, and deletions. Alignment with reference sequences from GenBank showed high similarity with *Cephalopachus bancanus* (88.12%-89.76%) based on BLAST NCBI analysis and lower similarity with *Carlito*

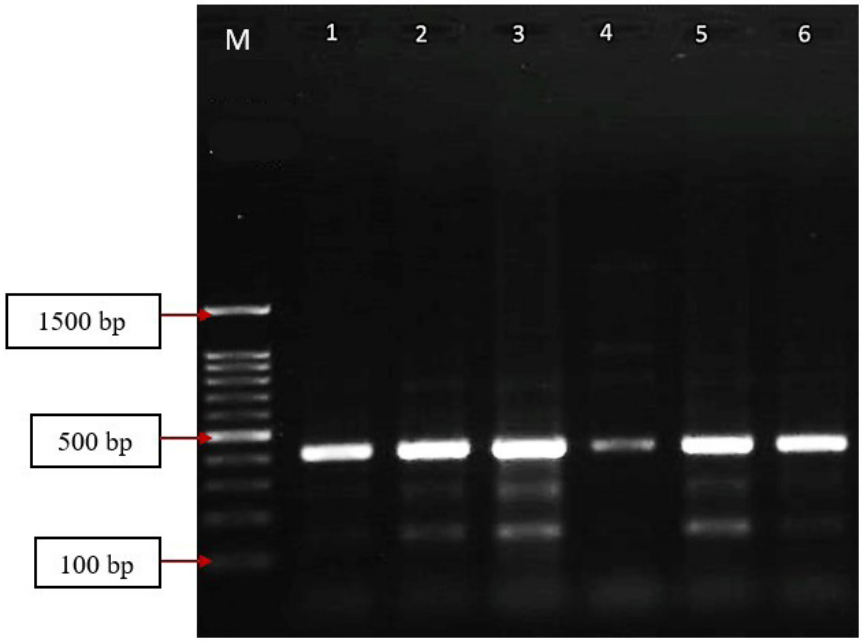

Figure 3. Agarose gel electrophoresis of mtDNA D-loop fragments (~420 bp) from six *Cephalopachus bancanus saltator* individuals, showing successful amplification for subsequent sequencing

Table 6. Total base pairs of the D-loop sene in Tarsier sequences from Belitung Island

Sample name and code	Total base pairs (bp)	Sample origins
Tarsier Belitung 1 (BEL 1)	424	Bukit Peramun, Air Selumar Village, Belitung
Tarsier Belitung 2 (BEL 2)	418	
Tarsier Belitung 3 (BEL 3)	423	
Tarsier Belitung 4 (BEL 4)	419	
Tarsier Belitung 5 (BEL 5)	419	Batu Mentas, Kelekak Datuk Village, Belitung
Tarsier Belitung 6 (BEL 6)	421	

syrichta (77.8%-78.21%). These similarity levels indicate intraspecific variation within *Cephalopachus bancanus* and support the taxonomic placement of the Belitung population within this species group.

BLAST results (Table 7) show that D-loop gene sequence data in *Tarsius* are still very limited and rarely studied. As a result, most species in the family Tarsiidae lack basic D-loop data in GenBank, which hinders subsequent phylogenetic and conservation studies. This gap is likely due to sampling bias, as studies tend to focus on more accessible species. Remote subpopulations such as *Cephalopachus bancanus* saltator are thus underrepresented. Therefore, expanded sampling and the addition of reference sequences in GenBank are essential to support accurate genetic identification and effective conservation planning.

3.5. Alignment Result

The alignment results for the six samples against GenBank data, including genetic mutations, are presented in Table 8.

The alignments reveal several mutations occurring in the nucleotide sequence, including deletion, substitution, and insertion mutations. Deletion mutations arise from the loss of one or more base pairs in the DNA sequence. Meanwhile, substitution mutations occur when another base pair replaces one base pair in the DNA sequence.

The sequencing of the mitochondrial D-loop region in six *Cephalopachus bancanus* saltator individuals from Belitung Island revealed substantial nucleotide variation. Alignment results showed 368 conserved sites out of 424 bp and 51 variable sites, comprising 7 parsimony-informative and 44 singleton sites, are presented in

Table 7. Homology Search BLAST NCBI Sekuen BEL 1 – BEL 6

Sample	Description	Query cover (%)	E value	Per. Ident (%)	Accession
BEL 1	*Tarsius bancanus* mitochondrion, complete genome	98	2e-134	88.12	NC_002811.1
	Carlito syrichta mitochondrion, complete genome	98	9e-58	77.80	NC_012774.1
BEL 2	*Tarsius bancanus* mitochondrion, complete genome	99	6e-144	89.50	NC_002811.1
BEL 3	*Tarsius bancanus* mitochondrion, complete genome	98	1e-145	89.76	NC_002811.1
	Carlito syrichta mitochondrion, complete genome	98	9e-58	77.80	NC_012774.1
BEL 4	*Tarsius bancanus* mitochondrion, complete genome	100	4e-141	89.07	NC_002811.1
	Carlito syrichta mitochondrion, complete genome	100	9e-58	77.80	NC_012774.1
BEL 5	*Tarsius bancanus* mitochondrion, complete genome	99	1e-140	89.05	NC_002811.1
	Carlito syrichta mitochondrion, complete genome	99	4e-61	78.21	NC_012774.1
BEL 6	*Tarsius bancanus* mitochondrion, complete genome	99	6e-144	89.55	NC_002811.1
	Carlito syrichta mitochondrion, complete genome	99	4e-61	78.21	NC_012774.1

Table 9. These mutations included base substitutions (both transitions and transversions), deletions, and insertions—indicating a relatively dynamic mutational landscape within this population.

3.6. Genetic Distance Analysis

The genetic distance results for the six test samples and the sequence database are presented in Table 10.

Genetic distances among Belitung samples were relatively low (0.0074-0.0370), supporting a close evolutionary relationship among individuals. However, the genetic distances to *Carlito syrichta* (0.1555-0.1777) indicate a clear divergence at the genus level, in line with current taxonomic classifications. The relatively short genetic distances among Belitung individuals, combined with the high haplotype diversity, suggest that this population has not undergone a severe bottleneck and still retains considerable genetic health.

Genetic distances among samples BEL 1 to BEL 6 were relatively close to the complete mitochondrial genome of *Tarsius bancanus* (NC_002811.1). The genetic distance for BEL 1, BEL 5, and BEL 6 was 0.1037 relative to *Tarsius bancanus* NC_002811.1, while the genetic distance of BEL 2, BEL 3, and BEL 4 to *Tarsius bancanus* NC_002811.1 was 0,0963. BEL 1 and BEL 3, as well as BEL 4 and BEL 5, shared the closest genetic similarities (distance = 0.0074). The most distant genetic relationships were between BEL 2 and BEL 4, and between BEL 1 and BEL 5-BEL 6, with a genetic distance of 0.0296.

Table 8. Results of alignment and gene mutation

Sample code	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
BEL 1	C	A	-	C	T	C	A	A	-	-	C	A	A	T	C	C
BEL 2	C	A	-	C	T	C	A	A	-	C	A	-	A	T	C	C
BEL 3	C	A	A	C	T	C	A	A	-	-	C	A	A	T	C	C
BEL 4	C	A	-	C	T	C	A	A	T	C	C	C	A	T	C	C
BEL 5	C	A	A	C	T	C	A	A	-	-	C	C	A	T	C	C
BEL 6	C	A	-	C	T	C	A	T	-	C	A	C	A	T	C	C
NC_002811.1	C	A	-	C	T	C	A	A	-	-	C	T	A	T	C	T

Table descrption: BEL 1- BEL 6 are the six test samples, and NC_002811.1 is the *Tarsius bancanus* mitochondrion complete genome from GenBank. Number in box description: 1. Deletion mutation; 2. Conserved site; 3. Substitution mutation 4. Insertion mutation

Table 9. Variation of nucleotide bases and total GAP

Sample code	Conserved sites	Variation		Variable sites	Total *gap*
		Pi	S		
BEL 1	368	7	44	51	8
BEL 2	368	7	44	51	8
BEL 3	368	7	44	51	8
BEL 4	368	7	44	51	8
BEL 5	368	7	44	51	8
BEL 6	368	7	44	51	8

Pi: Parsimony-informative site; S: Singleton site; Total gap: Insertion and Deletion (Indel) events

Table 10. Genetic distance

	1	2	3	4	5	6	7	8
BEL 1								
BEL 2	0.0222							
BEL 3	0.0074	0.0148						
BEL 4	0.0222	0.0296	0.0148					
BEL 5	0.0148	0.0222	0.0074	0.0074				
BEL 6	0.0296	0.0074	0.0222	0.0370	0.0296			
NC_002811.1	0.1037	0.0963	0.0963	0.0963	0.1037	0.1037		
NC_012774.1	0.1629	0.1555	0.1703	0.1703	0.1777	0.1629	0.1629	

BEL 1 – BEL 6 represent the six tested samples. NC_002811.1 = *Tarsius bancanus* mitochondrion complete genome, and NC_012774.1 corresponds to *Carlito syrichta* mitochondrion complete genome. 1-8 indicates the genetic distance between samples

3.7. Haplotype Network Analysis

The haplotype network analysis of the mitochondrial D-loop region revealed that each of the six *Cephalopachus bancanus saltator* individuals from Belitung Island represented a unique haplotype, with no haplotype sharing observed. This pattern demonstrates exceptionally high haplotypic diversity within the insular population, consistent with the haplotype diversity value (Hd = 1.0) obtained in this study (Figure 4).

3.8. Phylogenetic Analysis

Phylogenetic reconstruction using the neighbor-joining method revealed two main clades (Figure 5). One clade comprised individuals BEL 2 and BEL 6, with strong bootstrap support (93%). In contrast, the second clade included the remaining four individuals and formed two subclades (BEL 1–BEL 3 and BEL 4–BEL 5) with moderate support (68%-69%). *Carlito syrichta* (NC_012774.1) was clearly separated as an outgroup,

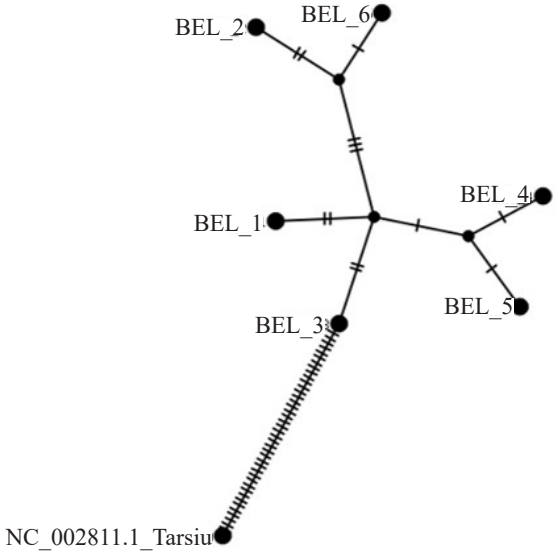

Figure 4. A haplotype network of Tarsiers from Belitung Island, constructed using the median-joining method in the PopART software

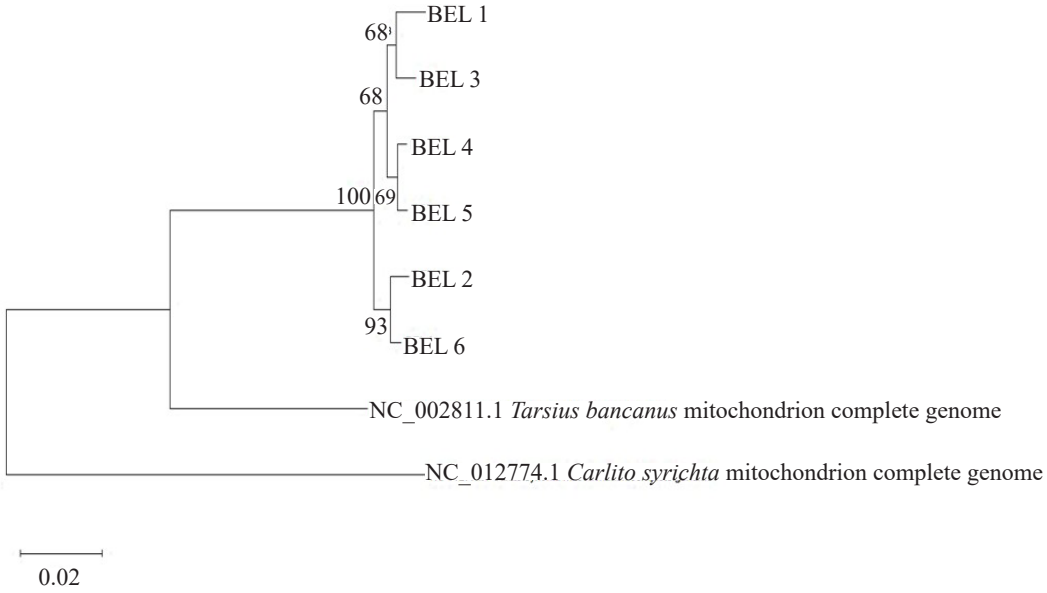

Figure 5. Phylogenetic tree reconstruction of Tarsier from Belitung Island with outgroup, using the Neighbor-Joining algorithm in the Mega Program with 1000 Bootstrap times

supporting the monophyly of the *Cephalopachus bancanus* samples.

The two well-supported clades observed in the phylogenetic tree suggest a potential historical separation or limited gene flow between sampling sites, possibly reflecting micro-allopatric divergence or ecological barriers. This finding aligns with the hypothesis that insular tarsier populations often experience genetic structuring due to geographical constraints and habitat patchiness.

3.9. Haplotype Diversity Analysis

The results of the haplotype diversity analysis for the six samples, which are divided into two populations, are presented in Table 11.

4. Discussion

The mitochondrial D-loop analysis revealed high haplotype diversity (Hd = 1.0) in *Cephalopachus bancanus* saltator from Belitung Island, with each sampled individual possessing a distinct haplotype. This level of variation, despite the population's limited range, is comparable to patterns reported in other insular primates with geographically isolated populations (Oklander and Soto-Calderon 2024). The phylogenetic reconstruction revealed two moderately supported clades, which may reflect variation among sampled individuals rather than definitive population subdivision. However, because mitochondrial markers represent only maternal lineages, nuclear DNA data will be required to confirm whether these subdivisions reflect true population structure. It should be noted that haplotype diversity values derived from small sample sizes and hypervariable mitochondrial regions should be interpreted cautiously.

The phylogenetic clustering did not correspond to sampling localities, as individuals from Bukit Peramun and Batu Mentas were interspersed across clades rather than forming site-specific groupings.

Table 11. Haplotype diversity value

Population	n	Genetic diversity		
		Hn	Hd	π
Bukit Peramun, Air Selumar Village, Belitung	4	4	1	0.01606
Batu Mentas, Kelekak Datuk Village, Belitung	2	2	1	0.01446

n is the number of species; Hn is the number of haplotypes; Hd is haplotype diversity; π is nucleotide diversity

This pattern provides no clear evidence of population subdivision between the two sampling sites and does not permit inference regarding the presence or absence of gene flow. The observed structure may reflect shared ancestral haplotypes, recent common ancestry, or limited phylogenetic resolution resulting from the small sample size and reliance on a single mitochondrial marker. Additional sampling and the inclusion of nuclear markers will be necessary to evaluate fine-scale population structure within Belitung Island.

When compared with other *C. bancanus* populations across Sumatra, Borneo, and the Natuna Islands, the Belitung population shows a comparable or even higher level of haplotype variation (Widayanti and Solihin 2007; Zahidin *et al*. 2019). In contrast, several mainland populations exhibit reduced diversity, likely due to habitat loss and demographic contraction. The distinct haplotype composition and moderate genetic distances from other reported populations may represent early stages of lineage divergence, similar to patterns of incipient speciation documented in other geographically isolated tarsiers in Southeast Asia (Shekelle *et al*. 2017). This underlines the evolutionary significance of the Belitung population in the broader context of island biogeography, where repeated cycles of sea-level change have shaped patterns of isolation and diversification (Woodruff 2010; Louys *et al*. 2021).

The evolutionary implications of these findings should be interpreted with caution. The presence of multiple haplotypes in the sampled individuals may indicate the absence of a recent severe bottleneck; however, this inference is limited by the small sample size and should be considered preliminary (Dixo *et al*. 2009). Ongoing deforestation and mining on Belitung Island have the potential to fragment habitats further, thereby increasing the risk of genetic erosion through drift and inbreeding. Although these impacts were not directly assessed in this study, such pressures could affect the long-term genetic integrity of this insular population, highlighting the need for broader genetic sampling in future conservation assessments.

From a management perspective, the results do not yet support formal recognition of the Belitung population as a distinct conservation unit. However, the restricted range and the haplotypic variation observed in the sampled individuals suggest that this population may warrant further evaluation as a potential conservation unit, pending confirmation using additional mitochondrial markers, complete mitogenome data, and/or multilocus nuclear genetic data (Moritz 1994; Allendorf *et al*. 2013).

In the interim, conservation actions such as maintaining habitat connectivity, restoring degraded areas, and implementing long-term genetic monitoring may be considered as precautionary measures. Such approaches could help preserve genetic diversity and ecological persistence while more comprehensive genetic data are developed (Hvilsom *et al*. 2022).

Finally, this study has important limitations. The sample size is small, geographic coverage is restricted to two localities, and only a single mitochondrial marker was used. Broader sampling, the inclusion of nuclear DNA markers, and integration with ecological and behavioral data will be essential to refine our understanding of population structure and to strengthen conservation planning. Despite these constraints, the findings provide critical baseline data for conservation genetics and contribute to the growing body of evidence that insular tarsier populations warrant heightened protection as distinct evolutionary entities.

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References

Allendorf, F.W., Luikart, G., Aitken, S.N., 2013. *Conservation and the Genetics of Populations*, second ed. Wiley-Blackwell, USA.

Bhoyar, L., Mehar, P., Chavali, K., 2024. An overview of DNA degradation and its implications in forensic caseworks. *Egypt J Forensic Sci*. 14, 15. https://doi.org/10.1186/s41935-024-00389-y

Dixo, M., Metzger, J.P., Morgante, J.S., Zamudio, K.R., 2009. Habitat fragmentation reduces genetic diversity and connectivity among toad populations in the Brazilian Atlantic Coastal Forest. *Biological Conservation*. 142, 1560-1569. https://doi.org/10.1016/j.biocon.2008.11.016

Groves, C., Shekelle, M., 2010. The Genera and Species of Tarsiidae. *Int J Primatol*. 31, 1071-1082. https://doi.org/10.1007/s10764-010-9443-1

Gustafsson, C.M., Falkenberg, M., Larsson, N.G., 2016. Maintenance and expression of mammalian mitochondrial DNA. *Annu Rev Biochem*. 85, 133-160. https://doi.org/10.1146/annurev-biochem-060815-014402

Hvilsom, C., Segelbacher, G., Ekblom, R., Fischer, M.C., Laikre, L., Leus, K., O´Brien, D., Shaw, R., Sork, V., 2022. *Selecting Species and Populations for Monitoring of Genetic Diversity*. IUCN. Gland, Switzerland.

Kowalczyk, M., Staniszewski, A., Kamińska, K., Domaradzki, P., Horecka, B., 2021. Advantages, possibilities, and limitations of mitochondrial DNA analysis in molecular identification. *Folia Biol (Kraków)*. 69, 101-111.

Kusuma, Y.W.C., Surya, M.I., Kurniawati, S., Yulita, K.D.S., Risna, R.A., Sudarmonowati, E., Matsuo, A., Kurita, K., Suyama, Y., Isagi, Y., 2024. Genetic diversity and structure of *Hopea bilitonensis*, an endemic Dipterocarp from Belitung Island, Indonesia. *Journal of Asia-Pacific Biodiversity*. 17, 400-405. https://doi.org/10.1016/j.japb.2024.01.008

Louys, J., Curnoe, D., Tong, H., 2021. The evolutionary biogeography of Southeast Asian mammals. *Biological Reviews*. 96, 266-293. https://doi.org/10.1111/brv.12652

Matsui, A., Rakotondraparany, F., Munechika, I., Hasegawa, M., Horai, S., 2009. Molecular phylogeny and evolution of prosimians based on complete sequences of mitochondrial DNAs. *Gene*. 441, 53-66. https://doi.org/10.1016/j.gene.2008.08.024

Moritz, C., 1994. Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology & Evolution*. 9, 373-375. https://doi.org/10.1016/0169-5347(94)90057-4

Oklander, L.I., Soto-Calderón, I.D., 2024. Applications of primate genetics for conservation and management. *Annual Review of Anthropology*. 53, 371-395. https://doi.org/10.1146/annurev-anthro-041422-114003

Satiyarti, R.B., Anggita, R., 2018. Endogamy marriage mitochondrial DNA variation of North Cigintung Garut isolates. *Biosfer: J Tadris Biol*. 9, 72-83. https://doi.org/10.24042/biosf.v9i1.2883

Schmitz, J., Ohme, M., Zischler, H., 2002. The complete mitochondrial sequence of *Tarsius bancanus*: Evidence for an extensive nucleotide compositional plasticity of primate mitochondrial DNA. *Mol Biol Evol*. 19, 544-553. https://doi.org/10.1093/oxfordjournals.molbev.a004110

Shekelle, M., Yustian, I., 2020. *Cephalopachus bancanus* ssp. saltator. The IUCN red list of threatened species 2020: e.T39765A17992312. https://dx.doi.org/10.2305/IUCN.

Shekelle, M., Groves, C.P., Maryanto, I., Mittermeier, R.A., 2017. Two new tarsier species (Tarsiidae, Primates) and the biogeography of Sulawesi, Indonesia. *Primate Conserv*. 31, 61-69.

Sophian, A., Syukur, A., 2021. Analysis of purity and concentration of isolated DNA in making raw DNA of rat species. *Eruditio*. 1, 1-5. https://doi.org/10.54384/eruditio.v1i2.75

Syafutra, R., Alikodra, H.S., Iskandar, E., 2017. Distribution and population of mentilin (*Cephalopachus bancanus bancanus*) in Bangka Regency. *Curr Res J Biol Sci*. 9, 9-15. https://doi.org/10.19026/crjbs.9.3421

Widayanti, R., Solihin, D.D., 2007. Kajian penanda genetik T*arsius bancanus* dan *Tarsius spectrum* dengan sekuen D-loop parsial DNA mitokondria. *Biota*. 170-176. https://doi.org/10.24002/biota.v12i3.2804

Widayanti, R., 2008. Kajian molekuler daerah D-loop parsial pada DNA mitokondria *Tarsius bancanus*. *Media Kedokteran Hewan*. 24, 86-91.

Wirdateti., Wulandari, S.W., Kuswandi, P.C., 2015. Penanda genetik *Tarsius* (*Tarsius* ssp.) dengan menggunakan gen Cytochrome Oxidase I (COI) DNA mitokondria (mtDNA) melalui metode sekuensing. *J Biol Indones*. 11, 275-284.

Woodruff, D.S., 2010. Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity. *Biodiversity and Conservation*. 19, 919-941. https://doi.org/10.1007/s10531-010-9783-3

Yustian, I., Merker, S., Muehlenberg, M., 2009. Luas Daerah Jelajah dan Estimasi Kepadatan Populasi *Tarsius bancanus saltator* di Pulau Belitung. *J Biol Indones*. 5, 411-421.

Zahidin, M.A., Abd Jalil, N., Naharuddin, N.M., Abd Rahman, M.R., Gani, M., Abdullah, M.T. 2019. Partial mtDNA sequencing data of vulnerable *Cephalopachus bancanus* from the Malaysian Borneo. *Data in brief*. 25, 104133. https://doi.org/10.1016/j.dib.2019.104133