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The first complete mitochondrial genome of Sumatran striped rabbit *Nesolagus netscheri* (Schlegel, 1880), and its phylogenetic relationship with other Leporidae

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***Nesolagus netscheri*, a Sumatran striped rabbit, is one of the rarest rabbits in the Leporidae family, and its genetic information is still limited. This study provides the first mitochondrial genome and molecular systematic characterization of the Sumatran striped rabbit, *Nesolagus netscheri*, Indonesia's rarest rabbit. It consists of a circular double-stranded DNA of 16,709 bp. It showed that the mitochondrial genome structure of *N. netscheri* is similar to that of *N. timminsi*. The mitochondrial genome of *N. netscheri* contained 22 transfer RNA (tRNA) genes, and all tRNA except for trnS1 showed a characteristic cloverleaf secondary structure. Evidence was found that the *atp8* gene of *N. netscheri* is under positive selection pressure. The phylogenetic analysis shows Leporidae was monophyletic, with *Nesolagus* at the basal. The study indicates a split between *N. netscheri* and *N. timminsi* in the Late Pleistocene around 0.43 million years ago. This research is a fundamental reference for the conservation of the rarest lagomorph species and provides important information for future evolutionary studies in the Leporidae family.**

Keywords Leporidae, Mitochondrial genome, Sumatran striped rabbit (*Nesolagus netscheri*), Phylogenetic

Nesolagus is a rabbit genus that comprises three distinct species: the Annamite striped rabbit (*Nesolagus timminsi*), the Sumatran rabbit (*Nesolagus netscheri*), and the extinct *N. sinensis*[1]. The *N. netscheri* is a lagomorph species found endemically in Sumatra, Indonesia[2,3]. Because insufficient information is available to estimate population size, range, and density, the International Union for Conservation of Nature (IUCN) has classified this species as Data Deficient[4]. The *N. netscheri* has been a legally protected species in Indonesia since then, and it continues to be protected under the recently updated Indonesian law. It has been classified as the rarest lagomorph owing to the small number of museum specimens and the rarity of historical observations[5].

The relationship between *N. netscheri* and *N. timminsi* adds complexity to the genus's evolutionary context because geographically they are separated and both have significant threats from habitat loss and fragmentation[3,6]. Despite its historical recognition and occasional sightings, there remain substantial gaps in understanding the evolutionary relationships of *N. netscheri* within the *Nesolagus* genus and the broader family Leporidae, indicating a need for molecular phylogenetic studies to elucidate the evolutionary history and genetic diversity of *N. netscheri*. A comprehensive understanding of these aspects is crucial, as it would provide insights into the evolutionary processes that have shaped the species and inform conservation strategies.

Mitochondrial (mtDNA) genomes provide a wealth of genetic markers that are crucial for conservation efforts, particularly for species like the Sumatran striped rabbit (*Nesolagus netscheri*). The complete sequencing of mtDNA can uncover specific genetic markers, such as cytochrome c oxidase subunit I (COI) and cytochrome b, which is widely recognized as a universal DNA barcode for animal species identification[7–9]. This marker is particularly valuable for assessing genetic diversity and population structure, allowing conservationists to monitor the health of populations and identify distinct genetic lineages that may require targeted conservation

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efforts[10–14]. For instance, in the case of the Sumatran striped rabbit, identifying unique mtDNA haplotypes could inform habitat management and restoration strategies, ensuring that genetic diversity is preserved in fragmented landscapes. Additionally, mtDNA can reveal information about historical population dynamics and gene flow, critical for understanding how habitat fragmentation affects species like the Sumatran striped rabbit. By analyzing variations in mtDNA, researchers can identify genetic bottlenecks and assess the impacts of habitat loss on gene flow between populations[15,16]. This information is particularly relevant in the context of Sumatra's rapidly changing landscape, where deforestation and agricultural expansion have led to isolated populations that may be at risk of inbreeding[17–20].

The use of mtDNA markers can thus help prioritize conservation actions, such as creating wildlife corridors to enhance connectivity between fragmented habitats[21]. Moreover, the high mutation rate and maternal inheritance of mtDNA make it an ideal candidate for studying evolutionary relationships and phylogenetic analyses within Leporidae[9,10,22,23]. For example, the identification of specific mitochondrial markers can aid in distinguishing between closely related species or subspecies, providing insights into their evolutionary history and informing conservation strategies that consider the unique genetic makeup of each population[9].

This research is the first successful characterization of the mitochondrial genome of *N. netscheri*, which has not been previously published or available in any database. This study aims to characterize the mitogenome of *N. netscheri* and reconstruct the evolutionary tree in the family Leporidae using a novel mitochondrial genome sequence. This study provides valuable information, including molecular markers, which can be utilized to address conservation needs, considering the limited extent of studies on rabbits. These findings will provide valuable information for phylogenetic, evolutionary, and population genetic studies. Additionally, findings can potentially be important to conserving the rarest rabbit, *N. netscheri*.

Results and discussion

Mitogenome composition and organization

The complete assembly of *N. netscheri*'s circular mitochondrial genome was generated by the Oxford Nanopore Technolgy (ONT) long reads. The length of this final assembly was 16,709 bp, with a coverage of 5021×. It is 3 bp smaller than the other *N. timminsi* mitogenome (NC_063946.1). The complete mitochondrial genome sequence of Sumatran striped rabbit *Nesolagus netscheri* has been deposited in GenBank under the accession number PQ047138. This genome size is within the range of available Leporidae mitochondrial genome (Table 1.). As in other mammals[24], the mitogenome of *N. netscheri* encodes 37 classical mitochondrial genes (13 protein-coding genes [PCGs], 22 tRNAs, 2 rRNAs, and 1 D-loop) (Fig. 1). The complete mitogenome had a significant A + T bias in its nucleotide composition (30.05% A, 31.8% T, 13.3% C, and 24.4% G). In this mitogenome, the H strand transcribed twelve PCGs, two rRNAs, and thirteen tRNAs, while the L strand transcribed the remaining ten genes (Table 2). This transcription pattern is the same as that of the genus *N. timminsi*. *N. timminsi* and *N. netscheri* belong to the same genus, and their gene orders are similar (Supplementary File 1).

Protein-coding genes

Standard initiation codons (ATN) were used for the initiation of all PCGs, and conventional stop codons (TAG or TAA) were used for the termination of 9 out of 13 PCGs. The genes *ND3*, *ND4*, and *COX3* all terminated with incomplete stop codons (either T or TA; Table 2), while the *CYTB* gene had an alternate putative stop codon (AGG). The start codons found in the PCGs are consistent with the standard vertebrate mitochondrial start codons, except for ATT. There have been comparable reports of this terminal codon among various Leporidae[28,31,33]. The same phenomenon has been observed with the regular and common stop codons TAG and TAA in mammals[24]. AGG is an uncommon stop codon; however, it has been identified in other Leporidae species[28,31,33] and other vertebrates in previous study[34]. The *COX3*, *NAD3*, and *NAD4* genes in vertebrate mitochondrial genomes, including Leporidae[28,31,33], frequently exhibit reduced stop codons. These reduced stop codons have been reported to be addressed through post-transcriptional polyadenylation[35].

Species	Genbank access	Size	References
Lepus sinensis	NC_025316	17,438	22
Lepus hainanus	NC_025748	17,472	Unpublished
Lepus yarkandensis	NC_050569	17,011	25
Lepus tolai	NC_025748	17,472	22
Brachylagus idahoensis	NC_064132	17,021	26
Lepus europaeus	OM993420	16,679	27
Romerolagus diazi	MW927505	17,400	28
Nesolagus timminsi	NC_063946	16,712	Unpublished
Lepus oiostolus	MT376741	17,370	29
Lepus americanus	NC_024043	17,042	30
Oryctolagus cuniculus	MN296708	16,740	31
Sylvilagus sp	ON456163	16,456	Unpublished
*Ochotona rufescens**	ON859136	16,408	32

Table 1. List of Leporidae mitochondrial genomes chosen to reconstruct the phylogenetic tree. *Outgroup.

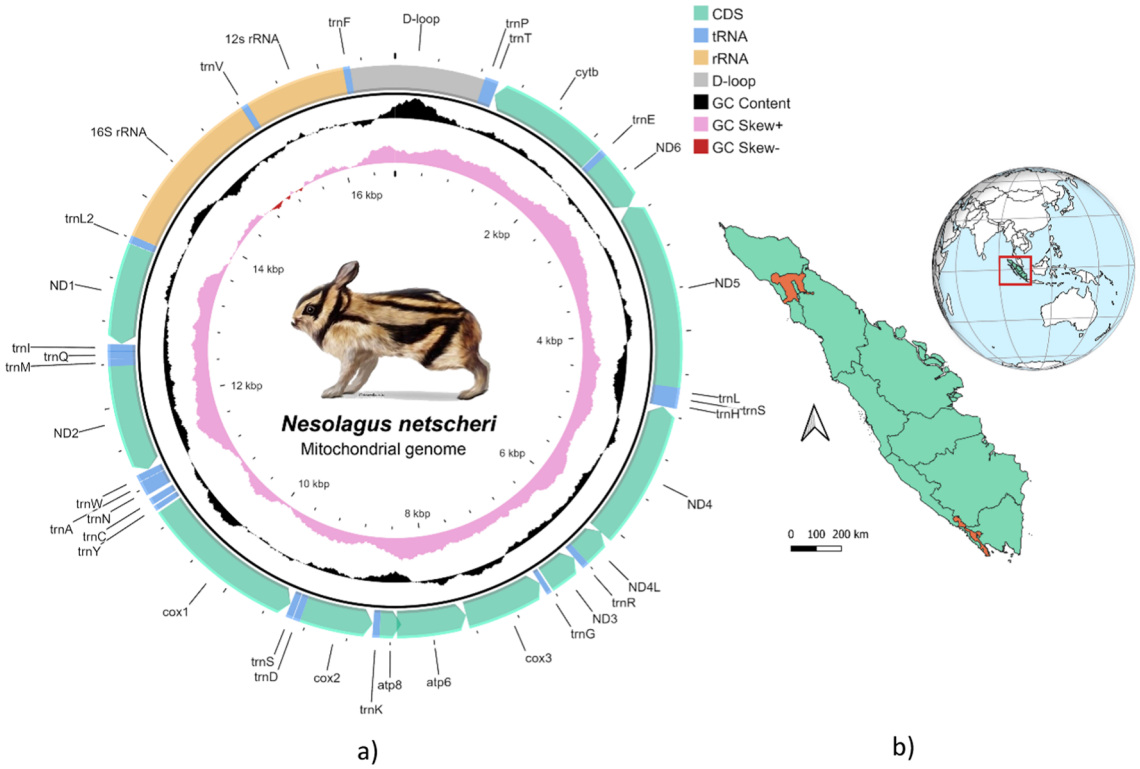

Fig. 1. (**a**) Circular representation of the mitochondrial genome of *Nesolagus netscheri*. The colored blocks indicate different gene types, with genes encoded on the light and heavy strands in counterclockwise and clockwise orientations, respectively. The black inner circle represents the GC content of the mitogenome, while the pink and red regions indicate GC skew. The rabbit illustration was adapted from https://scentsindonesia.com; (**b**) Geographic distribution of N. netscheri (orange regions) within its habitat. The map was created using QGIS version 3.40.1 Bratislava (https://www.qgis.org).

Relative synonymous codon usage values for *N. netscheri* are presented in Fig. 2. The PCGs contained 3,510 codons. The most prevalent codons in these mitogenomes encode Leu, Ile, Gly, and Thr. In contrast, the least frequently observed were those that encoded Trp. (Fig. 2).

Transfer and ribosomal RNA genes

The mitochondrial genome of *N. netscheri* had a total of 22 transfer RNA (tRNA) genes. The length of the tRNA genes varied from 54 bp (trnG) to 72 bp (trnN). A typical 'cloverleaf' secondary structure was inferred in all tRNA genes, with the exception of trnS1 (see Fig. 3). This is a commonly observed characteristic in genes for tRNA in mammals[36,37] and vertebrates[38]. The lack of an arm in trnS1 may potentially have a functional purpose in the process of structural compensation with other structures[39]. The 16 S RNA (rrnL) is positioned between tnrL2 and trnV and has a length of 1,580 bp, whereas the 12 S RNA (rrnS) is placed between trnV and trnF and has a length of 950 bp. The positions of the control region and rRNA genes (rrnS and rrnL) in the *N. netscheri* mitochondrial genome are similar to those already identified for other leporid mitogenomes[28,31,33].

The Ka/Ks ratio is an important parameter for quantifying selection pressure[40]. To assess the effect on mitochondrial PCGs, we investigated the Ka/Ks ratios for *N. netscheri* and two leporid species, *Lepus europaeus* and *Nesolagus timminsi*, which were used as reference species. The 13 PCGs showed varying Ka/Ks ratios, which suggests that the genes are exposed to a range of functional constraints (Fig. 4). The data indicates that all the PCGs are undergoing evolutionary changes due to purifying selection (<1), except for atp8. The lowest calculated value of Ka/Ks, which indicates selected pressure, was found for the *nd4l* gene in the Genus *Nesolagus* (Ka/Ks = 0.025). This suggests that nd4l faces the highest level of selective pressure and evolves at the slowest rate among the genes analysed. Purifying selection in mammalian mitochondrial DNA (mtDNA) is evidenced by studies that demonstrate a consistent pattern of selective pressure against deleterious mutations. For example, Stewart et al. highlighted that strong purifying selection operates during the transmission of mtDNA across generations, effectively maintaining the integrity of mitochondrial genomes by favoring the survival of less mutated variants[41]. Similarly, Tsai et al. reported that reducing the dosage of mitochondrial DNA polymerase enhances the elimination of defective mitochondrial genomes, indicating that purifying selection plays a critical role in maintaining mtDNA quality during oogenesis[42]. Furthermore, Ennis et al. found that the complete mitochondrial genome of Baird's tapir exhibited patterns of purifying selection in its protein-coding genes, reinforcing the notion that mtDNA is subject to evolutionary constraints across various mammalian

Name	Position		Size (bp)	Strand	Codon		Intergenic Nucleotide (bp)
	From	To			Start	Stop	
trnP	850	915	66	L			0
trnT	916	982	67	H			-1
CYTB	982	2121	1140	H	ATG	AGG	3
trnE	2125	2193	69	L			0
ND6	2194	2718	525	L	ATG	TAA	-4
ND5	2715	4535	1812	H	ATT	TAG	-9
trnL	4527	4596	70	H			0
trnS1	4597	4655	59	H			0
trnH	4656	4724	69	H			0
ND4	4725	6102	1378	H	ATG	T–	-7
ND4L	6096	6392	297	H	ATG	TAA	1
trnR	6394	6460	67	H			0
ND3	6461	6807	347	H	ATT	TA-	-1
trnG	6808	6875	54	H			0
COX3	6876	7659	784	H	ATG	TA-	-1
ATP6	7659	8339	681	H	ATG	TAA	-43
ATP8	8297	8500	204	H	ATG	TAA	1
trnK	8502	8569	68	H			3
COX2	8573	9256	684	H	ATG	TAA	0
trnD	9257	9325	69	H			3
trnS2	9329	9397	69	L			1
COX1	9399	10,940	1542	H	ATG	TAA	18
trnY	10,959	11,024	65	L			0
trnC	11,025	11,094	70	L			32
trnN	11,127	11,199	73	L			0
trnA	11,200	11,266	67	L			2
trnW	11,269	11,336	68	L			3
ND2	11,340	12,383	1044	H	ATT	TAA	-1
trnM	12,383	12,453	71	H			0
trnQ	12,454	12,525	72	L			-4
trnI	12,522	12,592	71	H			0
ND1	12,593	13,549	957	H	ATG	TAA	2
trnL	13,552	13,625	74	H			1
16 rRNA	13,627	15,206	1580	H			-2
trnV	15,205	15,271	67	H			0
12 rRNA	15,272	16,221	950	H			0
trnF	16,222	16,288	67	H			0
D-loop	16,289	849	1270				0

Table 2. Annotation of the *N. netscheri* mitochondrial genome.

species[43]. These studies underscore the importance of purifying selection in preserving the functional integrity of mitochondrial genomes in mammals.

The atp8 gene in the genus *Nesolagus* exhibits a Ka/Ks ratio of 1.22, indicating positive selection and a rapid rate of evolution. This suggests its role in adaptation to ecological challenges. The atp8 gene exhibits the same pattern of high Ka/Ks ratios in various mammals, including *Rattus*[44], Chiroptera[45], caviomorph rodents[46], as well as between *Puma concolor*[47]. It has been reported in other studies that positive selection also affects mitochondrial genes, particularly in animals that are adapted to harsh environments[23,48–50]. In extreme environments, positive selection in ATP synthase genes enhances metabolic efficiency and energy production, as seen in species inhabiting high-altitude or low-oxygen conditions[51,52]. For tropical mammals, *atp8* has been identified as critical for adaptation to the metabolic demands of dense rainforest ecosystems, which are characterized by high biodiversity and fluctuating conditions[53,54]. Studies on bats, cetaceans, and tropical chickens highlight the evolutionary significance of *atp8* in overcoming environmental stressors specific to their habitats, such as low oxygen, high energy demands, or thermal adaptation[55,56]. In the context of *Nesolagus*, which inhabits the tropical forests of Sumatra, the positive selection of *atp8* may enhance its capacity to adapt to the unique challenges of its environment, including dense vegetation, resource competition, and fluctuating climatic conditions[46,52,57–60]. These findings collectively underscore the ecological and evolutionary importance of the *atp8* gene in facilitating adaptation to harsh environments.

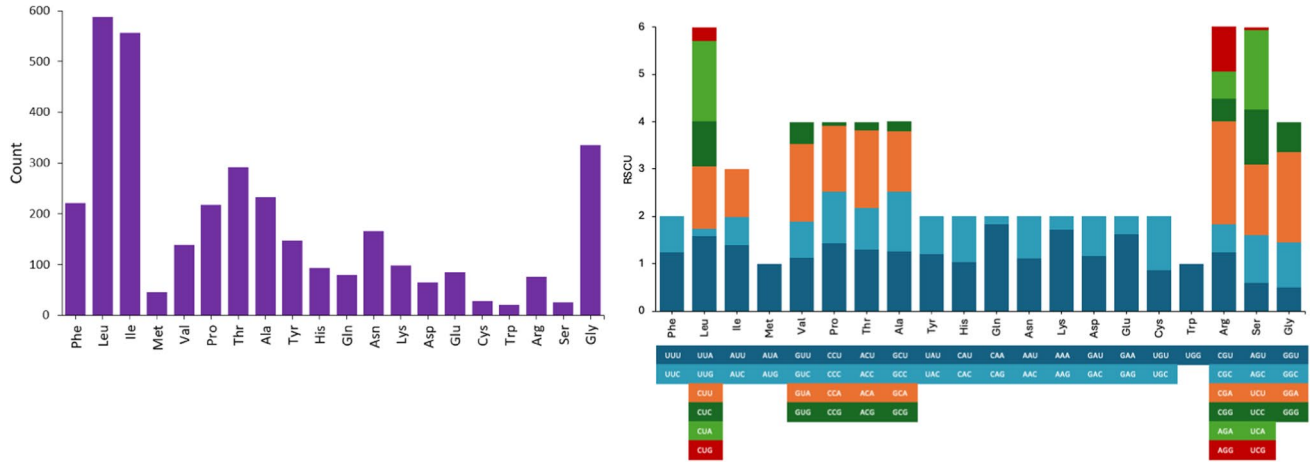

Fig. 2. Relative synonymous codon usage (left) and codon distribution (right) in the mitochondrial protein-coding genes of *N. netscheri*.

Phylogenetic of *N. netscheri*

Mitochondrial DNA is a commonly employed genetic marker for understanding molecular systematic in animals[61]. To better comprehend the evolutionary relationships within the Leporidae family, we collected concatenated nucleotide sequences of 13 PCGs and two ribosomal RNA (rRNA) genes from 13 leporids. The topologies of the phylogenetic trees obtained from the Bayesian inference (BI) and maximum likelihood (ML) analyses were nearly identical, as illustrated in Fig. 5. Both trees generated from the two models were supported by robust statistics values.

The phylogenetic analysis confirmed the monophyly of the family Leporidae (posterior probability = 0.99; bootstrap = 100), with strong support for the *Nesolagus* genus (1; 100), the genus *Lepus* (1; 100), and the group comprising *Oryctolagus cuniculus*, *Brachylagus idahoensis*, and *Sylvilagus* sp. (1; 91), forming a single single and well-supported clade. In the Leporidae family, *Nesolagus* is positioned basally as a sister group to a clade consisting of *Oryctolagus cuniculus*, *Brachylagus idahoensis*, *Sylvilagus* sp., *Romerolagus diazi*, and *Lepus* spp. The topology in our study is consistent with previous research that examined cranial morphology in Leporidae[62,63] and molecular data[64,65]. The data analysis shows a split between *N. netscheri* and *N. timminsi* during the Late Pleistocene (95%HPD, 0.43 mya ± 0.19). This study's divergence time matches the study of *Nesolagus sinensis* fossils from the Early Pleistocene period[1]. These fossils have been reported to be more primitive and directly related to the currently existing *Nesolagus*. In further studies, it is necessary to include the mitogenome of additional genera such as *Pentalagus*, *Bunolagus*, *Poelagus*, *Caprolagus*, and *Pronolagus* into phylogenetic analysis to perform a more comprehensive investigation of Leporidae phylogeny.

Conclusions

Mitochondrial genomes are increasingly utilized as valuable markers in phylogenetic, population genetic, and evolutionary studies. This paper reports and annotates the first complete mitochondrial genome of the elusive Sumatran striped rabbit (*Nesolagus netscheri*). There was 16,709 bp of circular double-stranded DNA. The mitochondrial genome's organization and gene arrangement is similar to the one described for its sister taxon, *N. timminsi*. According to our Bayesian-based phylogenetic tree, the genus *Nesolagus* is at the basal of the Leporidae tree, and *N. netscheri* diverged in the Late Pleistocene (0.43 Mya). This research provides vital genetic information essential for developing effective conservation strategies for this rabbit. In the context of Sumatra's unique and threatened forests, the mitochondrial genome data is critical for assessing genetic diversity, identifying evolutionary adaptations, and informing strategies such as inbreeding or bottleneck detection and translocation. This new information can be used as a reference mitogenome for future mitochondrial diversity studies to diagnose the species' population status. It is a crucial point for adaptation to environmental change because of its limited habitat and distribution. Other Leporidae mitochondrial genomes are still required to provide a more thorough evolutionary history, and population genetic research and monitoring are needed to support effective conservation management—particularly in the case of Sumatran striped rabbits.

Materials and methods

Sample collection and DNA extraction

A tissue sample from the leg muscular of *Nesolagus netscheri* was taken from a specimen from Setiawan et al. research[2]. The tissue sample was collected immediately after the animal's death to reduce DNA degradation caused by post-mortem damage. The sample was promptly frozen to ensure its preservation. Additionally, sterilized tools and gloves were used during sample handling to minimize the risk of exogenous contamination. Permission to access genetic resources for the Sumatran rabbit sample has been obtained from the Ministry of Environment and Forestry (no. SK.154/KSDAE/SET.3/KSA.2/8/2023). No live animal was involved in this research. This individual was reportedly caught in a forest within the Dempo mountain, South Sumatra province,

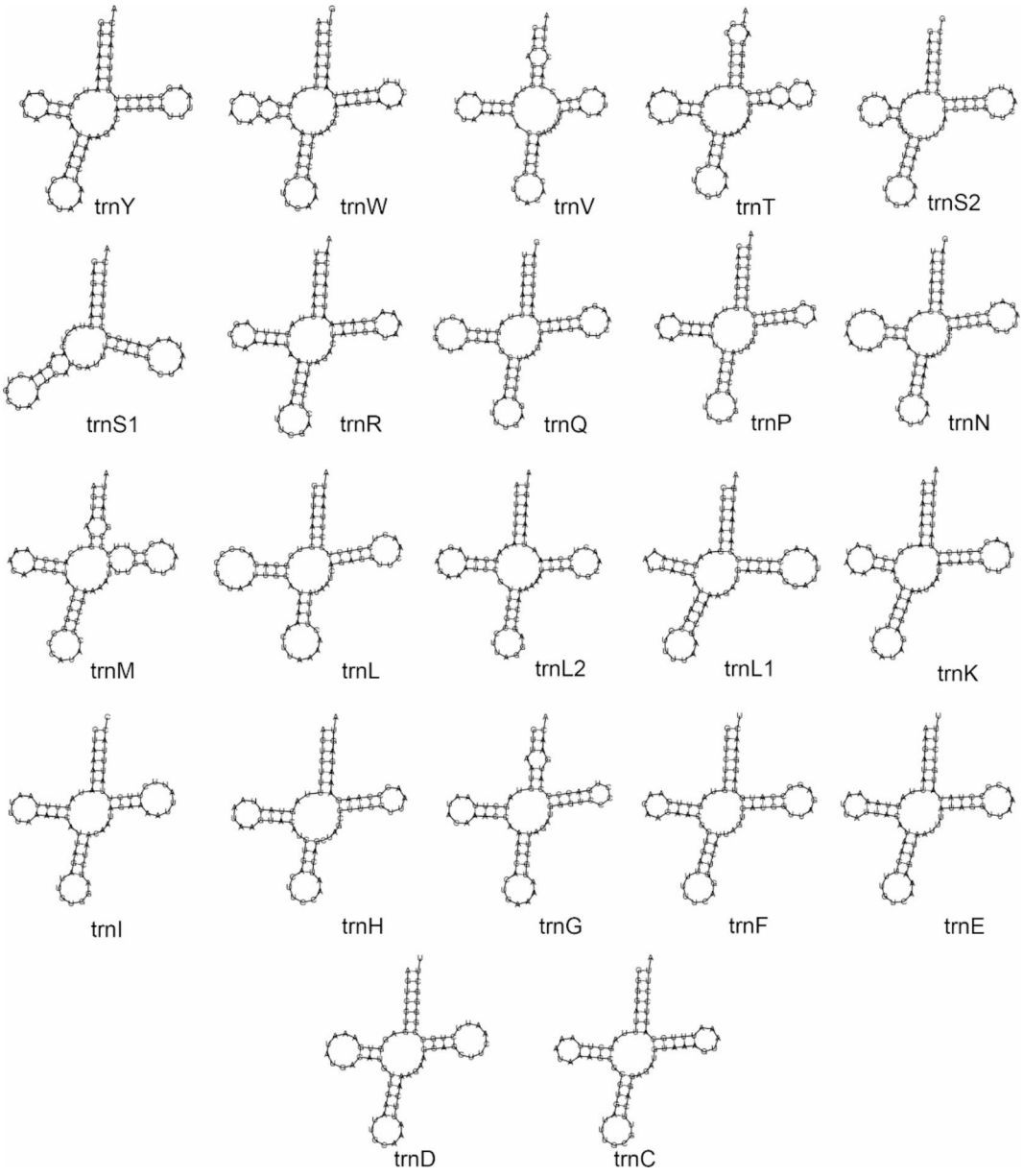

Fig. 3. Secondary structure of tRNAs in the mitochondrial genome of *N. netscheri*.

Indonesia. The tissue sample was extracted using the Qiagen Blood and Tissue Kit. Then the DNA samples were quantified using a Qubit Fluorometer.

Mitogenome sequencing and assembly

Following Genome DNA Extraction, genome DNA was sequenced using PromethION Flow Cell (Oxford Nanopore Technologies) and Ligation Sequencing Kit V14 SQK-LSK114 to prepare the genomic DNA library. A total of 1000 ng in 48µL of gDNA sample was used to produce the library. The first step is the DNA repair and end-prep step using NEBNext FFPE DNA Repair Mix and Ultra II End-prep Enzyme Mix. In the next step, the sample is given adapter ligation using Ligation Adapter reagent, Ligation Buffer, and NEBNext Quick Ligation Module. The genomic DNA library was sequenced on a PromethIon 24 device with super accuracy (SUP) base-calling mode and running for approximately 72 h. A total number of 14,512,100 reads were generated and made available in FASTQ. All these reads were employed to conduct de novo assembly of the mitogenome of *N. netscheri*.

Mitogenome annotation and analysis

The Guppy base caller ONT v.3.2.4 was utilized to determine the base sequence from the raw data accurately. Only sequences with a Phred score greater than 13, indicating good quality, were chosen for the de novo mitogenome assembly using Oxford Nanopore Technology[66,67]. The assembly was performed utilizing the Flye

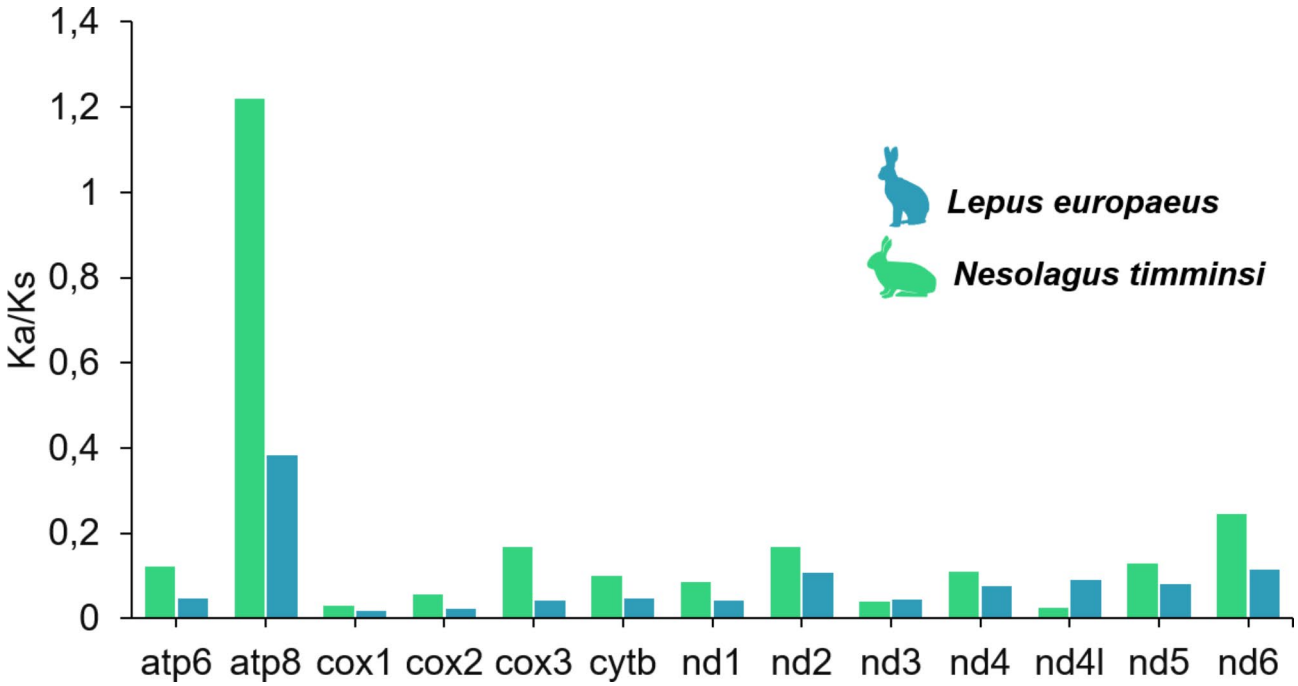

Fig. 4. Selective pressure analysis of the protein-coding genes (PCGs) of *N. netscheri*. By employing two distinct leporid mitogenomes as references, the Ka/Ks ratios were determined for each of the 13 PCGs.

program v.2.5[68] in the mitogenome assembly mode and functioned as baits to identify all potential mitochondrial sequences using Minimap2[69]. A subsequent set of ONT assemblies was generated and subsequently refined over four iterations applying the same dataset using racon[70] and medaka (https://github.com/nanoporetech/medaka).

The mitogenome sequences that were assembled were annotated using MITOS2[71]. The tRNA scan-SE 1.21 software was employed to detect tRNA genes, using the genetic code specific to vertebrate mitochondria[72]. A visual illustration of the mitogenome of *N. netscheri* was drawn using the Proksee[73], an online tool for visualizing mitochondrial data. The Ka_Ks calculator was utilized to quantify the ratio of nonsynonymous to synonymous substitution rates (Ka/Ks) for all 13 protein genes in three leporids, using MEGA X[74]. Bayesian and Maximum Likelihood phylogenetic trees were constructed using the 13 concatenated protein-coding genes (PCGs) of Leporids mitogenomes, as shown in Table 1. The best evolutionary model for analysis has been identified using jModeltest 2[75] to be the GTR model of sequence evolution, which includes a discrete gamma distribution and a proportion of invariable sites to account for variation in rates among sites (GTR+ Γ + I). The Maximum-Likelihood (ML) phylogenetic was inferred using IQ-TREE (version 2.1) via the IQ-TREE web server with[76] model selection and ultrafast bootstrap approximation (1000 iterations) based on the alignment of 13 PCGs. Beast 2.7.6 [77] was used to estimate divergence times based on PCGs. The time tree was constructed using two calibrated points: the divergence of Lepus americanus and Lepus europaeus, which occurred 8.6 mya[78], and the divergence of *L. americanus* and *Lepus tolai*, which was estimated based on fossil evidence to have occurred 0.78 mya[79]. The divergence time analysis employed an uncorrelated lognormal relaxed molecular clock and Yule process, with a total of 50 million generations. The convergence of the analysis was corroborated using Tracer[80]. All parameters show an ESS greater than 200, confirming sufficient sampling and reliability. The software TreeAnnotator was used to discard the initial 25% of trees and calculate the nodes' ages along with their 95% credible intervals. The produced tree was viewed using FigTree Version 1.4.4.

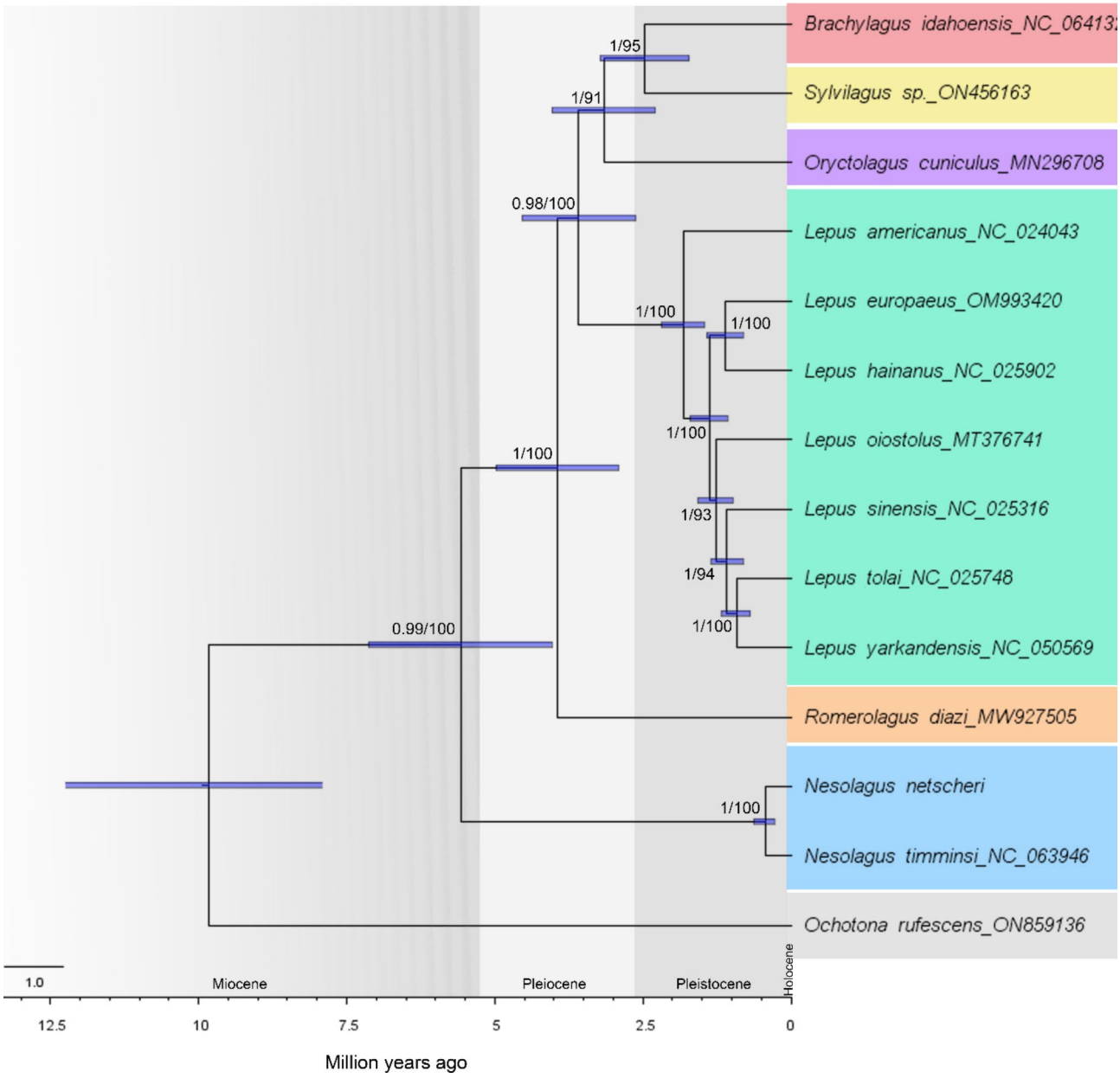

Fig. 5. The phylogenetic tree of Leporidae is based on 13 PCGs (11,396 bp), which is estimated using the Bayesian relaxed-molecular clock method. Posterior probability values from Bayesian inference and percentages of bootstrap from maximum likelihood analyses are listed on each node, respectively. Purple bars were used to show the 95% HPD for each node, with each genus represented by a different color.

Data availability

The new complete mitochondrial genome sequence of the Sumatran striped rabbit Nesolagus netscheri has been deposited in the GenBank of NCBI (https://www.ncbi.nlm.nih.gov/) database under the accession number PQ047138.

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Author contributions

D.S Conceptualized and designed the study, performed the genetic analysis, and wrote the the manuscript., N.R contributed to the sequencing and annotation of the mitochondrial genome, T.A Assisted with the bioinformatics analysis and data visualization. I.L performed genetic data analysis and sequencing. I.Y visualized the data and coordinated the fieldwork and sample collection. , A.S coordinated the fieldwork and sample collection and wrote the manuscript. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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