

Complete Mitochondrial Genome and Protein-Coding Gene Evolution of *Naemorhedus goral* in Wanglang National Nature Reserve

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Abstract

The complete mitochondrial genome of a single *Naemorhedus goral* individual collected after natural death in Wanglang National Nature Reserve, Sichuan Province, was sequenced, assembled, and annotated. The circular genome is 16,554 bp and contains 13 protein-coding genes (PCGs), two rRNA genes, 22 tRNA genes, and one control region; its A + T content is 61.8%. Molecular identification assigned the specimen to *N. goral*. All 13 PCGs showed $dN/dS < 0.5$, and 93.85% of the analyzed gene-taxon comparisons were < 0.1 . COX1 was the most conserved gene, whereas ATP8 had the highest mean dN/dS . Codon usage showed an A/C-ending preference, with 18 preferred codons ($RSCU > 1.2$), including CUA, CGA, and UCA. Maximum-likelihood and neighbor-joining analyses placed the sequence within Caprinae, although the immediate branching order differed between methods. Complete mitogenomes of *N. goral* have been reported previously; the present study contributes an independently archived sequence from Wanglang and a reproducible comparative analysis.

Introduction

Naemorhedus goral is a forest-dwelling member of Bovidae distributed across mountainous and rugged habitats in Asia. Mitochondrial genomes are widely used for specimen identification, comparative genomics, and phylogenetic hypothesis testing because they are compact, maternally inherited, and readily recovered from genomic sequencing data. However, mitochondrial DNA represents a single maternally inherited locus and, especially when obtained from one specimen, cannot by itself estimate population structure, gene flow, effective population size, or adaptive variation. Accordingly, a complete mitogenome from one individual is most appropriately treated as a specimen-level genetic record and as an input for broader multi-individual analyses^{[2][3]}.

Several complete mitochondrial genomes of *N. goral* and a chromosome-level nuclear genome are already available^{[1][4]}. This study therefore does not claim the first species-level mitogenome. Its specific contribution is a complete mitochondrial sequence from a geographically documented Wanglang specimen, integrated with molecular identification, genome annotation, codon-usage analysis, selection analysis, and exploratory phylogenetic reconstruction. Formal whole-mitogenome haplotype comparison and population sampling were outside the scope of this study; consequently, the Wanglang sequence is not presented as a distinct regional haplotype or as evidence for population-level conservation parameters.

Results

Molecular Identification and Genome Annotation Validation

BLAST comparisons of six mitochondrial genes showed 99.87–100% identity to *N. goral* reference sequences (E-value = 0.0). Molecular identification assigned the specimen to *N. goral*. Transmembrane prediction of COX1 identified a 516-amino-acid protein with 11 predicted transmembrane helices; no

secretory signal peptide was detected. Secondary-structure predictions for the 13 proteins were dominated by alpha-helices, with inter-protein variation. All proteins were predicted to be membrane associated, and the GPI-anchor score was 0.0. Twenty-three SSR loci were identified, with ND4 containing the largest number (four). Three-dimensional models for ND2, COX1, ND4L, and ND6 had mean pLDDT values > 0.80, whereas ATP6, ND4, and ND5 had lower scores (0.50–0.55), consistent with less confidently modeled regions. InterProScan recovered expected conserved domains, and the predicted COX1 heme-binding pocket included His48 and His94.

Table 1
Morphological characteristics of the Wanglang specimen and reference taxa

Criteria for Identification	Sample Characteristics	Features of <i>Naemorhedus goral</i>	Features of <i>Pseudois nayaur</i>	Identification Support
Body Size	Slender skeleton, overall relatively small proportion	Relatively small body size	Relatively large body size	<i>Consistent with N. goral</i>
Hair Color of Lower Limbs	Light yellowish brown	Light-colored	Darker	<i>Consistent with N. goral</i>
Anterior Longitudinal Stripe	No black longitudinal stripes	No black longitudinal stripes	With black longitudinal stripes	<i>Consistent with N. goral</i>
Mitochondrial Genome Structural Features				

The mitochondrial genome of *N. goral* is 16,554 bp and has the typical circular organization of vertebrate mitogenomes, comprising 13 PCGs, two rRNA genes, 22 tRNA genes, and one non-coding control region. The genome is compact, with short intergenic spacers and several gene overlaps. Base composition is AT-biased (A + T = 61.8%), and genes are asymmetrically distributed between the heavy and light strands. Most PCGs use standard vertebrate mitochondrial initiation codons and terminate with TAA or an incomplete T/TA stop codon. The approximately 940-bp control region lies between tRNA-Pro and tRNA-Phe and contains conserved sequence blocks and termination-associated motifs involved in mitochondrial transcription and replication^[2]. The overall organization is consistent with published *N. goral* and other caprine mitogenomes^{[1][5][6][7]}.

Prediction of tRNA Secondary Structures

A total of 22 tRNA genes were identified, ranging from 58 to 75 bp and collectively covering all amino acids required for mitochondrial translation. Except for tRNA-Ser(GCT), the predicted tRNAs formed the canonical cloverleaf structure with an aminoacyl arm, D-arm, anticodon arm, TpsiC arm, and variable loop. tRNA-Ser(GCT) had a lower score (54.5) and lacked a D-arm, a common structural feature of vertebrate mitochondrial tRNA-Ser. Infernal scores for the other tRNAs exceeded 76.3, well above the confidence threshold of 30, supporting the reliability of the predictions^[8].

Gene Composition and Codon Usage Characteristics

Amino acid composition analysis revealed that leucine dominates among the 13 PCGs of *N. goral* mitochondria, averaging 15.8% and reaching the highest value in ND3. Histidine is entirely absent from ND6 and ND3, while cysteine is completely missing in ND2 and ATP8^[9]. Codon usage bias analysis demonstrated a low GC and high A/U base composition in the mitochondrial genome, with an average GC content of 0.404 and a third-position GC3s average value of 0.347. The effective number of codons, or ENC, averaged 39.77 and ranged from 31.90 to 43.79, confirming strong codon bias. The GC content at different codon positions followed a decreasing trend of GC1 > GC2 > GC3, with values of 0.447, 0.401, and 0.353, respectively, yielding an overall GC content of 40.4%. A total of 18 preferred codons with relative synonymous codon usage, or RSCU, greater than 1.2 were identified. Among these, 14 ended with adenine, accounting for 77.78%, and 4 ended with cytosine, comprising 22.22%, whereas no preferred codons ended with uracil or guanine. For all codons with RSCU above 1, the third-position base composition was dominated by adenine at 48.28%, followed by cytosine at 34.48% and uracil at 17.24%, with guanine-ending codons being completely absent. The most strongly preferred codons were CUA for leucine with an RSCU of 2.25, CGA for arginine with an RSCU of 2.19, and UCA for serine with an RSCU of 2.12, reflecting extreme biases for specific codons^{[10][11]}. Codons ending with adenine or cytosine predominate, whereas guanine-ending codons and those containing CG dinucleotides such as AGG, CCG, and TCG are nearly universally avoided, reflecting a typical preference for A/C-ending codons and avoidance of G-ending motifs^[9].

Selective Pressure Analysis

Selective pressure was evaluated using CDS alignments of the 13 mitochondrial PCGs from the Wanglang *N. goral* and selected caprine relatives, with dN/dS ratios calculated for each gene comparison^[12]. All estimated ratios were < 0.5, and 93.85% were < 0.1, indicating that purifying selection predominated across the analyzed PCGs^{[13][14]}. The strength of constraint varied among genes: ATP8 had the highest mean dN/dS (0.1727) and therefore the weakest inferred purifying constraint, whereas COX1 had the lowest mean dN/dS (0.0069; mean dN = 0.0047) and was the most conserved gene. These estimates describe comparative gene-level constraints in the analyzed dataset and should not be interpreted as population-level selection parameters.

Phylogenetic Analysis

Concatenated CDS alignments of the 13 PCGs were analyzed using maximum-likelihood (ML) and neighbor-joining (NJ) methods^{[13][15]}. Both trees placed the Wanglang *N. goral* sequence within Caprinae, but the immediate branching order among Naemorhedus, Capra, Pseudois, Budorcas, Saiga, and related taxa differed between the ML and NJ reconstructions (Figs. 7 and 8). The figures therefore do not support a single robust sister relationship between *N. goral* and *P. nayaaur*. The phylogenetic result is interpreted as exploratory because taxon sampling was limited and mitochondrial data represent one maternally inherited locus^{[5][6][7][16]}.

Discussion

Complete mitochondrial genomes of *N. goral* and a chromosome-level nuclear reference have already been reported, so the value of the present study lies in adding a geographically documented specimen-level sequence from Wanglang National Nature Reserve and analyzing it through a consistent identification, annotation, structural, codon-usage, selection, and phylogenetic workflow^{[1][4]}. BLAST comparisons across six mitochondrial genes produced 99.87–100% identity to *N. goral* references, and the complete mitogenome was consequently assigned to *N. goral*. Agreement across several independent mitochondrial regions reduces the likelihood that the identification depends on a single short marker. Nevertheless, mitochondrial DNA traces the maternal lineage only; therefore, the result is appropriately interpreted as a robust molecular assignment of this specimen rather than a population-level taxonomic or demographic conclusion.

The 16,554-bp genome contains the standard vertebrate mitochondrial complement of 13 protein-coding genes, two rRNA genes, 22 tRNA genes, and one control region. Its compact organization, asymmetric distribution of genes between the heavy and light strands, short intergenic spacers, gene overlaps, and use of complete or incomplete stop codons are all characteristic of mammalian mitogenomes^[2]. The overall A + T content of 61.8% also agrees with the AT-rich composition commonly observed in caprine mitochondria. Incomplete T or TA termination codons are generally completed by post-transcriptional polyadenylation, and their occurrence therefore does not indicate incomplete assembly. The approximately 940-bp control region between tRNA-Pro and tRNA-Phe contains the expected regulatory motifs, further supporting the completeness and reliability of the genome annotation. No major gene rearrangement was detected, indicating that the Wanglang sequence retains the conserved mitochondrial architecture reported for *N. goral* and related Bovidae^{[1][5][6][7]}.

The RNA and protein predictions provide additional functional support for the annotation. Twenty-one tRNAs formed the canonical cloverleaf structure, whereas tRNA-Ser(GCT) lacked a D-arm, a well-known structural exception in vertebrate mitochondrial tRNAs; the high Infernal scores for the remaining tRNAs indicate that most gene boundaries were confidently identified^[8]. The predicted proteins were dominated by alpha-helices and were membrane associated, as expected for subunits of oxidative phosphorylation complexes embedded in the mitochondrial inner membrane. COX1 was predicted to contain 11 transmembrane helices and a conserved heme-binding pocket involving His48 and His94, consistent with its central catalytic role and its strong sequence conservation. The absence of a signal peptide and a GPI-anchor prediction is also compatible with mitochondrial rather than secretory-pathway targeting. Differences in pLDDT among proteins should be interpreted cautiously: the high scores for ND2, COX1, ND4L, and ND6 support stable modeled cores, whereas the lower values for ATP6, ND4, and ND5 may reflect flexible regions, limited template information, or model uncertainty rather than genuine loss of function. The 23 detected SSR loci, including four in ND4, may be useful candidates for future marker development, but their polymorphism and reproducibility must be validated in multiple individuals before they can be applied to population studies.

Amino-acid composition and synonymous codon usage both reflect the strong compositional constraints of the mitogenome. Leucine was the most abundant amino acid, averaging 15.8% across the 13 PCGs, which is consistent with the high proportion of hydrophobic residues required in membrane-spanning respiratory-chain proteins. The genome had an overall GC content of 40.4%, a GC3s value of 0.347, and an average ENC of 39.77, with GC1 > GC2 > GC3. These values indicate appreciable codon-usage bias, particularly at third codon positions. Eighteen codons had RSCU values above 1.2; 14 ended in adenine and four in cytosine, while no strongly preferred codon ended in guanine or uracil. CUA, CGA, and UCA showed the highest RSCU values. Thus, the observed A/C-ending preference is compatible with the broader AT-rich genome because codon bias is shaped not only by total nucleotide composition but also by strand-specific mutation pressure, the mitochondrial genetic code, and the restricted mitochondrial tRNA pool^{[9][10][11]}. These data describe compositional and translational tendencies, but they do not by themselves demonstrate adaptive optimization of translation.

The dN/dS analysis indicates that purifying selection is the dominant evolutionary force acting on the 13 mitochondrial PCGs. All estimates were below 0.5, and 93.85% of the analyzed gene-taxon comparisons were below 0.1, showing that nonsynonymous substitutions are generally removed more rapidly than synonymous substitutions^{[12][13][14]}. COX1 had the lowest mean dN/dS value (0.0069) and the lowest mean dN (0.0047), which agrees with the structural evidence for a highly conserved catalytic protein in cytochrome c oxidase. ATP8 had the highest mean dN/dS value (0.1727) and therefore the weakest inferred purifying constraint among the analyzed genes. This relative elevation is biologically plausible because ATP8 is short and often evolves faster than core oxidative-phosphorylation subunits; however, its value remains well below one and provides no evidence of positive selection. Moreover, dN/dS estimates for short mitochondrial genes can be sensitive to alignment quality, taxon choice, sequence divergence, and the substitution model. The gene-level rankings should therefore be regarded as comparative patterns within this dataset rather than fixed species-wide selection coefficients.

The phylogenetic analyses placed the Wanglang sequence within Caprinae, supporting the molecular identification and the overall taxonomic consistency of the assembly. However, the maximum-likelihood and neighbor-joining trees differed in the immediate branching order among *Naemorhedus*, *Pseudois*, *Capra*, *Budorcas*, *Saiga*, and related taxa. Consequently, the present data do not justify a single definitive sister relationship between *N. goral* and *P. nayaur*. Such topological differences can arise from limited taxon sampling, unequal evolutionary rates, model choice, and the fact that the mitochondrial genome behaves as one maternally inherited, non-recombining locus^{[5][6][7][13][15][16]}. A more robust reconstruction will require broader sampling of *Naemorhedus* and other caprine lineages, explicit partitioning and model testing, comparison of complete mitogenomes, and independent nuclear-genome evidence.

Taken together, the results show that the Wanglang specimen carries a structurally typical, AT-rich *N. goral* mitogenome with conserved tRNA organization, membrane-associated oxidative-phosphorylation proteins, pronounced codon bias, and pervasive purifying selection. The deposited sequence can support specimen identification, comparative annotation, primer design, and the development of candidate molecular markers for monitoring and conservation research. At the same time, the study is

based on one naturally deceased individual. It therefore cannot estimate mitochondrial haplotype diversity, population structure, gene flow, effective population size, adaptive variation, or conservation-management units, and it does not demonstrate that the Wanglang sequence is a distinct regional haplotype. Future research should compare this sequence directly with all available *N. goral* mitogenomes, sample multiple individuals and localities, validate the predicted SSRs, and integrate mitochondrial data with nuclear SNPs, morphology, geography, and ecological information. Such a design would convert the present specimen-level genomic record into a stronger basis for population assessment and evidence-based conservation management.

Conclusions

We assembled and annotated a 16,554-bp mitochondrial genome from one *N. goral* specimen from Wanglang National Nature Reserve. The sequence contains the standard 37 mitochondrial genes and a control region, is AT-rich, and shows pronounced codon-usage bias. Comparative analyses indicate purifying selection across all 13 PCGs, with COX1 the most conserved and ATP8 the least constrained in the analyzed dataset. Molecular identification assigns the specimen to *N. goral*.

This sequence provides an independently archived specimen-level mitogenome from Wanglang and a reference for subsequent comparative work. Because existing *N. goral* mitogenomes have already been published and only one individual was analyzed here, the study does not claim a novel species-level mitogenome, a distinct regional haplotype, or population-level conservation inference. Broader conclusions will require direct comparison with available mitogenomes and multi-individual nuclear and mitochondrial sampling.

Materials and Methods

Sample Collection and DNA Extraction

A muscle-tissue sample was collected from a single *N. goral* individual found naturally deceased in Wanglang National Nature Reserve, Sichuan Province. Collection complied with applicable wildlife-protection regulations and involved no live-animal procedures. The tissue was placed in liquid nitrogen and subsequently stored at -80 degrees C while avoiding repeated freeze-thaw cycles. The collection and use of the specimen for scientific research were authorized by the administrative bureau of Wanglang National Nature Reserve. Genomic DNA was extracted using SDS-proteinase K digestion followed by phenol-chloroform extraction. DNA purity and concentration were assessed with a NanoDrop 2000 (A260/A280 = 1.8-2.0) and Qubit 3.0 fluorometer, and integrity was evaluated by 1% agarose-gel electrophoresis.

Sequencing and Assembly

A qualified library was sequenced on an Illumina NovaSeq 6000 platform using paired-end 150-bp reads. Raw data were assessed with FastQC v0.11.9. The mitochondrial genome was assembled de novo with

GetOrganelle v1.7.5 using the mitochondrial-genome target and a maximum of 100 iterations^[17]. The circular assembly graph was inspected with Bandage v0.8.1.

Annotation and Comparative Analysis of the Mitochondrial Genome

The complete mitochondrial genome was annotated using MITOS^[18] followed by manual curation in Geneious Prime v2023.2. Gene boundaries and annotations for PCGs, rRNAs, and tRNAs were checked manually, and the genome map was generated with Proksee. tRNA structures were evaluated with tRNAscan-SE v2.0 and visualized with RNAfold. Amino-acid composition, codon usage, and dN/dS were analyzed for the 13 PCGs using MEGA 11 and DnaSP v6. For phylogenetic analysis, concatenated PCG CDS sequences from *N. goral* and selected caprine relatives were aligned with MAFFT v7.490 and analyzed using ML and NJ methods in MEGA 11. SSRs were screened with MISA v2.1. TMHMM 2.0, SignalP 6.0, and DeepLoc-2.1 were used for transmembrane-helix, signal-peptide, and subcellular-localization predictions, respectively.

Declarations

Author Contributions: Xiao Min conducted the experiments and drafted the manuscript. Xiao Min, Zhang Jingjing, and Dai Yuxin performed data curation, formal analysis, and software analyses; Dai Yuxin also contributed to methodological validation. Mu Sijing organized the data and prepared the manuscript format. Huang Junzhong and Zhao Lianjun provided resources. Liu Hao administered the project and acquired funding. Hua Donglai conceptualized and designed the study, conducted the investigation, supervised the project, and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data Availability Statement

The mitochondrial genome sequence analyzed in this study has been deposited in the NCBI GenBank database under accession number PZ631067. Other datasets generated or analyzed during this study are available from the corresponding author upon reasonable request.

Additional Information

Competing Interests: The authors declare no competing interests.

Ethics Declaration

All experimental protocols involving dead-wild animal samples were approved by the Institutional Animal Care and Use Committee of Mianyang Teachers' College. All methods were carried out in accordance with the relevant guidelines and regulations of the Wildlife Protection Law of the People's Republic of China and the internal management regulations of Wanglang National Nature Reserve. The muscle tissue sample used in this study was collected from a single *Naemorhedus goral* individual found naturally deceased in Wanglang National Nature Reserve, Sichuan Province. Permission for the collection and scientific use of the specimen was granted by the administrative bureau of Wanglang National Nature Reserve, coordinated through our co-authors Mr. Huang Junzhong (Sichuan Xiaohogou Provincial Nature Reserve) and Mr. Zhao Lianjun (Director and Senior Engineer of Sichuan Wanglang National Nature Reserve Management Bureau). No live animals were involved in this research, and no animals were deliberately killed or harmed for the purposes of this study.

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Figures

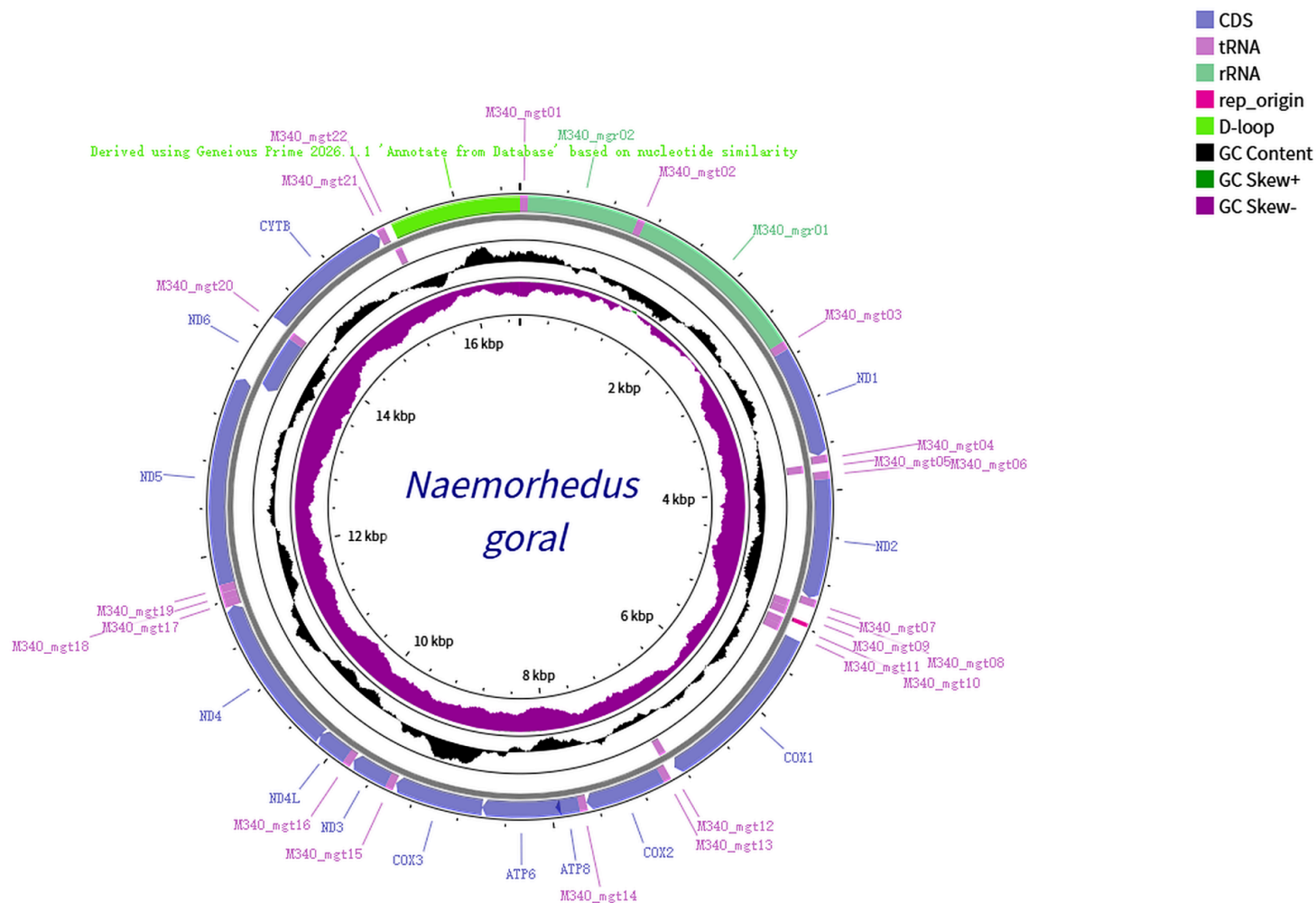


Figure 1

Circular map of the *N. goral* mitochondrial genome

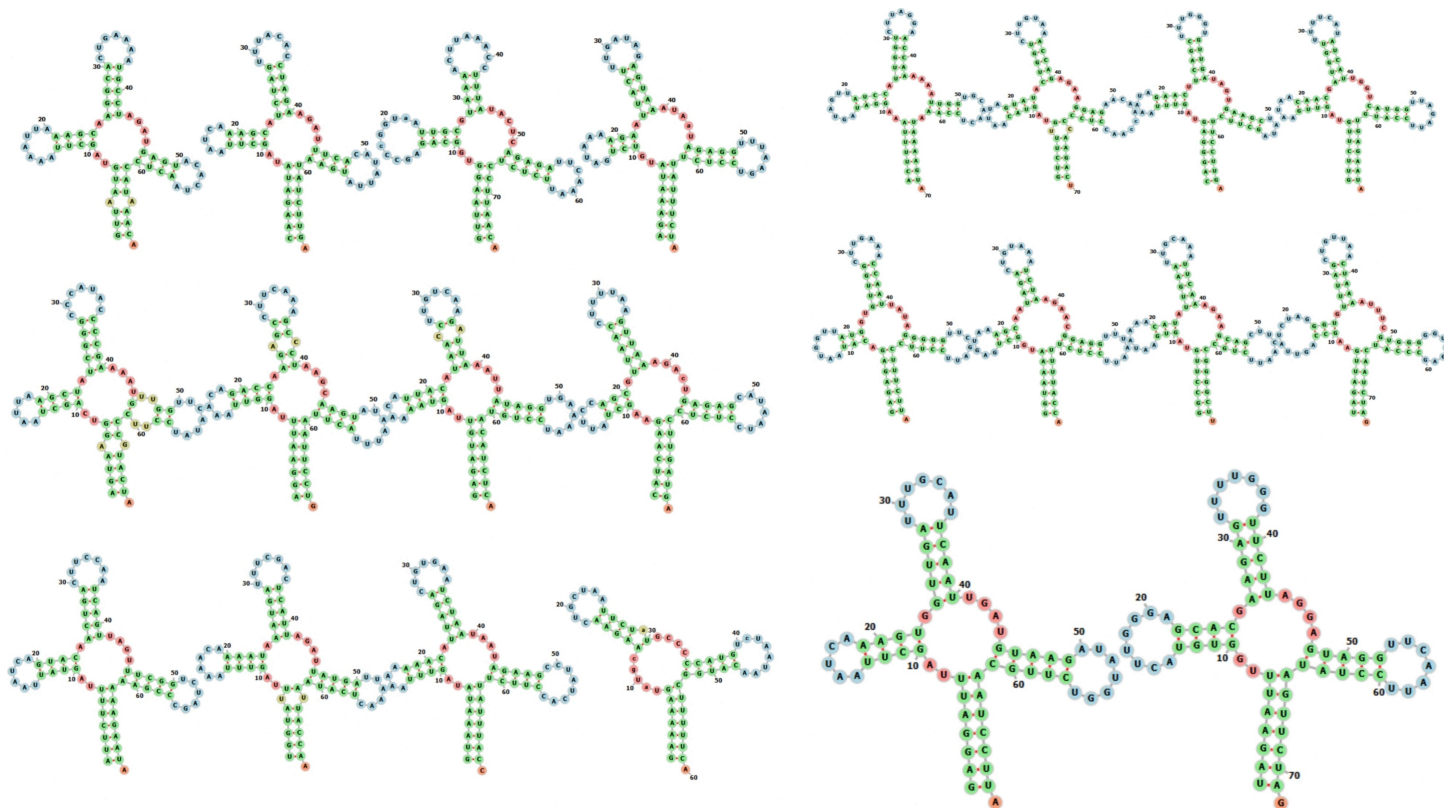


Figure 2

Predicted secondary structures of the mitochondrial tRNAs

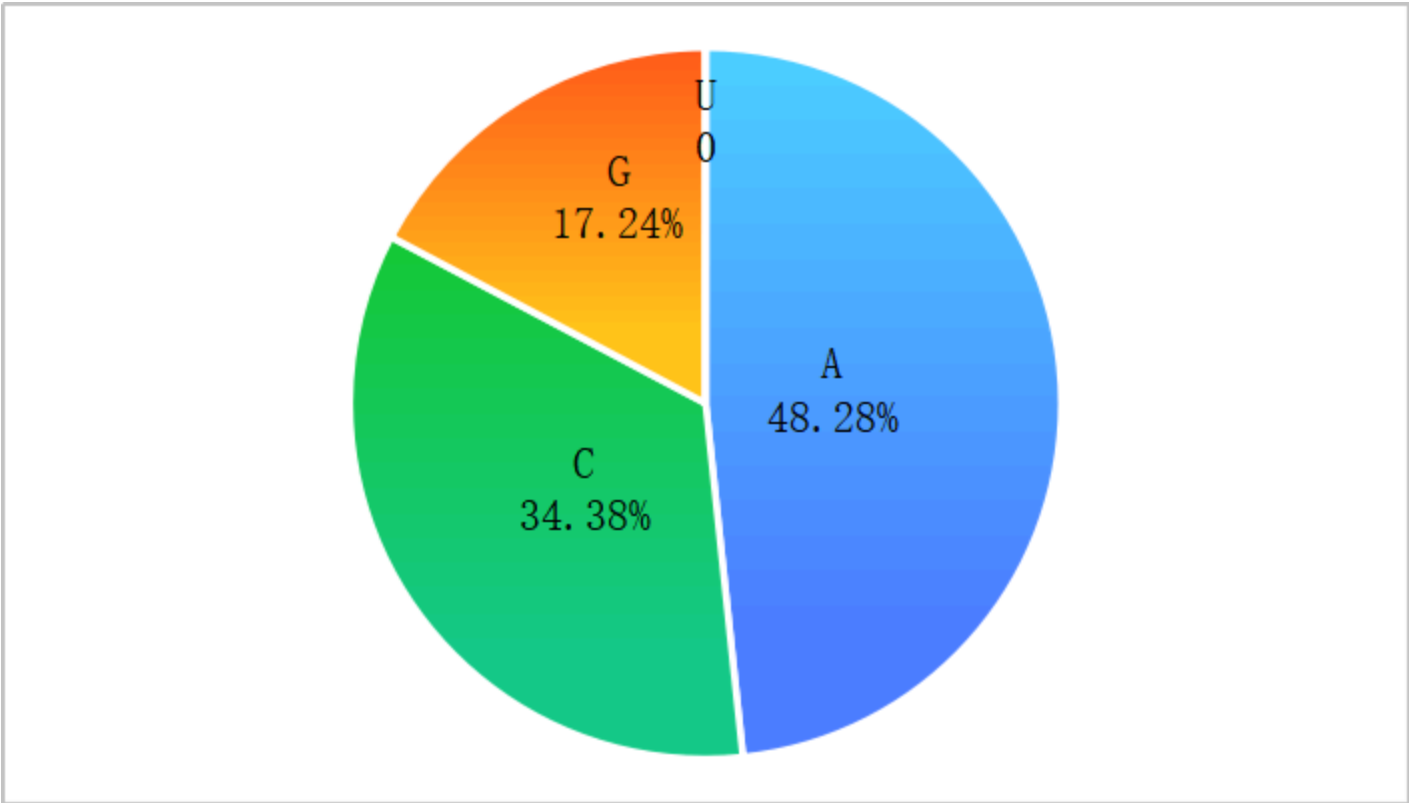


Figure 3

Third-position base composition of codons with RSCU > 1

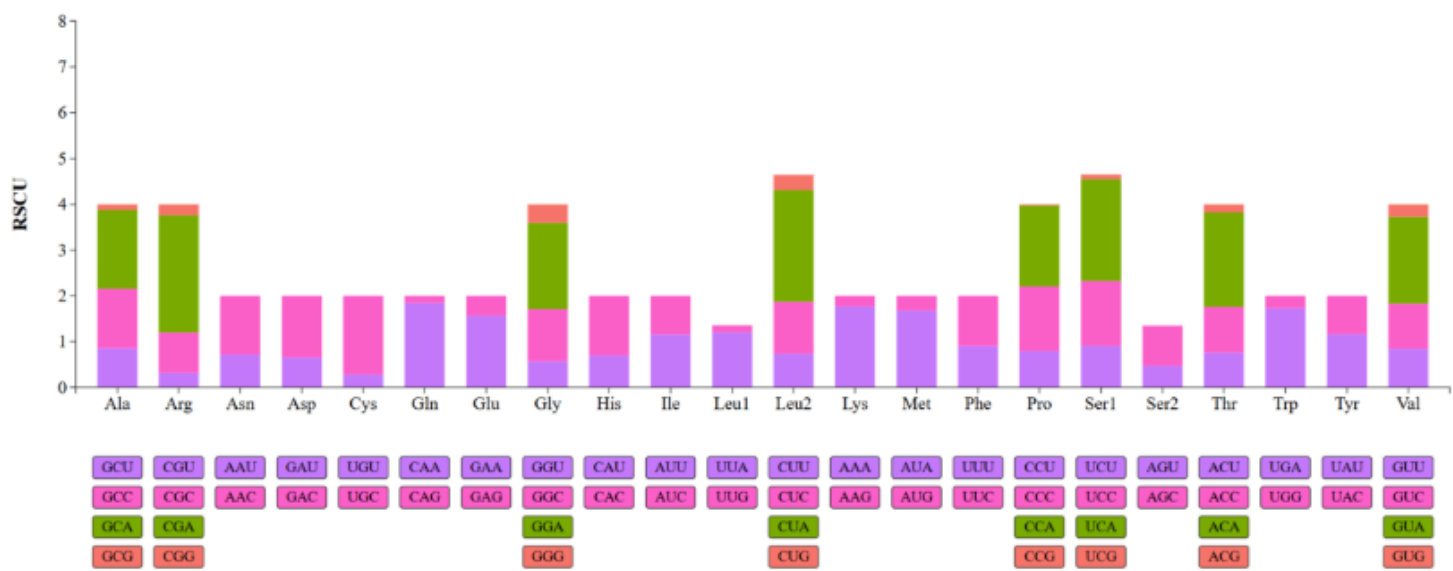


Figure 4

Visualization of Codon Usage Bias Analysis

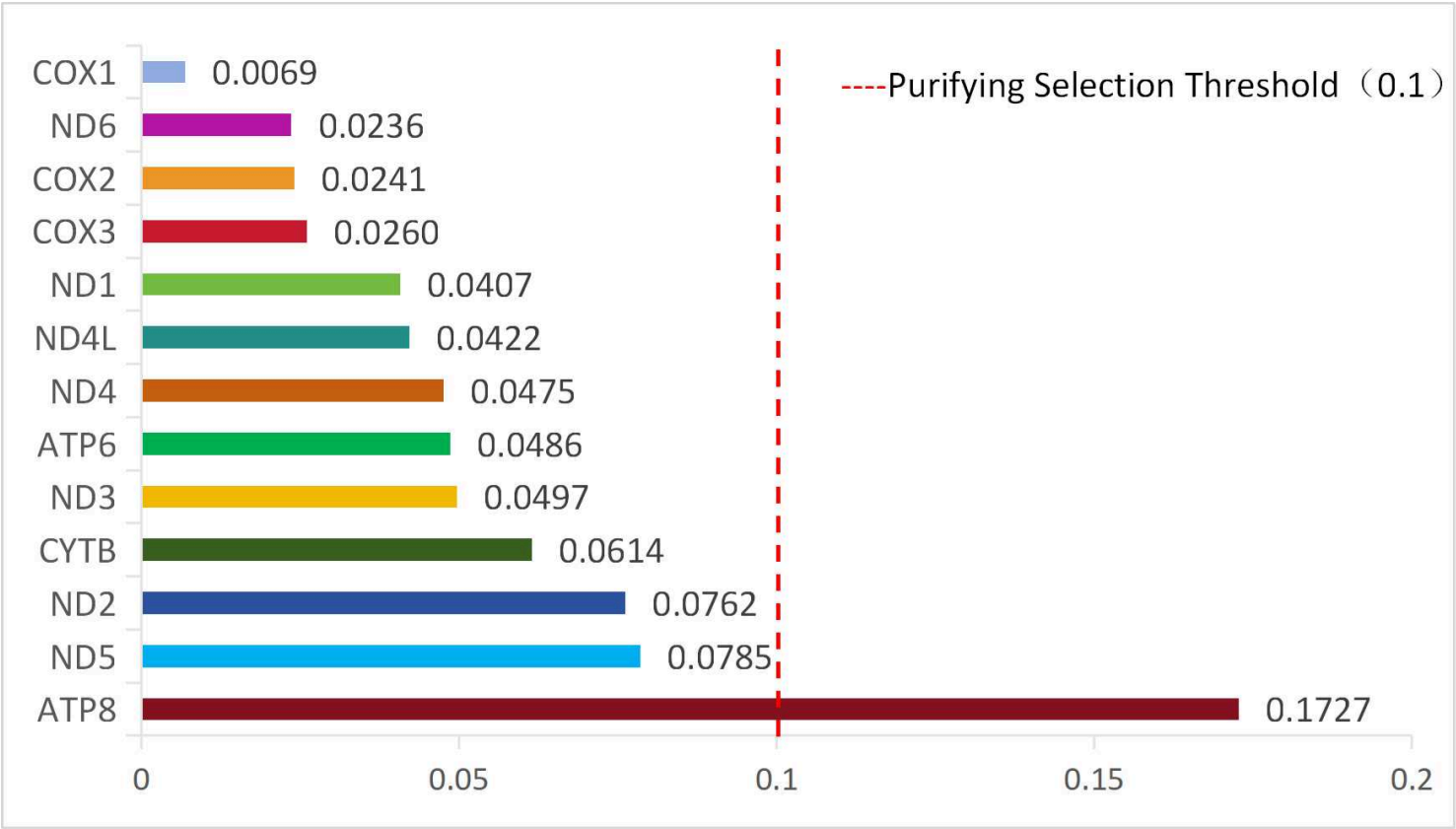


Figure 5

Mean Comparison of dN/dS Values Across Genes

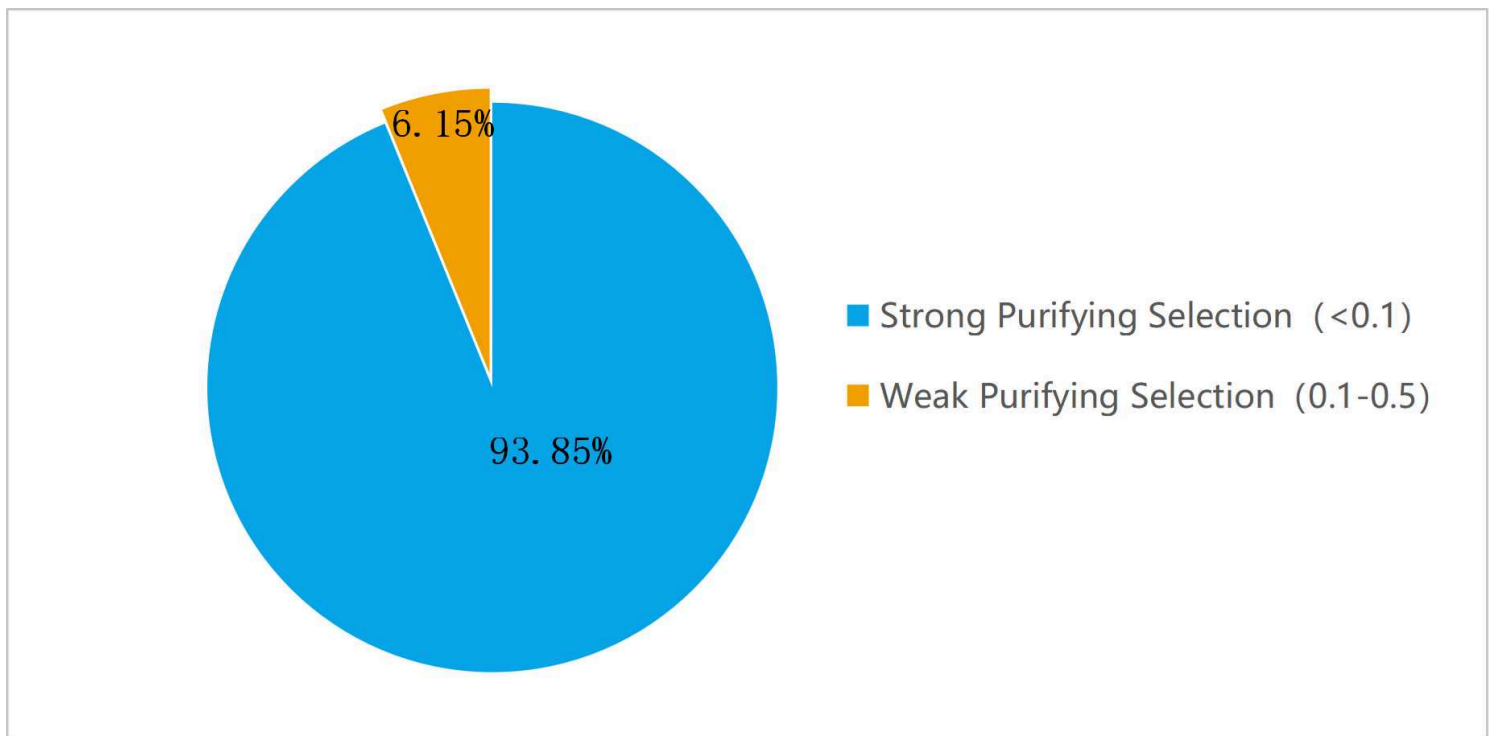


Figure 6

Overview of selective pressure intensity across all analyzed samples.

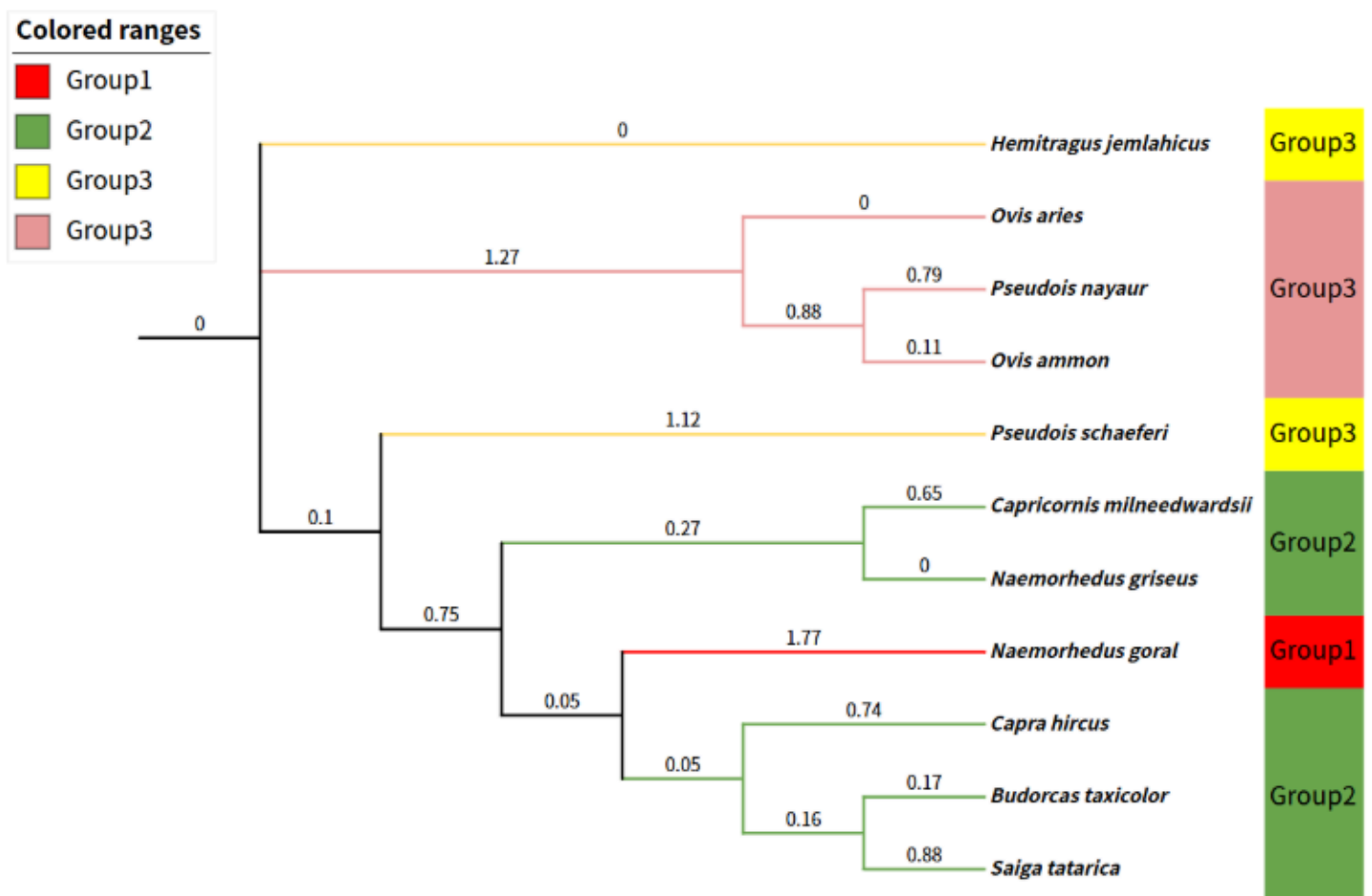


Figure 7

Phylogenetic Analysis Based on Mitochondrial Genome ML Phylogenetic Tree

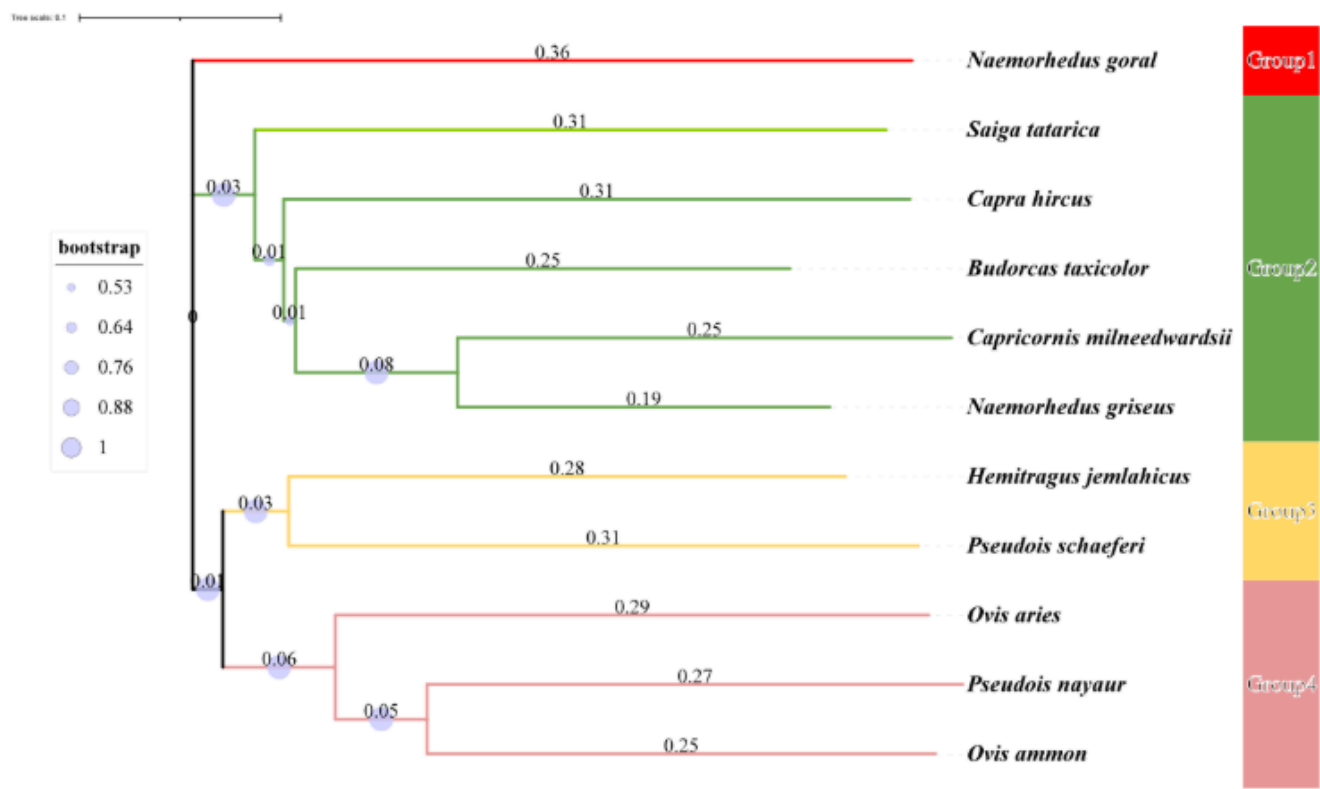


Figure 8

Phylogenetic Analysis Based on Mitochondrial Genome NJ Phylogenetic Tree