

## Short communication: Complete mitochondrial genome sequence of Bali cattle (*Bos javanicus*)

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**Abstract.** Jakaria J, Dharmawanthi AB, Dairoh, Ulum MF, Anwar S, Noor RR. 2025. Short communication: Complete mitochondrial genome sequence of Bali cattle (*Bos javanicus*). *Biodiversitas* 26: 1545-1552. Bali cattle (*Bos javanicus*), one of the most popular indigenous cattle breeds in Indonesia, was domesticated from the wild Banteng. This study aimed to determine the complete mtDNA sequence and assess its genetic variation utilizing the Illumina NovaSeq6000 platform, marking the first time reporting the complete mitochondrial genome sequence of Bali cattle. Blood samples were collected from BBIB Singosari, Malang, East Java. DNA was extracted using the DNA Extraction Kit protocol (Promega). The total mitochondrial genome was annotated using the MITOS2 software. The result of this study showed that mitochondrial DNA (mtDNA) has a total length of 16,712 bp and comprises 37 genes with a typical structure. These include 13 protein-coding genes, 22 transfer RNAs, 2 ribosomal RNAs, and a D-loop region. The nucleotide composition of the genome is 33.6% Adenine (A), 27.1% Thymine (T)/uracil (U), 25.8% Cytosine (C), and 13.4% Guanine (G). Phylogenetic analysis based on the complete mitochondrial genome sequences distinguished into three clades of cattle breed. Including *B. javanicus* (GenBank data), *B. taurus*, *B. indicus* and Bali cattle groups showed notable genetic variations. The results of this work offer genomic information capable of supporting the next investigations on the genetic structure and evolutionary background of Bali cattle.

**Keywords:** Bali cattle, mtDNA, phylogenetic analysis, sequencing

### INTRODUCTION

The mitochondrial genome (mtDNA) is the DNA present in the mitochondria that is only passed from the mother to the offspring (Vadakedath et al. 2023). mtDNA has been recognized as a valuable marker for mapping maternal lineages, researching evolutionary relationships, and learning about population history and migration patterns because of its inheritance devoid of recombination (Ferreira and Rodriguez 2024). Using mtDNA, which is widely used to investigate origins (Di Lorenzo et al. 2015) and genetic diversity (Abdul-Muneer 2014; Rong et al. 2018; Huang et al. 2023) and domestication history of species (Gupta et al. 2015). Among the unique traits of mtDNA that make it a reliable tool for tracking lineages and understanding population dynamics over time are maternal inheritance, high mutation rate, and lack of recombination (Veira et al. 2016). Through mtDNA analysis, the genetic diversity related to reproduction, livestock distribution, and selection can be identified. mtDNA genome variation can detect unique haplogroups, reveal

patterns of genetic structure and genetic drift both within and between populations, and measure the level of gene flow, thus providing more complete knowledge regarding demographic and evolutionary processes.

Numerous partial and total studies have been conducted on mtDNA in cattle. Most studies on mitochondria are limited to one location or subset of mitochondrial DNA. However, owing to recent advances in sequencing technology, it is now possible to precisely and rapidly sequence the entire mtDNA genome. Compared to studies of individual genes or areas, sequencing the complete mtDNA genome has improved the capacity and precision of phylogenetic analysis, enabling more precise determination of taxonomic connections, even at deep levels. Several studies on the total mtDNA genome have been published for several cattle breeds in Brazil. These cattle include local Sardinian cattle (Petretto et al. 2022), Japanese Shorthorn cattle (Mannen et al. 2020), dairy cattle (Fortuna et al. 2024), and Peruvian Creole (Arbizu et al. 2022) and Gaur cattle (Kamalakkannan et al. 2020), Yunling (Xia et al. 2019), and African Taurine (Dorji et al. 2022). Comparative references

for studies on the evolution, genetic diversity, and environmental adaptation of different cow populations can be derived from past data. Bali cattle (*Bos javanicus* (d'Alton, 1823)) are distinguished by their remarkable resilience to adverse environmental conditions and their high reproductive efficiency (Pribadi et al. 2015; Sudrajad et al. 2020; Freitas et al. 2021; Widyas et al. 2022) and exceptional carcass quality, achieving a dressing percentage of up to 56% (Hafid 2020).

The unique traits of Bali cattle establish this breed as an essential genetic resource. This research highlights the importance of examining its mitochondrial genome to gain a deeper understanding of genetic diversity and to improve conservation and breeding efforts. It encourages more effective strategies for preserving and utilizing Bali cattle. MtDNA research on Bali cattle can also help investigate their evolutionary relationships. Most mtDNA studies in Bali cattle have focused on specific regions such as the mtDNA d-loop region (Jakaria et al. 2019), 12SrRNA (Suselowati et al. 2023) and 16SrRNA (Misrianti et al. 2022), respectively. However, more accurate maternal phylogeny can be obtained by examining the genetic structure of Bali cattle using the total mtDNA genome sequence.

Bali cattle are genetic resources that must be maintained and preserved; therefore, identifying their genetic uniqueness and evolutionary adaptation through the application of the total mtDNA genome is very important. The application of the total mtDNA genome in Bali cattle is the first study to use Next-Generation Sequencing (NGS) technology to sequence Bali cattle mtDNA. This study is important because Bali cattle are valuable genetic resources known for their extraordinary adaptability, high reproductive efficiency, and superior carcass quality. The complete documentation of genetic information in Bali cattle is crucial for effective conservation research and sustainable breeding initiatives. The objective of this study was to analyze the diversity of the total mtDNA genome sequence in Bali cattle in order to identify potential genetic enhancements.

## MATERIALS AND METHODS

### Animals, DNA extraction, and sequencing

The blood samples for the analysis were obtained from male Bali cattle kept at BBIB Singosari, Malang, East Java, Indonesia. Blood was collected from the jugular vein using a projecting needle; then, the blood was stored in a 10 mL EDTA tube containing an anticoagulant. DNA was extracted from DNA extraction from blood samples using the Wizard® Genomic DNA Purification Kit (Promega, USA). DNA concentration was measured using a Nanodrop Thermo Scientific 2000c spectrophotometer (Wilmington, Delaware, USA). DNA library preparation was performed using the TruSeq Nano DNA Library Preparation Kit to measure the quality and quantity of DNA to meet the required standards (100 ng/μL). The total mtDNA genome was sequenced on the Illumina NovaSeq 6000 platform (Illumina, Seoul, Korea). Raw sequencing data were analyzed for quality, including read quality, total bases, total reads, GC content, and other basic statistics, to identify potential

problems. FastQC v0.11.7, was used to conduct preliminary quality evaluations of the raw data (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>), and adaptor sequences and low-quality reads were removed with Trimmomatic v0.38 using stringent parameters, only retaining reads where at least 90% of the bases had a Phred score of 20 or higher (<http://www.usadellab.org/cms/?page=trimmomatic>). The results of the raw data evaluation of the total mtDNA genome sequence were then converted into the FASTQ reading format using the bcl2fastq software. The mtDNA genome sequence was then assembled de novo using NOVOplasty software (<https://github.com/ndierckx/NOVOPlasty>). The resulting gene data in the form of ribosomal RNA (rRNA), transfer RNA (tRNA), and protein-coding genes were annotated and predicted based on the MITOS2 web server (<http://mitos2.bioinf.uni-leipzig.de/index.py>).

### tRNA structure and codon usage analysis

The tRNA structure analysis was predicted using Mitos web server and tRNAscan-SE v.2.0 (<https://lowelab.ucsc.edu/tRNAscan-SE/index.html>), including anticodons and secondary structures. The Forna tool (<http://rna.tbi.univie.ac.at/forna/>) was utilized to visual the result of tRNA structured. The amino acids distribution and the Relative Synonymous Codon usage (RSCU) in the Bali cattle mtDNA were analyzed using a bioinformatics tool (<https://jamiemcgowan.ie/bioinf/index.html>).

### Genetic distance and phylogenetic analysis

The present study identified the genetic relationships of Bali cattle by means of mitochondrial genome sequences from five more *Bos* species accessible in GenBank. Genetic distance and phylogenetic analyses were conducted using the mitogenome of Bali cattle along with five bovine mitogenomes: *Bos taurus* (FJ971086.1), *Bos taurus* (NC\_006853), *Bos indicus* (AY126697.1), *Bos javanicus* (FJ997262.1) and *Bos javanicus* (NC012706.1). A comparison of the genetic distance between Bali cattle and other published cattle breeds, namely *B. taurus*, *B. indicus*, and *B. javanicus*, from the NCBI dataset was analyzed using the pairwise distance approach. The Neighbor-Joining (NJ) model in MEGA11 software (Tamura et al. 2021) was used to analyze the phylogenetic relationship between the mtDNA genome of Bali cattle and other cattle breeds (NCBI dataset).

## RESULTS AND DISCUSSION

### MtDNA genome sequence of Bali cattle

The total mitochondrial (mtDNA) genome of Bali cattle was 16,712 base pairs (bp) in length (NCBI with PV387265). The total length of the Bali cattle mtDNA genome, which is larger than that of other cattle populations, is due to the repetitive motifs in the D-loop region (Jakaria et al. 2019). These motifs contribute to variations in mitochondrial genome length across various species.

**Table 1.** Length of sequence of each gene in Bali cattle mtDNA

Genes	Type	Strand	Position		Size (bp)
			Min	Max	
trnP	tRNA	L	15,727	15,792	66
trnT	tRNA	H	15,659	15,727	69
cytb	gene	H	14,515	15,654	1,140
trnE	tRNA	L	14,442	14,510	69
nad6	gene	L	13,914	14,441	528
nad5	gene	H	12,110	13,930	1,821
trnL	tRNA	H	12,040	12,109	70
trnS1	tRNA	H	11,979	12,038	60
tRNA-His	tRNA	H	11,909	11,978	70
nad4	gene	H	10,531	11,908	1,378
nad4l	gene	H	10,241	10,537	297
trnR	tRNA	H	10,171	10,240	70
nad3	gene	H	9,824	10,169	346
trnG	tRNA	H	9,755	9,823	69
cox3	gene	H	8,971	9,754	781
atp6	gene	H	8,291	8,971	681
atp8	gene	H	8,130	8,330	201
trnK	tRNA	H	8,062	8,128	67
cox2	gene	H	7,375	8,058	684
trnD	tRNA	H	7,305	7,373	69
tRNA-Ser	tRNA	L	7,230	7,300	71
cox1	gene	H	5,688	7,232	1,545
trnY	tRNA	L	5,619	5,686	68
tRNA-Cys	tRNA	L	5,552	5,618	67
trnN	tRNA	L	5,447	5,519	73
tRNA-Ala	tRNA	L	5,377	5,445	69
tRNA-Trp	tRNA	H	5,309	5,375	67
nad2	gene	H	4,267	5,308	1,042
tRNA-Met	tRNA	H	4,198	4,266	69
trnQ	tRNA	L	4,124	4,195	72
trnI	tRNA	H	4,058	4,126	69
nad1	gene	H	3,102	4,057	956
trnL2	tRNA	H	3,025	3,099	75
rrn16	rRNA	H	1,453	3,024	1,572
tRNA-Val	tRNA	H	1,386	1,452	67
rrn12	rRNA	H	430	1,385	956
trnF	tRNA	H	364	429	66
Control region	D-loop	-	(15,793- 16,172)	(1-363)	1,283

Notes: H: Heavy strand; L: Light strand; bp: base pair

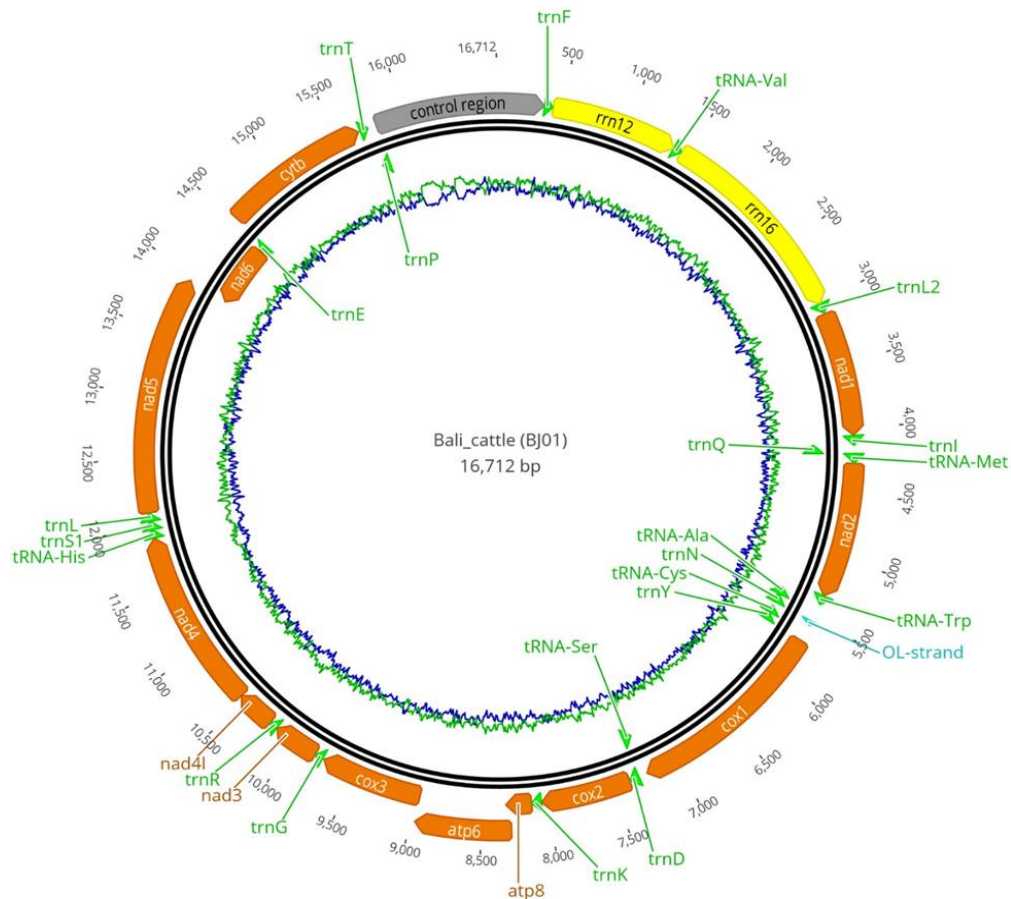
The overall base composition of the genome was 33.6% Adenine (A), 27.1% Thymine (T/uracil, U), 25.8% Cytosine (C), and 13.4% Guanine (G). The total AT content of mtDNA was 60.7%, and the GC content was 39.3%. This composition is in accordance with the trends observed in other bovine species, including Uruguayan native cattle (Liu et al. 2020) and Qaidam cattle (Guo et al. 2017). The mtDNA genome contains 37 genes that are prevalent in the mitochondrial genomes of mammals. There were 13 protein-coding genes, twenty-two transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes in the entire mtDNA genome. Compared with the mitochondrial genomes of Indian Gaur (Kamalakkannan et al. 2020), Malayan Gaur (Rosli et al. 2019), and Indian Mithun (Prabhu et al. 2019), the overall genome size and gene organization were found to be significantly conserved, providing a reassuring consistency. Nine of the 37 genes, including nad6 and eight

tRNA genes (*trnP*, *trnE*, *tRNA-Ser*, *trnY*, *tRNA-Cys*, *trnN*, *tRNA-Ala*, and *trnQ*) were found on the Light (L) strand, whereas the remaining 28 genes were found on the Heavy (H) strand. A detailed gene map of the mitochondrial genome is shown in Figure 1, and a full gene list is provided in Table 1.

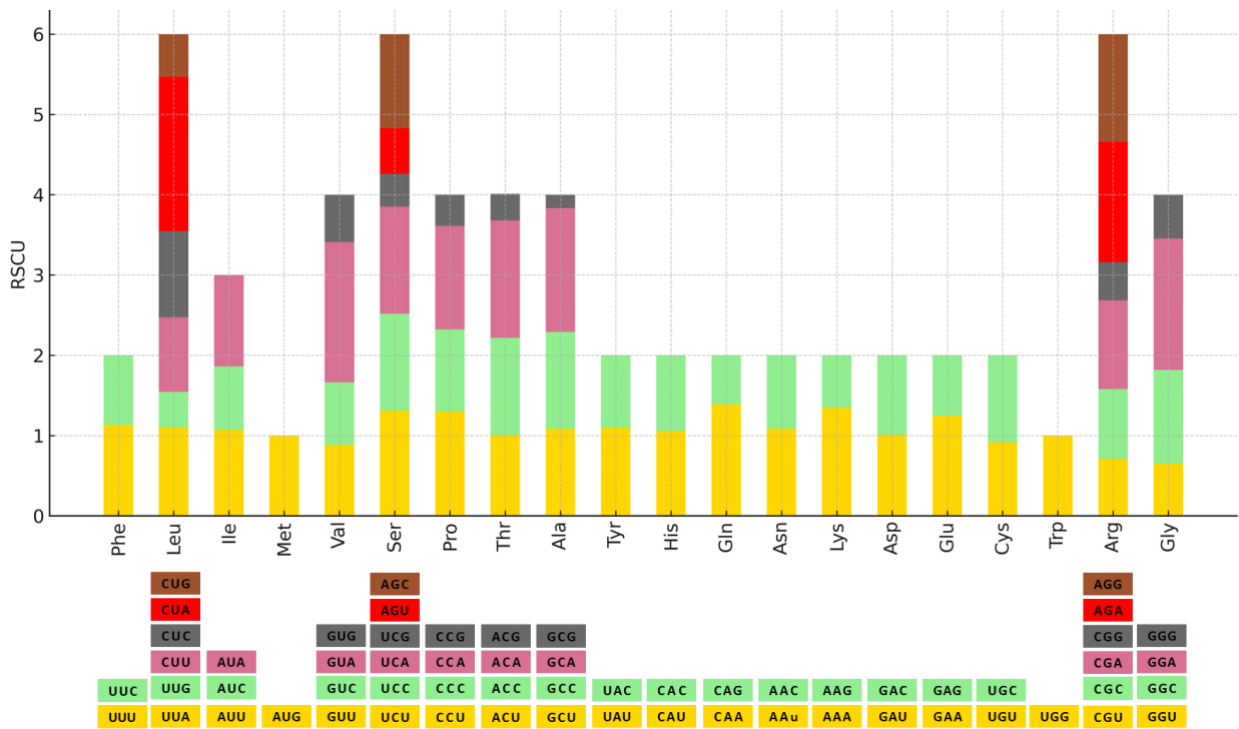
Based on mitochondrial DNA genome sequence analysis, Bali cattle produced 13 protein-coding genes (CDS) from a total of 37 genes. The CDS gene category was divided into 7 NADH dehydrogenase subunits (*nad1*, *nad2*, *nad3*, *nad4*, *nad4l*, *nad5*, and *nad6*), 3 cytochrome c oxidase genes (*cox1*, *cox2*, and *cox3*), 2 ATP synthase genes (*atp6* and *atp8*), and 1 cytochrome b gene (*cytb*). Thirteen CDS genes had a total length of 11,400 bp, with *atp8* having the shortest size (201 bp) and *nad5* having the longest size (1,821 bp). The results obtained in this study were from previous studies on other cattle related to mitochondrial protein-coding genes in the entire cattle genome. Wang et al. (2016) had been reported the results of CDS gene composition similar to Bali cattle in the mitochondrial genome of native Korean cattle. This indicated structural and functional conservation. The same study on Zhangmu cattle identified 13 CDS genes with the same structure (Guo et al. 2018). Among the PCGs in the Bali cattle mtDNA genome, four codons with the highest Relative Synonymous Codon Usage (RSCU) value were CUA (1.92), GUA (1.75), GGA (1.64) and GCA (1.54). These codons were leucine (CUA), valine (GUA), glycine (GGA), and alanine (GCA) (Figure 2). Those results could be known that among the synonymous alternative codons of each amino acid, the codons with adenine at third base codon position were preferred.

A total of 22 transfer RNA (tRNA) genes were found in the mitochondrial genome of Bali cattle. The tRNA genes had varying lengths ranging from 60 bp (*trnS1*) to 75 bp (*trnL2*), with a total length of 22 tRNA genes of 1,512 bp. tRNA genes predominantly were located on the heavy strand (H-strand) including *trnP*, *trnT*, *trnL*, *trnS1*, *tRNA-His*, *trnR*, *trnG*, *trnK*, *trnD*, *tRNA-Trp*, *tRNA-Met*, *trnI*, *trnL2*, *tRNA-Val*, and *trnF*. The remaining tRNA genes include *trnE*, *trnY*, *tRNA-Cys*, *trnN*, *tRNA-Ala*, and *trnQ* were located on the Light strand (L-strand). Based on MITOS2 and tRNAscan-SE analysis, the tRNA genes of Bali cattle were formed cloverleaf secondary structure, excepting the *trnS1* and *trnK* (Figure 3). The unusual structure in *trnS1* and *trnK* because of the loses and unstable dihydrouridine (DHU) arm loops. The unusual tRNA structure of this study was also reported in Peruvian Creole cattle (Arbizu et al. 2022), Gaur and Mithun (Ren et al. 2018; Deb et al. 2021), and Qaidam cattle (Guo et al. 2017).

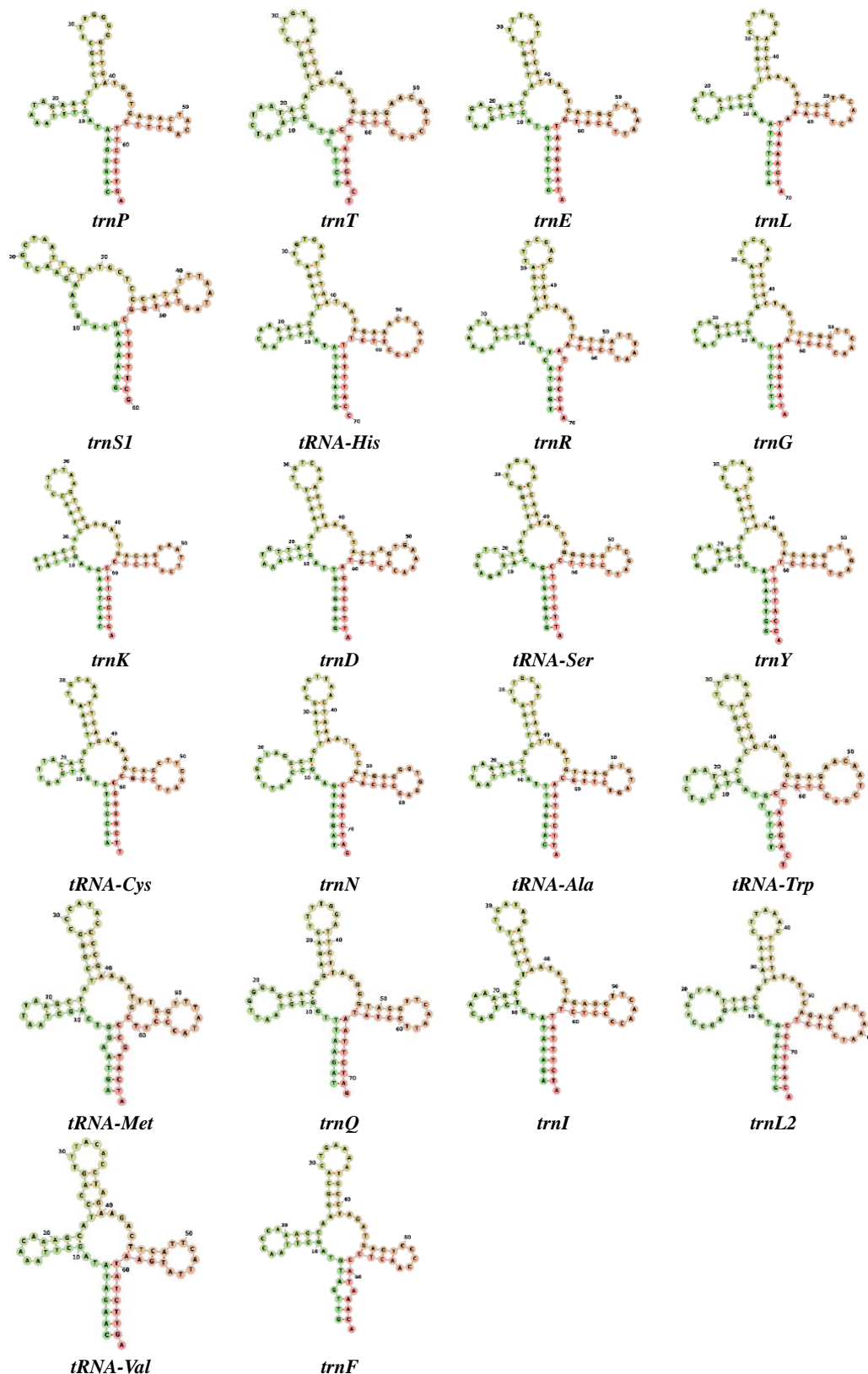
This study also produced two ribosomal RNA (rRNA) genes, 12S rRNA (965 bp) and 16S rRNA (1,572 bp), with a total length of 2,528 bp. The structure and length of the tRNA and rRNA gene sequences produced in the mitochondrial genome of Bali cattle were from other cattle breeds. Variation in gene size in the mitochondrial genome of cattle is caused by evolutionary pressure on populations.



**Figure 1.** The 28 genes on the H-strand are represented by colored blocks outside the circle on the mitochondrial genome map of Bali cattle, whereas the 9 genes on the L-strand are represented by colored blocks inside the circle



**Figure 2.** The Relative Synonymous Codon Usage (RSCU) value of the Bali cattle with codons are plotted on the X-axis and visualized by different colors



**Figure 3.** Predicted secondary structures of the 22 transfer RNA (tRNA) genes in Bali cattle

In both Bali and other cattle breeds, the tRNA gene has a total length ranging from 1,500 to 1,600bp. In addition, the control region (D-loop) in the mitochondrial genome of Bali cattle has a length of 1,283 bp. Bali cattle have a

longer D-loop than other cattle because of the presence of a 22 bp repetitive nucleotide sequence. The repetitive sequence 5'-GTA CAT AAT ATT AAT GTA ATA A-3' is repeated 9 times and is exclusively found in Bali cattle

(Jakaria et al. 2019). The size of the D-loop, a non-coding region crucial for mitochondrial replication and transcription, varied across various cattle breeds. The 1,283 bp D-loop sequence found in Bali cattle matched the range of cattle sequences recorded in past research. The D-loop length of Pesisir cattle is 680 bp (Putri et al. 2019), while East Asian cattle have a shorter sequence length (922 bp) than those in this study (Lee et al. 2012). The different length of the D-loop sequence in every cattle species indicates that genetic variations, environmental elements and population varieties probably shape the genetic variations. The structure and size of the mitochondrial genome in Bali cattle indicate a fairly high level of conservation and variation that provides information about its evolutionary history.

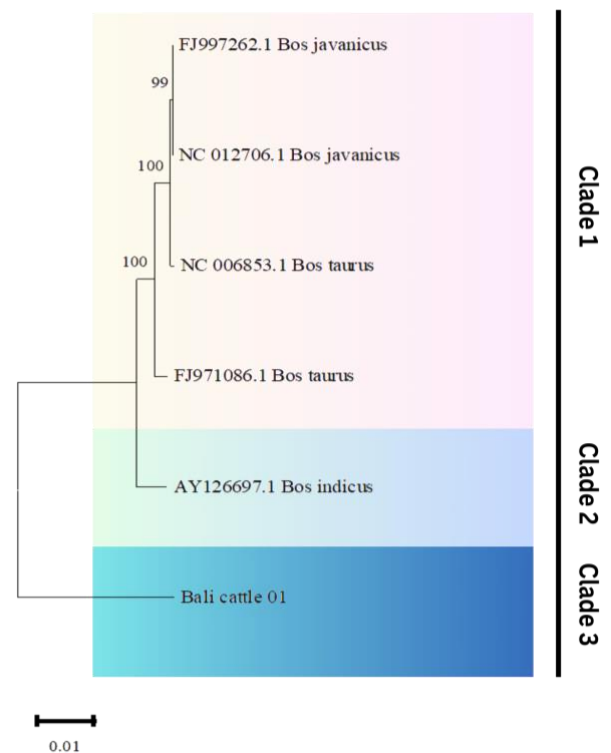
### Genetic distance and phylogenetic analysis

The results of the genetic distance analysis and relationships between Bali cattle and other cattle species are presented in Figure 4 and Table 2. Bali cattle showed a genetic distance between 0.05405 and 0.05530 with other *Bos* species. Genetic distance analysis showed that Bali cattle were relatively close genetically to *B. taurus* (FJ971086.1) and *B. indicus*, with values of 0.05405 and 0.05413, respectively. Table 2 also shows that Bali cattle had similar closeness values to *B. javanicus* (0.05485), indicating a significant relationship. Bali cattle are known to be the result of the domestication process of *Banteng* (*B. javanicus*).

Figure 4 shows the results of the phylogenetic analysis of the mitochondrial genome of Bali cattle compared to other species using the Neighbor-Joining (NJ) method. Phylogenetic trees showed the presence of clades as groups of organisms of common ancestral descent. A very high level of confidence in the phylogenetic analysis of the tree can be obtained through the observation of high bootstrap values (100%) in the review of each clade or group based on common ancestors (Hoang et al. 2018). The phylogenetic tree showed the presence of three main clades (Figure 4).

Bali cattle are split from the *B. indicus* clades (clade 2), *B. javanicus* and *B. taurus* (clade 1), based on the three main clades developed. *B. taurus* and *B. indicus* represent European and Indian cattle in different clades. *B. javanicus* has a strong genetic relationship with the individuals being compared. These phylogenetic results indicate a unique

evolutionary process between Bali cattle and other *Bos* species. The large genetic distance between Bali cattle and *B. javanicus* can be caused by the influence of a long-term domestication process, genetic drift, and environmental influences involving the ability to adapt to the environment (Hunter 2018; Andersson and Purungganan 2022). This also suggests the prospect of a distinct genetic variation contribution between Bali cattle and Banteng. These mechanisms may help explain the genetic variations separating Bali cattle from wild cattle, thereby stressing their special genetic identity inside the *Bos* species.



**Figure 4.** Phylogenetic tree of Bali cattle, *B. taurus*, *B. indicus*, and *B. javanicus* from total mitochondrial genome sequences with 1000 Bootstraps support

**Table 2.** Genetic distance of Bali cattle based on complete mtDNA genomes

Species	1	2	3	4	5	6
1 Bali_cattle_01 (this_study)	-					
2 <i>Bos_taurus</i> _(FJ971086.1)	0.05405	-				
3 <i>Bos_taurus</i> _(NC_006853.1)	0.05530	0.00569	-			
4 <i>Bos_indicus</i> _(AY126697.1)	0.05413	0.01081	0.01201	-		
5 <i>Bos_javanicus</i> _(FJ997262.1)	0.05485	0.00541	0.00093	0.01172	-	
6 <i>Bos_javanicus</i> _(NC_012706.1)	0.05485	0.00541	0.00093	0.01172	0.00000	-

In conclusion, this study offers important new perspectives on the genetic organization of Bali cattle and presents the first complete mitochondrial genome of cattle. The mtDNA analysis revealed significant phylogenetic differences between Bali cattle and other *Bos* species, including *B. taurus*, *B. indicus*, and *B. javanicus*. These findings underscore the particular genetic uniqueness of Bali cattle inside the *Bos* species and stress their genetic variations from their wild ancestors. The results of this study add significant genomic data that will guide and inspire future studies on the evolutionary background of Bali cattle, opening up new avenues for research in the field.

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