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Complete Mitochondrial Genome Analysis Reveals the Genetic Diversity of Madura Cattle (*Bos javanicus* × *Bos indicus* Hybrid)

N. Khairunnisa[1], A. B. Dharmayanthi[2], R. R. Noor[3], & J. Jakaria[3,*]
[1] Graduate Students of Animal Production and Technology Science, Graduate School, IPB University, Bogor, Indonesia
[2] Research Centre for Biosystematics and Evolution, National Research and Innovation Agency, Bogor, Indonesia
[3] Department of Animal Production and Technology, Faculty of Animal Science, IPB University, Bogor, Indonesia
Jl. Agatis, Kampus IPB Dramaga Bogor 16680, West Java, Indonesia
*Corresponding author: Jakaria@apps.ipb.ac.id
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ABSTRACT

Madura cattle are a significant local beef breed in Indonesia, but information about their genetic origins is limited, especially since no studies have fully characterized their mitochondrial DNA. This research aims to sequence the entire mitochondrial genome of Madura cattle and explore their phylogenetic relationships and diversity relative to reference bovine lineages. The study used five blood samples from Madura bulls at Singosari Artificial Insemination Center (BBIB). DNA was extracted from the blood, then the mitochondrial genome was amplified via long-range PCR and sequenced with Oxford Nanopore Technology. Across the five samples, quality-filtered reads ranged from 5061 to 11785, with mean read lengths of 5030 to 7475 bp and mean quality scores of 14.1 to 14.3. Bioinformatics analysis included genome assembly, gene annotation, nucleotide composition, and phylogenetic analysis. The findings showed that the Madura cattle mitochondrial genome is between 16338 and 16451 base pairs (GenBank accession PZ509169) and contains 37 genes: 13 protein-coding, 22 tRNA, 2 rRNA, and 1 control region. Nucleotide analysis indicated positive AT-skew and negative GC-skew in most protein-coding genes. Phylogenetic analysis revealed that Madura cattle cluster into two maternal groups, one aligned with *Bos javanicus* and the other with *Bos indicus*. These results suggest that the maternal lineage of Madura cattle is complex and may reflect historical introgression involving *Bos javanicus* and *Bos indicus*. However, further nuclear genomic analysis is needed to clarify genome-wide ancestry and admixture patterns.

Keywords: Bovidae; Madura cattle; maternal origin; mitochondrial whole genome; phylogenetics

INTRODUCTION

Madura cattle have been recognized by the Indonesian Ministry of Agriculture as one of Indonesia's local breeds, originating from Madura Island and its surrounding areas. Madura cattle are among Indonesia's valuable local livestock genetic resources that require protection and conservation (National Standards Agency of Indonesia, 2020). The conservation of Indonesian local cattle is important because these breeds possess unique genetic backgrounds, adaptive traits, and potential breeding value. Recent genetic and genomic studies have emphasized that genetic information is essential for supporting sustainable utilization, conservation, and breeding strategies for the Indonesian local cattle population (Sutarno & Setyawan, 2015). The Madura cattle population on Madura Island makes a significant contribution, meeting approximately 24% of the demand for beef cattle in East Java (Nurgiartiningsih & Budiarto, 2016). Additionally, Madura cattle play a vital role as a symbol of identity and culture for the Madura community (Nurgiartiningsih & Budiarto, 2016).

The population of crossbred Madura cattle on Madura Island over the past few decades has reached 53.3% of the total cattle population on the island (Nurgiartiningsih & Budiarto, 2016). This poses a potential threat to the genetic diversity of local livestock. Uncontrolled crossbreeding is also believed to be a major threat to the sustainability of Indonesia's local livestock. Uncontrolled crossbreeding with exotic breeds has also been reported as a factor that may contribute to genetic erosion and reduced quality in Indonesian local cattle populations (Sutarno & Setyawan, 2015).

To prevent the loss of local genetic resources, comprehensive genetic information on local cattle breeds is needed as baseline data for developing conservation programs and breeding strategies (Petretto *et al*., 2022; Pramod *et al*., 2018; Xia *et al*., 2019a). Mitochondrial DNA analysis is frequently used to assess intra- and interspecies diversity, trace maternal lineages, and examine evolutionary relationships among various

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species (Anwar *et al*., 2025). Popular mtDNA markers have been used in analyses of Bali cattle breeds (Jakaria *et al*., 2019), Thailand dairy cattle (Ariyaraphong *et al*., 2021), Alpine cattle (Granado *et al*., 2021), and Podolian cattle (Di Lorenzo *et al*., 2018), albeit only partially. Currently, intensive research on mitochondrial genomes is being conducted to examine genetic diversity in Uruguayan cattle (Liu *et al*., 2020), Georgian mountain cattle (Kunelauri *et al*., 2022), Shigaste humped cattle (Guo .*et al*., 2018), Yunling cattle (Xia *et al*., 2019b), Sardinian cattle (Petretto *et al*., 2022), Native Korean cattle (Wang *et al*., 2016), Guangxi cattle (Xia *et al*., 2019a), and Bali cattle (Anwar *et al*., 2025; Jakaria *et al*., 2025). Mitochondrial analysis of Madura cattle has been conducted, but only using partial markers such as D-loop (Hartatik *et al*., 2015), cytB (Prihandini *et al*., 2020), and COI (Utami *et al*., 2023). Although these markers provide useful preliminary information, they are insufficient to describe complete mitochondrial genome organization, nucleotide composition, codon usage, tRNA structure, control region variation, and whole mitogenome-based phylogenetic relationships. No studies have been reported on the analysis of the complete mtDNA genome of Madura cattle. Therefore, this study addresses this gap by providing the first complete mitochondrial genome characterization of Madura cattle using long-read Oxford Nanopore Technology. This study aims to fully characterize the mitochondrial genome of Madura cattle, reveal their genetic diversity, and examine their phylogenetic relationships with other cattle breeds worldwide.

MATERIALS AND METHODS

Ethical Approval

This research has been approved by the ethics committee of the National Research and Innovation Agency (BRIN) of the Republic of Indonesia under approval number 071/KE.02/SK/03/2024

Experimental Design

This study used five blood samples from Madura bulls collected at the Singosari Artificial Insemination Center (BBIB) in Malang, East Java, Indonesia. Blood samples were collected via the jugular vein, with volumes of 5-8 mL. DNA extraction and PCR amplification were performed at the Genomics Laboratory of the National Research and Innovation Agency (BRIN) in Cibinong, Bogor, while mitochondrial genome sequencing was conducted at PT Genomik Solidaritas Indonesia (GSI) in South Jakarta. This study was carried out in several stages, comprising DNA extraction, amplification, sequencing, bioinformatics analysis, and data analysis.

DNA Extraction

Mitochondrial DNA was extracted using the Quick-DNA HMW MagBead Kit (Zymo Research) following the manufacturer's protocol. DNA concentration was measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific, USA); extracted DNA concentrations ranged from 9.68 to 22.21 ng/μL. The extracted DNA was then used for further analysis.

Mitochondrial Genome Amplification

The mitochondrial genome was amplified by a long-range PCR approach using the GoTaq Long PCR Master Mix (Promega). Amplification was performed using two pairs of forward and reverse primers. The first primer pair consisted of forward 5′-ATG AGT TGG TAG TTT CGG TTG G-3′ and reverse 5′-AGT TGA TTG TTG GGG GTA GAG C-3′, while the second primer pair consisted of forward 5′-ACC AAA CAG AAC GCC TAA ACG-3′ and reverse 5′-GCT ATG AAG AAG AGG GCA AAT G-3′ (Anwar *et al*., 2025), each targeting approximately 9 kb fragments. PCR amplification was performed under the following cycling conditions: pre-denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 63 °C for 30 s, and extension at 68 °C for 5 min, with a final extension at 68 °C for 5 min. PCR products were verified by agarose gel electrophoresis using 0.5 × Tris-Borate-EDTA (TBE) buffer an**d** FluoroSafe DNA stain, and visualized under a UV transilluminator to confirm successful amplification prior to sequencing.

Mitochondrial Genome Sequencing

The DNA amplification products were subsequently processed for library preparation using the SQK-LSK110 Ligation sequencing Kit (Oxford Nanopore Technologies, UK). Each sample was barcoded using the Native Barcoding Kit v14 (SQK-NBD114), enabling multi-sample sequencing in a single run. The barcoded DNA was pooled and loaded onto an R10.4.1 flow cell for sequencing using the GridION device (Oxford Nanopore Technologies, UK). Basecalling was performed with Guppy in high-accuracy mode to generate FASTQ sequencing data. The data were subsequently separated by sample barcode using the MinKNOW software for demultiplexing.

Mitochondrial Genome Assembly

The sequencing data obtained were processed using several bioinformatics tools. Adapter and barcode trimming were performed using Porechop version 0.2.4 (Wick, 2017), while read quality filtering was performed using NanoFilt version 2.3.0 (De Coster *et al*., 2018) with a minimum quality threshold of Q10.

The filtered reads were then mapped to the *Bos javanicus* (Pq130485.1) and *Bos indicus* (OQ886063.1) reference genomes using Minimap2 version 2.28 with the *map-ont* parameter. The mapping results were saved in Binary Alignment Map (BAM) format and used to generate consensus sequences using iVar version 1.4.2 (Grubaugh *et al*., 2019) with the following parameters: minimum quality score Q20, minimum base frequency 0.6, minimum indel frequency 0.8, and minimum coverage depth 30×. iVar was used because this study

applied an amplicon-based targeted sequencing approach, and iVar enables consensus sequence generation from mapped amplicon reads using defined thresholds for base quality, allele frequency, indel frequency, and coverage depth. Sequencing statistics were evaluated after adapter trimming and quality filtering. Across the five samples, quality-filtered reads ranged from 5061 to 11785, total bases ranged from 25.46 to 79.68 Mb, mean read lengths ranged from 5030 to 7475 bp, N50 values ranged from 8987 to 9028 bp, and mean quality scores ranged from 14.1 to 14.3. The resulting consensus sequence was then curated by aligning it with the D-loop sequence obtained via Sanger sequencing from the same sample. The D-loop region used for Sanger sequencing was amplified using the forward primer 5′-GGA AGA AAC TGT AGT CTC AC-3′ and the reverse primer 5′-CAT CTA GGC ATT TTC AGT GC-3′. The trimming process leading to the formation of the consensus sequence was performed using the Galaxy bioinformatics platform (usegalaxy.eu). The complete mitochondrial genome sequences were then annotated using the Mitos2 software (Bernt *et al*., 2013) to identify the position and sizes of genes, including protein-coding genes (PCGs), rRNA, tRNA, and the control region (CR). The complete mitochondrial genome sequence generated in this study is available under GenBank accession number PZ509169.

Mitochondrial Genome Analysis

The complete mitochondrial genome sequences were visualized as circular genome maps using Geneious Primer v2025.0.2 (Kearse, 2012). Genetic distance analysis was performed using the Kimura 2-parameter (K2P) substitution model (Kimura, 1980) in Geneious Primer v2025.0.2. Analysis of Relative Synonymous Codon Usage (RSCU) and calculation of AT-skew and GC-skew were performed based on the nucleotide composition of the mitochondrial genome using the formula AT-skew = (A – T)/(A+T) and GC-skew = (G – C)/(G + C) were performed using bioinformatics tools available at web bioinformatics (jamiemcgowan.ie/bionf/index.htm). Visualization of tRNA secondary structures was performed using Forna (www.rna.tbi.univie.ac.at/forna/).

Phylogenetic tree reconstruction was performed by aligning sequences with MAFFT (Katoh *et al*., 2019) and the phyml method in Geneious Prime v2025.0.2 with 1000 bootstraps. Reference mitochondrial genome sequences used Zebu cattle (*Bos indicus*) accession ID NC005971.1, Ongole cattle (*Bos indicus*) accession ID NC006853.1, Bali cattle (*Bos javanicus*) accession ID PQ130485.1, CERZA Bali cattle (*Bos javanicus* CERZA) accession ID JN632606.1, Bali cattle (*Bos javanicus*) accession ID PV387265.1, Gaur (*Bos gaurus*) accession ID MT360653.1, Aurochs cattle (*Bos primigenius*) accession ID NC013996.1, Gayal (*Bos frontalis*) accession ID MW763078.1, and Yak (*Bos grunniens*) accession ID OR086006.1. Meanwhile, *Bubalus bubalis* (accession NC049568.1) was used as the outgroup.

RESULTS

Mitochondrial Genome Structure of Madura Cattle

The Madura cattle mitochondrial genome has a total length of 16338 to 16451 base pairs (bp), consisting of 1 control region (D-loop) and 37 genes, including 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes, and 13 protein-coding genes (PCGs). The protein-coding genes consist of three cytochrome c oxidase (COX1, COX2, and COX3), two subunits of ATP synthase (ATP6 and ATP8), and seven subunits of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6). The result of full-length mtDNA genome sequencing of Madura cattle showed that the sequence was the shortest at 16338 bp (Ma1), the longest at 16451 bp (Ma2), and the second longest at 16386 bp (Ma3), while Ma4 and Ma5 were 16,338 bp and 16,339 bp, respectively. The length variation among samples was mainly associated with differences in the control region or D-loop due to the presence of a tandem repeat motif with the motif 5′-ATA AGT ACA TAA TAT TTA TGT A-3′ in samples Ma2 and Ma3. The majority of genes are distributed on the heavy strand (H-strand); 28 of the 37 genes are on the H-strand, and 9 genes are on the light strand (L-strand), namely 8 tRNA genes (trnA, trnC, trnE, trnN, trnP, trnY, trnS2, and trnQ) and one protein-coding gene (ND6) (Figure 1 and Table 1). The start codons found in protein-coding genes (PCGs) are predominantly ATG and ATA. The stop codons found in the Madura cattle PCGs are AGA (cytB), TAA (ND6, ND5, ND4L, ATP6, ATP8, COX2, and COX1), and incomplete stop codons, namely TA- (ND1) and T (ND4, ND3, COX3, and ND2). The nucleotide composition of the complete mitochondrial genome was dominated by adenine and thymine, with adenine 33.47%, thymine 27.11%, cytosine 26.01%, and guanine 13.40%. The A+T content was 60.58%, whereas the G+C content was 39.42%, indicating an A+T-rich mitochondrial genome. Two amino acid substitutions were detected in the mitochondrial protein-coding genes of Madura cattle, including phenylalanine to leucine (F to L) in ATP8 at position 24 and valine to isoleucine (V to I) in ND5 at position 172.

AT-Skew and GC-Skew Composition

The total length of protein-coding genes (PCGs) in Madura cattle is 11403 bp, or 69.79% of the total mitochondrial genome. Analysis of the nucleotide composition of the protein-coding genes (PCGs) in the Madura cattle mtDNA genome shows a positive AT-skew and a negative GC-skew (Figure 2). The AT-skew values for the mitochondrial protein-coding genes of Madura cattle (ND1, ND2, COX2, ATP8, ATP6, ND3, ND4L, ND4, ND5, and cytB) yielded positive values, indicating that the adenine (A) content is higher than thymine (T), while the other three genes (COX1, COX3, and ND6) had negative values, indicating a predominance of thymine (T). However, the COX1 and COX2 genes had values close to zero, indicating that adenine and thymine were nearly balanced in these

Table 1. Structure and characterization of the complete mitochondrial DNA of Madura cattle

Name	Product	Strand	Position		Size (bp)	Start codon	Stop codon	Anti codon
			Min	Max				
Control Region	-	-	15430	16386	957	-	-	-
trnP	tRNA-Pro	L	15364	15429	66	-	-	TGG
trnT	tRNA-Thr	H	15295	15365	71	-	-	TGT
cytB	cytB	H	14152	15291	1140	ATG	AGA	-
trnE	tRNA-Glu	L	14079	14147	69	-	-	TTC
nad6	ND6	L	13551	14078	528	ATG	TAA	-
nad5	ND5	H	11747	13567	1821	ATA	TAA	-
trnL1	tRNA-Leu	H	11677	11746	70	-	-	TAG
trnS1	tRNA_ser	H	11616	11675	60	-	-	GCT
trnH	tRNA-His	H	11546	11615	70	-	-	GTG
nad4	ND4	H	10168	11545	1378	ATG	T--	-
nad4l	ND4L	H	9878	10174	297	ATG	TAA	-
trnR	tRNA-Arg	H	9808	9877	70	-	-	TCG
nad3	ND3	H	9461	9806	346	ATA	T--	-
trnG	tRNA-Gly	H	9392	9460	69	-	-	TCC
COX3	COX3	H	8608	9391	784	ATG	T--	-
ATP6	ATP6	H	7928	8608	681	ATG	TAA	-
ATP8	ATP8	H	7767	7967	201	ATG	TAA	-
trnK	tRNA-Lys	H	7699	7765	67	-	-	TTT
COX2	COX2	H	7012	7695	684	ATG	TAA	-
trnD	tRNA-Asp	H	6943	7010	68	-	-	GTC
trnS2	tRNA-Ser	L	6867	6937	71	-	-	TGA
COX1	COX1	H	5325	6869	1545	ATG	TAA	-
trnY	tRNA-Tyr	L	5256	5323	68	-	-	GTA
trnC	tRNA-Cys	L	5189	5255	67	-	-	GCA
trnN	tRNA-Asn	L	5084	5156	73	-	-	GTT
trnA	tRNA-Ala	L	5014	5082	69	-	-	TGC
trnW	tRNA-Trp	H	4946	5012	67	-	-	TCA
nad2	ND2	H	3904	4945	1042	ATA	T--	-
trnM	tRNA-Met	H	3835	3903	69	-	-	CAT
trnQ	tRNA-Gln	L	3761	3832	72	-	-	TTG
trnI	tRNA-Ile	H	3695	3763	69	-	-	GAT
nad1	ND1	H	2739	3694	956	ATG	TA-	-
trnL2	tRNA-Leu	H	2662	2736	75	-	-	TAA
rrn 16	16S rRNA	H	1162	2661	1500	-	-	-
trnV	tRNA-Val	H	1023	1089	68	-	-	TAC
rrn 12	12S rRNA	H	67	1021	955	-	-	-
trnF	tRNA-Phe	H	1	66	66	-	-	GAA

two genes. The GC-skewness values for the 12 protein-coding genes (ND1, ND2, COX1, COX2, COX3, ATP6, ATP8, ND3, ND4L, ND4, ND5, and cytB) are negative, indicating that cytosine (C) content exceeds guanine (G) content.

Relative Synonymous Codon Usage (RSCU)

Analysis of Relative Synonymous Codon Usage (RSCU) in the protein-coding genes (PCGs) of the Madura cattle mtDNA genome showed variation in synonymous codon usage. Analysis of the Relative Synonymous Codon Usage (RSCU) in protein-coding genes (PCGs) of the Madura cattle mtDNA genome shows a strong preference for codons ending in Adenine (A). The highest RSCU values were observed for CGA (Arg, 3.182), CTA (Leu, 2.808), GTA (Val, 2.172), TCA (Ser, 2.161), and ACA (Thr, 1.934). Based on codon counts, the most frequently represented amino acids were leucine (Leu, 532 codons), isoleucine (Ile, 481 codons), threonine (Thr, 304 codons), serine (Ser, 261 codons), and alanine (Ala, 211 codons) (Figure 3 and Table 2).

Transfer RNA (tRNA) Genes and Secondary Structure

Twenty-two tRNA genes were identified, with a total length of approximately 1515 bp. The length of each gene ranged from 60 bp (tRNA-Ser) to 75 bp (tRNA-Leu2). Based on the tRNA structure visualization results, Madura cattle tRNA forms a cloverleaf secondary structure. However, several mitochondrial tRNAs show deviations from this structure, namely trnS1 (tRNA-Ser1) and trnK (tRNA-Lys) (Figure 4).

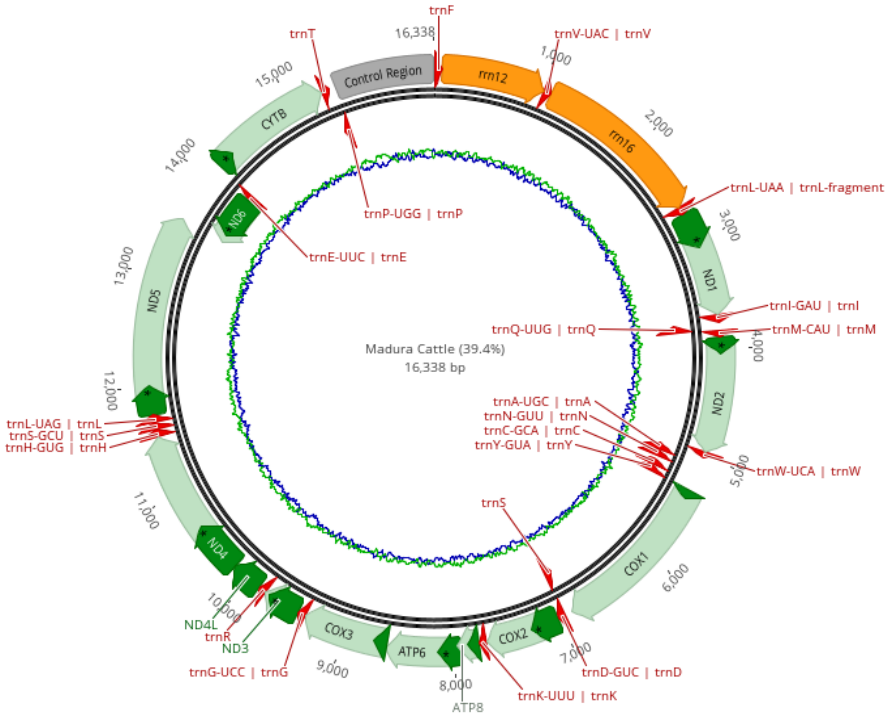


Figure 1. Annotation of the complete mitochondrial genome of Madura cattle

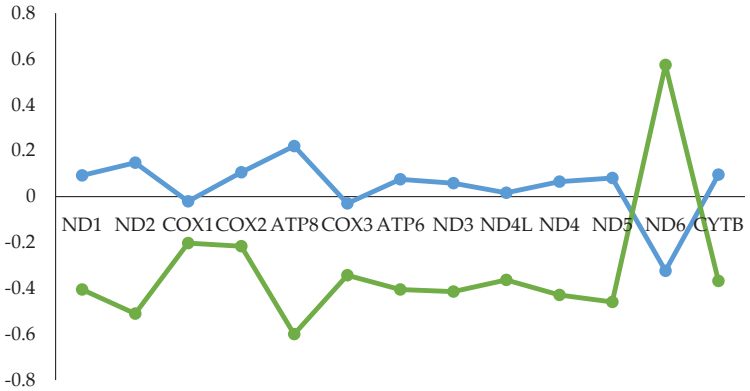


Figure 2. AT-skewness and GC-skewness profiles of 13 Protein-coding genes (PCGs) in Madura cattle. Note: AT SKEW, GC SKEW

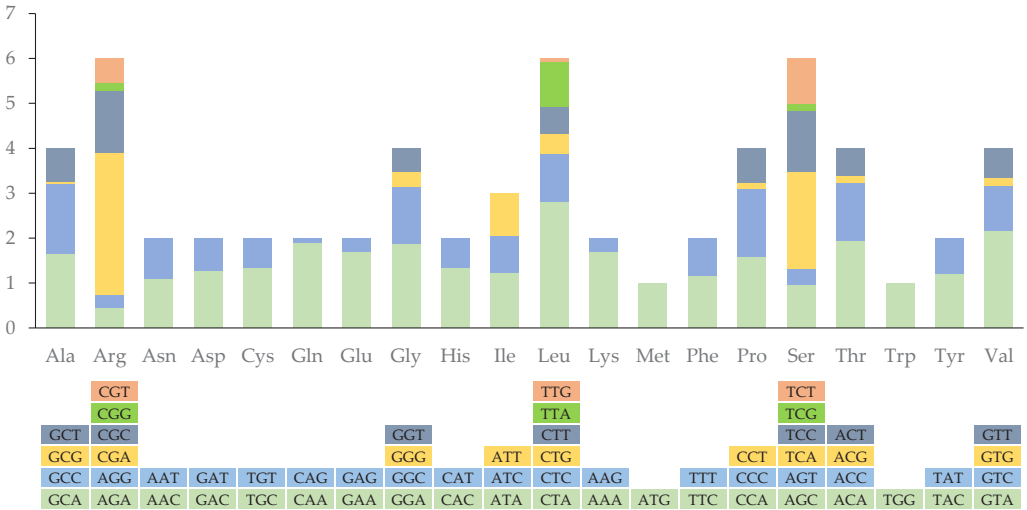


Figure 3. Relative Synonymous codon usage (RSCU) of mitochondrial protein-coding genes (PCGs) and tRNA genes in Madura cattle

Table 2 Relative Synonymous Codon Usage (RSCU) values and codon counts in the mitochondrial genome sequence of Madura cattle

No.	Codon	Amino acid	Codon count	RSCU	No.	Codon	Amino acid	Codon count	RSCU
1	TTT	Phenylalanine (Phe)	87	0.845	32	GCG	Alanine (Ala)	3	0.057
2	TTC	Phenylalanine (Phe)	119	1.155	33	TAT	Tyrosine (Tyr)	46	0.780
3	TTA	Leucine (Leu)	87	0.981	34	TAC	Tyrosine (Tyr)	72	1.220
4	TTG	Leucine (Leu)	7	0.079	35	CAT	Histidine (His)	33	0.660
5	CTT	Leucine (Leu)	55	0.620	36	CAC	Histidine (His)	67	1.340
6	CTC	Leucine (Leu)	95	1.071	37	CAA	Glutamine (Gln)	83	1.908
7	CTA	Leucine (Leu)	249	2.808	38	CAG	Glutamine (Gln)	4	0.092
8	CTG	Leucine (Leu)	39	0.440	39	AAT	Asparagine (Asn)	76	0.910
9	ATT	Isoleucine (Ile)	152	0.948	40	AAC	Asparagine (Asn)	91	1.090
10	ATC	Isoleucine (Ile)	130	0.811	41	AAA	Lysine (Lys)	88	1.692
11	ATA	Isoleucine (Ile)	199	1.241	42	AAG	Lysine (Lys)	16	0.308
12	ATG	Methionine (Met)	41	1.000	43	GAT	Aspartic acid (Asp)	19	0.731
13	GTT	Valine (Val)	25	0.662	44	GAC	Aspartic acid (Asp)	33	1.269
14	GTC	Valine (Val)	38	1.007	45	GAA	Glutamic acid (Glu)	70	1.707
15	GTA	Valine (Val)	82	2.172	46	GAG	Glutamic acid (Glu)	12	0.293
16	GTG	Valine (Val)	6	0.159	47	TGT	Cysteine (Cys)	7	0.667
17	TCT	Serine (Ser)	44	1.011	48	TGC	Cysteine (Cys)	14	1.333
18	TCC	Serine (Ser)	59	1.356	49	TGG	Tryptophan (Trp)	8	1.000
19	TCA	Serine (Ser)	94	2.161	50	CGT	Arginine (Arg)	6	0.545
20	TCG	Serine (Ser)	7	0.161	51	CGC	Arginine (Arg)	15	1.364
21	CCT	Proline (Pro)	35	0.769	52	CGA	Arginine (Arg)	35	3.182
22	CCC	Proline (Pro)	69	1.516	53	CGG	Arginine (Arg)	2	0.182
23	CCA	Proline (Pro)	72	1.582	54	AGT	Serine (Ser)	15	0.345
24	CCG	Proline (Pro)	6	0.132	55	AGC	Serine (Ser)	42	0.966
25	ACT	Threonine (Thr)	46	0.605	56	AGA	Arginine (Arg)	5	0.455
26	ACC	Threonine (Thr)	98	1.289	57	AGG	Arginine (Arg)	3	0.273
27	ACA	Threonine (Thr)	147	1.934	58	GGT	Glycine (Gly)	22	0.512
28	ACG	Threonine (Thr)	13	0.171	59	GGC	Glycine (Gly)	54	1.256
29	GCT	Alanine (Ala)	39	0.739	60	GGA	Glycine (Gly)	81	1.884
30	GCC	Alanine (Ala)	82	1.555	61	GGG	Glycine (Gly)	15	0.349
31	GCA	Alanine (Ala)	87	1.649					

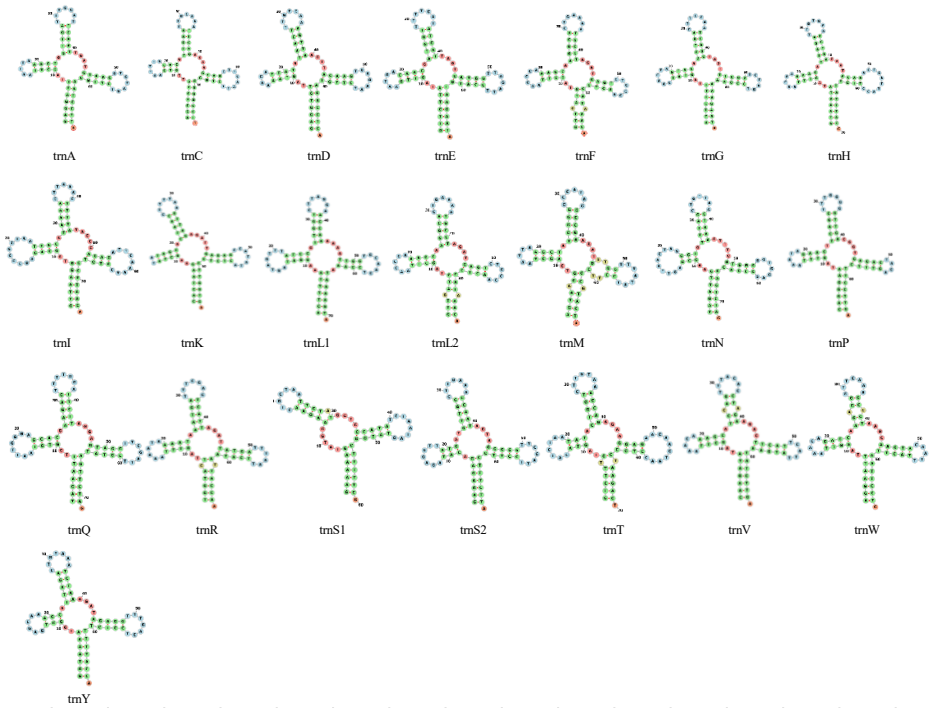


Figure 4. Predicted secondary structures of mitochondrial tRNAs in Madura cattle. Most tRNAs formed the typical cloverleaf structure consisting of the acceptor stem, D-arm, anticodon arm, TΨC arm, and variable loop, whereas trnS1 and trnK showed structural deviations in the DHU arm.

No.	Cattle breed (ID)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Madura cattle 1		0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
2	Madura cattle 2	0.069		0.000	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
3	Madura cattle 3	0.070	0.001		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002
4	Madura cattle 4	0.000	0.069	0.070		0.000	0.000	0.000	0.000	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
5	Madura cattle 5	0.000	0.070	0.070	0.000		0.000	0.000	0.000	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
6	*Bos indicus* (OQ886063.1)	0.002	0.069	0.069	0.002	0.002		0.000	0.000	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
7	*Bos indicus* (NC005971.1)	0.001	0.070	0.070	0.001	0.001	0.002		0.000	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
8	*Bos indicus* (AY126697.1)	0.001	0.070	0.070	0.001	0.001	0.001	0.000		0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002
9	*Bos taurus* (NC006853.1)	0.015	0.072	0.072	0.015	0.015	0.001	0.016	0.016		0.002	0.002	0.002	0.002	0.002	0.002	0.002
10	*Bos primigenius* (NC013996.1)	0.015	0.070	0.070	0.015	0.015	0.015	0.015	0.015	0.005		0.002	0.002	0.002	0.002	0.002	0.002
11	*Bos grunniens* (OR086006.1)	0.062	0.060	0.060	0.062	0.062	0.015	0.061	0.061	0.063	0.062		0.002	0.002	0.002	0.002	0.002
12	*Bos gaurus* (MT360653.1)	0.068	0.042	0.043	0.068	0.068	0.062	0.068	0.068	0.069	0.068	0.059		0.002	0.002	0.002	0.002
13	*Bos javanicus* (PV387265.1)	0.070	0.001	0.000	0.070	0.070	0.068	0.069	0.069	0.071	0.070	0.060	0.043		0.000	0.000	0.002
14	*Bos javanicus* (JN632606.1)	0.070	0.002	0.000	0.070	0.070	0.070	0.070	0.070	0.072	0.070	0.060	0.043	0.000		0.000	0.002
15	*Bos javanicus* (PQ130485.1)	0.069	0.001	0.000	0.069	0.070	0.069	0.070	0.070	0.072	0.070	0.060	0.043	0.000	0.000		0.002
16	*Bos frontalis* (MW763078.1)	0.064	0.037	0.037	0.064	0.063	0.063	0.064	0.064	0.063	0.063	0.060	0.016	0.037	0.038	0.037	

Figure 5. Genetic distance between Madura cattle and reference cattle breeds from GenBank based on the complete mitochondrial genome, with genetic distance values (below the diagonal) and standard deviations (above the diagonal)

Genetic Distance of Madura Cattle

The mitochondrial genetic distance analysis based on complete mitogenome sequences showed distinct maternal relationships among the five Madura cattle samples and reference cattle breeds (Figure 5). Madura cattle 1, 4, and 5 showed very low mitochondrial genetic distances to *Bos indicus*, ranging from 0.000 to 0.002, whereas Madura cattle 2 and 3 showed higher distances to *B. indicus*, ranging from 0.069 to 0.070. In contrast, Madura cattle 2 and 3 showed very low mitochondrial genetic distances to *Bos javanicus*, ranging from 0.000 to 0.001, while Madura cattle 1, 4, and 5 showed higher distances to *B. javanicus* of approximately 0.070. These results indicate the presence of two mitochondrial maternal lineages among the analyzed Madura cattle samples.

Phylogenetic Analysis

Phylogenetic reconstruction based on the complete mitochondrial genome revealed that Madura cattle formed two distinct clusters with high bootstrap support. Madura cattle 1, 4, and 5 clustered with *B. indicus,* supported by bootstrap values of 99-100. The second cluster consisted of Madura cattle 2 and 3, which grouped with *B. javanicus* accessions, also with bootstrap values of 99-100. The separation between the *B. indicus*-related and *B. javanicus*-related maternal lineages was also supported by a bootstrap value of 97 (Figure 6).

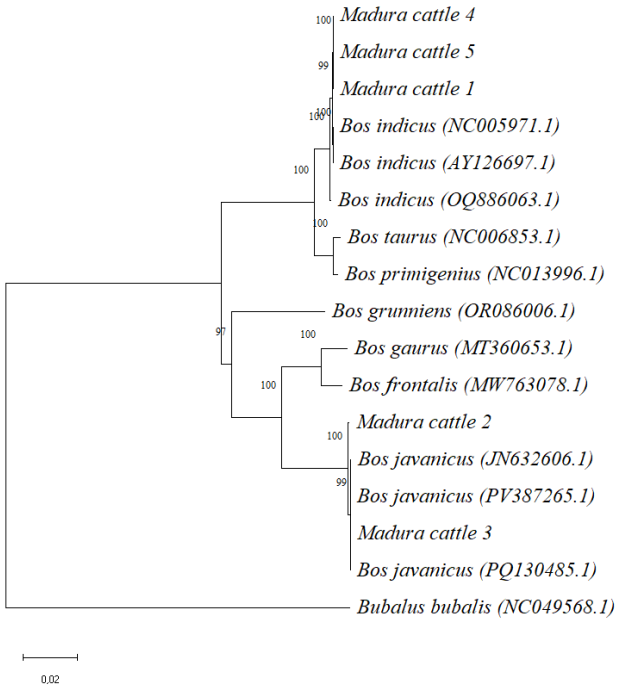


Figure 6. Phylogenetic tree based on complete mitochondrial genome sequences of Madura cattle and reference bovine species. Numbers at internal nodes indicate bootstrap support values based on 100 replicates. *Bubalus bubalis* was used as the outgroup.

DISCUSSION

The mitochondrial genome features of Madura cattle, including sequence length, gene organization, and strand distribution, are consistent with those reported in *Bos taurus* (Estrada *et al*., 2024), *B. indicus* (Arya *et al*., 2025), *B. javanicus* (Jakaria *et al*., 2025), *Bos grunniens* (Gu *et al*., 2007), *Bos gaurus* (Kamalakkannan *et al*., 2020), and *Bison bison* (Douglas *et al*., 2011). All of which share a conserved mitochondrial genome length of approximately 16.3 kb, indicating a stable genomic architecture across vertebrates. Protein-coding genes (PCGs) in the mtDNA of Madura cattle show variation in the use of start and stop codons. The start codons in Madura cattle show no differences compared to those of *B. javanicus* (Anwar *et al*., 2025), *Bos taurus* (Arbizu *et al*., 2022), and *B. grunniens* (Gu *et al*., 2007). Meanwhile, the stop codons of the Madura cattle tend to be more varied compared to their codons, namely the stop codon AGA (cytB), TAA (ND6, ND5, ND4L, ATP6, ATP8, COX2, and COX1), TA- (ND1), and T- (ND4, ND3, COX3, and ND2).

The majority of vertebrates use TAA as the primary stop codon in their mitochondrial DNA (mtDNA) genomes. In the standard genetic code, the AGA and AGG codons code for the amino acid arginine, but in vertebrate mtDNA, they function as stop codons (Zhang *et al*., 2022). The codon AGA also functions as an alternative codon that acts as a translation terminal signal, particularly in the cytB gene, and has been reported in several vertebrates, such as *B. javanicus* (Anwar *et al*., 2025), *Mustela lutreola* (Skorupski, 2022), *B. taurus* (Estrada *et al*., 2024), *Mesechinus miodon* (Kong *et al*., 2016), and *Myotis muricola* (Yoon & Park, 2015). The pattern of incomplete stop codon usage (TA- and T--) in Madura cattle is also commonly found in other vertebrates; these codons become complete termination codons (TAA) after undergoing post-transcriptional polyadenylation at the 3′ end of the mRNA (Skorupski, 2022), which is the addition of a poly (A) tail to the mRNA, thereby forming a complete stop codon sequence (Ojala *et al*., 1981).

No differences were found in the use of stop codons in the mtDNA genome of Madura cattle compared to *B. javanicus* (Anwar *et al*., 2025). However, differences were found in the stop codons of the genes ND3, COX3, and ND2 in *B. taurus,* especially TA- (Estrada *et al*., 2024), whereas the stop codons in Madura cattle for those genes are T--. The Madura cattle stop codon differs from that of Bos grunniens, specifically the AGA stop codon, which is absent and replaced by TAG (Gu *et al*., 2007).

The complete mitochondrial genomes of Madura cattle showed gene content and organization generally comparable to those of other Bovidae species. The difference in sequence length is due to the presence of a tandem repeat motif in the control region (D-loop) with the motif 5′-ATA AGT ACA TAA TAT TTA TGT A-3′ in samples Ma2 and Ma3, which was not found in samples Ma1, Ma4, and Ma5. The repeat motif in Ma2 was duplicated 6 times, whereas in Ma3 it was duplicated only 3 times. The same repeat motif was also found in *B. javanicus* (Anwar *et al*., 2025; Jakaria *et al*., 2025). This motif was found only in the species *B. javanicus.* Therefore, it has great potential to serve as a marker for *B. javanicus* (Jakaria *et al*., 2025). To date, the function of repetitive motifs in the D-loop region remains poorly understood. However, it is hypothesized that these motifs act as an independent regulatory region that may enhance the efficiency of mtDNA replication and transcription, providing an adaptive advantage through greater flexibility in regulating energy production and cellular metabolism (Kamalakkannan *et al*., 2020).

The nucleotide composition of the PCGs shows that 60.08% consists of adenine (A) and Thymine/uracil (T/U). Translation results indicate that the amino acid sequence of Madura cattle is generally similar to that of *B. indicus,* except for the ND3 gene, which is identical to that of *B. javanicus.* Two amino acid mutation sites were found to be unique to Madura cattle, consisting of a change in the ATP8 and ND5 genes from the amino acid phenylalanine (F) to leucine (L) at position 24, and a mutation from the amino acid valine (V) to isoleucine (I) at position 172.

The AT-skew values tend to be positive, and the GC-skew values tend to be negative, indicating a pattern of base composition asymmetry that reflects a nucleotide composition bias toward the bases adenine(A) and cytosine (C). This is consistent with the general characteristics of mtDNA genomes in mammalian groups, including the Bovidae family (Priyono *et al*., 2020). At the gene level, AT-skew values vary among genes. In Madura cattle, the COX1, COX3, and ND6 genes exhibit negative AT-skew values, whereas the genes encoding the proteins (ND1, ND2, COX2, ATP8, ATP6, ND3, ND4L, ND4, ND5, and cytB) have positive values. The same pattern has been reported in *B. javanicus* (Anwar *et al*., 2025), *B. indicus* (Arya *et al*., 2025), *Moschiola indica* (Sarvani *et al*., 2018), and *Bubalus bubalis* (Kumar De *et al*., 2019). However, in *Bubalus quarlesi*, the AT-skew pattern exhibits a distinct characteristic; a negative AT-skew value was detected only in the ND6 gene, while other protein-coding genes maintained a positive AT-skew (Priyono *et al*., 2020). These pattern differences indicate lineage-specific variations in mitochondrial nucleotide composition bias among Bovidae, which likely reflect differences in historical mutation pressure, mtDNA replication dynamics, or distinct maternal evolutionary backgrounds between the genera (Kamalakkannan *et al*., 2020).

The RSCU pattern in Madura cattle indicates unequal usage of synonymous codons in mitochondrial protein-coding genes. Several codons with relatively high RSCU values ended with adenine, including CGA, CTA, and GTA, suggesting a tendency toward A-ending codon preference. A similar pattern was reported in Bali cattle (*B. javanicus*), where the codons with the highest RSCU values were CUA/CTA (Leu, 1.92), GUA/GTA (Val, 1.75), GGA (Gly, 1.64), and GCA (Ala, 1.54), and codons with adenine at the third codon position were preferentially used (Jakaria *et al*., 2025). Therefore, the RSCU pattern of Madura cattle is generally consistent with the A-ending codon preference reported in Bali cattle, although the dominant codons were not identical. The frequent representation of leucine and serine in Madura cattle also agrees with the general amino acid usage pattern commonly observed in bovine mitochondrial protein-coding genes. In Madura cattle, leucine and isoleucine were the two most frequent amino acids, with 532 and 481 codons, respectively. Arya *et al*. (2025) also analyzed RSCU patterns and selection pressure in the complete mitochondrial genome of Badri cattle (*B. indicus*) and reported that mitochondrial protein-coding genes were mainly shaped by purifying selection. This suggests that codon usage variation in bovine mitogenomes may be influenced not only by nucleotide composition bias, but also by functional constraints related to mitochondrial protein translation (Parvathy *et al*., 2022).

A total of 22 tRNA genes were identified with a total length of 1515 bp. The tRNA anticodons found correspond to those commonly reported in vertebrates (Jakaria *et al*., 2025). Based on tRNA structure visualization, the Madura cattle tRNA forms a cloverleaf secondary structure consisting of an acceptor stem,

D-arm, anticodon arm, TΨC arm, and variable *loop.* Several mitochondrial tRNAs show deviations from this structure, namely trnS1 (tRNA-Ser1) and trnK (tRNA-Lys) (Figure 4). The structure of trnS1 and trnK differs from other tRNAs due to the loss or instability of the loop on the dihydrouridine (DHU) arm (Watanabe *et al*., 2014). These findings are consistent with the characteristics of tRNA in vertebrates; the cloverleaf structure and deviations in trnS1 and trnK were also found in *Bos gaurus* (Kamalakkannan *et al*., 2020), *B. javanicus* (Jakaria *et al*., 2025), and *B. taurus* (Estrada *et al*., 2024).

Phylogenetic reconstruction and genetic distance results using the full mitochondrial genome showed that some Madura cattle samples clustered with *B. indicus,* while others clustered with *B. javanicus.* This topology indicates the presence of distinct mitochondrial lineages within the analyzed Madura cattle samples, reflecting a complex maternal background related to both *B. indicus* and *B. javanicus* lineages. This clustering pattern is also consistent with the theory of mitochondrial genome inheritance, which is passed down exclusively through the maternal line (Ladoukakis & Zouros, 2017). The *B. indicus*-related lineage may be associated with the historical dissemination of indicine cattle across Asia through trade, migration, and livestock management practices (Pitt *et al*., 2019), whereas the *B. javanicus*-related lineage may reflect maternal introgression from banteng-derived cattle populations (Wang *et al*., 2026). Because mitochondrial DNA is maternally inherited, these findings describe the maternal history of Madura cattle and should be complemented by nuclear genomic data to resolve genome-wide ancestry and admixture patterns further.

CONCLUSION

The complete mitochondrial genome of Madura cattle was successfully characterized using Oxford Nanopore Technology, revealing the typical bovine mitochondrial organization and mitochondrial variation in the D-loop and protein-coding regions. Genetic distance and phylogenetic analysis consistently identified two maternal lineages, with one closely related to *Bos javanicus* and the other to *Bos indicus*. This finding provides baseline information on the maternal genetic diversity of Madura cattle and may support future genetic characterization and conservation. Further studies using broader sampling and nuclear genomic data are needed.

CONFLICT OF INTEREST

J. Jakaria, an associate editor at the Tropical Animal Science Journal, was not involved in the decision-making process for this publication. The authors also declare no conflicts of interest related to this study.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

While preparing this work, the authors utilized Grammarly to enhance the writing structure and grammar. Following this, we reviewed and edited the content as necessary and assumed full responsibility for the published material.

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