BMC Veterinary Research

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# *De-novo* assembled mitochondrial genome of Bhadawari buffalo (*Bubalus bubalis*) reveals close divergence to Egyptian buffalo

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## Abstract

**Background** The objective of this study was to assemble the mitochondrial genome of Bhadawari buffalo and do phylogenetic analysis of it. We assembled the complete mitochondrial sequence of Bhadawari buffalo *de novo* from short Illumina sequences generated from the paired-end library. Phylogenetic analysis was done on 24 assembled mitochondrial genomes from the Bovidae family using the Maximum Likelihood method and General Time Reversible Substitution Model.

**Results** The complete circularized mitochondrial assembly of Bhadawari consists of 16,358 bp and this encodes 36 genes (13 Protein coding 22 tRNA and 2 rRNA sequences). The phylogenetic analysis with other published mitochondrial assemblies revealed a divergence time of Bubalis and Syncerus from the Bos group about 6.4 million years from today. The divergence of Syncerus-Bubalis, Mediterranean occurred at about 4.17 MYA and 1.84 MYA respectively. The Indian and Egyptian buffaloes were grouped in a subclade while Chinese and Iraqi buffaloes were in another. The Indonesian and Indian buffaloes diverged from the Chinese subclade by about 0.72 MYA.

**Conclusions** The study suggests an independent selection and improvement of the Asiatic buffaloes in different geographical locations 10,000–40,000 years from today which coincides with the domestication period of buffaloes. The Bhadawari and Egyptian water buffalo revealed a more recent divergence.

**Keywords** Bhadawari Buffalo, Mitochondrial genome, *De Novo* genome assembly, Phylogeny, Divergence time

## Background

Mitochondria are organelles involved in many cellular activities including energy metabolism, apoptosis, physiological cell signalling, reactive oxygen species production etc [1]. Compared to nuclear genome, mitochondrial genome is more in number in the cells. Mitochondria varies in number of base pairs, number and function of genes present in them among different living organisms. The origin of mitochondria is explained by various theories and its divergence among species created a difference in genes present, expression of genes and various cellular mechanisms influenced by them [2]. The type of organ from which the sample was taken can affect the expression of the mitochondrial genome in addition to species [3]. Other than isolating the mitochondrial genome separately, the next-generation sequencing techniques have now made it possible to extract the mitogenome from the high-quality WGS data [4].

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The mitochondria of many buffalo species have been assembled and annotated till date. The Water buffalo mitochondrial genome has been reported by merging three water buffalo mitochondrial sequences using sequences of the PCR amplified product from the same [5]. To uncover the amino acid changes indicating evolutionary divergence between these species, they compared this protein's coding genes with those of other species to find a divergence between cow and water buffalo before 20 million years. In another study the mitochondrial genome of 29 Egyptian river buffaloes were sequenced and assembled separately using the mitochondrial reference genome of Bubalus bubalis from gene bank [6]. The 29 mitogenomes were grouped as 24 haplotypes and haplotype diversity was analysed to find the evolution of Egyptian buffalo from multiple migrants rather than single unique species.

Bubalus genus is a member of the Bovidae family which thought to have originated from the wild Buffaloes (*Bubalus arnee*) [7] which is now in endangered category [8]. The river buffaloes later on spread from Asia to wide geographic locations including Greece, Italy, (Mediterranean) and Egypt (Egyptian) [9]. The buffalo population is widely distributed in Asian countries with India harbouring a large diversity of 20 breeds. Chinese buffaloes have a suggested origin from South Asia and India [10]. The Indonesian Anoa buffaloes with species *Bubalus depressicornis* and *Bubalus quarlesi* [11]. The African buffaloes are said to originate in the Central Africa and evolving there to different forms like forest buffaloes, savannah buffaloe etc [12]. The molecular evolutionary studies conducted in buffalo genomes till date are mostly based on mitochondrial D-loop owing to its higher mutation rate [13, 14] and autosomal microsatellites [15]. Recent studies have even used already available whole genome NGS data from public platforms to assemble the Cape buffalo mitogenome assembly for estimating the divergence of its lineage [16]. Another approach in the swamp buffalo from Kalinga Province, Philippines was to generate high-quality PAC Bio data from scratch for developing a de novo genome assembly [17]. The comparison studies using *de novo* mitochondrial assemblies generated from whole genome sequenced data are rare.

Bhadawari is one of India's native buffalo breeds which is found in Madhya Pradesh (Bhind and Morena districts) as well as the Uttar Pradesh (Agra and Etawah districts) [18]. It is renowned for its exceptional capacity to produce milk with a high fat content, copper-colored body, and white ring on the side of the neck. This buffalo's population is in decline and must be conserved. We assembled Bhadawari buffalo mitochondrial genome *de novo* from Illumina short read data of 45X coverage. The Bhadawari buffalo mitochondria of 16, 358 bp was annotated and found to contain genes coding for 2 rRNA, 22 tRNA and 12 protein coding genes. The phylogenetic relations between Bhadawari buffalo mitochondrial genome and other available buffalo genomes were also analysed.

## Materials and methods

### Ethics statement

All the procedures in the present experiment adhered to the ethical standards set by the Institutional Animal Ethics Committee (IAEC) at NDRI, Karnal, Haryana, India (Reg. No. 1705/GO/Re/SL/13/CPCSEA). The authors confirm that animal care and use complied with the relevant standard operating protocols established by the IAEC to ensure the protection of animals for scientific purposes.

### Blood collection, DNA extraction

Blood samples were collected from the field (Etawah district of Uttar Pradesh, which is the breeding tract of the animal) from a female Bhadawari buffalo with the consent of the farmer under the supervision of a trained veterinarian. The chosen animal had medium body size, copper coloured body, as per breed descriptions given by National Bureau of Animal Genetic Resources (NBAGR), the national nodal agency for breed description. The animal was selected as per NBAGR description [19].

Genomic DNA was isolated using Phenol Chloroform isolation method [20].

### Whole genome sequencing

The DNA sample with good quantity and purity was selected for sequencing. The DNA was sequenced with Illumina HighSeq technology with a coverage of 54 X. Sequenced data was obtained as 150 bp paired end data. Data was subjected to quality control using FastQC version 0.11.9. The quality-controlled adapter free data was proceeded for further analysis.

### De novo assembly of mitochondria, annotation

The initial seed generation for *de novo* mitochondrial assembly was done using NORGAL (de Novo ORGAneLe extractor) which mines the mitochondrial genome from the WGS data by performing a cycle of *de novo* assembly and selection of longest high- depth contig [21]. From all the seeds generated by NORGAL, the best hit was selected based on length and was used seed extension and assembly of Bhadawari mitochondrial genome using NOVOPLASTY [22].

### Annotation of mitochondrial genome assembly

The GE-Seq tool on MPI-MP CHLOROBX website (https://chlorobox.mpimp-golm.mpg.de) was used for the genome annotation. The threshold for protein search identity was 55%, and that of rRNA, tRNA, and DNA search identity was 85%. Mitochondrial circular map was

drawn using Organellar Genome DRAW (OGDRAW) (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html).

### Phylogeny

Assembled mitochondrial genome of 26 representative population including 13 *Bubalus bubalis* (Water buffalo and Swamp buffalo), 2 *Bubalis arnee*, 3 *Syncerus caffer* (Cape buffalo), 1 *Bubalus carabanensis*, 1 *Bubalus quarlesi*, 1 *Bubalus depressicornis*, 2 Bos (Indigenous and taurine cattle), 1 *Bison bison*, 1 *Capra hircus* and 1 *Ovis aries* were downloaded from NCBI and the sequences were aligned using Multiple Alignment using Fast Fourier transform MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/). The maximum likelihood Phylogenetic tree was constructed using MEGA 11 [23] with General Time reversible Model [24] with gamma distribution algorithm using bootstrapping value of 1000. The *Capra hircus* was set as an outgroup to find the divergence time using the *Bos indicus* and *Bos taurus* median divergence time by RelTime method [25].

## Results

### Data and mitochondrial genome assembly

About 175 Gb raw data was obtained after Illumina short read sequencing with 488 million reads. Adapter sequences were removed and after quality control 156 Gb data was used for seed generation. 146 seeds were generated by NORGAL. The depth comparison and seed output are shown in Fig. 1.

The best hit with 97.4% Identity and a length of 3,277 bp was used as seed for NOVOPLASTY. The Bhadawari buffalo genome was assembled to 16,358 bp in length, with a base composition of: A:35%, T:26.4%, G:13.8% and C: 26.5%. The GC content is found to be 40.5%.

### Mitochondrial genome annotation

This genome was found to encode 37 genes including 13 Protein coding genes, 22 tRNA and 2 rRNA sequences. Circularized Bhadawari assembly is annotated (Fig. 2). The predicted genes are shown in Table 1. Out of the 13 protein coding genes in the Bhadawari buffalo genome, seven are NADH dehydrogenase subunits, two are ATPase subunits, three are Cytocrome Oxidase subunits and one is Cytochrome B. The gene coding NADH dehydrogenase vary in length from 1821 bp (NADH5) to 297 bp in NADH4 in this genome assembly. Smallest is found to be the ATPase coding gene with 201 bp length. The tRNA coding length ranged from 60 bp in tRNA -Ser to 75 bp in tRNA Leu. The gene coding 16 S rRNA found to be 1569 bp and 12 S rRNA is 957 bp.

Upon BLAST search against the other major publicly available *Bubalus bubalis* mitochondrial sequences showed, 97.52% identity with Murrah breed, 99.94% identity with Nili-Ravi breed, 99.97% identity with Egyptian isolate 99.88% with Mediterranean breed. The percentage identity to *Bubalus arnee* was 99.87% from Assam isolate, 99.84% was with Chhattisgarh isolate. The percentage of identity to the *Bubalus depressicornis* mitochondrion was 97.4%.

### Phylogenetic analysis of mitochondrial genome of *Bhadawari* buffalo

In the maximum likelihood based phylogenetic analysis of the selected breeds, the phylogenetic tree (Fig. 3) showed two main clades one with Bovinae family (Cattle, Bison and Buffalo) the other with the Ovinae. Bovinae again subdivided to Bos, Bison and Bubalis. The clade which included the buffalo breeds was divided into different sub clades and each subclade divided from nodes to form branches containing the *Syncerus caffer* group,

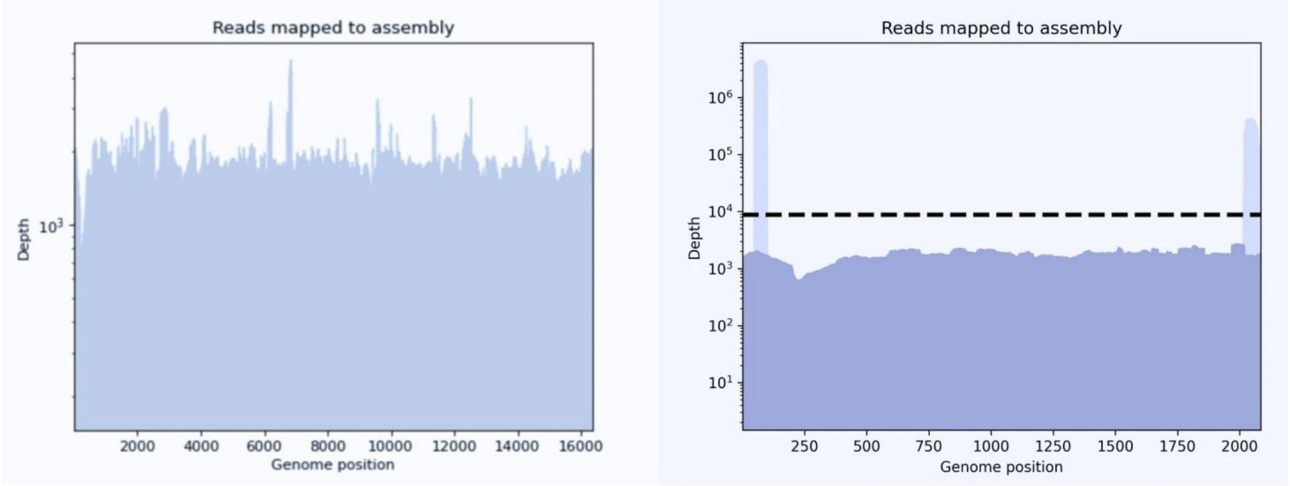


**Fig. 1** Depth Comparison: Detailed comparison of the mitochondrial genome's subsequence (light grey) and the nuclear sequence (dark grey). The nuclear depth threshold (ND threshold), which corresponds to the 99.8% of the nuclear depths (light grey), is shown as a dashed line

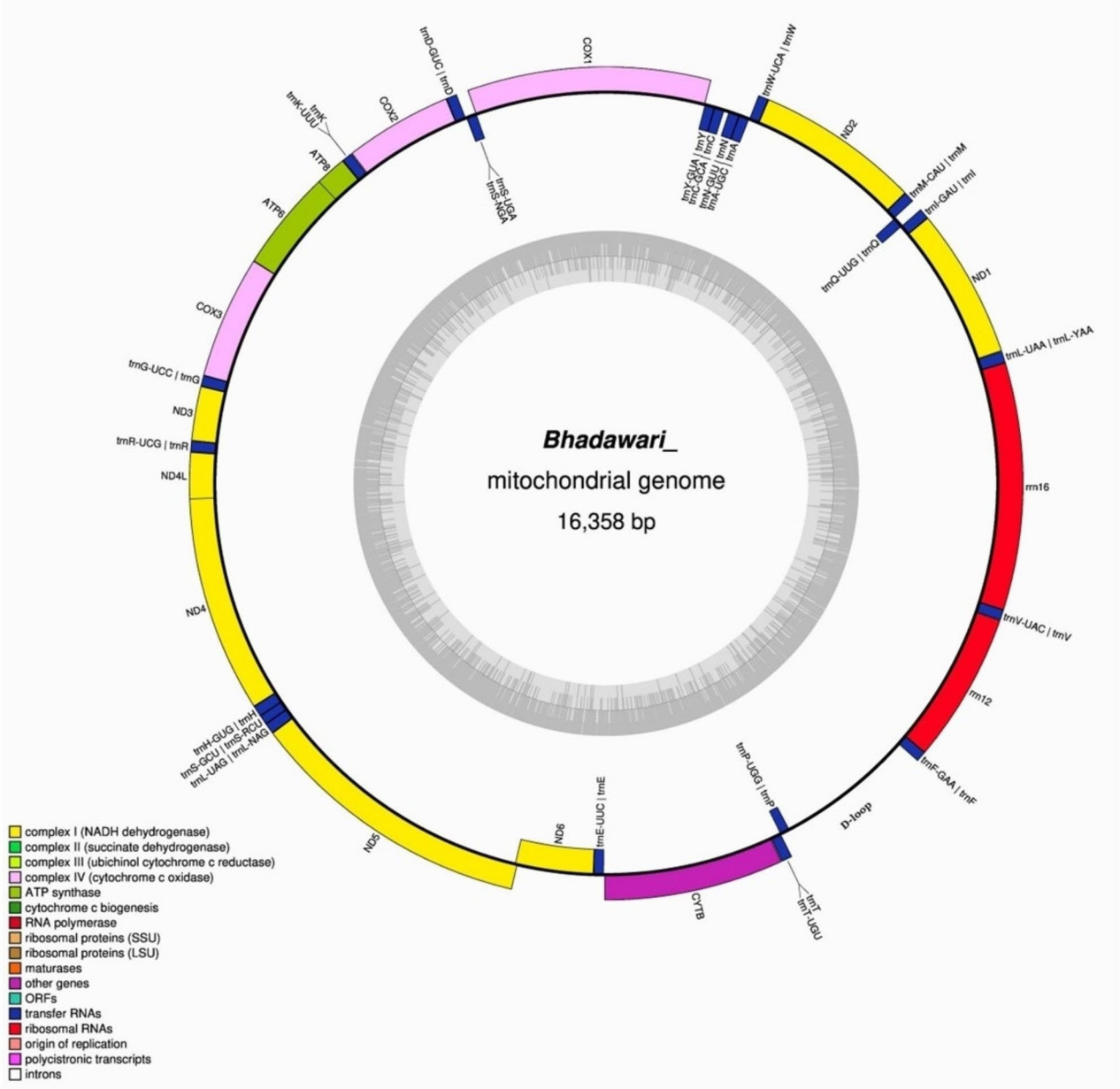


**Fig. 2** Mitochondrial genome annotation of Bhadawari buffalo (*Bubalus bubalis*)

Mediterranean buffalo, Indian and Egyptian buffalo group, and other including a large subclade with rest of the breeds. This larger subclade again diverged to two branches with one containing Chinese breeds (Gangxi, Fuzong), breed from Iraq (Basrah) and Indonesian buffaloes (*Quarlesi* and *depressicornis*). The Indian buffaloes including Murrah breed diverged from a single branch.

## Discussion

Even after the introduction of economical sequencing technologies and genomic evaluation tools, many indigenous breeds like the Bhadawari breed remained out of focus for the scientific community which is leading these breeds to an endangered status in the near future. Even though the fact that the mitochondrial genome assemblies have limitations due to maternal inheritance, substitution rates, and genome rearrangements [26, 27] the phylogeny results of this study could be validated with nuclear markers of the breed as future steps. The uniqueness and historic value could give the breed more significance in terms of conservation and inclusion in future breeding programs. However, the results are based on one head of sample from Bhadawari buffalo (threatened breed buffalo) combining this sample with others.

The size of the mitochondrial genome assembly of Bhadawari buffalo is found to be like the mitochondrial

**Table 1** Gene profile in Bhadawari Buffalo mitochondrial genome. H and L represent heavy and light strand respectively

| Name of the gene | Strand | Start | End | Size |
|---|---|---|---|---|
| tRNA-Leu-YAA | H | 766 | 840 | 74 |
| NADH dehydrogenase subunit 1(*ND1*) | H | 843 | 1798 | 955 |
| tRNA-Ile-I | H | 1799 | 1867 | 68 |
| tRNA-Q-Gln | L | 1865 | 1936 | 71 |
| tRNA-M-Met | H | 1939 | 2007 | 68 |
| NADH dehydrogenase subunit 2(*ND2*) | H | 2008 | 3049 | 1041 |
| tRNA-W-Trp | H | 3050 | 3116 | 66 |
| tRNA-Ala | L | 3118 | 3186 | 68 |
| tRNA-N-Asn | L | 3188 | 3260 | 72 |
| tRNA-C-Cys | L | 3293 | 3359 | 66 |
| tRNA-Y-Tyr | L | 3360 | 3426 | 66 |
| Cytocrome oxidase subunit I (*COX1*) | H | 3428 | 4972 | 1544 |
| tRNA-S-Ser-NGA | L | 4970 | 5040 | 70 |
| tRNA-D-Asp | H | 5046 | 5114 | 68 |
| Cytocrome oxidase subunit II (*COX2*) | H | 5116 | 5799 | 683 |
| tRNA-K-Lys | H | 5803 | 5869 | 66 |
| ATPase subunit 8(*ATP8*) | H | 5872 | 6072 | 200 |
| ATPase subunit 6(*ATP6*) | H | 6033 | 6713 | 680 |
| Cytocrome oxidase subunit III(*COX3*) | H | 6713 | 7496 | 783 |
| tRNA-G-Gly | H | 7497 | 7565 | 68 |
| NADH dehydrogenase subunit 3 (*ND3*) | H | 7566 | 7911 | 345 |
| tRNA-R-Arg | H | 7913 | 7981 | 68 |
| NADH dehydrogenase subunit 4 L(*ND4L*) | H | 7983 | 8278 | 295 |
| NADH dehydrogenase subunit (*ND4*) | H | 8272 | 9649 | 1377 |
| tRNA-H-His | H | 9650 | 9720 | 70 |
| tRNA-S-Serine-RCU | H | 9721 | 9780 | 59 |
| tRNA-L-Leu-NAG | H | 9781 | 9851 | 70 |
| NADH dehydrogenase subunit 5 (*ND5*) | H | 9852 | 11,672 | 1820 |
| NADH dehydrogenase subunit 6 (*ND6*) | L | 11,656 | 12,183 | 527 |
| tRNA-E-Glu | L | 12,184 | 12,252 | 68 |
| Cytochrome B | H | 12,257 | 13,396 | 1139 |
| tRNA-T-Thr | H | 13,400 | 13,469 | 69 |
| tRNA-P-Pro | L | 13,469 | 13,534 | 65 |
| tRNA-F-Phe | H | 14,462 | 14,530 | 68 |
| 12 S rRNA | H | 14,531 | 15,485 | 954 |
| tRNA-V-Val | H | 15,488 | 15,554 | 66 |
| 16 S rRNA | H | 15,555 | 765 | 1568 |

genome assembled from water buffalo in other studies – 16,355 bp [5], Egyptian water buffalo- 16,359 bp [28], South African cape buffaloes (Syncerus *caffer*) – 16357-16,362 bp [29]. This conservation in the mitochondrial genome size can be seen in other large ruminants including Bos taurus with 16, 338 bp and Bos indicus 16,339 bp [30]. Indian gaur (*Bos gaurus*) also has an assembled mitochondrial size of 16,345 bp with a comparable GC content of 39.3% [31] and Mithun (*Bos frontalis*) with 16,346 bp total length and GC content of 39.3% [32]. This nucleotide bias towards AT has already been confirmed in other related taxa including cattle [27].

In the phylogenetic analysis, the Bovinae clade, which encompasses Cattle, Bison, and Buffalo, exhibits additional subdivisions and diversification among buffalo breeds, indicating distinct evolutionary events. This is indicative of the fact that the indigenous buffalo breeds of India might have undergone separate selection and improvement in different geographic locations. The analysis grouped Bhadawari breed and the Egyptian buffalo in single branch while *Bubalus arnee* isolates from Chhattisgarh and Assam, NiliRavi and Murrah buffalo breeds were placed in a separate branch in this group which diverged at different point of time to specific breed groups.

The divergence time was estimated by keeping the divergence between Bos indicus and Bos taurus of 0.469 MYA (0.1–0.8 MYA) [33–35]. From the tree (Fig. 4), the divergence of Bubalis and Bos occurred about 6.40 MYA. This comes in between the value range 4.93 million years

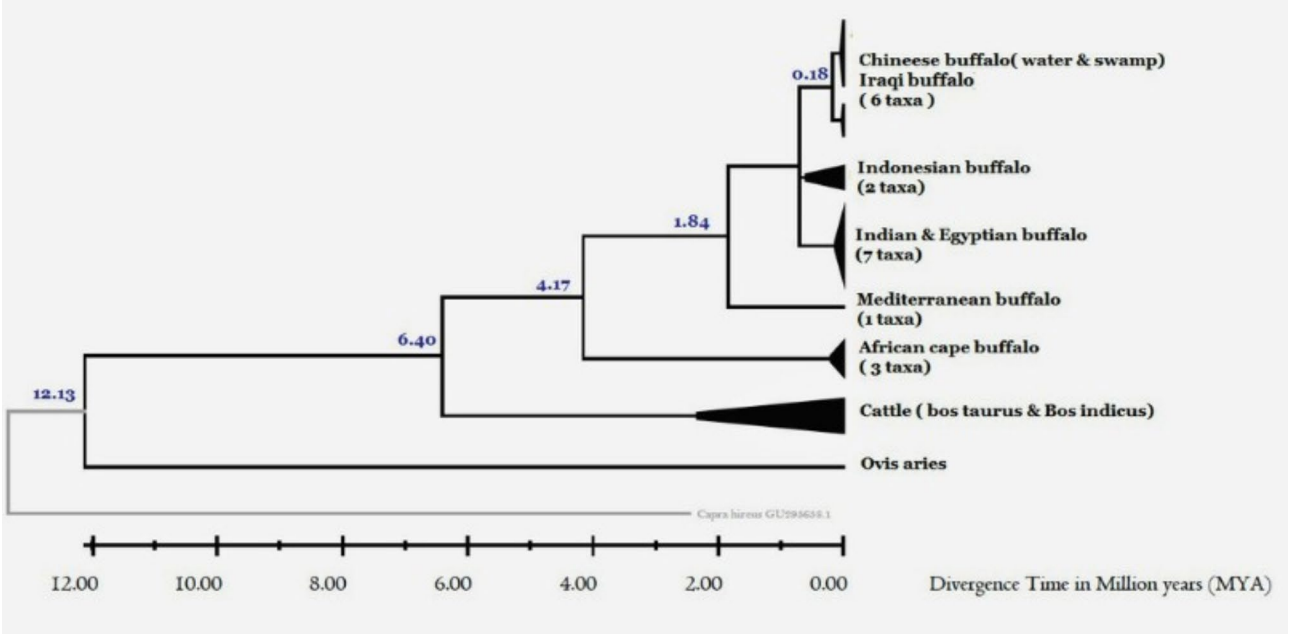


**Fig. 3** The timescale phylogenetic tree after collapsing nodes of cluster size 7. Divergent time are expresses in million years before each node with precision to 2 decimal places. *Capra hircus* is considered as the outgroup

from analysing the phylogenetic relationship of Bovini using microsatellite loci [36] and time period of 7.4 million years calculated using whole genome data of Bangladeshi river buffalo [37].

In the same subclade of Bos group, the divergence oof Bison from bos occurred 2.36 MYA. This time period is similar to that mentioned in the phylogenetic analysis conducted in a previous study using biogeographic inferences and molecular datings [38]. The African buffalo (*Syncerus caffer*) diverged 4.17 MYA. The divergence time between Mediterranean water buffalo and other water buffalo breeds was calculated to be 1.84 million years from today using the maximum likelihood tree. The Indian buffaloes, Indonesian buffaloes and Egyptian buffaloes diverged from the other Asian breeds considered here about 0.72 million years ago, including those from China and Iraq. This includes the separation of Swamp buffaloes and water buffaloes and their evolution in different geographical areas. This time period coincides with suggested divergence time range (10,000 to 1.7 MYA) of River and Swamp buffaloes [36, 39]. This time period can be considered as the divergence time of Asiatic buffaloes and their evolution in different geographic zones as suggested in range of 900 – 860 kyr in some whole mitogenome studies [40]. The divergence time between Murrah and other breeds was in range 10,000–40,000 years as per the tree. Interestingly the Egyptian buffalo also showed a divergence time in this range. The Indian buffaloes later continued evolution and selection in different geographical regions to form distinct breeds as seen today which is comparatively recent as suggested from other mitochondrial studies [41]. This roughly coincides with the domestication events of water buffaloes which supposed to have happened 6300 years before present somewhere in northwestern India [42]. The historical evidences suggest the migration of Indian riverine buffaloes across Asia to Egypt and then to Italy [43, 44].

Our findings support the concept that buffaloes diverged and dispersed around the globe, followed by gradual selection and development to form distinct breeds as observed today. The Mediterranean buffalo is found to show a far ancient divergence in comparison to other breeds. The divergence of the Chinese-Iraqi group, the Indonesian Mountain and Lowland anoa group, and the Indian-Egyptian group nearly coincided, and they later evolved and gained distinctive phenotypic and genotypic characteristics based on demographic situations. The inclusion of newly assembled Bhadawari buffalo and Egyptian buffaloes on a single branch of the phylogenetic tree suggests that Egyptian and Indian buffaloes diverged more recently than other Asian classes such as those from China, Indonesia, and Iraq, as well as those from the Mediterranean. Later on, domestication leading to selection and breeding for specific purposes must have generated well defined breed specific traits as of the present time.

## Conclusions

The first complete de-novo mitochondrial genome assembly of Bubalus bubalis (Bhadawari) comprising 16,358 bp size and encoding 36 genes is presented here. The phylogenetic analysis using 24 mitochondrial

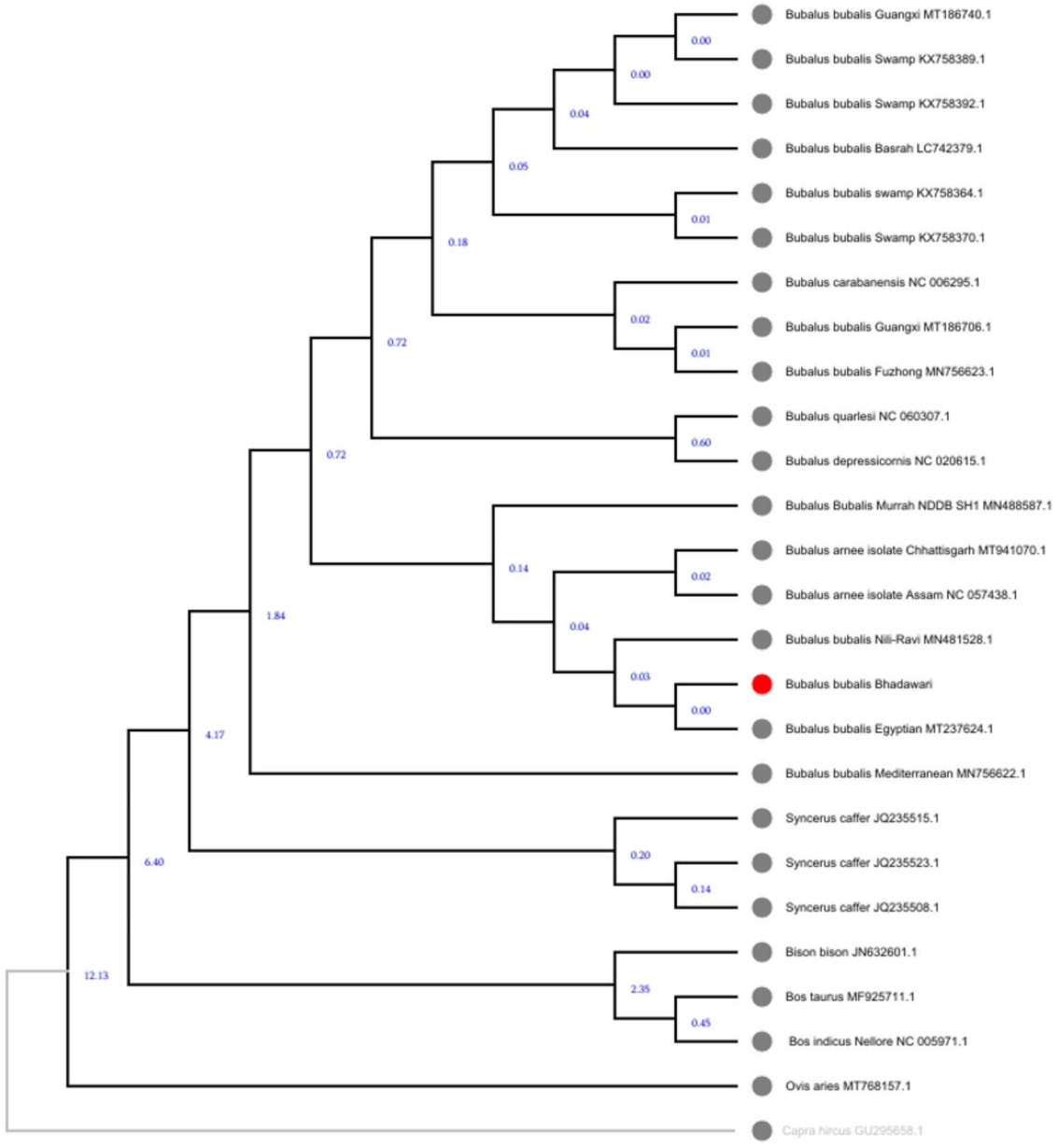


**Fig. 4** Time tree calculated using Reltime method. The node with red diamond represents the taxons (*Bos taurus* and *Bos indicus*) selected for calibration of time. The divergence time is expressed by figures on the middle of the node. *Capra hircus* was selected as the out group The divergence time is expressed by the figures on the branch

assemblies from Bovidae family including the newly assembled Bhadawari mitochondrial genome was suggestive of the divergent time between different buffalo types/breeds. This investigation grouped Chinese- Iraqi buffaloes and Indian -Egyptian buffaloes to one subclade. The results of this study especially the time range of divergence is in compromise with previous molecular and microsatellite studies involving buffalo mitochondria. As per the current phylogenetic study using 24 mitogenome assemblies, the newly assembled Bhadawari buffalo mitochondria upon divergence analysis shared a recent divergence pattern compared to other breeds.

**Acronyms**

| | |
|---|---|
| MYA | Million Years Ago |
| GC | Guanine-Cytocine |
| AT | Adenine-Thymine |
| tRNA | Transfer RNA |
| rRNA | Ribosomal RNA |
| NADH | Nicotinamide adenine dinucleotide (NAD) + hydrogen |
| BLAST | Basic Local Alignment Search Tool |
| NORGAL | De Novo ORGAneLle extractor) |
| Gb | Giga base |
| MEGA | Molecular Evolutionary Genetics Analysis |

| | |
|---|---|
| MAFFT | Multiple Alignment using Fast Fourier Transform |
| NCBI | National Center for Biotechnology Information |
| OGDRAW | OrganellarGenomeDRAW |
| GE-Seq | Gene Expression Sequencing |
| MPI-MP CHLOROBX | Max Planck Institute of Molecular Plant Physiology (MPI-MP) CHLOROBOX |
| NORGAL | De Novo ORGAneLle extractor |
| IAEC | Institutional Animal Ethics Committee |
| KYA | Kilo years ago |
| WGS | Whole genome sequencing |

**Acknowledgements**
We thank the Director, ICAR-NDRI, Karnal and Director, ICAR-CIRB, Hisar, for providinglogistic support and funds for the completion of the research work.

**Author contributions**
AS: Writing—initial draft, Methodology, Formal analysis, Software. VV: Conceptualization, Project administration, Funding acquisition, Writing, review and editing, Formal analysis. RKG: Methodology, Software. RA: review & editing. GG: review & editing. The final manuscript was read and approved by all the authors.

**Funding**
This work was supported by grants from ICAR-NPBI, Hisar, India (F.No: 2–4/PC (B/BUDG./24).

**Data availability**
All data generated or analyzed during this study are included in the manuscript.

## Declarations

**Conflict of interest**
The authors declare no conflict of interests.

**Competing interests**
The authors declare no competing interests.

**Ethics approval**
All the procedures in the present experiment adhered to the ethical standards set by the Institutional Animal Ethics Committee (IAEC) at NDRI, Karnal, Haryana, India (Reg. No. 1705/GO/Re/SL/13/CPCSEA). The authors confirm that animal care and use complied with the relevant standard operating protocols established by the IAEC to ensure the protection of animals for scientific purposes.

**Consent for publication**
All authors give their consent for the publication of this manuscript.

Received: 5 December 2024 / Accepted: 17 March 2025
Published online: 25 March 2025

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