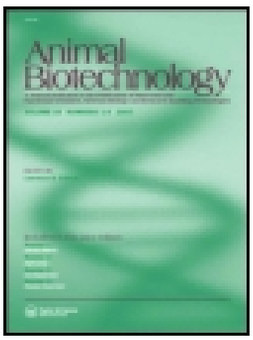

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ORIGINAL ARTICLE

Complete mitogenome reveals genetic divergence and phylogenetic relationships among Indian cattle (*Bos indicus*) breeds

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ABSTRACT

Indigenous cattle of India belong to the species, *Bos indicus* and they possess various adaptability and production traits. However, little is known about the genetic diversity and origin of these breeds. To investigate the status, we sequenced and analyzed the whole mitochondrial DNA (mtDNA) of seven Indian cattle breeds. In total, 49 single-nucleotide variants (SNVs) were identified among the seven breeds analyzed. We observed a common synonymous SNV in the COII gene (m.7583G > A) of all the breeds studied. The phylogenetic analysis and genetic distance estimation showed the close genetic relationship among the Indian cattle breeds, whereas distinct genetic differences were observed between *Bos indicus* and *Bos taurus* cattle. Our results indicate a common ancestor for European Zwergzebu breed and South Indian cattle. The estimated divergence time demonstrated that the *Bos indicus* and *Bos taurus* cattle lineages diverged 0.92 million years ago. Our study also demonstrates that ancestors of present zebu breeds originated in South and North India separately ~30,000 to 20,000 years ago. In conclusion, the identified genetic variants and results of the phylogenetic analysis may provide baseline information to develop appropriate strategies for management and conservation of Indian cattle breeds.

KEYWORDS

Bos indicus; *Bos taurus*; genetic divergence; mitochondrial DNA; single-nucleotide variant

Introduction

Cattle were the most important species among the 14 large wild terrestrial species fulfilled the conditions for successful domestication. Archeological evidence, historic documents and genetic analysis have recorded the domestication of cattle and their consequent spread over the world, as well as later migrations. Domestication of cattle occurred autonomously in the Near East and the Indian subcontinent between 10,000 and 8,000 years prior, gave rise to the two major domestic taxa viewed today, humpless *Bos taurus* (taurine) and humped *Bos indicus* (zebu), respectively.[1,2] Afterward, cattle accompanied human migrations, which advanced the dissemination of domestic cattle of taurine, indicine, or mixed origin over Asia, Africa, Europe and the New World.[3]

Archeological findings of the earliest European Neolithic ranches suggest that European taurine cattle have been brought into Europe as a result of the arrival of early pastoralists from the Near East through the Mediterranean coasts and along the Danube.[4] Mitochondrial DNA (mtDNA) sequence can be a viable tool to the establishment of the sites of domestication. A hotspot of zebu cattle mtDNA diversity (haplogroups I1 and I2) in the Indian peninsula with respect to the encompassing regions denotes domestication of *Bos indicus* happened in India, followed by migrations toward Southwest Asia, China and Southeast Asia.[5,6] Similarly, *Bos taurus* has the highest diversity in Southwest Asia, with four different mtDNA haplogroups (T, T1, T2 and T3).[7,8] Migrations in other continents frequently brought about contact among taurine and zebu cattle, which helped to the appearance of hybrid breeds of southwest Africa,[1,9] Asia,[10] China[11,12] and, much later, the Americas.[13,14]

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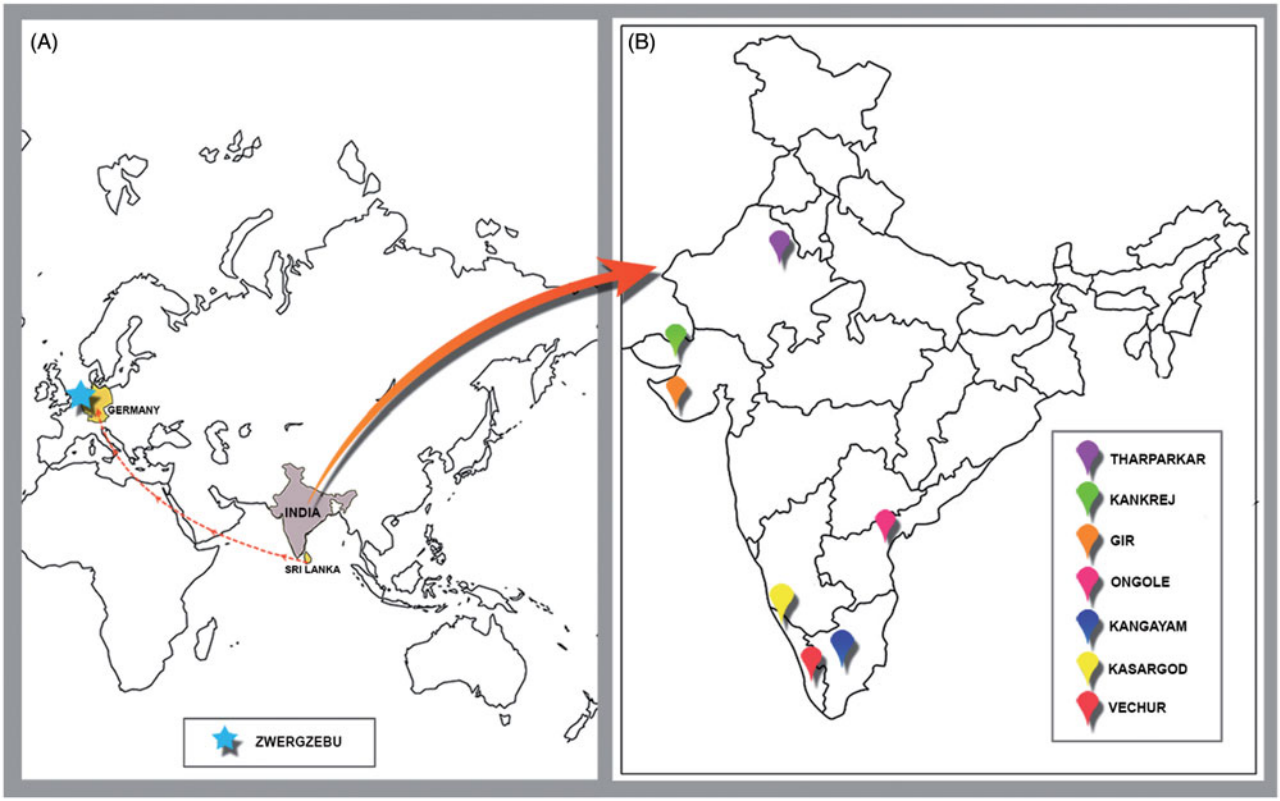

Figure 1. Location of the reference *Bos indicus* cattle, Zwergzebu and the Indian *Bos indicus* breeds used for the collection of samples in the present study. (A) The part of world map shows location of Zwergzebu. This breed was founded in Germany and is believed to have originated from zebu cattle of Sri Lanka. (B) The map of India displays respective breeding tracts of the cattle breeds used for the blood collection.

Table 1. Primer sequences used for the amplification of complete mitochondrial genome of Indian cattle.

Sl. No	Primer name	Sequence (5′-3′)	Start position	End position	Annealing temperature (°C)
1	BMito F1	GTTGATGTAGCTTAACCCAA	1	20	51
2	BMito R1	AGAAGGTGGACCCAATGATG	9249	9230	55
3	BMito F2	ATCGGAGGAGCTACACTTGC	8446	8465	57
4	BMito R2	GGGGCCTGCGTTTATATATTG	16330	16310	57

Fossil remnants of zebu cattle have been discovered from Mehrgarh region, a proto-Indus civilization site in Baluchistan in southwest Pakistan and were dated at 8000 years before present (YBP).[15] The faunal remains at Mehrgarh I, along with a figurine from Mehrgarh II[16] demonstrated the earliest evidence for a zebu breed. Proof gathered from the archaeological sites of Harappa and Mohenjo-daro shows that domestic zebu was common throughout the Indus Valley region ~5000 YBP.[17,18] Recent mtDNA studies indicate that, presumably the Indus Valley was the center of origin for the mtDNA I1 haplogroup and a primary center of zebu domestication.[6] Morphological contrasts between cattle illustrated in the rock art of South India and iconography of Indus Valley civilizations,[19] presence of peculiar cattle-oriented Neolithic culture and zooarchaeological data[20] propose that South India was a secondary center for zebu domestication. The frequency and dissemination of the I2 haplogroup within Uttar Pradesh and the Ganges region suggest a feasible secondary recruitment center of local wild female aurochs into ancestors of domestic zebu cattle within Northern India.

Later, a huge and different range of agro-ecological zones in India have assisted to evolve a number of indigenous cattle breeds. These breeds developed over centuries under low levels of selection followed in traditional animal husbandry practices. As a result, Indian cattle adapted to the harsh native environment became resistant to tropical diseases and external parasites and could sustain on low-quality roughages and grasses. There is a wide variation in size, coat color, appearance, horn and milk production level between North Indian and South Indian breeds. However,

studies are limited in genetic diversity and relationship between these breeds. Presently, our understanding of the early history of cattle domestication and genetic diversity is based mainly on analysis of mtDNA.[21] MtDNA markers provide a genetic basis for appropriate strategies of management and conservation of indigenous breeds. Recently, we demonstrated complete mtDNA sequencing of Indian cattle for the first time.[22] In the present study, we have characterized between-breed genetic diversity and further, explored the relationship among Indian cattle breeds.

Methods

Animals and sample collection

In the current study, six types of distinct zebu cattle breeds, which include milch breed (Gir), dual-purpose (milk and draft) breeds (Tharparkar, Kankrej and Ongole), draft breed (Kangayam) and a dwarf breed (Vechur) from Northern and Southern parts of India, were selected for complete mtDNA sequencing. An unrecognized cattle population of Southern India, 'Kasargod' cattle was also included in the study due to their small size like Vechur breed (Figure 1). The representative animal with original autochthonous phenotype from each breed was selected from the respective breeding tract. Blood samples from each breed ($n = 3$) were collected from a jugular vein in 10 ml Vacutainer tubes with EDTA and stored at −20 °C until DNA extraction. Genomic DNA was isolated blood samples using Xpress DNA blood isolation kit (MagGenome Technologies Pvt. Ltd, Cochin, India).

MtDNA sequencing

Two pairs of primers were designed from reported complete mtDNA sequence of *Bos taurus* spp. (AF492351) to amplify the mtDNA of cattle breeds under study (details of primers are listed in Table 1). The primers were designed in a way that, the first pair of primers for amplifying the initial ~9.2 kb of the mitogenome and second pair for remaining ~7.8 kb of the genome with an overlap of about 0.7 kb. The amplification was done using Long-range PCR (LongAmp™ Taq PCR Kit; New England Biolabs, Ipswich, MA, USA) as per the manufacturer's instructions. The PCR products were electrophoresed on a 0.8% agarose gel and appropriate amplicons were gel eluted using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Furthermore, the eluted amplicons were quantified by Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and breed pool was constructed.

For each sample, 500 ng of the purified amplicon was sheared to 500 bp size using M220 focused-ultrasonicator (Covaris, Woburn, MA, USA). NGS library was prepared by ligating specialized adapters in both ends of each fragment using NEBNext® Ultra™ II DNA Library Prep kit (Illumina, San Diego, CA, USA). Quantity and quality of prepared libraries were measured using Qubit Fluorometer and Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), respectively. Good quality libraries were subjected to sequencing on Illumina HiSeq 2500 platform (Illumina). The generated data were analyzed for quality and then aligned with reference mitochondrial genome of *Bos indicus*.

MtDNA sequence analysis

Individual reads were processed by trimming the fastq files for adapters with cutadapt.[23] Then the reads were mapped against a reference sequence, *Bos indicus* (Zwergzebu breed) complete mitochondrion (Genbank Accession id: AF492350) using the Burrows–Wheeler Alignment (BWA) Tool.[24] The aligned files were then processed with samtools[25] to call consensus sequences and variants. Variants were filtered for zygosity, mapping quality of 30 and read depth of 1000.

Phylogenetic analysis and genetic divergence time estimation

mtDNA sequence of Gir, Tharparkar, Kankrej, Ongole, Kangayam, Vechur and Kasargod cattle was compared with published mitochondrial genomes of *Bos indicus* and *Bos taurus* cattle breeds from NCBI database. The reference sequence *Bos indicus*, Zwergzebu (AF492350), Nellore (NC005971), *Bos taurus*, Korean native (AY526085), Holstein-Friesian (DQ124418), Ukrainian Grey (GQ129208), Hungarian Grey (GQ129207), Fleckvieh (AF492351), Romagnola (FJ971080), Cabannina (EU177867) breeds were included in this study. Sequences of *Bos primigenius* (GU985279 & NC013996), American Bison (*Bison bison*, GU947006), Yak (*Bos mutus*, NC025563), Gaur (*Bos gaurus*, NC024818), African buffalo (*Syncerus caffer*, NC020617) and Water buffalo (*Bubalus bubalis*, AY702618) were also used for the analysis. Maximum Likelihood-based phylogenetic analysis had been performed with consensus sequences of each species/breed using MEGA ver7.0.[26] The genetic distance was calculated based on Maximum Composite Likelihood Model method.[27]

Evolution time tree has been inferred based on sequence divergence between species and breeds by RelTime method.[28] The divergence time between Goat

(*Capra hircus*, KP662714) and Sheep (*Ovis aries*, KU575247) was set to 8.18-12.62 MYA as standard.[29,30]

Results

Complete mitochondrial genome of Indian cattle

The complete mtDNA of six distinct breeds (Gir, Kankrej, Tharparkar, Ongole, Kangayam and Vechur) and one unrecognized cattle (Kasargod cattle) of *Bos indicus* species were sequenced (Figure 2). The total data generated was ~400Mb/sample, of which ~90% of bases have quality ≥Q30. More than 90% pre-processed reads aligned to the reference *Bos indicus* mtDNA genome (Zwergzebu breed, Genbank Accession id: AF492350). More than 99% of the mitochondrial genome was covered by 1000× and mean sequencing depth of the genome exceeds 25,000× for all samples (Table 2).

Mitochondrial genetic variability

The variants identified mapped to the mitochondrial genome for all seven breed is shown in Figure 2 and Supplementary Table. Total identified variants were varied from 4 to 29 in the breeds analyzed (Table 3). Only single-nucleotide variants (SNVs) were found in the samples. We demonstrated a total of 49 unique SNVs (Table 4 and Figure 2) and 32 variants (65% of

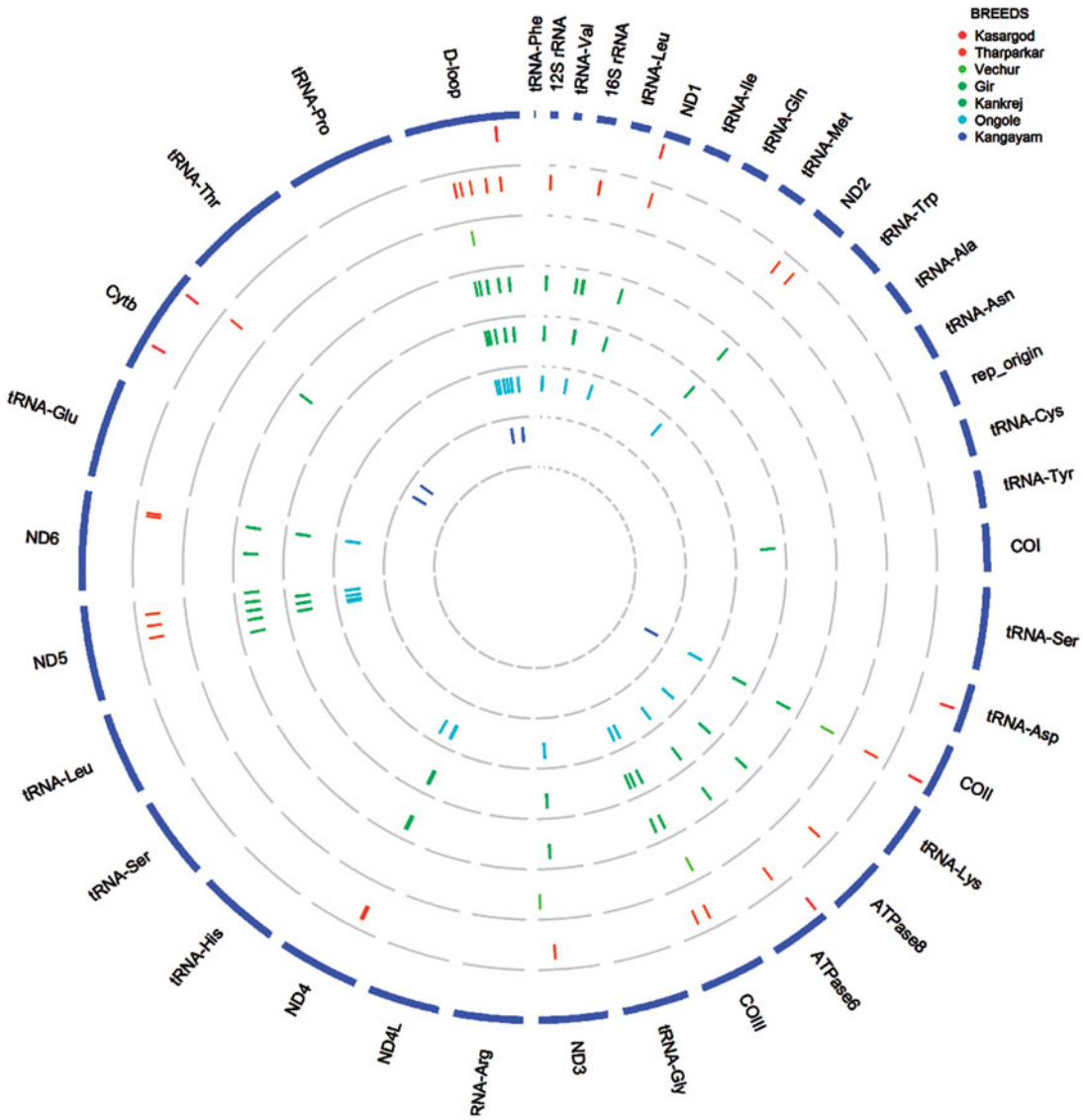

Figure 2. Schematic representation of the cattle mitochondrial genome. Genomic organization and structural features of cattle mtDNA are depicted in an outer circular genomic map. Protein-coding and rRNA genes are interspersed with 22 tRNA genes. Inner seven circles represent seven breeds of *Bos indicus* cattle. SNVs associated with genes, as well as breeds were also shown in the map.

Table 2. Data summary.

Sl No.	Breed	Total reads	>Q30 bases (%)	Alignment (%)	Bases covered ≥1000×	Average read depth (×)
1	Gir	1,793,494	92.39	97.95	16,331	27,880.14
2	Tharparkar	1,724,680	90.24	93.65	16,330	27,761.00
3	Kankrej	1,976,916	88.48	92.88	16,331	28,022.86
4	Ongole	2,509,266	90.34	94.87	16,332	28,117.71
5	Kangayam	2,106,914	89.60	83.48	16,331	27,552.71
6	Kasargod	1,905,232	89.76	91.73	16,329	27,668.71
7	Vechur	1,854,314	89.83	96.05	16,330	27,825.00

Total data, coverage and read depth summary of samples at various depths.

Table 3. Number of SNVs associated with mitogenome of Indian cattle breeds.

Sl No	Breed	Number of SNVs/breed
1	Gir	29
2	Tharparkar	26
3	Kankrej	26
4	Ongole	26
5	Kangayam	5
6	Vechur	4
7	Kasargod	7

The North Indian breed, Gir possessed maximum number of SNVs and the least number of SNVs were associated with the South Indian breed, Vechur.

the total variations discovered) map to the protein coding genes. Of these, five were non-synonymous variants. Among the protein-coding genes, NADH Dehydrogenase subunit 5 (ND5) gene possessed six SNVs. The D-loop region of the mitochondrial genome contained 11 variants. The tRNA genes and NADH Dehydrogenase subunit 4L (ND4L) gene were found to be identical in all breeds analyzed.

The North Indian cattle breeds (Gir, Kankrej and Tharparkar) showed more variants (an average of 27 variants) than the South Indian breeds (average variants = 10.5) with the reference *Bos indicus* genome (Zwergzebu breed) (Table 3). Among all the breeds, Gir displayed the highest number of SNVs (29) followed by Kankrej, Tharparkar and Ongole with 26 SNVs each. The South Indian breed, Vechur has the least number of SNVs (4). All Indian cattle breeds analyzed in this study have SNV (m.7583G > A) in COII gene. Except Vechur, other breeds, Gir, Kankrej, Ongole, Tharparkar and Kangayam displayed SNV at 16148th nucleotide position in the D-loop region (m.16148A > G). A total of 20 SNVs were commonly observed in North Indian breeds and Ongole cattle. The SNV m.2271T > C in 16S rRNA was observed exclusively in North Indian cattle breeds. Kangayam and Kasargod cattle showed a common SNV (m.144468T > C) in *Cytb* at 14468th nucleotide position of mtDNA. The Vechur breed did not share a common SNV with other South Indian breeds. Unique variants in each breed were also found and the numbers are, Gir = 6, Kankrej = 3, Tharparkar = 3, Ongole = 4, Kangayam = 2, Vechur = 3 and Kasargod = 4.

Of the total 49 unique SNVs detected in the seven Indian breeds, NADH dehydrogenase group possessed the maximum number of SNVs (18) compared with other genes. Except two variants, these SNVs were mainly present in North Indian breeds and Ongole cattle. South Indian cattle, that is, Vechur and Kasargod displayed a single SNV in NADH Dehydrogenase subunit 3 (ND3 m.9790T > C) and NADH Dehydrogenase subunit 1 (ND1 m.2800G > A), respectively, and Kangayam breed did not show any polymorphism in the NADH dehydrogenase group. North Indian breeds (e.g., Gir, Kankrej and Tharparkar) and the South Indian breed, Ongole showed an SNV at m.7831C > T in the ATPase8 gene. However, Kasargod cattle showed a nonsynonymous variant at ATPase6 m.7963G > A. In our study, 12S rRNA m.360C > T and 16S rRNA m.2212A > G SNVs were detected in Gir, Kankrej, Tharparkar and Ongole breeds. However, except Kasargod cattle (tRNA-Asp m.6980A > G), no variation has been found in tRNAs of mtDNA of Indian cattle.

Genetic distances and breed relationships

Maximum likelihood-based phylogeny analysis (MEGA ver7.0) consistently grouped the mitochondrial sequences of the subfamily *Bovinae* into four lineages: (1) Water Buffalo (*Bubalus bubalis*) and African buffalo (*Syncerus caffer*); (2) Gaur (*Bos gaurus*); (3) Wild Yak (*Bos mutus*) and Bison (*Bison bison*); and (4) *Bos taurus* and *Bos indicus* (Figure 3). The calculation of genetic distance of mtDNA between different species and breeds showed the range value between 0 and 0.138 (Table 5). The genetic distance between *Bos taurus* and *Bubalus bubalis* was observed to be high (0.138) and the genetic distance between *Bos indicus* (Zwergzebu) and *Bubalus bubalis* was 0.137. The South Indian cattle like Vechur, Kangayam and Kasargod displayed close genetic relationship (genetic distance value of zero) with the reference *Bos indicus*

Table 4. Position of SNVs present in mtDNA of Indian cattle (*Bos indicus*) breeds.

Breeds	Total SNVs	SNV position	Gene	Alteration		AA change	Feature
				REF	ALT		
VC, KD, KG, OG, KK, TH, GR	1	7583	COII	G	A		Synonymous
KD, KG, OG, KK, TH, GR	1	16148	D-loop	A	G		
OG, KK, TH, GR	20	15778	D-loop	C	T		
		8151	ATPase6	G	A		Synonymous
		15869	D-loop	T	C		
		15722	D-loop	C	T		
		13265	ND5	T	C	p.Ile507Thr	Nonsynonymous
		9117	COIII	G	A	p.Val171Ile	Nonsynonymous
		13008	ND5	T	C		Synonymous
		9708	ND3	T	C		Synonymous
		12738	ND5	C	A		Synonymous
		8939	COIII	A	G		Synonymous
		4625	ND2	C	A		Synonymous
		7831	ATPase8	C	T		Synonymous
		10525	ND4	C	T		Synonymous
		15721	D-loop	C	T		
		360	12S rRNA	C	T		
		10570	ND4	G	A		Synonymous
		16014	D-loop	T	C		
		2212	16S rRNA	A	G		
		2953	ND1	T	C		Synonymous
		14006	ND6	G	A		Synonymous
GR, KK, TH	1	2271	16S rRNA	T	C		
KD, KG	1	14468	Cytb	T	C		Synonymous
KD	4	6980	tRNA-Asp	A	G		Synonymous
		2800	ND1	G	A		Synonymous
		15129	Cytb	G	A	p.Ala327Thr	Non-synonymous
		7963	ATPase6	G	A	p.Val13Ile	Non-synonymous
TH	3	15224	Cytb	C	T		Synonymous
		3998	ND2	G	A		Synonymous
		13987	ND6	A	G		Synonymous
VC	3	8849	COIII	T	C		Synonymous
		9790	ND3	T	C		Synonymous
		15801	D-loop	T	C		
GR	6	14991	Cytb	T	C		Synonymous
		1366	16S rRNA	C	T		
		13546	ND5	C	A	p.Leu601Ile	Non-synonymous
		12369	ND5	C	T		Synonymous
		2323	16S rRNA	C	T		
		13787	ND6	C	T		Synonymous
KK	3	9230	COIII	C	T		Synonymous
		15764	D-loop	C	T		
		5590	COI	T	C		Synonymous
OG	4	15938	D-loop	T	C		
		11035	ND4	T	C		Synonymous
		12810	ND5	T	C		Synonymous
		15866	D-loop	G	A		
KG	2	15865	D-loop	A	G		
		14930	Cytb	T	C		Synonymous

A total of 49 single-nucleotide variants (SNVs) were observed among the breeds. VC: Vechur; KD: Kasargod; KG, Kangayam; OG: Ongole; KK: Kankrej; TH: Tharparkar; GR: Gir.

cattle (Zwergzebu). The genetic distance among *Bos taurus* cattle, namely, Holstein-Friesian, Hungarian Grey and Ukrainian Grey were also observed to be zero. In this study, we observed a proximate genetic relationship among Nellore (Brazilian breed), Kankrej and Tharparkar. However, Gir showed a genetic distance of 0.001 with Kankrej and Tharparkar breeds. The genetic distance between available *Bos primigenius* (GU985279) sequence and *Bos taurus* was less (0.004) compared with the distance (0.014) between *Bos primigenius* and *Bos indicus*.

In the phylogenetic tree among the cattle species, *Bos indicus* and *Bos taurus* cattle were present in distinct separate clades with a genetic distance of 0.015 (Figure 3). A phylogenetic relationship based on mtDNA revealed that the South Indian cattle and North Indian cattle were clustered in two separate clades with a genetic distance of 0.002. The South Indian breed, Ongole was found to be close to the North Indian breed, Kankrej.

Genetic divergence time of Bos indicus and Bos taurus cattle

In the current study, we used complete mitochondrial genome sequences to estimate the divergence time

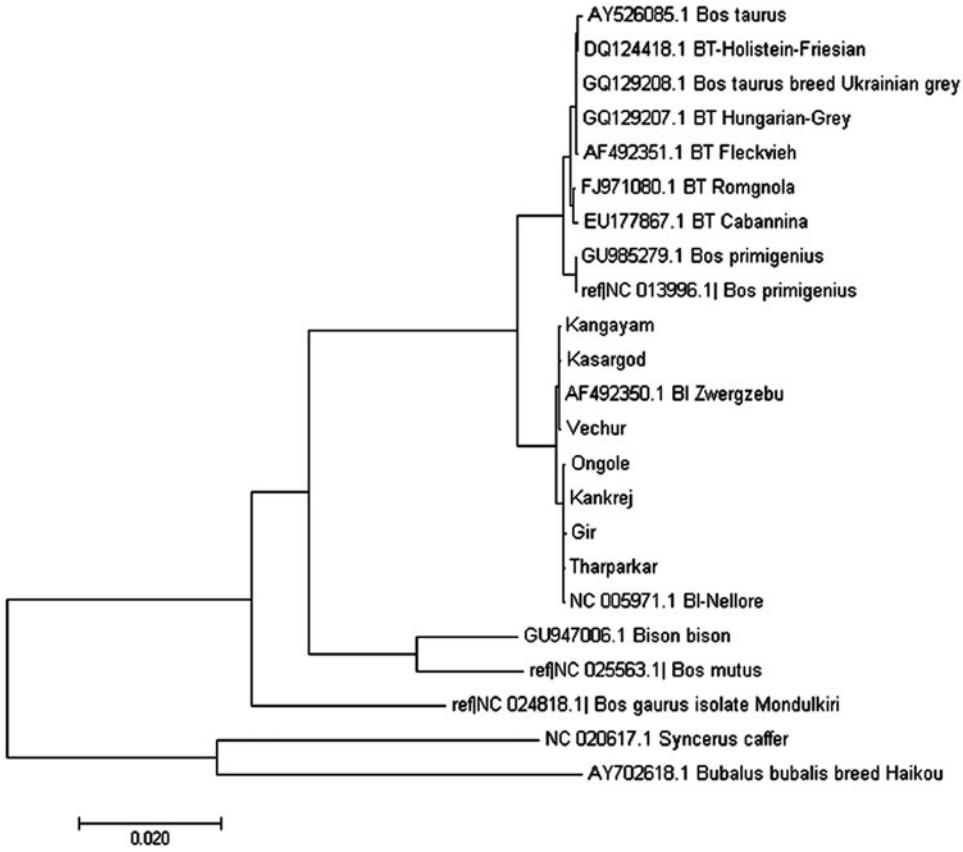

Figure 3. Phylogenetic analysis of cattle. Phylogenetic relationship was analyzed using Maximum Likelihood method (MEGA ver7.0) by comparison of complete mtDNA sequences of different species and breeds.

between *Bos taurus* and *Bos indicus* and also within *Bos taurus* and *Bos indicus* clusters (Figure 4). Divergence times were estimated based on sequence divergence between species by the RelTime method. By fixing the divergence time between *Capra hircus* and *Ovis aries*, we demonstrated that divergence between subfamilies Bovinae and Caprinae had happened at 13.31 million years ago (MYA). The first divergence within the Bovini tribe occurred 9.87 MYA with the splitting of the buffalo or the subtribe Bubalina (*Bubalus* and *Syncerus* spp.) from the non-buffalo or the subtribe Bovina. The divergence of *Bubalus bubalis* and *Syncerus caffer* clusters has been estimated at 6.35 MYA. The divergence of cattle group (*Bos taurus* and *Bos indicus*) from other genera has been dated to 4.34 MYA. We estimated the divergence time of genus *Bos gaurus* from other genera, *Bos mutus* and *Bison bison* as 3.44 MYA and the split between *Bos mutus* and *Bison bison* has been dated to 1.66 MYA.

The divergence of *Bos taurus* and *Bos indicus* clusters has been estimated to be 0.92 MYA. Our analysis showed that the divergence time among *Bos indicus* lineages as 0.10 MYA to form two distinct clusters (South Indian and North Indian cattle groups). Different breeds or their ancestors within these two *Bos indicus* clades were diverged between 30,000 and 10,000 years ago. It was observed that the ancestor of the South Indian cattle diverged to form Kangayam, Vechur and Kasargod cattle ~30,000 years ago. Interestingly, we found a common ancestor for *Bos indicus* South Indian breed and European dwarf breed, Zwergzebu. Ancestor of North Indian cattle diverged to different breeds, namely, Tharparkar, Kankrej and Gir ~20,000 years ago. It was also demonstrated from the mitochondrial genome data that, the South Indian breed, Ongole and North Indian breeds (Gir, Tharparkar, Kankrej) had a common ancestor 20,000 years ago. Furthermore, the study revealed that the wild auroch (*Bos primigenius* with accession number: GU985279) and ancestor of *Bos taurus* cattle were separated at 0.18 MYA. Italian *Bos taurus* cattle breeds (Romganola and Cabannina) were separated from other *Bos taurus* breeds at 0.12 MYA.

Discussion

The divergence between *Bos taurus taurus* and *Bos taurus indicus* is estimated to have occurred from a common ancestor, the aurochs (*Bos primigenius*)

Table 5. Genetic distance estimation based on Maximum Composite Likelihood Model method.

	Bos primigenius	*Bison bison*	*Syncerus caffer*	*Bubalus bubalis*	*Bos mutus*	*Bos gaurus*	*Bos taurus*	Kanga-yam	Ongole	Kankrej	Gir	Vechur	Tharp-arkar	Kasarg- od	Holstein-Friesian	Ukrainian grey	Romagnola	Hungarian Grey	Fleckvieh	Cabannina	Nellore	Zwergzebu
Bos primigenius																						
Bison bison	0.062																					
Syncerus caffer	0.134	0.129																				
Bubalus bubalis	0.136	0.132	0.096																			
Bos mutus	0.062	0.029	0.128	0.135																		
Bos gaurus	0.067	0.059	0.128	0.137	0.06																	
Bos taurus	0.004	0.062	0.135	0.138	0.064	0.068																
Kangayam	0.014	0.061	0.133	0.136	0.061	0.066	0.015															
Ongole	0.014	0.061	0.134	0.137	0.061	0.066	0.015	0.002														
Kankrej	0.014	0.061	0.134	0.137	0.062	0.067	0.015	0.002	0													
Gir	0.015	0.061	0.134	0.137	0.062	0.067	0.015	0.002	0.001	0.001												
Vechur	0.014	0.061	0.133	0.136	0.061	0.066	0.015	0	0.002	0.002	0.002											
Tharparkar	0.015	0.061	0.133	0.137	0.062	0.067	0.015	0.002	0.001	0	0.001	0.002										
Kasargod	0.014	0.061	0.133	0.136	0.061	0.066	0.015	0	0.002	0.002	0.002	0.001	0.002									
Holstein-Friesian	0.004	0.062	0.134	0.138	0.063	0.067	0.001	0.014	0.014	0.015	0.015	0.014	0.015	0.014								
Ukrainian grey	0.004	0.062	0.134	0.137	0.063	0.067	0.001	0.014	0.014	0.014	0.015	0.014	0.015	0.014	0							
Romagnola	0.003	0.061	0.134	0.137	0.062	0.067	0.002	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.002	0.002						
Hungarian Grey	0.004	0.062	0.134	0.137	0.063	0.067	0.001	0.014	0.014	0.014	0.015	0.014	0.015	0.014	0	0	0.002					
Fleckvieh	0.004	0.062	0.134	0.137	0.063	0.067	0.001	0.014	0.014	0.014	0.015	0.014	0.014	0.014	0.001	0	0.002	0				
Cabannina	0.004	0.062	0.134	0.137	0.063	0.067	0.003	0.014	0.014	0.014	0.015	0.014	0.014	0.014	0.002	0.002	0.001	0.002	0.002			
Nellore	0.015	0.061	0.133	0.137	0.062	0.067	0.015	0.002	0.001	0	0.001	0.002	0	0.002	0.015	0.015	0.014	0.015	0.014	0.014		
Zwergzebu	0.014	0.061	0.133	0.137	0.061	0.066	0.015	0	0.002	0.001	0.002	0	0.002	0	0.014	0.014	0.014	0.014	0.014	0.014	0.002	

The calculation of genetic distance of mtDNA between different species and breeds showed the range value between 0 and 0.138.

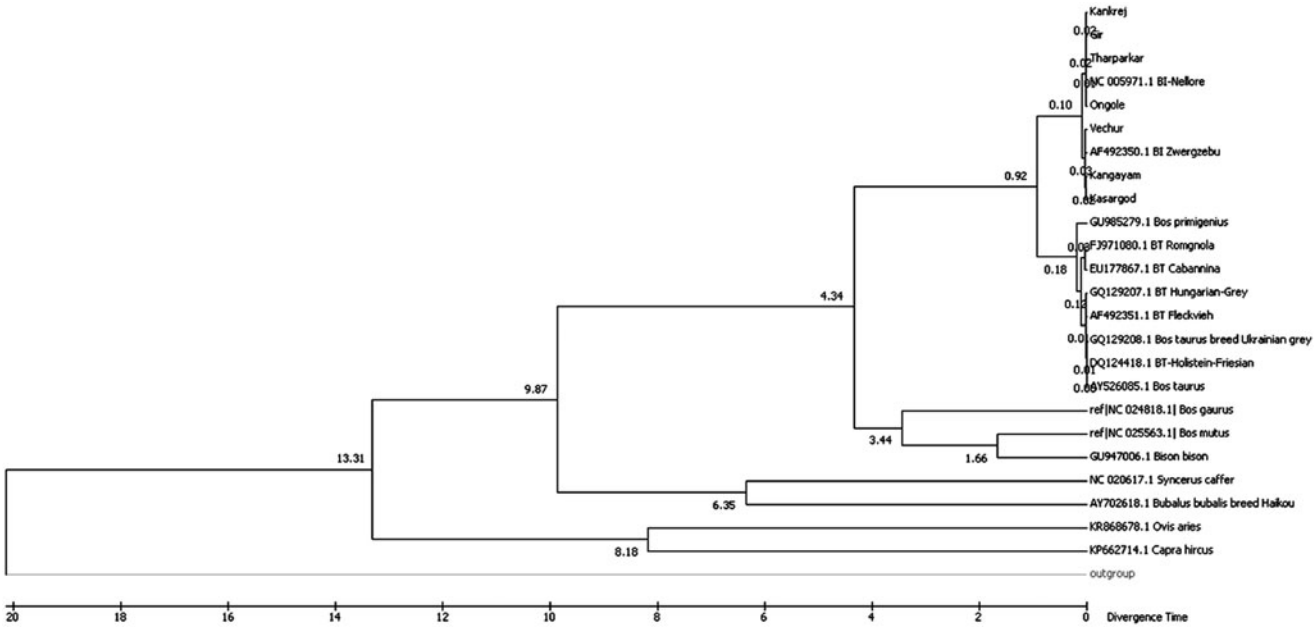

Figure 4. Assessment of genetic divergence time by the RelTime method. The complete mitochondrial genome sequence was used to estimate the divergence within subfamily Bovinae. Numbers above the nodes represents estimated genetic divergence time.

between 0.33 MYA[31] and 2 MYA.[32] After the divergence, cattle groups have gathered distinct genetic variations, which have come up with highly differentiated phenotypes. Presently, India has 40 distinct zebuine cattle breeds with different adaptability and production traits (ICAR-NBAGR, India). Considering the importance of cattle in Indian agriculture, here we have studied the complete mtDNA sequence to find out genetic diversity and relationship among these breeds. MtDNA is accounted as a popular genetic marker for the genetic diversity and evolutionary studies due to its high genetic variability, clonal inheritance and near-neutrality.[33]

To our knowledge, this is the first report of a complete mitochondrial genome sequence of Indian cattle. Previous studies on Indian cattle reported an analysis of mitochondrial D-loop region or microsatellite markers.[34,35] MtDNA is maternally inherited and is closed circular double-helix DNA, and the length of the sequence is ~16,500 bp.[36] The arrangement of genes in the mitochondrial genome of studied breeds was in accordance with prior studies in other cattle outside India.[37]

The nucleotide sequence data for polymorphic sites in mtDNA have been widely used for the studies of molecular evolution, the genetic structure of populations and the estimation of genetic relationships between and within in species.[38] In cattle, mtDNA variants reportedly affect milk fat yield, carcass composition, growth rate and fertility traits.[39,40] In the present study, we demonstrated mtDNA polymorphisms at the protein-, rRNA- and tRNA-coding gene level in the Indian cattle breeds by comparing with published complete cattle mitochondrial genomes. Among the protein coding genes, ND4L gene was found to be conserved in all the breeds analyzed. COI and COII genes showed single SNV and the COII variant was found in all the breeds used in the study. A single nucleotide substitution of m.7583G > A without any amino acid change was noticed in COII gene of all the breeds studied compared with mitochondrial genomes of reference, *Bos indicus* Zwergzebu and *Bos taurus* Holstein-Friesian. Further studies are required with more number of samples to confirm if the identified variant is actually a polymorphism (SNP). Once it is established, the variation might be useful as a genetic marker for the identification of Indian cattle breeds. We noticed that Kankrej was the only breed which displayed variant in the COI gene. COI gene has been reported to have a conserved sequence of mtDNA on livestock[41] and it is used for evolutionary studies[42] and analysis of the origin of livestock.[43] Among the Cytochrome group, *Cytb* gene showed more number of variations, that is, five SNVs. The relatively high variations in *Cytb* was also previously reported in cattle.[12, 44]

NADH dehydrogenase (ubiquinone) is one of the "entry enzymes" of cellular respiration or oxidative phosphorylation in the mitochondria.[45] In the current study, NADH dehydrogenase group possessed the maximum number of SNVs compared with other

genes. A recent report shows that single nucleotide polymorphism (ND1 m.3907 C > T, ND2 m.4351 G > A and ND2 m.5218 C > T) present in the Tibetan yaks was positively associated with high-altitude adaptation.[46] However, ND1 variant (m.3638 A > G) present in *Bos taurus* cattle resulted in the termination of transcription and it was negatively associated with high-altitude adaptation. Therefore, mitochondrial ND1 and ND2 can be considered as candidate genes associated with adaptation to high-altitude environments. In future, studies are required to find out the role of NADH dehydrogenase subunit genes in *Bos indicus* cattle.

We demonstrated an SNV in ATPase8 gene of North Indian cattle breeds (Gir, Kankrej and Tharparkar) and in one South Indian breed, that is, Ongole. Earlier, Qin and coworkers[47] reported the association of milk yield with ATPase6/ATPase8 genes in Holstein cows. As the North Indian breeds used in the present study produce comparatively higher milk yield than South Indian breeds, these genes may be tried to use as markers for identification of superior germplasm from Indian cattle. In our study, among the breeds analyzed, Gir is the highest milk producer and its mtDNA contains two SNVs at 16S rRNA m.1366C > T and 16S rRNA m.2323C > T. Literature reports that polymorphic sites on rRNA genes have been linked with milk traits in Holstein cows.[48] Earlier report demonstrated an association between polymorphism of mitochondrial tRNA-Asn gene with growth traits in *Bos indicus* Nellore cattle.[40] However, except Kasargod cattle, no variation has been found in tRNAs from mitogenome of Indian cattle studied. We noticed a highly polymorphic D-loop region (11 SNVs) in all the breeds studied. The literature suggests an association of D-loop variation with carcass traits such as longissimus muscle area and beef marbling score,[49] milk yield, fat content and estimated milk energy[50] and calving rates.[51]

Maximum Likelihood-based phylogenetic analysis agreed with the genetic distance calculated based on Maximum Composite Likelihood Model. It showed a strong separation between Bubalina and Bovina subtribes and an effective support for separation of different genera confirming previous studies from molecular data.[52–54] Morphological studies[55] and comparison of mitochondrial[56,57] or nuclear[58] sequences suggest clustering of yak with the bison. Our genetic distance estimation and phylogenetic tree construction results are in agreement with the above studies. In the phylogenetic tree cattle breeds were divided into two main clades; *Bos taurus* and *Bos indicus*. Previous studies based on nuclear ribosomal genes[59] and mitochondrial D-loop sequences[9] confirmed close relationship of taurine and zebu cattle *(Bos indicus*). Our study showed the division of *Bos indicus* clade into subclades containing North Indian and South Indian cattle breeds. The reference to European dwarf zebu cattle (Zwergzebu) was placed in the South Indian group. Our analysis demonstrates that, compared with North Indian breeds, South Indian breeds except Ongole showed less sequence variation with Zwergzebu cattle. The dwarf breed Vechur cattle showed the least number of SNVs and thus our results suggest a common ancestor for South Indian cattle and Zwergzebu breed. Zwergzebu cattle was founded in Germany and the breed is believed to have originated from zebu cattle of Sri Lanka and the Caucasus region.[60] As the geographic location of Sri Lanka is close to India, there is the likelihood of migration of the ancestral population of these breeds between these countries.

Although Ongole is a South Indian cattle, it is included among the gray-white cattle of the north and it has great similarity with the Gaolao breed and Bhagnari type of cattle in North India. Genetic distance, phylogenetic tree and SNV analyses of the present study also confirm the relationship of Ongole breed with North Indian breeds. The Brazilian breed, Nellore was originated from Ongole breed and it was first acknowledged in Brazil in the late 1800s. In the present study, Nellore was positioned among the North Indian breeds in the phylogenetic tree. To the best of our knowledge, Vechur is the shortest cattle identified and which is found in Kerala, a Southern state of India. Other similar dwarf cattle population called as Kasargod is also present in the Kerala state, but it is not recognized till now. Interestingly, in this study, Kasargod was found to be more close to Kangayam (a taller draft breed of nearby state) in the phylogenetic tree. Therefore, further studies are warranted to elucidate the genetic background of Kasargod population. We observed that the main *Bos taurus* clade was divided into wild *Bos primigenius* group and a group containing taurine cattle breeds. As the complete mtDNA sequence of wild aurochs (Genbank Accession id: GU985279 and NC013996) used in the present phylogenetic analysis was of European origin, these animals were found to be close to taurine cattle breeds in the phylogenetic tree obtained. Earlier, other researchers also reported the presence of northern and central European Bos *primigenius* and *Bos taurus* mtDNA in the same clade, which assist the single origin hypothesis.[7,10]

The current investigation demonstrated genetic divergence time between and within *Bos indicus* and *Bos taurus* cattle using complete mitochondrial

genome analysis. We demonstrated the divergence time between subfamilies Bovinae and Caprinae as 13.31 MYA. Earlier, Van Laere and coworkers[61] calculated the divergence time between these subfamilies as ~18 MYA by studying pseudoautosomal boundary (PAB) region of the genome. Reports suggest the first divergence within the Bovini occurred between 5 and 10 MYA with the splitting of the buffalo or the subtribe Bubalina from the non-buffalo or the subtribe Bovina.[62–64] We also obtained the same divergence time using the RelTime method. About 4–5 MYA, the Bovina subtribe was still a single widespread species. Then Bovina split into three major groups: Domestic cattle, Bison/Yak and Gaur/Mithun/Banteng and this occurred very rapidly. Estimated divergence times indicated that the *Bos indicus* and *Bos taurus* cattle lineages separated 0.92 MYA. Previously, the divergence of the *Bos taurus* and *Bos indicus* clusters has been estimated to be approximately 2.0 MYA based on sequences of mitochondrial control region (CR).[7,31,65] This divergence time was also estimated to be 2–3 MYA based on the fossil records. Researchers reported that the divergence between the Zebu and Taurine breeds was traced back to 0.2 to 1.0 MYA.[66,67] Before the domestication of Zebu cattle, the divergence between Zebu cattle and European breeds occurred and this hypothesis was also supported by the β-chain structure of hemoglobin. The deep divergence between *Bos taurus* and *Bos indicus* mtDNA sequences points to two independent primary domestication events from genetically discrete aurochs groups, each possibly with a subspecies status.[9,66]

Hiendleder and coworkers[32] determined divergence times among the *Bos indicus* and *Bos taurus* lineages were 0.202 and 0.227 MYA, respectively. Our result suggests that within divergence of zebu and taurine cattle happened at 0.1 and 0.18 MYA, respectively. Approximately 30,000 to 20,000 years before, ancestors of present zebu breeds were originated in South and North India, separately. This is the first report of time divergence among Indian cattle breeds. The average divergence times between Red Chittagong of Bangladesh versus Indian Sahiwal, Hariana and Ongole cattle and the values were 22,700, 24,800 and 26,900 YBP, respectively.[68] The literature suggests that domestication of cattle occurred independently in the Near East and the Indian subcontinent between 10,000 and 8,000 years ago. Therefore, as per our result and zooarchaeological data,[20] we can propose that Southern India acted as a center for domestication of Indian cattle along with Indus valley of North-Western India.

Conclusions

The present study elucidates the genetic diversity of native Indian cattle breeds using complete mtDNA sequence. To our knowledge, this is the first investigation of genetic variations across the whole mitochondrial genome of Indian cattle. The genetic distance, phylogenetic tree and divergence time analyses definitely differentiated cattle populations as per their historical origins and represented the genetic distinctiveness of Indian breeds. Indigenous cattle breeds picked up the genetic diversity through selection over centuries and depict the most efficient systems in their respective breed tracts. However, in the recent decades, the population size of some of the indigenous breeds has diminished due to several reasons including disregard of their genetic strengths and their genetic dilution through uncontrolled crossbreeding and interbreeding. This investigation gives an understanding of the history and genetic structure and, in future which may help in prioritization and designing of the conservation plans.

Ethical statement

The present study did not require an approval from the animal ethics committee as no animal experiment was performed.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability

The complete mtDNA sequences of cattle breeds were deposited in NCBI's Sequence Read Archive under BioProject ID:PRJNA408207 with the following Biosample accession numbers of SRR6059069, SRR6059070, SRR6059071, SRR6059072, SRR6059073, SRR6059074 and SRR6059075.

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