



Single nucleotide polymorphism in cytochrome B oxidase gene among indigenous cattle breeds of Tamil Nadu

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Bovine mitochondrial DNA (mtDNA) is a circular, coiled, multiple-copied and extra-nuclear genome with a high mutation and evolution rate of roughly five to ten times higher than nuclear DNA, making it extremely diverse within a species and essential material for phylogenetic and genetic diversity research in animal breeding (Chen *et al.* 2000). Unlike genomic DNA, mtDNA lacks recombination and introns; but have unique maternal inheritance, effective repair mechanisms and high mutation rate due to the mutagenic properties of reactive oxygen species (the harmful by-products of oxidative phosphorylation), an error-prone polymerase and limited mtDNA repair (Andalib *et al.* 2017). The complete nucleotide sequence of the bovine mitochondrial genome was 19,338 bp, which comprised of 13 protein-coding genes, 12S and 16S ribosomal RNA, 22 transfer RNA genes of the inner mitochondrial membrane, and a displacement loop (D-loop), which regulates transcription and replication of the coding DNA strand of the mtDNA (Clayton 1991).

Cytochrome b oxidase (CYTB) is one of the unique protein-coding genes with extensive intra-species and inter-species phylogenetic information due to its rapid evolutionary rate and high sequence diversity, with a larger variation ratio than other functional genes (Ciftci *et al.* 2013, Othman *et al.* 2017, Tarekegn *et al.* 2018, Hartatik *et al.* 2019, Rahmatullaili *et al.* 2019). Indigenous breeds of Tamil Nadu are known for draughtability, hence it becomes pertinent to choose an important mitochondrial gene – Cytochrome b oxidase, which is involved in energy metabolism. Till date, the role of CYTB other than phylogenetic analysis is not well established among indigenous cattle breeds in India. Hence, the validation of SNP obtained from WGS data and their distribution in indigenous population of Tamil Nadu was undertaken.

Sample collection: Blood samples (288) were collected

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aseptically from unrelated animals belonging to five cattle breeds, viz. Alambadi (16), Bargur (99), Kangayam (54), Pulikulam (58) and Umblachery (61), true to breed type from their respective conservation units of Tamil Nadu.

DNA isolation and template DNA preparation: Genomic DNA was isolated from the blood samples as described by Sambrook and Russel (2001). The template DNA with a concentration of approximately 50 ng/μl was prepared and stored at -20°C till further processing.

Whole genome sequencing (WGS) data was subjected to identify the unique single nucleotide polymorphisms (SNPs) in all the protein-coding regions of mitochondrial genome. Out of 273 SNPs identified in 13 protein coding regions, a missense SNP (14,716 C>T) at CYTB was chosen and genotyped using Tetra-primer ARMS-PCR among indigenous cattle breeds of Tamil Nadu.

Validation of SNP obtained from WGS data in Cytochrome b oxidase (CYTB): The gene sequence of cytochrome b in *Bos indicus* was obtained from the National Centre for Biotechnological Information (www.ncbi.nlm.nih.gov) in FASTA format. The Gene ID with accession number, gene length, number of designed primers and reference assembly range for the mitochondrial CYTB gene were 2885974 – YP_052709, 1140, 4 and 14517 to 15656, respectively. ExpASy tool was used to know the change in amino acid in the protein assembly of CYTB gene sequences.

Designing of primers: The primers for tetra-primer-ARMS-PCR were designed using online tool, primer3 (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). Details of primers designed for the CYTB gene are given in Table 1. The designed primers were analysed and blasted using Oligo Analyzer™ 1.0.3 tool and the reference sequence of representative genomes of NCBI, to identify the specificity of the target respectively.

The synthesised oligonucleotide primers (supplied in lyophilised form) were further diluted in the ratio of 1:10 using nuclease-free water to give a final concentration of 10 pmol/μl.

Tetra-primer ARMS PCR amplification of DNA: PCR

Table 1. Primers designed for the CYTB gene

Gene	Primer sequences (5' to 3')	Annealing temperature (°C)	Length of primer (bp)
CYTB (14716 C>T)	FO TTC CAG CCC CAT CAA ACA TTT C	56	22
	RO TGT GAG CAG AAG GAT TAC TCC G	56.4	22
	FI CAA CAG CAT TCT CCT CTG TGAC	55.7	22
	RI TCA CGT CTC GGC AGA TAT TGA	55.7	21

Note: FO, Forward outer; RO, Reverse outer; FI, Forward inner and RI, Reverse inner primers.

cocktail for tetra primer-ARMS PCR was prepared using all the components except the template for a set of reactions containing 5 µl of 2× master mix, 0.5 µl each of forward and reverse outer primers, 0.75 µl of inner forward primers, 1.25 µl of inner reverse primers, 1 µl of nucleus free water and 1 µl of DNA template with a total of 10 µl reaction mixture. The amplification was carried out in a Bio-Rad C1000 Touch™ thermocycler with following conditions: initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 54.7°C for 30 sec and extension at 72°C for 30 sec; and final extension 72°C for 8 min and hold at 4°C.

Analyzing the PCR product by agarose gel electrophoresis: The size of the PCR amplicon was verified in a horizontal submarine gel electrophoresis with 3% agarose gel. About 3 µl of the PCR product was run along with a 50 bp DNA ladder (Gene Ruler™, Fermentas). The PCR products were checked under a UV trans-illuminator and documented by a gel documentation system (Bio-Rad, USA). The expected product sizes were 305 bp and 188 bp for homozygous (wild); 305 bp, 188 bp and 159 bp for heterozygotes; and 305 bp and 159 bp for homozygous (mutant) genotypes.

Statistical analysis: The Chi-square (χ^2) test of goodness of fit was carried to check whether the population was in Hardy-Weinberg equilibrium or not (Falconer and Mackay 1996). Phylogenetic analysis was carried out by MEGA-X software for all the sequences of indigenous cattle

breeds along with *Bos indicus* and *Bos taurus* reference genome. Tajima's D statistics or the test of neutrality was carried out and the values were interpreted (Tajima 1989).

PCR amplification of CYTB gene: The total length of the CYTB gene was 1140 bp. It is an intron-less gene and the size of the PCR amplicon of the two outer primers in the CYTB gene was 305 bp. The analysis of WGS revealed one mutation in the exon of the CYTB gene and was characterized by a C>T transition at 200th position (i.e. 67th codon) and non-synonymous one replacing the amino acid threonine to isoleucine.

SNP genotyping of CYTB gene: The gene and genotype frequencies of SNP of CYTB gene are mentioned in Table 2.

All three possible genotypes (CC, CT and TT) with frequencies (0.96, 0.019 and 0.019) were observed only in Kangayam breed, which might be due to more number of animals or presence of genetic variability in the breeding tract when compared to other indigenous breeds of Tamil Nadu; and CC wild type genotype alone was reported in other four breeds. The tetra primer-ARMS PCR fragments showed the presence of CC, CT and TT genotypes (Fig. 1.)

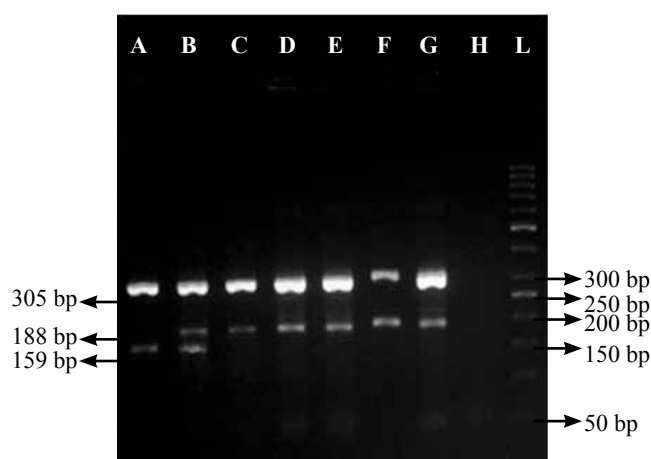


Fig. 1. PCR amplification of CYTB gene showing the presence of CC, CT and TT genotypes. Lanes A and B, Kangayam; Lane C, Alambadi; Lane D, Bargur; Lane E, Pulikulam; Lanes F and G, Umblachery; Lane H, NTC and Lane L, 50 bp ladder.

The wild type C allele was found to be fixed in all breeds in the study except Kangayam and the minor allele (T) was found to be distributed with the lowest frequency of 0.03 in Kangayam cattle, which might be an indication of selection acting against it. Further, the addition of more number of

Table 2. Gene and genotype frequencies of Cytochrome b oxidase gene

SNP location	Breed	Number of animals	Gene frequency		Genotype frequency		
			C (188 bp)	T (159 bp)	CC	CT	TT
200 C>T	Alambadi	16	1.0	0	1.0	0	0
	Bargur	99	1.0	0	1.0	0	0
	Pulikulam	58	1.0	0	1.0	0	0
	Umblachery	61	1.0	0	1.0	0	0
	Kangayam	54	0.97	0.03	0.96	0.019	0.019
χ^2 value			288.80**				

**, highly significant.

samples might throw better insight into the distribution of alleles in the Kangayam cattle breed and would enable us to validate the selection pressure.

Due to the unavailability of the phenotypic data on draught characters for Kangayam breed of cattle, the function of the mutation in the study could not be elucidated. Moreover, the results obtained in this study could not be compared due to paucity of literature for Cytochrome b oxidase gene in bovines and majority of the published literature were limited to phylogenetic and species-specific DNA barcode analyses.

In addition, chi-square (χ^2) test revealed that the population with respect to CYTB locus was not in Hardy-Weinberg equilibrium ($p < 0.01$) in Kangayam breed of cattle.

Phylogenetic analysis of CYTB gene: The results of phylogenetic analysis for Cytochrome b oxidase gene are depicted in Fig. 2. It revealed that indigenous breeds of Tamil Nadu were found to be under the same genetic lineage of *Bos indicus* reference genome (Nelore) and *Bos taurus* reference genome diverged separately for CYTB gene. The significant negative Tajima's D values (-2.40) might indicate the possibilities of a greater number of animals after a recent bottleneck among the recognized indigenous cattle breeds of Tamil Nadu.

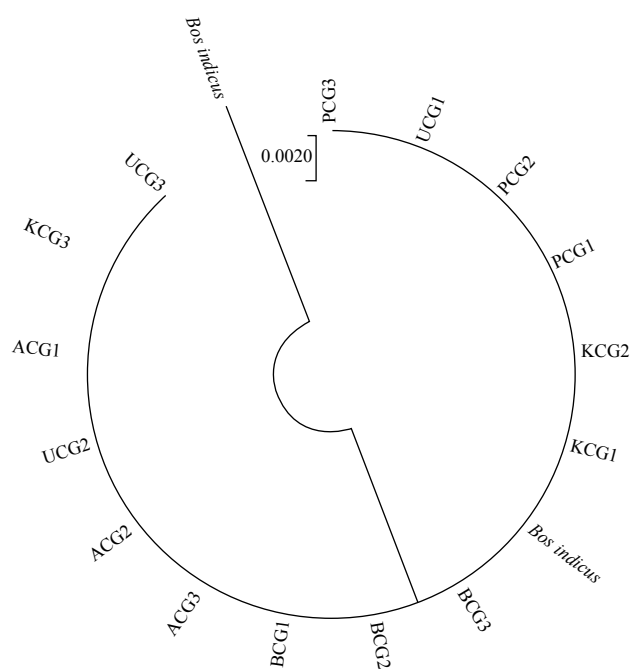


Fig. 2. Unrooted Neighbor-joining tree for *Bos indicus* breeds of Tamil Nadu and *Bos taurus* cattle. ACG1, ACG2 and ACG3 – Alambadi; BCG1, BCG2 and BCG3 – Bargur; KCG1, KCG2 and KCG3– Kangayam; PCG1, PCG2 and PCG3– Pulikulam; UCG1, UCG2 and UCG3– Umblachery; *Bos indicus* and *Bos taurus* – Reference genomes.

From this study, it could be concluded that either natural selection favouring draughtability was operating since evolution of these breeds or long-term conservation strategies followed by State and Central Governments over the decades which would have led to increased number of

all indigenous cattle breeds of Tamil Nadu from the genetic bottleneck.

SUMMARY

The present study was carried out to validate the single nucleotide polymorphism (SNP) in mitochondrial gene-cytochrome b oxidase at 14,716 bp (C to T transition) position obtained from whole genome sequence data of indigenous cattle breeds at Department of Animal Genetics and Breeding, Madras Veterinary College, Tamil Nadu. A total of 288 animals, viz. Alambadi (16), Bargur (99), Kangayam (54), Pulikulam (58) and Umblachery (61) were genotyped. The wild type allele C was observed to be fixed in all the breeds except Kangayam cattle. The gene frequency of C and T alleles and genotype frequencies of CC, CT and TT were estimated in Kangayam breed of cattle and chi-square test revealed the population in Hardy-Weinberg disequilibrium at CYTB locus. Phylogenetic analysis showed that the indigenous cattle breeds were present under the same genetic ancestry of *Bos indicus* reference genome, while the *Bos taurus* reference genome diverged separately. From this study, the possibilities of selective sweep or population expansion after a recent bottleneck among the indigenous cattle breeds of Tamil Nadu could be concluded.

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