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# Mitochondrial DNA polymorphisms in Nepalese Achhami cattle

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## Abstract

Achhami cattle is claimed to be the world's smallest cattle which is found in Achham district of Sudur Paschim Province of Nepal. A study was carried out to investigate the polymorphism in the control region of the mitochondrial DNA polymorphism in these Achhami cattle. Thirty-seven blood samples were collected from different pocket areas of in Achham district. Our study revealed that Achhami cattle lie significantly within the indicine haplogroups rather than taurine (34 out of 37 samples) manifesting the later introgression by taurine cattle population. The taurine haplogroup, found within Achhami was different than Lulu cattle, which might be from the independent domestication event. Within indicine haplogroup, I1 type haplogroup (64.7%) was found dominant over I2 type haplogroup (35.3%). Achhami cattle revealed its uniqueness as it segregates from Indian cattle for indicine type as well as Chinese cattle for taurine type. In order to understand its ancestry, the whole genome should be studied together with the consideration of more population of cattle from the Asian region.

**Key words:** mitochondrial DNA, haplotype types, origin, Achhami cattle, Nepal

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## Introduction

Nepal, being rich in animal genetic resources both in terms of diversity and numbers, is one of the countries with the highest livestock density per unit of cultivated land (Ablington, 1992; Ghimire, 2011). Achhami breed of cattle is one of the most notable indigenous cattle breeds of Nepal. It is a zebu breed (*Bos indicus*) originated in Achham district from the far western region of Nepal and distributed in Bajhang, Bajura, and Doti districts of the same region. It is claimed to be the smallest breed of the world less than one meter in height at the withers (Epstein, 1977; Neopane and Pokharel, 2005). They are suitable for the hilly region and can be reared in very low input systems and used mainly for milk production. The status of Achhami cattle was categorized under endangered criteria for domestic animal genetic resources developed by IUCN (Neopane and Pokharel, 2005). The cattle are now raised only for the sake of the social values of the animal (Mahato and Gorkhali, 2009). Due to the likelihood of the extinction of the pure Achhami cattle population, Nepal Government has prioritized the conservation of this breed. So far, the study of the Achhami cattle population has only been carried out at phenotypic and chromosomal level (Dhakal, 2008). Genetic characterization of the unique genetic materials possessed by the breed may be an important parameter to set the priority of conserving this breed (Gorkhali *et al.,* 2020).

Mitochondrial DNA (mtDNA) is instrumental in identifying maternal inheritance and is highly variable within species. Specifically the control region of mtDNA evolves very rapidly compared with nuclear DNA. Furthermore, it also tells the recent demographic processes acting on the populations (Bruford *et al.,* 2003). Mitochondrial DNA has been widely used to explore the maternal origin of the cattle by various researchers. A mitochondrial DNA (mtDNA) studies demonstrate that the Asian cattle may have three origins: *Bos taurus*, *Bos indicus* and *Bos grunniens*, of which *B. taurus* and *B. indicus* have the major influence (Yu *et al.,* 1999). Some molecular work has been done in Lulu cattle (Rana, 1996; Takeda *et al.,* 2004), which is known as the only humpless cattle (*Bos taurus*) in Nepal, whereas very little is known about Achhami. Unlike the metacentric Y-chromosome of *Bos taurus* were detected in Lulu cattle, Achhami harbored submetacentric most probably telocentric Y-chromosomes of *B. indicus* (Dhakal, 2008). Inadequate information is the hindrances for the development of strategies for the utilization and conservation of these cattle.

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Mitochondrial DNA (mtDNA) substitutions in cattle are widely used for the elucidation of evolutionary relationships (Bradley *et al.,* 1996; Loftus *et al.,* 1994; Mannen *et al.,* 1998). Loftus *et al.* (1994) have provided molecular evidence for a pre-domestic divergence between European and Indian cattle using mtDNA Displacement (D)-loop variation. In this present study, we investigated the mtDNA control region polymorphisms in Achhami cattle to gain deeper insight into diversity within the breed.

## Materials and Methods

### Sample collection

Blood samples of thirty seven animals were collected from four pocket areas of Achhami cattle namely Baijanath, Mastamndu, Ghugurkot and Jalpadevi in Achham district. Samples were collected from cattle judged to be true to type with the phenotypic characteristics of that population. The individuals selected had unrelated parents and grandparents based on the information provided by owners and also were cross-checked with their neighbors. Blood samples collected in tubes containing EDTA and were stored at -40$^{o}$C until further processing.

### Amplification, purification and DNA sequencing

Genomic DNA was extracted by standard phenol/chloroform method (Köchl *et al.,* 2005). The complete D-loop was amplified by using forward primer L15737: 5'-CTGCAGTCTCACCATCAACC-3' (Loftus *et al.,* 1994) and reverse primer H992: 5'-GATTATAGAACAGGCTCCTC-3'. The numbers in the primer name indicate the homologous positions of the primers 3' end on the mtDNA complete sequence of *B. taurus* (Anderson *et al.,* 1982). L and H refer to the light and heavy strands of the mtDNA respectively. Both primers for amplification and an internal primer (L16161: 5'-AATTACCATGC CGCGTGA-3') were used for sequencing. PCR was performed in a 50 µL reaction mixture containing Nuclease-free water (16 µl per reaction, for a final volume of 50 µl), PCR Master Mix (25 µl per reaction), primers (2 µl of each 10 µM primer working solution per reaction), and sample DNA (5 µl of 100 ng of genomic DNA) following 35 cycles of 1min at 94 °C, 30 s at 58 °C, and 90 s at 72 °C. Nine hundred twenty-one (921) base pairs of the control region of mtDNA (D-loop) were directly amplified and sequenced. The sequences were deposited in the GenBank under accession GenBank MT348396-MT348431, MT332098-MT332099.

### Sequence analysis and polymorphism detection

Nine hundred twenty-one base pairs from complete D-loop from each of the 37 Achhami cattle were edited using *Chromas* version 2.23 and aligned with the established representative reference sequences for cattle (Anderson et al., 1982) (Indicine: GenBank accession number: L27733.1, haplogroup I1 (NCBI Accession number: NC_005971, AY126697, EU177868) and haplogroup I2 (NCBI Accession number: EU177869, EU177870, AF492350) (Achilli *et al.,* 2008; Hiendleder *et al.,* 2008) and taurine: GenBank accession number: V00654.1) using Cluster *W* included in the program *MEGA* Version 5.0 (Tamura *et al.,* 2011). The published sequences for cattle including *Bos indicus*, *Bos taurus* and *Bos grunniens* from different regions and bordering countries were chosen from the NCBI GenBank for comparison and to determine the possible gene flow into the Achhami cattle. Some sequences of yaks (*Bos grunniens*) were also considered in order to understand the introgression of yak gene into Achhami cattle and to know the ranges of habitat of the Achhami cattle.

**Table 1:** Genebank sequences from Asian countries

| Country | Accession number | References |
|---|---|---|
| China | EU281346–EU281543, EF524120– EF524185;<br>AY521076–AY521098, AY521100–AY521136, AY902382–AY902405 | (Jia *et al.,* 2010)<br>(Lai *et al.*, 2006) |
| Tibet, China | AY378114 - AY378120;<br>EU281505 - EU281511 | (Cai *et al.,* 2007)<br>(Qi *et al.,* 2010) |
| Nepal | AB065125, AB065126, AB065128, AB085921 - AB085923;<br>L27722, L27723;<br>AB085925, AB065119 - AB065124, AB065129 - AB065131 | (Takeda *et al*., 2004)<br>(Loftus *et al.,* 1994)<br>(Fujise *et al.,* 2003) |
| India | AY378133 – AY 378137<br>L27722, L27723, L27733, L27732, DQ887765 | (Sharma *et al.,* 2015)<br>(Loftus *et al.,* 1994) |
| Yak | KM658599.1, KJ463418.1,MK124955.1,MN398192.1 | (Chu *et al.,* 2016) |

**Phylogenetic reconstruction**

The genetic distances among the control region sequences were estimated (Tamura & Nei, 1993). Unrooted neighbor-joining (NJ) tree was constructed by MEGA Version 5.0 package (Tamura *et al.,* 2011). Nucleotide diversity (Pi) within cattle breed and haplotype diversity (Hd) of breed were performed in DnaSP Version 3 (Rozas & Rozas, 1999).

## Results

***Genetic diversity***

A total of twenty-four haplotypes found in thirty-seven samples of Achhami cattle and the haplotype diversity was 0.965±0.016; both revealed high diversity within the population. Nucleotide diversity was very low in the population (0.01199 ± 0.00297). The mtDNA control region HVI sequences were found to be highly polymorphic with fifty-four variable sites across the 921 bp of the alignment.

***Haplogroup classification and phylogenetic analysis***

The Neighbor-Joining (NJ) tree drawn against reference sequences showed that out of thirty-seven individuals, three were found to be closer to taurine type and the majority (thirty four) were indicine type. The phylogenetic analysis indicated that the 37 mtDNA sequences fall in two distinct lineages: *Bos indicus* and *Bos taurus* (Figure 1). Most of the mtDNA haplotypes (91.9%, n=34) showed tendency to cluster together to *Bos indicus* and distinction was discernible into zebu sequences and 8.1% type (n=3) showed zebu phenotypes with *mtDNA Bos taurus*. This result indicates that cattle mtDNA profile are not always indicative of their phenotypes (Srirattana *et al.*, 2017) which is also true for Nepalese Lulu cattle (Takeda *et al.*, 2004). The tree reported that Achhami cattle already segregated from the wild origin, B. taurus (Aurochs) accession no: NC 013996.1 and B. indicus (Banteng) accession no: AF 162489.1, resulting distinct clade. The NJ tree drawn against yak populations revealed that none of the individuals were close to any of the existing yak populations, the other lineage of cattle found in Nepal.

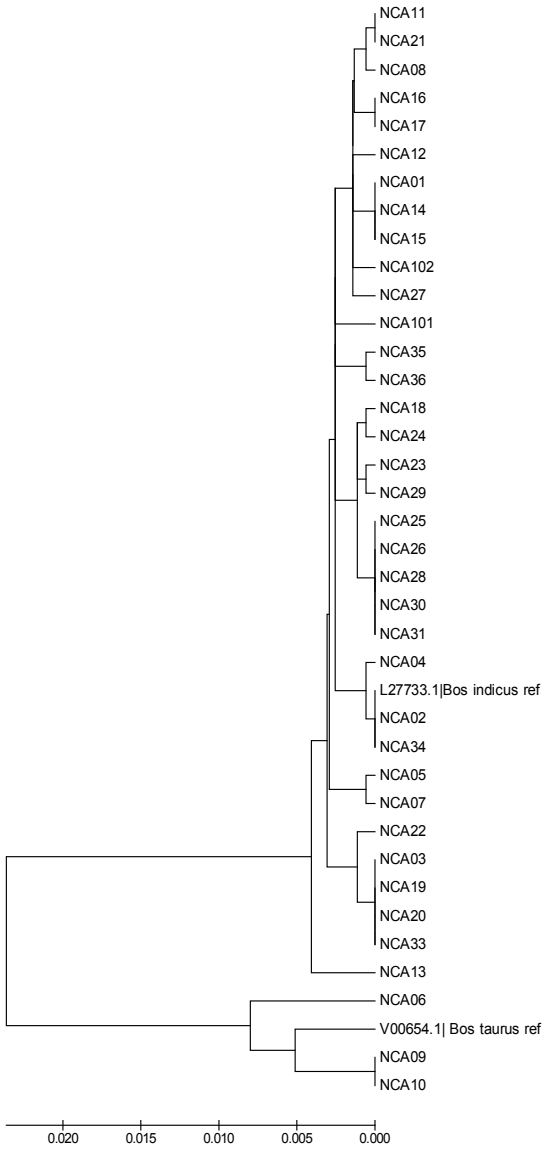


**Figure 1:** The phylogenetic tree of the 37 Achhami cattle mtDNA D-loop region sequence constructed with reference sequence for *Bos indicus* and *Bos taurus*

***Variation in the maternal lineage***

The *Bos indicus* type (n=34) were then analyzed for the presence of different haplogroups in the presence of reference sequences for haplogroup I1 and haplogroup I2 *( Lai et al., 2006, Wang et al., 2016)* both haplogroups for *Bos indicus* type, I1 and I2, were found in this population (Figure 2). Among them, haplogroup I1 was the predominant (64.7%, n= 22) group followed by I2 (35.3%, n= 12). The result thus exhibits no uniqueness in the Achhami cattle in terms of haplogroup diversity.

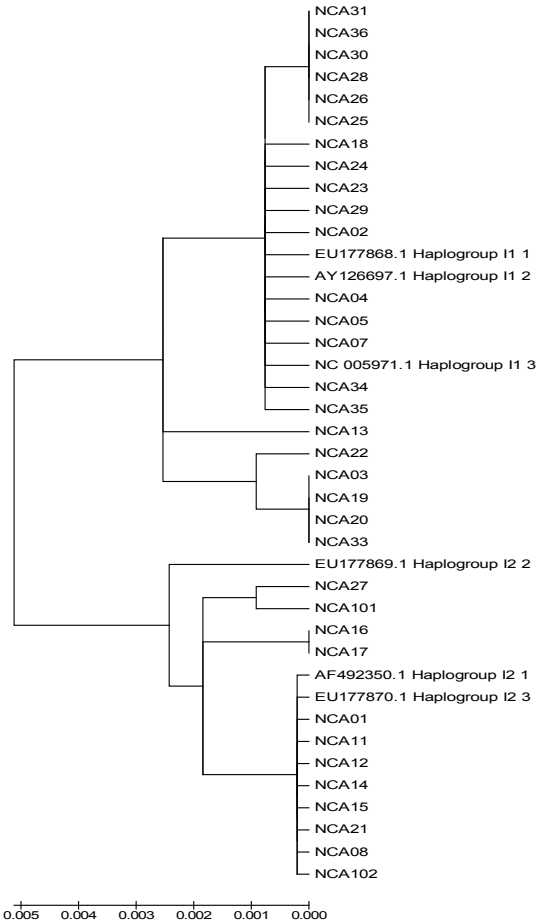


**Figure 2:** Phylogenetic trees of the 34 Achhami cattle mtDNA dloop region sequences which fall on Bos indicus and reference sequences for haplogroups I1 and I2

***Variation in Achhami cattle and other cattle breeds in Asian Region***

The sequences were then aligned and analysed with the available gene bank data of the Chinese cattle breed of both taurine and indicine type. The result suggested the pairing of indicine type sample with Indian breed and pairing of taurine type sample with Chinese, Chinese/Tibetan and Nepalese breed (Lulu).

## Discussion

In this study, we conducted a phylogenetic analysis of Achhami cattle to obtain information about the genetic origin. Achhami cattle population was majorly identified as indicine mtDNA. I1 haplogroup was dominant over I2 haplogroup in indicine type. This result supports that Haplogroup I1 is dominant haplogroup in Asian cattle population while I2 is localized mainly in Nepal, India and South west China (Yunnan, Guizhou and Tibet) suggesting the northward dispersal of I2 haplogroup from Indian and Nepal cattle in Asian continent (Jia *et al.*, 2010; Srirattana *et al.,* 2017). Furthermore, Haplotype I2 is proven to be more ancient clade than I1 (Jia *et al.,* 2010) and the present result is concurrent with the Indus Valley claimed to be the zebu domestication centre (Chen *et al.,* 2010). Most of the cattle in the south Asian region are zebu cattle (Bos indicus) which are suitable for hot tropical climate having heat resistance and disease resistance.

Achhami cattle also demonstrates the presence of taurine mitochondria in the physiological zebu background of Achhami cattle (B. indicus), illustrating a discordance between phylogenetic relationships based on morphology and mtDNA. The phenotype of cattle is therefore not always related to their mtDNA profile (Srirattana *et al.,* 2017). There have been an increasing number of incidences in the literature of discordances between mtDNA and population phyologenies (Loftus *et al.,* 1994; Yu *et al.,* 1999). These discrepancies usually result from one or a number of the following factors: evolutionary rate heterogeneity, secondary introgression from another species, the stochastic loss of mitochondrial lineages or the recent hybridization with the taurine breed in order to adapt in the lower altitude and increase the production. Some domesticated cattle breeds developed from a hybrid of *B. taurus* and *B. indicus* because the two subspecies can interbreed freely *(Kikkawa et al.,* 2003). Achhami cattle breed, however, might not be the admixture of *Bos taurus*, *Bos indicus* and *Bos grunniens* as each breed is dominated by one origin (Yu *et al.,* 1999) and unlike Nepalese Lulu cattle, it does not follow the transhumance farming system to hybridize with different indicine and taurine cattle and yak depending on their movement to various elevations (Takeda *et al.,* 2004). This is also proven by the separate clade made between Achhami cattle, Lulu cattle and Yak mtDNA sequences and also with existing wild cattle, Bateng. Nonetheless, these cattle inhabited in inaccessible remote area where there is less chance to cross these cattle with high yielding exotic cattle and in addition, people in this area are poor and farming system is based on traditional knowledge.

Besides maternal origin, the phylogenetic analysis of Achhami cattle also revealed various information regarding its genetic diversity and possible gene flow. Achhami cattle mtDNA exhibited the presence of larger genetic diversity within the small population group since this breed consists of both *B. taurus* and *B. indicus* types mtDNA (Lai *et al.,* 2006).

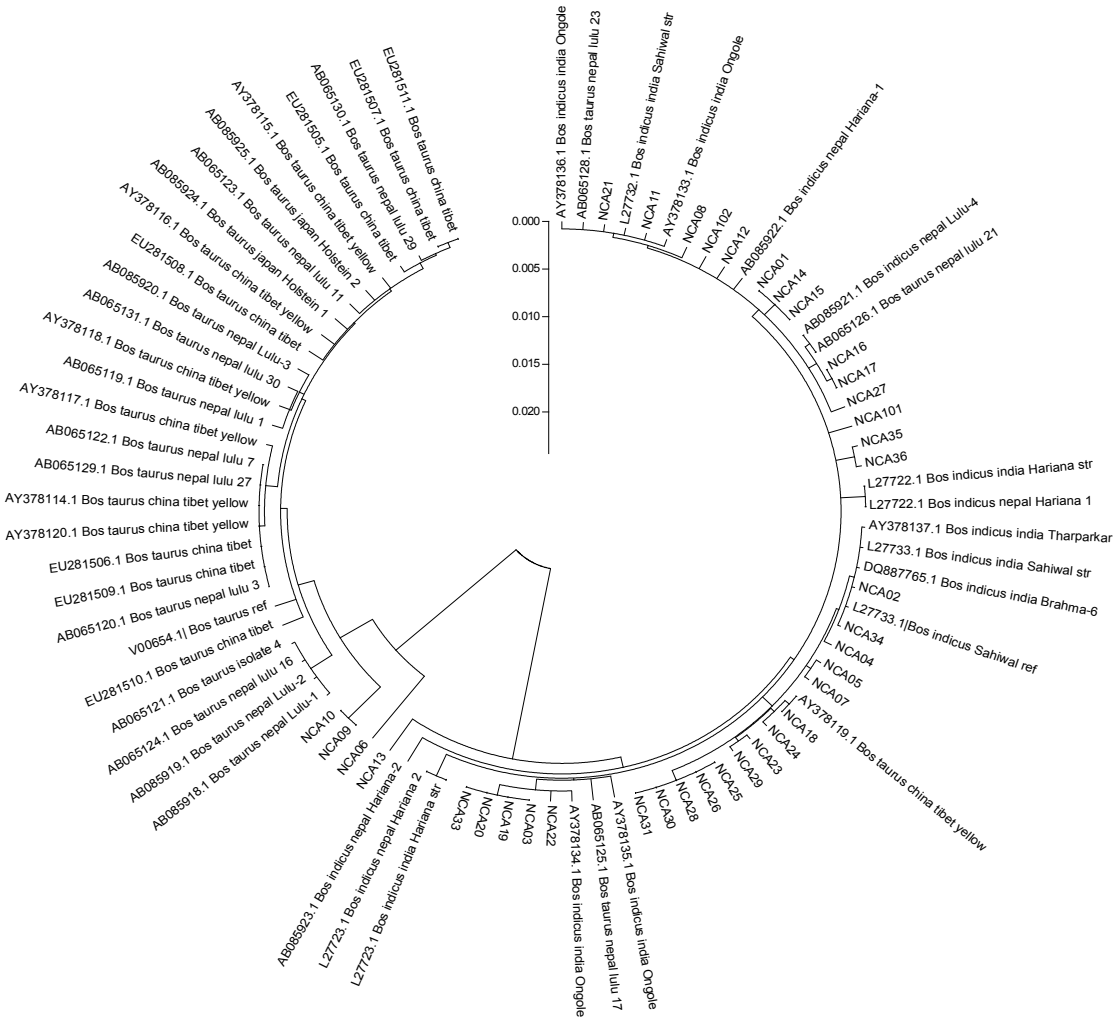


**Figure 3:** Phylogenetic trees of the 37 Achhami cattle mtDNA dloop region sequences with the sequences from cattle of China, Tibet, India and Nepal (Lulu)

## Conclusion

This study identified the Nepalese Achhami cattle in the far western region of Nepal were normally indicine cattle which might have assisted in upward dispersal of indicine gene to the Chinese cattle in Southern China. A research on Y-chromosome polymorphism is warranted to understand the real scenario of the ancestry of this cattle population. Further researches using advanced technology will help to clearly detail the genetic makeup and potential of Achhami cattle.

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## Conflict of interest

The authors have no conflict of interest to declare.

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