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Short Communication

EXTRA NUCLEAR DNA CONTROL REGION AND CYTOCHOME B GENE BASED PHYLOGENY OF KAIL SHEEP BREED OF AZAD JAMMU AND KASHMIR: IMPLICATIONS TOWARDS CONSERVATION

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ABSTRACT

In the present study, we analyzed two mitochondrial DNA (mtDNA) segments i.e. partial (564 bp) control region (D-loop) and complete (1140 bp) *Cytochrome b* (*Cyto b*) gene in Kail sheep breed raised in Azad Jammu and Kashmir (AJ&K). The phylogenetic analysis of partial mtDNA (564bp) control region revealed two distinct mitochondrial maternal lineages (haplo-groups A and B). However, the haplogroup A was the major lineage found in domestic Kail sheep. The phylogenetic pattern developed from the mtDNA control region was confirmed by the analysis of complete *Cyto* b. This study confirmed the results of previous reports in the region that the mitochondrial maternal lineages A and B are predominantly found in domestic sheep breeds in Asian countries. Furthermore, this preliminary study provides useful information of phylogenetic analysis and future conservation strategies of Kail sheep in the AJ&K region.

Key words: Mt DNA control region, Cytochrome b, Haplogroups, Phylogeny, Kail Sheep, Azad Jammu and Kashmir (AJ&K).

INTRODUCTION

Kail sheep is an important breed among Kajli, Pahari, and Poonchi sheep of Azad & Jammu Kashmir (AJ&K) area. AJ&K contributed 0.22 million of sheep population out of 29.1 million sheep in Pakistan (Economic Survey, 2013-14). Kail sheep is reared in Neelum and Lipa valleys of AJ&K mostly for mutton and wool production. Livestock is the main source of income in the elevated areas that are more spread out and majority of the rural population depends on it. Due to recent increase in the population of this region, the preferment in the livestock sector is being indispensable to furnish meat and wool for survival.

There are some studies conducted with focus on the phenotypic, genetic and phylogeographical characteristics of the sheep breeds in the Pakistan (Ahmed *et al*., 2014, Babar *et al*., 2014, Wajid *et al.*, 2014). To evaluate the genetic diversity in Kail sheep, we investigated the partial mitochondrial DNA (mtDNA) control region and entire Cytochrome b (*Cyto* b) gene sequences. Mitochondrial DNA (mtDNA) is a significant marker that has been extensively used in molecular evolutionary studies, population genetic analysis, and classification of animals (Wajid *et al*., 2013).

To date, five phylogenetically divergent mtDNA maternal lineages, A, B, C, D, and E, has been identified in various domestic sheep breeds from large geographical dispersed locations in all over the world (Meadows *et al*., 2011). The two most frequently ovine haplogroups are A and B found in every geographic region where sheep are domesticated. The haplogroup A is predominantly identified in various sheep breeds in Asian countries like Pakistan, China, India and Middle East. Haplogroup B is more recurrent in European domestic sheep (Meadows *et al.,* 2007). The genetic studies based on mtDNA control region revealed that lineage B type sheep might have been originated from European mouflon (*Ovis musimon*) (Hiendleder *et al*., 2002). While no wild ancestor of lineage A has been recognized to date, and no evidence has been determined to support that Urial and Argali wild sheep might be the originators (Meadows *et al.,* 2011), however according to a recent study the relationship of domestic sheep and Urial has been explained (Hussain *et al.,* 2015) . Heplogroup C is the third most widespread and has been reported from Middle East and Asia, Caucasus, Iberian Peninsula and Fertile Crescent and in a low frequency from native sheep of Portugal (Tapio *et al*., 2006). The two recently identified haplogroups D and E are originated only in Turkey and Caucasus regions (Meadows *et al*., 2007; Tapio *et al*., 2006). Partial sequences of the mitochondrial D-loop region have been used to explain the origin and mtDNA lineages pattern in domestic livestock species.

This study was designed to examine the genetic diversity and phylogentic analysis of indigenous Kail sheep breed in AJ&K. The phylogenetic analysis was performed to examine the maternal lineage in Kail sheep using two mitochondrial segments, partial mtDNA control region supported by complete *Cyto* b gene sequences. This study would aid in future in designing

proper breeding strategies for conservation and better utilization of this important genetic resource of AJ&K.

MATERIALS AND METHODS

In the present study, we investigated the Kail sheep of AJ&K based on mtDNA control region and Cytochrome b gene. Five mL blood was collected from six unrelated Kail sheep from their home tract in EDTA containing tubes and frozen in -20°C until DNA extraction. The genomic DNA extraction was carried out as previously described by Hussain *et al*. (2013). The extracted DNA was quantified and stored in -20°C for further use.

To amplify the partial mtDNA control region (564 bp) and complete *Cyto b* gene (1140bp), the specific primers were designed and used as described in Table 1. All the primers were optimized and the PCR reactions were done in 25 µl total volume containing genomic DNA (50 ng), 10 pmol both forward and reverse primers, 5x $(NH_4)_2SO_4$ buffer, 100 µM dNTPs mix, 1.5 mMol $MgCl_2$ and 5 units of *Taq* DNA polymerase. The PCR reactions were completed in thermocycler (Bio-Rad) with the conditions: First initial denaturation 95 ºC for 5 minutes, and then followed by 35 cycles with denaturation at 95 °C for 30 sec, annealing ranged 60-50 °C, decreasing with -1.0 per cycle for 30 sec, extension at 72 °C for 45 sec, followed by final extension at 72 °C for 10 minutes. The products were run on 1% gel electrophoresis and purified through alcohol precipitation method. The sequencing was performed using automated Genetic Analyzer 3130*XL* (Applied Biosystems). Nucleotide sequences of six Kail sheep have been deposited in GenBank under accession number of KU569711-KU569716 for control region and KU569705-KU569710 for *Cyto b* gene.

The sequences were aligned and edited through BioEdit Version 7.0.9.1 software (Hall, 1999). DnaSP version 5 was used for data analysis including haplotype diversity and nucleotide diversity (Librado and Rozas, 2009). A phylogenetic tree was made using the Neighbor-Joining method in MEGA-6 software employing the Kimura 2-parameter model of nucleotide substitution and 1000 bootstrap replications (Tamura *et al*., 2013). The phylogenetic analysis was performed by using 481 bp fragment of the first hypervariable segment (HVI) of control region. The reference sequences from five previously identified haplogroups namely A, B, C, D, and E were used as observed by Meadows and colleagues (2007, 2011) in mtDNA partial control region and *Cytochrome b* gene. With all the reported sequences from each lineage A, B, C, D and, E retrieved from GenBank NCBI-USA inferring the haplogroup determination.

RESULTS AND DISCUSSION

Current investigation is the preliminarily report of genetic study of AJ&K sheep breed by analyzing mitochondrial D-loop and *Cyto b* gene. The genetic study of Kail sheep was previously reported by Ahmed *el at.*, (2014) using microsatellite DNA markers. The present study was to assess the genetic diversity of Kail sheep and to obtain the useful information on their maternal origin. To infer the phylogenetic analysis, the study had included mtDNA sequences of other domestic and wild sheep breeds as well. In the study, the mtDNA highly variable segment of control region was analyzed in order to study the diversity and phylogeny of Kail sheep. The complete *Cyto b* gene of mtDNA was also analyzed to reconfirm the phylogenetic pattern developed by control region. Genetic diversity in term of haplotype number, haplotype and nucleotide diversities was calculated using bioinformatics tools. In the study, 564 bp long mtDNA control region in six Kail sheep samples were sequenced and analyzed that revealed 23 polymorphic sites of which 18 were singleton while the remaining 5 were parsimony informative. Total three haplotypes were observed in six mitochondrial control region sequences with haplotype diversity 0.0733 ±0.155 and nucleotide diversity was observed as 0.01548 ±0.00676. The most frequent haplotype was observed in three individuals. Similarly the complete *Cyto b* gene (1140 bp) was sequenced of six samples to reinforce the genetic diversity level and the phylogenetic pattern developed by the mtDNA control region. Five variable sites (amino acid changes) were observed in *Cyto b* gene sequences in all sequences. All the variable sites were singleton in nature. A total of four haplotypes were identified in six *Cyto b* sequences. The haplotype diversity was observed 0.800±0.172 while nucleotide diversity was 0.00146±0.00055.

The phylogenetic tree using Neighbor-Joining method based on partial mtDNA control region (481bp) of Kail sheep rooted with wild sheep (Urial) revealed two mitochondrial lineages A and B (Figure 1). The haplogroup A was predominantly found in Kail sheep as compare to haplogroup B with a single individual. Other haplogroups C, D, and E were not detected in Kail sheep. Our results confirmed the results of earlier studies that the haplogroup A is predominantly distributed in Asian countries (Meadows *et al*., 2007). The similar conclusion was supported by phylogenetic analysis through complete *Cyto* b gene sequences (1140bp), validating the phylogenetic status (Figure 1B). All the sequences were clustered with haplogroup A except a single haplotype H3, which fall in haplogroup B. Both phylogenetic trees showed the consistent clustering pattern of Kail sheep isolates. The limitation of the study was the unavailability of haplogroup report for comparison in any other sheep breeds of AJ&K. However, the presence of two mtDNA lineages (i.e. haplogroup A and B) is characteristic of

Kail sheep, which is in agreement with other studies suggesting the both lineages are found predominantly in Asian population.

The genetic data of Kail sheep is potentially significant for future conservation and management program for this important sheep breed of AJ&K. This breed is not only a valued reservoir of unique diversity, but also plays an important role in economics of local people. This study would pave a path for further DNA based studies on Kail sheep for better understanding of its genomic architecture.

Table 1. List of primers used for the amplification of partial mtDNA control region and complete Cytochrome b gene

Region	Primers	5'-3' Sequences	Product
mtDNA control region	D-loop-1	Forward:CCAGAGAAGGAGAACAACCAA Reverse: GGGTATTAAACTGCTTGACCG	658
mtDNA Cytochrome b	*Cyto b*-I	Forward: CATGGAATCTAACCATGACCAA Reverse: CTCTTCCTCCACGAAACAGG	676
	Cyto b-II	Forward: CGATTTTTCGCCTTTCACTT Reverse: GAAGGAGAACAACCAACCTCC	677

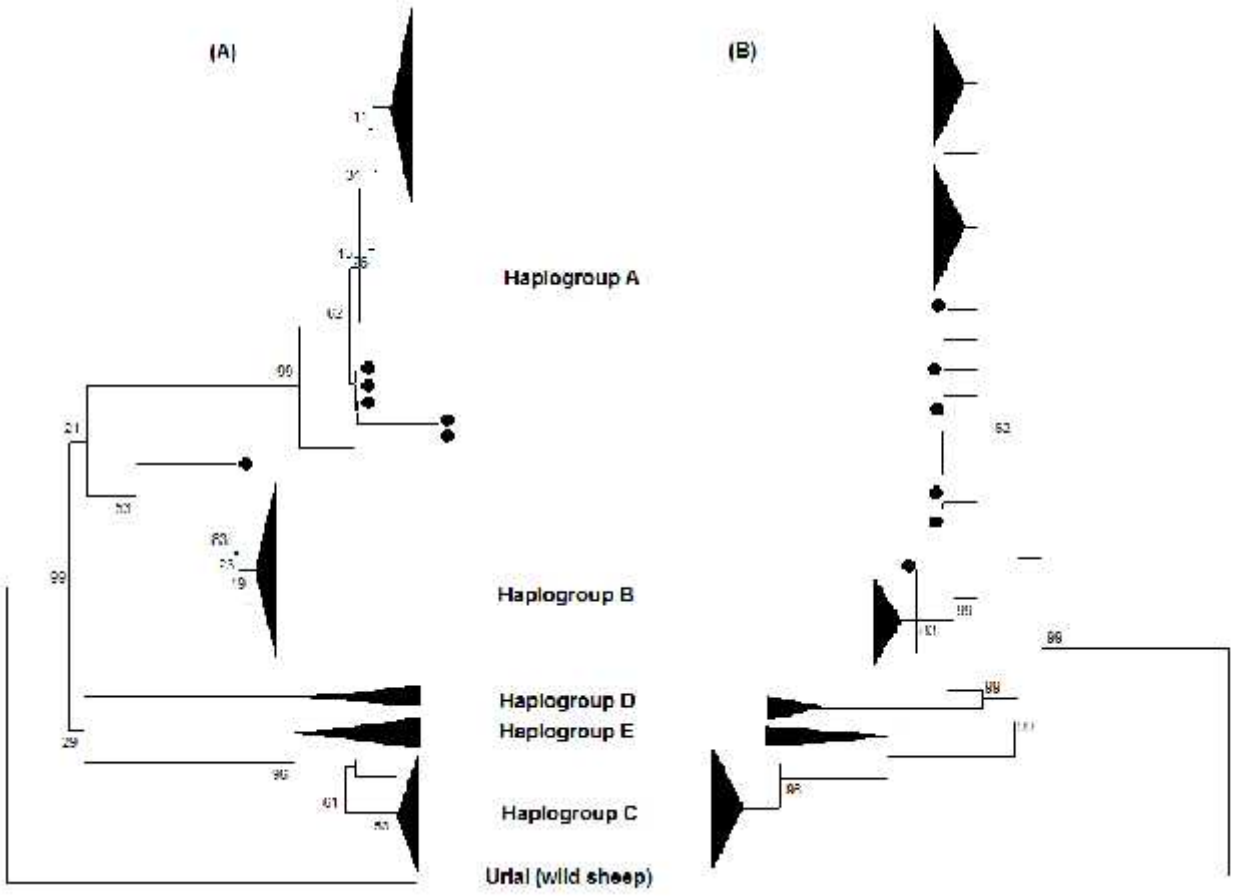

Figure 1. Neighbor-Joining tree of Kail sheep based on partial mitochondrial control region (481bp) (A), and complete Cytochrome b gene (1140 bp) (B) constructed by MEGA6.1. Both figure showed compose of Kail sheep with sequences of five internationally reported lineages i.e. A, B, C, D, and E. The Kail sheep sequences are represented in black circle. The sequence of wild sheep (Urial, *Ovis vignei*) has been used as outer group.

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