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Mitochondrial DNA Variation in Indigenous Sheep (*Ovis aries*) Breeds of Nepal

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ABSTRACT: *Nepal borders India in the south and China in the north. Four distinct indigenous domestic sheep breeds, i.e. Bhyanglung in the alpine region, Baruwal in the high hills, Kage in the mid hills and Lampuchhre in the low lands are distributed in the country. In this study, the mitochondrial DNA control region of 111 sheep from these four breeds was directly sequenced to determine their genetic variations and phylogenetic relationships. High mitochondrial DNA diversity among these breeds was observed and all haplotypes were classified into three haplogroups (A to C). Among the four breeds, three residing in middle to high hills had all three haplogroups while Lampuchhre sheep in low land only carried haplogroups A and B. This study revealed that a south-western route of gene flow in sheep might have come from China to India via Nepal. It can be concluded that these indigenous sheep breeds have isolated breeding paths. Lack of crossbreeding among Nepalese sheep breeds is unique, and it is important for the decision making on utilization and conservation of Nepalese sheep genetic resources.*

Keywords: *Control region, mitochondrial DNA, Nepalese indigenous breed, sheep (Ovis aries)*

INTRODUCTION

Domestic sheep (*Ovis aries*) have played a significant role in the economy of small and marginal farmers, especially in developing countries, as they are a potential source of meat, wool, milk, hide and manure. Even though Nepal is a small country with a territory of 147,181 square kilometres; it has a wide range of climates, mainly affected by different altitudes. Following the variations in agro-ecological zones and climates, Nepalese sheep have evolved into four different breeds: Bhyanglung in the alpine region (3000 masl to 5000 masl), Baruwal in the high hills (2000 masl to 3000 masl), Kage in the middle hills (600 masl to 1500 masl) and Lampuchhre in the low lands (sea level to 600 masl). These breeds are phenotypically distinct from each other (Neopane *et al.*, 2008).

Diversity of mitochondrial DNA (mtDNA) has been widely used to assess the origin, phylogeny and population structure of sheep breeds all over the world (Hiendleder *et al*., 1998a, 1998b, 2002; Meadows *et al*., 2005, 2007; Pedrosa *et al.,* 2005, 2007; Pereira *et al*., 2006; Tapio *et al*., 2006; Oner *et al*., 2013), specifically in China (Guo *et al*., 2005; Luo *et al*., 2005; Chen *et al.,* 2006; Wang *et al*., 2007; Sulaiman *et al*., 2011; Zhao *et al*., 2011) and

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India (Pardeshi *et al.*, 2007; Arora *et al.,* 2013; Singh *et al.*, 2013). In contrast, information on phylogeography of Nepalese sheep mtDNA is scarce. Some investigation has been done on the morphological characteristics of Nepalese sheep breeds (Tsundoda *et al.*, 1989; Neopane *et al.,* 2008) and also on the genetic relationship between native sheep breeds based on blood protein typing and karyotyping (Dohge *et al.*, 1989; Tsundoda *et al.*, 1989), which revealed a relatively high genetic variability within the breeds. Nevertheless, genetic studies have indicated that morphological characteristics of livestock breeds may provide incomplete or misleading information on their evolutionary history (Lei *et al.*, 2004). The variable structure of control region of mtDNA makes it possible to solve the problem of genetic polymorphism and origin, mainly because mtDNA displays a simple maternal inheritance without recombination and a relatively rapid evolution rate (Hiendleder *et al.*, 1998b). Haplotype and nucleotide diversity of mtDNA are two important indices for assessing genetic polymorphism and differentiation (Pereira *et al.*, 2006). Five maternal lineages (HapG A to E) have already been delineated in sheep mtDNA. Lineage HapG A was of Asian origin, HapG B predominated in Europe while HapG C, D and E originated in the Near East (Meadows *et al.*, 2007). To date, no mtDNA sequence has been reported from any Nepalese sheep breed. The objective of this study was therefore to investigate the genetic diversity and structure of four Nepalese sheep breeds using mtDNA control region sequence variation.

METHODOLOGY

Population sampling

Blood samples from four Nepalese sheep breeds (Bhyanglung, Baruwal, Kage and Lampuchhre) were considered for the study. Samples were collected from sheep that were judged to be true to type with the phenotypic characteristics of that breed. The individuals selected had unrelated parents and grandparents based on the information provided by the owners and also cross-checked with their neighbours. A total of 111 individuals from different locations were sampled (Figure 1) and the blood was stored at -40^{o}C until further processing. The details of breeds, geographic regions and sample sizes are given in Table 1.

Table 1. Four Nepalese sheep breeds from different geographic regions

Breed/population	Location	No. of samples sequenced
Bhyanglung	Alpine region (Mustang, Jumla)	29
Baruwal	High hills (Rasuwa, Lamjung, Jumla)	29
Kage	Mid hills (Kathmandu, Lalitpur, Kavre)	30
Lampuchhre	Low lands (Rupendehi, Siraha)	23
Total		**111**

DNA amplification and sequencing

Total genomic DNA was extracted from whole blood using standard phenol/chloroform extraction protocol followed by ethanol precipitation (Sambrook *et al.*, 2001). Eight hundred and seventeen base-pair (bp) long mtDNA control region of sheep was amplified using primers: 15388F (5'-GCC CCA CTA TCA ACA CCC AAA G-3') and CD-774R (5'-AAT GGG CGA TTT TAG ATG AGA TGG C-3') and sequenced using primers: CR018-for (5'-

ATC ATT ATC AAC GAT AC-3') and CR653-rev (5'-GAA GAA AGA ACC AGA TGC CT-3') following the procedure of Luo *et al.* (2005). Polymerase chain reaction (PCR) amplification was carried out in 50 µl reaction mixtures. The PCR thermocycling condition included an initial denaturing step at 95°C for 5 min followed by 35 amplification cycles (94°C for 50 s, 57°C for 60 s and 72°C for 60 s) and a final extension at 72°C for 10 min. Amplified mtDNA control region fragments were directly sequenced.

Analysis of sequence data

The raw sequencing profiles of mtDNA control region from each of the 111 sheep samples were manually edited using program *Chromas* version 2.23. Cleaned sequences were aligned using the Cluster *W* algorithm included in program *MEGA* version 4.0 (Tamura *et al*., 2007) to identify different haplotypes. Ten sheep mtDNA control region reference sequences belonging to the five known haplogroups (Meadows *et al*., 2011) were also included in the analysis, to facilitate the recognition of haplogroup status of each individual (Table 2). A neighbour-joining (*NJ*) tree was constructed for identified haplotypes together with all reference sequences based on the Kimura 2-parameter model implemented in the program *MEGA* 4.0. All gaps in the sequences were completely excluded from analysis. The robustness of internal branches was estimated based on 1000 bootstrap replications Haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987) for each sheep breed were also estimated using program *DnaSP* version 4.10 (Rozas *et al*., 2003).

Table 2. Reference sequences for different haplogroups of sheep mtDNA (HapG A to E) (Meadows *et al*., 2011)

HapG	GenBank accession no.
HapG A	HM236174.1, HM236175.1
HapG B	HM236176.1, HM236177.1
HapG C	HM236178.1, HM236179.1
HapG D	HM236180.1, HM236181.1
HapG E	HM236182.1, HM236183.1

RESULTS AND DISCUSSION

Sequence variation

The mtDNA control region sequences from 111 Nepalese sheep samples sequenced in this study had four one-nucleotide insertions/deletions (indels), all involving thymine nucleotide. Except these minor indels, the observed length variations in these fragments were caused by different copy numbers of a 76 bp tandem repeat (5'-CGT ATA TTA GTA TTA ATG TAA TAT AGA CAT TAT ATG TAT AAA GTA CAT TAA ATG ATT TAC CCC ATG CAT ATA AGC A-3'). Most of the Nepalese sheep mtDNA control region sequences (96.4%; 107/111) had four tandem repeats whereas only four sequences (3.6%; two from Baruwal and two from Kage) carried three tandem repeats and latter were excluded from further analysis. The remaining 107 sequences had 776 to 779 valid nucleotides excluding the two PCR primers. They were highly polymorphic with 132 variable sites, of which 36 were singleton variable sites (polymorphic sites appearing in only one animal) (27.3%) and 96 were parsimony informative sites (which appear in more than one animal) (72.7%). Out of total variable sites, 128 variants were transitions and four variants were transversions.

Haplogroup identification and phylogenetic analysis

Sixty four haplotypes were identified from 107 mtDNA control region sequences of the four Nepalese sheep breeds (Table 3 and Figure 2). More haplotype variation in mtDNA implies that more females than males have been used for breeding over time. This may be reflective of maternal inheritance as the selection for indigenous breeds is mostly male-oriented. This conclusion is also supported by the occurrence of high number of singletons in Nepalese sheep mtDNA sequences, suggesting the contribution from a large number of females carrying highly divergent mtDNA sequences (Hassan *et al.*, 2009).

Table 3. Haplotypes and their distribution in the four Nepalese sheep breeds

Haplotype	Haplogroup	Frequency	Breed			
			Bhyanglung	Baruwal	Kage	Lampuchhre
Hap_1	A	**5**		5		
Hap_2		7		7		
Hap_3		3		3		
Hap_4		1		1		
Hap_5		2		2		
Hap_6		1		1		
Hap_7		1		1		
Hap_8		1		1		
Hap_9		1	1			
Hap_10		1	1			
Hap_11		1	1			
Hap_12		4	4			
Hap_13		2	2			
Hap_14		2	2			
Hap_15		2	2			
Hap_16		2	2			
Hap_17		1	1			
Hap_18		1	1			
Hap_19		2	2			
Hap_20		5			5	
Hap_21		1			1	
Hap_22		1			1	
Hap_23		1			1	
Hap_24		1			1	
Hap_25		3			3	
Hap_26		1			1	
Hap_27		1			1	
Hap_28		1			1	
Hap_29		1			1	
Hap_30		1			1	
Hap_31		1			1	
Hap_32		2				2
Hap_33		2				2
Hap_34		1				1
Hap_35		2				2

Hap_36		1					1		
Hap_37		1					1		
Hap_38		1					1		
Hap_39		4					4		
Hap_40		1					1		
Hap_41		1					1		
Hap_42		4					4		
Hap_43		1					1		
Hap_44		1					1		
Hap_45	B	4			4				
Hap_46		1			1				
Hap_47		2	2						
Hap_48		1	1						
Hap_49		1	1						
Hap_50		1	1						
Hap_51		1				1			
Hap_52		1				1			
Hap_53		2				2			
Hap_54		1					1		
Hap_55	C	1			1				
Hap_56		1	1						
Hap_57		1	1						
Hap_58		1	1						
Hap_59		1	1						
Hap_60		1	1						
Hap_61		2				2			
Hap_62		1				1			
Hap_63		2				2			
Hap_64		1				1			

No haplotype was shared between breeds, suggesting that a high level of genetic diversity was present within each of these breeds. This unique pattern of haplotype distribution may also be attributed to total reproductive isolation due to harsh geographical structure of the country and unique husbandry practices (migratory farming system) in the alpine region and high hills of the country (Gorkhali *et al.*, 2006), that allow farmers to select animals with unique phenotypic characters associated with adaptability to different ecological conditions.

Using already identified (Meadows *et al.*, 2011) sheep mtDNA haplogroup reference sequences, three haplogroups (A, B and C) were identified among all indigenous Nepalese sheep (Figure 1 and Figure 2) with 44, 10 and 10 haplotypes, respectively. Among them, haplogroup A was predominant (68.8%) followed by haplogroups B (15.6%) and C (15.6%) (Tables 3 and 4). Except Lampuchhre, which had only haplogroups A and B, other three breeds possessed all three haplogroups (A, B and C). There was no specific haplogroup distribution pattern in breeds or different ecological regions. Haplogroup C sequences, which were first identified in Chinese sheep (Guo *et al.*, 2005; Luo *et al.*, 2005) and also detected late at low frequency in Middle Eastern and European sheep breeds (Pedrosa *et al.*, 2005, 2007; Pereira *et al.*, 2006; Tapio *et al.*, 2006; Oner *et al.*, 2013), were found in the three breeds distributed in the hills. This result suggested a probable gene flow from northeast to southwest but not reach the low lands of Nepal. Similar results were also observed in Indian

sheep, in which haplogroups A and B were predominant in most of the breeds whereas haplogroup C was at very low frequency (<1%) and only present in two breeds of

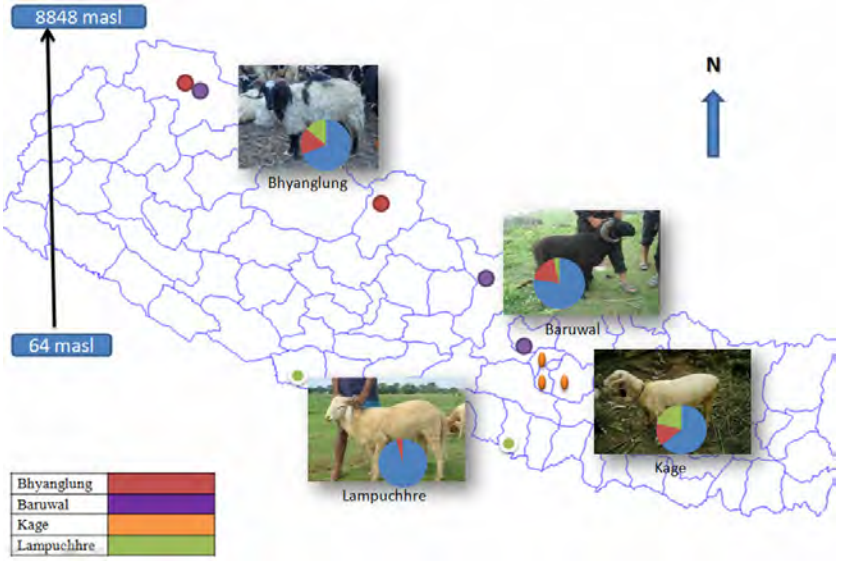

Fig. 1. Geographical distribution of samples and of the three mtDNA haplogroups A, B and C in Nepalese sheep breeds. Pie charts show the distribution of different haplogroups: HapG A (blue), HapG B (red) and HapG C (green)

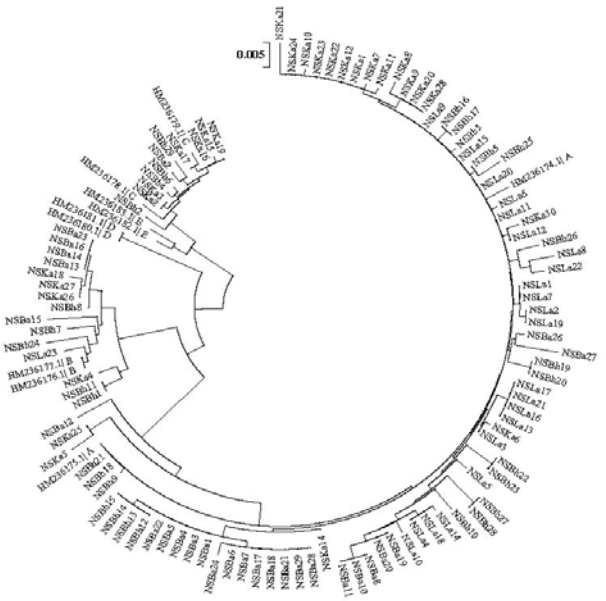

Fig. 2. The neighbor-joining phylogenetic tree constructed for 107 Nepalese sheep mtDNA sequences together with the 10 reference sequences belonging to the five known mtDNA control region haplogroups (A to E). All three major branches for lineages A, B and C were supported by > 99% bootstrap replications

north-western India (Pardeshi *et al.*, 2007; Arora *et al.*, 2013; Singh *et al.*, 2013). Nepalese sheep also showed a weak population sub-structuring as observed based on the distribution of three mtDNA haplogroups, which could have resulted from ancient trading between the neighboring countries and free borders between the adjacent vicinities (Gorkhali *et al.*, 2014).

Genetic diversity

The genetic diversity estimated on the basis of mtDNA control region sequences was similar among the four Nepalese sheep breeds. Number of haplotypes found in each breed ranged from 11 to 20 depending on the number of samples. The haplotype diversity ranged between 0.883 and 0.970 while nucleotide diversity ranged from 0.009 to 0.029 (Table 4). These observations were comparable with other sheep breeds/populations in the region (Chen *et al.*, 2006; Pardeshi *et al.*, 2007; Sulaiman *et al.*, 2011; Arora *et al.*, 2013; Singh *et al*,, 2013).

Table 4. Diversity and distribution of mtDNA haplogroups in Nepalese sheep breeds

Breed/ population	No. of haplotypes	Haplogroups			Haplotype diversity (h±SD)	Nucleotide diversity (π±SD)
		A	B	C		
Bhyanglung	20	19	5	5	0.970±0.017	0.029±0.004
Baruwal	11	21	5	1	0.883±0.036	0.022±0.004
Kage	19	18	4	6	0.958±0.024	0.029±0.004
Lampuchhre	14	22	1		0.941±0.030	0.009±0.003
Total	64	80	15	12	0.985±0.004	0.025±0.002

Overall haplotype diversity is high in Nepalese sheep. The high level of intra-population diversity and the weak geographical sub-structuring suggest that all three geographically independent domestication events for mtDNA HapG A, B and C have contributed to the genetic diversity of Nepalese sheep. Furthermore, this can be attributable to strong gene flow induced by historical human movements.

In general, the haplogroup prevalence in present study is consistent with the previous studies on domestic sheep breeds in Asia (Luo *et al.*, 2005; Chen *et al.*, 2006; Pardeshi *et al.*, 2007; Wang *et al.*, 2007; Zhao *et al.*, 2011; Arora *et al.,* 2013; Singh *et al.*, 2013). Since haplogroup A is most predominant in Asia, it is difficult to understand the direction of its gene flow. In case of haplogroup C, the gene flow from China towards India via Nepal can be an acceptable explanation as the gene flow of haplogroup C was from northeast towards southwest in the hilly areas but not to the low lands of Nepal.

CONCLUSIONS

Nepalese sheep revealed extensive and high mtDNA haplotype diversity. Haplogroups A to C were present in all the breeds found in low to high hills of the country except for Lampuchhre breed in low lands. The results also unveiled the origin of haplogroup C with a unique geographic distribution pattern, indicating its gene flow from China towards India via Nepal. Hence, it can be concluded that all indigenous Nepalese sheep breeds have isolated

breeding paths. Lack of crossbreeding among breeds is unique and important for the utilization and conservation of Nepalese sheep genetic resources.

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