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# Genetic Diversity and Phylogenetic Analysis of the mtDNA D-loop Region in Tibetan Sheep

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**ABSTRACT :** Seventeen haplotypes were detected from the complete mitochondrial DNA control region sequences analyzed from eighty individuals of two Tibetan domestic sheep breeds. The nucleotide composition of all the sequences was 33.0% A, 29.7%T, 22.9%C and 14.4%G; G+C was 37.3%. The length of the sequences ranged from 1,107 bp to 1,259 bp. The difference between them was primarily due to 3-5 copy numbers of a 75 bp tandem repeat sequence. The NJ phylogenetic tree (the number of replications of bootstrap test is 1,000) presented three major domestic sheep lineages, which suggested that modern Tibetan sheep breeds are derived from three maternal sources. (**Key Words :** mtDNA D-loop, Maternal Sources, Tibetan Sheep, Genetic Diversity)

## INTRODUCTION

Mitochondrial DNA is the genetic material that exists outside the nucleus of eukaryotic cells. It has a double-linked annular structure which is covalent and close. Including coding and non-coding regions, its length ranges from 15 kb to 20 kb in different species. The non-coding region is the control region. The rate of mtDNA evolution is about 5 to 10 times faster than nuclear DNA, and its genes do not usually recombine (Upholt et al., 1977). Animal mtDNA is deemed to strictly follow maternal inheritance and is highly variable within a species, so mtDNA is an important material for phylogenetic inference and for analyzing genetic diversity (Wolf et al., 1999).

The climate and landform of Tibet are different from other areas of China. Traffic from other parts of China is blocked, so the region's livestock are seldom influenced by external breeds. In this study we investigated two sheep breeds indigenous to Tibet which have not been studied or crossbred yet. By using the entire sequence information of the mtDNA D-loop, we analyzed the genetic diversity of two breeds and inferred their phylogenetic status. Therefore, the results are useful for the conservation and utilization of Chinese sheep genetic resources.

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## MATERIALS AND METHODS

### Sample collection and DNA extraction

Ear samples from eighty individuals (40 from each breed) of two Tibetan sheep breeds were collected from local Tibetan sheep production centers according to the simple random sampling method. The breeds were chosen from the Linzhou and Gangba areas of Tibet. DNA was extracted from these specimens using phenol/chloroform as described by Sambrock et al. (2001).

### Amplification, purification, cloning and DNA sequencing

The sequences of the primers for the mtDNA control region between $tRNA^{Phe}$ and $tRNA^{Pro}$ are forward: 5'CTCACCATCAACCCCCAAAGC3'; reverse: 5' TCATCTAGGCATTTTCAGTG 3' (Hiendleder et al., 2002). The PCR amplification reaction system consisted of genomic DNA 50 ng, dNTPs 200 μM, primers 10 pmol, $MgCl_2$ 250 μM, Taq DNA polymerase 1 U. The reaction profiles included an initial denaturation at 95°C for 5 min, followed by 30 cycles, each consisting of 30 sec denaturation at 94°C, 45 sec primer annealing at 55°C, 60 sec extension at 72°C, and then a final 8 min extension at

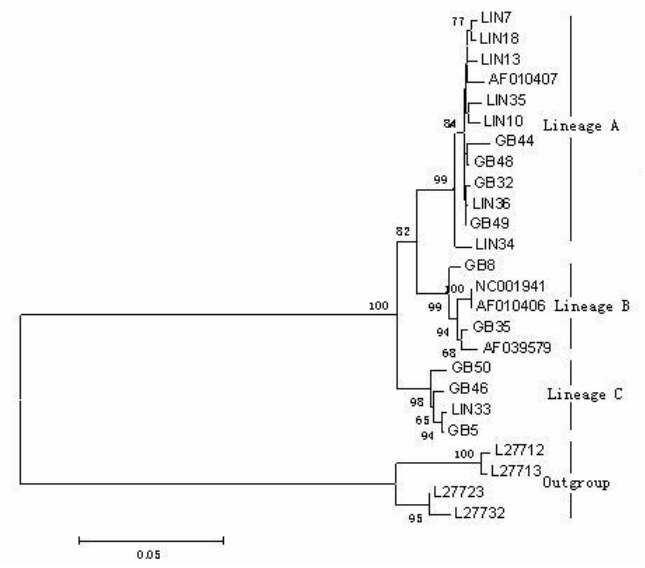


**Figure 1.** The NJ phylogenetic tree of all the mtDNA haplotypes of Tibetan sheep and a goat as an outgroup. LIN stands for Linzhou sheep, and GB for Gangba sheep. The numbers following LIN or GB are the individual number of the two Tibetan breeds. Others numbers are the access*ion* numbers from GenBank for European sheep and cattle. A, B and C stand for lineages A, B and C respectively. D stands for an outgroup.

72°C. The PCR products were electrophoresed through 2.0% (wt/vol) agarose gel which was stained with ethidium bromide solution.

The amplified products were purified with a DNA purification kit according to the manufacturer's instructions (TW-Biotech. CO., LTD.). The purified fragments were cloned into pGM-T easy vector and subsequently transformed into *E. coli* Top10. After 16 to 20 h, the single clone was cultured, and the recombinant plasmid was obtained. The recombinant DNA was extracted and then sequenced using an ABI model 3,730 automated sequencer. The unique polymorphisms in the results were confirmed by resequencing of independent clones.

**Data analysis**

All the sequences were multiple aligned using the BioEdit 7.0 software. Identical sequences were considered as the same haplotype. The polymorphism of the haplotypes was analyzed with the DnaSP 4.1 software. The neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA 2.0 program (Kumar et al., 2001) with a Kimura 2-parameter model and the bootstrap value was 1,000. At the same time, we selected the complete sequences of *B. taurus* and *B.indicus* from GenBank as controls.

## RESULTS

**Sequencing analysis of the complete control region**

With the sequence of AF010406 (GenBank accession number) as a criterion, all the sequences were aligned and seventeen haplotypes were obtained from the sequenced individuals (GenBank accession numbers: DQ903190-DQ903200 and DQ903202-DQ903207). The haplotype diversity was 1.0. The average number of nucleotide differences was 33.81. Except for minor deletions and insertions, 114 variable sites were obtained in the sequences, including 109 transitions and 5 transversions. The nucleotide composition of all the haplotypes was 33.0% A, 29.7%T, 22.9%C and 14.4%G. The nucleotide diversity was 3.06%. The mean pair-wise genetic distance of all the haplotypes calculated using the Kimura 2-parameter model was 0.034.

**Haplotype analysis in Chinese Tibetan sheep**

The length of the sequences varied considerably between 1,107 bp and 1,259 bp, but most of the sequences were 1,181 bp. The length variations were caused by different copy numbers of a 75 bp tandem repeat except for minor insertions and deletions. The copy numbers of the tandem repeat were present randomly in the haplotypes, so they could not be seen as being characteristic of the haplotypes. The variations in the three haplotypes mainly occurred in the region of high variability (from 640 bp to 1,140 bp), and haplotype C had greater variation than haplotypes A and B. *Fu's* test showed that the populations had not been expanded historically ($0.10>p>0.05$).

**Phylogenetic analysis of Tibetan sheep**

The neighbor-joining, UPGMA and minimum evolution (ME) dendrograms were generated based on the sequences obtained in this study together with the sequences from GenBank using the MEGA 2.0 program (Bootstrap value is 1,000). The results were identical. We only presented the NJ phylogenetic tree (Figure 1). Three distinct lineages were observed. *B.taurus* and *B.indicus* were first gathered as an outgroup. *O. musimon* and European sheep were in the same lineage show*ing* that the relationship between them was close. In addition, the number of individuals in lineage A was more than in lineage*s* B and C. Lineage C was only present in Tibetan sheep breeds, but not in European sheep and *O. musimon.*

## DISCUSSION

Mitochondrial DNA has been very widely used by geneticists to analyze the phylogenetic relationships at inter- or intra- species level in cattle, swine, goats and water buffalo. It has also been used to investigate the genetic variation of species (Watanbe et al., 1989; Tanaka et al., 1996; Giuffra et al., 2000; Sultana et al., 2003; Sasazaki et al., 2006). The composition of the nucleotides in this study was similar to that of Hiendleder's (1998a). The shortest

sequence length was 1,107 bp, and the longest 1,259 bp. The difference among the sequences was caused by 3-5 copies of a 75 bp tandemly repeated sequence. The number of individuals contained in lineage A was more than in lineages B and C, showing that the D-loop haplotypes in most Tibetan sheep were A. Only Tibetan sheep were present in lineage C, but not European sheep or *O. musimon*. This indicated that lineage C is only present in China. mtDNA D-loop sequencing by Guo et al. (2005) provided evidence of a novel maternal lineage in six native Chinese sheep breeds. Our results show the presence of the same lineage in Tibetan sheep.

The use of the mtDNA PCR-RFLP and sequence analysis methods has led to the prevailing opinion that domestic sheep breeds are derived from two maternal resources (Wood and Phua, 1996; Hiendleder et al., 1991, 1998b, 1999, 2002). The limit of their study is that the genetic material was collected only from European, African, New Zealand and central Asian, but not Chinese sheep breeds. China has numerous domestic sheep breeds, but until now they have not been studied comprehensively for genetic diversity or phylogenetic analysis at the molecular level. Li (1993) pointed out that Mongolian and Tibetan sheep were the ancestors of Chinese sheep, and that they were distinct in appearance. Linzhou and Gangba sheep are the oldest breeds in the remote areas of Tibet. They are barely influenced by external breeds, so they could represent the genetic background of Tibetan sheep. They have not been studied since the breeds came into being except at the morphological level. In this study, we are the first to investigate their genetic diversity at the molecular level. The results are useful for the further utilization of Tibetan sheep resources. Three lineages could be identified among the haplotypes in this study, which indicated that Tibetan sheep breeds were derived from three maternal sources. This finding is not consistent with previous research. Our study only involved a molecular investigation. Additional archaeological and geographical evidences are required to reach a definite conclusion. The results should be useful to help determine the origins of modern domestic sheep breeds.

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## REFERENCES

Giuffra, E., J. M. H. Kijas, V. Amarger, O. Carlborg, J. T. Jeon and L. Andersson. 2000. The origin of the domestic pig: Independent domestication and subsequent introgression. Genetics. 154:1785-1791.

Guo, J., L. X. Du, Y. H. Ma, W. J. Guan, H. B. Li, Q. J. Zhao, X. Li and S. Q. Rao. 2005. A novel maternal lineage revealed in sheep (Ovis aries). Anim. Genet. 36:331-336.

Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei. 2001. Molecular evolutionary genetics analysis software (Version 2.1). Bioinformatics.

Li, Z. N. 1993. Breeding sheep in China. Agriculture press, Beijing, China.

Hiendleder, S. 1998a. A low rate of replacement substitutions in two major Ovis aries mitochondrial genomes. Anim. Genet. 29:116-122.

Hiendleder, S., Bernhard Kaupe, Rudolf Wassmuth and Axel Janke. 2002. Molecular analysis of wild and domestic sheep questions current nomenclature and provides evidence for domestication from two different subspecies. Proc. R. Soc. Lond. B. 269:893-904.

Hiendleder, S., K. Mainz, Y. Plante and H. Lewalski. 1998b. Analysis of mitochondrial DNA indicates that domestic sheep are derived from two different ancestral maternal sources: no evidence for contributions from urial and argali sheep. J. Hered. 89:113-120.

Hiendleder, S., S. H. Phua and W. Hecht. 1999. A diagnostic assay discriminating between two major Ovis aries mitochondrial DNA haplogroups. Anim. Genet. 30:211-213.

Hiendleder, S., W. Hecht, V. Dzapo and Wassmuth. 1991. Ovine mitochondrial DNA: restriction enzyme analysis, mapping and sequencing data. Anim. Genet. 23:151-160.

Rozas, J., J. C. Sanchez-Delbarrio, X. Messeguer and R. Rozas. 2005. DNA Sequence Polymorphism Version 4.10.1.

Sambrook, J., E. F. Fritsch and T. Maniatis. 2001. Molecular cloning: a laboratory manual (third edition). Cold Spring Harbor, New York: Cold spring Harbor Laboratory Press.

Sasazaki, S., S. Odahara, C. Hiura, F. Mukai and Mannen, 2006. H. Mitchondrial DNA variation and genetic relationships in Japanese and Korean cattle. Asian-Aust. J. Anim. Sci. 19(10):1394-1398.

Sultana, S., H. Mannen and S. Tsuji. 2003. Mitochondrial DNA diversity of Pakistani goats. Anim. Genet. 34:417-421.

Tanaka, K., C. D. Solis, J. S. Masangkay, K. Maeda, Y. Kawamoto and T. Namikawa. 1996. Phylogenetic relationship among all living species of the genus *Bubalus* based on DNA sequences of the cytochrome b gene. Biochem. Genet. 34:443-452.

Tobias Polzin and Siavash Vahdati. 2004. Network4.1.0.8. Fluxus Technology Ltd.

Upholt, W. B. and I. B. Dawid. 1977. Mapping of mitochondrial DNA of individual sheep and goats: rapid evolution in the D loop region. Cell 11:571-583.

Watanabe, T., J. S. Masangkay, S. Wakana, N. Saitou and T. Tomita. 1989. Mitochondrial DNA polymorphism in native Philippine cattle based on restriction endonuclease cleavage patterns. Biochem. Genet. 27:431-438.

Wolf, C., J. Rentsch and P. Hubner. 1999. PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. J. Agric. Food Chem. 7:1350-1355.

Wood, N. J. and S. H. Phua. 1996. Variation in the control region sequence of the sheep mitochondrial genome. Anim. Genet. 27:25-33.