Meta Gene 8 (2016) 14–20

Contents lists available at ScienceDirect

Meta Gene

journal homepage: www.elsevier.com/locate/mgene

# Genetic diversity estimates point to immediate efforts for conserving the endangered Tibetan sheep of India

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ARTICLE INFO

*Article history:*
Received 16 September 2015
Revised 26 November 2015
Accepted 4 January 2016
Available online 19 January 2016

*Keywords:*
Bottleneck
Genetic diversity
Heterozygote deficiency
Microsatellite markers
Tibetan sheep

ABSTRACT

Tibetan is a valuable Himalayan sheep breed classified as endangered. Knowledge of the level and distribution of genetic diversity in Tibetan sheep is important for designing conservation strategies for their sustainable survival and to preserve their evolutionary potential. Thus, for the first time, genetic variability in the Tibetan population was accessed with twenty five inter-simple sequence repeat markers. All the microsatellites were polymorphic and a total of 148 alleles were detected across these loci. The observed number of alleles across all the loci was more than the effective number of alleles and ranged from 3 (*BM6506*) to 11 (*BM6526*) with 5.920 ± 0.387 mean number of alleles per locus. The average observed heterozygosity was less than the expected heterozygosity. The observed and expected heterozygosity values ranged from 0.150 (*BM1314*) to 0.9 (*OarCP20*) with an overall mean of 0.473 ± 0.044 and from 0.329 (*BM8125*) to 0.885 (*BM6526*) with an overall mean 0.672 ± 0.030, respectively. The lower heterozygosity pointed towards diminished genetic diversity in the population. Thirteen microsatellite loci exhibited significant ($P < 0.05$) departures from the Hardy–Weinberg proportions in the population. The estimate of heterozygote deficiency varied from −0.443 (*OarCP20*) to 0.668 (*OarFCB128*) with a mean positive value of 0.302 ± 0.057. A normal 'L' shaped distribution of mode-shift test and non-significant heterozygote excess on the basis of different models suggested absence of recent bottleneck in the existing Tibetan population. In view of the declining population of Tibetan sheep (less than 250) in the breeding tract, need of the hour is immediate scientific management of the population so as to increase the population hand in hand with retaining the founder alleles to the maximum possible extent.



## 1. Introduction

Sheep biodiversity in India is characterized by high degree of endemism as variations in agro climatic conditions have led to the development of more than 40 breeds (Acharya, 1982). This vast ovine biodiversity is being eroded rapidly with more than 50% of sheep breeds currently under threat (Bhatia and Arora, 2005). Sheep provides employment and income to the socially and economically disadvantageous sections of the society being reared by the landless laborers and marginal farmers. Indigenous breeds must be considered as important reservoirs of non-exploited resources due to the presence of potentially unrecognized beneficial genetic variation. Moreover, autochthones breeds are the cultural properties due to their role in the agriculture tenures and in the social life of rural populations. Tibetan sheep is one such important breed of Indian North temperate Himalayan region. The sheep migrated to India with the Tibetan traders who used them as beasts of burden for transporting various merchandise besides wool (Government of Bengal, 1864). Independence of India in 1947 led to political and economic changes with concomitant cessation of their migration in the country.

Tibetan animals are of medium size, mostly white with black or brown face and brown and white spots on the body. The fleece is relatively fine and is among the best obtained from native ovine breeds of Indian subcontinent (Banerjee, 2009). The animals are unique being adapted to the harsh temperate climate and difficult terrains of Himalayas and survive even in the open housing system. Tibetan is regarded as one of the most rustic sheep breeds which thrives in conditions of extreme harshness and deprivation while providing meat and down for the people. They are the only source of livelihood in the area since agriculture is not suitable due to the geo-climatic characteristics. However, population of Tibetan sheep has been decreasing drastically in recent past (Banerjee, 2009) due to lack of regulated market, transport linkage, shrinkage of pasture land, increased inbreeding and occurrence of diseases. Population has gone down from 30,000 (Acharya,

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http://dx.doi.org/10.1016/j.mgene.2016.01.002

1982) to less than 250 in Sikkim (Livestock Census, 2012; Kumar, 2015). Therefore, urgent specific management and conservation measures are required not only for the restoration of depleted natural population due to endangered status but also for its biological and economical relevance and ecological importance. Knowledge of genetic variability is very important for establishing any conservation and management program. The use of highly variable molecular genetic markers, such as microsatellites, is one of the most powerful means for studying genetic diversity because of their high degree of polymorphism, abundance, random distribution across the genome, co-dominant inheritance and neutrality with respect to selection (Barcaccia et al., 2013; Putman and Carbone, 2014). These are being used to estimate the diversity of autochthonous sheep breeds all over the world (Ghazyl et al., 2013; Ceccobelli et al., 2015) including India (Bhatia and Arora, 2005; Sharma et al., 2010). Unfortunately, no study has been undertaken so far to investigate the genetic characteristics of Tibetan sheep using molecular biology techniques. This is critical as urgent conservation efforts are required to conserve and utilize the Tibetan sheep genetic resource.

Hence, the aim of the present study was firstly to estimate the genetic intra-breed variability of Tibetan sheep using 25 microsatellite markers and secondly to detect population bottleneck, if any.

## 2. Materials and methods

### 2.1. Sample collection and polymerase chain reaction

The breeding tract of Tibetan sheep is now confined to the dry alpine zone, North District of Sikkim state in India (Fig. 1). Population is confined into six flocks only which are located in the Phalung valley (4697 m, latitude 27° 56′ longitude 88° 35′). Blood samples were acquired from twenty Tibetan sheep (about 10% of existing population) from the breeding region (Fig. 1) following the guidelines of MoDAD (Measurement of Domestic Animal Diversity) program (FAO, 2004). The native sheep were evaluated for their phenotypic breed characteristics as per the breed descriptor and individuals from each flock were sampled. Owners were questioned in detail to minimize the sampling of closely related individuals. Blood samples (5–6 ml) were collected in vacutainer containing Ethylene diamine tetra acetic acid (0.5 mM, pH 8.0). Genomic DNA was extracted from whole blood using phenol-chloroform protocol (Sambrook et al., 1989).

### 2.2. Microsatellite genotyping

A panel consisting of 25 microsatellite markers was selected for the diversity analysis of Tibetan sheep population. These were chosen from literature related with sheep diversity studies aiming to analyze highly polymorphic markers spread across the genome. These markers also adhere to the guidelines of International Society for Animal Genetics and FAO (http://dad.fao.org/en/refer/library/guidelin/marker.pdf). Detailed information on primers is presented in Table 1. Forward primer of each marker was 5′ labeled with fluorescent dye, i.e. FAM, NED, PET and VIC. PCR amplification was performed in a reaction volume of 25 μl on i-cycler. Reaction mixture consisted of 50–100 ng of genomic DNA, 200 μM of each dNTP, 50 pM of each primer and 0.5 units of *Taq* DNA polymerase. The amplification was carried out using a Touchdown program for all microsatellite loci, which consists of initial denaturation of 95 °C for 1 min; 3 cycles of 95 °C for 45 s and 60 °C for 1 min, 3 cycle of 95 °C for 45 s and 57 °C for 1 min; 3 cycles of 95 °C for 45 s and 54 °C for 1 min, 3 cycles of 95 °C for 45 s and 51 °C for 1 min and 20 cycles of 95 °C for 45 s and 48 °C for 1 min. The PCR amplification was confirmed by electrophoresing the products in 1.8% agarose gel followed by staining with ethidium bromide (0.5 mg/ml). PCR products were multiplexed (Table 1) and genotyping was carried out on an automated ABI-3100 DNA sequencer (Applied Biosystems, USA) using LIZ 500 as the internal size standard (Applied Biosystems, USA). Allele sizing was done using GeneMapper™ software v 3.7. Stutter related scoring error, often seen in dinucleotide repeats, was absent and alleles could be scored unambiguously.

### 2.3. Data analysis

Basic genetic parameters including allele frequencies, observed (No) and effective number of alleles (Ne), observed (Ho) and expected heterozygosity (He) and heterozygote deficit ($F_{IS}$) in the whole population were calculated by analyzing the genetic data with GenAlEx 6.2 software (Peakall and Smouse, 2008). Tests of Hardy–Weinberg equilibrium and Ewens–Watterson Neutrality were applied using POPGENE 1.31 version (Yeh et al., 1999). Bottleneck events in the population were tested by three methods. The first method consisted of three excess heterozygosity tests developed by Cornuet and Luikart (1996): (i) Sign test, (ii) Standardized differences test, and (iii) a Wilcoxon

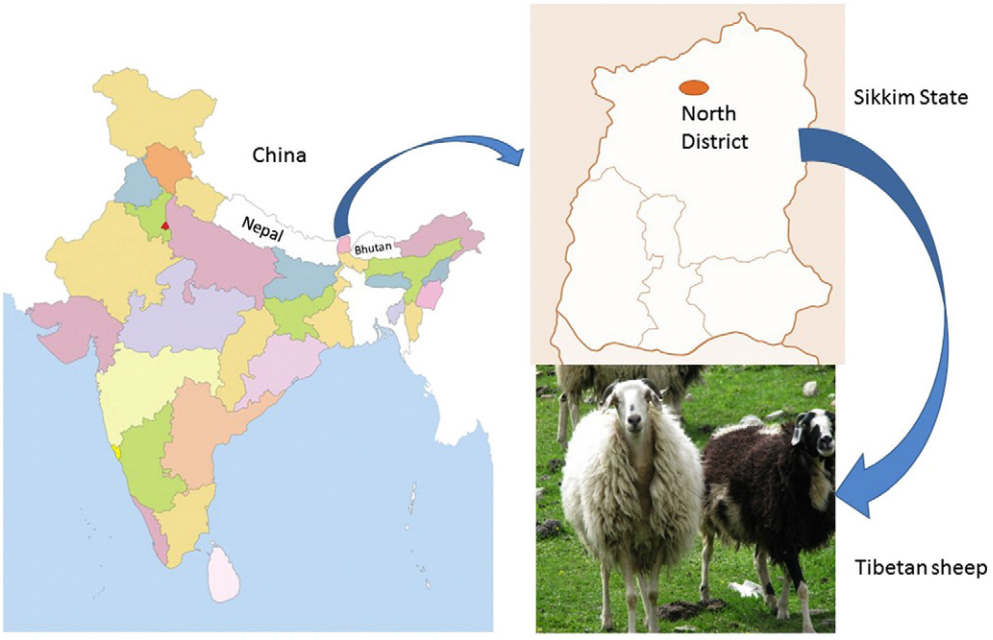


**Fig. 1.** Distribution of Tibetan sheep in Sikkim (India).