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

Rekha Sharma a,⁎, Brijesh Kumar b, Reena Arora a, Sonika Ahlawat a, A.K. Mishra a, M.S. Tantia a

a National Bureau of Animal Genetic Resources, Karnal 132 001, India
b ICAR Research Complex for NEH Region, Sikkim Centre, Gangtok 737102, India

A R T I C L E  I N F O

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

A B S T R A C T

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 assessed 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.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Sheep biodiversity in India is characterized by high degree of endemic 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, autochthonous 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. Tibetans are regarded as one of the most rustic sheep breeds which thrive 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, 1982)
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., 2014). These are being used to estimate the diversity of autochthonous sheep breeds all over the world (Ghazyl et al., 2013; Ceccobelli et al., 2014).

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 μM 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 (Fis) in the whole population were calculated by analyzing the genetic data with GenAIEx 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).](Image)
It is better to obtain as many individual data as possible to understand the current status of the population in terms of genetic diversity. However, in case of Tibetan sheep, only 215 animals are left (Kumar, 2015) which are reared in quite inaccessible region, the higher reaches of the North District of Sikkim state. Thus genomic data on twenty animals may be considered sufficient to evaluate genetic diversity of highly endangered sheep breed.

### 3.1. Genetic variability of microsatellite loci

All the markers were polymorphic and a total of 148 alleles were detected across the 25 loci. An exact test for genotypic linkage disequilibrium yielded no significant P values across the population, and therefore independent assortment of all the loci was assumed. Reasonable amount of polymorphism in Tibetan sheep breed was evident from the allele frequency data (available on request) with the mean number of alleles (MNA) of 5.920 ± 0.387 (Table 2). BM6526 showed the highest number of observed alleles per locus (11) while BM8125, OurFH72 and BM6506 showed the lowest (3). Expected number of alleles varied from 1.490 (BM8125) to 8.667 (BM6526) with the mean

| Microsatellite locus | Primer sequence (5' → 3') | Set | Chromosome location | Dye | Allele size range (bp) |
|---------------------|---------------------------|-----|---------------------|-----|-----------------------|
| BM0757              | F – tgg aaa caa tgt aaa cct ggg | 1   | 9                   | FAM | 182–198               |
| BM8125              | F – ctc tat ctg tgg aaa aag tgg g | 1   | 17                  | FAM | 111–115               |
| OurCP09             | F – cag aca cgg crr aac aac taa acg c | 1   | 17                  | NED | 80–112                |
| BM0827              | F – ggg cct gtc gta tgc tga g | 1   | 3                   | NED | 202–220               |
| OurHH47             | F – ctc acc gcc tta aat cta tat acg aac c | 1   | 18                  | VIC | 124–136               |
| CSSM47              | F – ctc tct ctc tca cta tca tgg | 2   | 2                   | VIC | 124–150               |
| MAF214              | F – aat gca gga gat ctt agc cag gga cg | 2   | 16                  | PET | 187–273               |
| OurCP20             | F – ggt ccc cgg gag gag gga acg g | 2   | 21                  | PET | 65–79                 |
| OurHH41             | F – gcc acc gta aat cta tat acg aac c | 2   | 10                  | NED | 120–130               |
| OurVH72             | F – ctc tct ctc tct tat tct ctc gc | 2   | 25                  | FAM | 123–127               |
| INRA63              | F – gac cca aaa ggt tgc tgc aca agc g | 3   | 14                  | FAM | 169–193               |
| OurAE129            | F – aat cca ggt tgg gaa cac ata ctc cag | 3   | 5                   | NED | 147–159               |
| OurCP34             | F – gct gaa cca gga tga tgc agg | 3   | 3                   | FAM | 102–122               |
| OurFH64             | F – cgg tcc act ctt gaa atg tat atc tgc | 4   | 4                   | NED | 117–133               |
| OurFCB128           | F – cag ctg ctc aac taa gac ata cat gcc | 3   | 2                   | PET | 91–121                |
| OurHH33             | F – aat tgc att cag tat ctt cta tcc cg | 4   | 4                   | NED | 117–133               |
| OurFP08             | F – cgg gat ctt ctc ggc taa tat gcc | 4   | 6                   | VIC | 115–131               |
| BM1314              | F – ctc ctc ctc ctc ctc cca aac | 5   | 22                  | NED | 157–173               |
| BM6506              | F – gca cgg ggt aac gac tgc gc | 5   | 1                   | FAM | 187–201               |
| CSDR247             | F – cag ctc gcc ggc act cag caa t | 5   | 14                  | NED | 215–239               |
| CSSM31              | F – cca aac tta gta ctc ttt aag aca | 5   | 9                   | FAM | 144–166               |
| OurFCB48            | F – gag tta gca gca tga ctc cag gag tca c | 5   | 17                  | PET | 144–164               |
| HSC                 | F – cgg cct ctt ctc ctt ctt ctt cct ctc c | 6   | X                   | FAM | 267–293               |
| BM6526              | F – cgg ctt ctc ctt ctt ctc ctt ctc ctc c | 6   | 26                  | VIC | 142–170               |
PIC = polymorphism information content. Pi is the frequency of the ith allele for the population & Sum pi^2 is the sum of the squared population allele frequencies.

uHe = unbiased expected heterozygosity = (2 N/(2 N − He)

He = expected heterozygosity = 1

Ho = observed heterozygosity = no. of Hets/N.

I = Shannon’s Information Index = Ln (pi).

Na = No. of different alleles.

of 3.70 (Table 2). Thus selection of microsatellites with a range of polymorphism reduced the risk of overestimating genetic variability, which might occur with the selective use of highly polymorphic loci. A microsatellite preferably should have at least 4 alleles to be useful for the evaluation of genetic diversity as per the standard selection of microsatellite loci (Barker, 1994). However, 3 alleles per locus have also been used to evaluate genetic diversity (Li et al., 2010). Therefore all the 25 microsatellites were retained for further analysis.

Allelic diversity (mean number of observed alleles per locus) higher than that observed in Tibetan sheep has been reported in some of the Indian sheep breeds (Arora et al., 2008; Kumarasamy et al., 2009; Radha et al., 2011; Sharma et al., 2010). However, MNA in Tibetan breed is higher than that observed in several other Indian sheep breeds viz. 4.94 in Madras Red sheep (Prema et al., 2008), 5.0 in Nilagiri sheep (Kayang et al., 2002) studies in addition to genetic diversity analysis.

### Table 2

| Locus     | Na | Ne | I  | Ho  | He  | uHe | Fst  | PIC  | z^2 value |
|-----------|----|----|----|-----|-----|-----|------|------|------------|
| BM0757    | 4  | 2.926 | 1.215 | 0.625 | 0.658 | 0.679 | 0.050 | 0.609 | 8.907
| BM8125    | 3  | 1.490 | 0.609 | 0.167 | 0.329 | 0.338 | 0.493 | 0.301 | 19.583
| BM0827    | 6  | 2.586 | 1.249 | 0.400 | 0.613 | 0.634 | 0.348 | 0.570 | 22.960
| CSSM47    | 4  | 1.800 | 0.855 | 0.222 | 0.444 | 0.471 | 0.500 | 0.411 | 21.130
| MA2174    | 6  | 4.800 | 1.651 | 0.250 | 0.792 | 0.826 | 0.684 | 0.760 | 36.078
| OarCP49   | 8  | 3.587 | 1.621 | 0.750 | 0.721 | 0.740 | −0.940 | 0.695 | 35.075
| OarCP20   | 5  | 2.658 | 1.198 | 0.900 | 0.624 | 0.640 | −0.443 | 0.576 | 13.388
| OarHH41   | 5  | 2.855 | 1.237 | 0.500 | 0.650 | 0.668 | 0.230 | 0.598 | 7.036
| OarHH47   | 6  | 4.781 | 1.662 | 0.421 | 0.791 | 0.812 | 0.468 | 0.762 | 45.735
| OarVH72   | 3  | 2.067 | 0.785 | 0.250 | 0.516 | 0.529 | 0.516 | 0.406 | 7.418
| BM6526    | 11 | 8.067 | 2.269 | 0.462 | 0.885 | 0.920 | 0.478 | 0.863 | 79.387
| INRA63    | 7  | 5.635 | 1.800 | 0.607 | 0.823 | 0.846 | 0.189 | 0.800 | 27.088
| OarAE129  | 4  | 2.941 | 1.221 | 0.600 | 0.660 | 0.733 | 0.091 | 0.610 | 6.800
| OarCP34   | 5  | 5.505 | 1.829 | 0.563 | 0.818 | 0.845 | 0.313 | 0.800 | 31.813
| OarFCB128 | 7  | 4.966 | 1.736 | 0.250 | 0.799 | 0.833 | 0.687 | 0.772 | 40.245
| HSC       | 5  | 3.375 | 1.353 | 0.722 | 0.704 | 0.724 | −0.026 | 0.654 | 28.439
| OarHH135  | 6  | 3.879 | 1.575 | 0.250 | 0.742 | 0.792 | 0.663 | 0.715 | 31.020
| OarHH64   | 6  | 4.346 | 1.578 | 0.412 | 0.770 | 0.793 | 0.465 | 0.732 | 42.689
| OarJMP209 | 9  | 5.333 | 1.871 | 0.750 | 0.813 | 0.833 | 0.077 | 0.788 | 59.949
| OarJMP08  | 7  | 4.042 | 1.607 | 0.412 | 0.753 | 0.775 | 0.453 | 0.716 | 33.819
| BM1314    | 5  | 1.681 | 0.797 | 0.150 | 0.405 | 0.415 | 0.630 | 0.368 | 20.497
| BM6506    | 3  | 1.629 | 0.644 | 0.250 | 0.386 | 0.396 | 0.333 | 0.329 | 5.788
| CSR2127   | 6  | 2.676 | 1.192 | 0.650 | 0.626 | 0.642 | −0.038 | 0.557 | 28.553
| CSSM31    | 8  | 3.580 | 1.818 | 0.842 | 0.813 | 0.835 | −0.036 | 0.787 | 20.559
| OarFCB48  | 7  | 2.935 | 1.383 | 0.368 | 0.659 | 0.677 | 0.441 | 0.620 | 27.125
| Mean      | 5.920 | 3.700 | 1.390 | 0.473 | 0.672 | 0.696 | 0.302 | 0.632 | 28.439
| SE        | 0.387 | 0.334 | 0.085 | 0.044 | 0.030 | 0.032 | 0.057 | 0.032 |

Na = No. of different alleles.

Ne = No. of effective alleles = 1/(Sum pi^2).

I = Shannon’s Information Index = −1 * Sum (pi * Ln (pi)).

Ho = observed heterozygosity = no. of Hets/N.

He = expected heterozygosity = 1 − Sum pi^2.

uHe = unbiased expected heterozygosity = (2 N/(2 N − 1)) * He.

Fst = Fixation Index = (He − Ho)/He = 1 − (Ho/He).

Pi = the frequency of the ith allele for the population & Sum pi^2 is the sum of the squared population allele frequencies.

PIC = polymorphism information content.

* Significant deviation from Hardy–Weinberg equilibrium (P < 0.05).

### 3.2. Heterozygosity and Hardy–Weinberg equilibrium

Genetic variability is also measured as the amount of actual or potential heterozygosity, which is presented in Table 2. Expected heterozygosity was higher than the observed heterozygosity at all the loci. The observed and expected heterozygosity values ranged from 0.150 (BM1314) to 0.842 (CSSM31) and from 0.329 (BM8125) to 0.885 (BM6526) with an overall mean of 0.473 ± 0.044 and 0.672 ± 0.030, respectively. Genetic variation of similar magnitude (0.46–0.55) has also been reported in the endangered Namaqu Afrikaner sheep indigenous to South Africa (Qwabe et al., 2015). Whereas, higher heterozygosity has been reported in the two endangered Spanish breeds, Churra tensina (0.659) and Churra lebrijana (0.674) despite their small population size (Calvo et al., 2011). Most of the Indian sheep breeds with sufficient population exhibit higher heterozygosity (Ho) than the Tibetan sheep (Arora et al., 2011). Tibetan belongs to the sheep breeds of North temperate region of India. It has the least diversity estimates among the breeds of North temperate region also (Table 3). Similarly, much higher
heterozygosity has been reported in several exotic sheep viz. Balearic sheep breeds (0.62, Pons and Landi, 2015), 12 sheep breeds of Croatia, Bosnia and Herzegovina (0.643–0.743, Salamon et al., 2014) and Turkish sheep breeds (0.66, Yilmaz and Sezenler, 2015). However, in assessing diversity estimates from different studies, it should be mentioned that the values are not directly comparable, as different microsatellite sets have been used by different workers. These values have only suggestive indication of diversity in the population.

Observed heterozygosity was lower than the expected showing a departure from Hardy–Weinberg equilibrium (HWE) and possibility of inbreeding. Significant deviation from HWE was observed in 13 out of 25 loci at P < 0.05 (Table 2). Departures from HWE of the similar magnitude were also reported in the endangered Spanish sheep breeds (Calvo et al., 2011), Italian (Cecchelli et al., 2015), Turkish (Yilmaz and Sezenler, 2015) and in various Indian sheep breeds (Radha et al., 2011). Various factors can contribute towards excess of homozygotes. First, the locus is under selection. Second, ‘null alleles’ may be present which are leading to a false observation of excess homozygotes. Third, inbreeding may be common in the population. Fourth, the presence of population substructure may lead to Wahlund’s effect. (Nei, 1987; Peter et al., 2007). Distinguishing among these is generally difficult (Christiansen et al., 1974). The likelihood of each of these explanations must be assessed from additional data, such as demographic information, i.e. population distribution. Null alleles are most unlikely to be segregating at all the loci. Similarly possible Wahlund's effects may not account significantly to the observed heterozygote deficit, as all the six flocks were at the same location. Ewens–Watterson Test for Neutrality revealed that all the microsatellite except OarH47 and INRA63 (Table S1, observed F values lie outside of the upper and lower limits of 95% confidence region of the expected F values) were neutral. Since 92% loci were neutral, selection as a cause of the decrease in observed heterozygosity was ruled out. Thus the difference between the observed and expected heterozygosity can be attributed to the non-random mating among the individuals of the population. This was also reflected in the positive FIS value (0.302 ± 0.057) which ranged from −0.443 to 0.687. Similar to our assumptions, the high FIS value (0.143) in the Spanish mouflon was considered as the major cause of the Hardy–Weinberg disequilibrium in that population (Calvo et al., 2011).

Higher heterozygote deficiency is generally observed in Indian sheep breeds; Magra (16%, Arora and Bhatia, 2006); Shahabadi (21.5%, Pandey et al., 2009); Rampur Bushair (22.7%, Pandey et al., 2008), and sheep breeds of Rajasthan–Nali (28.4%) and Chokla (28.6%) (Sodhi et al., 2006). However, Tibetan sheep (30.2%) exhibited the highest heterozygote deficiency among the Indian breeds, investigated so far. Authors agree that the major factor contributing towards the observed heterozygote deficiency in Tibetan sheep may be inbreeding due to extremely small population (<250) and ignorance of sheep owners about the scientific management. Under field condition, only natural mating is being practiced where the dominant male generally excludes subordinate males, and presumably sires most of the offspring. The general breeding practice in the region was to castrate most of the males. This lead to reduced effective population size/or mating between relatives and consequent genetic drift. The consanguinity produced by mating between relatives can be one of the principal causes for loss of heterozygotes, but has to be evaluated with caution, as inbreeding with significant deficit of heterozygote affect all or most of the loci in a similar way. Since eighty percent of loci depicted heterozygote deficit (Table 2), therefore we might consider consangunlity as the foremost cause of heterozygote deficiency. Salamon et al. (2014) have also concluded that breeding practice lead to the higher FIS value in the study involving 12 eastern Adriatic and western Dinaric native sheep breeds. Such a high level of inbreeding is risky as it could lead to genetic diseases and moreover can adversely affect animal fitness.

3.3. Genetic bottleneck analysis

Bottleneck influences the distribution of genetic variation within and among populations. Population of Tibetan sheep has gone down drastically, which indicates the possibility of demographic bottleneck. In recently bottlenecked populations, the majority of loci will exhibit an excess of heterozygotes, exceeding the heterozygosity expected in a population at mutation drift equilibrium. To estimate the excess of such heterozygosity Sign, Standardized differences and Wilcoxon sign rank tests were utilized. The actual mutation model of evolution followed by our microsatellites is not known, thus all the three models (IAM, TPM and SMM) were selected for running the Bottleneck program. The values of average heterozygosity (H) and their probabilities (H > H) in the Sign test, under three models of microsatellite evolution were calculated and used to measure the expected number of loci with heterozygosity excess (Table 4). The expected numbers of loci with heterozygosity excess were 14.77, 14.94 and 14.62 in IAM, TPM and SMM with probabilities of 0.13211, 0.10276 and 0.39403 respectively, meaning that the null hypothesis was accepted when using the Sign test. These results indicate that, due to mutation-drift equilibrium, the Tibetan population has not undergone a recent genetic bottleneck. The standardized difference test provided the T2 statistics equal to 2.252, 0.269 and −2.667 for the IAM, TPM and SMM models, respectively. The probability values were less than 0.05 for IAM and SMM, thus hypothesis of mutation-drift equilibrium was accepted under TPM only. Using the Wilcoxon rank test (a non-parametric test) the probability values were 0.00441 (IAM), 0.28009 (TPM) and 0.91775 (SMM) under these three models, indicating that the null hypothesis is accepted under TPM and SMM and thus the population under study has not undergone a recent bottleneck. It has been considered that the most useful markers for bottleneck detection are those evolving under IAM, and they provide guidelines for selecting sample sizes of individuals and loci (Cornuet and Luikart, 1996; Di Rienzo et al., 1994; Spencer et al., 2000); meanwhile, the TPM is thought to more closely simulate microsatellite mutation (Estoup and Cornuet, 2000). Unlike the SMM, which predicts all mutations corresponding to the increment or decrement of a single base-pair repeat, the TPM predicts the occurrence of an occasional multiple base-pair repeat (Di Rienzo et al., 1994). The strict SMM is obviously the most conservative model for testing for a significant heterozygosity excess caused by bottlenecks, because in some conditions it can produce a heterozygosity deficiency, and due to the heterozygosity excess it is always lower than other mutation models. Thus we have considered results from all the three tests together and it is clear that serious demographic bottlenecks have most probably not occurred in this breed.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked populations from stable populations (Luikart, 1997; Luikart and Cornuet, 1997). A graphical representation utilizing allelic class and proportion of alleles showed a normal ‘L’ shaped distribution (Fig. 2). The L shaped curve indicated the abundance of low frequency alleles with low frequency. This test discriminates many bottlenecked populations from stable populations (estoup and cornuet, 1997). To estimate the excess of such heterozygosity Sign, Standardized differences and Wilcoxon sign rank tests were calculated for heterozygosity excess (Table 4). The expected numbers of loci with heterozygosity excess were 14.77, 14.94 and 14.62 in IAM, TPM and SMM with probabilities of 0.13211, 0.10276 and 0.39403 respectively, meaning that the null hypothesis was accepted when using the Sign test. These results indicate that, due to mutation-drift equilibrium, the Tibetan population has not undergone a recent genetic bottleneck. The standardized difference test provided the T2 statistics equal to 2.252, 0.269 and −2.667 for the IAM, TPM and SMM models, respectively. The probability values were less than 0.05 for IAM and SMM, thus hypothesis of mutation-drift equilibrium was accepted under TPM only. Using the Wilcoxon rank test (a non-parametric test) the probability values were 0.00441 (IAM), 0.28009 (TPM) and 0.91775 (SMM) under these three models, indicating that the null hypothesis is accepted under TPM and SMM and thus the population under study has not undergone a recent bottleneck. It has been considered that the most useful markers for bottleneck detection are those evolving under IAM, and they provide guidelines for selecting sample sizes of individuals and loci (cornuet and luikart, 1996; di reinzo et al., 1994). The strict SMM is obviously the most conservative model for testing for a significant heterozygosity excess caused by bottlenecks, because in some conditions it can produce a heterozygosity deficiency, and due to the heterozygosity excess it is always lower than other mutation models. Thus we have considered results from all the three tests together and it is clear that serious demographic bottlenecks have most probably not occurred in this breed.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large proportion of alleles with low frequency. This test discriminates many bottlenecked populations from stable populations (Luikart, 1997; Luikart and Cornuet, 1997). A graphical representation utilizing allelic class and proportion of alleles showed a normal ‘L’ shaped distribution (Fig. 2). The L shaped curve indicated the abundance of low frequency alleles (<0.10) alleles. This finding suggested the absence of any detectably large, recent genetic bottleneck (last 40–80 generations) in declining

---

### Table 4

| Test/model | IAM | TPM | S.M.M. |
|------------|-----|-----|-------|
| Sign test (number of loci with heterozygosity excess) | 14.77 | 14.94 | 14.62 |
| Obs | 18 | 18 | 18 |
| P-value | 0.13211 | 0.10276 | 0.39403 |
| Standardized differences test | 2.252 | 0.269 | −2.667 |
| P-value | 0.001216 | 0.39403 | 0.00382 |
| Wilcoxon rank test (one tail for heterozygosity excess) | 0.00441 | 0.28009 | 0.91775 |

*Bottleneck (rejection of null hypothesis of mutation drift equilibrium).*
population, where the probability of low frequency allele’s loss was very high. Taken together all the results indicate the absence of bottlenecks in the recent past history of this breed.

Shrinkage of pasture, inbreeding, absence of scientific animal husbandry practices and management in very harsh climate have resulted in diminished profits for the Tibetan sheep herders. As a result, sheep rearing has failed to attract younger generations. Additionally, lack of proper policy is leading to rapid decline of Tibetan sheep population which are less than two hundred and fifty at present. Therefore, timely intervention is required to prevent extinction of this valuable breed of sheep, which helps in sustaining the livelihood of highlanders and the fragile agro-ecosystem. The information reported here is important for sheep breeders for the establishment of conservation strategies.

4. Conclusions

The present work for the first time generated the knowledge of existing genetic diversity in the endangered Tibetan sheep based on microsatellite analysis. It is a unique gene pool with good adaptation to the extremely cold and low oxygen conditions. Genetic parameters show a high value of inbreeding and therefore this breed should be monitored due to very low number of individuals that compose it. Still there is scope for reviving the breed because of high allele diversity which suggests that unique alleles present in this breed may not have been lost and the absence of bottleneck.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2016.01.002.

Acknowledgments

This work was financially supported by the Network Project on Animal Genetic Resources (ICAR).

References

Acharya, R.M., 1982. Sheep and goat breed of India. FAO Animal Production and Health Paper 30. FAO of United Nations, Rome, Italy.

Aroa, R., Bhatia, S., 2004. Genetic structure of muzaffarnagar sheep based on microsatellite analysis. Small Rumin. Res. 54, 227–230.

Aroa, R., Bhatia, S., 2006. Genetic diversity of Magra sheep from India using microsatellite analysis. Asian Australas. J. Anim. Sci. 19, 938–942.

Aroa, R., Bhatia, S., et al., 2008. Genetic variability in Jalalui sheep of India inferred from microsatellite data. Livest. Res. Rural Dev. 20 (Article no. 4 retrieved from www.cipav.org.co/lrrd20/1/aror2004.htm).

Aroa, R., Bhatia, S., et al., 2011. Diversity analysis of sheep breeds from Southern peninsular and Eastern regions of India. Trop. Anim. Health Prod. 43, 401–408.

Banerjee, S., 2009. Shift from transhumance and subtle livelihood patterns of the bhotia community and its impact on Tibetan sheep population in Sikkim (India). World Appl. Sci. J. 7, 1540–1546.

Barcaccia, G., Felicetti, M., et al., 2013. Molecular analysis of genetic diversity, population structure and inbreeding level of the Italian Lipizzan horse. Livest. Sci. 151, 124–133.

Barker, J.S.F., 1994. A global protocol for determining genetic distances among domestic livestock breeds. Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph and Ontario, Canada. 21, pp. 501–508.

Bhatia, S., Arora, R., 2005. Biodiversity and conservation of Indian sheep genetic resources—an overview. Asian Australas. J. Anim. Sci. 18, 1387–1402.

Bhatia, S., Arora, R., 2008. Genetic diversity in Kheri—a pastoralists developed Indian sheep using microsatellite markers. Indian J. Biotechnol. 7, 108–112.

Bottstein, D., White, R.L., et al., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32, 314–331.

Calvo, J.H., Alvarez-Rodriguez, J., et al., 2011. Genetic diversity in the Churra tenuina and Churrã tel jogue endangered Spanish sheep breeds and relationship with other Churra group breeds and spanish mouflon. Small Rumin. Res. 95, 34–39.

Cecconelli, S., Karsli, T., et al., 2015. Genetic diversity of cornigliese sheep breed using STR markers. Small Rumin. Res. 123, 62–69.

Christopher, F.B., Frydenberg, G., et al., 1974. Genetics of Zoarcas populations VI. Further evidence, based on age group samples, of a heterozygote deficit ESTIII polymorphism. Hereditas 77, 225–236.

Cornoet, J.M., Luikart, G., 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144, 2001–2014.

Di Rienzo, A., Peterson, A.C., et al., 1994. Mutational processes of simple-sequence repeat loci in human populations. Proc. Natl. Acad. Sci. U. S. A. 91, 3166–3170.

Estoup, A., Cornoet, J.M. 2000. Microsatellite evolution: inferences from population data. In: Goldstein, D.B., Schlötzer, C. (Eds.), Microsatellites: Evolution and Applications. Oxford University Press, Oxford, pp. 49–65.

FAO, 2004. Secondary guidelines for development of national farm animal genetic resources management plans for global management of cattle genetic resources using reference microsatellites global projects for the maintenance of domestic animal genetic diversity (MoDAD). http://www.fao.org/dad-is/.

Ghazvli, A., Mohktar, S., et al., 2013. Genetic diversity and distances of three Egyptian local sheep breeds using microsatellite markers. Zoology 3, 1–9.

Girish, H., Sivaselvam, S.N., et al., 2015. Molecular characterization of Nilagiri sheep (Ovis aries) of south India based on microsatellites. Asian Australas. J. Anim. Sci. 20, 633–637.

Government of Bengal, 1864. Proceedings of Bengal Government General. July 1864. In: Wake, H.C. (Ed.), Superintendent of Darjeeling, to Undersecretary to Govt. of Bengal. 277, pp. 47–57.

Gupta, N., Canna, T.A.S., 2007. Sheep Genetic Resources of India. Karan – A Finest Wool Breed. Monograph 50. National Bureau of Animal Genetic Resources, Karnal, India.

Gupta, S.C., Canna, T.A.S., et al., 2007. Sheep Genetic Resources of India—Kuree – An Endangered Breed. Monograph 49. National Bureau of Animal Genetic Resources, Karnal, India.

Kayang, B.B., Inoue-Murayama, M., et al., 2002. Microsatellite loci in Japanese quail and cross-species amplification in chicken and guinea fowl. Genet. Sel. Evol. 34, 233–253.

Kumar, B., 2015. Characterization of Tibetan sheep. Proceedings of 13th Annual Review Meet of Network Project on Animal Genetic Resources (ICAR), Karnal, India. 4.

Kumarasamy, P., Prema, S., et al., 2009. Molecular characterization of Coimbatore breed of sheep (Ovis aries) in South India. JUP. J. Genet. & Evol. 2, 56–65.

Livestock Census, 2012. BAHS—Basic Animal Husbandry Statistics: Department of Animal Husbandry, Dairying & Fisheries. Ministry of Agriculture, Government of India, Krishi Bhavan, New Delhi.

Luikart, G., 1997. Usefulness of Molecular Markers for Detecting Population Bottlenecks and Monitoring Genetic Change (Ph. D. thesis) University of Montana, Missoula, USA.

Luikart, G., Cornoet, J.M., 1997. Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conserv. Biol. 12, 228–237.

Luikart, G.L., Allendorf, F.W., et al., 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. J. Hered. 89, 238–247.

MacHugh, D.E., Shriver, M.D., et al., 1997. Microsatellite DNA variation and the evolution of domestication and phylogeography of taurine and zebu cattle (Bos taurus and Bos indicus). Genetics 146, 1071–1086.

Nee, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York USA.

Pandey, A.K., Sharma, R., et al., 2008. Genetic variability in Rampur–Busher sheep breed using microsatellite marker. Indian J. Anim. Sci. 78, 623–626.

Pandey, A.K., Sharma, R., et al., 2009. Variation of 18 STR loci in Shahabad sheep of India. Russ. J. Genet. 45, 1–7.

Peakall, R., Smouse, P.E., 2008. A heterogeneity test for fine-scale genetic structure. Mol. Ecol. Notes 17, 3389–3400.

Peters, C., Bruford, M., et al., 2007. Genetic diversity and subdivision of 57 European and Middle-Eastern sheep breeds. Int. Soc. Anim. Genet. 38, 37–44.

Pons, A.L., Landi, V., 2015. The biodiversity and genetic structure of balarese sheep breeds. J. Anim. Breed. Genet. 132, 268–276.

Pravosud, S., Kumarasamy, P., et al., 2009. Molecular characterization of Vembur sheep (Ovis aries) of south India based on microsatellites. Indian J. Sci. Technol. 2, 55–58.

Prema, S., Sivaselvam, S.N., et al., 2008. A note on genetic analysis in Madras Red sheep (Ovis aries) of India using microsatellite markers. Livest. Res. Rural Dev. 20, 181–185.

Putman, A.L., Carboni, I., 2014. Challenges in analysis and interpretation of microsatellite data for population genetic studies. Ecol. Evol. 4, 4399–4428.

Qvabe, S.O., Marle-Köster, E.V., et al., 2015. Genetic diversity and population structure of the endangered Namaqua Afrikaner sheep J. Anim. Breeding. Genet. http://dx.doi.org/10.1515/jbg.2015.030.

Radha, P., Sivaselvam, S.N., et al., 2011. Genetic diversity and bottleneck analysis of Kilakarsal sheep by microsatellite markers. Indian J. Biotechnol. 10, 52–55.

Salamon, D., Gutierrez-Gil, B., et al., 2014. Genetic diversity and differentiation of 12 eastern Adriatic and western Dinaric native sheep breeds using microsatellites. Animal 8, 200–207.

Fig. 2. Graphical representation of proportion of alleles and their distribution in Tibetan sheep.
Sambrook, J., Fritsch, E.F., et al., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Lab. Press, Cold Spring Harbour, NY.
Sharma, R., Pandey, A.K., et al., 2010. Microsatellite based diversity estimation of Changthangi—a high altitude sheep breed. Indian J. Anim. Sci. 80, 436–440.
Sodhi, M., Mukesh, M., et al., 2006. Characterizing Nalli and Chokla sheep differentiation with microsatellite markers. Small Rumin. Res. 65, 185–192.
Spencer, C.C., Neigel, J.E., et al., 2000. Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. Mol. Ecol. 9, 1517–1528.
Yeh, F.C., Yang, R.C., et al., 1999. POPGENE Version 1.31: Microsoft Window-based Free Software for Population Genetic Analysis. University of Alberta, Edmonton.
Yilmaz, O., Sezenler, T., 2015. Genetic relationships among four Turkish sheep breeds using microsatellites. Turk. J. Vet. Anim. Sci. 39, 576–582.