



Microsatellite and mitochondrial DNA based genetic diversity analysis of a lesser known Chitarangi sheep of north-western India

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ABSTRACT

Microsatellite and mitochondrial DNA based genetic characterization of Chitarangi sheep population inhabiting Fazilka and Muktsar districts of Punjab and Sriganaganar district and adjoining areas of Rajasthan was carried out, and genetic diversity measures were analysed. High estimates of allele diversity (9.875 ± 0.641) and gene diversity (0.712 ± 0.027) were observed across the population. A significant positive F_{IS} (0.096 ± 0.031) value suggested a deficiency in the number of heterozygotes in Chitarangi sheep. The population revealed presence of substantial genetic diversity and the typical L-type distribution of allelic frequencies indicated the absence of any recent bottlenecks in Chitarangi sheep and no mode shift was detected in this population. Majority of haplotypes identified through mitochondrial DNA based analysis of Chitarangi sheep grouped with major haplogroup A that is predominantly of Asian origin. The genetic characterization of Chitarangi sheep will help in devising suitable strategies for its genetic improvement, management and recognition at National level.

Keywords: Chitarangi, Diversity analysis, Microsatellite markers, Mitochondrial DNA, Sheep

Small ruminants are an important part of rural economy, especially in arid and semi-arid regions of India which is characterized by sparse vegetation, marginal land holding, frequent droughts and scarcity of water. As per 19th Livestock Census, the total sheep population of 65.06 million accounts for 12.7% of total livestock population in the country. There are 43 registered indigenous sheep breeds which are native to different agro-climatic conditions of the country. Apart from registered breeds many lesser known sheep populations exist in different region of the country which need to be properly characterized to assess complete sheep diversity in India. Chitarangi is an important lesser known sheep population inhabiting Fazilka and Muktsar districts of Punjab and Sriganaganar district and adjoining areas of Rajasthan. The phenotypic characterization revealed that Chitarangi is a medium to large sized sheep with carpet quality fleeces and is important for livelihood security of the sheep farmers of its habitat (Mishra *et al.* 2020).

No information was available on genetic diversity analysis of this sheep population. Microsatellite markers are widely used in genetics and are considered of high utility for the genetic characterization of the populations (Stahlberger-Saitbekova *et al.* 2001). Therefore, the present study was undertaken to assess the genetic diversity of Chitarangi sheep using microsatellite markers and mitochondrial DNA.

MATERIALS AND METHODS

Blood samples were collected from 48 unrelated Chitarangi animals from their distribution area. To ensure unrelatedness, the samples were collected from the flocks situated in different parts of the distribution area and only 1–2 samples were collected per flock. The genomic DNA was isolated using standard phenol/chloroform/isoamyl alcohol extraction method (Sambrook *et al.* 1989). Genetic diversity was assayed using a set of 24 microsatellite markers, of which 20 were taken from MoDAD list of FAO recommended for ovines (Bradley *et al.* 1997) and the remaining four markers (CSRD247, HSC, INRA63, MAF214 and OarCP49) were taken from the panel of markers for parentage verification tested at the 2001/02ISAG comparison test (Di Stasio 2001). The forward primer for each marker was fluorescently labelled with FAM, NED, VIC or PET dye. Amplification of the loci was performed in a 25 μ l final reaction volume containing at least 100 ng of genomic DNA, 5 pM of each primer, 1.5 mM $MgCl_2$, 200 μ M dNTPs, 0.5 U *Taq* DNA polymerase and 1X *Taq* buffer. A common touch down PCR programme, as suggested under MoDAD project (FAO 1996) without extension step was used for the amplification of all the 24 markers. PCR amplification consisted of 3 cycles of 45 sec at 95°C, 1 min at 60°C; 3 cycles of 45 sec at 95°C, 1 min at 57°C; 3 cycles of 45 sec at 95°C, 1 min at 54°C; 3 cycles of 45 sec at 95°C, 1 min at 51°C and 20 cycles of 45 sec at 95°C, 1 min at 48°C. The amplified products were resolved on 2% agarose gel and genotyped on an automated DNA

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Table 1. Genetic variability measures in Chitarangi sheep across different microsatellite markers

Locus	N _a	N _e	H _o	H _e	F _{IS}	PIC
BM757	6	2.543	0.542	0.607	0.107	0.60677
BM8125	6	1.868	0.521	0.465	-0.121	0.464627
BM827	8	3.651	0.609	0.726	0.162	0.726134
OarCP0049	13	5.640	0.771	0.823	0.063	0.822699
OarHH47	11	4.768	0.705	0.790	0.108	0.790289
CSSM47	8	1.612	0.489	0.380	-0.288	0.379804
MAF0214	10	3.046	0.618	0.672	0.080	0.67171
OarCP20	10	2.454	0.532	0.593	0.102	0.592572
OarHH41	10	4.249	0.717	0.765	0.062	0.764652
OarVH72	6	3.064	0.660	0.674	0.021	0.673608
BM6526	14	4.581	0.756	0.782	0.033	0.781727
OaCP34	6	3.082	0.563	0.676	0.167	0.675561
OarAE129	4	2.344	0.362	0.573	0.369	0.573339
OarFCB128	11	4.617	0.688	0.783	0.122	0.783416
HSC	11	4.904	0.674	0.796	0.153	0.796076
OarHH35	13	5.543	0.574	0.820	0.299	0.819604
OarHH64	7	3.027	0.432	0.670	0.355	0.669686
OarJMP29	11	4.235	0.750	0.764	0.018	0.76389
OarJMP8	10	5.290	0.854	0.811	-0.053	0.810982
BM1314	13	4.299	0.521	0.767	0.321	0.767361
BM6506	7	2.182	0.563	0.542	-0.038	0.541665
CSRD0247	13	7.143	0.822	0.860	0.044	0.86
CSSM31	16	8.828	0.896	0.887	-0.010	0.886722
OarFCB48	13	7.131	0.659	0.860	0.233	0.859761
Mean	9.875	4.171	0.636	0.712	0.096	0.712
SE	0.641	0.371	0.027	0.027	0.031	0.027

N_a, Observed number of alleles; N_e, Effective number of alleles [Kimura and Crow (1964)].

sequencer using LIZ 500 as internal lane standard (ABI PRISM). The raw data files were extracted by Gene Mapper software version 3. Popgen 3.2 (Yeh *et al.* 1999) and GenAlEx6.5 softwares (Peakall and Smouse, 2005) were used for the genetic diversity analysis. Polymorphism Information Content (PIC) of the microsatellite loci was estimated according to Botstein *et al.* (1980). Bottleneck events were estimated following two different approaches. The first was based on the heterozygosity excess consisting of sign test, standardized differences test and a Wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models, viz. infinite allele (IAM), stepwise mutation (SMM) and two-phase mutation model (TPM). The test was conducted using Bottleneck 1.2.01 software (Piry *et al.* 1999). The second was based on graphical representation of mode-shift equilibrium. The maternal lineages of Chitarangi sheep were identified by exploring the mitochondrial DNA diversity in 15 samples. A 1,246 bp fragment spanning part of the control region, the tRNA-Phe and 12S rRNA was amplified using F-AACTGCTTGACCGTACATAGTA and R-AAGGG-TATAAAGCACCGCC primers (Meadows *et al.* 2005). The amplified products were purified and sequenced in both forward and reverse directions using ABI Prism Big Dye Terminator 3.1 chemistry and ABI 3100 Automated DNA Sequencer (Applied Biosystems, USA). Multiple sequence alignment of the mtDNA sequences of Chitarangi sheep was performed with MEGA 7.0 (Kumar *et al.* 2016)



Fig. 1. Chitarangi male

after alignment to a minimum of 1041 bp. All positions containing gaps and missing data were eliminated from the dataset. DNA SPv6 (Rozas *et al.* 2017) was used to analyze polymorphic sites, number of haplotypes (h), nucleotide diversity (π) and haplotype diversity (Hd). Median joining network was generated using the Network 5.0.0.3 software (<http://www.fluxus-engineering.com>) to explore relationship among the observed haplotypes.

RESULTS AND DISCUSSION

Genetic diversity analysis: Different measures of within breed genetic variations, viz. actual number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o) and expected heterozygosity (H_e) along with polymorphism information content (PIC) of different

microsatellite loci and within population inbreeding estimates (F_{IS}) are given in Table 1. The microsatellite loci amplified were observed to be polymorphic in the investigated Chitarangi sheep population. All the markers were highly informative with average PIC value of 0.71. This indicated the usefulness of the markers for genetic diversity analysis. A total of 237 distinct alleles were identified across the 24 markers in Chitarangi sheep. The observed number of alleles ranged from 4 (OarAE129) to 16 (CSSM31) with a mean of 9.875. Effective number of alleles were lower than the observed number of alleles and ranged from 1.612 (CSSM47) to 8.828 (CSSM31) with a mean value of 4.171. The non-significant differences ($P>0.05$) between the average observed heterozygosity values (0.636) and the average expected heterozygosity values (0.712) suggested random mating in Chitarangi population and the population to be at HW equilibrium.

The estimates of allele diversity (mean number of observed alleles) and gene diversity (mean expected heterozygosity) implied the presence of substantial amount of genetic variability in this population. Singh *et al.* (2017) reported similar estimates of allele diversity (7.778) and gene diversity (0.669) in the Kajali sheep of Punjab, another lesser known sheep. The mean F_{IS} (within population inbreeding estimates) value was 0.096 which indicated deficiency in the number of heterozygotes in Chitarangi sheep. The observed positive F_{IS} in the investigated sheep population might be due to the use of fewer rams for the breeding purpose. The existence of population substructure (Wahlund effect) due to sampling from different flocks in different villages of the distribution area appears to be the most probable explanation. The most appropriate or exact reason for deficit of heterozygotes is difficult to predict due to non availability of pedigree information in the field conditions. The positive F_{IS} value for Chitarangi sheep is comparable to those reported previously for most of Indian sheep breeds, viz. Magra, Nali, Malpura, Jalauni, Kajali and Deccani. However, Ganjam, Chotanagpuri and Madgyal had F_{IS} values of about zero whereas, Marwari, Sonadi and Garole had much higher values of 0.231, 0.215 and 0.234, respectively (Arora *et al.* 2011a,b,c; Singh *et al.* 2017).

The genetic characterization of Chitrangi sheep by using microsatellite markers revealed substantial amount of genetic variability in this sheep population. This variability

can be utilized for the selection of animals for increasing their body weight. This will increase the meat productivity of the Chitrangi sheep. Thus, higher meat production will fetch more income to the small and marginal farmers rearing this sheep. The genetic variability observed in the present study is a step towards the registration of Chitarangi sheep as distinct breed at national level, conservation and genetic improvement.

Bottleneck analysis: Bottleneck hypothesis was explored in Chitarangi sheep. According to this hypothesis if the population has experienced recent reduction, effective population size exhibit a correlation with reduction of allele numbers and gene diversity. In a population at mutation-drift equilibrium, there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. If allele number reduces faster than the gene diversity in a population, it is experiencing bottleneck. The observed gene diversity is higher than the expected gene diversity which is computed from the observed number of alleles, under the assumption of a constant-size (equilibrium) population (Luikart *et al.* 1998). Three different tests, viz. sign rank, standardized differences and Wilcoxon tests under all the 3 models of microsatellite evolution (IAM, SMM and TPM) were employed to investigate whether Chitarangi sheep has undergone recent bottleneck (Table 2). The populations exhibiting significant excess in heterozygosity are considered to have experienced a recent genetic bottleneck. In our study, the heterozygosity excess was not significant under any of the models (IAM, TPM and SMM) using Wilcoxon rank test. Microsatellite loci are likely to evolve predominantly under SMM (Shriver *et al.* 1993). Under strict SMM, the heterozygosity excess resulting from population size expansion is either undetectable or heterozygosity deficiency is found (Maruyama and Fuesrt 1984). The heterozygosity excess is usually lower under the SMM model than IAM and even heterozygosity deficiency (negative values) may occur in SMM (Cornuet and Luikart 1996). This is in agreement with our finding (Table 2) in which negative T_2 value (-13.60) was obtained indicating that the population may be expanding with no recent bottleneck. Moreover, the Wilcoxon test, which is considered to be more reliable than the sign test and standardized differences test, showed no significant results for population bottleneck under all the three models, i.e. IAM, TPM and SMM. In addition, the

Table 2. Test for mutation drift equilibrium in the 24 microsatellite loci in Chitarangi sheep

Test	Parameters	IAM	TPM	SMM
Sign test	Observed no. of loci with H_e excess	17	7*	0*
	Expected no. of loci with H_e excess	14.36	14.21	14.06
	P value	0.19	0.003	0.00*
Standardized difference test	T^2 value	0.524	-4.241*	-13.60*
	P value	0.300	0.00001	0.000
Wilcoxon sign rank test	P value (two tail test for H_e excess and deficiency)	0.172	0.998	1.000

* $P<0.05$; IAM, Infinite allele model; SMM, Stepwise mutation model; TPM, Two-phase mutation model.

Table 3. Mitochondrial DNA diversity indices in Chitarangi sheep (N=15)

Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (μ)	Singleton variable sites	Parsimony informative sites
9	0.848	0.00266	14	13

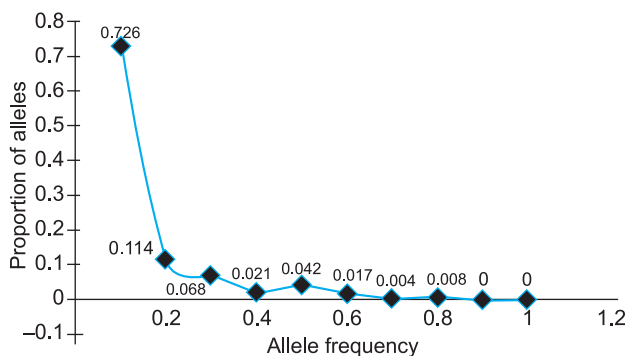


Fig. 2. Mode Shift analysis in Chitarangi sheep.

mode shift analysis (Luikart and Cornuet 1997) in the Chitarangi sheep population resulted in a typical L-shaped curve indicating the absence of bottleneck (Fig. 2). Thus, both the mode shift analysis and mutation drift equilibrium tests showed no recent reduction in the population size of this sheep population.

Mitochondrial DNA diversity: A 1246 bp fragment of mitochondrial DNA spanning part of the control region, tRNA-Phe coding region and 12S rRNA gene was amplified and sequenced in 15 samples of Chitarangi sheep. Upon sequence alignment, 9 haplotypes could be identified in Chitarangi sheep. These novel sequences were submitted to GenBank and accession numbers were obtained (MN073924-MN073932). The mitochondrial DNA diversity indices observed in Chitarangi sheep are given in Table 3.

The haplotype diversity in Chitarangi sheep (0.848) was higher than the overall haplotype diversity (0.603) reported by Arora *et al.* (2013) in 19 sheep breeds from 3 agroecological zones of India, but the value was lower than the value reported by Singh *et al.* (2013) (0.987) involving 12 breeds from four agro-climatic regions of India. The results were on similar lines for nucleotide diversity indices in our study when compared with previous two studies in Indian sheep. To assess the relationship within Chitarangi sheep and between known ovine haplogroups across the globe, a median joining network was constructed using Chitarangi sheep haplotypes and major mtDNA haplogroups representing five lineages (Lineage A to Lineage E) (Meadows *et al.* 2011). Majority of the Chitarangi sheep haplotypes grouped with lineage A and only 2 haplotypes clustered with lineage B (Fig. 3). This observation is in concordance with previous study in Indian sheep by Arora *et al.* (2013).

In conclusion, the molecular genetic analysis revealed substantial genetic variation in Chitarangi sheep. High measures of allele (9.875 ± 0.641) and gene diversity

(0.712 ± 0.027) were observed across this population and a significant positive F_{IS} (0.096 ± 0.031) value suggested deficiency in the number of heterozygotes in Chitarangi sheep. The mitochondrial diversity analysis reveals that majority of the Chitarangi sheep haplotypes grouped with lineage A and only 2 haplotypes clustered with lineage B.

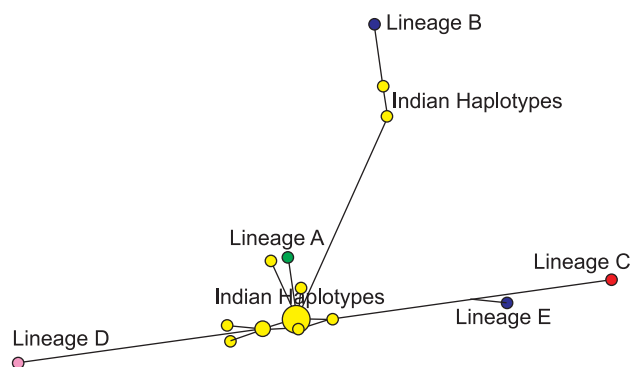


Fig. 3. Median Joining network of Chitarangi mitochondrial DNA. Indian haplotypes are represented by yellow coloured nodes and node size is proportional to haplotype frequency. Sequences representative of the five known ovine lineages (A-E) were taken from Meadows *et al.* (2011).

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