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GENETIC DIVERSITY ANALYSIS OF KAIL SHEEP BY USING MICROSATELLITE MARKERS

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ABSTRACT

The Kail sheep breed of Azad Jammu and Kashmir (AJ&K) was analyzed by using 11 microsatellite markers in order to estimate genetic diversity of this famous breed. This study aimed to provide information on the genetic structure of the Kail breed. In total 47 samples of Kail sheep were genotyped. We obtained 58 alleles, while average number of alleles was 5.2727 ranging from 3 (INRA32, MM12) to 8 (MAF70). The observed heterozygosity (Ho) and expected heterozygosity (He) values were 0.7659 and 0.7185 respectively, most likely as a result of the mating structure of the Kail sheep breed. High heterozygosity values in the investigated population revealed low level of inbreeding, low selection pressure. Within population inbreeding estimates (F_{IS} = 0.0525) further supported low rate of inbreeding. The mean polymorphism information content (PIC) value was 0.60, indicating that the set of used markers were highly informative and may be used in parentage identification. This is the first report of microsatellites based variation in Kail sheep breed and it may assist to construe the genetic characteristics and benefit to the future conservation programs.

Key words: Kail sheep, Azad Jammu and Kashmir, Microsatellite markers, Genetic Diversity

INTRODUCTION

The genetic studies have been generously expedited over the past decades on genetic diversity of small ruminants based on microsatellite markers. This marker has been proven to be valuable for genetic diversity studies (Ghazi *et al.*, 2013; Maletsanake *et al.*, 2013), linkage analysis (Pandey *et al.*, 2013), parentage testing (Kahnshour *et al.*, 2013) and population genetic studies (Tapio *et al.*, 2010) such as reconstruction of phylogenetic and relationship among populations. The use of microsatellites as a genetic marker in the field of molecular genetics is enlightening new feature of genetic variation, due to its elevated variability, highly imperative for detecting differences within populations and between individuals. Microsatellite typing can divulge degree of polymorphism and easy data interpretation make it valuable marker for molecular genetic studies (Buduram, 2004). Sheep are an important livestock species in Azad Jammu and Kashmir (AJ&K), Pakistan and play a major role in the economy of the country. There are 29.1 million sheep in Pakistan, out of which 0.22 million of sheep populations present in AJ&K. Kail breed is among four well defined sheep breeds in AJ&K like Kali, Pahari and Poonchi. They are mostly reared for meat and wool production (Khan *et al.*, 2007).

The present study was carried out to characterize the Kail sheep breed of AJ&K, Pakistan using the microsatellite markers, so that breed identification and effective conservational strategies could be adopted in future for this breed.

MATERIALS AND METHODS

Sampling and DNA extraction: Forty-seven blood samples were collected from unrelated Kail sheep from different Government and private breeding tracts in AJ&K. The isolation of genomic DNA was extracted by standard method as per the method described by Hussain *et al.*, 2013. The isolated DNA samples were assessed for their quantity and quality by spectrophotometric measurements (NanoDrop Thermo Scientific, Wilmington, DE) and also checked on 0.8% agarose gel electrophoresis.

Microsatellite amplification: A total of 11 microsatellite loci were selected, 10 recommended by Food and Agriculture Organization (FAO) (http://www.fao.org/dad-is/) and one (BM1818) recommended by International Society for Animal Genetics (ISAG) (http://www.isag.org.uk / Docs/ 2005_ Panels Markers Sheep Goats. pdf) based on their level of allelic diversity (Table 1). Five markers out of total 11 microsatellite markers were scrutinized as labeled markers: MAF70, BM1818, INRA32, ILSTS011 and BM1314 shown in Table 1. These markers were 5'-labeled with VIC, PET, NED and FAM fluorescence tagged. ABI PRISM 3130 genetic analyzer (Applied Biosystem, USA) was used to perform the fragment analysis of PCR products. A set of 6 microsatellite

markers analyzed were: OarAE101, OarVH72, MAF33, MM12, ETH152 and OarFCB48 illustrated in Table, 1, selected for this study used as unlabeled markers to perform the fragment length analysis of the PCR products using 12% non denaturing polyacrylamide gel (PAGE) in 1X TAE buffer, 50 bp DNA ladder (Fermentas, Maryland, USA) was used as a size standard. To visualize the PCR products, gels were stained with silver staining. PCR amplification through BioRad thermo cycler was carried out using reaction mixture of 25 µL containing 50 ng template DNA, 50 mM KCl, 10mM Tris-HCl, 2.5 mM dNTPs, 1.5 mM MgCl, 0.75 pmol/µL of forward/reverse primers and 0.1 µL of 5U *Taq* polymerase (Frementas, Maryland, USA) and distilled H_2O to make final volume. PCR condition were used as the initial denaturing at 95 °C for 4 minutes was followed by 35 cycles each for 30 s at 94 °C for denaturation, 45 s at 62-52°C for annealing and 45 s at 72 °C for extension followed by 10 minutes at 72 °C as final extension. The unlabeled markers products were electrophoresed on 12% non denaturing polyacrylamide gel in 1X TAE buffer at 120 volts for 7 hours.

Automated DNA genotyping: Genotyping of the microsatellite markers was carried out on an automated ABI PRISM 3130XL Genetic Analyzer (Applied Biosystem, USA) using LIZ 500 (Applied Biosystem, USA) as the internal size standard. Electropherograms were genotyped automatically with the GeneMapper™ software v3.7.

Polyacrylamide Gel electrophoresis: Genotyping of the unlabeled microsatellite markers was carried out through 12% non-denaturing polyacrylamide gel electrophoresis (PAGE) in 1X TAE buffer, 50 bp DNA ladder was used as a size standard. Allele sizing was done using Excel sheet through flow values of alleles compared with relative flow of 50bp DNA ladder run along with the samples.

Data Analysis: Allele identification of microsatellite markers was done using GeneMapper™ software (V 3.7 Applied Biosystem, USA). POPGEN v3.2 (Yeh *et al.*, 1999) was used for the calculation of allele frequency, observed number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He). Polymorphic information content (PIC) values were calculated using POWERSTAT V1.2.1 for the effectiveness of markers. Within breed inbreeding coefficient (Fɪs) was estimated using software GENPOP v 4.0.10 (Raymond and Rousset, 1995). The results of polyacrylamide gel electrophoresis (PAGE) were determined by the relative flow method and the genotypes scoring were performed manually. The online INCHWORM program was also used for the confirmation of size of the alleles, estimates the length of the alleles on the basis of electrophoresis mobility (http://molecularworkshop.com/programs/inchworm.html).

RESULTS

In the present study, 58 alleles were found across 11 investigated microsatellites loci. All the observed microsatellite markers were found to be polymorphic in Kail sheep breed. Considerable variation was observed in the number of alleles across 11 microsatellite markers in Kail sheep, with a mean of 5.2727 ranged from 3 (MM12, INRA32) to 8 (MAF70). Mean of effective number of alleles 3.9471was less than observed ranged from 2.0343 (MM12) to 5.8824 (MAF33). All the tested loci showed a considerable level of genetic variability in term of observed number of alleles (>2) (Crawford *et al.*, 1995). The mean values of observed and expected heterozygosities were 0.7659 and 0.7185, respectively (Table 2). Eight markers out of 11 showed higher observed heterozygosity than expected. ILSTS011 and OarFCB48 showed high observed heterozygosity (1.0000) while, INRA32 showed lowest (0.2000) and expected heterozygosity was ranging from 0.5149 (MM12) to 0.8405 (MAF33). The mean PIC and mean Shannon index were 0.69 and 1.4457 respectively. The mean inbreeding coefficient (F_{IS}) value by Weir and Cockerham method showed 0.0525 whereas based on this approach, five loci showed negative inbreeding values. The polymorphic information content (PIC) showed the informativeness of the loci, with an average of 0.69 across all loci. The BM1314 showed high PIC value (0.82) and MM12 showed lowest (0.42) (Table 2). The overall frequency of all observed alleles is shown in Fig 1.

Table 1. Primer name, sequences (forward and reverse), product size, annealing temperature and chromosome number of 11 microsatellite markers used in Kail sheep.

Marker	Primer sequence (5'-3')	Band size range (bp)	Tm (°C)	Chrom. No.
BM1314	TTCCTCCTCTTCTCTCCAAAC ATCTCAAACGCCAGTGTGG	149-179	54+55	22
BM1818	AGCTGGGAATATAACCAAAGG AGTGCTTTCAAGGTCCATGC	258-284	52+54	20
ETH152	TACTCGTAGGGCAGGCTGCCTG GAGACCTCAGGGTTGGTGACAG	157-169	67+64	05
ILSTS011	GCTTGCTACATGGAAAGTGC CTAAAATGCAGAGCCCTACC	269-283	54+54	09
INRA 32	AAACTGTATTCTCTAATAGCTAC GCAAGACATATCTCCATTCCTTT	160-195	52+54	11
MAF33	GATCATCTGAGTGTGAGTATATACAG GACTTTGTTTCAATCTATTCCAATTTC	121-141	55+52	09
MAF 70	GCAGGACTCTACGGGGCCTTTGC CACGGAGTCACAAAGAGTCAGACC	150-180	54+56	4
MM12	CAAGACAGGTGTTTCAATCT ATCGACTCTGGGGATGATGT	122	58+50	12
OarAE101	TTCTTATAGATGCAACTCAAGCTAGG TAAGAAATATATTTGAAAAAACTGTATCTCCC	99-123	54+54	-
OarFCB48	GAGTTAGTACAAGGATGACAAGAGGCAC GACTCTAGAGGATCGAAAAGAACCAG	146-166	60+60	17
OarVH72	CTCTAGAGGATCTGGAATGCAAAGCTC GGCCTCTCAAGGGGCAAGAGCAGG	114-140	66+71	25

Table2: Observed number of alleles (Na), effective number of alleles (Ne), Shannon index (I), observed heterozygosity (Ho), expected heterozygosity (He), Polymorphic information content (PIC) and within population estimates (F_{IS}) at 11 microsatellite markers genotyped in Kail sheep.

Markers	Na	Ne	I	Ho	He	PIC	F_{IS}
BM1314	5.0000	4.5455	1.5571	0.6000	0.7899	0.82	0.2308
BM1818	5.0000	2.5000	1.2275	0.6000	0.6076	0.57	0.0000
ETH152	6.0000	4.2553	1.6012	0.8000	0.6747	0.73	0.1458
ILSTS011	5.0000	4.2553	1.5162	1.0000	0.7747	0.73	-0.3072
INRA32	3.0000	2.3810	0.9433	0.2000	0.5873	0.55	0.6552
MAF33	7.0000	5.8824	1.8639	0.9250	0.8405	0.81	-0.1145
MAF70	8.0000	4.8411	1.7317	0.9750	0.8035	0.77	-0.2288
MM12	3.0000	2.0343	0.8166	0.6750	0.5149	0.42	-0.3276
OarAE101	5.0000	4.0609	1.4909	0.9750	0.7633	0.72	-0.2935
OarFCB48	5.0000	4.3956	1.5255	1.0000	0.7823	0.73	0.2945
OarVH72	6.0000	4.2667	1.6286	0.6750	0.7753	0.74	0.1184
Mean	5.2727	3.9471	1.4457	0.7659	0.7185	0.69	0.0525

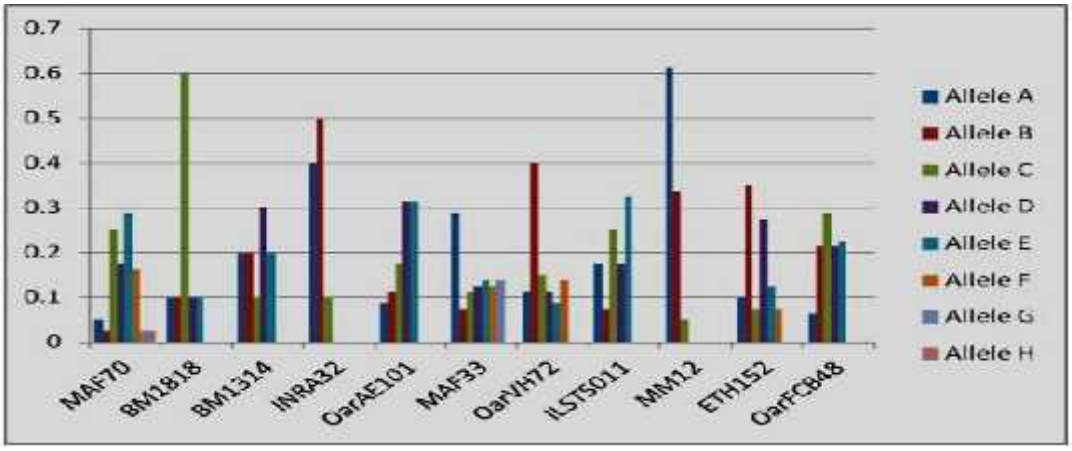

Fig. 1. Overall Allele Frequencies of all observed alleles in 11 microsatellite markers in Kail sheep.

DISCUSSION

There is very few information available on the genetic diversity of AJ&K sheep breeds. This is the first study of its kind on genetic structure of Kail sheep of Neelam and Leepa valley of AJ&K. FAO has recommended that microsatellite markers for genetic diversity studies should have more than four alleles. In this study only two loci (INRA32 and MM12) out of eleven showed three alleles. The total number of alleles per locus ranged from 3 (INRA32 and MM12) to 8 MAF70 with mean value of 5.2727, demonstrated considerable amount of genetic diversity in Kail sheep. This study can be compared with other Pakistani sheep breeds like Salt Range (6.85) and Lohi (5.97) (Hirbo *et al.*, 2006), Balkhi, Hashtnagi and Michni (3.80) (Ibrahim *et al.*, 2010) and Indian sheep Garolea breed (6.20) (Sodhi *et al.*, 2003), Jalauni breed (5.92) (Arora *et al.*, 2008), Vembur sheep (5.88) (Pramod *et al.*, 2009), Shahabadi sheep (5.56) (Pandey *et al.*, 2010), Nali sheep (5.52) (Sodhi *et al.*, 2006), Ganjam sheep (5.48) (Arora *et al.*, 2010), Chokla breed (5.32) (Sodhi *et al.*, 2006), Kheri sheep (5.30) (Bhatia *et al.*, 2005), Muzaffarnagri sheep (5.04) (Arora and Bhatia, 2004) and Medras Red sheep (5.00) (Prema *et al.*, 2008), Iranian sheep breeds (6.48) (Seidani *et al.*, 2009) and higher allelic diversities by Iraqi Hamdani sheep breed (14.17) (Al-Barzinji *et al.*, 2011), Najdi sheep of Saudi Arabia (9.11) (Musthafa *et al.*, 2012), Egyptian sheep breeds (10.30) (EI-Nahas *et al.*, 2008), Spanish sheep breeds (13.30) (Calvo *et al.*, 2011) and Bhutan sheep breeds (13.38) (Dorji *et al.*, 2010). The effective number of alleles ranged from 2.0343 (MM12) to 5.8824 (MAF33) with mean value of 3.9471. This value is higher than two Indian sheep breeds Nali and Chokla with 3.338 and 3.271 respectively (Sodhi *et al.*, 2006).

A substantial difference was seen between observed heterozygosity (H_O) (0.7659) and expected heterozygosity (He) (0.7185) in all microsatellite markers, most likely as a result of the mating structure in Kail sheep. Under assumption, the significant difference between H_O and He showed the random mating within population. This considerable difference indicated some loss of diversity in this breed, perhaps due to population decline and increased inbreeding. This is also evinced by a very low within population inbreeding estimates F_{IS} value (0.0525). The geographical structure of Kail sheep give some consideration that the farmers permitted the animals to be bred naturally, no controlled mating is practice in their flocks. Both rams and ewes are grazed and housed together so no selection pressure was subjected on animals, additionally conceivably, due to lack of any directional selection. The inbreeding coefficient F_{IS} revealed low mean of 0.0525 ranged from 0.3278 (MM12) to 0.6552 (INRA32). Five loci out of all studied 11 loci revealed negative values indicating excess of heterozygotes in the Kail population. The remaining six loci showed positive F_{IS} values, which had scaled the overall mean of heterozygote deficit. Higher rate of inbreeding was observed in Hamdani sheep (0.469) (Al-Barzinji *et al.*, 2011), Sarda sheep (0.19) (Pariset *et al.*, 2003), Chokla (0.299) and Nali (0.397) (Sodhi *et al.*, 2006), Najdi sheep (0.13) (Mushafa *et al.*, 2012), Magra sheep (0.16) (Arora and Bhatia, 2006), Kheri sheep (0.128) (Bhatia and Arora, 2008) and the Muzzafarnagari sheep (0.058) (Arora and Bhatia, 2004). Another parameter, investigative of genetic variation is polymorphism information content (PIC). The PIC revealed high mean of 0.69 across all loci ranged from 0.82 (BM1314) to 0.42 (MM12). PIC is used for the measurement of informativenesss of the markers and it ranges from 0 to 1. The PIC value of the loci if 1 or close to1 with many number of alleles are normally desired for genetic diversity studies (Bostein *et al.*, 1980). All the microsatellite markers showed a consistent polymorphism (PIC > 0.5) except MM12 (PIC = 0.42). However, these markers are extensively used in sheep genetic diversity studies throughout. The Shannon index information also showed the mean value 1.4457, reflecting the species richness and also relative density.

In conclusion, the results of this study illustrated significant genetic variability in the population of Kail sheep in Neelam and Leepa valley of AJ&K. The genetic diversity of Kail sheep will be precious in formulating efficient management and conservation policies for this precious genetic resource.

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