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Full Length Research Paper

Genetic diversity in Balkhi, Hashtnagri and Michni sheep populations using SSR markers

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Morphological and genetic diversity among the three neighboring sheep breeds native to Central valley of Khyber Pukhtunkhwa (KP, Pakistan) was investigated. A total number of 138 non relative individuals of Balkhi (46), Hashtnagri (44) and Michni (48) was sampled for morphological as well as molecular characters using 31 ovine specific SSR markers. Morphological observations and morphometric traits varied significantly among different sheep breeds. Balkhi having usually brown or white colour, with a tucked up fat tail was the larger breed. Hashtnagri is a medium sized breed; body covered with white wool, having long white tail, with a tail switch. The body colour of Michni sheep was usually brown or some times white. This breed is comparatively small in size with longer fat tail, hanging near (33.3%) or below (66.7%) hock. Total number of 119 alleles was identified with mean number of 3.8 alleles per locus, ranging from 2 to 8. Twelve unique alleles were identified in Michni population at different loci. Average gene diversity was higher in Michni (0.561). Inbreeding estimate (F_{IT}) was significantly higher (27.1%) among three breeds and was highest between Balkhi and Hashtnagri (31%), similarly highest gene flow (Nm = 60.4) and lowest population differentiation (F_{ST} = 4.3%) was estimated between these two breeds. Maximum genetic distance was observed between Balkhi and Michni; however, Balkhi was genetically closed to Hashtnagri population. Balkhi and Michni were assigned at high accuracy to their respective population; however, the identity of Hashtnagri is obscure.

Key words: Balkhi, Hashtnagri, Michni, simple sequence repeat (SSR) markers, morphological characteristics, genetic diversity.

INTRODUCTION

A total of 33 native sheep breeds are reported in Pakistan (khan et al., 2007), of these seven (3 fat-tailed and 4 thin-tailed) are native to Khyber Pukhtunkhwa (KP). The fat-tailed breeds (Balkhi, Hashtnagri and Michni) are in the central and generally plain portion of the province. Balkhi, is however, scattered throughout the province, as well as in Punjab and Afghanistan. The said breeds are characterized in the past, but by the virtue of sharing a common breeding tract, it may provides the possibility of interbreeding which consequently leads to the breed to stands at risk of extinction and once the breed is lost it can not be recovered (FAO, 2007).

Balkhi is a heavy breed its colour is usually brown/tan, sometimes black or white in colour, possesses tucked-up fat tail. They are mainly raised for mutton production.

Their population in Khyber Pukhtunkhwa (KP) is about

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Abbreviations: SSR, Simple sequence repeat; **Nm,** gene flow; F_{IT}, inbreeding estimate; F_{IS}, within population inbreeding estimates; F_{IT}, total inbreeding estimates; F_{ST}, measurement of population differentiation; **SDS,** sodium dodecyl sulphate; **EDTA,** ethylene diamine tetraacetic acid; **dNTPs,** deoxynucleotides; **PCR,** polymerase chain reaction; **KP,** Khyber Pukhtunkhwa.

0.3 million heads (GOP, 2006). It is an invading breed whose rams are extensively used for breeding purposes with other native breeds especially in the central valley of Khyber Pukhtunkhwa (KP). Hash-tnagri, a medium-sized and generally white coated with small to medium size head, which is partially or wholly black or tan in colour. They possess short legs and hanging fat-tail with a small switch hanging over the tail. They produce carpet quality wool with a fibre diameter of 35 µm (Khan et al., 2003). Michni is a medium size breed; brown or white in colour with small head, an elongated and thin neck, long body and pendulous fat-tail, hanging below the hocks. The tail is characteristically overlapped at the distal end.

Livestock diversity is changing rapidly mainly due to changes in market demand, as crosses with more proficient offspring gives more productivity to the farmer. It is thus required to conserve the genetic diversity among native livestock populations. The morphological and genetic structure of native sheep breeds is still poorly understood. The level of genetic diversity among these breeds will provide information for successful breeding improvement programs. Molecular characterization is a powerful tool to consider the genetic variation existed within and among breeds. These markers by virtue of being highly polymorphic, co-dominant, evenly distributed and neutral in nature have been used in genome characterization (Soysal et al., 2005; Navani et al., 2002; Pandey et al., 2006) and estimating genetic variation among breeds of various livestock species (Buchanan et al., 1994; MacHugh et al., 1998; Saitbekova et al., 1999; Diez-Tascon et al., 2000; Li et al., 2000; Arranz et al., 1998, 2001; Barker et al., 2001; Kim et al., 2002; Mukesh et al., 2004; Sodhi et al., 2006). The objectives of this study are to determine the morphemical variation and the genetic diversity using 31 SSR markers (FAO, 2005) among these native and neighbouring breeds.

MATERIALS AND METHODS

Morphological characterization and general trait measurement

Total 138 individuals of three breed that is, Balkhi (46), Hashtnagri (44) and Michni (48) were sampled randomly from distinct locations in their breeding tract for morphological characterization. All of the morphological appearance and general trait measurements that is, head length , mouth width, ears colour, neck length and girth, body length, tail length and diameter, colour of different body parts etc was recorded on the Performa, developed as per guide line from FAO (1986).

Blood sampling for DNA isolation

Animals selected for morphological characterization were sampled for blood collection. 3 ml sample was collected from each individual. For this purpose jugular vein was punctured using disposable syringe and the blood was transferred to the labelled vacutainer. Samples were stored at - 20°C until DNA isolation.

Isolation of genomic DNA from blood

Genomic DNA was isolated from whole blood samples using standard phenol chloroform extraction procedure along with DNA extraction buffer containing SDS (1%), sucrose (160 mM), Tris base (100 mM), EDTA (80 mM) and proteinase-K (0.5 µg/ml).

Polymerase chain reaction

All PCR reactions were carried out in 15 µl reaction volumes containing ~100 ng total genomic DNA, 0.5 µM of each primer, 200 µM of dNTPs, 50 mM KCl, 10 mM Tris, 2.0 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase. Amplification condition were; an initial denaturation at 95°C for 7 min, followed by 35 cycles each consisting of a denaturation step of 30 sec at 94°C, annealing step of 30 s and an extension step of 1 min at 72°C. The last cycle was followed by 10 min extension at 72°C. All amplification reactions were performed using palmcycler PCR system (Corbett research) programmable thermocycler. Each of the 31 Ovine specific SSR markers (MAF65, OarFCB193, OarJMP29, OarJMP58, OarFCB304, BM8125, OarFCB128, OarCP34, OarVH72, OarHH47, DYMS1, SRCRSP1, SRCRSP5, SRCRSP9, MCM140, MAF33, MAF209, INRA63, OarFCB20, BM1329, MAF214, ILSTS11, MCM527, OarFCB226, ILSTS28, MAF70, BM1824, OarAE129, HUJ616, OarCP38 and ILSTS5) (Table 1) was amplified in every individual DNA sample. Annealing temperature was adjusted accordingly for each primer. A total of 4340 reactions were done on PCR. The products of PCR were analyzed on 3% agarose gel along with fermentas low range DNA ladder marker.

Statistical analysis

Field data containing the phenotypic observations and morphological trait measurement was analyzed using computer software program SPSS V10.0. Genotype for each individual was scored manually from gel. Observed number of alleles and allele frequencies (Nei, 1973) were computed using POPGENE software version 1.32 (Yeh et al., 1999). Frequencies of null alleles were calculated using software GENEPOP version 4.0 (Raymond and Rousset, 1995). To estimate and test gene diversities and fixation indices that is, F_{IS} (within population inbreeding estimates), F_{IT} (total inbreeding estimates) and F_{ST} (measurement of population differentiation) proposed by Weir and Cockerham (1984) computer software program FSTAT version 2.9.3.2 was used (Goudet, 2001). The significance level ($p < 0.05$) was determined from permutation tests in FSTAT program. The GENE CLASS software was used to calculate the proportion of individuals correctly assigned to their population of origin by different statistical approaches that is Bayesian method (Rannala and Mountain, 1997), frequency based method (Paetkau et al., 1995) and distance based method that is, Nei's standard distance (1972), Nei's minimum distance (1973), Nei's et al., (1983), Cavalli-Sforza and Edwards cord distance (1967) and shared allele distance (Goldstein et al., 1995).

RESULTS

Fat-tailed breeds (Balkhi, Hashtnagri and Michni) maintained in flocks under different shepherding system throughout central part of the province were studied. Morphological examination of the specimens pertaining to these breeds were carried out at farm, however, blood

Table 1. Morphometric traits (means ± SE) of sheep belonging to Balkhi, Hashtnagri and Michni breed in central Khyber Pukhtunkhwa (KP).

Parameter	Mean ± SE			Significance
	Balkhi	Hashtnagri	Michni	
Head length	25.9 ± 0.25^{c}	23.8 ± 0.25^{b}	22.4 ± 0.17^{a}	***
Mouth width	6.4 ± 0.10^{a}	6.7 ± 0.10^{a}	7.1 ± 0.09^{b}	***
Neck length	24.8 ± 0.35^{b}	22.8 ± 0.31^{a}	22.0 ± 0.24^{a}	***
Neck girth	37.2 ± 0.63^{b}	33.2 ± 0.51^{a}	32.5 ± 0.53^{a}	***
Body length	75.4 ± 0.78^{c}	73.0 ± 0.65^{b}	68.1 ± 0.51^{a}	***
Heart girth	97.0 ± 2.07^{b}	92.5 ± 0.78^{b}	84.5 ± 0.81^{a}	***
Belly girth	112.9 ± 1.13^{c}	106.0 ± 0.91^{b}	96.9 ± 0.92^{a}	***
Body depth (Heart)	40.5 ± 0.34^{b}	39.3 ± 0.37^{b}	36.9 ± 0.33^{a}	***
Body depth (Belly)	43.4 ± 0.36^{b}	42.6 ± 0.30^{b}	40.0 ± 0.29^{a}	***
Rump front	21.4 ± 0.29^{c}	19.9 ± .21^{b}	18.1 ± 0.28^{a}	***
Rump back	23.2 ± 0.55^{c}	20.4 ± 0.24^{b}	17.4 ± 0.18^{a}	***
Rump length	18.5 ± 0.32^{c}	17.3 ± 0.25^{b}	15.2 ± 0.16^{a}	***
Rump area (cm^2)	418.1 ± 13.55^{c}	349.4 ± 6.89^{b}	269.9 ± 4.56^{a}	***
Tail length	26.8 ± 0.79^{a}	39.0 ± 1.09^{b}	42.0 ± 0.68^{c}	***
Tail diameter	85.8 ± 1.83^{b}	56.3 ± 1.33^{a}	52.7 ± 1.04^{a}	***
Wither height	82.3 ± 0.66^{c}	75.7 ± 0.59^{b}	70.2 ± 0.60^{a}	***
Rump height	83.9 ± 0.62^{c}	77.8 ± 0.50^{b}	72.3 ± 0.52^{a}	***

***$p < 0.001$. Different superscripts on values (tukey test) show differences at the same row.

samples were collected for DNA analysis using SSR markers at the laboratory at Institute of Biotechnology and Genetic Engineering (IBGE), Khyber Pukhtunkhwa (KP) Agricultural University Peshawar.

Morphological comparison

Table 1 represents significant variation among the three neighbouring sheep breeds for all morphometric traits. Balkhi breed was heaviest in size, manifested by larger size of head length, neck length, neck girth, body length, heart girth, belly girth, body depth (heart), body depth (belly), rump front, rump back, , rump length, rump area (cm^2), tail diameter, wither height and rump height; among the three breeds. Balkhi posses tucked-up fatty rump tail, therefore had lower length. Michni on the other hand, has overlapping tail, hanging below hocks, almost touching the ground. Michni can be distinguishingly characterised by wider mouth than other two neighbouring breeds.

The breeds had some qualitative morphological distinction as well. Balkhi has exclusively flat forehead, slightly or fully convex nose; however, bulging forehead can be rarely found in Michni and Hashtnagri breeds. All of the animals belonging to three breeds were characteristically polled; however, one third of the Balkhi males had scurs. Back was straight in most of Balkhi and Hashtnagri animals; however, half of the Michni (47.9%) carried arched backs.

Tail length and structure varied among the three breeds. Fat tail was bi-lobed, tucked up, short in length hanging above hock in Balkhi. Hashtnagri tail was longer than Balkhi and was hanging mostly (84.8 %) near hock, sometimes (15.2%) below hock. A short thin tail switch, white in colour, is one of the distinguishing features in Hashtnagri sheep, which sometimes, longer enough to touch the ground. Fat-tail was broad and characteristically overlapped at the lower terminal, mostly (66.7%) hanging below hock in Michni animals.

Balkhi and Michni breed are characteristically brown (camel colour), however, variation to white dark brown is also a possibility. Hashtnagri has no consistent colour pattern, but is mostly white. Colour variation in Hashtnagri was observed: black and brown spots were common on the facial and head parts. Ear colour had been inconsistent; however black colour was dominant in Hashtnagri and brown in Balkhi and Michni.

Loin and back was covered with white wool in Hashtnagri. Balkhi sheep are predominantly covered with hairs, however, animals with wool cover on loin, back and limbs are found. Limbs were covered with wool in 50% of Hashtnagri animals. In most of the Michni animals (83.3%) loin and back were covered with wool, however,

other body parts were completely devoid of wool.

Genetic diversity

All the recommended 31 SSR primers, which are claimed to produce polymorphic band in sheep populations, amplified successfully. Aggregate number of 119 alleles across 31 SSR Loci in three sheep populations was identified. Mean number of alleles per locus was 3.8, ranging from 2 (BM1824, ILSTS5, ILSTS11 and OarVH72) to 8 (BM1329 and OarFCB304).

The three sheep populations shared a considerable number (76%) of alleles. A total of 91 alleles were shared among the three sheep populations with a mean of 2.9, ranging from 0 (OarCP38) to 6 (OarFCB304). Hashtnagri and Michni populations shared more number of alleles followed by Balkhi and Michni and Balkhi and Hashtnagri breeds (100; 95; 91), having mean shared-allele per locus as 3.2, 3.1 and 2.9, respectively. OarFCB304 and MAF70 were among the most prolific loci and shared maximum number of alleles among different combinations (Table 2). Michni carried 12 unique alleles, whereas Balkhi (BM1329) and Hashtnagri (OarFCB304) carried one each across all loci. BM1329 was the most mutated locus, yielded three unique alleles in Michni population. Among others loci BM8125, HUJ616 yielded two unique alleles, BM1824, MAF65, OarCP38, OarFCB20 and YMS1 yielded one unique allele each in Michni population.

Average gene diversity among three sheep populations across all loci was estimated to be 0.545 and ranged from 0.016 (BM1824) to 0.761 (MAF70) (Table 2). Gene diversity was higher at loci having higher number of alleles. Average gene diversity was higher in Michni population (0.561) which ranged between 0.049 (BM1824) and 0.813 (MAF70). In Balkhi sheep-population, the average gene diversity was estimated to be 0.547, ranged from 0.204 (OarVH72) to 0.753 (MAF70). Average gene diversity was estimated to be low (0.533) in Hashtnagri population as compared to other populations. In this population the value ranged from 0.078 (OarCP38) to 0.755 (OarFCB304).

Allele size and frequency

Different alleles and their frequencies obtained through amplifying genome fragments using SSR primers in three sheep breeds are presented in Table 3. At sixteen loci (OarVH72, BM1824, OarJMP29, OarHH47, SRCRSP1, MAF33, MAF209, INRA63, OarAE129, HUJ616, OarFCB193, MCM140, OarFCB226, BM8125, OarFCB304 and YMS1), the same allele was at highest frequency in all the three sheep populations. At six loci (MAF65, ILSTS5, SRCRSP5, OarFCB20, ILSTS28 and MAF70), the highest frequency at the same allele was observed in Balkhi and Hashtnagri. In Balkhi and Michni populations, the frequency for the same allele was found highest at two loci (ILSTS11 and BM1329). Hashtnagri and Michni also had same alleles at highest frequency at six loci (MCM527, OarCP38, OarJMP58, SRCRSP9, OarCP34 and MAF214). At marker OarFCB128, different alleles were at high frequency in different populations. The frequency of unique alleles was low in Balkhi and Hashtnagri populations (0.027 and 0.012, respectively), however, in Michni the frequency of three unique alleles was greater than 20% with highest at marker MAF65 (allele A). Null alleles were found at high frequency in Balkhi population at twelve loci (MAF65, OarJMP29, OarCP34, OarHH47, MAF33, MAF209, OarFCB193, OarJMP58, MCM140, OarFCB226, BM8125 and YMS1), however it ranged from 0.097 (SRCRSP9) to 0.577 (ILSTS11).

In Hashtnagri sheep the frequency of null alleles ranged from 0.017 (BM1329) to 0.959 (OarCP38), at nine loci the frequency was highest as compare to other two breeds (ILSTS11, SRCRSP1, SRCRSP5, INRA63, OarAE129, OarCP38, OarFCB128, SRCRSP9 and BM1329). In Michni population null alleles were found at highest frequency at six loci (ILSTS5, OarFCB20, HUJ616, MAF214, ILSTS28 and MAF70) ranged from 0.001 (BM8125) to 0.830 (ILSTS5).

Population structure of three sheep populations

Gene diversity analysis is developed primarily to estimate the inter- and intra-population genic variations with respect to entire genome of the organism. It is important to use large number of loci which are ideally a random sample from the total genome. It is usually expressed in the proportions of the subcomponents. It is similar to the Shannon Information Index, I, which is also concerned with partitioning of the genetic variation of the total population. However, Shannon Information Index does not carry much meaning; whereas gene diversity indicates the genetic variability of a population and can be related to a number of codons differences per locus.

F-statistics is primarily concerned with relationship between the genotype frequencies in the total populations and in the sub population for a single locus. This is usually expressed in ratio of different type of gene diversities, or simply; it is the ratios of gene diversities of heterozygosities rather than the correlation of uniting gametes.

F_{IT} is the total inbreeding estimates among the subpopulations; F_{IS} is the same value within subpopulation and F_{ST} is measurement of differentiation among subpopulations.

The fixation indices F_{IT} and F_{ST} for estimating population differences for all SSR Loci between different breeds are presented in Table 4. Overall mean inbreeding estimate

Table 2. Number of total amplified, shared and unique alleles and gene diversity at each locus in three sheep populations.

Loci	Number of Alleles	Shared alleles				Unique alleles			Gene diversity			
		B-H	B-M	H-M	B-H-M	B	H	M	Total	B	H	M
BM1329	8	4	4	4	4	1		3	0.710	0.650	0.724	0.757
BM1824	2	1	1	1	1			1	0.016	-	-	0.049
BM8125	6	3	3	4	3			2	0.595	0.486	0.652	0.646
HUJ616	4	2	2	2	2			2	0.359	0.399	0.268	0.409
ILSTS11	2	2	2	2	2				0.384	0.499	0.309	0.344
ILSTS28	5	4	4	4	4				0.674	0.721	0.617	0.683
ILSTS5	2	2	2	2	2				0.464	0.444	0.511	0.438
INRA63	3	3	3	3	3				0.622	0.646	0.628	0.591
MAF209	3	3	3	3	3				0.484	0.539	0.412	0.501
MAF214	5	3	3	5	3				0.628	0.464	0.740	0.679
MAF33	3	3	3	3	3				0.575	0.644	0.648	0.432
MAF65	3	2	2	2	2			1	0.463	0.380	0.352	0.658
MAF70	7	4	5	6	4				0.761	0.753	0.716	0.813
MCM140	4	3	3	4	3				0.582	0.565	0.611	0.569
MCM527	3	3	3	3	3				0.611	0.597	0.618	0.618
OarAE129	3	2	3	2	2				0.248	0.214	0.226	0.304
OarCP34	3	3	3	3	3				0.605	0.587	0.641	0.587
OarCP38	3	0	0	2	0			1	0.359	-	0.078	0.639
OarFCB128	4	4	4	4	4				0.692	0.743	0.667	0.667
OarFCB193	4	4	4	4	4				0.639	0.663	0.660	0.593
OarFCB20	3	2	2	2	2			1	0.518	0.515	0.442	0.596
OarFCB226	4	4	4	4	4				0.729	0.747	0.744	0.696
OarFCB304	8	6	7	6	6		1		0.763	0.729	0.755	0.804
OarHH47	3	3	3	3	3				0.580	0.599	0.597	0.543
OarJMP29	3	3	3	3	3				0.600	0.614	0.638	0.547
OarJMP58	4	4	4	4	4				0.695	0.736	0.629	0.719
OarVH72	2	2	2	2	2				0.277	0.204	0.367	0.260
SRCRSP1	3	3	3	3	3				0.550	0.530	0.604	0.515
SRCRSP5	3	3	3	3	3				0.482	0.436	0.478	0.531
SRCRSP9	4	3	3	4	3				0.593	0.668	0.599	0.513
YMS1	5	3	4	3	3			1	0.641	0.629	0.591	0.704
Mean/locus	3.8	2.9	3.1	3.2	2.9				0.545	0.547	0.533	0.561
SD	1.6	1.1	1.2	1.2	1.1				0.166	0.177	0.195	0.166

Key: B: Balkhi; H: Hashtnagri; M: Michni.

of 27.1% was significantly higher and ranged at different loci from -25.7% (OarFCB304) to 98.4% (ILSTS5). F_{IT} estimates between two different sheep populations were also significantly different from zero. Between Balkhi and Hashtnagri (31%) the valuewas highest followed by Balkhi and Michni (26.6%) and Hashtnagri and Michni (23.9%). The genetic differen-tiation (F_{ST}) at all loci across the three populations was significantly different and valued 0.066, which means that the proportion of total genetic differentiation between the breeds is 6.6% and among the individuals of all the three breeds is 93.4%. F_{ST} values between different populations was also higher ($P < 0.05$). Mean genetic differences (F_{ST}) between Balkhi and Hashtnagri was the lowest (4.3%), between Balkhi and Michni was 7.6% and between Hashtnagri and Michni was 8.1%. Mean gene flow between the three neighbouring breeds was high (N_m = 12.71). However, gene flow when estimated between two breeds separately; was highest (N_m = 60.414) bet-ween Balkhi and Hashtnagri; followed by Balkhi and Michni (N_m = 19.738) and Michni and Hashtnagri (N_m = 14.836).

Genetic distance

Table 5 shows that Balkhi and Hashtnagri were the closest

Table 3. Size and frequencies of SSR-amplified and null alleles present on genomes of three different sheep populations.

Locus/allele	Size (bp)	Allele frequency			Locus/allele	Size (bp)	Allele frequency		
		B	H	M			B	H	M
OarVH72					**ILSTS11**				
A	160	0.886	0.761	0.848	A	290	0.571	0.186	0.783
B	175	0.114	0.239	0.152	B	300	0.429	0.814	0.217
Null		0.151	0.151	0.000	Null		0.577	0.876	0.255
BM1824					**ILSTS5**				
A	190	1.000	1.000	0.975	A	190	0.686	0.524	0.311
B	200			0.025	B	195	0.314	0.476	0.689
Null				0.000	Null		0.561	0.681	0.830
MAF65					**OarJMP29**				
A	95			0.318	A	140	0.244	0.261	0.204
B	100	0.763	0.779	0.25	B	145	0.535	0.489	0.625
C	110	0.237	0.221	0.432	C	155	0.221	0.25	0.17
Null		0.459	0.457	0.449	Null		0.299	0.262	0.278
OarCP34					**OarHH47**				
A	120	0.21	0.487	0.548	A	110	0.375	0.302	0.139
B	125	0.579	0.276	0.129	B	160	0.514	0.546	0.625
C	135	0.21	0.237	0.323	C	170	0.111	0.151	0.236
Null		0.385	0.303	0.227	Null		0.434	0.287	0.300
SRCRSP1					**SRCRSP5**				
A	145	0.643	0.523	0.639	A	155	0.193	0.193	0.63
B	150	0.167	0.337	0.279	B	160	0.727	0.693	0.25
C	155	0.19	0.139	0.081	C	165	0.079	0.114	0.12
Null		0.213	0.357	0.200	Null		0.290	0.305	0.005
MAF33					**MAF209**				
A	135	0.477	0.454	0.704	A	100	0.046	0.045	0.136
B	140	0.302	0.227	0.023	B	110	0.558	0.739	0.67
C	150	0.221	0.318	0.273	C	125	0.395	0.216	0.193
Null		0.402	0.324	0.143	Null		0.507	0.324	0.259
INRA63					**OarFCB20**				
A	185	0.383	0.267	0.212	A	110	0.5	0.684	0.444
B	190	0.433	0.512	0.576	B	120	0.5	0.316	0.458
C	200	0.183	0.221	0.212	C	135			0.097
Null		0.300	0.381	0.372	Null		0.147	0.200	0.296
MCM527					**OarAE129**				
A	180	0.43	0.454	0.454	A	95	0.077		0.111
B	200	0.465	0.409	0.409	B	110	0.885	0.875	0.833
C	210	0.105	0.136	0.136	C	120	0.038	0.125	0.056
Null		0.239	0.318	0.318	Null		0.129	0.327	0.251
OarCP38					**HUJ616**				
A	260		0.04	0.25	A	140	0.732	0.844	0.757
B	300			0.263	B	145	0.268	0.156	0.135
C	380		0.96	0.487	C	150			0.081
Null			0.959	0.451	D	155			0.027
					Null		0.081	0.017	0.114
OarFCB193					**OarJMP58**				
A	100	0.097	0.214	0.26	A	180	0.163	0.537	0.417

Table 3. Contd.

B	110	0.516	0.511	0.58	B	185	0.326	0.146	0.226
C	120	0.177	0.19	0.02	C	190	0.326	0.256	0.202
D	130	0.21	0.083	0.14	D	200	0.186	0.061	0.155
Null		0.344	0.228	0.285	Null		0.206	0.055	0.121
OarFCB128					**SRCRSP9**				
A	90	0.209	0.139	0.52	A	160	0.357	0.579	0.667
B	110	0.349	0.198	0.18	B	165	0.262	0.216	0.056
C	125	0.267	0.512	0.16	C	170	0.381	0.159	0.189
D	130	0.174	0.151	0.14	D	310		0.045	0.089
Null		0.239	0.283	0.169	Null		0.097	0.156	0.006
MCM140					**OarFCB226**				
A	120		0.023	0.267	A	100	0.238	0.198	0.398
B	180	0.075	0.209	0.035	B	110	0.345	0.337	0.352
C	200	0.537	0.558	0.593	C	120	0.179	0.186	0.125
D	230	0.387	0.209	0.105	D	130	0.238	0.279	0.125
Null		0.520	0.392	0.081	Null		0.308	0.303	0.181
ILSTS28					**MAF214**				
A	160	0.295	0.163	0.178	A	105		0.091	0.1
B	165	0.256	0.279	0.478	B	120	0.695	0.227	0.322
C	170	0.359	0.535	0.233	C	170	0.232	0.398	0.444
D	180	0.09	0.023	0.111	D	220	0.073	0.204	0.011
Null		0.119	0.054	0.209	E	300		0.079	0.122
					Null		0.000	0.000	0.045
BM8125					**MAF70**				
A	140	0.076	0.105	0.012	A	120	0.244	0.41	0.223
B	150	0.674	0.523	0.537	B	125	0.384	0.244	0.191
C	200	0.25	0.209	0.075	C	140	0.139	0.244	0.287
D	210			0.05	D	190	0.151		0.096
E	450			0.237	E	210	0.081	0.064	0.096
F	510		0.163	0.087	F	500		0.013	0.032
Null		0.205	0.028	0.001	G	520		0.026	0.074
					Null		0.000	0.000	0.043
OarFCB304					**BM1329**				
A	180	0.439	0.39	0.326	A	160	0.378	0.323	0.181
B	190	0.232	0.183	0.233	B	170	0.054	0.132	0.069
C	200	0.037	0.158	0.128	C	210	0.027		
D	240	0.037	0.049	0.116	D	220	0.446	0.176	0.347
E	300	0.073	0.195	0.081	E	400	0.095	0.368	0.083
F	430	0.037		0.058	F	430			0.292
G	490		0.012		G	700			0.014
H	510	0.146	0.012	0.058	H	720			0.014
Null		0.000	0.000	0.000	Null		0.000	0.017	0.000
YMS1									
A	170	0.029		0.033					
B	175	0.314	0.193	0.293					
C	180	0.514	0.568	0.424					
D	185	0.143	0.238	0.185					
E	195			0.065					
Null		0.166	0.043	0.090					

B: Balkhi; H: Hashtnagri; M: Michni; Null: frequency of null alleles.

Table 4. Pair wise estimates of F-statistics and gene flow (Nm) at each of 31 SSR loci between three sheep breeds.

Locus	Overall			B and H			B and M			H and M		
	F_{IT}	F_{ST}	Nm	F_{IT}	F_{ST}	Nm	F_{IT}	F_{ST}	Nm	F_{IT}	F_{ST}	Nm
MAF65	0.599*	0.237*	1.227	0.907*	- 0.037	696.851	0.458*	0.246*	1.256	0.580*	0.288	1.168
OarFCB193	0.419*	0.009	10.866	0.401*	0.003	21.656	0.420*	0.022	10.146	0.440*	0.008	15.703
OarJMP29	0.197*	- 0.001	30.473	0.128*	- 0.011	188.458	0.264*	- 0.004	46.729	0.203*	0.010	20.761
OarJMP58	0.088	0.060	4.897	0.136*	0.114	3.505	0.107	0.047	7.941	0.012	0.012	20.527
OarFCB304	- 0.257	0.017*	14.089	- 0.263	0.027*	13.588	- 0.239	0.014*	21.178	- 0.268	0.011	25.153
BM8125	0.159*	0.057*	5.026	0.269*	0.031*	10.862	0.278*	0.085*	4.657	- 0.051	0.056*	7.030
OarFCB128	0.300*	0.083	3.096	0.307*	0.045*	7.905	0.237*	0.072	5.025	0.366*	0.154	2.434
OarCP34	0.317*	0.113*	2.523	0.426*	0.107*	3.578	0.375*	0.208	1.754	0.117	0.010	18.414
OarVH72	- 0.085	0.019	12.551	- 0.029	0.042	9.037	- 0.054	- 0.004	77.346	- 0.157	0.015	20.781
OarHH47	0.528*	0.016	9.702	0.656*	- 0.015	73.587	0.607*	0.049	6.673	0.327*	0.018	13.987
YMS1	0.271*	0.006	17.469	0.316*	0.006	21.741	0.297*	- 0.005	44.786	0.208*	0.017	16..342
SRCRSP1	0.424*	0.011	13.398	0.433*	0.023	12.131	0.308*	0.008*	20.966	0.522*	0.000	27.107
SRCRSP5	0.451*	0.212	1.316	0.570*	- 0.016	192.889	0.435*	0.296	1.133	0.390*	0.271	1.283
SRCRSP9	0.186*	0.071	4.093	0.311*	0.062	6.07	0.146*	0.126	3.187	0.089	0.020	15.231
MCM140	0.277*	0.069	4.173	0.481*	0.025	11.384	0.169*	0.111	3.584	0.192*	0.068	5.698
MAF33	0.198*	0.057	5.010	0.286*	- 0.003	40.844	0.286*	0.099	3.997	0.014	0.081	5.024
MAF209	0.312*	0.032	7.675	0.379*	0.050	7.223	0.322*	0.042	8.322	0.229*	0.001	33.519
INRA63	0.408*	- 0.001	17.432	0.395*	- 0.003	29.788	0.394*	0.018	11.999	0.434*	- 0.014	82.505
OarFCB20	0.584*	0.034	6.708	0.581*	0.044	6.867	0.528*	- 0.014	37.932	0.631*	0.059	5.816
BM1329	- 0.082	0.098*	3.084	- 0.044	0.093	4.32	- 0.254	0.081	5.15	0.049	0.119	3.323
MAF214	- 0.055	0.116*	2.581	0.094	0.179*	2.141	0.004	0.149*	2.654	- 0.249	0.027	13.929
ILSTS11	0.646*	0.318	0.774	0.740*	0.259	1.334	0.452*	0.083	4.653	0.702*	0.517	0.452
MCM527	0.481*	- 0.015	142.733	0.464*	- 0.013	126.176	0.464*	- 0.013	126.176	0.514*	- 0.018	0
OarFCB226	0.201*	0.012	14.399	0.170*	- 0.012	214.131	0.236*	0.014	17.261	0.197*	0.032	10.494
ILSTS28	0.212*	0.052*	5.522	0.126*	0.026*	12.371	0.233*	0.040	8.762	0.265*	0.085	4.634
MAF70	- 0.100	0.033*	8.323	- 0.048	0.043*	8.854	- 0.082	0.033	11.227	- 0.166	0.024	14.434
BM1824	0.000	0.012	14.625	-	-	-	- 0.003	0.009	19.5	0.001	0.015	19.5
OarAE129	0.600*	- 0.005	14.775	0.593*	0.006	15.884	0.481*	- 0.022	61.134	0.745*	- 0.003	13.459
HUJ616	0.262*	0.013*	12.007	0.214*	0.020	13.07	0.312*	0.015	15.477	0.245*	0.004	21.72
OarCP38	-	-	1.050	-	-	-	-	-	-	0.233*	0.280*	1.05
ILSTS5	0.984*	0.113	2.405	0.973*	0.027	8.932	1.000*	0.227	1.531	0.977*	0.068	5.097
Mean	0.271*	0.066*	12.710	0.310*	0.043*	60.414	0.266*	0.076*	19.738	0.239*	0.081*	14.836

F_{IT} = total inbreeding estimates; F_{ST} = measure of population differentiation; Nm = gene flow; *p<0.05 from permutation tests in FSTAT program;
B: Balkhi, H: Hashtnagri; M: Michni.

Table 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among different sheep breeds.

Breed	Balkhi	Hashtnagri	Michni
Balkhi	---	0.898	0.868
Hashtnagri	0.107	---	0.878
Michni	0.141	0.130	---

Table 6. Percentage of individuals correctly assigned to their population of origin based on Bayesian, frequency and genetic distance assignment methods using 1000 simulations.

Breed	Bayesian criteria	Frequency criteria	Distance criteria				
			D_S	D_M	D_A	D_C	D_{AS}
Balkhi	78.26	95.65	89.13	76.09	78.26	82.61	91.30
Hashtnagri	9.09	70.45	61.36	88.64	4.55	2.27	22.73
Michni	97.87	97.87	95.74	93.62	95.74	89.36	82.89
Overall	62.77 (86/137)	88.32 (121/137)	82.48 (113/137)	86.13 (118/137)	60.58 (83/137)	59.12 (81/137)	66.42 (91/137)

D_S = Nei's standard distance; D_M = Nei's minimum distance; D_A = Nei's average distance; D_C = cord distance (C Sforza and Edwards) D_{AS} = shared allele distance (Goldstein et al., 1995).

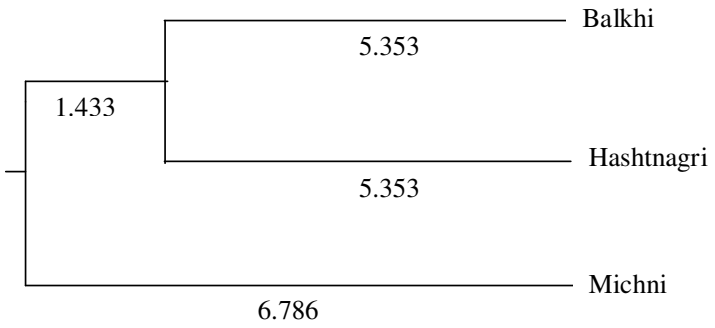

Figure 1. Phylogenetic tree of three sheep populations.

among the three populations. Hashtnagri had some degree of proximity with Michni; however, Michni and Balkhi had the maximum genetic distance. A phylogentic tree was derived from Nei genetic distance from values available in Table 6 using the neighbour joining method (Figure 1). It depicts that Balkhi and Hashtnagri populations are comparatively closer genetically, however Michni population is slightly distant.

Genetic assignment of sampled individuals to their respective breed population

Individual genotype obtained through PCR using SSR primers were analysed using "Gene Class" software assessed the probability for assigning individual/group to a reference population (Cornuet et al., 1999). To this end various methods are employed; including Bayesian (Rannala and Mountain, 1997), frequency based (Paetkau et al., 1995) and distance based methods. Distance based method assign the candidate using five different methods: Nei's standard distance (1972), Nei's minimum distance (1973), Nei's D_A (1983), cord distance (Cavalli-Sforza and Edwards, 1967) and shared-allele distance (Goldstein et al., 1995).

Assigning percentage of sampled specimens to their respective breed population has been presented in Table 6. Sampled specimens from Michni breed were assigned at high accuracy: ranged from 82.89 to 97.87% from different methods. Approximately 76 to 96% Balkhi individuals were sampled correctly as computed by different methods. Results for Hashtnagri have been more inconsistent as computed under different methods. The accuracy of sampled individuals ranged from 2.27 to

88.64%.

DISCUSSION

Colour of Pakistani sheep breeds is mostly white; however, brown, red or black colour sheep are also common (Hasnain, 1985). Hashtnagri is usually white with either partially or completely black or tan head, as reported by Khan et al., (2003). Contrary to Khan et al. (2003), Michni were usually brown in colour, however, a few white animals were also observed. Blakhi were usually brown however white specimen was found in some flocks.

In all of morphometric traits variation existed, indicating phenotypic variation among these breeds. Balkhi is a recognised as heavy breed (khan et al., 2003), having large tucked up fat tail/rump compared to Michni and Hashtngari. Tail in Michni hangs below hock and in some cases almost touches the ground. Tail in Hashtangri and Michni is more or less folded at the lower end, however, has different structures. Hashtnagri has an additional tails-witch hanging at the flap over (Hasnain, 1985). Face legs and belly were devoid of wool in Balkhi and Michni animals.

The markers used under the current study are highly specific ovine markers suggested by FAO (2005). Knowledge about the amount and distribution of genetic diversity in populations and their evolutionary history can be used to make conservation policy for endangered species (Feng, 2000). Although Hashtnagri and Mchini shared maximum (85%) alleles than other breeds combinations (Balkhi and Michni: 82%; Balkhi and Hashtnagri: 76%), but were found genetically distant in comparison to Balkhi and Hashtnagri. Similarly, Balkhi and Hashtnagri despite their less sharing alleles had closest genetic relation. Two Indian neighbouring sheep breeds: Nali and Chokla, share 70.4% of their alleles and reported to have close genetic similarity (Sodhi et al., 2006). Michni population can be distinctly characterised based on their higher number of unique alleles and higher genetic diversity. Among the 12 unique alleles 3 can serve as genetic markers because of their frequencies which exceed 0.2 (Kim et al., 2002). Baltic sheep breed have been reported to posses 36 unique alleles at 15 microsatellite loci (Grigaliunaite et al., 2003). In other studies, Indian, Swiss and Spanish sheep breeds have been reported to be the carrier of unique alleles but in lower frequencies insufficient for declaring as genetic markers (Sodhi et al., 2006; Saitbekova et al., 2001; Arranz et al., 2001). OarFCB193 amplified a unique allele of 107bp with a frequency of 92% in Mouflon sheep (Saitbekova et al., 2001). Michni population being the smallest of the three, concentrated in limited habitat and are their larger number are raised in comparatively pure flocks, can be easily focused for conservation and development. Molecular results of the current study also suggest emphasising Michni conservation on priority.

Average gene diversity in Michni was comparatively higher: which is a function of its higher number of alleles (Moioli et al., 2001). BM1329 and OarFCB304 were highly polymorphic loci giving 8 alleles each in sheep population under-study. Other scientific workers reported OarFCB304 as highly polymorphic with 5 alleles in Afshari sheep (Qanbari et al., 2007), 7 in Iranian sheep breeds (Khanian and Banabazi, 2006) 19 in merino sheep (Diez-Tascon et al., 2000) and 46 in Chinese sheep breeds (Dongyan et al., 2007). Null alleles are those allele that fail to multiply during PCR using a given microsatellite primer due to mutation at the primer site (Callen et al., 1993; Pemberton et al., 1995). These are non-functional mutants, frequent at few loci in Michni (6 versus 9 versus 12; in Michni, Hashtnagri and Balkhi) suggesting greater focus on Michni conservation.

Per pair F_{ST} value equals 0.05 indicates moderate differentiation and those lower than 0.05 indicate low differentiation between populations/breeds (Hartl, 1980). The current F_{ST} suggests that Michni population has sufficient distinction from Balkhi and Hashtnagri populations; however, differentiating Hashtnagri from Balkhi is obscure. Total inbreeding estimates (F_{IT}) was high between Balkhi and Hashtnagri populations, indicative of high rate of inbreeding in these populations which is evident by the heterozygotes deficiency in these populations.

The highest rate of gene flow further supports proximal interbreeding leading to weakly identified phenotypic features and genetic similarities between the neighbourring breeds: Balkhi and Hashtnagri; sharing the same breeding tract (Beja-Pereira et al., 2003). Field data is evident of the fact that Balkhi is the invading breed, mostly used for cross breeding in Hashtnagri flocks. Nali and chokla are two such breeds where inadvertent breeding between the neighboring breeds lead to weak identification due to an enhanced gene flow between them (Sodhi et al., 2006).

The principles underlying assigning individual to the population are based on computing likelihood of a genotype utilizing either its allelic frequencies in the candidate population (Frequency-based method) or probability density of population allele frequencies at independent loci in each population (Bayesian approach). Distance based-method utilizes the respective genetic distance of an individual to different populations and assign to the closest one (Waser and Strobeck, 1998; Davies et al., 1999; Bjornstad and Roed, 2002; Fan et al., 2002; Koskinen, 2003) using different methods: Nei's standard (1972), Nei's minimum (1973), Nei's et al., (1983), chord distance (Cavalli-Sforza and Edwards, 1967) and shared-allele distance (Goldstein et al., 1995).

Values for number of loci, number of alleles, sample size and heterozygosity is directly correlated to assignment accuracy (Bjornstad and Roed, 2002; Fan et al.,

2002). Overall results obtained for individual assignment to their respective populations in the current study varied when computed through different methods. The reliability of the three methods depends upon the study conditions. According to Cornuet et al. (1999), Bayesian method is considered superior over frequency based and genetic distance methods. However, if the population deviates from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium, distance-based method is preferred over Bayesian method. D_{AS} distance method may perform better if genetic diversity within the population is high in an evolutionary spread population (Goldstein et al., 1995). Conditions of the current study indicate the suitability of Bayesian method, which indicates that specimen sampled for Michni were assigned with 98% accuracy, followed by Balkhi with 78%. Individual sampled for Hashtnagri breed had very poor assignment accuracy (9%). Inter-population genetic differentiation influences the assignment accuracy and can be obtained at higher level in well-differentiated population (Bjornstad and Roed, 2002).

In conclusion, specimens from Michni and Balkhi breeds exist in the original form in the central part of Khyber Pukhtunkhwa (KP). Despite significant morphological differences between the three breeds Hashtnagri lost its genetic identity due to excessive gene flow resulted from the invading effect of Balkhi rams. The study, however, suggests maximum conservational emphasis on Michni, which possessed 12 unique alleles.

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