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Genetic Diversity of Endangered Sheep Breed of Valley Swat, Pakistan, Based on Microsatellite Analysis

Naveed Ullah[a], Muhammad Tariq Zeb[b*], Mirza Ali Khan[c], Muhammad Tahir Khan[d], Sibghat Ullah[e], Muhammad Nauman-ul-Islam[f], Azizullah Noor[g], Ihsanullah Khan[h], Muhammad Idrees[i], Sohail Ahmad[j], Ihsan uddin[k]

[a,j]*Department of Animal Breeding and Genetics, The University of Agriculture, Peshawar, Pakistan.*
[b,k]*Center of Microbiology and Biotechnology, Veterinary Research and Disease Investigation Center, Swat, Pakistan.*
[c]*Center of Microbiology and Biotechnology, Veterinary Research Institute, Peshawar, Pakistan.*
[d]*Department of Bioinformatics, Mohammad Ali Jinnah University, Islamabad, Pakistan.*
[e]*Foot and Mouth Disease Research Centre, Veterinary Research Institute, Peshawar, Pakistan.*
[f]*Livestock Research and developmental Station surezai, Peshawar, Pakistan.*
[g]*Hazara University, Mansehra, Pakistan.*
[h]*Livestock and Dairy Development Department, Khyber Pakhtunkhwa, Peshawar, Pakistan.*
[i]*Center for Biotechnology and Microbiology, The University of Swat, Pakistan.*
[b]*Email: drtariqzeb@yahoo.com*

Abstract

Morphologically, Kutta breed is black coat color sheep, with short tail and having thick wool fiber diameter. Weight differences in Kutta sheep exists as animals found in plain areas are slightly heavier and bigger than those found in hilly and remote areas of upper Kalam and Malamjaba of valley Swat with an average body weight of 23.2 ±0.34 kg. Aim of the study is to provide information on the genetic structure of the Kutta breed. A set of 31 FAO recommended microsatellite (SSR) markers was used to access the genetic variability in 21 DNA samples extracted.

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* Corresponding author.

The breed showed high level of genetic diversity. Out of the total 31 FAO recommended markers, 23 were fully amplified during Polymerase Chain reactions (PCR), having a total of 151 different alleles obtained, with an effective allele of (6.57) per locus. Similarly high level of average heterozygosity (0.77) and gene diversity (0.80) was observed. The within breed estimate (FIS) in breed was found extremely low (0.002). Shannon information Index (I) was found (1.65). The mean Chi-square value for the Hardy Weinberg Equilibrium (0.10) indicates that the population has lost its equilibrium on the overall basis. Majority of the markers were found neutral when test on Evens-Watterson, which suggest that decreases in heterozygosity in the population was not due to selection. Test for mutation drift equilibrium in the population showed that the population had preserved its effective size and has not experienced genetic bottleneck at least in the recent past.

Keywords: Kutta sheep; Endangered; Microsatellite; Inbreeding estimate; Heterozygosity**.**

1. Introduction

Sheep survive very well under harsh climatic condition. It is well adapted to various climatic conditions i.e. mountainous habitat to desert. Sheep was domesticated between 9000 and 11000 years ago. It is assumed that domesticated sheep descended from the wild sheep of Asia and Europe. Domestic sheep evolved from two wild stocks viz. European Moufflon *(Ovismusimon)* and Eastern moufflon *(Ovisorientalis).*Among the local European breed; remarkable population of moufflon is still found in certain parts of Europe. Both of the Moufflon stocks are considered as ancestor of domestic sheep [14]. Pakistani sheep breeds are assumed to be descended from Urail of Afghanistan, Argali of India and Marco polo of China however, recent cytological evidences decline it. Moreover, evidence of domestication exist in Bolan valley of Mehargar [2]. A total 28 indigenous breeds of sheep are found in Pakistan. Punjab and Khyber Pakhtunkhwa have seven each; Balochistan and Azad Jammu and Kashmir (AJK) have four each while Sindh and Northern Areas have three breeds each. In Pakistan sheep are kept for wool and mutton. However, majority Pakistani sheep's have coarse wool texture [17]. Commercially important sheep breeds for commercial purposes in Pakistan are Rambouillet, Awassi, Kaghani, Damani, Kachhi and Lohi [17].

Kutta sheep is characteristically black coat; thin short tail sheep with an average weight of 23.2±0.34 kg. Utilization of the breed is limited to wool production that is used for manufacturing hand woven cloth; locally called "Lamsay & sharri". The breed show remarkable resistance to enterotoxamia and Foot Mouth diseases. Delicious taste of its meat quality has another bright aspect which need to be highlighted [1]. However, as a result of indiscriminate crossing with other exotic breeds of sheep, the population is at extremely endangered. According to recently assessment report (September-October, 2012), less than 300 specimens remain in the entire region of Swat, of them 274 were ewes and only 23 were breeding rams. As nearly 20% of the documented breeds are considered to be under the threat of extinction. So far, of 7600 breeds documented in the databank animal Genetic Resources (AnGr) 11% got extinct and only 38% are safe from extinction [18]. Kutta is also among those breed which is going to extinct [1]. Keeping in view the endangered status of Kutta sheep; the present study was designed to genotypically characterize Kutta sheep using FAO recommended SSR markers. This will facilitate the conservation process for the breed.

2. Material and methods

2.1 Blood sampling for DNA isolation

Animal belonging to pure breed of Kutta sheep were selected from their breeding tract for blood collection. Three milliliter of blood sample was collected randomly from each individual. For this purpose jugular vein was punctured using disposable syringe and the blood was transferred to the labeled vacutainer. Before DNA isolation, the samples were carefully placed in a refrigerator at -20 ºC.

2.2 Genomic DNA extraction from blood and PCR

DNA was extracted from blood following phenol chloroform extraction procedure. The details of procedure given elsewhere [12]. PCR reaction volume was 15 µL with 100ng total genomic DNA, 0.25 µM of each primer (forward and reverse), 200 µM of dNTP, 1 X PCR buffer (Fermentas), 1.5mM MgCl2and 1.0 unit of Taq DNA polymerase. Amplification condition were an initial denaturation at 94 ºC, followed by 30 cycles each consisting of a denaturation step of 1 minute at 94 ºC, annealing step of 1 minute, and an extension step of 2 minutes at 72 ºC. The last cycle was followed by 10 minutes extension at 72 ºC. All amplification reactions were carried out using Gene Amp PCR System 2700 (Applied Biosystem). For each and every DNA sample 31 ovine specific SSR markers (given in supplementary Table 1) were applied. The PCR product was analyzed on 10% polyacrylamide gel along with 1 Kbp DNA ladder. The gel was stained in ethidium bromide solution for twenty minutes and then was visualized under UV light. Each allele bands were manually scored from the gel.

2.3 Statistical Analysis

Variations at different SSR markers among pure Kutta were analyzed using different computer software program. Each individual genotype banding pattern was scored manually from PAGE banding pattern. Observed and expected heterozygosity, effective number of alleles, observed number of alleles, allele's frequencies and linkage disequilibrium between various combinations of loci was calculated using POPGENE software version 1.32 [21]. Allelic richness and estimates of inbreeding at different loci were taken to calculate gene diversity using [8]. Frequencies of null alleles were calculated using GENEPOP version 4.0 [15]. Polymorphism information (PIC) was calculated for each locus with the following mathematical equation [6] using allele frequency data.

$$PIC = 1 - \sum_{i=1}^{n} p_i^2 - 2\left[\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} p_i^2 p_j^2\right]$$

3. Results

Twenty one unrelated animals true to Kutta breed were selected randomly from their breeding tract. Blood samples from selected individuals were analyzed for 31 SSR markers at Institute of Biotechnology and Genetic Engineering, The Agricultural University Peshawar to characterize the population at molecular level. The level of genetic diversity found in Kutta population of Swat valley is presented. Total of 151 different alleles were examined at 23 polymorphic loci in which 109.3 with an average of 4.766 in Kutta population (Table 1). Observed number of alleles (Na) ranged from 4 in (in BM1824 HUJ616 and (OARVH 72 primers) to 9 (in

OARJMP58 primer). Effective number of allele (Ne) was very less than (Na) ranging from 2.348 (in OARFCB226) to 7.606 (in MAF70) (Table 1). Shannon information index (I) value ranged from 1.103 (in primer BM1824) to 2.108 (in primer MAF 70) with an average of 1.645 (Table 1). The Observed heterozygosity at different loci was different ranging from 0.222 (in OARFCB226) to 1 (in ILSTS5, SRCRSP1, MAF70, and MCM527) as indicated in Table 1. However, expected heterozygosity ranged from 0.590 in primer OARFCB226 to 0.900 in primer SRCRSP9. The overall average heterozygosity was 0.767 ranging from 0.574 in (OARFCB226) to 0.868 (MAF70). However, the observed heterozygosity (0.797) was almost similar to the expected heterozygosity (0.798) (Table 1). At about eight loci the expected heterozygosity exceeded the observed heterozygosity that included SRCRSP9, INRA063, SRCRSP5, OARFCB304, BM1824, ILSTS11 and ILSTS28 (Table 1).

Table 1: Sample size, Number of observed and effective alleles, Shannon information index, Observed, Expected and Average Heterozygosity at 23 Microsatellite loci in Kutta sheep.

Locus	**Sample size**	**Na**	**Ne**	**I**	**Ho**	**He**	**Hav**
SRCRSP9	8	8	6.400	1.960	0.625	0.900	0.844
MAF214	19	7	5.014	1.734	0.842	0.822	0.800
OARCP38	11	8	6.368	1.933	0.818	0.883	0.843
INRA063	8	6	4.129	1.560	0.750	0.808	0.758
SRCRSP5	12	8	6.698	1.969	0.583	0.888	0.850
ILSTS5	12	7	3.600	1.536	1.000	0.754	0.722
OARFCB304	17	8	6.084	1.903	0.706	0.861	0.836
OARFCB128	19	7	4.126	1.633	0.947	0.778	0.758
DYMS1	18	7	5.734	1.826	0.944	0.849	0.826
OARCP34	20	7	5.063	1.766	0.900	0.823	0.802
BM8125	19	5	4.057	1.496	0.895	0.774	0.753
BM1824	8	4	2.509	1.103	0.500	0.641	0.602
OARJMP58	19	9	6.623	2.010	0.947	0.876	0.849
SRCRSPI	17	7	4.313	1.616	1.000	0.791	0.768
MAF70	16	9	7.606	2.108	1.000	0.895	0.868
MCM527	19	7	5.429	1.779	1.000	0.838	0.816
ILSTS11	18	8	5.268	1.795	0.611	0.833	0.810
ILSTS28	15	6	4.500	1.603	0.800	0.805	0.778
BM1329	20	5	4.324	1.539	0.950	0.788	0.769
HUJ616	5	4	2.778	1.168	0.800	0.711	0.640
MAF209	16	5	3.160	1.370	0.687	0.706	0.684
OARFCB226	18	5	2.348	1.133	0.222	0.590	0.574
OARVH72	10	4	3.226	1.275	0.800	0.726	0.690
MEAN	15	6.565	4.766	1.645	0.797	0.798	0.767

With inbreeding the expected homozygosity increases at the expense of heterozygosity. The overall inbreeding estimate is indicated as (Fis) which was 0.002 for the Kutta population understudy (Table 2).The mean PIC value of the population was 0.678 per locus; ranging from 0.218 in primer (OARFCB226) to 0.850 in (MAF70) (Table 2 and Table 4). This value indicates that the set of marker used for this population has high degree of relevance for providing information regarding the genetic diversity of this population. The mean chi-square value for the Hardy Weinberg Equilibrium (0.097) indicates that the population has lost its equilibrium in general (Table 2). However, out of 23 loci 19 were in the state of equilibrium. The four loci viz. ILSTS28, BM1824, INRA063 andHUJ616 had exhibited a high degree of disequilibrium 0.361, 0.451, 0.458 and0.796 respectively (Table 2 and Table 4).

Table 2: Genetic diversity, Allelic richness, Inbreeding estimate, Polymorphism Information Content and HWE P value at 23 Microsatellite loci in Kutta sheep.

Locus	**G_{div}**	**A_R**	**Fis**	**PIC**	**HWE P**
SRCRSP9	0.920	6.456	0.320*	0.801	0.049
MAF214	0.822	5.004	-0.025	0.747	0.018
OARCP38	0886	5.950	0.077	0.814	0.002
INRA063	0.813	4.837	0.077	0.646	0.458
SRCRSP5	0.902	6.058	0.353*	0.815	0.012
ILSTS5	0.742	4.580	-0.347	0.572	0.036
OARFCB304	0.866	5.611	0.185	0.748	0.006
OARFCB128	0.773	4.703	-0.225	0.644	0.000
DYMS1	0.846	5.358	-0.116	0.768	0.000
OARCP34	0.821	5.149	-0.096	0.736	0.000
BM8125	0.770	4.306	-0.101	0.677	0.005
BM1824	0.652	3.492	0.233	0.301	0.451
OARJMP58	0.870	5.922	-0.089	0.808	0.000
SRCRSPI	0.785	4.613	-0.274	0.678	0.001
MAF70	0.898	6.483	-0.114	0.850	0.001
MCM527	0.833	5.165	-0.200	0.775	0.000
ILSTS11	0.840	5.158	0.272*	0.742	0.049
ILSTS28	0.805	4.632	0.006	0.689	0.361
BM1329	0.784	4.437	-0.211	0.727	0.000
HUJ616	0.700	4.000	-0.143	0.580	0.796
MAF209	0.706	4.117	0.027	0.643	0.002
OARFCB226	0.601	3.471	0.630*	0.218	0.000
OARVH72	0.722	3.773	-0.108	0.628	0.001
MEAN	0.798	4.925	0.002	0.678	0.097

Test for non-random association between alleles belonging to different loci showed that 22 of the allele combinations were at frequency deviating significantly from expected ($p < 0.05$) (Table 3).

Table 3: Linkage disequilibrium among different combinations of alleles at significance level ($p<0.05$)

Locus	Alleles	Locus	Alleles	Co-relation	Chi-square	P-value
SRCRSP9	C	INRA063	E	2.0000	8.00	0.0047
SRCRSP9	G	BM1824	C	-1.3333	5.33	0.0209
MAF214	A	OARFCB128	D	0.5000	4.25	0.0393
MAE214	A	OARFCB128	G	0.5000	4.25	0.0393
MAF214	A	MAF70	I	0.5000	4.00	0.0455
OARCP38	A	BM1824	C	-2.0000	8.00	0.0047
OARCP38	D	BM1824	B	-2.7080	14.67	0.0001
OARCP38	D	BM1824	C	4.6904	44.00	0.0000
OARCP38	E	BM1824	C	-2.0000	8.00	0.0047
OARFCB128	D	ILSTS11	H	0.5000	4.00	0.0455
OARFCB128	G	ILSTS11	H	0.5000	4.00	0.0455
OARFCB128	A	OARFCB226	E	0.5000	4.00	0.0455
OARCP34	A	MAF70	A	0.5000	4.00	0.0455
OARCP34	A	MAF70	C	0.5000	4.00	0.0455
BM1824	C	HUJ616	C	-2.0000	8.00	0.0047
OARJMP58	F	SRCRSP1	A	0.5000	4.00	0.0455
OARJMP58	F	SRCRSP1	C	0.5000	4.00	0.0455
OARJMP58	A	ILSTS11	A	0.5000	4.25	0.0393
OARJMP58	C	ILSTS11	A	0.5000	4.25	0.0393
SRCRSP1	A	OARFCB226	E	0.5000	4.00	0.0455
SRCRSP1	B	OARFCB226	A	0.5155	4.25	0.0392
SRCRSP1	C	OARFCB226	E	0.5000	4.00	0.0455

Allele frequencies at 23 polymorphic loci in Kutta population are given in Table 4. Null alleles also called dis-functional allele, results due incomplete amplification during PCR were absent at four loci (ILSTS5, DYMS1, SRCRSP1 and MAF 70).

Null alleles were present at very low rate in just six loci (INRA063, OARCP34, OARJMP58, ILSTS28, BM1329 and MAF209); however, majority is of higher value (twelve loci) with highest frequency of 0.264 at marker OARFCB226 (Table 4).

Table 4: Alleles frequency at different loci and Size of alleles in base pair observed at different loci in Kutta population

Locus	**Null**	**Allele size range**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
SRCRSP9	0.214	331-457	0.062	0.062	0.125	0.062	0.125	0.187	0.250	0.125	
MAF214	0.200	177-240	0.078	0.052	0.289	0.157	0.157	0.026	0.236		
OARCP38	0.169	351-457	0.181	0.045	0.136	0.181	0.045	0.045	0.181	0.181	
INRA063	0.076	184-263	0.062	0.062	0.312	0.187	0.312	0.062			
SRCRSP5	0.186	158-211	0.166	0.208	0.041	0.166	0.125	0.125	0.125	0.041	
ILSTS5	0.000	200-240	0.041	0.041	0.291	0.083	0.416	0.083	0.041		
OARFCB304	0.100	151-200	0.029	0.250	0.147	0.147	0.235	0.117	0.088	0.029	
OARFCB128	0.015	116-174	0.052	0.342	0.078	0.052	0.105	0.315	0.052		
DYMS1	0.000	134-209	0.055	0.222	0.111	0.138	0.222	0.194	0.055		
OARCP34	0.090	105-153	0.125	0.200	0.125	0.050	0.050	0.325	0.125		
BM8125	0.189	316-381	0.105	0.342	0.131	0.131	0.289				
BM1824	0.125	174-210	0.125	0.562	0.250	0.062					
OARJMP58	0.057	120-176	0.026	0.157	0.026	0.236	0.105	0.078	0.184	0.078	0.105
SRCRSP1	0.000	135-182	0.029	0.029	0.029	0.205	0.352	0.176	0.176		
MAF70	0.000	166-219	0.156	0.062	0.156	0.093	0.062	0.093	0.093	0.187	0.093
MCM527	0.142	145-199	0.052	0.026	0.131	0.210	0.157	0.157	0.263		
ILSTS11	0.188	257-334	0.027	0.222	0.250	0.027	0.222	0.138	0.027	0.083	
ILSTS28	0.090	316-398	0.066	0.233	0.233	0.300	0.033	0.133			
BM1329	0.084	209-263	0.350	0.175	0.125	0.150	0.200				
HUJ616	0.198	135-152	0.500	0.100	0.100	0.300					
MAF209	0.083	166-204	0.500	0.156	0.156	0.093	0.093				
OARFCB226	0.264	122-162	0.083	0.611	0.194	0.083	0.027				
OARVH72	0.106	250-351	0.450	0.250	0.150	0.150					

Whether the population had gone bottleneck in recent past or not was tested by quantitative geographical method that elaborated the allele frequency spectra [11].

The microsatellite alleles were divided into 10 frequency classes in plot. Although alleles with low frequencies (0.01-0.1) were found abundantly, however, alleles distribution exhibited the normal L- shaped (Figure 1).

The graphical illustration clearly showed that the population had not undergone bottleneck at least in the recent past.

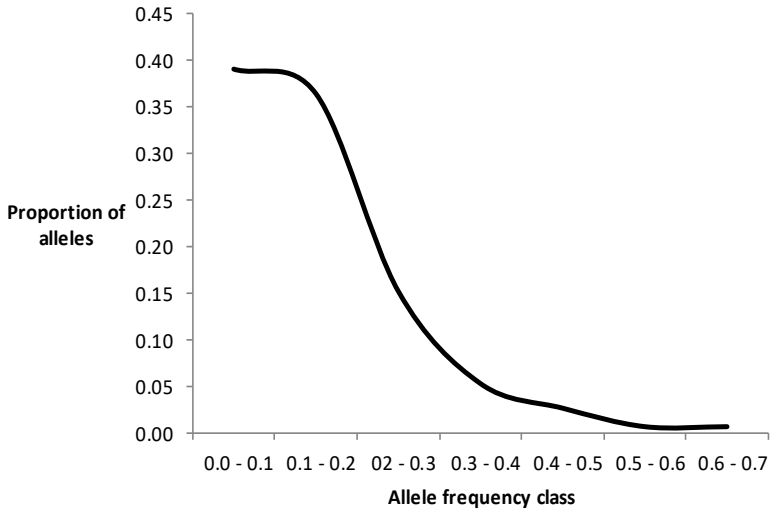

Figure 1: Normal (L shape) distribution of alleles in allele frequency classes**.**

4. Discussion

Color of Pakistani sheep breeds is mostly white, however brown, red or black color sheep are also common [17]. Morphology describing, Kutta sheep is black coated small sized thin tail breed. Males are horned and female are polled. In 2007 this breed has been classified as endangered species [1]. Because of this, it is necessary to gather information of the population's genetic diversity distribution of the population to effectively design the conservation policy for this endangered species.

Various alleles in diversity parameters were quite a high in the present study for Kutta sheep breed (Table 1); that is accordance with other domestic sheep breeds [3, 7, 16]. Kutta sheep breed yielded a total of 151 alleles in a sample population for 23 marker loci, averaging 6.565 observed and 4.766 expected alleles compared to Vembur sheep having a total of 147 alleles in 25 marker loci with a mean of 5.88 alleles per locus in the population [3]. Effective number of alleles (Ne) ranged from 2.348 (OARFCB226) to (MAF70) compared to effective allele mean per locus 0.405 in Shandi and Wadi breeds of China [22]. The observed number of allele in Garole sheep was (6.2) which is similar to the present study (ranged from four in (BM1824, HUJ616 and OARVH72) to nine (OARJMP58)) [13].

Kutta sheep have substantial genetic variation on the basis of gene diversity and average number of allele per locus. The gene diversity was (0.798), similar to the values reported for sheep breeds of Iran (Sanjabi 0.77, Zardi 0.77, Kajol 0.76 and Kolu 0.76) [19], Garole sheep 0.603 [20], Nali sheep 0.651, Chokla sheep 0.657 [13].

The average heterozygosity over all loci in Kutta sheep was (0.767). The high value of average heterozygosity within the breed could be attributed to high number of alleles (151) in present study [9]. In Kutta population the observed heterozygosity (0.797) was almost similar to the expected heterozygosity (0.798). At about seven loci,

the expected heterozygosity exceeded the observed heterozygosity i.e (SRCRSP9, INRA063, SRCRSP5, OARFCB304, BM1824, ILSTS11, and ILSTS28). In Kutta sheep breed the expected heterozygosity (0.798) was high from average heterozygosity (0.767) evidences for overall loss in heterozygosity [4].The observed number of allele was quite high than effective alleles at all of the loci, which justified the predominance of some alleles at almost each locus in a population [10]. However, in assessing diversity estimates from different studies, one should be careful not compare different values with each other, because different microsatellite markers have been used by different researchers. Therefore, these give just a suggestive indication of diversity in the population

The overall inbreeding estimates (FIS) in Kutta sheep was (0.002) indicating a very low rate of inbreeding in the population. Higher heterozygosity values highlighted inbreeding of low level, slightly or without selection pressure and large number of alleles. The similar low values of FIS were also found in Muzzafarnagri sheep breed (0.058) [5]. In Ganjam sheep inbreeding estimate value was also found very low (0.087).

Mean chi square value in Kutta sheep for HWE was (0.097) which indicates that the population has lost its equilibrium. Four loci i.e ILSTS28, BM1824, INRA063 and HUJ616 had exhibited a high degree of receptive disequilibrium (0.361, 0.451, 0.458, and 0.796). These results suggest ban of indiscriminate crossing with other exotic and local breeds to preserve this precious germplasm. This breed is under constant threat of invasion from Rambouillet and Bulkhi sheep breeds.

5. Conclusion and recommendation

In conclusion the specimen size is drastically reduced in a recent past and efforts should be mobilized to conserve it before complete extinction of the breed might occur. It is one of the local sheep breed which possessed high genetic diversity. Its population has shrunken to an endangered level and drastic efforts are required for its in-situ and in-vivo conservation for future use. Policy may be designed and be strictly implemented not to permit cross breeding in the pure flocks at the habitat.

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7. Conflict of interest

The authors confirm that there is no conflict of interest.

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