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Molecular Characterization of Balochi Sheep by using Microsatellite Markers in Pakistan

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ABSTRACT

Balochi sheep is one among the established sheep breeds of Balochistan province in Pakistan. As far as its size is concerned, it is medium sized, having fat tail and inhabits in the Northern regions in Balochistan. Molecular studies are the base of breed characterization. Hence, nearly 25 unrelated (including both male and female) animals of Balochi breed were sampled for DNA extraction. Some specific markers (15 out of available 30 SSR markers) were employed in the present study to highlight genetic polymorphism. The PCR was utilized for amplification of individual DNA samples. All of the 15 SSR markers were amplified. After gel documentation, a total number of 97 alleles were recognized having 1 to 5 polymorphic forms (OARFCB193, OARJMP29, MAF33) to 4 (OARHH47, DYMS1, SRCRSP5). For total loci, number of alleles averaged 2.1162±0.3769. Shannon's Information index (I) 0.6184±0.2447 and the effective number of alleles (Ne) averaged 1.6251±0.4604. Average observed, expected and average heterozygosities were found to be 0.5815±0.1059, 0.4330±0.5811 and 0.4331±0.5811, respectively. It was noted that the F-statistic was ranging from 0.2166 to 0.95182 for the microsatellite markers employed in the study. Expected reduction in heterozygosity was higher than mean value in case of most of the markers with lower standard errors showing the prevalence of homozugous Balochi sheep population. The main cause reason behind prevalence of homozygous population might be inbreeding as only few rams had been used for breeding the flocks. This study would provide basis for breed characterization and lead to breed improvement program.

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MJ conducted research. MAB and MMT supervised the work. AS and JA helped in write up. AF and YHB helped in write up and reviewed the article.

Key words
Balochi sheep, Genetic characterization, Molecular markers, Genetic diversity

INTRODUCTION

Genetic diversity studies are based on DNA molecular markers which are commonly employed to understand polymorphism. Among the first developments, PCR based markers were Random Amplified Polymorphic DNA (RAPD) in which sequences of DNA were amplified using short oligonucleotide, from 10 to 12 base pair (bp) long, for the amplification of random DNA sections (Williams *et al*., 1990).Overall, the reliability of RAPD markers is low due to the success of amplification of any DNA fragment which could be due to many factors such as DNA template quality, PCR conditions, reagents and equipment (Edwards and Mc-Couch, 2007). Restriction Fragment Length Polymorphisms (RFLP's) have been the first genomic DNA-based molecular markers produced from a specific application of Southern blot analysis.

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Amplified fragment length polymorphisms (AFLPs) are more sophisticated than RAPD markers, which provide the amplification of restriction fragments ligated to specific adaptors. Only a portion of restriction fragments within the range of 50 and 350 bp in size can be amplified by PCR and visualized on polyacrylamide gels. Large genomes usually require additional selective bases to the primers to reduce the number of co-amplified bands (Zhang and Hewitt, 2003). In animal cells, besides the nuclear DNA, Mitochondrial DNA (mtDNA) is also found. The study of this kind of DNA in population genetic is famous because it can only be transferred from mother to off springs in most species. The second feature of this DNA is that it grows faster than nuclear DNA, however, having small size about 15 to 20 kilobase (kb) in length, containing around 37 genes which are found in duplicates in the cells of the eukaryotic organisms (Othman *et al*., 2015). Microsatellite markers contain DNA section, which is only a few nucleotides long (2 to 6 bp), it occurs frequently many times in tandem (CACACACACACACACA) (Teneva, 2009). Microsatellites loci are also recognized

as simple sequence repeats (SSR's) and sequence tagged microsatellites (STMS) (Mburu and Hanotte, 2005). The recurrent fragment can be a mono, di, tri or tetra nucleotide; however, the most common is di-repeats. Molecular markers are suggested in order to overcome the limitations related with RFLP and RAPD (Garcia *et al*., 2004). These markers serve as a useful tool for animal classification and genetic distance estimation (Baker and Bradley, 2006).

This is first time designed to study Balochi sheep breed at molecular level, using a set of 15 ovine specific SSR markers, in Balochistan. The findings of the current study may help in understanding the scope of genetic variability and would be helpful in making better approaches for conservation and future development in sheep breeding programmed.

MATERIALS AND METHODS

Balochi sheep (n=30) of both sexes (from different pedigrees) present on different sites were sampled for their whole blood to extract DNA and it was kept in view that they should not be genetically related to each other in order to have diversity of samples. The animals under study were present at Sheep Research Center Yetabad at Loralai, Balochistan Pakistan and they were randomly selected for sampling. The whole blood samples were obtained in specific tubes (vacutainers) having anti-coagulant and their volumes measured to be nearly 7 millilitters. The anticoagulant contained in vacutaniers was ethylene diamine tetra acetic acid (EDTA). After collection the samples were carried to the processing labs in cold containers avoiding them from extreme heat conditions which could have otherwise destroyed the samples. The blood samples were kept cold and caution was taken to prevent exposing them to extreme temperatures keeping in view standard sampling carrying protocols. The collected samples then were stored at -20°C until they were processed for DNA extraction and further analysis at the Hi-Tech Laboratory in the Centre for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta.

DNA extraction

For DNA extraction from the blood, 300µl of blood and 1 ml of lysis buffer were taken. After that samples were vertexed for five minutes. After vertex mixing samples were centrifuged at 13000 rpm for fifteen minutes. The process was repeated 2 to 3 times. White colorless pellet formed at the bottom of the Eppendrof tube, discarded the remaining solution save the pellet. Then we added 20µl protienase kinase (PK), 80µl Sodium Dodecyl Sulfate (SDS) and 250µl buffer solution and vertex mixed for five minutes. Now the samples were left overnight at 58ºC for digestion in water bath, afterwards added 300µl PCI and vertexed for five minutes. After vertexing, the sample was centrifuged at 13000 rpm for fifteen minutes at 4°C. Then top aqueous layer was separated in a new tube and small amount of isopropanol was added with a ratio of 1:1 and incubated at room temperature for 5 min. Samples were centrifuged at 13000rpm for 10 min at 4ºC. For washing, 200µl ethanol was added in samples and centrifuged at 13000 rpm for 10 min at 4 °C, ethanol was discarded and the pellet was air dried. After adding 150-200 µl water, the DNA was stored at -20ºC for further analysis.

Polymerase chain reaction (PCR)

All PCR reactions (DNA sample of 30 Balochi sheep) were carried out in 25 pl reaction vessels containing 100 ng total genomic DNA, 0.5 pM of each primer, 200 pM of dNTPs, 50mMKCl, 10 mM Tris, 2.0 mM $MgCl_2$ and 1.0 unit of Taq DNA polymerase. Different conditions for amplification used were denaturation, annealing and extension respectively at 95°C for 7 min, followed by 30 cycles, for 30 sec. at 94°C for 30 sec (annealing) and an extension step of 1 min at 72°C. The final cycle was followed by l0 min extension at 72°C. All amplification reactions were performed using Palm Cycler PCR system (Corbett Research) Programmable Thermo cycler. For the amplification of each individual DNA sample all the 15 Ovine specific SSR markers (OarFCB128, OarCP34, OarJMP58, OarAE129, BM1329, BM8125, HUJ616, DYMS1, SRCRSP9, OarFCB226, SRCRSP5, ILSTS11, and ILSTS28) were employed (Table I). The annealing temperature for each primer was fixed accordingly. A total of 2240 reactions were done on PCR and the products of PCR were separated on 5% agarose gel using Thermo Scientific 0' Range Ruler 5bp DNA ladder.

Statistical analysis

The data obtained were fed into POPGENE software in binary format as ASCII files and analysis were carried out that generated several parameters including observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index, observed homozygosity (obs. Hom.), observed heterozygosity (obs. Het.), expected homozygosity (Exp. Hom.), expected heterozygosity (Exp. Het.) and average Heterozygosity (Ave. Het.) (Kimura and Crow, 1964; Nei, 1973). Other genetic diversity related estimates and fixation indices, FIS (within population inbreeding estimates), FIT (total inbreeding estimates) and FST (measurement of population differentiation) proposed by Wright (1978) were also computed.

Table I. List of ovine markers employed in current research work.

Primer	Primer Sequence (5′ → 3′)
OarFCB128	
	F: ATAAAGCATCTTCTCTTTATTTCCTCGC
	R: CAGCTGAGCAACTAAGACATACATGCG
OarCP34	
	F: GCTGAACAATGTGATATGTTCAGG
	R: GGGACAATACTGTCTTAGATGCTGC
OarCP38	
	F: CAATTTGGTGCATATTCAAGGTTGC
	R: GCAGTCGCAGCAGGCTGAAGAGG
OarJMP58	
	F: GAAGTCATTGAGGGGTCGCTAACC
	R: CTTCATGTTCACAGGGTCAGGG
OarFCB304	
	F: CCCTAGGAGCTTTCAATAAAGAATCGG
	R: CGCTGCTGTCAACTGGGTCAGGG
OarAE129	
	F: AATCCAGTGTGTGAAAGACTAATCCAG
	R: GTAGATCAAGATATAGAATATTTTTCA ACACC
BM1329	
	F: TTGTTTAGGCAAGTCCAAAGTC
	R: AACACCGCAGCTTCATCC
BM8125	
	F: CTCTATCTGTGGAAAAGGTGGG
	R: GGGGGTTAGACTTCAACATACG
HUJ616	
	F: TTCAAATACACATTGACAGGG
	R: GGACCTTTGGCAATGGAAGG
DYMS1	
	F: AACAACATCAAACAGTAAGAG
	R: CATAGTAACAGATCTTCCTACA
SRCRSP9	
	F: AGAGGATTGGAAATGGAATC
	R: GCACTCTTTTCAGCCCTAATG
OarFCB226	
	F: CTATATGTTGCCTTTCCCTTCCTGC
	R: GTGAGTCCCATAGAGCATAAGCTC
SRCRSP5	
	F: GGACTCTACCAACTGAGCTACAAG
	R: GTTTCTTTGAAATGAAGCTAAAGCAATG C
ILSTS11	
	F: GCTTGCTACATGGAAAGTGC
	R: CTAAAAGTCAGAGCCCTACC
ILSTS28	
	F: TCCAGATTTTGTACCAGACC
	R: GTCATGTCATACCTTTGAGC

RESULTS AND DISCUSSION

Banding pattern of 15 ovine SSR markers against DNA sample of Balochi sheep breed in Balochistan, using 50bp DNA ladder (50-1350bp) on 5% agarose gel are shown in Figure 1.

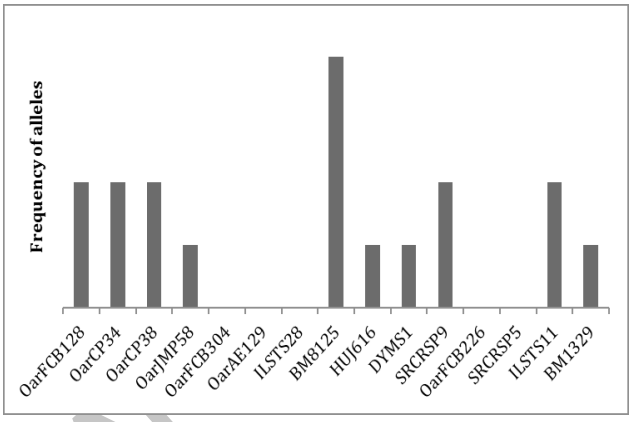

Fig. 1. Allelic polymorphism found in Balochi sheep.

The figure illustrates that Primer 1 (OarFCB128) amplified two bands (50,45 bp) suggesting two alleles, Primer 2 (OarCP34) amplified two bands (45,43 bp) suggesting two alleles, Primer 3 (OarCP38) amplified two bands (50, 55bp) suggesting two alleles, primer 4 (OarJMP58) amplified one band (60 bp) suggesting only one allele, Primers 5 (OarFCB304), primer 6 (OarAE129) and primer 7 (BM1329) amplified no bands, suggesting no alleles and primer 8 (BM8125) amplified four bands (45, 50, 60 and 100 bp) suggesting four alleles.

Primer 9 (HUJ616) amplified one band (50 bp) suggesting one allele, Primer 10 (DYMS1) amplified two bands (60 and 65bp) suggesting two alleles, Primer 11 (SRCRSP9) amplified only one band (45 bp) suggesting one allele, Primers 12 (OarFCB226) and 13(SRCRSP5) showed no amplification of bands, signifying no alleles, Primer 14 (ILSTS11) amplified two bands (90, 100 bp) suggesting two alleles, and Primer 15 (ILSTS28) amplified one band (above 100 bp) suggesting only one allele.

As shown in Figure 2, minimum and maximum polymorphism was observed in case of primer BM1329 and primer BM8125, respectively.

Various characteristics of genic polymorphism (Table II), homozygosity and heterozygosity (Table III) along with F-statistics (Table IV) have shown phenomenal attributes, genetic diversity and multiple allelomorphism in Balochi sheep characterizing particular breed markers that might be helpful in identification and breed improvement programs.

Table II. Genic variation statistics for all locii.

Locus	Sample size	na	ne	I
OARFCB128	124	3	1.1027	0.2245
OarCP34	124	4	1.239	0.4227
OarCP38	124	4	1.4293	0.6398
OarJMP58	124	5	1.1592	0.333
OarFCB304	124	3	2.1541	0.891
OarAE129	124	4	1.3554	0.5737
BM1329	124	4	1.7096	0.74
BM8125	124	4	2.3234	0.9616
HUJ616	124	4	1.6786	0.8085
DYMS1	124	2	1.7009	0.6024
SRCRSP9	124	4	2.8182	1.164
OarFCB226	124	4	2.0773	0.9326
SRCRSP5	124	4	2.1699	0.9117
ILSTS11	124	3	1.8638	0.7823
ILSTS28	124	4	2.5738	1.132
Mean	124	3.7333	1.8237	0.7413
St. Dev		0.7037	0.5226	0.2761

na, Observed number of alleles; * ne, Effective number of alleles [Kimura and Crow (1964)]; * I, Shannon's Information index [Lewontin (1972)]

Table III. Heterozygosity statistics for all loci.

Locus	n	Obs. Hom	Obs_ Het	Exp_ Hom*	Exp_ Het*	Nei**	Ave_ Het
OARFCB128	124	0.9355	0.0645	0.9061	0.0939	0.0931	0.0936
OarCP34	124	0.7903	0.2097	0.8055	0.1945	0.1929	0.193
OarCP38	124	0.7742	0.2258	0.6972	0.3028	0.3003	0.298
OarJMP58	124	0.8548	0.1452	0.8615	0.1385	0.1374	0.1381
OarFCB304	124	0.5968	0.4032	0.4599	0.5401	0.5358	0.535
OarAE129	124	0.7097	0.2903	0.7356	0.2644	0.2622	0.2609
BM1329	124	0.4677	0.5323	0.5816	0.4184	0.4151	0.4151
BM8125	124	0.1935	0.8065	0.4258	0.5742	0.5696	0.5652
HUJ616	124	0.7097	0.2903	0.5924	0.4076	0.4043	0.4048
DYMS1	124	1	0	0.5846	0.4154	0.4121	0.4122
SRCRSP9	124	0.5806	0.4194	0.3496	0.6504	0.6452	0.6449
OarFCB226	124	0.4032	0.5968	0.4772	0.5228	0.5186	0.518
SRCRSP5	124	0.3065	0.6935	0.4565	0.5435	0.5392	0.5366
ILSTS11	124	0.5	0.5	0.5328	0.4672	0.4634	0.4616
ILSTS28	124	0.5968	0.4032	0.3836	0.6164	0.6115	0.6072
Mean	124	0.628	0.372	0.590	0.410	0.407	0.406
St. Dev		0.229	0.229	0.1755	0.1755	0.174	0.173

*Expected homozygosity and heterozygosity were computed using Levene (1949), ** Nei's (1973) expected heterozygosity.

Table IV. F-statistics and gene flow for all loci.

Locus	Sample size	Fis	Fit	Fst	Nm*
OARFCB128	124	0.3036	0.312	0.012	20.5411
OarCP34	124	-0.0988	-0.0881	0.0097	25.4356
OarCP38	124	0.2423	0.2553	0.0171	14.3387
OarJMP58	124	-0.0643	-0.0573	0.0066	37.5956
OarFCB304	124	0.2439	0.2446	0.001	247.9324
OarAE129	124	-0.109	-0.1068	0.002	123.0179
BM1329	124	-0.2885	-0.284	0.0035	70.6853
BM8125	124	-0.4316	-0.415	0.0116	21.2962
HUJ616	124	0.2813	0.2816	0.0004	648.2206
DYMS1	124	1	1	0.0008	311.75
SRCRSP9	124	0.3493	0.3495	0.0004	616.0954
OarFCB226	124	-0.1525	-0.1497	0.0024	104.4611
SRCRSP5	124	-0.2903	-0.2866	0.0029	86.8323
ILSTS11	124	-0.0798	-0.0753	0.0042	59.6357
ILSTS28	124	0.3363	0.3403	0.0061	40.8444
Mean	124	0.0815	0.0856	0.0045	55.4463

FIS (within population inbreeding estimates), FIT (total inbreeding estimates) and FST (measurement of population differentiation).

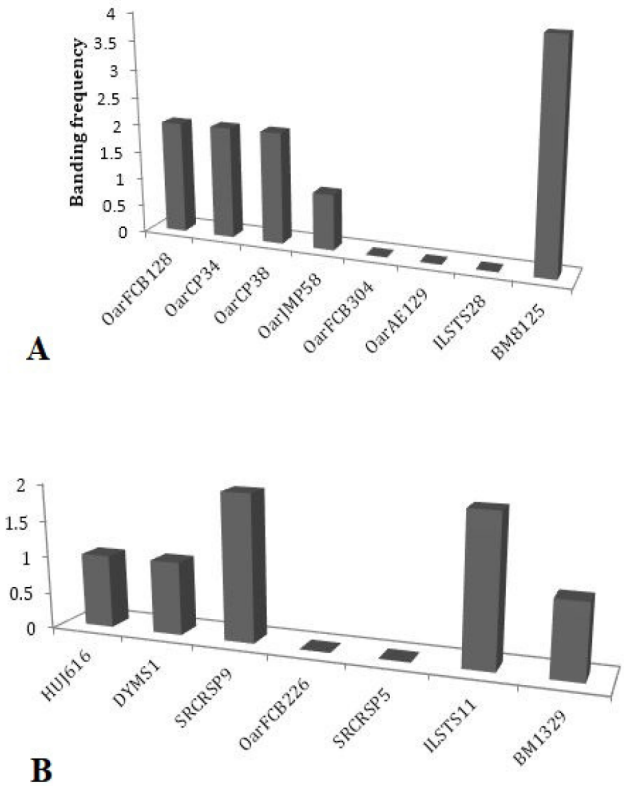

Fig. 2. Banding pattern for primers 1-8 (A) and 9-15 (B).

During the present study the mean number of observed alleles (Na) was 3.7333 ±0.869 (range 1.0-4.0), while the average number of effective alleles (Ne) was 1.8237±0.064 (range 1.0-3.509) which is significantly less than reported by Musavi *et al*. (2011) 6.296 and 4.394 in Hazaragie sheep, Ahmed *et al*. (2014) 5.2727 and 3.9471 in Kail sheep, 63 and 3.658 in Indian Bellary sheep, 8.76 and 4.54 and 8.64 and 4.57 in Indian sheep breed Changthangi and Munjal. The Shannon lndex (I) found in the present study was 0.7413 ± 0.353 which is markedly less than those reported by other researchers such as 0.87 (Musavi *et al*., 2011); 1.445 (Ahmed *et al*., 2014) and 1.419 in Harnai sheep.

The average observed heterozygosity (Ho) in the population under study was 0.372+0.219 which can be compared with the findings of various workers viz; 0.825 by Musavi *et al*. (2011), 1.445 by Ahmed *et al*. (2014), 0.712 by Yama *et al*. (2011). Mean expected heterozygosity (He) was 0.405±0.219 during the study which was higher than Ho at most of the loci; however, the primers ILSTS28, OarCP38, OarFCB34, HUJ616, DYMS1, SRCRSP9, OARFCB128 showed higher He then Ho. The finding 0.405 and 0.372 of the present study were less than those reported by Musavi *et al*. (2011) 0.778, Ahmed *et al*. (2014) 0.7185, Wajid *et al*. (2014) 6.23 and 6.255 in Balochi and Rakhshani breeds, respectively.

The average heterozygosity and gene diversity were found to be 0.4056±0.209 and 0.4067±0.209, respectively during the study. These values were quite smaller than those reported by Musavi *et al*. (2011) 0.757 and 0.772, whereas, higher values for gene diversity were reported by Dalvit *et al*. (2008) 0.806, 0.801, 0.796 and 0.801 in four Italian sheep breeds Alpagota, Brogna, Foza and Lamon, respectively value of F-statistic found during the study ranging from 0.2851 to 0.9132 for SSR markers, with an average value of 0.0856±0.021, showed that majority of the marker yielded higher than average expected reduction in heterozygosity. The standard errors found during the study were usually low indicating that homozygosity prevails in the population under study. This might be due to intense inbreeding in this flock of Balochi sheep.

Al-Barzinji *et al*. (2011) reported 0.469 inbreeding value in Hamdani sheep, whereas in contrast Musavi *et al*. (2011) reported negative (-0.069) value for inbreeding estimates. However positive, but less than ours (0.0525) and (0.253) inbreeding estimates were reported by Ahmed *et al*. (2014) in the Kail sheep of Azad Jammu and Kashmir and Indian Bellary sheep populations, respectively. The findings of Wajid *et al*. (2014) about the inbreeding estimates of Balochi and Rakhshani sheep breeds of Balochistan were 0.0292 and 0.0084, respectively.

CONCLUSIONS

The Balochi is a unique sheep that due its local adaption have evolved some peculiar characteristics and needs more attention. The presence and absence of horns and the fleece color were not the effect of adaptations to the environment but have a communal' importance and therefore, could not be revealing of the genetic relationships. The latest use of microsatellite markers in various sheep breeds showed to be very beneficial for the determination of genetic relationships among populations. It is concluded from the study that a set of 15 FAO recommended microsatellite markers, most of them have shown their effectiveness for the significant genetic diversity in the population of Balochi sheep breed in Balochistan. The results of the current study may be helpful contribution in the understanding of genetic structure of the indigenous breeds of the province. Furthermore, the results may be helpful for the comparison and conservation of local genetic resource. Balcohi breed improvement program needs to be launched to improve the breed in the native place.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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