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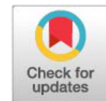
Genetic Diversity in Bibrik Sheep of Pakistan Elucidated through Molecular Characterization



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Authors' Contribution
MJA conducted the research work and wrote the article. MMT supervised the research work. NR helped in analysis and lab work. MR helped in sampling and blood collection. AF, ISS and MA helped in write up. MJ, AF, MAB and SI helped in review and editing of the manuscript. IJ helped in literature review.

Key words
Bibrik sheep, Genetic diversity, Molecular characterization, DNA markers

ABSTRACT

Bibrik sheep is found vastly in Baluchistan province in Pakistan. Randomly sampled animals of this breed were subjected to genetic analysis using ovine molecular markers where fifteen sheep SSR genetic markers were employed for describing multiple-allelomorphism. The average observed number of alleles (Na), effective number of alleles (Ne) and Shannon's Information Index were 2.07 ± 0.79, 1.86 ± 0.69 and 0.54 ± 0.29, respectively. Heterozygosity was described by the average, observed and expected heterozygosities whose values were found to be 0.56 ± 0.27, 0.44 ± 0.27 and 0.43 ± 0.26, respectively. Slightly higher magnitude of observed heterozygosity indicated that forces affecting Hardy-Weinburg Equilibrium were not much effective in these flocks and outbreeding was common phenomenon that reduced the inbreeding in the flocks as inbreeding tends to promote homozygosity. The range in observed heterozygosity was very wide for different molecular markers. These measurements underlined the absence of inbreeding in the flocks that commonly causes inbreeding depression. The presence of genetic diversity was apparent and these markers would be employed for identification of Bibrik sheep through DNA fingerprinting. It was concluded that present assessment of genetic diversity could be used as tool for making future breeding plans but large scale enumeration is required for the Bibrik sheep.

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INTRODUCTION

Genetic characterization of animal genetic resources using molecular markers is vital to fetch important information regarding genetic makeup of animals, understand population structure, estimating genetic parameters and to make them patent. These tools provide data about polymorphism of genetic origin, genetic diversity and lead to understand genetic differentiation and development of populations. Presently breeds are identified and distinguished on the basis of breed specific markers hence importance of molecular markers to characterize genetic resources cannot be neglected (Baker and Bradely, 2006). Nowadays molecular markers are commonly used to understand genetic phenomenon, describe breeds and varieties both in animals and plants. Various types of molecular techniques are being used such as RAPD, AFLP, RFPLP, SSR, SNP etc. Microsatellite markers which are commonly used for different breeds are well recognized and FAO has prepared set of identifiable molecular markers for each species. Various ovine markers are available to identify breed specific markers in order to distinguish and purify the breeds genetically and study multiple allelomorphism. Population composition, genetic distances, heterozygosities and other genetic parameters are investigated to understand population diversity and structure. A number of studies have been conducted by researchers all around the world in this regard. 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 pairs (bp) for amplification of random DNA sections (Williams *et al*., 1990). Overall, 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 polymorphism (RFLP) has been the first

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genomic DNA-based molecular marker produced from a specific application of southern blot analysis (Kumar *et al*., 2018). As RFLP and RAPD have some short comings, so other molecular markers are preferred (Garcia *et al*., 2004). Worldwide studies are vastly available regarding molecular/ genetic characterization of animals. A few studies in Pakistan have also been conducted about genetic characterization of sheep (Kari sheep by Sohail *et al*., 2010; Mengali, Balochi, Beverigh and Harnai sheep by Tariq *et al*., 2012; Harnai by Ali *et al*., 2016). The current study was planned to perform genetic characterization of Bibrik sheep of Balcoshitan through molecular markers.

MATERIALS AND METHODS

Whole blood of unrelated randomly selected Bibrik sheep including both male and female was collected from different flocks in their breeding tract (25 samples each from Multi-Purpose Sheep Research Station, Yet Abad and Research Station Tomagh, District, Loralai, Balochistan, Pakistan). Whole blood (7 ml) collected from each animal in vacutainer tubes previously containing the anticoagulant ethylene-diamine-tetra-acetic acid (EDTA) was safely transported to HiTech analytical laboratory of the Centre for Advanced Studies in Vaccinology and Biotechnology for further processing. The blood samples were stored at -20°C until DNA isolation was performed. The analysis was conducted at Hi-Tech Laboratory (CASVAB), University of Balochistan, Quetta.

DNA was extracted from the whole blood by using genomic DNA purification kit (Puregene-Gentra System,

Table I. List of ovine markers used in present study.

No.	Marker gene	Chromosome	Primer Sequence 5'-3'
1	OarFCB128	OAR2	F: ATAAAGCATCTTCTCTTTATTTCCTCGC
			R: CAGCTGAGCAACTAAGACATACATGCG
2	OarCP34	OAR3	F: GCTGAACAATGTGATATGTTCAGG
			R: GGGACAATACTGTCTTAGATGCTGC
3	OarCP38	OAR10	F: CAATTTGGTGCATATTCAAGGTTGC
			R: GCAGTCGCAGCAGGCTGAAGAGG
4	OarJMP58	OAR26	F: GAAGTCATTGAGGGGTCGCTAACC
			R: CTTCATGTTCACAGGGTCAGGG
5	OarFCB304	OAR19	F: CCCTAGGAGCTTTCAATAAAGAATCGG
			R: CGCTGCTGTCAACTGGGTCAGGG
6	OarAE129	OAR5	F: AATCCAGTGTGTGAAAGACTAATCCAG
			R: GTAGATCAAGATATAGAATATTTTTCAACACC
7	BM1329	OAR6	F: TTGTTTAGGCAAGTCCAAAGTC
			R: AACACCGCAGCTTCATCC
8	BM8125	OAR17	F: CTCTATCTGTGGAAAAGGTGGG
			R: GGGGGTTAGACTTCAACATACG
9	HUJ616	OAR13	F: TTCAAATACACATTGACAGGG
			R: GGACCTTTGGCAATGGAAGG
10	DYMS1	OAR20	F: AACAACATCAAACAGTAAGAG
			R: CATAGTAACAGATCTTCCTACA
11	SRCRSP9	CHI12	F: AGAGGATTGGAAATGGAATC
			R: GCACTCTTTTCAGCCCTAATG
12	OarFCB226	OAR2	F: CTATATGTTGCCTTTCCCTTCCTGC
			R: GTGAGTCCCATAGAGCATAAGCTC
13	SRCRSP5	OAR18	F: GGACTCTACCAACTGAGCTACAAG
			R: GTTTCTTTGAAATGAAGCTAAAGCAATG C
14	ILSTS11	OAR9	F: GCTTGCTACATGGAAAGTGC
			R: CTAAAAGTCAGAGCCCTACC
15	ILSTS28	OAR3	F: TCCAGATTTTGTACCAGACC
			R: GTCATGTCATACCTTTGAGC

Denaturation 94 °C; annealing 72 °C; extension 72 °C

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USA). All PCR reactions (DNA sample of 50 Bibrik sheep) were carried out in 25pl reaction volume containing 100 ng total genomic DNA, 0.5 pM of each primer, 200 pM of dNTPs, 50 mM KCl, 10 mM Tris, 2.0 mM $MgCl_2$ and 1.0 unit of DNA Taq polymerase. Different conditions used for amplification cycles were 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 sec 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) a programmable thermo cycler. Various SSR markers used were OarFCBl28, OarCP34, OarCP38, OarJMP58, OarFCB304, OarAEl29, BMl329, BM8125, HUJ616, DYMS1, SRCRSP9, OarFCB226, SRCRSP5, ILSTSll and ILSTS28 (Table I). The annealing temperature for each primer was fixed accordingly. A total of 3075 reactions were accomplished on PCR and the products of PCR were analysed on 5% agarose gel by employing the Thermo Scientific 0 Range Ruler 5bp DNA ladder.

The genetic parameters including observed number of alleles (Na), effective number of alleles (Ne), Shannon's Information Index (SI), observed homozygosity (obs. hom.), observed heterozygosity (obs. het.), expected homozygosity (exp. hom.), expected heterozygosity (exp. het.) and average heterozygosity (ave. het.) were calculated using POPGENE32 software version 1.32 (Nei, 1973). To estimate genetic diversities and Fixation Indices that is, FIS (within population inbreeding estimates), FIT (total inbreeding estimates) and FST (measurement of population differentiation), the same software (POPGENE32) was used. Distribution of alleles of different gene markers is shown in Figure 1.

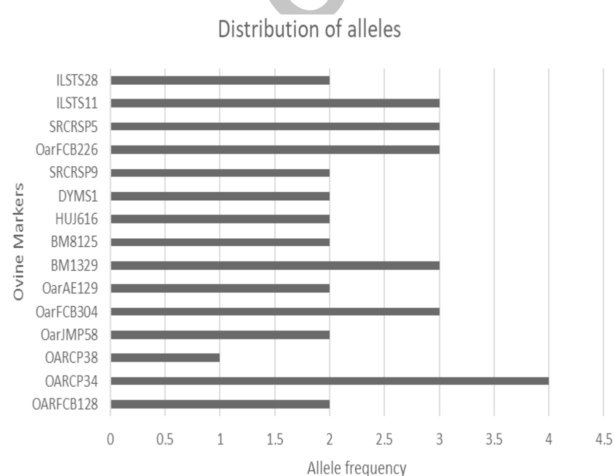

Fig. 1. Distribution of alleles of different gene markers.

RESULTS AND DISCUSSION

From 15 ovine SSR markers run parallel to DNA samples of Bibrik sheep breed using 5bp DNA ladder (10 - 100bp) on agarose (5%) gel different number of bands were obtained corresponding to number alleles (ranging between 1 to 4). Appearance of different number of alleles is indicator of genetic polymorphism in the Bibrik sheep. The number of suggested alleles as indicated by the bands produced on the agarose against each primer indicated that multiple allelomorphism was present in Bibrik sheep. The OarFC128 primer produced 3 bands on the gel image (40, 50, 60 bp) signifying 3 alleles, OarCP34 generated 2 bands indicating two alleles, OarCP38 produced 2 bands on gel showing two alleles, OarJMP58 produced 2 bands suggesting two alleles, OarFCB304 produced 1 band indicating 1 allele, Oar129 has two bands showing two alleles, BM8125 produced three bands indicating three alleles, ILSTS28 generated 4 band indicating four alleles, and ILSTS11 amplified 2 bands suggesting 2 alleles (Table II).

Table II. Population genetic parameters and genetic diversity of Bibrik sheep.

Locus	Na	Ne	I	Ho	He	H_{avg}	Nei
OARFCB128	3.0	1.01	0.46	0.49	0.52	0.51	0.51
OARCP34	2.0	1.07	0.78	0.81	0.19	0.18	0.18
OARCP38	2.0	1.99	0.32	0.35	0.66	0.65	0.65
OarJMP58	2.0	2.29	0.56	0.59	0.41	0.40	0.40
OarFCB304	1.0	1.19	0.90	0.93	0.07	0.06	0.06
OarAE129	2.0	1.51	1.11	1.0	0.0	0.0	0.0
BM1329	2.0	1.83	0.67	0.70	0.29	0.29	0.29
BM8125	3.0	2.03	0.81	0.84	0.16	0.15	0.15
HUJ616	2.0	2.18	0.19	0.22	0.78	0.77	0.77
DYMS1	1.0	1.28	0.012	0.04	0.96	0.95	0.95
SRCRSP9	2.0	1.56	0.52	0.55	0.46	0.45	0.45
OarFCB226	2.0	3.15	0.62	0.65	0.35	0.34	0.34
SRCRSP5	1.0	1.31	0.33	0.36	0.64	0.63	0.63
ILSTS11	2.0	2.35	0.34	0.37	0.63	0.62	0.62
ILSTS28	4.0	3.17	0.45	0.48	0.52	0.51	0.51
Mean	2.1 ±0.8	1.86 ±0.69	0.54 ±	0.56 ±0.27	0.44 ±0.27	0.43 ±0.26	0.43 ±0.27

Na, number of alleles observed; Ne, effective number of alleles; I, shannon's information index; Ho, observed heterozygosity; He, expected heterozygosity; Havg, average heteorzygosity; Nei, gen distance

Out of fifteen ovine molecular markers used in the present study, all yielded variable number of bands observed in the gel image and a total number of alleles

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obtained was 31 (on the average > 2 alleles), with range of 1 (OarFCB304, DYMS1, SRCRSP5) to 4 (ILSTS28) recognizing polymorphism in the Bibrik sheep population. The Na, Ne and SI averaged 2.067 ± 0.799, 1.859 ± 0.687 and 0.537 ± 0.289, respectively (Table II). The mean (±sd) observed, expected and average heterozygosities were found to be 0.557 ± 0.271, 0.443 ± 0.271 and 0.433 ± 0.269, respectively. The observed heterozygosity was in range of 0.039 (DYMS1) to 1.000 (OarAE129), expected heterozygosity 0.00 (OarAE129) to 0.961 (DYMS1) and average heterozygosity 0.00 (OarAE129) to 0.951 (DYMS1) for different molecular markers (Table II).

F-statistics is indicative of expected amount of heterozygosity in a population which may be the degree of reduction in heterozygosity. The observed heterozygosity in case of various markers as indicated by observed F value that ranged from 0.232 to 0.931 and averaged 0.592 ± 0.232. The lower standard errors specified the prevalence of homozygosity to some extent in Bibrik population indicating intense inbreeding that showed the use of only few sires (rams) in the population that were closely related to ewes (Table III).

Table III. F-statistics for Bibrik breed of sheep found in Balochistan.

Locus	K	F_{obd}	F_{min}	F_{max}	F_{avg}	s.e	Lower CI-95	Upper CI-95
OARF-CB128	2	0.93	0.50	0.92	0.93	0.02	0.50	0.92
OARCP34	4	0.29	0.25	0.75	0.53	0.06	0.28	0.74
OARCP38	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
OarJMP58	2	0.72	0.50	0.92	0.82	0.03	0.50	0.92
OarFCB304	3	0.46	0.33	0.82	0.64	0.05	0.35	0.82
OarAE129	2	0.69	0.50	0.92	0.81	0.03	0.50	0.92
BM1329	3	0.56	0.33	0.82	0.69	0.04	0.35	0.82
BM8125	2	0.75	0.50	0.92	0.84	0.02	0.50	0.92
HUJ616	2	0.71	0.50	0.93	0.82	0.03	0.50	0.92
DYMS1	2	0.68	0.50	0.92	0.80	0.04	0.50	0.92
SRCRSP9	2	0.83	0.50	0.92	0.87	0.01	0.50	0.92
OarFCB226	3	0.49	0.33	0.81	0.66	0.05	0.34	0.81
SRCRSP5	3	0.51	0.33	0.89	0.70	0.05	0.34	0.89
ILSTS11	3	0.48	0.33	0.82	0.65	0.04	0.34	0.82
ILSTS28	2	0.76	0.50	0.92	0.84	0.02	0.50	0.92
Mean		0.59± 0.23	0.39± 0.14	0.82± 0.23	0.71± 0.22	0.03	0.39± 0.14	0.39± 0.14

K, number of heterozygous loci; F obd, F observed; F min, F minimum; F max, F maximum; F avg, F average; F denoted F-statistics; CI, confidence interval.

DISCUSSION

Population genetic parameters are indicators of various phenomenon in the population and help in understating population structure. The phenomenon of polymorphism is generally related to presence of number of multiples alleles of a gene. In current study Na was 2.067±0.799 and Ne was 1.859±0.687 which was less than reported values in previous studies on other sheep breeds in the world (Ali *et al*., 2016 in Harnai breed 2.448±0.869; Ahmad *et al*., 2014 in Kail sheep as 5.273; Musavi *et al*., 2011 in Hazargi sheep 6.296; Yadave *et al*., 2011 in Indian sheep as 8.64; Kumar *et al*., 2018 as 6.7). The Ne reported by these researchers in respective breeds were also higher than those values found in the present study except for 1.70 by Ali *et al*. (2016) 3.94 by Ahmad *et al*. (2014), 4.394 by Musavi *et al*. (2011), 4.57 Yadave *et al*. (2011), 3.658 by Kumar *et al*. (2018). SI as calculated in present research (0.537) was also lower than as reported in the previous studies: 0.59 (Ali *et al*., 2016), 1.58 (Musavi *et al*., 2011), 1.445 (Ahmad *et al*., 2014) 1.419 (Kumar *et al*., 2018) in various sheep breeds. Muneeb *et al*. (2012) also reported obs. het., exp. het., polymorphic information content and SI, as 0.67 ± 0.19, 0.75 ± 0.14, 0.71 ± 0.16 and 1.69 ± 0.51, respectively in Najdi sheep.

The first step of revealing the genetic diversity is to prefer using polymorphic microsatellite markers (Alvarez *et al*., 2004). As far as obs. het. was concerned it averaged 0.557±0.271. Ali *et al*. (2016) and Kumar *et al*. (2018) reported lower obs. het. (0.336 and 0.512, respectively) while other studies have presented higher values of obs. het as compared to present findings 0.825 by Musavi *et al*. (2011), 0.712 by Yama *et al*. (2011), 1.445 by Ahmed *et al*. (2014). Exp. het. in present findings (0.443) was lower than as reported by Ali *et al*. (2016), Ahmed *et al*. (2014), Wajid *et al*. (2014), Musavi *et al*. (2011), Shariffi *et al*. (2009) reported as 0.602, 0.623, 0.718, 0.772, 0.77, respectively.

The ave. het. (0.433) and genetic diversity (0.433) values as found in the present study differed from other studies. Koray *et al*. (2020) reported obs. het. in Karayaka sheep as 0.171 (Giresun) to 0.376 (Ordu) and 0.757 (Samsun) to 0.845 (Ordu) in four area of Turkey, respectively. Ali *et al*. (2016) has shown avg. het. as 0.347 and Nei as 0.347 in Harnai sheep, Musavi *et al*. (2011) has reported higher values of avg. het. as 0.757 and 0.772, and Dalvit *et al*. (2009) reported different and higher values in four Italian sheep breeds as 0.806, 0.801, 0.796 and 0.801. These results are possibly due to the differences in sampling populations in terms of the population's breeding strategy, genotypes and region's climatic conditions. Thus, adaptation may have caused this allelic richness and

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diversity.

The F statistics found in present study indicated that half of the markers showed higher than average reduction in heterozygosity in Bibrik population. This reduction is leading to homozygous Bibrik population which is alarming situation as no selection would produce effective improvement in homozygous populations and inbreeding intensity is also on the increase leading to fixation of characters and causing inbreeding depression in various traits. Prevalence of homozygosity was also indicated by lower standard errors.

CONCLUSION

It is suggested that prompt decisions should be taken to avoid intense inbreeding in the population and outcrossing is recommended to improve the breed by using distantly related or totally unrelated males. The poor growth rate can be managed by rotating males and frequent changes in breeding males by keeping the flocks open. Mass selection might be adopted for increase in size of the animals that would ultimately lead to improved production from this breed. Also purity of the breed should have maintained by taking care of intense inbreeding or close breeding. It is also recommended that molecular markers are needed to identify and make the breed a patent. This animal genetic resource might be conserved and propagated by adopting latest molecular technologies in the form of biological markers which would identify breed specific markers to test the purity of the breed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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