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ASSESSMENT OF GENETIC DIVERSITY IN BALOCHI AND RAKHSHANI SHEEP BREEDS OF BALOCHISTAN USING MICROSATELLITE DNA MARKERS

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ABSTRACT

This study was conducted to analyze the genetic diversity and variability of two sheep breeds (Balochi and Rakhshani), from Balochistan province of Pakistan, through the use of 11 microsatellite markers recommended by FAO. All the screened loci were polymorphic and 70 alleles in total were observed in all studied loci with average polymorphic information content equal to 0.57, showing that the microsatellite panel used was highly informative. The result divulged high level of genetic variability in each of the two investigated sheep breeds, allele diversity in Balochi 4.5455 and Rakhshani was 4.0909; gene diversity in Balochi 0.5927 and Rakhshani was 0.6182. High heterozygosity value indicated low level of inbreeding, low or no selection pressure and large number of alleles. Further support in this regard was observed by inbreeding estimate (Balochi $F_{IS} = 0.0292$ and Rakhshani $F_{IS} = 0.0084$) in our sheep population. High level of genetic differentiation between Balochi and Rakhshani sheep breeds was evident from high genetic differentiation estimates ($F_{ST} = 0.1884$). The pair wise comparison between both breeds at each locus in term of number of alleles shared (36%, 25/70) reflected the variation between them. The Nei's genetic distances ($D_S = 1.3001$ and $D_A = 0.2725$) and gene flow (Nm = 1.0767) further indicating the genetic variation between Balochi and Rakhshani sheep breeds. These data depicts the effectiveness of FAO recommended microsatellite markers for estimation of genetic diversity in Balochi and Rakhshani sheep breeds of Pakistan and may be helpful for comparison with other reported data and for better understanding and breed conservation efforts locally and worldwide.

Key words: Sheep breeds; genetic diversity; microsatellite makers; Balochi breed; Rakhshani breed.

INTRODUCTION

Sheep have been estimated to inhabit fertile areas of Globe some 9000 years back and considered among few earliest domesticated species of livestock. This was further supported by mitochondrial lineage studies, which revealed that sheep was evolved from ancestors of wild mouflon (Chen *et al.* 2006; Chessa *et al.* 2009). Afterward due to various divergences such as human migration, ecological environment, climate change and selection policies for varied objectives led over 2351 breeds of sheep found on land today (DAD-IS, 2010).

This study included two sheep breeds Balochi and Rakhshani of Balochistan province of Pakistan. Balochistan province comprises of arid and semi arid land makes about 44% of the total geographical area of the country which embraces many precious livestock breeds and play pivotal role in the socio-cultural and socio-economic life of the livestock keepers in area. Sheep is among the most important domesticated species of livestock in Pakistan which play a significant role in the well being of small farmers and landless people. Pakistan is the 10th largest sheep producing country in the world after China and India and having 28 Sheep (*Ovis aries*) breeds (Khan *et al.* 2008).

Microsatellite markers have been widely used in studies of the genetic diversity and characterization of sheep breeds (Arranze *et al.* 2001; Rendo *et al.* 2004; Peter *et al.* 2004; Dalvit *et al.* 2008; Kevorkian *et al.* 2010). Microsatellites are stretches of DNA that consist of tandem repeats of a specific nucleotides sequence, consist of mono, di, tri or tetra repeats in the genome. These stretches are frequently found in genomes (Tautz and Renz, 1984) and are easily amplified by PCR using primers designed at flanking ends of these microsatellite markers (Weber and May, 1989). Electrophoresis is the tool of choice to identify these microsatellite alleles reflecting different number of repeats (Buchanan and Thue, 1998). They are numerous advantages which have been able to declare these markers as important tool in various areas of research, e. g., genome mapping studies (Kappes *et al.* 1997), linkage (Schmutz *et al.* 1995) and population studies (Buchanan *et al.* 1996). All the advantages being as useful marker due to easy amplification, small quantity of required DNA, easy and automated analytical procedure, possibility of multiple alleles detection, high mutation rates, the neutrality and co-dominance nature and their abundance in all eukaryotes (Cannon *et al.* 2001; FAO, 2007). Present study was designed to estimate the unexplored genetic diversity of two sheep breeds (Balochi and Rakhshani) of province Balochistan using FAO recommended microsatellite DNA markers with the aim whether these markers can be useful for estimation of genetic diversity of our local sheep breeds and to generate the useful data for further analysis and comparison as well as designing strategies for breed conservation locally and Worldwide.

MATERIALS AND METHODS

Blood collection and DNA Extraction: Different animals having no blood relation with typical phenotypic features known for both breeds were selected. Blood samples were not taken from sibs deliberately, though parentage record was unavailable, so as to keep the sample number limited from an area. In total, 50 blood samples from true representative individuals of Balochi and Rakhshani breeds were collected (25 samples from each breed) in 200 µL EDTA (Ethylenediamine tetra-acetic acid) containing falcon tubes from different Government livestock farms and their respective home tracts in Pakistan.

Standard method of DNA extraction as described in (Sambrook and Russel, 2001) was performed on all blood samples. The concentration of extracted DNA and its purity were estimated by gel electrophoresis and spectrophotometeric analysis based on 260 nm and 280 nm absorbance. The final concentration of DNA was brought to 50 ng/uL and stored at -40 C^0 before further use.

Genotyping of Microsatellite Markers: A set of total of 11 microsatellite markers (loci) distributed across the *Ovis aries* genome and showing polymorphism in sheep were selected and tested for diversity analysis for present study. Of the total microsatellite markers (loci) analyzed 5 were: MAF70, BM1818, INRA32, ILSTS011 and BM1314 used as labeled markers (Table 1). The forward primer of these markers (loci) was 5´-labelled with FAM, PET, NED or VIC fluorescence tag, in order to perform fragment analysis of the PCR products in ABI PRISM 3130 genetic analyzer (Applied Biosystem, USA). A set of 6 microsatellite markers (loci) analyzed were: OarAE101, OarVH72, MAF33, MM12, ETH152 and OarFCB48 (Table 1), selected for this study used as unlabeled markers to perform the fragment length analysis of the PCR products using 12% non denaturing polyacrylamide gel (PAGE) in 1X TAE buffer, 50 bp DNA ladder was used as a size standard. To visualize the PCR products gels were silver stained (Bassam *et al.* 1991). PCR amplification was performed on BioRad thermo cycler using reaction mixture of 25 µL containing 50 ng templates DNA, 50mM KCl, 10mM Tris-HCl, 2.5 mM dNTPs, 1.5 mM MgCl, 0.75 pmol/µL of forword/ reverse primers and 0.1 µL of 5U *Taq* polymerase (Frementas, USA). PCR condition were used as the initial denaturing at 95 C^0 for 4 minutes was followed by 35 cycles each for 30 sec at 94 °C for denaturation, 45 sec at 62-52°C for annealing and 45 sec at 72 °C for extension followed by 10 minutes at 72 C^0 for final extension. The unlabeled markers products were electrophoresed on 12% non denaturing polyacrylamide gel in 1X TAE buffer at 120 volts for 7 hours.

Statistical Analysis: To analyze the genetic variation in microsatellite loci in Balochi and Rakhshani sheep populations, in term of number of alleles as observed and expected number of alleles, observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and Estimates Shannon (1949) information index as a measure of genetic diversity was carried out across the 11 loci using POPGENE version 1.31 (Yeh and Yong, 1999). To evaluate the extent of difference within and among populations, the fixation index F_{IS} was calculated as estimator of inbreeding.

GENEPOP version 4.0 (Raymond and Rousset, 1995) was for the calculation of F-statistics (F_{ST}, F_{IT}, F_{IS}). The F_{ST} is the effect of subpopulation (S) compared to the total population (T), F_{IT} is the inbreeding co-efficient of an individual (I) relative to the subpopulation (S) and F_{IS} is the inbreeding co-efficient of an individual (I) relative to total population (T). The method used by Weir and Cockerham (1984) for estimation of F_{IT}, F_{ST} and F_{IS} was employed for each locus in this study. Estimation of gene was calculated using fromula F_{ST} ($F_{ST} = 0.25(1-F_{ST})/F_{ST}$) as described by Slatkin and Barton (1989). The allele frequencies were utilized for the calculation of the polymorphic information content (PIC) values using POWERSTAT V1.2.1 used to determine the usefulness of markers. The results of polyacrylamide gel electrophoresis were analyzed by the relative flow method. The genotypes were scored manually, the size of the alleles was calculated online using INCHWORM program (http://www.molecularworkshop.com/program/inchworm.html) which estimates the length of the molecule, based on the electrophoresis mobility.

RESULTS

Genotyping data from 11 microsatellites loci was used to assess the genetic structure and differentiation in Baloci and Rakhshani sheep breeds. A total of 70 microsatellite alleles were identified across the genome in both breeds for 11 analyzed microsatellite loci. All the loci were polymorphic (having ≥ 2 alleles). The mean number of alleles (MNA) observed for all loci was 4.5455 in Balochi and 4.0909 in Rakhshani. The number of alleles per locus ranged from 2 (MAF33 in Balochi and ETH152 in Rakhshani) to 8 (ILSTS011 in Balochi). While the effective number of alleles (Ne) was less than the observed number of alleles of mean 2.9699 and 2.9659 in Balochi and Rakhshani respectively (Table 2). The number of alleles at different loci served as a measure of genetic variability in both breeds. The observed heterozygosity was perceived less than expected heterozygosity in both breeds (Table 2), the mean observed the expected heterozygosity were respectively 0.5927 and 0.6230 in the Balochi while the corresponding parameters were 0.6182 and 0.6255 respectively in Rakhshani. The observed heterozygosity was ranged from 0.0400 (INRA32) to 1.0000 (OarVH72 and OarFCB48) in Balochi while the consequent parameter in Rakhshani ranged from 0.0400 (MM12) to 1.0000 (OarAE101, MAF33 and OarFCB48). The expected heterozygosity ranged from 0.2229 (MM12) to 0.7951 (ILSTS011) in Balochi while in Rakhshani it ranged from 0.2743 (ETH152) to 0.8212 (MAF70). The average heterozygosity in both breeds ranged from 0.2960 (MM12) to 0.7588 (MAF70) with mean of 0.6118 (Table 2).

The polymorphic information content (PIC) is another most indicative parameter of genetic variations, follow this parameter all the loci may be considered as high informative. The present study indicated the PIC values ranged from 0.21 (MM12) to 0.76 (MAF70) with mean 0.55 in Balochi while in Rakhshani this value was varied from 0.23 (ETH152) to 0.78 (MAF70) with mean of 0.57 (Table 2). Based on this value, virtually 73% of the microsatellite markers were observed highly informative (PIC > 0.50), 18% were logically informative (PIC > 0.25 & < 0.50) while only 9% were less polymorphic/informative (PIC < 0.25), these observed figures were same for both breeds. This PIC analysis further indicated high utility of used set of markers for genetic diversity analysis.

The inbreeding coefficients for all loci within population across Balochi and Rakhshani breeds are given in table 4. Both Balochi and Rakhshani sheep population showed significant (P<0.05) heterozygote deficit with mean 0.0292 in Balochi and 0.0084 in Rakhshani sheep breed. Observed F_{IS} in Balochi breed ranged from -0.6129 (MAF33) to 0.9438 (INRA32) while the corresponding parameter in Rakhshani breed ranged from -0.8629 (MAF33) to 1.0000 (OarVH72). The mean value of F_{IT} was 0.1968 (total inbreeding estimate) ranged from -0.3441 in OarFCB8 to 0.8752 in MM12. The mean F_{ST} (estimate of population differentiation) was observed 0.1884, was ranged from 0.0465 in MAF70 to 0.5381 MM12.

Table 1. Microsatellite markers, Primer sequences, Type of repeat, location, Accession numbers and References

S. N	Loci	Primer sequences	Type of repeats	Ch. No.	G. Bank Accesion No.	Reference
1	OarFCB48	F: GAGTTAGTACAAGGATGACAAGAGGCAC R: GACTCTAGAGGATCGCAAAGAACCAG	(GT) 10	17	M82875	(Buchanan *et al.*, 1994)
2	OarVH72	F: CTCTAGAGGATCTGGAATGCAAAGCTC R: GGCCTCTCAAGGGGCAAGAGCAGG	(GT) 14	25	L12548	(Pierson *et al.*, 1993)
3	MAF70	F: CACGGAGTCACAAAGAGTCAGACC R: GCAGGACTCTACGGGGCCTTTGC	(AC) 39	4	M77199	(Buchanan and Crawford,1992)
4	OarAE101	F: TTCTTATAGATGCACTCAAGCTAGG R:TAAGAAATATATTTGAAAAAACTGTATCTCCC	(TG) 22	6	L13692	(Montgomery *et al.*, 1993)
5	MAF33	F: GATCTTTGTTTCAATCTATTCCAATTTC R: GATCATCTGAGTGTGAGTATATACAG	(AC) 18	9	M77200	(Buchanan and Crawford,1992)
6	BM1818	F: AGCTGGGAATATAACCAAAGG R: AGTGCTTTCAAGGTCCATGC	(TG)13	20	G18391	(Bishop *et al.*, 1994)
7	ILSTS011	F: GCTTGCTACATGGAAAGTGC R: CTAAAATGCAGAGCCCTACC	(CA) 11	9	L23485	(Brezinsky *et al.*, 1993)
8	MM12	F: CAAGACAGGTGTTTCAATCT R: ATCGACTCTGGGGATGATGT	-	9	-	-
9	INRA 032	F: ACACATACACACACACGCACA R: TGGCTGCTCAAAAAATAGCA	(AC)14	11	X67823	(Vaiman *et al.*, 1994)
10	BM1314	F: TTCCTCCTCTTCTCTCCAAAC R: ATCTCAAACGCCAGTGTGG	(AC) 18	22	G18433	(Bishop *et al.*, 1994)
11	ETH152	F: TACTCGTAGCGCAGGCTGCCTG R: GAGACCTCAGGGTTGGTGATCAG	(CA)17	5	Z14040	(Steffen *et al.*, 1993)

Table 2. Summary of number of alleles observed and expected, Heterozygosity Statistics and polymorphic information content for All Loci.

Loci	Size range	Balochi				PIC	Rakhshani				PIC A.H.	
		No. of alleles		Heterozygosity			No. of alleles		Heterozygosity			
		Na	*Ne*	*Ho*	*He*		*Na*	*Ne*	*Ho*	*He*		
MAF 70	141-159	5.0000	3.4819	0.8800	0.7273	0.76	7.0000	5.1230	0.7600	0.8212	0.78	0.7588
BM1818	252-280	5.0000	4.0453	0.6800	0.7682	0.67	6.0000	3.3784	0.7200	0.7184	0.73	0.7284
INRA 32	166-188	4.0000	3.4435	0.0400	0.7241	0.60	4.0000	2.4510	0.3200	0.6041	0.59	0.6508
ILSTS011	270-289	8.0000	4.5290	0.5200	0.7951	0.72	4.0000	2.8217	0.7600	0.6588	0.63	0.7124
BM1314	144-161	4.0000	2.7594	0.5200	0.6506	0.54	5.0000	3.6765	0.8800	0.7429	0.68	0.6828
OarAE101	96-110	4.0000	2.5100	0.4800	0.6139	0.57	4.0000	3.9683	1.0000	0.7633	0.70	0.6748
OarVH72	135-167	7.0000	4.0453	1.0000	0.7682	0.71	3.0000	2.9343	0.0000	0.6727	0.59	0.7060
MAF33	137-169	2.0000	1.8911	0.7600	0.4808	0.36	3.0000	2.1589	1.0000	0.5478	0.43	0.5040
MM12	96-112	3.0000	1.2794	0.1200	0.2229	0.21	3.0000	1.5964	0.0400	0.3812	0.32	0.2960
ETH152	168-219	3.0000	1.7147	0.5200	0.4253	0.38	2.0000	1.3676	0.3200	0.2743	0.23	0.3428
OarFCB48	141-181	5.0000	2.9691	1.0000	0.6767	0.60	4.0000	3.1486	1.0000	0.6963	0.62	0.6728
Mean		4.5455	2.9699	0.5927	0.6230	0.55	4.0909	2.9659	0.6182	0.6255	0.57	0.6118

Notes: (*Na*) Observed and (*ne*) expected number of alleles, (*Ho*) obsevered and (*He*) expected heterozygosity, (PIC) polymorphic information content, A.H. average heterozygosity and Expected heterozygosity were computed using Levene (1949)

Table 3. Summary of F-Statistics and Gene Flow (Nm) estimate for each locus across Balochi and Rakhshani

Locus	Fit	Fst	Nm
MAF70	-0.0304	0.0465	5.1270
BM1818	0.1349	0.0999	2.2537
INRA32	0.7597	0.1311	1.6568
ILSTS011	0.1561	0.0607	3.8717
BM1314	0.0283	0.0522	4.5399
OarAE11	0.1104	0.1887	1.0745
OarVH72	0.3898	0.1384	1.5564
MAF33	-0.1702	0.3298	0.5081
MM12	0.8752	0.5381	0.2146
ETH152	0.3744	0.4894	0.2608
OarFCB48	-0.3441	0.0957	2.3624
Mean	0.1968	0.1884	1.0767

Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst

Table 4. Inbreeding estimates (FIS) in Balochi and Rakhshani Breeds of sheep population

Locus	Balochi	Rakhshani
MAF70	-0.2346	0.0557
BM1818	0.0967	-0.0227
INRA32	0.9436	0.4595
ILSTS011	0.3326	0.1772
BM1314	0.1844	-0.2088
OarAE11	0.2021	-0.3369
OarVH72	-0.3284	1.0000
MAF33	-0.6129	-0.8629
MM12	0.4505	0.8929
ETH152	-0.2476	-0.1905
OarFCB48	-0.5078	-0.4654
Mean	0.0292	0.0084

DISCUSSION

The study of genetic variation has a significant role in developing rational breeding strategies for economical animal species (Maudet *et al.* 2002). The microsatellite markers have been investigated in farm animals for estimating genetic variations among breeds and among closely related breeds. This study investigated the genetic variability in Balochi and Rakhshani sheep breeds using microsatellite genotypic data. All the 11 microsatellite markers were observed polymorphic in both breeds. The mean number of alleles ranged from 4.5455 in Balochi and in Rakhshani was 4.0909. ILSTS011 highly polymorphic loci giving 8 alleles in Balochi, other scientific workers reported the same result in Spanish sheep breeds (Alvarez *et al.* 2004), and larger than 2 in Hashtangri, Mchini and Balkhi, Khyber Pakhtoonkhwa (KP), Pakistan (Ibrahim *et al.* 2010). On the other hand the highly polymorphic loci in Rakhshani was MAF70 giving 7 alleles, the same as in Hashtangri, Mchini and Balkhi, Khyber Pakhtoonkhwa (KP), Pakistan (Ibrahim *et al.* 2010).

The pair wise comparison between both breeds at each locus in term of number of alleles shared (36%, 25/70) reflected the variation between Balochi and Rakhshani. However the three breeds of Khyber Pakhtoonkhwa (KP), Pakistan, Hashtangri, Mchini and Balkhi showed high maximum alleles than in our study. Hashtangri and Mchini shared maximum 85%, Balkhi and Mchini 82% and Balkhi and Hashtangri shared 76% alleles. The two Indian neighboring sheep breeds Chokla and Nali shared 70.4% alleles (Sodhi *et al.* 2006).

Observed heterozygosity averaged over loci was 0.5927 in Balochi and in Rakhshani the value of corresponding parameter was 0.6182, high values 1.0000 was observed in each locus OarVH72 and OarFCB48 in Balochi and MAF33, OarAE101 and OarFCB48 in Rakhshani. The mean observed value in both breeds is comparable with the Romanian sheep (Milk line Palse, Meat line Palse, Karabash and Botosani Karakul) having 0.61 (Kevorkian *et al.* 2010). The expected heterozygosity that is considered to be a finer estimator of the genetic variability (Kim *et al.* 2002), observed from 0.2229 in (MM12) to 0.7951 (ILSTS011) in Balochi and from 0.2743 (ETH152) to 0.8212 (MAF70) in Rakhshani with mean 0.6230 and 0.6255 in Balochi and Rakhshani respectively. The high expected heterozygosity observed at ILSTS011 (0.7951) in Balochi the same locus is also studied in Italian sheep (Gentile di Puglia, 0.7560), Hungarian sheep (Bulgarian, 0.7600) (d'Angelo *et al.* 2009; Kusza *et al.* 2010) and in Rakhshani the high value observed 0.8212 (MAF70), the same locus is also studied in other sheep, in Romanin sheep (0.7600), Italian sheep (Gentile di Puglia, 0.8830) and Hungarian sheep (Bulgarian, 0.8670), (Kevorkian *et al.* 2010; d'Angelo *et al.* 2009; Kusza *et al.* 2010].

The high average value for PIC (0.55 and 0.57, Balochi and Rakhshani respectively) displayed by the panel of 11 microsatellite markers in both sheep breeds supported the appropriateness of the used set of markers for genetic diversity analysis (Barker, 1994). The high value was observed 0.76 (MAF70) in Balochi and 0.78 at the same marker in Rakhshani, comparable with Romanian sheep (0.77) (Kevorkian *et al.* 2010).

Within population, inbreeding estimate (F_{IS}) value is negative for three loci (MAF33, ETH152 and OarFCB48) in both breeds which indicates heterozygosity, other loci are negative (MAF70 and OarVH72) in Balochi and (BM1818, BM1314 and OarAE11) in Rakhshani. The mean F_{IS} values observed were 0.0292 and 0.0084 in Balochi and Rakhshani respectively. The inbreeding estimate (F_{IS}) in most literature surveyed indicated different level of inbreeding value as 0.0660 in Spanish breeds (Alvarez *et al.* 2004), 0.1900 in Sarda sheep (Pariset *et al.* 2003), 0.0330 in Turkish sheep breeds (Soysal *et al.* 2005), 0.0580 in Indian Muzzafarnagri sheep breeds (Arora and Bhatia, 2004), 0.1590 in Magra (Arora and Bhatia, 2004) and high rate of inbreeding reported by Sodhi and co-worhers (2005) in Nali and Chokla sheep breeds 0.3970 and 0.2990 respectively. The average genetic differentiation (F_{ST} = 0.1884) between two breeds contributed by 11 loci showed somewhat high degree of genetic differentiation as compared to two Indian sheep breeds Nali and Chokla (F_{ST} = 0.083) which showed a certain degree of differentiation at 19 loci. The difference between both breeds is 18.82% also showed high difference as compare to corresponding Indian breeds (8.3%). The value of corresponding parameter estimated by other workers was 0.17 in Swiss sheep breeds (Stahlberger-Saitbekova *et al.* 2001) and 0.082 in Romanian sheep breeds (Milk line Palse, Meat line Palse, Karabash and Botosani Karakul) (Kevorkian *et al.* 2010). This was also evident from considerable low level of gene flow (Nm = 1.0767) in our sheep breeds compared to two Indian sheep breeds Nali and Chokla (Nm = 3.896). The Nei's (1978) genetic distance and genetic identity between the sheep breeds Balochi and Rakhshani were 1.3001 and 0.2725 respectively. The high genetic distance showed the divergence between the two sheep breeds.

In conclusion we report that above mentioned, FAO recommended microsatellite makers have shown their effectiveness for the estimation of genetic diversity among our local sheep breeds of Balochistan and these results may be helpful and may contribute to have an understanding of genetic structure of our local sheep breeds. Furthermore, the data produced may prove helpful for comparison and breed conservation efforts locally and worldwide.

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