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GENETIC DIVERSITY OF HARIANA AND HISSAR CATTLE FROM PAKISTAN USING MICROSATELLITE ANALYSIS

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ABSTRACT

Genetic diversity of Hariana and Hissar cattle breeds of Pakistan was investigated by using 30 bovine microsatellite markers proposed by the Food and Agriculture Organization and the International Society for Animal Genetics. The study was aimed at assessing the current genetic variability to understand whether the two cattle breeds are genetically different. Out of 30 loci, 27 microsatellite loci amplified well and exhibited polymorphisms. Polymorphic information content (PIC) value was 0.749 in Hariana and 0.719 in Hissar. A total of 128 alleles were detected across the 27 loci with an average of 4.59 and 4.37 alleles per locus in Hariana and Hissar breeds, respectively. The observed number of alleles (N_a) ranged from 2 (CSRM60) to 7 alleles (BM1818; ETH10) in Hissar cattle, and 3 (INRA032; MM12) to 6 (BM1818; ETH10; INRA023; SPS115) in Hariana cattle. The effective number of alleles (N_e) were in the range of 1.905 (HEL1) to 3.774 (TGLA126) in Hariana and 1.513 (ETH225) to 5.708 (BM1818) in Hissar cattle. The estimated mean observed (H_o) and expected heterozygosity (H_e) were 0.51 and 0.67 in Hariana vs. 0.47 and 0.63 in Hissar cattle, respectively. Heterozygote deficiency analysis revealed that both the populations exhibited significant deviations from Hardy Weinberg (HW) equilibrium ($P<0.05$) at some of the loci. There was a significant deficit of heterozygotes (F_{IS}) in both the breeds, ranging from 25.2% in Hariana to 25.9% in Hissar. The average F_{IS} values for most of the loci in both breeds were significantly different ($P<0.05$) from zero. These results reveal that although Hariana and Hissar breeds shared the common breeding tract, they are genetically different enough as separate breeds.

Key words: Hariana, Hissar, Pakistan indigenous cattle, microsatellite, genetic diversity.

INTRODUCTION

Besides milk and meat production, cattle in Pakistan have traditionally been bred to produce bullocks for ploughing. Two of the draught zebu cattle breeds present in the eastern part of the country are Hariana and Hissar. These breeds are famous for their excellent draught power capacity, endurance and agility at work. There is some evidence that Hariana are the descendants of cattle brought from the northern passes in present-day Pakistan by Vedic Aryan people as they migrated into India during the second millennium BC (Kulke and Rothermund, 1990). Animals of this breed are generally tall, with a grey or white coat. They have long narrow face and flat forehead. Horns are short, curved and emerged laterally from the outer angles of the poll, curving upwards and inwards in aged animals. Hissar cattle have their origin from Indian state "Hissar" but are found in fair number in areas adjoining Indian border including districts of Bahawalnagar and Bahawalpur in the Punjab province of Pakistan. Porter (2002) mentioned that Hissar originated during 1815-1898 AD from Kankraj, Hariana, Gir, Nagori, Ongole, Tharparkar and Krishna Valley cattle breeds and were selected for type and fast trotting around 1899-1912 AD. Hissar animals are of moderately dark gray color with even darker color around neck, shoulders, hump and legs in males. They have moderate head, with flat forehead and small horns which emerge laterally and balanced to the extent that they give appearance of half circle.

These two breeds have coexisted in almost the same geographical areas for the last several years. It is vague as to what extent genetic exchange between these two breeds has taken place during their differentiation. Elucidation of genetic variability and genetic relationship among breeds has direct relevance with the issues of sustainable use of domestic animal genetic resources. To avoid further loss of important genetic resources, an objective breed classification based on genetic uniqueness is of priority (Hall and Bradley, 1995).

In the last few years, the usefulness of micro-satellite markers for the estimation of genetic diversity and relationships among cattle breeds has been documented in numerous studies (Dorji *et al.,* 2003; Jordana *et al.,* 2003; Metta *et al.,* 2004; Mukesh *et al.,* 2004). Microsatellite markers, due to their co-dominant and multi-allelic attributes, prove to be efficient in genetic diversity studies, and have become markers of choice in characterization of cattle breeds (Edwards *et al.,* 2000; Canon *et al.,* 2001). However, the genetic structure for Pakistani zebu cattle breeds has not been assessed. The present study was, therefore, undertaken

to assess the current genetic diversity and level of differentiation between Hariana and Hissar cattle breeds inhabiting the same geographical area using 30 bovine specific microsatellite markers from the list of Measurement of Domestic Animal Diversity (MoDAD) markers recommended by Food and Agriculture Organization (FAO) and the International Society for Animal Genetics (ISAG) (FAO, 1998).

MATERIALS AND METHODS

DNA extraction and microsatellite genotyping

Blood samples (5 mL) from unrelated 10 Hariana and 25 Hissar cattle were collected randomly from their respective breeding tracts (for Hissar from District Bahawalnagar and Minchanabad, and for Hariana from District Bahawalnagar and Sargodha) and Government livestock experiment station, Kullorekot in March 2007**.** Genomic DNA were isolated following the standard protocol involving proteinase K digestion and phenol:chloroform extraction (Grimberg *et al.*,1989). The set of 30 primers for polymerase chain reaction (PCR) amplification of microsatellite loci were synthesized from e-oligos, Gene Link, USA.

PCR amplification of microsatellite loci was carried out in 20 µL reaction volume consisting of 2.5 mM $MgCl_2$, 250 mM dNTPs, 30 ng of each primer, 15 ng of template DNA, and 0.5 U of *Taq* DNA polymerase (Fermentas, USA). PCR reaction was carried out in CreaCon T-cy thermal cycler. The PCR conditions employed were: initial denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of 30 seconds at 94^{o}C, 30 seconds at annealing temperature of each marker and 1 minute extension at 72^{o}C. The final extension step at 72^{o}C was prolonged for 5 minutes. The PCR amplification products were electrophoresed at 30V in 4% high resolution agarose gel, having resolving power of 2-20 bp from Genechoice, CLP®, USA. The fingerprints were visualized and documented using Uvipro Gel Documentation System, UVtec, UK.

Statistical analysis

For each locus and breed, commonly derived statistics from the microsatellite genotypic data *viz.*, allelic frequencies, observed number of alleles, mean effective number of alleles, exact test for deviations from Hardy-Weinberg equilibrium, observed and expected heterozygosities and Nei's unbiased genetic distances (Nei, 1978) were estimated by POPGENE version 1.31 (Yeh and Yang, 1999). The polymorphic information content (PIC) values were analyzed by MOLKIN version 3.0 (Gutierrez *et al.*, 2005). Breed differentiation was estimated by Wright (1978), fixation indices for each locus across breeds were determined according to the variance based on the method of Weir and Cockerham (1984), using the FSTAT program version 2.9.3.2 (Goudet, 2002). The extent of global inbreeding was further studied with the same software by estimating the F_{IS} values and their significance levels within each breed.

RESULTS AND DISCUSSION

In this study, genetic variation in 35 individuals belonging to Hariana (n = 10) and Hissar (n = 25) breeds was analyzed using 30 microsatellite loci. Out of the 30 microsatellite loci, 27 loci amplified successfully and produced definite banding patterns. All amplified loci were polymorphic, showing percentage of polymorphic loci (PPL) as 100%. This high PPL value indicates the usefulness of these markers in population studies. Similarly, polymorphic information content (PIC) value was 0.749 in Hariana and 0.719 in Hissar. It was also seen in the taurine (Bradley *et al.*, 1994; Canon *et al.*, 2001; Maudet *et al.*, 2002) and indicus (Kumar *et al.*, 2003; Metta *et al.*, 2004; Mukesh *et al.*, 2004) breeds investigated earlier using microsatellite markers. The high PIC value indicated that the breeds are more heterogenous with no selection for certain traits. The high allelic diversity observed is probably due to no selection pressure for the improvement of draught characters. These findings are in agreement with Muralidhar (2003), who used 10 microsatellite loci and obtained PIC values ranging from 0.150 to 0.790 in Ongole and from 0.13 to 0.80 in Deoni cattle.

The estimated parameters pertaining to genetic polymorphism in two breeds *viz.*, observed and effective number of alleles, and observed and expected heterozygosity are presented in Table 1. A reasonable amount of variability in both the breeds is discernible from the allele frequency data. A total of 128 alleles were detected across the 27 loci with an average of 4.59 and 4.37 alleles per locus (mean number of alleles in Hariana and Hissar breeds, respectively. The number of observed alleles ranged from 2 at locus CSRM60 to the highest of 7 alleles at loci BM1818 and ETH10 in Hissar, and 3 (INRA032, MM12) to 6 (BM1818, ETH10, INRA023, SPS115) in Hariana. The observed number of alleles demonstrated that almost all the microsatellite loci utilized in the present study were sufficiently polymorphic. Both breeds showed that by the increase of number of alleles at different loci, there was increase in mean genetic diversity in population and supported by Moioli *et al.* (2001).

The effective number of alleles (N_e) is an estimate of the number of alleles with equal frequencies corresponding to a particular PIC value. The mean effective number of alleles was more in Hissar cattle (2.89) than Hariana (2.87) in the present study. The N_e values were in range of 1.905 (HEL1) to 3.774 (TGLA126) in Hariana and 1.513 (ETH225) to 5.708 (BM1818) in Hissar cattle. The observed numbers of alleles in this study are less than those presented by Mukesh *et al.* (2004) for Sahiwal (5.2) and Hariana

(6.5). The value of mean effective number of alleles is also less in this study compared to 3.42 in Hariana and 2.95 in Sahiwal reported by Mukesh *et al.* (2004). The allele sizes obtained at each locus in both the indigenous breeds were comparable to each other and also in agreement with the data published for taurine cattle breeds (Canon *et al.*, 2001).

The observed mean (H_o) and expected (H_e) heterozygosity were 0.51 and 0.67 in Hariana vs. 0.47 and 0.63 in Hissar cattle, respectively. The observed mean heterozygosity was lower than the expected heterozygosity for both breeds. The diversity analysis demonstrated considerable level of genetic variation in both breeds, with Hariana breed displaying somewhat higher levels of genetic polyporphism compared to Hissar breed (Table 1). This implies that higher amount of genetic variability can be exploited in these small-sized breeds for individual identification, parentage verification or segregation analysis. Takezaki and Nei (1996) pointed out that average heterozygosity must be between 0.3 and 0.8 in a breed to be a useful marker for measuring genetic variation. The present results for mean heterozygosity in these breeds are within this range. The fairly comparable levels of H_o have been

Table 1: Microsatellite alleles (Na-observed, Ne-expected), heteozygosity (Ho-observed, He-expected) and allele size range at each locus in Hariana and Hissar cattle

S. No.	Locus	Allele range (bp)	Hariana				Hissar			
			Na	Ne	Ho	He	Na	Ne	Ho	He
1.	BM1818	248-278	6	3.70	0.60	0.76	7	5.70	0.44	0.84
2.	BM1824	176-197	5	3.51	0.50	0.75	5	4.48	0.52	0.79
3.	BM2113	122-156	4	2.89	0.60	0.69	4	2.78	0.60	0.65
4.	CSRM60	79-115	4	2.35	0.50	0.60	2	1.72	0.60	0.42
5.	CSSM66	171-209	4	2.04	0.60	0.53	5	2.41	0.44	0.59
6.	ETH3	103-133	4	3.17	0.50	0.72	3	2.03	0.52	0.52
7.	ETH10	207-231	6	3.22	0.50	0.72	7	3.84	0.60	0.75
8.	ETH152	181-211	4	3.07	0.30	0.71	3	2.32	0.48	0.58
9.	ETH185	214-246	5	2.81	0.70	0.67	5	2.59	0.72	0.62
10.	ETH225	131-159	4	2.15	0.10	0.56	3	1.51	0.00	0.34
11.	HEL1	99-119	4	1.90	0.40	0.50	4	2.24	0.40	0.56
12.	HEL9	141-173	5	2.70	0.50	0.66	4	1.84	0.28	0.46
13.	HEL13	178-200	4	2.66	0.40	0.65	5	3.18	0.40	0.70
14.	ILSTS005	176-194	5	3.22	0.50	0.72	5	2.96	0.48	0.67
15.	ILSTS006	277-309	5	2.63	0.60	0.65	6	4.13	0.56	0.77
16.	INRA005	135-149	4	2.32	0.90	0.60	3	2.13	0.68	0.54
17.	INRA023	195-225	6	3.03	0.40	0.70	4	2.92	0.36	0.67
18.	INRA032	160-204	3	2.63	0.60	0.65	4	2.51	0.44	0.61
19.	INRA035	100-124	4	2.59	0.50	0.64	4	2.63	0.52	0.63
20.	INRA037	112-148	4	2.56	0.50	0.64	5	3.26	0.48	0.70
21.	INRA063	167-189	5	2.70	0.50	0.66	4	2.13	0.44	0.54
22.	MM12	101-145	3	2.59	0.30	0.64	4	1.90	0.44	0.48
23.	SPS115	234-258	6	3.17	0.60	0.72	5	3.21	0.60	0.70
24.	TGLA53	143-191	5	3.17	0.40	0.72	4	2.99	0.44	0.68
25.	TGLA122	136-184	5	3.12	0.60	0.71	3	2.79	0.48	0.65
26.	TGLA126	115-131	5	3.77	0.50	0.77	5	3.64	0.32	0.74
27.	TGLA227	75-105	5	3.57	0.70	0.75	5	4.04	0.48	0.76
	Mean		4.59	2.87	0.51	0.67	4.37	2.89	0.47	0.63
	SD		0.84	0.49	0.15	0.06	1.18	0.95	0.13	0.11

N_a = Mean observed number of alleles; N_e = Mean effective number of alleles; H_o = Mean observed heterozygosity; H_e = Mean expected heterozygosity.

reported in 12 west/central African cattle breeds as 0.506-0.697 (Ibeagha-Awemu *et al.,* 2004); for Hariana cattle of India as 0.53 (Mukesh *et al.*, 2004) and for three Chinese cattle breeds and two reference cattle populations as 0.532-0.771 (Mao *et al.*, 2007). The average H_e estimation in this study is fairy comparable with 0.241-0.881 for Hallikar breed (Kumar *et al.*, 2006); 0.717 in Kherigarh cattle (Pandey *et al.,* 2006a) and 0.68 in Kenkatha breed (Pandey *et al.,* 2006b).

Heterozygote deficiency analysis revealed that both breeds exhibited significant deviations from Hardy Weinberg (HW) equilibrium ($P<0.05$) at some of the loci. Although, it is difficult to envisage the exact basis of this deviation, yet the presence of low-frequency null alleles segregating at these loci may be a possible reason. As a result, some heterozygotes are genotyped as homozygotes and a few individuals may fail to amplify any allele. This deviation could also be linked to fairly high positive F_{IS} (within-population inbreeding estimate) values obtained in both the breeds (Table 2). The deficiency of heterozygotes among indigenous cattle population is, on the other hand, an indicator of inbreeding among cattle breeds.

Table 2: Estimation of variation within a breed (F_{IS}) at 27 loci

S. No.	Locus	Breed	
		Hariana	Hissar
1.	BM1818	0.229*	0.482*
2.	BM1824	0.348	0.349*
3.	BM2113	0.136	0.084*
4.	CSRM60	0.182	-0.412*
5.	CSSM66	-0.125*	0.269*
6.	ETH3	0.318*	0.000
7.	ETH10	0.323	0.209*
8.	ETH152	0.591*	0.177
9.	ETH185	-0.033*	-0.152*
10.	ETH225	0.830*	1.000*
11.	HEL1	0.209*	0.296*
12.	HEL9	0.256	0.406*
13.	HEL13	0.405*	0.433*
14.	ILSTS005	0.323	0.294*
15.	ILSTS006	0.085	0.281*
16.	INRA005	-0.543	-0.263
17.	INRA023	0.446*	0.469*
18.	INRA032	0.085	0.288*
19.	INRA035	0.237	0.181
20.	INRA037	0.231	0.326*
21.	INRA063	0.256*	0.193
22.	MM12	0.550*	0.093*
23.	SPS115	0.176	0.149
24.	TGLA53	0.459*	0.358*
25.	TGLA122	0.169	0.272
26.	TGLA126	0.366	0.573*
27.	TGLA227	0.080	0.380
	Overall	0.252	0.259

*The values are significantly different from zero ($P<0.05$).

The overall estimates for F statistics were significantly ($P<0.05$) different from zero. Table 2 shows the within-population inbreeding estimates (F_{IS}) at each microsatellite locus. There was a significant deficit of heterozygotes in both the breeds, ranging from 25.2% in Hariana to 25.9% in Hissar. The average F_{IS} values for most of the loci in both breeds were significantly different ($P<0.05$) from zero. The shortage of heterozygotes and excess of homozygotes ($F_{IS}>0$) might be attributed to a number of factors including assortative mating, linkage with loci under selection, population heterogeneity or null alleles.

These results indicate that the existence of a fairly moderate genetic diversity among indigenous cattle breeds across these loci makes it possible to use these breeds to improve draught traits. The microsatellite loci used in the present study further validate their use for evaluation of genetic diversity in *Bos indicus* breeds.

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