Genetic diversity analysis of Indian buffalo breeds By microsatellite markers

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ABSTRACT

The present study was conducted on 20 unrelated animals each of Bhadawari and Murrah breeds to estimate genetic diversity parameters by microsatellite markers. Ten microsatellites markers recommended by FAO for buffalo diversity were used. The number of alleles in both the breeds per locus ranged from 3 to 6. The average numbers of alleles were 4.2 ± 0.38 for Bhadawari and 4.2 ± 0.44 for Murrah breed. The mean observed heterozygosity was 0.49 for Bhadawari and 0.53 for Murrah breed. The average expected heterozygosity was 0.57 and 0.59 for Bhadawari and Murrah breed, respectively. The observed heterozygosity values in both breeds were lowest for the loci ILSTLS008 and CSSM029. The overall mean heterozygosity in both breeds was 0.51. The average PIC value for all loci was 0.51 in Bhadawari and 0.52 in Murrah breeds. In the present study, 70 per cent of the microsatellite markers were found to be highly informative. Seven loci showed deviations from the HWE for the loci ILSTLS008, BRN, CSSM029, CSSM019, CSSM036, CSSM047 and CSSM038. The mean FIS, FIT and FST for all loci was – 0.12, 0.16 and 0.04, respectively. The standard genetic distance (Ds) between Bhadawari and Murrah breeds was 0.13 and the genetic identify was 0.88.

INTRODUCTION

India is gifted with rich genetic resources in terms of its buffalo breeds. Despite the major contribution in country's total milk production, the genetic improvement and germplasm conservation of indigenous buffalo breeds have not been given due attention. Many of our pure breeds which have been playing an important role in sustaining farmer's economy have become endangered.

For the overall improvement in the breeds and to meet future challenges there is an urgent need to characterize our buffalo breeds. Detailed knowledge of genetic variation within and among different breeds is very important for understanding and improving traits of economic importance. Rapid developments in molecular biology has made it possible to detect genetic polymorphism at the DNA sequence level. Out of various DNA markers, microsatellite markers are highly polymorphic, distributed throughout the genome, locus specific and co-dominant. They have an edge over other genetic markers for comparative studies of evolution, genetic variation, parentage assessment and gene flow. Microsatellite markers have been used for studying polymorphism and genetic diversity in many livestock species (Barker et al., 1997, Haeringen et al., 1998, Martin Burriel et al., 1999).

Murrah is the premier milch breed of India. It is being used for genetic upgrading of local buffaloes in many parts of the world. Bhadawari breed is known for its high milk fat content. Bhadawari and Murrah breeds have been characterized on the basis of their morphology to some extent. The genetic characterization of these breeds using microsatellite markers will throw light on their evolution, phylogenetic relationship and genetic variability existing among them. The present study was taken up with the objectives to assess DNA polymorphism in Murrah and Bhadawari breeds and to evaluate genetic diversity between and within the two breeds using microsatellite markers.

MATERIALS AND METHODS

The present study was conducted on twenty animals each of Murrah and Bhadawari breeds maintained at Govermental Livestock Farm, Hisar and at Uttar Pardesh Pandit Deen Dyal Uppadhaya Pashu Chikitsa Vigyan Evam Gau – Anusandhan Sansthan, Mathura (U.P), respectively. Genomic DNA was extracted from blood using standard protocol. Ten microsatellites primers from the bovine genome were chosen as per the guidelines of FAO (http://www.fao.org/dad-is/) viz., ILSTS030, ILSTLS08, BRN, CSSM057, CSSM041, CSSM029, CSSM019, CSSM036, CSSM047, CSSM038.

Various PCR parameters like concentration of gDNA, dNTPs, Taq polymerase and MgCl2 and annealing

temperature were optimized to obtain a specific amplified product. The PCR was carried out in a 25 µl reaction volume. For amplification, initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing temperature varies for each primer for 1 min and extension at 72°C for 1 min for 40 cycles and final extension also at 72°C for 15 mins and hold at 4°C infinitely.

The amplified products were resolved in a 6% denaturing urea polyacrylamide gel (ATTO sequencing gel apparatus). The resolved amplified products in denaturing PAGE were detected using silver staining (Bassam et al. 1991). The various genetic parameters for analysis of population substructure and genetic distances were obtained using POPGENE version 1.31 (Yeh et al. 1999). The effective numbers of alleles (Ne) were estimated as described by (Kimura and Crow, 1964). PIC values was calculated as described by Botstein et al. (1980). Relative levels of gene flow and inbreeding were estimated as per the formula of (Slatkin and Barton, 1989). The statistics for Ewens Watterson neutrality test was calculated using the algorithm given by Manly (1985). The genetic distance and genetic identity between breeds were estimated using standard genetic distance (Ds) method (Nei; 1972) using the infinite allele model (Kimura and Crow, 1964).

RESULTS AND DISCUSSION

Observed and effective number of alleles: The observed number of alleles (Na) varied from 2 (ILSTLS008) to 6 (CSSM036) in Bhadawari and Murrah breed with an average of 4.2 ± 0.38 and 4.2 ± 0.44, respectively (Table.1). The overall number of alleles across both breeds ranged from 3 (ILSTLS008, BRN and CSSM029) to 6 (ILSTS030, CSSM019 and CSSM036) with an average of 4.6 ± 0.39, indicating ILSTS008 as the least and ILSTS030, CSSM019 and CSSM036 as most polymorphic locus. Effective number of alleles (ne) varied from 1.3 to 4.3 and 1.7 to 5.1 for loci ILSTL008 and CSSM036, in Bhadawari and Murrah breeds, respectively. Effective number of alleles for all the loci was less than the observed number of alleles. The total number of alleles detected from the 10 loci was 46 in both breeds.

The locus ILSTS030 showed the same number of alleles as reported by Arora et al. (2004) in Bhadawari buffalo and Navani et al. (2002) in a panel of 25 buffaloes (Murrah, Nili Ravi and Mehsana). Identical number of alleles (3) was also observed at locus CSSM057 and 6 at locus CSSM019 in Murrah as were reported in Bhadawari by Arora et al. (2004) whereas in Bhadawari 5 allele at locus CSSM057 and 4 allele at locus CSSM019 were observed. Navani et al. (2002) reported overall 4 alleles in a panel of 25 buffaloes for the locus ILSTS008, while in present study overall 3 alleles were observed.

Effective number of alleles is a more useful component in population genetic studies than the observed number (Crow and Kimura, 1970), since most of the alleles may be represented by only once or twice in a population and contributed very little to the genetic variance in the population. Considerable variation in the distribution of allele frequencies between loci has been reported in other buffalo breeds (Arora et al. 2004; Kataria et al. 2006; Navani et al. 2002; Mishra et al. 2008; Kathiravan et al. 2008) similar to present study.

Table 1. Observed (Na) and effective (Ne) number of alleles at different loci in Bhadawari and Murrah breeds

		Bhadawari (n=20)		Murrah (n=20)		Overall (n=40)	
S.N.	**Locus**	**Na**	**Ne**	**Na**	**Ne**	**Na**	**Ne**
1.	ILSTS030	5.0	2.9	5.0	2.1	6.0	2.6
2.	ILSTLS008	2.0	1.3	2.0	1.7	3.0	1.6
3.	BRN	3.0	2.2	3.0	2.0	3.0	2.1
4.	CSSM057	5.0	3.2	3.0	2.0	5.0	2.7
5.	CSSM041	4.0	1.9	4.0	2.6	4.0	2.3
6.	CSSM029	3.0	2.1	3.0	2.0	3.0	2.0
7.	CSSM019	4.0	2.0	6.0	2.8	6.0	2.4
8.	CSSM036	6.0	4.3	6.0	5.1	6.0	4.8
9.	CSSM047	5.0	2.5	5.0	3.5	5.0	4.0
10.	CSSM038	5.0	3.3	5.0	3.2	5.0	3.9
	Mean	4.2±0.39	2.6±0.28	4.2±0.45	2.7±0.33	4.6±0.40	.8±0.33

Allele frequency: In Bhadawari breed, allele frequencies ranged from 0.02 (A, at locus ILSTS030) to 0.87 (B at locus ILSTLS008). The allele frequencies in Murrah breed ranged from 0.25 (E at locus ILSTS030) to 0.70 (B at locus ILSTLS008). The overall allele frequencies ranged from 0.01 (A at locus ILSTS030) to 0.78 (B at locus ILSTS008). Considerable variation in the distribution of the allele frequencies between loci and between breeds was observed.

In Bhadawari breed, locus CSSM036 showed a maximum of six alleles with frequencies 0.32, 0.10, 0.07, 0.27, 0.17 and 0.05, respectively. Locus ILSTS008 had minimum number of two alleles viz. A and B with frequency 0.13 and 0.87, respectively. In Murrah breed, loci CSSM019 and CSSM036 showed maximum number of six alleles with frequencies 0.05, 0.15, 0.15, 0.55, 0.07, 0.02 and 0.250, 0.10, 0.12, 0.22, 0.22 and 0.07, respectively. Minimum number of two alleles were observed at locus ILSTLS008 i.e. B and C with frequencies 0.700 and 0.300, respectively.

Heterozygosity: For Bhadawari breed, the mean observed heterozygosity (Ho) was 0.49 ±0.098 and it ranged from 0.05 to 0.95 at loci CSSM029 and BRN, respectively. Average expected Levene's and Nei's heterozygosity (He) were 0.58 ±0.050 and 0.57 ±0.05 ranging from 0.23 to 0.79 and 0.23 to 0.77 for loci ILSTLS008 and CSSM036, respectively (Table 2). The mean observed heterozygosity value was less than mean expected heterozygosity. Average observed heterozygosity was high for all loci except ILSTLS008 and CSSM029. The values of observed heterozygosity in Murrah breed varied from 0.00 to 0.80 with an average of 0.53 ±0.99. The mean expected Levene's and Nei's heterozygosity were 0.61 ±0.04 and 0.59 ±0.04 ranging from 0.43 to 0.83 and 0.42 to 0.80 for the loci ILSTSLS008 and CSSM036, respectively. In both the breeds, mean observed heterozygosity was 0.51 ±0.09 and it ranged from 0.02 (locus ILSTLS008, CSSM029) to 0.87 (locus BRN). Mean expected heterozygosity (Levene's and Nei's) was observed 0.61 ±0.05 and 0.61 ±0.04, respectively. The value ranged from 0.36 to 0.80 and 0.36 to 0.79, respectively at loci ILSTLS008 and CSSM036. It was observed that at most of the loci the value of observed heterozygosity was less than that of the expected heterozygosity.

The low level of heterozygosity for this locus may be due to the presence of null alleles, which result from failure of amplification of certain alleles due to mutations in the primer binding sites (Callen et al., 1993). Further, the locus may be under selection leading to a lack of heterozygotes. The average observed and expected (Nei, 1978) heterozygosity reported in their study were 0.48 and 0.51 for swamp buffalo and 0.56 and 0.58 for riverine buffalo. The expected heterozygosity values obtained in this study are comparable for both Bhadawari and Murrah buffaloes as reported by Barker et al. (1997) for Riverine buffalo.

However, the observed & expected heterozygosity obtained in this study are comparably less than those reported in Riverine buffaloes by Navani et al. (2002); Arora et al. (2004); Soysal et al. (2005); Kataria et al. (2006); Jakhesara et al. (2008). The values obtained are considerably similar to the Marathwada buffalo reported by Kathiravan et al., (2008). The observed and expected heterozygosity for Murrah are comparable to those reported by Kataria et al. (2006) but in Bhadawari less observed heterozygosity was obtained than those reported by Arora et al. (2004). Slight differences in the heterozygosity can be explained, on the basis of the small sample number taken in the study.

Higher amount of genetic variability in the form of high heterozygosity values can be exploited even in populations of small sizes and employed in planning breeding strategies. In this context, Barker (1999) reported that breed with highest average heterozygosity should be preferred in choosing breeds that have equal priority.

Polymorphism information content (PIC)

The average PIC value in Bhadawari breed was 0.51 ±0.05 and it ranged from 0.20 to 0.73for the loci ILSTLS008 and CSSM036, respectively (Table 3). The average PIC in Murrah breed was 0.53 ±0.05 and it varied from 0.34 for ILSTLS008 to 0.78 for CSSM036 locus. In both breeds, range of PIC varied from 0.33 to 0.76 for the loci ILSTLS008 and CSSM036, respectively, with the mean value of 0.55 ±0.04.

The PIC values for loci in the two buffalo populations were lower than those reported for cattle by Kemp et al., (1995). In the present study, loci ILSTS030 and CSSM057 showed less PIC values but for loci CSSMO019 high value was obtained compared to those reported by Arora et al., (2004) in Bhadawari breed.

Table 2 : Observed (Ho) and expected (He) heterozygosity at different loci in Bhadawari, Murrah and Overall

Locus	Bhadawari			Murrah			Overall		
	Ho	He		Ho	He		Ho	He	
		Levene's	Nei's		Levene's	Nei's		Levene's	Nei's
ILSTS030	0.75	0.68	0.65	0.55	0.54	0.53	0.65	0.63	0.62
ILSTLS008	0.06	0.23	0.23	0.00	0.43	0.42	0.03	0.36	0.36
BRN	0.95	0.55	0.54	0.80	0.51	0.50	0.87	0.53	0.52
CSSM057	0.50	0.71	0.69	0.50	0.51	0.50	0.50	0.64	0.62
CSSM041	0.50	0.50	0.49	0.75	0.64	0.62	0.62	0.57	0.56
CSSM029	0.05	0.54	0.53	0.00	0.51	0.50	0.03	0.52	0.51
CSSM019	0.45	0.51	0.50	0.70	0.66	0.64	0.57	0.59	0.58
CSSM036	0.90	0.79	0.77	0.80	0.83	0.80	0.85	0.80	0.79
CSSM047	0.39	0.62	0.60	0.80	0.74	0.72	0.60	0.76	0.75
CSSM038	0.40	0.72	0.70	0.40	0.71	0.69	0.40	0.76	0.75
Average	0.49 ± 0.10	0.58 ± 0.05	0.57 ± 0.05	0.53 ± 0.10	0.61 ± 0.04	0.59 ± 0.04	0.51 ± 0.09	0.61 ± 0.04	0.61 ± 0.04

Table 3. : PIC values at different loci in Bhadawari and Murrah breeds

Sr.No.	Locus	PIC		
		Bhadawari (n = 20)	Murrah (n = 20)	Overall (n = 40)
1	ILSTS030	0.59	0.49	0.56
2	ILSTLS008	**0.20**	**0.33**	**0.33**
3	BRN	0.44	0.40	0.42
4	CSSM057	0.63	0.40	0.55
5	CSSM041	0.43	0.56	0.51
6	CSSM029	0.43	0.4 2	0.42
7	CSSM019	0.45	0.61	0.55
8	CSSM036	0.73	0.78	0.76
9	CSSM047	0.56	0.67	0.71
10	CSSM038	0.65	0.65	0.70
	mean	0.51 ± 0.05	0.53 ± 0.05	0.55 ± 0.04

The average PIC value for overall population is almost same for all the loci selected in this study and loci reported by Arora et al. (2004). However, average PIC values for the selected loci is less than those selected by Mishra et al. (2008) and Jakhesara et al. (2008) for Assamese buffaloes and Mehsana buffalo, respectively. Since all the markers except ILSTLS008 (low PIC in both the breeds) are highly or reasonably informative, these can be used to assess genetic diversity in other indigenous buffalo breeds as well.

Hardy-Weinberg equilibrium (HWE): Expected genotypic frequencies under random mating were computed using the POPGENE software. The chi-square (^{2}) and likelihood ratio (G^2) test were performed to examine Hardy-Weinberg equilibrium at each locus. According to the probability of ^{2} and G^2 tests, ILSTS030, CSSM057 and CSSM041 were found in Hardy-Weinberg equilibrium. Deviations from the HWE were observed in both breeds for the seven loci viz. ILSTLS008, BRN, CSSM029, CSSM019, CSSM036, CSSM047, and CSSM038. It was observed that both breeds were not in HWE at most of the loci indicating non random mating and inbreeding in the breed

F-Statistics and gene flow: Within breed differentiation estimated by Wright's fixation index (F_{IS}) was observed for all the loci. The estimated value of F_{IS} ranged from -0.75 to 0.90 for BRN and CSSM029 loci, respectively in Bhadawari breed with a mean of 0.17 ± 0.15 (Table 4). The F_{IS} value for four loci viz. ILSTS030, BRN, CSSM041 and CSSM036 were negative. In Murrah breed, F_{IS} value ranged from -0.60 to 1.00 for BRN and ILSTLS008 loci, respectively, with the mean value of 0.14 ± 0.15. Six loci viz. ILSTS030, BRN, CSSM057, CSSM041, CSSM019 and CSSM047 showed negative value in Murrah breed. Between breed differentiation ($F_{IS,}$ F_{IT} and F_{ST}) and gene flow (N_em) for all loci is presented in Table. Fixation index F_{IS} values ranged from -0.68 to 0.95 for loci BRN and CSSM029 with a mean of 0.12 ± 0.16. F_{IS} values were found negative for loci, ILSTS030, BRN, CSSM041, CSSM019 and CSSM036. The F_{IT} values varied from -0.68 to 0.95 for loci BRN and CSSM029 respectively, with a mean value of 0.16 ± 0.16. The F_{IT} values were negative for loci ILSTS030, BRN, CSSM041, and CSSM036. F_{ST} ranged from 0.00 to 0.12 for loci CSSM029 and CSSM047, respectively, with a mean value of 0.04 ± 0.01. The estimates of gene flow (N_em) between Bhadawari and Murrah breed for each locus were also studied (Table 4). The estimated values for individual locus ranged from 1.89 to 76.32 for loci CSSM047 and CSSM029 with a mean of 5.65 ± 1.78. Arora et al. (2004) observed F_{ST} value of 0.05 between Bhadawari and Tarai buffalo breeds which are comparable to the observed in the present study. This low level of differentiation may be due to fragmentation of a larger population into smaller isolated subpopulations. Another reason may be a high migration rate between the two populations.

Ewens Watterson Neutrality Test

Statistical data for Ewens Watterson test for neutrality for ten loci are presented in Tables for Bhadawari, Murrah and in overall population, respectively. The observed sum of the squared allele frequencies (observed F), i.e. homozygosity was compared with the 95 per cent confidence intervals for

Table 4 : Within and between breed differentiation indices and gene flow

S.No	Locus	Within breed FIS		Between breed differentiation			
		Bhadawari	Murrah	F_{IS}	F_{IT}	F_{ST}	N_em
1	ILSTS030	-0.14	-0.03	-0.09	-0.05	0.04	5.78
2	ILSTLS08	0.77	1.00	0.92	0.93	0.09	2.39
3	BRN	-0.75	-0.60	-0.68	-0.67	0.00	59.42
4	CSSM057	0.28	-0.00	0.16	0.20	0.05	5.01
5	CSSM041	-0.03	-0.21	-0.13	-0.19	0.02	14.30
6	CSSM029	0.90	1.00	0.95	0.95	0.00	76.32
7	CSSM019	0.09	-0.09	-0.01	0.01	0.02	12.68
8	CSSM036	-0.17	0.01	-0.08	-0.07	0.00	57.27
9	CSSM047	0.36	-0.11	0.10	0.21	0.12	1.89
10	CSSM038	0.43	0.42	0.42	0.46	0.07	3.34
	mean	0.17 ± 0.15	0.14 ± 0.15	0.12 ± 0.16	0.16 ± 0.16	0.04 ± 0.01	5.65 ± 1.78

the expected sum of the squared allele frequencies (expected F). The 95 per cent confidence intervals and standard errors for observed F were calculated using 1000 simulated samples. In Bhadawari breed locus CSSM041 showed higher observed homozygosity (0.51) than expected homozygosity (0.50) whereas in Murrah breed, loci ILSTS030 and CSSM019 showed higher observed homozygosity (0.47 and 0.36) than expected homozygosity (0.42 and 0.35). In the overall population, only one locus CSSM019 showed higher observed homozygosity (0.42) than expected homozygosity (0.41). The overall test for neutrality (pooled across the two breeds) showed that observed F values of three microsatellite loci (CSSM047, CSSM036 and CSSM038) out lied the lower and upper limits of the 95 percent confidence region (Table 4). The values indicate that most of the markers were neutral and lie in non coding region of the genome values.

Further, if a population is composed of two subpopulations, each from a random mating population, but with different frequencies of an allele, then in the total population there will be more homozygotes and fewer heterozygotes than would be predicated by the Hardy- Weinberg ratio. This is the 'Wahlund Effect', i.e., reduction in observed heterozygosity when compared to expected (Smith, 1998). Null alleles may also lead to false observation of excess homozygotes. Therefore, it can be concluded that there is inbreeding in the two populations under field conditions. This observation is supported by the fact that the overall observed heterozygosity was less (0.51) for both populations as compared to the reported by Arora et al., (2004); Navani et al., (2002); Kataria et al., (2006) and Jakeshesara et al., (2008) for reverine buffaloes.

Shared alleles: The shared alleles in two breeds can also be used to discriminate the breeds, which showed high inter-breed variation. Loci BRN, CSSM041, CSSM029, CSSM036, CSSM047 and CSSM038 showed 100 percent sharing of alleles. Least sharing (33.33 %) of alleles between two breeds was observed at locus ILSTLS008. On an average, 82.67 per cent of alleles were shared between both the breeds.

Genetic distance: The Nei's original genetic distance (D_s) between both breeds was observed to be 0.13 while the genetic identity was 0.88. The Nei's unbiased genetic distance and genetic identity between these was 0.11 and 0.89, respectively. Considering the mutation rate of microsatellite as 10^{-4} per generation, the number of generation of divergence between these two buffalo populations was estimated to be 652 generations, indicating that Bhadawari and Murrah originated from the same buffalo population about 652 generation ago.

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