Characterization of Indian Riverine Buffaloes by Microsatellite Markers

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ABSTRACT : Six breeds of riverine buffalo viz. Murrah, Mehsana, Jaffrabadi, Nagpuri, Nili-Ravi and Bhadawari were characterized using FAO-recommended cattle specific microsatellite markers. Among the total of twenty microsatellite markers screened to explore genomic variability of six buffalo breeds, only ten were polymorphic in nature. Four out of ten polymorphic microsatellite loci were rated as informative. The numbers of alleles detected ranged from 2 to 7, with a mean of 5.5±0.07 per microsatellite marker. The most polymorphic marker was BM1818 with a total of 7 alleles present at this locus. One breed specific marker was found in each of Mehsana (BM1818) and Bhadawari (ILSTS030) and four were found in Jaffarabadi (BM1818, ILSTS030, ILSTS054 and ILSTS011). Genetic distance (Ds) between the Mehsana and Bhadawari breed was the maximum (0.29), followed by Murrah and Mehsana (0.27), and Nili-Ravi and Bhadawari (0.26). The lowest Ds was found between the Jaffrabadi and Nagpuri breeds which was only 0.05. The highest divergence time of 1318 years was established between Mehsana and Bhadawari breeds whereas it was found to be lowest (272 years) between the Jaffrabadi and Nagpuri breeds. (**Key Words :** Characterization, Buffalo, Genetic Distance, Microsatellite)

INTRODUCTION

India is considered as the home tract of some of the best buffalo breeds and has been the center of dispersion of good specimen of buffaloes for improvement of the species elsewhere in the world dominating the world trade in export of reputed breeds from the country. India has about 97.7 million buffaloes (FAO, 2004) with nine well-recognized breeds (Murrah, Nili-Ravi, Surti, Jaffrabadi, Bhadawari, Mehsana, Nagpuri, Toda and Pandharpuri) based on their phenotypic characteristics, production performances and ecogeographical distribution. But, these breeds constitute only 30% of total buffalo population of India and the remaining does not belong to any well-defined breeds and categorized as nondescript (Sethi, 2001). Considering milk production the only criterion, local breeds are indiscriminately crossed with Murrah buffaloes leading to a steady decline in the genetic diversity present in terms of different qualities like heat tolerance, feed conversion ratio, high fat percentage etc.

Recent developments in molecular biology and statistics have opened the possibility of identifying and using genomic variation for the characterization of livestock. There are two main categories of genomic information that can be used for this purpose. They are the genes with known effects on the expression of certain protein and genes with effects detected on the characteristic in statistical terms. The first category, which also known as candidate gene approach used extensively for livestock improvement but has limited use in characterization because of low level of polymorphism. The second group of markers is based on polymorphic sequences of the DNA, which corresponds to genes with detectable variation by means of RFLP, microsatellites or other similar molecular systems (Beattie, 1994). Microsatellite markers, by virtue of their codominant and multiallelic nature prove to be efficient in genetic diversity studies, pedigree evaluation and genetic mapping as compared to other molecular markers like RAPD, RFLP and ISSRs (Nagaraju et al., 2001). Microsatellites have become markers of choice in characterization of breeds of several species like cattle, buffalo, goat, pig, chicken etc. (Edwards et al., 2000; Canon et al., 2001; Chenyambuga et al., 2004; Li et al., 2004; Selvi et al., 2004; Fan et al., 2005; Olowofeso et al., 2005; Yoon et al., 2005). Thus, the present study was carried to characterize the Indian buffalo breeds with different microsatellite markers.

MATERIALS AND METHODS

Experimental animals

The present study was carried out on 240 Indian

Riverine buffaloes consisting of 40 unrelated animals of each of six different breeds such as Murrah, Mehsana, Jaffarabadi, Nagpuri, Nili-Ravi and Bhadawari. These were located either in Govt. Livestock Farms or in the native breeding tract of the breeds. Animals were unrelated and selected randomly for the present study.

Breeds

A brief description of the breeds under study has been elucidated below.

Murrah : Murrah is the most important breed of buffalo. The original home of this breed is mainly in Punjab, Haryana and Delhi where they are found in the pure form. However this breed has spread over all parts of the country and is being bred either in pure form or is being used for grading up of local non-descript buffaloes. Distinguishing character of this breed is deep massive frame with short broad back and comparatively light neck and head. Well-developed udder, broad hoofs, tightly curved horns and drooping quarters are important characters of this breed. Murrah buffaloes are one of the most efficient milk and butter fat producers not only in India but also probably in the world.

Mehsana : They are found in Mehsana district of Gujarat from where it derives its name. They are also found in Banaskantha, Sabarkantha, Gandhinagar and Ahmedabad dustrict. The breed is considered to have originated from regular inter-breeding between Murrah and Surti. It is a medium sized breed with low-set deep body. Skin color is mostly jet black and occasionally brown gray with some white markings on the face, legs or tips of the tail.

Jaffarbadi : Jaffarbadi buffaloes are found in their pure form in the Gir forest and neighborhood of Jaffarabad in Saurastra region in Gujarat state. The breed is also known as Bhavanagari. Distinguishing characters of this breed are very prominent forehead and heavy horns, which are inclined to droop on each side of the neck and then turn up at the points but not in such a tight curve as in Murrah buffalo. Body is longer but not so compact. They have well-developed udder and generally black in color.

Nili-Ravi : Nili and Ravi are two types of buffalo found in the valleys of rivers Sutlej and Ravi in Punjab particularly in Ferozpure district. There is no much difference between the two types and now they are officially treated as one breed. Distinguishing characters of this breed are having a medium sized deep frame body. The animals are massive in appearance and have white markings on forehead, face, muzzle, legs and tail. Horns are small with a high coil and neck is long, thin and fine. They are usually black in color and some are brown also. This breed is recognized to be one of the best breeds of buffalo in India next only to Murrah.

Nagpuri : These animals are mostly found in Central and South India particularly in Nagpur, Wardha, Akola, Amrawati in Maharashtra. Nagpuri buffalo are a small sized buffalo with a long flat-curved horns and color is usually black, occasionally white marking are observed on the face, legs and switch. The face is long and thin with a straight profiles. The neck is longer with heavy brisket.

Bhadawari : They are found in the ravines of Yamuna, Chambal and Utangan rivers spread over in Uttar Pradesh and Madhya Pradesh. Bhadawari buffaloes are medium sized with wedge-shaped body. Color pattern of the body varies from blackish-copper to light copper with typical white color ring (chevron), locally called as "Kanthi" at the lower side of the neck. Color of legs is usually like wheat straw, which is peculiar to this breed.

Sample collection

Approximately 10 ml venous blood was collected from each animal in polypropylene tubes containing ACD as anticoagulant and brought to laboratory under low temperature. Finally, all the samples were kept at -20°C till DNA was isolated.

Genomic DNA extraction

High molecular weight genomic DNA was prepared using phenol-chloroform extraction method (Clamp et al., 1993) with minor modifications. The quantity and quality of DNA was evaluated on spectrophotometer and through 0.8% agarose gel electrophoresis.

Selection of microsatellite markers

The microsatellite marker analysis of the buffalo DNA was carried out using FAO recommended heterologous cattle primer pairs (Bradley et al., 1997). A total of twenty primer pairs of bovine origin were screened across six breeds of buffalo. Each marker was tested on twelve random DNA samples of six breeds of buffalo (two from each breed) and two random samples of cattle as control. The markers were screened for their conservation (amplification of a product) and polymorphism in buffaloes. The highly polymorphic markers were further studied on large number of DNA samples.

Detection of microsatellite alleles

The parameters like $MgCl_2$ and annealing temperature were optimised to obtain a specific amplified product in sufficient quantity. The reaction volume was kept constant at 10 μl. DNA samples (15 ng in 6 μl volume) were loaded sequentially into the reaction tubes held on ice. Subsequently, 4 μl of the ice-cold PCR mix containing α-^{33}P dCTP was added to each reaction tube and finally, placed in Thermal Cycler (Biometra) for PCR amplification.

Table 1. Distribution of microsatellite alleles in riverine buffaloes

Locus	Breeds	Allele	Frequency	MgCl$_2$ (mM)	Tm (°C)
HEL5	Murrah	113,116	0.500,0.500	1.5	52
	Mehsana	109,111,113,116	0.083,0.167,0.417,0.333		
	Jaffarabadi	111,113	0.500,0.500		
	Nagpuri	109,111	0.500,0.500		
	Nili-Ravi	109,111	0.500,0.500		
	Bhadawari	109,111	0.500,0.500		
BM1818	Murrah	195,236	0.500,0.500	1.5	56
	Mehsana	195,218,230,236	0.500,0.083,0.334,0.083		
	Jaffarabadi	186,191,214	0.083,0.417,0.500		
	Nagpuri	191,230	0.583,0.417		
	Nili-Ravi	191,230	0.500,0.500		
	Bhadawari	191,230	0.500,0.500		
ILSTS030	Murrah	118,120	0.500,0.500	1.5	56
	Mehsana	118,120	0.500,0.500		
	Jaffarabadi	124,127	0.500,0.500		
	Nagpuri	118,120	0.500,0.500		
	Nili-Ravi	118,120	0.500,0.500		
	Bhadawari	122,124	0.500,0.500		
ILSTS011	Murrah	223,225,230	0.667,0.083,0.250	1.5	58
	Mehsana	223,230	0.583,0.417		
	Jaffarabadi	233,235,237	0.250,0.167,0.583		
	Nagpuri	223,225,230	0.250,0.083,0.667		
	Nili-Ravi	223,230	0.583,0.417		
	Bhadawari	223,230	0.750,0.250		

The M13 sequencing ladder (G, A, T and C) was generated for size marking of the PCR products, by running it in the same gel. Sequenase Version 2.0 DNA Sequencing Kit (USB, #US-70770) was used for sequencing of M13 DNA by chain-termination method (Sanger et al., 1977). The PCR amplified products were separated by denaturing sequencing gel electrophoresis (7%) using Sequi-GEL-GT electrophoresis apparatus. Finally, autoradiography was performed to detect various microsatellite bands for identification of different alleles present in six buffalo population.

Statistical analysis

Fingerprinting :Genotype of every animal was recorded manually from the autoradiographs. The M13 sequencing ladder, run in the same gel, was used for size marking of the PCR products. Genotyping involved the recording of the homozygous or heterozygous state of the animal, as well as the size of the respective alleles. Ultimately, the frequencies of different alleles were estimated in different breeds following gene-counting method.

Genetic distance : Genetic distance between the breeds were estimated by Nei's standard genetic distance (Ds) (Nei, 1972) :

$$D = -\ln\left(\frac{\sum_m \sum_i p_{1mi} p_{2mi}}{\left[\sum_m \sum_i p_{1mi}^2\right]^{\frac{1}{2}} \left[\sum_m \sum_i p_{2mi}^2\right]^{\frac{1}{2}}} \right)$$

where m is summed over loci, *i* over alleles at the m-th locus, and p_{1mi} is the frequency of the i-th allele at the m-th locus in population 1.

Time of divergence

The time of divergence were calculated from the equation Ds = 2at, where Ds is the Nei's standard genetic distance, a is rate of mutation and t is the time of divergence in generations. As the mutation rate for buffalo has not been determined, the mutation rate of 1.1×10^{-4} determined for sheep (Craford and Cuthbertson, 1996) has been used.

RESULTS AND DISCUSSION

Microsatellite finger printing

A total of 20 primer pairs (out of 30 FAO recommended cattle specific primers, Bradley et al., 1997) were screened across six breeds of buffalo for polymorphism study. It was observed that out of these 20 primers, 10 produced polymorphic banding pattern and 8 were found to be monomorphic whereas with 2 primers, lack of amplification was observed. Four out of ten polymorphic microsatellite loci were rated as informative indicating high degree of polymorphism. Subsequently, those four primers were used to explore genetic diversity amongst different breeds of buffalo. Out of four microsatellite markers, BM-1818 was found to be most polymorphic revealing 7 alleles across the breeds. The numbers of alleles detected from this microsatellite locus were varied from 2 in Murrah, Nagpuri,

Table 2. Nei's genetic distance and divergence time amongst various buffalo breeds (Upper triangular matrix demonstrates Nei's genetic distance while lower triangular matrix indicates time of divergence (Years))

	Murrah	Mehsana	Jaffarabadi	Nagpuri	Nili-Ravi	Bhadawari
Murrah	-	0.27	0.13	0.14	0.15	0.23
Mehsana	1,227	-	0.15	0.13	0.19	0.29
Jaffarabadi	590	681	-	0.05	0.13	0.14
Nagpuri	636	590	272	-	0.13	0.22
Nili-Ravi	681	863	590	590	-	0.26
Bhadawari	1,045	1,318	636	1,000	1,181	-

Nili Ravi and Bhadawari to 4 in Mehsana. Other microsatellite markers like HEL5 revealed 2 alleles each in Murrah, Jaffrabadi, Nagpuri, Nili Ravi and Bhadawari whereas 4 alleles in Mehsana, ILSTS030 showing 2 alleles in all the breeds and ILSTS011 depicting 2 alleles each in Mehasena, Nili-Ravi and Bhadawari whereas 3 alleles each in Murrah, Nagpuri and Jaffrabadi. However, an average of 5.5±0.07 alleles per locus was established in Indian buffaloes. Arora et al. (2004) found the mean number of alleles per locus across the two Indian buffalo breeds named Bhadawari and Tarai to be 4.7. Meanwhile Ganai and Yadav (2001) demonstrated 5.37±0.78 alleles per locus in Indian goats using same set of microsatellite markers, which revealed very high informativeness of microsatellites over the species. However, Food and Agriculture Organization (FAO) suggests that five different alleles per locus are required for estimation of genetic differences between breeds. The mean number of alleles in the present study is in accordance with the FAO recommendations.

Frequency distribution

The number of alleles and frequencies at four informative marker loci for all six breeds of Riverine buffaloes are presented in Table 1. The frequencies of different alleles across the locus were ranging from 0.083 to 0.75. However, in most of the cases, the frequencies were found to be moderate (0.5) indicating allelic predisposition of specific loci in different buffalo population. In some breeds, novel alleles were also been observed delineating their rare existence in the population, making it as a resource population for breed conformancy.

Breed specificity

BM1818 revealed one breed specific allele of 218 bp in Mehsana buffalo, which was not observed in other breeds. Similarly, ILST030 derived one specific allele of 122 bp in Bhadawari buffalo while in Jaffarbadi breed, six breed specific alleles were identified with ILSTA011 (233, 235 and 237 bp), BM1818 (186 and 214 bp) and ILSTS030 (126 bp) microsatellite. A monomorphic locus ILSTS054 showed allele size of 94 bp in all the breeds except Jaffarabadi where an allele of 84 bp was found to be present predominantly. Thus, this allele of 84 bp can be used as breed specific markar in Jaffrabadi buffalo. Hansen et al. (2002) also reported a number of potential breed specific microsatellite alleles for Canadienne, Brown Swiss, Holstein and Jersey cattle.

Genetic distance

The genetic distance (Ds) between two breeds was calculated according to Nei's standard genetic distance formula (Table 2). It has been found that Ds between Mehsana and Bhadawari breed was maximum (0.29), followed by between Murrah and Mehsana (0.27) and between Nili-Ravi and Bhadawari (0.26). The lowest Ds (0.05) was estimated between the Jaffrabadi and Nagpuri breed. Ultimately, it was observed that the Bhadawari breed was distantly related from all the other breeds which is in consistent with its unique characteristics like very high butter fat content, grey or greyish black body colour, presence of 2 white lines called chevron at the lower side of the neck which have made the breed distinct from other Indian breeds of buffaloes. Arora et al. (2004) established the genetic distance between two Indian buffalo breeds viz. Bhadawari and Tarai to be 0.155. Genetic distance estimates in other species were also demonstrated by several workers (Ganai and Yadav, 2001 in goats; Hansen et al., 2002 in cattle) applying a number of microsatellite markers to establish breed to breed evolutionary relationship within a species.

Time of divergence

The highest magnitude of 1318 years of time of divergence was calculated between Mehsana and Bhadawari breed of buffaloes while the lowest estimate (272 years) was observed between Jaffrabadi and Nagpuri breed (Table 2). In most of the breed pairs, the time of divergence was determined as more than 500 years, which probably make the breeds unique to their specific phenotypic characteristics. However, it may be enunciated that most of the Indian buffalo breeds may be of common origin, which are possessing some common characteristics like coat colour, horn type and body features etc. mostly resembling to Murrah buffalo. From the historical perspective, it has been observed that Murrah may be one of the oldest Indian buffalo breed from which some other

Indian breeds have been evolved through crossing of native local breeds with Murrah and rigorous selective breeding depending on the likelihood of the desired characteristics. But, to conclude precisely, chromosome-wise densely mapped microsatellite markers are to be exhaustively analysed in random bred large population. In the present study, the time of divergence was calculated on the basis of sheep reference data as the information on buffalo was lacking in the literature. Hence, to obtain the accurate measure, the rate and time of mutation over the generations with respect to buffalo is to be established precisely, which not only delineate the accurate phylogenetic relationship of buffalo with other species but also enable to explain the breed to breed evolutionary relationship with emphasis to time of speciation.

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