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Assessment of Genetic Variability in Two North Indian Buffalo Breeds Using Random Amplified Polymorphic DNA (RAPD) Markers

M. Sodhi, M. Mukesh*, A. Anand[1], S. Bhatia and B. P. Mishra

National Bureau of Animal Genetic Resources, P.O. Box-129, Karnal-132001, Haryana, India

ABSTRACT : Murrah and NiliRavi are the important North Indian buffalo breeds occupying the prominent position of being the highest milk producers. These breeds are more or less similar at morphological as well as physiological levels. The technique of RAPD-PCR was applied in the present study to identify a battery of suitable random primers to detect genetic polymorphism, elucidation of the genetic structure and rapid assessment of the differences in the genetic composition of these two breeds. A total of 50 random primers were screened in 24 animals each of Murrah and NiliRavi buffaloes to generate RAPD patterns. Of these, 26 (52%) primers amplified the buffalo genome generating 263 reproducible bands. The number of polymorphic bands for the 26 chosen RAPD primers varied from 3 (OPG 06 and B4) to 26 (OPJ 04) with an average of 10.1 bands per primer and size range of 0.2 to 3.2 kb. DNA was also pooled and analyzed to search for population specific markers. Two breed specific RAPD alleles were observed in each of Murrah (OPA02 and OPG16) and NiliRavi (OPG09) DNA pools. RAPD profiles revealed that 11 (4.2%) bands were common to all the 48 individuals of Murrah and NiliRavi buffaloes. Pair-wise band sharing calculated among the individual animals indicated considerable homogeneity of individuals within the breeds. Within breed, band sharing values were relatively greater than those of interbreed values. The low genetic distance (Nei's) value (0.109) estimated in this study is in accordance with the origin and geographical distribution of these breeds. The RAPD analysis indicated high level of genetic similarity between these two important North Indian buffalo breeds. (**Key Words :** Buffalo, RAPD Markers, DNA Polymorphism, Band Sharing, Genetic Relationship)

INTRODUCTION

India possesses the best and diversified germplasm of buffalo represented by ten well-defined breeds. Indian buffaloes contribute about 50% of the total milk produced but constitute less than half of the cattle population. Among the buffaloes, Murrah occupies prominent position being the highest milk producer and has been widely used for improving native buffalo populations in India and also imported by several countries for improvement programmes of their native breeds. The home tract for Murrah is mainly considered as southern part of Haryana comprising districts of Rohtak, Jind, Hisar and Gurgaon. However, this breed has now spread to almost all parts of the country. NiliRavi is yet another important North Indian buffalo breed whose breeding tract is spread all along the sutlej river on the Indo-Pak border (Nivsarkar et al., 2000). NiliRavi is more or less similar to Murrah except for some white markings on extremities and walled eyes. Both these breeds are of particular importance owing to their capacity to resist tropical diseases, withstand climatic influences and also have better efficiency to utilize coarse feed and fodder.

Similarities between Murrah and NiliRavi have remained a case of riddle for animal geneticists for quite some time. Also of concern is development of suitable strategy for conservation of these valuable germplasm based on the genetic diversity. Genetic characterization of native breeds is the first step in prioritization of breeds for conservation. In the recent past, RAPD-PCR approach has been used successfully in numerous studies to reveal informative polymorphism in several species and characterization of genetic variability among breeds of species (Caetano-Anolles et al., 1991; Hunt et al., 1992; Kantanen et al., 1995; Mohd-Azmi et al., 2000; Shilin et al., 2001; Appannavar et al., 2002; Saifi et al., 2004; Sharma et al., 2004; Suprabha et al., 2005). Technically RAPD assay is simple, rapid, sensitive and low cost method for producing much polymorphic information of the target genome.

Therefore, it would be of immense importance to investigate the two most important Indian breeds of buffalo

* Corresponding Author: M. Mukesh. Tel: +91-184-2267918, Fax: +91-184-2267654, E-mail: manishi@nbagr.ernet.in

[1] Division of Neurology, Post Graduate Institute of Medical Education and Research, Chandigarh-160012, India.

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Table1. RAPD primers with their sequences, screened in the two buffalo breeds and characteristics of their amplification products

Primers	Sequences (5' to 3')	GC content (%)	Total number of bands detected	Origin of amplification		Size of products (kbp)
				Murrah	NiliRavi	
OPA02	TGCCGACCTG	70	15	15	14	0.2-1.6
OPBA02	TGCTCGGCTC	70	06	06	06	0.3-2.0
OPD12	CACCGTATCC	60	14	14	14	0.5-2.5
OPG09	CTGACGTCAC	60	08	08	08	0.5-2.5
OPG10	AGGGCCGTCT	70	16	14	14	0.5-3.0
OPG16	AGCGTCCTCC	70	05	05	04	0.3-1.6
OPI05	TGTTCCACGG	60	04	04	04	0.3-1.0
OPJ04	CCGAACACGG	70	26	24	26	0.3-2.3
OPJ06	TCGTTCCGCA	60	07	07	06	0.4-2.0
OPJ12	GTCCCGTGGT	70	11	11	11	0.3-3.0
OPJ16	CTGCTTAGGG	60	13	13	13	0.5-3.0
OPK07	AGCGAGCAAG	60	17	17	17	0.3-3.2
OPK19	CACAGGCGGA	70	09	09	09	1.0-2.0
OPM04	GGCGGTTGTC	70	04	04	04	0.5-2.0
OPM06	CTGGGCAACT	60	03	03	03	0.3-1.6
OPM07	CCGTGACTCA	60	08	08	08	0.5-2.5
OPM08	TCTGTTCCCC	60	10	10	10	0.3-3.0
OPM11	GTCCACTGTG	60	12	12	12	0.4-2.0
OPO01	GGCACGTAAG	60	05	05	05	0.5-3.0
OPZ04	AGGCTGTGGT	60	21	21	21	0.4-3.1
OPZ05	TCCCATGGTG	60	07	07	07	1.0-2.0
OPZ09	CACCCCAGTC	70	19	19	19	0.4-3.0
ILO1127	CCGCGCCGGT	90	09	09	09	0.4-2.0
ILO65	CCGGTGTGGG	80	05	05	05	0.4-2.0
P4	GCATGCGATC	60	03	03	03	0.3-2.0
P6	ACGTCGAGCA	60	06	06	06	0.4-2.5

at DNA level to assess the extent of genetic relatedness using the technique of PCR based random amplified polymorphic DNA (RAPD) (Welsh et al., 1990; Williams et al., 1990). The present study was undertaken to achieve rapid screening of large number of random oligo primers for their ability to detect genetic polymorphism and subsequently examine within and between breed genetic variability in Murrah and NiliRavi buffaloes. The phenetic analysis of RAPD data by 26 primers revealed the extent of genetic similarities among the two buffalo breeds.

MATERIALS AND METHOD

Samples and DNA extraction

Fresh blood samples (6-9 ml) were collected in EDTA vacuutainer tubes from 24 randomly chosen animals each of Murrah and NiliRavi buffalo breeds from their respective breeding tracts in the states of Haryana and Punjab. Genomic DNA from each blood samples was isolated following the procedure of Clamp et al. (1993). In brief, cells were lysed using 2×volume of lysis solution (155 mM Ammonium Chloride, 10 mM Potassium Bicarbonate, 0.1 mM EDTA, pH 8.0) followed by proteinase K digestion in the extraction buffer (10 mM Tris pH 8.0, NaCl 400 mM, EDTA 2 mM, SDS 0.2%) at 56°C for over night. The mixture was subjected to phenol, phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) extractions. The final aqueous phase was mixed with 2×volumes of ice-cold absolute alcohol followed by 1/10th volume of 3 M sodium acetate (pH 5.2) to precipitate the extracted DNA. The DNA pellet was rinsed twice with 70% ethanol, dried and resuspended in TE buffer (10 mM Tris. Cl, 1 mM EDTA pH 8.0). The quality and quantity of the extracted DNA was checked by spectrophotometric method and agarose gel electrophoresis. DNA samples were adjusted to a concentration of 20-25 ng/µl with nuclease free water for RAPD-PCR analysis.

PCR primers

A total of 50 random primers were screened for detecting RAPD-PCR based polymorphism in the two buffalo breeds. Of these, 46 primers from Operon Technologies (Alameda, CA, USA) and 4 tested by Gwakisa et al. (1994) and Kantanen et al. (1995) were employed in the present study. The primers were chosen arbitrarily and each primer was 10 nucleotide in length with G+C content in the range of 60-70%.

DNA amplification

PCR reaction was performed in 25 µl reaction volume containing 20 ng of genomic DNA, 2.5 µl PCR buffer, 50 ng oligo primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.0

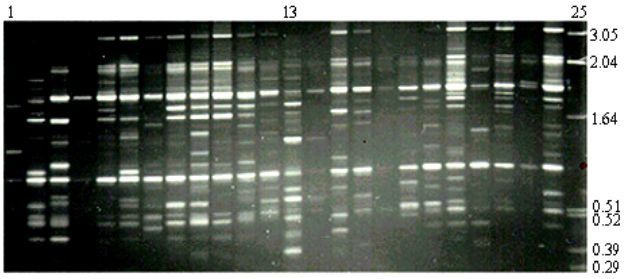

Figure 1. RAPD profiles obtained in Murrah and NiliRavi animals with primer OPZ 04. Lane 1-12: Murrah; Lane 13-25: NiliRavi; Lane-25: Molecular weight ladder (1 kb).

U Taq DNA polymerase (M/s Promega Corporation, WI, USA). PCR amplification was carried out in Perkin Elmer Cetus thermal cycler, programmed for initial denaturation at 94°C for 2 min followed by 40 cycles each with denaturation at 94°C for 1 min, annealing at 41°C for 1 min and extension at 72°C for 1 min. The final extension step was carried out 72°C for 5 min. The PCR amplified DNA samples were resolved on 1.5% agarose gel (M/s Sigma Aldrich, USA) in 1×TBE (Tris-Boric Acid-EDTA) buffer at 80 V for 4-5 h. 1 kb DNA ladder (M/s Gibco BRL, USA) was used in each gel as molecular size standard. For reproducibility, reactions for each sample/primer combinations were performed thrice and primers giving reproducible patterns were used for final scoring. To identify the breed specific RAPD alleles, DNA from all the 24 individuals of each breed was bulked and used as template for amplification.

Statistical analysis

The PCR amplified DNA bands were scored across the lanes as discrete variables. Each band in the RAPD profile was treated as an independent locus with two alleles. Scoring was done manually as '1' for the presence and '0' for the absence across all the lanes/samples for a given RAPD profile. Only distinct and prominent RAPD bands were scored for the generation of binary matrix. The RAPD profiles were analyzed by means of band sharing between all possible pairs and the percentage of common bands was determined. To evaluate the extent of similarity between RAPD fingerprints, the pair wise similarity was calculated as a fraction of shared bands (band sharing values) among the animals within or between the two buffalo breeds using the formula of Jefferys and Mortan (1987).

$$BS = 2(Bxy)/(Bx+By)$$

Where Bxy, is the number of DNA fragments shared by both the individuals x and y, Bx and By are the total number of fragments possessed by the individual x and y. The Nei's (1972) standard genetic distance (D) was calculated to estimate the genetic distance between Murrah and NiliRavi breeds. For cluster analysis, similarity matrix was calculated by treating each fragment as a unit character. Subsequently the similarity measures were subjected to unweighted pair-group method analysis (UPGMA) to depict the relationships amongst animal of the two breeds. The analysis was performed using NTSYSpc ver. 1.80 software (Exeter Software, New York, USA).

Table 2. The frequency (%) of the RAPD alleles at polymorphic loci in the two buffalo breeds

Locus	Fragment size kbp	% of RAPD alleles	
		Murrah	NiliRavi
OPA02	0.47	26	0
OPJ04	0.45	0	66
	0.35	0	39
OPG16	1.3	56	0
	0.8	26	100
	1.2	8	100
OPG10	1.6	100	16
	1.4	100	8
	1.3	100	4
OPBA01	2.1	100	50

RESULTS AND DISCUSSION

Out of the 50 primers screened, 26 primers (52%) amplified the buffalo genome generating polymorphic and reproducible RAPD banding pattern. The remaining 24 primers were not included in the analysis because of poor/no amplification (13 primers) or producing too complex and unstable products (11 primers). This could be attributed to the formation of heteroduplexes or non-specific amplification where primer/template homology is not perfect. The nomenclature, sequences and percentages of GC content of the 26 RAPD primers analyzed are listed in Table 1.

RAPD profiles with selected 26 primers were reproducible and exhibited strong and well-defined bands under similar reaction conditions. The extent of polymorphism generated from the target genome in terms of polymorphic band pattern varied for different primers. The number of polymorphic bands for the 26 informative RAPD primers varied from 3 (OPG06 and B4) to 26 (OPJ04) with a total of 263 score able bands and an average of 10.1 bands per primer. DNA fragment size of these RAPD profiles ranged from 0.2 to 3.2 kb. Figure 1 depicts the representative electrophoretic pattern of RAPD bands at primer OPZ04 in Murrah and NiliRavi buffaloes.

To search for the population specific markers, DNA pools of each of the breed was prepared and amplified with individual primers. Two breed specific RAPD alleles were observed in each of Murrah (0.47 kb at OPA02 and 1.3 kb at OPG16) and NiliRavi (0.45 and 0.35 kb at OPJ04).

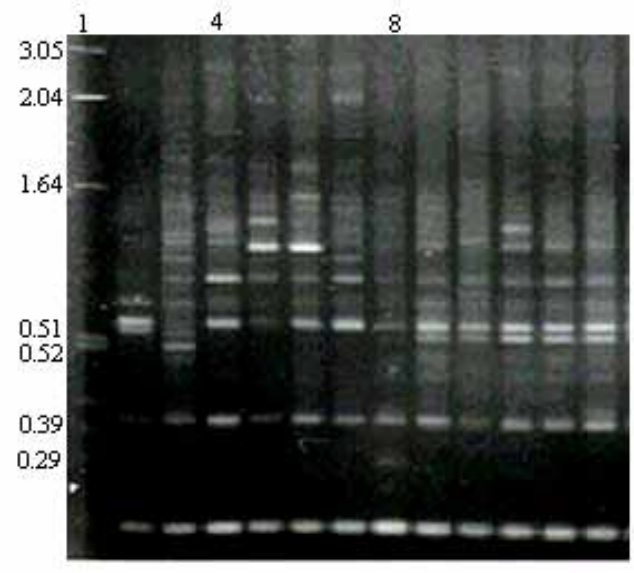

Figure 2. RAPD pattern revealed in pooled and individual DNA samples of two buffaloe breeds at locus OPA02. Lane 1: Molecular weight marker (1 kb); Lane 2: Pooled genomic DNA of NiliRavi animals; Lane 3: Pooled genomic DNA of Murrah animals; Lane 4-7: Individual DNA samples of NiliRavi; Lane 8-13: Individual DNA samples of Murrah.

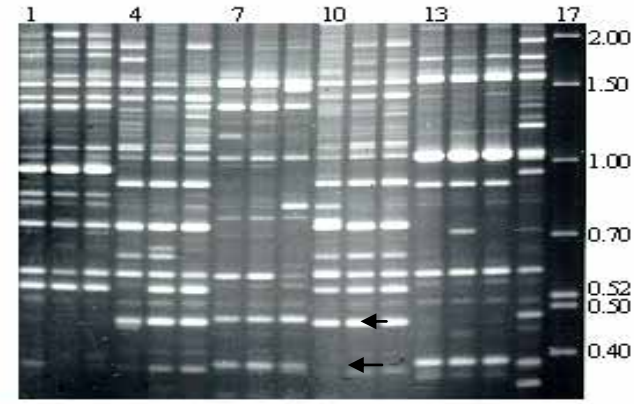

Figure 3. RAPD pattern revealed in pooled and individual DNA samples of two buffaloe breeds at locus OPJ04. Lane 1-3, 7-9, 13-16: Individual DNA samples of Murrah; Lane 4-6, 10-12: Individual DNA samples of NiliRavi; Lane 17: Molecular weight marker (Bio marker EXT, M/s Bioventures).

However, when the primers were analyzed at individual level, the frequencies of these apparently population specific RAPD alleles were found to be 0.26, 0.56, 0.66, and 0.39 respectively for 0.47, 1.3, 0.45, and 0.35 kb fragments (Table 2). RAPD profiles indicating the presence of population specific allele at OPA02 in Murrah and at OPJ04 in NiliRavi are depicted in Figure 2 and 3.

A comparative study of RAPD profiles showed that out of total 263 fragments, 11 (4.2%) bands were common in all the samples whereas, 31 (11.8%) fragments were common in more than 80 percent of Murrah and NiliRavi individuals.

Table 3. Band sharing values within and between Murrah, NiliRavi buffaloes at 26 markers

Primers	Murrah N = 24	NiliRavi N = 24	Murrah-NiliRavi N = 48
OPA02	0.89	0.76	0.69
OPBA02	0.86	0.79	0.76
OPD12	0.78	0.81	0.77
OPG09	0.81	0.74	0.68
OPG10	0.76	0.79	0.70
OPG16	0.84	0.68	0.65
OPI05	0.73	0.75	0.74
OPJ04	0.77	0.73	0.75
OPJ06	0.88	0.81	0.76
OPJ12	0.75	0.68	0.64
OPJ16	0.84	0.75	0.72
OPK07	0.86	0.78	0.68
OPK19	0.90	0.74	0.72
OPM04	0.83	0.73	0.65
OPM06	0.88	0.79	0.75
OPM07	0.86	0.82	0.76
OPM08	0.73	0.70	0.76
OPM11	0.86	0.82	0.80
OPO01	0.90	0.78	0.75
OPZ04	0.89	0.86	0.79
OPZ05	0.80	0.77	0.72
OPZ09	0.82	0.76	0.70
ILO1127	0.85	0.75	0.71
ILO65	0.81	0.73	0.69
B4	0.78	0.71	0.70
B6	0.76	0.78	0.72
Average	0.82	0.76	0.72

Analysis at individual primer level revealed that some of the RAPD alleles were present in 100% individuals of either breed but simultaneously shared by few of the individuals of other breed as well. For example, at locus OPG10, out of 16 RAPD bands, three fragments (1.6, 1.4 and 1.3 kb) common in all the 24 Murrah animals were also present in 16, 8 and 4% animals of NiliRavi respectively. Similarly for primer OPBA01, out of the 15 RAPD fragments, one band of 2.1 kb, was present in each of the Murrah and 12 of NiliRavi animals. At another locus OPG16, out of 5, fragments of 0.8 and 1.2 kb were present in all 24 animals of NiliRavi whereas only 25 and 8% of Murrah animals revealed the presence these two bands respectively (Table 2).

Pair wise average band sharing calculated within the Murrah and NiliRavi buffaloes were 0.82 and 0.76 percent, respectively (Table 3), which indicated considerable degree of homogeneity within the two populations. Whereas, the average inter breed band sharing values were estimated to be 0.72 that is relatively lower than the within breed band sharing. The overall high percentage of band sharing between the two breeds revealed high level of genetic relatedness. UPGMA analysis between the individuals of the two breeds revealed the presence of eight clusters at 68% similarity index (Figure 4). Fifteen Murrah (63%) and

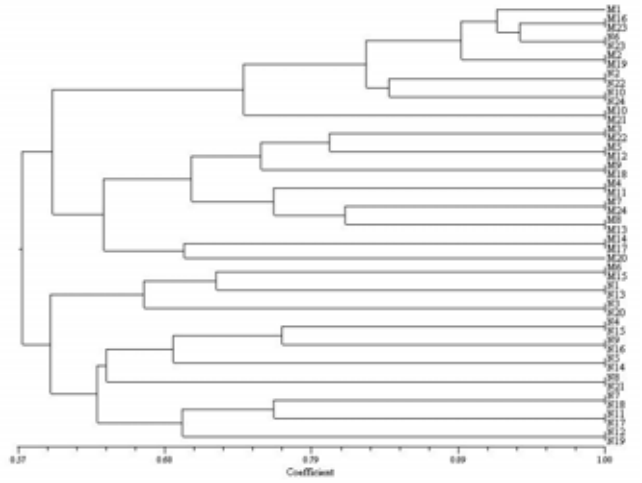

Figure 4. Phenogram resulting from analysis of 263 RAPDs depicting relationships between 48 animals of Murrah and NiliRavi buffalo breeds. The units on the scale are relative levels of branching of nodes based on genetic similarity measures.

16 NiliRavi (66%) animals were grouped exclusively in clusters 2, 3 and 5, 6, 7, 8 respectively, whereas the individuals of both the breeds shared clusters 1, 4. Nei's original measure of genetic distance (1972) between the two breeds was estimated to be 0.109, which indicate that Murrah and NiliRavi are closely related. The close genetic similarity between the two buffalo breeds is in line with their origin and geographical distribution. Similar low genetic distance values between the two breeds were obtained using the heterologous bovine microsatellite markers (unpublished data).

The present study further reveals the suitability of RAPD as a sensitive, inexpensive and convenient technique to detect the level of genetic relationship at molecular level amongst buffalo breeds for which not many polymorphic markers are available. The ease with which informative markers can be found in RAPD analysis without the prior sequence information has added feature over other means of revealing polymorphism such as RFLP and microsatellite analysis. Still, its use has been limited, primarily due to poor reproducibility and sensitivity to amplification conditions (Reidy et al., 1992; Macpherson et al., 1993). However, such difficulties have been avoided in the present work to a large extent by subjecting all the amplification reactions for each sample/primer combination thrice and only the reproducible and clear patterns were further used for scoring the similarity index. The extension of such an approach to other buffalo breeds may be employed for rapid analysis of much needed population structure to develop future conservation and breed improvement strategies.

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