

Genetic diversity analysis and cytogenetic profiling of Assamese buffaloes from North-East India

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Received: 10 April 2009; Accepted: 30 September 2009

ABSTRACT

In the present study cytogenetic profiling and genetic characterization using microsatellite genotyping was carried out on Assamese swamp buffalo population. Microsatellite data analysis for 23 markers indicated substantial genetic variation existing in the buffalo population. Sufficient allelic diversity was observed with 133 alleles present across different loci. The genetic diversity analysis of Assamese buffaloes displayed moderate to high level of within population variability in terms of mean number of alleles per locus (5.78) and heterozygosity estimates, viz. mean observed ($H_o = 0.618$) and expected heterozygosity ($H_e = 0.665$). The Assamese buffalo population showed low heterozygote deficiency ($F_{IS} = 0.029$). Mean polymorphic information content (PIC) across the 23 loci was 0.611, which indicated suitability of the marker panel for diversity analysis in these buffaloes. Bottleneck analysis indicated no recent genetic bottleneck in the Assamese buffalo population. Further, cytogenetic screening of 46 Assamese buffaloes indicated riverine ($2n=50$) or hybrid ($2n=49$) status. None of the animals had a typical pure swamp karyotype ($2n=48$). Thus, majority of the investigated animals were characterized as riverine type, which is in contrast to the general classification of Assamese buffaloes as swamp type derived from their morphological and behavioral similarity to swamp buffaloes.

Key words: Assamese buffalo, Genetic diversity, Karyotype, Microsatellite

The genetic diversity of Indian buffaloes is represented by 10 acknowledged breeds besides many lesser known populations of significant regional importance. The domestic river buffalo (*Bubalus bubalis*) has acquired a prominent status of being a leading dairy animal in India representing the largest buffalo population in the world (97 million heads; Livestock census 2003) and displaying diverse gene pool ranging from high yielding dairy type to semi-domesticated type buffaloes (Assamese and Toda). Most of the buffaloes of the north-eastern and eastern region resemble swamp type in their external morphological features, low milk yield, utility as draught animal and small to medium body size. Buffaloes found along the Brahmaputra river valley region in Asom represent a unique population persisting and producing in a typical *khunti* system. The buffaloes of Asom have been described as swamp type (Tamuly *et al.* 1982).

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These animals are exceptionally suitable for ploughing paddy fields and providing draught power for varied agricultural activities under the local production system. Also, their milk is valued for quality and fetches higher prices. The wild relatives of these buffaloes, known as *Bubalus arnee* and considered as the founder of domesticated buffaloes, are found in the wild-life reserves in the state. Asom and other north eastern states of India form the land link with east Asian countries, which are the home of swamp buffaloes (Cockrill 1974) and are thus assumed to share common buffalo germplasm. Regarding the demarcation of spread of river and swamp type buffaloes, no separation line (north-south, east-west) within India has been defined, though the existence of swamp type buffaloes has been documented (Bidhar *et al.* 1986, Yadav *et al.* 1988). The present investigation was undertaken to characterize the Assamese buffaloes distributed in the Brahmaputra valley, river islands and bordering wildlife sanctuaries.

MATERIALS AND METHODS

Information on Assamese buffaloes regarding its habitat, distribution and managerial practices were collected through preset questionnaires. Data regarding various

morphometric traits was recorded on 29 adult buffaloes from the various locations in Brahmaputra valley, near wildlife sanctuaries and organized farm. Blood samples were collected from unrelated animals of both sexes from various locations in Central Asom of the breeding tract for genetic characterization based on cytogenetic profiling and microsatellite genotyping.

Cytogenetic analysis

Blood samples in heparin tubes were collected from 46 Assamese buffaloes (20 males + 26 females) from different locations of the breeding tract by jugular venipuncture and shipped to laboratory for further leukocyte culture. Blood leukocyte cultures were established in RPMI 1640 culture medium fortified with fetal calf serum, antibiotics and pokeweed mitogen. The cultures were harvested using standard procedure for the preparation of metaphase chromosome spreads. At least 25 metaphase spreads were analyzed per animal to determine its chromosomal constitution. Representative karyotypes were prepared using the *Genus* software.

Microsatellite based genotyping and data analysis: Blood samples from 41 unrelated Assamese buffaloes from different locations of the breeding tract were collected from jugular vein into EDTA containing vacutainer tubes. Genomic DNA was extracted from whole blood following standard phenol – chloroform extraction method (Sambrook and Russell 2001). A set of 23 microsatellite markers originally identified in cattle and tested for diversity analysis in buffaloes (Navani *et al.* 2002) were utilized for the present study. PCR amplification of microsatellite loci was carried out in 25 μ l reaction volume containing 1.5mM MgCl₂, 200 mM dNTPs, 50 ng of forward and reverse primer, ~ 100 ng of genomic DNA and 0.5 Unit of Taq DNA polymerase. PCR was carried out in PTC – 200 Thermal cycler using cycling conditions: 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at precise annealing temperature, 1 min at 72°C and for 10 min at 72°C. The PCR products were resolved on 6% denaturing polyacrylamide gels and sized using a 10 bp ladder. Gels were stained by silver staining (Bassam *et al.* 1991) and genotypes scored manually for each animal.

Computation and data analysis for determination of within breed genetic variability was carried out using standard MS analysis software. Observed number of alleles, effective number of alleles, observed and expected heterozygosity estimates were computed after Nei (1973) using POPGENE software (Yeh *et al.* 1999). Polymorphism information content (PIC) was calculated using allele frequencies according to the formula given by Botstein *et al.* (1980). Departure from Hardy-Weinberg equilibrium based on Chi-square test was performed using POPGENE software. Heterozygote deficiencies at each locus and linkage disequilibrium among the loci were computed using FSTAT software (Goudet 2002). The population was tested for

genetic bottleneck by the qualitative graphical test of mode shift in allele frequency distribution using BOTTLENECK program (Piry *et al.* 1999).

RESULTS AND DISCUSSION

Assamese buffaloes are largely distributed in the upper, lower and central Brahmaputra valley in Sibsagar, Dibrugarh, Jorhat, Kamrup, Marigaon, Sonitpur, Golaghat, Barpeta, Nalbari, Darrang, Goalpara, Cachar, Karimganj, and Hailakandi districts (Fig. 1). The buffaloes are reared traditionally in an extensively managed nomadic system under zero input conditions, locally termed as *khunti* system. The system is typified as open housing of large herds of buffaloes by pooling together buffaloes of several farmers in temporary camps, mostly in the river banks/islands or near the forest areas which is named as a “*Khunti*”. No housing was provided to the adult animals while young calves were



Fig. 1. Breeding tract (spotted) of Assamese buffaloes.

Table 1. Body measurements (cm) of adult Assamese buffaloes

Parameters	Mean $\pm$ SE	Range
Height at withers (cm)	137.8 $\pm$ 1.2	130-149
Body length (cm)	148.3 $\pm$ 1.4	134-161
Heart girth (cm)	190.0 $\pm$ 1.8	170-208
Face length (cm)	54.0 $\pm$ 0.7	44-62
Tail length (cm)	82.9 $\pm$ 1.2	70-95
Horn length (cm)	63.5 $\pm$ 1.8	45-89
Horn base (cm)	21.9 $\pm$ 0.6	16-27

provided thatched housing. Various body conformation parameters recorded for Assamese buffaloes (Table 1) from various locations revealed that Assamese buffaloes are medium sized animals. The body colour is primarily black with varying shades from dark to grayish black. Hairs are brown tinged with some albino animals (light pink colour) also observed in the herds. The horn pattern is mostly of macrocerous type, being nearly straight and turning at the ends. Spirocerous type is also found, being curved upward to form a circle. Assamese buffaloes are primarily reared for milk and sale of young male calves, which are used for carting and agricultural operations. Milk yield varies between 0.5 and 6 litre/day with fat percentage averaging about 8.5%.

Cytogenetic characteristics of Assamese buffaloes: Cytogenetic analysis of Assamese buffaloes was performed to ascertain whether these buffaloes are swamp or riverine type based on the distinctive karyotypic features of the 2 types of buffaloes. Majority of the animals (41) investigated possessed a somatic chromosome count of 50 while only 5 had a riverine X swamp hybrid karyotype ($2n=49$). None of the 46 animals investigated was a pure swamp type characterized by 48, XX or 48, XY (Bongso *et al.* 1984, Sat 1987). This is a significant finding as it describes investigated Assamese buffaloes as largely riverine type, which is in contradiction to the general description of Assamese buffaloes as swamp type (Amonge *et al.* 1998, Das *et al.* 2005). Similar observations were also reported by Yadav *et al.* (1998), who screened 15 Assamese buffaloes and found that 14 were riverine type ($2n=50$) and only one male was a riverine $\times$ swamp hybrid ($2n=49$).

The karyotype of riverine type of buffalo comprises 24 pairs of autosomes and a pair of sex chromosomes. Of the 24 pairs of autosomes, 5 pairs were biarmed (metacentric/submetacentric) and 19 pairs were acrocentric. Representative metaphase spreads and karyotypes of a male riverine type Assamese buffalo ($2n=50$) and a female riverine $\times$ swamp hybrid type ($2n=49$) are presented in Fig 2. The swamp $\times$ river hybrid type not only indicated a reduced

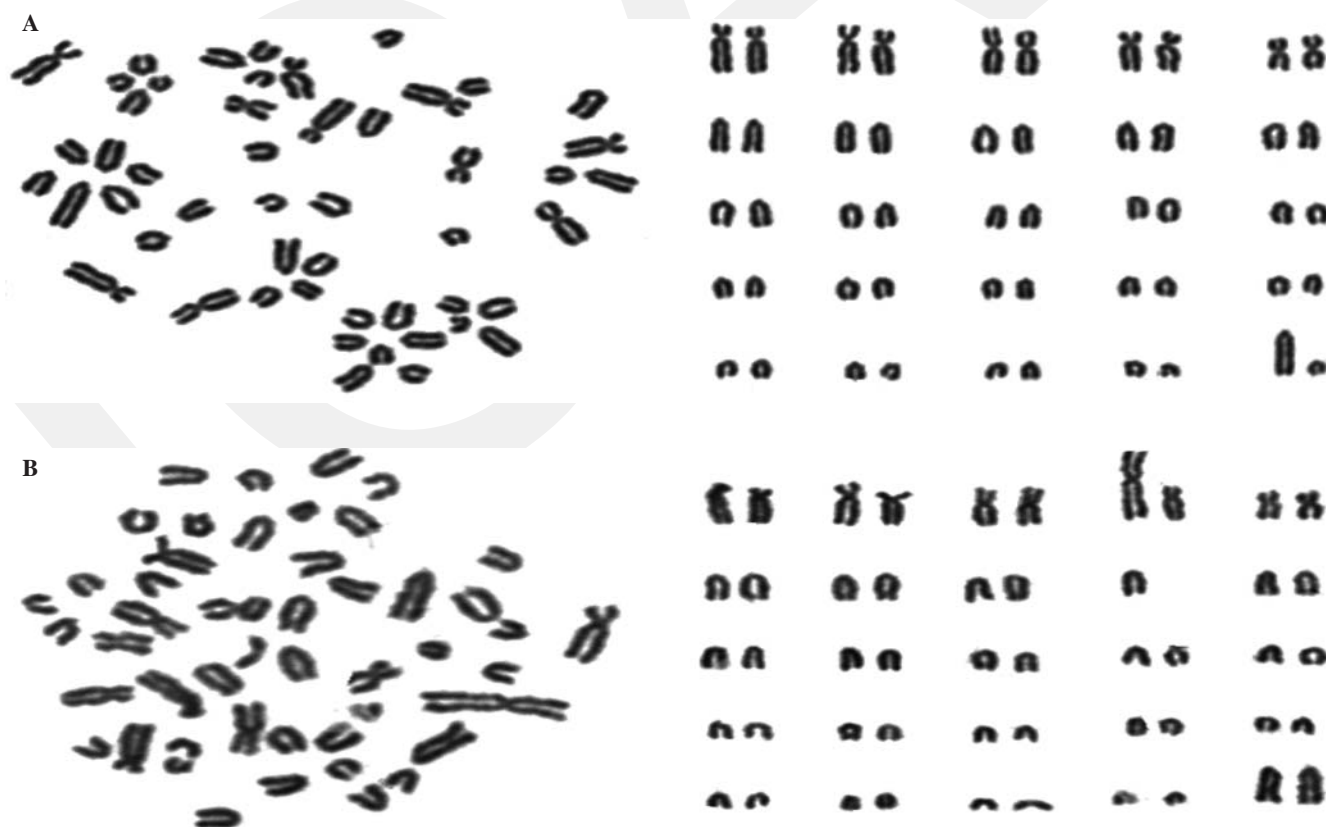


Fig 2. Metaphase spread and representative karyotypes of Assamese buffaloes – A: $2n=50$ (Riverine type), B: $2n=49$ (Riverine X Swamp hybrid type)

Table 2. Microsatellite marker based diversity estimates in Assamese buffaloes

Locus	Observed number of alleles	Effective number of alleles	Heterozygosity			PIC	F _{IS}
			H _o	H _e	Nei's H _e		
ILSTS033	7	3.77	0.846	0.749	0.735	0.698	-0.152
CSRM060	7	3.27	0.846	0.707	0.694	0.651	-0.22
ILSTS030	4	2.83	0.720	0.660	0.646	0.574	-0.114
ILSTS052	9	3.81	0.640	0.753	0.738	0.700	0.132
CSSM066	6	3.45	0.680	0.725	0.710	0.656	0.043
ILSTS058	8	4.45	0.885	0.790	0.775	0.749	-0.141
ILSTS056	7	3.60	0.583	0.738	0.722	0.691	0.192
ILSTS026	5	3.42	0.583	0.723	0.708	0.658	0.176
HEL013	8	6.47	0.625	0.864	0.846	0.826	0.261
ILSTS029	5	1.88	0.589	0.477	0.468	0.441	-0.152
ILSTS028	5	2.32	0.720	0.580	0.569	0.504	-0.266
ILSTS061	8	3.40	0.731	0.720	0.706	0.682	-0.036
ILSTS060	3	1.98	0.708	0.504	0.494	0.391	-0.434
ILSTS019	3	1.43	0.346	0.305	0.300	0.277	-0.156
ILSTS011	6	4.72	0.539	0.803	0.788	0.756	0.316
ILSTS068	5	4.54	0.731	0.795	0.780	0.745	0.063
ILSTS036	5	3.81	0.84	0.753	0.738	0.691	-0.139
ILSTS008	6	3.54	0.435	0.733	0.717	0.673	0.394
ILSTS025	6	2.96	0.692	0.675	0.662	0.622	-0.046
BM1818	6	3.35	0.423	0.716	0.702	0.655	0.397
ILSTS095	7	3.99	0.227	0.766	0.749	0.714	0.697
ILSTS031	4	2.34	0.731	0.583	0.572	0.518	-0.278
ILSTS034	3	1.22	0.154	0.182	0.178	0.171	0.137
Mean	5.78	3.33	0.618	0.665	0.652	0.611	0.029

diploid chromosome number (2n=49) but also enlarged size of one of the fourth chromosome of swamp type, which is characteristic of swamp origin (Fig. 2). This swamp buffalo chromosome resulted from the tandem fusion of river buffalo chromosomes 4 (4p) and 9 (DiBerardino and Iannuzzi 1981, Chowdhary *et al.* 1989) and is distinctly larger than any biamed pair of river buffaloes and is thus a marker chromosome for differentiating river, swamp and their crosses. In fact 3 types of buffaloes exist in the state of Asom. The wild type buffalo exists in forest sanctuaries, the second one is the domestic Assamese buffalo and the third type is the cross between the 2 (hybrid), occurring primarily in the vicinity of their coexistence. The sire of such hybrids is said to be the wild type, which breeds with domestic females when taken to the sanctuary for grazing. This supports the origin of doemstic hybrid buffalo. Mathur *et al.* (1995) described wild buffaloes in Kaziranga National Park of Asom into 3 categories: (i) wild inside the park, (ii) hybrid feral inside the park, and (iii) hybrid domestic in cattle camps on the adjacent islands in river Brahmaputra. Similar categorization of wild Asiatic buffaloes has been reported in Nepal where genetic discrimination has been attempted to distinguish wild from mixed ancestry (hybrid) and domestic buffaloes (Flamand *et al.* 2003). The hypothesis of the evolution of domesticated swamp and river buffaloes from ancestral swamp-like animals represented by wild Asiatic buffalo (*Bubalus arnee*) was supported by mtDNA data (Lei *et al.*

2007). However to authenticate such an assumption, there is definitely a need to screen the wild Asiatic buffaloes.

Microsatellite based genetic diversity analysis

Blood samples were collected from 41 Assamese buffaloes and processed for microsatellite data analysis using 23 microsatellite markers. The analysis indicated substantial genetic variation existing in the population. Various diversity measures were estimated from microsatellite data set, viz. mean observed allele per locus- 5.78, mean effective allele number - 3.33, mean observed heterozygosity (H_o) - 0.618, mean expected heterozygosity (H_e) - 0.665, and mean F_{IS} - 0.029 (Table 2). The microsatellite allelic data revealed reasonable amount of polymorphism at all the loci investigated in Assamese buffaloes. The mean observed number of alleles estimated in this study for Assamese buffalo population was higher than that reported for Bhadawari, Tarai buffaloes (Arora *et al.* 2004) and Pandharpuri, Nagpuri buffaloes (Mishra *et al.* 2008). However, comparable mean number of alleles per locus was reported in other Indian riverine buffalo breeds (Kumar *et al.* 2006), though using a different set of microsatellite markers. The mean observed heterozygosity obtained in this study is in close agreement with the range reported in other Indian buffalo breeds (Arora *et al.* 2004, Kumar *et al.* 2006, Mishra *et al.* 2008). The observed heterozygosity was higher than the expected heterozygosity at 12 loci indicating heterozygosity excess

Table 3. Test for Hardy-Weinberg equilibrium at 23 microsatellite loci in Assamese buffaloes

Locus	DF	Chi Square	P-value
ILSTS033	21	64.24	0.000
CSRM060	21	10.54	0.971
ILSTS030	6	18.62	0.004
ILSTS052	16	36.75	0.434
CSSM066	15	36.10	0.002
ILSTS058	18	48.26	0.010
ILSTS056	21	33.96	0.037
ILSTS026	10	21.10	0.020
HEL013	18	43.33	0.032
ILSTS029	10	2.39	0.992
ILSTS028	10	5.27	0.873
ILSTS061	18	65.15	0.000
ILSTS060	3	5.15	0.161
ILSTS019	3	0.99	0.802
ILSTS011	15	43.95	0.000
ILSTS068	10	17.68	0.061
ILSTS036	10	13.17	0.215
ILSTS008	15	26.54	0.033
ILSTS025	15	11.65	0.705
BM1818	15	29.32	0.015
ILSTS095	21	112.33	0.000
ILSTS031	6	9.28	0.158
ILSTS034	3	7.32	0.062

in more than 50% of the markers studied. Within population inbreeding estimate (F_{IS}) was positive with a mean value of 0.029, which is much less than that reported for other indigenous breeds of buffaloes (Arora *et al.* 2004, Mishra *et al.* 2008). Low heterozygote deficiency in Assamese buffaloes indicated a wide genetic base, which could possibly arise from the frequent introgression of wild blood in the domestic buffaloes. Assamese buffaloes thus had higher genetic variation derived from its gene diversity as judged in comparison to the genetic variation observed in other indigenous buffalo populations. Mean polymorphic information content (PIC) across the 23 loci was 0.611, which indicated suitability of the marker panel for diversity analysis in these buffaloes. Twelve out of 23 investigated loci revealed deviations from Hardy Weinberg equilibrium (Table 3). Among these 12 loci, 4 were showing heterozygosity excess with the remaining 8 showing heterozygosity deficiency. Heterozygote deficiency may arise due to several reasons including null alleles. A high heterozygote deficiency of >60% observed in ILSTS95 could be due to null allele/genotyping error. Allele frequencies in Assamese buffaloes followed the normal L-shaped distribution (Fig. 3) indicative of the absence of any recent bottleneck.

This is the first report of genetic diversity analysis of Assamese buffaloes, which indicated the existence of substantial within population genetic diversity and absence of genetic bottleneck in the recent past in this population.

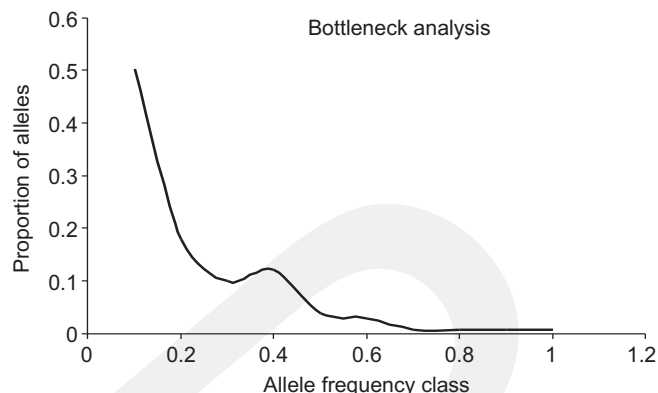


Fig 3. Bottleneck analysis in Assamese buffaloes showing L-shaped allele frequency curve based on microsatellite data.

Cytogenetic screening of buffaloes from different locations of Central Asom established majority to be riverine type with small numbers of river $\times$ swamp hybrid type suggestive of the existence of some swamp type germplasm also. Screening of more animals and further comparison with other sub-populations of Assamese buffaloes, buffalo populations of other north-eastern states and river buffalo breeds would establish the genetic and evolutionary relationship in classification of the riverine and swamp type buffaloes.

ACKNOWLEDGEMENTS

The authors wish to thank the Director, NBAGR, Karnal, for support in carrying out the research work and ICAR, New Delhi for the financial support. The technical support by Shri Naresh Kumar, NBAGR and staff of AAU, Khanapara, Guwahati, in collecting blood samples is duly acknowledged.

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