Genetic characterization and diversity assessment in ‘Bhangor’ indigenous swamp buffalo population using heterologous microsatellite markers

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ABSTRACT

‘Bhangor’ newly identified swamp buffalo population from North East Indian, was characterized using microsatellite markers. Genomic DNA was isolated from blood samples of 76 unrelated animals, 15 microsatellite markers (CSSM33, BM1818, CSRMS60, HEL13, ILSTS019, ILSTS025, ILSTS028, ILSTS029, ILSTS033, ILSTS036, ILSTS056, ILSTS058, ILSTS061, ILSTS089 and ETH003) were found to be highly polymorphic in the population of the selected markers. A total of 114 alleles were observed, which ranged from 3 in CSRM60 and ILSTS025 locus to 12 in ILSTS056 and ILSTS061. The mean effective number of alleles across all polymorphic loci was found to be 3.76. The overall mean expected heterozygosity and unbiased expected heterozygosity values were 0.67 and 0.68, ranging from 0.067 (ILSTS025) to 0.85 (ILSTS058) and 0.068 (ILSTS025) to 0.86 (ILSTS058), respectively. Within the population, the inbreeding estimates ($F_{IS}$) ranged between −0.4352 and 0.804, with an average $F_{IS}$ of 0.114 ± 0.033. The outcome for infinite allele model (IAM), two-phase model (TPM) and test for mode shift revealed the absence of any recent bottleneck in the investigated buffalo population. The population was found to be in optimum diversity based on polymorphic microsatellite markers. With fast changing agro-climatic conditions; there is an urgent need to characterize the nondescript livestock populations.

KEYWORDS
Genetic diversity; microsatellite; buffalo

Introduction

India possesses the largest buffalo population in the world, which includes a rich diversity of riverine and swamp buffaloes adapted to different agro-biodiversity regions. There are 20 registered buffalo breeds and many nondescript populations distributed across the country. The swamp buffalo are easily distinguishable primarily due to their distinct morphology and karyotype. So far, buffaloes of the north-eastern states of India have been reported to be swamp type based on their phenotypic characteristics. Swamp buffaloes are smaller and have lower milk yields than river buffaloes. They are mainly found in Eastern Asia and are primarily raised for draught power. The indigenous buffalo of Tripura are known as ‘Bhangor’ by local farmers, these have been characterized based on cytogenetic analysis as swamp. It has a population of approximately 7000 as per the latest 20th livestock census, 2019.

Tripura is hilly region, bounded on three sides with Bangladesh international border. Livestock in the state is mainly livelihood oriented and generally owned by small and marginal farmers. Buffaloes are reared mainly for draft power in the paddy fields and are known as ‘living tractors of the east’. The buffalo live in swamps, where they feed on grass, the hooves are large and extended apart which help in easier walking through deep mud. Milk yield varies between 1.5 and 2.5 liters per day milking is not practice in the region and milk is left for the young ones. The buffaloes are an important source of meat protein in the region, especially during traditional festivals. Swamp buffalo more closely resemble wild water buffalo and are used as draft animals in rice paddies throughout the Southeast Asia. The present day mechanization of farmland operations and indiscriminate breeding practices threatens the swamp buffalo populations. Destruction of natural grazing lands is one of the major concerns.

In the North Eastern part of India buffaloes have generally been described as swamp type based on their physical characteristics. In this region admixture of both riverine and swamp along with hybrids are present. Also, this region is home to Asiatic wild buffalo...
(Bubalus arnee), the progenitor of domesticated water buffalo.\textsuperscript{6,7} Recently, cytogenetic studies on indigenous swamp buffalo populations of Meghalaya and Tripura states of India have been carried out.\textsuperscript{8,9} Genetic diversity analysis of livestock populations is important for breed identification, characterization, and studying population structure dynamics for conservation management. DNA polymorphisms are powerful tools for molecular analysis and are used to understand evolution and diversity.\textsuperscript{10} Microsatellites or simple sequence repeats (SSRs) are considered as markers of choice for the assessment of genetic diversity in livestock populations.

Microsatellite markers have been widely used for the characterization of water buffalo diversity. Microsatellite marker based characterization and analysis of diversity in different livestock breeds are available in literature.\textsuperscript{11–17} Microsatellite marker data highlighted population relationships consistent with their geographical distribution and historical spread in south-east Asian swamp buffalo populations.\textsuperscript{18} Analysis of 26 Asian swamp buffalo populations showed the highest genetic variability in Thai buffaloes ($H_o = 0.573$).\textsuperscript{19} Although, phenotypic characterization of Bhangor buffaloes has been documented, there is no information available on the genetic diversity analysis of this population.\textsuperscript{4} The genetic characterization of Bhangor buffalo gains significance for the maintenance of genetic diversity and prevention of germplasm erosion of indigenous genetic stock. The present study was undertaken to characterize and evaluate the status of indigenous ‘Bhangor’ buffalo population using microsatellite markers. It would also help in understanding conservation aspects for indigenous buffalo population.

### Results

A total of 15 microsatellite loci were found to be highly polymorphic and successfully amplified in Bhangor buffalo population. Various within breed variability measures estimated for each locus in Bhangor buffalo viz., number of observed alleles ($n_o$), effective number of alleles ($n_e$), observed ($H_o$) and expected heterozygosity ($H_e$) are presented in Table 1. Sufficient allelic diversity was observed with a total number of 114 distinct alleles recorded by genotyping 76 adult buffaloes, with an average of 7.60 ± 0.68 alleles per locus. Maximum number of 12 alleles was observed at locus ILSTS056 and ILSTS061, while minimum 3 alleles were recorded at CSRM60 and ILSTS025 locus. The mean effective number of alleles (Ne) across all polymorphic loci was found to be 3.76 ± 0.39, it ranged from 1.07 (ILSTS025) to 6.67 (ILSTS058), which was within the range observed in other Indian riverine buffalo populations.\textsuperscript{10,13}

The average estimate of expected heterozygosity ($H_e$) was 0.67 ± 0.05, the highest value observed at ILSTS058 locus (0.85) and lowest at ILSTS025 (0.06), while unbiased estimate (Nei’s $H_e$) was 0.68 ± 0.05. Shannon’s information index values was found to be 0.1740 (ILSTS025), whereas the highest value was 2.054 (ILSTS061). The average polymorphism information content (PIC) value indicates the informative-ness of different microsatellite markers studied and is also an important measure for DNA polymorphism. The average PIC value was 0.63. Table 1 depicts the details about the observed and expected number of alleles, heterozygosity (expected, observed and unbiased) and PIC values for each locus. Within-population inbreeding estimates ($F_{IS}$) for Bhangor buffalo population ranged between −0.4352 and 0.804 with an average $F_{IS}$ of 0.114 ± 0.033. The qualitative graphical method mode-shift analysis revealed the normal L-shaped distribution of allele frequencies, suggesting the absence of a recent genetic bottleneck in the Bhangor buffalo population.

### Discussion

The present study is the first attempt toward the genetic characterization of Bhangor buffaloes using microsatellite markers. The polymorphism of a gene/allele is prerequisite for it to be useful in genetic analysis of population. Sufficiently high allelic diversity was observed with a total number of 114 distinct microsatellite alleles across 15 loci. The observed number of alleles ($n_o$) and mean number of alleles are good indicators of genetic variation in a local population, the observed numbers of alleles was higher than the effective numbers of alleles for the studied loci. The mean observed numbers of alleles were found to be a little higher in the present study than that reported in Toda (5.4), Nagpuri (5.24), Chilika (4.68), Jaffarabadi (4.76), Bhadawari (4.7), Tarai (4.7), Marathwada (4.48), Pandharpuri (6.2) and Banni (5.75) buffaloes\textsuperscript{14,20–24} but is lower than the swamp population of Manipur (8.9) and Nagaland (9.1).\textsuperscript{5} While studying Thai swamp buffalo population average 4.7 alleles were observed with 10 loci.\textsuperscript{25} The 20 microsatellite markers analyzed showed a mean number of alleles of (7.28) ranging from 6 (ILSTS005) to 17 (ETH003) in Turkish water buffalo.\textsuperscript{26} The higher number of alleles secured implies increased allelic
diversity present in the population. However 11–26 alleles per locus are also reported in other Indian water buffalo breeds.\textsuperscript{13} These allelic differences may be attributed to the population under study, microsatellite markers studied, and the genetic polymorphism existing within the population itself. The effective number of alleles across all loci in Bhangor population was found to be $3.76 \pm 0.39$, where as in Assam and Manipur sample with same set of Loci it was $4.65 \pm 0.70$ and $4.30 \pm 0.39$, respectively.\textsuperscript{5}

The average number of alleles obtained in the present study (7.60) was in line with FAO recommendation that suggests an analysis of at least 5 alleles per locus for genetic diversity based studies in livestock. However except for the locus CSR60 and ILSTS025 having 3 alleles, rest all the markers demonstrated a good amount of polymorphism. Sukla et al. 2006 reported an average estimate of 5.5 alleles per locus for six Indian buffalo breeds based on 10 polymorphic microsatellite loci.\textsuperscript{27} Similarly, an average of 5.15 alleles per locus was reported by Ozkan et al. in buffaloes of Turkey\textsuperscript{28} and with same set of loci, Assam and Manipur sample showed 9.46 and 9.26 alleles per locus, respectively.\textsuperscript{5} The effective number of alleles across all loci was lower than the observed values. The alleles with lower/rare frequency were considered as novel ones and these alleles may be assigned to population.

The average expected heterozygosity was 0.670 observed across all polymorphic loci for the population, which is a good measure to assess genetic diversity in a population. A similar average for Ho and He was observed in Colombian buffalo.\textsuperscript{29} Kumar et al. reported ranges of 0.63–0.71 and 0.71–0.78 for the observed and the expected heterozygosity, respectively, indicating higher expected than observed heterozygosity in eight studied Indian breeds.\textsuperscript{30} Substantially high average heterozygosity values pointed toward the existence of considerable genetic variability in the Bhangor buffalo population and suitability of the markers as well. Mishra et al. 2015 has studied buffalo population from North East India, and has reported the mean observed heterozygosity values as 0.624, 0.587, 0.513, 0.533, 0.701 and 0.530 in upper Assamese, north Assamese, Manipuri, Mizoram, Nagaland and lower Assamese buffalo population, respectively.\textsuperscript{5}

The Nei’s He estimate ranged in conformation with other Indian riverine buffaloes population, Murrah, Mehsana, Toda, Surti, Pandharpuri, Nilli Ravi, Tarai, Bhadawari, Jaffarabadi and Nagpuri buffaloes.\textsuperscript{11,15,20,30,31} Shannon’s information index values were found to be maximum in Bhadawari, Jaffarabadi and Nagpuri buffaloes.\textsuperscript{11,15,20,30,31} The locus ILSTS025 showed PIC estimate less than 0.5 are considered as important for genetic diversity based analysis. The average PIC value was 0.63. The locus ILSTS025 showed PIC estimate less than the threshold of 0.5 (Table 1). Similar observations have been reported earlier in the Nagpuri\textsuperscript{22} and South Kanara buffalo\textsuperscript{12} populations of India. Comparatively higher PIC value were reported in following Indian population Pandharpuri buffalo 0.73,\textsuperscript{20} Mehsana buffalo 0.73\textsuperscript{33} and Chilika buffalo 0.66.\textsuperscript{23} In contrast a higher PIC value of 0.933 was reported in Egyptian buffalo.\textsuperscript{34}

### Table 1. Markers wise allelic diversity in Bhangor buffalo population.

| Locus   | Observed number of alleles | Effective number of alleles | Shannon’s information index I | Observed heterozygosity $H_o$ | Expected heterozygosity $H_e$ | Nei expected heterozygosity $H_{ne}$ | Allele size (bp) | PIC value | Coefficient of inbreeding Fis |
|---------|---------------------------|----------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------------|-----------------|-----------|-------------------------------|
| CSSM33  | 8                         | 4.340                      | 1.715                         | 0.840                         | 0.770                         | 0.757                                 | 157-179         | 0.785     | 0.740                          | -0.0915     |
| BM1818  | 9                         | 4.614                      | 1.742                         | 0.850                         | 0.784                         | 0.804                                 | 244-300         | 0.804     | 0.776                          | -0.0845     |
| CSR60   | 3                         | 1.865                      | 0.725                         | 0.611                         | 0.674                         | 0.684                                 | 174-200         | 0.684     | 0.622                          | -0.4352     |
| HEL13   | 9                         | 3.070                      | 1.412                         | 0.968                         | 0.674                         | 0.684                                 | 174-200         | 0.684     | 0.622                          | -0.4352     |
| ILSTS019| 6                         | 2.207                      | 1.093                         | 0.129                         | 0.547                         | 0.556                                 | 175-182         | 0.556     | 0.538                          | 0.7640      |
| ILSTS025| 3                         | 1.072                      | 0.174                         | 0.069                         | 0.067                         | 0.068                                 | 113-144         | 0.068     | 0.03                           | -0.0265     |
| ILSTS028| 10                        | 5.095                      | 1.831                         | 0.656                         | 0.804                         | 0.816                                 | 143-175         | 0.816     | 0.750                          | 0.1835      |
| ILSTS029| 8                         | 2.379                      | 1.332                         | 0.677                         | 0.580                         | 0.589                                 | 156-170         | 0.589     | 0.545                          | -0.1688     |
| ILSTS033| 6                         | 3.165                      | 1.404                         | 0.560                         | 0.684                         | 0.698                                 | 140-152         | 0.698     | 0.629                          | 0.1813      |
| ILSTS036| 6                         | 3.189                      | 1.370                         | 0.691                         | 0.664                         | 0.700                                 | 124-172         | 0.700     | 0.646                          | 0.1034      |
| ILSTS056| 12                        | 5.143                      | 2.020                         | 0.500                         | 0.823                         | 0.823                                 | 144-182         | 0.823     | 0.785                          | 0.3793      |
| ILSTS058| 8                         | 6.672                      | 1.958                         | 0.714                         | 0.850                         | 0.866                                 | 130-144         | 0.866     | 0.848                          | 0.1598      |
| ILSTS061| 12                        | 5.253                      | 2.054                         | 0.774                         | 0.819                         | 0.832                                 | 137-161         | 0.832     | 0.802                          | 0.0546      |
| ILSTS089| 7                         | 4.133                      | 1.604                         | 0.391                         | 0.758                         | 0.775                                 | 114-128         | 0.775     | 0.697                          | 0.4838      |
| ETH003  | 7                         | 4.056                      | 1.652                         | 0.167                         | 0.753                         | 0.786                                 | 109-133         | 0.786     | 0.741                          | 0.7788      |
| Mean    | 7.600                     | 3.769                      | 1.472                         | 0.533                         | 0.670                         | 0.684                                 | 0.533           | 0.684     | 0.533                          | 0.114       |
| SE      | 0.689                     | 0.396                      | 0.131                         | 0.077                         | 0.052                         | 0.053                                 | 0.077           | 0.053     | 0.033                          |           |
While lower values were reported in Cuban water buffalo 0.495 PIC \(^{35}\) and in Gullian buffaloes of Iran 0.61 PIC values, \(^{36}\) Turkish buffalo 0.655 \(^{26}\) indicating their suitability for genetic diversity analysis in buffalo. Genetics studies using three microsatellite markers (CSSM66, ILSTS61, and ILSTS17) showed presence of 9 alleles across the three loci in Indonesian swamp buffalo, loci ILSTS61 had a high PIC compared to the other loci. \(^{37}\)

For studying the evolutionary relationship of closely related populations microsatellite markers are of choice, to measure the genetic difference between different populations using the difference in allelic frequencies of several loci in these populations. Relatedness of population is shown by the frequencies of shared alleles. Table 2 summarizes the genetic distance estimate between Murrah, Nilli Ravi, Manda, Chilika, Kalahandi, Manipur and Assam, breeds based on uniform microsatellite data. The high genetic distance measure between the riverine populations supports a strong substructuring of the diversity. However, a moderate genetic distance measure was observed with the Manipur and Assam populations (FST = 0.083, 0.097; \(p = 0.072\) respectively), suggesting some gene flow between these populations (Nm = 1.127, 1.568 respectively). Earlier phylogenetic analysis studies with mitochondrial D-loop region grouped Bhangor buffalo haplotypes with Manipuri and Chilika swamp buffalo of India and with Chinese and Carabao haplotypes. \(^{4}\)

Given the estimates of the observed and effective number of alleles, heterozygosity and PIC parameters, these marker loci may be used for carrying out the genetic studies on Bhangor population. Thus, the result suggests that there is substantial genetic variation and heterozygosity across the studied loci in the indigenous Bhangor buffalo population as assessed on the basis of polymorphic microsatellite markers.

**Conclusion**

The analysis presented in this study provides the first preliminary data on the genetic diversity of the Bhangor non-descript swamp buffalo population from Tripura. Microsatellite analysis revealed high level of polymorphism and informativeness of the studies markers in genetic analysis in local population. These markers may be further used for breed characterization and to assist the conservation of genetic diversity of the Indian buffaloes.

**Materials and methods**

**Ethics statement**

The study was approved by the ICAR-National Bureau of Animal Genetic Resources, Karnal, ICAR/NBAGR/IRC-2.11. All methods were carried out in accordance with guidelines and regulations of the concerned committee. Morphometric data of the animals were collected based on preset questionnaires prepared by ICAR-NBAGR and informed consent was obtained from the farmers during the pilot survey conducted in Tripura state.

**Sample collection and genomic DNA isolation**

A volume of 10 ml blood sample was collected from 76 animals from several villages located in the remote area of the districts West Tripura, Khowai, Dhalai, Unakoti, Gomati, and North Tripura. Samples were collected under aseptic conditions through jugular vein puncture in sterile polypropylene tubes containing EDTA (Ethylene diamine tetra acetate) as anticoagulant. Genomic DNA was isolated following standard phenol-chloroform extraction method. \(^{38}\) The genomic DNA samples were evaluated for their purity, quality and concentration using agarose gel documentation and NanoDrop spectrophotometer (absorbance ratio 260/280 nm).

**Selection of markers, PCR amplification and microsatellite typing**

A total of 24 heterologous bovine microsatellite markers \(^{10}\) were utilized to genotype the sampled individuals. The 5’ end of forward primer of each microsatellite marker was labeled with one of the fluorescent dyes, viz., FAM (Blue), VIC (Green), NED (Yellow) or PET (Red) to assess the fragment length of genotyped PCR product with automated DNA sequencer (ABI 3100). PCR amplification was carried out in thermal cycler in a final reaction volume of 15 μl containing 10 pmol/μl of each primer, 10 mM of each dNTP, 1.5 mM MgCl2 and 1.2 U Taq polymerase (Invitrogen, California), after optimization of annealing temperature for each microsatellite locus.

| Population       | Bhangor | Murrah | Manda | Chilika | Kalahandi | Manipur | Assam |
|------------------|---------|--------|-------|---------|-----------|---------|-------|
| Bhangor          | 0.689   | 0.21   | 0.161 | 0.095   | 1.127     | 1.568   |
| Murrah           | 0.21    | 0.856  | 0.458 | 0.182   | 2.174     |
| Manda            | 0.143   | 0.21   | 0.003 | 0.025   | 0.145     |
| Chilika          | 0.149   | 0.143  | 0.12  | 0.04    | 1.346     |
| Kalahandi        | 0.146   | 0.149  | 0.12  | 0.04    | 1.446     |
| Manipur          | 0.083   | 0.157  | 0.157 | 0.139   | 0.145     |
| Assam            | 0.097   | 0.152  | 0.198 | 0.136   | 0.117     | 0.124   | 0     |
PCR program used for amplification included initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 52–58°C for 1 min and final extension at 72°C for 10 min. The amplified products are clearly seen in the Agarose gel, and no nonspecific amplification or PCR failure was observed. After multiplexing of different dye-labeled amplified markers, the pooled samples were run on ABI automated DNA sequencer along with internal control LIZ standard. The data was extracted using GENEMAPPER software documenting the allele sizes for each marker in each animal. However, only 15 microsatellite loci (CSSM33, BM1818, CSRM60, HEL13, ILSTS019, ILSTS025, ILSTS028, ILSTS029, ILSTS033, ILSTS036, ILSTS056, ILSTS058, ILSTS061, ILSTS089 and ETH003) were found to be polymorphic for Bhangor population and hence were selected for studying genetic diversity. The information about these microsatellite markers along with corresponding primer sequences annealing temperature and chromosomal location are depicted in Table 3.

### Statistical analysis

The GenAlEx software (version 6.503) was employed to calculate different within-population diversity measures viz, mean number of alleles per locus (n_a), effective number of alleles per locus (n_e), observed heterozygosity (H_o) and coefficient of genetic diversity (H_e) of Nei for microsatellite loci analyzed in Bhangor buffaloes. Within-population-inbreeding estimates (F_IS) were calculated using FSTAT computer program (version 2.9.3.2). Polymorphism information content (PIC) values were estimated through allele frequencies using the following equation:

\[
PIC = 1 - \sum_{i=1}^{k} p_i^2 - \sum_{i=1}^{k-1} p_i \sum_{j=i+1}^{k} p_j^2
\]

Hardy–Weinberg equilibrium test was conducted and Bottleneck 1.2.01 software was used to conduct sign test, standardized differences test and Wilcoxon sign-rank test to detect genetic bottleneck. For evaluation of genetic relationship between Bhangor buffalo with other buffalo population, the microsatellite data generated from same set of loci for Murrah, Nilli-ravi, Manda, Chilika, Kalahandi and swamp population of Manipur and Assam was used.

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**Author contributions**

KVS and RSK designed the study, KVS and RD managed resources populations and performed blood sampling, KVS and MS performed microsatellite data analysis. KVS wrote the manuscript, all authors reviewed the manuscript.

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**Data availability statement**

The datasets generated and analyzed during the current study are available in the institute NBAGR repository, and will be shared as and when required.

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