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Genetic Characterization and Diversity Assessment in 'Bhangor' Indigenous Swamp Buffalo Population using Heterologous Microsatellite Markers

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Research Article

Keywords: genetic diversity, microsatellite, molecular markers, Bhangor, SSR

Posted Date: March 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-294166/v1

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Abstract

The present study was aimed at genetic characterization and diversity analysis of newly identified swamp buffalo population 'Bhangor' using the Food and Agriculture Organization (FAO) recommended bovine microsatellite markers. Genomic DNA was isolated from blood samples of 76 unrelated animals. Of the 24 markers, 15 markers (CSSM33, BM1818, CSRM60, HEL13, ILSTS019, ILSTS025, ILSTS028, ILSTS029, ILSTS030, ILSTS033, ILSTS036, ILSTS056, ILSTS058, ILSTS061, ILSTS089 and ETH003) were found to be highly polymorphic in the population. A total of 114 alleles were secured, with an overall average of 7.60 alleles per locus. The number of alleles ranged from 3 (CSRM60 and ILSTS025) to 12 (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. The average PIC estimate across all polymorphic loci was 0.63. The population was found to be in optimum diversity based on polymorphic microsatellite markers. This is the newly characterized buffalo population from north-east India.

Introduction

India is one of the mega-diversity zones, with a rich diversity of buffaloes both riverine and swamp. A total of 17 breeds of buffaloes are registered in India [1]. It includes a total population of 108.7 million, animals that about 60% of the total buffalo population in the world. Most of which are riverine buffalo the elite milch breeds, contributing 50% to the national milk production of India.

Geographically Tripura is land-locked state major portion lies inside Bangladesh. With an area of 10,491 km2 (4,051 sq mi) it is the third-smallest state, situated between the river valley of Myanmar and Bangladesh. The geographical terrain by and large consists of parallel hills and ridges running from the northwest to the southeast direction, with alternating narrow valleys. The present day mechanization of farmland operations and indiscriminate breeding practices threatens the genetic diversity in livestock populations. Moreover, with fast changing agroclimatic conditions, there is an urgent need to characterize the nondescript livestock populations at the molecular level. So far buffaloes of the north-eastern states of India have been studied except for Tripura and are generally considered as swamp type based on their phenotypic resemblance to swamp type buffaloes. The indigenous buffalo of Tripura are known as '*bhangor'* or '*manipuri'* by local people, as per the latest livestock census its population is approx 7000 [2]. These swamp buffaloes are reared mainly for draft power in the paddy fields and their population is declining. Genetic diversity analysis of livestock populations is important for breed identification, characterization, and studying population structure dynamics for conservation management. Molecular characterization and analysis of genetic diversity using microsatellite markers in different buffalo breeds in India [3–11] and world buffalo population [12–14] are available in the scientific literature. Microsatellites are considered as markers of choice for the assessment of genetic diversity in livestock populations. In order to analyze and conserve within- and between-population genetic variability, Food and Agriculture Organization (FAO) has recommended a set of microsatellite markers for different livestock species (www.fao.org) [15].

The present study was undertaken to characterize and evaluate genetic diversity in the 'Bhangor' buffalo population from Tripura North-east India using FAO recommended microsatellite markers. Bhangor buffalo were characterized phenotypically and cytogenetically [16] however, characterisation in terms of genetic diversity was not studied yet. The genetic characterization of Bhangor buffalo gains significance for the maintenance of genetic diversity and prevention of germplasm erosion of indigenous livestock.

Results

The adult animals are generally gray or light-black in colour. The horn is curved like sickle with a broad base which is mostly corrugated. The horn tip pointed upward and backwards. The ears are horizontally placed. The forehead is mostly convex. The adult animals are medium built and compact. Lower leg portion between hoofs and knee is of white to greyish-white in

colour. The tail switch is generally black. The udder is bowl shaped and poorly developed, with small, cylindrical teats with pointed tips. The buffaloes have typical white markings on both sides of muzzle and lower jaw (Figure no 1–2). Animals had one white patch on the lower part of the neck region which is very prominent in young ones. Animal are primarily used in paddy fields. Milking is not done thought milk yield varies between 1.5 to 2.5 liters per day. The fat percentage is high averaging about 8.5%. The buffaloes are also used as source of meat protein, especially during traditional festivals. North-eastern part of India is the region where admixture of both riverine and swamp along with hybrids are present [22]. Recently our lab has reported existence of indigenous swamp buffalo population in Meghalaya state of India based on cytogenetic analysis [23].

A total of 15 microsatellite loci were found to be polymorphic and successfully amplified in Bhangor buffalo population. Locus wise allele frequency distribution and fragment sizes are presented in Table 2. A total of 114 alleles were recorded for 15 loci by genotyping 76 adult buffaloes, thus giving an average of 7.60 ± 0.68 alleles per locus. A maximum number of alleles were observed for ILSTS056 and ILSTS061 (12 alleles each) and minimum number of alleles for CSRM60 and ILSTS025 (3 alleles). The mean effective number of alleles (N_e) across all polymorphic loci was found to be 3.76 ± 0.39. Ne ranged from 1.07 (ILSTS025) to 6.67 (ILSTS058). The average estimate of expected heterozygosity (H_e) was 0.67 ± 0.05 while unbiased estimate (Nei's H_e) was found to be 0.68 ± 0.05. The expected heterozygosity estimate was highest for ILSTS058 locus (0.85) and lowest for ILSTS025 (0.06). On the other hand, Nei's H_e estimate ranged from 0.068 (ILSTS025) to 0.86 (ILSTS058). Lowest Shannon's information index values were found to be 0.1740 (ILSTS025) and 0.725(CSRM60), whereas the highest value was 2.054 (ILSTS061), total 8 loci were found having index value above 1.48 with an overall mean of 1.47 ± 0.131. The average PIC estimate of 0.63 was observed across all polymorphic loci in the population. The PIC estimate ranged from 0.03 (ILSTS025) to 0.84 (ILSTS058). Table 2 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 (FIS) for buffalo ranged between 0.171 and 0.495 with an average FIS of 0.059 ± 0.033.

In mutation drift equilibrium, heterozygosity excess/deficiency under the models generated by the BOTTLENECK is presented in Table no 3. Infinite allele model (IAM), and stepwise mutation model (SMM) under sign test showed deficiencies in 8 and 22 loci, respectively. Standardized difference and Wilcoxon test revealed significant heterozygosity excess under the Infinite allele model. The qualitative graphical method mode-shift analysis revealed the normal L-shaped distribution of allele frequencies as depicted in Figure no 3, 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. Sufficiently high allelic diversity was observed with a total number of 114 distinct microsatellite alleles across 15 loci. Tripura is landlocked state and its physiography is characterised by hills and valleys. Buffaloes are distributed in the hills as well as valley/plain regions of different parts of the state. The state has nine major rivers, over 400 wetlands, and swamps, providing a natural habitat for swamp buffaloes to wallow [24]. The total buffalo population of Tripura is estimated around 7000 (20th Livestock Census, 2019), 50% of which is found in Unakoti district alone. Livestock in the state is mainly livelihood oriented and generally owned by small and marginal farmers. Population of the animal is decreasing over the years due to mechanisation of agriculture work and change in crop cultivation pattern. Destruction of natural grazing lands is one of the major concerns. The world famous indigenous Murrah buffalo has been brought in Tripura in the year 2013 for research and developmental purposes, to be explored for boosting milk production [25].

The observed number of alleles (Na) and mean number of alleles are indicators of genetic variation in a population. The observed numbers of alleles (Na) were higher than the effective numbers of alleles (Ne) for all the loci studied. The mean observed and effective numbers of alleles were found to be a little higher in the present study than that reported in Toda, Jaffrabadi (4.76), Bhadawari (4.7), Tarai (4.7), Chilika (4.68), Marathwada (4.48), Nagpuri, Pandharpuri and Banni buffaloes [4, 6, 7, 10, 26–29] but is lower than the swamp population of Manipur and Nagaland [22]. The higher number of allele secured implies increased allelic diversity present in the population. However 11 to 26 alleles per locus are also reported in

Indian water buffalo [5]. These allelic differences may be attributed to the population under study, microsatellite markers studied, and the genetic polymorphism existing within the population itself.

Similarly,

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. Under the present study locus CSRM60 and ILSTS025 (3 alleles) 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 [30]. Similarly, an average of 5.15 alleles per locus was reported by Ozkan et al. in buffalo breed of Turkey [31]. 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 heterozygosity estimate of 0.670 was observed across all polymorphic loci for the population. The expected heterozygosity estimate was highest for ILSTS058 locus (0.85) and lowest for ILSTS025 (0.06). All the loci, except ILSTS025, exhibited high expected gene diversity, which are good measures to assess genetic diversity in a population. A similar average for Ho and He was observed in Colombian buffalo [32]. 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.

PIC value indicates an estimate associated with a particular marker for detecting polymorphism and it ranges from 0 to 1. Markers with PIC value greater than 0.5 are considered important for genetic diversity based analysis [25]. Microsatellite loci ILSTS025 showed PIC estimate less than the threshold of 0.5 (Table 2). The other markers were informative and important for genetic diversity and paternity based analysis in Bhangor population. 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 Bhangor buffalo as assessed on the basis of 15 polymorphic microsatellite markers.

Material And Methods

Sample collection and genomic DNA isolation

Habitat, distribution, management practices, utility, performance and socio-economic status of the farmers rearing '*Bhangor*' buffalo was collected through preset questionnaires during the pilot survey conducted in Tripura state. Blood samples were collected from unrelated animals of both sexes from various villages in the breeding tract in the districts of West Tripura, Khowai, Dhalai, Unakoti, Gomati, and North Tripura. Blood samples were collected under aseptic conditions through jugular vein puncture in sterile polypropylene tubes containing anticoagulant i.e., ethylene diamine tetra acetate (EDTA). Genomic DNA was isolated following standard phenol-chloroform extraction method [17]. The genomic DNA samples were evaluated for their purity, quality and concentration using agarose gel documentation and NanoDrop spectrophotometer (absorbance ratio 260/280nm).

Selection of markers, PCR amplification and Microsatellite typing

Bhangor buffaloes were genotyped using a battery 24 recommended microsatellite markers/loci (www.fao.org) present on the bovine genome. The information about these DNA markers is depicted in Table 1. 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). However, only 15 microsatellite loci (CSSM33, BM1818, CSRM60, HEL13, ILSTS019, ILSTS025, ILSTS028, ILSTS029, ILSTS030, 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 markers used in this study were dinucleotide repeats which are

more polymorphic than tri-nucleotides. The amplified products are clearly seen in the Agarose gel, and no non-specific amplification or PCR failure was observed.

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, 1.5mM $MgCl_2$ and 1.2U Taq polymerase (Invitrogen, California), after optimization of annealing temperature for each microsatellite locus. 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 [18].

Statistical analysis

The GenAlEx software (version 6.503) [19] was employed to calculate different within-population diversity measures viz, mean number of alleles per locus (Na), effective number of alleles per locus (Ne), observed heterozygosity (Ho) and coefficient of genetic diversity (He) of Nei for microsatellite loci analyzed in Bhangor buffaloes. Within-population-inbreeding estimates (FIS) were calculated using FSTAT computer program (version 2.9.3.2) [20]. Bottleneck 1.2.01 software was used to conduct sign test, standardized differences test and Wilcoxon sign-rank test to detect whether buffalo population has undergone any recent reduction in the effective population size or genetic bottleneck [21].

Conclusion

The analysis presented in this study provides the first preliminary data on the genetic diversity of the non-descript swamp population ‘Bhangor’ from Tripura. Microsatellite markers used in the present study were highly polymorphic and revealed the effectiveness of studied markers in explaining the existing diversity levels, population structure and inbreeding status in the population. Bhangor buffaloes are distributed in a small area with less population size; various diversity indices suggest sufficient genetic variability in this population for its sustenance. These markers may be further used for breed characterization and to assist the conservation of genetic diversity of the Indian buffaloes.

Declarations

ACKNOWLEDGEMENTS

The authors wish to thank the Director, ICAR-NBAGR, Karnal, for support in carrying out the research work. The authors wish to thank the Director, Joint Directors and Veterinary Officers of Department of A.H. & Veterinary Services, Govt. of Tripura for their active support in carrying out the field work and assistance during the collection of blood samples of buffaloes.

Author contributions

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

Competing interests

The authors declare no competing interests.

Ethics statement

1. The study was approved by the ICAR-National Bureau of Animal Genetic Resources, Karnal. 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.

2. All methods were carried out in accordance with relevant guidelines and regulations of ICAR-NBAGR. Animal blood samples were collected by a trained veterinarian.

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Tables

Table no 1

Details of heterologous bovine microsatellite markers used to study genetic diversity in Bhangor buffalo population.

Loci	Primer sequence (5'–3')	Chromosome	Annealing Temperature
CSSM33	F-CACTGTGAATGCATGTGTGTGAGC R-CCCATGATAAGAGTGCAGATGACT	17 (17)	65
BM1818	F-AGCTGGGAATATAACCAAAGG R-AGTGCTTTCAAGGTCCATGC	23 (2p)	58
CSRM60	F-AAGATGTGATCCAAGAGAGAGGCA R-AGGACCAGATCGTGAAAGGCATAG	10(11)	55
HEL13	F- TAAGGACTTGAGATAAGGAG R- CCATCTACCTCCATCTTAAC	11	52
ILSTS019	F-AAGGGACCTCATGTAGAAGC R-ACTTTTGGACCCTGTAGTGC	29 (5p)	55
ILSTS025	F-GTTACCTTTATATAAGACTCCC R-AATTTCTGGCTGACTTGGACC	2	55
ILSTS028	F-TCCAGATTTTGTACCAGACC R-GTCATGTCATACCTTTGAGC	11	55
ILSTS029	F-TGTTTTGATGGAACACAGCC R-TGGATTTAGACCAGGGTTGG	3 (6)	55
ILSTS030	F-CTGCAGTTCTGCATATGTGG R-CTTAGACAACAGGGGTTTGG	2 (2q)	55
ILSTS033	F-TATTAGAGTGGCTCAGTGCC R-ATGCAGACAGTTTTAGAGGG	12 (13)	55
ILSTS036	F-GAGTATTATGCTTGGGAGGC R-AGACAGGATGGGAAGTCACC	11	55
ILSTS056	F-GCTACTGAGTGATGGTAGGG R-AATATAGCCCTGGAGGATGG		55
ILSTS058	F-GCCTTACTACCATTTCCAGC R-CATCCTGACTTTGGCTGTGG	17	55
ILSTS061	F-AAATTATAGGGGCCATACGG R-TGGCCTACCCTACCATTTCC	15	55
ILSTS089	F-AATTCCGTGGACTGAGGAGC R-AAGGAACTTTCAACCTGAGG	29(5p)	55
ETH003	F-GAACCTGCCTCTCCTGCATTGG R-ACTCTGCCTGTGGCCAAGTAGG	19 (3p)	58

Table no 2

Markers wise allelic diversity in Bhangor buffalo population.

Locus	Observed number of alleles *Na*	Effective number of alleles *Ne*	Shannon's Information Index *I*	Observed heterozygosity H_o	Expected heterozygosity H_e	Allele size (bp)	Nei expected heterozygosity H_e	PIC value
CSSM33	8	4.340	1.715	0.840	0.770	157-179	0.785	0.740
BM1818	9	4.614	1.742	0.850	0.784	244-300	0.804	0.776
CSRM60	3	1.865	0.725	0.091	0.464	92-132	0.475	0.432
HEL13	9	3.070	1.412	0.968	0.674	174-200	0.685	0.627
ILSTS019	6	2.207	1.093	0.129	0.547	175-182	0.556	0.538
ILSTS025	3	1.072	0.174	0.069	0.067	113-144	0.068	0.03
ILSTS028	10	5.095	1.831	0.656	0.804	143-175	0.816	0.750
ILSTS029	8	2.379	1.332	0.677	0.580	156-170	0.589	0.545
ILSTS033	6	3.165	1.404	0.560	0.684	140-152	0.698	0.629
ILSTS036	6	3.189	1.370	0.615	0.686	124-172	0.700	0.646
ILSTS056	12	5.143	2.020	0.500	0.823	144-182	0.823	0.785
ILSTS058	8	6.672	1.958	0.714	0.850	130-144	0.866	0.848
ILSTS061	12	5.523	2.054	0.774	0.819	137-161	0.832	0.802
ILSTS089	7	4.133	1.604	0.391	0.758	114-128	0.775	0.697
ETH003	7	4.056	1.652	0.167	0.753	109-133	0.786	0.741
Mean	7.600	3.769	1.472	0.533	0.670		0.684	
SE	0.689	0.396	0.131	0.077	0.052		0.053	

Table no 3

Mutations drift equilibrium, heterozygosity excess/deficiency under different mutation models in Bhangor buffalo population.

Test	Parameters	IAM	SMM
Sign Test	Observed no. of loci with H_e excess	16	2
	Expected no. of loci with H_e excess	14.15	14.22
	Loci with heterozygosity deficiency	8	22
	p-value	0.2911	0.000
Standardized difference test	T2 value	0.562	-8.835
	p-value	0.287	0.287
Wilcoxon sign rank test	*p* (one tail for H deficiency)	0.855	0.000
	p-value (one tail for H excess)	0.151	1.000
	p-value (two tail test for H_e excess or deficiency)	0.302	0.00001

Estimation based on 1000 replications; He: heterozygosity excess expected; p: probability; IAM: infinite allele model; SMM: step-wise mutation model.

Figures

Figure 1

Bhangor adult Female buffalo

Figure 2

Bhangor adult male buffalo

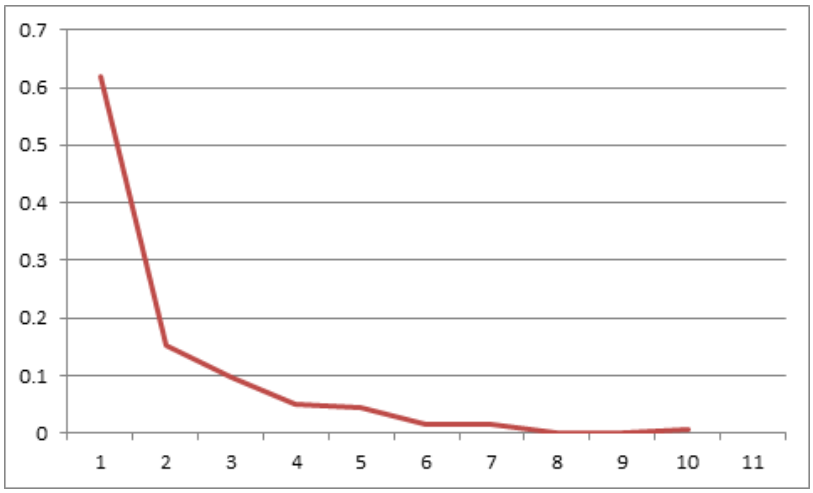

Figure 3

L- Shaped mode-shift graph showing lack of bottleneck in Bhangor population