Research paper

STR markers based genetic diversity evaluation of Chilika buffalo of Odisha state

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ABSTRACT

The study was conducted to genetically characterize the Chilika buffalo breed of Odisha state using microsatellite (STR) markers allelic diversity. Blood samples of 48 unrelated animals were collected randomly from different regions of breeding tract and DNA was extracted from whole blood using standard protocol. Genetic diversity was assessed using 23 heterologous bovine microsatellite markers previously reported, through PCR amplification and fragment analysis on an automated DNA sequencer. A sufficient allelic diversity was observed with a mean of 3.97 alleles per locus. The mean observed heterozygosity in the population was 0.593, suggesting animals under random mating. Additionally, three quantitative tests were employed namely the sign test, standardized difference test, and Wilcoxon sign rank test along with a qualitative test for mode shift distortion of allelic frequencies, which showed that the population has not undergone any genetic bottleneck in the recent past. The results indicate Chilika buffalo though a small population has been maintaining sufficient genetic diversity.

Key Words: allelic diversity, bottleneck, Chilika buffalo, microsatellite markers, genotyping
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INTRODUCTION

India has a huge diversity of buffalo population from diverse geographical and agro-climatic zones comprising more than 50% of world's buffalo population, accounting for highest buffalo milk production in the world (Kathiravan et al., 2012). In India, along with the 13 registered buffalo breeds/populations, there are many non-descript buffalo populations, with significant contribution to milk as well as meat production, besides being important part of draft power in agricultural operations. These breeds/populations are well adapted to the agro-climatic conditions of their respective breeding tracts (Kataria et al., 2009). Among the 13 registered buffalo breeds, Chilika is one of the *sui generis* riverine buffalo breed of India, found around Chilika Lake in Odisha mainly in Puri, Ganjam, and Khorda districts. Chilika has its abode in the largest brackish lagoon of Asia, 'Chilika Lake' after which the buffalo breed has been named (Patro et al., 2003). As per breed-wise survey of Department of Animal Husbandry & Dairying, total population of the Chilika buffalo is estimated to be around 3,000 (http://dahd.nic.in/documents/statistics/livestock-census). Local farmers rear these buffaloes due to their unique potential to convert the wild brackish water vegetation of Chilika Lake to nutritionally superior milk and this forms the major part of their income source with zero input (Singh et al., 2017).

Furthermore, this breed shares home tract with several other buffalo breeds in the proximity. In recent times due to breed improvement policy of the Odisha state government, high milk yielding

Murrah breed has been introduced in the region around native tract of Chilika buffalo. This infiltration may pose a threat to the unique germplasm of 'Chilika' and other nearby breeds; and possibly distort the native population's genetic structure. Additionally, the introgression of improver breeds like Murrah in this area could initiate vicious circle because of their non-adaption to the local conditions. Such an introgression will not only dilute this important buffalo germplasm but also disturb the Chilika Lake ecosystem as outside breeds introduced are mostly stall fed. Moreover, those breeds won't be as resistant to fasciolosis and many other parasitic infections as observed among the native Chilika buffaloes. Thus, in order to mitigate these ecological disruptions and conserve the Chilika breed, there is an urgent need to demarcate the genetic structure of this unique germplasm as a part of monitoring the small population for maintaining sufficient genetic diversity.

Among various genetic markers, the microsatellite (also known as STR, SSR) are the choice of markers to study population genetics since they are not only highly polymorphic with absence of null alleles but also distributed globally in the genome (Dodgson et al., 1997). These markers show locus specificity and can be analysed using simple PCR amplification (Kataria et al., 2009) and amenable to genotyping using automated sequencer. These properties of microsatellite marker's enable the analysis of several parameters of population genetics such as populations' structure, relatedness between them, and bottlenecks. In the present study genetic diversity and bottleneck in Chilika buffalo population was assessed using data generated on 23 heterologous bovine microsatellite markers. The fluorescent labeled specific primers were used for evaluating the allele sizes of various loci among different Chilika animals within the breed.

MATERIALS AND METHODS

Blood samples collection and DNA isolation

Blood samples of 48 unrelated animals of Chilika buffalo breed were collected randomly from the breeding tract of Odisha state of India. It was ensured to collect samples from both side land area surrounding Chilika Lake selecting the animals true to breed and ensuring them being from unrelated animals, covering almost 20 different villages of Krushnaprasad and Brahmagiri blocks of Puri district and Bhusandpur block of Khorda district. Genomic DNA was isolated from blood samples using standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

PCR amplification and genotyping of STR markers

PCR amplification of microsatellite (simple tandem repeats, STR) loci was carried out after testing quality and quantity of DNA samples on agarose gel as well as by measuring OD260 and OD280 on a Nanodrop (Thermo Fisher Scientific). A set of 23 microsatellite markers reported earlier (Kataria et al., 2009) for the genetic diversity analysis of buffaloes were used in the present study (Table 1). The forward primer for each marker was fluorescently labelled with FAM, NED, VIC or PET dyes. Polymerase chain reaction (PCR) was performed in a total reaction volume of 25 µl, using the thermal conditions as followed 94°C for 2 min, 32 cycles of 94°C for 1 min, specific annealing temperature for 1 min (Table 1), 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified PCR products checked on agarose gel containing different dyes were then electrophoresed together after multiplexing in different sets, considering the expected size and dye labels, in an automated DNA sequencer along with GS500LIZ (Applied Biosystems, USA) as an internal lane control.

Data analysis

After acquiring the raw run data obtained after fragment analysis on automated DNA sequencer, the allele size data for each sample was then extracted using *GENEMAPPER* software (Imle, 2005) and further analyzed for heterozygosity; allele numbers, observed and expected; *Fis* for inbreeding or loss of heterozygosity locus –wise, by using *GenAlEx* 6.5 software (Peakall and Smouse, 2015). Polymorphism information contents (*PIC*) values for each marker were derived by using the

Table 1. Details of heterologous bovine microsatellite markers used to study genetic diversity in Chilika buffalo

Microsatellite Locus	Primers Sequence	Allele Size Range in Buffalo	Annealing Temperature (°C)
CSRM60	For- 5'-AAGATGTGATCCAAGAGAGAGGCA-3'		
	Rev-5'-AGGACCAGATCGTGAAAGGCATAG-3'	160-188	55.0
ILSTS026	For- 5'-CTGAATTGGCTCCAAAGGCC -3'		
	Rev-5'-AAACAGAAGTCCAGGGCTGC-3'	141-159	55.0
HEL13	For-5'-TAAGGACTTGAGATAAGGAG-3'		
	Rev-5'-CCATCTACCTCCATCTTAAC -3'	166-192	55.0
ILSTS030	For- 5'-CTGCAGTTCTGCATATGTGG -3'		
	Rev-5'-CTTAGACAACAGGGGTTTGG-3'	146-158	55.0
ILSTS033	For- 5'-TATTAGAGTGGCTCAGTGCC -3'		
	Rev-5'-ATGCAGACAGTTTTAGAGGG-3'	126-138	58.4
ILSTS019	For- 5'-AAGGGACCTCATGTAGAAGC -3'		
	Rev-5'-ACTTTTGGACCCTGTAGTGC-3'	161-181	56.0
ILSTS058	For- 5'-GCCTTACTACCATTTCCAGC -3'		
	Rev-5'-CATCCTGACTTTGGCTGTGG-3'	118-170	55.0
ILSTS056	For- 5'-GCTACTGAGTGATGGTAGGG -3'		
	Rev-5'-AATATAGCCCTGGAGGATGG-3'	140-172	55.0
ILSTS089	For- 5'-AATTCCGTGGACTGAGGAGC -3'		
	Rev- 5'-AAGGAACTTTCAACCTGAGG -3'	118-126	61.0
CSSM66	For- 5'-ACACAAATCCTTTCTGCCAGCTGA -3'		
	Rev-5'-AATTTAATGCACTGAGGAGCTTGG-3'	168-202	55.0
ILSTS095	For-5'-GAAAGATGTTGCTAGTGGGG-3'		
	Rev-5'- ATTCTCCTGTGAACCTCTCC -3'	197-205	58.0
ILSTS029	For- 5'-TGTTTTGATGGAACACAGCC -3'		
	Rev-5'-TGGATTTAGACCAGGGTTGG-3'	140-168	54.0
ILSTS028	For- 5'-TCCAGATTTTGTACCAGACC -3'		
	Rev-5'-GTCATGTCATACCTTTGAGC-3'	141-169	55.0
ILSTS025	For- 5'-GTTACCTTTATATAAGACTCCC -3'		
	Rev-5'-AATTTCTGGCTGACTTGGACC-3'	116-136	56.0
ILSTS052	For- 5'-CTGTCCTTTAAGAACAAACC -3'		
	Rev-5'-TGCAACTTAGGCTATTGACG-3'	147-179	55.0
ILSTS060	For- 5'-TAGGCAAAAGTCGGCAGC -3'		
	Rev-5'-TTAAGGGGACACCAGCCC-3'	160-188	65.0
BM1818	For- 5'-AGCTGGGAATATAACCAAAGG -3'		
	Rev-5'-AGTGCTTTCAAGGTCCATGC-3'	241-253	56.0
ILSTS061	For- 5'-AAATTATAGGGGCCATACGG -3'		
	Rev-5'-TGGCCTACCCTACCATTTCC-3'	105-133	55.0
CSSM33	For- 5'-CACTGTGAATGCATGTGTGAGC-3'		
	Rev-5'-CCATGATAAGAGTGCAGATGACT -3'	155-177	61.0
CSSM19	For- 5'-TTGTCAGCAACTTCTTGTATCTTT -3'		
	Rev-5'-TGTTTTAAGCCACCCAATTATTTG -3'	131-161	55.0
CSSM57	For- 5'-GTCGCTGGATAAACAATTTAAAGT -3'		
	Rev-5'-TGTGGTGTTTAACCCTTGTAATCT -3'	103-131	60.0
CSSM47	For- 5'-TCTCTGTCTCTATCACTATATGGC -3'		
	Rev-5'-CTGGGCACCTGAAACTATCATCAT-3'	127-163	55.0
CSSM45	For- 5'-TAGAGGCACAAGCAAACCTAACAC -3'		
	Rev-5'-TTGGAAAGATGCAGTAGAACTCAT -3'	102-122	60.0

formula given by Botstein et al. (1980). *PIC* takes into consideration the number of alleles a marker locus has and the frequency of these alleles for each individual marker. Mode shift analysis was carried out to generate information about population having or not suffered from any bottle neck during recent past with the help of online available *Bottleneck* software (Cornuet et al., 1997).

RESULTS AND DISCUSSION

Previously, microsatellite markers based genetic diversity analyzed using PAGE gel has been reported in Chilika buffaloes (Mishra et al., 2009), but in the present study samples collected were

Table 2. Genetic variability measures in Chilika buffalo across different microsatellite markers

S.No.	Marker	Alleles*		Heterozygosity*		PIC	Fis
		Na	Ne	Ho	He		
1.	ILSTS89	7.000	3.695	0.756	0.729	0.683501	-0.036
2.	ILSTS95	4.000	1.884	0.277	0.469	0.385811	0.411
3.	CSSM47	13.000	7.030	0.848	0.858	0.842725	0.012
4.	ILSTS33	5.000	3.648	0.652	0.726	0.675518	0.102
5.	ILSTS60	7.000	1.842	0.739	0.457	0.427463	-0.617
6.	BM1818	11.000	4.062	0.667	0.754	0.724284	0.116
7.	ILSTS19	6.000	1.630	0.050	0.387	0.371061	0.871
8.	ILSTS25	7.000	4.213	0.585	0.763	0.736868	0.232
9.	ILSTS56	13.000	3.732	0.435	0.732	0.696795	0.406
10.	HEL013	11.000	6.443	0.795	0.845	0.827747	0.058
11.	ILSTS28	12.000	4.172	0.638	0.760	0.725551	0.160
12.	ILSTS058	17.000	6.991	0.723	0.857	0.844065	0.156
13.	ILSTS61	7.000	2.759	0.489	0.638	0.602802	0.233
14.	CSSM19	9.000	4.615	0.652	0.783	0.755011	0.167
15.	CSSM57	9.000	2.818	0.659	0.645	0.594232	-0.022
16.	ILSTS52	9.000	3.659	0.800	0.727	0.685734	-0.101
17.	CSSM45	11.000	5.275	0.432	0.810	0.788447	0.467
18.	CSSM66	16.000	7.337	0.622	0.864	0.850177	0.280
19.	ILSTS26	11.000	2.152	0.340	0.535	0.521193	0.364
20.	ILSTS29	14.000	6.027	0.622	0.834	0.815543	0.254
21.	ILSTS30	10.000	2.866	0.778	0.651	0.597307	-0.195
22.	CSRM60	10.000	2.563	0.617	0.610	0.562282	-0.012
23.	CSSM33	7.000	2.043	0.457	0.511	0.49117	0.106
	Mean	9.826	3.976	0.593	0.693	0.661099	0.69322

**Na-observed number of alleles, Ne-effective number of alleles, Ho-observed heterozygosity, He-expected heterozygosity, PIC-polymorphism information contests, fiss-inbreeding coefficient*

from the animals randomly distributed across eastern as well as western areas of Chilika Lake and also fluorescent labelled specific primers were used and genotyped on automated DNA sequencer.

A set of 23 heterologous bovine microsatellite markers, reported earlier (Kataria et al. 2009) used to successfully amplify 48 random DNA samples of Chilika buffalo. Different variability measures within breed estimated for each locus in Chilika buffalo viz., number of observed alleles (*Na*), effective number of alleles (*Ne*), observed (*Ho*) and expected heterozygosity (*He*) are given in Table 2. A high allelic diversity was observed in Chilika buffalo with a total number of 226 distinct alleles across 23 loci (Figure 1). Most of the loci exhibited high level of allelic polymorphism as observed number of alleles varied from 4 (ILSTS95 locus) to 17 (ILSTS058 locus) at different loci with a mean number of 9.82 alleles (Table 2). The effective numbers of alleles were less than the observed values across all the loci and ranged between 1.63 (ILSTS019 locus) to 7.33 (CSSM66 locus) with a mean of 3.97. The allelic values were higher than reported in previous studies conducted on Indian buffaloes (Navani et al., 2002; Mishra et al., 2009; Mishra et al., 2010) and therefore it signifies higher allelic diversity in Chilika buffaloes. On the other hand mean number of different alleles and effective number of alleles along with expected and observed heterozygosity of Chilika buffalo were comparable with that of reports on few other buffalo breeds

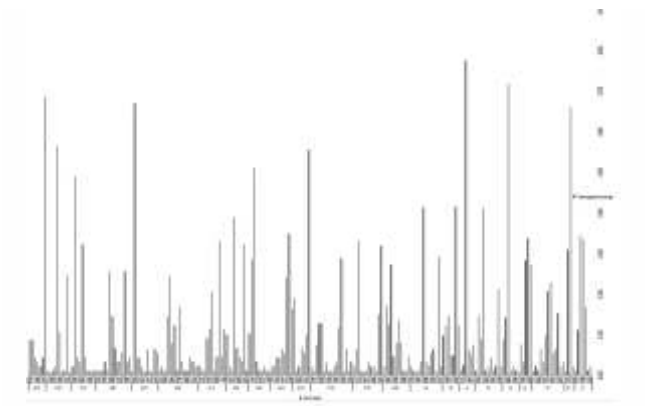

Figure 1. Allele frequencies observed across 23 microsatellite loci (listed in Table 2) in Chilika buffalo

Table 3. Test for null hypothesis for mutation drift equilibrium under three mutation model (IAM, TPM and SMM) using sign rank, standardized differences and Wilcoxon tests in Chilika buffalo

Test	Parameter	IAM	TPM	SMM
Sign Test	Observed no. of loci with He excess	13	6	2
	Expected no. of loci with He excess	13.82	13.64	13.6
	p-value	0.44	0.00129*	0*
Standardized Difference Test	T2	-0.43	-5.695	-15.538
	p-value	0.33346	0*	0*
Wilcoxon Sign Rank Test	p-value (two tail test for He excess and deficiency)	0.86967	0.00307*	0*

(p < 0.01)*

(Vijh et al., 2008). The range of observed heterozygosity was found to be 0.277-0.848 for ILSTS95 and CSSM47 locus. Similarly, expected heterozygosity was ranging from 0.387-0.864 for ILSTS19 and CSSM66 locus. The higher value of expected heterozygosity (0.693) in this breed might possibly be due to the presence of number of different alleles even at small frequencies in Chilika population. Polymorphism Information Contents (PIC) values ranged from 0.37 (ILSTS019) to 0.85 (CSSM66) with a mean of 0.66, indicating suitability of markers used for genetic diversity analysis.

The sufficiently high average values for Na (9.826), He (0.693) and PIC (0.66) displayed by the panel of 23 microsatellite markers further supported their suitability for genetic diversity analysis in other buffalo breeds as well. In the present investigation, heterozygote deficiency analysis was evaluated on the basis of obtained Fis (within-population-inbreeding estimate) values. If the Fis value is more than zero then it represents the condition of heterozygote deficiency for that particular locus and several loci had positive value i.e. more than zero revealing heterozygote deficiency (Table 2). Several loci contributed to this deficiency (17/23), but five loci contributed maximally for heterozygote deficiency, which were ILSTS95, ILSTS19, ILSTS56, CSSM45 and ILSTS26 with values of 0.411, 0.871, 0.406, 0.467 and 0.364, respectively. A number of factors affect the level of heterozygosity but among them the inbreeding, presence of population sub-structure (Wahlund effect), and null alleles are the prominent reasons. The justification of single specific factor affecting heterozygosity is tedious matter (Barker et al., 2001) since lack of heterozygosity might not be only due to aforesaid reasons alone but also may result from cumulative effect of more than one factors. Additionally, in the field area non-availability of pedigree data is a big problem when uncontrolled natural service is followed for breeding the animals, so lack of heterozygosity might also be due to the inadvertent sampling of few related animals, still in this study just five of the markers mentioned above showed high Fis values, rests were showing sufficient heterozygosity extent.

The bottleneck analysis in Chilika buffalo was demonstrated in this study on the basis of heterozygosity excess by using different approaches, the first approach is based on the principle of heterozygosity excess, which works upon the level of heterozygosity in a manner that expected equilibrium gene diversity should be lower than the observed gene diversity in a recently bottlenecked population and it is calculated on the basis of observed number of alleles with the

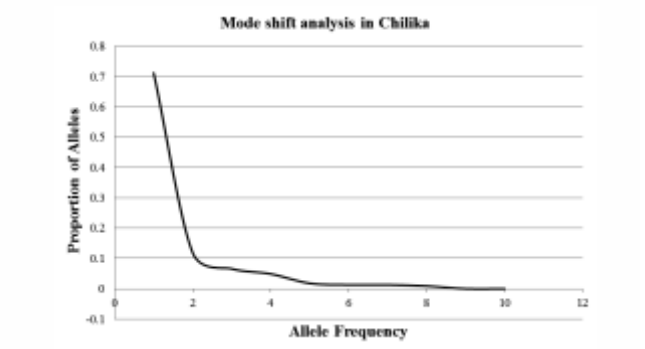

Figure 2. Mode shift analysis in Chilika buffalo showing normal L-shaped distribution of allelic frequencies without suffering any recent bottleneck

assumption of a constant-size (equilibrium) population (Luikart et al., 1998). Three different test parameters viz. sign rank, standardized differences and Wilcoxon tests were employed under all the 3 models, Infinite Allele Model (IAM), Stepwise Mutation Model (SPM) and Two Phase Model (TPM) of microsatellite evolution to investigate whether Chilika has experienced any recent bottleneck (Table 3). Results of sign rank test under IAM mutation model revealed value of 13.82 as expected number of loci with heterozygosity excess on the other hand the observed number of loci with heterozygosity excess was 13. In the case of TPM and SMM, the expected and observed loci with heterozygosity excess were 13.64 and 6; 13.6 and 2, respectively. The values for three tests conducted for the Chilika buffalo significantly deviated and thereby null hypothesis of mutation drift equilibrium was rejected indicating the absence of genetic bottleneck in the recent past. The probability values for sign rank test under IAM (p = 0.44000), was not significantly different but for TPM (p = 0.00129) and SMM (p = 0.00000) were significantly different (Table 3). This again supports the rejection of null hypothesis.

Another influential assessment of qualitative graphical method which is based on mode-shift distortion was also used in this study to visualize the allele frequency distribution as an indicator for genetic bottleneck. The results indicated the absence of any recent bottlenecks in Chilika buffalo and no mode shift (Luikart and Cornuet, 1997) was found in the population (Figure 2), which is similar to previous reports on Chilika and other buffalo breeds (Kataria et al., 2010).

The present outcomes thus emphasize and re-verify the efficacy of these microsatellite markers to assess the existing genetic variability in one of the important buffalo breeds of Odisha state of India. Number of diversity analysis parameters suggests abundance of genetic variability in Chilika buffalo for its sustenance and the results obtained will be useful for its future-breeding programme. Further, it will be interesting to explore microsatellite based diversity analysis in other Odisha buffalo breeds like Paralakhemundi, Kalahandi, Sambalpuri and Manda which would be quite valuable in assessing the genetic variability among different buffalo breeds/populations to assess the genetic relationships among them and thus quite effective in designing/planning the different breeding and conservation strategies.

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