

Cytogenetic profiling and mitochondrial DNA analysis reveal existence of swamp buffalo population in Manipur state

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ABSTRACT

India is the home of best milch buffalo breeds in the world. Majority of them are riverine type found in almost the entire country extending from north to south and west. However buffalo populations in the eastern and north-eastern region are yet to be precisely classified, though these have been generally considered to be swamp type based on their phenotypic resemblance to swamp type buffaloes. Manipuri buffaloes, essentially reared for draught and meat purposes have been characterized phenotypically and cytogenetically in this study. Morphometric data collected from 56 adult buffaloes from hilly and plain areas revealed differences in their body measurements. Cytogenetic analysis of 40 randomly selected animals (7 males and 33 females) from both the locations revealed diploid chromosome count of 48, twenty-three pairs of autosomes and a pair of sex chromosomes, typical of swamp type buffaloes. Most distinctive karyotypic feature of swamp buffalo observed in all the animals was the size of the fourth pair of metacentric chromosome. Comparison of mitochondrial D-loop sequence of these buffaloes with representative riverine and swamp type buffaloes further corroborated this conclusion. This report, thus, is the first confirmed documentation of existence of pure swamp type buffaloes in Manipur state.

Key words: Karyotype, Manipuri swamp buffalo, mtDNA analysis, Swamp buffalo

The domestic river buffalo (*Bubalus bubalis*) stands next only to cattle in its utility, mainly through its enormous contribution in the form of milk, meat, draught, hide and manure. India possess a diverse gene pool of buffaloes ranging from high-yielding dairy type (Murrah and Nili-Ravi) to extensively managed draught type buffaloes (Assamese and Toda). Majority of the buffaloes of the north-eastern and eastern region of India resemble swamp buffaloes in their external morphological features, low milk yield, utility in draught work in marshy lands and small body size. No demarcation line in the distribution of river and swamp type buffaloes (north-south, east-west) within India was established, though the existence of swamp type buffaloes in eastern and north-eastern part of India has been acknowledged (Bidhar *et al.* 1986, Yadav *et al.* 1988).

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The buffaloes from Asom have generally been described as swamp type, exclusively based on their morphological similarity (Tamuly *et al.* 1982, Das *et al.* 2005). However, there has been no systematic study to characterize the buffalo germplasm of the north-east region of India, which is possibly the region for the river-swamp division. Also this region is the home of the Asiatic wild buffalo (*Bubalus arnee*), which is thought to be the progenitor of the domesticated water buffalo (Mathur *et al.* 1995, Lei *et al.* 2007). Buffaloes in Manipur state of north-eastern India have significant contribution towards draught power and as meat animal. The present study was thus undertaken to characterize and establish the status of the Manipuri buffaloes, an important yet unexplored buffalo genetic resources of the north-east India.

MATERIALS AND METHODS

Information on Manipuri buffalo regarding its habitat, distribution and management practices was collected through preset questionnaires. Data regarding various morphometric traits was recorded on 56 adult buffaloes from the hilly and valley areas of Manipur state comprising the districts of Senapati, Chandel and Thoubal. Blood samples were

collected from unrelated animals of both sexes from various villages in the breeding tract for cytogenetic and mitochondrial DNA analysis.

Cytogenetic analysis

Blood leukocyte cultures for 40 Manipuri buffaloes (7 males + 33 females) randomly selected from different locations (both hill and valley region) of the breeding tract 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.

Analysis of mitochondrial D-loop sequence variation

DNA samples were isolated following standard phenol-chloroform isolation procedure (Sambrook and Russel 2001). Mitochondrial D-loop region was amplified using 16 representative unrelated DNA samples of Manipuri buffaloes. The D-loop region of ~ 1.1 kb was amplified using specific primers reported by Sutarno and Lymbery (1997). The primer sequences were: Mito (D-Loop) F: 5' TAGTGCTAATACCAACGGCC 3'; Mito (D-Loop) R: 5' AGGCATTTTCAG TGCCTTGC 3'. The PCR cycling conditions were: initial denaturation at 94°C for 6 min followed by 30 cycles of 94°C for 45 sec; 60°C for 45 sec; 72°C for 1 min with final extension at 72°C for 10 min. The PCR products were analysed on 1% TAE-agarose gel.

Sequencing and sequence analysis

Direct sequencing of specific amplified PCR product was employed to analyze unknown polymorphism in the mitochondrial D-loop region. Representative samples of Manipuri buffalo population were sequenced with forward or reverse primers and compared to detect sequence variations if any. Individual chromatograms were edited and

ambiguous and skipped bases were disregarded. The edited nucleotide sequences (729 bp each) were aligned and compared amongst the buffalo samples to identify point mutations/variations. Multiple alignment of the edited nucleotide sequences was performed using 'MegAlign' program of 'Lasergene' software package. Mt-DNA D-loop haplotype sequences of Manipuri buffaloes were compared with that of different buffalo populations: Murrah Accession Nos. (AY475204–AY475218), Chinese buffaloes - 29 haplotypes (Lei *et al.* 2007), Carabao and Mediterranean buffaloes – 10 haplotypes (Kierstein *et al.* 2004), while *Bos taurus* (Accession No. AY526085) was used as an out-group. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.* 2007).

RESULTS AND DISCUSSION

Manipuri buffaloes are distributed in the hilly as well as valley/plain regions of different parts of Manipur state. Based on the habitat and distribution, Manipuri buffaloes are locally classified into 2 types, viz. hill type locally termed as "*Tamgi-eroi*" mostly distributed in Chandel, Churachandpur, Ukhrul, Senapati and Tamenglong districts while the valley type locally named as "*Chingi-eroi*" are scattered in Imphal East, Imphal West, Bishnupur and Thoubal districts of the state. The population of Manipuri buffaloes is estimated around 0.77 lakh (Livestock Census 2003). The population trend indicates a staggering decline of about 45% during last 20 years. This vertical decrease in the buffalo population of the state is a subject of concern especially when the overall buffalo population of the country is showing a rising trend all through these years.

The Manipuri buffaloes in the hilly region are mainly used

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

Trait	Manipur-Hill (N=19)		Manipur-Valley (N=37)	
	Mean	Range	Mean	Range
Height at wither**	124.16±1.07	118–134	133.69±1.00	118–147
Body length**	122.68±2.09	104–134	129.53±1.75	110–150
Heart girth**	199.26±1.82	182–213	188.95±2.27	170–215
Face length ^{NS}	43.37±0.81	38–54	44.61±0.64	37–52
Tail length ^{NS}	68.68±1.53	56–83	69.83±1.52	33–84
Horn length ^{NS}	55.11±2.28	42–75	58.59±1.79	41–79
Distance between hip bones**	18.79±0.66	15–24	21.19±0.32	18–26
Horn width at base**	11.42±11.42	9–19	9.62±0.27	8–13
Paunch girth**	207.42±1.80	197–218	183.69±2.09	165–203
Ear length ^{NS}	22.00±0.43	18–26	22.31±0.96	11–28

**Highly significant; ^{NS}Not significant.



Fig. 1. Manipuri buffaloes showing typical white markings.

for draught and meat purpose while the animals in the plains/valley are used primarily for agricultural operations, viz. ploughing and puddling the wet rice fields and carting. Adult male or female buffaloes are mostly used as single animal to plough fields with locally designed ploughs. In the hilly terrain, these buffaloes are also used to pull logs of wood from the mountainous terrain where these buffaloes can walk and climb at ease. In the hilly region, these buffaloes are also used for meat purpose and the farmers never milk their buffaloes. Animals are reared primarily under zero-input

system. No housing is provided in the hilly tract while in the valley, animals are provided *thatched* house open on all sides except for roof. In the hilly region, buffaloes are largely let loose in the forests for grazing and stay in the forests even at night. Only during the rice cultivation season (May to July) the buffaloes are kept at home and used for ploughing the rice fields. Ploughing is done mostly during morning hours for 3 to 4 hours and one adult buffalo can plough about 1/4 acre of land. The load carrying capacity is about 5 to 6 quintal and one adult male Manipuri buffalo can pull around 40 cu

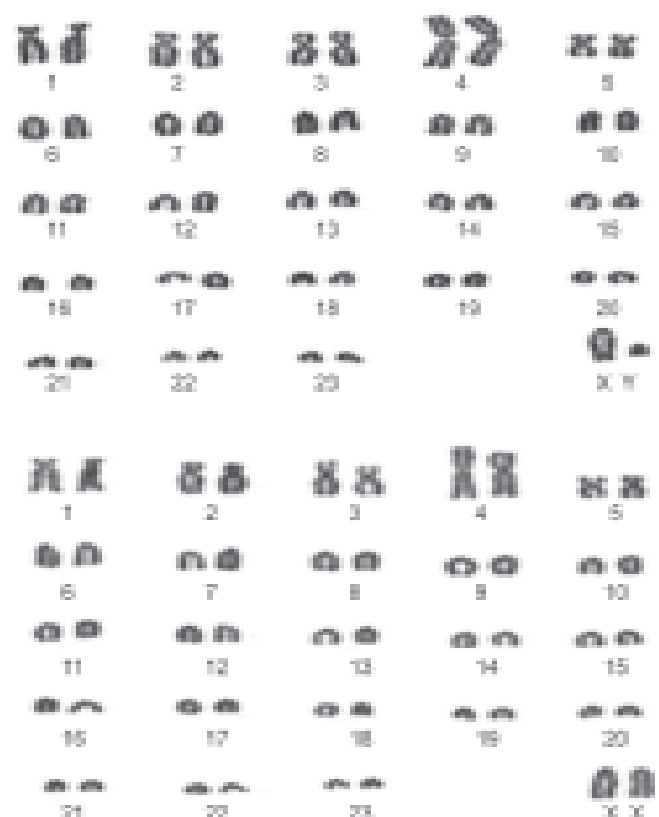


Fig. 2. Metaphase spread and karyotype of Manipuri male (A) and female (B) buffaloes showing $2n=48$ chromosomes typical of swamp type buffaloes.

ft log while a female buffalo can pull 10 to 20 cu ft. Both sexes are used for agricultural work. During paddy cultivation season, farmers even hire Manipuri buffalo for ploughing and leveling of wet rice fields. For rest of the period the animals remain in forests and rarely provided any additional feed or fodder except occasional salt lick.

Various body measurements of the two types of Manipuri buffaloes (hill and valley type) were recorded and evaluated. The Manipuri buffaloes are generally grey to greyish-black in colour. The horn is sickle shaped (curved) with broad horn base and mostly corrugated. The ear is horizontally placed with hairs on inner and outer margins. The forehead is mostly convex. The buffaloes are medium built and compact with strong legs. Typical white or grayish-white colour is observed between hoofs and knee in majority of animals. The tail switch is generally black. The udder is bowl shaped and poorly developed. The teats are small and cylindrical with round or pointed tips. The hill type animals are comparatively shorter, as reflected in their mean body length and height at withers ($P<0.01$). However, they are stouter than their valley counterparts with significantly higher ($P<0.01$) heart and paunch girth (Table 1). The Manipuri buffaloes have typical white markings on either side of muzzle and lower jaw. Two white stripes, one in the neck and another slightly above the brisket, represent another typical feature of Manipuri buffaloes (Fig. 1). Some animals had one white band in the neck region while in some the lower white band is split type.

Cytogenetic characteristics of Manipuri buffaloes

Distinctive karyotypic features established for swamp and river buffaloes were exploited to ascertain whether Manipuri buffaloes belong to the riverine or swamp category. Forty blood samples of both sexes, collected from varying locations of Manipur hill and plain regions, were utilized for cytogenetic analysis of Manipuri buffaloes. All the 40 animals exhibited a diploid count of $2n=48$ chromosomes, which is typical of swamp type buffaloes (Bongso *et al.* 1984, Sat 1987). This comprised 23 pairs of autosomes and a pair of sex chromosomes. Of the 23 pairs of autosomes, 5 were biarmed (metacentric/submetacentric) and 18 were

Table 2. Multiple alignment of different Manipuri mtDNA haplotypes showing sequence variations

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#Domain=Data;
[
      1111 1111111111 1112222333 45556677
      1245666777 7788990000 0122344446 6882468036 03470511
      1284569025 6903351268 9012501462 7344884560 47972546
#MN50-HAP14  TCGTGGACCA CTCGATCAAT GATGATCCCC CGTTGCGGCG AGTCCAC
#MN5-HAP1     CTA.AA..T..T.A..... AGCA.C.TT..A..AT.A...C..T.T
#MN8-HAP2     T.A.AAGTTG.....TGG.A.....T.TTACCATA.....GAC..TGT
#MN10-HAP3    T.G.....G..A.....C..C..T
#MN11-HAP4    T.....G.....T.....G.....T.....T.....
#MN12-HAP5    T.....G.....T.....G.....T.....T.....
#MN13-HAP6    T.....G.....G.....T.TT.....T.....
#MN18-HAP8    T.....C.....T.....T.....T.....
#MN19-HAP9    T.A.AAGTTG.....GG.A.....T.TTACCATA.....GAC..TGT
#MN22-HAP10   T.....T.....T.....T.....T.....
#MN24-HAP11   T.....G.....T.....T.....T.....
#MN29-HAP12   T.....G.....T.....T.....T.....
#MN33-HAP13   T.....T.....T.....T.....T.....

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acrocentric. Representative metaphase spreads and karyotypes of a female and male Manipuri buffalo are presented in Fig. 2. Further, the size of the fourth pair of metacentric chromosome in all the investigated Manipuri buffaloes were typical of swamp type resulting from the tandem fusion of chromosomes 4 (4p) and 9 (DiBerardino and Iannuzzi 1981, Chowdhary *et al.* 1989) of riverine type. Cytogenetic analyses thus confirmed the swamp status of Manipuri buffaloes of north-east India.

Analysis of mitochondrial DNA D-loop sequence variation

Sequence analysis of mt-DNA D-loop region (729 bp) belonging to 16 Manipuri animals revealed a total of 14 distinct haplotypes. The number of polymorphic sites within Manipuri buffaloes was 48 while the number of singletons was 14 (Table 2). A total of 14 D-loop haplotypes were detected in Manipuri, while 15 reference sequences of Murrah (AY475204 - AY475218) revealed 11 distinctive haplotypes. None of the Murrah and Manipuri haplotypes overlapped with each other indicating distinct haplotypic structure of each population (Fig. 3). This is evident from the river or swamp origin of the two buffalo populations. The frequencies of different D-loop haplotypes of Manipuri buffaloes

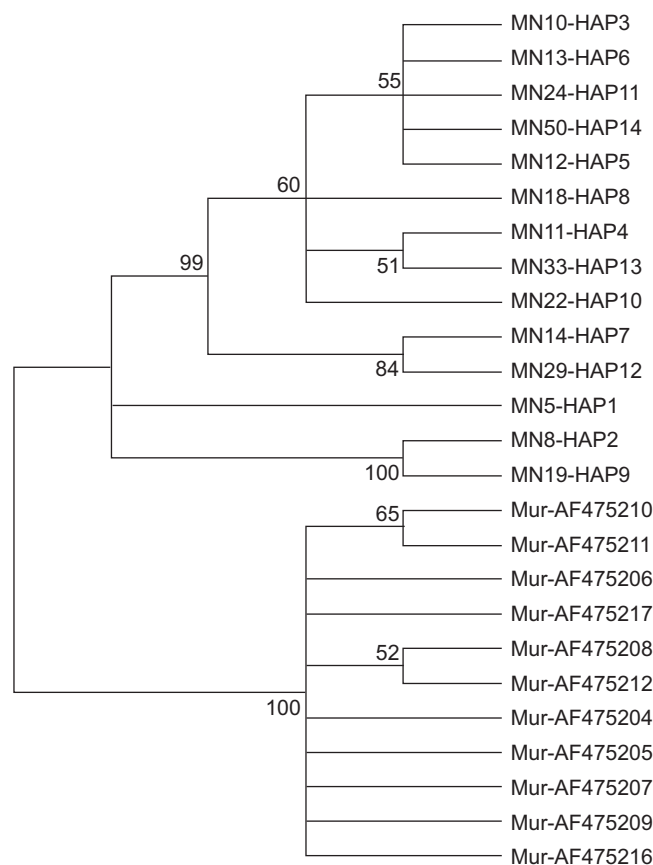


Fig. 3. Phylogenetic analysis of mtDNA haplotypes of Manipuri and Murrah buffalo populations following NJ procedure with 1000 bootstrap re-sampling

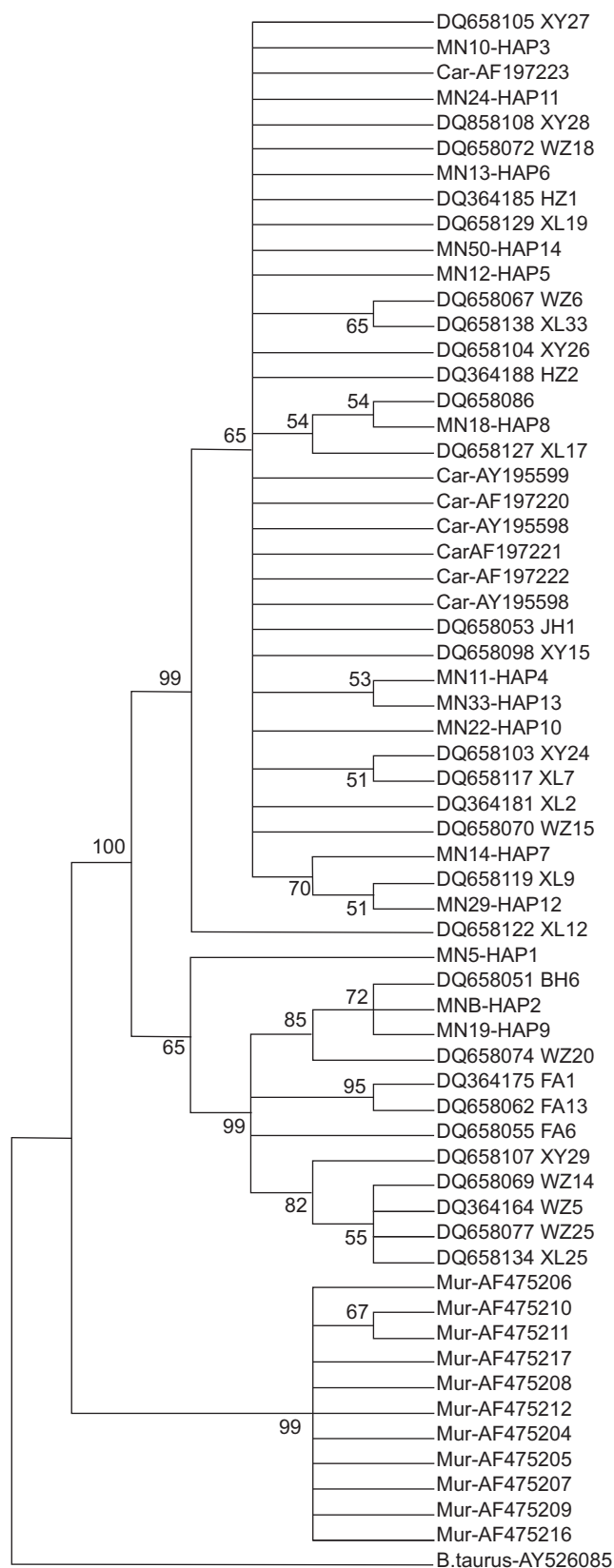


Fig. 4. Phylogenetic analysis of different mtDNA haplotypes of various riverine and swamp buffalo populations following NJ procedure with 1000 bootstrap re-sampling

indicated equal frequency (0.0625) for each of the haplotypes except MN19-HAP9 whose frequency was estimated to be 0.188.

The phylogenetic analysis of mt-DNA D-loop region clustered all Manipuri buffalo haplotypes in one clade with 100% bootstrap value while the Murrah buffaloes were found to be distinctly different and constituted a different cluster (Fig 3). Within the swamp buffalo clade, two internal branches with reasonable bootstrap values were observed. The Manipuri buffaloes are grouped along with the haplotypes reported for Chinese swamp buffaloes and also the Carabao haplotypes (Fig 4). This is in consistent with the recent findings that Chinese domestic swamp buffaloes have two distinct maternal lineages, of which lineage B have been explained by occasional introgression of wild buffalo into domestic swamp buffalo (Lei *et al.* 2007). Interestingly the Manipuri buffalo haplotypes investigated in this study clustered into both the reported swamp lineages, lending further support to the hypotheses of two separate lineages for swamp buffaloes. No specific grouping of haplotypes for the hill and valley Manipuri buffaloes into distinct classes was observed in the phylogenetic tree thus indicating a common origin for both the populations. Both phylogenetic trees positioned the river and swamp haplotypes into specific and distinct grouping, which confirmed the unequivocal classification of Manipuri buffaloes as true swamp type. Further analysis of larger number of samples, also involving other buffalo populations including Asiatic wild buffalo will clarify the phylogeography of swamp and river buffalo populations, their origin and evolution in the Indian sub-continent and demarcate the swamp-river divide in the north-east region of the country. In conclusion, this is the first report on Manipuri buffaloes as pure swamp type. Since there is a severe decline of this buffalo population, it warrants immediate intervention to further propagate and conserve this unique germplasm.

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