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Genetic variation and relationships among eight Indian riverine buffalo breeds

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Abstract

Twenty-seven microsatellite loci were used to define genetic variation and relationships among eight Indian riverine buffalo breeds. The total number of alleles ranged from 166 in the Toda breed to 194 each in the Mehsana and the Murrah. Significant departures from the Hardy–Weinberg equilibrium were observed for 26 locus-breed combinations due to heterozygote deficiency. Breed differentiation was analysed by estimation of F_{ST} index (values ranging from 0.75% to 6.00%) for various breed combinations. The neighbour-joining tree constructed from chord distances, multidimensional scaling (MDS) display of F_{ST} values and Bayesian clustering approach consistently identified the Toda, Jaffarabadi, and Pandharpuri breeds as one lineage each, and the Bhadawari, Nagpuri, Surati, Mehsana and Murrah breeds as admixture. Analysis of molecular variance refuted the earlier classification of these breeds proposed on the basis of morphological and geographical parameters. The Toda buffaloes, reared by a tribe of the same name, represent an endangered breed from the Nilgiri hills in South India. Divergence time of the Toda buffaloes from the other main breeds, calculated from Nei's standard genetic distances based on genotyping data on seven breeds and 20 microsatellite loci, suggested separation of this breed approximately 1800–2700 years ago. The results of the present study will be useful for development of rational breeding and conservation strategies for Indian buffaloes.

Keywords: conservation, divergence, genetic distance, heterozygosity, Indian buffalo, microsatellite

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Introduction

The water buffalo (*Bubalus bubalis*) contributes immensely to the agricultural economy through milk, meat, hides and draught power. A larger part of the human population depends on domestic water buffalo than on any other livestock species in the world (FAO & UNEP 2000). This species was distributed from southern Asia to Europe during the Pleistocene. Later on with increasing dry climatic conditions, its distribution was restricted to the Indian subcontinent and Southeast Asia (Cockrill & Mahadeven 1974; Nachtsheim & Stengel 1977). The water buffalo has been divided into two broad lineages on the basis of their gross behaviour and morphology (Macgregor 1939): the river buffalo, found in the Indian subcontinent and further west to the Balkans and Italy; and the swamp buffalo, distributed from Assam in India through Southeast Asia to the Yangtze valley of China in the east. There have been conflicting suggestions on domestication of this species. It is not yet settled whether separation of the two lineages, represented by river and swamp buffaloes, preceded their domestication (Cockrill 1974; Chen & Li 1989; Amano *et al*. 1994; Baker *et al*. 1997a, b; Lau *et al*. 1998; Ritz *et al*. 2000; Tanaka *et al*. 1995, 1996; Kierstein *et al*. 2004). It is generally believed that buffaloes were first domesticated in the Indus civilization some 5000 years ago (Cockrill 1974). However, Chen & Li (1989) have suggested that this species was domesticated in China during the fifth millennium BC.

River buffaloes are the mainstay of the dairy industry in India. Out of 90 million heads, approximately 70% buffaloes

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Fig. 1 Sampling sites of various Indian buffalo breeds. Numbers correspond to the following sampling sites with number of animals sampled in parentheses: 1, Surati (48); 2, Mehsana (48); 3, Jaffarabadi (47); 4, Toda (48); 5, Pandharpuri (48); 6, Nagpuri (48); 7, Bhadawari (48); 8, Murrah (48).

have been grouped as nondescript since these animals do not resemble any of the nine morphologically well-characterized breeds, namely Nili-Ravi, Murrah, Bhadawari, Mehsana, Jaffarabadi, Surati, Nagpuri, Pandharpuri and Toda. Generally small and marginal farmers rear buffaloes and the herd size is very small; large farms are rare. Various breeds have been developed mainly as a result of farmers' 'wisdom' over the past thousands of years; herd books and breed registration are not in vogue. Genetic improvement for milk production is limited to selection of bulls based upon their dams' yield and such bulls are primarily used through natural mating. Breed-wise census data are not available. However, grading up and substitution of local breeds by the Murrah, a well-known dairy breed originally from North India, is a common practice (George *et al*. 1988). The Toda breed (Nair *et al*. 1986), maintained by a tribe of the same name in the Nilgiri hills of South India, is endangered due to disappearance of pasture lands and upward social mobility of this tribe, reducing the population of this breed to less than 1000 heads. To design rational breeding strategies for optimum utilization and conservation of available genetic variability in Indian buffaloes, it is essential to understand their genetic architecture and relationships among various breeds. Genomic studies in buffaloes are scanty (Navani *et al*. 2002). In the present study, we have analysed microsatellite variation and relationships among eight Indian riverine buffalo breeds.

Materials and methods

Blood sampling and DNA isolation

Unrelated animals with typical phenotypic features known for a given breed were selected from several villages in the respective breeding tracts (Fig. 1). Although no parentage records were available, sampling from sibs was avoided deliberately by restricting the number of samples from a given hamlet. Three hundred and eighty-three animals representing eight well-recognized breeds were included in this study. Blood samples were collected from the jugular vein into ethylenediamine tetra-acetic acid (EDTA)-containing vacuutainer tubes. DNA was extracted from fresh blood following organic extraction method (Sambrook & Russel 2001).

Microsatellite loci studied

We have tested and characterized 495 cattle microsatellite primer pairs on a panel of 24 unrelated Murrah buffaloes and have estimated the level of heterozygosity for these markers (unpublished). All these markers were originally selected from BOVMAP database (available at www.marc.usda.gov/). Out of these, 27 moderately to highly polymorphic microsatellite loci distributed across 17 cattle chromosomes were selected for the present study (Table 1).

Table 1 Summary statistics of microsatellite markers as determined on a panel of 24 unrelated Murrah buffaloes

Locus	BTA	Number of alleles	Allele size range	Heterozygosity	Ann. temp. (°C)	Accession no.
BMS4012	1	6	95–105	0.29	58	G19068
BMS4016	1	7	129–157	0.96	58	G19058
BMS2519	2	5	104–118	0.75	58	G19007
BMS2847	8	5	206–216	0.71	58	G18988
ILSTS089	29	5	119–127	0.81	58	L37239
MSBQ	27	6	122–138	0.77	56	UniSTS:250824
AFR227	6	5	101–121	0.90	58	X83436
CA004	15	9	138–168	0.75	58	U32910
BMS1724	9	6	149–173	0.43	58	G18692
TGLA159	4	7	227–241	0.73	58	UniSTS:250994
BM1352	13	7	99–115	0.64	58	G18664
BM4513	14	5	128–144	0.86	58	G18507
RM372	18	6	123–141	0.81	56	G29112
BMS1226	13	7	154–174	0.86	58	G18635
0TGLA36	12	7	122–136	0.73	58	UniSTS:250943
BMS1316	12	6	113–129	0.71	58	G18654
ILSTS058	17	9	136–160	0.75	58	L37225
BL1029	14	5	144–158	0.82	58	UniSTS:251027
CSSM047	8	9	138–172	0.65	58	U03821
BL1036	14	6	177–191	0.86	56	UniSTS:251340
BL1134	10	5	100–118	0.46	58	UniSTS:251331
BMS518	6	6	152–166	0.78	58	G18861
BMS462	16	7	118–136	0.77	58	G18864
BM757	9	9	189–208	0.88	58	G18473
BMS2325	11	5	120–128	0.83	58	G18764
BMS2116	27	6	102–120	0.57	56	G18922
BMS1747	14	9	91–107	0.77	58	G18696

BTA, cattle chromosome.

Microsatellite genotyping

Polymerase chain reaction (PCR) analysis for microsatellites was performed using GeneAmp PCR system 9700 (Applied Biosystems) with one of the two primers for a given locus labelled with one of the four fluorescent dyes: NED, PET, VIC and FAM. Reaction components included Ampli*Taq* Gold PCR Master Mix (Applied Biosystems, Roche Molecular Systems, Inc.), forward primer (1 pM), reverse primer (1 pM), template (50 ng), and the final reaction volume was made to 10 μL with double distilled water. Thermocycling conditions were as follows: initial denaturation for 5 min at 95 °C followed by 30 cycles of denaturation for 1 min at 94 °C, 45 s at the respective annealing temperatures (see Table 1), elongation at 72 °C for 1 min. The final elongation time was extended to 7 min at 72 °C. Amplified PCR products were analysed using the ABI 3730 automated DNA sequencer (Applied Biosystems), and GENEMAPPER version 3.5 software (Applied Biosystems) was used to obtain allele designations.

Statistical analysis

Allele frequency, number of alleles, observed and expected heterozygosities, F_{ST} (Weir & Cockerham 1984) and genetic distances (Nei's standard genetic distances and chord distances) were calculated using MICROSATELLITE ANALYSER (MSA) version 3.15 (Dieringer & Schlötterer 2003). Tests for deviation from Hardy–Weinberg equilibrium (HWE) at each locus for each breed were performed using GENEPOP version 3.1 (Raymond & Rousset 1995). *P* values were corrected for multiple comparisons by applying a sequential Bonferroni correction (Rice 1989). Chord distances among breeds were utilized to construct neighbour-joining tree using PHYLIP, version 3.5 (Felsenstein 1993) and the tree was visualized using TREEVIEW version 1.6.6 software (Page 1996). F_{ST} values between all possible breed pairs were displayed by multidimensional scaling (MDS) using SPSS 10.0.5 (SPSS Inc.) software; the stress value was 0.12.

Breed differentiation was further investigated using Bayesian clustering approach implemented in STRUCTURE program (Pritchard *et al*. 2000). This program generates clusters of individuals based on their multilocus genotypes. We used an admixture model with a burn-in period of 1 000 000 iterations and 100 000 Markov chain Monte Carlo (MCMC) repetitions to calculate the probable number of genetic clusters (*K*).

AMOVA (analysis of molecular variance) was performed using ARLEQUIN, version 2.001 (Schneider *et al*. 2002).

Table 2 Summary statistics showing total and mean number of alleles and mean heterozygosity in eight Indian buffalo breeds

Parameters	Bhadawari	Nagpuri	Surati	Pandharpuri	Toda	Mehsana	Murrah	Jaffarabadi
Total number of alleles	193	178	179	182	166	194	194	177
Mean number of alleles	7.15	6.60	6.63	6.74	6.15	7.19	7.19	6.56
Observed heterozygosity	0.71	0.70	0.69	0.63	0.66	0.67	0.69	0.70
Expected heterozygosity	0.77	0.75	0.75	0.75	0.71	0.76	0.78	0.76

Table 3 Hardy–Weinberg equilibrium deviated loci in eight Indian buffalo breeds

Bhadawari	Nagpuri	Surati	Pandharpuri	Toda	Mehsana	Murrah	Jaffarabadi
BMS462	BMS4012	BMS4012	BMS4012	ILSTS089	BMS4012	BMS1724	BMS1724
	BMS1724	BMS1724	MSBQ		BMS1724	BL1134	BL1134
		BMS462	CA004		BMS1747	BMS462	BMS1747
			BMS1724		BL1036		
			BL1029				
			BMS518				
			BMS462				
			BM757				
			BMS 1747				

Population structures were defined on the basis of buffalo breed classification described by Cockrill (1981) and phylogenetic clusters obtained by us. A hierarchical analysis of variance was carried out to partition total variance into variance components attributable to interindividual and/or interbreed differences. Variance components were then used to compute fixation indices and their significance were tested at 1000 permutations as described by Excoffier *et al*. (1992).

Under a stepping-stone model of migration (Kimura 1953; Kimura & Weiss 1964), isolation by distance (IBD) is generally expected if the change in diversity is balanced by genetic drift and migration. A number of methods have been suggested for identifying IBD by different researchers. In the present study, we tested this by plotting Nei's standard genetic distances against geographical distances. The significance of correlations was determined using Mantel tests (Mantel 1967) as implemented in IBD version 2.1 (Jensen *et al*. 2005).

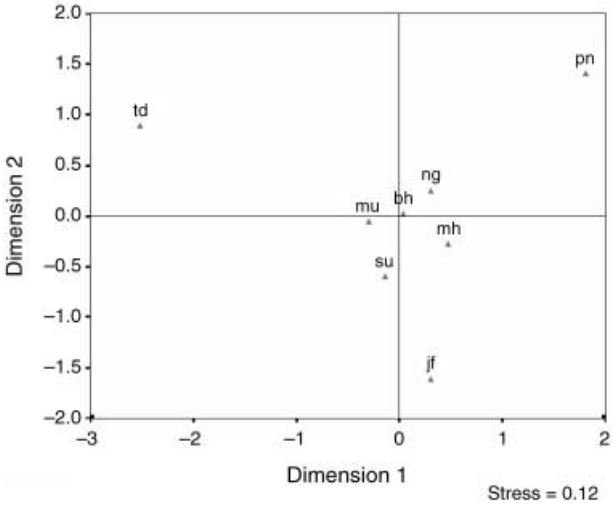

Fig. 2 Multidimensional scaling plot of pairwise F_{ST} values between Indian buffalo breeds. bh, Bhadawari; ng, Nagpuri; su, Surati; td, Toda; pn, Pandharpuri; mh, Mehsana, mu, Murrah; jf, Jaffarabadi.

Results

A total of 383 animals representing eight breeds of Indian buffaloes (Fig. 1) were analysed using 27 microsatellite markers. Two hundred and eighty-eight alleles were observed for 27 loci and the total number of alleles per breed ranged from 166 in the Toda to 194 each in the Mehasna and the Murrah (Table 2). The number of alleles per locus per breed ranged from 4 to 13. The observed and expected heterozygosity averaged over all loci varied from 0.63 to 0.71 and 0.71 to 0.78, respectively. To check for HWE at 27 loci across eight breeds, a total of 216 tests were conducted. Twenty-six breed-locus combinations were not in equilibrium (Table 3). Out of the 27 loci studied, 12 showed lack of equilibrium in one or more breeds. BMS1724 locus was mostly in disequilibrium except in the Bhadawari and Toda breeds. Amongst various breeds, the Pandharpuri had the maximum number of nine loci that deviated from HWE.

Nei's standard genetic distances and F_{ST} values for all the breed pairs were estimated (data not shown). Genetic distance ranged from 0.025 between the Surati and the

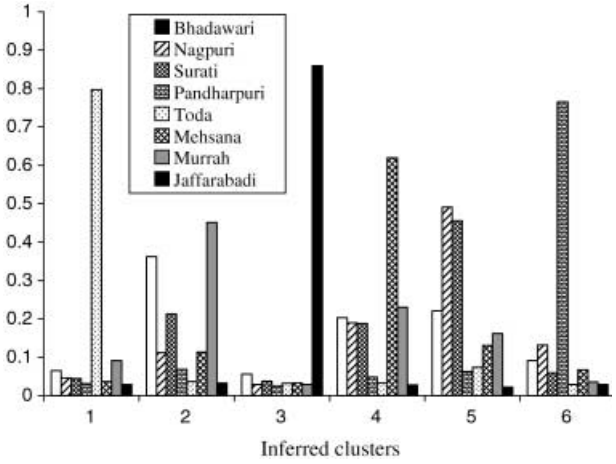

Fig. 3 Proportion of membership coefficient of Indian buffalo breeds into six inferred clusters obtained from STRUCTURE.

Nagpuri to 0.186 between the Toda and the Pandharpuri. The effect of population subdivision (F_{ST}) has been displayed by multidimensional scaling (MDS) in Fig. 2. Briefly, F_{ST} values ranged from 0.0075 (Bhadawari-Murrah) to 0.0604 (Toda-Pandharpuri). Thus, 0.75% to 6.00% (with an average of 3.4%) of the microsatellite variability is explained by subdivision of population into breeds while the remaining variability is within breeds. F_{ST} values for most breed combinations were lesser than 0.05 indicating low genetic differentiation between these breeds. The MDS display showed four clusters with a stress value of 0.12: the Toda, Jaffarabadi, and Pandharpuri breeds represented one cluster each, and the Bhadawari, Nagpuri, Surati, Mehsana and the Murrah formed a single cluster near the origin. This clustering was consistent with lineage allocation based upon the neighbour-joining tree (data not shown) constructed from chord distances.

The genetic distances between different breeds were small. To confirm whether four inferred clusters of buffalo breeds were genetically distinct, we further performed Bayesian clustering analysis using STRUCTURE. The latter analysis showed division of genetic variation into six clusters. Figure 3 shows the proportion of an individual genome from each breed that contributed to each of the six clusters under a model with the highest posterior probability. Clusters 1, 3 and 6 had contributions mainly from the Toda, Jaffarabadi and Pandharpuri animals, respectively, with a very high membership coefficient. Cluster 4 had 60% of the Mehsana. In addition, approximately 20% each of the Murrah, Bhadawari Nagpuri and Surati animals contributed to this cluster. The Nagpuri and Surati animals were almost equally shared in cluster 5 whereas the Murrah and the Bhadawari were predominant members of cluster 2.

Cockrill (1981) has classified the buffalo breeds of India on the basis of their geographical distribution and gross morphology. He has described 15 breeds in five groups based on their geographical distribution in India, namely, *Murrah group*: Murrah and Nili-Ravi; *Gujarat*: Surati, Mehsana and Jaffarabadi; *Uttar Pradesh*: Bhadawari and Tarai; *Central India*: Nagpuri, Manda, Jerangi, Kalahandi, Sambalpur and Pandharpuri; and *South India*: Toda and South Kanara. Out of these, the Nili-Ravi, Murrah, Bhadawari, Mehsana, Jaffarabadi, Surati, Nagpuri, Pandharpuri and the Toda are well-recognized breeds. Further based on morphology, Cockrill (1981) grouped these recognized breeds in two different categories: (i) the Bhadawari, Nagpuri, Surati, Pandharpuri, and the Toda, and (ii) the Nili-Ravi, Murrah, Mehsana, and the Jaffarabadi. To test the validity of this classification, we performed an AMOVA. When no grouping was assumed, 97.85% of the total variation was found to be within breeds and the remaining 2.15% was among breeds ($P < 0.01$). When buffalo breeds were grouped according to their geographical location and gross morphology, among-group variation was merely 0.18% and 0.02%, respectively. However, this value was 0.46% for the grouping based upon clusters obtained from the MDS display and neighbour-joining tree.

To enable us to estimate the divergence time of different buffalo breed clusters identified in this study, we tested the assumption whether our genotyping data depicted equilibrium with respect to change in diversity resulting from drift and migration. We used Nei's standard genetic distances calculated from our data for 27 microsatellite loci across eight breeds. Correlation between genetic distance and geographical distance was not significant ($r = 0.4056$; $P < 0.084$; Fig. 4A) indicating lack of evidence for IBD. It may be recalled that the Pandharpuri breed showed lack of HWE for nine loci (Table 3). In contrast, all the remaining breeds were out of HWE for a much smaller number of loci. It is worth noticing that despite having reasonable average number of alleles, this breed had the least heterozygosity (Table 2). We re-estimated Nei's standard genetic distances by excluding the Pandharpuri breed and seven loci (BMS4012, ILSTS089, BMS1724, BL1036, BL1134, BMS462 and BMS1747) from our data set that were not in HWE in at least one or more of the remaining seven breeds. Now we found a significant positive correlation between genetic and geographical distances ($r = 0.7078$; $P < 0.0410$; Fig. 4B) indicating equilibrium between drift and migration in these data sets. The latter were used to estimate divergence time between the Toda and the remaining six buffalo breeds using the formula, $D = 2\alpha t$ (Nei 1976), where D is Nei's standard genetic distance, α is the assumed mutation rate, and t being the time of divergence. The mutation rate was assumed to be 1.4×10^{-4}/locus/gamete according to Crawford & Cuthbertson (1996). Assuming a generation interval of 6 years for this species, we obtained estimates of 1800–2700 years, with a modal value around 2200 years, for divergence time between the Toda and other six breeds.

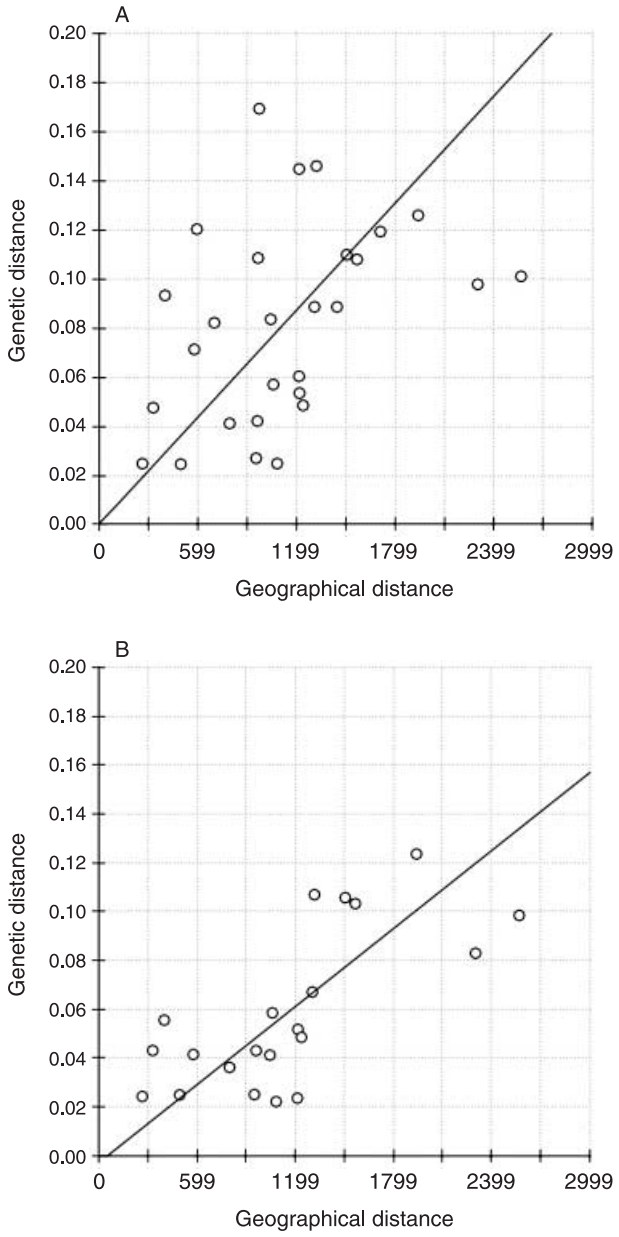

Fig. 4 Scatter plots of pairwise Nei's standard genetic distance vs. geographical distance: (A) eight breeds and 27 loci, and (B) seven breeds and 20 loci.

Discussion

Our study presents the first comprehensive genetic analysis of the Indian riverine buffaloes using microsatellite markers and provides information on relationships among various buffalo breeds in one of the most important regions of the world with respect to buffalo production.

The genetic analysis of 27 microsatellite loci showed that all eight buffalo breeds have a reasonably high level of diversity as reflected by average heterozygosity estimates of 0.63 to 0.71 in contrast to the heterozygosity level of 0.40 to 0.58 in swamp buffaloes (Baker *et al*. 1997a) and in European cattle breeds (Moazami-Goudarzi *et al*. 1997). We have found a total of 26 locus-breed combinations deviating from HWE with nine such loci in the Pandharapuri breed. Departure from HWE was always due to heterozygote deficiency that may result from one or more of the following reasons. (i) Presence of a null allele: a null allele is an allele that fails to multiply during PCR using a given microsatellite primer due to mutation at the primer site (Callen *et al*. 1993; Pemberton *et al*. 1995). (ii) Small sample size: rare genotypes are less likely to be included in the samples. (iii) Wahlund effect, i.e. presence of fewer heterozygotes in a population than predicted on account of population subdivision. Breeding strategies in farm animal species generally favour the prevalence of sires or sire lines selected for behavioural or economic traits leading to increased consanguinity. Such a breeding system produces reduced heterozygosity within a subpopulation in a breed. In the absence of use of extensive artificial insemination in buffaloes decrease in heterozygosity due to increased consanguinity is less likely for most of the breeds studied by us. However, it may be worth noticing that the average heterozygosity was the lowest in the Pandharpuri despite reasonable mean number of alleles observed in this breed. This may be indicative of substructuring within the Pandharpuri breed.

Estimation of genetic subdivision in buffaloes showed that the average proportion of genetic differentiation among breeds was 3.4%. This value was comparable to that obtained for diversity studies of the wild Asian water buffalo in Nepal (Flamand *et al*. 2003) but was low in comparison to other diversity studies in livestock species using microsatellites — 7–11% for the European cattle (MacHugh *et al*. 1998; Kantanen *et al*. 2000; Canon *et al*. 2001), 8% in horses (Canon *et al*. 2000), and 13% in pigs (Martinez *et al*. 2000). The low genetic differentiation is consistent with the fact that rigorous selection through use of artificial insemination has been absent in this species in India unlike in other livestock breeds in developed countries.

The neighbour-joining tree constructed from chord distance matrix, and the MDS display of pairwise F_{ST} values revealed at least four distinct clusters, one each represented by three breeds, namely, the Toda, Jaffarabadi and the Pandharpuri, and a fourth cluster representing an admixture of the remaining five breeds. Although genetic distances between various breed pairs were small, presence of these four clusters is further strongly supported by Bayesian clustering analysis. The Murrah is a well-recognized dairy breed of North India. In our analysis, this breed is represented not as a unique cluster but distributed with most of the other buffalo breeds of northern, central and western India. This observation is consistent with the wide usage of this breed for grading-up of the local breeds in other parts of the country. The Jaffarabadi, Surati, and

the Mehsana belong to the northwestern state of Gujarat. Our results show most of the Jaffarabadi animals as a distinct cluster away from the Surati and the Mehsana. The Jaffarabadi breed has been developed in and around the Gir forest, a habitat of the famous Gir lions. It is possible that a very massive head and large body size of this breed in contrast to all other breeds have been an outcome of severe struggle for survival of these animals against the predator. It is pertinent to note that buffaloes in these areas have been subject to continuous predation even after domestication due to overlapping of their grazing areas with the habitat of lions. Crossbreeding of the domesticated females with the wild males from the Gir forest might have also led to continuous introgression and thus contributed to the development of morphological features of the present-day Jaffarabadi breed. Further, clustering analyses show that classification of the Jaffarabadi along with the Mehsana and Murrah breeds based upon morphological parameters by Cockrill (1981) is also not tenable. Similarly, our findings are in sharp contrast with apparent morphological grouping of the Bhadawari, Nagpuri, Pandharpuri and Toda breeds (Cockrill 1981). Most of the Pandharpuri and Toda animals represent two different clusters in themselves as revealed by the present study.

The Toda buffaloes are reared as a semiwild species by the Toda tribe (Rivers 1906) in the Nilgiri hills in South India. The Toda people are a unique tribe since this is the only tribe in India with buffalo-based dairying as their primary occupation (Nair *et al*. 1986). The buffalo occupies a central place in their social, religious and cultural life. Unlike other buffalo-rearing rural communities in India, the buffalo is a sacred animal for this tribe. There is no knowledge on the origin and or arrival of this tribe in the Nilgiri hills or for that matter on their buffaloes. Given the complete dependence of their culture on buffalo production, it is tempting to suggest that occupation of Nilgiri hills by this tribe must have been closely linked with introduction of buffalo husbandry in these areas. In the light of our estimates of the divergence time of approximately 1800–2700 years of the Toda buffaloes from other main breeds in India, it is possible that the Toda people might have probably occupied these hills around that time. However, keeping in view the poor reliability of divergence time estimates from microsatellite data one would need anthropological evidence to substantiate this proposition. Sequencing of the D-loop region of mitochondrial DNA of the Toda and other breeds may further be helpful in confirmation of divergence time estimates obtained by us.

In conclusion, we have provided the first comprehensive insight into the genetics of the Indian riverine buffalo breeds using microsatellite markers. We have distinguished four clusters among these breeds. Clusters obtained by us refute the earlier classification of these breeds proposed by Cockrill (1981) on the basis of morphological and geographical parameters. Studies of genetic diversity and phylogenetic relationships are important for devising rational priorities for conservation decisions. However, additional information on cultural, agrarian, environmental and economic aspects need to be evaluated while prioritizing breeds for conservation purposes. Given the extremely small number of the Toda buffaloes, the uniqueness of this breed as revealed by microsatellite analysis in the present study, and the special cultural significance of this breed to the Toda tribe of South India, there is an immediate need to undertake necessary steps for conservation of this breed.

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