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Evaluation of Genetic Variation and Phylogenetic Relationship among North Indian Cattle Breeds

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ABSTRACT : In the present study, genetic analyses of diversity and differentiation were performed on four breeds of Indian zebu cattle (*Bos indicus*). In total, 181 animals belonging to Ponwar, Kherigarh, Gangatiri and Kenkatha breeds were genotyped for 20 cattle specific microsatellite markers. Mean number of alleles observed per locus (MNA) varied between 5.75 (Kenkatha) to 6.05 (Kherigarh). The observed and expected heterozygosity for the breeds varied from 0.48 (Gangatiri) to 0.58 (Kherigarh) and 0.65 (Kenkatha) to 0.70 (Kherigarh), respectively. F_{IS} estimates of all the breeds indicated significant deficit of heterozygotes being 28.8%, 25.9%, 17.7% and 17.7% for Gangatiri, Ponwar, Kherigarh and Kenkatha, respectively. The F_{ST} estimates demonstrated that 10.6% was the average genetic differentiation among the breeds. Nei's genetic distance DA and Cavalli- Sforza and Edwards Chord distance (D_C) and the phylogenetic tree constructed from these reflected the close genetic relationship of Gangatiri and Kenkatha, whereas Ponwar appears to be more distant. (**Key Words** : Cattle, Genetic Variation, India, Microsatellite)

INTRODUCTION

India possesses the largest livestock population in the world including the highest number of 185.18 million cattle (Livestock Census, 2003). The indigenous zebu cattle (*Bos indicus*) are characterized by prominent hump, a long face, upright horn or drooping ears, dewlap and slender legs. Color varies from white to grey and black (Acharya and Bhat, 1984). Zebus have relatively lower basal metabolic rate and better capacity of heat dissipation. Therefore, they easily adapt to tropical heat and have also developed resistance to diseases, especially tick born diseases.

Potentially there is much unrecognized beneficial genetic variation present in the rare, especially the semi managed breeds and populations, which form important reservoirs of non-exploited resources. There is a tendency for world wide animal production to be based on a few, highly selected breeds which is causing pressure leading to a reduction in number of local breeds (Blott et al., 1998; Barker, 1999). In India, increasing number of cattle breeds is showing the declining trend in population. These breeds were very popular and widespread till first half of last century as draft or dual purpose breeds, but registered a consistent reduction in their number afterwards due to three major factors: mechanization in agriculture, urbanization, and competition from high yielding cross bred cattle populations. Thus, it is essential that the resources (personal and financial) available be best used to ensure that as much valuable genetic diversity as possible survives into the future. Consequently, first step in assessing genetic conservation needs is development of baseline information: evaluation of their genetic variability and their distribution among the populations.

The biological unit for conservation in domesticated animals is usually the breed. When we are selecting breeds for conservation it may be important not just to consider taxonomic distinctness or between population variations but also to take measures of within population diversity. Such measures could be included into a diversity index and population selected for conservation on the basis of this index. Microsatellite markers had been widely used to analyze phylogenetic relationships among various animal groups and different breeds (Bradley et al., 1994; Edwards et al., 2000; Pandey et al., 2006). Microsatellite loci comprise an attractive potential source of information about population histories and evolutionary processes, as these loci permit simple and accurate typing in combination with high levels of polymorphism and widespread distribution in the genome.

In order to develop objective criteria for conservation of the cattle breeds of northern region viz. Ponwar,

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Table 1. Characteristics of microsatellite loci analyzed in four Indian zebu cattle

Marker	Primer sequences	Chromosome number	Annealing temp. (°C)	No of alleles	Size range (bp)
ETH225	gatcaccttgccactatttcct, acatgacagccagctgctact	9	57	10	142-162
ETH10	gttcaggactggccctgctaaca, cctccagcccactttctcttctc	5	55	8	208-222
ILSTS006	tgtctgtatttctgctgtgg, acacggaagcgatctaaacg	7	56	8	277-307
HEL1	caacagctatttaacaagga, aggctacagtccatgggatt	15	55	9	101-121
ILSTS011	gcttgctacatggaaagtgc, ctaaaatgcagagccctacc	14	58	4	261-269
MM8	cccaaggacagaaaagact, ctcaagataagaccacacc	2	55	8	123-145
BM1818	agctgggaatataaccaaagg, agtgctttcaaggtccatgc	23	58	9	256-286
INRA005	caatctgcatgaagtataaatat, cttcaggcataccctacacc	12	55	7	137-151
MM12	caagacaggtgtttcaatct, atcgactctggggatgatgt	9	55	10	100-136
INRA063	atttgcacaagctaaatctaacc, aaaccacagaaatgcttggaag	18	55	6	178-194
INRA035	atcctttgcagcctccacattg, ttgtgctttatgacactatccg	16	55	10	104-124
CSRM60	aagatgtgatccaagagagaggca, aggaccagatcgtgaaaggcatag	10	55	11	97-121
HAUT27	ttttatgttcatttttgactgg, aactgctgaaatctccatctta	26	55	6	147-159
ILSTS030	ctgcagttctgcatatgtgg, cttagacaacaggggtttgg	2	55	6	145-155
ILSTS054	gaggatcttgattttgatgtcc, agggccactatggtacttcc	21	55	9	133-151
ILSTS033	tattagagtggctcagtgcc, atgcagacagttttagaggg	12	55	9	137-163
ILSTS005	ggaagcaatgaaatctatagcc, tgttctgtgagtttgtaagc	10	55	5	180-192
HEL9	cccattcagtcttcagaggt, cacatccatgttctcaccac	8	59	8	150-170
BM1824	gagcaaggtgtttttccaatc, cattctccaactgcttccttg	1	55	6	180-198
ILSTS034	aagggtctaagtccactggc, gacctggtttagcagagagc	5	57	14	142-212

Kherigarh, Gangatiri and Kenkatha, we collected data for 20 microsatellite loci in these breeds to determine genetic relationship between them.

MATERIALS AND METHODS

Sample collection and DNA extraction

Blood samples were collected from total of 181 individuals representing four cattle breeds: 40 samples from Ponwar breed (P) and 47 each of Kherigarh (K), Gangatiri (G) and Kenkatha breeds (Kn), respectively. In Sampling we attempted to avoid closely related animals and sampled the animals that met the standards for each breed. All these breeds were sampled from their breeding tracts in different districts of Uttar Pradesh state of India. Ponwar cattle were sampled from Puranpur and Madhotanda subdivision of Pilibhit district. Kherigarh cattle were sampled from Nihasan and Pallia subdivision of Lakhimpur- Kheri district. Gangatiri cattle were sampled from Ballia district and Kenkatha was sampled from Banda district. DNA was extracted from blood samples as per the standard protocol (Maniatis et al., 1982).

Microsatellite analysis

A set of 20 microsatellite markers (Table 1) recommended for cattle in FAO's DADIS MoDAD programme were utilized for generating microsatellite genotyping data. Since microsatellite markers are co-dominant, 181 samples correspond to 362 alleles for each microsatellite locus. An amalgamation of 20 co-dominant loci and 181 samples were projected to create 7240 allelic data for the population included in this study. Polymerase Chain Reaction (PCR) was performed utilizing 50-100 ng genomic DNA in a 25 µl reaction volume using PTC-200 PCR machine (MJ Research Inc., MA, USA). The PCR reaction cycle was accomplished by denaturation for 1 min at 95°C, 30 cycles of '95°C for 1 min, precise annealing temperature of primer for 1 min, 72°C for 1 min' and finally extension at 72°C for 5 min. The PCR products were resolved on 6% denaturing polyacrylamide gels (Sequi GT System, Bio-Rad) and silver stained according to protocol given by Bassam et al. (1991). All the microsatellite markers showed reproducible and discernable bands. Exact allelic size was determined by direct comparisons with the 10 bp ladder (Invitrogen, Life Technologies, CA, USA). Size of the alleles was calculated online using 'INCHWORM' programme (http://www.molecular workshop.com/programs/inchworm.html).

Statistical analysis

Allele frequency, the mean number of alleles per locus, observed heterozygosity, and heterozygosity expected from Hardy-Weinberg assumptions for each locus were computed using the POPGENE software package (Yeh et al., 1999). The two measures of heterozygosity are highly correlated, but our study focused on the expected heterozygosity since it is considered to be a better estimator of the genetic variability present in a population. The computer program FSTAT (Goudet, 1995) was used to obtain the estimates of inbreeding coefficients and population sub division based on F-statistics (Weir and Cockerham, 1984). Nei's standard genetic distance (D) (Nei, 1978), Nei's genetic distances D_A

Table 2. Genetic variation in 4 Indian cattle breeds including observed (N_a) and expected (N_e) alleles per locus, observed (H_o) and expected (H_e) heterozygosity

Locus	Ponwar		Kherigarh		Gangatiri		Kenkatha	
	$N_a(N_e)$	$H_o(H_e)$	$N_a(N_e)$	$H_o(H_e)$	$N_a(N_e)$	$H_o(H_e)$	$N_a(N_e)$	$H_o(H_e)$
ETH225	7 (2.40)	0.25 (0.59)	8 (2.68)	0.57 (0.63)	8 (2.40)	0.43 (0.59)	6 (2.10)	0.38 (0.53)
ETH10	8 (4.03)	0.45 (0.76)	4 (1.97)	0.27 (0.50)	3 (2.29)	0.24 (0.57)	4 (2.63)	0.45 (0.63)
ILSTS006	5 (3.29)	0.62 (0.71)	5 (3.81)	0.70 (0.75)	5 (2.86)	0.33 (0.66)	8 (3.39)	0.39 (0.71)
HEL1	5 (2.33)	0.59 (0.58)	6 (2.80)	0.58 (0.65)	8 (3.00)	0.56 (0.67)	5 (2.32)	0.56 (0.58)
ILSTS011	3 (2.09)	0.48 (0.53)	4 (2.46)	0.47 (0.60)	3 (2.38)	0.50 (0.59)	2 (1.53)	0.31 (0.35)
MM8	7 (2.47)	0.31 (0.60)	7 (4.11)	0.68 (0.76)	5 (2.36)	0.23 (0.58)	6 (2.89)	0.76 (0.66)
BM1818	6 (2.14)	0.35 (0.54)	8 (3.77)	0.61 (0.74)	7 (2.82)	0.30 (0.65)	6 (2.90)	0.55 (0.66)
INRA005	4 (3.51)	0.54 (0.73)	6 (3.75)	0.67 (0.74)	5 (4.09)	0.63 (0.76)	5 (4.49)	0.62 (0.79)
MM12	7 (3.51)	0.53 (0.73)	8 (5.82)	0.48 (0.84)	8 (4.02)	0.40 (0.76)	8 (4.16)	0.67 (0.77)
INRA063	5 (2.11)	0.53 (0.53)	6 (1.96)	0.49 (0.49)	3 (2.13)	0.47 (0.54)	4 (1.42)	0.32 (0.30)
INRA035	7 (5.34)	0.78 (0.82)	8 (5.48)	0.70 (0.83)	8 (5.93)	0.62 (0.84)	8 (5.47)	0.76 (0.83)
CSRM60	7 (3.47)	0.34 (0.73)	5 (3.54)	0.56 (0.73)	6 (3.09)	0.40 (068)	8 (3.51)	0.38 (0.72)
HAUT27	5 (3.10)	0.22 (0.69)	5 (2.73)	0.36 (0.64)	5 (1.71)	0.18 (0.42)	4 (2.37)	0.23 (0.58)
ILSTS030	4 (3.16)	0.30 (0.69)	5 (4.76)	0.81 (0.79)	4 (2.31)	0.32 (0.57)	4 (2.61)	0.72 (0.62)
ILSTS054	6 (3.92)	0.55 (0.75)	6 (4.24)	0.57 (0.77)	7 (3.44)	0.67 (0.72)	6 (4.55)	0.83 (0.79)
ILSTS033	5 (2.61)	0.63 (062)	4 (2.63)	0.51 (0.63)	7 (2.40)	0.62 (0.59)	5 (2.80)	0.40 (0.65)
ILSTS005	5 (4.19)	0.77 (0.77)	5 (3.52)	0.68 (0.73)	5 (4.01)	0.80 (0.76)	5 (3.45)	0.74 (0.72)
HEL9	6 (4.86)	0.80 (0.81)	8 (3.69)	0.66 (0.74)	7 (5.13)	0.54 (0.82)	5 (4.64)	0.55 (0.79)
BM1824	6 (2.89)	0.59 (0.66)	3 (2.25)	0.51 (0.56)	4 (2.30)	0.62 (0.57)	5 (2.15)	0.43 (0.54)
ILSTS034	11 (7.42)	0.57 (0.89)	10 (5.79)	0.65 (0.84)	11 (5.20)	0.64 (0.82)	11 (4.33)	0.58 (0.78)
Mean	5.95 (3.44)	0.51 (0.69)	6.05 (3.59)	0.58 (0.70)	5.95 (3.19)	0.48 (0.66)	5.75 (3.19)	0.53 (0.65)
SD	1.73 (1.31)	0.17 (0.10)	1.82 (1.90)	0.13 (0.10)	2.11 (1.17)	0.17 (0.11)	2.02 (1.12)	0.18 (0.14)

(Nei, 1983) and Cavalli-Sforza and Edwards Chord distance D_C (Cavalli-Sforza and Edwards, 1967) were estimated using MICROSATELLITE ANALYZER (MSA) version 3.15 (Dieriger and Schlotterer, 2003). Pairwise distance matrix based on the proportion of the shared alleles with populations as taxonomic unit was utilized to construct UPGMA tree using PHYLIP version 3.5 (Felsenstein, 1993) and the tree was visualized using TREEVIEW version 1.6.6 software (Page, 1996). Breed differentiation was further investigated using Bayesian clustering approach implemented in STRUCTURE program (Pritchard et al., 2000). Individual animals were assigned to different clusters based on their multilocus genotypes. Admixture model was used 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. The program STRUCTURE was first run with K = 1 to K = 5, so as to model the whole data set. The best value of ln Pr (X/K) was obtained for K = 3 (-9,915.4). These three inferred populations would correspond to the "ancestral" populations from which our current breeds were derived.

RESULTS AND DISCUSSION

The twenty investigated microsatellites represented 15 autosomal chromosomes in cattle. Total number of alleles at each locus and their size range are presented in Table 1. The observed and effective number of alleles and observed and expected heterozygosity are presented in Table 2. A total of 163 alleles were observed at the twenty loci analyzed. All the loci were polymorphic (raw microsatellite data and allele frequencies are available from corresponding author). Mean number of alleles (MNA) observed per locus were between 5.75 (Kenkatha) and 6.05 (Kherigarh) while number of effective alleles were between 3.19 (Kenkatha, Gangatiri) and 3.59 (Kherigarh) in these cattle breeds. MNA per locus in the individual breed varied from 3 (ILSTS011) to 11 (ILSTS034) in P, 3 (BM1824) to 10 (ILSTS034) in K, 3 (ETH10, ILSTS011 and INRA063) to 11 (ILSTS034) in G and 2 (ILSTS011) to 11 (ILSTS034) in Kn. Although several alleles were found uniquely in each cattle breed, they are unlikely to be useful as breed markers due to their low frequencies in the studied sample size.

The mean observed (H_o) and expected (H_e) heterozygosity values averaged over loci showed an overall pattern similar to that obtained for MNA per locus. The observed and expected heterozygosity of all the breeds varied from 0.48 (G) to 0.58 (K) and 0.65 (Kn) to 0.70 (K), respectively. The results of microsatellite analysis revealed relatively high degree of heterozygosity in all the four populations. The expected mean hetyerozygosities varied from 0.65 to 0.70 indicating that there are no appreciable differences in the level of genetic variability among these cattle breeds. Kherigarh had the largest genetic variability followed by Ponwar, Gangatiri and Kenkatha in the descending order. Surprisingly even in comparatively small population such as Ponwar (10,000 heads) and Kherigarh

Table 3. Heterozygote deficiency (F_{IS}) in four cattle breeds

Locus	Ponwar	Kherigarh	Gangatiri	Kenkatha
ETH225	0.581	0.109	0.271	0.280
ETH10	0.416	0.467	0.585	0.276
ILSTS0	0.129	0.071	0.498	0.454
HEL1	-0.027	0.107	0.174	0.025
ILSTS1	0.102	0.222	0.148	0.111
MM8	0.493	0.111	0.613	-0.144
BM1818	0.354	0.182	0.536	0.167
INRA00	0.254	0.092	0.184	0.218
MM12	0.277	0.432	0.483	0.124
INRA63	0.014	0.009	0.133	-0.066
INRA35	0.057	0.160	0.267	0.087
CSRM60	0.528	0.226	0.412	0.481
HAUT27	0.680	0.435	0.565	0.614
ILSTS3	0.567	-0.013	0.448	-0.163
ILSTS5	0.273	0.258	0.061	-0.040
ILSTS3	-0.001	0.186	-0.051	0.387
ILSTS0	-0.001	0.060	-0.048	-0.028
Hel09	0.010	0.108	0.345	0.307
BM1824	0.112	0.094	-0.082	0.198
ILST034	0.352	0.222	0.213	0.255
Mean	0.259	0.177	0.288	0.177
SE	0.050	0.030	0.049	0.046

(15,000 heads) the mean expected heterozygosity (H_e) was 0.69 and 0.70 respectively. H_e of four breeds was comparable to the H_e reported for other Indian breeds including Sahiwal (0.61), Haryana (0.66), Red Kandhari (0.64), Deoni (0.69) and Hallikar (0.785) (Mukesh et al., 2004; Sodhi et al., 2005; NaveenKumar et al., 2006).

Heterozygote deficiency (F_{IS}) estimates for all the breeds indicated significant deficit of heterozygotes, being 25.9%, 17.7%, 28.8% and 17.7% in P, K, G and Kn, respectively (Table 3). Similarly heterozygote deficiency has been observed in some other Indian native cattle; Sahiwal (32.6%), Hariana (21.1%) and Deoni (17.2%) (Mukesh et al., 2004) and Red Kandhari (27.8%) (Sodhi et al., 2005). Numerous factors, such as inbreeding, genetic hitchhiking, null alleles (non amplifying alleles) and the occurrence of population substructure (Wahlund effect) has been established as reasons for heterozygote deficiencies in populations (Nei, 1987). The inbreeding detected in this population is likely to be a manifestation of diminished population size coupled with lack of sufficient number of breeding males in the breeding region. Male calves of six to twelve months of age are traded to farmers outside the breeding region to be exploited in agricultural operations and transportation after castration, thus leading to their genetic death. As a result reproductable males are significantly reduced in the breeding tract. Moreover semen of these breeds is also not available in their respective area. Altogether the effective population size is curtailed and breeding between relatives stimulates inbreeding and genetic drift. Thus the fundamental cause for heterozygote deficiency in these cattle breeds is likely to be inbreeding prompted by the above expressed issues and demonstrated by the overall positive f-value.

Table 4. Global F-statistics across four cattle breeds

Locus	F_{IT}	F_{ST}	F_{IS}
ETH225	0.522	0.325	0.292
ETH10	0.581	0.271	0.429
ILSTS0	0.353	0.078	0.298
HEL1	0.290	0.230	0.078
ILSTS1	0.176	0.026	0.154
MM8	0.274	0.039	0.245
BM1818	0.469	0.241	0.301
INRA00	0.207	0.032	0.181
MM12	0.379	0.072	0.331
INRA63	0.063	0.031	0.034
INRA35	0.159	0.015	0.146
CSRM60	0.474	0.110	0.409
HAUT27	0.586	0.038	0.570
ILSTS3	0.254	0.105	0.167
ILSTS5	0.203	0.080	0.133
ILSTS3	0.329	0.216	0.144
ILSTS0	0.003	0.006	-0.003
Hel09	0.267	0.079	0.204
BM1824	0.114	0.035	0.082
ILST034	0.325	0.093	0.256
Mean	0.301	0.106	0.142
SE	0.036	0.021	0.032

Overall means for the F statistics across all the four breeds (Table 4) were significantly different from zero; F (F_{IT}) = 0.301±0.036 (total inbreeding estimate), f (F_{IS}) = 0.223±0.031 (within population inbreeding estimate) and θ (F_{ST}) = 0.106±0.021 (measure of population differentiation).

Table 5. Genetic distance between 4 Indian zebu breeds on the basis of 20 markers

	Ponwar	Kherigarh	Gangatiri	Kenkatha
Ponwar	0	0.20136	0.24467	0.24893
Kherigarh	0.360	0	0.19379	0.23797
Gangatiri	0.399	0.363	0	0.18159
Kenkatha	0.400	0.399	0.338	0

Above diagonal D_A Nei's genetic distance (Nei 1983), below diagonal D_C Chord distance (Cavealli-Sforza and Edwards, 1967).

Global analysis indicated that on an average breed had a 22.3% ($p<0.05$) deficit of heterozygotes whereas, the total population had 30.1% ($p<0.05$) deficit of heterozygotes. It should be noted that levels of apparent breed differentiation were modest in UP cattle breeds. The average genetic differentiation among breeds, measured as F_{ST} values was 10.6%. This is comparable with the 11% differentiation observed between Marathwada cattle breeds of India (Sodhi et al., 2005), 11.2% in seven European cattle (Mac-Hugh et al., 1998) and 10.7% in 20 north European cattle breeds (Kantanen et al., 2000). However, even this moderate differentiation is comparatively higher more than 9% observed in Swiss cattle (Schmid et al., 1999).

In a simulation study Takazaki and Nei (1996) studied the efficiency of several genetic distance measures, such as D_A (Nei, 1983), D_C (Cavalli-Sforza and Edwards, 1967), D_{SW} (Shriver et al., 1995) and $\delta\mu^2$ (Goldstein et al., 1995), when applied to microsatellites and found that the accuracy of the Cavalli- Sforza and Edwards Chord distance (D_C) and Nei's D_A distance were generally higher than other distances. Accuracy of the dendrogram obtained from such distances, however can only be confirmed for nodes with bootstrap values above 0.70 (Lanyon, 1985) and the nodes with bootstrap values below 0.50 were not significant. Nei's D_A distances ranged from 0.182 (between Gangatiri and Kenkatha) to 0.249 (between Ponwar and Kenkatha) and D_C ranged from 0.338 (between Gangatiri and Kenkatha) to 0.400 (between Ponwar and Kenkatha) (Table 5). High degree of genetic divergence was observed for P from G and Kn by both the distance methods. Genetic divergence was highest between Ponwar and Kenkatha and lowest in Gangatiri and Kenkatha cattle breeds. Phylogenetic tree based on both the distance methods with 10,000 bootstraps showed similar pattern. Gangatiri and Kenkatha joined together with 80.6% and 71.4% bootstrap values followed by Kherigarh with 100% bootstrap value and finally by Ponwar (Figure 1). Ponwar cattle were found to be genetically distinct from all the three breeds analyzed in the present study, which coincides with the geographic location of these cattle populations. Ponwar and Kherigarh breeds are concentrated in northern part while Gangatiri and Kenkatha are distributed in southern parts of the state. Also, it has to be mentioned that morphologically too, Ponwar had been bred to have predominantly black coat colour, while the other three breeds are with white coat colour.

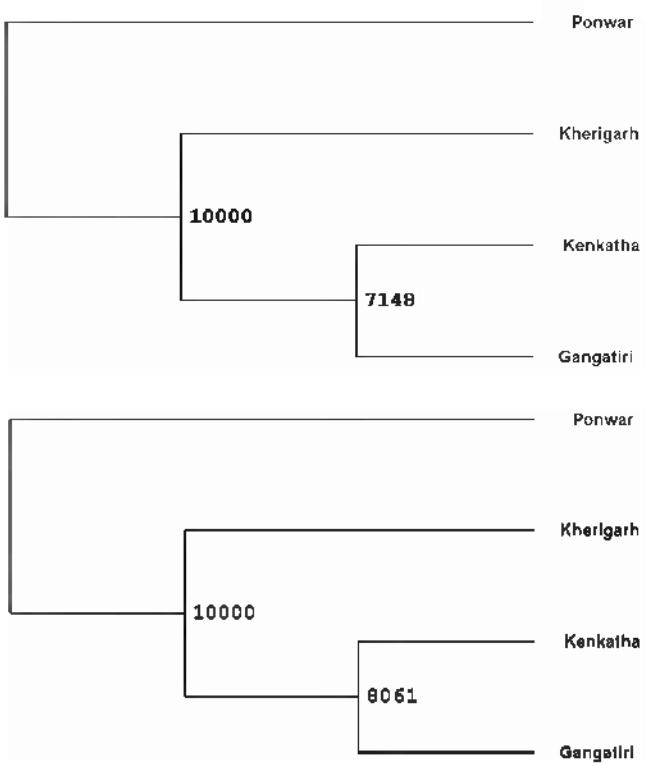

Figure 1. Tree showing the genetic relationships among cattle populations using Nei's distance (above) and Cavalli-Sforza and Edwards distance (below). The number in the branch indicates the percentage occurrence in 1,000 bootstrap replicates.

The program STRUCTURE computes the allelic frequencies expected in each locus for the inferred populations and also the proportion of membership of the four sampled breeds in each of the three inferred populations (Table 7). The first inferred population was basically formed by Ponwar individuals, the second by Kherigarh and the third inferred population was formed by Gangatiri and Kenkatha individuals, thus supporting the mode of clustering obtained from distance based methods. 95% of Gangatiri and 97.7% individuals of Kenkatha were found to represent the cluster 3. Theoretically, this population structure would be a consequence of the genetic background of the original populations from which present day breeds were derived. However, the inferred populations do not necessarily correspond to real ancestral populations, and they can be determined simply by the sampling scheme (Pritchard et al., 2000).

Nei's standard genetic distance (D) was used to estimate the divergence time between these breeds using the formula,

Table 6. Estimated divergence time of the four cattle breeds on the basis of 20 microsatellite loci

Breeds	Generations	Years
Ponwar-Kherigarh	971.4	7,721
Ponwar-Gangatiri	1,392.5	11,136,
Ponwar-Kenkatha	1,322.1	10,576
Kherigarh-Gangatiri	864.3	6,912
Kherigarh-Kenkatha	1,325.7	10,606
Gangatiri-Kenkatha	1,021.8	8,174

Table 7. Proportion of membership of each of the four cattle breeds in each of the inferred clusters using the program STRUCTURE

Source populations	Inferred clusters		
	1	2	3
Ponwar	0.968	0.020	0.012
Kherigarh	0.016	0.964	0.020
Gangatiri	0.014	0.037	0.950
Kenkatha	0.015	0.008	0.977

$D = 2at$ (Nei, 1976), where, 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 (Crawford and Cuthbertson, 1996). Assuming a generation interval of 8 years (Barker et al., 1997) we obtained divergence time estimates varying from 6,912 years to 11,136 years (Table 6).

In conclusion our results show relatively high genotypic diversity within north Indian zebu cattle breeds. Among the four populations divergence of Ponwar appears to be earlier in time than other three breeds. The collected data allows an insight into the genetic structure of the analyzed populations, which reveals the relatedness of Gangatiri and Kenkatha, while Ponwar is genetically distinct from all the other three breeds.

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