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Original Article

Low Differentiation despite High Genetic Diversity in Five Zebu Cattle (*Bos Indicus*) Breeds Native to North and Western Parts of India

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Abstract

Information on genetic structure and variability of livestock breeds is critical for efficient conservation plans. Here we present molecular characterization of 5 Indian grey colored cattle breeds from northern and western parts of India on the basis of 21 FAO recommended microsatellite markers. Kankrej breed has its native tract in the state of Gujarat, Tharparkar, Nagori and Mewati in Rajasthan and Hariana in the state of Haryana. All the five breeds had high diversity values. The average number of alleles per locus varied between 9.00 (Tharparkar) to 11.81 (Hariana). Gene diversity (expected heterozygosity) was high and varied from 0.707 in Tharparkar to 0.777 in Hariana. F_{IS} values were very low (almost zero) in all the five breeds, with mean F_{IS} values ranging from 0.00 to 0.007. Phylogenetic analysis by Neighbour Joining tree based on Nei's genetic distance and Cavalli-Sforza Edwards chord distances generated identical phylogenies, which are in accordance with historical and geographical data. The two breeds from Rajasthan viz., Tharparkar and Nagori clustered together and were joined by the third breed from Rajasthan (Mewati). Hariana breed from adjoining Haryana state joined the tree last while Kankrej from Gujarat state was quite distinct in the cluster. AMOVA revealed very low breed differentiation among the five breeds. Only 1.57% variation was due to between breed differences and the remaining 98.43% due to individuals within breeds. PCA, MDS and STRUCTURE analysis revealed no breed structure. A very high migration (average 11.26

immigrants per generation) was estimated between the five breeds. The study thus reveals very low genetic differentiation despite high genetic variability in the five investigated breeds.

Keywords: Gene diversity; Phylogenetic analysis; Neighbour joining tree; Breed structure; Genetic differentiation

Introduction

Man derives various direct and indirect benefits from livestock. There is a strong necessity in conserving livestock biodiversity for potential future benefits, for the ecosystem services it offers, for the recreation we draw from farm animals and obviously for ethical considerations. Of all the farm animal species, about a dozen are of major economic significance to man. The most notable among these is the cattle due to its number, universal existence and wide ranging contributions. The vast and varied reservoir of Indian cattle genetic resources is represented by 30 acknowledged breeds and a large number of hitherto uncharacterized populations [3]. This vast number of indigenous zebu breeds evolved as a result of the country's enormous cultural and ecological diversity. These Indian indigenous cattle breeds have been broadly classified as dairy, draft and dual type depending upon their utility for milk production or agricultural work or both.

The five grey colored cattle breeds viz. Hariana, Mewati, Nagori, Tharparkar and Kankrej originated and adapted to the varying agroclimatic conditions of northern and western parts of India. Though Hariana, Mewati and Nagori have been acknowledged as distinct breeds due to their isolated breeding tracts yet there is a large similarity in their body conformation and morphometric characterististics [3]. These breeds have been contributing to the agrarian economy of the region over the years. The common outstanding features of these Indian cattle breeds include adaptability to extreme climatic conditions, subsistence on poor feed and better capabilities to withstand environmental stress/diseases. These characteristics make these native zebu breeds a rich source of highly evolved gene pool of immense economic importance. Kankrej has been recognised, as a milch breed, Hariana and Tharparkar as dual-purpose breeds while Mewati and Nagori are excellent draft breeds with lower milk production efficiency. In recent years the population of all these breeds, except Kankrej, has gone down considerably and the situation is alarming. The primary factors contributing to this sharp decline of indigenous cattle genetic variability are adoption of crossbreeding for enhanced milk productivity,

mechanization of agricultural operations diminishing the utility of bullocks, shrinking of common grazing land and several other factors.

A variety of aspects of a population are valuable including its phenotypic traits (monogenic and polygenic), reproductive characteristics, geographic distribution, origin and habitat etc. The genetic description of populations, breeds and species allows the evaluation of genetic variability, a fundamental aspect in deciding breeding strategies and genetic conservation programs [48]. The estimation of genetic variability is uniquely valuable in highly specialized livestock breeds since the use of modern assisted reproduction techniques, such as artificial insemination and embryo transfer, can speedily decrease the genetic variability of the population. This is crucial for populations to evolve and adapt to the continuously changing environments. Molecular markers have been extensively utilized to assess this variability since they present information on every region of the genome, notwithstanding the level of gene expression. Microsatellites (highly polymorphic simple sequence repeats) are presently the most preferred and used molecular markers, mainly because of the possibility of combining their analysis with the polymerase chain reaction (PCR). These markers have been exploited to explicate bovine domestication and migration patterns [55, 16] and to characterize cattle populations globally [14, 50 and 12].

Evaluation of genetic diversity and genetic relationship between native cattle breeds of India has been taken up recently. The present study is an attempt to assess the existing diversity in Indian cattle breeds as per globally accepted criterion [59] of identifying genetically distinct breeds. In this study, we describe the genetic diversity and relationships amongst the five native, grey colored cattle breeds adapted to arid and semi arid agro-climatic regions of northern and western India to serve as guide for decisions regarding conservation and management.

Materials and Methods

Blood sample collection and DNA extraction

Blood samples were collected from 232 animals from five different grey cattle breeds. The numbers of individuals sampled include 46 from Hariana, 44 from Kankrej, 43 from Mewati, 49 from Nagori and 50 from Tharparkar. Although no parentage records are kept by farmers in the field, animals were selected from different villages after interviewing the farmers in detail to ensure unrelatedness. Genomic DNA was isolated from blood samples as described by [29].

Microsatellite genotyping

A total of 21 FAO recommended bovine microsatellite markers were chosen for the present study. The PCR amplification was carried out in 25 µl reaction volume containing 1.5 mM MgCl2, 200 µM dNTPs, 50 ng each of forward and reverse primer, ~100 ng of genomic DNA and 0.5 U of Taq DNA polymerase (Bangalore Genei, India). The PCR products were visualized on 2% agarose gels with 1× Tris-acetate-EDTA buffer containing 200 ng/ml of ethidium bromide. Genotyping was performed on ABI 3100 Avant automated DNA sequencer, with LIZ 500 (Applied Biosystems) as the internal lane standard (size standard). Post-PCR multiplexing was used to genotype 3-5 loci concurrently, depending on the PCR product size and dye label of the primers used. Sizing and allele calling were performed by using Genotyper version 3.6 software (Applied Biosystems). The allele data thus generated were used for further statistical analyses.

Statistical analysis

Various diversity indices like observed number of alleles, allele frequency, observed and expected heterozygosity, population differentiation (F_{ST}) [7], global F-statistics and heterozyogote deficiency were calculated using Microsatellite Analyzer version 4.05 [11]. Possible divergence from Hardy-Weinberg expectations was determined running the GENEPOP version 3.1 [41]. Gene flow, defined as the number of reproductively successful migrants among populations, was calculated from Nei's coefficient of gene differentiation as described in Slatkin and Barton [43]. Analysis of molecular variance was performed using ARLEQUIN version 3.0 [34]. The relationship among the 5 breeds was analyzed using two different approaches. Firstly, genetic divergence between the breeds was estimated according to Nei [40] and Cavalli-Sforza and Edwards [36] using Microsatellite Analyzer version 3.15 [11].

Pair-wise chord distances among breeds were utilized to derive dendrogram and radiation tree respectively using PHYLIP version 3.5 [25] and the tree was visualized using TREEVIEW version 1.6.6. Bootstrap resampling (n=10,000) was performed to test the robustness of the topologies. Secondly, the geometric relationship between the breeds was examined using two ordination techniques viz. multidimensional scaling and principal components analysis. Pair-wise F_{ST} values between all possible breed pairs were displayed by multidimensional scaling (MDS) using SPSS version 10.5. Pair-wise chord distance measures between individual animals were utilized to perform principal components analysis using SPSS version 10.5.

Breed differentiation was further investigated using Bayesian clustering approach as implemented in STRUCTURE program version 2.2 [30]. This method uses multilocus genotypes to infer the fraction of population/individual genetic ancestry that belongs to a cluster, for a given number of clusters (K). We performed 20 runs for each K value at 2–8. 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. In this study, each sampled population was assigned to one cluster if its average proportion of membership was >0.50, or was considered to be of a markedly admixed ancestry if its average proportion of membership to each cluster was <0.50, although this criterion is somewhat arbitrary.

Results and Discussion

Genetic diversity, along with the degree of differentiation, was examined using microsatellite markers within and among the five native grey colored cattle breeds from the northern and western states of India. Allelic frequencies are available upon request. The diversity parameters are presented in Tables 1-3.

Within Breed Variability

A total of 319 alleles were detected across the 21 microsatellite loci investigated in the 5 breeds. Analysis of microsatellite data suggested that all the 5 cattle breeds from the northern and western parts of India displayed a high within-population genetic variation. This is certainly a favorable factor when planning conservation and improvement programs. Hariana cattle had the highest total number of alleles (248; range 7-17) across the 21-microsatellite markers evaluated. The corresponding allelic count in Kankrej, Mewati, Nagori and Tharparkar cattle were 217 (6-18), 200 (4-19), 205 (4-21) and 189 (5-21), respectively (Table 1).

Table 1 Observed and effective number of alleles and Private alleles in 5 Indian cattle breeds

	Hariana			Kankrej			Mewati			Nagori			Tharparkar		
Locus	N	Ne	PA	N	Ne	PA	N	Ne	PA	N	Ne	PA	N	Ne	PA
CSRM60	12	3.66	3	10	3.25	0	8	2.27	0	9	1.93	1	7	2.66	0
ETH10	9	4.16	0	10	4.45	1	8	3.90	0	7	3.81	1	6	3.59	1
ILSTS11	8	2.37	2	8	2.28	2	4	2.14	0	6	1.92	1	6	1.59	1
TGLA122	14	8.80	1	13	7.77	2	10	7.07	0	12	6.28	0	10	5.30	0
INRA05	7	4.24	0	8	5.55	1	7	5.33	0	7	4.67	0	6	3.43	0
INRA63	7	3.09	1	8	2.33	1	7	2.16	0	7	2.40	0	5	3.01	0
TGLA227	10	3.23	1	7	2.63	0	9	2.33	0	8	2.51	1	8	2.47	1
CSSM08	11	4.40	0	8	5.25	0	7	3.57	3	7	3.53	1	6	4.21	2
HEL05	10	5.15	2	8	4.20	0	8	4.77	1	9	3.68	1	8	4.66	0
ILSTS05	13	6.15	2	12	5.42	1	9	5.45	0	9	4.82	0	11	4.72	1
ILSTS33	13	4.09	3	8	3.07	2	9	3.28	0	8	2.43	0	7	3.31	0
INRA35	15	6.77	5	11	5.53	0	11	5.31	0	9	5.14	1	8	4.86	0
BM1824	7	2.62	1	6	2.70	0	5	2.53	0	4	2.07	0	5	1.94	0
CSSM66	13	6.55	1	11	4.72	0	10	4.07	0	12	5.15	1	10	4.75	1
ETH03	10	4.02	3	8	2.76	1	7	2.64	0	6	2.78	1	7	2.98	0
ETH225	10	2.78	0	9	2.79	0	8	2.11	0	10	2.46	2	7	1.57	1
MM12	16	4.42	2	13	4.32	0	13	4.05	0	11	3.23	0	9	3.34	0
CSSM33	16	8.69	1	15	7.46	1	19	10.05	1	21	8.30	3	21	8.66	1
HEL01	17	5.29	0	18	8.93	0	15	3.64	0	18	7.31	0	20	9.71	1
HEL09	13	8.98	0	13	8.74	1	11	7.67	0	12	8.38	0	11	7.55	0
ILSTS34	17	5.51	6	13	4.55	0	15	5.64	2	13	5.01	1	11	3.37	0
Mean	11.81	5.00		10.33	4.70		9.52	4.29		9.76	4.18		9.00	4.18	
Loci in	13			14			17			18			18		9

HWE														

N= observed number of alleles; Ne = effective number of alleles; PA = private alleles

The mean allelic diversity (number of alleles per locus) varied from 9.00 in Tharparkar (range 5-21), 9.52 in Mewati (4-19), 9.76 in Nagori (4-21), 10.33 in Kankrej (6-18) to 11.81 in Hariana (7-17). As a general observation the gene frequencies and the evenness of allele frequencies varied greatly between populations and the loci vary greatly in number of alleles (presumably reflecting differences in the mutation rate across loci). The number of alleles per locus in the 5 investigated breeds is higher than those previously reported for 22 acknowledged indigenous breeds and 5 hitherto uncharacterized populations from India, which varied from 3.88 to 9.12 using microsatellite markers [5]. The allelic diversity computed in the five breeds was also comparable with other Indian breeds [6, 18 and 51]. The allelic diversity in the five Indian native cattle breeds is considerably higher than that reported for a majority of cattle breeds from different parts of the world [1, 10, 13, 22, 23, 24, 27, 31, 32, 35, 49, 53 and 60]. Comparable or higher allelic diversity has been reported in 9 European breeds [28], 27 Chinese breeds [20] and 10 Brazilian breeds [2]. This may be due to the use of denaturing polyacrylamide sequencing gels and silver staining in earlier studies, which possibly does not discover all the alleles, especially the alleles differing by few nucleotides. This is duly reflected in the findings of Sodhi *et al.* [44] and Sodhi *et al.* [46], who used PAGE-silver staining and automated DNA sequencer for genetic characterization of Tharparkar breed using the same microsatellite markers and DNA from the same animals. Using PAGE-silver staining, only 6.2 alleles per locus were identified in contrast to 9.0 using automated DNA sequencer. Higher allelic diversity reported in 9 European breeds [28], 27 Chinese breeds [20] and 10 Brazilian breeds [2] were also achieved using automated DNA sequencer. Elevated allelic diversity in Indian cattle might also be attributed to lack of any appreciable planned selection pressure due to negligible use of AI under filed conditions and thus signifies the existence of larger effective population sizes of the explored Indian cattle breeds. In general higher number of alleles per locus has been detected in zebuine breeds as compared to taurine breeds [2]. The mean effective number of alleles (Ne) estimated in the 5 breeds varied from 4.18 (Nagori and Tharparkar) to 5.00 (Hariana) (Table 1). The mean effective number of alleles in all the 5 breeds is far lower (even less than 50%) than the actual mean observed number of alleles per locus. This implies that we have a set of alleles with very different frequencies. Alleles with frequencies away from the even 'average' contribute very

little to the effective number of alleles. Heterozygosity is of prime interest in evaluating genetic variation in natural populations. It can elucidate a great deal about the structure and even history of a population. The mean observed heterozygosity (Ho) estimated across 21 microsatellite markers in 5 breeds was very high ranging from 0.721 (Tharparkar) to 0.788 (Nagori) (Table 2).

Table 2 Heterozygosity and Polymorphism information content (PIC) of the five cattle breeds

Locus	Hariana			Kankrej			Mewati			Nagori			Tharparkar		
	Ho	He	PIC	Ho	He	PIC	Ho	He	PIC	Ho	He	PIC	Ho	He	PIC
CSRM60	0.652	0.735	0.695	0.705	0.700	0.650	0.628	0.566	0.513	0.469	0.486	0.445	0.640	0.631	0.5896
ETH10	0.696	0.768	0.726	0.818	0.784	0.742	0.605	0.752	0.705	0.694	0.745	0.693	0.800	0.729	0.6732
ILSTS11	0.457	0.584	0.538	0.500	0.568	0.523	0.465	0.540	0.475	0.408	0.485	0.443	0.300	0.375	0.3454
TGLA122	0.870	0.896	0.876	0.791	0.882	0.858	0.884	0.869	0.842	0.936	0.850	0.824	0.800	0.820	0.7887
INRA05	0.891	0.772	0.728	0.864	0.829	0.795	0.721	0.822	0.786	0.776	0.794	0.756	0.740	0.716	0.6685
INRA63	0.630	0.684	0.638	0.523	0.577	0.538	0.581	0.544	0.504	0.531	0.589	0.534	0.660	0.675	0.6029
TGLA227	1.000	0.698	0.641	0.955	0.627	0.560	0.907	0.577	0.491	1.000	0.608	0.526	0.920	0.601	0.5275
CSSM08	0.689	0.782	0.741	0.841	0.819	0.784	0.884	0.728	0.683	0.714	0.724	0.672	0.640	0.770	0.7243
HEL05	1.000	0.815	0.781	0.907	0.771	0.731	0.977	0.800	0.767	1.000	0.736	0.698	0.959	0.794	0.761
ILSTS05	0.870	0.847	0.819	0.932	0.825	0.795	0.977	0.826	0.803	0.796	0.801	0.762	0.840	0.796	0.759
ILSTS33	0.587	0.764	0.721	0.659	0.682	0.618	0.674	0.704	0.647	0.612	0.594	0.538	0.680	0.705	0.6662
INRA35	0.870	0.866	0.841	0.659	0.839	0.811	0.674	0.824	0.790	0.735	0.820	0.786	0.820	0.802	0.7646
BM1824	0.739	0.625	0.549	0.659	0.636	0.559	0.651	0.613	0.541	0.551	0.521	0.440	0.420	0.491	0.4486
CSSM66	0.761	0.857	0.832	0.841	0.797	0.759	0.791	0.763	0.729	0.796	0.814	0.783	0.780	0.798	0.7606
ETH03	0.761	0.759	0.714	0.750	0.645	0.577	0.581	0.628	0.547	0.571	0.646	0.573	0.680	0.671	0.6147
ETH225	0.652	0.648	0.622	0.636	0.649	0.618	0.581	0.532	0.508	0.653	0.600	0.561	0.360	0.368	0.35
MM12	0.652	0.782	0.747	0.750	0.777	0.739	0.837	0.762	0.723	0.714	0.698	0.644	0.680	0.708	0.653
CSSM33	0.902	0.896	0.876	0.841	0.876	0.854	0.977	0.915	0.898	0.898	0.892	0.874	0.960	0.901	0.8832
HEL01	0.732	0.821	0.799	0.674	0.898	0.880	0.488	0.734	0.712	0.682	0.873	0.855	0.766	0.907	0.8891
HEL09	0.976	0.899	0.878	0.837	0.896	0.8744	0.881	0.880	0.855	0.787	0.890	0.869	0.894	0.877	0.8536

ILSTS34	0.732	0.829	0.804	0.805	0.790	0.752	0.780	0.833	0.803	0.867	0.809	0.776	0.809	0.711	0.6707
Mean	0.768	0.777	0.761	0.759	0.755	0.740	0.740	0.724	0.713	0.788	0.819	.709	0.721	0.707	0.7117

Ho = Observed heterozygosity; He = expected heterozygosity; PIC =polymorphism information content

Similarly the expected heterozygosity or gene diversity in the investigated 5 breeds was high, varying between 0.707 (Tharparkar) and 0.819 (Nagori). The genetic variability computed in the five breeds was either higher or comparable with other Indian breeds, ranged from 0.460 to 0.789 in Indian indigenous breeds [5], 2 breeds; Ghumusari and Hariana [18], Hill Cattle [6] and 4 Indian cattle breeds including Ghumusari and Bhinjharpuri [51]. In the reported 23 observed heterozygosity values for different Indian cattle breeds, 23% were lower than 0.50, 30.4% varied between 0.50 to 0.60, 39.1% between 0.60 and 0.70 and only 4.3% were above 0.70. Similarly, of the 25 diversity values reported for different Indian cattle breeds, 8% were below 0.50, 80% varied from 0.60 to 0.70 and 12% were above 0.70. Heterozygosity estimates, in general, were moderate to high (>0.45) in different breeds investigated worldwide [1, 2, 8, 13, 21, 22, 23, 24, 27, 33, 42, 49, 58, 60], but lower than estimated in the 5 breeds in this study. The observed and expected heterozygosities in all the 5 breeds are almost similar suggesting that mating is approximately random within in each breed. Higher estimates of observed and expected heterozygosities observed in the 5 breeds are indicative of low inbreeding, which is also substantiated by close to zero mean inbreeding coefficients (F_{IS}) across 21 loci in all the five breeds (ranged between –0.025 to 0.007) (Table 3).

Table 3 Inbreeding coefficient (F_{IS}) and deviations from Hardy Weinberg Equilibrium frequency (p-values)

Locus	**Hariana**		**Kankrej**		**Mewati**		**Nagori**		**Tharparkar**	
	F_{IS}	HWE (P-Value)	F_{IS}	HWE	F_{IS}	HWE	F_{IS}	HWE	F_{IS}	HWE
CSRM60	0.108	0.000	-0.012	0.458	-0.116	0.387	0.030	0.381	-0.020	0.567
ETH10	0.090	0.180	-0.050	0.744	0.193	0.001*	0.064	0.134	-0.103	0.946
ILSTS11	0.214	0.001*	0.115	0.006	0.134	0.084	0.155	0.001*	0.196	0.001*
TGLA122	0.024	0.305	0.098	0.039*	-0.023	0.640	-0.108	0.810	0.019	0.286

INRA05	-0.161	0.772	-0.048	0.742	0.118	0.013*	0.018	0.168	-0.040	0.603
INRA63	0.074	0.052	0.089	0.023*	-0.076	0.886	0.094	0.179	0.017	0.481
TGLA227	-0.444	1.000	-0.537	1.000	-0.587	1.000	-0.659	1.000	-0.543	1.000
CSSM08	0.114	0.207	-0.033	0.587	-0.222	0.996	0.009	0.422	0.165	0.023
HEL05	-0.236	1.000	-0.185	0.966	-0.230	1.000	-0.368	1.000	-0.216	1.000
ILSTS05	-0.033	0.047	-0.137	0.999	-0.191	1.000	0.001	0.677	-0.061	0.878
ILSTS33	0.228	0.000*	0.028	0.017*	0.036	0.497	-0.036	0.616	0.031	0.116
INRA35	-0.010	0.636	0.211	0.002*	0.178	0.002*	0.100	0.060	-0.027	0.653
BM1824	-0.190	0.033*	-0.042	0.739	-0.070	0.349	-0.063	0.118	0.140	0.239
CSSM66	0.107	0.046*	-0.062	0.689	-0.043	0.737	0.018	0.275	0.017	0.448
ETH03	-0.008	0.023*	-0.171	0.958	0.070	0.377	0.112	0.155	-0.019	0.185
ETH225	-0.013	0.374	0.014	0.337	-0.100	0.940	-0.095	0.859	0.017	0.578
MM12	0.163	0.000*	0.030	0.003*	-0.106	0.170	-0.029	0.297	0.035	0.004*
CSSM33	-0.014	0.072	0.035	0.004*	-0.074	0.988	-0.012	0.475	-0.071	0.555
HEL01	0.104	0.000*	0.246	0.000*	0.333	0.000*	0.216	0.000*	0.151	0.000 *
HEL09	-0.093	0.985	0.060	0.081	-0.007	0.518	0.111	0.000*	-0.025	0.463
ILSTS34	0.112	0.000*	-0.026	0.470	0.057	0.201	-0.077	0.260	-0.144	0.968
Mean	0.007		-0.018		-0.035		-0.025		-0.023	

*Deviate significantly from the Hardy Weinberg Equilibrium expected frequency ($p<0.05$)

Hardy-Weinberg Equilibrium

Hardy Weinberg equilibrium (HWE) calculations were determined for each population, combining the allele frequency data for all 21 markers used. The results of he HWE test are shown in Table 3. There were a total of 105 HWE tests (21 loci in 5 populations). A total of 25 locus-population combinations were statistically significant ($P < 0.05$) (Table 3). These deviations comprised 8 loci in Hariana; 7 loci in Kankrej; 4 in Mewati and 3 each in Nagori and Tharparakr. In a majority of the locus-population combinations deviating from HWE frequencies (22/25), the observed hetrozygosities were below the

expected heterozygosities. Significant deviation from HWE is a general phenomenon and has been detected in several cattle breeds from different parts of the world [1, 4, 6, 8, 9, 10, 17, 18, 23, 27, 45, 47, 51, 52, 54 and 56]. It is, though, difficult to envisage the exact basis of this departure, however, the presence of low- frequency null alleles segregating at these loci may be a likely reason. Other factors, which contribute to HWE deviations, include inbreeding, genetic hitchhiking and occurrence of population substructure (Wahlund effect), which result in heterozygote deficiencies [39]. Private alleles were detected in all the five breeds, viz. 34 in Hariana, 13 in Kankrej, 7 in Mewati, 14 in Nagori and 9 in Tharparkar (Table 1). However, only 2 out of the 77 private alleles were detected in frequencies >0.05 (both in Hariana).

Genetic variation and relationship between breeds

Two genetic distances were used for the comparison of breeds: Nei's genetic distance [40] and Cavalli-Sforza and Edwards genetic distances [36] based on allelic frequencies of microsatellite loci. Pair wise genetic distances calculated by the two methods are shown in Table 4 while F_{ST} and Nm values are presented in Table 5.

Table 4 Pairwise estimates of genetic distance between populations

Breeds	**Hariana**	**Kankrej**	**Mewati**	**Nagori**	**Tharparkar**
Hariana	--	0.24535	0.24223	0.26985	0.30372
Kankrej	0.08079	--	0.23970	0.26873	0.29554
Mewati	0.07786	0.07589	--	0.23736	0.28188
Nagori	0.09645	0.10427	0.08124	--	0.26370
Tharparkar	0.12571	0.11987	0.11051	0.09467	--

Cavalli's pair-wise genetic distances (above diagonal) and Nei's genetic distance (D_A, below diagonal)

Table 5 Pairwise estimates of F_{ST} (above diagonal) and Nm (below diagonal) of 5 Indian cattle breeds

Breeds	Hariana	Kankrej	Mewati	Nagori	Tharparkar
Hariana	**	0.0180	0.0144	0.0261	0.0428
Kankrej	13.644	**	0.0087	0.0340	0.0444
Mewati	17.111	28.486	**	0.0229	0.0394
Nagori	9.329	7.103	10.667	**	0.0275
Tharparkar	5.591	5.381	6.095	8.841	**

The significance of F_{ST} values was tested by using a permutation test. All pair wise F_{ST} values were significant. The smallest F_{ST} value (0.0087) was obtained between Kankrej and Mewati cattle. Low F_{ST} values were also obtained between Hariana and Mewati (0.0144) and between Hariana and Kankrej (0.0180). The largest F_{ST} (0.044) was obtained between Kankrej and Mewati cattle.

Estimates of Nei's pair-wise genetic distances and Cavalli's genetic distances were all significantly different from zero indicating that all breeds can be considered as genetically independent. Distance-based phylogenetic analysis was used to describe the relationships between the 5 breeds. Identical phylogenetic relationships were obtained for the two distance measures (Fig. 1).

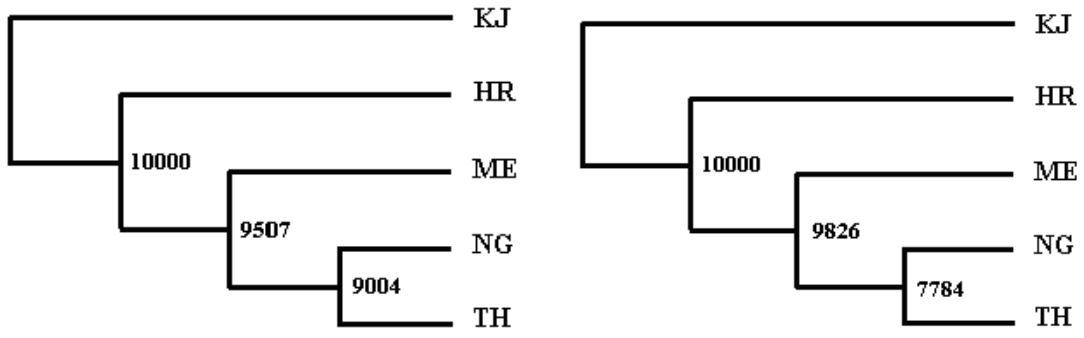

Fig. 1 Neighbour Joining tree based on Nei's genetic distance (left) and Cavalli-Sforza Edwards chord distance (right) (10000 bootstraps)

The highest genetic distances were observed between Tharparkar-Hariana using both Nei's (0.126) as well as Cavalli's genetic distances (0.304). Among the five investigated breeds the lowest distances were observed between Mewati and Kankrej by both Nei's (0.076) and Cavalli's method (0.240).

The phylogenetic reconstruction based on pair-wise genetic distance measures (Nei's and Cavalli's) following Neighbour Joining procedure revealed the clustering of Tharparkar and Nagori at a single node (Fig. 1). The tree topology was confirmed by the relatively high bootstrap values (10000). Mewati joined the cluster next followed by Hariana and Kankrej. This is comprehensible in view of the fact that Tharparkar, Nagori and Mewati belong to the same province (Rajasthan) and the farmers use bulls of these breeds interchangeably irrespective of the respective breeding tract as there is no breed society or breeding policy to check such indiscriminate breeding. Though Hariana is morphologically more comparable to Mewati and Nagori, yet it joins the tree later due to its distinctive breeding tract in the neighboring province of Haryana. Kankrej is geographically and morphologically quite distinct from the other 4 breeds and its distinct position in the phylogeny is precise.

Multidimensional scaling (MDS) and principal components analysis (PCA) were further utilized to predict the genetic structure among the five cattle breeds. The basic purpose of both these techniques is to reduce the number of variables and to detect structure in the relationship between variables. The multidimensional scaling display (MDS) of pair-wise F_{ST} values showed the close proximity of Mewati, Hariana and Kankrej while Nagori and Tharparkar were found to form distinct clusters (Fig. 2).

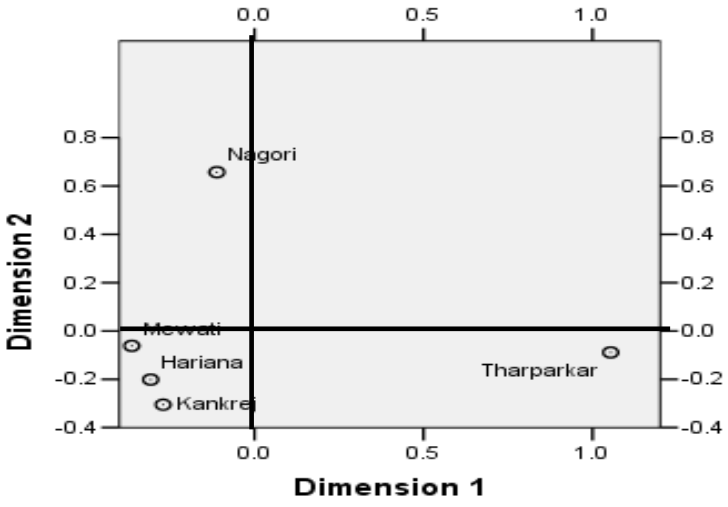

Fig. 2 Multidimensional scaling plot of pair-wise F_{ST} values (s-stress=0.00040)

The stress value was found to be 0.00040. Principal component analysis based on inter individual chord distance estimates demonstrated that the first three principal components together explained 37.14% (PC1 = 27.81, PC2 = 5.36 and PC3 = 3.97%) of the total variance. The lower proportion of variance explained by the first three principal components could be attributed to comparatively more number of principal components having eigen values greater than one. The three-dimensional scattergram obtained after principal components analysis illustrated the intermingling of animals of all the five breeds (Fig. 3).

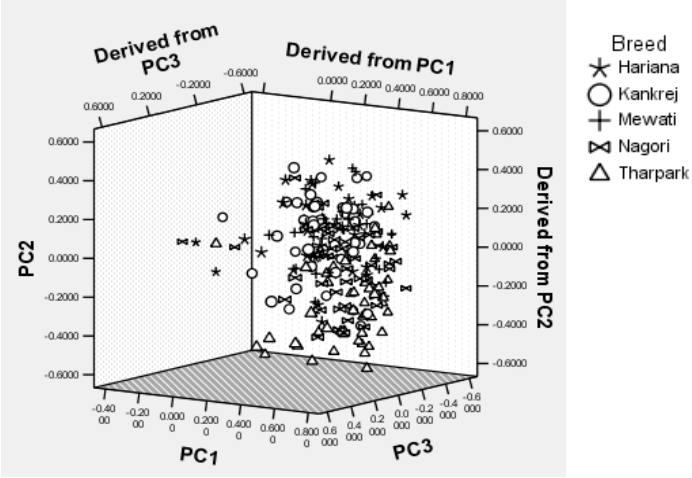

Fig 3. Principal component analysis based on pair-wise inter individual chord distance (First 3PCs explain 37.14% of total variance; PC1 = 27.81%; PC2=5.36% PC3 = 3.97%)

Population Differentiation

To validate the clustering obtained by phylogenetic and ordination analysis, AMOVA (analysis of molecular variance) was executed (Table 6).

Table 6 Analysis of molecular variance

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	4	67.57	0.1088 Va	1.57
Within populations	459	3125.72	6.8086 Vb	98.43
Total	463	3192.72	6.9174	

Fixation Index F_{ST}: 0.01572

When no grouping was assumed, 98.43% of the total variation was found to be within breeds while the remaining 1.57% was found to be among different breeds (P<0.01). Present day subdivision of the five Indian grey cattle into breeds is, thus, low and much smaller than that reported for 20 Northern European breeds (F_{ST} = 0.107) [27]; 7 other European breeds (F_{ST} = 0.112) [15]; 18 local cattle breeds from Spain, Portugal, and France (F_{ST} = 0.07) [24]; 7 French breeds with 23 loci (F_{ST} = 0.08) [10]; eight Southwest European beef cattle breeds (F_{ST} = 0.068) [26]; 3 Indian breeds (F_{ST} = 0.117 and D 0.113) [37, 38]; 5 Korean native breeds (F_{ST} = 0.11) 21 and 10 Brazilian breeds [F_{ST} = 0.098; 2]. These values were also smaller than those obtained for Ghumusari and Hariana (F_{ST} = 0.072) [18] and four Indian cattle populations (F_{ST} = 0.044) [51]. However, low differentiation, similar to present study, between Kenkatha and Gaolao breeds of Indian cattle has been recently detected (F_{ST} = 0.0219) [47].

Genetic Admixture and Exchange of animals

Bayesian clustering analysis was performed to assign individuals to different clusters using STRUCTURE program. Several independent runs were performed for each K with K=2 to K=8 in order to identify the appropriate K and to verify the consistency of the estimates across runs [19]. Using the model-based program STRUCTURE [30], the highest values of L' (K) and ΔK were obtained consistently with K = 3, showing that K = 3 was the optimal number of clusters for the cattle populations studied. The value of criterion used (D.ΔK) was about 19 for K=3 and was lower for all other values of K (2-8). Table 7 summarizes the proportion of shared ancestry among the five genetically defined clusters.

Table 7 Population membership of individuals of different populations in the three inferred clusters based on STRUCTURE analysis

		Inferred clusters		
Population	N	1	2	3
Hariana	46	0.395	0.259	0.346
Kankrej	44	0.373	0.282	0.346
Mewati	43	0.312	0.281	0.407
Nagori	49	0.267	0.355	0.378
Tharparkar	50	0.252	0.484	0.265

The distribution exhibited markedly admixed ancestry as the average proportion of membership to each cluster was well below the assumed threshold value of 0.50 indicating absence of relevant genetic structure among the five cattle populations investigated. This is interesting in view of the fact that despite exhibiting very high genetic diversity, there is very low genetic differentiation and genetic structuring in the five investigated cattle breeds. Lack of genetic structuring in the five breeds is duly corroborated by very high rate of migration among the breeds. The mean migration rate (Nm=number of migrants per generation) observed across the 21-microsatelite markers was very high (11.26), which ranged between 6.475 for CSSM66 to 37.289 for TGLA227. Pair wise migration across the breeds revealed highest gene flow between Kankrej- Mewati (28.49) followed by Hariana-Mewati (17.11) (Table 5). Tharparkar, in general, had comparatively lower gene flow with other breeds, perhaps due to its isolated and distant breeding tract. Such a high migration is understandable in view of the breeding practices essentially operative in the breeding tracts of these breeds. Farmers purchase good-looking bulls of Hariana, Mewati, Nagori (the three breeds resemble to a great extent in their physical characteristics) and Tharparkar and use these for natural mating in the breeding areas of the same as well as different breeds. Thus there is free and intentional gene flow between these breeds due to their morphological similarities. Kankrej on the other hand is morphologically distinct from the other four breeds but is a migratory breed. During its migration in summer, it passes through the breeding tracts of Mewati, Nagori and Hariana breeds. As all the breeds are managed under free grazing conditions in open fields, exchange of gene pool through crossing is practically likely. Such high migration across breeds is thus responsible for very low differentiation between the investigated breeds, as it has been postulated that populations with migration rate of more than one migrant per generation (Nm=2 and 4) exhibit no fixation [57].

Conclusion

This study, thus, presents a comprehensive analysis of the 5 grey colored cattle breeds from North and western parts of India. The geographically contiguous breeds revealed very high values of genetic diversity parameters viz. number of alleles per locus, observed and expected heterozygosity, and polymorphism information content as assessed from 21 microsatellite markers. Despite high genetic diversity values, there was very low differentiation among the breeds as revealed by F_{ST}, AMOVA, Multidimensional scaling, Factorial component analysis and STRUCTURE analysis. This genetic diversity analysis of closely related cattle breeds will help in conservation prioritization and in making plans that reconcile their genetic improvement with maintenance of genetic variation.

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