DHR International Journal Of Biomedical and Life Sciences (DHR-IJBLS)
ISSN 2278-8301, Vol. 3(1), 2012
Available online *http://www.doublehelixresearch.com/DHRIJBLS*

Original Article

Genetic Diversity and Bottleneck Analysis of Indigenous Grey Cattle Breeds of India Based on Microsatellite Data

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Abstract

Bovine microsatellite data of 21 loci was used for the assessment of genetic diversity and bottleneck analysis of 10 indigenous grey cattle breeds from different agroclimatic regions of India. A total of 448 alleles were observed across 21 loci and the observed number of alleles varied from 11 (INRA 05) to 35 (ILSTS-05). The overall observed and expected heterozygosity was 0.722 and 0.802, the PIC and Shannon index values were 0.779 and 2.065 indicating high genetic diversity. The mean number of alleles was 12.33 across 10 grey cattle breeds. The maximum observed heterozygosity was observed in Hariana and minimum in Hill Cattle breed. The F_{ST} estimates revealed 92.5% of the total genetic variation within breed, while 7.5% of the total genetic variation was among breeds.

Three different tests, *viz*., Sign rank, Standardized differences and Wilcoxon tests using 3 models of microsatellite evolution (IAM, SMM and TPM) were employed to investigate whether any of the ten Indian grey cattle populations have undergone recent bottleneck or were in mutation drift equilibrium. The tests revealed no genetic bottleneck in recent past and this was confirmed by mode shift test which gave L-shaped distribution for proportion of alleles.

Keywords: Cattle; Microsatellite markers; Genetic diversity; Conservation, F_{ST}.

Introduction

The indigenous cattle breeds have evolved over centuries and become locally adapted to the environments prevailing in the breeding tracts. The indigenous breeds have acquired specific adaptations and gene combinations, *viz*, disease resistance, adaptation to harsh climatic conditions, and exploitation of poor-quality feeds, which generally are not found in high-producing exotic breeds of cattle. *Zebu* cattle are being crossed with the exotic cattle to increase the production potential. However, the resulting crosses fail to adapt hot and humid climates [12]. As a result, some of the native draft breeds are on the verge of extinction and have been genetically eroded.

Thus, there is worldwide recognition of the need for the conservation of livestock diversity and for characterization of breeds and populations including their genetic differentiation and relationships. Of the many genetic markers now available, microsatellite loci are best suited for answering these questions [10] because of their high variability, high mutation rate, large number, distribution throughout the genome, codominant inheritance and

neutrality with respect to selection [3].

Microsatellites are highly polymorphic and hence large number of alleles due to varying number of tandem repeats, a feature attributed to their relatively high mutation rate (10^{-2}) per generation [5]. The objective of the present investigation was to estimate genetic variability in different breeds of cattle and evaluate genetic attributes of these populations/breeds.

Materials and Methods

Collection of Blood and Extraction of DNA_: A total of 460 blood were collected randomly from genetically unrelated animals of Hariana, Nagori, Tharparkar, Mewati, Binjharpuri, Ghumusari, Kangayam, Hill Cattle, Punganur and Kankrej cattle breeds from their respective native breeding tracts. Whole blood (7-8 ml) was collected from jugular vein in EDTA coated Vacutainer tubes and transported to laboratory at 0^{o}-5^{o}C. Genomic DNA was extracted from blood samples as per Sambrook and Russell [24].

Microsatellite Genotyping

A total of 21 bovine specific microsatellite markers recommended in MoDAD project of FAO [8, 9] were utilized to generate data. The 5' end of forward primers was labeled with either FAM (Blue), VIC (Green), NED (Yellow/Black) or PET (red). The PCR amplification was carried out in 25 µl reaction volume containing 1.5 mM $MgCl_2$, 200 µM dNTPs, 50 ng each of forward and reverse primer, 50-100 ng of genomic DNA and 0.5 Units of Taq DNA polymerase (Bangalore Genei, India). PCR products were loaded

on to a 2% agarose gel, electrophoresed and visualized over UV light after ethidium bromide staining to detect the amplification. Genotyping was performed on automated DNA sequencer (Applied Biosystems, ABI 3100 Avant). 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

The observed and expected heterozygosity estimates were calculated after Levene [15] and Nei [21] as implemented in POPGENE software [28]. The χ^2 and G tests for deviation from Hardy-Weinberg equilibrium in the 10 cattle populations were conducted. Heterogeneity of deviations from Hardy-Weinberg equilibrium among the microsatellite loci was examined by treating the deviations as correlation coefficient and tested accordingly [2]. The PIC value was estimated as:

$$PIC = 1 - \left(\sum_{i=1}^{n} P_i^2\right) - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2\, pi^2\, pj^2$$

where *n* is the number of alleles P_i is the frequency of the i^{th} allele at a locus in a population. *Pj* is the frequency of the j^{th} allele at a locus in a population.

Ewens-Watterson test was performed to test the neutrality for microsatellite markers; the statistics F (sum of square of allelic frequency) and limit (upper and lower) at 95% confidence region for the test were calculated using the algorithm by Manly [17] utilising 1000 simulations and implemented in POPGENE software [28]. Tests for pair wise linkage (genotypic) disequilibrium among the microsatellite loci were done using FSTAT version 2.9.3 [11] for 21 microsatellite loci. F-statistics were determined after Weir and Cockerham [25] as used in F-STAT software with Jackknifing procedure applied over loci in deriving significance levels. These parameters of population structure are defined as the correlations between pairs of genes (i) within individuals (F) (ii) between individuals in the same population (θ), and (iii) within individuals within populations (f), and are analogous to Wright's [27] F_{IT}, F_{ST} and F_{IS} , respectively. The mutation drift equilibrium was tested using Bottleneck software [23] using all the three Mutation models.

Result and Discussion

Various measures of genetic variation in terms of allele number, heterozgosity, information index and PIC values are presented in Table 1.

Table 1 The overall allelic pattern in terms of Number of alleles, effective number of alleles and observed heterozygosity are presented in the summary as below and Polymorphic Information Content (PIC) for ten indigenous grey cattle breeds

Locus Sample	na*	ne*	Obs_Het	Exp_Het*	PIC
CSRM60	28	4.767	0.709	0.791	0.774
ETH10	18	4.679	0.744	0.787	0.757
ILSTS11	15	2.359	0.450	0.577	0.537
TGLA122	23	9.127	0.864	0.891	0.881
INRA05	11	5.701	0.805	0.826	0.801
INRA63	17	2.848	0.608	0.650	0.612
TGLA227	24	3.023	0.724	0.670	0.626
CSSM08	14	4.801	0.578	0.793	0.762
HEL05	18	6.133	0.889	0.838	0.821
ILSTS05	35	8.626	0.816	0.885	0.874
ILSTS33	27	5.451	0.689	0.818	0.798
INRA35	26	8.202	0.765	0.879	0.867
BM1824	12	2.865	0.569	0.652	0.592
CSSM66	18	6.644	0.738	0.850	0.833
ETH03	19	4.073	0.700	0.755	0.722
ETH225	22	3.878	0.598	0.743	0.726
MM12	26	6.507	0.682	0.847	0.832
CSSM33	29	10.282	0.908	0.904	0.895
HEL01	20	9.549	0.671	0.896	0.887
HEL09	14	8.081	0.864	0.877	0.864
ILSTS34	32	10.362	0.801	0.905	0.898
Mean	21	6.093	0.722	0.802	0.779
S.E	1.46	0.56	0.026	0.021	0.024

A total of 448 microsatellite alleles were observed in 21 loci and 460 animals belonging to the ten breeds with mean number of 21.33 alleles per locus. Overall allelic pattern across populations is shown in Fig 1.

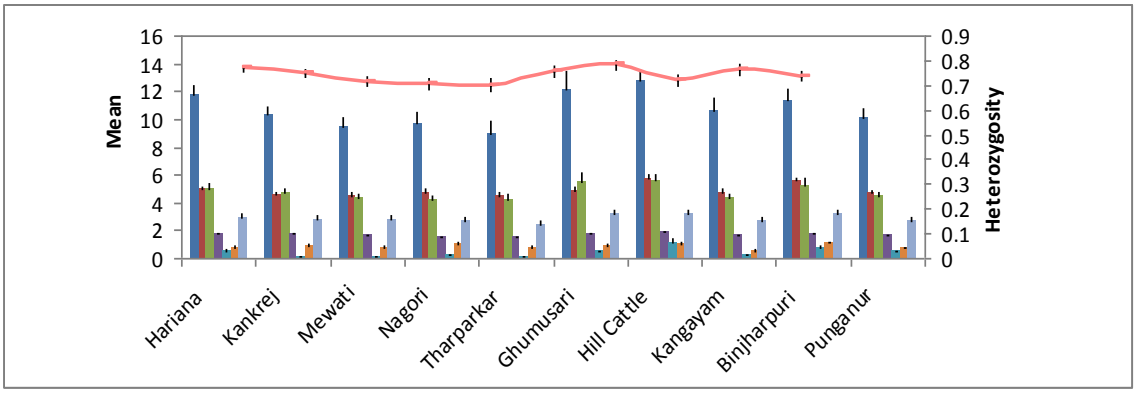

Fig. 1 The overall allelic pattern in terms of number of alleles, effective number of alleles and observed heterozygosity across populations

The most polymorphic marker was ILST05 with a total of 35 alleles and the least polymorphic marker was INRA05 with 11 alleles each. The mean numbers of alleles were not significantly different in the ten studied populations. The observed heterozygosity (H_o) ranged from 0.682 (Hill Cattle) to 0.768 (Hariana), while the expected heterozygosity (H_e) ranged from 0.700 (Tharparkar) to 0.781 (Hill cattle). The comparison of average H_{exp} and average H_{obs} values did not show marked differences in the studied populations. Shannon's Information Index [16], which measures the level of diversity, was sufficiently high with an overall mean of 2.065. The Polymorphic Information Content (PIC) values greater than 0.5 with an overall mean 0.779 confirm their utility for diversity analysis. This was also seen in the earlier investigated taurine and indicus breeds using microsatellite markers [4, 6, 18, 13, 19, 20]. The F_{ST} values is 0.075 which signifies moderate degree of population differentiation among different breeds of Cattle (7.5%). The mean F_{IS} was found to be 0.033 which is not significantly different from zero. The effective number of migrants was estimated as 4.062 pointing to large gene flow and continuity among different cattle breeds. The results of F-statistics are shown in Table 2.

Table 2 Weir & Cockerham (1984) estimation of F_{IT} (CapF), F_{ST} (theta) and F_{IS} (smallF) and Nm (Number of Migrants)

Locus	Capf (F_{IT})	Theta (F_{ST})	Smallf (F_{IS})	Nm
CSRM60	0.111	0.079	0.035	2.851
ETH10	0.059	0.044	0.016	4.879
ILSTS11	0.222	0.03	0.198	6.170
TGLA122	0.033	0.031	0.002	6.454
INRA05	0.03	0.046	-0.017	4.498
INRA63	0.068	0.038	0.031	5.339
TGLA227	-0.064	0.154	-0.259	1.442
CSSM08	0.281	0.135	0.168	1.607
HEL05	-0.056	0.047	-0.108	4.383
ILSTS05	0.086	0.094	-0.008	2.424
ILSTS33	0.161	0.043	0.124	4.918
INRA35	0.134	0.042	0.096	5.004
BM1824	0.137	0.114	0.026	1.884
CSSM66	0.138	0.061	0.082	3.517
ETH03	0.078	0.056	0.024	3.788
ETH225	0.208	0.161	0.056	1.285
MM12	0.2	0.056	0.152	3.852
CSSM33	-0.003	0.018	-0.021	9.484
HEL01	0.265	0.185	0.098	1.129
HEL09	0.017	0.021	-0.004	8.522
ILSTS34	0.125	0.121	0.004	1.873
Mean	0.105	0.075	0.033	4.062

Nm Gene flow estimated from F_{ST}=0.25(1- F_{ST})/ $F_{ST.}$

The overall test for neutrality across the ten populations (Table 3) revealed that observed F values were within the lower and upper limits of the 95 % confidence interval for most of the loci using 1000 simulations.

Table 3 The Ewens-Watterson test for Neutrality at 21 microsatellite loci in ten cattle breeds

Locus	n	k	Obs. F	Min F	Max F	Mean*	SE*	L95*	U95*
CSRM60	920	28	0.210	0.036	0.943	0.159	0.004	0.086	0.322
ETH10	914	18	0.214	0.056	0.964	0.238	0.009	0.124	0.470
ILSTS11	920	15	0.424	0.067	0.970	0.276	0.011	0.146	0.558
TGLA122	914	23	0.110	0.044	0.953	0.189	0.005	0.102	0.392
INRA05	890	11	0.175	0.091	0.978	0.359	0.018	0.183	0.707
INRA63	908	17	0.351	0.059	0.965	0.250	0.009	0.131	0.493
TGLA227	920	24	0.331	0.042	0.951	0.179	0.004	0.095	0.352
CSSM08	896	14	0.208	0.071	0.971	0.297	0.013	0.153	0.583
HEL05	846	18	0.163	0.056	0.961	0.234	0.008	0.127	0.451
ILSTS05	914	35	0.116	0.029	0.928	0.120	0.002	0.071	0.235
ILSTS33	906	27	0.183	0.037	0.944	0.159	0.004	0.089	0.317
INRA35	910	26	0.122	0.039	0.947	0.165	0.004	0.091	0.305
BM1824	918	12	0.349	0.083	0.976	0.334	0.018	0.166	0.699
CSSM66	916	18	0.151	0.056	0.964	0.242	0.010	0.124	0.488
ETH03	920	19	0.246	0.053	0.962	0.230	0.008	0.119	0.483
ETH225	920	22	0.258	0.046	0.955	0.200	0.007	0.105	0.421
MM12	906	26	0.154	0.039	0.946	0.168	0.004	0.090	0.344
CSSM33	910	29	0.097	0.035	0.940	0.147	0.003	0.083	0.289
HEL01	888	20	0.105	0.050	0.958	0.211	0.006	0.110	0.411
HEL09	898	14	0.124	0.071	0.972	0.298	0.014	0.150	0.601
ILSTS34	886	32	0.097	0.031	0.933	0.132	0.002	0.074	0.256

* These statistics were calculated using 1000 simulated samples.

Only for 3 microsatellite loci (INRA63, HEL1 and HEL9), the observed F values were marginally outside the upper and lower limits of the 95 % confidence interval which point towards the selection of the region which may be responsible for some economic trait.

Genetic bottleneck occurs when population experiences a severe reduction in effective population size. This may influence distribution of genetic variation within and among populations. Loss of genetic diversity may reduce the survival potential of small populations to respond to adverse conditions [1] and increased inbreeding may reduce population viability [14, 22, 26]. The three tests (sign test, standard

difference test and wilcoxon rank test) under these three models (IAM, TPM and SMM) for heterozygosity excess can detect the recent bottleneck up to 50-250 generations.

In the present study, ten cattle breeds, sign rank test under IAM model accepted the null hypothesis, whereas under SMM and TPM (except Mewati, Tharparkar, Ghumusari and Binjharpuri) models of microsatellite evolution, the mutation drift equilibrium was rejected favoring heterozygotic deficiency instead of heterozygotic excess (Table 4).

Table 4 Test for Null Hypothesis under three Microsatellite Evolution Models, (genetic bottleneck analysis)

	Infinite alleles model (IAM)			**Two-phase model (TPM)**			**Stepwise mutation model (SMM)**		
	Expected	**Observed**	**P Value**	**Expected**	**Observed**	**P Value**	**Expected**	**Observed**	**P Value**
Hariana	12.73	12	0.453	12.5	6	0.004**	12.31	3	0.000
Kankrej	12.59	12	0.478	12.54	6	0.004**	12.28	3	0.000
Mewati	12.55	12	0.485	12.57	10	0.178 ^{NS}	12.41	4	0.000
Nagori	12.57	13	0.519	12.46	8	0.040*	12.37	2	0.000
Tharparkar	12.5	15	0.188	12.39	10	0.201^{NS}	12.4	5	0.001
Ghumusari	12.55	12	0.486	12.38	10	0.201 ^{NS}	12.38	5	0.001
Hill Cattle	12.69	12	0.459	12.64	7	0.012*	12.37	2	0.000
Kangayam	12.6	13	0.523	12.42	8	0.042*	12.23	3	0.000
Binjharpuri	12.66	13	0.534	12.44	9	0.097 ^{NS}	12.34	3	0.000
Punganur	12.63	12	0.472	12.46	8	0.041*	12.35	2	0.000

Sign test: Number of loci with heterozygosity excess (probability)

* These statistics were calculated using 1000 simulated samples.

** Significance

Bottleneck analysis as depicted in Table 1 indicates that the observed heterozygosity excess (He) is less than the expected excess heterozygosity (Hee) in 1,000 simulations based on allele frequency and heterozygosity for all breeds (except Nagori, Tharparkar, Kangayam and Binjharpuri) revealing absence of genetic bottleneck . When a population experiences a reduction of its effective size, the allele number is reduced faster than the heterozygosity, i.e. the observed heterozygosity is larger than the heterozygosity expected from the observed allele number when the locus is at mutation-drift equilibrium.

The standardized differences test is a parametric test and takes into account the magnitude of heterozygosity excess/deficiency [7]. The standardized differences test revealed positive T2 values under IAM (except Kangayam; -0.020) in all the ten breeds, although all were statistically not-significant (comparing to table value). In all the ten breeds, it was found that T2 values are negative under TPM and SMM and all were statistically significant (Table 5) but the negative sign reveals heterozygotic deficiency and not heterozygotic excess. This reveals absence of genetic bottleneck using quantitative test.

Table 5 Standardized differences test to evaluate various cattle breeds for mutation drift equilibrium under different models

	Infinite alleles model (IAM)		Two-phase model (TPM)		Stepwise mutation model (SMM)	
Breeds	**T2**	**P Value**	**T2**	**P Value**	**T2**	**P Value**
Hariana	0.275	0.392 ^{NS}	-4.744	0.000^{q}	-13.822	0.000 ^{q}
Kankrej	1.148	0.125 ^{NS}	-2.914	0.002 ^{q}	-10.944	0.000 ^{q}
Mewati	0.569	0.285 ^{NS}	-3.589	0.000 ^{q}	-11.161	0.000 ^{q}
Nagori	0.584	0.280 ^{NS}	-3.846	0.000 ^{q}	-12.367	0.000 ^{q}
Tharparkar	1.541	0.062 ^{NS}	-2.087	0.018 ^{q}	-9.048	0.000 ^{q}
Ghumusari	0.86	0.195 ^{NS}	-3.781	0.000 ^{q}	-10.864	0.000 ^{q}
Hill Cattle	0.678	0.249 ^{NS}	-4.394	0.000 ^{q}	-12.43	0.000 ^{q}
Kangayam	-0.02	0.492 ^{NS}	-5.52	0.000 ^{q}	-14.313	0.000 ^{q}
Binjharpuri	1.231	0.109 ^{NS}	-3.05	0.001 ^{q}	-10.944	0.000 ^{q}

- Negative values in standardized differences test signifies heterozygotic deficiency
  q significant deviation due to heterozygotic deficiency

This result was further reinforced by Wilcoxon signed-rank test which revealed significant P-value for the one-tail test of heterozygosity excess. The probability values with wilcoxon rank test for IAM, TPM and SMM indicated the acceptance of mutation drift equilibrium in all breeds under all the models while rejected in Tharparkar under IAM (Table 6).

Table 6 Wilcoxon signed-rank test to evaluate various cattle breeds for mutation drift equilibrium under different models

Wilcoxon-rank test (probability for one tail heterozygosity excess)

Breeds	IAM	TPM	SMM
Hariana	0.406	0.995	1.000
Kankrej	0.064	0.985	1.000
Mewati	0.329	0.931	1.000
Nagori	0.178	0.989	1.000
Tharparkar	0.030	0.856	1.000
Ghumusari	0.079	0.911	1.000
Hill Cattle	0.178	0.982	1.000
Kangayam	0.216	0.992	1.000
Binjharpuri	0.069	0.899	1.000
Punganur	0.354	0.996	1.000

All are non significant except Tharparkar (P<0.05).

The qualitative test for mode shift utilizing the Proportion of allele was performed in all the ten cattle breeds. The Proportion of alleles at low frequency was highest resulting in L-shaped curve, which denotes the absence of a recent genetic bottleneck (Fig. 2).

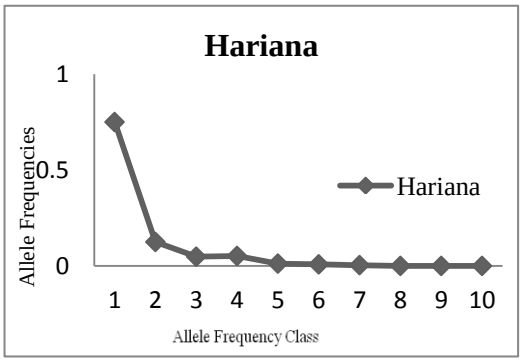

Fig. 2 Representative picture of Mode- Shift Test using Graphic Representation

Acknowledgements

The authors acknowledge their sincere thanks to the Director, NBAGR and Dr. B. Prakash Principal Scientist, NBAGR, for providing all possible facilities and encouragement in carrying out this study.

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