RESEARCH ARTICLE

Microsatellite DNA typing for assessment of genetic variability in Tharparkar breed of Indian zebu (*Bos indicus*) cattle, a major breed of Rajasthan

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Abstract

The present study estimates genetic variability with a set of 25 microsatellite markers in a random sample of 50 animals of Tharparkar breed of Indian zebu (*Bos indicus*) cattle. Tharparkar is a dual-purpose breed, valued for its milk as well as draught utility, and is adapted to the inhospitable Thar desert conditions of Rajasthan typified by summer temperature hovering above 50°C, sparse rainfall and vegetation, and scarcity of even drinking water. The observed number of alleles ranged from 4 (ETH3, ILSTS030, INRA5, INRA63 and MM8) to 11 (HEL9 and ILSTS034), with allelic diversity (average number of observed alleles per locus) of 6.20. Observed and expected heterozygosity ranged from 0.25 (INRA63) to 0.77 (ETH10), and from 0.51 (HEL5 and HAUT27) to 0.88 (HEL9) respectively. Wide range of genetic variability supported the utility of these microsatellite loci in measurement of genetic diversity indices in other Indian cattle breeds too. Various average genetic variability measures, namely allele diversity (6.20), observed heterozygosity (0.57), expected heterozygosity (0.67) and mean polymorphism information content (0.60) values showed substantial within-breed genetic variability in this major breed of Rajasthan, despite accumulated inbreeding as reflected by high average inbreeding coefficient (F_{IS} = 0.39). The Tharparkar population has not experienced a bottleneck in the recent past.

[Sodhi M., Mukesh M., Prakash B., Ahlawat S. P. S. and Sobti R. C. 2006 Microsatellite DNA typing for assessment of genetic variability in Tharparkar breed of Indian zebu (*Bos indicus*) cattle, a major breed of Rajasthan. *J. Genet.* **85**, 165–170]

Introduction

The vast and varied cattle genetic resources of India are identified in the form of 30 documented breeds of zebu cattle (*Bos indicus*) (Acharya and Bhat 1984) besides many populations still uncharacterized and undefined. Conservation of genetically unique breeds/populations is of top priority to prevent loss of genetic diversity within each domestic species. In view of the massive costs involved, it is not practicable to launch conservation and improvement programmes for each livestock breed. In the absence of appropriate grouping of breeds, clustering derived from molecular characterization of general genetic variability will provide valuable evidence to rank/prioritize the breeds in terms of phylogenetic distinctness. This will help breeders to implement rational decisions for conservation and improvement of valuable germplasm. Indigenous cattle breeds are considered, for diverse reasons, as treasured genetic resources that tend to disappear as a result of new market demands, crossbreeding or breed replacements, and mechanized agricultural operations. There is a terrible risk that most breeds may perish before they have been exclusively recognized and exploited (FAO 2000). This is predominantly true for Indian zebu cattle breeds which have already suffered significant loss of economic importance and extensive decline in their population size primarily owing to indiscriminate crossbreeding.

Among the currently used molecular marker systems for genetic characterization, microsatellites are most preferred because of their extremely informative polymorphic nature, their abundance in the genome, and the ease of amplification and typing by PCR. These markers have been used to explain bovine domestication and migration patterns (Bradley

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Keywords. genetic bottleneck; microsatellite markers; genetic diversity; Tharparkar cattle.

et al. 1994; Loftus *et al*. 1994; Bruford *et al*. 2003) and to characterize cattle populations (Kantanen *et al*. 2000; Tambasco *et al*. 2000; Beja-Pereira *et al*. 2003; Dorji *et al*. 2003; Jordana *et al*. 2003; Metta *et al*. 2004; Mukesh *et al*. 2004). Microsatellite genetic markers contribute diverse types of information pertinent for making decisions regarding conservation of livestock (Sunnucks 2000).

Wide-ranging, but still meagre, efforts, especially in developing countries, are in progress to characterize livestock at the phenotypic as well as molecular-genetic level, and to document their production environments, utilization and status. Genetic characterization of Tharparkar breed of Indian cattle is our contribution to the ongoing global endeavour of genetic characterization of cattle genetic resources. Tharparkar is a dual-purpose breed valued for its milk as well as draught utility and adapted to the inhospitable Thar desert conditions of Rajasthan and parts of Gujarat extending up to Rann of Kutch, typified by summer temperature hovering above 50°C, scarce rainfall and vegetation, and scarcity of even drinking water. Over the years elite animals of this breed have been frequently used for upgrading local low-yielding cattle breeds. Comprehensive information on Tharparkar cattle, including geographical distribution, management practices, breed characteristics, and production and reproduction performance traits, has been collected through surveys in the native tracts (Nivsarkar *et al*. 1992). The present study gives an account of the existing within-breed genetic variability in Tharparkar cattle and the generated data can be used to determine genetic relationships with other indigenous as well as exotic cattle breeds.

Materials and methods

Sampling and DNA isolation

Blood samples were randomly collected from 50–55 genetically unrelated animals of Tharparkar cattle from different villages of Jaisalmer and Jodhpur districts of Rajasthan (figure 1) in line with MoDAD recommendations (FAO 1998). Genomic DNA was extracted from whole blood using proteinase K digestion followed by standard phenol–chloroform extraction procedure.

Microsatellite markers

A panel of 25 bovine-specific microsatellite markers (BM1818, ETH3, ETH10, ETH152, ETH185, ETH225, HEL1, HEL5, HEL9, ILSTS005, ILSTS006, ILSTS011, ILSTS030, ILSTS033, ILSTS034, ILSTS054, INRA005, INRA035, INRA063, MM8, CSSM60, CSSM66, HAUT24, HAUT27, ILST002) recommended in the MoDAD project of FAO (1996) for cattle genetic diversity studies was selected for genetic characterization and revealing the extent of genetic diversity in Tharparkar cattle.

Figure 1. Breeding tract of Tharparkar cattle.

PCR-based microsatellite DNA typing

PCR was carried out in 25-μ1 reaction volume containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 50 ng of each primer, ~100 ng of template DNA and 0.5 U of *Taq* DNA polymerase (Promega, Madison, USA) using PTC-100 thermocycler (MJ Research, Watertown, USA). PCR cycling conditions were: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at annealing temperature (52–64 °C) of each primer, 1 min at 72°C, and final extension of 10 min at 72°C. PCR-amplified products were resolved on 6% urea–PAGE denaturing sequencing gel at 75 W (Sequi Gen GT apparatus. Bio-Rad, Hercules, USA) and visualized by silver staining (Bassam *et al*. 1991). Allele sizes were estimated using a 10-bp ladder (Invitrogen Life Technologies, Carlsbad, USA). Genotype of each individual animal at 25 different loci was recorded by direct counting.

Analysis of molecular data

Genotype of each individual animal was determined and recorded from the silver-stained gels for each microsatellite locus. Different measures of within-breed genetic variations, namely number of alleles, allele frequencies, effective number of alleles (n_e), observed heterozygosity (H_o), expected heterozygosity (H_e), were estimated using the POPGENE software package (Yeh *et al*. 1999) to evaluate variability at DNA level. Polymorphism information content (PIC) for each locus was calculated according to Botstein *et al*. (1980).

Departure from Hardy–Weinberg proportions and linkage disequilibrium were determined using exact probability tests provided in GENEPOP version 3.1 a (Raymond and

Rousset 1995). A Monte Carlo method (Guo and Thompson 1992) was applied to compute unbiased estimates of the exact probabilities (*P* values). Length of chain was set to be 50,000 iterations. The within-population inbreeding estimate (F_{IS}) at each microsatellite locus were estimated using the FSTAT version 2.9.3.2 computer program (Goudet 2002). The level of significance ($P < 0.05$) was determined from permutation test with the sequential Bonferroni procedure applied over all loci.

To detect whether the Tharparkar population has experienced a recent reduction in the effective population size or a genetic bottleneck, three different approaches were followed. In the first approach, based on heterozygosity excess, three different tests, namely a 'sign test', a 'standardized differences test' and a 'Wilcoxon sign-rank test', were employed under different models of microsatellite evolution like the infinite allele (IAM), stepwise mutation (SMM) and two-phased (TPM) models of mutation. The second approach was the graphical representation of the mode-shift indicator proposed by Luikart and Cornuet (1997). These two approaches were conducted using Bottleneck v1.2.02 software (http://www.ensam.inra.fr/URLB; Cornuet and Luikart (1996)). The third approach was the *M*-ratio conducted by using the m_p_val.exe program (Garza and Williamson 2001). (*M*-ratio $M = 1/4\ k/r$; Garza and Williamson 2001) is the ratio of the number of alleles present at a locus (k) to the range of allele sizes in base pairs for the same locus (r). To test whether an *M*-ratio is lower than expected, 10,000 replicates were simulated. The number of times that the simulated *M* is higher than the calculated *M* represents the statistical significance of the *M*-ratio reduction.

Results and discussion

All the loci were amplified successfully and exhibited substantial levels of genetic diversity estimates. These microsatellite markers have also been reported to be polymorphic in taurine (Bradley *et al*. 1994; Loftus *et al*. 1994, 1999; Kantanen *et al*. 2000; Beja-Pereira *et al*. 2003; Bruford *et al*. 2003; Jordana *et al*. 2003) as well as zebu cattle (Tambasco *et al*. 2000; Dorji *et al*. 2003; Kumar *et al*. 2003; Metta *et al*. 2004; Mukesh *et al*. 2004). The observed effective number of alleles, PCR product size range, observed and expected heterozygosity, and PIC value of each microsatellite marker investigated are presented in table 1. Allele frequency profile for each of the microsatellite markers is depicted in figure 2.

Table 1. Number of alleles (n_o observed, n_e effective), heterozygosity (H_o observed, H_e expected), polymorphism information content (PIC), size-range and within-population inbreeding estimates (F_{IS}) at microsatellite loci in Tharparkar cattle.

	Locus	Size range (bp)	n_o	n_e	H_o	H_e	PIC	F_{IS}
1.	BM1818†	254–294	6.0	1.38	0.65	0.70	0.66	0.498*
2.	CSSRM66	177–209	9.0	4.57	0.44	0.79	0.77	0.056
3.	CSSM60†	182–202	8.0	3.04	0.64	0.67	0.65	0.448*
4.	ETH3	109–133	4.0	2.00	0.66	0.51	0.49	−0.487
5.	ETH10	210–246	6.0	3.66	0.77	0.74	0.72	−0.047
6.	ETH152†	190–204	5.0	2.87	0.62	0.67	0.48	0.823*
7.	ETH185†	208–242	6.0	2.75	0.37	0.64	0.56	0.310*
8.	ETH225	130–170	7.0	2.26	0.50	0.57	0.47	0.116
9.	HAUT24†	109–133	7.0	4.59	0.78	0.80	0.79	0.390*
10.	HAUT27†	140–162	6.0	2.02	0.45	0.51	0.46	0.330*
11.	HEL1	102–120	7.0	3.24	0.67	0.70	0.69	−0.048
12.	HEL5†	145–165	6.0	2.02	0.32	0.51	0.44	0.371*
13.	HEL9†	144–170	11.0	7.94	0.57	0.88	0.83	0.163*
14.	ILSTS002	122–138	6.0	2.93	0.50	0.67	0.61	0.254*
15.	ILSTS05	182–198	5.0	3.70	0.71	0.73	0.57	0.036
16.	ILSTS006†	276–296	6.0	4.02	0.44	0.76	0.51	0.431*
17.	ILSTS011†	250–270	6.0	3.46	0.46	0.72	0.59	0.362*
18.	ILSTS030†	158–184	4.0	2.57	0.60	0.62	0.57	1.000*
19.	ILSTS033†	132–154	6.0	3.16	0.69	0.69	0.61	0.122*
20.	ILSTS034	148–210	11.0	3.88	0.56	0.75	0.74	0.256
21.	ILSTS054	126–156	5.0	4.40	0.64	0.78	0.73	0.567*
22.	INRA5†	132–146	4.0	2.67	0.67	0.63	0.51	0.177*
23.	INRA35	102–114	6.0	3.27	0.67	0.71	0.70	0.064
24.	INRA63†	170–188	4.0	2.26	0.25	0.56	0.54	0.550*
25.	MM8	132–148	4.0	2.14	0.66	0.53	0.43	0.050
	Mean		6.2	3.23	0.57	0.67	0.60	0.140

*Mean estimates from Jackknife over loci; $P < 0.05$.

†Loci showing significant deviation from Hardy–Weinberg equilibrium.

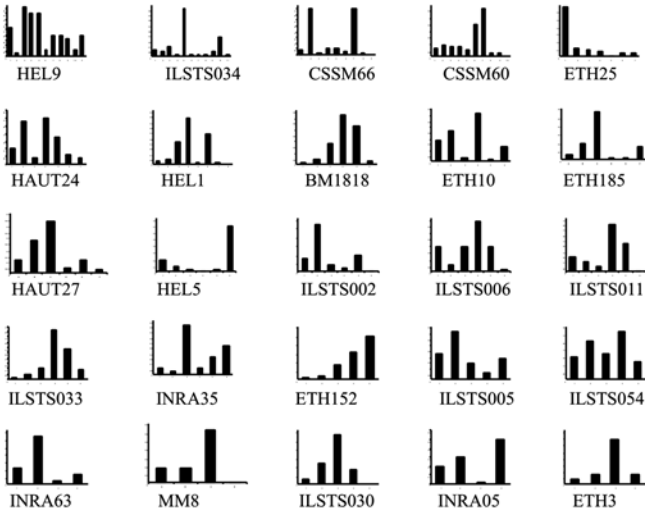

Figure 2. Allelic frequency distribution across the 25 microsatellite markers in Tharparkar cattle.

A total of 155 distinct alleles were detected across the 25 loci. The observed number of alleles ranged from 4 (ETH3, ILSTS030, INRA5, INRA63 and MM8) to 11 (HEL9 and ILSTS034), with allelic diversity (average number of observed alleles per locus) of 6.20. The overall effective number of alleles was less than the observed value across all the loci and ranged between 1.38 (BM1818) to 7.94 (HEL9), with a mean of 3.23. Observed and expected heterozygosity ranged from 0.25 (INRA63) to 0.77 (ETH10), and from 0.51 (HEL5 and HAUT27) to 0.88 (HEL9) respectively. PIC for each locus ranged from 0.43 at MM8 to 0.83 at HEL9, with an average of 0.60. Reasonably high PIC values observed for most of the markers are indicative of the usefulness of microsatellites for biodiversity evaluation in this breed.

Examination of the population genotype frequencies and heterozygote deficiency indicated that most of the loci agreed with Hardy–Weinberg expectations ($P > 0.05$). This deviation could also be attributed to rather high positive F_{IS} (within-population inbreeding estimate) estimates obtained (table 1). Exact test assessment for genotypic linkage disequilibrium yielded no significant P values across the population, and hence independent assortment was inferred.

There was a significant deficit of heterozygotes at a few loci (table 1) and the average F_{IS} values for majority of the loci were significantly different ($P < 0.05$) from zero. This shortage of heterozygotes and excess of homozygotes in Tharparkar cattle ($F_{IS} > 0$) might be attributed to a number of factors, namely sample relatedness, linkage with loci under selection (genetic hitchhiking), population heterogeneity, or null alleles (nonamplifying alleles).

The conclusion from the bottleneck analysis is absence of any bottleneck in Tharparkar cattle in the recent past. The first approach based on heterozygosity excess works on the principle that in a recently bottlenecked population, the observed gene diversity is higher than the expected equilibrium gene diversity (H_{eq}), which is computed from the observed number of alleles (k), under the assumption of a constant-size (equilibrium) population. None of the calculated P values (table 2) was significant ($P > 0.05$), demonstrating that the null hypothesis of mutation–drift equilibrium is fulfilled in this population. A second approach, a qualitative geographical method, detected no mode shift in the frequency distribution of alleles and a normal L-shaped curve was observed, where the alleles with the lowest frequencies (0.01–0.1) were observed to be most abundant (figure 3).

When a population experiences a bottleneck event, rare alleles are lost more often by drift than the common alleles during a population size reduction and k is reduced.

Table 2. Number of loci with heterozygosity excess/deficiency and probabilities obtained from three microsatellite evolution models for bottleneck test.

	Test	Exc. H exp	Exc. H obs	Def. H obs	P
1.	Sign test				
	IAM	12.93	20	5	0.02032
	TPM	12.98	18	7	0.13626
	SMM	12.98	11	14	0.02710
2.	Standardized differences test (T2 values)				
	IAM	3.043			0.00117*
	TPM	0.761			0.22320
	SMM	−3.712			0.00010*
3.	Wilcoxon test (probabilities - one tail for H excess)				
	IAM				0.00037*
	TPM				0.10496*
	SMM				0.97875

*Deviation from the mutational equilibrium $P < 0.05$.

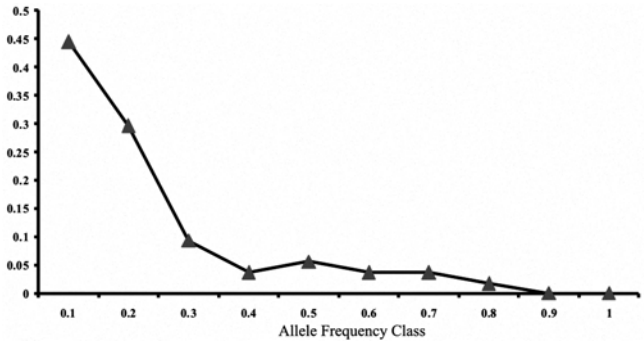

Figure 3. Mode-shift analysis for test for genetic bottleneck in Tharparkar cattle.

However, allele loss does not always occur at the extremes of the allele size distribution, so the range in allele sizes (r) will not be reduced at the same rate as k. Consequently the M-ratio declines in the case of a bottleneck event. However the M-ratio for the Tharparkar population was 0.6234, which was not statistically significant at the 5% level, indicating absence of a bottleneck event. The concordance in the results of three approaches revealed the absence of a recent-past demographic reduction in the Tharparkar population.

The study thus presents valuable insight into the existing genetic variability in Tharparkar cattle. The high degree of variability demonstrated in Indian Tharparkar cattle, notwithstanding its recent shrinking population size, implies that this population is a rich reservoir of genetic diversity. This fact together with its demonstrated functional superiority and its distinctive environmental adaptation consolidate the significance of its conservation as a valuable pure breed and its utilization in agricultural exploitation as a source for indigenous cattle improvement to achieve better production. The extension of the ongoing attempt at molecular-genetic characterization to other indigenous cattle breeds for determining genetic relationships will help in prioritizing the breeds on the basis of their genetic makeup and phylogenetic ranking for effective conservation and improvement programmes.

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Received 21 September 2005