RESEARCH ARTICLE

Genetic diversity studies of Kherigarh cattle based on microsatellite markers

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Abstract

We report a genetic diversity study of Kherigarh cattle, a utility draught-purpose breed of India, currently declining at a startling rate, by use of microsatellite markers recommended by the Food and Agriculture Organization. Microsatellite genotypes were derived, and allelic and genotypic frequencies, heterozygosities and gene diversity were estimated. A total of 131 alleles were distinguished by the 21 microsatellite markers used. All the microsatellites were highly polymorphic, with mean (± s.e.) allelic number of 6.24 ± 1.7, ranging 4–10 per locus. The observed heterozygosity in the population ranged between 0.261 and 0.809, with mean (± s.e.) of 0.574 ± 0.131, indicating considerable genetic variation in this population. Genetic bottleneck hypotheses were also explored. Our data suggest that the Kherigarh breed has not experienced a genetic bottleneck in the recent past.

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Introduction

Livestock of a given species are often derived from a single domestication event, followed by genetic and phenotypic radiation through natural and artificial selection, typically resulting in great genetic diversity among breeds. This diversity is perceptible in the form of local adaptation to stressful and unpredictable environments, resistance to many of the leading indigenous diseases, and vastly improved and specialized production ability. Nearly all wild ancestors of livestock are either extinct or exist in small populations with limited genetic diversity. Thus, contrary to the situation in many agricultural plant species, most or all genetic diversity in livestock exists within and between the available domesticated farm animals. Consequently, extensive, but still inadequate, efforts are in progress to characterize livestock at phenotypic and genetic levels, and to document their production environments, utilization and status.

Measuring diverse attributes of a population is important to its characterization, taking into account phenotypic traits, reproduction, geographic distribution, origin and habitat. The genetic characterization of populations, breeds and species allows evaluation of genetic variability, a fundamental element in working out breeding strategies and genetic conservation plans. Molecular markers have been comprehensively exploited to access this variability as they contribute information on every region of the genome, regardless of the level of gene expression. Microsatellites (highly polymorphic simple sequence repeats) are presently the most favoured molecular markers, essentially owing to the option of blending their analysis with use of the polymerase chain reaction (PCR). Microsatellites have been effectively exploited to understand bovine domestication and migration pattern (Bradley *et al*. 1994; Loftus *et al*. 1994; Edwards *et al*. 2000) and to evaluate genetic diversity and relationships among cattle populations (MacHugh *et al*. 1997; Canon *et al*. 2001; Kim *et al*. 2002; Maudet *et al*. 2002; Dorji *et al*. 2003; Jordana *et al*. 2003; Metta *et al*. 2004; Mukesh *et al*. 2004).

The enormous and diverse cattle genetic resources of India are illustrated by the 30 documented breeds of zebu cattle (Acharya and Bhat 1984) besides numerous populations yet to be characterized and defined. Many Indian cattle breeds enter the draught category, because cattle development in India has principally rested on production of bullock energy required for conventional agricultural operations and load

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pulling. Present-day cattle breeds have evolved through mutation and genetic drift, as well as artificial selection (Barker 1999). The drive for enhanced animal production using a few superbly selected breeds has instigated rapid erosion in the number of local breeds through crossbreeding or breed replacements.

The Kherigarh breed of Indian zebu cattle (*Bos indicus*) has evolved as a draught breed over centuries and has become adapted to harsh native environments, acquiring resistance to tropical diseases and ability to survive on low-quality roughage and grasses. The breed is primarily employed for agricultural operations, for carrying loads, and for transportation. The breeding tract of this breed encompasses Nighasan and Pallia blocks of Lakhimpur Kheri district (27N54 80E48) of Uttar Pradesh state, which lies in the foothills of the Himalayas. The breed is primarily maintained by small, marginal and landless labourers. Bullocks are fast and good for light draught (carting) and agricultural operations. The estimated size of the Kherigarh population in the entire breeding tract is just 15,000 head and declining (NBAGR 2003). This calls for urgent conservation and improvement strategies for this breed.

The present study is part of an ongoing global endeavour for genetic characterization of livestock genetic resources. The population structure, genetic variability and genetic bottlenecks in Kherigarh cattle have been evaluated using 21 microsatellite markers from the United Nations Food and Agriculture Organization (FAO) recommended list for the measurement of domestic animal diversity (FAO 1995).

Materials and methods

Sample collection

Blood samples were acquired from 50 randomly selected and unrelated Kherigarh animals from the breeding region (figure 1) following the guidelines of MoDAD (Measurement of Domestic Animal Diversity; FAO 1995) programme. To ensure unrelatedness of animals in the absence of reliable pedigree accounts, animals were selected from distinct villages after interviewing the owners in detail. Blood samples (5–6 ml) were collected from the jugular vein of the animal in vacutainers containing EDTA as anticoagulant.

Molecular techniques

DNA was extracted from whole blood using standard protocols (Sambrook *et al*. 1989). The DNA isolation procedure involved lysis of RBCs, digestion of protein using proteinase K, and precipitation of protein using phenol : chloroform : isoamyl alcohol. A set of 21 microsatellite markers (table 1) recommended for cattle in FAO's DADIS MoDAD programme were utilized for generating microsatellite genotyping data in a panel of 47 animals. Since microsatellite markers are codominant, 47 samples correspond to 94 alleles for each microsatellite locus. An amalgamation of 21 codominant loci and 47 samples were projected to create 1974 allelic data for the population included in this study.

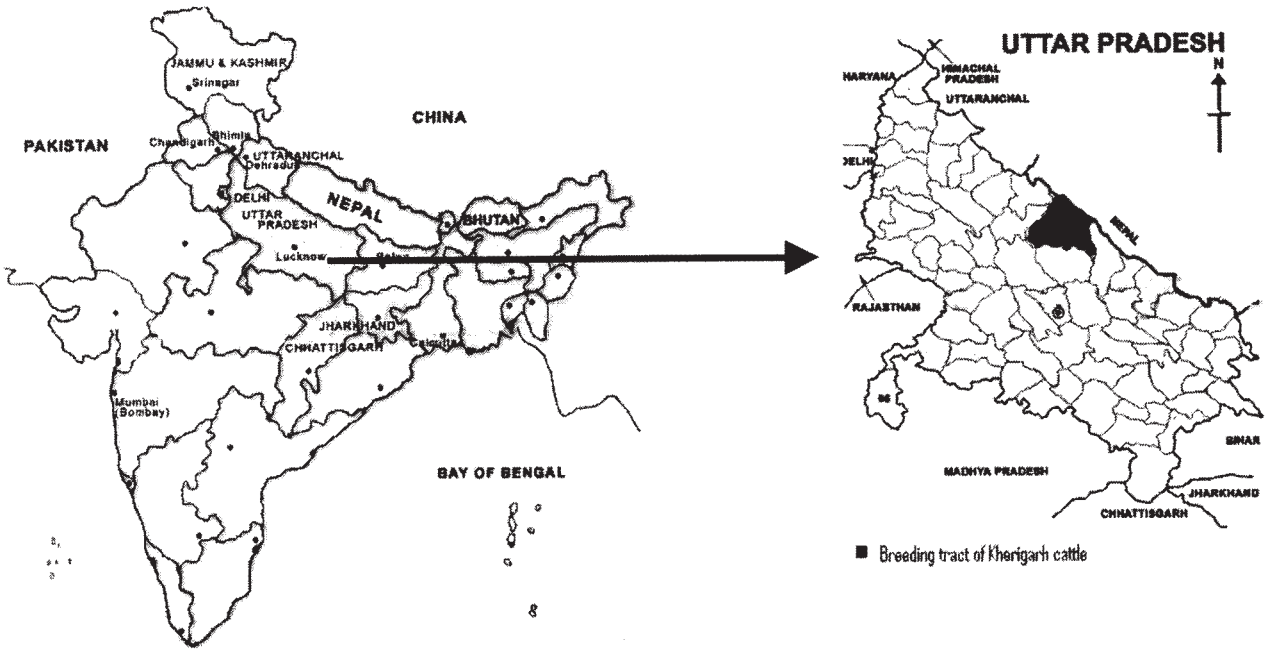

Figure 1. Map depicting breeding tract of Kherigarh cattle in India.

Table 1. Microsatellite markers, sequences, location and annealing temperature.

Marker	Primer sequences	Chromosome number	Annealing temp. (°C)
MM8	CCCAAGGACAGAAAAGACT, CTCAAGATAAGACCACACC	2	55
INRA063	ATTTGCACAAGCTAAATCTAACC, AAACCACAGAAATGCTTGGAAG	18	55
ILSTS030	CTGCAGTTCTGCATATGTGG, CTTAGACAACAGGGGTTTGG	2	55
BM1818	AGCTGGGAATATAACCAAAGG, AGTGCTTTCAAGGTCCATGC	23	58
CSRM60	AAGATGTGATCCAAGAGAGAGGCA, AGGACCAGATCGTGAAAGGCATAG	10	55
ILSTS054	GAGGATCTTGATTTTGATGTCC, AGGGCCACTATGGTACTTCC	21	55
ILSTS005	GGAAGCAATGAAATCTATAGCC, TGTTCTGTGAGTTTGTAAGC	10	55
HEL5	GCAGGATCACTTGTTAGGGA, AGACGTTAGTGTACATTAAC	21	55
ILSTS006	TGTCTGTATTTCTGCTGTGG, ACACGGAAGCGATCTAAACG	7	56
ILSTS011	GCTTGCTACATGGAAAGTGC, CTAAAATGCAGAGCCCTACC	14	58
INRA035	ATCCTTTGCAGCCTCCACATTG, TTGTGCTTTATGACACTATCCG	16	55
ILSTS033	TATTAGAGTGGCTCAGTGCC, ATGCAGACAGTTTTAGAGGG	12	55
HEL9	CCCATTCAGTCTTCAGAGGT, CACATCCATGTTCTCACCAC	8	59
BM1824	GAGCAAGGTGTTTTTCCAATC, CATTCTCCAACTGCTTCCTTG	1	55
ETH225	GATCACCTTGCCACTATTTCCT, ACATGACAGCCAGCTGCTACT	9	57
HAUT24	CTCTCTGCCTTTGTCCCTGT, AATACACTTTAGGAGAAAAATA	22	52
ILSTS034	AAGGGTCTAAGTCCACTGGC, GACCTGGTTTAGCAGAGAGC	5	57
HAUT27	TTTTATGTTCATTTTTTGACTGG, AACTGCTGAAATCTCCATCTTA	26	55
HEL1	CAACAGCTATTTAACAAGGA, AGGCTACAGTCCATGGGATT	15	55
MM12	CAAGACAGGTGTTTCAATCT, ATCGACTCTGGGGATGATGT	9	55
INRA005	CAATCTGCATGAAGTATAAATAT, CTTCAGGCATACCCTACACC	12	55

PCR was performed with 50–100 ng of genomic DNA in a 25-μl reaction volume using a PTC-200 PCR machine (M. J. Research, Watertown, USA). The PCR procedure involved initial denaturation at 95°C for 1 min, 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, Hercules, USA) and sized using a 10-bp ladder (Invitrogen, Life Technologies, Carlsbad, USA) as standard for sizing. Gels were stained by silver staining (Bassam *et al*. 1991), and genotypes scored manually.

Statistical analysis

Observed and expected heterozygosity estimates were computed after Nei (1973), as executed in POPGENE software (Yeh *et al*. 1999). The observed number of alleles and effective number of alleles were also evaluated using POPGENE software. Allelic frequencies were utilized for assessing polymorphic information content (PIC), a measure of informativeness of a marker, calculated according to Botstein *et al*. (1980) using the given formula

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} x_i^2 - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2x_i^2 x_j^2,$$

where k is the number of alleles and x_i and x_j are the frequencies of the ith and jth alleles respectively.

Departure from Hardy–Weinberg equilibrium was derived using the exact test of POPGENE (Yeh *et al*. 1999). Heterogeneity of deviations from Hardy–Weinberg equilibrium among the microsatellite loci was investigated by considering the deviations as correlation coefficients and tested accordingly (Barker *et al*. 2001). Heterozygote deficiencies were estimated as $F_{\mathrm{IS}} = (H_{\mathrm{o}} - H_{\mathrm{e}})/H_{\mathrm{e}}$, where H_{o} and H_{e} are the observed and expected frequency of heterozygotes respectively.

Linkage disequilibrium among the microsatellite loci was analysed employing F-STAT version 2.9.3, an updated version of 1.2 (Goudet 1995), for the 21 microsatellite loci. To test for evidence of a recent genetic bottleneck, the program BOTTLENECK (Piry *et al*. 1999) was used.

Results and discussion

All of the 21 microsatellite loci, which have been identified to be polymorphic in a variety of *Bos taurus* and *Bos indicus* breeds (MacHugh *et al*. 1997; Edwards *et al*. 2000; Kim *et al*. 2002; Dorji *et al*. 2003; Jordana *et al*. 2003; Metta *et al*. 2004; Mukesh *et al*. 2004), amplified successfully in our Kherigarh samples and produced definite banding patterns from which individual genotypes could be ascertained. Linkage disequilibrium was not detected between the investigated loci. Therefore all the loci were retained for the analysis.

Across the 21 microsatellites studied, a total of 131 distinct alleles were identified (genotypic distributions available from the authors on request). The allele frequency data revealed a reasonable amount of polymorphism (table 2). The number of observed alleles varied between 4 (ILSTS011, ILSTS033, BM1824) and 10 (ILSTS034), with overall mean number of alleles per locus of 6.24 (±1.7 s.e.). FAO has specified a minimum of four distinct alleles per locus for proficient judgment of genetic differences between breeds.

Table 2. Measures of genetic variation in Kherigarh cattle.

Locus	n_o	n_e	Shannon's information index	PIC	Heterozygosity*			Heterozygote deficiency (F_{IS})
					Observed	Expected	Nei's	
MM8	7	4.11	1.637	0.726	0.681	0.765	0.757	0.099
INRA063	7	1.96	1.024	0.458	0.489	0.495	0.489	0.000
ILSTS030	5	4.62	1.569	0.7498	0.809	0.792	0.783	−0.0321
BM1818	9	4.07	1.775	0.7338	0.630	0.7622	0.754	0.1639
CSRM60	5	3.64	1.448	0.688	0.564	0.735	0.725	0.2221
ILSTS054	6	4.21	1.594	0.730	0.553	0.771	0.763	0.2746
ILSTS005	5	3.52	1.373	0.666	0.681	0.724	0.716	0.0493
HEL5	5	2.93	1.252	0.603	0.261	0.673	0.659	0.6040
ILSTS006	5	3.81	1.428	0.692	0.697	0.749	0.738	0.0554
ILSTS011	4	2.46	1.059	0.530	0.468	0.601	0.594	0.2122
INRA035	7	4.75	1.741	0.764	0.717	0.798	0.790	0.0913
ILSTS033	4	2.63	1.095	0.543	0.511	0.626	0.619	0.1751
HEL9	9	6.52	1.998	0.828	0.636	0.856	0.847	0.2483
BM1824	4	3.17	1.248	0.627	0.575	0.692	0.684	0.1607
ETH225	8	2.60	1.426	0.596	0.565	0.623	0.616	0.0821
HAUT24	5	3.01	1.269	0.610	0.432	0.677	0.668	0.3527
ILSTS034	10	6.30	2.033	0.824	0.711	0.851	0.841	0.1547
HAUT27	6	2.81	1.248	0.589	0.386	0.652	0.645	0.4006
HEL1	6	2.80	1.295	0.604	0.583	0.652	0.643	0.0930
MM12	8	5.59	1.868	0.799	0.435	0.830	0.821	0.4705
INRA005	6	3.75	1.476	0.689	0.674	0.742	0.733	0.0800
Mean	6.24	3.77	1.469	0.669	0.574	0.717	0.709	
s.e.	1.7	1.24	0.293	0.097	0.131	0.091	0.090	

n_o, Observed number of alleles; n_e, effective number of alleles (Kimura and Crow 1964).
Shannon's information index (Lewontin 1972); PIC, polymorphic information content.
*Expected heterozygosity was computed using Levene's (1949) and Nei's (1973) expected heterozygosity.

By this criterion, all 21 microsatellites employed in this study showed ample polymorphism for evaluating genetic variation within breed and exploring genetic differences between breeds. The observed number of alleles for all the 21 loci exceeded the effective number of alleles which varied from 1.96 (INRA063) to 6.52 (HEL9) with mean (± s.e.) of 3.77 ±1.24 (table 2).

Genetic markers showing PIC values higher than 0.5 are normally considered as informative in population-genetic analyses (Botstein *et al*. 1980). The average (± s.e.) PIC in our sample was 0.669 ± 0.097. Consequently, with the exception of INRA063, all loci were informative. This was also seen in the taurine and indicus breeds investigated earlier using microsatellite markers (Bradley *et al*. 1994; Canon *et al*. 2001; Maudet *et al*. 2002; Kumar *et al*. 2003; Metta *et al*. 2004; Mukesh *et al*. 2004).

Mean (± s.e.) observed heterozygosity, averaged over the 21 loci, was 0.574 ± 0.131, which was lower than the expected heterozygosity (table 2). Average expected heterozygosity (Nei 1973) within the Kherigarh population ranged from 0.495 (INRA063) to 0.856 (HEL9), with overall mean (± s.e.) of 0.717 ± 0.091. Kherigarh cattle thus seem to harbour a good amount of genetic variation. The average observed heterozygosity estimation in this study (0.574±0.131) is marginally lower than that shown in seven Italian cattle breeds (0.60 – 0.68; Del Bo*et al*. 2001) and five Swiss cattle breeds (0.60 – 0.69; Schmid *et al*. 1999). Fairly comparable levels of heterozygosity were reported in Deoni cattle breed (0.59) of India (Mukesh *et al*. 2004) and 12 west/central African cattle breeds (0.506–0.697; Ibeagha-Awemu *et al*. 2004). However, lower heterozygosities (0.42 and 0.53) and lower numbers of alleles than in Kherigarh have been reported in two Indian zebu cattle breeds, namely Sahiwal and Hariana (Mukesh *et al*. 2004), whose populations are in rapid decline in India. With the lower observed number of alleles (Hariana 6.5 and Sahiwal 5.2), inbreeding estimates (F_{IS}) were higher, 0.211 in Hariana and 0.326 in Sahiwal cattle (Mukesh *et al*. 2004). Although the observed number of alleles in Kherigarh cattle (6.24) is lower than in Hariana cattle (6.5), F_{IS} (0.188) does not show a proportionate increase. This suggests that the Kherigarh breed retains considerable genetic variability and only moderate levels of inbreeding, despite its declining population in the breeding region.

Our within-population inbreeding estimate (F_{IS}) is significantly positive, as derived from table-wide randomizations ($P < 0.05$). The F_{IS} estimates ranged between −0.032 and 0.604, with average of 0.188. Thus, on an average, there is a substantial shortfall (18.8%) of heterozygotes in the Kherigarh population. All of the 21 microsatellite markers, except INRA063 and ILSTS030, contributed to this observed heterozygote shortage. Numerous factors, such as inbreeding, genetic hitchhiking, null alleles (nonamplifying alleles)

and occurrence of population substructure (Wahlund effect), have been established as reasons for heterozygote deficiency in populations (Nei 1987). The confinement of the Kherigarh breed to a small geographical area and shortage of breeding bulls in the population could be reasons for the deficiency of heterozygotes. But the fundamental cause of low heterozygosity in Kherigarh cattle breed is likely to be inbreeding. All the examined loci, except ILSTS030, HEL9 and MM12, were observed to be neutral when probed with the Ewens-Watterson neutrality test (Manly 1985), suggesting that homozygosity in Kherigarh cattle is unlikely to be the result of selection (data not shown, available on request). Null alleles are largely unlikely to be segregating at all the loci. Likewise, prospective Wahlund effects (localities with subpopulations) may not account significantly for the observed heterozygote deficit.

The inbreeding detected in this population is likely to be a manifestation of diminished population size and confinement to a small breeding territory, coupled with insufficient number of breeding males in the breeding region. The Kherigarh cattle population has shrunk to just about 15,000 head in the entire breeding tract (NBAGR 2003). Moreover, male calves of six to 12 months of age are traded to farmers outside the breeding region to be used in agricultural operations and transportation after castration, thus leading to their genetic death. As a result breedable males are significantly reduced in the breeding tract. Altogether the effective population size is curtailed and breeding between relatives stimulates inbreeding and genetic drift.

When a population goes through a bottleneck rare alleles tend to be lost and the average number of alleles per locus, or allelic diversity, is reduced. Heterozygosity, however, is not reduced proportionally, because rare alleles contribute little to heterozygosity. The difference between allelic diversity and heterozygosity is used as the basis for statistical tests to detect presence of recent genetic bottleneck (Piry *et al*. 1999). The allele frequency spectrum visualized by the qualitative graphical method of Cornuet and Luikart (1996) is shown in figure 2. The microsatellite alleles were organized into 10 frequency classes, which permit checking whether the distribution followed the normal L-shaped form, where alleles with low frequencies (0.01 – 0.1) are the most numerous. The observed distribution suggests that the breed did not encounter a genetic bottleneck in the recent past.

The significant level of variability in Kherigarh breed, notwithstanding its diminished population size, is indicative of a valuable reservoir of genetic diversity in this breed. This fact, coupled with its evident environmental adaptation, emphasizes the importance of genetic regulation and conservation of this indigenously evolved draught breed and its sustainable utilization. It is now critical to initiate planned and organized breeding, as our measured inbreeding coefficient (F_{IS}) indicates moderate levels of inbreeding in the population. High-priority action is also necessary considering the husbandry practices exercised by local farmers, which may further weaken the diversity levels through breeding of relatives. To make a start, a Kherigarh breed society should be formed, which should be educated and supported for comprehensive safeguarding and upgrading of the breed to make it economically sustainable. Exodus of purebred animals from the breeding tract should be curbed and availability of proven males as well as frozen semen of the breed should be ensured in the breeding tract to limit loss of genetic diversity.

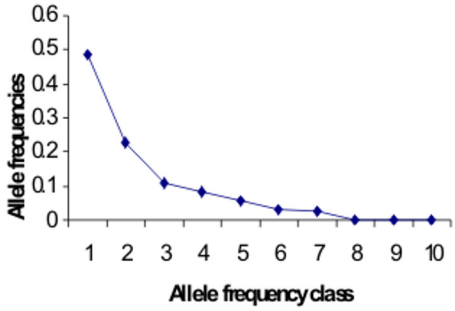

Figure 2. L-shaped mode-shift graph showing lack of recent genetic bottleneck in Kherigarh population.

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