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Evaluation of Genetic Variability in Kenkatha Cattle by Microsatellite Markers

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ABSTRACT : Kenkatha cattle, a draft purpose breed, which can survive in a harsh environment on low quality forage, was explored genetically exploiting FAO-suggested microsatellite markers. The microsatellite genotypes were derived by means of the polymerase chain reaction (PCR) followed by electrophoretic separation in agarose gels. The PCR amplicons were visualized by silver staining. The allelic as well as genotypic frequencies, heterozygosities and gene diversity were estimated using standard techniques. A total of 125 alleles was distinguished by the 21 microsatellite markers investigated. All the microsatellites were highly polymorphic with mean allelic number of 5.95±1.9 (ranging from 3-10 per locus). The observed heterozygosity in the population ranged between 0.250 and 0.826 with a mean of 0.540±0.171, signifying considerable genetic variation. Bottleneck was examined assuming all three mutation models which showed that the population has not experienced bottleneck in recent past. The population displayed a heterozygote deficit of 21.4%. The study suggests that the breed needs to be conserved by providing purebred animals in the breeding tract. (**Key Words :** Cattle, Genetic Variation, Kenkatha, Microsatellite)

INTRODUCTION

India has several indigenous cattle populations associated with different geographical areas. The enormous and diverse cattle genetic resources of India are signified in the form of 30 documented breeds of zebu cattle (Acharya and Bhat, 1984) besides numerous populations yet uncharacterized and undefined. These have been formed by centuries of human and natural selection. Breeds have been selected to fit a wide range of environmental conditions and human needs. The genetic diversity found in domestic breeds allows farmers to develop new characteristics in response to changes in environment, diseases or market conditions. Indigenous zebu breeds often possess gene combinations and special adaptations (such as disease resistance, adaptation to harsh conditions and poor quality feeds, etc.) not found in other breeds. Among these, majority are draught breeds as cattle development in India principally rested on the production of bullocks required for conventional agricultural operations and load pulling.

The rich biological heritage of farm animal is fast getting eroded and trends in the last few decades are alarming. There is a marked decline in the population of unique animals conforming to the true attributes of native breeds. Widespread use of cross breeding, destruction of traditional production systems and a general thrust towards management systems which rely on greater inputs placed this precious germ pool under threat. It is only since last decade that concerted conservation efforts have really been made to preserve the genetic diversity of cattle in India. Importantly, it is necessary to determine which breeds should be conserved (using objective criteria) because loss of variation will restrict the options available to meet future unknown requirements.

Diverse attributes of a population are effective in its characterization, taking account of phenotypic traits (monogenic and polygenic), reproduction, geographic distribution, origin and habitat. Although these breeds have been classified as separate breeds on phenotypic traits, allelic diversity and genetic relationship are unknown. Genetic characterization of populations, breeds and species allows the evaluation of genetic variability, a fundamental element in working out breeding strategies and genetic conservation plans. Microsatellites have been effectively exploited to elucidate bovine domestication and migration prototype (Bradley et al., 1994; Edwards et al., 2000) and to evaluate genetic diversity (Selvi et al., 2004; Li et al., 2005; Yoon et al., 2005) 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).

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

Marker	Primer sequences	Chromosome number	Annealing temp. (°C)
ILSTS006	tgtctgtatttctgctgtgg, acacggaagcgatctaaacg	7	56
BM1824	gagcaaggtgtttttccaatc, cattctccaactgcttccttg	1	55
INRA063	atttgcacaagctaaatctaacc, aaaccacagaaatgcttggaag	18	55
BM1818	agctgggaatataaccaaagg, agtgctttcaaggtccatgc	23	58
ILSTS054	gaggatcttgattttgatgtcc, agggccactatggtacttcc	21	55
ILSTS034	aagggtctaagtccactggc, gacctggtttagcagagagc	5	57
INRA005	caatctgcatgaagtataaatat, cttcaggcataccctacacc	12	55
HAUT27	ttttatgttcatttttgactgg, aactgctgaaatctccatctta	26	55
ILSTS033	tattagagtggctcagtgcc, atgcagacagttttagaggg	12	55
HEL9	cccattcagtcttcagaggt, cacatccatgttctcaccac	8	59
CSRM60	aagatgtgatccaagagagaggca, aggaccagatcgtgaaaggcatag	10	55
ILSTS011	gcttgctacatggaaagtgc, ctaaaatgcagagccctacc	14	58
ILSTS005	ggaagcaatgaaatctatagcc, tgttctgtgagtttgtaagc	10	55
ETH10	gttcaggactggccctgctaaca, cctccagcccactttctcttctc	5	55
HEL1	caacagctatttaacaagga, aggctacagtccatgggatt	15	55
INRA035	atcctttgcagcctccacattg, ttgtgctttatgacactatccg	16	55
MM8	cccaaggacagaaaagact, ctcaagataagaccacacc	2	55
ILSTS030	ctgcagttctgcatatgtgg, cttagacaacaggggtttgg	2	55
MM12	caagacaggtgtttcaatct, atcgactctggggatgatgt	9	55
ETH225	gatcaccttgccactatttcct, acatgacagccagctgctact	9	57
HEL5	gcaggatcacttgttaggga, agacgttagtgtacattaac	21	55

Kenkatha breed of Indian zebu cattle (*Bos indicus*) evolved as a draft breed over centuries under low levels of breeding management and, as a result of natural selection and human intervention has become adapted to harsh native environment and sustenance on low quality roughages and grasses. The breed is primarily employed for agricultural operations, for carrying load and transportation. It is distributed mainly in and around Banda (25N28 80E20) district of Uttar Pradesh and Lalitpur and Tikamgarh districts of Madhya Pradesh State. The name of the breed is derived from the Ken river of the area. Animals are small in size and have grey and white body colour. Head is short and broad.

The present study is an attempt to study genetic variation of Kenkatha cattle of India, using DNA marker technology. The population structure, genetic variability and genetic bottleneck in Kenkatha cattle have been evaluated using twenty-one microsatellite markers.

MATERIAL AND METHODS

Sample collection

Blood samples were collected from fifty random Kenkatha animals following the guidelines of MoDAD (Measurement of Domestic Animal Diversity) (FAO, 1995) programme. Great care was taken to sample individuals that are not directly related. Firstly distinct villages were selected from the breeding tract (Banda district of Uttar Pradesh and Lalitpur, Tikamgarh districts of Madhya Pradesh State). Secondly animal owners were interviewed in detail regarding the pedigree of the animal for selecting unrelated random animals. Blood samples (5-6 ml) were collected from jugular vein of animal in vacutainers containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant.

Molecular techniques

Genomic DNA was isolated following standard process with slight modifications. 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 co-dominant, 47 samples correspond to 94 alleles for each microsatellite locus. An amalgamation of 21 co-dominant loci and 47 samples were projected to create 1,974 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 procedure comprised 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) and sized using a 10 bp ladder (Invitrogen, Life Technologies, CA, USA) as standard for sizing. Gels were stained using silver staining (Bassam et al., 1991) and genotypes scored manually. Size of the alleles was calculated online using 'INCHWORM' programme (http://www.molecularworkshop.com/programs/inchworm.html).

Table 2. Measures of genetic variation in Kenkatha cattle

Locus	N_o	N_e	PIC	Heterozygosity			Heterozygote deficiency, f (F_{IS})
				Observed	Expected	Nei's	
ILSTS006	8.0	3.3011	0.6645	0.3696	0.7047	0.6971	0.478
BM1824	5.0	2.1871	0.4856	0.4130	0.5487	0.5428	0.249
INRA063	5.0	2.6988	0.5631	0.6170	0.6362	0.6295	0.031
BM1818	7.0	4.7505	0.7599	0.5532	0.7980	0.7895	0.309
ILSTS054	6.0	4.6505	0.7513	0.8261	0.7936	0.7850	-0.041
ILSTS034	10.0	4.4770	0.7544	0.6047	0.7858	0.7766	0.233
INRA005	5.0	4.4853	0.7404	0.6207	0.7907	0.7771	0.218
HAUT27	4.0	2.3467	0.5115	0.2500	0.5805	0.5739	0.572
ILSTS033	5.0	2.7702	0.5807	0.4000	0.6462	0.6390	0.384
HEL9	5.0	4.7190	0.7543	0.5789	0.7986	0.7881	0.278
CSRM60	9.0	4.4457	0.7518	0.3778	0.7838	0.7751	0.521
ILSTS011	3.0	2.4985	0.5327	0.3333	0.6065	0.5998	0.453
ILSTS005	6.0	3.7413	0.6889	0.7381	0.7415	0.7327	0.005
ETH10	5.0	2.8960	0.6123	0.5000	0.6622	0.6547	0.247
HEL1	6.0	2.3283	0.5367	0.5610	0.5775	0.5705	0.029
INRA035	8.0	5.4362	0.7918	0.7556	0.8252	0.8160	0.085
MM8	5.0	2.7400	0.5723	0.7561	0.6429	0.6350	-0.179
ILSTS030	4.0	2.6065	0.5491	0.7234	0.6230	0.6163	-0.163
MM12	9.0	4.2618	0.7362	0.6739	0.7738	0.7654	0.130
ETH225	6.0	2.1038	0.5008	0.3830	0.5303	0.5247	0.280
HEL5	4.0	2.1559	0.5912	0.3077	0.5431	0.5362	0.437
Mean	5.95	3.4095	0.6395	0.5401	0.6854	0.6774	0.214
St.Dev	1.88	1.0851	0.101	0.1707	0.1002	0.0989	

Statistical analysis

Observed and expected heterozygosity estimates were computed after Nei (1973) as executed in POPGENE software (Yeh et al., 1999). The observed and effective numbers of alleles were also evaluated applying POPGENE software. Allelic frequencies were utilized for assessing Polymorphic Information Content (PIC) values as per Botstein et al. (1980). The PIC value was estimated as per formula given below

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

Where k is the number of alleles and x_i and x_j are the frequencies of the i^{th} and j^{th} alleles respectively.

Departure from Hardy-Weinberg equilibrium was derived using the exact test of POPGENE. Heterogeneity of deviations from Hardy-Weinberg equilibrium among the microsatellite loci was investigated by considering the deviations as correlation coefficient and tested accordingly (Barker et al., 2001). Heterozygote deficiencies were articulated as $F_{IS} = (H_o-H_e)/H_e$ where, H_o and H_e are the observed and expected frequency of heterozygotes, respectively.

Linkage (Genotypic) disequilibrium among the microsatellite loci was analyzed employing F-STAT version 2.9.3, an update version 1.2 (Goudet, 1995) for 21 microsatellite loci. Finally, the bottleneck hypothesis was explored exercising BOTTLENECK 1.2.01 software (Cornuet and Luikart, 1996).

RESULTS AND DISCUSSION

The investigated 21 bovine microsatellites represent 16 autosomal chromosomes of cattle (Table 1) and all amplified well in Kenkatha cattle. Five pairs of microsatellite loci ILSTS054- HEL5, INRA005-ILSTS033, ILSTS034-ETH10, MM8-ILSTS030 and MM12-ETH225 were present on chromosome number 21, 12, 5, 2 and 9 respectively. Thus we analyzed these pair of loci for the presence of linkage disequilibrium using FSTAT. It has been conclusively proven that these loci are not in linkage disequilibrium and hence all were used for estimating genetic parameters.

All these 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 and produced definite banding patterns from which individual genotypes could be ascertained. Genetic variability parameters of Kenkatha cattle viz., observed and effective number of alleles, observed, expected and Nei's expected heterozygosity, polymorphic information content (PIC) and heterozygote deficiency at each of the 21 microsatellite loci are included in Table 2.

Across the 21 microsatellites scrutinized a total of 125 distinct alleles were identified in Kenkatha cattle (genotypic

Table 3. Heterozygosity excess/deficiency under different mutation models (Heterozygosity Method) in Kenkatha cattle population

Models	Sign Test	Standardized	Wilcoxon test
IAM	Hee = 12.33	T2 = 1.863	P (one tail for H deficiency): 0.99292
	Hd = 6	P = 0.03122	P (one tail for H excess): 0.00789
	He = 15		P (two tails for H excess and deficiency): 0.01578
	P = 0.16861		
TPM	Hee = 12.53	T2 = -0.605	P (one tail for H deficiency): 0.35397
	Hd = 11	P = 0.27261	P (one tail for H excess): 0.65864
	He = 10		P (two tails for H excess or deficiency): 0.70793
	P = 0.18251		
SMM	Hee = 12.43	T2 = -4.444	P (one tail for H deficiency): 0.00974
	Hd = 15	P = 0.00000	P (one tail for H excess): 0.99123
	He = 6		P (two tails for H excess or deficiency): 0.01947
	P = 0.00445		

Parameters for T.P.M: Variance = 30.00 Proportion of SMM in TPM = 70.00%; Estimation based on 1,000 replications.
Hee: Heterozygosity excess expected; Hd: Heterozygosity deficiency; He: Heterozygosity excess; P: Probability; IAM: Infinite allele model. TPM: Two phase model. SMM: Stepwise mutation model.

distributions available from the authors on request). The allele frequency data revealed a reasonable amount of polymorphism in Kenkatha cattle (Table 2). The number of observed alleles varied between 3 (ILSTS011) and 10 (ILSTS034) with an overall mean number of 5.95±1.9 alleles per locus. All the 21 microsatellites employed in this study signified ample polymorphism and their suitability 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 2.10 (ETH225) to 4.75 (BM1818) with a mean of 3.41±1.09 (Table 2).

The average PIC estimate was 0.639±0.101. Genetic markers demonstrating PIC value higher than 0.5 are considered informative in population genetic analysis (Botstein et al., 1980). Consequently, with the exception of BM1824 all the loci were really informative like in 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).

The observed heterozygosity averaged over the 21 loci was 0.540±0.171 which was lower than the expected heterozygosity (Table 2). The average expected heterozygosity (Nei, 1973) within the Kenkatha population ranged from 0.530 (ETH225) to 0.825 (INRA035) with an overall mean of 0.685±0.100. Kenkatha cattle, thus, possess considerable measure of genetic variation derived from its gene diversity as estimated against the genetic variation described in several breeds. The average observed heterozygosity estimation in this study (0.540±0.171) is marginally lower than illustrated in seven Italian cattle breeds 0.6-0.68 (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 twelve west/central African cattle breeds 0.506-0.697 (Ibeagha-Awemu et al., 2004). However, lower heterozygosity (0.42) and reductions in number of alleles than Kenkatha have been recounted in Sahiwal cattle breed of India (Mukesh et al., 2004) whose populations is on a rapid decline in India.

There is a negative correlation between observed heterozygosity (Sahiwal, 0.42; Gangatiri, 0.46; Hariana 0.53; Kenkatha 0.54 and Deoni 0.59) and F_{IS} (Sahiwal, 0.32; Gangatiri, 0.31; Hariana, 0.21; Kenkatha, 0.21 and Deoni, 0.17) in Indian Zebu cattle (Mukesh et al., 2004; Sharma et al., 2006). H_o shows increasing trend with the decline in F_{IS} of these breeds. This clearly indicates that Kenkatha cattle retain considerable genetic variability and moderate level of inbreeding, notwithstanding its declining population in the breeding region.

Within-population inbreeding estimate f (F_{IS}) was significantly positive as derived from table wide randomizations ($p<0.05$). The f-estimates ranged between -0.179 and 0.572 with an average of 0.214. Thus, on an average, deficiency (21.4%) of heterozygote existed in the Kenkatha population. All the 21 microsatellite markers, except ILSTS054, MM8 and ILSTS030 contributed to this observed heterozygote shortage. All the scrutinized loci were observed to be neutral (except ILSTS054, INRA005 and HEL9) when probed with Ewens-Watterson neutrality test (Manly, 1985) indicating that homozygosity in Kenkatha might not be an outcome of selection (data not shown, available on request). Null alleles are largely unlikely to be segregating at all the loci. Likewise prospective Wahlund effects may not account significantly for the observed heterozygote deficit as breeding tract of this breed is continuous and is not differentiated into separate pockets by geographical barriers. It is right time to initiate planned and organized breeding, as F_{IS} is indicative of moderate level of inbreeding in the population.

Bottleneck occurs when populations experience severe, temporary reduction in size. It influences the distribution of genetic variation within and among populations. Cornuet

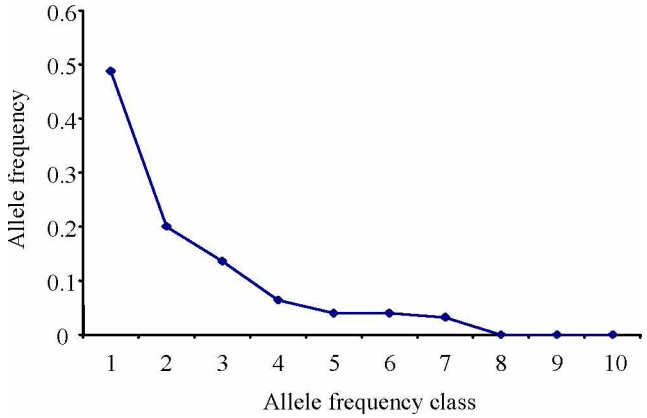

Figure 1. Mode shift curve depicting lack of bottleneck in Kenkatha cattle.

and Luikart. 1996 introduced heterozygosity excess as a method for detection of bottlenecks. This method is based on the premise that populations experiencing recent reduction in size develop an excess of heterozygosity at selectively neutral loci relative to the heterozygosity expected at mutation-drift equilibrium. Three tests were used to look for bottleneck in the Kenkatha cattle viz Sign test, Standardized difference test and Wilcoxon rank test. Sign test is a nonparametric test. The standardized difference test and Wilcoxon rank tests are parametric and more useful if the number of loci is more than twenty. In our study we utilized twenty one loci and three models - Infinite allele model (IAM), Two phase model (TPM) and Stepwise mutation model (SMM). IAM and SMM represent the two extreme models of mutation (Chakraborty and Jin. 1992). It is known that most of the loci are expected to evolve in an intermediate way (TPM). Results of Sign test show absence of bottleneck utilizing TPM and SMM as He is significantly higher than Hee (Table 3). In Wilcoxon rank test probability values for one tail for H excess (He) is 0.00789, 0.65864 and 0.99123 in IAM, TPM and SMM respectively. Except in IAM model these probability values are more than 0.05. Thus accepting null hypothesis and showing no bottleneck in the population as per two models. In standardized difference test if T2 values are lower than 1.645 (value from table of normal distribution) null hypothesis of population at mutation drift equilibrium is accepted. Except in IAM, null hypothesis is accepted as T2 is -0.605 in TPM and -4.444 in SMM. In addition, allele frequency spectra was visualized (Figure 1) through the qualitative graphical method of Cornuet and Luikart (1996). The microsatellite alleles were organized in to 10 frequency classes, which permit checking whether the scattering followed the normal L-shaped form, where alleles with low frequencies (0.01-0.1) are the most numerous. With the help of quantitative as well as qualitative methods it can very well be concluded that Kenkatha population has not undergone genetic bottleneck in the past. Bottleneck has been reported in two sub strains of Japanese black cattle by Sasazaki et al. (2004).

In conclusion, using neutral genetic markers we showed that despite unplanned breeding, this breed still has sufficient genetic variability. Hence the genetic variation that has persisted in Kenkatha population could provide a valuable source of genetic material that may be used for meeting the demands of future breeding programmes. High priority action is necessary considering the husbandry practices exercised by local farmers, which may further weaken the diversity levels through the breeding of relatives. To make a start, breed society needs to be formed, which should be educated and supported for the comprehensive safeguarding and upgrading of the breed to make it economically sustainable in the present agricultural scenario of the country. Exodus of purebred males from the breeding tract need to be curbed and availability of proven males as well as frozen semen of the breed be ensured in the breeding tract.

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