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Research Article

GENETIC CHARACTERIZATION AND BOTTLENECK STUDIES IN KATHIAWARI HORSE BREED OF INDIA

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ABSTRACT

To assist the conservation efforts and breeding strategies, the first genetic characterization of the Kathiawari breed of horse was undertaken and 24 microsatellite markers were used to genotype 50 unrelated Kathiawari horses. The observed heterozygosity for the Kathiawari breed was 0.6509 ± 0.1874 with the expected heterozygosity of 0.6448 ± 0.1544. The average number of alleles per locus was 5.166 ± 1.493 while polymorphism information content was 0.591. Wright's fixation index, F_{IS} (0.1155 ± 0.0252) indicated high level of heterozygote deficiency which suggested a high level of inbreeding. This basic study indicated the existence of substantial genetic diversity in the Kathiawari horse population. Significant genotypic linkage disequilibrium was detected across the population, suggesting evidence of linkage between loci. A normal 'L' shaped distribution of mode–shift test, non-significant heterozygote excess on the basis of different models, as revealed from Sign, Standardized differences and Wilcoxon sign rank tests as well as non-significant *M* ratio value suggested that there was no recent bottleneck in the existing Kathiawari breed population, which is important information for equine breeders. This study also revealed that the Kathiawari, a Indian horse breed can be differentiated from some other exotic breeds of horses on the basis of these microsatellite primers.

Key words: Kathiawari horse, microsatellite, bottleneck, breed characterization

India has a rich biodiversity of equines in the form of six distinct indigenous horse (*Equus caballus*) breeds, namely Kathiawari, Marwari, Spiti, Zanskari, Bhutia and Manipuri, in addition to indigenous donkeys and wild asses (Yadav *et al.*, 2001). These horse breeds are well adapted to different agroclimatic regions and possess certain unique characteristics. Kathiawari breed of horse is found in Saurashtra region of Gujarat State. The animals of this breed are well known for their canter gait popularly known as '*Rawaal*'. Kathiawari is well known for its pace and speed, relative disease resistance, possesses good endurance power and faithfulness to owner which attracts the royal community of India.

However, owing to indiscriminate breeding and lack of sound breeding policies, the breed's characteristics are being diluted and presently only few thousand true Kathiawari horses are in existence (Singhvi, 2001). To avoid further loss of potential unique genes, and to preserve the genetic diversity within the breed, an objective breed classification based on genetic uniqueness is of priority (May, 1990, Hall and Bradly, 1995). Characterization at the morphological and genetic levels is the first step towards formulating breeding policies and prioritising the breeds for conservation in an effective and meaningful way. Recently an array of DNA based markers has been developed to carry out studies on genetic variation (Bradley *et al.*, 1996, Canon *et al.*, 2000). Among these, microsatellites are considered to be the most suitable marker system for evaluating breeds for genetic diversity, owing to their abundance in the mammalian genome, high level of polymorphism, codominant inheritance and amenability for automation (Takezaki and Nei 1996). Except for phenotypic characterization, no genetic characterization studies have been carried out in Kathiawari horses. The present study involved molecular characterization based on microsatellite markers to detect historical population bottlenecks, if any, in the Kathiawari horse breed.

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MATERIALS AND METHODS

Sample collection and DNA isolation: Vacutainers tubes containing ethylenediamine-tetra acetic acid (EDTA) as an anticoagulant were used to collect blood samples from 50 unrelated horses from four different districts of Gujarat. Genomic DNA was isolated and purified from white blood cells using method of proteinase K digestion and standard phenol/chloroform/ isoamyl alcohol extraction and absolute ethanol precipitation (Sambrook *et al.*, 1989).

Microsatellite amplification: Samples were genotyped for a set of 24 microsatellites (Table 1) recommended for genetic characterization of equines by the International Society for Animal Genetics (ISAG). PCR was carried out in 25 µl reaction containing 100 ng template DNA, 10 pmol each primer, 200 µM dNTPs, 10 mM Tris HCl (pH 9.0), 50 mM KCl, and 0.5 U of Taq polymerase (Bangalore Genei Pvt. Ltd., India) using Thermal Cycler (Eppendorf, Germany). Different primer pairs were used with 1.5–2.0 mM $MgCl_2$ concentration. Thermal conditions for amplification included initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at optimum temperature (58-63°C) for 45 sec, and extension at 72°C for 45 sec each, with a final extension at 72°C for 10 min. The PCR products were electrophoresed at 80 volts in a 2% agarose gel and stained with ethidium bromide (0.5 mg/ ml). The amplified products observed under ultra violet light (300 nm) transilluminator were further resolved in 7% urea polyacrylamide denaturing sequencing gel on Sequi-GT system 30 × 38 cm (Bio-Rad Laboratories, USA). Alleles were visualized by silver staining, following Bassam *et al.* (1991). The sizes of amplified products were estimated using a 10 bp molecular weight marker (Invitrogen, USA). Genotypes of individual horses at the different polymorphic loci were recorded by direct counting.

Computation and statistical analysis: Allele frequencies for each locus were calculated with $2n$=100 for Kathiawari horses and can be obtained from authors on request. Heterozygosity (Nei 1978) and other genetic diversity variables were calculated using POPGENE computer package (Yeh *et al.* 1999). Polymorphism information content (PIC) values were calculated by using the method described by Botstein *et al* (1980). The probability of random mating in the population was estimated by Chi-square (X^2) and likelihood ratio (G^2) tests to examine Hardy-Weinberg equilibrium (HWE) at each locus. The tests for departure from Hardy-Weinberg proportions and linkage disequilibrium were performed using exact probability tests provided in GENEPOP version 3.1 a (Raymond and Rousset 1999). A Monte Carlo method (Guo and Thompson 1992), with the length of chain set to be 50000 iterations, was used to compute unbiased estimates of the exact probabilities (P values). Ewens–Watterson neutrality test was performed to test the neutrality of microsatellite markers, the statistics for the test were calculated using the algorithm given by Manly (1985), using 1000 simulated samples.

Bottleneck events in the population were tested by three methods. The first method consisted of three excess heterozygosity tests developed by Cornuet and Luikart (1996) like sign test, standardized differences test and a wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models such as Infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model of mutation (TPM). The second method was the graphical representation of the mode-shift indicator originally proposed by Luikart *et al.* (1998). Loss of rare alleles in bottlenecked populations was detected when one or more of the common allele classes have a higher number of alleles than the rare allele class (Luikart *et al.* 1998). This test was re-scaled so that frequency distribution of the allele frequency classes in each population would be based on equal 0.05 increments. These two methods were conducted using Bottleneck v1.2.02 (http://www.ensam.inra.fr/URLB). The third method was the M ratio conducted by applying the m_p_val.exe program (Garza and Williamson 2001). 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) is called the M ratio ($M = ¼\ k/r$; Garza and Williamson 2001). The M ratio is stable if the population

size was stable for a long time. 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. However, lost alleles do 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 event of a bottleneck event. The *M* ratio was calculated for the whole population and contrasted with that under equilibrium. To test whether an *M* value is lower than expected, 10000 replicates were simulated. The number of times that the simulated *M* is higher than the calculated *M* represents the statistical significance of the *M* value reduction.

RESULTS AND DISCUSSION

Genetic diversity: A total of 124 alleles were detected across the 24 loci analyzed. The number of alleles per marker varied from 2 (NVHEQ54) to 8 (HMS03, VHL20 and NVHEQ18) and the across loci Ho value ranged from 0.1400 (NVHEQ54) to 0.8600 (LEX20) while the He value varied from 0.1315 (NVHEQ54) to 0.8480 (VHL20). The PIC value ranges from 0.122 (NVHEQ54) to 0.820 (VHL20). The heterozygosity for whole dataset were 0.6509 for Ho and 0.6448 for He. Whereas, PIC value for whole dataset was 0.5910. The average numbers of alleles were 5.166±1.493 for observed alleles and 3.1746±1.1481 for effective number of alleles. Allele frequencies and range of allele size for all loci are given in Table 1. The allele numbers and heterozygosity levels observed across the studied loci revealed high level of genetic variability in Kathiawari horse. The within-population inbreeding estimate (F_{IS}) was 0.1155±0.0252.

The neutrality test of each marker tested by Ewens-Watterson test for neutrality suggested that all the microsatellite loci except LEX20, NVHEQ29, HTG14 and AHT04 (Table 2) were neutral and unlinked to any selected trait as observed F value lie outside of the upper and lower limits of 95% confidence region of expected F value). The Chi-square and likelihood ratio tests performed to examine Hardy Weinberg Equilibrium (HWE) at each locus indicates that eighteen loci were deviating from HWE. Sixteen loci (NVHEQ79, ASB02, NVHEQ05, NVHEQ21, NVHEQ54, LEX20, NVHEQ11, NVHEQ29, NVHEQ40, HTG07, HTG14, AHT04, HTG06, HTG15,NVHEQ82 and NVHEQ70) showed higher observed heterozygosity than the expected values (Table 2). Heterozygote deficiency analysis revealed significant deviation from HWE ($P < 0.05$) at some of the loci. It is, however, difficult to envisage the exact basis of this departure, although the presence of low frequency null alleles segregating at these loci may be a possible reason. An exact test for genotypic linkage disequilibrium yielded 538 significant *P* value across the population is suggestive of a lack of independent assortment for the loci.

Bottleneck study: Since the population of Kathiawari horses true to their breed has gone down drastically and it is possible that demographical bottlenecks might have occurred. Because bottlenecks influence the distribution of genetic variation within and among populations, the genetic effects of reduction in population size require evaluation. In the present study, evidence for a bottleneck was not detected in any of three methods. The result of test for null hypothesis under three microsatellite evolution models to test the bottleneck in the Kathiwari population is given in Table 3. The value of average heterozygosity (*He*) and their probability ($H > He$) in the Sign test, under three models of microsatellite evolution (IAM, SMM and TPM) were calculated and used to measure the expected number of loci with heterozygosity excess which was 13.57 for IAM under null hypothesis (Table 3). The probability value in this case was 0.00002 and thus rejects the null hypothesis indicating bottleneck under this model. However the expected numbers of loci with heterozygosity excess were 14.08 and 14.11 in TPM and SMM with probabilities 0.7496 and 0.2498 respectively, meaning that the null hypothesis was not rejected when using the Sign test. These results indicate that due to mutation drift equilibrium, the Kathiawari population has not undergone a recent genetic drift.

Table 1

Total number alleles (k), heterozygosity observed (Ho) and expected (He), polymorphism information content (PIC), chromosome location and F_{IS} Index of the microsatellites analyzed in the Kathiawari horse breed

Microsatellite loci	Total number of alleles (k)	Heterozygosity		PIC	Chromosomal location	F_{IS} Index
		Ho	He			
NVHEQ-79	5	0.6400	0.5331	0.476	17	-0.2126
ASB-02	5	0.8200	0.7194	0.664	15	-0.1514
HMS-03	8	0.7755	0.7814	0.745	09	-0.0027
HMS-07	6	0.7069	0.7516	0.704	01	0.0513
NVHEQ-05	4	0.5306	0.4906	0.441	20	-0.0926
NVHEQ-21	4	0.5745	0.5452	0.485	20	-0.0650
NVHEQ-54	2	0.1400	0.1315	0.122	Unknown	-0.0753
VHL-20	8	0.8235	0.8480	0.820	30	0.0192
LEX-20	6	0.8600	0.7782	0.737	01	-0.1163
NVHEQ-18	8	0.6604	0.7409	0.701	10	0.1002
NVHEQ-11	6	0.8478	0.7470	0.699	19	-0.1474
UCDEQ-425	6	0.2553	0.6989	0.648	28	0.6308
NVHEQ-29	5	0.8200	0.7762	0.731	04	-0.0672
NVHEQ-40	6	0.6538	0.6139	0.551	11	-0.0754
HTG-07	4	0.5385	0.5364	0.440	04	-0.0136
HMS-02	5	0.6304	0.7200	0.663	10	0.1148
HTG-14	4	0.7400	0.7374	0.682	Unknown	-0.0137
AHT-04	6	0.9048	0.7837	0.739	24	-0.1684
HTG-06	4	0.6977	0.6487	0.571	15	-0.0881
HTG-15	3	0.5094	0.4638	0.377	Unknown	-0.1089
NVHEQ-82	5	0.7727	0.6424	0.575	03	-0.2168
HTG-04	5	0.5273	0.5808	0.511	09	0.0839
NVHEQ-100	4	0.4583	0.5090	0.468	01	0.0900
NVHEQ-70	5	0.7347	0.6960	0.639	03	-0.0665
All loci	124	0.6509	0.6448	0.591		0.1155 ± 0.0252

The standardized difference test provides that T_i (probability) statistics equal to 4.516 (0.00) 2.70 (0.00346) and -0.308 (0.3790) for the IAM, TPM and SMM model respectively. The probability values were less than 0.05 for IAM and TPM, thus hypothesis of mutational drift equilibrium was not rejected under SMM only. Using Wilcoxon rank test (a non-parametric test) the probability values were 0.00 (IAM), 0.00033 (TPM) and 0.61585 (SMM) under these three models, indicating that null hypothesis is not rejected under SMM only and the population under study has not undergone a recent bottleneck.

The Mode-shift indicator test was also utilized as a second method to detect potential bottlenecks, as the non-bottleneck populations that are near mutation-drift equilibrium are expected to have a large population of alleles with low frequency. The graphical representation utilizing allelic class and proportion of alleles showed normal 'L' shaped distribution (Fig 1). This distribution clearly reinforced the result that the studied population has not experienced a recent bottleneck. The *M* ratio for Kathiawari population was 0.6872 and this value was not significant at the 0.05 level, further indicating the absence of bottleneck events in the recent past history of this breed.

During the research described in this paper we carried out the first genetic analysis of the

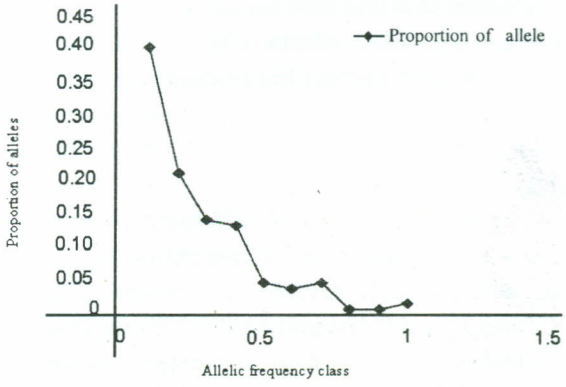

Fig 1. Graphical representation of proportion of alleles and their contribution in Kathiawari horses at twenty five microsatellite loci.

Table 2

Chi-square and G-square probabilities (95% confidence level), observed *F*, and upper (U95) and lower (L95) 95% confidence limits of expected *F* values across 22 polymorphic loci in Kathiawari horses

Microsatellite locus	Hardy Weinberg Equillibrium test			Ewens-Watterson Neutrality test		
	Degrees of freedom	Probability of X^2	Probability of G^2	Observed F	L95	U95
NVHEQ79	10	0.2510	0.1081	0.4722	0.2604	0.8312
ASB02	10	0.0558	0.0217	0.2878	0.2662	0.8320
HMS03	28	0.0028	0.0376	0.2266	0.1860	0.6570
HMS07	15	0.0059	0.1444	0.2549	0.2347	0.7918
NVHEQ05	06	0.9311	0.8851	0.5144	0.3086	0.9017
NVHEQ21	06	0.2314	0.2889	0.4606	0.3049	0.8977
NVHEQ54	01	0.6238	0.5013	0.8698	0.5050	0.9802
VHL20	28	0.0008	0.0004	0.1603	0.1849	0.6182
LEX20	15	0.0000	0.0000	0.2296	0.2302	0.7790
NVHEQ18	28	0.0000	0.0000	0.2661	0.1867	0.6666
NVHEQ11	15	0.0000	0.0001	0.2611	0.2351	0.7606
UCDEQ425	15	0.0000	0.0000	0.3085	0.2349	0.7673
NVHEQ29	10	0.0110	0.0022	0.2316	0.2658	0.8308
NVHEQ40	15	0.1793	0.1617	0.3920	0.2337	0.7552
HTG07	06	0.8142	0.7755	0.4687	0.3051	0.9251
HMS02	10	0.0325	0.0187	0.2878	0.2661	0.8358
HTG14	06	0.0494	0.0117	0.2700	0.3178	0.9036
AHT04	15	0.3717	0.1253	0.2256	0.2304	0.7596
HTG06	06	0.0489	0.0047	0.3588	0.2961	0.8886
HTG15	03	0.4326	0.4486	0.5406	0.3608	0.9628
NVHEQ82	10	0.0000	0.0001	0.3649	0.2575	0.8301
HTG04	10	0.2224	0.2236	0.4245	0.2706	0.8463
NVHEQ100	06	0.0119	0.0109	0.4963	0.3105	0.8997
NVHEQ70	10	0.0048	0.0029	0.3111	0.2688	0.8457

Kathiawari breed using DNA markers. The polymorphism of the microsatellites reported in the literature for horse breeds other than India mostly range from 0.66 to 0.75 for Ho and from 0.64 to 0.77 for He (Wimmers *et al.*, 1998; Bjornstad *et al.*, 2000, Canon *et al.*, 2000, Cunnnigham *et al.*, 2001, Luis *et al.*, 2002, Bruzzone *et al.* 2003, Aberle *et al.*, 2004, Achmann *et al.*, 2004, Galov *et al.*, 2005, Royo *et al.*, 2005) where as for Indian horse breeds the values range from 0.53 to 0.62 for Ho and from 0.65 to 0.78 for He. The observed heterozygosity for the Kathiawari breed was 0.6509±0.1874 and the expected heterozygosity was 0.6448±0.1544 while the average number of alleles per locus was 5.166±1.493, these values being similar to the data published for other horse breeds. The mean value of within-population inbreeding estimate (F_{IS} =0.1155±0.0252) indicated the clear heterozygote deficiency and moderate of inbreeding in the population (Table 1).

Table 3

Test for null hypothesis under three microsatellite evolution models

Test/Model		IAM	TPM	SMM
Sign test: number of loci with heterozygosity excess (probability)	a) Expected	13.57 (0.00002)*	14.08 (0.07496)	14.11 (0.24980)
	b) Observed	23.00	18.00	12.00
Standard difference test: *Ti* Value (probability)		4.516 (0.00000)*	2.700 (0.00346)*	-0.308 (0.37903)
Wilcoxon rank test (probability of heterozygosity excess)		0.00000*	0.00032*	0.61585

* Rejection of null hypothesis / bottleneck.

Behl *et al.* (2006) used 25 microsatellite marker for genetic characterization of Zanskari breed of horse. The within-population inbreeding estimates (F_{IS}) indicate moderate levels of inbreeding. The sign test and standardized differences test of these data revealed absence of any significant heterozygotic excess in both infinite allele model (IAM) and sequential allele model (SMM), demonstrating that the Zanskari breed of horse has not experienced any recent genetic bottleneck.

Gupta *et al.* (2005) studied genetic diversity and bottleneck studies in the Marwari horse breed using 26 different microsatellite pairs with 48 DNA samples from unrelated horses. The estimated mean allelic diversity was 5.9 with a total of 133 alleles. A high level of genetic variability within this breed was observed in terms of high values of mean (± S. E.) effective number of alleles (3.3±1.27), observed heterozygosity (0.5306±0.22), expected Levene's heterozygosity (0.6612±0.15), expected Nei's heterozygosity (0.6535±0.14), and polymorphism information content (0.6120±0.03). Low values of Wright's fixation index, F_{IS} (0.2433±0.05) indicated low levels of inbreeding. A bottleneck study showed a normal 'L' shaped distribution of mode–shift test, non-significant heterozygote excess on the basis of different models, as revealed from Sign, Standardized differences and Wilcoxon sign rank tests as well as non-significant *M* ratio value suggested that there was no recent bottleneck in the existing Marwari breed population. Chauhan *et al.* (2004) used 25 microsatellite markers for genetic characterization of Indian Spiti horses and found average value of 0.6237 for Ho and 0.6689 for He. Three loci (AHT16, AHT44 and UM012) were observed to be monomorphic whereas other loci were highly polymorphic with a total of 102 alleles.

Summarizing the information above, we can conclude that the high genetic variability observed in Kathiawari horses can be utilized in planning breeding strategies in the small population of Kathiawari horses. The present panel of microsatellites evaluated in Kathiawari horses showed a very high heterozygosity and PIC and therefore, this set of microsatellite may be reliably used for genetic characterization and genetic diversity studies in other breeds of horse as well. Bottleneck data clearly suggested that the population has not undergone to any recent bottleneck event. The present work contributes to the knowledge of population structure, genetic characterization and assessment of existing genetic diversity in the Kathiawari horse population. Further genetic analysis of other Indian horse breeds and their comparison need to be carried out to determine the phylogenetic evolutionary relationship and genetic distance among the indigenous equine breeds. The strong inference that the Kathiawari breed has not undergone major bottlenecks is also important for equine breeders and conservationists, as it suggest that any unique allele present in this breed may not have been lost.

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