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Genetic characterization of horse breeds (*Equus caballus*) of Western India

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ABSTRACT

Assessment of genetic diversity and population structure of population through molecular markers allows determining their extinction risk and to manage and conserve the respective population. The present study was undertaken for population genetic analysis at a molecular level of horse breeds for planning conservation, utilization and improvement in the livelihood. Gujarat has three recognized horse breeds Kathiawari, Marwari and Kachchhi-Sindhi. The genetic characterization and differentiation analysis among and within these breeds was analyzed using 26 microsatellite markers out of which 25 markers were polymorphic while one was monomorphic. The present study indicated that Marwari and Kathiawari horse breeds are genetically nearer as compared to Kathiawari—Kachchhi-Sindhi and Marwari—Kachchhi-Sindhi. The observed number of alleles in all three breeds ranged from 2 (NVHEQ21) to 16 (ASB17). The within population in breeding estimate (FIS) was less than zero, indicating absence of inbreeding in Kathiawari, Marwari and Kachchhi-Sindhi breed. The Wilcox text, sign test and standardized difference test in present study revealed the presence of significant heterozygotic excess in both IAM and SMM model demonstrating that these three breeds of horse has showed recent genetic bottleneck.

Keywords: Bottle neck, Heterozygosity, Kathiawari, Marwari and Kachchhi-Sindhi, Microsatellites

An equine diversity in India is reflected in the form of 7 distinct horse breeds namely Kathiawari, Marwari, Spiti, Zanskari, Bhutia, Manipuri and recently recognized Kachchhi-Sindhi horse (Anonymous, 2017). Western part Gujarat is rich in livestock diversity represented by 2 cattle, 4 buffalo, 2 sheep, 5 goat, 2 camel, 3 horse breeds and several breeds still awaited to characterize and recognized. Gujarat has three recognized horse breeds Kathiawari, Marwari and recently recognized as seventh horse breed of India and third horse breed of Gujarat—Kachchhi-Sindhi, which is believed to have descended from Sindhi—a breed of Sindh province of Pakistan.

Kathiawari breed of horse is found in Saurashtra region of Gujarat State and is very similar to that of Arabian horse. This breed is famous for their canter gait popularly known as "Rawaal" *chal*. Kathiawari breed is also known for its pace and speed, relative disease resistance, possesses good endurance power and faithfulness to owner which attracts the royal community of India. Marwari or Malani is a breed of horse from the Marwar (or Jodhpur) region and also found in the Gujarat region. The Marwari horse has evolved from one of the worlds' ancient breeds. Marwari horse is used for riding and packing and this breed also known for their sturdiness, swiftness, elegance and beauty. Kachchhi-Sindhi is breed of horse found in Kachchh region of Gujarat state and also found in Rajasthan, Punjab and Sindh region of Pakistan. It is used for light draught and agricultural work and is known for sturdiness and endurance power (Rewad).

In Gujarat state, according to the Livestock Census 2007, the total horse population was 13,417. The horse population trend was declining by 25.91% from 2003 to 2007. The breed wise population enumerates Kathiawari and Marwari were 9,377 and 2,747, respectively. Owing to indiscriminate breeding and lack of sound breeding policies, the breed's characteristics are being diluted (Singhvi, 2001). As the Kathiawari breed is integrated under threatened category, conservation of this valuable germplasm is a major concern of state government. Molecular characterization is essential prerequisite for meaningful breeding policies and conservation of these breeds. Several studies on establishing genetic relationships and differentiation based on microsatellite markers have been reported in different species (Berber *et al.* 2014). Microsatellites are the marker of choice for evaluation of genetic diversity of population and for population differentiation (Takezaki and Nei 1996, Goldstein and Schlotterer 1999). Thus, it will help breeders to manage, conserve and improve the valuable germplasm. The present study was undertaken to evaluate the genetic diversity using a set of 26 microsatellite markers and also evaluated for any recent genetic bottleneck in three horse breeds belonging to western India.

MATERIALS AND METHODS

Blood samples and DNA isolation: Blood samples (50) from each breed (Kathiawari, Marwari and Kachchhi-Sindhi) were collected from their breeding tract in Gujarat.

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Genomic DNA was isolated by John's method (John *et al.* 1991) and Genomic DNA extraction kit (HiPura^{TM} Mammalian Genomic DNA Purification Spin Kit).

Microsatellite analysis: A set of 26 horse microsatellite markers, divided into 2 multiplex panels were used for PCR amplification. PCR was carried out in the final volume of 15 µl containing 5 × AMP Mix, emPCR Additive, emPCR Enzyme Mix. Thermal conditions for amplification included initial denaturation at 98°C for 3 min, followed by 30 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 75 sec, and extension at 72°C for 30 sec each, with a final extension at 72°C for 5 min for panel 1 and annealing at 55°C for panel 2. The amplified PCR products were resolved by gel electrophoresis on 1.5–2.0% agarose gel containing ethidium bromide. The amplified product were mixed with deionized formamide based dye, denatured at 95°C and chilled immediately on ice and further genotyping was done by capillary electrophoresis on ABI PRISM ® 310 genetic analyzer.

Statistical Analysis: The data on genotypic frequencies were subjected to statistical analysis using the population genetics software GenAlEx 6.41 (http://biology.anu.edu.au/GenAlEx/Welcome.html) and MS toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). The horse breeds were also evaluated for any recent genetic bottleneck with sign test and standardized differences test, which test the population for significant heterozygosity excess or deficit under SMM as well as IAM model as described by Cornuet and Luikart (Cornuet and Luikart 1996).

RESULTS AND DISCUSSION

In present study, all the microsatellites selected were similar to previously used by Gupta *et al*. (2012), Chauhan *et al.* (2011), Behl *et al.* (2006) and Koringa *et al.* (2008) in equine diversity studies to evaluate the genetic diversity among three horse breeds. The value of diversity measures at each locus is given in Table 1. Out of 26 microsatellite markers, 25 markers were found polymorphic in all three breeds of horses. Only one marker HTG14 was observed to be monomorphic in all three breeds of horses.

The number of alleles in polymorphic loci ranged from 4 (HTG6, HMS1, NVHEQ05) to 12 (AHT4, HTG10) in Kathiawari breed of horse. In Marwari breed, the number of alleles ranged from 2 (NVHEQ21) to 13 (ASB17), while, in Kachchhi-Sindhi breed, the number of alleles ranged from 4 (NVHEQ21) to 13 (LEX3). The mean observed

Table 1. Observed (No) and expected (Ne) number of alleles, observed (Ho) / Expected (He) heterozygosity and Polymorphism information content (PIC) at different loci in Kathiawari, Marwari and Kachchhi-Sindhi breeds of horse

Breed	Kathiawari				Marwari				Kachchhi-Sindhi				PIC
Locus	No	Ne	Ho	He	No	Ne	Ho	He	No	Ne	Ho	He	
VHL20	9	4.082	0.720	0.755	10	3.849	0.740	0.748	10	6.203	0.680	0.839	0.8338
HT44	8	2.148	0.660	0.534	6	2.312	0.567	0.573	7	3.230	0.660	0.690	0.5755
AHT4	12	6.676	0.880	0.850	10	4.390	0.772	0.780	10	6.859	0.780	0.854	0.8516
HMS7	6	3.855	0.680	0.741	7	4.859	0.794	0.802	9	6.127	0.820	0.837	0.8104
HTG6	4	2.506	0.780	0.601	4	3.003	0.667	0.674	10	4.184	0.700	0.761	0.783
AHT5	7	3.814	0.940	0.738	7	4.252	0.765	0.773	8	4.118	0.633	0.757	0.8089
HMS6	5	3.092	0.700	0.677	5	3.994	0.750	0.757	7	5.482	0.714	0.818	0.7455
ASB23	10	1.777	0.480	0.437	9	3.420	0.708	0.715	9	3.882	0.680	0.742	0.6261
ASB2	9	3.719	0.813	0.731	8	5.394	0.815	0.823	12	7.268	0.833	0.862	0.8200
HTG10	12	6.410	0.900	0.844	11	6.850	0.854	0.863	12	6.693	0.860	0.851	0.8607
HTG7	5	2.741	0.860	0.635	5	3.336	0.700	0.707	6	2.873	0.729	0.652	0.7277
HMS3	9	5.754	0.860	0.826	8	3.808	0.737	0.745	8	5.751	0.755	0.826	0.8258
HMS2	7	3.114	0.694	0.679	9	3.228	0.690	0.697	10	4.183	0.755	0.761	0.7857
ASB17	10	3.159	0.720	0.683	13	5.025	0.801	0.809	13	4.378	0.680	0.772	0.8314
LEX3	8	5.532	0.673	0.819	9	6.553	0.847	0.856	13	6.141	0.612	0.837	0.8695
HMS1	4	2.573	0.740	0.611	6	4.421	0.774	0.782	7	3.689	0.646	0.729	0.7507
CA425	10	3.618	0.740	0.724	7	3.504	0.715	0.722	11	4.766	0.860	0.790	0.7191
NVHEQ29	6	4.946	0.780	0.798	7	3.475	0.712	0.720	7	3.197	0.640	0.687	0.7369
NVHEQ21	6	2.496	0.960	0.599	2	2.000	0.500	0.505	4	2.123	0.980	0.529	0.4413
NVHEQ11	6	3.811	0.700	0.738	6	2.166	0.538	0.544	6	2.826	0.600	0.646	0.6213
NVHEQ05	4	1.791	0.380	0.442	4	2.378	0.579	0.585	5	2.381	0.540	0.580	0.4889
NVHEQ100	8	2.431	0.680	0.589	10	4.006	0.750	0.758	11	6.094	0.837	0.836	0.7333
HTG14	1	1.000	0.000	0.000	1	1.000	0.000	0.000	1	1.000	0.000	0.000	0
NVHEQ70	6	1.895	0.440	0.472	6	2.684	0.627	0.634	8	2.825	0.755	0.646	0.5672
NVHEQ82	5	3.642	0.740	0.725	6	3.096	0.677	0.684	8	3.723	0.620	0.731	0.689
NVHEQ40	9	3.885	0.367	0.743	6	1.801	0.445	0.450	7	2.952	0.480	0.661	0.6133
Mean	7	3.479	0.688	0.654	7	3.646	0.746	0.674	8.4	4.344	0.687	0.719	
SE	0.50	0.284	0.041	0.035	0.5	0.271	0.042	0.034	0.55	0.326	0.035	0.034	

No, observed number of alleles; Ne, effective number of allele; Ho, observed heterozygosity; He, expected heterozygosity; PIC, Polymorphism information content.

number of alleles were 7.0±0.50, 7±0.53 and 8.4±0.55 for Kathiawari, Marwari and Kachchhi-Sindhi breeds respectively. The mean effective number of alleles was 3.479±0.284 for Kathiawari, 3.646±0.271 for Marwari, 4.344±0.326 for Kachchhi-Sindhi breed of horse. In pooled population including all three breeds the number of alleles ranged from 2 (NVHEQ21) to 16 (ASB17). Locus ASB17 was highly polymorphic with 16 alleles. The mean observed and effective number of alleles across all markers were 10.385±0.577 and 4.645±0.382, respectively. Number of alleles reflects the polymorphism of loci, which was sufficiently high in all the horse breeds. Values of observed (No) and effective (Ne) number of alleles were similar to Marwari breeds (Gupta *et al.* 2012), Zanskari breed of horse (Behl *et al.* 2006). Effective numbers of alleles were found significantly lower than the observed number of alleles indicating large number of alleles at low frequency. Similar finding was also reported in Spiti horses (Chauhan *et al.* 2011); in Zanskari breed of horse (Behl *et al.* 2006); in Marwari horse breed (Gupta *et al.* 2012).

The mean observed heterozygosity for Kathiawari, Marwari and Kachchhi-Sindhi breed of horse were 0.688±0.041, 0.746±0.042 and 0.687±0.035, respectively with overall mean observed heterozygosity as 0.709±0.035 across all three breeds. The observed heterozygosity was found to be higher than the expected heterozygosity in Kathiawari (0.688±0.041 v/s 0.654±0.035) and Marwari (0.746±0.042 v/s 0.674±0.034) breeds whereas lower in Kachchhi-Sindhi (0.687±0.035 v/s 0.0719±0.034) breed of horse. The overall mean expected heterozygosity was 0.732±0.647. Greater than 0.6 heterozygosities reflect higher genetic variability present in these breeds. Similarly, greater than 0.71 heterozygosities were reported in Arab-Barb, Arabian, Barb, Thoroughbred and French Trotter horse breeds (Berber *et al.* 2014), 0.675, 0.657 and 0.666 for Barb, Arab-Barb, and Arabian Pur-Sang breeds (Ahmed *et al*. 2018). The observed heterozygosity was higher than the expected heterozygosity indicates either certain amount of inbreeding or breed dilution. The polymorphism information content (PIC) values ranged from 0.4413 (NVHEQ21) to 0.8695 (LEX3). The majority of markers have PIC values higher than required value of 0.6, except HT44 (0.5755), NVHEQ05 (0.4889) and NVHEQ70 (0.5672). The high PIC values of markers reflect informativeness in polymorphism and suitability in genetic diversity studies in equine. Koringa *et al.* (2008) have also reported high estimate of PIC marker in Kathiawari breed of horse.

The mean estimates of F statistics were 0.015±0.039 (F_{IT}), –0.044±0.038 (F_{IS}), and 0.058±0.008 (F_{ST}). The pair wise F_{ST} distance among three populations were also estimated (Table 2). In F statistics, the within population inbreeding estimates (F_{IS}) deviation from Hardy-Weinberg equilibrium (HWE), revealed that the absence of inbreeding in Kathiawari, Marwari and Kachchhi-Sindhi breed and population of these three breeds is under selective mating. Gupta *et al.* (2012), Behl *et al.* (2006) and Chauhan *et al.* (2011) reported little amount of inbreeding in Marwari, Zanskari, Zanskari pony and in Spiti horses, respectively. The negative value of F_{IS} (–0.045) indicates excess of heterozygosity so, inbreeding can be ruled out. The most probable causes for same are migration, selective mating and extensive cross breeding between the breeds of neighbouring region. The lower F_{ST} values indicated that breed differentiation is poor and that might be due to higher gene flow among breeds. Berber *et al*. (2014) reported the genetic differentiation between five different breeds was significant (P<0.01) with high F_{ST} value based on the infinitesimal model. The Nei's genetic distance values between Kathiawari and Marwari, Kathiawari and Kachchhi-Sindhi, Marwari and Kachchhi-Sindhi were 0.106, 0.331 and 0.286, respectively. This indicates Kachchhi-Sindhi breed is genetically more distant from the Kathiawari and Marwari breeds.

Table 2. Nei's genetic distances and F_{ST} estimates between three horse breeds

Breed	Kathiawari	Marwari	Kachchhi-Sindhi
Kathiawari	–	0.0389	0.1053
Marwari	0.106	–	0.0893
Kachchhi-Sindhi	0.331	0.2860	–

Above diagonal $F_{ST,}$ below diagonal Nei's genetic distance.

In present study, Mutation drift equilibrium was studied to address the problem of bottleneck. The Wilcoxon's test, Sign test and Standardized difference test under Infinite Allele Model (IAM) and Stepwise Mutation Model (SMM) were used to determine whether populations under genetic bottleneck or not. BOTTLENECK v1.2.02 software (Piry *et al.* 1999) was used to detect genetic bottleneck. The bottleneck P values for all the three tests under IAM and SMM mutation model were significant indicating that all three breeds (Kathiawari, Marwari and Kachchhi-Sindhi) have undergone genetic bottleneck in the recent past. In mutation drift equilibrium study, bottleneck P values for all the three tests under IAM and SMM mutation model were significant indicating that all three breeds (Kathiawari, Marwari and Kachchhi-Sindhi) have undergone genetic bottleneck in the recent past as null hypothesis was rejected but graphical representation utilizing allelic class and proportion of allele showed a normal 'L' shaped distribution in all three breeds of horses (Fig. 1). The present finding in graphical method concludes that a population has not experienced recent genetic bottleneck. Koringa *et al.* (2008) and Gupta *et al*. (2012) had also reported no recent genetic bottleneck in Kathiawari and Marwari horse breed whereas Chauhan *et al.* (2011) had reported recent genetic bottleneck in Kathiawari horse breed.

The data was also subjected to AMOVA (Analysis of Molecular Variance) for estimation of overall breed differentiation and it indicated 15% variations among breeds and 85% variations among individuals within the breed. Similarly, only 8.8% variations between Barb, Arab-Barb and Arabian Pur-Sang horse breeds while 91.2% of

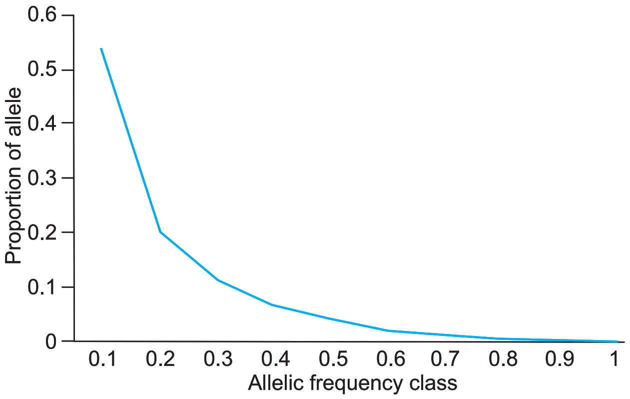

Fig. 1. Bottleneck analysis in Kathiawari, Marwari and Kachchhi-Sindhi Horse.

variations among individuals within breed (Ahmed *et al.* 2018). DNA based genetic marker shows an important application to estimate genetic similarity within and between breeds and genetic distance among different breeds of livestock. This study can also be useful for breed characterization studies as well as in selection programmes as it is equally important for utilization of germplasm resources. The level of observed genetic diversity of horse can be used to decide which populations should be prioritized for conservation purposes especially when the head counts are showing steep decline.

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