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Genetic relationships of five Indian horse breeds using microsatellite markers

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The genetic relationships of five Indian horse breeds, namely Marwari, Spiti, Bhutia, Manipuri and Zanskari were studied using microsatellite markers. The DNA samples of 189 horses of these breeds were amplified by polymerase chain reaction using 25 microsatellite loci. The total number of alleles varied from five to 10 with a mean heterozygosity of 0.58 ± 0.05. Spiti and Zansakari were the most closely related breeds, whereas, Marwari and Manipuri were most distant apart with Nei's D_A genetic distance of 0.071 and 0.186, respectively. In a Nei's D_A genetic distances based neighbour joining dendrogram of these breeds and a Thoroughbred horse outgroup, the four pony breeds of Spiti, Bhutia, Manipuri and Zanskari clustered together and then with the Marwari breed. All the Indian breeds clustered independently from Thoroughbreds. The genetic relationships of Indian horse breeds to each other correspond to their geographical/environmental distribution.

Keywords: genetic diversity, horse breeds, India, microsatellite markers

Introduction

The Indian horse breeds are distinct not only because of their adaptation to different agro-climatic conditions prevailing in the country, but also because they have unique traits such as sturdiness, stiffness, endurance potential, relative disease resistance etc. However, the changed scenario after development of the road network and mechanisation combined with indiscriminate breeding with exotic or nondescript animals has led to drastic decline in the populations of these breeds. Since, presently only a few thousand true breeding horses of each of these breeds are available (Singhvi, 2001; Yadav *et al.*, 2001), it is necessary to evolve strategies for their conservation. The evaluation of genetic diversity/relationships among livestock breeds is an important prerequisite for developing cost-effective and meaningful breed conservation/improvement programmes. The microsatellite DNA markers, due to their highly polymorphic nature, have been extensively employed in the analysis of genetic diversity amongst breeds of various livestock species including horses (Bjornstad *et al.*, 2000; Cañón *et al.*, 2000; Kelly *et al.*, 2002; Tozaki *et al.*, 2003; Achmann *et al.*, 2004; Aberle *et al.*, 2004; Solis *et al.*, 2005; Glowatzki-Mullis *et al.*, 2006). The present study was undertaken to characterise five Indian horse breeds for genetic variation and to establish relationships amongst them using a set of 25 microsatellite markers. The Thoroughbred horses, which are most common exotic horses in India, were also included in our study as an outgroup.

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Material and methods

Samples

The blood samples were collected from 189 horses of five Indian horse breeds from their respective areas of distribution (Figure 1). The breeds involved and their sample sizes were: Marwari (42), Spiti (32), Bhutia (26), Manipuri (47) and Zanskari (42). The blood samples were also collected from Thoroughbred (24) horses from Haryana state. The Marwari horses are native to Marwar region of Rajsthan province and are supposed to have been evolved to fulfil the needs of erstwhile local princely state. The present population of Marwari horses is estimated to be less than 3000 (Singhvi, 2001; Singh *et al.*, 2002). The other four Indian breeds included in the study are small sized and classified as ponies (Bhat *et al.*, 1981). These pony breeds have close resemblance with Tibetan ponies. The Zanskari ponies are found in Zanskar and Ladakh areas of Jammu and Kashmir. They are small-sized animals with compact bodies and strong legs. They are known for their hardiness and well adapted to work at these high altitude areas of the Himalayas located

Figure 1 The areas of distribution of five Indian horse breeds.

between 3000 to 5000 m altitude. They are mainly used for transportation and agricultural operations. The Zanskari breed is at the verge of extinction as only a few hundred horses of this breed exist now (Yadav *et al.*, 2001). The Spiti and Bhutia ponies with similar characteristics are also found in same kind of agro-climatic conditions. Though, the Spiti horses are distributed in Lahaul/Spiti, Kinnour and Pangi areas of Himachal Pradesh, they are mainly bred in a few hamlets of Pin valley using traditional selection practices for identifying males for breeding. There present population is estimated to be less than 3000 (Katoch *et al.*, 2004; Behl *et al.*, 2005). The Bhutia ponies with their estimated population of less than 5000 are distributed in the Middle/Eastern Himalayas all along the Tibet border reared by the Bhutia tribe (Bhat *et al.*, 1981; Yadav *et al.*, 2001). The Manipuri ponies with a present population of 2327, are found in Manipur province in north-east India. The Manipuri ponies are referred to as original polo ponies. They are evolved from ponies brought from Tibet around 1200 years ago (Anonymous, 2006). All these Indian horse breeds have been listed as threatened breeds.

The genomic DNA was isolated from collected samples by standard procedure of digestion with proteinase-K, separation with phenol/chloroform/isoamylalcohol and precipitation with ethanol. The isolated DNA samples were stored at −20°C and working dilutions were stored at 4°C.

PCR amplification

The genomic DNA was amplified by polymerase chain reaction (PCR) using 25 equine microsatellite loci (Table 1) using the protocol described in Crawford *et al.* (1995). The amplified DNA fragments were analysed on 7% denaturing polyacrylamide gel and detected by silver staining (Bassam *et al.*, 1991). Alleles were scored manually against DNA size markers and known samples used as standards on every gel.

Statistical analysis

The allele frequencies, observed/effective number of alleles and observed/expected heterozygosities for each locus were calculated using POPGENE computer program (Yeh *et al.*, 1999). The polymorphism information content (PIC) was calculated as described by Botstein *et al.* (1980). The tests for departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between loci were performed using exact probability tests provided in GENEPOP version 3.4 (Raymond and Rousset, 1995). Monte Carlo method (Gou and Thompson, 1992) was applied to compute unbiased estimate of the exact probabilities (*P* value). Length of chain was set to be 50 000 iterations with critical *P* value adjusted to 0.05 on population level.

Using variance based method of Weir and Cockerham (1984), population differentiation by F statistics was computed using FSTAT version 2.9.3 computer program

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Table 1 *PCR product size range (bp), observed number of alleles, observed heterozygosity, polymorphism information content and F_{ST} for 25 microsatellite loci in five Indian horse breeds*

Locus	Allele size range (bp)	Observed no. of alleles	Observed heterozygosity	Polymorphism information content (PIC)	F_{ST} (θ)
HTG4	131–143	7	0.60	0.73	0.072
HTG6	78–102	6	0.56	0.76	0.111
HTG7	116–128	7	0.55	0.80	0.062
HTG8	176–192	8	0.68	0.83	0.025
HTG10	88–114	7	0.58	0.73	0.093
HTG14	127–139	7	0.61	0.71	0.049
HTG15	128–146	7	0.62	0.82	0.062
AHT4	142–164	10	0.60	0.84	0.06
AHT5	126–140	7	0.52	0.82	0.061
HMS2	216–236	9	0.63	0.84	0.067
HMS3	149–169	9	0.65	0.82	0.077
HMS6	157–169	6	0.50	0.80	0.086
HMS7	168–186	9	0.63	0.86	0.038
VHL20	85–109	9	0.52	0.86	0.083
LEX20	196–208	6	0.58	0.81	0.054
NVHEQ5	149–161	7	0.60	0.82	0.056
NVHEQ11	120–130	6	0.51	0.80	0.028
NVHEQ18	118–134	8	0.66	0.85	0.062
NVHEQ29	91–103	7	0.59	0.82	0.076
NVHEQ40	146–156	5	0.55	0.77	0.031
NVHEQ100	185–203	8	0.58	0.82	0.026
NVHEQ21	151–161	6	0.49	0.74	0.083
NVHEQ54	176–186	6	0.50	0.74	0.127
UCDEQ425	238–250	7	0.53	0.81	0.078
ASB2	89–107	8	0.55	0.85	0.077

(Goudet, 2001). Mean and standard deviation of F statistics parameters, θ, F, f, that are analogous to Wright's (1969) F_{ST} F_{IT} and F_{IS}, respectively, were obtained across breeds by jackknifing procedure over loci (Weir, 1990). The level of significance ($P < 0.05$) was determined from permutation test with sequential Bonferroni procedures applied over all loci. The Nei's D_A genetic distances (D_A, Nei *et al.*, 1983) and Reynolds' genetic distances (D_R, Reynolds *et al.*, 1983) between pairs of populations and neighbour joining tree between breeds were generated with POPULATIONS package (Langella, 2002). The phylogenetic tree was visualised using TREEVIEW computer program (Page, 1996).

Principal component analysis was performed for each population from allele frequency data according to the procedures described by Cavalli-Sforza *et al.* (1994). The data for individual genotypes were prepared by scoring a '0' if a particular allele was absent, '1' if it was present in one copy and '2' if it was homozygous. To further decipher the question that how many breeds are actually present, the data was analysed using STRUCTURE computer program (version 2, Pritchard *et al.*, 2000) applying Markov chain Monte Carlo method without admixture using a burn in period of 30 000 iterations and data was collected after 10^6 iterations assuming number of breeds (*K*) between 1 and 6.

Results and discussion

All the loci reported in the study amplified successfully and produced unambiguous banding patterns from which individual genotypes could be assessed. Estimated parameters pertaining to genetic variation viz. observed/effective number of alleles and observed/expected heterozygosity, polymorphism information content (PIC) in the studied Indian horse breeds are summarised in Tables 1 and 2. A reasonable amount of polymorphism in all the five breeds is discernible from allele frequency data. A total of 183 alleles were detected across the 25 loci with mean number of alleles varying from 5.40 ± 1.04 in Spiti ponies to 5.80 ± 1.32 in Zanskari ponies. The total number of alleles across all five breeds ranged from 135 in Spiti ponies to 145 in Zanskari ponies. The overall PIC values varied from 0.71 at locus HTG14 to 0.86 at loci HMS7 and VHL20 across all five Indian horse breeds. The observed number of alleles and fairy high PIC values demonstrated that almost all the microsatellite loci utilised in the present study were sufficiently polymorphic suggesting their suitability in evaluation of Indian horse breeds. The PCR product size range varied from 78 to 102 bp at locus HTG6 to 238 to 250 bp at locus UCDEQ425. The allele sizes obtained at each locus across the studied Indian breeds were in agreement with the data published for Asian and European horse breeds (Cañón *et al.*, 2000; Bjornstad and Roed, 2001; Bjornstad *et al.*, 2003; Curik *et al.*, 2003; Tozaki *et al.*, 2003; Aberle *et al.*, 2004; Achmann *et al.*, 2004). The effective number of alleles were distinctly less than the observed

Table 2 *The population genetic variability in five Indian horse breeds evaluated using 25 microsatellite loci*

Breed	Sample size	Mean no. of alleles		Mean heterozygosity		F_{IS}
		Observed	Effective	Observed	Expected	
Marwari	42	5.72 ± 1.51	4.58 ± 1.13	0.58 ± 0.08	0.78 ± 0.06	0.147 (0.047)
Spiti	36	5.40 ± 1.04	4.72 ± 1.02	0.56 ± 0.07	0.79 ± 0.05	0.189 (0.052)
Bhutia	26	5.64 ± 0.86	4.62 ± 0.89	0.55 ± 0.07	0.79 ± 0.04	0.194 (0.051)
Manipuri	47	5.60 ± 1.04	4.52 ± 0.85	0.55 ± 0.07	0.78 ± 0.04	0.279 (0.061)
Zanskari	42	5.80 ± 1.32	4.94 ± 1.18	0.61 ± 0.06	0.78 ± 0.05	0.219 (0.055)
Thoroughbred	24	4.64 ± 0.64	3.85 ± 0.49	0.53 ± 0.06	0.65 ± 0.03	0.009 (0.027)

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values across all loci in all the five breeds with mean values ranging from 4.52 ± 0.85 in Manipuri horses to 4.94 ± 1.18 in Zanskari horses.

The mean observed heterozygosity values ranged from 0.55 ± 0.07 in Bhutia and Manipuri horses to 0.61 ± 0.06 in Zanskari horses. The obsereved heterozygosity was lower than the expected heterozygosity in all the five breeds. The mean expected heterozygosity did not vary much between the studied breeds varying in a narrow range of 0.78 (Marwari, Manipuri and Zanskari) to 0.79 (Spiti and Bhutia). Heterozygote deficiency analysis revealed that all the five populations exhibited significant deviation from HWE ($P < 0.05$) at many loci. It is though difficult to explain the exact basis of this departure; however, this may be attributed to the lower population of size varying from a few hundred to a few thousand for all these breeds. The presence of low frequency null alleles segregating at these loci may be other possible reason. This deviation could also be linked to positive F_{IS} (within population inbreeding estimates) values obtained in all the breeds.

Mean estimates of F statistics obtained from jackknifing over loci (Weir, 1990) were: f (F_{IS}) = 0.206 ± 0.033, θ (F_{ST}) = 0.065 ± 0.021 and F (F_{IT}) = 0.245 ± 0.041. The overall estimates of F statistics were significantly ($P < 0.01$) different from zero. There was significant deficit of heterozygotes in all the breeds, ranging from 14.7% in Marwari to 27.9% in Manipuri. The average F_{IS} values for of these breeds were significantly different from zero. Global analysis indicated that the studied breeds had a 20.6% deficit of heterozygotes ($P < 0.01$), whereas the total population had 24.5% deficit of heterozygotes ($P < 0.01$) (Table 3). The main cause for shortage of heterozygotes and excess of homozygotes ($F_{IS} > 0$) seems to be the inbreeding/non-random mating arising from small population sizes and extensive use of only a few breeding studs in these breeds. The locus under selection (genetic hitchhiking), null alleles (non-amplifying alleles) or presence of population sub-structure (Wahlund effect) may be the other possible reason for lack of heterozygotes in a population (Nei, 1987).

The D_A and D_R genetic distances between pairs of populations are given Table 4. None of the five Indian horse breeds was found to be closely associated with Thoroughbreds with an average D_A and D_R of 0.217 and 0.067, respectively. Within Indian breeds the Marwari and Manipuri with a D_A and D_R of 0.186 and 0.058 were most distant apart. In fact, the genetic distances suggest that the Marwari breed was most distinguishable within the studied Indian horse breeds. The Marwari horses are medium sized with an average height of 154.19 ± 0.32 cm (Singh *et al.*, 2002). The Marwari breed was primarily developed for survivability and endurance in desert type environment by crossbreeding the local stock with Arabian horses. They

Table 3 *Within population inbreeding estimates (F_{IS}) in five Indian horse breeds*

Locus	Marwari	Spiti	Bhutia	Manipuri	Zanskari
HTG4	0.002	−0.045	−0.042	0.112**	0.121**
HTG6	0.157**	0.287**	0.382**	0.403**	0.006*
HTG7	0.338**	0.247**	0.343**	0.301**	0.169**
HTG8	−0.046	−0.031	−0.036	0.214**	0.096**
HTG10	0.219**	0.188**	0.157**	0.167**	0.207**
HTG14	0.004	−0.036	−0.039	0.131**	0.118**
HTG15	0.186**	0.222**	0.225**	0.232**	0.271**
AHT4	0.146**	0.287**	0.216**	0.362**	0.194**
AHT5	0.327**	0.337**	0.400**	0.353**	0.33**
HMS2	0.321**	0.256**	0.261**	0.241**	0.300**
HMS3	−0.005	0.238**	0.288**	0.261**	0.201**
HMS6	0.436**	0.297**	0.360**	0.352**	0.241**
HMS7	−0.036	−0.032	−0.045	0.211**	0.222**
VHL20	−0.004	0.481**	0.456**	0.383**	0.349**
LEX20	0.197**	0.368**	0.271**	0.337**	0.213**
NVHEQ5	0.167**	0.305**	0.257**	0.203**	0.277**
NVHEQ11	−0.017	−0.036	0.004	0.412**	0.346**
NVHEQ18	0.177**	0.24**	0.198**	0.194**	0.297**
NVHEQ29	0.235**	0.309**	0.292**	0.152**	0.276**
NVHEQ40	−0.049	−0.037	−0.048	0.261**	0.176**
NVHEQ100	−0.048	−0.046	−0.035	0.319**	0.261**
NVHEQ21	0.32**	0.298	0.279	0.352**	0.307**
NVHEQ54	0.006*	−0.049	−0.048	0.353**	0.082*
UCDQ425	0.355**	0.330**	0.377**	0.341**	0.188**
ASB2	0.293**	0.339**	0.384**	0.332**	0.238**
Mean (s.d.)	0.147 (0.047)**	0.189 (0.052)**	0.194 (0.051)**	0.279 (0.061)**	0.219 (0.055)**

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Table 4 *Nei's D_A genetic distances (lower triangle) and Reynolds genetic distances (upper triangle) between five Indian horse breeds and Thoroughbred horses outgroup using 25 microsatellite loci*

Breed	Marwari	Spiti	Bhutia	Manipuri	Zanskari	Thoroughbred
Marwari	–	0.041	0.053	0.058	0.052	0.061
Spiti	0.155	–	0.016	0.021	0.012	0.067
Bhutia	0.175	0.098	–	0.024	0.028	0.064
Manipuri	0.186	0.126	0.134	–	0.038	0.070
Zanskari	0.181	0.071	0.119	0.133	–	0.074
Thoroughbred	0.209	0.236	0.213	0.212	0.213	–

can be expected to be fairly distant from other four pony breeds on the basis of physical characteristics and adaptability to environment.

Within pony breeds, the maximum D_A and D_R found were only of the order of 0.133 and 0.038 between Zanskari and Manipuri. The least D_A and D_R were found to be 0.071 and 0.012, between Spiti and Zanskari indicating their close genetic relatedness. These results were also reflected in neighbour-joining tree, based on D_A genetic distances, developed after 1000 bootstraps of the data where all the pony breeds joined first then with the Marwari with good statistical support (Figure 2). All the Indian breeds clustered independently from Thoroughbreds. The Spiti and Zanskari ponies joined first then with other two pony breeds of Bhutia and Manipuri with high statistical support. The lower genetic distances found between the pony breeds can be expected as animals of Zanskari, Spiti, Bhutia and Manipuri are small sized (less than 12 hands) ponies with similar physical characteristics (Bhat *et al.*, 1981; Katoch *et al.*, 2004). All these pony breeds are supposed to be evolved from the Tibetan ponies. Moreover, principal component analysis showed a tight cluster of Zanskari, Spiti, Bhutia and Manipuri pony breeds well separated from Marwari horses. All the Indian breeds were clearly distinguishable from the Thoroughbred horses (Figure 3).

Further, to study the population structure of Indian horse breeds, the data was analysed using STRUCTURE computer program. The models with assumed number of breeds, $K = 1$, 2 or 3 gave insufficient posterior probabilities,

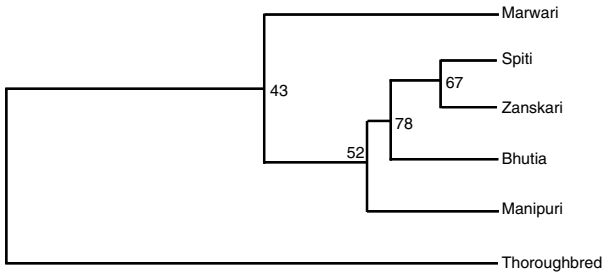

Figure 2 The neighbour joining dendrogram showing the genetic relationships among the five Indian horse breeds and Thoroughbred horses based on Nei's unbiased D_A distances (Nei *et al.*, 1983) using microsatellite markers. The numbers at nodes are values for 1000 bootstrap resampling of the data.

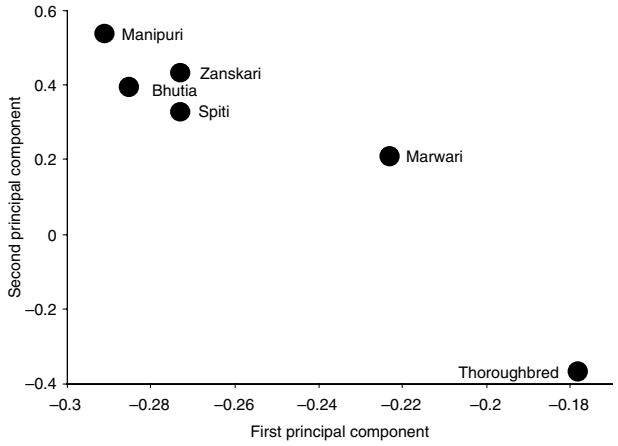

Figure 3 PCA of the transformed allele frequencies from 25 microsatellite loci typed in five Indian horse breeds and Thoroughbred horse outgroup. The first PC accounted for 69.8% of the underlying variation and the second PC condenses 8.8% of the variation.

Pr(K/X) and the model with $K = 4$ was substantially better than models with even larger K. At $K = 4$, ln Pr(K/X) also stabilised to about minimum values (Table 5). When individual horses were clustered assuming number of breeds to be four, about 90% of individuals belonging to Zanskari, Spiti and Bhutia were assigned to one cluster (Table 6), whereas, majority of the Marwari and Thoroughbred individuals were assigned to their respective clusters. Though, Manipuri horses formed a separate cluster 12% of the Manipuri horses clustered with common cluster of Zanskari, Spiti and Bhutia horse. These results point towards the genetic closeness of pony breeds of India. These findings contribute to the knowledge of genetic structure of these endangered breeds and should aid in evolving efficient conservation/breeding strategies for the Indian horse breeds.

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Table 5 *Estimated posterior probabilities of K number of assumed breeds for sampled individuals with genotype X*

K	ln Pr(K/X)	Pr(K/X)
1	2625	~0
2	2281	~0
3	2126	~0
4	2063	0.989
5	2061	0.006
6	2055	0.00 003

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Table 6 *Proportion of membership of each of five Indian breeds and Thoroughbred horses in each of the four clusters*

	Inferred cluster			
Breed	1	2	3	4
Marwari	0.869	0.073	0.014	0.031
Spiti	0.067	0.886	0.006	0.041
Bhutia	0.019	0.913	0.005	0.063
Manipuri	0.006	0.121	0.000	0.873
Zanskari	0.029	0.904	0.009	0.058
Thoroughbred	0.012	0.016	0.959	0.013

and Biostatistics, College of Veterinary and Animal Sciences, Palampur, Himachal Pradesh; (6) Dr M. Ragnekar and Dr Z. Ahmed, Field Research Laboratory, Defense Research and Development Organisation, Leh, Jammu and Kashmir.

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