RESEARCH NOTE

Genetic characterization of Indian Spiti horses

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Introduction

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. Spiti horses are slow moving, sure-footed, and are found mainly in the hilly areas of the Himalayan range, mostly in the districts of Lahaul-Spiti and Kinnaur in the state of Himachal Pradesh, India. Spiti horses not only thrive under such harsh conditions but are also used as pack animals. Unfortunately, the population numbers of this breed are decreasing at an alarming rate and it is thought to be close to extinction. Consequently, there is an urgent need to characterize this valuable breed to set priorities for its conservation.

Molecular characterization is an essential prerequisite to developing an effective and meaningful conservation programme for Spiti horses. Several DNA-based technologies have been developed in the last decade to type polymorphic loci, including random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), variable number of tandem repeats (VNTRs), single-strand conformational polymorphism (SSCP), and methods that make use of polymorphisms of short tandem repeats called microsatellites. Among the array of molecular markers, microsatellites are considered especially suitable for biodiversity evaluation, owing to their codominant inheritance, high heterozygosity, ease and reliability of scoring, ubiquitous presence throughout the genome, and high degree of polymorphism (Takezaki and Nei 1996). The aim of the present work was to assess genetic variation in the Spiti breed of Indian horses, using 25 microsatellite markers that had been observed in earlier studies to be polymorphic in exotic breeds of horses (Ellegren *et al*. 1992; van Haeringen *et al*. 1994, 1998; Ewen and Matthews 1995; Eggleston-Stott *et al*. 1996, 1997; Meyer *et al*. 1997; Swinburne *et al*. 1997, 2000; Coogle and Bailey 1998; Lindgren *et al*. 1999; Kakoi *et al*. 1999).

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Materials and methods

Blood samples and DNA isolation

Blood samples from 24 genetically unrelated animals of Spiti breed were collected from Spiti and Pin valleys of Lahaul-Spiti district and Government Livestock Farm, Leri, Himachal Pradesh. Ten to fifteen ml of blood per animal was collected aseptically by jugular venipuncture into sterile vacutainers coated with EDTA (0.5 mM, pH 8.0) as the anticoagulant. Genomic DNA was isolated and purified from white blood cells using proteinase K digestion and standard phenol/chloroform/isoamyl alcohol extraction and absolute ethanol precipitation (Sambrook *et al*. 1989).

Microsatellite analysis

Twentyfive microsatellite markers that were known to be polymorphic in exotic horse breeds (Ellegren *et al*. 1992; van Haeringen *et al*. 1994, 1998; Ewen and Matthews 1995; Eggleston-Stott *et al*. 1996, 1997; Meyer *et al*. 1997; Swinburne *et al*. 1997, 2000; Coogle and Bailey 1998; Kakoi *et al*. 1999; Lindgren *et al*. 1999) were selected for genetic characterization (table 1). Polymerase chain reaction (PCR) was carried out in 25 µl reaction mixture containing optimum $MgCl_2$ concentration (table 1), 10 mM Tris HCl (pH 8.0), 50 mM KCl, 0.01% (w/v) gelatin, 100 ng template DNA, 200 µM dNTPs, 50 ng of each primer, and 0.5 U of *Taq* polymerase (Bangalore Genei, Bangalore, India) using Thermal Cycler (Perkin-Elmer). The thermal cycling conditions included an initial denaturation at

Table 1. Microsatellite loci, their primer sequences and PCR conditions used.

Microsatellite locus	Primer sequences (forward and reverse)	Annealing temperature (°C)	$MgCl_2$ conc. (mM)	Reference
AHT16	5′-ATGTTGTGCAAATGGGATGA-3′ 5′-TGCCCATTGATTGATGATTG-3′	60	1.5	Swinburne *et al.* (1997)
AHT 17	′- CCCCATAACCACAAGTGAGG-3′ 5′-GAAGTGGGAGAGTCGGTAAGG-3′	60	1.5	Swinburne *et al.* (1997)
AHT44	5′-GAAAAGGAGAAAGGATGCCC-3′ 5′-ATGAGAGAGGCCAACCAGG-3′	60	1.5	Swinburne *et al.* (2000)
UM002	5′-AGTGGCAGCATAAAGATG –3′ 5′-TTTTGGTCCTTGTAGGAG-3′	56	1.5	Meyer *et al.* (1997)
UM004	5′-AGGTCAGGTTCACTTTTTC-3′ 5′-AGGTCACTGTGCCTAGTTG-3′	56	1.5	Meyer *et al.* (1997)
UM005	5′-CCCTACCTGAAATGAGAATTG-3′ 5′-GGCAAAAGATCAGGCCAT-3′	56	1.5	Meyer *et al.* (1997)
UM007	5′-GGGAATAGAGAAAGGTGAAG-3′ 5′-TTAGAGTTCCTGCTCCTCC-3′	56	1.5	Meyer *et al.* (1997)
UM010	5′-TACAGCCATTGGAAATCTTAC-3′ 5′-CACCATTACATTTTCCCAG-3′	56	1.5	Meyer *et al.* (1997)
UM011	5′-TGAAAGTAGAAAGGGATGTGG-3′ 5′-TCTCAGAGCAGAAGTCCCTG-3′	56	1.5	Meyer *et al.* (1997)
UM015	5′-AGTCTGGCTGAGGATACTG-3′ 5′-GGTGAGAAAGGAGATAAATG-3′	56	1.5	Meyer *et al.* (1997)
UM021	5′-CGTCCACTTAGGACAATGTAG-3′ 5′-ATGCACAGCAAGATGCAG-3′	56	1.5	Meyer *et al.* (1997)
HTG 2	5′-GATTGGCAACAGATGTTAACTCGG-3′ 5′-CCCCATGAGAACTAACAATGTTAG-3′	55	1.5	Lindgren *et al.* (1999)
HTG4	5′-CTATCTCAGTCTTGATTGCAGGAC-3′ 5′-CTCCCTCCCTCCCTCTGTTCTC-3′	55	1.5	Ellegren *et al.* (1992)
HTG5	5′-TGCTAAGCCTCAG CACATACA-3′ 5′-TGGAAATAAGGTTAGCAGGGATGC-3′	55	1.5	Ellegren *et al.* (1992)
HTG6	5′-CCTGCTTGGAGGCTGTGATAAGAT-3′ 5′-GTTCACTGAATGTCAAATTCTGCT-3′	55	1.5	Ellegren *et al.* (1992)
HTG8	5′- CAGGCCGTAGATGACTACCAATGA-3′ 5′- TTTTCAGAGTTAATTGGTATCACA-3′	55	1.5	Ellegren *et al.* (1992)
HTG 20	5′-CTGGTTTACCTTCCCTACAG-3′ 5′-CCAATGGTTCCTCTGAGAAG-3′	55	1.5	Lindgren *et al.* (1998)
VHL20	5′-CAAGTCCTCTTACTTGAAGACTAG-3′ 5′-AACTCAGGGAGAATCTTCCTCAG-3′	60	2.0	van Haeringen *et al.* (1994)
VHL123	5′-CCTCCTTCACAGTGAAGTGC-3′ 5′-GAGTATATAGCTCCAGACCTC-3′	57	1.5	van Haeringen *et al.* (1998)
UCDEQ62	5′-AAACTGAGCACCAGACTC-3′ 5′-GATGGATACTCCTGTAGCA3′	55	1.5	Eggleston-Stott *et al.* (1996)
UCDEQ502	5′-CCTTGGGCTTTAGCAACT-3′ 5′-CCATTGGAAACTGAGAGG-3′	55	1.7	Eggleston-Stott *et al.* (1997)
TKY16	5′-GGTTATGGTTTGGTATCTGTC-3′ 5′-AAAACAATGGCTTCCTGGTCA-3′	55	1.5	Kakoi *et al.* (1999)
TKY19	5′-CTTCTGCTGATTCCTGAATG-3′ 5′-GGATCTCCTTAAATGGAACA-3′	55	1.5	Kakoi *et al.* (1999)
LEX68	5′-AAATCCGAGCTAAAATGTA-3′ 5′-TAGGAAGATAGGATCACAAGG-3′	56	1.5	Coogle and Bailey (1998)
VIAS-H64	5′-CTAGTTTATGAGCTGAGCCACTC-3′ 5′-GTGGAGAACTTTGTATTCTCCTCT-3′	55	1.5	Ewen and Matthews (1995)

95°C for 2 min, followed by 32 cycles of 1 min at 95°C, 1 min at annealing temperature (table 1), and 1 minute at 72°C. A final elongation step was carried out at 72°C for 15 min. On completion of the reaction, PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and observed under ultraviolet light (300 nm) for amplification. The amplified products were mixed with 3× formamide-based dye (95% formamide, 5% xylene cyanol, 5% bromophenol blue, 10 mM NaOH), denatured at 95°C, chilled immediately on ice and further resolved by electrophoresis through 6% denaturing urea–polyacrylamide sequencing gel of 30 cm × 38 cm. (Sequi-GT system, Bio-Rad Laboratories) in 1× TBE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA) at 80 watts. The standard used to estimate the product sizes was 100 ng of 10 base-pair DNA ladder (Life Technologies, Delhi, India). The resolved alleles were visualized by silver staining the polyacrylamide gel. The genotype of each individual animal at each locus was recorded manually from the silver-stained gel.

Data analysis

The data on genotypic frequencies were subjected to statistical analysis using the population genetics software POPGENE version 1.31 (Yeh *et al*. 1999), and genetic variation was estimated to evaluate intrabreed variability in terms of observed and effective number of alleles, observed and expected heterozygosity, polymorphism information content (PIC) (Botstein *et al*. 1980) and gene diversity (F_{IS}) for each microsatellite locus. The allele number and approximate allele size range for each locus were determined manually from the silver-stained gels. Allelic frequencies for each polymorphic locus were calculated with $2n = 48$.

Results and discussion

Three loci (AHT16, AHT44 and UM021) were observed to be monomorphic, whereas for marker UM015 we observed a large number of nonspecific alleles of unexpected sizes that could not be scored. The remaining 21 microsatellite loci were highly polymorphic, with a total of 102 alleles scored (table 2). The values of diversity measures at each polymorphic locus are given in table 3. The observed number of alleles ranged from two (HTG2) to eight (UM007) with a mean (± standard error) of 4.8 ± 0.30 alleles per locus. The effective number of alleles ranged from 1.8 (HTG2) to 5.1 (TKY19) with a mean (± SE) value of 3.2 ± 0.18. In other horse breeds, Ellegren *et al*. (1992) reported three and five alleles at HTG2 and HTG6 loci, respectively, Meyer *et al*. (1997) observed five and six alleles at UM002 and UM005 loci, respectively, and 10 and six alleles were observed at VHL20 (van Haeringen *et al*. 1994) and TKY19 (Kakoi *et al*. 1999). The numbers of alleles observed in the studied population are in agreement with the data of Meyer *et al*. (1997) and Kakoi *et al*. (1999). The variable numbers of alleles and their frequencies at different loci indicate high

Table 2. Allelic frequency distribution at polymorphic microsatellite loci.

Allele/locus	AHT17	UM002	VHL123	UM010	UM005	HTG5	LEX68
A	0.2292	0.0625	0.0625	0.0417	0.0417	0.2083	0.6042
B	0.1875	0.0208	0.1042	0.0833	0.5625	0.1250	0.1250
C	0.3750	0.0208	0.1250	0.1042	0.1458	0.4167	0.0625
D	0.1667	0.1667	0.4167	0.1250	0.1250	0.1458	0.2083
E	0.0417	0.3333	0.2917	0.6458	0.0833	0.1042	
F		0.3958			0.0417		
Allele/locus	VHL20	UM004	HTG6	TKY16	TKY19	UCDEQ502	HTG8
A	0.1042	0.1042	0.0625	0.2500	0.0909	0.0435	0.2368
B	0.0625	0.1250	0.2708	0.3542	0.1818	0.4348	0.4474
C	0.2292	0.1875	0.1042	0.2708	0.1364	0.2174	0.3158
D	0.1458	0.1458	0.3333	0.1250	0.0909	0.2826	
E	0.0833	0.4375	0.1042		0.2727	0.0217	
F	0.3750		0.1250		0.2273		
Allele/locus	VIAS-H64	UM011	UCDEQ62	HTG4	UM007	HTG2	HTG20
A	0.2727	0.0625	0.0417	0.4130	0.0435	0.3125	0.0682
B	0.1136	0.1875	0.4583	0.3043	0.0217	0.6875	0.7045
C	0.1364	0.0208	0.5000	0.2826	0.0652		0.0909
D	0.0909	0.2917			0.1739		0.1364
E	0.3864	0.3958			0.4565		
F		0.0417			0.0870		
G					0.1087		
H					0.0435		

Table 3. Allele size range, number of alleles (observed n_o, effective n_e), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), gene diversity (F_{IS}) and average gene diversity estimates of polymorphic microsatellite loci in Spiti horses.

Microsatellite locus	Allele size range (bp)	No. of alleles			H_e			
		n_o	n_e	H_o	Levene's	Nei's	PIC	Gene diversity
AHT17	126–144	5.0	3.9	0.5417	0.7580	0.7422	0.7004	0.2702
UM002	242–262	6.0	3.3	0.6667	0.7145	0.6997	0.6470	0.0471
UM004	110–122	5.0	3.6	0.5417	0.7411	0.7257	0.6891	0.2536
UM005	212–222	6.0	2.7	0.6250	0.6498	0.6363	0.6049	0.0177
UM007	110–148	8.0	3.7	0.6957	0.7498	0.7335	0.7070	0.0515
UM010	108–122	5.0	2.2	0.5833	0.5594	0.5477	0.5176	– 0.0650
UM011	158–182	6.0	3.5	0.6250	0.7323	0.7170	0.6700	0.1283
HTG2	102–105	2.0	1.8	0.4583	0.4388	0.4297	0.3374	– 0.0667
HTG4	128–140	3.0	2.9	0.5652	0.6715	0.6569	0.5831	0.1396
HTG5	75–95	5.0	3.8	0.5833	0.7509	0.7352	0.6980	0.2066
HTG6	82–108	6.0	4.4	0.7500	0.7908	0.7743	0.7416	0.0314
HTG8	155–180	3.0	2.8	0.5263	0.6615	0.6440	0.5708	0.1828
HTG20	134–158	4.0	1.9	0.4091	0.4831	0.4721	0.4403	0.1335
VHL20	88–108	6.0	4.2	0.8333	0.7801	0.7639	0.7313	– 0.0909
VHL123	144–156	5.0	3.5	0.7083	0.7261	0.7109	0.6653	0.0037
UCDEQ62	152–170	3.0	2.2	0.7500	0.5496	0.5382	0.4316	– 0.3935
UCDEQ502	154–168	5.0	3.1	0.5652	0.6966	0.6815	0.6243	0.1706
TKY16	114–130	4.0	3.6	0.5417	0.7385	0.7231	0.6717	0.2509
TKY19	144–162	6.0	5.1	0.7727	0.8245	0.8058	0.7778	0.0410
LEX68	152–164	4.0	2.3	0.5833	0.5842	0.5720	0.5243	– 0.0197
VIAS-H64	136–164	5.0	3.8	0.7727	0.7537	0.7366	0.6956	– 0.0491
Average		4.8	3.2	0.6237	0.6836	0.6689	0.6204	0.1244
SEM		0.3030	0.1885	0.0243	0.0227	0.0022	0.0249	0.0333

genetic variability in Spiti equids. This forms the basis of all the diversity indices for estimation of genetic distances and construction of phylogenetic trees with other indigenous horse breeds.

The observed heterozygosity values across 21 polymorphic marker loci ranged from 0.4091 (HTG20) to 0.7727 (TKY19 and VIAS-H64) with a mean (± SE) of 0.6237 ± 0.02. Levene's and Nei's expected heterozygosity values varied from 0.4388 and 0.4297 (HTG2) to 0.8245 and 0.8058 (TKY19) respectively, with mean (± SE) values of 0.6836 ± 0.02 and 0.6689 ± 0.002. van Haeringen *et al*. (1994) also reported higher values of heterozygosity, in the range of 0.73 to 0.80, at VHL20 among Quarter, Standardbred, Miniature and Thoroughbred horses. Kakoi *et al*. (1999) observed a high value of heterozygosity (0.65 to 0.72) in Thoroughbred and Hokkaido native horses at locus TKY19. Higher values of heterozygosity at different loci in the Spiti horses indicate that the population has preserved different alleles at different loci. The mean (± SE) PIC for all loci in the Spiti population was 0.6204 ± 0.11, ranging from 0.3374 for HTG2 to 0.7778 for VHL20. Most markers had a PIC value higher than 0.5. These high values describe the informativeness of these markers in the polymorphism and genetic diversity. Since all markers were highly or reasonably informative, they can probably be used to assess genetic diversity in other indigenous equine breeds as well.

Within-population inbreeding estimates (F_{IS}) at each microsatellite locus are also presented in table 3, and a relatively low level of heterozygote deficit (0.1244) was observed, which could be due to moderate level of inbreeding. The high genetic variability observed in Spiti horses can be utilized in planning breeding strategies in the small populations of Spiti horses. The present panel of microsatellites evaluated in Spiti horses showed a very high heterozygosity and polymorphism and, therefore, this set of microsatellites may be reliably used for genetic diversity studies in other breeds of horses as well.

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