



Characterization and microsatellite analysis for genetic diversity and bottlenecks of Spiti donkey

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Received: 1 October 2013; Accepted: 25 April 2017

ABSTRACT

The Spiti donkeys distributed in Spiti and Yangthang regions of Himachal Pradesh were characterized for morphometric characteristics, management practices and utility. They were also evaluated for genetic diversity and bottlenecks using microsatellite markers. Spiti donkeys remain comparatively smaller in size with compact bodies usually covered with a thick coat of long hairs. The height at withers of male and female Spiti donkeys was 88.59 ± 3.27 and 88.65 ± 3.30 cm, respectively. The genomic DNA isolated from the blood samples collected from the 46 animals of this breed was amplified by PCR and resolved for alleles on automated DNA sequencer. The observed number of alleles ranged from 4 (VHL20, HTG6) to 10 (HTG7), with a mean of 6.33 ± 1.72 alleles per locus. The observed heterozygosity ranged from 0.48 (NVHEQ54) to 0.89 (HTG7), with a mean value of 0.72 ± 0.13 . The mean F_{IS} was 0.048 indicating moderate level of inbreeding. The normal 'L' shaped distribution of allelic frequency indicated towards absence of any recent genetic bottlenecks in the Spiti donkeys.

Key words: Bottleneck, Microsatellite genotyping, Morphometric characteristics, Spiti donkeys

The donkey (*Equus asinus*) is a sure footed, docile and hard working animal that is used extensively for pack purposes especially in difficult terrains. Despite its usefulness, the donkey has remained a neglected species, often overlooked by researchers and policy planners (Varshney and Gupta 1994, Behl *et al.* 2009). Little information is available regarding types or breeds of donkeys available in our country, their phenotypic characteristics and management practices (Singh *et al.* 2007, Behl *et al.* 2013). Some distinct populations of indigenous donkeys like Spiti and Laddakhi donkeys are known to exist in our country. The present study was undertaken to characterize the Spiti donkeys in their native tract to ascertain their present status, geographical localization, morphometric characteristics, management practices followed to rear them and their utility. They were also evaluated for within breed genetic diversity and recent genetic bottlenecks using microsatellite markers.

MATERIALS AND METHODS

Physical characterization: Information on physical characteristics, management practices, reproduction traits, utility etc. was collected by interviewing farmers and measuring a total of 70 animals from 3 villages in Spiti subdivision of Himachal Pradesh using a questionnaire

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developed at National Bureau of Animal Genetic Resources. The data were statistically analysed as described in Snedecor and Cochran (1989).

Microsatellite analysis: Blood samples were collected from forty-six genetically unrelated animals of Spiti breed. The genomic DNA was isolated by standard procedure of digestion with proteinase K, extraction with phenol/chloroform and precipitation with ethanol. The isolated genomic DNA samples were stored at -20°C and working dilutions were stored at 4°C . The genomic DNA was amplified by PCR using a set of twelve heterologous microsatellite markers of horse origin (Table 1). The primers were labeled with HEX (green) and FAM (blue) fluorescent dyes to facilitate multiplexing and the resolution of alleles on automated sequencer.

Within breed genetic variation parameters of observed and effective number of alleles, observed and expected heterozygosity at each locus were calculated using POPGENE computer program version 1.31 (Yeh *et al.* 1999). The polymorphism information content (PIC) at each locus was calculated according to Botstein *et al.* (1980). The probability of exclusion (PE) at each locus was determined as described by Jamieson and Taylor (1997). The exact test for Hardy-Weinberg disequilibrium at each locus and linkage disequilibrium between pairs of loci were computed using GENETOP software (Raymond and Rousset 1995) after applying 100 batches of 1000 iterations to calculate the exact probabilities. Within breed gene

diversity (F_{IS}) and level of significance was computed using FSTAT computer program, version 2.3.9 (Goudet 2001). The Spiti donkeys were also evaluated for any recent genetic bottleneck by sign test, standardized differences and Wilcoxon rank test under infinite allele model (IAM), sequential mutation model (SMM) and two step mutation model (TMM) using BOTTLENECK computer program (Piry *et al.* 1999).

RESULTS AND DISCUSSION

Distribution: Spiti donkeys are mainly distributed in the trans Himalayan regions of Spiti and Yangthang in Lahaul-Spiti and Kinnour districts of Himachal Pradesh having altitude range between 3000–4300 metres above mean sea level. The climate remains cold and arid, characterized by dry and mild summer without monsoon rains. Heavy snowfall and severe cold is observed in winters. The total donkey population as per 2007 livestock census in Lahaul-Spiti and Kinnour districts was 2007 and 2361, respectively.

Management practices: In each household, 1–3 donkeys were reared. They were housed in open space fenced by piled stones near the house during day time in summers. During the night and winters, they were housed in animal house along with other livestock which was mostly part of the house on the ground floor. They were raised both on grazing and stall feeding. They were allowed to graze in nearby waste lands and pastures in summer months. In winter months, when this region remains completely under snow cover, they were provided stored dry fodder. They

were well adapted to fodder scarcity endemic to this region in winter months. They were also provided with some amount of concentrate depending on availability and amount of work undertaken by the animal. The concentrate mainly consisted of black pea, barley, pea husk, wheat or even commercial animal feed available in the market.

Physical characteristics: Spiti donkeys are comparatively smaller in size, strongly built with compact bodies, straight backs and strong legs. The body normally covered with a thick coat of long hairs (Figs 1–2). The main coat colours were brown (58%), brown with lighter bellies (16%), dark brown/black (14%) and light brown with whitish bellies (12%). White markings were observed around muzzle (68%) and eyes (12%). The dorsal cross was visible only in lighter shades.

Head was comparatively shorter and broader with a mean face lengths of 32.1 ± 1.47 and 31.5 ± 1.03 cm, in male and female animals, respectively. The nasal bone was straight to slightly convex. The ears were comparatively smaller, straight, pointing upwards with slightly lateral orientation having lengths of 21.39 ± 1.13 and 21.5 ± 0.81 cm in adult male and female animals. The head was supported by strong neck having mean lengths of 31.1 ± 2.21 (male) and 30.15 ± 1.52 cm (female).

Body length for male and female animals was 91.0 ± 2.88 and 90.96 ± 2.52 cm, respectively. Height at withers of male and female Spiti donkeys was 88.59 ± 3.27 and 88.65 ± 3.30 cm, respectively. The mean heart girth was recorded as 100.5 ± 5.02 and 98.58 ± 4.23 cm in male and female animals, respectively. The tail extends up to hocks with mean length of 54.21 ± 7.63 and 55.56 ± 9.26 cm in male and female animals, respectively. The tail switch was not distinguishable due to presence of thick hair on rest of the tail.

The estimated weights of adult (above 3 years) male and female animals were 75.12 ± 9.57 and 75.69 ± 9.85 kg, respectively. In most of the morphometric parameters, no significant differences were observed between male and female Spiti donkeys except for canon circumference of the fore leg and hoof circumferences ($P < 0.05$). The important morphometric characteristics of male and female



Figs 1–2 **1.** Male donkey of Spiti breed. **2.** Female donkey of Spiti breed.

Table 1. Primer sequences and annealing temperatures for 12 microsatellite loci employed for genetic characterization of Spiti donkeys

Locus	Primer sequence	Annealing temperature (°C)
COR07	GACTTGCCCTGGCTTTGAGTCGTGTTGGATGAAGCGAATGA	58
COR18	AGTCTGGCAATATTGAGGATGTAGCAGCTACCCTTTGAATACTG	58
COR71	CTTGGGCTACAACAGGGAATACTGCTATTTCAAACACTTGGA	58
AHT4	AACCGCCTGAGCAAGGAAGTCCCAGAGAGTTTACCCT	60
HTG6	CCTGCTTGGAGGCTGTGATAAGATGTTCACTGAATGTCAAATTCTGCT	58
HTG7	CCTGAAGCAGAACATCCCTCCTTGATAAAGTGTCTGGGCAGAGCTGCT	58
HTG10	CAATTCCC GCCCCACCCCGGCATTTTATTCTGATCTGTACATTT	58
HTG15	TCTTGATGGCAGAGCCAGGATTTGAATGTCACCATGCGGCACATGACT	58
HMS2	ACGGTGGCAACTGCCAAGGAAGCTTGCAGTCGATGTGTATTAATG	58
NVHEQ54	AGATGTCCACCTTCTCGCTGCGGGGCTTTTAGGAGGTAACATA	60
VHL20	CAAGTCTCTTACTTGAAGACTAGAACTCAGGGAGAATCTTCCTCAG	60
VHL209	TCTTACATCTTCCATTACAACATATGATACATATGTACGTGAAAGGAT	57

Spiti donkeys are given in Table 2. When compared to the donkeys of Sindhi breed distributed in Rajasthan (Behl *et al.* 2013), both male and female animals of Spiti breed were found to be significantly ($P<0.01$) smaller in wither height, heart girth, face length and estimated body weight.

Utility: Spiti donkeys were mainly used as pack animals for transportation of dung and manure from the pastures to the villages or fields, fuel-wood and fodder from the nearby forests, construction material, trekking and camping materials for tourists etc. They are able to carry upto 100 kg of load in this rugged terrain with hypoxic conditions. Some income is also generated by selling these animals. However, due to rapid mechanization, their population is decreasing alarmingly and requires serious efforts for conservation and improvement to save this breed from further dilution/extinction.

Microsatellite analysis for genetic diversity and bottlenecks: Genetic evaluation of a breed is a major prerequisite in any breed conservation or improvement programme. On the global front, an increasing number of breeds of donkeys are now evaluated for diversity using microsatellite markers (Aranguren-Mendez *et al.* 2001, Jordana *et al.* 2001, Ivankovic *et al.* 2002).

All the loci reported in this study amplified successfully and produced unambiguous allele patterns in Spiti donkeys. The values for diversity measures found in Spiti donkeys are given in Table 3. The PCR product size range varied from 78–87 bp at locus VHL20 to 253–271 bp at locus

COR18. The observed number of alleles ranged from 4 (VHL20, HTG6) to 10 (HTG7), with a mean (\pm SD) of 6.33 ± 1.72 alleles per locus. The effective number of alleles ranged from 2.03 (VHL209) to 4.84 (HTG7), with a mean value of 3.35 ± 0.94 . The observed heterozygosity values across the twelve loci ranged from 0.48 (NVHEQ54) to 0.89 (HTG7), with a mean value of 0.72 ± 0.13 . The expected heterozygosity varied from 0.51 (VHL209) to 0.80 (HTG7 and AHT4), with a mean value of 0.69 ± 0.09 . The allele number and heterozygosity values observed across the studied loci indicated presence of reasonably high levels of genetic variability in Spiti donkeys. The allele numbers and heterozygosity levels were consistent with data from previous studies (Aranguren-Mendez *et al.* 2001, Jordana *et al.* 2001).

The mean PIC for these loci assessed from allele frequency data was 0.68 ± 0.09 , ranging from 0.51 (VHL209) to 0.79 (HTG7 and AHT4). These PIC values suggested that these loci were sufficiently polymorphic reflecting their suitability for genetic variability studies. The PE values ranged between 0.3 (VHL209) to 0.61 (HTG7) with a combined PE at 12 loci of 0.9995. Since, all markers were reasonably informative, they may be employed to assess genetic variability in other indigenous donkey breeds as well.

In exact test for Hardy-Weinberg disequilibrium at each locus, only one of the loci (NVHEQ54) deviated significantly ($P<0.01$) from Hardy-Weinberg proportions.

Table 2. The important morphometric characteristics of adult Spiti donkeys

Parameter	Male			Female		
	Mean \pm SD (cm)	n	Range (cm)	Mean \pm SD (cm)	n	Range (cm)
Body length	91.10 \pm 2.88	29	86-97	90.96 \pm 2.52	26	85-95
Height at wither	88.59 \pm 3.27	29	84-95	88.65 \pm 3.30	26	80-94
Heart girth	100.5 \pm 5.02	28	90-115	98.58 \pm 4.23	26	90-107
Neck length	31.10 \pm 2.21	29	27-36	30.15 \pm 1.52	26	28-33
Face length	32.10 \pm 1.47	29	29-15	31.5 \pm 1.03	26	30-34
Ear length	21.39 \pm 1.13	28	19-23	21.5 \pm 0.81	26	20-23
Tail length	54.21 \pm 7.63	28	39-68	55.56 \pm 9.26	25	37-70
Canon length (FL)	19.93 \pm 0.84	29	18-21	19.96 \pm 0.77	26	19-22
Canon length (HL)	26.10 \pm 1.35	29	23-28	26.4 \pm 1.0	25	24-28
Canon circumference (FL)*	12.04 \pm 0.71	27	11-13	11.57 \pm 0.70	26	10-13
Canon circumference (HL)	12.79 \pm 0.85	19	11-14	12.7 \pm 0.92	20	11-14
Pastern length (FL)	8.66 \pm 0.72	29	7-10	8.62 \pm 0.57	26	8-10
Pastern length (HL)	8.68 \pm 0.56	25	8-10	8.6 \pm 0.65	25	8-10
Pastern circumference (FL)	11.51 \pm 0.85	27	10-13	11.27 \pm 0.78	26	10-13
Pastern circumference (HL)	12.36 \pm 1.21	11	10-15	11.94 \pm 1.61	18	10-15
Hoof length (FL)	5.83 \pm 0.54	29	5-7	5.73 \pm 0.45	26	5-6
Hoof length (HL)	5.53 \pm 0.51	19	5-6	5.45 \pm 0.50	21	5-6
Hoof circumference (FL)*	20.88 \pm 1.01	25	19-23	20.25 \pm 1.03	24	18-22
Hoof circumference (HL)*	20.67 \pm 2.02	12	18-26	19.18 \pm 1.13	17	17-21
Estimated body weight (kg)	75.12 \pm 9.57	31	58.35-99.84	75.69 \pm 9.85	29	54.35-103.11

FL, fore leg; HL, hind leg. *Difference between male and female significant at $P<0.05$.

Table 3. Allele size range, observed (n_o) and effective (n_e) number of alleles, observed (H_o) and expected (H_e) heterozygosity, polymorphism information content (PIC), gene diversity estimates (F_{IS}) and exclusion probabilities (PE) at 12 microsatellite loci in spiti donkeys

Locus	Allele size range	n_o	n_e	H_o	H_e	PIC	F_{IS}^1	PE
COR07	161-172	6	2.63	0.65	0.63	0.62	-0.028	0.348
COR18	79-85	6	4.45	0.72	0.78	0.78	0.050	0.568
COR71	253-271	6	3.74	0.80	0.74	0.73	-0.039	0.521
AHT4	132-157	8	4.74	0.78	0.80	0.79	-0.009	0.595
HTG6	183-201	4	2.93	0.78	0.67	0.66	-0.184*	0.410
HTG7	225-239	10	4.84	0.89	0.80	0.79	-0.116*	0.617
HTG10	78-90	7	3.39	0.87	0.71	0.70	-0.234**	0.501
HTG15	78-87	5	3.49	0.80	0.72	0.71	-0.122	0.477
HMS2	84-103	8	2.89	0.65	0.66	0.65	0.015	0.422
NVHEQ54	132-144	6	2.30	0.48	0.57	0.57	0.148	0.358
VHL20	118-135	4	2.74	0.70	0.64	0.63	-0.087	0.355
VHL209	189-203	6	2.03	0.52	0.51	0.51	0.032	0.300
Mean \pm SD		6.33 \pm 1.72	3.35 \pm 0.94	0.72 \pm 0.13	0.69 \pm 0.09	0.68 \pm 0.09	0.048 \pm 0.11	0.9995 ²

* $P < 0.05$, ** $P < 0.01$. ¹Derived after 1000 iterations, ²Cumulative probabilities.

All pairs of loci were also tested for linkage disequilibrium. Only 12 pairs out of 78 combinations showed significant linkage. However, due to absence of information on their chromosomal location, as heterologous markers were used, nothing can be said conclusively about linkage between these 12 pairs.

The mean genetic diversity estimate (F_{IS}) of only 0.048, calculated after 1000 iterations of the data, indicated only moderate level of inbreeding with 8 loci showing heterozygote excess and 4 loci showing heterozygote deficit. The heterozygote excess values were significant only at 3 loci (HTG6, HTG7 and HTG10), whereas, heterozygote deficit was not significant at any locus (Table 3).

The Spiti donkeys were also evaluated for any recent genetic bottlenecks. In the sign test, the probabilities of 0.289, 0.531 and 0.115 under IAM, TPM and SMM of getting significant number of loci with heterozygosity excess indicated absence of any recent genetic bottlenecks in Spiti donkeys. In standardized differences test, T_2 values of 0.559 and -2.657 under TPM and SMM also indicated absence of any recent genetic bottleneck in this population. Similar results were observed in Wilcoxon rank test, where probabilities for heterozygote excess of 0.271 and 0.916 under TPM and SMM indicated mutation drift equilibrium in Spiti donkeys. Only standardized difference, test under IAM with T_2 value of 2.382 and Wilcoxon rank test under IAM with a probability in tail for heterozygote excess of 0.0034 indicated absence of mutation drift equilibrium. However, all other above mentioned tests and normal 'L' shaped distribution of allelic frequency strongly indicated the absence of any recent genetic bottlenecks in Spiti donkeys. The information generated on this neglected but a useful animal will be helpful in drawing attention of the researchers and planners for developing improvement and conservation strategies for these animals.

ACKNOWLEDGEMENTS

We are thankful to the Animal Husbandry Department of Himachal Pradesh especially the Director for their help in undertaking this study. Mr. P.C. Sharma, Veterinary Pharmacist, State Veterinary Hospital, Shimla and Mr. Subhash Chander, T-4, NBAGR are also gratefully acknowledged for technical assistance in undertaking this survey.

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