RESEARCH NOTE

Genetic characterization of Zanskari breed of horse

RAHUL BEHL[1]*, JYOTSNA BEHL[1], NEELAM GUPTA[1], S. C. GUPTA[1], S. P. S. AHLAWAT[1], MAHESH RAGNEKAR[2] and Z. AHMED[2]

[1]*National Bureau of Animal Genetic Resources, P.O. Box 129, GT By-Pass Road, Karnal 132 001, India*
[2]*Field Research Laboratory, Defence Research and Development Organization, c/o 56 APO, India*

Introduction

India is endowed with some magnificent breeds of horses that have evolved and adapted to different geographical and ecological conditions. Special mention is made of horses of the Zanskar region for their exceptional ability to survive and perform under the harsh climatic conditions of their high-altitude habitat (between 3000 and 5000 metres). These horses, with small, strongly built and compact bodies, are known for their ability to survive on scarce food in the winter snow-bound months and yet have incredible stamina and strength. However, poor management of these animals has led to dilution of their characteristic features to some extent. The ever-increasing road network and mechanization have also led to steep decline in their population and immediate conservation measures are required (Bhat *et al*. 1981; Singhvi 2001; Yadav *et al*. 2001). In a breed improvement or conservation programme genetic evaluation of the concerned breed is a major prerequisite. Microsatellite markers, which are highly polymorphic, dispersed throughout all eukaryotic nuclear genomes, follow codominant inheritance, amplified easily by polymerase chain reaction (PCR), and scored on urea–polyacrylamide gels with a fair degree of reliability, are the markers of choice for evaluation of genetic diversity of a population (Takezaki and Nei 1996; Goldstein and Schlotterer 1999). Increasing numbers of breeds of horse are now evaluated for diversity and differentiation employing microsatellite markers (Bjornstad *et al*. 2000; Canon *et al*. 2000; Kelly *et al*. 2002; Tozaki *et al*. 2003; Aberle *et al*. 2004; Achmann *et al*. 2004; Morais *et al*. 2005; Solis *et al*. 2005; Zabek *et al*. 2005; Glowatzki-Mullis *et al*. 2006). Reports are now also available on some of the Indian horse breeds (Chauhan *et al*. 2004; Gupta *et al*. 2005). The present study was undertaken to genetically evaluate Zanskari horses for genetic diversity and to evaluate whether they have experienced any recent genetic bottlenecks. The Zanskari breed of horse was characterized for within-breed diversity using a set of 25 microsatellite markers, and the results indicate that substantial genetic diversity exists in this breed. 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.

Materials and methods

Blood samples and DNA isolation

Blood samples were collected from 42 genetically unrelated animals of Zanskari breed from its breeding tract in Jammu and Kashmir state. About 10 ml of blood per animal was collected aseptically into EDTA (0.5 mM, pH 8.0) coated Vacutainers. Genomic DNA was isolated by the standard procedure of proteinase K digestion, phenol / chloroform / isoamyl alcohol extraction and absolute ethanol precipitation. The isolated genomic DNA was stored at −20°C and working dilutions were stored at 4°C.

Microsatellite analysis

Genomic DNA was amplified by PCR using a set of 25 horse microsatellite markers (table 1). The PCR protocol was based on Crawford *et al*. (1995). Each 25-μl reaction consisted of DNA (about 100 ng), primers (60 ng each), dNTPs (40 mM each), 10× buffer (10 mM Tris, 50 mM KCl, 0.1% gelatin; pH 8.4, 2.5 μl), $MgCl_2$ (1.5 mM) and *Taq* DNA polymerase (0.75 U). The thermocycling conditions included an initial denaturation at 95°C for 2 min, followed by 30 cycles of 45 s at 95°C, 45 s at annealing temperature (table 1),

*For correspondence. E-mail: behl1969@rediffmail.com.

Keywords. Zanskari horse; microsatellite markers; genetic variability; bottlenecks.

and 45 s at 72°C. A final elongation step was carried out at 72°C for 10 min. The amplified products were resolved by electrophoresis through 7% denaturing urea–polyacrylamide gel and visualized by silver staining. PCR product sizes were

Table 1. Primer sequences, chromosome numbers, and annealing temperatures for 25 microsatellite loci employed for genetic characterization of Zanskari horse.

Locus	Primer sequence	Chromosome number	Annealing temperature (°C)
HTG4	CTATCTCAGTCTTGATTGCAGGAC CTCCCTCCCTCCCTCTGTTCTC	9	58
HTG6	CCTGCTTGGAGGCTGTGATAAGAT GTTCACTGAATGTCAAATTCTGCT	15	58
HTG7	CCTGAAGCAGAACATCCCTCCTTG ATAAAGTGTCTGGGCAGAGCTGCT	4	58
HTG8	CAGGCCGTAGATGACTACCAATGA TTTTCAGAGTTAATTGGTATCACA	9	58
HTG10	CAATTCCCGCCCCACCCCCGGCA TTTTTATTCTGATCTGTCACATTT	21	58
HTG14	CCAGTCTAAGTTTGTTGGCTAGAA CAAAGGTGAGTGATGGATGGAAGC	22	60
HTG15	TCTTGATGGCAGAGCCAGGATTTG AATGTCACCATGCGGCACATGACT	5	55
AHT4	AACCGCCTGAGCAAGGAAGT CCCAGAGAGTTTACCCT	24	60
AHT5	ACGGACACATCCCTGCCTGC GCAGGCTAAGGGGGCTCAGC	8	60
HMS2	ACGGTGGCAACTGCCAAGGAAG CTTGCAGTCGATGTGTATTAAATG	10	58
HMS3	CCAACTCTTGTGCACATAACAAGA CCATCCTCACTTTTTCACTTTGTT	9	60
HMS6	GAAGCTGCCAGTATTCAACCATTG CTCCATCTTGTGAAGTGTAACTCA	4	60
HMS7	CAGGAAACTCATGTTGATACCATC TGTTGTTGAAACATACCTTGACTGT	1	60
VHL20	CAAGTCCTCTTACTTGAAGACTAG AACTCAGGGAGAATCTTCCTCAG	30	60
LEX20	GGAATAGGTGGGGGTCTGTT AGGGTACTAGCCAAGTGACTGC	1	60
NVHE05	CGCATGTGCTTCCCCTCAC CCTCTTTCCACGCAATCACTCTA	20	60
NVHEQ11	GGCCCCACCCACTAAATATCACTG CGGGGTCTTGGAAATTTATGAAGG	19	59
NVHEQ18	GGAGGAGACAGTGGCCCCAGTC GCTGAGCTCTCCCATCCCATCG	10	60
NVHEQ29	GAGATTTTGCCCCAAAGGTTA CTCTTCTTTCTTCCCCAGGTCT	Unknown	60
NVHEQ40	TGGCATCTGAATGGAGAATG GATTATGATGCTACAGGGGAAAG	11	60
NVHEQ100	CCAAAGCAGAACATGTGAAGTT TGGCATAGATGTTAGCTAAGTGA	1	59
NVHEQ21	CCAGAACCTGGACTGAACAGTGTC GAATGTGCTTGATGCAGAAGAAGG	Unknown	60
NVHEQ54	AGATGTCCACCTTCTCGCTG CGGGGCTTTTAGGAGGTAACTA	Unknown	60
UCDQ425	AGCTGCCTCGTTAATTCA CTCATGTCCGCTTGTCTC	28	55
ASB2	CCTTCCTGTAGTTTAAGCTTCTG CACAACTGAGTTCTCTGATAGG	15	60

Table 2. Allele size range, number of alleles (observed n_o, effective n_e), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC) and gene diversity estimates (F_{IS}) at 25 microsatellite loci in Zanskari horse.

Microsatellite locus	Allele size range (bp)	n_o	n_e	H_o	H_e	PIC	F_{IS}
HTG4	131–141	5	3.73	0.64	0.73	0.71	0.121
HTG6	88–100	4	3.56	0.71	0.72	0.70	0.006
HTG7	118–128	5	3.92	0.62	0.74	0.73	0.169
HTG8	178–192	6	5.45	0.74	0.82	0.81	0.096
HTG10	94–110	6	4.02	0.59	0.75	0.74	0.207
HTG14	129–137	5	3.69	0.64	0.73	0.71	0.118
HTG15	128–140	5	4.64	0.57	0.78	0.78	0.271
AHT4	142–164	9	6.97	0.69	0.86	0.85	0.194
AHT5	126–138	6	5.48	0.55	0.82	0.81	0.330
HMS2	222–236	7	6.67	0.59	0.85	0.85	0.300
HMS3	151–163	7	6.03	0.67	0.83	0.83	0.201
HMS6	159–167	5	4.05	0.57	0.75	0.75	0.241
HMS7	168–186	9	7.01	0.67	0.86	0.86	0.222
VHL20	93–107	7	6.29	0.55	0.84	0.84	0.349
LEX20	196–206	5	4.10	0.59	0.76	0.75	0.213
NVHEQ5	151–161	6	5.64	0.59	0.82	0.82	0.277
NVHEQ11	120–130	6	5.01	0.52	0.80	0.80	0.346
NVHEQ18	118–134	7	6.55	0.60	0.85	0.84	0.297
NVHEQ29	91–103	5	4.74	0.57	0.79	0.78	0.276
NVHEQ40	146–154	5	4.54	0.64	0.78	0.77	0.176
NVHEQ100	185–199	6	5.15	0.59	0.81	0.80	0.261
NVHEQ21	151–159	4	3.20	0.48	0.69	0.68	0.307
NVHEQ54	178–186	4	3.07	0.62	0.67	0.65	0.082
UCDEQ425	242–250	5	4.81	0.64	0.79	0.79	0.188
ASB2	89–101	6	5.33	0.62	0.81	0.81	0.238

estimated by comparing electrophoretic mobility with that of DNA size markers and previously known samples. The alleles were scored manually from the silver-stained gel.

Data analysis

The within-breed genetic variation parameters observed and effective number of alleles, observed and expected heterozygosity, and gene diversity (F_{IS}) at each microsatellite locus were calculated using the POPGENE computer program version 1.31 (Yeh *et al*. 1999). The polymorphism information content (PIC) (Botstein *et al*. 1980) at each locus was also calculated. The exact test for linkage disequilibrium between pairs of loci was computed using GENEPOP software (Raymond and Rousset 1995).

The Zanskari horse was also evaluated for any recent genetic bottlenecks by sign test and standardized differences test which test the population for presence of any significant heterozygotic excess under IAM as well as SMM models as described by Cornuet and Luikart (1996) using the Bottleneck computer program (Piry *et al*. 1999). These methods are based on the premise that populations experiencing recent severe reduction in size develop an excess of heterozygosity at selectively neutral loci, relative to heterozygosity expected at mutation–drift equilibrium.

Results and discussion

The values of diversity measures at each locus are given in table 2. Observed number of alleles ranged from 4 (HTG6, NVHEQ21 and NVHEQ54) to 9 (AHT4 and HMS7), with a mean (± s.e.) of 5.80 ± 1.32 alleles per locus. PCR product size range varied from 88–100 at locus HTG6 to 242–250 at locus UCDEQ425. Effective number of alleles ranged from 3.07 (NVHEQ54) to 7.01 (HMS7), with a mean (± s.e.) value of 4.95 ± 1.18. Observed heterozygosity values across the 25 polymorphic marker loci ranged from 0.48 (NVHEQ21) to 0.74 (HTG8), with a mean (± s.e.) of 0.61 ±0.06. Expected heterozygosity varied from 0.67 (NVHEQ54) to 0.86 (AHT4), with mean (± s.e.) of 0.78 ± 0.05. These data are consistent with data from previous studies (Ellegren *et al*. 1992; Guerin *et al*. 1994; Coogle *et al*. 1996; Breen *et al*. 1997; Eggleston-Stott *et al*. 1997; Roed *et al*. 1997, 1998; Canon *et al*. 2000; Tozaki *et al*. 2003; Aberle *et al*. 2004). The allele numbers and heterozygosity levels observed across the studied loci indicate presence of reasonably high level of genetic variability in Zanskari horse. The within-population inbreeding estimate (F_{IS}) of 0.219 ± 0.087 suggests moderate level of inbreeding.

The mean (± s.e.) PIC for all loci assessed from the allele frequency data was 0.78 ± 0.06, ranging from 0.65 for NVHEQ54 to 0.86 for HMS7. All the loci used in this study had PIC values significantly higher than the required value

of 0.6 (Buchanan *et al*. 1993), pointing to the high degree of informativeness of these markers in evaluation of genetic diversity. Since linked loci have diminished usefulness for population-genetic studies, we also tested for linkage disequilibrium between pairs of loci with exact tests. No significant disequilibrium was detected ($P > 0.05$) for pairwise comparisons of loci, including three unmapped loci or for the two loci on chromosomes 4 and 15 or for three loci on chromosome 9, reiterating their suitability for parent exclusion or individual identification purposes.

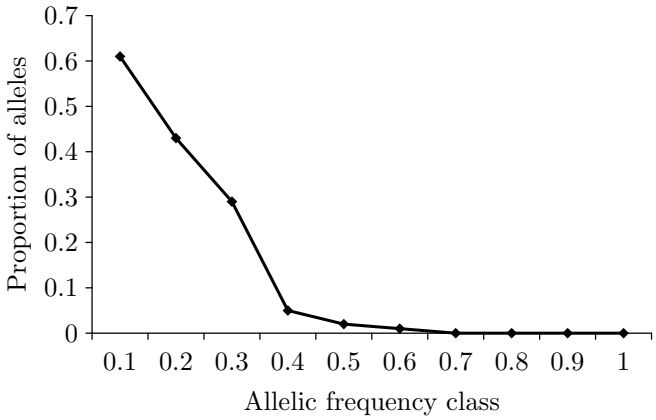

Figure 1. Normal L-shaped curve of distribution of proportion of alleles in different allelic frequency classes.

The observed heterozygosity exceeds the average of the corresponding distribution of heterozygosities expected at equilibrium for about 15 (14.6 and 14.83 under IAM and SMM, respectively) of the 25 loci in our sample of Zanskari horses. The probability ($P = 0.053$) of getting 14.6 loci with heterozygosity excess under IAM, and static T2 value of 1.872 under this model favour the null hypothesis that the population is in mutation–drift equilibrium. The results obtained under SMM showing a probability ($P = 0.092$) of obtaining 14.83 loci with heterozygosity excess and T2 value of 2.973 also favour the null hypothesis of mutation–drift equilibrium. In a normal population in mutation–drift equilibrium, the proportion of alleles with lower frequency is larger than the proportion of alleles with higher frequencies. A normal L-shaped distribution of a plot of allelic frequency class versus proportion of alleles (figure 1) reinforces the result that the Zanskari horse population has not experienced any recent bottleneck. Though the population has drastically decreased in number in recent times (Singhvi 2001; Yadav *et al*. 2001) the population size has not been reduced to such an extent, even in the recent past, as to induce detectable genetic bottlenecks. Moreover, the breeding area of Zanskari horses is spread over a reasonably vast area and the practice of bringing good studs from neighbouring areas helps in maintaining adequate genetic variability even when the population size is small. This is in contrast to the situation for Spiti horses, where the breeding tract is restricted to a small area of the Pin valley and their breeding is also restricted to a very small closed population, which has led to genetic bottlenecks (Behl *et al*. 2005). The results obtained in this study may be helpful to both planners and breeders in planning breeding or conservation strategies for the magnificent Zanskari horse.

References

Aberle K. S., Hamann H., Drogemuller C. and Distl O. 2004 Genetic diversity in German draught horse breeds compared with a group of primitive, riding and wild horses by means of microsatellite DNA markers. *Anim. Genet.* **35**, 270–277.

Achmann R., Curik P., Dovc P., Kavar T., Bodo I., Habe F. *et al*. 2004 Microsatellite diversity, population subdivision and gene flow in Lipizzan horse. *Anim. Genet.* **35**, 385–392.

Behl R., Gupta N., Behl J. and Gupta S. C. 2005 Genetic bottleneck studies on Spiti horses using microsatellite markers. *Centaur* **21**, 40–43.

Bhat P. N., Bhat P. P., Khan B. U., Goswami O. B. and Singh B. 1981 *Animal genetic resources of India,* pp. 87–88. National Dairy Research Institute Press, Karnal.

Bjornstad G., Gunby E. and Roed K. H. 2000 Genetic structure of Norwegian horse breeds. *J. Anim. Breed. Genet.* **117**, 307–317.

Botstein D., White R. L., Skolnick M. and Davis R. W. 1980 Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am. J. Hum.* Genet. **32** 314–331.

Breen M., Lindgren G. and Binns M. M. 1997 Genetical and physical assignment of equine microsatellites — first integration of anchored markers in horse genome mapping. *Mamm. Genome* **8**, 267–273.

Buchanan F. C., Littlejohn R. P., Galloway S. M. and Crawford A. M. 1993 Microsatellites and associated repetitive elements in the sheep genome. *Mamm. Genome* **4**, 258–264.

Canon J., Checa M. L., Carleos C., Vega-Pla J. L., Vallejo M. and Dunner S. 2000 The genetic structure of Spanish Cellic horse breeds inferred from microsatellite data. *Anim. Genet.* **31**, 39–48.

Chauhan M., Gupta A. K. and Dhillon S. 2004 Genetic characterization of Indian Spiti horses. *J. Genet.* **83**, 291–295.

Coogle L., Roid R. and Bailey E. 1996 Equine dinucleotide repeat loci LEX015 – LEX024. *Anim. Genet.* **27**, 217–218.

Cornuet J. M. and Luikart G. 1996 Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001–2014.

Crawford A. M., Dodds K. G., Ede A. J., Pierson C. A., Montgomery G. W., Garmonswa H. G. *et al*. 1995 An autosomal linkage map of sheep genome. *Genetics* **140**, 703–724.

Eggleston-Stott M. L., Valle A. D., Bautista M., Dileanis S., Wictum E. and Bowling A. T. 1997 Nine equine dinucleotide repeats at microsatellite loci UCDEQ136, UCDEQ405, UCDEQ412, UCDEQ425, UCDEQ437, UCDEQ467, UCDEQ487, UCDEQ502 and UCDEQ505. *Anim. Genet.* **28**, 370–383.

Ellegren H., Johansson M., Sandberg K. and Andersson L. 1992 Cloning of highly polymorphic equine microsatellites in horse. *Anim. Genet.* **23**, 133–142.

Glowatzki-Mullis M. L., Muntwyler J., Pfister W., Marti E., Rieder S., Poncet P. A. and Gaillard C. 2006 Genetic diversity among horse populations with a special focus on the Franches-Montagnes breed. *Anim. Genet.* **37**, 33–39.

Goldstein D. B. and Schlotterer C. 1999 *Microsatellites: evolution and applications.* Oxford University Press, Oxford.

Gupta A. K., Chauhan M., Tandon S. N. and Sonia 2005 Genetic

diversity and bottleneck studies in the Marwari horse breed. *J. Genet.* **84**, 295–301.

Guerin G., Bertaud M. and Amigues Y. 1994 Characterization of seven new horse microsatellites: HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 and HMS8. *Anim. Genet.* **25**, 62.

Kelly L., Postiglioni A., De Andres D. F., Vega-Pla J. L., Gagliardi R., Biagetti R. and Franco J. 2002 Genetic characterization of Uruguayan Creole horses and analysis of relationships among horse breeds. *Res. Vet. Sci.* **72**, 69–73.

Morais J., Oom M. M., Malta-Vacas J. and Luis C. 2005 Genetic structure of an endangered Portuguese semiferal pony breed, the Garrano. *Biochem. Genet.* **43**, 347–364.

Piry S., Luikart G. and Cornuet J.-M. 1999 Bottleneck: a computer program for detecting recent reductions in effective population size using allele frequency data (available from http://www.ensam.inra.fr/URLB/Bottleneck). *J. Hered.* **90**, 502–503.

Raymond M. and Rousset F. 1995 Genepop, Version 3.4, Population genetic software for exact tests and ecumenicism (http://biomed.curtin.edu.au/genepop/). *J. Hered.* **86**, 248–249.

Roed K. H., Midthjell L., Bjornstad G. and Olsaker I. 1997 Equine dinucleotide repeat microsatellites at NVHEQ5, NVHEQ7, NVHEQ11, NVHEQ18 and NVHEQ24 loci. *Anim. Genet.* **28**, 381–382.

Roed K. H., Midthjell L. and Bjornstad G. 1998 Eight new equine dinucleotide repeat microsatellites at NVHEQ26, NVHEQ29, NVHEQ31, NVHEQ40, NVHEQ43, NVHEQ90, NVHEQ98 and NVHEQ100 loci. *Anim. Genet.* **29**, 470.

Singhvi N. M. 2001 Conservation and management of equines. *Indian J. Anim. Genet. Breed.* **23**, 292–295.

Solis A., Jugo B. M., Meriaux J. C., Iriondo M., Mazon L. I., Aguirre A. I. *et al*. 2005 Genetic diversity within and among four South European native horse breeds based on microsatellite DNA analysis: implications for conservation. *J. Hered.* **96**, 670–678.

Takezaki N. and Nei M. 1996 Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**, 389–399.

Tozaki T., Takezaki N., Hasegawa T., Ishida N., Kurusawa M., Saitou N. and Mukoyama H. 2003 Microsatellite variation in Japanese and Asian horses and their phylogenetic relationship using a European horse outgroup. *J. Hered.* **94**, 374–380.

Yadav M. P., Ghei G. C. and Tandon S. N. 2001 Equine genetic resources in India and their conservation. *Indian J. Anim. Genet. Breed.* **23**, 296–301.

Yeh F. C., Boyle T., Rongcal Y., Ye Z. and Xian J. M. 1999 POPGENE version 3.31, a Microsoft Windows based freeware for population genetic analysis (http://www.ualberta.ca/~fyeh/). University of Alberta, Edmonton, Canada.

Zabek T., Nogai A., Radko A., Nogaj J. and Slota E. 2005 Genetic variation of Polish endangered Bilgoraj horses and two common horse breeds in microsatellite loci. *J. Appl. Genet.* **46**, 299–305.

Received 23 February 2006