



## Genetic characterization of Ladakhi donkeys using microsatellite markers

RAHUL BEHL<sup>1</sup>, S K NIRANJAN<sup>2</sup>, JYOTSNA BEHL<sup>3</sup>, HIMANI SHARMA<sup>4</sup>, M S TANTIA<sup>5</sup>, REENA ARORA<sup>6</sup>,  
PUNEET RANJAN<sup>7</sup>, VIJAY K BHARATI<sup>8</sup>, M IQBAL<sup>9</sup>, R K VIJH<sup>10</sup> and ARJAVA SHARMA<sup>11</sup>

ICAR-National Bureau of Animal Genetic Resources, Karnal, Haryana 132 001 India

Received: 26 July 2018; Accepted: 27 August 2018

### ABSTRACT

The donkeys of Ladakh region of Jammu and Kashmir, are well adapted to the cold, arid and hypoxic conditions of this region. The genomic DNA, isolated from 25 blood samples collected from these donkeys, were amplified by PCR using FAM and HEX labeled primers and resolved for alleles on automatic DNA sequencer. Total 20 loci of the horse origin were tested and only 13 loci gave scorable results. Rest of the loci either did not amplify well (HMS3, HMS7, ASB17 and COR22) or showed less than four alleles (HMS5, HMS6, HTG4), in the studied population. At the 13 loci included in the final analysis, the PCR product size ranged from 79–85 bp at locus HTG6 to 257–275 bp at locus COR18. The observed number of alleles varied from 4 (HTG15, HTG6, HTG10 and VHL20) to 9 (HTG7 and COR71) with a mean of  $5.92 \pm 1.80$ . The observed heterozygosity ranged from 0.44 (VHL209) to 0.90 (AHT5) with a mean of  $0.76 \pm 0.13$ . The mean genetic diversity estimate ( $F_{IS}$ ) was  $-0.076$ . The cumulative exclusion probability (PE) of these loci was 0.999838 indicating their suitability for parentage testing in these donkeys. The sign test, standardized differences test, the Wilcoxon test using the allelic frequency data under two phased mutation model and sequential mutation model at the studied loci as well as normal 'L' shaped distribution of the allelic frequency indicated the absence of any recent genetic bottleneck in donkeys of Ladakh region. When these donkeys were compared to Spiti donkeys of Himachal Pradesh and Brown type donkeys of Andhra Pradesh on the basis of allelic frequency data at these loci, they showed Nei's minimum genetic distances of 0.115 and 0.165, respectively, from these population.

**Key words:** Diversity, Donkey, Ladakh, Microsatellites

The Ladakh region of Jammu and Kashmir is situated at an altitude ranging from 2300 to 5000 m above sea level with temperature varying from  $-13.5^{\circ}\text{C}$  to  $26.2^{\circ}\text{C}$  (at Leh) with an average annual rainfall of about 10 mm. The donkeys of this region are well adapted to cold, arid and hypoxic environment of this region. The donkeys of Ladakh region in Jammu and Kashmir have light brown to dark brown coat colour with lighter bellies (Fig. 1). The mean height at withers of these animals is  $94.27 \pm 3.75$  and  $93.85 \pm 3.8$  cm in adult males and females, respectively (ICAR-NBAGR Newsletter, 2017). Due to improved road connectivity and mechanization, the donkey population of Ladakh region, as of other parts of the country, has reduced drastically in recent times and now stands at mere 5667 (Livestock census 2012). Immediate attention and conservation measures are required to save these animals, well adapted to extreme environmental conditions with feed and fodder scarcity endemic to this region. In a breed conservation or improvement programme genetic evaluation of the concerned breed is a major pre-requisite.

Present address: <sup>1,6,10</sup>Principal Scientist (behl1969@rediffmail.com, tantiams@gmail.com, rameshkvijh@gmail.com), <sup>11</sup>Director (arjava@yahoo.com), <sup>2</sup>Senior Scientist (sakethniranjan@gmail.com).

The microsatellite markers, which are highly polymorphic, dispersed throughout the eukaryotic genome, follow codominant inheritance, can be easily amplified using polymerase chain reaction and resolved easily, are the markers of choice for evaluation of the genetic diversity of a population (Takezaki and Nei 1996, Goldstein and Shlotterer 1999). Increasing number of donkey populations have been now evaluated for genetic diversity using heterologous microsatellite markers of horse origin (Aranguren-Mendez *et al.* 2001, Jordana *et al.* 2001, Ivankovic *et al.* 2002, Colli *et al.* 2013, Rosenbam *et al.* 2015, Behl *et al.* 2017a and b). The present study was undertaken to evaluate donkeys of Ladakh region for genetic diversity and bottlenecks and compare them for genetic distances with two other donkey populations of India using microsatellite markers.

### MATERIALS AND METHODS

Blood samples (25) were collected from the Leh district of Jammu and Kashmir in EDTA coated vacutainers. 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  $-20^{\circ}\text{C}$  and working



Fig. 1. Male Ladakhi donkey.

dilutions were stored at 4°C.

The genomic DNA was amplified by PCR using heterologous microsatellite markers of horse origin. Each 25 µl reaction consisted of DNA (about 100 ng), primers (7.5 pmol each), dNTPs (200 µM each), 10× buffer (50 mM KCl, 10 mM tris-HCl, 0.1% gelatin), MgCl<sub>2</sub> (1.5 mM) and Taq DNA polymerase (1 unit). The thermocycling conditions included an initial denaturation at 94°C for 2 min followed by 30 cycles of 45 sec at 92°C, 45 sec at annealing temperature (Table 1) and 1 min at 72°C. A final extension step was carried at 72°C for 15 min. The primers were labeled with HEX and FAM to facilitate resolution of alleles on automated DNA sequencer.

The within-breed genetic diversity 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 parentage exclusion (P) at each locus was determined as described by Jamieson and Taylor (1997). The cumulative probability of parentage exclusion (PE) was calculated as

$$PE = 1 - (1-P_1) (1-P_2) (1-P_3) \dots (1-P_K)$$

The exact test for Hardy-Weinberg equilibrium at each locus and linkage disequilibrium between pairs of loci was calculated using GENEPOP software (Raymond and Rousset 1995, Rousset 2008) after applying 100 batches of 1000 iterations to calculate the exact probabilities. The within-breed gene diversity (F<sub>IS</sub>) and its level of significance was computed as described by Weir and Cockerham (1984) after 260 randomizations of the data using FSTAT computer program, version 2.9.3 (Goudet 2001). The data were also analysed for any recent genetic bottlenecks, after 1000 replications of the data, using BOTTLENECK computer program (Piry *et al.* 1999). Nei's minimum (D<sub>N</sub>, Nei 1973) and Reynolds weighted (D<sub>R</sub>, Reynolds *et al.* 1983) genetic distances between pairs of populations were calculated by POPULATIONS computer program (Langella *et al.* 2002).

## RESULTS AND DISCUSSION

The 13 loci used in the final analysis, amplified well, produced unambiguous allele patterns and showed more than four alleles. In all, 20 loci were tested. The loci that either did not amplify well (HMS3, HMS7, ASB17 and COR22) or showed less than four alleles (HMS5, HMS6, HTG4,) were not included in the final analysis.

At the thirteen loci, the PCR product size ranged from 79–85 bp at locus HTG6 to 257–275 bp at locus COR18. The observed number of alleles varied from 4 (HTG15, HTG6, HTG10 and VHL20) to 9 (HTG7 and COR71) with a mean of 5.92±1.80. The effective number of alleles ranged

Table 1. Annealing temperature (°C), PCR product size range (bp), Observed (N<sub>O</sub>) and effective (N<sub>e</sub>) number of alleles, observed (H<sub>O</sub>) and expected (H<sub>e</sub>) heterozygosity, polymorphism information content (PIC), probability of exclusion (PE) and F<sub>IS</sub> at 13 heterologous microsatellite loci in Ladakhi donkeys

| Locus   | Annealing temperature (°C) | Size-range (bp) | N <sub>O</sub> | N <sub>e</sub> | H <sub>O</sub> | H <sub>e</sub> | PIC       | PE                    | F <sub>IS</sub>     |
|---------|----------------------------|-----------------|----------------|----------------|----------------|----------------|-----------|-----------------------|---------------------|
| HTG15   | 58                         | 129–135         | 4              | 2.47           | 0.65           | 0.61           | 0.56      | 0.336                 | -0.075              |
| HTG6    | 58                         | 79–85           | 4              | 2.95           | 0.67           | 0.67           | 0.64      | 0.414                 | 0.012               |
| AHT5    | 59                         | 120–158         | 7              | 4.15           | 0.90           | 0.78           | 0.75      | 0.557                 | -0.161              |
| HTG7    | 59                         | 132–154         | 9              | 4.43           | 0.79           | 0.80           | 0.77      | 0.591                 | 0.007               |
| HTG10   | 58                         | 93–101          | 4              | 2.90           | 0.86           | 0.68           | 0.60      | 0.384                 | -0.273              |
| HMS2    | 58                         | 227–243         | 6              | 5.36           | 0.87           | 0.84           | 0.80      | 0.627                 | -0.031              |
| AHT4    | 58                         | 136–157         | 7              | 4.74           | 0.87           | 0.81           | 0.78      | 0.598                 | -0.080              |
| NVHEQ54 | 61                         | 184–203         | 6              | 4.19           | 0.82           | 0.78           | 0.75      | 0.548                 | -0.051              |
| COR18   | 56                         | 257–275         | 7              | 4.48           | 0.83           | 0.79           | 0.76      | 0.572                 | -0.041              |
| VHL209  | 55                         | 78–88           | 5              | 1.60           | 0.44           | 0.38           | 0.36      | 0.216                 | -0.148              |
| COR7    | 59                         | 161–171         | 5              | 3.62           | 0.68           | 0.74           | 0.69      | 0.478                 | 0.081               |
| COR71   | 59                         | 164–205         | 9              | 3.50           | 0.88           | 0.73           | 0.70      | 0.516                 | -0.212 <sup>#</sup> |
| VHL20   | 58                         | 79–95           | 4              | 2.66           | 0.68           | 0.64           | 0.56      | 0.351                 | -0.068              |
| Mean    |                            |                 | 5.92±1.80      | 3.62±1.06      | 0.76±0.13      | 0.71±0.12      | 0.67±0.12 | 0.999838 <sup>§</sup> | -0.076              |

\*Locus deviated significantly from Hardy Weinberg equilibrium (P<0.05); <sup>§</sup>Cumulative PE; <sup>#</sup>Significant excess of heterozygotes (P<0.05).

from 1.60 (VHL209) to 5.36 (HMS2) with a mean of  $3.62 \pm 1.06$ . The observed heterozygosity ranged from 0.44 (VHL209) to 0.90 (AHT5) with a mean of  $0.76 \pm 0.13$ . The expected heterozygosity ranged between 0.38 (VHL209) to 0.84 (HMS2) with a mean of  $0.71 \pm 0.12$  (Table 1).

The mean PIC for all loci assessed from allele frequency data was  $0.67 \pm 0.12$ , ranging from 0.36 (VHL209) to 0.80 (HMS2) indicating their suitability for genetic variability studies in Indian donkey breeds. The cumulative PE of 0.999838 at these 13 loci indicated the suitability of this set of 13 loci for parent exclusion studies in these donkeys.

In exact test for Hardy-Weinberg disequilibrium at each locus, only one loci (NVHEQ54) deviated significantly ( $P < 0.05$ ) from Hardy-Weinberg equilibrium. All possible pairs of loci were also tested for linkage disequilibrium with only 3 pairs (HTG6 and HTG4, HTG7 and VHL209, HTG10 and VHL20) out of 78 pairs showed significant ( $P < 0.05$ ) linkage disequilibrium. However, nothing can be said conclusively about these 3 pairs because loci being heterologous their chromosomal locations were not known.

The  $F_{IS}$  estimates for individual loci revealed that although 10 out of the studied 13 loci showed heterozygosity excess but at only one loci (COR71) heterozygosity excess was significant ( $P < 0.05$ ) (Table 1). The overall  $F_{IS}$  values of  $-0.076$  were also not significantly different from zero.

These donkeys were also evaluated for any recent genetic bottlenecks. In the sign test which tests the probability of getting significant number of loci with heterozygote excess, the probability values of 0.142 and 0.563 under two phased mutation model (TPM) and step-wise mutation model (SMM), respectively, indicated absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh (Cornuet and Luikart 1996). In standardized differences test,  $T_2$  values of 0.943 and  $-1.561$  under TPM and SMM also indicated absence of any recent genetic bottleneck in Brown type donkeys of Andhra Pradesh. The Wilcoxon test also showed absence of any recent genetic bottlenecks with probability values of 0.137 and 0.420 for heterozygosity excess under TPM and SMM. Although the probability values (0.051) under sign test for getting significant number of loci with heterozygote excess,  $T_2$  values (2.507) of standardized differentiation test and probability values (0.013) of heterozygote excess under infinite allele model (IAM) pointed towards recent genetic bottleneck but the IAM is not considered to be the appropriate model for evaluating bottlenecks employing microsatellites. Further, the normal 'L' shaped (Fig. 2) curve of allelic frequency distribution in mode shift test (qualitative test) also pointed towards absence of any recent genetic bottleneck in Ladakhi donkeys. Though, the population of Ladakhi donkeys (5667, Livestock Census, 2012) along with overall population of donkeys of Jammu and Kashmir has decreased drastically in recent times, the open mating practice and mixing of these donkeys in pastures during summer months may have contributed to the absence of any genetic bottleneck in these donkeys. The genetic bottlenecks in a population are generally detected

Table 2. Nei's minimum genetic distances ( $D_N$ , above diagonal) and Reynolds weighted genetic distances ( $D_R$ , below diagonal) between three Indian donkey populations using 13 heterologous microsatellite loci

| Donkey population | Ladakhi | Spiti | APB <sup>§</sup> |
|-------------------|---------|-------|------------------|
| Ladakhi           | –       | 0.115 | 0.165            |
| Spiti             | 0.139   | –     | 0.218            |
| APB               | 0.194   | 0.261 | –                |

<sup>§</sup>Brown type donkeys of Andhra Pradesh

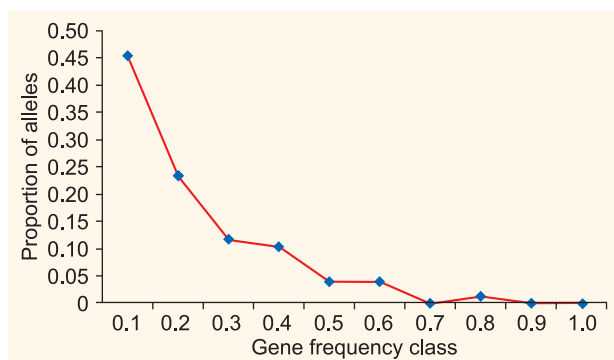


Fig. 2. Normal 'L' shaped plot of allelic frequency class with proportion of alleles showing absence of any recent genetic bottlenecks in Ladakhi donkeys.

when the effective population size is reduced to 20–30 individuals only (Luikart *et al.* 1998).

Based on the allele frequency data at 11 common loci, these donkeys were also compared with Spiti donkeys of Himachal Pradesh and Brown type donkeys of Andhra Pradesh. Ladakhi donkeys showed least  $D_N$  and  $D_R$  of 0.115 and 0.139 from Spiti donkeys (Table 2). Both these donkey types are reared in similar environmental conditions and their distribution area is near to each other/ geographically contiguous. Spiti and Brown type donkeys of Andhra Pradesh were most distant populations with  $D_N$  and  $D_R$  genetic distances of 0.218 and 0.261.

#### ACKNOWLEDGEMENTS

We gratefully acknowledge the kind help received from the Director and staff of Animal Husbandry Department of Jammu and Kashmir. Help received from Mr. Rakesh Kumar of NBAGR is also gratefully acknowledged.

#### REFERENCES

- Aranguren-Mendez J, Jordana J and Gomez M. 2001. Genetic diversity in Spanish donkey breeds using microsatellite DNA markers. *Genetics Selection Evolution* **33**: 433–42.
- Behl R, Niranjan S K, Behl J, Tantia M S, Arora R, Dharma Rao M V, Reddy P P, Vijn R K and Sharma A. 2017a. Genetic characterization of Brown type donkeys of Andhra Pradesh. *Indian Journal of Animal Sciences* **87**: 1102–05.
- Behl R, Sadana D K, Behl J, Banerjee P, Joshi J, Vijn R K, Attri P N, Nadda S and Joshi B K. 2017b. Characterization and microsatellite analysis for genetic diversity and bottlenecks of Spiti donkeys. *Indian Journal of Animal Sciences* **87**: 1221–25.

- Botstein D, White R L, Skolnick M and Davies R W. 1980. Construction of a genetic linkage map in man using restriction length polymorphism. *American Journal of Human Genetics* **32**: 314–31.
- Colli, Perrotta G, Negrini R, Bomba L, Bigi D, Zambonelli P, Verini S A, Liotta L and Ajmore-Marron P. 2013. Detecting population structure and recent demographic history in endangered livestock breeds: the case of the Italian autochthonous donkeys. *Animal Genetics* **44**: 69–78.
- Cornuet J M and Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allelic frequency data. *Genetics* **144**: 2001–14.
- Goldstein D B and Schlotterer C. 1999. *Microsatellites: evolution and applications*. Oxford University Press, Oxford.
- Goudet J. 2001. Fstat, version 2.9.3. A program to estimate and test gene diversities and fixation indices (<http://www.unil.ch/popgen/softwares/fstat.html>). Lausanne University, Lausanne, Switzerland.
- ICAR-NBAGR Newsletter. 2017. Characterization of Ladakhi donkeys. *ICAR-NBAGR Newsletter* **14**(1): 2.
- Ivankovic A, Kavar T, Caput P, Mioc B, Pavic V and Dovc P. 2002. Genetic diversity of three donkey populations in the Croatian coastal region. *Animal Genetics* **33**: 169–77.
- Jamieson A and Taylor C S. 1997. Comparison of three probability formulae for parentage exclusion. *Animal Genetics* **28**: 397–400.
- Jordana J, Folch P and Aranguren J A. 2001. Microsatellite analysis of genetic diversity in Catalonian donkey breeds. *Journal of Animal Breeding and Genetics* **118**: 57–63.
- Langella O. 2002. POPULATIONS, version 1.2.31. Available from <http://www.pge.cnrs-gif.fr/bioinformatics/populations/>
- Livestock Census. 2012. 19<sup>th</sup> Livestock Census. Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India.
- Luikart G, Allendorf F W, Cornuet J M and Sherwin W B. 1998. Distortion of allele frequency distribution provides a test for recent population bottlenecks. *Journal of Heredity* **89**: 238–47.
- Nei M. 1973. The theory and estimation of genetic distance. *Genetic Structure of Populations*. (Ed.) Morton N E. University Press of Hawaii, Honolulu. Pp 45–54.
- 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 (<http://www1.montpellier.inra.fr/CBGP/softwares/Bottleneck>). *Journal of Heredity* **90**: 502–03.
- Raymond M and Rousset F. 1995. Genepop, version 3.4. Population genetic software for exact tests and ecumenicism (<http://biomed.curtin.edu.au/genepop>). *Journal of Heredity* **86**: 248–49.
- Reynolds J, Weir B S and Cockerham C C. 1983. Estimation of the coancestry coefficients: basis for a short term genetic distance. *Genetics* **105**: 769–79.
- Rosenbam S, Costa V, Al-Araimi N, Kefena E, Abdel-Moniem A S, Abdalla M A, Bakhiet A and Pereira-Beja A. 2015. Genetic diversity of donkey populations from putative centers of domestication. *Animal Genetics* **46**: 30–36.
- Rousset F. 2008. Genepop, 007: a complete reimplementation of the Genepop software for Windows and Linux (<http://genepop.curtin.edu.au>). *Molecular Ecology Resources* **8**: 103–06.
- Takezaki N and Nei M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**: 389–99.
- Weir B S and Cockerham C C. 1984. Estimating F statistics for analysis of population structure. *Evolution* **38**: 1358–70.
- 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 (<https://sites.ualberta.ca/~fyeh/popgene.html>).