Asian-Aust. J. Anim. Sci.
Vol. 20, No. 1 : 25 - 30
January 2007

www.ajas.info

Individual Identification and Breed Allocation with Microsatellite Markers: An Evaluation in Indian Horses

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ABSTRACT : The capability of microsatellite markers for individual identification and their potential for breed assignment of individuals was evaluated in two Indian horse breeds. The strength of these individual assignment methods was also evaluated by increasing the number of loci in increments of five. The probability of identity of two random horses from the two breeds at all twenty five studied loci was as low as 1.08×10^{-32} showing their suitability to distinguish between individual horses and their products. In the phylogenetic approach for individual assignment using Nei's genetic distances, 10.81% of horses associated with breed other than the major cluster of the source breed horses when all twenty five microsatellite loci were implemented. Similar results were obtained when the maximum likelihood approach for individual assignment was used. Based on these results it is proposed that, although microsatellite markers may prove very useful for individual identification, their utility for breed assignment of horses needs further evaluation. (**Key Words :** Individual Identification, Breed Assignment, Microsatellite Markers, Indian Horses)

INTRODUCTION

Due to their highly polymorphic nature microsatellite DNA markers, have revealed remarkable capacity for utilization in the analysis of phylogenetic relationships amongst populations in different species including horses (Bjornstad et al., 2000; Canon et al., 2000; Tozaki et al., 2003; Aberle et al., 2004) and their usefulness in kinship analysis/parentage testing is well proven (Marklund et al., 1994; Bowling et al., 1997; Tozaki et al., 2001; Cho and Cho, 2004). Recently, their utility has been speculated in breed assignment of a single animal using individual genotype information (Bjornstad and Roed, 2001; 2002).

A test for discrimination among individual animals and their assignment to a breed is essential for effective and accurate selection/management of livestock breeds and the authentication of the quality and the origin of livestock products. However, effectiveness of such individual specific demarcation procedures will be affected by several factors such as genetic differentiation between the breeds in question, degree of reproductive isolation etc. (Cornuet et al., 1999). The present study was undertaken to evaluate microsatellite markers for individual identification and to assess their potential for breed allocation of individual animals using both a phylogenetic approach and a method based on maximum likelihood estimates in Indian horses. In India six breeds of horses are described falling into two main groups; one group represented by the ponies of the Himalayan region, namely Zanskari, Spiti, Bhutia and Manipuri, and the other group represented by breeds adapted to hot arid regions like Marwari and Kathiawari (Bhat et al., 1981). Since, the breeds of the two groups are quite similar in characteristics (Bhat et al., 1981; Pundir, 2001; Singh et al., 2002; Katoch et al., 2004); Spiti and Marwari horses were chosen as the test breeds representing the Himalayan ponies and the desert type horses, respectively.

MATERIALS AND METHODS

Samples

Forty-two blood samples from Marwari horses and thirty-two blood samples from Spiti horses were collected from their breeding tracts in the Indian provinces of Himachal Pradesh and Rajsthan, respectively. Care was taken to collect blood samples from unrelated horses conforming to the respective breed characteristics. Genomic

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Received February 13, 2006, Accepted July 13, 2006

Table 1. Primer sequences, annealing temperatures, PCR product size range and observed number of alleles of 25 microsatellite loci used in the study

Microsatellite locus	Primer sequence	Annealing temp. (°C)	PCR product size range (bp)	Observed number of alleles
HTG4	CTATCTCAGTCTTGATTGCAGGAC CTCCCTCCCTCCCTCTGTTCTC	58	131-139	5
HTG 6	CCTGCTTGGAGGCTGTGATAAAGAT GTTCACTGAATGTCAAATTCTGCT	58	84-100	5
HTG 7	CCTGAAGCAGAACATCCCTCCTTG ATAAAGTGTCTGGGCAGAGCTGCT	58	116-126	5
HTG 8	CAGGCCGTAGATGACTACCAATGA TTTTCAGAGTTAATTGGTATCACA	58	176-192	8
HTG 10	CAATTCCCGCCCCACCCCCGGCA TTTTTATTCTGATCCTGTCACATTT	58	94-114	6
HTG 14	CCAGTCTAAGTTTGTTGGCTAGAA CAAAGGTGAGTGATGGATGGAAGC	60	127-139	6
HTG 15	TCTTGATGGCAGAGCCAGGATTTG AATGTCACCATGCGGCACATGACT	55	128-146	7
AHT4	AACCGCCTGAGCAAGGAAGT CCCAGAGAGTTTACCCT	60	142-164	9
AHT5	ACGGACACATCCCTGCCTGC GCAGGCTAAGGGGGCTCAGC	60	126-138	5
HMS2	ACGGTGGCAACTGCCAAGGAAG CTTGCAGTCCGAATGTGTATTAAATG	58	218-236	9
HMS3	CCAACTCTTGTGCACATAACAAGA CCATCCTCACTTTTTCACTTTGTT	60	151-159	8
HMS6	GAAGCTGCCAGTATTCAACCATTG CTCCATCTTGTGAAGTGTAACTCA	60	159-167	4
HMS7	CAGGAAACTCATGTTGATACCATC TGTTGTTGAAACATACCTTGACTGT	60	168-186	8
VHL20	CAAGTCCTCTTACTTGAAGACTAG AACTCAGGGAGAATCTTCCTCAG	60	93-109	8
LEX20	GGAATAGGTGGGGGTCTGTT AGGGTACTAGCCAAGTGACTGC	60	196-208	6
NVHEQ5	CGCATGTGCTTCCCCTCAC CCTCTTTCCACGCAATCACTCTA	60	149-161	7
NVHEQ11	GGCCCCACCCACTAAATATCACTG CGGGGTCTTGGAAATTTATGAAGG	59	120-130	6
NVHEQ18	GGAGGAGACAGTGGCCCCAGTC GCTGAGCTCTCCCATCCCATCG	60	118-134	8
NVHEQ29	GAGATTTTGCCCCAAAGGTTA CTCTTCTTTCTTCCCCAGGTCT	60	91-103	7
NVHEQ40	TGGCATCTGAATGGAGAATG GATTATGATGCTACAGGGGAAAG	60	146-156	5
NVHEQ100	CCAAAGCAGAACATGTGAAGTT TGGCATAGATGTTAGCTAAGTGA	59	185-203	8
NVHEQ21	CCAGAACCTGGACTGAACAGTGTC GAATGTGCTTGATGCAGAAGAAGG	60	151-161	6
NVHEQ54	AGATGTCCACCTTCTCGCTG CGGGGCTTTTAGGAGGTAACTA	60	178-186	4
UCDEQ425	AGCTGCCTCGTTAATTCA CTCATGTCCGCTTGTCTC	55	238-250	7
ASB2	CCTTCCTGTAGTTTAAGCTTCTG CACAACTGAGTTCTCTGATAGG	60	89-105	7

DNA was isolated by the standard phenol/chloroform procedure and DNA samples were stored at -20°C and/or at 4°C.

Polymerase chain reaction

The genomic DNA was amplified by polymerase chain reaction using twenty-five microsatellite primers (Table 1) as described in Behl et al. (2002). Amplified DNA

Table 2. Probability of identity of two individuals chosen at random from within a breed (G1) and from different breeds (G2) at twenty five microsatellite loci in two Indian horse breeds

Microsatellite loci	G1		G2
	Marwari	Spiti	
HTG4	0.161	0.125	0.090
HTG 6	0.171	0.121	0.097
HTG 7	0.126	0.111	0.045
HTG 8	0.052	0.058	0.033
HTG 10	0.123	0.106	0.106
HTG 14	0.164	0.152	0.063
HTG 15	0.049	0.054	0.046
AHT4	0.043	0.055	0.037
AHT5	0.128	0.082	0.086
HMS2	0.045	0.056	0.040
HMS3	0.048	0.067	0.045
HMS6	0.112	0.144	0.098
HMS7	0.053	0.039	0.027
VHL20	0.062	0.059	0.061
LEX20	0.121	0.063	0.044
NVHEQ5	0.092	0.076	0.042
NVHEQ11	0.060	0.069	0.053
NVHEQ18	0.102	0.042	0.027
NVHEQ29	0.073	0.077	0.021
NVHEQ40	0.088	0.098	0.098
NVHEQ100	0.038	0.063	0.036
NVHEQ21	0.105	0.159	0.073
NVHEQ54	0.173	0.161	0.065
UCDEQ425	0.067	0.086	0.063
ASB2	0.047	0.078	0.050
Total	7.42×10^{-28}	5.38×10^{-28}	1.08×10^{-32}

fragments were analysed on 7% polyacrylamide gel and detected by silver staining (Yang et al., 1999). Alleles were scored manually against standard DNA size markers which were run alongside the samples in each gel. To maintain homogeneity in allele scoring, three reference samples from Marwari horses were also analyzed along with the Spiti ponies.

Statistical analysis

Allele frequencies for each locus were calculated with 2n = 64 and 84 for Spiti and Marwari horses, respectively. The genetic diversity between the breeds was calculated according to Nei (1978) using POPGENE computer package (Yeh et al., 1999). The shared allele distances between populations were calculated by the method described by Jin and Chakraborty (1994). In this method, the average proportion of shared alleles between populations is computed over all possible combinations of individuals sampled. The probability of identity of two individuals chosen at random within a breed (G1) is given by:

$$G1 = \prod_{i=1}^{r}\left[\sum_{j=1}^{n_i} q_{ij}^{4} + 4\sum\sum q_{ij}^{3}.q_{ij}^{2}\right]$$

with q_{ij} being the frequency of the j[th] allele and i[th] locus in a population.

The probability of identity of two individual horses belonging to two breeds was calculated as follows:

$$G2 = \prod_{i=1}^{r}\left[\sum_{j=1}^{n_i} q_{ij}^{2}.q'^{2}_{ij} + 4\sum\sum q_{ij}.q'_{ij}.q_{ik}.q'_{ik}\right]$$

where, q and q' being the frequencies of corresponding alleles.

The neighbour-joining tree was generated from the genetic distance matrix using PHYLIP 3.6b (Felsenstein, 2004) with individuals as operational taxonomic units. The phylogenetic tree was visualized using TreeView (Page, 1996). The number of animals allocated to correct breed cluster was used to assess the assignment success.

The phylogenetic procedure for resolving the breed of origin outlined above does not predict the certainty of breed assignment of a particular individual. By using the allele frequency distribution of the multilocus genotype data and implementing a maximum likelihood approach, breed allocation of individuals and certainty of these allocations were estimated by WHICHRUN 4.1 (Banks and Eichert, 2001). Incorporating jack-knife iterations, this procedure samples individuals one at a time and recalculates the allele frequency in the absence of each genotype before determining the most likely source population of the particular individual. To resolve the stringency of an allocation WHICHRUN utilizes the log of odds (LOD) ratio for the two most likely source populations. The breed allocation was restricted to apply only for the assignments that had a LOD ratio of at least two, to reduce the chance of an error to 1/100 or less.

The strength of these individual assignment methods was also investigated by increasing the number of implemented loci randomly in increments of five. The strength of the twelve loci incorporated in PE Applied Biosystems Stockmark System (Foster City, CA, USA) was also examined.

RESULTS AND DISCUSSION

The PCR product size range and total number of alleles observed at all twenty five loci used in the study are given in Table 1. The allelic frequency data can be obtained from the authors. The probability of identity of two individual animals (G1), taking in to consideration all the twenty-five loci, was 7.42×10^{-28} for Marwari horses and 5.38×10^{-28} for Spiti horses. The probability of identity of randomly picked individuals (G2) from different breeds at these loci was 1.08×10^{-32}. The G1 values were quite low at 8.77×10^{-14} and 7.78×10^{-14}, even with the twelve microsatellite loci PE

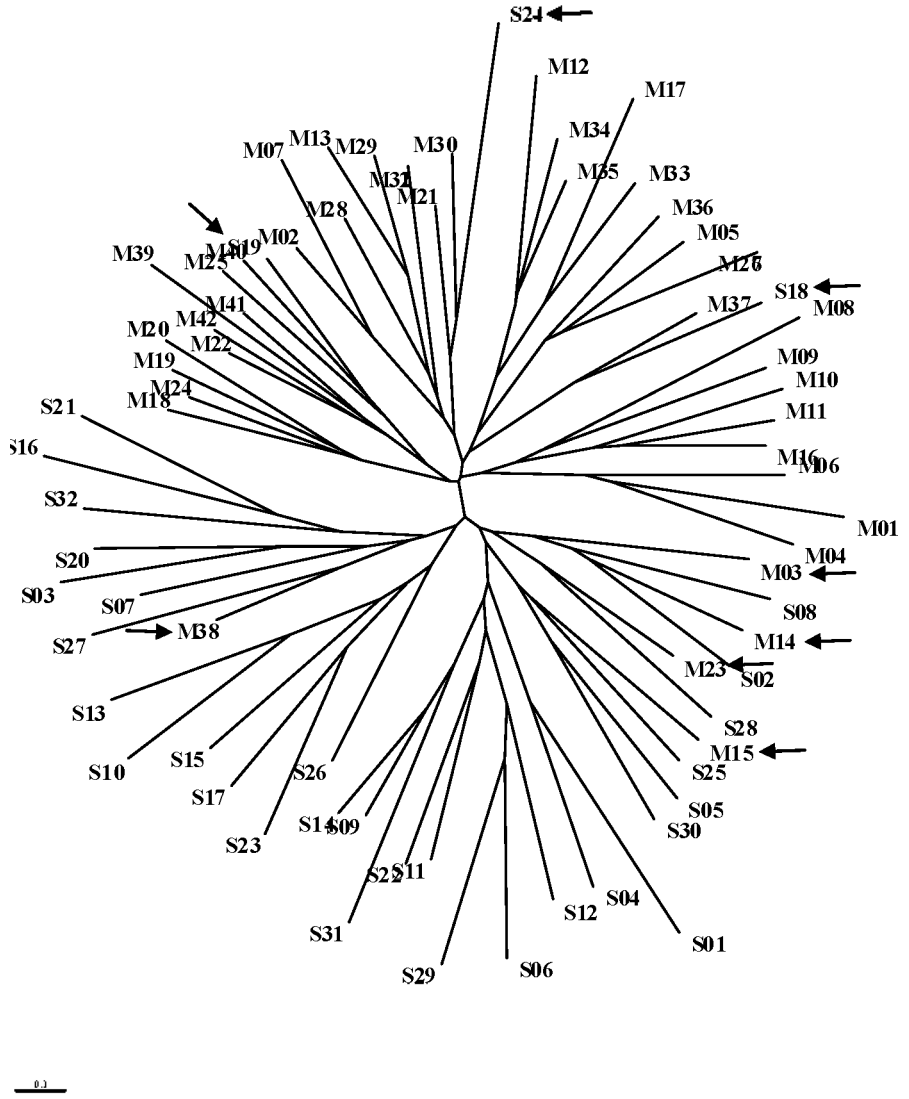

Figure 1. Radial presentation of the neighbour joining dendrogram generated by PHYLIP and viewed by TREEVIEW using allele frequency data of seventy four native Indian horses of Spiti (S, n = 32) and Marwari (M, n = 42) breeds.

Biosystems Stockmark kit. for Marwari and Spiti horses, respectively. The G2 value at these loci was 1.92×10^{-15} (Table 2). These values showed their suitability to distinguish between individual horses belonging to either two different or even the same breed.

Besides distinguishing between individuals in breeding/ conservation programmes. the breed allocation of the individuals is equally important to discriminate between pure breds and breed crosses for skillful breed management. Moreover, great importance is attached to the breed of a horse in terms of its market value. If a method could be developed for authentication of breed it would be of great help to horse breeders. Bjornstad and Roed (2001), based on their study in Norwegian horses. have proposed that microsatellite markers could be potential candidates for such analysis. Since, horse breed societies in India generally do not maintain the breed registries such a method is greatly required. To address this issue. the potential of microsatellite markers to allocate an individual horse to a particular breed was evaluated.

In the phylogenetic approach with Nei's distances for individual assignment. of the seventy four horses. eight (10.81%) associated with a breed other than the major cluster of the source breed individuals when all twenty five microsatellites were taken into consideration (Figure 1). To evaluate how assignment precision was influenced by number of implemented loci, their number was increased in increments of five randomly (Figure 2). An error rate of about 25 percent was observed when the twelve microsatellites used in PE Biosystems Stockmark Kit were implemented.

Similar results were also obtained when a maximum likelihood approach for individual assignment was used. About 50% horses were allocated to wrong breed when the

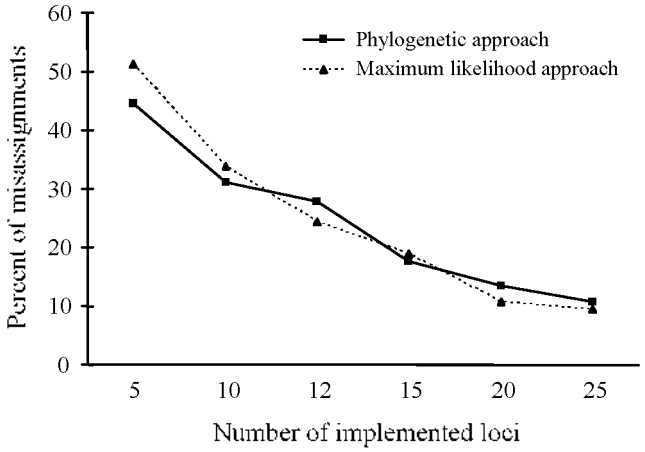

Figure 2. Percent of misassingned animals of two Indian horse breeds namely, Spiti (S) and Marwari (M) after breed allocation with a phylogenetic approach using Nei's distances and maximum likelihood analysis having a stringency of LOD scores of greater than two. The number of implemented loci was increased in increments of five in the given order and twelve loci used in the PE Biosystems Stockmark Kit are shown in italics (*HTG4*, *HTG6*, *HTG7*, HTG8, *HTG10*, HTG14, HTG15, *AHT4*, *AHT5*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *VHL20*, LEX20, NVHEQ5, NVHEQ11, NVHEQ18, NVHEQ29, NVHEQ40, NVHEQ100, NVHEQ21, NVHEQ54, UCDEQ425, *ASB2*).

five microsatellites were implemented with a desirable stringency of LOD score of more than two. By restricting breed allocation to a LOD ratio of at least two, a particular assignment will have a 1/100 chance of error or less. Even when all 25 loci were implemented an error rate of 9.46% was observed with the same stringency. In our study a slightly lower precision rate for breed allocation was observed than the rate observed by Bjornstad and Roed (2001), wherein, the phylogenetic approach and the analysis based on the likelihood estimates (LOD>2) allocated 95 and 96% horses to the correct breed, respectively.

The two breeds chosen for this study i.e. Spiti and Marwari inhabit the high altitude cold regions of Himachal Pradesh and the desert area of Rajsthan, respectively. Since a horse adapted to the hot and arid environment is unlikely to survive and reproduce in the intensely cold weather conditions and *vice versa*, it can be ruled out that the results observed in this study were affected by intermixing of breeds due to migration. However, lower genetic distance values have been observed amongst a large number of horse breeds of the world (Bjornstad et al., 2000; Canon et al., 2000; Kelly et al., 2002; Bjornstad et al., 2003) indicating lower demarcation amongst the horse breeds as such compared to the breeds of other livestock species like cattle or sheep. This can be attributed to the fact that the horse was domesticated several thousand years later than cattle or sheep (Bokonyi, 1996). The Nei's standard genetic distance and shared allele distance between the Spiti and Marwari horses was found to be 0.22 and 0.15. Since, the genetic differentiation between populations is likely to influence the capacity to assign individuals to their source populations (Cornuet et al., 1999), the higher proportion of misallocated horses in our study compared to other livestock breeds (Buchanan et al., 1994; MacHugh et al., 1998; Blott et al., 1999) can be expected. In conclusion, it can be said that though the microsatellite markers may prove to be very useful markers for individual identification, however, their utility for breed assignment of horses needs to be further evaluated.

ACKNOWLEDGEMENTS

We gratefully acknowledge the valuable help received from the following persons/agencies in obtaining samples. i) Dr. Gurmej Singh, then Incharge, Network Project, NBAGR, Karnal (Haryana); ii) Dr. Sanjeet Katoch, Associate Professor, Department of Animal Breeding, Genetics and Biostatistics, College of Veterinary and Animal Sciences, Palampur (Himachal Pradesh); iii) Col. Umaid Singh and Mr. Gajendrapal Singh Posana of Marwari Horse Society, Jodhpur and iv) Marwar Horse Breeding and Research Institute, Jodhpur (Rajsthan).

REFERENCES

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

Banks, M. A. and W. Eichert. 2001. WHICHRUN, version 4.1, a computer programme for population assignment based on multilocus genotype data. Bodega Marine Laboratory, University of California at Davies, Bodega Bay, CA, USA.

Behl, R., R. Kaul, N. Sheoran, J. Behl, M. S. Tantia and R. K. Vijh. 2002. Genetic identity of two Indian pig types using microsatellite markers. Anim. Genet. 33:158-159.

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

Bjornstad, G. and K. H. Roed. 2001. Breed demarcation and potential for breed allocation of horses assessed by microsatellite markers. Anim. Genet. 32:59-65.

Bjornstad, G. and K. H. Roed. 2002. Evaluation of factors affecting breed assignment precision using microsatellite data from horse breeds and simulated breed crosses. Anim. Genet. 33:264-270.

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

Bjornstad, G., N. O. Nilsen and K. H. Roed. 2003. Genetic relationship between Mongolian and Norwegian horses? Anim. Genet. 34:55-58.

Blott, S. C., J. L. Williams and C. S. Haley. 1999. discriminating

among cattle breeds using genetic markers. Heredity. 82:613-619.

Bokonyi, S. 1984. Horse. In: Evolution of Domesticated Animals (Ed. I. L. Mason). Longman, London. pp. 162-173.

Bowling, A. T., M. L. Eggleston-Stott, G. Byrns, R. S. Clark, S. Dileanis and E. Wictum. 1997. Validation of microsatellite markers for routine horse parentage testing. Anim. Genet. 28:247-252.

Buchanan, F. C., L. J. Adams, R. P. Littlejohn, J. F. Maddox and A. M. Crawford. 1994. Determination of evolutionary relationships among sheep breeds using microsatellites. Genomics. 22:397-403.

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

Cho, G. J. and B. W. Cho. 2004. Microsatellite DNA typing using 16 markers for parentage verification of the Korean native horse. Asian-Aust. J. Anim. Sci. 17:750-754.

Cornuet, J.-M. S. Piry, G. Luikart, A. Estoup and M. Solignac. 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genet. 153: 1989-2009.

Felsenstein, J. 2004. PHYLIP, version 3.6b, phylogeny inference package. Department of Genome science, University of Washington, Seattle.

Jin, L. and R. Chakraborty. 1994. Estimation of genetic distance and coefficient of gene diversity from single-probe multilocus DNA fingerprinting data. Mol. Biol. Evol. 11:120-127.

Katoch, S. P. K. Dogra, Y. P. Thakur and K. Gupta. 2004. Phenotypic characterization of Spiti horse in its breeding tract -body measurements. Centaur. 20:45-48.

MacHugh, D. E., R. T. Loftus, P. Cunningham and D. G. Bradley. 1998. Genetic structure of seven cattle breeds assessed using 20 microsatellite markers. Anim. Genet. 29:333-340.

Marklund, S., H. Ellegren, S. Erikson, K. Sandberg and L. Anderson. 1994. Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. Anim. Genet. 25:19-23.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genet. 89:583-590.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Appl. Biosci. 12:357-358.

Pundir, R. K. 2001. Physical and morphological characteristics of Spiti horses. Indian J. Anim. Sci. 71:381-382.

Singh, M. K., M. P. Yadav and N. T. .Mehta. 2002. Breed characteristics of Marwari and Kathiawari horses. Indian J. Anim. Sci. 72:319-323.

Tozaki, T., H. Kokoi, S. Mashima, K. I. Hirota, T. Hasegawa, N. Ishida, N. Miura, N. H. Choi-Miura and M. Tomita. 2001. Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. J. Vet. Med. Sci. 63:1191-1197.

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

Yang, L., S. H. Zhao, K. Li, Z. Z. Peng and G. W. Montgomery. 1999. Detemination of relationship among five indigenous Chinese goat breeds with six microsatellite markers. Anim. Genet. 30:452-455.

Yeh, F. C., R. Yang and T. Boyle. 1999. POPGENE, version 1.31, a microsoft windows based freeware for population genetics analysis. University of Alberta, Alberta, Canada.