

Short tandem repeat based analysis of genetic variability in Munjal—the threatened sheep population of Northwestern India

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ABSTRACT

This paper examines the genetic variability at 25 microsatellite loci in Munjal - an important sheep of northwestern semi-arid region of India. Various genetic diversity estimates revealed high level of genetic variation within the investigated sheep population. The mean number of observed alleles (allele diversity) per microsatellite marker was 8.64 and that of effective alleles was 4.57. The average observed and expected heterozygosity (gene diversity) values were 0.712 and 0.744 respectively. The mean polymorphic information content value (0.721) further reflected high level of polymorphism across the loci. High heterozygosity values suggested low level of inbreeding and large number of alleles. Within the population, inbreeding estimate (F_{IS} = 0.034) further supported low level of inbreeding in the population. The Mode shift test showed that reduction in effective population size or a recent genetic bottleneck (40–80 generations) was unlikely in this sheep breed. Considerable level of genetic variability observed in the present study can be utilized in planning future breeding strategies of Munjal sheep especially in view of its threatened population status. In addition, information generated in the study can further be utilized for studying differentiation and relationships among different Indian sheep breeds for the selection of priority breeds at national level to maximize genetic diversity to be conserved for the benefit of future generations.

Keywords: Genetic variability, Microsatellite loci, Munjal sheep

Sheep diversity in India is reflected by 42 distinct breeds distributed throughout the country, which have evolved through natural selection for adaptation to specific agro-ecological conditions, ranging from the high altitude of the Himalayas to the deserts of Rajasthan as well as humid-coastal areas of India. Besides these recognized sheep breeds there are several other populations like Munjal investigated under the present study. Most of these breeds/populations are well adapted to the harsh climate, long-distance migration, tropical diseases, poor nutrition and shortage of drinking water. Complete characterization of these indigenous breeds is imperative as they are the only remaining sources of putative alleles of economic values that might be lost through multiple factors including unchecked increase, uncontrolled intermixing amongst them, infusion of exotic germplasm due to haphazard crossbreeding, absence of planned strategies for their conservation and sheer negligence (Bhatia and Arora 2005). Moreover, genetic characterization of native breeds is a first step in prioritization of breeds for conservation.

Here, we present genetic analysis of Munjal sheep—primarily a mutton-type sheep, popular among the farmers for its better reproductive efficiency. It is supposed to have originated through the breeding of Nali and Lohi sheep and is found in districts of Hisar, Ambala, Karnal of Haryana; Patiala, Bathinda of Punjab; and Ganganagar, Hanumangarh of Rajasthan (Basuthakur 1998, Mason 1988). Kushwaha *et al.* (1999) studied the characteristics of Munjal sheep in parts of Rajasthan and Punjab. Poonia (2008) established under organized farm conditions that Munjal sheep is very economical sheep population of the country due to its earlier maturity, faster growth and shorter lambing interval as compared to Magra, Malpura and Muzaffarnagri sheep breeds. In view of threatened status of the population (Yadav *et al.* 2009) the genetic variability analysis using short tandem repeats would provide information important in view of setting its conservation and improvement strategies.

MATERIALS AND METHODS

Blood samples of 50 unrelated animals of Munjal sheep were collected from its breeding tract (Karnal, Bathinda and Hanumangarh) using purposive sampling. Not more than 4 samples were collected from one flock. Care was taken to

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avoid every possibility of mixing of blood with nondescript or other breeds by taking samples from distinct flocks exhibiting breed specific characteristics. DNA was extracted from the white blood cells using a standard phenol/chloroform/isoamyl alcohol extraction protocol (Sambrook *et al.* 1989). A battery of 25 microsatellite markers (Table 1) based on the list of MoDAD (FAO) by Bradley *et al.* (1997) and Di Stasio (2001) was utilized to generate data on 50 DNA samples of the Munjal sheep. The forward primer for each marker was fluorescently labelled with either FAM, NED, VIC or PET dye. Amplifications of the loci was performed in a 25µl final reaction volume containing at least 100ng of genomic DNA, 5 pM of each primer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.5 U Taq polymerase and 1x Taq buffer. A common "Touchdown" PCR programme used for amplification of all the twenty five markers involved 3 cycles of 45 sec at 95°C, 1 min at 60°C; 3 cycles of 45 sec at 95°C, 1 min at 57°C; 3 cycles of 45 sec at 95°C, 1 min at 54°C; 3 cycles of 45 sec at 95°C, 1 min at 51°C and 20 cycles of 45 sec at 92°C, 1 min at 48°C (FAO 1996). Amplification was confirmed on 2% agarose gel and the genotyping were carried out on automated DNA sequencer using LIZ 500 as internal lane standard. Data were collected and analyzed using Gene Mapper Software. Genetic variation was quantified by calculating observed and effective number of alleles, observed heterozygosity, expected heterozygosity and within breed heterozygotes deficiency (F_{IS}) following GenAlEx6 software (Peakall and Smouse 2005). Polymorphism information content (PIC) of microsatellite loci was estimated according to Botstein *et al.* (1980). The tests for deviations from the Hardy-Weinberg equilibrium were derived using GENEPOP-3.2a (Raymond and Rousset 1995). The bottleneck hypothesis was investigated using BOTTLENECK-1.2.02 (Cornuet and Luikart 1996).

RESULTS AND DISCUSSION

Microsatellite markers, their sequences, type of repeat, size range, location and accession numbers etc. are presented in Table 1. Different measures of genetic variation in Munjal sheep population are presented in Table 2. Allele frequencies ranged from 0.013 to 0.888. The number of alleles observed across the studied microsatellite loci varied from 4 (BM6506) to 14 (INRA63) with a mean of 8.64 ± 2.60 . The effective number of alleles, being distinctly lower than the observed number of alleles ranged between 1.264 (CSSM47) to 8.399 (OarJMP29) with an average of 4.57 ± 1.64 . The observed heterozygosity (H_o) ranged from 0.175 (CSSM47) to 0.925 (CP20) and the expected heterozygosity (H_e) from 0.209 (CSSM47) to 0.881 (JMP29). The mean observed heterozygosity (0.712), though lower than the mean expected heterozygosity (0.744), was not significantly different ($P > 0.05$). This finding indicated maintenance of random mating structure by the investigated sheep population. Heterozygote deficiency analysis revealed significant

deviation from HWE ($P \leq 0.05$) at several loci. The exact basis of this departure is difficult to explain, however, the presence of low frequency null alleles segregating at these loci may be a possible reason. Another reason for this deviation could be the high positive F_{IS} (within population inbreeding estimates) values obtained for several loci. Presence of population substructure (Wahlund effect) may also lead to deviation from HWE. The high mean PIC value showed that most of the loci were highly informative indicating high polymorphism across the loci with an overall mean of 0.721 and ranging from 0.210 (CSSM47) to 0.994 (Oar JMP29), thus suggesting suitability of these markers for genetic diversity analysis in Indian sheep too (Kemp *et al.* 1995). The Munjal sheep showed substantial genetic variation based on their allele (8.64) and gene diversity (0.744) measures. The average genetic variation ($H_o = 0.744$) in Munjal sheep was higher than that reported for the genetic variation in Jalauni ($H_o = 0.681$; Arora *et al.* 2008) and Magra ($H_o = 0.694$, Arora and Bhatia 2006), Garole ($H_o = 0.665$, Sodhi *et al.* 2003) and Muzaffarnagri ($H_o = 0.697$, Arora and Bhatia 2004) breeds of Indian sheep. However, direct comparisons could not be made as in the reported studies genotyping was done by the silver staining methodology.

Seven of the 25 loci studied showed significant heterozygote deficiency in the Munjal sheep population (Table 2). Within population inbreeding estimates (F_{IS}) for the investigated loci was 0.034 with a range from -0.513 (BM6506) to 0.637 (OarHH64). Heterozygote deficiency found in Munjal sheep could be attributed to one or more of the following factors: segregation of non-amplifying alleles, Wahlund effects, scoring biases, assortative mating (sample relatedness), linkage with loci under selection (genetic hitchhiking), population heterogeneity or inbreeding. Positive F_{IS} value suggested inbreeding to be one of the main causes for lack of heterozygotes in Munjal sheep. The apparent lack of heterozygotes (positive F_{IS} values) may be attributed to Wahlund effect (population substructure) or inbreeding. Segregation of non amplifying (null) alleles is unlikely to affect this value as it is derived from 25 microsatellite markers. Although the main cause for high genetic homogeneity or lack of heterozygotes might be ascribed to inbreeding (overall positive F_{IS} value), the possibility of Wahlund effect (population substructure) may also not be ruled out due to pooling samples (within breed) from different breeding flocks i.e. different villages in the same area. Further, in view of absence of pedigreed data under field conditions, the effect of relatedness of few samples otherwise deemed unrelated during collection may not be denied. In the absence of detailed past information about this breed it is, however, difficult to identify precisely which previous demographic factors affected the current genetic structure of this breed. Low level of inbreeding ($F_{IS} = 0.034$) observed in the present sheep population suggests unplanned and indiscriminate breeding. This may be due to the fact that its

Table 1. Microsatellite markers, their sequences, type of repeat, size range, location and accession numbers

Locus	Primer sequence	Type of repeat	Size range (bp)	Chronological no.	GenBank Acc. no.
BM757	tgg aaa caa tgt aaa cct ggg ttg agc cac caa gga acc	(GT) ₁₇	178-198	9	G18473
BM827	ggg ctg gtc gta tgc tga g gtt gga ctt gct gaa gtg acc	-	214-224	3	U06763
BM1314	ttc ctc ctc ttc tct cca aac atc tca aac gcc agt gtg g	-	141-161	22	G18433
BM6506	gca cgt ggt aaa gag atg gc agc aac ttg agc atg gca c	-	189-199	1	G18455
BM6526	cat gcc aaa caa tat cca gc tga agg tag aga gca agc agc	-	140-170	26	G18454
BM8125	ctc tat ctg tgg aaa agg tgg g ggg ggt tag act tca aca tac g	-	105-121	17	G18475
CSRD247	gga ctt gcc aga act ctg caa t cac tgt ggt ttg tat tag tca gg	(AC) _n	203-237	14	EU009450
CSSM31	cca agt tta gta ctt gta agt aga gac tct cta gca ctt tat ctg tgt	AAAA(CA) ₇ TA(CA) ₂₅	162-182	23	U03838
CSSM47	tct ctg tct cta tca cta tat ggc ctg ggc acc tga aac tat cat cat	(TG) ₁₂ TATGTA(TG) ₄	120-160	2	U03821
HSC	ctg cca atg cag aga cac aag a gtc tgt ctc ctg tct tgt cat c	-	267-285	20	M90759
INRA63	gac cac aaa ggg att tgc aca agc aaa cca cag aaa tgc ttg gaa g	(AC) ₁₃	165-203	14	X71507
MAF214	aat gca gga gat ctg agg cag gga cg ggg tga tct tag gga ggt ttt gga gg	-	187-231	16	M88160
OarAE129	aat cca gtg tgt gaa aga cta atc cag gta gat caa gat ata gaa tat ttt tca aca cc	(AC) ₁₄	141-169	5	L11051
OarCP20	gat ccc ctg gag gag gaa acg g ggc att tca tgg ctt tag cag g	(AC) ₁₄	67-79	21	U15695
OarCP34	gct gaa caa tgt gat atg ttc agg ggg aca ata ctg tct tag atg ctg c	(AC) ₁₇ TTGCGTGT(CA) ₄	108-122	3	U15699
OarCP49	cag aca cgg ctt agc aac taa acg c gtg ggg atg aat att cct tca taa gg	(AC) ₁₇	80-110	17	U15702
OarFCB48	gag tta gta caa gga tga caa gag gca c gac tct aga gga tgc caa aga acc ag	(TG) ₁₁ CA(TG) ₃	142-164	17	M82875
OarFCB128	cag ctg agc aac taa gac ata cat gcg att aaa gca tct tct ctt tat ttc ctc gc	(GT) ₆ GC(GT) ₁₅	97-123	2	L01532
OarHH35	aat tgc att cag tat ctt taa cat ctg gc atg aaa ata taa aga gaa tga acc aca cgg	(TG) ₁₇	111-139	4	L12554
OarHH41	tcc aca ggc tta aat cta tat agc aac c cca gct aaa gat aaa aga tga tgt ggg ag	(AC) ₂₃	118-140	10	L12555
OarHH47	ttt att gac aaa ctc tct tcc taa ctc cac c gta gtt att taa aaa aat atc ata cct ctt aag g	(AC) ₃₂	124-146	18	L12557
OarHH64	cgt tcc ctc act atg gaa agt tat ata tgc cac tct att gta aga att tga atg aga gc	(TG) ₁₇	120-134	4	L12558
OarJMP8	cgg gat gat ctt ctg tcc aaa tat gc cat ttg ctt tgg ctt cag aac cag ag	(gT) _n	115-129	6	U35059
OarJMP29	gta tac acg tgg aca ccg ctt tgt ac gaa gtg gca aga ttc aga ggg gaa g	(CA) ₂₁	86-144	24	U30893
OarVH72	ctc tag agg atc tgg aat gca aag ctc ggc ctc tca agg ggc aag agc agg	(AC) ₁₄	121-133	25	L12548

Table 2. Genetic diversity indices across 25 microsatellite markers in Munjal sheep

Locus	Number of alleles		Heterozygosity		Information Index	PIC	F _{IS}	P value
	Observed(n _a)	Effective (n _e)	Observed (H _o)	Expected (H _e)				
BM757	5	4.040	0.650	0.753	1.477	0.711	0.136	0.0673
BM827	5	3.882	0.553	0.742	1.447	0.698	0.256	0.0045
BM1314	10	7.145	0.419	0.860	2.104	0.845	0.512	0.0000
BM6506	4	2.372	0.875	0.578	0.986	0.499	-0.513	1.0000
BM6526	9	3.489	0.744	0.713	1.642	0.691	-0.042	0.7226
BM8125	9	3.274	0.615	0.695	1.541	0.665	0.114	0.0964
CSRD247	10	3.579	0.700	0.721	1.580	0.680	0.029	0.3841
CSSM31	9	4.285	0.868	0.767	1.791	0.744	-0.133	0.6665
CSSM47	6	1.264	0.175	0.209	0.512	0.210	0.162	0.1228
HSC	8	5.045	0.615	0.802	1.770	0.774	0.232	0.0021
INRA63	14	7.295	0.769	0.863	2.280	0.851	0.109	0.0356
MAF214	10	4.051	0.900	0.753	1.709	0.721	-0.195	0.0261
OarAE129	10	3.810	0.750	0.738	1.602	0.696	-0.017	0.5881
OarCP20	5	2.795	0.925	0.642	1.238	0.599	-0.440	0.7223
OarCP34	8	4.568	0.769	0.781	1.729	0.752	0.015	0.4268
OarCP49	13	6.362	0.800	0.843	2.093	0.825	0.051	0.2147
OarFCB48	10	6.504	0.900	0.846	2.039	0.829	-0.064	0.1957
OarFCB128	9	4.971	0.795	0.799	1.774	0.770	0.005	0.4755
OarHH35	11	5.808	0.550	0.828	1.980	0.807	0.336	0.0000
OarHH41	8	5.272	0.875	0.810	1.822	0.786	-0.080	0.4288
OarHH47	9	4.501	0.800	0.778	1.750	0.784	-0.029	0.6529
OarHH64	8	4.124	0.275	0.758	1.678	0.729	0.637	0.0000
OarJMP8	13	8.399	0.825	0.881	2.296	0.711	0.063	0.5399
OarJMP29	13	8.399	0.825	0.881	2.296	0.994	0.063	0.1283
OarVH72	6	3.390	0.900	0.705	1.379	0.654	-0.277	0.9723
Mean	8.640	4.566	0.712	0.744	1.672	0.721	0.034	0.3389

population status in the breeding tract is threatened and consequently few rams are used for the whole and nearby villages in the breeding region.

Sign test revealed substantial differences between the number of loci observed and expected with heterozygosity excess (Table 3). Three out of 25 studied microsatellite loci had heterozygosity excess, while remaining showed significant ($P \leq 0.01$) heterozygosity deficiency under SMM. The dataset showed mutation drift equilibrium under TPM model. Standardized difference test showed significant heterozygosity deficiency ($T_2 = -9.728$, $P \leq 0.01$) in Munjal sheep population under SMM, while Wilcoxon test revealed that the population had undergone recent bottleneck assuming

the IAM. The mode shift test, however, revealed mutation drift equilibrium in this sheep population (Fig. 1). All above statistical tests used to study mutation drift equilibrium revealed significant heterozygosity deficiency in Munjal sheep population under SMM and mutation drift equilibrium under TPM. The mutation model for most of the loci is probably intermediate between the IAM and SMM (De Rienzo *et al.* 1994). The statistical tests conclude that the Munjal sheep population had not faced any recent genetic bottleneck.

The long term management and *ex-situ* as well as *in-situ* restoration efforts for a breed/population require the understanding of its genetic structure. Hence, outcome of

Table 3. Statistical tests for mutation drift equilibrium at 25 microsatellite loci under different mutation models in Munjal sheep

		IAM	TPM	SMM
Sign test	Expected number of loci with heterozygosity excess	14.98	14.88	14.69
	Observed number of loci with heterozygosity excess	19	12	3**
Standardized difference test	T ₂ values	1.819*	-2.201*	-9.728**
Wilcoxon test	One tailed test for heterozygosity excess	0.00403**	0.83061	0.99999

* $P \leq 0.05$, ** $P \leq 0.01$.

IAM, Infinite allele model; TPM, two phase mutation model; SMM, strict one- step step-wise mutation model.

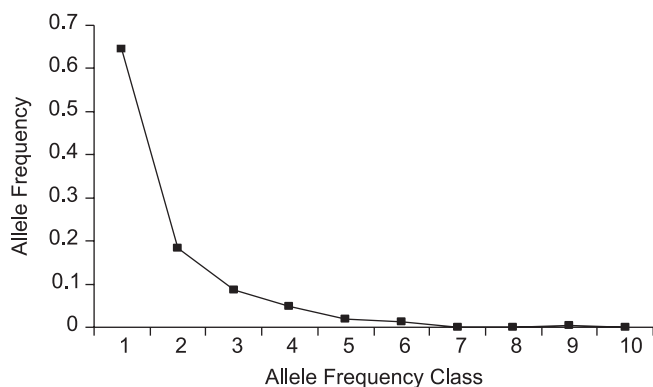


Fig 1. L-shaped curve depicting no mode-shift in Munjal sheep.

this study will facilitate the recognition and maintenance of this unique indigenous gene pool for current and future generations. Our results suggested that there is substantial genetic variation and polymorphism across the studied loci in Munjal sheep. The population though has low level of inbreeding; nevertheless, the study suggested scope for its further genetic improvement by undertaking appropriate breeding strategies to further reduce and circumvent inbreeding in the population. Availability of sufficient rams of superior quality in the breeding tract needs to be ensured to minimize the genetic dilution of this sheep population. Establishing a nucleus flock and implementing as open nucleus breeding system with exchange of rams between nucleus herd and farmers flock will help in its genetic improvement as well as conservation. Further, integrating genetic improvement programmes with market oriented production strategies will not only raise the economy of its rearers but would also ensure sustainable conservation of this important Indian sheep which is otherwise exhibiting threatened population status.

REFERENCES

- Arora R and Bhatia S. 2004. Genetic structure of Muzaffarnagri sheep based on microsatellite analysis. *Small Ruminant Research* **54**: 227–30.
- Arora R and Bhatia S. 2006. Genetic diversity of Magra sheep from India using microsatellite analysis. *Asian-Australasian Journal of Animal Science* **19**: 938–42.
- Arora R, Bhatia S, Sehrawat A, Maity S B and Kundu S S. 2008. Genetic variability in Jalauni sheep of India inferred from microsatellite data. *Livestock Research for Rural Development* **20**: article#4.
- Basuthakur A K. 1988. *Sheep Research Production and Marketing in India*. pp. 117. Inter-India Publications, New Delhi.
- Bhatia S and Arora R. 2005. Biodiversity and conservation of Indian sheep genetic resources- an overview. *Asian-Australasian Journal of Animal Science* **18**: 1387–1402.
- Bradley D G, Fries R, Bumstead N, Nicholas F W, Cothran E G, Ollivier L and Crawford A M. 1997. Report of an Advisory group of the International Society of Animal Genetics as a contribution to assist FAO prepare for the modad Project, *DADIS*. FAO: 1–15.
- Botstein D, White R L, Skolnick M and Davis R W. 1980. Construction of genetic linkage maps in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32**: 314–31.
- Cornuet J M and Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetic* **144**: 2001–14.
- Di Rienzo A, Peterson A C, Garza J C, Valdes A M, Slatkin M and Freimer N B. 1994. Mutational process of simple-sequence repeat loci in human populations. *Proceedings of National Academy of Science, USA* **91**: 3166–70.
- Di Stasio L. 2001. Applied genetics in sheep and goats. Panel of markers for parentage verification tested at the 2001/02 ISAG comparison test, www.isag.org.uk/pdf/2005_PanelsMarkersSheepGoats.pdf.
- FAO. 1996. Global project for the Maintenance of Domestic Animal Genetic Diversity (MoDAD). <http://www.fao.org/dad-is/>
- Mason I L. 1988. *World Dictionary of Livestock Breeds*. CAB International, pp. 297.
- Kemp S J, Hishida O, Wambugu J, Rink A, Longeri M L, Ma R Z, Da Y, Lewin H A, Barendse W and Teale A J. 1995. A panel of polymorphic bovine, ovine and caprine microsatellite markers. *Animal Genetics* **26**: 299–306.
- Kushwaha B P, Riyazuddin, Singh R N and Parthasarathy S. 1999. Characteristics of Munjal sheep. *Animal Genetic Resources Information* **25**: 27–31.
- Peakall R and Smouse P E. 2005. GENALEX-6: Genetic Analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Note* **6**: 288–95.
- Poonia J S. 2008. Reproductive performance of Munjal sheep. *Indian Journal of Small Ruminants* **14**(1): 121–23.
- Raymond M and Rousset F. 1995. GENEPOP-3.2a, Population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**: 248–49.
- Sambrook J, Fritsch E F and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Press, New York.
- Sodhi M, Mukesh M, Arora R, Tania M S and Bhatia S. 2003. Genetic structure of Garole- a unique Indian micro sheep assessed using microsatellite markers. *Indian Journal of Dairy Science* **56**: 167–73.
- Yadav D K, Arora R, Bhatia S and Singh G. 2009. Evaluation of Munjal sheep: A journey from identity crisis to extinction threat. *Compendium of invited lectures and abstracts of National Symposium on Livestock Biodiversity Conservation & Utilization: Lessons from Past and Future Perspectives*. pp. 200. National Bureau of Animal Genetic Resources, Karnal, India.