



Genetic discrimination of Muzaffarnagri and Munjal sheep of northwestern semi arid zone of India based on microsatellite markers and morphological traits

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ABSTRACT

Muzaffarnagri and Munjal are both mutton-type and highly course-wooled sheep of north-western-semi arid zone of India. Muzaffarnagri is a descript sheep breed while Munjal is not recognized as a breed at the national level. The present study examines the genetic differentiation based on microsatellite markers and morphometric traits using multivariate analysis. A total of 194 Munjal and 294 Muzaffarnagri sheep were described for 7 body measurements for morphometric characterization. The study included animals aged between 2- to 8-teeth, raised under field conditions in semi-arid environment. The univariate analysis revealed that Munjal sheep were larger and heavier than Muzaffarnagri sheep. The sexual dimorphism (m/f) was 1.13 (Munjal) and 1.11 (Muzaffarnagri). Munjal males were 37% heavier than females, whereas Muzaffarnagri males were 27% heavier than females. The coefficient of variation of all traits ranged from 4.14 to 33.67%. Flock and age effects established high heterogeneity among females of different flocks in both breeds/populations, whereas in males these effects showed homogeneity between flocks in Muzaffarnagri sheep and heterogeneity in Munjal sheep. Step-wise discriminant analysis revealed that paunch girth followed by tail length was more discriminating for the 2 sheep breeds/populations. The Mahalanobis distance (3.65, female; 4.56, male) between 2 sheep breeds was high and significant. Nearest Neighbour Discriminant Analysis showed that 86.31% of Muzaffarnagri females and 90.48% of Munjal males were classified into their source population. Microsatellite markers data showed high level of allelic richness (>6.0) and genetic diversity (>0.7) for both breeds/populations. Bayesian cluster analysis also indicated distinct clusters with average membership coefficient of 0.974 and 0.972 for Muzaffarnagri and Munjal sheep respectively. The results based on morphological and microsatellite data revealed that these sheep belong to genetically distinct groups. The findings emerged on genetic and phenotypic analyses would serve as important inputs for designing conservation and improvement strategy for these sheep breeds/populations.

Key words: Discriminant analysis, Microsatellite markers, Morphometric, Munjal sheep, Muzaffarnagri sheep, Semi arid, Sheep, Trait

Sheep biodiversity in India is characterized by high degree of endemism and variations in agro-climatic conditions of the different regions. India is having 39 registered sheep breeds. Muzaffarnagri and Munjal, mutton-type and course-wooled sheep of northwestern semi arid zone of India, are considered breeds/populations at risk with population size below 10,000 and exhibiting a declining trend (Kumar *et al.* 2006, Yadav *et al.* 2010). There is need to characterize and document indigenous livestock breeds to plan their conservation, improvement and sustainable utilization. Phenotypic comparison based on morphological characters can provide to an extent a reasonable representation of genetic differences among breeds. Multifactorial analyses of morphological traits were found suitable to assess variation within and can discriminate different population types when all measured morphological

variables are considered simultaneously (Traoré *et al.* 2008). The initial efforts of either phenotypic or genetic characterization of Indian sheep are mostly restricted to single breed/population (Arora and Bhatia 2004, Kumar *et al.* 2006, Singh *et al.* 2007, Yadav *et al.* 2009, 2011, 2011a). However, Arora *et al.* (2011) studied genetic relationships among some Indian sheep breeds/populations based on microsatellite markers. Little attention from researchers in India has been given to the breed compared to other sheep breeds using multivariate analysis methods. Yadav *et al.* (2013) reported only such study in Indian sheep. Combined use of microsatellite and morphometric data in characterization of Indian livestock breeds is still lacking. The present study is an attempt to investigate the genetic differentiation/distinctness of 2 sheep breeds/populations based on microsatellite data and morphometric traits. The general aim was to compare their morphometric traits, and to establish morphological and genetic standards.

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MATERIALS AND METHODS

Morphometric data: Morphological measurements were taken on randomly selected 194 Munjal sheep (152 females, 42 males) and 294 Muzaffarnagri sheep (241 females, 53 males) from 32 and 36 flocks respectively. Animals having 2 to 8 permanent incisors were included in the study. The 7 morphometric traits, viz. height at withers (HW), body length (BL), chest girth (CG), paunch girth (PG), ear length (EL), tail length (TL) and body weight (BW) were measured. Body weights (kg) were recorded with a weighing machine while other traits were measured with a measuring tape (cm) after making the animal stand squarely on an even ground. All measurements were carried out by the same person to avoid inter-individual variations.

Genetic data: The blood samples of 40 animals of each Muzaffarnagri and Munjal sheep were collected randomly in their distribution area. DNA was extracted from the white blood cells using a standard phenol/chloroform/isoamyl alcohol extraction protocol. Twenty-five microsatellite markers were used in the study (Table 1). The forward primer for each marker was fluorescently labeled with either FAM, NED, VIC or PET dye. Amplifications of the loci were performed as described elsewhere (Arora *et al.* 2011). Allele sizing was done using GENEMAPPER software.

Data analysis: The FSTAT ver 2.9.3 program (Goudet *et al.* 1995) was used to calculate observed number of alleles (N_a), observed heterozygosity (H_o), gene diversity (H_e)

expected heterozygosity (H_s and H_T) and the estimator of genetic differentiation (G_{ST}). Structure version 2.3.1 (Prichard *et al.* 2000) was used to analyze the genetic structure of the populations using the Bayesian Markov Chain Monte Carlo approach assuming $K=2$. Ten independent runs were performed for each K . The analysis was performed by means of the admixture model with correlated allele frequencies with a burn in period of 100000 and 100000 iterations for data collection.

Descriptive statistics for the morphometric traits were obtained using JMP Genomics 4.1 software of SAS (2009). Means separation was done using t-test of the same statistical package at probability level of 5%. The influence of age and flock on the body traits measured was assessed fitting a model which includes the age effect with 4 levels (2-teeth, 4-teeth, 6 teeth and 8-teeth) and flock effect with 32 levels in Munjal and 36 levels in Muzaffarnagri sheep. Step-wise discriminant procedure was applied using PROC STEPDISC to determine which morphological traits have more discriminant power than others. The relative importance of the morphometric traits in discriminating the 2 breeds/populations was assessed using the level of significance ($P<0.05$) and partial R^2 values ≥ 0.01 . The CANDISC procedure was used for calculating the Mahalanobis distances of the morphological traits, and derived canonical functions. The ability of these canonical functions to assign each individual sheep to its breed/

Table 1. Diversity indices across 25 microsatellite loci in Muzaffarnagri and Munjal sheep

Locus	Allelic richness		Gene diversity (H_e)		H_s	H_T	G_{ST}
	Muzaffarnagri	Munjal	Muzaffarnagri	Munjal			
BM757	4.810	4.938	0.755	0.763	0.759	0.788	0.036
BM827	4.319	4.930	0.660	0.755	0.707	0.724	0.023
BM1314	8.000	9.222	0.857	0.882	0.869	0.894	0.028
BM6506	4.288	3.416	0.463	0.582	0.522	0.557	0.061
BM6526	6.641	7.714	0.766	0.722	0.744	0.752	0.010
BM8125	6.221	6.882	0.556	0.705	0.631	0.640	0.015
CSRD247	6.134	7.126	0.673	0.730	0.702	0.727	0.035
CSSM31	10.700	8.165	0.781	0.777	0.779	0.878	0.113
CSSM47	7.523	3.844	0.637	0.212	0.424	0.447	0.052
HSC	6.997	6.996	0.800	0.815	0.807	0.819	0.015
INRA63	7.161	11.460	0.799	0.876	0.837	0.843	0.007
MAF214	6.871	7.803	0.835	0.764	0.799	0.837	0.045
OarAE129	5.563	7.107	0.571	0.747	0.659	0.677	0.027
OarCP20	6.158	4.422	0.494	0.650	0.572	0.585	0.022
OarCP34	6.335	7.175	0.795	0.791	0.793	0.790	-0.004
OarCP49	9.680	9.803	0.879	0.854	0.866	0.884	0.019
OarFCB128	7.052	7.159	0.799	0.809	0.804	0.806	0.002
OarFCB48	8.613	8.822	0.856	0.858	0.857	0.873	0.019
OarHH35	8.396	8.818	0.813	0.842	0.827	0.846	0.022
OarHH41	6.980	7.245	0.702	0.821	0.761	0.781	0.025
OarHH47	8.544	7.491	0.767	0.788	0.777	0.806	0.035
OarHH64	6.599	6.999	0.667	0.773	0.720	0.721	0.001
OarJMP8	6.845	6.071	0.776	0.754	0.765	0.779	0.019
OarJMP29	6.937	10.998	0.804	0.893	0.848	0.853	0.006
OarVH72	5.746	5.080	0.712	0.713	0.712	0.778	0.084
Mean	6.925	7.187	0.728	0.755	0.742	0.763	0.029

population was calculated as the percentage of correct assignment of each breed/population using the DISCRIM procedure.

RESULTS AND DISCUSSION

The 25 microsatellite loci used in the present study were polymorphic in both the breeds/populations (Table 1). The allelic richness for Muzaffarnagri and Munjal sheep was 6.925 and 7.187 respectively. The gene diversity value for Muzaffarnagri was 0.728 and for Munjal 0.755. The overall H_S and H_T values for both breeds/populations were 0.742 and 0.763 respectively. The coefficient of gene differentiation (G_{ST}) was 0.029. Structure analysis gave an average membership coefficient (q) of 0.974 and 0.972 for Muzaffarnagri and Munjal sheep respectively. The results revealed that both Muzaffarnagri and Munjal sheep possess sufficient genetic diversity in terms of allelic richness as well as gene diversity. The high values of allelic richness were comparable to some European and Middle –Eastern sheep breeds (Peter *et al.* 2007). The H_S and H_T , two estimators of genetic and gene diversity of the investigated sheep breeds/populations were more than 0.7. In general, these levels of genetic diversity ($H_S=0.742$, $H_T=0.763$) in the Indian sheep tested in the present study were comparable to the values reported in other Swiss sheep breeds ($H_S=0.63$ and $H_T=0.75$, Saitbekova *et al.* 2001). The genetic differentiation based on G_{ST} was only 2.9%. Arora *et al.* (2011) also reported a low F_{ST} value of 4.8% and genetic distance (D_A ; Nei *et al.* 1983) of 0.151 between these 2 sheep breeds/populations.

Descriptive statistics of the body biometry of the 2 sheep breeds/populations are presented in the Table 2. The univariate analysis revealed that the body measures of the 2 breeds/populations were significantly ($P<0.05$) different except height at withers. The morphometric traits of Munjal sheep were significantly ($P<0.05$) higher than those of their Muzaffarnagri counterparts other than tail length, suggesting Munjal sheep were larger and heavier than Muzaffarnagri sheep. The body biometry estimates obtained in this study are comparable to values reported for these sheep breeds/populations (Kushwaha *et al.* 1999 and Mandal *et al.* 2000). These mean values would allow for the defining of the adult morphological standard of the 2 breeds from both the mean point of view and variability (Table 2). The measure of sexual dimorphism (m/f) is shown in Table 2. Overall sexual dimorphism in Munjal and Muzaffarnagri sheep was 1.13 and 1.11 respectively. Munjal males were 37% heavier than females, whereas Muzaffarnagri males were 27% heavier than females. The coefficient of variation of all traits in two breeds ranged from 4.14% to 33.67% with only EL, TL and BW higher than 10% which corresponds with a mean homogeneity within the breed. Similar results were reported in sheep breeds of Karnataka (Yadav *et al.* 2013). Table 2 also showed the significance of the age of the animal and flock effects on the analyzed traits in males and females. The sexes were analyzed separately due to differences in sample size and, more importantly, to the high sexual dimorphism found. In females, the flock effect was highly significant ($P<0.0001$) while the age effect had highly significant effect

Table 2. Raw means, coefficient of variation, and significance of flock and age effects for each of the morphological traits in different sheep breeds

Breed	Body traits	Females				Males				Sexual dimorphism (m/f)
		Statistics		Fixed effects		Statistics		Fixed effects		
		Mean	CV	Flock	Age	Mean	CV	Flock	Age	
Munjal	BL	75.60 ^a	5.44	***	***	83.64 ^c	6.56	NS	NS	1.11
	HW	73.24 ^a	4.14	***	*	80.38 ^c	5.56	*	NS	1.10
	CG	83.78 ^a	5.74	***	***	91.76 ^c	5.18	*	**	1.10
	PG	86.90 ^a	6.69	***	***	94.33 ^c	6.52	*	NS	1.09
	EL	18.47 ^a	32.70	***	NS	18.81 ^c	33.67	**	NS	1.02
	TL	42.02 ^b	19.82	***	NS	47.90 ^d	22.07	**	NS	1.14
	BW	43.95 ^a	14.22	***	***	60.05 ^c	16.72	**	**	1.37
Muzaffarnagri	BL	73.54 ^b	6.25	***	**	80.32 ^d	7.32	NS	**	1.09
	HW	73.60 ^a	5.32	***	**	80.68 ^c	6.10	NS	**	1.10
	CG	78.51 ^b	5.73	***	***	84.32 ^d	6.97	NS	**	1.07
	PG	79.33 ^b	7.23	***	***	84.51 ^d	7.87	NS	**	1.07
	EL	18.34 ^a	10.73	***	NS	19.49 ^c	10.54	NS	NS	1.06
	TL	49.77 ^a	11.99	***	NS	55.49 ^c	14.11	NS	NS	1.11
	BW	39.61 ^b	14.98	***	***	50.21 ^d	21.25	**	***	1.27

BL, body length; HW, height at withers; CG, chest girth; PG, paunch girth; EL, ear length TL, tail length; BW, body weight; CV, coefficient of variation (%); ^{ab}means (female) and ^{cd}means (male) in the same column with different superscripts are significantly different; *Significance level: $P<0.05$; ** significance level: $P<0.01$; *** significance level: $P<0.0001$.

Table 3. Step-wise selection summary of traits

Sex	Step	Trait entered	Partial R ²	F-value	P>F	Wilk's lambda	P<lambda	Average squared canonical correlation	P>ASCC
Female	1	PG	0.291	160.69	<0.0001	0.70873	<0.0001	0.291	<0.0001
	2	TL	0.246	127.31	<0.0001	0.53430	<0.0001	0.465	<0.0001
	3	BL	0.035	14.32	0.0002	0.51534	<0.0001	0.484	<0.0001
	4	HW	0.042	17.02	<0.0001	0.49369	<0.0001	0.506	<0.0001
	5	CG	0.014	5.79	0.0166	0.48641	<0.0001	0.513	<0.0001
	6	EL	0.009	3.80	0.0520	0.48167	<0.0001	0.518	<0.0001
Male	1	PG	0.370	54.62	<0.0001	0.63000	<0.0001	0.369	<0.0001
	2	TL	0.262	32.64	<0.0001	0.46501	<0.0001	0.534	<0.0001
	3	HW	0.078	7.74	0.0066	0.42854	<0.0001	0.571	<0.0001
	4	BL	0.133	13.84	0.0003	0.37141	<0.0001	0.628	<0.0001
	5	CG	0.065	6.22	0.0145	0.34717	<0.0001	0.652	<0.0001

($P < 0.0001$) only for 3 (CG, PG and BW) out of the 7 analyzed traits in both breeds/populations, showing a high heterogeneity among females of different flocks. In males of Munjal sheep, the flock effect resulted significant effects except BL and age effect was nonsignificant except CG and BW, whereas males of Muzaffarnagri sheep resulted nonsignificant flock effect except BW and significant age effect except for EL and TL. In view of significant flock effect females appear different between flocks. Likewise, the results show homogeneity between flocks in Muzaffarnagri males and heterogeneity between flocks in Munjal males. The possible influencing factors could be the fewer males than females, preferential selection as well as management of males over females and spread of Munjal flocks over wider area than Muzaffarnagri flocks (Kumar *et al.* 2006, Yadav *et al.* 2010, 2011a). The high variability observed in phenotypic traits is congruent with high within-population genetic diversity observed in these sheep breeds/populations (Yadav *et al.* 2011, Arora and Bhatia 2004).

The results of the step-wise discriminant analysis are presented in Table 3. All the measured variables were significant ($P < 0.0001$) in both the sexes except EL. However, PG followed by TL had more discriminant power than the other morphometric traits. The best discriminant function model used in this study included only 2 morphological measures (PG and TL) out of the 6 pre-selected in both sexes. This implies that these 2 morphological traits are more important and informative and could be sufficient in differentiating between the 2 sheep breeds/populations than getting several additional measurements on all 6 traits. These could be used to assign the 2 sheep breeds into distinct populations, thereby reducing the errors of selection in future breeding and selection programmes. Table 4 gives the canonical variable generated (CAN1) in canonical discriminant analysis which was significant ($P < 0.0001$). Canonical loadings that measure the simple linear correlation between each independent variable and canonical variable are presented in the same table. The Mahalanobis distance (within parentheses) and classification of the breeds/populations

into their genetic source are shown in Table 5. The pair-wise distance between the 2 sheep breeds was highly significant ($P < 0.0001$). The genetic distance theoretically describes the differences between breeds. Phenotypic differences are maintained in part by the reduction of gene flow among populations separated by large distances. The pair-wise distance in males (4.56) was higher than females (3.65). This could be facilitated by the fact that male animals are phenotypically more pure than female as the farmers keep 1 or 2 true to breed rams in their flocks for breeding and rest of the male animals are sold around 6–9 months of age (Kumar *et al.* 2006, Yadav *et al.* 2010). One more reason could be attributed to lesser number of male animals as

Table 4. Standardized coefficients or the canonical discriminant function, the canonical correlation, the Eigen value and percentage total variance accounted for

Traits	Discriminant variate (CAN1)	
	Female	Male
PG	0.984	1.241
TL	-.841	-0.810
Adjusted canonical correlation	0.681	0.729
Approximate standard error	0.026	0.047
Eigen value	0.872	1.150

Table 5. Mahalanobis distance (within parentheses) between the 2 sheep breeds and percentage of individual sheep classified into genetic group

Sex	Breed	Munjal	Muzaffarnagri
Female	Munjal	82.24 (0)	17.76 (3.65)
	Muzaffarnagri	13.69 (3.65)	86.31(0)
	Error level	0.18	0.14
	Priors	0.50	0.50
Male	Munjal	90.48 (0)	9.52 (4.56)
	Muzaffarnagri	18.87 (4.56)	81.13 (0)
	Error level	0.10	0.19
	Priors	0.50	0.50

compared to females. Nevertheless, the above results are substantiated by the classification results.

Most Munjal sheep (82.24% females and 90.48% males) were classified into their source population followed by Muzaffarnagri sheep (86.31% females and 81.13 % males). However, while 17.76% of Munjal ewes were misclassified as Muzaffarnagri ewes, 13.69% of Muzaffarnagri ewes were wrongly assigned as Munjal ewes. Similarly in rams, 9.52% of Munjal were misclassified as Muzaffarnagri and 18.87% of Muzaffarnagri were wrongly assigned as Munjal rams. More than 80% of sheep were classified into their source genetic group in both breeds/populations. The high morphological distance between the 2 sheep breeds/populations, coupled with high correct assignment to their respective source groups is an indication that they belong to different breeds. Our results are similar to the reports of some of the workers (Yakubu and Akinyemi, 2010, Yadav *et al.* 2013). The morphometric analysis results are in agreement to those obtained from microsatellite data analysis. Structure analysis using the Bayesian clustering algorithm for K=2 gave values of average membership coefficient (q) as 0.974 and 0.972 for Muzaffarnagri and Munjal sheep respectively. These high values of membership coefficient revealed that Muzaffarnagri and Munjal are distinct populations showing little or no admixture. Traditional populations are thought of as distinct types evolved as a result of geographical isolation and cultural separation of the communities keeping the animals (Rege 2002). As socio-economic status of the communities rearing these sheep is more or less similar (Kumar *et al.* 2006, Yadav *et al.* 2011), geographical separation seems to be dominating factor in their distinct identity.

The general aim of conservation of animal genetic resources is to maintain within and across breed diversity. Across-breed diversity is classically considered as a major criterion to be taken into account when setting priorities for conservation of domestic animal breeds. A comprehensive phenotypic and genetic characterization (Kumar *et al.* 2006, Yadav *et al.* 2010, 2011a, 2012, Arora and Bhatia 2004, Yadav *et al.* 2011) of these sheep breeds/populations would enable their accurate comparison with other sheep breeds, thereby assisting in the development of conservation and improvement programmes. The present study consolidates information arising from genetic analysis and morphological differentiation. According to Kunene *et al.* (2009), such phenotypic and genetic data when sensibly combined into a single database could be critical for decisions regarding indigenous sheep breeds' exploitation and conservation. The present information on the genetic and phenotypic differentiation of Munjal and Muzaffarnagri sheep breeds/populations could therefore be exploited in designing more appropriate strategies for their breeding, selection, management and conservation.

The genetic and morphometric comparison led to defining of the adult morphological standard of the 2 breeds/populations from both the mean point of view as well as variability. Paunch girth and tail length could best be used

to show morphological variation in these 2 breeds/populations. Flock and age effects established high heterogeneity among females of different flocks in both breeds/populations whereas in males these effects showed homogeneity between flocks in Muzaffarnagri sheep and heterogeneity in Munjal sheep. The authors suggest that these 2 parameters may be used with high reliability to discriminate between the 2 sheep breeds/populations. Besides, the panel of 25 microsatellite markers could successfully segment the 2 breeds/populations into distinct groups. Therefore, based on morphological divergence, supported by microsatellite data, we concluded that Munjal and Muzaffarnagri are distinct sheep breeds/populations. To conserve the current genetic pool of these unique ovine genetic resources, introgression and crossbreeding must be prohibited.

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