Indian Journal of Animal Sciences **87** (9): 1097–1101, September 2017/Article

Microsatellite based genetic diversity estimation in Kajali sheep and its phylogenetic relationship with other indigenous sheep breeds

S SINGH[1], A K MISHRA[2], V VOHRA[3], K N RAJA[4], Y SINGH[5], K M SINGH[6], I GANGULY[7] and R ARORA[8]

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

Received: 2 March 2017; Accepted: 27 March 2017

ABSTRACT

Microsatellite based characterization of Kajali sheep population found in Punjab and adjoining regions was carried out and genetic diversity measures were analysed. High measures of allele (7.778±0.80) and gene diversity (0.66±0.03) were observed across this population. A significant positive F_{IS} (0.23±0.03) value suggested a deficiency in the number of heterozygotes in Kajali sheep which may be attributed to population sub-structuring into different colour variants (White and Kali Kajali). The population revealed presence of genetic diversity and there was no significant heterozygosity excess indicating the absence of genetic bottleneck in the recent past. The phylogenetic study analysis with 18 other Indian sheep breeds revealed that Kajali sheep clustered in same node with Munjal sheep (bootstrap value of 22%). However, Kajali and Munjal sheep are phenotypically distinct from each other. The genetic characterization of Kajali sheep will help in devising suitable strategies for its genetic improvement, management and recognition at national level.

Key words: Characterization, Diversity analysis, Kajali sheep, Microsatellite markers

India is rich in ovine biodiversity, possessing about 4.5% of sheep population of the world. According to 19th livestock census (2012), the sheep population of India is 65.06 million, which contribute 12.7% of total livestock population of our country. However, only about 25% of the country's estimated sheep population which comprises 40 registered breeds has been exploited so far, through survey on native distribution tracts and/or their molecular characterization. Besides, about 47 lesser-known sheep groups categorized into breeds/populations (19), strains (11), varieties (5) and recent derivatives (12) had been reported (Bhatia and Arora 2010). In order to assess the complete ovine diversity available in our country, these nondescript sheep groups need to be properly characterized and documented. This would enable unique/economically important ovine populations to be identified, documented and recognized at the national level, eventually leading to their conservation and improvement. Kajali sheep is one such lesser known sheep population widely distributed in Punjab and adjoining area. The Kajali animals are large in size with well-built body having roman nose, long and pendulous ears and characteristic long tail touching up to the ground.

The unique phenotypic appearance especially colour pattern on face, roman nose, ear size and shape and tail length are able to distinguish this valuable germplasm from other extant sheep breeds of this region. Although reports are available on the morphometric characteristics of Kajali sheep (Mishra *et al*. 2016, Mishra *et al*. 2017), their molecular genetic characterization is totally lacking. Therefore, the present study was undertaken to assess the genetic diversity measures of Kajali sheep using microsatellite markers.

MATERIALS AND METHODS

Blood samples were collected from 42 unrelated animals of Kajali sheep in its breeding tract. The genomic DNA from the blood samples was isolated using a standard phenol/chloroform/isoamyl alcohol extraction method (Sambrook *et al*. 1989). A set of 18 microsatellite markers based on the list of MoDAD (FAO) reported by Bradley *et al*. (1997) and Di Stasio (2001) were utilized to generate data on 42 DNA samples of the Kajali sheep. The details of microsatellite markers, primer sequences, size range, genebank accession number and chromosomal location is given in Table 1. The forward primer for each marker was fluorescently labelled with FAM, NED, VIC or PET dye. Amplification of the loci was performed in a 25 μl final reaction volume containing at least 100 ng of genomic DNA, 5 pM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs,

Present address: [1]Senior Scientist (sssanjeev197@gmail.com), [2]Principal Scientist (anilmishra65@gmail.com), [3]Senior Scientist (vohravikas@gmail.com), [4]Scientist (drknraja@yahoo.co.in), [7]Senior Scientist (drindrajit@gmail.com), [8]Principal Scientist (rejagati@yahoo.co.in). [5]Deputy Director (ysinghvet @gmail.com), KVK, Majra, Mohali . [6]Ph D Student (maheshsngh7 @gmail.com), Animal Genetics and Breeding, ICAR-NDRI, Karnal.

Table 1. Microsatellite markers used in the study of sheep

Locus	Primer sequence	Size range (bp)	Genebank accession no.	Chromosome no.
BM6506	F -gCA CgT ggT AAA gAg ATg gC R - AgC AAC TTg AgC ATg gCA C	191–199	G18455	1
OarCP20	F -gAT CCC CTg gAg gAg gAA ACg g R - ggC ATT TCA Tgg CTT Tag CAg g	72–88	U15699	21
OarFCB48	F -gAg TTA gTA CAA ggA TgA CAA gAg gCA C R - gAC TCT AgA ggA TCg CAA AgA ACC Ag	146–152	M82875	17
OarVH72	F -CTC Tag Agg ATC Tgg AAT gCA AAg CTC R - ggC CTC TCA Agg ggC AAg AGC Agg	121–133	L12548	25
OarHH47	F -TTT ATT gAC AAA CTC TCT TCC TAA CTC CAC C R - gTA gTT ATT TAA AAA AAT ATC ATA CCT CTT AAg g	124–144	L12557	18
BM757	F - Tgg AAA CAA TgT AAA CCT ggg R - TTg AgC CAC CAA ggA ACC	172–186	G18473	9
BM1314	F -TTC CTC CTC TTC TCT CCA AAC R - ATC TCA AAC gCC AgT gTg g	141–167	G18455	22
BM8125	F -CTC TAT CTg Tgg AAA Agg Tgg g R - ggg ggT Tag ACT TCA ACA TAC g	109–119	G18475	17
OarHH35	F -AAT TgC ATT CAg TAT CTT TAA CAT CTg gC R - ATg AAA ATA TAA AgA gAA TgA ACC ACA Cgg	107–137	L12554	4
OarHH64	F -CgT TCC CTC ACT ATg gAA AgT TAT ATA TgC R - CAC TCT ATT gTA AgA ATT TgA ATg AgA gC	127–135	L12558	4
OarJMP8	F -Cgg gAT gAT CTT CTg TCC AAA TAT gC R - CAT TTg CTT Tgg CTT CAg AAC CAg Ag	115–139	U35059	6
OarJMP29	F -gTA TAC ACg Tgg ACA CCg CTT TgT AC R - gAA gTg gCA AgA TTC AgA ggg gAA g	109–143	U30893	24
BM827	F -ggg CTg gTC gTA TgC TgA g R - gTT ggA CTT gCT gAA gTg ACC	210–218	U06763	3
OarHH41	F -TCC ACA ggC TTA AAT CTA TAT AgC AAC C R - CCA gCT AAA gAT AAA AgA TgA TgT ggg Ag	120–140	L12555	10
CSSM31	F -CCA AgT TTA gTA CTT gTA AgT AgA R - gAC TCT CTA gCA CTT TAT CTg TgT	146–180	U03838	23
CSSM47	F -TCT CTg TCT CTA TCA CTA TAT ggC R - CTg ggC ACC TgA AAC TAT CAT CAT	148–152	U03821	2
CSRD0247	F -ggA CTT gCC AgA ACT CTg CAA T R - CAC TgT ggT TTg TAT TAg TCA gg	211–239	EU009450	14
MAF0214	F -AAT gCA ggA gAT CTg Agg CAg ggA Cg R - ggg TgA TCT TAg ggA ggT TTT ggA gg	185–229	M88160	16
OarCP0049	F -CAg ACA Cgg CTT AgC AAC TAA ACg C R - gTg ggg ATg AAT ATT CCT TCA TAA gg	83–139	U15702	17
BM6526	F -CAT gCC AAA CAA TAT CCA gC R - TgA Agg Tag AgA gCA AgC AgC	191–199	G18454	26
OarCP34	F - gCT gAA CAA TgT gAT ATg TTC Agg R - ggg ACA ATA CTg TCT Tag ATg CTg C	112–126	U15699	3
INRA0063	F -gAC CAC AAA ggg ATT TgC ACA AgC R - AAA CCA CAg AAA TgC TTg gAA g	173–199	X71507	14
OarAE129	F -AAT CCA gTg TgT gAA AgA CTA ATC CAg R - gTA gAT CAA gAT ATA gAA TAT TTT TCA ACA CC	133–159	L11051	5
OarFCB128	F -CAg CTg AgC AAC TAA gAC ATA CAT gCg R - ATT AAA GCA TCT TCT CTT TAT TTC CTC GC	106–130	L01532	2

0.5 U *Taq* DNA polymerase and 1× *Taq* buffer. A common touch down PCR programme, as suggested under MoDAD project (FAO 1996) without extension step was used for the amplification of all the eighteen markers. PCR amplification consisted of 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 95°C, 1 min at 48°C. The amplified products were resolved on 2% agarose gel and genotyped on an automated DNA sequencer using LIZ 500 as internal lane standard (ABI PRISM). The raw data files were extracted by Gene Mapper software version 3. Popgen3.2 (Yeh *et al*. 1999) and GenAlEx6.5 (Peakall and Smouse 2005) softwares were used for the genetic diversity analysis. Polymorphism Information Content (PIC) of the microsatellite loci was estimated

according to Botstein *et al*. (1980). In the present study, to estimate the bottleneck events in the population, two different approaches were followed. The first approach based on the heterozygosity excess consisted of three tests: sign test, standardized differences test and a Wilcoxon sign-rank test. The probability distribution was established using 1000 simulations under three models: infinite allele (IAM), stepwise mutation (SMM) and two-phase mutation model (TPM). The test was conducted using Bottleneck 1.2.01 software (Piry *et al.* 1999). Another test that was used to depict the bottleneck analysis was based on graphical representation of mode-shift equilibrium.

RESULTS AND DISCUSSION

The genetic variability measures of Kajali sheep (Figs 1, 2) across the 18 microsatellite markers are depicted in Table 2. The allele frequencies across all the loci ranged from 0.012 to 0.731 (data not shown). A total of 140 alleles were identified across the 18 markers in Kajali sheep. The observed number of alleles ranged from 3 (BM8125) to 15 (OarCP20) with a mean of 7.77±0.80. Effective number of alleles was lower than the observed number of alleles and ranged from 1.81 (OarHH64) to 9.47 (CSSM47) with a mean value of 3.98±0.58. The average observed heterozygosity values (0.522) compared to the average expected heterozygosity values (0.669) did not show significant differences (P>0.05) which suggested random mating in Kajali population. The estimates of allele diversity (mean number of observed alleles) and gene diversity (mean expected heterozygosity) implied the presence of substantial amount of genetic variability in the Kajali sheep population. Similar trends were reported in the Koraput sheep breed of Odisha (another lesser known sheep) by Singh *et al.* (2015). They observed higher measures of allele (9.33) and gene diversity (0.76) in this population.

Table 2. Genetic variability measures in Kajali sheep across different microsatellite markers

Locus	Na	Ne	Heterozygosity		PIC	F_{IS}
			Observed	Expected		
BM757	4.00	1.912	0.444	0.477	0.408	0.068
BM8125	3.00	1.949	0.432	0.487	0.381	0.112
OarHH47	9.00	5.935	0.727	0.831	0.808	0.125
BM6526	8.00	2.557	0.610	0.609	0.587	–0.001
OarCP34	5.00	3.219	0.650	0.689	0.646	0.057
CSRD0247	6.00	3.269	0.575	0.694	0.644	0.172
MAF0214	5.00	2.058	0.500	0.514	0.473	0.027
OarCP0049	9.00	5.787	0.756	0.827	0.805	0.086
OarHH35	7.00	3.378	0.613	0.704	0.666	0.129
OarHH64	7.00	1.817	0.192	0.450	0.430	0.572
OarJMP8	6.00	2.604	0.138	0.616	0.555	0.776
OarFCB48	4.00	2.510	0.000	0.602	0.548	1.000
OarCP20	15.00	8.733	0.762	0.885	0.957	0.140
CSSM31	9.00	2.524	0.424	0.604	0.578	0.297
OarVH72	12.00	8.036	0.800	0.876	0.729	0.086
CSSM47	13.00	9.475	0.765	0.894	0.885	0.145
OarFCB128	6.00	2.274	0.500	0.560	0.535	0.107
INRA0063	12.00	3.717	0.500	0.731	0.708	0.316
Mean	7.778	3.986	0.522	0.669	0.63	0.234
SD	0.803	0.588	0.054	0.035	0.04	0.065

Na, Observed number of alleles; Ne, effective number of alleles [Kimura and Crow (1964)]. Expected heterozygosity was calculated by Levene (1949).

In Kajali sheep population, the microsatellite loci analysed were observed to be polymorphic with an average of ≥3 alleles per locus. All the markers were highly informative with PIC value ≥ 0.5, except BM757, BM8125, MAF0247 and OarHH64 which were reasonably informative with PIC values between 0.25 - 0.499 (Bostein *et al*. 1980). Thus, 78% markers were highly informative and 22% were reasonably informative, indicating that these markers are quite useful for the genetic diversity analysis. Seventeen out of eighteen loci had excess of homozygotes.

Within population heterozygote deficit (F_{IS}) values ranged from –0.001 (BM6526) to 1 (OarFCB48) with a mean value of 0.23, thereby exhibiting a significant deficit of heterozygotes. The observed positive F_{IS} in the investigated sheep population might be due to the non-random mating and use of fewer rams for the breeding purpose. However, the high gene diversity estimates observed in the present study does not support inbreeding. As this value is derived from 18 microsatellite markers it is unlikely to be affected by segregation of non amplifying (null) alleles. The existence of population substructure (Wahlund effect) due to sampling from different flocks in different villages of the distribution area appears to be the most probable explanation. The exact reason of this 23% deficit of heterozygotes is difficult to predict due to non availability of pedigree information in the field conditions.

The positive F_{IS} value for Kajali sheep is comparable to those reported previously for most Indian sheep breeds (Arora *et al.* 2011b), except for Ganjam and Chotanagpuri sheep from eastern agro-ecological region and Madgyal from southern peninsular region, other breeds exhibited a positive F_{IS} value. Further the positive F_{IS} values reported in other Indian sheep breeds varied from a minimum of 7.0% in Patanwadi to a maximum of 23.4% in Garole (Arora *et al.* 2011a, Arora *et al.* 2011c).

Bottleneck analysis: Bottleneck hypothesis was explored in Kajali Sheep. According to the hypothesis if the population that has experienced the recent reduction, effective population size exhibit a correlation in reduction of allele numbers and gene diversity. In a population at

Figs 1–2. **1.** Kajali male. **2.** Kajali female.

Table 3. Different test for mutation drift equilibrium in the 18 microsatellite loci in Kajali sheep

Test	Parameter	IAM	TPM	SMM
Sign Test	Observed no. of loci with He excess	12	8	6
	Expected no. of loci with He excess	10.65	10.89	10.67
	p-value	0.35	0.13	0.02*
Standardized difference test	T2 value	0.684	-2.317	-7.911
	p-value	0.246	0.010**	0.000**
Wilcoxon sign rank test	p-value (two tail test for He excess and deficiency)	0.417	0.468	0.007

**P<0.01; *P<0.05.

mutation-drift equilibrium, there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. Allele number if reduces faster than the gene diversity then in such a population experiencing bottleneck, the observed gene diversity higher than the expected gene diversity which is computed from the observed number of alleles, under the assumption of a constant-size (equilibrium) population (Luikart *et al.* 1998). Three different tests, viz. sign rank, standardized differences and Wilcoxon tests under all the 3 models of microsatellite evolution (IAM, SMM and TPM) were employed to investigate whether Kajali sheep has undergone recent bottleneck (Table 3). Most often, gene diversity excess has been demonstrated only for loci evolving under the infinite allele model. If the locus evolves under the strict stepwise mutation model, there can be situations where this gene diversity excess is not observed (Cornuet and Luikart 1996). To check whether a population exhibits a significant number of loci with gene diversity excess three tests viz., sign test, Standardized differences test and Wilcoxon test were conducted. The values for three tests conducted for the Kajali sheep were significant and thereby null hypothesis of mutation drift equilibrium was rejected. Thus, Kajali sheep revealed moderate genetic diversity within population although the population was deviating from mutation drift equilibrium (except IAM), there was no significant heterozygosity excess indicating the absence of genetic bottleneck in the recent past.

Another powerful test of qualitative graphical method based on mode-shift distortion was also utilized to visualize the allele frequency spectra as an indicator for genetic bottleneck. The results indicated the absence of any recent bottlenecks in Kajali sheep and no mode shift (Luikart and Cornuet 1997) was detected in the population (Fig. 3).

Phylogenetic analysis: Microsatellite allele frequency data of nineteen (19) sheep breeds/populations of our country were used to calculate genetic distance estimates and phylogenetic tree (Fig. 4) was constructed using an Unweighted Pair Group method with Arithmetic mean algorithm (UPGMA) Nei *et al.* (1983). The study revealed that breeds from north-western arid and semi-arid region were closely related, as they clustered together. Marwari, Sonadi and Chokla farmed one cluster. Breeds from the southern penissular (Deccani and Madgyal) and eastern region (Garole, Ganjam and Chhotanagpuri) region were separated from rest of the breeds. Kajali sheep under study clustered in same node with Munjal sheep (bootstrap value of 22%), another lesser known mutton type sheep distributed in near vicinity of the breeding tract of Kajali sheep. However, Kajali and Munjal sheep are phenotypically distinct from each other. The low bootstrap value between Kajali and Munjal also indicates low confidence of phylogenetic tree.

The present study revealed considerable genetic diversity in Kajali sheep population in terms of allele number and gene diversity. However, a deficit in heterozygotes (F_{IS}=23%) observed may be attributed to within population sub-structuring. Kajali sheep is well adapted to the native breeding tract and has potential impacts on the regional economy. The results of this study contribute to the

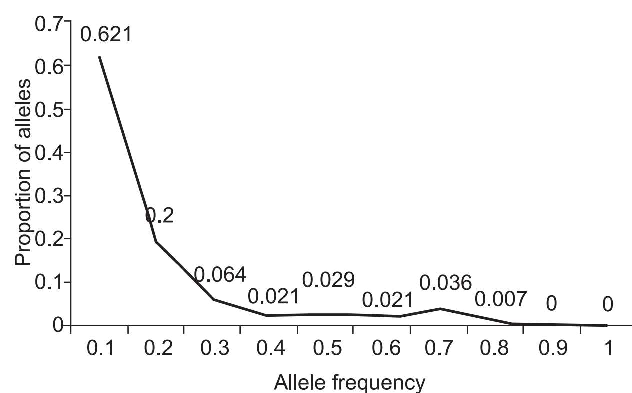

Fig. 3. Mode shift analysis in Kajali sheep.

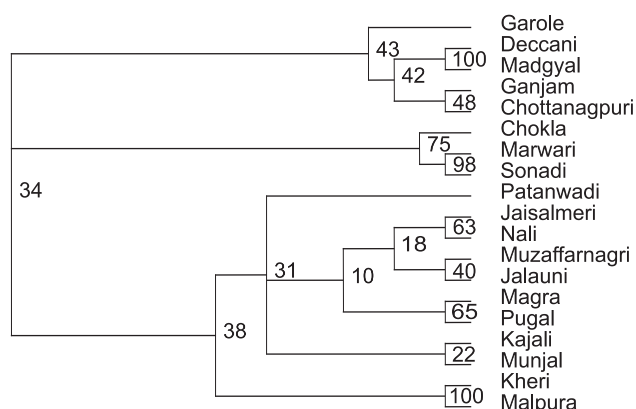

Fig. 4. Phylogenetic analysis by Nei's DA (1983) UPGMA method.

knowledge of genetic diversity available in lesser known ovine germplasm of India and it will help in devising suitable strategies for its genetic improvement, management and its recognition at the national level.

ACKNOWLEDGEMENT

The authors thanks the Director, ICAR- National Bureau of Animal Genetic Resources, Karnal for providing the necessary facilities for the research work. Thanks are also due to the Department of Animal Husbandry, Punjab for their full cooperation during the entire, research duration and survey.

REFERENCES

Arora R, Bhatia S, Mishra B P, Jain A and Prakash B. 2011a. Diversity analysis of sheep breeds from Southern peninsular and Eastern regions of India. *Tropical Animal Health and Production* **43**(2): 401–08.

Arora R, Bhatia S, Mishra B P and Joshi B K. 2011b. Population structure in Indian sheep ascertained using microsatellite information. *Animal Genetics* **42**(3): 242–50.

Arora R, Bhatia S, Yadav D K and Mishra B P. 2011c. Current genetic profile of sheep breeds/populations from Northwestern semi arid zone of India. *Livestock Science* **135**: 193–98.

Bhatia S and Arora R. 2010. Lesser known ovine germplasm- An under evaluated asset of India. *Indian Journal of Animal Sciences* **80**(9): 880–90.

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.

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.

Cornuet J M and Luikart G. 1996. Analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**: 2001–14.

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/ 2005Panels Markers Sheep Goats.pdf.

FAO. 1996. Global Project for the Maintenance of Domestic Animal Genetic Diversity (MoDAD). http://www.fao.org/dad-is/.

Kimura M and Crow J F. 1964. The number of alleles that can be maintained in a finite population. *Genetics* **49**: 725–38.

Levene H. 1949. On a matching problem in genetics. *Annals of Mathematical Statistics* **20**: 91–94.

Livestock census (Nineteenth). 2012. Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India.

Luikart G, Allendorf F W, Cournuet J M and Sherwin W B. 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity* **89**: 238–47.

Luikart G and Cornuet J M. 1997. Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conservation Biology* **12**: 228–37.

Mishra A K, Raja K N, Vohra V, Singh S and Singh Y. 2016. Phenotypic traits and performance of Kajali Sheep: a lesser known ovine genetic resource of Punjab, India. *Indian Journal of Animal Sciences* **86**(11): 1279–82.

Mishra A K, Raja K N, Vohra V, Singh S and Singh Y. 2017. Principal component analysis of the biometric traits to explain body conformation in Kajali sheep of Punjab, India. *Indian Journal Animal Sciences* **87**(1): 93–98.

Peakall R and Smouse P E. 2005. GENALEX-6.5: Genetic Analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Note* **6**: 288–95.

Piry S, Luikart G and Cornuet J M. 1999. Bottleneck: A computer programme for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**: 502–03.

Sambrook J, Fritsch E F and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Singh S, Raja K N, Ganguly I, Arora R and Kataria R S. 2015. Genetic characterization of Koraput sheep of Odisha. *Indian Journal of Animal Sciences* **85**(6): 667–69.

Yeh F C, Yang R C and Boyle T. 1999. POPGENE 32 - Version 1.31. Population genetics software. http.//www.ualberta.ca/~fyeh/fyeh.