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Elucidating the genetic diversity using SSR based markers in Gojri buffalo

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ABSTRACT

A population based study was conducted in a large sample of Gojri buffalo, a less known dairy buffalo from Northern India, to assess its genetic variations using 25 heterologous simple sequence repeats (SSR) marker loci. Primers for markers used in the study were labelled either with VIC, NED, PET or FAM dye. Genotyping of each sample was performed by sequencing the PCR amplicons and thereby, estimating diversity indices based on frequency of different allele sizes. Gojri buffalo had an average of 8.2 alleles per locus with 3.65 mean effective number of locus. The polymorphic information content (PIC) values for studied SSR markers ranged from 0.11–0.81, indicating that all the markers, except ILSTS 19, were informative and suitable for the diversity analysis in the buffalo population. The average observed heterozygosity (Ho) and unbiased expected heterozygosity (uHe) estimate were 0.67 and 0.70, respectively in the population with majority of the markers showing Hardy Weinberg equilibrium. A higher expected heterozygosity in Gojri population indicates presence of sufficient genetic diversity, and a higher overall mean of Shannon's information index (1.5) support these findings. Moreover, both genetic Bottleneck and Mode Shift analysis indicated absence of genetic bottleneck in the recent past among the studied Gojri population. Population inbreeding estimates (F_{IS}=0.029) indicated an average deficiency of 2.9% and suggests no probable inbreeding in the population. It can be concluded that there is presence of sufficient genetic variations in Gojri population and this information can augment in designing its breeding and conservation programme.

Key words: Characterization, Genetic diversity, Gojri buffalo, Microsatellite, SSR markers

Elucidating genetic variations in a population can help to delineate the genetic differences that exist within a population or are acquired through evolutionary forces and natural selection. Indian buffaloes (*Bubalus bubalis*) have rich genetic diversity which is reflected in 15 recognized breeds. Although a sufficient genetic diversity has been reported among the major buffalo breeds (Tantia *et al*. 2006, Vijh *et al*. 2008, Mishra *et al*. 2009, Kathiravan *et al*. 2009) of India, still it is interesting to generate information about the genetic structure of lesser known buffalo populations particularly those which are having good dairy potential, and to interpret these meaningful genetic variations which could contribute to evaluation of genetic relationships and diversity among the Indian buffaloes. One such less known buffalo population has been identified as Gojri buffaloes having their breeding tract in Punjab and Himachal Pradesh (Vohra *et al*. 2012). Simple sequence repeats or microsatellites as coined by Litt and Luty (1989) are highly polymorphic and are presently the most favoured molecular markers having numerous advantages over the other type of genetic markers. Microsatellite markers can be successfully used to explore genetic diversity among Indian bovines, and in the past they have also been effectively exploited to understand bovine domestication and migration pattern (Loftus *et al*. 1994, Acousta *et al*. 2012, Vohra *et al*. 2017). Determination of genetic variation in a population and its comparative evaluation with related populations can help in meticulous planning of its breeding programme and in designing strategies for their conservation.

In this study an attempt was made to explore the population structure and estimate within population genetic variations in Gojri buffaloes for the first time using specific SSR markers recommended for diversity analysis (FAO 2011).

MATERIALS AND METHODS

Population studied and sample size: Gojri buffalo derive its name from the Gujjar community, who are responsible for rearing and maintaining this germplasm. Gojri buffaloes primarily bred in Punjab and Himachal Pradesh. Mohali, Roopnagar, Pathankot districts of Punjab and Nurpur, Jassur, Chawari, Jyot, Sahu, Rakh, Bharmaur and Tissa areas in Kangra and Chamba division of Himanchal Pradesh is considered its home tract. Physical appearance of Gojri

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buffaloes include black skin colour with brown hairs, white patches are present on forehead of some animals. Horns are medium to large sized (Fig. 1) with curved orientation which moves backwards and then towards front to complete the loop (Vohra *et al*. 2012). A total of 48 random blood sample were collected from Gojri buffaloes, sampling was done such that the entire native tract of this population is covered. Care was taken that no two related individuals were included in sampling, i.e. all the individual had different sire and dam and were genetically unrelated as far as possible. Genomic DNA was isolated from the blood samples using phenol-chloroform extraction method as described by Sambrook and Russel (2001). The purity of genomic DNA was checked by spectrophotometer. Genomic DNA samples having the $OD_{260/280}$ ratio in the range of 1.7–1.9 were used for further diversity analysis.

Fig. 1. Adult Gojri buffalo.

PCR amplification and SSR genotyping: Polymerase chain reaction based amplification was carried out using fluorescent labelled primers. Forward primer of each microsatellite marker was 5'-labelled with either FAM (Blue), VIC (Green), NED (Yellow) or PET (Red) fluorescence tags. Details of the SSR markers used in the study are presented in Table 1. PCR amplification of each SSR loci was carried out in 15 µl reaction volume consisting of 200 µM of each dNTP, 5 Pm of each primer, 1.5 Mm $MgCl_2$ and 1.2 U Taq polymerase. Amplification was performed using MASTERCYCLER EP with initial denaturation at 95°C for 2 min followed by 30 cycles of 94°C for 60 sec, annealing temperature (Table 1) for 60 sec and a final extension for 10 min at 72ºC. After completion of PCR, amplification was confirmed by running a small aliquot of PCR product on 1.5% agarose gel. About 0.5 µl of PCR amplicons were mixed with 9.0 µl of Hi Di formamide and 0.5 µl Liz standard and analyzed on automated DNA analyzer.

Statistical analysis: Allele sizing for different DNA fragments was carried out utilizing the GeneScan software (version 5.0, Applied Bio system). Diversity indices and within breed diversity were estimated using POPGENE 32 software (Yeh *et al*. 1999) and GenAlEx V5.0 (Peakall and Smouse 2001). Allele frequencies estimated were utilized for assessing polymorphic information content (PIC) as per Botstein *et al*. (1980). To compute unbiased estimates of exact probabilities (P value) for departure from Hardy-Weinberg equilibrium among the microsatellite loci was also investigated. Genetic bottleneck tests for detecting departures from expectations under mutation-drift equilibrium, the two different approaches were followed. In first approach, the test was conducted using Bottleneck 1.2.01 software (Piry *et al*. 1999) based on three tests namely sign test, standardized differences test and a Wilcoxon sign-rank test and probability distribution was established using 1,000 simulations under three models, viz. infinite allele (IAM), stepwise mutation (SMM) and two-phase mutation model (TPM). Second approach to bottleneck analysis was based on graphical representation of mode-shift equilibrium.

RESULTS AND DISCUSSION

Genetic variation and diversity analysis of a livestock population is a crucial step in its characterization, as it allows the assessment of unexplored genetic variability and structure of a population. Assessment of genetic variability present in a dairy breed or population is a basic and a mandatory step in designing its genetic improvement programs and framing its conservation priorities. Present study explored genetic variations in detail for Gojri buffalo population using SSR markers.

SSR marker and its polymorphism information content (*PIC*): All marker loci were evaluated according to their PIC information and all the 25 markers exhibited sufficiently high level of polymorphism. The overall mean PIC was 0.65 while it ranged from 0.81 (CSSM57) to 0.11 (ILSTS19). PIC value greater than 0.50 is considered highly informative whereas a value between 0.25–0.50 gives reasonably informative markers, while markers with less than 0.25 are slightly informative (Bostein *et al.* 1980). The overall mean PIC value was comparable with the reports of Martinez *et al*. (2006) in Murrah buffaloes. It was reported slightly higher in Nagpuri buffaloes by Kataria *et al*. (2009) and Bhuyan *et al*. (2010).

Estimation of genetic diversity indices: A total of 207 alleles in the population. The average number of alleles per locus was 8.2 and out of 25 studied markers, two (ILSTS019 and ILSTS033) revealed low allelic diversity with less than 5 alleles (Table 2). The mean number of alleles (MNA) in population over a range of loci is considered to be a reasonable indicator of allelic variation. The MNA per locus in our study was comparable to the reports of Kathiravan *et al*. (2010) in South Kanara buffaloes; Marques *et al.* (2011) in Brazilian buffaloes; Martinez *et al.* (2006) and Bhuyan *et al.* (2010) in Murrah buffaloes. Barker *et al.* (1997) reported that the number of alleles detected, increases with the increase in sample size, therefore larger the sample size higher is the MNA in the population. The overall mean observed heterozygosity value in our study was 0.67 and ranged from 0.12 (ILSTS 19) to 0.95 (ILSTS 52). This indicated that for ILSTS19 locus almost all the animals (88%) were homozygous, carrying identical SSR whereas for ILSTS52 locus 95.8% animals were heterozygous in

Table 1. SSR markers, primer sequence, allele size, chromosomal location, dye (fluorescence tags) and annealing temperature used for genetic diversity analysis in Gojri buffalo population

SSR marker	Sequence	Allele size (bp)	Chromosome location	Dye	Annealing temperature (ºC)
BM1818	For-5'-AGCTGGGAATATAACCAAAGG-3' Rev-5'-AGTGCTTTCAAGGTCCATGC-3'	229–279	28	FAM	56
CSRM060	For-5'-AAGATGTGATCCAAGAGAGAGGCA-3' Rev-5'-AGGACCAGATCGTGAAAGGCATAG-3'	92–136	10	VIC	55
CSSM019	For-5'-TTGTCAGCAACTTCTTGTATCTTT-3' Rev-5'-TGTTTTAAGCCACCCAATTATTTG-3'	131–161	01	NED	55
CSSM033	For-5'-CACTGTGAATGCATGTGTGTGAGC-3' Rev-5'-CCCATGATAAGAGTGCAGATGACT-3'	149–175	17	PET	65
CSSM045	For-5'- TAGAGGCACAAGCAAACCTAACAC-3' Rev-5'-TTGGAAAGATGCAGTAGAACTCAT-3'	102–122	02	FAM	60
CSSM047	For-5'-TCTCTGTCTCTATCACTATATTGC-3' Rev-5'-CTGGGCACCTGAAACTATCATCAT-3'	127–162	03	NED	55
CSSM057	For-5'-GTCGCTGGATAAACAATTTAAAGT-3' Rev-5'-TGTGGTGTTTAACCCTTGTAATCT-3'	102–130	09	FAM	60
ETH003	For-5'-GAACCTGCCTCTCCTGCATTGG-3' Rev-5'-ACTCTGCCTGTGGCCAAGTAGG-3'	96–192	03	NED	65
HEL013	For-5'-TAAGGACTTGAGATAAGGAG-3' Rev-5'-CCATCTACCTCCATCTTAAC-3'	158–198	11	VIC	55
ILSTS019	For-5'-AAGGGACCTCATGTAGAAGC-3' Rev-5'-ACTTTTGGACCCTGTAGTGC-3'	169–185	29	VIC	55
ILSTS025	For-5'-GTTACCTTTATATAAGACTCCC-3' Rev-5'-AATTTCTGGCTGACTTGGACC-3'	110–144	11	FAM	56
ILSTS026	For-5'-CTGAATTGGCTCCAAAGGCC-3' Rev-5'-AAACAGAAGTCCAGGGCTGC-3'	131–153	02	NED	56
ILSTS028	For-5'-TCCAGATTTTGTACCAGACC-3' Rev-5'-GTCATGTCATACCTTTGAGC-3'	143–173	03	PET	55
ILSTS029	For-5'-TGTTTTGATGGAACACAGCC-3' Rev-5'-TGGATTTAGACCAGGGTTGG-3'	140–180	21	PET	55
ILSTS030	For-5'-CTGCAGTTCTGCATATGTGG-3' Rev-5'-CTTAGACAACAGGGGTTTGG-3'	146–158	02	PET	55
ILSTS033	For-5'-TATTAGAGTGGCTCAGTGCC-3' Rev-5'-ATGCAGACAGTTTTAGAGGG-3'	126-138	12	FAM	58
ILSTS036	For-5'-GAGTATTATGCTTGGGAGGC-3' Rev-5'-AGACAGGATGGGAAGTCACC-3'	122–172	14	NED	55
ILSTS052	For-5'-CTGTCCTTTAAGAACAAACC-3' Rev-5'-TGCAACTTAGGCTATTGACG-3'	135–179	02	PET	55
ILSTS056	For-5'-GCTACTGAGTGATGGTAGGG-3' Rev-5'-AATATAGCCCTGGAGGATGG-3'	132–178	17	PET	56
ILSTS058	For-5'-GCCTTACTACCATTTCCAGC-3' Rev-5'-CATCCTGACTTTGGCTGTGG-3'	118–182	17	NED	55
ILSTS060	For-5'-TAGGCAAAAGTCGGCAGC-3' Rev-5'-TTAAGGGGACACCAGCCC-3'	150–204	19	VIC	56
ILSTS061	For-5'-AAATTATAGGGGCCATACGG-3' Rev-5'-TGGCCTACCCTACCATTTCC-3'	109–165	23	FAM	61
CSSM066	For-5'-ACACAAATCCTTTCTGCCAGCTGA-3' Rev-5'-AATTTAATGCACTGAGGAGCTTGG-3'	142–210	29	VIC	55
ILSTS089	For-5'-AATTCCGTGGACTGAGGAGC-3' Rev-5'-AAGGAACTTTCAACCTGAGG-3'	106–144	12	FAM	55
ILSTS095	For-5'-GAAAGATGTTGCTAGTGGGG-3' Rev-5'-ATTCTCCTGTGAACCTCTCC-3'	187–219	11	VIC	58

nature. The overall mean observed heterozygosity values reported were lower, 0.476 in Murrah buffaloes by Martinez *et al*. (2006), 0.45 in Nagpuri buffaloes by Kataria *et al*. (2009), 0.487 in Chilika buffalo by Mishra *et al*. (2009), 0.441 in Brazilian buffalo by Marques *et al*. (2011), and 0.46 in Cuban water buffaloes by Acousta *et al*. (2012). In the Gojri buffaloes, the expected heterozygosity ranged from 0.11 (ILSTS19) to 0.83 (CSSM57). The highest expected heterozygosity reflected presence of more variation in Gojri population. The observed mean expected heterozygosity values in Gojri buffaloes were comparable to that of Murrah buffalo reported by Kumar *et al*. (2006)

Table 2. Genetic diversity indices estimate for each SSR locus in Gojri buffalo population

Locus	N	Na	Ne	I	Ho	He	uHe	F_{IS}	PIC
BM1818	41	6	3.386	1.421	0.341	0.705	0.713	0.515	0.662
CSRM60	48	8	2.559	1.226	0.875	0.609	0.616	–0.436	0.546
CSSM19	48	6	3.648	1.537	0.729	0.726	0.734	–0.004	0.695
CSSM33	46	7	2.685	1.351	0.609	0.628	0.634	0.030	0.599
CSSM45	31	6	3.566	1.467	0.581	0.720	0.731	0.193	0.677
CSSM47	46	11	5.364	1.951	0.783	0.814	0.823	0.038	0.793
CSSM66	33	9	5.089	1.823	0.667	0.803	0.816	0.170	0.777
CSSM57	48	11	6.202	2.003	0.896	0.839	0.848	–0.068	0.819
ETH003	46	16	2.633	1.566	0.522	0.620	0.627	0.159	0.595
HEL13	46	8	5.062	1.782	0.761	0.802	0.811	0.052	0.775
ILSTS19	48	3	1.134	0.262	0.125	0.118	0.120	–0.057	0.113
ILSTS25	41	8	3.420	1.455	0.634	0.708	0.716	0.104	0.666
ILSTS26	33	6	4.094	1.524	0.788	0.756	0.767	–0.043	0.715
ILSTS28	47	6	4.072	1.522	0.809	0.754	0.763	–0.072	0.713
ILSTS29	32	10	5.057	1.896	0.750	0.802	0.815	0.065	0.781
ILSTS30	47	6	3.174	1.430	0.830	0.685	0.692	–0.212	0.650
ILSTS33	48	3	2.429	0.962	0.542	0.588	0.595	0.079	0.503
ILSTS36	48	12	3.483	1.641	0.750	0.713	0.720	–0.052	0.680
ILSTS52	48	8	3.623	1.580	0.958	0.724	0.732	–0.324	0.693
ILSTS56	44	8	2.411	1.261	0.523	0.585	0.592	0.107	0.555
ILSTS58	47	11	4.244	1.809	0.723	0.764	0.773	0.054	0.742
ILSTS60	47	13	2.410	1.413	0.660	0.585	0.591	–0.127	0.558
ILSTS61	48	11	4.092	1.802	0.750	0.756	0.764	0.007	0.733
ILSTS89	46	6	4.000	1.508	0.804	0.750	0.758	–0.072	0.709
ILSTS95	30	8	3.340	1.507	0.267	0.701	0.712	0.619	0.656
Mean	43.48	8.28	3.647	1.508	0.67	0.69	0.70	0.029	0.656

Sample size (N), number of alleles (No-Observed, Ne-Effective), Shannon's index (I), heterozygosity (Ho-observed, He–expected, uHe–unbiased estimate of expected), polymorphism information content (PIC), within-population inbreeding estimates (F_{IS}).

and Nagpuri buffaloes by Kataria *et al*. (2009). The locus CSSM57 showed highest heterozygosity and was found to be polymorphic loci. The least polymorphic loci (ILSTS 19) exhibited low heterozygosity, supporting the fact that genetic diversity depends on allelic variations. The overall mean Shannon's information index was 1.5 and it ranged from 0.26 (ILSTS19) to 2.0 (CSSM57), thus showing higher gene diversity in the population.

In our study, Gojri buffaloes revealed the presence of sufficient genetic diversity within the population. The mean within-population inbreeding estimates (F_{IS}) was 0.029 for Gojri buffalo indicated an average deficiency of 2.9%. $F_{IS.}$ ranged from –0.436 (CSRM 60) to 0.61 (ILSTS95) and 11 of 25 loci had negative F_{IS}. The markers loci were tested individually for Hardy-Weinberg equilibrium. Sixteen out of 25 loci were nonsignificant, while 9 loci (BM1818, CSRM60, CSSM66, ILSTS26, ILSTS33, ILSTS36, ILSTS60, ILSTS95 and ETH003) deviated significantly from Hardy-Weinberg equilibrium. It is, though, difficult to predict the exact basis of this departure, however, the presence of low frequency null alleles segregating at these loci may be a possible reason or these markers (or loci) could be under selection pressure. On the contrary, Vijh *et al*. (2014) studied HWE at 22 loci tested in 12 populations for deviation and found that only 2 loci (BM1818 and ILSTS38) were not in the HWE among Indian water buffaloes.

Genetic bottleneck analysis: Test for genetic bottleneck did not show any significant reduction of effective population size in the recent past. The graphical presentation of mode shift analysis (Fig. 2) showed the distribution of allelic frequencies with respect to proportion of alleles in Gojri buffaloes and the population followed a normal L-shaped curve suggesting that the population did not encounter genetic bottleneck in the recent past. The proportion of alleles having high frequency in the population were minimal. Similar observation of no bottleneck had been reported in Banni buffalo breeds by Mishra *et al*. (2009). Three different tests namely Sign test, Standardized differences test and a Wilcoxon sign-rank test, were used

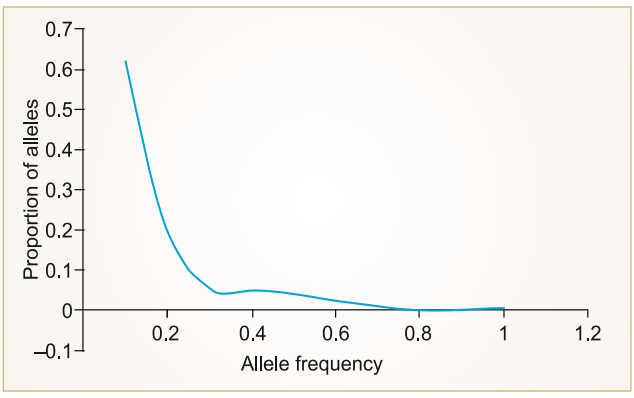

Fig. 2. Mode shift analysis in Gojri buffalo.

to test for the departure from mutation drift equilibrium based on heterozygosity excess or deficiency. According to mutation drift equilibrium tested in Gojri buffalo (Table 3), based on P value, the sign test showed that IAM, TPM models were significant; in standardized difference test, SMM and TPM models were significant; and through Wilcoxon sign rank test, IAM model was significant. On the basis of these tests conducted in Gojri buffalo we can conclude that null hypothesis of mutation-drift equilibrium cannot be accepted.

Gojri is a unique and structured buffalo population of Northern India and possess sufficient genetic diversity within population with an average deficiency of 2.9%, did not show any genetic bottleneck in the recent past, and population is free from inbreeding effect. The information generated shall elucidate and contribute to the knowledge of genetic diversity present in lesser known dairy buffalo populations of the country and shall stem the future basis for comparative diversity analysis in Indian buffalo populations. Further, this shall help policy planners in designing breeding and conservation programmes for Gojri buffalo.

Table 3. Different test for mutation drift equilibrium in the 25 microsatellite loci in Gojri buffalo

Test	Parameter	IAM	TPM	SMM
Sign test	Observed no. of loci with He excess	14.72	14.83	14.80
	Expected no. of loci with He excess	19	6	13
	P value	0.05*	–4.29	–12.73
Standardized difference test	T_2 value	0.60	–4.29	–12.73
	P value	0.27	0.00**	0.00**
Wilcoxon sign rank test	P value (one tail test for He excess)	0.04*	0.99	0.85

**P<0.01.

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