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Genetic diversity status of only registered cattle breed of Chhattisgarh–Kosali

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ABSTRACT

Assessment of diversity is essential for germplasm characterization and management. Kosali is the first and only registered breed of Chhattisgarh state which immensely contributes to the local economy of 70% farmers. Genomic microsatellite markers being valuable tool for estimating genetic diversity were selected for exploring existing genetic variability in Kosali cattle population. The standard metrics of genomic diversity detected high variability in this breed. All the loci were polymorphic resulting in 297 alleles. Mean values of observed and expected number of alleles were 11.423±0.877 and 4.989±0.372, respectively. Similarly, higher mean values of observed heterozygosity (0.693±0.031) also corroborated with the allelic diversity. Mean expected heterozygosity (0.765±0.02) under Hardy-Weinberg equilibrium (HWE) was higher than the observed values indicating some deviations from assumptions of the model. It can be attributed to the forces such as inbreeding. In fact a positive F_{IS} value of 0.088±0.038 indicated some heterozygote deficiency in the population. Bottleneck analysis indicated that Kosali cattle have not suffered any population bottleneck event during evolutionary trajectory. This study is first to report the genetic diversity status of Kosali cattle based on microsatellite markers.The results imply the necessity of management programs in order to conserve the existing genetic variation and to avoid any escalation of inbreeding.

Keywords: Cattle, Chhattisgarh, Genetic diversity, Inbreeding, Kosali cattle, Microsatellite, Mode shift analyses

India emerges out to be the cattle king with a cattle population of 192.49 million (20th Livestock Census 2019). Indian zebu cattle have evolved over the centuries through domestication events and natural selection resulting in vast diversity of breeds (Pandey *et al.* 2006). Management of our native germplasm has become a major challenge as most of our breeds are low producers. These populations are facing genetic dilution due to multiple factors like increasing mechanization in agriculture, over emphasis on some high producing breeds, market forces and many unforeseen factors in different parts of the country. Thus, there is a need to develop an action plan for conservation and sustainable utilization of indigenous cattle breeds by utilizing available technologies for their management.

Characterization and registration is primarily required to prevent loss of unique gene pool and to preserve maximum amount of genetic diversity. Evaluation of genetic variability emerges out as a fundamental element in working out breeding strategies and genetic conservation plans (Sharma *et al*. 2006). Microsatellites are extremely useful in conservation genetics because of the high degree of polymorphism, which makes them informative and provides great discriminating power (Sharma *et al.* 2018, Karsli and Balcioglu 2019). Microsatellites have become markers of choice to evaluate the genetic diversity and study relationship in Indian cattle breeds (Sodhi *et al*. 2005, Sharma *et al*. 2013, Prakash and Deepika 2014, Sharma *et al*. 2015, Radhika *et al*. 2017, Joshi *et al*. 2018).

Currently India has 50 registered breeds of cattle (www.nbagr.res.in) with Kosali being 36th recognized breed (Accession No.INDIA_CATTLE_2600_KOSALI_03036). It is the first and only recognized breed from Chhattisgarh. Estimated population of Kosali cattle is around 31.32 lakhs (Jain *et al*. 2018). Kosali is small sized, draft purpose cattle breed. Kosali cattle was phenotypically characterized and registered as a breed in 2012 using the standard methodology developed by ICAR-NBAGR, Karnal, Haryana, India. However, no information is reported on genetic characterization so far. Therefore, this study reports diversity estimates of Kosali population using bovine microsatellite markers recommended by Food and Agricultural Organization (FAO/ISAG 2004).

MATERIALS AND METHODS

Breed distribution, characteristics and sampling: Topologically, Chhattisgarh is divided in three agro-climatic conditions, the Northern hills (5 districts), the Central plains (15 districts) and the Bastar pleatu (7 districts). Kosali breed

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is mostly concentrated in the central plain region which accounts for 50% of total geographical area of this state. Breeding tract lies in between 19.8 to 22.7 degrees north latitude and 80.3 to 83.6 degrees east longitude.

Skin colour of Kosali animals is mainly brownish, blackish white and grayish black. Coat colour is predominantly red (54%) followed by white (36%), black and other mixtures (10%). Mostly observed colour of muzzle, eyelash and tail is black. Short, glossy and straight hairs are predominantly found. Horn shape is slightly inward curved but stumped. Ears are horizontal being parallel to the ground (Fig. 1).

Fig. 1. Kosali cattle.

Blood samples were collected from Kosali cattle in their home tract following the guidelines of MoDAD (Measurement of Domestic Animal Diversity) programme (FAO 1995). Blood samples (5–6 ml) from jugular vein of 48 unrelated animals were collected in vacutainer containing Ethylene diamine tetra acetic acid (0.5 mM, *p*H 8.0) as anticoagulant. Care was taken to select unrelated Kosali animals true to the breed characteristics.

DNA extraction, quantification and amplification: Genomic DNA was isolated using standard protocol of proteinase K digestion followed by phenol-chloroform-isoamyl alcohol (25 : 24 : 1) extraction (Sambrook *et al.* 1989) and ethanol precipitation. Quality and quantity of extracted DNA was checked in 0.8% agarose gel and Nanodrop spectrophotometer, simultaneously. Concentration of DNA was adjusted to 50 ng/µL, and quality of the product was evaluated by determining the 260/280 nm absorbance ratios, which were > 1.8. Genomic DNA samples and working DNA samples were stored at –20°C and 4°C respectively.

Microsatellite genotyping: A total of 26 unlinked bovine microsatellite loci, selected from the list of microsatellite markers recommended for diversity estimation for cattle population by FAO/ISAG (www.fao.org/3/a-aq569e.pdf) were used in this study. The 5′ end of each primer was labeled either with FAM, VIC, NED or PET fluorescent dyes to facilitate multiplexing for allele genotyping on automated DNA sequencer. Polymerase chain reaction (PCR) was carried out in 10 µl reaction volume which consisted of 10–50 ng of DNA, 2.5 pM of each primer and Dream Taq Green PCR maser mix consisting of 0.2 mM of each dNTP and 2 mM of $MgCl_2$. The amplification protocol consisted of initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, annealing at specific temperature for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were checked on 2% agrose gel having ethidium bromide (0.5 mg/ml) by visualization under ultraviolet light. PCR products belonging to a panel were multiplexed according to band intensity. The genotyping reaction consisted of 1 µl of PCR product, 8.9 µl of Hi-Di formamide and 0.1 µl of GeneScan-500 LIZ size standard. Subsequently, genotyping was carried out on an automated ABI-3100 DNA sequencer.

Data analysis: Allele sizing was done using GeneMapper^{TM} software v3.7. Basic genetic parameters including allele frequencies, observed (Na) and effective number of alleles (Ne), observed (Ho) and expected heterozygosity (He) and heterozygote deficit (F_{IS}) in the whole population were calculated by analyzing the genetic data with GenAlEx 6.2 software (Peakall and Smouse 2008). Hardy-Weinberg equilibrium (HWE) and Ewens-Watterson Neutrality analysis were performed using POPGENE 1.31 version (Yeh *et al.* 1999). Bottleneck software version 1.2.02 (http://www.ensam.inra.fr/URLB) was selected to test the bottleneck hypothesis under a stepwise-mutation model (SMM), two-phased model of mutation (TPM), and infinite allele model (IAM). This method is used for testing the departure from mutation-drift equilibrium based on heterozygosity excess or deficiency. For the analyses, 1,000 iterations were used, with results over loci extracted from three different tests like sign test, standardized difference test and Wilcoxon rank test developed by Cornuet and Luikart (1996). The method of graphical presentation involving mode shift indicator was used to detect presence of skewed distributions of allele frequencies in the population (Luikart *et al.* 1998).

RESULTS AND DISCUSSION

Six panels of 26 microsatellite (SSR) markers successfully described the genetic variability within the only registered breed of Chhattisgarh. All microsatellite loci selected for estimating diversity were polymorphic. A large number of alleles were observed across them which reflected in the sum total of 297 alleles. Independent assortment of selected loci was considered as no significant linkage disequilibrium was detected at the 26 genotyped loci on the basis of an exact test for genotypic linkage disequilibrium.

FAO guidelines for molecular characterization of genetic resources recommend a minimum of 4 distinct alleles per locus for proficient judgment of genetic differences between breeds (FAO 2011). All the 26 microsatellites used in this study corroborated with this recommendation as all were having at least 5 alleles (Table 1). They manifested sufficient polymorphism for evaluating genetic variation within a breed and exploring genetic differences between breeds. CSSM33 showed the highest number of observed alleles per locus (20) while TGLA227 and ILSTS05 showed the lowest (5). High mean observed number of alleles (11.423±0.877) pointed towards the high diversity existing

Table 1.Standard metrics of genomic diversity and allele size range observed in Kosali cattle

Locus	N	Allele size range (bp)	Na	Ne	I
CSSM66	43	151–207	15	4.759	1.991
ETH10	43	195–217	8	2.645	1.230
ILSTS06	42	279–303	9	4.117	1.615
TGLA122	43	133–179	16	7.953	2.345
TGLA227	43	83–89	5	1.775	0.883
BM1824	44	180–194	6	2.890	1.295
CSSM60	44	92–114	9	3.959	1.712
ILSTS11	42	259–269	7	2.811	1.290
INRA05	43	136–144	6	4.340	1.552
INRA63	43	180–188	6	2.599	1.273
ETH03	39	62–142	16	5.895	2.266
HEL05	42	119–195	14	4.489	1.836
ILSTS33	42	121–165	16	6.093	2.148
INRA35	40	100–150	9	4.451	1.693
ILSTS05	42	182–190	5	4.366	1.526
CSSM08	43	180–202	12	3.683	1.621
CSSM33	43	132–188	20	7.769	2.465
ETH225	42	136–168	12	7.946	2.216
TGLA53	43	140–184	17	4.543	2.091
CSSM45	42	50–114	16	6.798	2.212
HEL09	37	140–168	11	7.186	2.137
ILSTS54	42	126–144	9	5.297	1.789
MM08	42	104–142	12	5.870	2.043
MM12	41	102–126	9	3.242	1.568
HEL01	43	42–116	13	5.383	1.995
ILSTS34	42	114–202	19	8.842	2.525
Mean	42.115		11.423	4.989	1.820
SE	0.295		0.877	0.372	0.083

Na, No. of different alleles; Ne, No. of effective alleles = 1/(Sum pi^2); I, Shannon's information index = –1* Sum (pi*Ln (pi); where pi is the frequency of the i^{th} allele for the population.

in the population. Expected number of alleles varied from 1.775 (TGLA227) to 8.842 (ILSTS34) with a mean of 4.989±0.372. Allelic diversity of similarly higher magnitude had been reported for cattle breeds of Odisha; Motu (10.04) by Pandey *et al*. (2011) and Bhinjharpuri (11.43) and Ghumusari (12.19) by Prakash and Deepika (2014). Most of the other Indian cattle breeds have lower values for mean observed number of alleles. Sharma *et al*. (2015) reported allelic diversity of several breeds such as Gangatiri (9.190), Gaolao (9.143), Hariana (6.57), Kenkatha (9), Kherigarh (9.23), Mewati (7.76), Ongole (7.66) and Ponwar (8.85). Similarly, lower allele diversity was also reported for Deoni (5.82) and Red Kandhari (5.86) by Sodhi *et al*. (2005); Purnea (8.87) by Sharma *et al*. (2013); Vechur (8.46) by Radhika *et al*. (2017) and Nagori (8.2) by Joshi *et al*. (2018). Indigenous cattle breeds that were analyzed using automatic DNA sequencer have only been compared to rule out any discrepancy arising due to the difference in technique of genotyping.

Based on the Shannon's Index (I), once again higher diversity status was assigned to Kosali population which ranged from 0.883 (TGLA227) to 2.525 (ILSTS34) with a mean value of 1.820±0.083. The Shannon's Index is an information statistic index, which assumes all types are represented in a sample and that they are randomly sampled. It combines both evenness and richness in a single measure (Moges *et al.* 2016). All the markers except TGLA227 had I values of more than 1, so they can be potentially used for diverse genetic applications including linkage mapping, individual identification and parentage testing.

Estimated mean values for observed (Ho) and expected (He) heterozygosity were 0.693±0.031 and 0.765±0.02, respectively (Table 2). Ho values ranged from 0.349 (CSSM08) to 0.952 (HEL05) whereas, He was between 0.437 (TGLA227) and 0.887 (ILSTS34). The average observed heterozygosity was to the tune of values reported for several other Indian cattle breeds, viz. Motu (0.660) by Pandey *et al*. (2011); Ghumusari (0.683) by Prakash and Deepika (2014); Ponwar (0.696) and Purnea (0.688) by Sharma *et al*. (2015). Whereas, it was higher than values reported for several other Indian cattle breeds, viz. Deoni (0.57) and Red Kandhari (0.47) by Sodhi *et al*. (2005); Golao (0.616), Hariana (0.604), Mewati (0.579) and Ongole (0.459) by Sharma *et al*. (2015); Vechur (0.621) by Radhika *et al*. (2017) and Nagori (0.61) by Joshi *et al*. (2018). It can very well be concluded that Kosali cattle had substantial genetic variation based on its gene diversity in addition to the average number of alleles per locus. Gene diversity is composed of two elements, the number of alleles and the abundance of the alleles. Accordingly, higher value of mean expected heterozygosity was observed in Kosali cattle population.

Observed heterozygosity was lower than that expected for a population under Hardy-Weinberg equilibrium (HWE) and hence, possibility of inbreeding. Significant deviation from HWE was indeed observed at 11 loci at $P<0.001$ (Table 2). Various factors in a population can lead to deviation from HWE which can be systematic forces such as selection, migration and mutation, and dispersive forces such as genetic drift and inbreeding. Heterozygote deficiency in the population was also reflected in the positive F_{IS} value (0.088) which ranged from –0.225 to 0.549. The value of F_{IS} can range from –1 (all individual heterozygotes) to +1 (no observed heterozygotes) measuring the mean reduction in heterozygosity of an individual due to non-random mating within a population (Tantia *et al*. 2018). A small but positive value of F_{IS} indicated occurrence of sufficient heterozygotes in the population, at present. However, it also indicated the need of scientific management of cattle breeding so as to avoid further escalation in inbreeding magnitude. Negative in breeding coefficient was observed at 10 loci for Kosali cattle out of 26 investigated loci, indicating possibility of out breeding. An overall heterozygote deficiency of 8.8% exists in Kosali cattle population which is much lower than many Indian cattle breeds. Higher values of heterozygote deficiency had been reported for Red Kandhari (27.8%), Deoni (17.9%) by Sodhi *et al*. (2005) and Gaolao (14.6%), Ongole (22.1%) by Sharma *et al*. (2015). Odisha cattle

Table 2. Heterozygosity statistics for all loci in Kosali cattle along with Hardy Weinberg Equilibrium test

Locus	N	Ho	He	UHe	F_{IS}	ChiSq	Probability	Significance
CSSM66	43	0.837	0.790	0.799	–0.060	167.314	0.000	***
ETH10	43	0.651	0.622	0.629	–0.047	59.704	0.000	***
ILSTS06	42	0.500	0.757	0.766	0.340	46.470	0.113	NS
TGLA122	43	0.814	0.874	0.885	0.069	78.885	0.999	NS
TGLA227	43	0.512	0.437	0.442	–0.172	17.974	0.055	NS
BM1824	44	0.614	0.654	0.661	0.062	12.273	0.658	NS
CSSM60	44	0.773	0.747	0.756	–0.034	65.129	0.002	**
ILSTS11	42	0.619	0.644	0.652	0.039	23.123	0.337	NS
INRA05	43	0.791	0.770	0.779	–0.027	6.430	0.972	NS
INRA63	43	0.558	0.615	0.622	0.093	19.408	0.196	NS
ETH03	39	0.615	0.830	0.841	0.259	193.225	0.000	***
HEL05	42	0.952	0.777	0.787	–0.225	230.665	0.000	***
ILSTS33	42	0.929	0.836	0.846	–0.111	94.133	0.961	NS
INRA35	40	0.350	0.775	0.785	0.549	76.406	0.000	***
ILSTS05	42	0.833	0.771	0.780	–0.081	13.276	0.209	NS
CSSM08	43	0.349	0.729	0.737	0.521	232.059	0.000	***
CSSM33	43	0.930	0.871	0.882	–0.068	144.910	0.994	NS
ETH225	42	0.667	0.874	0.885	0.237	100.700	0.004	**
TGLA53	43	0.698	0.780	0.789	0.105	253.371	0.000	***
CSSM45	42	0.762	0.853	0.863	0.107	188.578	0.000	***
HEL09	37	0.622	0.861	0.873	0.278	85.681	0.005	**
ILSTS54	42	0.738	0.811	0.821	0.090	32.954	0.614	NS
MM08	42	0.833	0.830	0.840	–0.004	114.349	0.000	***
MM12	41	0.659	0.692	0.700	0.048	21.462	0.974	NS
HEL01	43	0.767	0.814	0.824	0.057	137.682	0.000	***
ILSTS34	42	0.643	0.887	0.898	0.275	245.179	0.000	***
Mean	42.115	0.693	0.765	0.775	0.088			
SE	0.295	0.031	0.020	0.021	0.038			

Ho, Observed heterozygosity = No. of heterozygotes / N; He, Expected heterozygosity = 1–Sum pi^2; UHe = Unbiased expected heterozygosity = (2N/(2N–1)) × He; F_{IS}, Fixation Index = (He–Ho) / He = 1–(Ho/He); Where pi is the frequency of the i^{th} allele for the population and Sum pi^2 is the sum of the squared population allele frequencies; *P<0.05; **P<0.01; ***P<0.001; NS, non-significant.

breeds had F_{IS} estimates closer to Kosali cattle–Motu (11.3%) delineated by Pandey *et al*. (2011) and Ghumusari (10.7%) reported by Prakash and Deepika (2014). The negative consequences of genetic erosion and inbreeding depression have been amply documented and may be manifested by loss of viability, fertility and disease resistance, and the frequent occurrence of recessive genetic diseases (Taberlet *et al.* 2008). Fortunately, situation is not alarming for Kosali cattle due to higher level genetic diversity and low magnitude of inbreeding depression.

Genetic signatures of recent reduction in population size were investigated using three different models. The probability values obtained under these models using three different statistical tests are presented in Table 3. The bottleneck analysis was under the assumption of mutation-drift equilibrium, where the allele frequency shows that the given population is not being in equilibrium, due to a recent reduction of the effective population size.Any population that experienced a recent bottleneck (40–80 generations) will show higher than expected (equilibrium) heterozygosity for a large number of loci (Sharma *et al*. 2017).

Probability values calculated for Sign test, Standardized difference test and Wilcoxon test under IAM model were above 0.05, which indicated that the populations were in mutation drift equilibrium and there was no reduction in population size. However, Sign test and Standardized difference test do indicated bottleneck event under TPM and SMM models. Piry *et al*. (1999) recommended assumption of two-phase mutation model (TPM) for calculating heterozygosity excess significance over all loci (p) by the Wilcoxon's test for microsatellite data. Following this recommendation, no bottleneck signature can be described in the population. A second approach was also employed to confirm these observations. A mode shift in the allele-frequency spectrum did not detect a recent

Table 3. Population bottleneck analysis for Kosali cattle population

Model used		IAM	TPM	SMM
Sign test (No. of loci with heterozygosity excess)	Exp	15.60	15.47	15.34
	Obs	16	7*	4*
	P value	0.5213	0.0007	0.0000
Standardized differences test	T2 value	1.025	–4.137*	–12.815*
	P value	0.1527	0.0000	0.0000
Wilcoxon test (one tail for H excess)	P value	0.1039	0.9971	1.0000

*Rejection of null hypothesis (P<0.05).

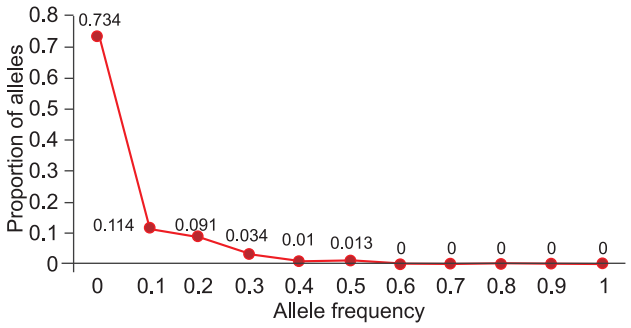

Fig. 2. Graphic representation of proportion of alleles and their distribution in Kosali Cattle.

bottleneck in Kosali population. It is expected for a non-bottlenecked population to have a large proportion of alleles with low frequency that are near mutation-drift (Sharma *et al*. 2017). A graphical representation utilizing allelic class and proportion of alleles showed a normal 'L' shaped distribution (Fig. 2). The L-shaped curve indicated the abundance of low frequency (<0.10) alleles suggesting non-occurrence of any bottleneck event in the population.

In conclusion, Kosali cattle population harbours high degree of genetic variability, as demonstrated by various within population diversity estimates calculated on the basis of genomic microsatellite markers. Kosali breed is remarkably adapted to the stressed environmental conditions of the region, where pastures are diminishing and shortage of dry and green fodder is prevalent. Frequent droughts, inappropriate management skills and dominance of small holdings are the reasons for insufficient feed and fodder. Kosali is reared under extensive production system with very little inputs. The percentage of landless, sub-marginal and marginal farmers accounts for more than 50% of rural population. These animals emerged as a major source of sustenance for under privileged people residing in Chhattisgarh. Primary requirements for overall improvement of Kosali cattle breed are organized breeding strategies, management and conservation for which population diversity data deciphered in current paper is imperative.

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