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Assessment of with-in breed diversity in Hallikar cattle (*Bos indicus*) through microsatellite markers

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Abstract

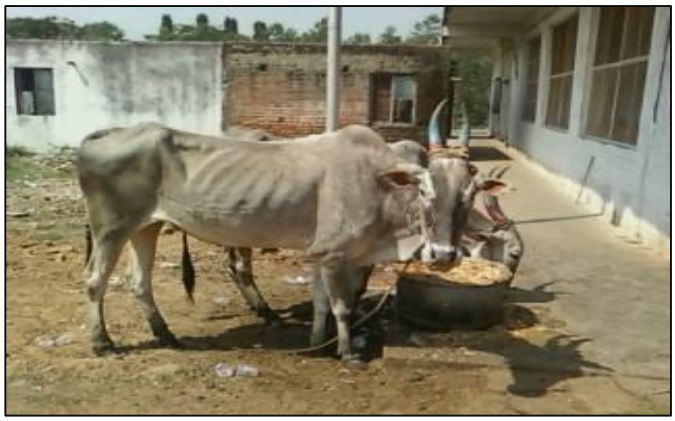
Hallikar cattle, a popular draft breed of India, were assessed genetically using five microsatellite markers, as recommended by FAO. All the screened loci were polymorphic and a total of 39 alleles were observed across the analyzed loci. The mean number of alleles was found to be 7.8±1.92 with a range of 5 to 10. The allele size ranged from 122 to 302 bp. The frequency distribution of microsatellite alleles in the breed was from 0.0104 to 0.7138. The estimated expected heterozygosity value was 0.6896±0.1403 and the PIC was 0.6565±0.1378. The population was not in Hardy-Weinberg equilibrium proportion due to systematic and dispersive processes acting upon. The within population inbreeding estimate was 0.1331, indicating deficiency of heterozygosity considerably in population of Hallikar cattle. The panel of microsatellites used was highly informative for molecular characterization and could be used for exploitation of genetic diversity of the related breeds for conservation. No mode shift was detected in the frequency distribution of alleles and a normal L-shaped curve was observed, indicating that the population is non-bottlenecked and stable with respect to population size.

Keywords: Hallikar cattle, Heterozygosity, Microsatellites, PIC

Introduction

Hallikar cattle are considered as one of the premier draft breeds of India, popularly known as the "champion of draft breeds". It is a typical Mysore type breed, found distributed mainly in Mysore, Mandya, Bangalore, Kolar, Tumkur, Hassan and Chitradurga districts of Karnataka (Nivsarkar *et al*., 2000). The breed has got a long history of over 600 years and hence forms the base for the origin of most of the present day south Indian cattle breeds. The Hallikar cattle is a medium-sized animal with compact and muscular body. Grey to dark grey-coloured coat (Fig. 1), prominent bulgy forehead and backward carrying long horns (emerging near each other at the base) are the unique physical features of the breed. The average speed of the bullocks is 3 kilometer per hour with the pulling power of 0.91 hp. As per the Livestock Census (Report, 2007), the population of Hallikar cattle was found to be 1.998 million with the breedable females of 0.75 million.

Fig 1. A typical Hallikar bullock



Considering the historical prospective, unique features and utility, it has been decided to study the genetic structure of the breed using microsatellite markers which are short tandem repeats, exhibiting high degree of polymorphism and distributed throughout the genome. Moreover, molecular characterization of breed through microsatellite analysis is one of the basic requirements for taking up conservation measures.

Materials and methods

The analysis was carried out in a sample of 48 unrelated Hallikar cattle in the breeding tract of Karnataka state. Genomic DNA was isolated by standard phenol-chloroform method (Sambrook *et al*., 1989). Isolated DNA samples were assessed for its quality and quantity by Spectrophotometric measurement.

Amplification of microsatellite loci

A total of five microsatellite markers (ETH10, ETH225, ILSTS006, ILSTS011 and TGLA122) were selected as per the suggestions of FAO in the Secondary Guidelines for Development of National Farm Animal Genetic Resources Management Plans for Global Management of Cattle Genetic Resources using reference microsatellites (FAO, 2004). The details of markers are furnished in Table 1. These markers were amplified using thermal cycler (Applied Biosystem, 2027 Thermal cycler). PCR reaction mixture (15µl) containing 50-100 ng of template DNA; 1.5 mM $MgCl_2$; 5 picomoles each of forward and reverse primers; 1 unit of taq DNA polymerase and 200 mM dNTPs was prepared. Amplification was carried out with initial denaturation at 94ºC for 5min followed by 35 cycles of denaturation (94ºC for 45 sec), annealing (56ºC to 65ºC for 45 sec) and extension (72ºC for 45 sec).

Amplified PCR products were checked on two per cent agarose gel and visualized through UV transilluminator after staining with ethidium bromide. The samples which showed amplification were taken and mixed with 0.50µl of size standard fluorescent dye Gene Scan Liz 500 (Applied Biosystem) and made up the volume to 10µl with Hi-Di formamide. The samples were denatured for five

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Table1. Details of microsatellite markers

Name of the Marker	Sequence of primers	Annealing temperature (°C)	Chromosome position
ETH10	F 5' GTT CAG GAC TGG CCC TGC TAA CA 3' R 5' CTT CCA GCC CAC TTT CTC TTC TC 3'	64.2	5
ETH225	F 5' GAT CAC CTT GCC ACT ATT TCC T 3' R 5' ACA TGA CAG CCA GCT GCT ACT 3'	59.1	9
ILSTS006	F 5' TGT CTG TAT TTC TGC TGT GG 3' R 5' ACA CGG AAG CGA TCT AAA CG 3'	56.3	7
ILSTS011	F 5' GCT TGC TAC ATG GAA AGT GC 3' R 5' CTA AAA TGC AGA GCC CTA CC 3'	57.3	14
TGLA122	F 5' CCC TCC TCC AGG TAA ATC AGC 3' R 5' AAT CAC ATG GCA AAT AAG TAC ATA C 3'	59.1	21

minutes at 94ºC, snap-chilled on ice for five minutes and then run on ABI 3730XL Genetic analyzer. The fragment size and genotype of alleles were performed by the Gene Mapper™ version 4.0, followed by manual verification.

Statistical Analysis

The alleles were designated with alphabets (A) in ascending order of size and extered in POPGENE 1.31 programme (Yeh *et al*., 1999). The parameters such as number of alleles, effective number of alleles, allele frequencies, observed and expected heterozygosities, Hardy-Weinberg equilibrium and F-statistics were estimated. The polymorphism information content (PIC) was calculated using Nei's formula (Nei, 1978). To determine whether this population exhibiting a significant number of loci with heterozygosity excess, the gene frequencies are subjected to a BOTTLENECK (Cornuet & Luikart, 1996) analysis.

Results and discussion

The parameters estimated in Hallikar cattle, such as number, size and frequency of microsatellite alleles are furnished in Table 2.

All the five microsatellite loci screened were found to be polymorphic (100%). The number of observed alleles ranged from 5 (ILSTS011) to 10 (TGLA122), with overall mean of 7.8±1.92. A total of 39 alleles were observed at these polymorphic loci in the breed. The mean number of alleles observed in the study is higher than the number reported for Sahiwal (5.2) and Deoni (5.9) breeds of cattle of India (Mukesh *et al.*, 2004). In another study, the mean number of alleles per locus was found to be 5.82 in Red Kandhari and 5.86 in Deoni breeds (Sodhi *et al*., 2005).

The size of microsatellite alleles in Hallikar cattle ranged from 122 (ETH225) to 302 (ILSTS006) bp, which is in accordance with the initial studies on N'Damas (*Bos taurus*) and Boran (*Bos indicus*) cattle (Kemp *et al*., 1995). These alleles occurred at a minimum frequency of 0.0104 (122 bp alleles at ETH225) to a maximum of 0.7188 (168 bp allele at the same locus ETH225). The 168 bp allele at the ETH225 locus was found to be the predominant allele (72 per cent) in the population, followed by 262 bp allele at ILSTS011 locus (51 per cent), 210 bp allele at ETH10 locus (38 per cent) and 292 bp allele at ILSTS006 locus (31 per cent). However, a wider range of allele frequency from 0.0119 (158 bp allele at ILSTS033) to a maximum of 0.9375 (194 bp allele at ETH152) was observed in Ongole cattle (Karthickeyan *et al.*, 2008), another breed found in Southern India. In general, the number and sizes of microsatellite alleles observed fall within the range mentioned in Secondary Guidelines for Development of National Farm Animal Genetic Resources Management Plans (FAO, 2004).

Table 2. Allele number, size and frequency at five microsatellite loci in Hallikar cattle

Locus	Observed No. of alleles	No. of effective alleles	Allele sizes (bp) and their frequencies									
ETH10	7	3.4434	202	204	206	208	210	212	216			
			0.0109	0.0109	0.0543	0.2500	0.3804	0.2826	0.0109			
ETH225	8	1.8701	122	136	138	144	146	152	156	168		
			0.0417	0.0312	0.1042	0.0104	0.0104	0.0208	0.0625	0.7188		
ILSTS006	9	4.2563	286	288	290	292	294	296	298	300	302	
			0.0106	0.0213	0.0851	0.3191	0.3085	0.1596	0.0213	0.0106	0.0638	
ILSTS011	5	3.0321	260	262	264	266	268					
			0.1622	0.5135	0.0946	0.0676	0.1622					
TGLA122	10	6.1604	136	140	142	144	150	152	154	158	162	164
			0.1667	0.0208	0.0417	0.2292	0.1667	0.0625	0.0625	0.0208	0.2083	0.0208
Mean / Range	7.8 ± 1.92	3.7525 ± 1.60	Allele range = 122 to 302 bp; Allele frequency = 0.0104 to 0.7188									

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The results such as PIC, Chi-square values, heterozygosities and within-population inbreeding are presented in Table 3. The PIC value is the statistical assessment of information of markers. This value was ranging from 0.4463 (ETH225) to 0.8175 (TGLA122) with a mean PIC of 0.6565±0.1378 in Hallikar cattle. All loci exhibited high PIC values of more than 0.5 indicating polymorphic nature of these loci in the breed. The mean PIC value is almost similar to the Krishna Valley (0.6205) cattle (Karthickeyan *et al.*, 2006) and also comparable to that of Umblachery (0.5625), Kangayam (0.5628) and Ongole (0.5584), the other Indian draught cattle (Karthickeyan *et al.*, 2007; Karthickeyan *et al.*, 2008; Karthickeyan *et al.*, 2009) breeds available in southern region. The PIC value depends upon the number of alleles and their relative frequencies in the respective locus. In general, the higher mean PIC value obtained in the study reflects that the markers used are highly informative and suitable for diversity analysis.

Table 3. Polymorphism information content, Chi-square values, heterozygosities and heterozygosity deficiency at five microsatellite loci in Hallikar cattle

LOCUS	PIC	Hardy - Weinberg Equilibrium	Heterozygosity		Within - population inbreeding estimate (F_{IS})
			Observed	Expected	
ETH10	0.6565	98.5481**	0.6304	0.7096	0.1116
ETH225	0.4463	53.1895**	0.3542	0.4653	0.2388
ILSTS006	0.7298	77.0488**	0.4681	0.7651	0.3882
ILSTS011	0.6326	19.6179*	0.7027	0.6702	- 0.0485
TGLA122	0.8175	42.9563^{NS}	0.8333	0.8377	0.0052
Mean	0.6565 ± 0.1378		0.5977 ± 0.1896	0.6896 ± 0.1403	0.1331

**Significant (p≤0.05), **Highly significant (p≤0.01), ^{NS} Not significant (p≤0.05)*

Table 4. Bottleneck analysis in Hallikar cattle

Model		IAM	TPM	SMM
Sign rank test Number of loci with heterozygosity excess	Expected	2.98	2.97	2.94
	Observed	0.33036	0.67149	0.01186
Standardized differences test T2 values		0.612	- 0.993	- 4.507
Wilcoxon test Probability of heterozygosity excess		0.3125	0.40625	1.00000

IAM-Infinite allele model; TPM-Two phase model; SMM-Stepwise mutation model

The Chi-square ($\chi 2$) test for Hardy-Weinberg equilibrium revealed that the Hallikar population is not in equilibrium with respect to four out of five loci screened. The disequilibrium exhibited in four loci might be due to unobserved null alleles in those loci which have not been, hitherto, identified by other means (Peter *et al.*, 2005). Further, these deviations may also be due to both systematic and dispersive processes operating in the population.

The overall means for observed and expected heterozygosities were 0.5977±0.1896 and 0.689±0.1403 respectively with the ranges of 0.3542 (ETH225) to 0.8333 (TGLA122); and 0.4653 (ETH225) to 0.8377 (TGLA122). Except ETH225 locus, all the other loci exhibited relatively higher expected heterozygosity which reflects the existence of variation within the breed. The mean expected heterozygosity value is in agreement with that of Krishna Valley (0.6569), Kangayam (0.6183), Umblachery (0.6139) and Ongole (0.6079) cattle (Karthickeyan *et al.*, 2006; Karthickeyan *et al.*, 2007; Karthickeyan *et al.*, 2008; Karthickeyan *et al.*, 2009) and also comparable to that of Sahiwal (0.06), Hariana (0.66) and Deoni (0.70), the other Indian cattle (Mukesh *et al.*, 2004) breeds; whereas low average heterozygosity (0.46±0.1) was also reported in Ongole cattle (Metta *et al.*, 2004). The high heterozygosity values observed in the present study indicate higher amount of genetic variability that could be exploited in the population of Hallikar cattle.

The within-breed diversity was estimated using the F_{IS} (Within Population Inbreeding Estimate; Wright's Fixation Index) values as a measure of heterozygote deficiency. The overall F_{IS} was found to be 0.1331 i.e., on the positive side. Out of five loci, only one locus was found to have outbred as reflected from negative value. But the overall positive value obtained in this study may be attributed to use of sires selected from the same herd.

Identifying populations that have experienced a severe reduction in size (i.e., bottleneck) is important because bottleneck can increase rate of inbreeding, loss of genetic variation, fixation of deleterious alleles and increase the probability of population extinction. Three mutation models namely, infinite allele model (IAM), two phase model (TPM), step-wise mutation model (SMM) using the programme Bottleneck were worked out and presented in Table 4. The Hallikar cattle population is non-bottlenecked as evidenced from the bottleneck analysis, i.e., it has not undergone any recent reduction in the effective population size and remained at mutation-drift equilibrium. In the present study, no mode-shift was detected in the frequency distribution of alleles and a normal L-shaped curve was observed which is shown in Fig.2.

Conclusions

The study revealed the polymorphic nature of microsatellite loci screened in Hallikar breed of cattle. The overall mean polymorphic information content of 65 per cent indicates that these markers are highly informative and suitable for characterization of domestic animal

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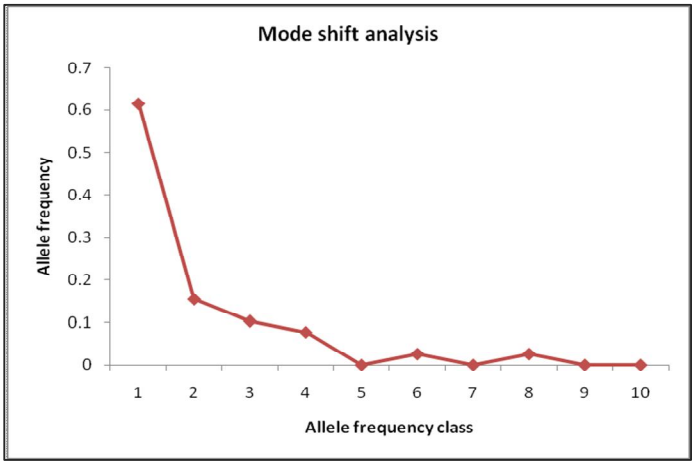
Fig 2. Graphical representation of allele frequency and their contribution in Hallikar cattle at five microsatellite loci



biodiversity. It also opens up the scope for exploiting the genetic variability in the population for conservation. Comparative analysis with other Indian draught cattle breeds will determine the genetic distance and evolutionary relationship of this breed with other cattle breed of India. The mutation-draft equilibrium indicates that the Hallikar cattle population has not undergone any recent reduction in size.

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