

Molecular Characterization of Hallikar Breed of Cattle Using Microsatellite Markers

S. Naveen Kumar, M. R. Jayashankar*, C. S. Nagaraja, M. G. Govindaiah
R. Saravanan¹ and S. M. K. Karthickeyan¹

Department of Animal Breeding, Genetics and Biostatistics, Veterinary College, Bangalore-560 024, India

ABSTRACT : Molecular characterization of Hallikar, the native cattle breed of Karnataka, was undertaken using 19 cattle specific, highly polymorphic microsatellite markers recommended by FAO. The genomic DNA was subjected to PCR amplification and alleles were resolved through six per cent denaturing PAGE with a 10 bp DNA ladder followed by silver staining. Genotyping of animals was done based on allele size. The number of alleles ranged from three to nine with allele sizes ranging from 102 bp to 294 bp. These alleles were distributed in the frequency range between 0.0306 and 0.8673 in the population. The mean observed number of alleles was 6.368 ± 1.4225 . The mean observed and expected heterozygosities were 0.7515 ± 0.1734 and 0.7850 ± 0.1381 , respectively. The high heterozygosity observed implies presence of higher genetic variability within Hallikar breed. The PIC (Polymorphism Information Content) values ranged from 0.2322 (ETH152) to 0.8654 (ETH225). The percentage of polymorphic loci obtained was 100 as all the 19 microsatellite markers were found to be polymorphic. Except for ETH152, all the other loci had high PIC values, indicating that these markers are highly informative for characterization of Hallikar breed. The population was tested for Hardy-Weinberg equilibrium at 19 microsatellite loci, and at 74 per cent of the loci the population was found to be in disequilibrium. (*Asian-Aust. J. Anim. Sci.* 2006. Vol 19, No. 5 : 622-626)

Key Words : Hallikar, Draft Cattle, Microsatellite, Heterozygosity

INTRODUCTION

India has diversified and unique cattle genetic resources having 30 well recognized breeds that constitute 7.75 per cent (FAO, 1995) of the total cattle breeds of the world. Hallikar is the pride cattle breed of Karnataka having a history of 600 years. Hallikar animals are world famous for their excellent 'draught power capacity', endurance and discipline at work. This breed is considered as the progenitor of the Amrithmahal, Khillar and Kangayam breeds. The animals of this breed are fast track animals used extensively for dry land agricultural operations and for transportation in rural areas.

In the recent past, there is perception amongst the farmers and breeders in the breeding tract about the deterioration in the form, size, quality, growth, reproduction and production potentialities of Hallikar cattle breed due to changes in the utility and cropping pattern, breeding objectives and agrobiodiversity of the breeding tract. The first step for the sustainable use of domestic animal genetic resources is the gathering of information about the genetic variability through characterization of breeds.

The microsatellites, which are tandem repeats of short

DNA sequences, have proved to be most sensitive markers for population genetic studies. In view of their high polymorphism, high heterozygosity, Mendelian co-dominant inheritance, ubiquity throughout the genome and ease of scoring by PCR, microsatellites are considered as powerful DNA markers for genetic characterization of native breeds (May, 1990).

This study was undertaken with the objective of characterizing Hallikar breed of cattle using microsatellite markers.

MATERIALS AND METHODS

Experimental animals

Hallikar animals are of medium size in body with beautifully and elegantly placed horns with pointed tips. The head is graceful and proportionately built with concave forehead and face tapering up to muzzle. They have a well-developed hump and dewlap. The color of these animals varies from white to light gray with slightly darker shoulders and hindquarters, especially in breeding bulls. White markings or irregular patches around the eyes and cheeks, and neck or shoulder region are the distinctive features of this breed. Animals are quite temperamental, fiery and active and are very good for quick transportation either on metal or slush rural roads. The home tract of Hallikar is spread over Chitradurga, Chikamagalur, Tumkur, Mysore, Mandya, Hassan and Bangalore (Rural) districts and adjoining areas.

* Corresponding Author: M. R. Jayashankar. Tel: +91-80-23411483, Fax: +91-80-23410509, E-mail: mrjshankar@yahoo.com

¹ NBAGR Core Lab, Department of Animal Genetics and Breeding, Madras Veterinary College, TANUVAS, Chennai-600 007, India.

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Table 1. Allele number, size and frequency at nineteen microsatellite loci in Hallikar cattle

Locus	T° A*	Observed No. of alleles	Allele sizes (bp) and their frequencies								
ILSTS005	58°C	7	192	194	196	198	200	202	208		
			0.1633	0.0714	0.1531	0.1429	0.1837	0.1633	0.1224		
ILSTS006	55°C	8	280	282	284	286	288	290	292	294	
			0.0714	0.0408	0.1122	0.2653	0.2245	0.1633	0.0408	0.0816	
ILSTS011	58°C	5	266	268	270	272	274				
			0.0816	0.1224	0.2347	0.2653	0.2959				
ILSTS030	58°C	6	154	156	158	160	162	164			
			0.2245	0.0816	0.1939	0.1837	0.1122	0.2041			
ILSTS033	55°C	7	148	150	152	154	156	158	160		
			0.0612	0.1735	0.1939	0.0918	0.1429	0.2143	0.1224		
ILSTS054	55°C	8	144	146	148	150	152	154	156	158	
			0.1667	0.1146	0.0833	0.1875	0.1458	0.1146	0.1042	0.0833	
INRA005	55°C	6	118	120	122	126	128	130			
			0.3125	0.2292	0.1042	0.1875	0.1250	0.0417			
INRA032	55°C	5	124	126	128	130	132				
			0.048	0.0918	0.1531	0.4184	0.2959				
INRA035	58°C	5	118	120	122	124	126				
			0.0918	0.2755	0.2857	0.2347	0.1122				
INRA063	56°C	7	194	198	202	204	206	208	210		
			0.0714	0.2143	0.2347	0.1429	0.1429	0.0612	0.1327		
ETH003	57°C	5	120	122	124	126	128				
			0.0816	0.2959	0.2755	0.2755	0.0714				
ETH010	54°C	8	216	218	220	222	224	226	228	230	
			0.0408	0.0612	0.1837	0.1633	0.2959	0.0816	0.1429	0.0306	
ETH152	55°C	3	196	200	202						
			0.8673	0.0714	0.0612						
ETH225	58°C	9	150	152	154	156	158	160	162	164	166
			0.1224	0.0918	0.0510	0.0612	0.0918	0.1122	0.1224	0.1429	0.2041
HEL001	57°C	7	102	104	106	108	110	112	114		
			0.0612	0.1327	0.1224	0.2347	0.2041	0.0714	0.1735		
HEL005	57°C	6	160	162	164	166	168	170			
			0.1224	0.1837	0.2755	0.2449	0.1122	0.0612			
HEL009	57°C	7	154	156	158	160	162	164	166		
			0.1224	0.2959	0.2959	0.1327	0.0816	0.0408	0.0306		
BM1818	58°C	6	268	270	272	274	276	278			
			0.0714	0.0612	0.1735	0.2347	0.2143	0.2449			
BM2113	58°C	6	124	126	128	130	132	134			
			0.1224	0.1429	0.2245	0.2347	0.1531	0.1224			
Mean		6.3684									
SD		1.4225									

* T° A = Annealing temperature.

Blood samples were obtained from 50 Hallikar cattle maintained by farmers in the home tract.

Microsatellite analysis

Genomic DNA was extracted from venous blood by high salt method as described by Miller et al. (1988). Agarose gel electrophoresis (0.8%) was carried out for confirming the quality of the isolated DNA. Good quality DNA was used for further study.

Nineteen microsatellite markers recommended by FAO (1996) were used for characterizing the Hallikar breed. They were ILSTS005, ILSTS006, ILSTS011, ILSTS030, ILSTS033, ILSTS054, INRA005, INRA032, INRA035,

INRA063, ETH003, ETH010, ETH152, ETH225, HEL001, HEL005, HEL009, BM1818 and BM2113.

The amplification reactions were carried out in 0.2 ml microfuge tubes using a programmable thermal cycler (PTC 200, MJ Research, USA). Each 20 µl reaction mixture contained 2 µl PCR assay buffer, 0.6 µl MgCl₂ (1.5 mM), 1 µl each of Forward and Backward primer (5 pmol/reaction), 0.8 µl dNTPs (100 mM each), 0.15 µl Taq DNA polymerase (5 units/µl), 2 µl template DNA (50-100 ng) and triple glass distilled water to make up to 20 µl. The contents were vortexed. A PCR programme with an initial denaturation at 94°C for 3 min followed by 29 cycles of denaturation for

94°C for 45 sec with annealing temperature ranging from 51°C to 58°C (depending on the primer used, Table 1) for 45 sec and an extension duration of 45 sec at 72°C was used. Final extension was done at 72°C for 5 min followed by refrigeration at 4°C. Amplified PCR products were checked on one per cent agarose gel and visualized through UV illumination after ethidium bromide staining. The samples that showed amplification were resolved through six per cent denaturing polyacrylamide gel electrophoresis with 10 bp DNA ladder followed by silver staining. The gels were analyzed using Diversity Database software (Bio-Rad, USA) in a gel documentation system. Genotyping of animals was done based on allele size. The genotypes of the cattle were scored and the number and size of the alleles with their frequencies were calculated.

Statistical analysis

Mean, standard deviation and standard error were calculated as per Snedecor and Cochran (1989). Further analyses of data were done as follows:

Estimation of allele frequency : Allele frequency i.e., the occurrence of single or double bands on the gel was estimated manually by direct counting. Finally allele size, number of alleles, allele frequency and heterozygosity was calculated.

Estimation of heterozygosity : The expected heterozygosity or genetic diversity was measured (Nei, 1973) by the formula,

$$H_e = 1 - \sum P_i^2$$

where P_i - is the frequency of i^{th} allele.

The observed heterozygosity (H_o) was calculated as the actual percentage of heterozygosity occurring in the sample population.

$$H_o = \frac{\text{Number of heterozygotes}}{\text{Total number of samples}} \times 100$$

Estimation of polymorphism information content (PIC) : Polymorphism information content (PIC) was calculated by using the formula (Botstein et al., 1980) as:

$$\begin{aligned} PIC &= 1 - \left[\sum_{i=1}^k x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k 2x_i^2 x_j^2 \right] \\ &= \sum_{i=1}^{k-1} \sum_{j=i+1}^k 2x_i x_j - \sum_{i=1}^{k-1} \sum_{j=i+1}^k 2x_i^2 x_j^2 \\ &= 2 \sum_{i=1}^{k-1} \sum_{j=i+1}^k x_i x_j (1 - x_i x_j) \end{aligned}$$

Where,

k = number of alleles

x_i = allele frequency at the homozygous loci

$x_i x_j$ = allele frequency at the heterozygous loci

Test of Hardy-Weinberg equilibrium : A χ^2 -test of goodness of fit was carried out with the observed number and the expected number to check whether population was in Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

It is well established that highly polymorphic microsatellite marker, because of their high heterozygosity, Mendelian codominant inheritance, ubiquity through out the genome and ease of scoring by PCR, is being considered as powerful DNA marker for genetic characterization of livestock breeds, in addition to ranking of species/breeds according to their level of phylogenetic distinction (May, 1990). The present study aimed at characterization of Hallikar breed of cattle, using 19 microsatellite markers recommended by FAO.

Allele numbers, sizes and their frequencies

Number of alleles observed at a single locus ranged from 3 (ETH152) to 9 (ETH225) with allele sizes ranging from 102 bp to 294 bp (Table 1). The frequency distribution of alleles was in the range of 0.0306 to 0.8673. The mean number of alleles per loci was 6.368.

Manjunatha Prabhu (2004) studied microsatellite pattern of Amrithmahal cattle using 25 cattle specific primers. The allele number and allele size ranged between 2 to 8 and 89 bp to 302 bp, respectively, while the allele frequencies ranged from 0.06 to 0.38. Selvi et al. (2004) characterized Mafriwal, a synthetic dairy cattle breed of Malaysia, using 50 microsatellite markers. The observed number of alleles per locus ranged from 4 to 8 and the allele frequencies ranged from 0.02 to 0.52. The mean number of alleles per locus was 6.23. Using 20 microsatellites, Dorji et al. (2003) observed the mean number of alleles per loci to range from 7.2 to 8.9, in native Siri (*Bos indicus*) cattle populations of Bhutan. The allelic frequencies observed in the present study were higher than the frequencies reported by the various workers in indicus cattle.

Markers would be more informative, when the number of alleles at a given locus is more. Considering the number of alleles observed with the different primers in the present study, the usefulness of these primers in characterizing Hallikar breed need to be studied with more number of animals.

Heterozygosity

Heterozygosity is an appropriate measure of genetic

Table 2. Observed and estimated heterozygosity in Hallikar cattle

Microsatellite locus	Heterozygosity	
	Observed	Expected
ILSTS005	0.6735	0.8578
ILSTS006	0.7347	0.8334
ILSTS011	0.7143	0.7732
ILSTS030	0.9592	0.8258
ILSTS033	0.8980	0.8475
ILSTS054	0.8333	0.8739
INRA005	0.8333	0.7947
INRA032	0.6122	0.7111
INRA035	0.7755	0.7742
INRA063	0.8980	0.8403
ETH003	0.8571	0.7566
ETH010	0.8571	0.8271
ETH152	0.1429	0.2413
ETH225	0.7755	0.8811
HEL001	0.6939	0.8403
HEL005	0.7551	0.8073
HEL009	0.7755	0.7911
BM1818	0.6531	0.8083
BM2113	0.8367	0.8292
Mean	0.7515	0.7850
SD	0.1734	0.1381

variability within a population when populations are expanding (Hanslik et al., 2000). In the present study, the observed heterozygosity ranged from 0.1429 (ETH152) to 0.9592 (ILSTS030) where as the range for expected heterozygosity was 0.2413 (ETH152) to 0.8811 (ETH225) (Table 2).

The average observed heterozygosity at all the 19 loci studied was 0.7515 ± 0.1734 , nonsignificantly lower than average expected heterozygosity value of 0.7850 ± 0.1381 . Earlier reports in *Bos indicus* have also indicated higher heterozygosity values. The observed heterozygosity values in Siri cattle was found to range from 0.67 to 0.73 (Dorji et al., 2003), and from 0.654 to 1.000 in Amrithmahal cattle (Manjunatha Prabhu, 2004).

Using 30 microsatellite markers, Mateus et al. (2004) obtained average observed heterozygosity ranging from 0.5533 to 0.7430 in 10 native Portuguese cattle breeds, American Charolais and the Brazilian Caracu. Selvi et al. (2004) obtained a mean overall heterozygosity of 0.79 in Mafriwal, a synthetic dairy cattle breed.

The high heterozygosity values observed in the present study indicated the presence of large number of polymorphic loci in the Hallikar breed. This implies a higher amount of genetic variability that can be exploited even in populations of small sizes as also opined by Moioli et al. (2001).

Polymorphism information content (PIC)

PIC values indicate the informativeness of the microsatellite loci studied. In the present study the PIC

Table 3. Polymorphism information content (PIC) and test for Hardy-Weinberg equilibrium

Microsatellite locus	PIC	Hardy-Weinberg equilibrium	
		Chi square value	df
ILSTS005	0.8394	45.1463**	21
ILSTS006	0.8137	59.0222**	28
ILSTS011	0.7457	27.4550**	10
ILSTS030	0.8041	53.3924**	15
ILSTS033	0.8283	42.9961**	21
ILSTS054	0.8571	53.1038**	28
INRA005	0.7705	25.8743*	15
INRA032	0.6794	15.3397 ^{NS}	10
INRA035	0.7469	28.2807**	10
INRA063	0.8206	35.0929*	21
ETH003	0.7269	20.9162*	10
ETH010	0.8071	28.6207 ^{NS}	28
ETH152	0.2322	40.9084 ^{NS}	3
ETH225	0.8654	57.9174*	36
HEL001	0.8206	48.9658**	21
HEL005	0.7843	10.9933 ^{NS}	15
HEL009	0.7674	46.8803**	21
BM1818	0.7849	26.9413*	15
BM2113	0.8081	18.3810 ^{NS}	15

* Significant ($p \leq 0.05$), ** Significant ($p \leq 0.01$).

^{NS} Not significant ($p \geq 0.05$).

values for all the 19 microsatellite loci ranged from 0.2322 to 0.8654 (Table 3). Except locus ETH152, all the loci showed high PIC values suggestive of more polymorphic nature.

Cent percent polymorphic loci were obtained as all the 19 microsatellite loci used were found to be polymorphic. The polymorphism at the locus is created by increasing dinucleotide repeats and mutation. The present study proves that the cattle specific microsatellite markers used were highly polymorphic and hence highly informative for genetic characterization of cattle breeds.

The present findings are in accordance with the reports by other workers in indicus cattle. Muralidhar (2003) used ten microsatellite loci and obtained PIC values ranging from 0.15 to 0.79 in Ongole and from 0.13 to 0.80 in Deoni cattle, while the PIC values observed for ETH 010, ETH 225 and BM 2113 were 0.493, 0.140 and 0.682, and 0.661, 0.357 and 0.644, in Ongole and Deoni breed, respectively (Srinivas Sriramula, 2003).

Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium test was used to predict whether the population is stable or not. The observed genotypes were compared with expected genotypes in a χ^2 -test for goodness of fit.

In general the population studied was not in Hardy-Weinberg equilibrium proportions as 74 per cent of the loci were producing highly significant χ^2 values (Table 3). The deviation of 74 per cent of the loci from equilibrium may be due to many causes such as selection, genetic drift and

small sample size. Kim et al. (2002) have ascribed lack of genetic equilibrium to existence of null alleles, high mutation rates and size homoplasy of microsatellite loci from their study in Korean and Chinese goat breeds.

Hardy Weinberg disequilibrium was attributed by Dorji et al. (2003) to population subdivision following sampling from a range of distinct locations within the same broad geographical area. Similar reasons might be applied in the present study, as the samples were drawn from different locations, within the breeding tract of Hallikar breed. Whereas in Mafriwal cattle, Selvi et al. (2004) suggested that the practice of using frozen semen not only from bulls at the Institute but also from bulls kept at other farms may explain the deviation of Hardy Weinberg equilibrium.

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REFERENCES

- Botstein, D., R. L. White, M. Skolnick and R. W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32:314-331.
- Dorji, T., O. Hanotte, M. Arbenz, J. E. O Rege and W. Roder. 2003. Genetic Diversity of Indigenous Cattle Populations in Bhutan: Implications for Conservation. *Asian-Aust. J. Anim. Sci.* 16:946-951.
- Food and Agricultural Organization of the United Nations (FAO) 1995. World Watch List.
- Food and Agricultural Organization of the United Nations (FAO) 1996. Global projects for the maintenance of Domestic Animal Genetic Diversity (MoDAD). <http://www.fao.org/dad-is/>.
- Hanslik, S., B. Harr, G. Brem and C. Schlotterer. 2000. Microsatellite analysis reveals substantial genetic differentiation between contemporary New World and Old World Holstein-Friesian populations. *Anim. Genet.* 31:31-38.
- Kim, D. S., J. S. Yeo, J. W. Lee, J. W. Kim and C. B. Choi. 2002. Genetic diversity of goats from Korea and China using microsatellite analysis. *Asian-Aust. J. Anim. Sci.* 15:461-465.
- Manjunatha Prabhu, B. H. 2004. Phenotypic and Molecular Genetic Characterization of Amrithmahal Cattle. M.V.Sc. Thesis, University of Agricultural Sciences, Bangalore, India.
- Mateus, J. C., M. C. T. Penedo, V. C. Alves, M. Ramos and T. Rangel-Figueiredo. 2004. Genetic diversity and differentiation in Portuguese cattle breeds using microsatellites. *International Society for Animal Genetics, Anim. Genet.* 35:106-113.
- May, R. M. 1990. Taxonomy as destiny. *Nature.* 347:129-130.
- Miller, S. A., D. Dykes and H. F. Polesky. 1988. A simple salting out procedure for extraction of DNA from human nucleated cells. *Nucleic Acids Res.* 16:1215.
- Moioli, B., A. Georgoudis, F. Napolitano, G. Catillo, S. Luciola, Ch. Ligda and J. Boyazoglu. 2001. Genetic Diversity between Italian and Greek Buffalo populations. *AGRI.* 29:31-40.
- Muralidhar, M. 2003. Molecular Genetic Characterization of Ongole and Deoni cattle. M.V.Sc. Thesis, Acharya N.G. Ranga Agricultural University, Hyderabad, India.
- Nei, M. 1973. The Theory and Estimation of Genetic Distance. In: *Genetic Structure of populations* (Ed. N. E. Morton). University of Hawaii Press, Honolulu. pp. 45-54.
- Selvi, P. M., J. M. Panandam, K. Yusoff and S. G. Tan. 2004. Molecular Characterisation of the Mafriwal Dairy Cattle of Malaysia using Microsatellite Markers. *Asian-Aust. J. Anim. Sci.* 17:1366-1368.
- Snedecor, G. W. and W. G. Cochran. 1989. *Statistical Methods.* 8th Ed., Iowa State Univ. Press, Ames, Iowa, USA.
- Srinivas Sriramula. 2003. Molecular Genetic Characterization of Ongole and Deoni cattle using Microsatellite Markers. M.V.Sc. Thesis, Acharya N.G. Ranga Agricultural University, Hyderabad, India.