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GENETIC CHARACTERIZATION OF NON DESCRIPTIVE BREED (*Bos indicus*) OF MARATHWADA REGION USING GENETIC MARKERS

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ABSTRACT

Molecular characterization of cattle breeds is important for the prevention of germplasm erosion by cross breeding. The Indian zebu cattle have their significant role in evolution of present day cattle breeds. The present study was carried out to characterize non descriptive cattle breed of Marathwada region using microsatellite markers and mitochondrial DNA sequencing (Folmer region). Five microsatellite markers (ETH225, ETH10, SPS115, BM4028 and TGLA159) were selected to screen cattle population from seven districts of Marathwada respectively. The expected heterozygosity varied from 0.7410 (TGLA159) to 0.8722 (ETH10). The PIC values for polymorphic loci ranged from 0.919 to 0.937. The Fst value (0.1584) indicated moderate genetic differences in the cattle breed. The mitochondrial sequences of 21 samples indicate 99.9% homology with Bos indicus or Bos taurus species. The primary study indicates that there is presence of genetic variation between the non descriptive cattle population of Marathwada region.

KEYWORDS : *Bos indicus,* Microsatellite markers, Mitochondrial DNA, Polymorphism

Indian cattle are also known as zebu cattle (*Bos indicus*). These are broadly categorized into dairy, dual and draught purpose breeds depending on their utility. There are 30 documented breeds of zebu cattle in India. There are numerous yet to be characterized and defined cattle breed populations in various states of India including Maharashtra (Metta et al. 2004; Nivsarkar et al. 2000).

Zebu cattle are used in cross breeding programs as they can adapt to hot and humid climates (Koger 1980; Turner 1980). However, several of these breeds are now being bred out because of intensive cross breeding. As a result, some of the native draft breeds are on the verge of extinction. Hence, there is a need to conserve these breeds. The phenotypic characterization of domestic cattle is often affected by the environment and the genetic complexity. Many studies have been initiated to characterize the European cattle breeds (*Bos taurus*) using the molecular markers like microsatellite (Kantanen et al. 2000; Ciampolini et al. 1995; Peelman et al. 1998; Canon et al. 2001; MacHugh et al. 1998). Microsatellite markers being codominant and multiallelic in nature, prove to be efficient in genetic diversity studies, pedigree evaluation and genetic mapping as compared to other molecular tools like RAPD, RFLP and ISSRs (Nagaraju et al. 2001). Hence, microsatellites have become one of the preferred markers in cattle breed characterization (Canon et al. 2001; Edwards et al. 2000; Loftus et al. 1999; MacHugh et al. 1997).

Similarly, the mitochondrial cytochrome oxidase c subunit 1 (COI) gene is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom. The M1-M6 partition of the COI gene also referred to as the Folmer region has been recognized as an efficient identification tool for Metazoan species, turning it into the core fragment for DNA barcoding of the animals. Many studies have indicated that the deepest roots of cattle phylogeny occur between Indian cattle and European cattle (Bradley et al. 1998). In spite of the evolutionary significance of the Indian cattle breeds, the available literature on genetic characterization of these breeds using reliable molecular markers is scanty. Recently, (Kumar et al., 2003) carried out admixture analysis of South Asian cattle breeds revealing the influence of *Bos taurus* in the Indian sub-continent.

The aim of the present study was to characterize non descriptive breeds of cattle (known as Gavran in colloquial language) of Marathwada region at a molecular level by analyzing genetic variation within and among the seven populations using microsatellite markers and sequencing of COI gene.

MATERIALS AND METHODS

Sample Collection

42 samples of non descriptive cattle breed (non endangered species), six each from seven districts of Marathwada region viz. Aurangabad, Jalna, Parbhani, Beed, Latur, Nanded and Osmanabad were selected. The

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blood samples were drawn from the left ear vein of these animals for DNA isolation and further analysis. The blood collection was done by qualified Veterinarians, hence no specific permissions were required for these above seven locations (Ethical Committee Report No: IEC/IRB-No.11122011IR3).

DNA isolation

Total DNA was extracted from the blood samples by modifying Sambrook- Manniatis method (1989).

Microsatellite Genotyping PCR

Five microsatellite markers were selected from the database (http://www.fao.org/dad_is) recommended by the Food and Agriculture Organization and the International Society for Animal Genetics (FAO and ISAG) and NBAGR - National Bureau of Animal Genetic Resources, Karnal, India. PCR amplification was done on a thermal cycler (Eppendorf) in 15 µl reaction using 1.5 µl of 10X PCR Buffer (Invitrogen, USA), 1U Taq DNA Polymerase, primer mix of reverse 1.0 µl and forward 1.0µl (2.0 pmol each), 2.0 µl DNA template (60 ng) and DNAase free water to make a final reaction volume of 15 µl. The PCR conditions employed were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C, 30 sec at annealing temperature of 55°C and 30 sec extension at 72°C, then final extension at 72°C for 10 min. Amplicons obtained after PCR assays were recovered from agarose gels, purified using the Invitrogen PCR Product Purification kit and subjected to direct sequencing. Each panel was run in capillary electrophoresis on an ABI3130® genetic analyzer (Applied Biosystem, USA). Microsatellite fragment sizing was done using the software Gene MapperTM version 3.7 (Applied Biosystems, USA). The alleles were identified by the software and also manually verified to avoid any false positive.

The statistical analysis was carried using POPGENE 1.32v software (Yeh et al. 1999). The following parameters were computed: the percentage of polymorphic loci (P), observed and effective number of alleles per locus (Na, Ne), expected and observed heterozygosity (He, Ho), gene flow (Nm) and the inbreeding coefficient (Fis). The polymorphic information content (PIC) values were analyzed by the software MOLKIN version 3.0 (Gutierrez et al. 2005).

Mitochondrial COI Gene Sequencing

The primer set- LCO1490 and HCO2198 amplify a 708 bp fragment of the COI gene in a wide range of eukaryotic taxa (Folmer et al., 1994). 21 DNA samples, three each from seven populations were selected for the present investigation. The PCR reaction volume of 20 µL consisted of between 20 and 100ng DNA template, 50mM KCl, 10mM TrisHCl, 1.5mM $MgCl_2$, 0.05mM of each dNTP, 0.5µM of each primer and 1.25 U of AmpliTaq Gold 360 (Applied Biosystems, Foster City, CA, USA). The PCR conditions employed were: initial denaturation at 94°C for 10 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at annealing temperature of 45°C and 1 min extension at 72°C, then final extension at 72°C for 10 min. Amplicons obtained after PCR assays were recovered from agarose gels, purified using the Invitrogen PCR Product Purification kit and subjected to direct sequencing. Using the gene specific sequencing primers and ABI BigDye® Terminator v3.1 Cycle Sequencing reaction kit (Applied Biosystems, USA), the purified PCR amplicons were sequenced by ABI 3130 (Applied Biosystems) automated sequencer. The sequences obtained were subjected to BLAST analysis.

RESULTS AND DISCUSSION

The genetic variation in 42 individuals belonging to non descriptive cattle breed was analyzed using five microsatellite loci. The percentage of polymorphic loci was 100% in six populations except for Parbhani population (80%). This indicates that the microsatellite markers selected can be used to determine genetic differences in cattle breeds. Similar observation was made by Metta et al. (2004) regarding the effectiveness of microsatellite markers in cattle genetic characterization. The amount of heterozygosity is the most widespread and important biological informative measure of genetic variation in a population because individuals in diploid organisms are either heterozygous or homozygous at a particular loci (Nei 1987; Hedricks 2000). The overall mean values for observed heterozygosity (0.7619) and expected heterozygosity (0.8153) per locus indicated presence of heterozygosity in native cattle breed (table 1). Similar Ho (0.788) and He (0.819) values were observed by Deepika

Table 1: Genetic Variability of Selected Loci in Wild Population

Locus	Ho	He
ETH225-1	1.0000	0.8479
ETH10-1	0.6667	0.8722
SPS115-1	0.5714	0.8037
BM4028-1	0.9524	0.8118
TGLA159-1	0.6190	0.7410
Mean	0.7619	0.8153

Ho= Observed Heterozygosity,
He= Expected Heterozygosity

and Salar, 2013 in native cattle breeds of North and Western Parts of India. Whereas in Hariana and Hissar cattle breed of Pakistan (Ho =0.5, He= 0.6), Hallikar and Kangayam cattle breeds of India (Ho =0.6, He = 0.7) heterozygosity deficiency was observed (Rehman and Khan, 2009; Shekar et al., 2011; Karthickeyan et al., 2009) when compared with the cattle breed in the present study. The mean observed number of alleles per locus (Na= 10.400) and the mean effective number of alleles (Ne=5.1224) further confirmed the genetic variation in this breed (table 2). The effective number of alleles was 50% of the observed number of alleles. Similar values of Na (9.00 to 11.81) and Ne (4.18 to 5.00) were observed in five native cattle breeds of North and Western Parts of India (Deepika and Salar, 2013) whereas in Hariana, Hissar, Kangayam, Umblachery, Ongole and Deoni cattle breeds the Na value ranged from 4.04 to 4.5 and Ne values ranged from 2.0 to 2.19 (Rehman and Khan 2009; Karthickeyan et al., 2009; Karthickeyan et al., 2007; Metta et al., 2004). This suggests that the native non descriptive cattle breed has more genetic variation than the descriptive cattle breed.

Table 2: Microsatellite Loci Showing Observed, Effective Number of Alleles

Locus	Na	Ne
ETH225-1	11.0000	5.8026
ETH10-1	11.0000	6.7328
SPS115-1	10.0000	4.6421
BM4028-1	8.0000	4.8197
TGLA159-1	12.0000	3.6148
Mean	10.4000	5.122

Na= Observed number of alleles,
Ne= Expected number of alleles

Gene flow is the transfer of alleles of genes from one population to another. The migration into or out of a population may be responsible for a marked change in allele frequencies. This immigration may also result in the addition of new genetic variants to the established gene pool of a particular species or population. The Fis, Fst and Nm parameters indicate gene flow, important in determining the gene pool of the population. In present investigation, the values of Fis (-0.1374), Fst (0.1584) and Nm (1.3282) have clearly indicated a high level of gene flow (table 3). The mean Fis being negative indicates presence of heterozygosity according to Hardy Weinberg expectations. Similar negative Fis value of -0.048 and -0.084 was observed in Umblachery and Kangayam cattle breed respectively (Karthickeyan et al., 2007; Karthickeyan et al., 2009). This suggests surplus of heterozygotes in these cattle breed population. Fst values are used to determine the degree of genetic differentiation among populations. According to Wright guidelines (1978), the mean Fst value (0.1584) in the present study falls in the range of moderate to large genetic differentiation. Based on the Fst value, most of the genetic variation was within the populations of non descriptive cattle breed. The Fst value of 0.117 was recorded in Ongole and Deoni breeds (Metta et al., 2004). Thus, it indicates that native Indian cattle breed populations are moderately variable in their genetic makeup.

Table 3: Microsatellite Loci Showing F-Statistics and Gene Flow

Locus	Fis	Fst	Nm
ETH225-1	-0.3404	0.0986	2.2847
ETH10-1	0.0769	0.1518	1.3969
SPS115-1	0.1111	0.1806	1.1340
BM4028-1	-0.3793	0.1288	1.6917
TGLA159-1	-0.1304	0.2429	0.7790
Mean	-0.1374	0.1584	1.3282

Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst

Table 4: Polymorphic Information Content

Locus	PIC
ETH225-1	0.936822877299068
ETH10-1	0.930080020989112
SPS115-1	0.937728937728938
BM4028-1	0.919795078525237
TGLA159-1	0.937468505920887

A global value of Nm > 1 prevents random differentiation by genetic drift (Slatkin, 1987). The Nm value in this study was 1.3282, suggesting some factors other than genetic drift are involved in giving rise to differentiation among population. The present investigation indicates that some deterministic factors like availability of food and environmental conditions may have played an important role in genetic variability of cattle breed population and not genetic drift. Thus it is evident that the gene pool of non descriptive cattle breed is maintained.

The polymorphic information content (PIC) for the five microsatellite markers ranged from 0.919 to 0.937 (table 4). The PIC values in Kangayam (Karthickeyan et al., 2009), Hallikar (Shekar et al. 2011), Ongole, Deoni (Metta et al., 2004), Hariana and Hissar breeds (Rehman and Khan, 2009) were 0.5628, 0.6565, 0.79 to 0.80 and 0.749 to 0.719 respectively. The high PIC value indicates presence of polymorphism at all the five microsatellite markers. Thus, there is scope for preserving and maintaining genetic polymorphism in the cattle breed population.

A proper molecular toolbox for identifying vertebrate species should be considered as many useful loci as possible, especially when the currently available nuclear loci (18S and 28S) have low resolution at the species level.

Table 5: BLAST Results of Mitochondrial DNA Sequences

Sr. No.	Sample Name	Size of seque - nce (bp)	Query Coverage	Max Identity	NCBI BLAST Result	Accession Number
1	AU 02	687	96%	99%	Bos taurus isolate WPC25	KC153977
2	AU 15	698	93%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
3	AU 18	688	95%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
4	BECA 02	686	97%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
5	BECA 10	819	79%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
6	BECA 12	703	94%	97%	Bos taurus voucher ABR	JX218056
7	PA 05	687	94%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
8	PA 07	686	95%	99%	Bos taurus isolate WPC25	KC153977
9	PA 09	687	96%	98%	Bos taurus voucher ABR	JX218056
10	JA 09	660	98%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
11	JA 10	685	94%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
12	JA 13	685	97%	98%	Bos taurus voucher ABR	JX218056
13	LACA 02	680	97%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
14	LACA 05	688	91%	99%	Bos taurus voucher ABR	JX218056
15	LACA 08	695	94%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
16	NACA 10	685	97%	99%	Bos indicus isolate deqin	GU256940
17	NACA 16	688	93%	99%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
18	NACA 18	687	97%	98%	Bos taurus voucher ABR	JX218056
19	OSCA 04	861	76%	98%	Bos taurus voucher ABR	JX218056
20	OSCA 12	688	70%	89%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002
21	OSCA 15	695	94%	94%	Bos indicus voucher SGJB-Si (Seeri Breed)	JN417002

AU- Aurangabad, BECA- Beed, PA-Parbhani, JA- Jalna, LACA- Latur, NACA- Nanded, OSCA- Osmanabad

The amplification across a wide taxonomic range, the ease of sequence alignment and the variability pattern rendered by Folmer region of COI makes it a good candidate for genetic identification of cattle breeds. Luo et al. (2011) have shown in their study that CO1 barcoding region, the universal DNA barcode, is preferred among the mitochondrial protein-coding genes as a molecular diagnostic for eutherian species identification. The BLAST results indicated presence of both *B. indicus* and *B. taurus* maternal origin (table 5). Amongst 21 samples, eight showed 97% to 99% homology with B. taurus that is of European ancestry. While 13 samples were 89% to 99% homologous to *B. indicus* that is Indian Zebu species. This indicates that the non descriptive cattle breed of Marathwada region is mainly of Indian origin- *B. indicus*. The genome wide associated studies and quantitative trait locus studies done by (Bolormaa et al., 2013) have shown that *B. indicus* and *B. taurus* are only two surviving subspecies of the ancestral wild cattle species. The mtDNA analysis also indicates that *B. indicus* and *B. taurus* constitute the majority of the cattle populations in the world and were domesticated independently in India (Bradley et al. 1996; 1998). Thus, the non descriptive cattle breed of Marathwada region shows presence of both subspecies i.e. that *B. indicus* and *B. taurus*.

Summary

The present study on the genetic characterization of non descriptive cattle breed of Marathwada region has genetic variation within and among the seven populations. The genetic variation in natural population is maintained wherein there are forces other than genetic drift that maintains the genetic variation amongst and within them. The cattle breed belongs mainly to Indian origin, namely *Bos indicus* as it is evident from mitochondrial sequence analysis. These findings can be useful for determining inbreeding levels and DNA barcoding of the cattle breeds.

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