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Assessment of genetic diversity in native cattle breeds of Tamil Nadu through Y-chromosome microsatellite markers and allelic profiling

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Abstract
The Y-chromosome microsatellite DNA marker is used to understand the polymorphism of this marker in native cattle from Tamil Nadu, as the Y-chromosome marker serves as an analog line for males, while the mitochondrial DNA (mtDNA) line is relevant for females. The study involved 25 unrelated male animals from each breed, resulting in a total of 100 samples across the four breeds. The maximum number of alleles was two alleles identified at each Y-microsatellite locus, while the average allele count across populations was 1.1, and the total number of alleles was relatively low (ranging from 1 to 2 alleles). The heterozygosity values for seven breeds ranged from 0 to 53%. The INRA 062 locus had the highest heterozygosity value (53%) among the microsatellite loci in the Pullikulam cattle population, which are local cattle from southern part of Tamil Nadu. The PIC values for all microsatellites ranged from 0.10 to 0.29, with the highest value recorded in Umblacherry cattle.

Keywords: Y-chromosome microsatellite, cattle genetics, polymorphism, heterozygosity, Tamil Nadu breeds

Introduction
The genetic studies of native cattle of Tamil Nadu have interested since Tamil Nadu has a great genetic variation of native cattle. For instance, Kangayam cattle was the most popular native cattle comparing other breeds because these cattle have been known as the one of Banteng (*Bibos banteng*) descendant. In the other hand, we also could find others native cattle of Tamil Nadu that suspected to have genetic status descendent from Banteng (i.e., Kangayam and Umblacherry cattle) where might be introducing genetic by modern breeds like *Bos indicus* or *Bos taurus*. So, this was important more study for native cattle of Tamil Nadu regarding to genetic improvement or genetic preserved of native cattle. Because, it could prevent from decreasing or extinction of the specific characters of native cattle of Tamil Nadu.

The various techniques and research for the genetic characterization of indigenous cattle were performed based on blood type and protein (Namikawa *et al.*, 1980) [18], blood proteins and enzymes, and the amino acid β-chain from hemoglobin X. These results demonstrated that Kangayam cattle have a unique characteristic, such as the HbX allele in their blood, which has not been found in other breeds, especially Zebu and Taurine. Information regarding specific alleles or breed-specific alleles from molecular data on native cattle in Tamil Nadu remains limited. The specific allele or breed-specific allele from the short tandem repeat at locus INRA 023 of Kangayam cattle has been documented. Moreover, Kangayam cattle possess a specific allele at the HEL9 and INRA 035 microsatellite loci compared to the *Bos taurus* breeds (Simmental, Limousin, and Brangus). Thus, this study only yielded limited findings concerning the specific alleles of native cattle. Utilizing 16 microsatellite markers (Winaya, 2000) [32], DNA microsatellite analysis was able to identify the genetic relationships among native cattle from *Bos indicus*.

In this study, we focused on Y-chromosome microsatellite DNA markers to explore the polymorphism of this marker in native cattle populations.

Since Y-chromosome microsatellites are male-specific, we hoped this marker would demonstrate male genetic attributes. It is acknowledged that males play a significant role in the genetic transmission of traits from ancestors to their offspring. Additionally, Kangayam cattle, a descendant of Banteng represented by the indigenous breed of Tamil Nadu, must be preserved to avoid extinction. Moreover, the analysis of genome and population genetics led to Y-chromosome haplotype analysis, which is an important tool for studying populations in their natural context (Hurles and Jobling, 2001) [12].

The Y-chromosome is the only segment of the mammalian genome that is exclusively inherited from the paternal line, or patrilineality. As such, the Y chromosome serves as a unique marker for investigating the contributions of animals to the evolutionary history of male individuals within a species. For example, genetic markers associated with the Y-chromosome have significantly contributed to the understanding of human phylogeography (Hammer *et al.*, 1997) [10]. However, the data on Y-chromosome genetic populations in non-primates, such as horses, cattle, and sheep, remain quite limited due to the rarity of the marker on the Y-chromosome and the lack of sequence information (Petit *et al.*, 2002) [25], along with low levels of variation (Hellborg and Ellegren, 2004; Meadows *et al.*, 2004; Queney *et al.*, 2001) [11, 16, 26]. Therefore, it can be concluded that the various combinations of mutations throughout the male lineage represent a conserved and singular haplotype linkage that seldom deviates or shows bias. Analysing genetic variations in this area is expected to yield more accurate predictions regarding the characteristics of male cattle.

The Y-chromosome marker was required as a male analog line, as well as a mitochondrial DNA (mtDNA) line in females. The levels of polymorphism in the non-recombinant Y-chromosome region range from the rare occurrence of bi-allelic mutations at Single Nucleotide Polymorphisms (SNPs) to the more commonly found variations at minisatellite or microsatellite locus markers (short tandem repeat STR). Furthermore, SNP polymorphisms on the Y-chromosome are often identified in specific populations (Hammer *et al.*, 1997) [10].

Materials and Methods

Sample Collection and DNA isolation

The research study involved 25 unrelated male cattle for each breed, resulting in a total of 100 samples from four breeds. The animals were aged from 1.5 to 2.0 years, and all were male. The samples were collected from the native areas of farmers' fields for each breed, in addition to District Livestock Farms and Frozen Semen Stations in Tamil Nadu. For each male cattle, 10 mL blood samples were collected. The blood cells were obtained through a vacutainer from the jugular vein. These blood cells were subsequently mixed with 10% EDTA for preservation and as an anticoagulant until they were used for the isolation of the DNA genome.

The isolation of genomic DNA from blood cells was performed using the standardized protocol of the phenol-chloroform extraction method (Sambrook *et al.*, 1989) [27]. To evaluate the quality and quantity of the extracted DNA, horizontal submarine mini electrophoresis was conducted in 0.8% agarose, employing 0.5X TBE as the running buffer. The measurements were obtained by assessing the optical density (OD) at 260 and 280 nm wavelengths in a UV-VIS spectrophotometer.

Primer selection and standardization of PCR: The primer pairs utilized in this study were focused on the Y-chromosome locus microsatellite sequence of Bos taurus. The PCR amplifications were performed using a thermal cycler machine (PCR machine) that was programmed for each specific primer pair. The master mix composition was as follows: 60 ng of template DNA; 0.8 units of Taq polymerase; 0.2 mM of dNTPs; 0.5 µmol of primers (both forward and reverse); and 1 X PCR buffer containing 1.5 mmol of $MgCl_2$ (Table 1).

Genotyping of Microsatellite Markers

The genotyping of microsatellite markers was made by capillary electrophoresis using the automatic sequencer, and the data were captured using ABI 3130 of Applied Biosystems, India Data collection Software Version 4.0. The basic measures of genetic variation were computed using Microsatellite Analyzer version 3.15 (Dieringer *et al.*, 2003) [4]. The exact test for Hardy- Weinberg equilibrium was performed using Arlequin version 3.1 (Excoffier *et al.*, 2005) [7]. The Polymorphism information content was estimated using GenAlEx program (Peakall *et al.*, 2006) [23].

Statistical analysis

The bands that appear in the polyacrylamide gel, stained with silver at each locus and genotyping of microsatellite markers were assumed to represent the DNA microsatellite alleles. The diversity of these microsatellite alleles was determined by the differences in allele migration observed in the gel for each individual sample. Then, the frequency of each allele at each microsatellite locus was calculated using the formula.

$$X_i = \frac{2n_{ij} + \sum n_{ij}}{2n}, \ldots, j \neq 1$$

Where,

- Xi= Allele frequency i
- nij= Individual number for genotype ij
- n= allele number

The mean value of heterozygosity (h) is used to measure genetic diversity across all loci, including both polymorphic and monomorphic types. A locus is classified as polymorphic when the allele frequency obtained is equal to or below 0.99 (Nei, 1987) [19]. The equation for calculating the heterozygosity (h) for each locus is

$$h = 2n\frac{1 - \sum Xi^2}{2n - 1}$$

Where,

- h- Heterozygosity of locus
- Xi= Allele frequency of locus ith
- n=Number of individual samples

The phylogenetic trees were subsequently constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on 1000 bootstrap values (Sokal and Sneath, 1963) [28].

Results and Discussion

The heterozygosity and Polymorphic Information Content of Y-chromosome

The genetic diversity of native cattle breeds in Tamil Nadu was assessed by examining the types and quantities of alleles, heterozygosity, and Polymorphic Information Content (PIC) in Y-chromosome microsatellites. The maximum number of alleles observed was two, which were present at every microsatellite locus, while the average number of alleles across all populations (comprising seven cattle breeds) was 1.1, indicating a generally low overall allele count (ranging from 1 to 2 alleles) (Table 2).

The heterozygosity values for four breeds ranged from 0 to 53%. The highest microsatellite loci heterozygosity (h) was observed at the INRA 062 locus (53%) within the Umblacherry cattle population, as shown in Table 2. Meanwhile, the PIC values for all microsatellites fall between 0.00 and 0.38, with the highest value recorded in Kangayam cattle; however, this was considered low polymorphic according to Botstein *et al.*, (1980) (Table 2) [1].

Phylogenetic relationship

Based on the analysis of Y-chromosome microsatellite markers, the genetic relationship (phylogenetic) indicated that Kangayam cattle and Umblacherry cattle had a genetic distance of 71%, marking them as the closest. Despite this, they were still categorized within the same cluster. Meanwhile, Pullikulam cattle were found to be the nearest cluster to these three breeds. On the other hand, Bargur was classified as a distinct cluster compared to the breeds studied.

Y-chromosome microsatellite polymorphism

The polymorphism of microsatellite markers, as shown by the heterozygosity (h) value, was low, with values from 0 to 53%. This finding is different from the previous research by Li *et al.* (2007), which studied Ethiopian cattle using five microsatellite loci of the Y-chromosome (INRA124, INRA126, INRA189, BM861, and BYM-1), where the heterozygosity values ranged from 0.541 (54.1%) to 0.795 (79.5%). These results indicate that the genetic diversity of Ethiopian cattle remains relatively high compared to the native cattle of Tamil Nadu. Several potential factors may account for these differences. Because this study utilized microsatellite markers from the Y-chromosome, the low average number of alleles on the Y-chromosome could be affected by selection factors, mating systems, migration patterns, or other mechanisms that lead to a reduced number of males for effective population size (Meadows *et al.*, 2006) [15].

The research conducted by Ginja *et al.* (2009) [9] investigated genetic variations on the Y-chromosome, utilizing Portuguese cattle (Bos taurus) and Brahman cattle (Bos indicus) through SNPs and STRs (short tandem repeats) located on the Y-chromosome. The average heterozygosity value (h) for Portuguese cattle ranged from 0.09 (9%) to 0.30 (30%), while Brahman cattle had a value of 0 (0%). The Y chromosome was identified as a non-recombinant region, effectively receiving selective pressure due to a specific sequence on the chromosome, excluding pseudo-autosomal regions. This condition is markedly different from that of the autosomes and X chromosome, where recombination events can lead to new DNA sequences from various locus compositions (Nowak, 1991) [22].

According to FAO (2004) [8] guidelines, assessing genetic variation between breeds requires at least four different alleles per locus. Therefore, in this study, none of the loci were deemed appropriate for these criteria. Consequently, we suggest increasing the number of samples and expanding the geographical range for the analysis of genetic variation among breeds. It is assumed that a larger sample size in this research could lead to the discovery of additional alleles that may be classified as polymorphic alleles, in accordance with FAO (2004) [8] provisions. In fact, we have not yet identified more alleles. This situation could be influenced by other factors, such as inbreeding. Generally, the management of animal mating, particularly through artificial insemination (AI), tends to utilize a limited number of bulls. Conversely, the heterozygosity value exceeding 50% indicates that the genetic diversity within the population is relatively high. Thus, increasing the number of individuals in the population could enhance the observed diversity. It is hoped that specific candidate markers for Indonesian native cattle can be identified based on Y-chromosome microsatellite markers. This was important because the status of the males as a transmitter of specific genetic character was still needed, especially related to breeding management.

In this study, an intriguing phenomenon was also identified, where certain individuals of Kangayam cattle possess double alleles or multiple alleles, or are multi-allelic at similar loci, such as the INRA 057 and INRA 189 loci. Theoretically, this phenomenon should not occur, as Y-chromosomal microsatellite alleles are based on a haplotype model. However, according to Li *et al.* (2007), the events of multi-allelic forms resembling ladder-like DNA (ladder-like bands) are unique occurrences of Y-chromosome microsatellites, referred to as multi-copy or hemizygous events at microsatellite loci. Similarly, Edwards *et al.* (2000) [5] also found several multi-alleles at Y-chromosome microsatellite loci, specifically at INRA 126 in Bos taurus and Bos indicus cattle. Li *et al.* (2007) state that the presence of double or multiple alleles (multiple-copy alleles) at microsatellite loci is due to their location in the Pseudo Autosomal Regions (PAR) rather than in the Y-Specific Male (MSY) region. It is established that the PAR area is the terminal region of the Y-chromosome, with an inheritance model similar to that of the autosomal region. Consequently, males possess two copies of the gene or DNA sequence in their Y-chromosomal region; one set is located in the PAR, while the other set is paired with the X-chromosome. Therefore, males can inherit their alleles on the X-chromosome from their fathers, and females can inherit their alleles on the Y-chromosome from their fathers.

Perez-Lezaun *et al.* (1997) and Li *et al.* (2007) [14, 24] have shown that Y-chromosome microsatellites at the INRA 124 and INRA 126 loci can be amplified in females. This indicates that the alleles at these loci may lead to multi-allelic events, as these alleles were also detected on the X chromosome of females. Therefore, Perez-Lezaun *et al.* (1997) [24] advised against the use of these two microsatellites in the genetic characterization of incidents involving male introductions. Furthermore, Edwards *et al.* (2007) [6] reported that the INRA 126 microsatellite could be amplified in both male and female yak (Bos grunniens). This finding also suggested that the INRA 126 locus has a homologous sequence on the X chromosome. Thus, the

phenomenon of double or multi-copy alleles observed in this study exemplifies the uniqueness of Y-chromosome microsatellite alleles. Nevertheless, when assessing genetic variation, double or multiple-allele copies are still classified as a single allele type, as the additional allele is merely a duplicate or copy of the original allele. This occurrence can be attributed to the effects of segregation and recombination during chromosomal events, particularly in the pseudoautosomal region (PAR) of the Y-chromosome, where such unique events transpire.

The genetic relationship based on Y-chromosome microsatellite: According to the genetic relationship or phylogenetic analysis, it was found that Kangayam and Umblacherry cattle share the closest genetic distance (71%) when compared to other breeds (Figure 1). Nevertheless, these Tamil Nadu local cattle breeds were still grouped within a single cluster. This indicates that these breeds (Kangayam-Kangayam, Umblacherry, and Kangayam) exhibit genetic closeness based on the seven locus alleles of the Y-chromosome microsatellite that were analysed. In contrast, Pullikulam cattle, while in a different cluster, have the closest genetic distance to one cluster. Therefore, it can be inferred that Pullikulam cattle may also have a closer genetic relationship with Kangayam and Umblacherry cattle than with Bargur cattle. Although Bargur cattle are categorized in a separate cluster from the other Tamil Nadu breeds, it can be suggested that they might possess a specific locus of Y-chromosome microsatellite from Bos indicus (i.e., INRA 124, 126, and 189 INRA INRA). Generally, the phenotypes of Bargur cattle are similar to those of other indigenous breeds or Bos indicus, particularly in terms of coloured skin and humped appearance. Thus, it is assumed that a larger proportion of the genetics of Bargur cattle is derived from the Bos indicus breed. The Polymorphic Information Content (PIC) values of the Y-chromosome microsatellite DNA marker ranged from 0 to 0.37, with a mean PIC value between 0.10 and 0.27. Among the seven loci examined, none exhibited a PIC value exceeding 0.50. Therefore, as per Botstein *et al.* (1980) [1], the seven loci utilized in this study were classified as less informative for population genetics analysis. Additionally, Meadows *et al.* (2006) [15] reported low variability in the nucleotide sequences within a specific region of the Y-chromosome in certain animal species, including cattle. Consequently, further research is necessary to gather more insights regarding the polymorphic loci of the Y-chromosome microsatellite markers in native cattle from Tamil Nadu. However, based on previous studies, we anticipate that this research will contribute valuable information for understanding the genetic diversity of Indonesian native cattle.

Research conducted by Cai *et al.* (2006) [2] on native cattle in China, which focused on two microsatellite loci, UMN2404 and UMN0103, revealed that each marker contained only two alleles. This likely indicates a lineage from both Bos indicus and Bos taurus breeds, suggesting that Chinese cattle may be a hybrid of these two breeds. Furthermore, numerous studies have proposed that Chinese cattle are descendants of the Bos primigenius species (Chen *et al.*, 1995) [3]. It is believed that a Mongolian tribe, during their migration to China, also brought along cattle, which were domesticated from the wild Primigenius breed and subsequently crossed with Bos taurus breeds. This factor may explain the genetic mixing observed in Chinese native cattle. Similarly, Indonesian native cattle present a somewhat different scenario, where the introduction of Bos taurus and Bos indicus genes occurred due to political influences during the last few decades of governance in India.

Additionally, based on initial studies by Namikawa *et al.* (1980) [18] regarding blood types, as well as research on proteins (Noor *et al.*, 2000) [21], satellite and microsatellite DNA from autosomes (Winaya, 2000; Verkaar *et al.*, 2002, 2003; Nijman *et al.*, 2003; Ugla, 2008), and mitochondrial DNA (Verkaar *et al.*, 2002, 2003; Ugla, 2008; Mohamad *et al.*, 2012) [17, 20, 29, 30, 31, 32], it has been established that a significant genetic component of the native cattle in Tamil Nadu is derived from Banteng. This leads to the conclusion that Banteng and Indonesian native cattle share a close genetic relationship, as indicated by phylogenetic analysis of genetic distances. To accurately define the characteristics of Tamil Nadu's local cattle, we recommend using this reference, as it supports the notion that the genetic traits of Tamil Nadu's native cattle, including Kangayam, Umblacherry, Bargur, and Pullikulam, should exhibit a higher genetic contribution from Banteng. Furthermore, historical evidence from both fossil remains and documented records indicates that Banteng is one of the ancestral breeds of cattle globally.

Table 1. List of Microsatellite Markers Sequence and their Allele Size

Locus	Primer sequences (5'-3')	Allele Range (bp)	References
INRA008	F- GAG CCT GTG TGT GTA TAC AC R- GGC ACT TTC CTC TCC TGT CGC G	135-142	Vaiman *et al.*, 1994
INRA057	F- CCT AGC GAC TGT CCA AGC G R- CAC GGG CTG AGA ATT CAA AAC	120-134	Vaiman *et al.*, 1994
INRA062	F-TGT GCA GCA CCT TGT CTC C R-ACA TGC ATG TGC TTG TGT CG	150-167	Vaiman *et al.*, 1994
INRA124	F-GAT CTT TGC AAC TGG TTT G R-CAG GAC ACA GGT CTG ACA TG	130-141	Vaiman *et al.*, 1994
INRA126	F-TCT AGA GGA TCA AGG ATT TGT G R-AAT CCA TGG AAA GAT GCA CTG	181-195	Vaiman *et al.*, 1994
DYS199	F-AAT AGG TTG ACC TGA CAA TGG R-TCA TTT TAG GTA CCA GCT CTT	110-123	Underhill *et al.*, 1996
INRA189	F-TAC ACG CAT GTC CTT GTT TCG G R-CTC TGC ATC TGT CCT GGA CTG G	65-128	Kappes *et al.*, 1997

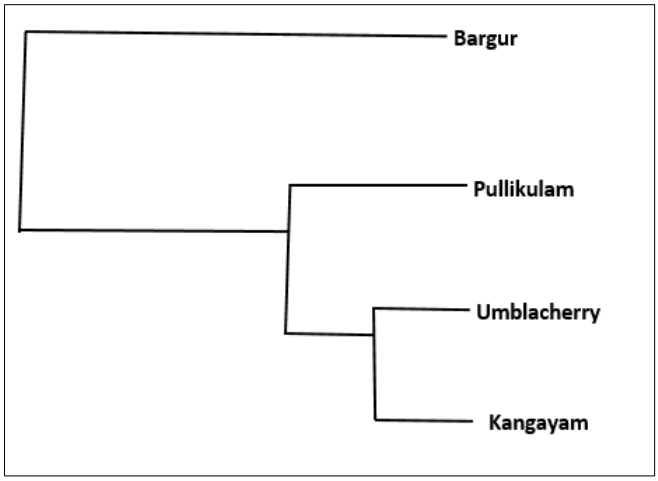

Fig 1: Phylogenetic relationship of Native cattle breeds of Tamil Nadu

Table 2: Observed number of alleles, Allele Frequency, Heterozygosity and Polymorphic Information content of Y-chromosome Microsatellite Markers

Breeds		Microsatellite Locus							Mean ±SD
		INRA008	INRA057	INRA062	INRA124	INRA126	DYS199	INRA189	
	N	25	25	25	25	25	25	25	-
Bargur	N_o	2	1	2	2	1	2	2	-
	Allele frequency	0.17	1.00	0.33	0.28	1.00	0.17	0.22	-
		0.83		0.67	0.72		0.83	0.78	-
	H	0.30	0.00	0.47	0.43	0.00	0.30	0.36	0.27± 0.20
	PIC	0.24	0.00	0.34	0.32	0.00	0.24	0.28	0.20± 0.14
Pullikulam	N_o	2	2	2	2	2	1	1	-
	Allele frequency	0.20	0.13	0.47	0.20	0.07	1.00	1.00	-
		0.80	0.87	0.53	0.80	0.93			-
	H	0.34	0.24	0.53	0.34	0.14	0.00	0.00	0.23± 0.19
	PIC	0.27	0.20	0.37	0.27	0.12	0.00	0.00	0.17± 0.14
Umblacherry	N_o	2	2	2	2	2	2	2	-
	Allele frequency	0.28	0.28	0.28	0.28	0.17	0.22	0.22	-
		0.72	0.72	0.72	0.72	0.83	0.78	0.78	-
	H	0.43	0.43	0.36	0.43	0.30	0.36	0.36	0.38± 0.05
	PIC	0.32	0.32	0.28	0.32	0.24	0.28	0.28	0.29± 0.03
Kangayam	N_o	2	2	2	2	2	2	2	-
	Allele frequency	0.33	0.11	0.17	0.44	0.11	0.17	0.11	-
		0.67	0.89	0.83	0.56	0.89	0.83	0.89	-
	H	0.47	0.21	0.30	0.52	0.21	0.30	0.21	0.32± 0.13
	PIC	0.34	0.18	0.24	0.38	0.18	0.24	0.18	0.25± 0.08
N- No of Samples, N_o- Observed Number of Alleles, H- Heterozygosity, PIC- Polymorphism Information Content									

Conclusion

The study of genetic variation in the indigenous cattle of Tamil Nadu through the molecular marker of Y-chromosome microsatellite DNA can shed light on not only the low variation found in this marker but also the phenomenon termed multiple alleles (multi-allelic) at certain loci on the Y-chromosome microsatellite. This situation, however, would not be problematic as long as there is no change in gene expression, as this marker is not a gene marker but a microsatellite marker (or accessory DNA), which will not impact protein expression.

The presence of native cattle in Tamil Nadu has been influenced by gene flow from Bos taurus and Bos indicus, including Kangayam, Umblacherry, Bargur, and Pullikulam cattle. Kangayam cattle, in particular, exhibit a greater character introduced by Banteng (Bibos banteng). It is suggested that native cattle may contain a proportion of Banteng genetics. However, this study indicates that the polymorphism of Y-chromosome microsatellite DNA is low. Therefore, further research is needed, considering a larger sample size and a broader geographical area. Kangayam and Umblacherry cattle are recognized for having the largest genetic contribution from Banteng, allowing both breeds to continue as native cattle of Tamil Nadu. Conversely, Bargur and Pullikulam cattle have a significant opportunity to enhance their existence as specific regional cattle, despite their genetic composition being more aligned with Bos indicus characteristics.

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