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Estimation of genetic variability parameters in Punganur cattle by microsatellite markers

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Abstract
Assessment of genetic variability in Punganur breed of cattle in Andhra Pradesh was carried out using 20 microsatellite markers. The mean number of alleles was 7.9±0.475 with a range of five to fourteen and allele size varied from 79 to 309 bp. The allelic frequency of microsatellite alleles varied from 0.0037 to 0.3824. Allelic richness obtained within the breed had a mean value of 6.17053 ± 0.29669. The mean observed and expected heterozygosity values were 0.0202 ± 0.0079 and 0.8384 ± 0.1946, respectively. The mean of within population inbreeding and Shannon index values were 0.9756 ± 0.0094 and 1.89044, respectively. Higher PIC (0.8171) value indicated the scope for maintaining variation in population. The Punganur cattle population studied was found to be deviated from Hardy-Weinberg equilibrium. High Inbreeding, shortfall of heterozygote's and little genetic variation was noticed in Punganur population.

Keywords: Inbreeding, microsatellite markers, molecular characterization

1. Introduction
A large and divergent range of agro-ecological zones in India resulted in the development of number of defined cattle populations. Cattle are the largest livestock species in India with 43 well recognised breeds (NBAGR 2018) [1]. Only 11.6% of total cattle (190.9 million) belong to pure indigenous breeds, whereas remaining 69.7% were classified as non-descript indigenous animals. The Southern Peninsular region of India is home to fourteen descriptive cattle breeds. Of which, Punganur cattle is the shortest humped dwarf cattle breed originating from Punganur area in Chittoor district of Andhra Pradesh. Punganur cattle are distributed in Chittoor and to some extent in West Godavari and Krishna districts. Genetic characterization of populations, breeds and species allows the evaluation of genetic variability, a fundamental element in working out breeding strategies and genetic conservation plans. Information about genetic diversity and differentiation of Punganur breed is essential to formulate appropriate conservation, breeding and sustainable management programmes in order to prevent extinction and genetic erosion of this breed. Microsatellite markers have been widely used for population genetic analyses of livestock species, as they are informative and can successfully elucidate the relationships between individuals and populations, assess within-breed genetic diversity and inbreeding, introgression from other species, genetic differentiation and admixture among breeds (Ghazy *et al.*, 2013) [3]. Hence, the present research work was carried out to study the genetic variability of Punganur breed using 20 microsatellite markers.

2. Materials and Methods
2.1 Experimental animals
A total of 50 blood samples were collected at random from Punganur cattle (Figure 1 and 2) maintained at Livestock Research Station, Palamaner (35 number) and Farmers herds (15 number). The experiment was conducted at The Department of Animal Genetics and Breeding, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati from March to December, 2017. Genomic DNA was isolated by following the standard protocol involving Proteinase K digestion and Phenol: chloroform extraction (Sambrook *et al.*, 1989) [4].

2.2 Microsatellite marker typing
A total of twenty microsatellites (BM1818, BM1824, BM2113, CSRM60, ETH10, ETH152, ETH225, HAUT27, HEL 1, ILSTS005, ILSTS006, INRA005, INRA035, INRA037, INRA063, MM12, SPS115, TGLA53 and TGLA227) were selected based on degree of polymorphism and genomic coverage (FAO 2004) [6] are given in Table 1.

The selected microsatellite markers complied with the recommendations of the FAO and the International Society for Animal Genetics (ISAG) [5]. Amplification of loci was performed in a final reaction volume 15 µl containing 100 ng of genomic DNA, 10 pM of each primer, 25 mM $MgCl_2$, 200 Mm, dNTPs, 5 U/µl of *Taq* polymerase and 10X Taq buffer (Table 2). Optimization of PCR reaction was carried out for PCR assay buffer, $MgCl_2$ and annealing temperature. PCR was carried out in Kyratec ® Thermal cycler (M/s JH BIO, Bangalore). A PCR programme with an initial denaturation at 94ºc for 5 minutes followed by 35 cycles of denaturation for 94ºc for 45 seconds with annealing temperature ranging from 52º c to 66ºc (depending upon the primer used) for 45 seconds and an extension duration of 45 seconds at 72º c was used. Final extension was done at 72ºc for 5 minutes followed by refrigeration at 4ºc (Table 3). Amplified PCR products were checked on agarose gel electrophoresis with 50bp DNA ladder. Genotyping of animals was done based on allele size. The genotypes were scored based on the presence of a single band (homozygotes) or double bands (heterozygotes) on the agarose gel slab.

Table 1: The primer sequences and chromosomal localization of the microsatellites used

Sl. No.	Microsatellite Locus	Primer sequence (5'-3')	Chromosome number	Accession number	Annealing temperature (^{O}c) (reported)
1	BM1818	F: AGCTGGGAATATAACCAAAGG R: AGTGCTTTCAAGGTCCATGC	23	G18391	57.5
2	BM1824	F: GAGCAAGGTGTTTTTCCAATC R: CATTCTCCAACTGCTTCCTTG	01	G18394	57.0
3	BM2113	F: GCTGCCTTCTACCAAATACCC R: CTTCCTGAGAGAAGCAACACC	02	M97162	57.0
4	CSRM60	F: AAGATGTGATCCAAGAGAGAGAGGCA R: AGGACCAGATCGTGAAAGGGCATAG	10	Z14042	63.5
5	ETH10	F: GTTCAGGACTGGCCCTGCTAACA R: CCTCCAGCCCACTTTCTCTTCTC	05	Z22739	66.0
6	ETH152	F: TACTCGTAGGGCAGGCTGCCTG R: GAGACCTCAGGGTTGGTGATCAG	05	Z14040 G18414	64.0
7	ETH185	F: TGCATGGACAGAGCAGCCTGGC R: GCACCCCAACGAAAGCTCCCAG	17	Z14042	66.0
8	ETH225	F: GATCACCTTGCCACTATTTCCT R: ACATGACAGCCAGCTGCTACT	09	Z14043	58.0
9	HAUT27	F: AACTGCTGAAATCTCCATCTTA R: TTTTATGTTCATTTTTTGGACTGG	26	X89252	52.0
10	HEL 1	F: CAACAGCTATTTAACAAGGA R: AGGCTACAGTCCATGGGATT	15	X65202	52.0
11	ILSTS005	F: GGAAGCAATGAAATCTATAGCC R: TGTTCTGTGAGTTTGGTAAGC	07	L23481	54.5
12	ILSTS006	F: TGTCTGTATTTCTGCTGTGG R: ACACGGAAGCGATCTAAACG	07	L23482	57.0
13	INRA005	F: CAATCTGCATGAAGTATAAATAT R: CTTCAGGCATACCCTACACC	10	X63793	52.5
14	INRA035	F: TTGTGCTTTATGACATATCCG R: ATCTTTGCAGCCTCCACATTG	16	X68049	57.5
15	INRA037	F: GATCCTGCTTATATTTAACCAC R: AAAATTCCATGGAGAGAGAAAC	10	X71551	53.0
16	INRA063	F: ATTTGCACAAGCTAAATCTAACC R: AAACCACAGAAATGCTTGGAAG	18	X71507	58.0
17	MM12	F: CAAGACAGGTGTTTCAATCT R: ATCGACTCTGGGGATGATGT	09	Z30343	53.0
18	SPS115	F: AAAGTGACACAACAGCTTCTCCAG R: AACGAGTGTCCTAGTTTGGCTGTG	15	FJ828564	61.5
19	TGLA53	F: GCTTTCAGAAATAGTTTGCATTCA R: ATCTTCACATGATATTACAGCAGA	16	-	58.0
20	TGLA227	F: CGAATTCCAAATCTGTTAATTTGCT R: ACAGACAGAAACTCAATGAAAGCA	18	-	59.0

Fig 1: Punganur bull

Fig 2: Punganur Cow

Table 2: Components of PCR mix

Sl. No	PCR components	Final Concentration	Quantity taken (µl) for microsatellites
1	PCR assay buffer A (10X) with KCl and without $MgCl_2$	10X	1.5
2	PCR assay buffer A (10X) with $(NH4)_2SO_4$ without $MgCl_2$ (15mM)	10X	1
3	$MgCl_2$	25 mM	0.9
4	dNTPs	200 µM	0.3
5	Forward primer	10 picomoles	1
6	Reverse primer	10 picomoles	1
7	*Taq* DNA polymerase	5 U/µl	0.1
8	Template genomic DNA	100 ng	1.5
9	Nuclease free water	-	Make up to 15

Table 3: Cyclic conditions used for amplification of the Microsatellite markers

Step	Process	Temperature (^{0}C)	Duration	No. Of cycles
1	Initial denaturation	94	5 minutes	1
2	Denaturation	94	45 seconds	35
3	Annealing	Varies for each locus (52 - 66)	45 seconds	
4	Extension	72	45 seconds	
5	Final extension	72	10 minutes	1
6	Refrigeration	4	Forever	

2.3 Statistical analysis for microsatellite data

Based on the size of the alleles obtained on the agarose gel electrophoresis, microsatellite allele frequencies, effective number of alleles (N_e), polymorphism information content (PIC), test for Hardy-Weinberg equilibrium, observed (H_o) and expected heterozygosity (H_e) and F-statistics (F_{IS}) were calculated using the POPGENE version 1.31 Yeh *et al.,* (199) [7]. Polymorphism information content was calculated using PIC calculator in the Google home page.

3. Results and Discussion

The parameters estimated out of microsatellite analysis in Punganur cattle such as number of alleles, effective number of alleles, range of allele sizes and range of allele frequency, chi-square value with respect to Hardy-Weinberg equilibrium, observed and expected Heterozygosity, Shannon index and within population inbreeding estimate is detailed in Table 4 and the photographs showing allele size of 20 microsatellite markers for Punganur cattle is shown in Figures 3(a) and 3(b).

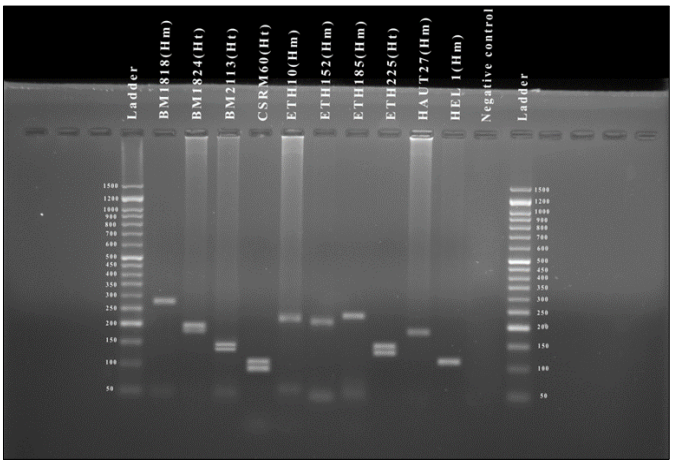

Fig 3(a): Agarose gel image showing allele size of ten microsatellite markers

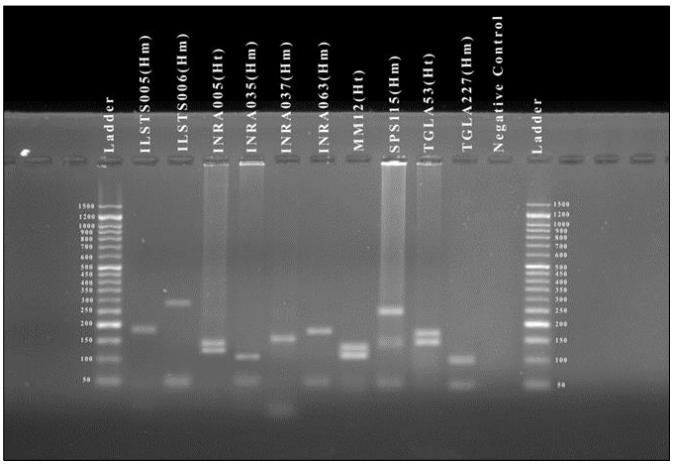

Fig 3(b): Agarose gel image showing allele size of ten microsatellite markers

3.1 Alleles at microsatellite loci

The genetic variation of Punganur cattle is characterized by a total of 158 alleles with allele size ranging from 79 bp (TGLA227) to 309 bp (ILSTS006) across 20 microsatellite loci (Figures 1 and 2) chosen and the allelic diversity differed from five (INRA005) to fourteen (TGLA227) with an overall mean of 7.9 ± 0.475 alleles indicating that all the microsatellite loci were sufficiently polymorphic and appropriate for genetic diversity analysis.

The total number of alleles (158) noticed in the present study in Punganur cattle was higher than those reported by Ngono Ema *et al.* (2014) [8] in Cameroonian indigenous cattle (139), Barani *et al.* (2015) [9] in Pulikulam cattle (142), Hussein *et al.* (2015) [10] in Sudanese Zebu cattle (74) and Vohra *et al.* (2017) [11] in Belahi cattle (149). The mean number of observed alleles (7.9) estimated in Punganur cattle were almost comparable with the previous studies of Chandra Shekar *et al.* (2011) [12] in Hallikar cattle (7.8), Ivankovic *et al.* (2011) [13] in Istrain cattle (9.1) and Barani *et al.* (2015) [9] in Pulikulam cattle (7.89).

Table 4: Genetic heterogeneity estimates at 20 microsatellite locus in Punganur cattle

Locus	N_o	N_e	Allele size	Allele frequency	H_o	H_e	Shannon index	FIS	PIC	HWE	
										Chi -square Value	df
BM1818	8	6.9193	250-279	0.0408-0.2041	0	0.8643	1.9950	1.0000	0.8384	385.623083	28
BM1824	8	5.7500	173-199	0.0408-0.2449	0.0408	0.8346	1.8921	0.9506	0.8039	365.8914	28
BM2113	6	3.9054	121-148	0.0686-0.3824	0.0392	0.7513	1.4993	0.9473	0.7045	201.699226	15
CSRM60	11	8.2565	90-116	0.0096-0.1731	0.0577	0.8874	2.1988	0.9344	0.8824	512.908951	55
ETH10	9	7.1429	179-217	0.0400-0.2200	0	0.8687	2.0701	1.0000	0.8556	457.948028	36
ETH152	7	6.2689	188-201	0.0612-0.2041	0	0.8491	1.8836	1.0000	0.8200	322.0673	21
ETH185	6	5.1194	214-244	0.1020-0.3061	0	0.8130	1.7133	1.0000	0.7778	264.267235	15
ETH225	6	4.9251	138-159	0.0408-0.2755	0.0408	0.8052	1.6649	0.9488	0.7662	248.960262	15
HAUT27	8	6.9594	146-163	0.0612-0.1837	0	0.8651	2.0028	1.0000	0.8394	381.743413	28
HEL 1	10	8.5445	101-132	0.0204-0.1633	0	0.8921	2.2007	1.0000	0.8707	539.5025	45
ILSTS005	6	5.1194	175-192	0.0816-0.2857	0	0.8130	1.7091	1.0000	0.7767	265.085907	15
ILSTS006	8	6.6880	285-309	0.0612-0.2449	0	0.8592	1.9681	1.0000	0.8331	381.427921	28
INRA005	5	5.1440	134-148	0.0120-0.3061	0.0.408	0.7446	1.3897	0.9446	0.6886	146.874148	10
INRA035	7	5.3002	101-112	0.0612-0.2449	0	0.8197	1.7574	1.0000	0.7842	365.752673	21
INRA037	6	5.2082	113-148	0.0816-0.2857	0	0.8163	1.7178	1.0000	0.7809	264.650693	15
INRA063	8	7.000	170-190	0.0816-0.2041	0	0.8660	2.2061	1.0000	0.8453	378.120630	28
MM12	10	7.5861	111-149	0.0306-0.2347	0.1429	0.8771	2.1544	0.8355	0.8547	330.867232	45
SPS115	8	5.3237	241-271	0.0408-0.3061	0	0.8205	1.8401	1.0000	0.7882	405.736156	28
TGLA53	7	6.2936	148-183	0.0612-0.2245	0.0408	0.8498	2.8864	0.9515	0.8210	296.286002	21
TGLA227	14	7.2979	79-107	0.00016-0.2857	0	0.8719	2.2392	1.0000	0.9218	869.502823	91
Mean	7.9±0.4	6.17±0.2		0.0096-0.3824	0.0202±0.007	0.8384±0.194	1.8904	0.9756±0.009	0.81232±0.0125		

**Highly significant ($p<0.01$)

The effective number of alleles (N_e) varied from 3.9054 (BM2113) to 8.5445 (HEL 1) with an overall mean of 6.17 ± 0.29669. The allelic richness observed in Punganur cattle was higher than those noticed by Kumar *et al.* (2014) [14] in Non-descriptive cattle (5.122), Barani *et al.* (2015) [9] in Pulikulam cattle (3.73) and Vohra *et al.* (2017) [11] in Belahi cattle (4.39).

3.2 Heterozygosity

The mean observed heterozygosity (H_0) was 0.02015 ± 0.0079 with a range from 0.00 (BM1818, ETH10, ETH152, ETH185, HAUT27, HEL 1, ILSTS005, ILSTS006, INRAO35, INRA037, INRA063, SPS115 and TGLA227) to 0.1429 (MM12). Deficiency of observed heterozygosity in the present study might be attributed to the non-random mating that occurred among the population. The observed heterozygosity values were lower, when compared to the expected heterozygosity for all loci in the present study was in agreement with the findings of earlier research workers Barani *et al.* (2015) [9] in Pulikulam cattle and Vohra *et al.* (2017) [11] in Belahi cattle, which might be due to fact that samples collected from the cattle which were maintained as closed related population and also due to presence of more homozygosity in the individual samples analyzed. Whereas the least expected heterozygosity value was 0.7446 (INRA005) and the highest was 0.8921 (HEL 1) followed by 0.8874 (CSRM60) with an average of 0.8384 ± 0.1946. Earlier studies of Vargas *et al.* (2016) [15] in Amazonian Macabea cattle (0.728) and Vohra *et al.* (2017) [11] in Belahi cattle (0.72) revealed lower mean values for expected heterozygosity compared to the values obtained in the present study. The shortage of observed heterozygosity in population might be due to the fact that the present population of Punganur cattle at Livestock research station, Palamner descended from the foundation stock of 56 cows and five bulls since the year 1994 and the progeny produced over 23 years (> four generations) of time span is a closed herd.

3.3 Within population inbreeding estimate

The inbreeding (F_{IS}) estimate describes the excess or deficit of heterozygotes within sub-population. The inbreeding estimates (F_{IS}) obtained in the present study were all positive and varied from 0.8355 (MM12) to 1.00 (BM1818, ETH10, ETH152, ETH185, HAUT27, HEL 1, ILSTS005, ILSTS006, INRA035, INRA037, INRA063, SPS115 and TGLA227) with a mean F_{IS} value of 0.9756 ± 0.0092 indicating an excess of homozygotes in the population, which, are comparable with Siwa (0.83) and Farafra (0.69) breeds (El-Sayed *et al.*, 2016) [16] and Punganur cattle (Devi *et al.*, 2017) [17]. In contrast to the present findings, high heterozygosity values were recorded by Karthickeyan *et al.* (2007) [18] in Umblachery breed (-0.0487) and Hussein *et al.* (2015) [10] in Sudanese Zebu cattle breeds Fuga (-0.317), Butana (-0.830) and Kenana (-0.195) cattle breeds of Sudan.The shortage of heterozygotes and excess of homozygotes in the population could be attributed to a number of factors *viz.*, wahlund effect, assosrative mating, and linkage with loci under selection (genetic hitchhiking), population heterozygosity, null alleles or inbreeding.

3.4 Shannon Index

The Shannon Index is a parameter for determining diversity index. The mean value for Shannon Index is 1.89044, which clearly indicates the higher amount of genetic variation in the population and there is more scope for conservation. Thiagarajan (2012) [19] reported the mean Shannon Index value of 2.1994 in Umblachery population.

3.5 Polymorphism Information Content

The polymorphism information content (PIC) values across the twenty microsatellite loci ranged from 0.6886 (INRA005) to 0.9218 (TGLA227) with an overall mean of 0.81232 ± 0.0125. All the markers selected for this study had PIC values higher than 0.5 which indicated the suitability of these markers for the genetic diversity analysis and also suggested the utility of the microsatellites in molecular characterization and there by exploitation of genetic variability of the population. Similar results were noticed in earlier reports of indigenous breeds of cattle by Chenna Kesvulu *et al.* (2009) [23] in Punganur cattle (0.809), Barani *et al.* (2011) [12] in Pulikulam cattle (0.8251).

3.6 Hardy–Weinberg equilibrium

The chi square test results of all the twenty loci revealed significant deviation of all loci from Hardy-Weinberg equilibrium. Population disequilibria similar to present study was also noticed at 12 out of 18 in Pulikulam cattle (Barani *et al*., 2015) [12] and three out of 28 loci in Macabea cattle (Vargas *et al*., 2016) [18]. Deviation of all the loci in Punganur cattle from Hardy-Weinberg equilibrium may be attributed to the presence of low frequency null alleles (non-amplifying alleles) segregating at all these loci, small sample size and inbreeding. High positive F_{IS} (within population inbreeding estimates) values and presence of population substructure (Wahlund effect) could be the reasons for the deviation at all loci.

4. Conclusion

The present study is an attempt to understand genetic variation of Punganur cattle breed using 20 microsatellite markers. The overall mean polymorphic information content of 81 percent, these markers are highly informative and suitable for characterization of domestic animal biodiversity. High Inbreeding, shortfall of heterozygotes and little genetic variation was noticed in Punganur population, suggesting that appropriate measures has to be adopted. The dwindling population of this breed requires immediate attention and conservation. Comparative analysis with other zebu breeds of cattle in India will lead to determination of genetic divergence among breeds of India. The information generated in the present study will pave the way for designing appropriate selection, breeding and conservation strategies for this unique cattle genetic resource of the region.

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