Indian Journal of Animal Sciences **94** (11): 942–947, November 2024/Article
https://doi.org/10.56093/ijans.v94i11.152528

Genetic diversity and phylogenetic status of Punganur cattle based on mtDNA analysis

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Received: 8 June 2024; Accepted: 20 September 2024

ABSTRACT

The Punganur is distinctive Indian cattle breed that has witnessed a decline in purity due to uncontrolled crossbreeding practices. This study aims to assess the genetic diversity and phylogenetic status of Punganur cattle by analyzing the mtDNA D-loop region. mtDNA D-loop sequences generated from 47 individuals belonging to Punganur breed, cattle similar to Punganur breed, other dwarf cattle breeds and 165 additional sequences representing *Bos taurus* and *Bos indicus* from the database were used for comparative analysis. 119 polymorphic sites and 130 haplotypes were observed, with an overall haplotype and nucleotide diversity of 0.988 and 0.028, respectively. Phylogenetic analysis indicated a lack of clear demarcation between Punganur and Punganur Type animals. A Holstein animal clustered with a Punganur Type (PP12) animal, suggesting a connection to the crossbreeding program in the country. Based on the mtDNA D-loop region, Punganur and Punganur Type animals could not be differentiated, emphasizing the need for additional molecular tools and a larger sample size to address this question adequately. This study highlights the urgency of preserving the genetic integrity of Punganur cattle and calls for further comprehensive analyses to elucidate their true phylogenetic relationships.

Keywords: D-Loop, Genetic diversity, Haplotype diversity, Mitochondrial DNA, Punganur cattle, Phylogenetic status

Cattle form a unique role in Indian community by virtue of its historical, cultural and economic values (Cunningham 1992). In the diverse landscape of Indian agriculture, dwarf cattle breeds have emerged as invaluable assets, offering advantages such as low maintenance costs, resilience against diseases, and adaptability to varying environmental conditions. This makes them particularly indispensable for the sustenance of small, marginal, and landless farmers (Srinivas and Ramesha 2017). The Punganur cattle, originating in the Chittoor district of Andhra Pradesh in southern region of India, exemplifies one such short-statured breed. This breed, as described by historical accounts from Shortt (1876), Wallace (1888), and Slater (1918), has been identified as a small variety within the broader category of Mysore cattle. Extensive crossbreeding with Mysore cattle (Littlewood 1936) and the infusion of exotic breeds such as Jersey, Holstein Friesian, and predominantly Kerry (Report 1989) have led to a considerable reduction in the number of pure Punganur cattle, reaching a critical low of approximately 70 (Pundir and Sahai 1997). A subsequent revival, with the population increasing to 2772 (Report 2014), has been attributed to concerted efforts aimed at rescue and conservation by both governmental initiatives and progressive farmers. In light of the growing emphasis on the conservation and development of the Punganur cattle breed, it becomes imperative to delve into the historical context, challenges faced, and the recent resurgence in numbers.

The mitochondrial DNA (mtDNA) serves as a valuable tool for investigating questions related to breed development and conservation, providing insights into genetic diversity and ancestry (Gupta *et al*. 2015). Being an extranuclear genome with a genome size of approximately 16 kb and multiple copies, the mtDNA possesses distinctive features such as haploidy, abundance in cells, small genome size, maternal inheritance, a higher mutation rate than nuclear DNA, and a propensity for change mainly through mutation rather than recombination (Sorenson *et al*. 1999). The displacement loop (D-loop) region has the ability to accumulate mutations at high and neutral rate as it is the non-coding region in mtDNA.

In the backdrop of the impending extinction of the Punganur cattle breed in Andhra Pradesh and the uncertainties regarding its purity based on existing literature,

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this study sought to unravel the genetic relationships and phylogenetic status of both Punganur and Punganur-like animals based on the D-loop region of mitochondrial DNA.

MATERIALS AND METHODS

Collection of samples: This study does not warrant permission from Institutional Animal Ethics Committee as no experimentation was conducted on animals. A total of 47 unrelated blood samples that were aseptically collected from different cattle breeds, including 28 Punganur, 5 Ongole, 5 Hallikar, 4 Bargur, 3 Malnad Gidda and 2 Vechur were obtained from various Veterinary clinicians. Among the Punganur samples, 14 were categorized as Punganur and 14 as Punganur Type animals based on their phenotypic characteristics, as per earlier documented descriptions (Fig.1).

DNA extraction: DNA isolation was carried out using high salt method (Montgomery and Sise 1990) with some

Fig. 1. Punganur and Punganur Type animals (male and female).

Table 1. Pair-wise theta (above diagonal) and Pi (below diagonal) values between different genetic groups

	PUN	PUTY	ONG	HLK	MG	VEC	BAR	THA	SIRI	RED	HAR	SHA
PUN		0.00593	0.00697	0.00698	0.00697	0.00697	0.00697	0.00754	0.00759	0.00745	0.00718	0.0069
PUTY	0.00470		0.00431	0.00593	0.00593	0.00592	0.00592	0.00674	0.00645	0.00667	0.00610	0.0059
ONG	0.00613	0.00470		0.00613	0.00612	0.00612	0.00612	0.00650	0.00621	0.00642	0.00630	0.0061
HLK	0.00613	0.00470	0.00580		0.00222	0.00319	0.00605	0.00364	0.00348	0.00360	0.00685	0.0032
MG	0.00613	0.00470	0.00579	0.00222		0.00222	0.00605	0.00253	0.00241	0.00249	0.00685	0.0022
VEC	0.00613	0.00470	0.00579	0.00288	0.00222		0.00604	0.02490	0.01997	0.00863	0.00684	0.0052
BAR	0.00613	0.00470	0.00579	0.00647	0.00647	0.00646		0.00688	0.00658	0.00680	0.00684	0.0060
THA	0.00665	0.00535	0.00631	0.00328	0.00253	0.01897	0.00736		0.01982	0.00834	0.00757	0.0249
SIRI	0.00667	0.00512	0.00583	0.00314	0.00241	0.01620	0.00704	0.01691		0.01980	0.00727	0.0199
RED	0.00656	0.00529	0.00623	0.00325	0.00249	0.00744	0.00727	0.00735	0.01689		0.00753	0.0086
HAR	0.00631	0.00484	0.00596	0.00685	0.00685	0.00684	0.00684	0.00757	0.00727	0.00753		0.0068
SHA	0.00613	0.00470	0.00579	0.00288	0.00222	0.00517	0.00646	0.01897	0.01620	0.00744	0.00684	

Note: PUN, Punganur; PUTY, Punganur Type; ONG, Ongole; HLK, Hallikar; MG, Malnad Gidda; VEC, Vechur; BAR, Bargur; THA, Tharparkar; SIRI, Siri; RED, Red Sindhi; HAR, Haryana; SHA, Sahiwal.

Table 2. Pair-wise K (above diagonal) and number of segregating sites (below diagonal)

	PUN	PUTY	ONG	HLK	MG	VEC	BAR	THA	SIRI	RED	HAR	SHA
PUN		0.00516	0.00717	0.00518	0.00747	0.00541	0.00641	0.01345	0.01202	0.00716	0.00660	0.00573
PUTY	17		0.00666	0.00431	0.00713	0.00469	0.00573	0.00664	0.01133	0.00664	0.00595	0.00501
ONG	20	17		0.00774	0.00431	0.00575	0.00582	0.01463	0.01323	0.00747	0.00729	0.00612
HLK	20	17	15		0.00910	0.00503	0.00649	0.01269	0.01089	0.00697	0.00651	0.00540
MG	20	17	15	3		0.00554	0.00508	0.01491	0.01375	0.00745	0.00723	0.00591
VEC	20	17	15	6	3		0.00489	0.01239	0.01105	0.00609	0.00551	0.00431
BAR	20	17	15	10	10	10		0.01356	0.01230	0.00686	0.00613	0.00526
THA	19	17	15	6	3	69	10		0.01763	0.01383	0.01364	0.01296
SIRI	20	17	14	6	3	63	10	57		0.01311	0.01229	0.01140
RED	19	17	14	6	3	21	10	21	57		0.01229	0.00650
HAR	20	17	14	11	11	11	11	11	11	11		0.00589
SHA	20	17	15	6	3	7	10	68	63	22	11	

Note: PUN, Punganur; PUTY, Punganur Type; ONG, Ongole; HLK, Hallikar; MG, Malnad Gidda; VEC, Vechur; BAR, Bargur; THA, Tharparkar; SIRI, Siri; RED, Red Sindhi; HAR, Haryana; SHA, Sahiwal.

modifications. Briefly, 0.5 mL of blood was mixed with RBC lysis buffer, mixed thoroughly to lyse the RBCs. The nuclear material was pelleted by centrifugation at 4000 rpm for 15 min, and the supernatant was discarded. The pellet was washed with RBC lysis buffer, mixed with 500 µL of lysis buffer (*p*H 8) and 10 µL of proteinase K and incubated at 56°C overnight. The mix was added with 50 µL of 2M NaCl. The phase was separated using chloroform-isoamyl alcohol and the DNA was precipitated using ethanol. The genomic DNA quality was verified using 1% agarose gel electrophoresis and quantified using NanoDrop™ 2000/2000c (Thermo Fisher Scientific).

PCR amplification and sequencing: The primers were designed targeting 1024 bp of the D-loop region of mtDNA (Accession No. V00654) with a length of 1024 bp. The Forward primer was 5'-CTGCAGTCTCACCATCAACC-3' and the reverse primer was 5'-GCCTGCGTTTATATATTGAC-3'. The primers specificity was confirmed using primer BLAST (Ye *et al*. 2012). The PCR amplifications of 10 µL reaction containing 5 µL of 2× master mix, 0.5 µL of forward and 0.5 µL of reverse primers, 3 µL of nuclease free water and 1 µL of DNA template/ sample were carried out in a thermal cycler (Applied Biosystems, Germany). The amplification involved an initial denaturation at 94°C for 3 min, followed by 35 cycles at denaturation 94°C for 5 min, annealing 60°C for 30 s, and extension 72°C for 60 s, with a final extension at 72°C for 10 min. The PCR products were

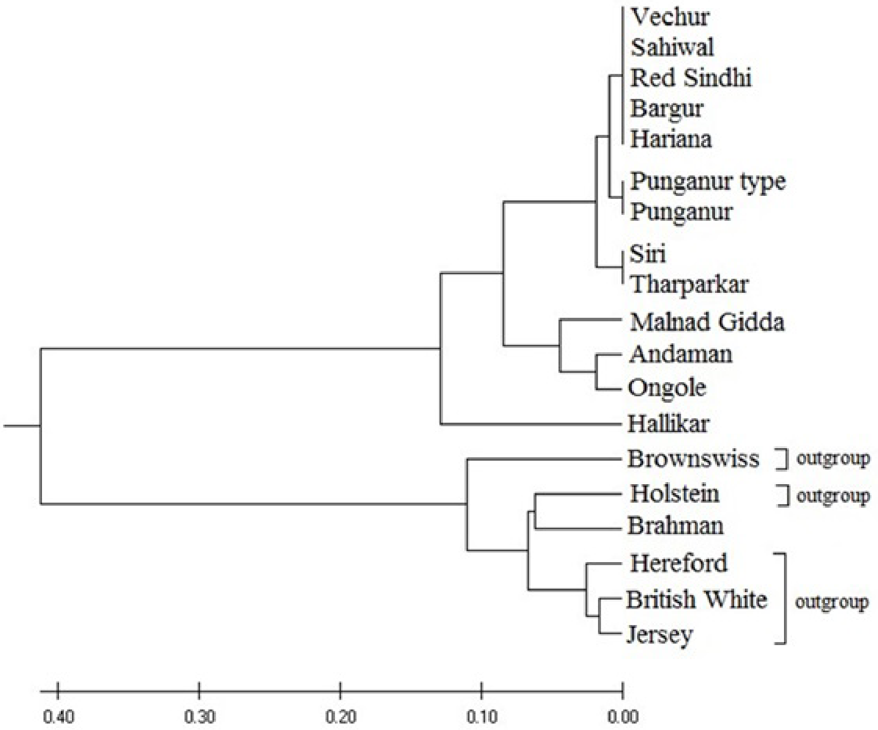

Fig. 2. UPGMA tree based on F_{ST} values between different cattle populations.

Table 3. Polymorphism and diversity indices obtained from 912 bp mtDNA D-loop sequences

Group	Punganur	Punganur Type	Ongole	Hallikar	Malnad Gidda	Vechur	Bargur	Tharparkar	Siri	Red Sindhi	Hariana	Sahiwal	Brahman
Sample Size (n)	14	14	9	5	3	3	4	19	29	14	4	3	15
Nucleotide diversity (pi)	0.00613	0.00470	0.00579	0.00288	0.00222	0.00517	0.00646	0.01897	0.01620	0.00744	0.00684	0.00591	0.00531
Theta W per site	0.00697	0.00592	0.00612	0.00319	0.00222	0.00517	0.00604	0.02453	0.01935	0.00863	0.00684	0.00591	0.00547
Theta W per sequence	6.289	5.346	5.519	2.880	2.000	4.667	5.455	19.456	16.042	6.918	6.000	5.333	4.921
Haplotypes	11	7	8	3	3	3	4	19	20	14	4	3	8
Haplotype diversity (HD)	0.967	0.813	0.972	0.700	1.000	1.000	1.000	1.000	0.924	1.000	1.000	1.000	0.895
No. of polymorphic/ segregating sites (S)	20	17	15	6	3	7	10	68	63	22	11	8	16
Avg. number of nucleotide differences (K)	5.527	4.242	5.222	2.600	2.000	4.667	5.833	15.041	13.433	5.967	6.000	5.333	4.781
Tajima's D	-0.50620	-0.85185	-0.25952	-0.66823	--	--	0.69482	-0.97964	-0.71412	-0.57862	0.00000	--	-0.11410

quality checked using 1.5% agarose gel and the successful amplifications were sent for sequencing at AgriGenome Labs. Pvt. Ltd., Kerala following Sanger's dideoxy chain termination method. In order to obtain good quality full length sequence, two internal sequencing primers were used in the sequencing reaction. The internal forward sequencing primer was 5'-CTGGTTCTTTCTTCAGGGCC-3' and the internal reverse sequencing primer was 5'-ATACCAAATGTATGACAGCC-3'.

The electropherograms were quality checked, assembled and analyzed using Codon Code Aligner software v6.0.2 (LI-COR, Inc., Lincoln, USA). After trimming the poor-quality ends, the final analysis was performed on 912 bp of the D-loop region. The sequences were aligned using MUSCLE in MEGA X software (Kumar *et al*. 2018). The DnaSP v6 (DNA Sequence Polymorphism) software (Rozas *et al*. 2017) was used for polymorphism and divergence analysis. Phylogenetic analysis was performed with MEGA X (Molecular Evolutionary Genetic Analysis) software to estimate the divergence of Punganur cattle with Vechur, Malnad Gidda, Bargur, Hallikar and Ongole cattle. A comprehensive analysis was conducted using 165 D-loop sequences from various *B. indicus* (13 breeds) and *B. taurus* (5 breeds) obtained from the NCBI database.

RESULTS AND DISCUSSION

Genetic diversity: The multiple alignments of 48 sequences revealed 181 gaps or missing sites, 612 monomorphic sites, and 119 polymorphic sites with 126 mutations, indicating multiple mutations at certain polymorphic positions. The pair-wise polymorphisms among Indian genetic groups were evaluated using the parameters 'Pi' and 'theta' (Table 1). The pair-wise divergence was estimated in terms of 'K' and number of segregating sites (Table 2). Population-wise polymorphism and divergence information is indicated in Table 3. The Tajima's D values (test of neutrality) were negative in all the populations except in Bargur and Hariana cattle (Table 3) indicating the potential for selective sweeps, though the Tajima's D values are not significant ($p>0.05$). The genetic differentiation of all cattle populations were given in terms of *Hs* (=0.94), H_{ST} (=0.05), *Ks* (=6.92), K_{ST} (=0.66), *Z* (=5870.50) and *Snn* (=0.39) indicated high differentiation among populations, supported by a permutation test ($p<0.001$). Intriguingly, the Chi-square test, assuming genetic undifferentiation, yielded non-significant results (Chi-square= 2906.239; $p>0.05$), challenging the notion of overall population differentiation. A refined analysis focussing on Punganur and Punganur Type groups alongside the genetic groups (Ongole, Hallikar, Vechur, Malnad Gidda and Bargur) neighboring the home tract of Punganur revealed significant differentiation between population (Chi-square= 260.722; $p<0.05$). However, when specifically assessing Punganur and Punganur Type groups, all differentiation parameters and the Chi-square value became non-significant (Chi-square = 12.000; p = 0.4457), suggesting a unique genetic homogeneity

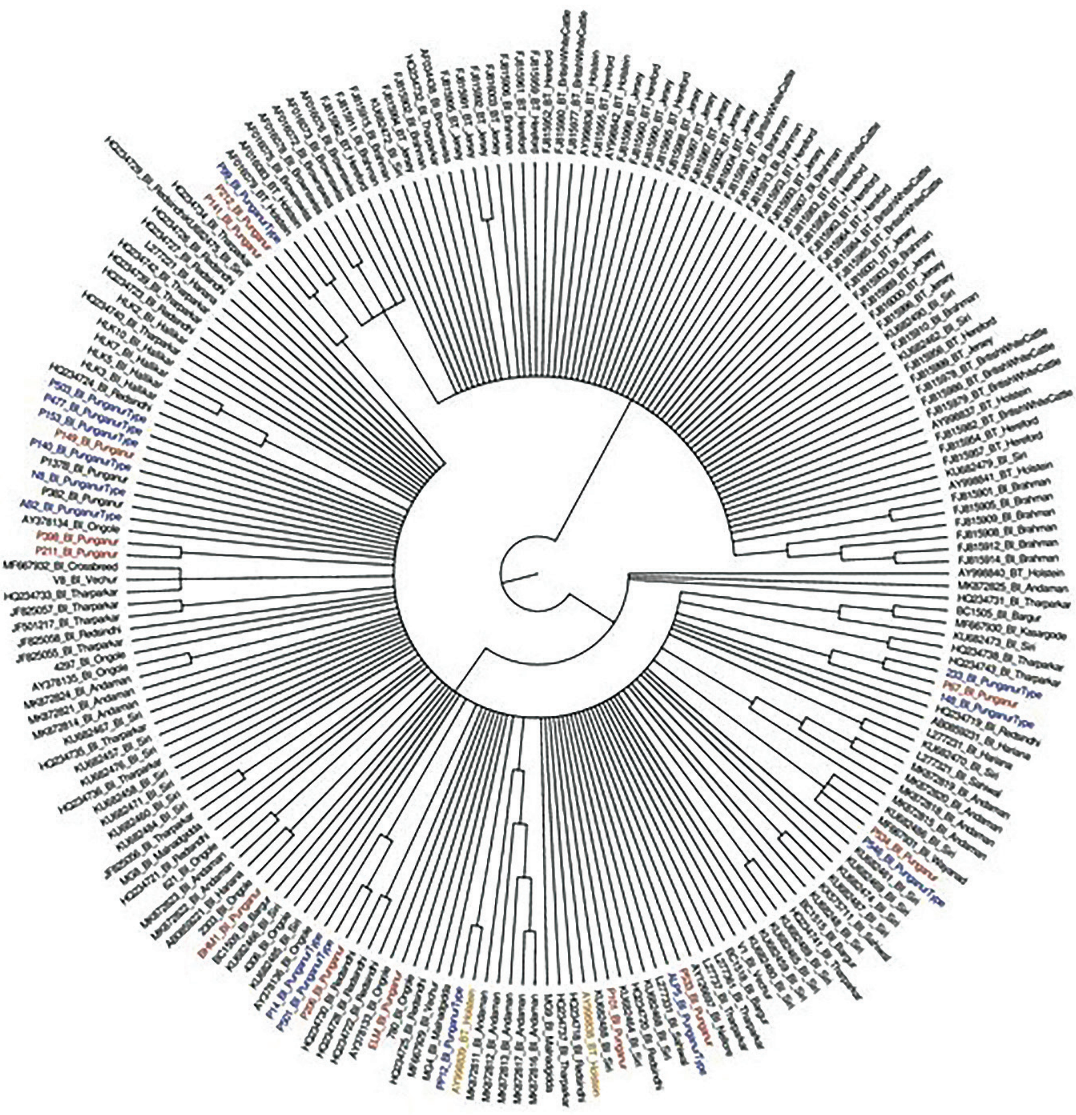

Fig. 3. Neighbor joining bootstrap consensus tree of different individual cattle.

within this subset. The overall gene flow between populations were estimated based on haplotype data in terms of G_{ST} (=0.05364) and based on sequence data in terms of F_{ST} (=0.70590) and N_{ST} (=0.71056). The effective numbers of migrants (*Nm*) was 8.82, 0.21 and 0.20 for G_{ST}, F_{ST} and N_{ST} respectively. A UPGMA tree constructed based on F_{ST} values further illustrated the genetic relationships among the studied populations (Fig. 2).

Phylogenetic analysis: The Neighbor joining tree generated and tested for tree topology with 1000 bootstrap replicates (Fig. 3) showed two major clusters, one dominated by *B. indicus* and another by *B. taurus*. Notably, a distinct group emerged featuring a Holstein individual, a Tharparkar individual, and an Andaman individual.

Historical records by Shortt (1876), Wallace (1888), Slater (1918), and Littlewood (1936) depict Punganur cattle as a small variant of Mysore cattle, expressing concerns about its near extinction. Survivors underwent crossbreeding with inferior cattle. The population of Punganur cattle was estimated to be 2772 in breed estimate report of 2013 and in 20th Livestock census, the population of Pure Punganur was reported as 9876 and Graded Punganur as 3399. These population dynamics raise concerns about purity of these animals. Further, the present-day Punganur cattle exhibit dissimilarities with Mysore cattle. In the present study, the populations of Punganur cattle were categorized into Punganur and Punganur Type based on their phenotypic appearance. The genetic polymorphism and divergence studies indicated that polymorphism exists in the Punganur, Punganur Type and other dwarf breeds. To the best of our knowledge, no study has assessed genetic polymorphism in Punganur cattle using mitochondrial DNA sequences. As diversified populations were used in the analysis, allele frequency based Chi-square test indicated that populations (dwarf, miniature breeds and other Indian cattle breeds) were undifferentiated whereas the haplotype and nucleotide-based test showed high differentiation. If the number of haplotypes is so large that the expected numbers of each haplotype from each locality are very small, under such circumstances, the Chi-square test is not powerful. Contrarily, analysis involving Punganur and other Indian cattle breeds reveals high differentiation. When restricted to Punganur and Punganur Type genetic groups, no differentiation was observed, suggesting a shared maternal source for the two genetic groups.

The F_{ST} values indicated no differentiation between Punganur and Punganur Type, Vechur and Sahiwal, a low genetic differentiation of Punganur with Bargur, Tharparkar, Red Sindhi, and Hariana, a moderate genetic differentiation with Hallikar (F_{ST}=0.11 to 0.13) and a great genetic differentiation of Punganur with Ongole. The negative F_{ST} values between Punganur and Punganur Type animals suggest that these two genetic groups cannot be differentiated using mitochondrial DNA markers. These results interestingly raise a question about whether the present day Punganur cattle and Mysore type cattle have similar origin. The observations in the present study are in concurrence with observation of Sharma *et al.* (2015) that within breed diversity is more in Indian breeds when compared to between breed diversity.

In phylogenetic analysis, certain Holstein individuals grouped together in *B. indicus* cluster. One of the Punganur Type individual (PP12) shared its recent common ancestor with Holstein Frisian animal. This could be a result of crossbreeding programme that is happening in the subcontinent as part of National Cattle Breeding Policy. No clear demarcation was observed between Punganur and Punganur Type animals. It should be noted that the phenotype of cattle is not always related to their mtDNA profiles (Srirattana *et al*. 2017). The lack of proper appraisal about the phenotype of this animal is a major hurdle in identification of pure Punganur animals. Hence, it is important that the community should arrive at a consensus about phenotypic features of this breed and verify for historical likely admixture with *B. taurus* blood using mitochondrial DNA before formulating a breeding plan for this breed.

The study revealed no genetic differentiation of the Punganur and Punganur Type and certain dwarf cattle breeds of India and low to moderate genetic differentiation with other Indian cattle breeds. A thorough verification of the historical likelihood of admixture with *B. taurus* blood using mitochondrial DNA is crucial before formulating any breeding plans for this endangered Indian cattle breed.

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