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Genetic polymorphism of *LHX4* gene and its association with growth and reproductive traits in goats of Odisha

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ABSTRACT

The purpose of this study was to investigate the genetic variation in exon 6 of the *LHX4* gene in Ganjam and Black Bengal goats. DNA was extracted from the buccal swab samples. By using specific primers, the target partial sixth exon of the *LHX4* gene was amplified (382 bp). PCR amplification and DNA sequencing analysis revealed two non-synonymous transitions namely c.44997A>G and c.45028 C>A which caused a change in amino acid from histidine to glutamic acid and valine to isoleucine, respectively. Genotype frequencies in the Ganjam population were 0.32 and 0.68 for GG and AA genotypes, whereas the same in the Black Bengal population were 0.10 and 0.90 for GG and AA genotypes, respectively. The heterozygote GA genotype was missing in both populations and significantly deviated from the Hardy-Weinberg equilibrium. The Black Bengal goat had a significantly smaller body size than the Ganjam goat. Black Bengal goats had a significantly greater twinning percentage than the Ganjam breed. They also had a significantly lower age at sexual maturity (ASM), age at first kidding (AFK), and shorter kidding intervals (KI) than the Ganjam goats underscoring their superior reproductive potential. Except for chest girth, the effect of genotypes on body weight and morphometric traits was determined to be statistically non-significant. The effect of genotype was found to be non-significant on the reproductive traits.

Keywords: Genetic polymorphism, *LHX4*, Morphometric traits, Reproductive traits

Goats provide a wide range of livestock products, viz. meat, milk, skin, fibre and manure and help improving their socio-economics (Singh *et al.* 2023). Goat farming employs around 70% of the nation's landless agricultural labourers, marginal farmers, and small farmers. They serve as the family's main source of animal protein aside from being a source of money and employment for them (Kumar *et al.* 2010).

The state of Odisha is home to two prominent goat breeds, the Black Bengal and Ganjam, which have a combined population of 21.49 lakhs as of 2019. While the Ganjam, Nayagarh, and Gajapati districts of Odisha are home to the most Ganjam goats, the Black Bengal goats are distributed in the regions adjoining Odisha and West Bengal. The Ganjam breed is primarily raised by Golla farmers in a semi-nomadic extensive system (Karna *et al.* 2020). By utilizing their enormous genetic potential, it is possible to significantly boost the productivity of these goats. This could be achieved by improving the genetic makeup of goats by breeding for fertility and growth (Karna *et al.* 2016). Finding suitable genetic markers having significant association with growth and reproductive traits is step in the right direction. The use of markers can improve the efficiency of selection by allowing the breeders to identify superior genotype accurately than traditional phenotypic selection (de Lima *et al*. 2020)

The *LHX4* gene belongs to the LIM homeodomain (LIM-HD) gene family, which consists of transcription factors involved in the regulation of developmental processes. *LHX4* plays a crucial role in the development of the pituitary gland, influencing the secretion of hormones such as growth hormone (GH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Its activity in regulating these hormones directly affects growth, reproductive functions (Haim-Pinhas *et al.* 2016, Yan *et al.* 2018, Samarsinghe *et al.* 2022). A detailed understanding of these animals' reproductive, growth, and morphometric features is necessary to implement the right selection strategy for their genetic improvement. Keeping the above facts in view, this investigation was executed to explore the variation in *LHX4* gene and association of the variants with growth, morphometric and reproductive traits in Ganjam and Black Bengal goats.

MATERIALS AND METHODS

Location and sampling of animals: Ganjam goats are

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a distinct population that is found in the fourth and fifth agroclimatic zones of India, namely the east and south-eastern coastal planes and the north-eastern ghats. The east and south-eastern coastal planes have a hot and humid climate, with mean maximum summer temperatures of 39°C and mean lowest winter temperatures of 11.5°C, as well as mean annual rainfall of 1577 mm and relative humidity ranging from 80 to 89%. Black Bengal goat samples were collected from the farmers of the Khurda and Baripada area as a representative population of the breeds. The morphometric information on Ganjam goats was collected from registered farmers' herds in the Ganjam field unit of the All India Coordinated Research Project (AICRP) on Goat Improvement from three field centres of Odisha: Chatrapur, Rambha, and Khallikote. Body weight and 11 morphometric characteristics namely, wither height (cm), brisket height (cm), body length (cm), chest girth (cm),head length (cm), horn length (cm), ear length (cm), tail length (cm), rump width (cm), rump height (cm) and neck circumference (cm) were measured as described by Karna *et al.* (2022). The reproductive traits recorded were age at sexual maturity (months), age at first kidding (months), kidding interval(months) and twinning record by interviewing the farmers.

Isolation of Genomic DNA: The buccal and nasal swabs were collected using a clean transport swab stick from unrelated, healthy, adult female Ganjam goats (N=50) and Black Bengal goats (N=50) over a year old during the year 2022. The genomic DNA was isolated using a DNA isolation kit (Qiagen) and purified from the buccal swabs using the spin protocol method. To determine the DNA yield, the concentration of DNA was measured in Nanodrop (QIAxpert). Then, concentration was assessed at 260 nm and 280 nm. DNA quality and integrity were assessed by running in 1% agarose gel for 10 min.

PCR amplification and sequence analysis: The target region of 382 bp in the sixth exonic region of the *LHX4* gene was amplified using the forward (5'GATTCTCTCAGAACTTGGCCAGACC3') and reverse primers (5'AGGAGGATGATCCATTTCATCGAGC3') reported by Li *et al*. 2008. Using a PCR kit from Qiagen, the target gene segments for the *LHX4* gene were amplified using PCR. PCR reactions were carried out in a final volume of 25 µL. The PCR reaction mixture was prepared with 12.5 µL of 2× master mix (Qiagen), 5.0 µL of genomic DNA with 80ng/µl concentration, 1.0 µL of forward primer, 1.0 µL of reverse primer and 5.5 uL of nuclease-free water. PCR was carried out with initial denaturation at 95°C for 4 min, followed by 30 cycles with denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 40 s, and the final extension at 72°C for 10 min. The PCR amplification was verified using horizontal gel electrophoresis with 5 µL of PCR product and 3 µL of gel loading dye in 1% agarose gel in 1X Tris-acetate-EDTA buffer. Sanger's dideoxy sequencing method was used for sequencing. The chromatogram analysis and alignment of DNA sequences using MEGA 11 (Tamura *et al.* 2021) and BIOEDIT software (Hall 1999) were done to identify SNPs in the aligned sequences.

Statistical analysis: The data on body weights, body dimensions, and reproductive traits were analysed by fitting a linear model keeping genotypes and breed as fixed effects to assess their effect on the growth, morphometric and reproductive traits. A Chi-square test was done to assess the association of multiple kidding with attributes such as breed and genotypes. The data analyses were done using SPSS software (version 27.0).

RESULTS AND DISCUSSION

Polymorphism in LHX4 gene: The present study explored the genetic variation in the 382 bp segment of the LHX4 gene encompassing the region of partial exon 6 was explored through PCR and DNA sequencing methods. DNA concentration ranged between 40 and 80 ng/ul and the target 382bp length of the LHX4 gene was amplified without any non-specific bands (Fig. 1). Through chromatogram analysis two additional SNPs were identified at 44997th position and 45028th position. (22th, 23 th position in the target sequence) respectively from the reference sequence (Accession no: NC 030823) at the location: Chromosome 16: 59,998,546-60,044376 (Fig. 2). Both the nucleotide substitution at the 22nd and 53rd position of the amplified segment, i.e. G>C and A>G resulted in the change of the codon CAG > CAC leading to the substitution of Glutamine > Histidine and codon ATT>GTT leading to the substitution of isoleucine>valine, respectively. A heterozygous genotype was found to be missing in both the Ganjam and Black Bengal goat populations. Out of the two, the substitution at the 53rd position in 382 bp segment of *LHX4* gene was found to be polymorphic in both the population. It was worthwhile to genotype the population and examine the relationship between this variation and characteristics related to body weight, morphometrics, and reproduction. This can also be taken into account when choosing animals for QTL (Quantitative trait loci) mapping and for the selection of animals based on MAS (marker-assisted selection). There are some reports concerning variation in exon 6 of the *LHX4* gene in goat breeds such as Inner Mongolia white cashmere, Shaanbei white cashmere, Laoshan, Xinong Sannen, Guanzhong, Boer, Haimen, and Xuhuai populations (Li *et al.*2008). The substitution of the G nucleotide at the position in place of the C nucleotide as

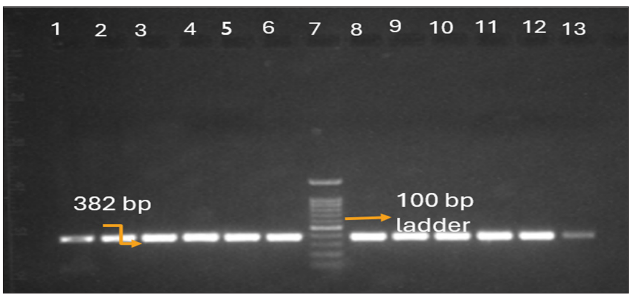

Fig. 1. PCR products of 382 base pair length (Lanes 1,2,3,4,5,6,8,9,10,11,12,13: 382 bp and 7: 100 bp length ladder).

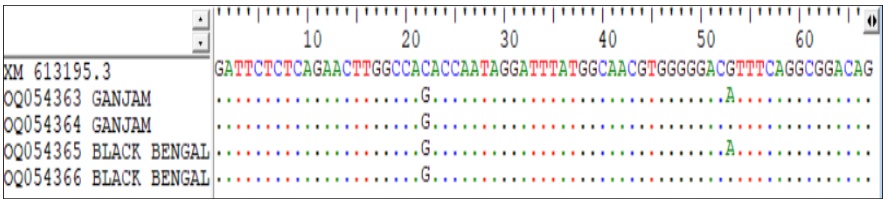

Fig. 2. Genetic variants of *LHX4* gene at 22 and 53 position with respective 382 bp segment having G variant and A/G variants the accession no's: OQ05436 5, OQ054366 for Black Bengal and OQ054363, OQ054364 for Ganjam.

reported by Li *et al*. (2008) was one entirely novel missense mutation, but the mutation was not polymorphic as in both the Ganjam and Black Bengal goats it was monomorphic. Polymorphism has also been reported in the same locus of the gene in the Chinese cattle breed by Ren *et al.* (2010). The variability in the gene has also been reported in other regions of Shannabei Cashmere goats by Yan *et al*. (2018), in Sheep by Zhao *et al*. (2017) and in cattle by Liu *et al*. (2011). GA type (heterozygous) was missing in both Ganjam and Black Bengal populations.

The sequences were submitted to genbank and NCBI accession numbers were obtained were OQ054363, OQ054364 for Ganjam goats and OQ054365, OQ054366 for Black Bengal goats.

Gene, genotype frequency, and population genetics parameters: Genotyping was done for 100 goats, 50 goats from each breed produced two genotypic variants (AA and GG) in both the Ganjam breed and Black Bengal breed of goat. Genotype frequency of the GG genotype was 0.32 and 0.10 in Ganjam and Black Bengal whereas the frequencies of the AA genotype were 0.68 and 0.90 in Ganjam and Black Bengal populations, respectively. A simple chi-square test was done to know the Hardy-Weinberg equilibrium in both breeds. Both the populations were found to be deviating significantly from the equilibrium ($P<0.05$) indicating that there may be a selection force operating which may be either natural or artificial or there might have been non-random mating of animals. But as reported by Li *et al.* (2008) Chinese goat breeds had three genotypes (AA, AG, GG) and it was following the H-W equilibrium. In Chinese cattle breed, Nanyang, Liu *et al.* (2010) reported four genotypes MM, NN, MN, and MH and the observed genotypic frequencies are 0.8,0.007,0.16,0.02 respectively. Yan *et al.* (2018) reported three genotypes II, ID and DD with 0.34,0.49, and 0.15 genotypic frequencies respectively.

The observed number of alleles in both populations was 2, and the effective number of alleles was 1.77 and 1.21 in Ganjam and Black Bengal breeds respectively. Observed homozygosity and heterozygosity were 1 and 0 respectively in both populations. expected homozygosity and heterozygosity were 0.56, 0.81, and 0.439,0.18 in Ganjam and Black Bengal goats, respectively. Shannons' information index was 0.62 and 0.32 for Ganjam and Black Bengal, respectively. Heterozygosity statistics revealed that the both Ganjam and Black Bengal population showed a small genetic diversity but comparatively, the Ganjam population was more diversified than the Black Bengal population. Nei's genetic identity and genetic distance were estimated to be 0.94, and 0.05 respectively between Ganjam and Black Bengal populations.

Phylogenetic tree analysis: The nucleotide sequence of the LHX4 gene in the Ganjam population (NCBI accession no: OQ054364 andOQ054363) showed 100% similarity with the Black Bengal (NCBI accession no: OQ054366 and OQ054365) population and almost 99% similarity with the sequence obtained from Inner Mongolia white cashmere and other Chinese goat breeds (NCBI accession no: EU331422.1). From the phylogenetic analysis (Fig. 3), it is revealed that even though Ganjam shares 99% similar genes to Inner Mongolia white cashmere and other related Chinese breeds, they do not come under the same cluster, so it revealed that there is an evolutionary distance between the Chinese goat population and goat population of Odisha.

Effect of genotype and breed on the growth, morphometry and reproductive traits: The mean and standard error of growth, morphometric and reproductive traits, organized by genotype and breed are presented in Table 1. It is quite evident that compared to Black Bengal goats, Ganjam goats had significantly ($P<0.01$) higher values for the most traits. However, for head length, no significant difference between the two goat populations was observed According to this comparison, Black Bengal goats were clearly a smaller breed than Ganjam goats.

Ganjam and Black Bengal goats showed highly significant differences for all three reproductive traits ($P<0.01$). Black Bengal goats had lower values for all the traits, revealing that they have higher reproductive potential than Ganjam goats with early sexual maturity, early age at first kidding, and small kidding intervals. Black Bengal goats showed a significantly high twinning percentage in the population studied compared to the Ganjam breed ($P<0.05$)

The mature body weight and dimensions of Ganjam goats in this study are significantly lower than those reported by Rao *et al.* (2010). The body weights and dimensions of the goats were comparable to previous publications with a somewhat larger sample size (Karna *et al.* 2016). It could be because the goats studied here are of a mixed-age population ranging from 1.5 to 5 years old, whereas previous publications only included adult weight, which could have resulted in a larger average than this sample. There was no information on this particular variant being associated with the growth traits in goats. However, in cattle, Ren *et al.* (2010) reported higher body weights in the GA genotype compared to the GG genotype.

There was no significant difference between the AA

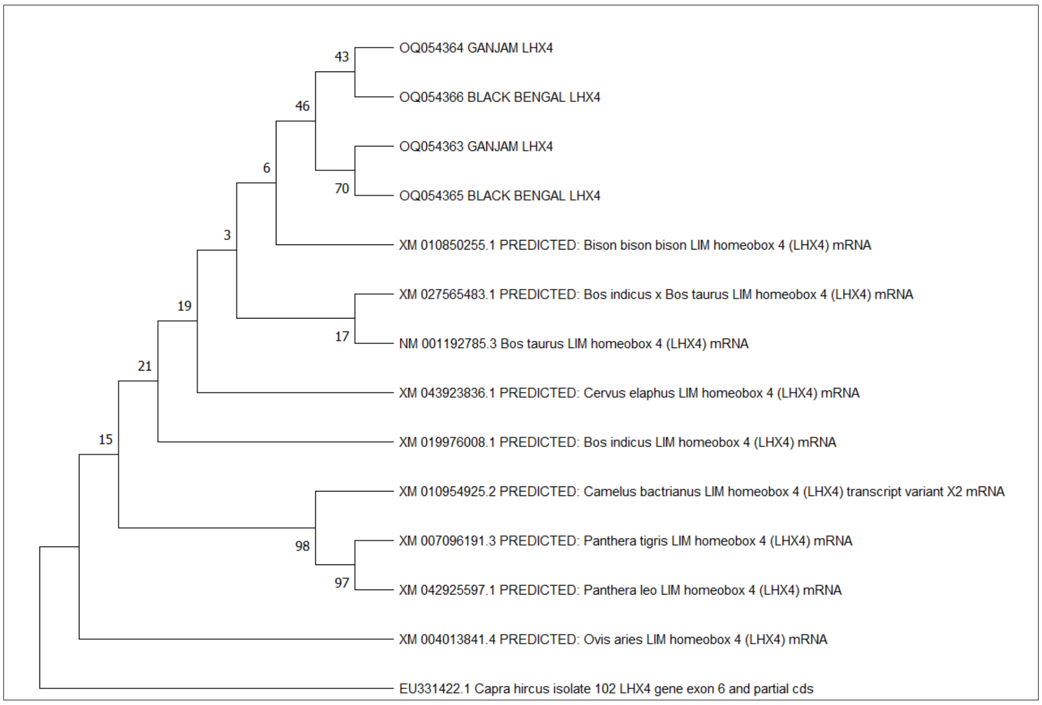

Fig. 3. Phylogenetic tree constructed by using nucleotide sequence of *LHX4* gene.

Table 1. Effect of breed and genotype on growth, morphometric and reproductive traits

Trait	Genotypes			Breed		
	AA	GG	P-value	Ganjam	Black Bengal	P-value
Body Weight (kg)	22.43±0.36	23.81±0.81	0.12	31.10±0.48	15.14±0.75	<0.01
Body length (cm)	56.00±0.81	56.81±1.82	0.68	64.59±1.08	48.22±1.68	<0.01
Wither Height (cm)	62.35±0.64	63.54±1.44	0.45	71.37±0.85	54.52±1.32	<0.01
Chest girth (cm)	65.15±0.53	67.86±1.20	<0.05	74.01±0.71	59.00±1.10	<0.01
Brisket Height (cm)	41.99±0.66	43.75±1.49	0.28	45.89±0.88	39.85±1.37	<0.01
Head length (cm)	16.50±0.31	16.31±0.70	0.81	16.08±0.41	16.73±0.65	0.40
Horn length (cm)	11.67±0.45	10.90±1.01	0.49	18.70±0.60	3.86±0.93	<0.01
Ear length (cm)	12.40±0.21	13.20±0.48	0.13	14.28±0.28	11.32±0.44	<0.01
Neck circumference (cm)	30.06±0.50	30.56±1.13	0.69	32.73±0.67	27.88±1.04	<0.01
Tail length (cm)	11.78±0.20	11.98±0.46	0.68	14.20±0.27	9.56±0.42	<0.01
Rump height (cm)	63.84±0.73	65.63±1.65	0.32	74.58±0.98	54.90±1.52	<0.01
Rump width (cm)	12.78±0.33	14.00±0.76	0.14	15.18±0.45	11.60±0.70	<0.01
Age at sexual maturity (months)	10.73±0.13	10.51±0.25	0.43	13.65±0.19	7.59±0.19	<0.01
Age at first kidding (months)	16.63±0.10	16.42±0.19	0.37	19.45±0.13	13.60±0.15	<0.01
Kidding interval (months)	8.75±0.05	8.81±0.11	0.63	9.46±0.78	8.11±0.09	<0.01

and GG genotypes with respect to three reproductive traits. Though the results seem to suggest that the AA genotype has a substantially higher rate (45.6% vs 23.8%) of multiple kidding as compared to the GG genotype and the result is statistically not significant (Chi-Square=3.24, df=1, P-value = 0.072). However, in the intronic region of the *LHX4* gene, a 12-base pair deletion polymorphism was reported to be associated with the litter size by Yan *et al.* (2018) in Chinese goats.

It can be inferred that the Ganjam breed seems to have more diversity with respect to the target genetic locus compared to the Black Bengal breed. The 'GG' genotype had a significant association with higher chest girth compared to the 'AA' genotype but the latter genotype had a relatively higher incidence of multiple kidding (45.5% vs 23.8%) though it was statistically not significant. However, investigation on a larger scale with a more diverse sample from both breeds, covering the whole breeding tract of both populations is necessary to establish causal association of *LHX4* gene variants with growth and reproduction traits.

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