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PM Rojan
Ph.D. Scholar and Assistant Professor, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

R Thirupathy Venkatachalapathy
Professor, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Marykutty Thomas
Assistant Professor, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

TV Aravindakshan
Senior Professor and Director, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

K Radha
Associate Professor, Department of Dairy Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

V Ramnath
Professor and Head, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Corresponding Author:
PM Rojan
Ph.D. Scholar and Assistant Professor, Centre for Advanced Studies in Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

High-resolution melting analysis of chromodomain helicase DNA binding protein 9 (CHD9) gene in goat breeds of Kerala, India

PM Rojan, R Thirupathy Venkatachalapathy, Marykutty Thomas, TV Aravindakshan, K Radha and V Ramnath

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Abstract
The objective of this study was to identify polymorphisms in chromodomain helicase DNA binding protein 9 (CHD9) gene and assess its potential impact on body weight and conformation traits within the Malabari and Attappady Black goat breeds in Kerala. Significant differences were observed in adult body weights, height at withers and chest girths between the Malabari and Attappady Black breeds. Genotyping using High-Resolution Melting (HRM) revealed AA, GG and AG genotypes of CHD9. The highest frequency of AA genotype was observed in both breeds. Both Malabari and Attappady black breeds were in Hardy-Weinberg equilibrium. Notably, the genotypic patterns of CHD9 did not exert a significant effect on body weight or other conformation traits in either breed.

Keywords: Body weight, CHD9 gene, Goat, Malabari and Attapaddy black

1. Introduction
In the context of India's agro-economic environment, goats play a crucial role, particularly in areas that experience climatic vagaries that can negatively impact crop production (Singh *et al.*, 2013) [1]. The diverse range of contributions made by goats, including meat, milk, hair, skin and dung, significantly strengthen the India's Livestock sector's gross domestic product, accounting for around nine per cent (Singh *et al.*, 2023) [2]. In India goats are primarily reared for the purpose of meat production, hence playing a substantial role in supporting livelihoods. It is worth noting that goat meat accounts for around 14 per cent of India's total meat production. Within this context, the Malabari breed, sometimes referred to as the Tellicherry goat, assumes a dual-purpose breed status. The Malabari breed exhibits characteristics such as moderate size, high fertility, impressive milk yield and the capacity to adapt to hot and humid environments. The Malabari breed is said to have emerged several centuries ago as a result of the interbreeding between indigenous wild goats and goats of Arab, Surti and Mesopotamian origins. Despite being mostly of white coloration, the Malabari goats have a wide range of coat colours. The geographical range of their distribution encompasses the state of Kerala and extends into adjacent areas of Tamil Nadu and Karnataka (Venkatachalapathy *et al.*, 2016) [3]. The Attappady black goat, a unique meat breed, originated in the hilly terrains of Attappady, which are situated between the Nilgiri and Muthikulam hills within the Western Ghats of Kerala. These medium-sized goats have evolved from local wild goats. The Attappady black goats have completely black coats, sometimes featuring unusual white patches on their foreheads and are distinguished by their slender and lean appearances (Stephen *et al.*, 2005; Rodricks *et al.*, 2023) [4-5]. These goats serve as symbolic representations of the complex relationship between the local ecosystem and the evolutionary process of livestock within the diverse agricultural biodiversity of India.
The chromodomain helicase DNA binding protein 9 (CHD9) gene is located on chromosome 18 in the goat genome. It is comprised of a single transcript comprising 36 exons in total. It has been implicated in osteogenesis and skeletal development (Shur *et al.*, 2006; Benayahu *et al.*, 2007; Newton and Pask, 2020) [6-8]. In pursuit of unravelling the genetic intricacies associated with body weight and conformation traits in goats, our present investigation delves into the identification of single nucleotide variants within the CHD9 gene.

Focusing specifically on Malabari and Attappady black breeds, this study seeks to shed light on the nuanced associations between genetic variations in CHD9 and the phenotypic characteristics of Malabari and Attappady black goat breeds.

2. Materials and Methods

2.1 Animal selection and recording of phenotypic data

The research was conducted on two distinct goat breeds, namely Malabari and Attapaddy black, which are found in the hot and humid tropical region of Kerala, India. These breeds differ in terms of coat colours, body size and conformation. The study involved the collection of data from a total of 170 Malabari and 65 Attappady black goats. These goats were maintained in the farms of the Kerala Veterinary and Animal Sciences University, as well as in the satellite field units of the ICAR-All India Co-ordinated Research Project (ICAR-AICRP) on Goat Improvement. The goats were reared under semi-intensive management system, which involved grazing for 5-6 hours and being fed with green fodder that was available seasonally. Additionally, the goats were provided with concentrate mixtures based on their individual status and age. The study involved the collection of body weight and body biometric measurements, including body length, chest girth and height at withers, from adult does aged between two and three years.

2.2 Extraction of genomic DNA and Polymerase chain reaction (PCR)

The genomic DNA extraction from blood samples involved employing the phenol-chloroform extraction method, with subsequent evaluation of purity and concentration using NanoDrop spectrophotometer. To ensure high-quality samples, one percent agarose gel electrophoresis was performed. Samples meeting the criteria of a 260/280 ratio ranging between 1.7 and 1.9, coupled with the presence of an intact band, were selectively chosen for subsequent analytical phases. The primers were designed in exon region of *Capra hircus* CHD9 (NCBI NC_030825.1) sequence using the Primer 3 (www.ncbi.nlm.nih.gov). Sequence specificity of the primers was confirmed by homology through a BLAST search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The primers of CHD9 were forward 5'ATGTGTTATCGCCACACTCTC3' and reverse primer 5'GGACTGCCATCTGCTACATC 3' ranging in the region of 23020072 bp 23020165 bp of chromosome 18 was used. The optimization of annealing temperatures for each gene fragment was performed through conventional gradient PCR, utilizing the Bio-Rad T100™ thermal cycler. The temperature range tested was between 57 °C and 65 ºC. The PCR products were subjected to gel electrophoresis using two per cent agarose to identify any additional (spurious) bands along with the GeneRuler 50 bp DNA (Thermo Scientific).

2.3 Genotyping by High Resolution Melting (HRM) analysis

The HRM reactions were conducted using the Eco Real-Time PCR System (Illumina) using Eco 48-well plates that were sealed using Eco adhesive seals. Each sample was amplified in duplicate as technical replicates. In each experimental trial, the control group consisted of a non-template control and a negative control (Nuclease-free water). The volume of the reaction was 10 µL. The reaction mixture comprises of 1µL of genomic DNA at a concentration of 50 ng/µL, 5 µL of Sso Fast Evagreen HRM Master mix at a concentration of 1X, 0.3 µL of each primer at a concentration of 10 pmol/µL and 3.4 µL of nuclease-free water. The PCR program started with an initial denaturation of 3 min at 95 °C, continued with 40 cycles of 30 s at 95 °C, 30 s annealing at 63.5 °C (CD6 gene) and 30s extension at 72 °C.

The HRM was conducted subsequent to the PCR. The experimental procedure entailed an initial denaturation step at 95 °C for a duration of 15 s, followed by rapid cooling to 55 °C for a duration of 15 s. Subsequently, the final denaturation was carried out by gradually elevating the temperature from 55 °C to 95 °C with a stepwise escalation of 0.1 °C. The capture of fluorescence data was ensued at every increment till reaching a temperature of 95 °C.

The normalisation of the melt curves was performed between completely hybridised and completely single-stranded regions, after the elimination of background fluorescence using the Ecostudy software that accompanies the qPCR instrument. The samples were plotted on their respective melting profiles. Negative first derivative plots and difference plots were constructed for the samples. The melting profiles of the samples were compared to that of the reference genotype, which was transformed into a horizontal line in the difference graph. Distinct clusters were seen in plots, indicating different genotypes. Representative samples from each genotype that were originally detected in the first HRM experiment were subsequently verified using commercially available Sanger sequencing (Eurofins Genomics India Pvt. Ltd., Bengaluru). The aforementioned verified samples were subsequently utilised as designated reference controls for HRM experiment performed on the remaining samples.

2.4 Statistical analysis

A two-sample independent t-test was used to assess the differences in the means of body weight, body length, height at withers and chest girth between two native breeds. The estimation of gene and genotype frequencies was carried out using the methodology outlined by Falconer (1996) [9]. The Chi-square test for Hardy Weinberg equilibrium (HWE) was conducted using the HW_TEST version 1.1 (Santos *et al.*, 2020) [10]. The estimation of population genetic parameters was carried out using Popgene Version 1.32. The study employed the general linear model in SPSS V.21.0 to analyse an association between genotypes and with body weight, body length, height at withers and chest girth. The post-hoc test utilised in this study was Duncan's multiple range test, which was employed to determine homogenous subgroups. The analysis employed as fixed linear model.

$$Y_{ijk} = \mu + R_i + C_j + e_{ijk}$$

Where, Y_{ijk} is the body weight/body length/height at withers/chest girthmeasured on ijkth animal, µ is the population mean, R_i is the fixed effect associated with ith centre (i = 1, 2, 3, 4, 5, 6, 7 for Malabari and i = 1, 2, 3 for Attappady black), C_j is the fixed effect associated with jth genotype (k =AA, Aa,aa) and e_{ijk} is the random error.

3. Results and discussion

3.1 Body weight

A statistically highly significant (p-value<0.01) difference in the body weights of adult Malabari (31.90±0.59 kg) and Attappady Black (25.41±0.50 kg) goats was observed. The Malabari goats demonstrated a wider range of adult body weights, spanning 16 to 52 kg, in comparison to the Attappady Black goats, which revealed a narrower range of

16 to 35 kg. The findings of this study highlight the substantial differences in body weights across the two native goat breeds. The findings align with the prior studies (Stephen *et al.*, 2005; Verma *et al.*, 2009; Venkatachalapathy *et al.*, 2016; Radhika *et al.*, 2018; Thomas, 2019; Rodricks *et al.*, 2023) [3-5, 11-13].

3.2 Body measurements

The height at withers exhibited a range of 51 to 82 cm for Malabari goats and 58 to 76 cm for Attappady Black goats. A highly significant difference ($p<0.01$) in height at withers was observed between Malabari (66.20 ± 0.40 cm) and Attappady Black (68.08 ± 0.39 cm) goats. The average chest girths for Malabari and Attappady Black goats were 72.39±0.49 cm and 70.65±0.53 cm, respectively, with a significant difference ($p<0.05$) between them. Chest girths ranged from 58 to 89 cm for Malabari goats and 59 to 82 cm for Attappady Black goats. Concerning body length, there was no significant difference between Malabari (62.80±0.56 cm) and Attappady Black goats (63.48±0.64 cm). Body length measurements spanned from 45 to 83 cm for Malabari goats and 54 to 76 cm for Attappady Black goats. These findings highlight various morphometric differences between the two distinct goat breeds under investigation. Previous studies have also revealed comparable results for body confirmation traits (Stephen *et al.*, 2005; Verma *et al.*, 2009; Venkatachalapathy *et al.*, 2016; Radhika *et al.*, 2018; Thomas, 2019; Rodricks *et al.*, 2023) [3-5, 11-13].

3.3 Analysis of CHD9

The 94 bp amplicon yielded three distinctive melt curves, corresponding to the genotypes AA, GG and AG. The validation of these genotypes was confirmed by sequencing representative amplicons that displayed these specific melt curves. The melt curve and sequence maps are displayed in Fig.1, Fig.2 and Fig.3.

The genotype and allele frequencies are detailed in Table 1. The highest frequency of AA genotype was observed in both breeds. Both Malabari and Attappady black breeds were determined to be in Hardy-Weinberg equilibrium concerning to this locus. Different genetic parameters estimated are listed in Table 2. Heterozygote deficit of -0.1631 was found in Attappady back. The Fst was 0.0071, suggesting a minimal level of genetic difference across the breeds.

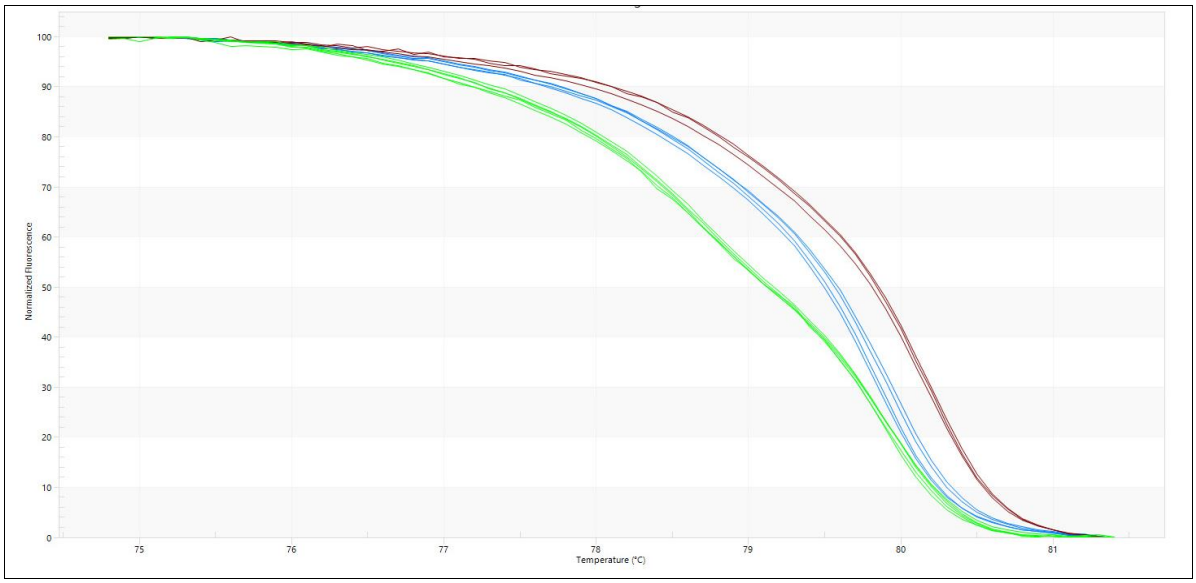


Fig 1: Normalized melt curve of HRM analysis, showing homozygous GG (brown), homozygous AA (blue) and heterozygous G/A (green) genotypes of CHD9

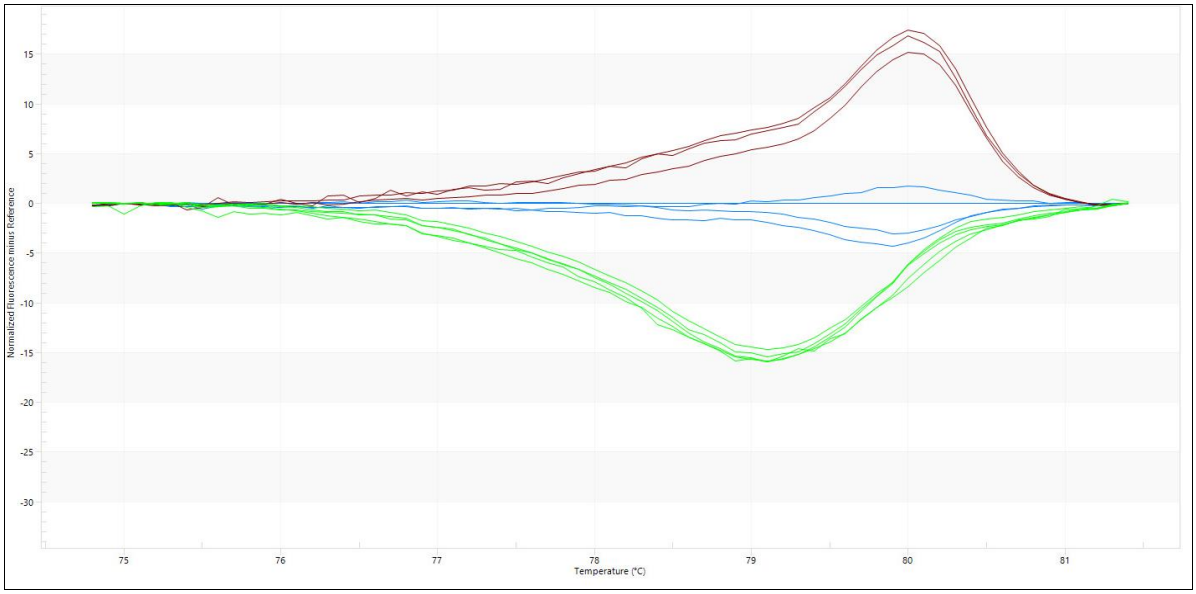
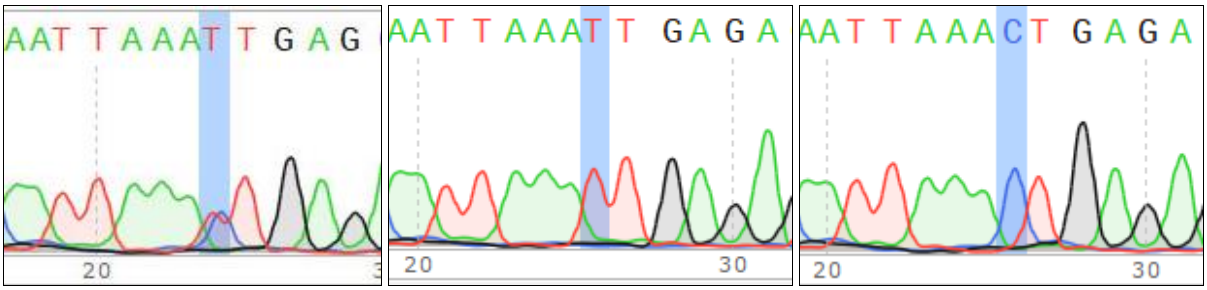


Fig 2: Difference curve of HRM analysis in CHD9, keeping homozygous AA genotype (blue) as reference



i* ii* iii*

Fig 3: Sequence map showing homozygous GG (i), homozygous AA (ii) and heterozygous G/A (iii) genotypes of CHD9 (* Reverse complement)

Table 1: Genotype and allele frequency of CHD9 based on HRM analysis in Malabari and Attappady black goat breeds

Breed and Parameters	Genotype			Allele frequency	
Malabari	**AA**	**AG**	**GG**	**A**	**G**
Observed numbers	87	64	14	0.7212	0.2788
Expected numbers	85.824	66.352	12.824	**Chi-square**	
Observed frequency	0.527	0.402	0.085	***p-value***	0.6489
Attappady black	**AA**	**AG**	**GG**	**A**	**G**
Observed numbers	38	24	1	0.7937	0.2063
Expected numbers	39.683	20.635	2.683	**Chi-square**	
Observed frequency	0.603	0.381	0.016	***p-value***	0.3630

Table 2: Population genetic parameters in Malabari and Attappady black goat breeds

Parameter	Malabari	Attappady black
Effective number of alleles	1.6726	1.4871
Shannon's index (I)	0.5918	0.5091
Nei's expected heterozygosity (H)	0.4021	0.3275
Wright's fixation index (Fis)	0.0354	-0.1631
Observed homozygosity	0.6121	0.6190
Observed heterozygosity	0.3879	0.3810
Expected homozygosity	0.5966	0.6698
Expected heterozygosity	0.4034	0.3302

3.4 Association of CHD9 with body weight and conformation traits

The study found that the genotypic variations of CHD9 did not have a notable impact on body weight and other conformation traits in either breed. Nevertheless, the effect of the center was significant on body weight, height at withers and body length in both breeds. In Malabari goats, the center had a significant effect on body weight, height at withers and body length at $p<0.01$ level. Conversely, in Attappady Black goats, the influence of the center was significant on body length at $p<0.01$ level and on body weight and height at withers at $p<0.05$ level.

The center had a significant effect ($p<0.01$) on chest girth, in Malabari breed. The highest measures were recorded at Tanur, Thrissur and Kottakkal. The animals in Thrissur exhibited the greatest height at withers, but the Malabari goats at Mannuthy and Tanur were seen to be the lengthiest. The centers Tanur, Thrissur and Kottakkal had the highest body weight among Malabari goats. The Thiruvazhamkunnu center had the highest measurements in terms of body weight, height at withers and body length for Attappady black goats. The least square means for the effects of center and genotypes on body weight, height at withers, chest girth and body length are presented in table 3 for the Malabari breed and table 4 for the Attappady black breed, respectively.

Table 3: Least square mean ±SE for the effects of center and genotype of CHD9 on body weight and conformation traits in Malabari goats

Effect	Body weight (kg)	n	Height at withers (cm)	Chest girth (cm)	Body length (cm)
Center					
1	29.31 ± 1.14^{ab}	30	65.53 ± 0.78^{ab}	69.27 ± 1.02^{a}	57.93 ± 0.81^{a}
2	27.47 ± 1.13^{ab}	30	67.47 ± 0.72^{b}	69.47 ± 0.99^{a}	57.70 ± 1.04^{a}
3	33.93 ± 1.07^{cd}	32	66.69 ± 0.86^{b}	73.63 ± 0.95^{bcd}	68.56 ± 0.78^{b}
4	35.62 ± 1.10^{d}	15	72.93 ± 1.23^{c}	75.53 ± 1.49^{cd}	73.13±1.28c
5	26.00 ± 1.30^{a}	16	67.44 ± 0.77^{b}	70.88 ± 1.40^{ab}	66.06 ± 1.25^{b}
6	38.13 ± 1.35^{d}	36	62.61 ± 0.81^{a}	75.83 ± 1.14^{d}	62.86 ± 0.79^{d}
7	30.92 ± 2.01^{bc}	10	63.60 ± 1.85^{a}	71.70 ± 1.97^{abc}	53.40 ± 1.07^{e}
Over all mean	31.90±0.59	169	66.20±0.40	72.38±0.49	62.80±0.56
CHD9 Genotypes					
GG	32.80±1.78	59	67.14±1.26	71.07±1.65	63.71±2.18
AG	30.54±0.89	84	66.11±0.72	71.11±0.73	62.11±0.97
AA	32.62±0.87	21	66.34±0.54	73.52±0.72	63.05±0.75
Total	31.81±0.60	164	66.32±0.41	72.37±0.50	62.74±0.57

Center-1. Vadakara, 2. Perambra, 3. Tanur, 4. Thrissur, 5. Thiruvazhamkunnu, 6. Kottakkal, 7. Mannuthy
Mean values with different superscript within a column within a factor differ significantly ($p<0.05$)

Table 4: Least square mean ±SE for the effects of center and genotype of CHD9 on body weight and conformation traits in Attappady black goats

Effect	Body weight (kg)	n	Height at withers (cm)	Chest girth (cm)	Body length (cm)
Center					
1	26.83±1.07[a]	25	69.20±0.66[a]	70.04±1.02	65.24±0.91[a]
2	24.43±0.48[a]	30	67.90±0.45[a]	71.60±0.53	60.03±0.56[b]
3	24.94±1.14[a]	10	65.80±1.14[b]	69.30±1.59	69.4±1.28[c]
Over all mean	25.41±0.50	65	68.08±0.39	70.65±0.53	63.48±0.64
CHD9 Genotypes					
GG	26	1	68.00±0.00	66.00±0.00	64.00±0.00
AG	24.63±0.96	24	68.17±0.57	70.08±0.89	63.63±0.99
AA	25.96±0.6	38	68.24±0.54	70.95±0.68	63.68±0.88
Total	25.44±0.52	63	68.21±0.39	70.54±0.54	63.67±0.65

Center 1. Thiruvazhamkunnu, 2. Attappady, 3. Mannuthy
Mean values with different superscript within a column within a factor differ significantly ($p<0.05$).

The examination of effect sizes pertaining to body weight, height at withers, body length and chest girth for genotype in Malabari goats revealed values consistently below 0.01 for the former three traits and below 0.05 for the latter. Similarly, for Attappady Black goats, the effect size remained below 0.05 across all traits, as indicated by the estimated partial eta squared values. This brings to light the possibility that the sample size employed in the study may have been insufficient to discern significant differences across genotypes (Serdar *et al.*, 2021) [14]. Another aspect that merits consideration is the influence of dominant effects, particularly when investigating homozygotes with low frequencies. This introduces a potential challenge because the heterozygous genotypes may be combined with the more common homozygous genotype, so creating a level of intricacy that might potentially undermine the robustness of the study's design.

The CHD9 is involved in the processes of chromatin remodeling and the differentiation of mesenchymal stem cells (Shur and Benayahu, 2005) [15]. Studies demonstrated that *CReMM* or CHD9 exhibits varying binding affinities to skeletal tissue-specific promoters, including core binding factor A1, osteocalcin, biglycan, collagen-II and myosin (Shur *et al.*, 2006) [6]. These findings suggest that CHD9 selectively engages with promoters that become active during the appropriate developmental stage of the tissue. The involvement in the process of differentiation of osteogenic precursors has been confirmed in previous studies (Benayahu *et al.*, 2007) [7]. The CHD9 protein, which functions as a chromatin remodeler and epigenetic regulator, has been associated with osteogenesis and the development of the skeletal system. Nevertheless, the precise roles it plays in these processes remain unclear. In recent studies, a novel role for CHD9 in osteogenesis has been introduced, emphasizing its capacity to regulate the expression of runt-related transcription factor 2 (Newton and Pask, 2020) [8].

4. Conclusion

The study identified and genotyped, G>A (chromosome 18, position 23020094) polymorphism in CHD9 gene in the populations of Malabari and Attappady Black goat breeds in Kerala. Genotyping using HRM revealed AA, GG and AG genotypes of CHD9 with the highest frequency of AA genotype in both breeds. Significant differences were observed in adult body weights, height at withers and chest girths between the Malabari and Attappady Black breeds. The genotypic patterns of CHD9 had no significant impact on body weight and conformation traits in either of the breeds.

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