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Identification of *GDF9* Promoter SNPs Affecting Lambing Rate in Australian-Hu Sheep

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ABSTRACT

Background: Litter size represents a key economic trait in Australian-Hu sheep (Australian White sheep ♂ × Hu sheep ♀) and determines breeding profitability. Growth differentiation factor 9 (*GDF9*) is recognized as a principal candidate gene influencing litter size in *ovis aries*. Previous research on Australian-Hu sheep has focused on associations between polymorphisms in the coding region of *GDF9* and litter size, with limited attention to the promoter region. To address this gap, this study examined the *GDF9* promoter region polymorphism in Australian-Hu sheep, analyzed its association with multiple lambing and evaluated its impact on promoter activity, aiming to provide a theoretical foundation for selecting molecular markers for multiple lambing breeding.

Methods: To investigate single nucleotide polymorphism (SNP) loci effects in *GDF9* promoter region on lambing rate of Australian-Hu crossbred sheep, 193 pregnant ewes were selected. Genomic DNA (gDNA) was extracted from blood, the *GDF9* promoter region was amplified by PCR and SNP sites were identified using Sanger sequencing. Association analyses between SNPs and lambing rate were conducted and a dual-luciferase reporter assay evaluated the SNPs' effects on *GDF9* transcriptional activity.

Result: In the *GDF9* promoter region of Australian-Hu Sheep, six linked SNP sites were identified: g.42117805C>T, g.42117824T>C, g.42117899G>A, g.42118070A>G, g.42118197T>G and g.42118272C>T. Five loci (g.42118070A>G, g.42118272C>T, g.42117805C>T, g.42117824T>C and g.42117899G>A) showed significant association with lambing rate ($P<0.05$), with heterozygous genotypes yielding higher lambing rate. Dual-luciferase reporter assays indicated that the (-517 to +3) region functions as the core promoter. Mutations at g.42118070A>G, g.42118197T>G and g.42118272C>T within this region reduced *GDF9* promoter transcriptional activity. These findings provide a basis for identifying molecular markers to screen for multiple lambing traits in Australian-Hu sheep.

Key words: Australian-Hu sheep, *GDF9*, Lambing rate, Promoter, SNP.

INTRODUCTION

The Xinjiang Uygur Autonomous Region is one of China's "Five Major Pastoral Areas," where lambing rates determine economic outcomes for local herders. Ewes typically experience seasonal estrus, resulting in an annual lambing cycle with a single lamb per event (Ma, 2014). Enhancing sheep reproductive performance is a pressing concern for the industry. Sheep farming in southern Xinjiang faces three challenges: low reproduction rates, inefficient farming practices and limited profitability (Dou and Zhang, 2016). The Australian-Hu crossbred sheep, a superior meat sheep hybrid, has been developed through crossbreeding between Australian White sheep and indigenous Hu sheep in China. The Australian White sheep, serving as the sire breed, show medium-to-high heritability (Zhang *et al*., 2024) and feature large body size, early sexual maturity, rapid growth rate and high-quality meat (Pewan *et al*., 2020). The maternal Hu sheep are noted for year-round estrus, high fertility, early maturity and robust adaptability (Geng *et al*., 2022). These sheep inherit the growth advantages of the paternal line and possess polyploidy-related superior genes, leading to improved reproductive performance and economic benefits compared to purebred sheep (Liu *et al*., 2018).

Prolificacy in sheep is a complex trait governed by multiple genes acting synergistically. The genetic mechanisms include major and minor effect genes and their interactions. Among genes influencing sheep reproductive traits, those with major effects are significant. Key factors for multiple lambing include bone morphogenetic protein receptor IB (BMPR-IB) (Sun *et al*., 2023), BMP15 (bone morphogenetic protein 15) and GDF9

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(growth differentiation factor 9) (Zamani *et al*., 2015; Maitra *et al*., 2015). GDF9, secreted by oocytes, regulates follicular growth and differentiation in early stages. It facilitates mitotic division of pre-granulosa cells within the ovarian tissue cavity, induces cumulus expansion and enhances expression of genes associated with cumulus expansion and ovulation. GDF9 is expressed throughout follicular development. In early phase, it sustains follicular growth and oocyte development, while in late phase, it promotes corpus luteum development (Liu *et al*., 2025). *GDF9*'s impact on lambing rate links to SNPs (Dimitrova *et al*., 2024). Eleven SNP sites have been identified in *GDF9*: G1 (260G>A), G2 (471C>T), G3 (477G>A), G4 (721G>A), G5 (978A>G), G6 (994G>A), G7 (1111G>A), G8 (1184C>T), *FecG*^{T}(1279A>C), *FecG*^{E}(1034G>T), *FecG*^{V}(934C>T) (Zheng, 2023; Dimitrova *et al*., 2024). G8 heterozygotes increase ovulation by 1.4, *FecG*^{T} mutation heterozygotes increase ovulation by 1.2 and lambing rate by 0.7, while G8 and FecGT mutation homozygotes impair primary follicle development. *FecG*^{E} mutations enhance ovulation and lambing rates, with homozygous mutations increasing ovulation and affecting sheep multiple-birth traits (Wang and Cao, 2011).

Previous research has focused on the *GDF9* coding region to explore relationships between SNPs and lambing rates, with little attention to promoter regions. In this study, we examined the association between SNPs in the *GDF9* promoter region of Australian-Hu sheep and lambing traits. We utilized promoter PCR amplification, lambing association analysis, recombinant plasmid construction and dual-luciferase assays to identify and evaluate SNP activity within the *GDF9* promoter region. This research investigated polymorphism in the *GDF9* promoter region of Australian-Hu sheep, analyzed its association with multiple lambing rate and assessed its influence on promoter activity. This study aims to provide a theoretical foundation for selecting molecular markers in breeding programs targeting prolific traits.

MATERIALS AND METHODS

Experimental materials

This study was conducted from January to September 2025 at the College of Life Sciences and Technology, Tarim University, Alar, China. The experimental cohort comprised 193 adult Australian-Hu sheep ewes in optimal condition with lambing records. The lambing rate was calculated as total live lambs born divided by experimental ewes (lambing rate = total number of live lambs/number of experimental ewes). The selected ewes were unrelated within three generations. All individuals were from the Hotan Australian-Hu sheep Breeding Base in the Xinjiang Uygur Autonomous Region, maintained under uniform husbandry conditions. Blood samples were obtained via venipuncture, with 5 mL of anticoagulated blood preserved at -20°C for analysis.

Cloning of the Australian-Hu sheep *GDF9* promoter region

Primers were designed based on sheep *GDF9* 5′ regulatory region sequence from GenBank (Table 1) for segmented amplification and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Genomic DNA (gDNA) was extracted from blood samples using a DNA extraction kit from TianGen Biochemical Technology Co., Ltd., Beijing, China. The promoter region sequence was amplified using the extracted gDNA template. The polymerase chain reaction (PCR) system comprised 50 µL total volume, including 25 µL of 2× Taq PCR Mix, 1 µL each of upstream and downstream primers, 1 µL of DNA template and 22 µL of ddH, O. PCR cycling conditions were: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute; followed by final extension at 72°C for 10 minutes. PCR products were stored at 4°C. The products underwent agarose gel electrophoresis to evaluate fragment length and integrity, then were sent to Sangon Biotech (Shanghai) for sequencing.

Analysis of *GDF9* promoter in Australian-Hu sheep

The promoter region was identified using BDGP software. Transcription factor binding sites were determined through JASPAR software. CpG islands were identified using MethPrimer software.

Construction of recombinant vectors for dual luciferase assay

Three pairs of specific primers incorporating restriction sites were designed (Table 1) and synthesized by Sangon

Table 1: *GDF9* promoter sequencing primers in Australian-Hu Sheep.

Primer	Primer sequences (5′ - 3′)	Tm (°C)	Length/bp	Purpose
P1	F: TCAGTCTTCTCCTCGGTTC	56	1237	Promoter region
	R: TGATTTGGAGGGTGAGAG			
P2	F: CCCAAATAAAGGCACCAAG	60	780	
	R: AAACAGAGCCAGGCAAAG			
P3	F: CTAGGGTACCACTGCACAGACTGGTTCTG	62	520 (-517 to +3)	Recombinant plasmid construction
	R: CTAGCTCGAGCATGGCTTGGAAGAATTAGC			
P4	F: CTAGGGTACCACTGCACAGACTGGTTCTG	62	991(-908 to +3)	
	R: CTAGCTCGAGCATGGCTTGGAAGAATTAGC			
P5	F: CTAGGGTACCCTGTTCACTGCCCAGGAGGA	60	1321(-1200 to +3)	
	R: CTAGCTCGAGGCAGCAAAACCAAAGGAAGAAT			

Underline indicates the restriction enzyme sites (GGTACC for *Kpn*I, CTCGAG for *Xho*I).

Biotech (Shanghai). PCR amplification of *GDF9* promoter segments yielded fragments of 520, 991 and 1321-bp. The PCR reaction system and protocol matched those previously described. PCR products were purified using a PCR product purification kit from TianGen Biochemical Tech. The purified target fragments and pGL3-Basic vector underwent double digestion. The recombinant plasmid was constructed using T4 ligase and transformed into DH5α competent cells (Tiangen, Beijing). The digestion system contained 1 μg of target fragment/vector, 1 μL *Kpn*I, 1 μL *Xho*I, 5 μL 10× FD Buffer and ddH_2O to 50 μL final volume. The pGL3-Basic vector was digested overnight at 37°C and purified using a PCR product purification kit per manufacturer's instructions. Positive clones identified through PCR screening were sent to Sangon Biotech for sequencing. Correctly sequenced positive plasmids were extracted using a plasmid midi-prep kit (Tiangen, Beijing). The constructed recombinant plasmids were designated as pGL3-520, pGL3-991 and pGL3-1321.

Point mutation vectors were generated using the aforementioned method. The recombinant plasmids constructed were designated as pGL3-991(mut0), pGL3-991(mut3) and pGL3-991(mut6).

Cell culture and transfection

Transfection was conducted using GenXP III transfection reagent (Beijing Probe Gene Technology Co., Ltd.). The internal control plasmid was pRL-TK. The experimental groups were: pGL3-Basic + pRL-TK, pGL3-1321 + pRL-TK, pGL3-991 + pRL-TK, pGL3-520 + pRL-TK, pGL3-991(mut0) + pRL-TK, pGL3-991(mut3) + pRL-TK and pGL3-991(mut6) + pRL-TK. Recombinant and empty plasmids were co-transfected with pRL-TK into 293T cells at a plasmid DNA ratio of 4:1. Each condition was replicated in triplicate. Cells were harvested 48 hours post-transfection and dual luciferase activity was assessed using the Dual Luciferase Report Gene Assay Kit (Yisheng Bio).

Data analysis

Sequence peak charts were analyzed using Chromas software to identify SNP sites and conduct genotyping analysis. Haploview 4.2 software analyzed linkage relationships among SNP sites. Chi-square tests using Excel and SPSS evaluated whether genotypes at each SNP locus adhered to Hardy-Weinberg equilibrium, with P>0.05 indicating equilibrium. Genotype and gene frequencies were calculated using Excel, from which polymorphism information content (PIC), expected heterozygosity (He) and effective number of alleles (Ne) were derived. One-way ANOVA was performed using SPSS to examine associations between genotypes at each SNP locus and lambing rate. Least significant difference (LSD) multiple comparisons were conducted, with P<0.05 considered significant and P<0.01 highly significant.

The relative activity of the promoter was quantified as the ratio of Firefly luciferase to Renilla luciferase activity. Renilla luciferase serves as an internal control to normalize Firefly luciferase signals, eliminating variations from cell transfection efficiency, sample loading and experimental artifacts, thus ensuring accurate promoter activity measurement. The data were analyzed using one-way ANOVA and visualized with GraphPad Prism 5.

RESULTS AND DISCUSSION

Cloning of the *GDF9* promoter region

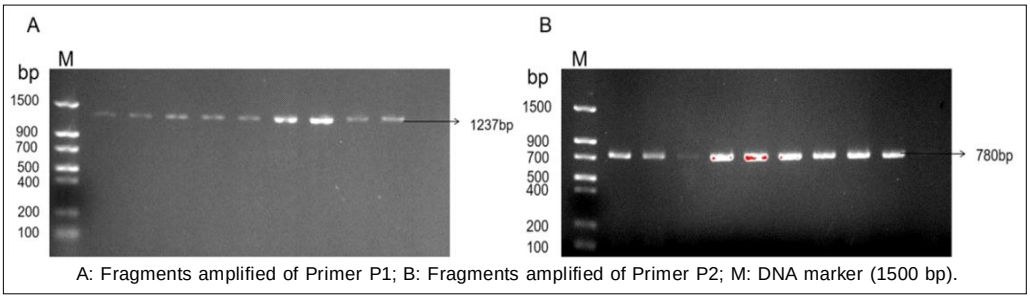
The *GDF9* promoter amplification was conducted using gDNA from Australian-Hu sheep as template, with primers P1 and P2 (Table 1) as described. Sequencing results verified that PCR product sizes matched the anticipated designs (Fig 1), confirming their sequencing suitability.

Analysis of *GDF9* promoter sequence

Sequencing of the *GDF9* promoter identified six mutation sites (Fig 2): g.42117805C>T, g.42117824T>C, g.42117899G>A, g.42118070A>G, g.42118197T>G and g.42118272C>T. Linkage analysis showed complete linkage among these sites (Fig 3), indicating strong linkage disequilibrium, with the predominant haplotype being C-T-G-A-T-C.

Analysis of *GDF9* sequence identified three potential promoter regions with scores above 0.85, located at -1092 to -1043, -633 to -584 and -270 to -221 bp. JASPAR software prediction showed multiple transcription factor-binding sites near these regions (Fig 4). MethPrimer analysis identified two CpG islands at positions -1953 to -1816 and -1801 to -1472 bp.

Sites g.42118070A>G, g.42117805C>T and g.421182 72C>T showed low polymorphism (PIC < 0.25), while g.421



A: Fragments amplified of Primer P1; B: Fragments amplified of Primer P2; M: DNA marker (1500 bp).

Fig 1: PCR amplification products of the Australian-Hu Sheep *GDF9* promoter.

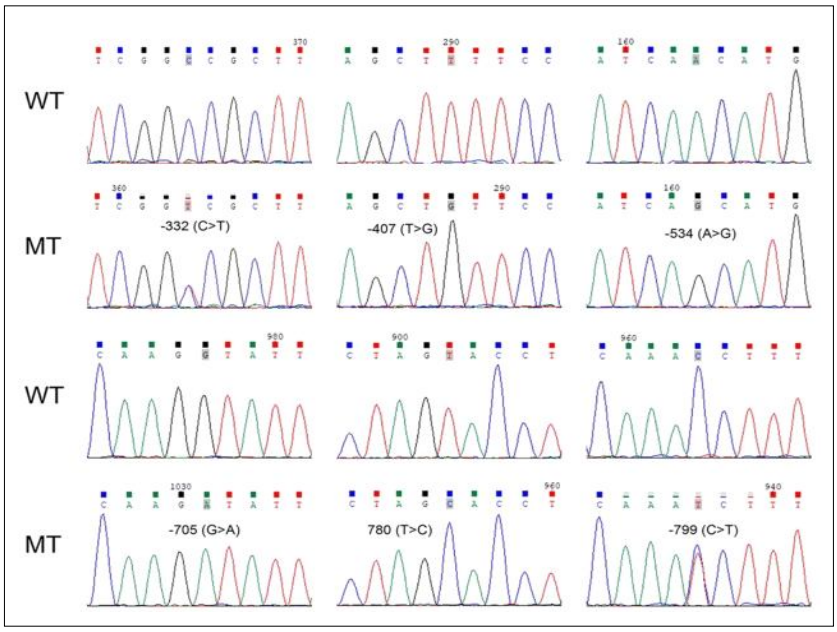


Fig 2: Sequencing results of the *GDF9* promoter mutation sites in Australian-Hu sheep.

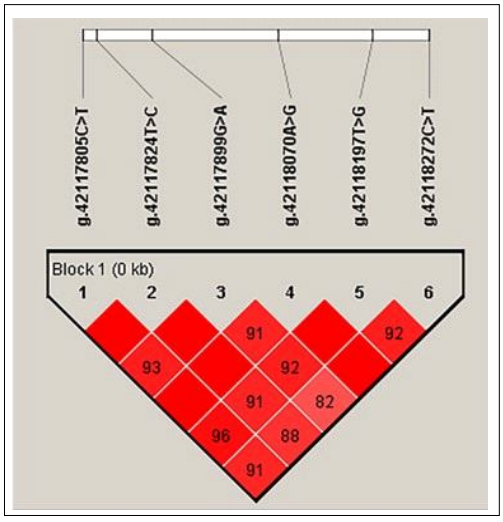


Fig 3: Linkage map of the *GDF9* promoter mutation sites in Australian-Hu sheep.

18197T>G, g.42117824T>C and g.42117899G>A showed moderate polymorphism (0.25 < PIC < 0.50). Chi-square tests showed g.42118070A>G, g.42118197T>G, g.42118 272C>T, g.42117805C>T, g.42117824T>C and g.42117899 G>A sites were in Hardy-Weinberg equilibrium (P>0.05) (Table 2).

Association analysis of *GDF9* SNPs with lambing productivity

The *GDF9* variants g.42118070A>G, g.42118272C>T, g.42117805C>T, g.42117824T>C and g.42117899G>A showed significant correlation with lambing rate in Australian-Hu sheep (P<0.05). The CT genotype at g.42118272C>T was associated with a 0.25 lamb increase compared to CC genotype. The AG genotype at g.42118070A>G showed a 0.31 lamb increase versus AA genotype. The GA genotype at g.42117899G>A was linked to a 0.22 lamb increase compared to GG genotype and TC genotype at g.42117824T>C showed a 0.23 lamb increase versus TT genotype. The frequency of TC genotype was 0.33 higher than CC genotype (Table 3).

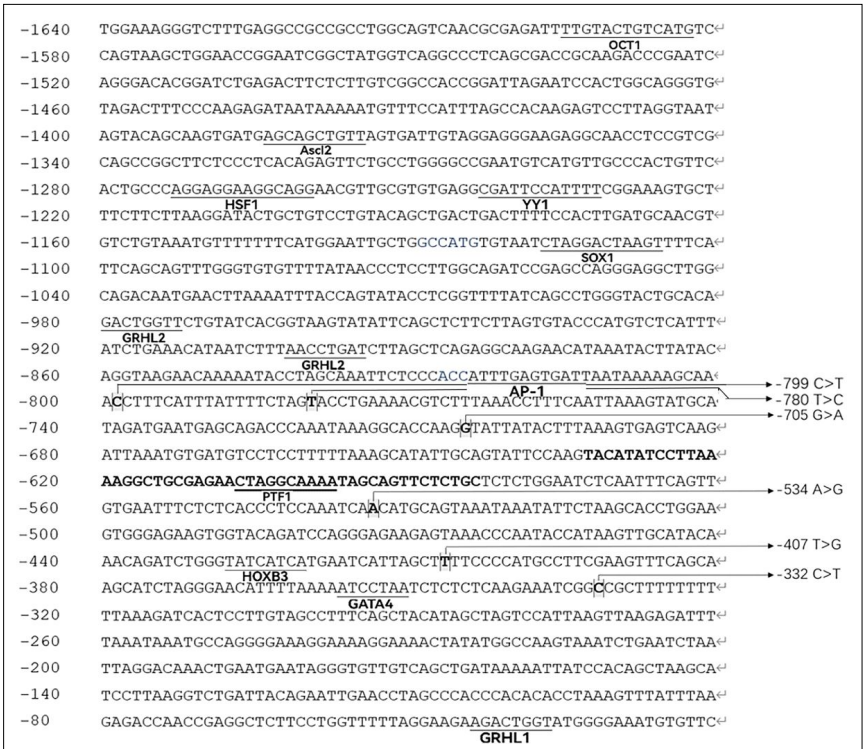
Construction of recombinant vectors

To examine SNP transcriptional activity, primer P3-5 (Table 1) was designed to amplify three segments encompassing the mutation site. These segments were cloned into reporter vector pGL3-Basic to identify core promoter regions, designated as pGL3-520, pGL3-991 and pGL3-1321. Fragments containing mutation sites (g.42118070A>G, g.42118197T>G, g.42118272C>T) and fragments containing mutation sites (g.42117805C>T, g.42117824T>C, g.42117899G>A, g.42118070A>G, g.42118197T>G, g.42118272C>T) were directionally cloned into dual luciferase reporter vector pGL3-Basic alongside wild-type fragment to identify critical transcription factor binding sites. These constructs were named pGL3-991(mut0), pGL3-991(mut3) and pGL3-991(mut6). Sequencing confirmed the target fragment sequences (Fig 5 and 6), verifying successful construction of the promoter region fragment and point mutation recombinant plasmid.

Activity analysis of the truncated fragment on *GDF9* promoter

The dual luciferase reporter plasmids pGL3-520, pGL3-991 and pGL3-1321, along with empty vector pGL3-Basic, wereco-transfected with control plasmid pRL-TK into 293T cells. After forty-eight hours, cells were harvested and

luciferase activity assessed. Results (Fig 7) showed that fluorescence activity of pGL3-520 was significantly higher than pGL3-Basic (p<0.001). pGL3-991 and pGL3-1321 did not exhibit significant difference in activity compared to pGL3-520, suggesting that primary transcription binding sites of the *GDF9* promoter are located within the (-517 to +3) region.



Black bold text shows the predicted *GDF9* promoter region; Underlined sequences represent transcription factor binding sites; Arrows and allele notations indicate SNPs within this region.

Fig 4: Predicted transcription factor binding sites for *GDF9* in Australian-Hu sheep.

Table 2: Genetic diversity index of SNPs in Australian-Hu sheep *GDF9* promoter.

Site	Genotype	Genotype frequency	Allele	Allele frequency	PIC	Ne	P
-332 (C>T)	CC	0.82	C	0.90	0.16	1.22	0.27
	CT	0.16	T	0.10			
	TT	0.02					
-407 (T>G)	TT	0.63	T	0.79	0.27	1.49	0.95
	TG	0.32	G	0.21			
	GG	0.05					
-534 (A>G)	A A	0.84	A	0.91	0.14	1.19	0.90
	AG	0.15	G	0.09			
	GG	0.01					
-705 (G>A)	GG	0.66	G	0.81	0.26	1.44	0.99
	GA	0.30	A	0.19			
	A A	0.04					
-780 (T>C)	TT	0.62	T	0.78	0.28	1.52	0.94
	TC	0.33	C	0.22			
	CC	0.05					
-799 (C>T)	CC	0.80	C	0.90	0.17	1.23	0.99
	CT	0.19	T	0.10			
	TT	0.01					

P>0.05 indicates conformity with the Hardy-Weinberg equilibrium (HWE).

Activity analysis of SNPs in *GDF9* promoter

Dual luciferase reporter plasmids pGL3-991(mut0), pGL3-991(mut3) and pGL3-991(mut6) were co-transfected with control plasmid pRL-TK into 293T cells. After forty-eight hours, cells were harvested and luciferase activity was assessed. Results in Fig 8 showed that luciferase activity of pGL3-991, pGL3-991(mut3) and pGL3-991(mut6) was significantly elevated ($p < 0.05$) compared to control plasmid pGL3-Basic. The pGL3-991(mut3) group showed a significant reduction in luciferase activity ($p<0.05$) compared to the pGL3-991 group, suggesting that g.42118070A>G, g.42118197T>G and g.42118272C>T sites may inhibit promoter transcriptional activity. No significant changes in luciferase activity were observed between pGL3-991(mut3) and pGL3-991(mut6) groups, indicating that the additional three mutation sites do not affect promoter activity.

Analysis of SNP site association with lambing performance

GDF9 is a key component of the transforming growth factor β (TGF-β) superfamily. It regulates early follicular development and oocyte maturation in sheep, with its deficiency causing follicular arrest and infertility. Research has shown that single nucleotide polymorphisms (SNPs)

Table 3: *GDF9* promoter genotype mutation sites associated with litter size in Australian-Hu sheep.

Gene	Site	Genotype	Sample size	Average litter size
GDF9	-332 C>T	CC	158	1.46±0.57[a]
		CT	31	1.71±0.59[b]
		TT	4	1.75±0.96[ab]
	-407 T>G	TT	122	1.46±0.58[a]
		TG	62	1.55±0.59[a]
		GG	9	1.78±0.67[b]
	-534 A>G	A A	161	1.46±0.57[a]
		AG	30	1.77±0.63[b]
		GG	2	1.50±0.71[a]
	-705 G>A	GG	127	1.44±0.57[a]
		GA	59	1.66±0.61[b]
		A A	7	1.43±0.54[a]
	-780 T>C	TT	119	1.43±0.58[a]
		TC	64	1.66±0.60[b]
		CC	10	1.50±0.53[a]
	-799 C>T	CC	155	1.45±0.56[a]
		CT	36	1.78±0.63[b]
		TT	2	1.50±0.71[a]

Different lowercase letters (a, b, c) after lambing rate values indicate significant differences between groups (P<0.05). Values with same letter are not significantly different.

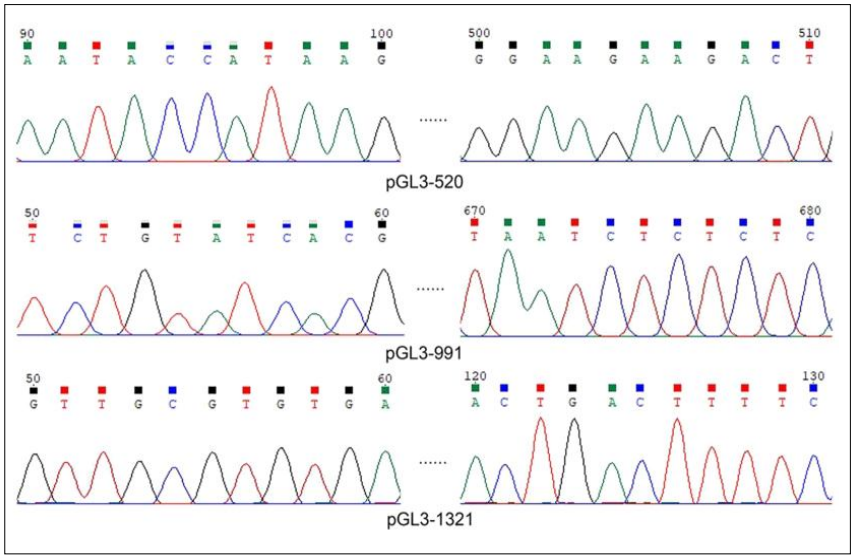


Fig 5: Sequencing results of truncated fragment recombinant plasmids for *GDF9* promoter in Australian-Hu sheep.

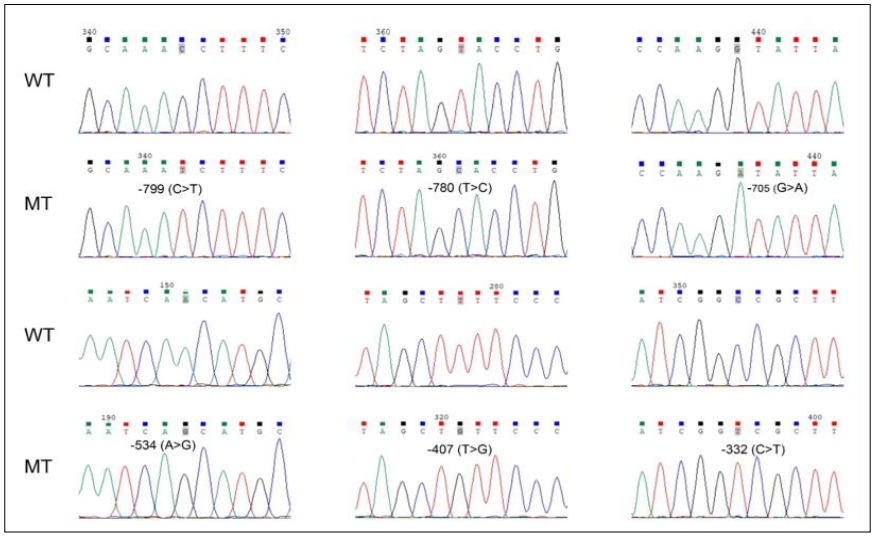
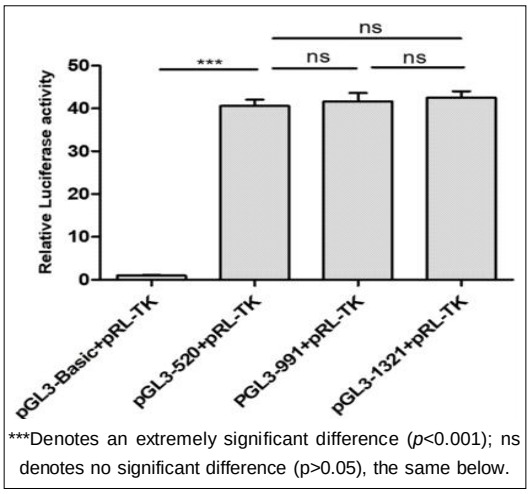
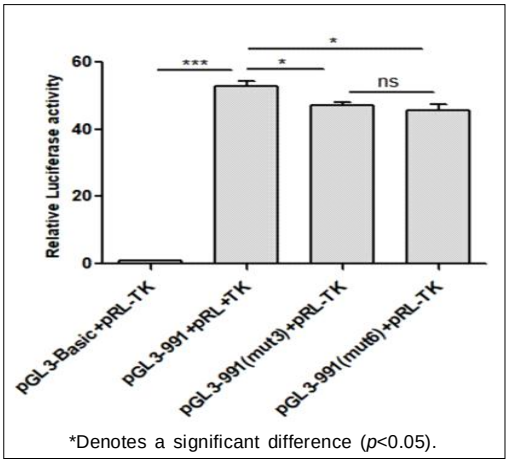


Fig 6: Sequencing results of the recombinant plasmids for the *GDF9* promoter point mutation in Australian-Hu sheep.



***Denotes an extremely significant difference (p<0.001); ns denotes no significant difference (p>0.05), the same below.

Fig 7: The effect of different truncated fragments on the transcriptional activity of the *GDF9* promoter in Australian-Hu sheep.



*Denotes a significant difference (p<0.05).

Fig 8: The effect of SNPs on the transcriptional activity of the *GDF9* promoter in Australian-Hu sheep.

in the *GDF9* coding region correlate with litter size in multiple-lamb sheep breeds, such as Hu sheep and Small-tailed Han sheep (Ling *et al*., 2025). However, studies of the promoter region of Australian-Hu sheep remain scarce.

Six single nucleotide polymorphisms (SNPs) were identified: g.42117805C>T, g.42117824T>C, g.42117899G>A, g.42118070A>G, g.42118197T>G and g.42118272C>T. Linkage analysis showed these sites were fully linked, forming a 0-kb strong linkage disequilibrium (LD) block, designated as Block1. The most prevalent haplotype was "C-T-G-A-T-C," exhibiting the highest frequency. This aligns with the "-534A/G, -407T/G and -332C/T complete linkage" pattern identified by Li *et al*. (2020) in the Hu sheep *GDF9* promoter region, indicating that the LD structure in the *GDF9* promoter may represent a conserved genetic pattern regulating reproductive traits in sheep. A mutation at one locus can alter transcription factor binding affinity, while other linked loci may enhance this regulatory effect by modifying the promoter region's secondary structure. This suggests *GDF9* in Australian-Hu sheep maintains its regulatory capacity over reproductive traits under hybridization conditions. Five SNPs (g.42118070A>G, g.42118272C>T, g. 42117805C>T, g.42117824T>C and g.42117899G>A) showed significant correlations with lambing rate in Australian-Hu Sheep (P<0.05), demonstrating "heterozygous genotypes yielding higher lambing rates."

The loci g. 42118197T>G, g. 42117824T>C and g. 42117899G>A demonstrated moderate polymorphism (0.25 < PIC < 0.50), suggesting these loci maintain genetic diversity within the Australian-Hu sheep population and are viable molecular markers for breeding selection. In contrast, loci g. 42118070A>G and g. 42117805C>T exhibited low polymorphism (PIC<0.25). This low polymorphism may result from long-term artificial selection for high lambing rates, leading to increased frequency of dominant alleles. Caution is needed in future breeding programs to prevent excessive loss of genetic diversity, as

reduced diversity may limit the population's adaptive potential and selection response-especially under changing environmental conditions in southern Xinjiang. Additionally, all associated loci adhered to Hardy-Weinberg equilibrium (P>0.05), indicating a stable genetic structure within the Australian-Hu sheep population, without significant shifts from artificial selection.

Bioinformatics analysis identified two CpG islands within the *GDF9* promoter region in Australian-Hu sheep, at -1953 to -1816 and -1801 to -1472 bp. Multiple predicted binding sites for reproduction-associated transcription factors were observed. GATA4, a critical transcription factor, regulates ovarian granulosa cell proliferation (Jin, 2016). SNPs near its binding site may affect GATA4 binding, influencing granulosa cell survival and follicular maturation, impacting lambing rate. These findings align with previous studies on sheep *GDF9* gene variations. Pan *et al*. (2016) reported significant associations between SNPs in the *GDF9* regulatory region and lambing rate in Small-tailed Han sheep, with the heterozygous genotype predominant. Wang *et al*. (2020) confirmed the synergistic regulatory effect of SNP linkage in the *GDF9* promoter region on reproductive traits in Luzhong meat sheep. This study identified a complete linkage pattern for six SNPs in the *GDF9* promoter region in hybrid Australian-Hu sheep and elucidated their synergistic effect on lambing rate, supplementing genetic regulatory evidence for reproductive traits in hybrid sheep.

Promoter region activity analysis

Through promoter truncated fragments of varying lengths (pGL3-520, pGL3-991 and pGL3-1321) in dual-luciferase reporter assays, the core promoter region (-517 to +3) was identified as the principal sequence driving gene transcription. This region contains multiple transcription factor-binding sites, including HOXB3, GATA4 and GRHL2. The extended regions (-908 to +3) and (-1200 to +3) facilitate binding of additional transcription factors for nuanced regulation of core transcription activity. The characterization of the *GDF9* promoter's core functional zone provides a crucial molecular target for elucidating mechanisms that modulate follicular development through transcriptional regulation, influencing the lambing rate.

Through analysis of point mutation activity using a dual luciferase reporter system, mutations g.42118070A>G, g.42118197T>G and g.42118272C>T were identified as critical sites affecting promoter transcriptional activity. These mutations are located within essential transcription factor-binding regions and their presence alters binding efficiency of transcription factors, suppressing *GDF9* transcription. In a study on Hu sheep and Bashibai sheep by Jin *et al*. (2016), mutations in the 5' regulatory region of *GDF9* gene altered the binding site of transcription factor OCT1. After co-transfection with an OCT1 expression vector, transcriptional activity of the mutant promoter was significantly reduced. These findings indicate that OCT1 exerts a pronounced inhibitory effect on promoter activity of the mutant *GDF9* gene in sheep, explaining the functional effects of the identified mutation sites. Furthermore, the 5' regulatory region of *GDF9* contains multiple binding sites for transcription factors associated with follicular development, such as GATA4 and YY1. The mutation identified may synergistically affect binding efficiency of multiple transcription factors, regulating *GDF9* expression. This hypothesis warrants further validation through subsequent EMSA or ChIP experiments.

Wang *et al*. (2024) investigated four Chinese sheep breeds, including Small-tailed Han sheep and Hu sheep and identified polymorphic sites within *BMP15* and *GDF9* genes. Mutations at these sites were significantly associated with lambing rate, affecting ovulation numbers. As critical paracrine factors secreted by oocytes, balanced activity of GDF9 and BMP15 is crucial in determining follicular development fate. Individuals with single mutations in BMP15 or GDF9 exhibit increased ovulation, potentially due to diminished dimerization capacity or reduced receptor-binding ability of mutated proteins (Hanrahan *et al*., 2004). When mutations in both BMP15 and GDF9 co-occur, secretion levels of these proteins are low, resulting in reduced mature BMP15 and GDF9 proteins, weakened functional interaction between BMP15 and GDF9 and diminished biological activity of BMP15/GDF9 heterodimers. This leads to impaired regulation of follicular development, culminating in increased ovulation rates in mutant ewes (Wang *et al*., 2020). This suggests that when using *GDF9* mutation sites for selecting prolificacy traits in sheep, it is necessary to consider the genetic background of breeds and interaction of ovarian microenvironmental factors to achieve precise improvement in reproductive performance.

CONCLUSION

This study identified six fully linked single nucleotide polymorphisms (SNPs) (g.42117805C>T, g.42117824T>C, g.42117899G>A, g.42118070A>G, g.42118197T>G and g.42118272C>T) in the *GDF9* promoter region. Five loci (g.42118070A>G, g.42118272C>T, g.42117805C>T, g.42117824T>C and g.42117899G>A) showed significant correlations with lambing rates in Australian-Hu Sheep (P<0.05). Three linked SNPs (g.42118070A>G, g.42118197T>G and g.42118272C>T) reduced the transcriptional activity of the *GDF9* promoter. These mutation sites may serve as molecular markers for multiple-lamb breeding in Australian-Hu Sheep, holding practical significance for improving meat sheep reproduction.

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Disclaimers

The views and conclusions in this article are solely those of the authors and do not represent the views of their affiliated institutions. The authors are responsible for the accuracy of the information provided, but do not accept liability for any losses resulting from use of this content.

Informed consent

All animal procedures and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare no conflicts of interest regarding this article's publication. No funding influenced the study design, data collection, analysis, publication decision, or manuscript preparation.

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