BF-2099
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RESEARCH ARTICLE Indian Journal of Animal Research

Integrative Analysis of *MTHFR* Gene Polymorphisms and Semen Quality Traits in Bulls: A Bioinformatics and Association Study

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10.18805/IJAR.BF-2099

ABSTRACT

Background: This section clearly introduces the goal of the study and the genetic focus (MTHFR gene and SNPs in relation to bull semen).

Methods: We conducted polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and Sanger sequencing of the MTHFR gene in bulls from four breeds: Sinan, Guanling, Australian Wagyu and Simmental cattle.

Result: Four SNPs were identified: g.41883859T > C (Exon 8), g.41884250G > A/g.41884310T > C (Exon 9) and g.41886545T > C (Intron 12). These SNPs displayed moderate polymorphism and conformed to Hardy-Weinberg equilibrium. Furthermore, strong linkage disequilibrium was observed among the four loci. The dominant haplotype H1 and the diplotype H1H1 were identified. Associations revealed that the g.41883859T > C TT genotype increased ejaculate volume but reduced sperm density, while the g.41884250G > A AA genotype improved sperm density and motility. The g.41884310T > C TT genotype enhanced motility parameters. The H1H1 diplotype was associated with superior motility. Bioinformatics analysis suggested that the g.41883859T > C polymorphism altered the MTHFR mRNA structure and MTHFR interacted with enzymes involved in one-carbon metabolism.

Key words: Bovine semen quality, Bull, *MTHFR* gene, Single-nucleotide polymorphisms.

INTRODUCTION

Advancements in breed selection and breeding technologies, such as artificial insemination (AI) and Multiple Ovulation and Embryo Transfer (MOET), are fundamental to modernizing the cattle industry. These technologies enhance reproductive efficiency, accelerating breeding progress and driving industry development (Li *et al*., 2025). In AI-based genetic improvement programs, semen traits are crucial for fertilization success and genetic progress. Accurate semen trait screening improves reproductive efficiency, accelerates genetic gain and enhances the performance and economic benefits of beef and dairy cattle (Modiba *et al*., 2022). For example, Biswal *et al*. (2025) emphasized osteopontin's role in semen quality and fertility rates in bulls, highlighting the importance of semen traits in AI fertility outcomes. Frozen semen production, a cornerstone of AI technology, requires maximizing sperm yield from genetically superior bulls, necessitating systematic fertility assessments at breeding stations. Targeted selection for superior semen traits is crucial for genetic improvement. Screening candidate genes, such as *MTHFR*, which is involved in folate metabolism and sperm motility regulation, helps identify functional genes through marker-assisted selection (MAS), advancing molecular breeding (Cui *et al*., 2016; Dai *et al*., 2009).

MTHFR is a key enzyme in the folate cycle, converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is crucial for homocysteine methylation to methionine (Fodinger *et al*., 2000). Folate-derived one-carbon units supply methyl groups essential for nucleotide synthesis and DNA replication (Giammarco *et al*., 2024). Folic acid is vital for DNA and protein synthesis, both critical for spermatogenesis. Folic acid deficiency impairs sperm quality by inducing DNA damage, abnormal androgen levels and sperm pH imbalances, disrupting normal spermatogenesis. Human studies link the *MTHFR* C677T polymorphism (a valine to alanine substitution in exon 4)

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How to cite this article: Wang, H., Yin, F.Y., Wang, X., Zhou, W., Wu, J., Liu, J., Zhao, Y., Zhu, Y., Ni, M., Xu, L. and Mo, B. (2026). Integrative Analysis of *MTHFR* Gene Polymorphisms and Semen Quality Traits in Bulls: A Bioinformatics and Association Study. *Indian Journal of Animal Research*. 1-9. doi: 10.18805/IJAR.BF-2099.

Submitted: 15-12-2025 **Accepted:** 10-02-2026 **Online:** 05-03-2026

to infertility, reducing *MTHFR* activity and disrupting folate metabolism, affecting DNA synthesis and methylation (Alfaleh *et al*., 2023; Aarabi *et al*., 2015; Afedo *et al*., 2020). DNA methylation loss is most pronounced in sperm from C677T homozygotes, impairing sperm function and embryo development (Aliakbari *et al*., 2020; Rotondo *et al*., 2021). In cattle, *MTHFR* mRNA expression is higher in adult testes than juveniles (Afedo *et al*., 2020) and the gene is essential for normal blastocyst development (Ishitani *et al*., 2020). Given the conserved role in spermatogenesis across species, *MTHFR* polymorphisms in cattle may influence semen quality through folate-dependent methylation.

While *MTHFR* polymorphisms are linked to reproductive function in humans and mammals, their association with semen quality in cattle is unclear. The impact of bovine *MTHFR* variants on sperm traits is important for cattle breeding. For instance, Borgohain *et al*. (2019) investigated fertility-related genes in bovine semen, highlighting their impact on sperm motility and acrosome integrity, while Revanasiddu *et al*. (2019) found that genetic variants in the ZNF280BY gene are significantly associated with semen quality traits in Murrah buffalo bulls. Identifying such relationships could provide novel molecular markers for selecting superior bulls and improving genetic gain in AI programs. This study aims to investigate SNPs in the bovine *MTHFR* gene and examine their correlation with semen quality traits, offering a theoretical foundation for advancing bovine breeding by enhancing sperm quality and supporting breeding bull selection.

MATERIALS AND METHODS

Experiments were conducted at the Key Laboratory of Animal Genetics, Breeding and Reproduction, College of Animal Science, Guizhou University and Semen was collected from the Guizhou Province breeding station, from August 2024 to June 2025. Fifty sexually mature bulls, representing four breeds (Simmental, n=22; Australian Wagyu, n=6; Guanling, n=12; Sinan, n=10), were studied. Bulls were kept under uniform management conditions with an average temperature of 16.0±1.5°C in October (Qianxi City, Bijie, Guizhou).

Semen was collected twice weekly for one month using artificial vagina techniques. Ejaculate volume was measured with temperature-controlled graduated tubes (Minitube International). Sperm quality was assessed using Minitube GmbH's equipment, including sperm density (Sperm Density Meter) and kinematic analysis (CASA system). The CASA system evaluated motility, progressive motility, rapid progressive motility, slow motility, static cells and circular motility, along with velocity parameters (curvilinear, straight-line and average path velocity). Sperm deformity was measured by Giemsa staining. Fresh semen was diluted with glycerol-egg yolk-citrate for freezing and stored in liquid nitrogen before thawing at 38°C for DNA extraction.

Genomic DNA was extracted using the Sperm DNA Isolation Kit (Top9238T, Beijing TopToda Biotechnology). DNA concentration was measured with a NanoDrop™ spectrophotometer (A260/A280 ≥1.8) and quality assessed by agarose gel electrophoresis. DNA samples were cryopreserved at -20°C until genotyping. The bovine *MTHFR* gene reference sequence (NC_037343) was retrieved from NCBI and thirteen primer pairs targeting *MTHFR* coding regions were designed using Primer 3 Plus (Table 1). PCR was performed in 20 μL reactions with 2× ES Taq Master Mix, primers and genomic DNA. Thermal cycling conditions were as follows: denaturation at 94°C for 2 min, 35 cycles of 94°C for 30 s, primer annealing at Tm and extension at 72°C for 30 s; final extension at 72°C for 2 min. PCR products were electrophoresed on 1.5% agarose gels, visualized with a GelDoc XR+ system and purified for Sanger sequencing. Sequence alignment was performed using SeqMan Pro, with SNPs detected based on chromatogram profiles and validated by triplicate sequencing.

The association between *MTHFR* genotypes/haplotypes and semen quality traits was analyzed using SPSS GLM (μ + Gi + Aj + Bk + eijk) with significance at $P<0.05$ and $P<0.01$ (Wijayanti *et al*., 2023). Post-hoc comparisons were made with Tamhane's T2 test. Genotypic distributions and genetic parameters (allele and genotype frequencies, effective alleles and polymorphism content) were computed using Nei's method (modified by Ren *et al*., 2021). Hardy-Weinberg equilibrium was assessed via χ^2 tests and linkage disequilibrium was analyzed using SHEsis. The mRNA secondary structure was predicted with RNAfold webserver, SOPMA and SWISSMODEL. Protein interaction was analyzed *via* STRING12.0 and Cytoscape and genetic structure was visualized with GSDS.

RESULTS AND DISCUSSION

SNP identification of the *MTHFR* gene

Agarose gel electrophoresis (1.5% w/v) of PCR amplicons confirmed specific amplification of target fragments. Bidirectional Sanger sequencing identified four SNPs in the bovine *MTHFR* gene (NC_037343).

SNP1 (g.41883859T > C): Exon 8, synonymous mutation.
SNP2 (g.41884250G > A): Exon 9, synonymous mutation.
SNP3 (g.41884310T > C): Exon 9, synonymous mutation.
SNP4 (g.41886545T > C): Intron 12.

All loci exhibited three genotypes (homozygous major/heterozygous/homozygous minor), validated by chromatogram peak symmetry (Fig 1).

Genetic indices of the *MTHFR* gene

Dominant genotypes at loci g.41883859T > C, g.41884250G > A, g.41884310T > C and g.41886545T > C were TC (0.42), GA (0.42), GG (0.42), TC (0.4) and TC (0.48), respectively. The dominant alleles were T (0.53), G (0.63), T (0.52) and T (0.56). These loci were moderately polymorphic (PIC = 0.25-0.5) (Table 2).

Linkage disequilibrium (LD) analysis

Based on LD criteria established in prior studies (Ardlie *et al*., 2002) LD analysis (Fig 2) revealed strong linkage disequilibrium (D2 > 0.800 and $r^2 > 0.330$) among the four loci.

Haplotype and diplotype analysis

Haplotype analysis identified nine distinct haplotypes and twelve diplotypes (Table 3). The predominant haplotype H1 (T-G-T-T, frequency = 0.50) and diplotype H1H1 (T/G/T/T, frequency = 0.30) emerged as the dominant genetic structures.

Association analysis of *MTHFR* gene SNPs and semen quality

At locus g.41883859T > C, the TT genotype exhibited significantly higher ejaculation volume and HAC but lower sperm density than the CC genotype (P<0.05). The AA genotype at g.41884250G > A showed higher sperm density and progressive motility compared to AG and GG

Table 1. Primer sequence information.

Primers	Primer sequence (5′-3′)	Product length/bp	Annealing temperature/°C	Gene accession number
MTHFR-1	F: CGGAGGGACTTAAGGCTGA R: GCCCCTCTGCCATTTGATGA	313	59	NC_037343.1
MTHFR-2	F: CATCTCTCACCTGACCCTGC R: TTCACCTTGCCTCCATTCCC	377	60	NC_037343.1
MTHFR-3	F: GGAGCCCCTCCTCAGAAAGA R: AAAGCAAGACCCCAGAGAGC	309	60	NC_037343.1
MTHFR-4	F: GCAGACACCAAGTTCAGCAA R: CTCACCTGTGCCTCTCCTTC	311	58	NC_037343.1
MTHFR-5	F: GTCACCACTTCAGCCTGTCC R: CTGAACTTCACCCCAGAGCC	423	60	NC_037343.1
MTHFR-6	F: CCGGCAGCTGGTGAAACTAT R: GACTTCCTGACCCACCCGA	303	60	NC_037343.1
MTHFR-7	F: TGCCTGTCCAGCTTGATGTT R: CGAAAGGCTCCTCCACACTC	302	60	NC_037343.1
MTHFR-8	F: GGGAGTCTGAGTGTGGAGGA R: TCCCTCTGTAAGCCCCTTCC	459	59	NC_037343.1
MTHFR-9	F: AGGCTAGGTCTCTGATGGGG R: TGAGCTCCCTTCCACTTTGAT	404	60	NC_037343.1
MTHFR-10	F: AGCGTCCAGTGTTTCAGAGC R: TTGGGCACTCTGTGATTGCT	303	60	NC_037343.1
MTHFR-11	F: CTGTCTGCACCCCAAGTCTG R: CCGGGCGAGAGGTAAAACAC	308	60	NC_037343.1
MTHFR-12	F: ACGTGCGATTATGCGTACCT R: AGACTCTCAGCTGAGCAGGA	597	60	NC_037343.1
MTHFR-13	F: CGCCTTCCTCATCTCCATCC R: GGTTTCAGTCCAAGGTCCGT	516	60	NC_037343.1

MTHFR: Methylenetetrahydrofolate reductase.

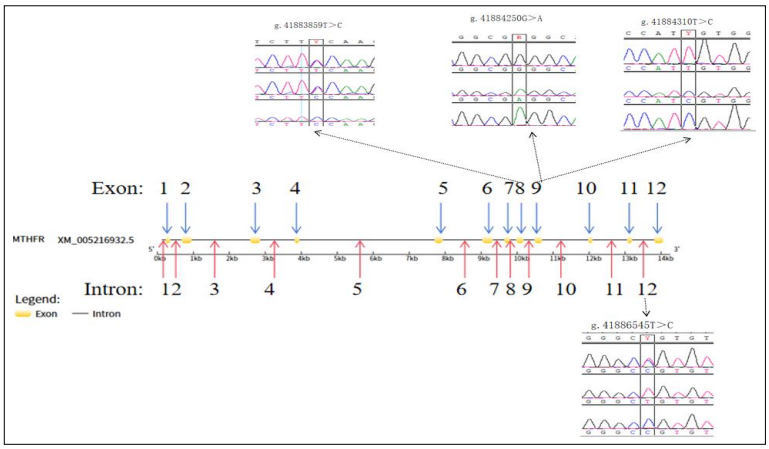


Fig 1: Structural diagram of the bovine MTHFR gene and peak maps of four SNPs.

genotypes (P<0.05). For g.41884310T > C, TC genotypes displayed reduced sperm motility, rapid progressive motility, VCL, VSL and VAP compared to TT (P<0.05). At g.41886545T > C, CC genotypes had lower ejaculation volume and static motility but higher sperm density compared to TT (P<0.05) (Table 4).

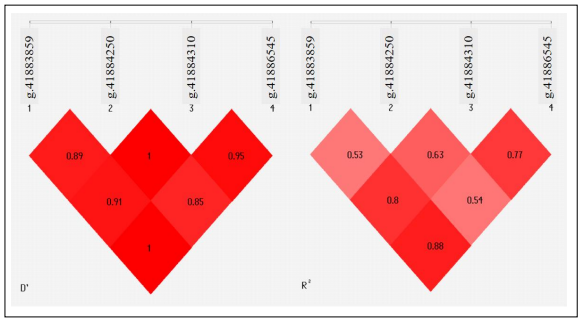


Fig 2: Linkage disequilibrium analysis of four loci in the bovine *MTHFR* gene.

Diplotype analysis

Diplotype H1H1 showed higher ejaculation volume than H2H3 (P<0.05). H2H2 exhibited greater sperm density compared to H1H1 and H1H3 (P<0.05). H1H1 demonstrated superior rapid progressive motility, VSL and VAP compared to H1H2 (P<0.05) (Table 5).

Predicted mRNA secondary structure of *MTHFR* gene

The SNPs g.41883859T > C, g.41884250G > A and g.41884310T > C altered the mRNA secondary structure, with ΔG values of -1,219.55 kcal/mol, -1,219.24 kcal/mol and -1,219.12 kcal/mol, respectively (Fig 3).

Prediction and analysis of MTHFR protein structures

The secondary structure of theMTHFR protein showed random coils (52.06%), α-helix (35.57%) and extended

Table 2: Population genetic characteristics of SNPs in the bovine *MTHFR* gene.

SNPs	Genotype frequency			Allele frequency		He	Ne	PIC	χ^2
g.41883859T>C	TT (16)	TC (21)	CC (13)	T	C	0.498	1.993	0.374	1.232
	0.32	0.42	0.26	0.53	0.47				
g.41884250G>A	GG (21)	GA (21)	AA (8)	G	A	0.466	1.873	0.358	0.491
	0.42	0.42	0.16	0.63	0.37				
g.41884310T>C	TT (16)	TC (20)	CC (14)	T	C	0.499	1.997	0.375	1.974
	0.32	0.4	0.28	0.52	0.48				
g.41886545T>C	TT (16)	TC (24)	CC (10)	T	C	0.493	1.972	0.371	0.034
	0.32	0.48	0.2	0.56	0.44				

Note: Expected heterozygosity (He), expected homozygosity (Ho), effective allele number (Ne), polymorphism information Content (PIC) and P-value of Hardy-Weinberg equilibrium (PHWE). *df* = 2, χ^2 (0.05) = 5.991, χ^2<5.991 to be in Hardy-Weinberg equilibrium.

Table 3: Haplotype and diplotype analysis of the bovine *MTHFR* gene.

Type	Genetype	SNP1	SNP2	SNP3	SNP4	Frequency
Haplotypes	H1 (50)	T	G	T	T	0.50
	H2 (34)	C	A	C	C	0.34
	H3 (9)	C	G	C	C	0.09
	H4 (2)	T	A	C	T	0.02
	H5 (1)	C	A	C	T	0.01
	H6 (1)	C	G	C	T	0.01
	H7 (1)	T	G	C	T	0.01
	H8 (1)	C	G	T	T	0.01
	H9 (1)	C	G	T	C	0.01
Diplotypes	H1H1 (15)	TT	GG	TT	TT	0.30
	H1H2 (14)	TC	GA	TC	TC	0.28
	H1H3 (4)	TC	GG	TC	TC	0.08
	H1H4 (1)	TT	GA	TC	TT	0.02
	H1H9 (1)	TC	GG	TT	TC	0.02
	H2H2 (6)	CC	A A	CC	CC	0.12
	H2H3 (4)	CC	GA	CC	CC	0.08
	H2H4 (1)	TC	A A	CC	TC	0.02
	H2H5 (1)	CC	A A	CC	TC	0.02
	H2H 6(1)	CC	GA	CC	TC	0.02
	H2H7 (1)	TC	GA	CC	TC	0.02
	H3H8 (1)	CC	GG	TC	TC	0.02

strands (12.37%) (Fig 4). The tertiary structure was primarily composed of random coils (Fig 5).

Interaction network analysis of MTHFR protein

MTHFR interacts with key enzymes involved in methylation metabolism, including MTR, BHMT, AMT and TYMS (Fig 6).

Among the four *MTHFR* gene SNP loci identified in this study, g.41883859T>C, g.41884250G>A and g.41884310T>C are synonymous mutations that do not alter amino acid sequences but may impact gene function by modifying mRNA structure or function (He *et al*., 2024). Secondary structure predictions for both wild-type and mutant *MTHFR* mRNA showed alterations in secondary structure and free energy, potentially affecting mRNA stability and translational efficiency, which may lead to altered MTHFR protein expression (Edwards *et al*., 2012). The g.41886545T>C

Table 4: Association analysis between *MTHFR* gene SNP loci and semen quality traits in cattle.

SNPs	Haplotype	Abnormal sperm rate%	Ejaculation volume [ml]	Sperm density [10^6/ml]	Total motility [%]	Progressive motility [%]	Rapid progressive motility [%]	Slow progressive motility [%]
g.41883859T>C	CC	16.54±1.72	5.13±0.6^b	15.30±1.17^a	73.50±2.48	66.99±2.69	34.71±2.28	30.93±2.28
	TC	16.29±1.53	6.09±0.53	13.61±1.04	70.85±2.20	64.02±2.39	34.51±2.02	28.44±2.02
	TT	16.13±1.81	7.83±0.63^a	11.89±1.23^b	73.36±2.60	65.17±2.82	37.65±2.39	26.87±2.39
g.41884250G>A	AA	18.99±2.58	5.79±0.97	15.57±1.73^a	74.73±3.44	68.91±3.73^a	38.37±3.39^a	29.03±3.42
	AG	16.66±1.56	6.37±0.58	13.77±1.05^b	69.21±2.08^b	62.08±2.26^b	32.63±2.05^b	28.45±2.06
	GG	14.65±2.02	6.61±0.76	13.33±1.36^b	75.10±2.71^a	67.75±2.93^a	36.92±2.66	29.32±2.68
g.41884310T>C	CC	16.55±1.67	5.17±0.64^b	15.25±1.12	74.42±2.27	67.79±2.49	36.98±2.20	29.55±2.24
	TC	15.64±1.49	6.41±0.572	12.68±1.01	68.6±2.02^b	61.48±2.22	31.7±1.96^b	28.57±1.99
	TT	15.93±2.24	7.51±0.86^a	13.57±1.51	77.2±3.04^a	68.83±3.33	40.83±2.95^a	27.33±2.99
g.41886545T>C	CC	17.64±2.14	4.97±0.75^b	16.03±1.46^a	71.41±3.11	65.45±3.37	33.09±2.89	31.04±2.84
	TC	15.86±1.30	5.92±0.46	13.53±0.88	71.91±1.89	64.86±2.05	35.53±1.76	28.12±1.72
	TT	16.13±1.80	7.83±0.63^a	11.89±1.22^b	73.36±2.61	65.17±2.83	37.65±2.43	26.87±2.38

SNPs	Haplotype	Non-progressive motility [%]	Static sperm [%]	Circular motility [%]	DSL [μm]	DCL [μm]	DAP [μm]	VCL [μm/s]	VSL [μm/s]
g.41883859T>C	CC	6.50±0.54	26.50±2.48	1.36±0.43	12.4±0.92	27.21±1.20	14.97±0.94	97.40±3.79	47.89±2.53
	TC	6.83±0.48	29.15±2.2	1.08±0.38	12.13±0.82	27.37±1.07	14.81±0.83	97.74±3.36	46.98±2.24
	TT	8.19±0.57	26.64±2.6	0.65±0.45	10.68±0.96	25.45±1.26	13.13±0.99	101.75±3.97	48.09±2.65
g.41884250G>A	AA	5.83±0.79	25.27±3.44	1.52±0.61	13.55±1.31	30.49±1.74	16.55±1.35	103.56±5.61^a	49.83±3.53
	AG	7.13±0.48	30.79±2.08^a	1.00±0.37	11.51±0.79	26.31±1.05	14.07±0.81	94.18±3.39^b	44.93±2.14
	GG	7.35±0.62	24.90±2.71^b	1.51±0.48	11.73±1.03	26.20±1.37	14.35±1.06	100.67±4.41	49.05±2.78
g.41884310T>C	CC	6.63±0.52	25.58±2.27	1.27±0.42	12.42±0.90	27.63±1.20	15.07±0.92	101.22±3.68	49.52±2.33
	TC	7.12±0.46	31.41±2.02^a	1.21±0.37	11.88±0.80	27.15±1.06	14.57±0.82	92.50±3.28^b	43.79±2.08^b
	TT	8.37±0.70	22.80±3.04^b	0.67±0.56	10.65±1.20	24.81±1.60	12.99±1.24	108.66±4.93^a	52.47±3.12^a
g.41886545T>C	CC	5.96±0.66^b	28.59±3.11	1.33±0.54	12.62±1.15	28.20±1.48	15.38±1.17	94.78±4.81	45.59±3.18
	TC	7.05±0.4	28.09±1.89	1.21±0.33	12.1±0.69	27.17±0.90	14.76±0.71	99.07±2.92	47.97±1.93
	TT	8.19±0.55^a	26.64±2.61	0.65±0.45	10.68±0.96	25.45±1.24	13.13±0.98	101.75±4.04	48.09±2.66

SNPs	Haplotype	VAP [μm/s]	BCF [Hz]	ALH [μm]	HAC	STR (=VSL/VAP)	LIN (=VSL/VCL)	WOB (=VAP/VCL)
g.41883859T>C	CC	55.37±2.55	12.80±0.75	0.94±0.03	0.25±0.01	0.86±0.01	0.49±0.02	0.57±0.01
	TC	54.93±2.26	12.16±0.67	0.96±0.03	0.26±0.01^b	0.85±0.01	0.48±0.01	0.56±0.01
	TT	55.84±2.67	10.85±0.79	0.99±0.03	0.28±0.02^a	0.86±0.01	0.47±0.02	0.55±0.01
g.41884250G>A	AA	58.40±3.61^a	12.41±1.11	1.05±0.05	0.27±0.02	0.85±0.01	0.48±0.02	0.57±0.02
	AG	52.41±2.18^b	12.13±0.67	0.92±0.03	0.25±0.01	0.85±0.01	0.48±0.01	0.56±0.01
	GG	57.05±2.84^b	12.10±0.87	0.95±0.04	0.27±0.02	0.86±0.01	0.49±0.02	0.57±0.01
g.41884310T>C	CC	57.36±2.42	12.48±0.73	0.98±0.03	0.27±0.01	0.86±0.01	0.49±0.01	0.57±0.01
	TC	51.52±2.16^a	12.02±0.65	0.92±0.03^b	0.24±0.01^b	0.85±0.01	0.47±0.01	0.56±0.01
	TT	60.33±3.24^b	10.87±0.97	1.02±0.04^a	0.31±0.01^a	0.87±0.01	0.48±0.01	0.56±0.01
g.41886545T>C	CC	53.35±3.23	12.84±0.94	0.95±0.04	0.25±0.02^b	0.85±0.01	0.48±0.02	0.56±0.02
	TC	55.89±1.96	12.17±0.57	0.96±0.02	0.26±0.01	0.86±0.01	0.49±0.01	0.57±0.01
	TT	55.84±2.71	10.85±0.79	0.99±0.03	0.28±0.02^a	0.86±0.01	0.47±0.02	0.55±0.01

Different lowercase letters (a, b) indicate significant differences (P<0.05) among genotypes at the same SNP locus.

variant is an intronic mutation that may affect gene transcription or translation by inducing alternative splicing or interacting with regulatory elements (Luo *et al*., 2019). Gao *et al*., (2014) reported that an intronic SNP (g.480C>T) in TNP2 was associated with seminal parameters in Holstein bulls (Gao *et al*., 2014). This could result from linkage disequilibrium (LD) between the SNP and neighboring functional variants, suggesting haplotype-mediated effects (Raza *et al*., 2020). LD analysis showed strong associations between the four loci, suggesting that these loci may co-evolve within a shared haplotype. Population genetic analysis revealed intermediate polymorphism levels (0.25<PIC<0.5) and Hardy-Weinberg equilibrium (P>0.05), indicating genetic stability and potential for selective breeding applications.

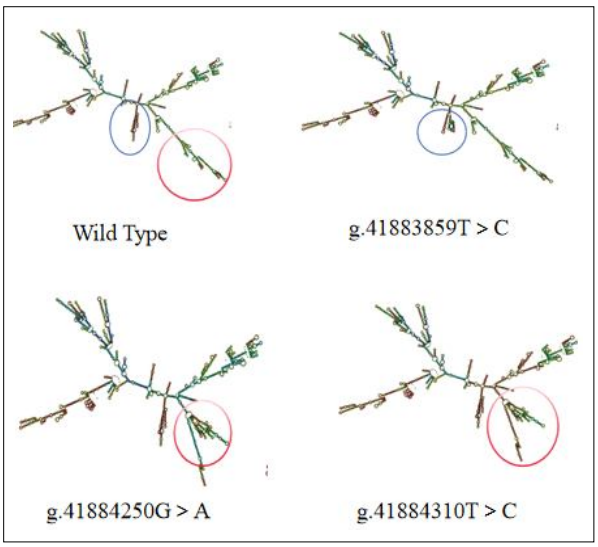


Fig 3: Prediction of mRNA secondary structures for g.41883859T>C; g.41884250G>A; g.41884310T>C of the *MTHFR* gene and the wild type.

The association analysis showed significant site specificity in the effect of *MTHFR* polymorphisms on semen quality. Specifically, sperm density was lower in individuals with the TT genotype at g.41883859T>C and g.41886545T>C compared to CC genotypes, similar to human *MTHFR* C677T genotypes (Ebisch *et al*., 2003). Adequate intake of vitamins B9 and B12 can improve semen parameters in males with the *MTHFR* polymorphism, especially in T allele carriers at C677T (Xie *et al*., 2019). Rebolledo *et al*., (2024) used CRISPR/Cas9 to create *MTHFR* C677C (CC) and C677T (TT) mouse models, identifying 360 differentially methylated regions (DMRs) in sperm from *MTHFR* 677TT mice, which were mostly hypomethylated. Folic acid supplementation reversed DNA methylation abnormalities, suggesting that dietary folic acid and vitamin B12 supplementation may improve sperm quality in cattle.

Individuals with the AA genotype at g.41884250G>A exhibited higher sperm density and enhanced rapid

Table 5: Association analysis between *MTHFR* gene diplotypes and semen quality in cattle.

Diplotypes	Abnormal sperm rate %	Ejaculation volume [ml]	Sperm density [10^6/ml]	Total motility [%]	Progressive motility [%]	Rapid progressive motility [%]	Slow progressive motility [%]
H2H2	18.47±3.34	5.69±1.03	17.2±1.64^a	76.82±3.89	70.91±4.30	33.60±3.60	35.21±4.08
H2H3	20.21±4.07	4.33±1.25^b	14.84±2.00	66.72±4.73	61.11±5.23	30.75±4.38	29.77±4.97
H1H2	16.72±2.34	6.41±0.72	14.13±1.15	66.26±2.72	58.62±3.01	29.55±2.52^b	28.04±2.86
H1H3	15.98±3.86	5.50±1.19	11.51±1.89^b	74.56±4.49	68.65±4.96	35.28±4.16	31.84±4.71
H1H1	15.61±2.42	7.28±0.74^a	12.95±1.19^b	75.98±2.81	68.08±3.11	38.18±2.61^a	29.16±2.95

Diplotypes	Non-progressive motility [%]	Static sperm [%]	Circular motility [%]	DSL [μm]	DCL [μm]	DAP [μm]	VCL [μm/s]	VSL [μm/s]
H2H2	5.91±0.79	23.18±3.89	2.11±0.67	13.13±1.44	28.29±1.63	15.84±1.45	97.42±5.64	48.53±3.70
H2H3	5.61±0.96	33.28±4.73	0.60±0.81	12.18±1.76	27.95±1.98	14.96±1.76	89.36±6.86	41.72±4.50
H1H2	7.64±0.55	33.75±2.72	1.04±0.47	11.14±1.01	25.96±1.14	13.69±1.01	90.28±3.95^b	42.24±2.59
H1H3	5.91±0.91	25.44±4.49	1.54±0.77	14.18±1.67	29.24±1.88	17.00±1.67	99.15±6.51	50.65±4.27
H1H1	7.9±0.57	24.02±2.81	0.74±0.48	10.98±1.04	24.86±1.18	13.31±1.05	103.78±4.08^a	50.82±2.68

Diplotypes	VAP [μm/s]	BCF [Hz]	ALH [μm]	HAC	STR (=VSL/VAP)	LIN (=VSL/VCL)	WOB (=VAP/VCL)
H2H2	56.29±3.73	12.75±1.19	0.98±0.05	0.25±0.02	0.86±0.01	0.50±0.02	0.58±0.02
H2H3	49.32±4.54	13.12±1.44	0.91±0.06	0.22±0.03^b	0.84±0.02	0.47±0.03	0.55±0.02
H1H2	49.64±2.61^b	11.47±0.83	0.91±0.04	0.24±0.02	0.85±0.01	0.47±0.02	0.55±0.01
H1H3	58.85±4.31	13.69±1.37	0.94±0.06	0.24±0.03	0.86±0.01	0.51±0.03	0.60±0.02
H1H1	58.39±2.7^a	11.29±0.86	0.98±0.04	0.29±0.02^a	0.87±0.01	0.49±0.02	0.57±0.01

Different lowercase letters (a, b) indicate significant differences (P<0.05) among genotypes at the same SNP locus.

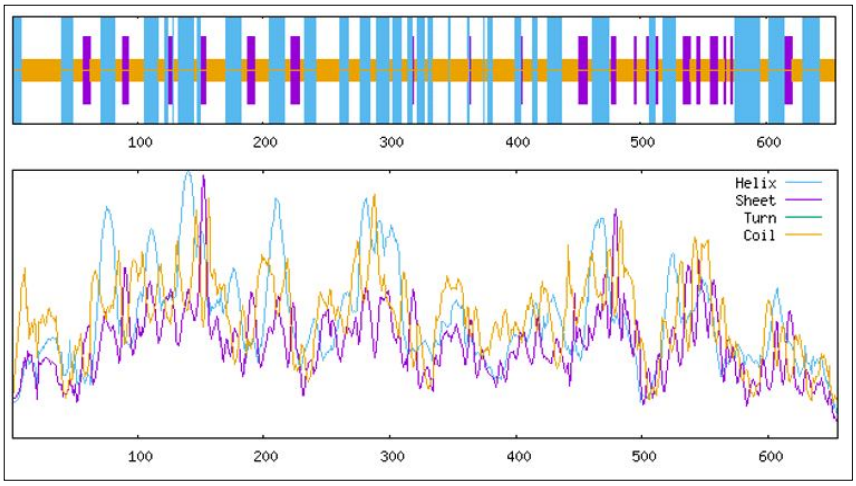


Fig 4: Secondary structure of the bovine MTHFR protein.

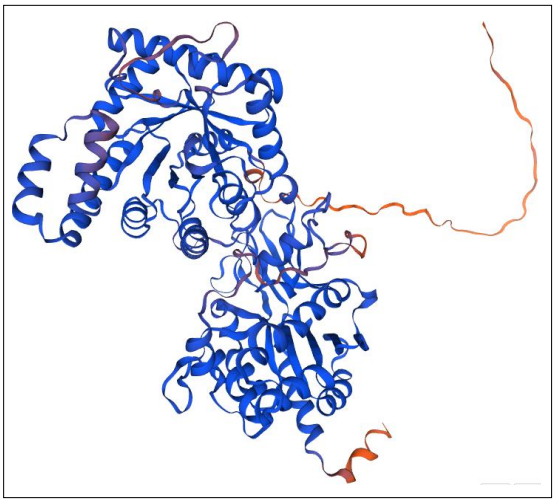


Fig 5: Tertiary structure of the bovine MTHFR protein.

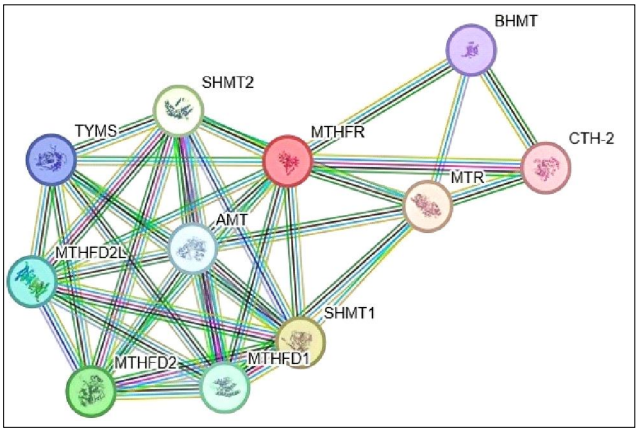


Fig 6: Interaction map of bovine MTHFR protein.

progressive motility. H1H1 diplotypes showed significantly higher rapid progressive motility, straight-line velocity (VSL) and average path velocity (VAP) compared to H1H2 diplotypes (P<0.05). Studies show that sperm with rapid progressive motility have higher fertilization success in AI (Hidalgo *et al*., 2021). Evaluating sperm motility parameters like VAP and VSL can better predict post-thaw semen quality than traditional motility measures (% mot) or progressive motility (% prog), making these parameters effective for pre-freezing semen screening (Defoin *et al*., 2008). Selecting higher-quality sperm improves fertility and reproductive outcomes in breeding programs (Carvalho *et al*., 2023). Therefore, molecular markers for the AA genotype at g.41884250G>A and the H1H1 haplotype should be used for selecting breeding bulls and pre-screening semen before cryopreservation.

Structural predictions showed that the bovineMTHFR protein adopts a random coil conformation. Protein interaction analysis identified interactions betweenMTHFR and enzymes involved in methylation metabolism, such as MTR and BHMT. *MTHFR* regulates methyl donor production (e.g., SAM), playing a role in epigenetic processes like sperm DNA methylation and histone modification, which are crucial for spermatogenesis and genomic stability (Osunkalu *et al*., 2020).

CONCLUSION

Specific polymorphisms in the bovine *MTHFR* gene, particularly the AA genotype at the g.41884250G>A locus and the H1H1 haplotype, are significantly associated with superior semen quality traits. These include higher sperm density, improved motility and parameters predictive of better cryopreservation outcomes and fertility. In contrast, the TT genotype at the g.41883859T>C and g.41886545T>C loci is linked to lower sperm density. These findings offer valuable molecular markers for bull selection and pre-screening semen in breeding programs.

ACKNOWLEDGEMENT

This work was supported by the Guizhou Provincial Special Fund for Science and Technology Achievements Transformation ([2024] 092); Guizhou Provincial Key

Laboratory of Livestock and Poultry Genetic Resources Innovation and Utilization (ZSYS[2025]034); Guizhou Province Outstanding Young Scientific and Technological Talent Program (YQK[2023]020), Guizhou academy of agricultural sciences ("JBGS"[2024] No. 02) and Guizhou Province Cattle Industry Technology System (GZRNCYJSTX-04).

Disclaimers

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

Informed consent

This study was approved by the Guizhou University Experimental Animal Ethics Committee (No. EAE-GZU-2023-E085) and all animal procedures and handling techniques were approved by the University Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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