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Bovine TMEM95 gene: Polymorphisms detecting in five Chinese indigenous cattle breeds and their association with growth traits

Xian Guo [a,b,1], Sihuan Zhang [c,1], Han Yang [c], Jie Pei [a,b], Xiaoyun Wu [a,b], Pengjia Bao [a,b], Chunnian Liang [a,b], Lin Xiong [a,b], Min Chu [a,b], Xianyong Lan [c,*], Ping Yan [a,b,*]

[a] Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu 730050, China
[b] Key Laboratory of Yak Breeding Engineering of Gansu Province, Lanzhou, Gansu 730050, China
[c] College of Animal Science and Technology, Northwest A&F University, Shaanxi Key Laboratory of Molecular Biology for Agriculture, Yangling, Shaanxi 712100, China

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ABSTRACT

Background: Transmembrane protein 95 (*TMEM95*) plays a role in male fertility. Previous studies showed that genes with a significant impact on reproductive traits can also affect the growth traits of livestock. Thus, we speculated that the genetic variation of *TMEM95* gene may have effects on growth traits of cattle.

Results: Two SNPs were genotyped. The rs136174626 and rs41904693 were in the intron 4 and 3′-untranslated region, respectively. The linkage disequilibrium analysis illustrated that these two loci were not linked. The rs136174626 was associated with six growth traits of Nanyang cattle, four traits of Luxi cattle, and three traits of Ji'an cattle. For rs41904693 locus, the GG individuals had greater body height and abdominal girth in Ji' an cattle than TT and TG individuals. In Jinnan cattle, GG and TT individuals had greater body height, height at hip cross, body length, and heart girth than TG individuals. The potential splice site prediction results suggest that the rs136174626 may influence the splicing efficiency of *TMEM95*, and the miRNA binding site prediction results showed that the rs41904693 may influence the expression of *TMEM95* by affecting the binding efficiency of Bta-miR-1584 and *TMEM95* 3′-UTR.

Conclusions: The findings of the study suggested that the two SNPs in *TMEM95* could be a reliable basis for molecular breeding in cattle.

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1. Introduction

Beef is rich in protein and amino acids, which can improve people's physical quality and disease resistance, thus, it is favored by different individuals. Beef is the third most consumed meat worldwide, accounting for about 25% of the meat market, lagging behind pork and poultry, and the proportion of beef in the meat market is increasing year-by-year. In order to improve the production level of beef cattle, all countries are selecting great breeds. Large beef cattle breed grow fast, thus, it can be slaughtered at a young age when the meat is tender. Therefore, the body size of beef cattle has caused widespread interest among breeders.

China has abundant resources of cattle breeds, which can be used as beef cattle breeding [1]. At present, molecular breeding methods are gradually applied to animal breeding, such as marker assisted selection (MAS), gene editing breeding, and genomic selection breeding [2,3,4]. Molecular breeding is based on important traits associated functional genes and genetic variations [5]. Therefore, it is highly essential to identify the crucial genetic variations and functional genes associating with cattle growth traits.

Transmembrane protein 95 (*TMEM95*) gene is one of the members of the TMEM family, which is located on *Bos taurus* chromosome 19 [6]. In 2014, a nonsense mutation in Fleckvieh cattle *TMEM95* gene (c.483C > A, p.Cys161X, rs378652941) was found to be associated with idiopathic male subfertility, while this mutation was not detected in 13 Chinese indigenous cattle breeds [7,8]. Further studies unveiled that *TMEM95* is located on the acrosomal

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* Corresponding authors.
E-mail addresses: lanxianyong79@nwsuaf.edu.cn (X. Lan), yanping@caas.cn (P. Yan).
[1] These authors contributed equally to this work.

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membrane of the sperm head, and the c.483C > A mutation reduces fertility by influencing the interaction of sperm the oocyte vestments [9,10,11,12,13].

Previous studies have demonstrated that genes having a significant impact on reproductive traits can also affect the growth traits of livestock, such as, sperm-associated antigen 17 (*SPAG17*) and growth differentiation factor 9 (*GDF9*) [14,15]. For instance, Pausch et al. used genome-wide association studies (GWAS) that found that the coding variant in acyl-CoA dehydrogenase very long chain (*ACADVL*) gene (p.Pro236Thr) was such a candidate functional mutation, causing male subfertility (it was identified together with *TMEM95* gene), while other studies pointed out that this mutation (p.Pro236Thr) was associated with growth traits of beef cattle [7,16]. Therefore, a mutation in *TMEM95* gene may have an influence on growth traits of beef cattle. In the present study, we detected genetic variations in Chinese cattle *TMEM95* gene and analyzed their associations with growth traits of beef cattle, in order to lay the foundation for cattle breeding.

2. Materials and methods

2.1. Ethics statement

The present research was approved by the Faculty Animal Policy and Welfare Committee of Northwest A&F University (Yangling, China). The experimental process was consistent with protocols of international guides for the ethical use of animals in research.

2.2. Samples collection

A total of 830 female cattle samples from 5 Chinese indigenous cattle breeds were collected, including Qinchuan cattle (n = 321, Shaanxi Province), Nanyang cattle (n = 221, Henan Province), Jinnan cattle (n = 195, Shanxi Province), Ji' an cattle (n = 63, Jiangxi Province), and Luxi cattle (n = 30, Shandong Province) [8]. All individuals were randomly selected, unrelated (through at least three generations), and healthy. The growth traits of the cattle were measured, including body weight, body height, body length, heart girth, hip width, daily gain, circumference of cannon bone, abdominal girth, height at hip cross, chest width, and chest depth. A total of 590 cattle have the data of growth traits (Table 1). The genomic DNA was isolated from ear tissues using phenol–chloroform DNA extraction method [17]. The quality of DNA was measured by the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Primer design, genetic variation screening, and genotyping

Based on the sequence of bovine *TMEM95* gene in National Center for Biotechnology Information database (NCBI Reference Sequence: NC_037346.1), a total of 5 pairs of primers were designed to screen novel genetic variations in cattle *TMEM95* gene, which covers all regions of cattle *TMEM95* gene (Screen primers 1–5 in Table 2, Fig. 1). After PCR amplification using DNA pool as template (contain 30 DNA samples), Sanger sequencing of PCR product (Sangon Biotech Co., Ltd., Shanghai, China) and BLAST were used to screen the novel variations. Then, Tetra-primer amplification refractory mutation system-PCR (T-ARMS-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), and forced PCR-RFLP methods were used to genotype individuals [16]. The primers of T-ARMS-PCR were designed using Primer1 website (http://primer1.soton.ac.uk/primer1.html) (Table 2) [18,19]. The other primers used in the present study were designed via Primer Premier software (version 5.0) (Table 2).

The rs136174626 was genotyped using forced PCR-RFLP due to the lack of naturally recognizable restriction enzyme cutting site. The rs41904693 was genotyped using PCR-RFLP method. The primer pairs of rs136174626-F/R and rs41904693-F/R were used to amplify the fragments containing rs136174626 and rs41904693 mutation loci (Table 2). After PCR amplification, the PCR products were digested by restriction enzymes *Taq*I (*Tth*HB8I) and *Vpa*K11BI (*Ava*II), respectively. Afterwards, the genotypes of the individuals were distinguished according to the agarose gel electrophoresis of enzyme-digested products. The product size and the corresponding genotypes are listed in Table 2. The PCR reactions and PCR programs were as same as our previous research [8].

2.4. Statistical analysis

Genotypic frequencies and allelic frequencies were calculated directly. Population genetic parameters (gene homozygosity, *Ho*; effective allele numbers, *Ne*; polymorphism information content, *PIC*) and Hardy-Weinberg equilibrium (*HWE*) were computed using MSR website (http://www.msrcall.com/Gdicall.aspx). The linkage disequilibrium and haplotypes were calculated using SHEsis (http://analysis.bio-x.cn/myAnalysis.php) [20]. The relationship between genotypes and growth traits of cattle were analyzed using the basic general linear model: $Y_{ij} = \mu + G_{ij} + e$, where Y_{ij} represents the measured data of growth traits, μ is the mean for each trait, G_{ij} denotes the effect of genotype, and e is the random error [8,21,22,23]. The association between genotypes and growth traits was assessed using SPSS 19.0 software (IBM, Armonk, NY, USA) via One-Way ANOVA followed by Post Hoc Multiple Comparisons (three genotypes) or Independent-Samples T Test (two genotypes) [21].

2.5. Functional prediction of genetic variation

Ensemble database (http://www.ensembl.org/index.html) was used to analyze the conservation of the genetic variations locus among species. ESE Finder was applied to predict the potential splice site [24]. RegRNA 2.0 web server was utilized for identifying regulatory RNA motifs and functional sites (such as miRNA binding sites) [25,26].

3. Results

3.1. Identification of genetic variations in cattle TMEM95 gene

Five PCR products were obtained after the PCR amplification using screen primer pairs (Fig. 1). Then, the PCR products were sent to a company for sequencing. According to the results of BLAST and sequence analysis, seven variations were revealed in cattle *TMEM95* gene, including intron variants NC_037346:g.27056355 G-A, g.27056359 G-A, g.27056493 C-A (rs136174626), and downstream gene variants g.27057161 T-G (rs41904693), g.27057175 G-T, g.27057314 G-T, and g.27057316 A-T. Among these variations, rs136174626 and rs41904693 can be searched in the Ensemble database. Considering the conservatism and applicability of the variations, this study genotyped the genotypes of the rs136174626 and rs41904693 in the studied populations. The rs136174626 and rs41904693 were in the intron 4 and 3′-untranslated region, respectively (Fig. 1). In the Ensemble database, these two SNPs have been identified in the Iranian *Bos taurus* from the NextGen Project. In the Ensemble database, rs136174626 was identified in seven Iranian Bos taurus. The frequencies (count) of AA, AC, and CC were 0.286 (2), 0.143 (1), and 0.571 (4), respectively. The minor allele frequency (MAF) of rs136174626 in the Iranian Bos taurus was 0.357. The rs41904693 was identified in eight

Table 1
The breeds, sample size, and descriptive summaries for the measurements of growth traits of the cattle used in this study.

Traits/Breeds (number)	Body measurement (Mean ± SD)			
	Nanyang cattle (*N* = 105)			
Age	6-month old	12-month old	18-month old	24-month old
Body weight (kg)	160.27 ± 18.91	223.24 ± 22.96	298.35 ± 30.89	368.04 ± 39.72
Body height (cm)	106.15 ± 5.09	114.12 ± 3.84	121.38 ± 6.48	126.42 ± 4.30
Body length (cm)	105.69 ± 5.75	117.11 ± 7.38	129.45 ± 7.12	138.08 ± 7.49
Heart girth (cm)	128.97 ± 7.27	141.39 ± 7.72	156.08 ± 8.38	168.94 ± 9.77
Hip width (cm)	18.33 ± 1.34	20.71 ± 1.61	23.15 ± 1.82	25.30 ± 2.28
Daily gain (kg)	0.72 ± 0.10	0.35 ± 0.12	0.42 ± 0.17	0.39 ± 0.11
	Luxi (*N* = 25)	Ji' an (*N* = 79)	Jinnan (*N* = 181)	Qinchuan (*N* = 200)
Body weight (kg)	434.64 ± 82.18	256.50 ± 53.37	/	
Body height (cm)	133.28 ± 5.94	107.22 ± 6.25	128.61 ± 6.33	129.71 ± 6.83
Body length (cm)	150.4 ± 12.70	119.08 ± 8.78	151.91 ± 11.45	137.45 ± 12.48
Heart girth (cm)	187.28 ± 12.22	151.61 ± 11.61	184.94 ± 14.85	179.44 ± 16.95
Circumference of cannon bone (cm)	18.44 ± 0.51	14.01 ± 0.74	/	/
Abdominal girth (cm)	213.24 ± 18.29	185.75 ± 15.62	/	/
Height at hip cross (cm)	/	107.70 ± 13.16	131.25 ± 7.32	/
Chest width (cm)	/	/	/	38.53 ± 5.36
Chest depth (cm)	/	/	/	64.50 ± 6.61

Table 2
PCR primer sequences of cattle *TMEM95* gene.

Primers	Primer sequences (5′–3′)	Note (Product size)
Screen-F1	CGAAGTGGAGTACCGCAGAC	Promoter region (760 bp)
Screen-R1	CCTCAGTGGTGGCTTAGGG	
Screen-F2	CTCAACTTAGCAGGACTATGGAA	Promoter, Exon1 and partial Exon 2
Screen-R2	GGCAAAGGCCGAGAATGT	(1398 bp)
Screen-F3	CCTGGACATTCTCGGCCTTT	Exon2 to Exon5 (900 bp)
Screen-R3	GGCCAACAGTACACCTCGAA	
Screen-F4	TGGAGGTGAACCATCCCTG	Intron3 to Exon6 (1396 bp)
Screen-R4	GGCCATTTGACAGCACGTTT	
Screen-F5	TCACCACCACTCCCACATC	Exon6 and gene downstream (861 bp)
Screen-R5	AACCCACTCCAGTATTCTTGC	
rs136174626-F	GTGAGTAAGAAAGGGAAGGGGTCG	Forced-PCR-RFLP (171 bp) *Taq* I (*Tth*HB8I)
rs136174626-R	CCTGGCAGGTTGAACAGTTG	CC = 22 + 149 bp: AA = 171 bp;
		AC = 171 + 22 + 149 bp.
rs41904693-F	TCTTTGTGTGCGGAACTGC	PCR-RFLP (401 bp) *Vpa*K11BI (*Ava*II)
rs41904693-R	ACCATCTGACACTGGGACTA	TT = 271 + 130 bp; GT = 271 + 198 + 72 + 130 bp; GG = 198 + 72 + 130 bp.
rs136174626-inner F	AGGGCGGGGGACAGCCAGACCCTTGGT	Product size for T allele: 188 bp
rs136174626-inner R	TCTGGACGATTGAACCACTTGGGGCTGAAC	Product size for G allele: 249 bp
rs136174626-outer F	ATCCTTCCTCCTGCCCTCGTGGCCCTTC	Product size of two outer primers: 380 bp
rs136174626-outer R	TATTTTCCTGGCACGGGGACTGCCCCTG	
rs41904693-inner F	TGCCGTGAGTAAGAAAGGGAAGGGGTGGC	Product size for C allele: 202 bp
rs41904693-inner R	CAAGTGCCCCAAGCCCCCGGCTTCCT	Product size for A allele: 156 bp
rs41904693-outer F	GGACCAAGGGGTGCCTAGGTCCTTGGGC	Product size of two outer primers: 303 bp
rs41904693-outer R	TCGGGGCCAACAGTACACCTCGAAGCCC	

Note: NCBI Reference Sequence: NC_037346.1.

Iranian Bos taurus. The frequencies (count) of TT, GG and GT were 0.375 (3), 0.500 (4), and 0.125 (1), respectively. The MAF of rs41904693 in the Iranian *Bos taurus* was 0.438. For rs136174626 locus, 808 individuals were genotyped, while for rs41904693 locus, 307 individuals were genotyped. Because the efficiency of PCR amplification and restriction enzymes were different, the numbers of the genotyped individuals for rs136174626 and rs41904693 were different. We attempted to identify the genotypes of individuals using T-ARMS-PCR and PCR-RFLP methods, whereas both rs136174626 and rs41904693 loci failed to be genotyped using T-ARMS-PCR. Thus, rs136174626 and rs41904693 were genotyped using forced PCR-RFLP and PCR-RFLP methods, respectively.

3.2. Population genetic parameters analysis

In the present study, both rs136174626 and rs41904693 had three genotypes (rs136174626: AA/AC/CC; rs41904693: TT/TG/GG). However, the genotype GG of rs41904693 locus was not detected in Qinchuan, Nanyang, and Luxi cattle (Table 3). After genotyping, the genetic parameters were calculated (Table 3). For rs136174626 locus, the MAF was 0.276 in the detected populations. The frequencies of AA (0.359 ~ 0.530) and AC (0.367 ~ 0.482) genotypes were close to each other, and those frequencies were higher than the frequency of CC genotype (0.068 ~ 0.159) in Qinchuan, Nanyang, and Jinnan cattle. The three genotypes were distributed uniformity in Luxi cattle, and their frequencies were 0.316, 0.368, and 0.316. In Ji' an cattle, the frequency of AC was up to 0.711, while the frequency of CC was only 0.058. The uneven distribution caused this locus to be seriously deviated from the Hardy-Weinberg equilibrium in Ji' an cattle population. For rs41904693, the MAF was 0.133 in the detected populations. The TT was the main genotype and T was the main allele in the detected population. For the rs136174626 and rs41904693, the *Ho* was greater than 0.5 in the detected populations, indicating that the percentage of individuals with homozygous genotypes was higher than that of the heterozygous genotype. The *Ne* of the populations for rs136174626 were close to 2, which demonstrated that the alleles of rs136174626 were evenly distributed in the detected

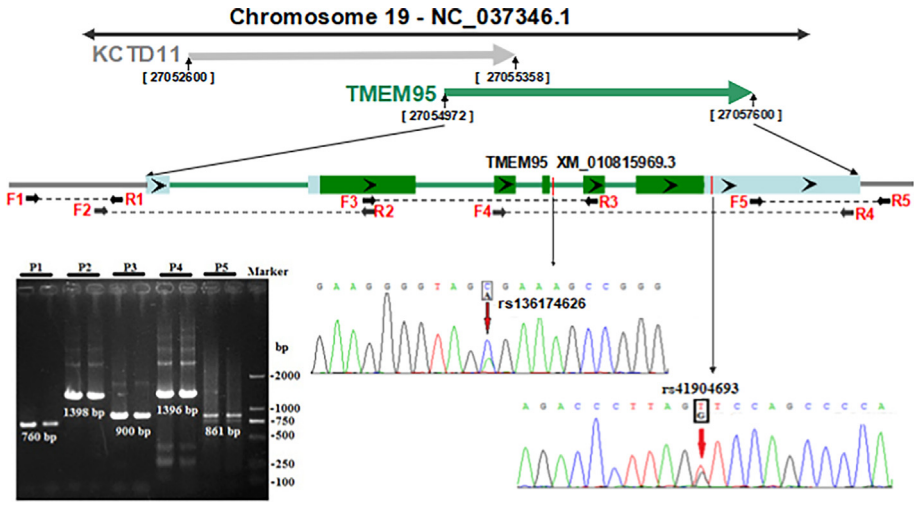

Fig. 1. Bovine *TMEM95* gene structure diagram, variations screening electrophoretogram and sequencing maps of genetic variations. P1–P5 represented the five screening primer pairs. The electrophoretic bands of different lengths were amplified by primers P1–P5 and the same bands represented two repetitions. The sequencing maps showed the two genetic variations genotyped in this study, rs136174626 and rs41904693 in *TMEM95* gene.

Table 3
Genotypic and allelic frequencies and genetic parameters of rs136174626 and rs41904693 in cattle.

Loci/Breeds (Numbers)	Genotypic frequencies			Allelic frequencies		Genetic parameters [a]			HWE [b]
rs136174626	AA	AC	CC	A	C	*Ho*	*Ne*	*PIC*	*P*-value
Qinchuan (*n* = 321)	0.530	0.367	0.103	0.713	0.287	0.591	1.692	0.325	0.070
Nanyang (*n* = 221)	0.516	0.416	0.068	0.724	0.276	0.600	1.666	0.320	0.536
Luxi (*n* = 19)	0.316	0.368	0.316	0.500	0.500	0.500	2.000	0.375	0.251
Ji' an (*n* = 52)	0.231	0.711	0.058	0.587	0.413	0.515	1.942	0.367	0.00075
Jinnan (*n* = 195)	0.359	0.482	0.159	0.600	0.400	0.520	1.923	0.365	0.952
rs41904693	TT	TG	GG	T	G	*Ho*	*Ne*	*PIC*	*P*-value
Qinchuan (*n* = 30)	0.733	0.267	/	0.867	0.133	0.769	1.301	0.204	0.399
Nanyang (*n* = 69)	0.507	0.493	/	0.754	0.246	0.629	1.591	0.302	0.007
Luxi (*n* = 30)	0.667	0.333	/	0.833	0.167	0.722	1.385	0.239	0.270
Ji' an (*n* = 63)	0.413	0.429	0.158	0.627	0.373	0.532	1.879	0.358	0.506
Jinnan (*n* = 115)	0.739	0.226	0.035	0.852	0.148	0.748	1.337	0.220	0.271

Note: [a]*Ho*, gene homozygosity; *Ne*, effective allele numbers; *PIC*, polymorphism information content. [b]*HWE*, Hardy-Weinberg equilibrium.

populations. The *PIC* values showed that the rs136174626 and rs41904693 belonged to medium (0.25 < *PIC* < 0.5) or low polymorphism (*PIC* < 0.25) in the detected populations (Table 3).

3.3. Analysis of linkage disequilibrium and haplotypes

The results of linkage disequilibrium analysis are mainly represented by D' and r^2 values. Compared with D', r^2 is less affected by sample size and allele frequency, thus, r^2 can better reflect the relationship between the two loci. When $r^2 = 0$, there was no association between the two loci. When $r^2 > 0.33$, an association was noted between the two loci. In the current study, the r^2 ranged from 0.027 to 0.126 (Table 4). Hence, these two loci were not linked in the analyzed populations.

Table 4
Linkage disequilibrium parameters (D' and r^2) between rs136174626 and rs41904693 in cattle.

Breeds	D' value	r^2 value
Qinchuan	0.685	0.036
Nanyang	0.225	0.027
Luxi	0.514	0.040
Ji' an	0.710	0.235
Jinnan	1.000	0.126

3.4. Effects of the SNPs on growth traits of cattle

The results of association analysis revealed that the genotypes of rs136174626 and rs41904693 were significantly associated with growth traits of cattle in some cattle breeds (Fig. 2, Fig. 3, Table 5, Table 6, insignificant data were not shown). For rs136174626 locus, the genotypes were associated with the body weight, body height, body length, heart girth, hip width, and weight daily gain of Nanyang cattle; body weight, body height, body length, and heart girth of Luxi cattle; body height and body length of Ji' an cattle ($P < 0.05$ or $P < 0.01$, Table 5). The individuals with CC and AC genotypes had significantly greater body weight, body height, and body length compared with individuals with AA genotype in Nanyang and Luxi cattle (Table 5, Fig. 2). For rs41904693 locus, the genotypes were found to be associated with the body height and abdominal girth of Ji' an cattle; the body height, body length, height at hip cross, and heart girth of Jinnan cattle ($P < 0.05$ or $P < 0.01$, Table 6). In Ji' an cattle, the GG individuals had greater body height and abdominal girth than TT and TG individuals. In Jinnan cattle, GG and TT individuals had greater body height, height at hip cross, body length, and heart girth than TG individuals (Table 6, Fig. 3).

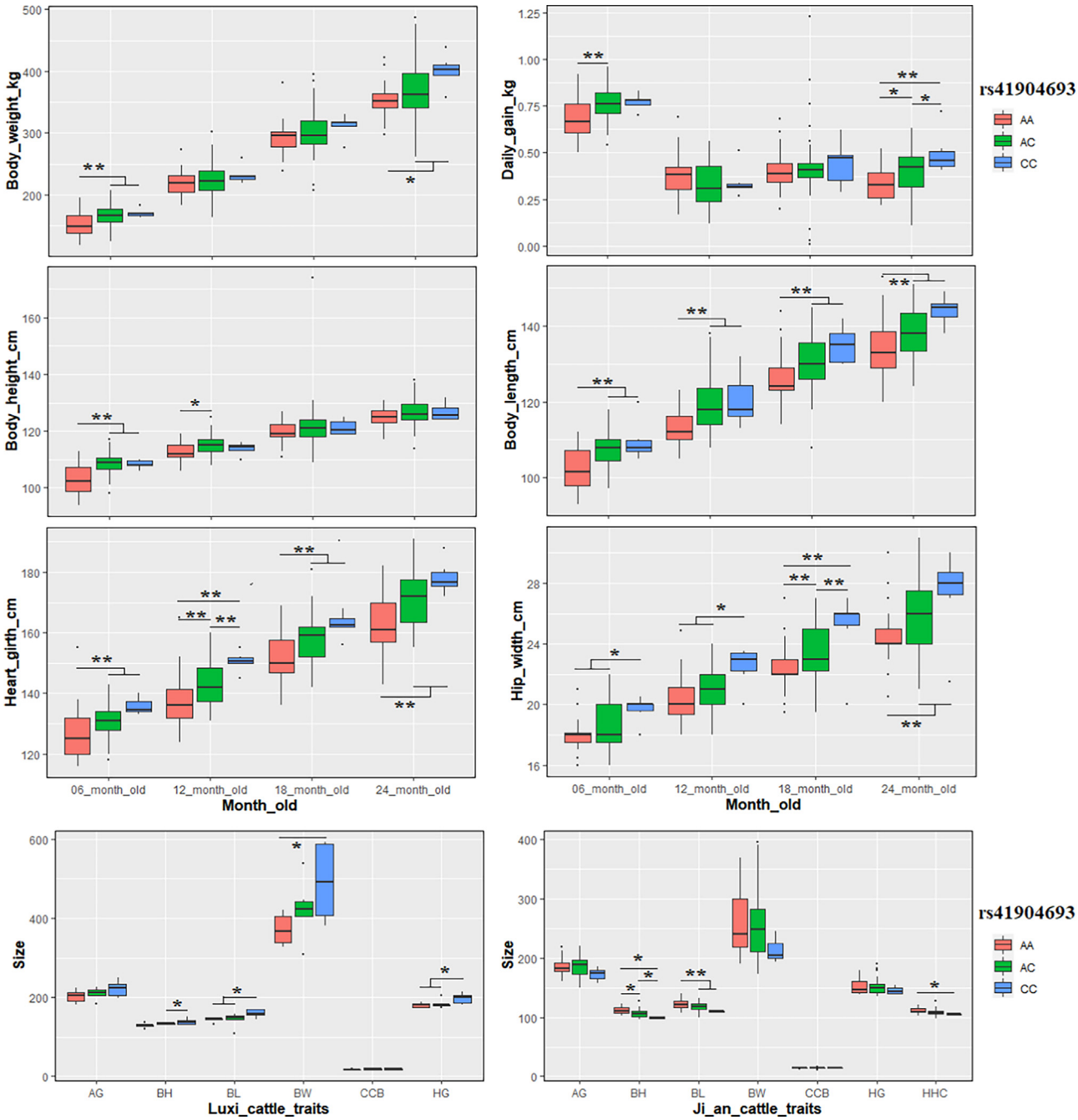

Fig. 2. The association of bovine *TMEM95* rs136174626 with growth traits of cattle. AG, Abdominal girth (cm); BH, Body height (cm); BL, Body length (cm); BW, Body weight (kg); CCB, Circumference of cannon bone (cm); HG, Heart girth (cm); HHC, Height at hip cross (cm). * and ** above the genotypes represented significant differences at $P < 0.05$ and $P < 0.01$ levels, respectively.

3.5. Functional prediction of rs136174626 and rs41904693 in TMEM95 gene

The conservation of the variants in *TMEM95* gene was predicted. For the rs136174626 locus, the A base was only found in the cow and Hybrid – Bos Taurus, and the locus was not very conservative among different species (Table 7). However, rs41904693 was conservative among different species, except for the G allele that was found in the Hybrid – Bos Indicus (Table 7). Since the location of the rs136174626 was close to Exon-4, we assumed that the mentioned mutation might influence the splicing of mRNA if this mutation located in a splicing site. The prediction by ESE Finder showed that the rs136174626 was located in a mammalian branch site (U2 type). The sequence changed from "TAGAGAA" to "TAGCGAA", and the predicted score changed from −1.36360 to 0.83290. The prediction of rs41904693 showed that the T to G mutation did not change the predicted score. Given that the rs41904693 was located in the 3' UTR of cattle *TMEM95* gene, we wondered whether this mutant would affect the binding of miRNA to target site. The miRNA binding sites' prediction results showed that both TT and GG genotypes could be recognized by miR-1584-5p, while the mutation changed the minimum free energy (mfe) (Fig. 4). Besides, the mutation changed the secondary structure of mRNA, which will have effect on miRNA binding (Fig. 4). Furthermore, the C

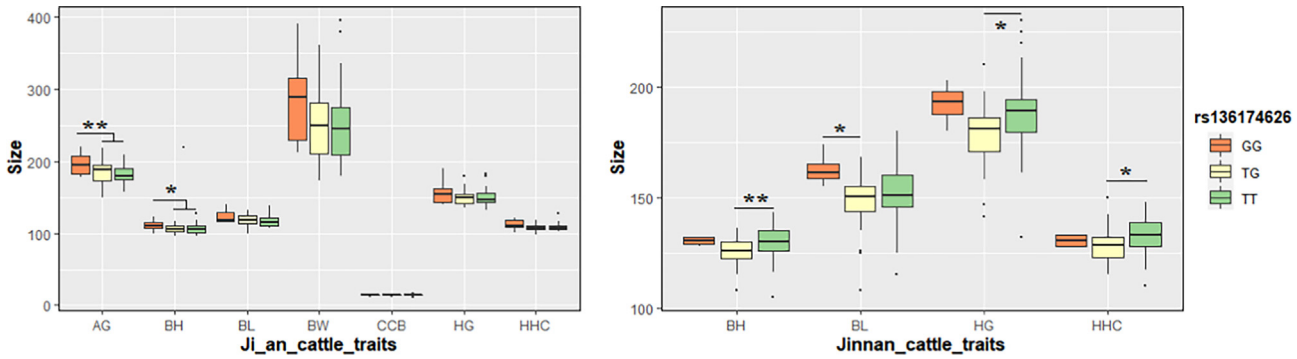

Fig. 3. The association of bovine *TMEM95* rs41904693 with growth traits of cattle. AG, Abdominal girth (cm); BH, Body height (cm); BL, Body length (cm); BW, Body weight (kg); CCB, Circumference of cannon bone (cm); HG, Heart girth (cm); HHC, Height at hip cross (cm). * and ** above the different genotypes represented significant differences at $P < 0.05$ and $P < 0.01$ levels, respectively.

Table 5
Association of bovine *TMEM95* rs136174626 with growth traits of cattle.

Breeds	Traits	Genotypes (Mean ± SD)			*P-values*
		AA	AC	CC	
Nanyang cattle (6 months old)	Body weight (kg)	$151.50^{b} \pm 3.79$	$165.28^{a} \pm 2.46$	$170.33^{a} \pm 2.83$	0.001
	Body height (cm)	$102.88^{b} \pm 0.91$	$108.47^{a} \pm 0.53$	$108.33^{a} \pm 0.62$	7×10^{-6}
	Body length (cm)	$102.44^{b} \pm 1.00$	$107.55^{a} \pm 0.63$	$109.67^{a} \pm 2.19$	2.1×10^{-5}
	Heart girth (cm)	$126.28^{b} \pm 1.51$	$130.66^{a} \pm 0.84$	$135.67^{a} \pm 1.12$	1.02×10^{-4}
	Hip width (cm)	$18.05^{b} \pm 0.19$	$18.39^{b} \pm 0.21$	$19.67^{a} \pm 0.36$	0.011
	Daily gain (kg)	$0.68^{b} \pm 0.02$	$0.75^{a} \pm 0.01$	$0.77^{ab} \pm 0.02$	0.006
Nanyang cattle (12 months old)	Body height (cm)	$112.56^{b} \pm 0.64$	$115.26^{a} \pm 0.54$	$113.83^{ab} \pm 0.87$	0.006
	Body length (cm)	$112.97^{b} \pm 0.84$	$119.32^{a} \pm 1.154$	$120.50^{a} \pm 2.91$	8.8×10^{-5}
	Heart girth (cm)	$137.78^{c} \pm 1.47$	$143.34^{b} \pm 1.04$	$150.50^{a} \pm 1.34$	1.52×10^{-4}
	Hip width (cm)	$20.40^{b} \pm 0.262$	$20.87^{b} \pm 0.24$	$22.50^{a} \pm 0.55$	0.013
Nanyang cattle (18 months old)	Body length (cm)	$126.09^{b} \pm 1.21$	$130.19^{a} \pm 1.08$	$135.00^{a} \pm 2.05$	0.006
	Heart girth (cm)	$151.22^{b} \pm 1.42$	$158.15^{a} \pm 1.14$	$162.67^{a} \pm 1.63$	1.09×10^{-4}
	Hip width (cm)	$22.61^{c} \pm 0.27$	$23.51^{b} \pm 0.24$	$25.00^{a} \pm 1.03$	0.003
Nanyang cattle (24 months old)	Body weight (kg)	$352.06^{b} \pm 4.72$	$372.97^{a} \pm 6.89$	$401.00^{a} \pm 10.97$	0.013
	Body length (cm)	$134.53^{b} \pm 1.33$	$138.63^{a} \pm 1.05$	$144.16^{a} \pm 1.55$	0.004
	Heart girth (cm)	$162.78^{b} \pm 1.60$	$171.75^{a} \pm 1.31$	$178.16^{a} \pm 2.30$	1×10^{-5}
	Hip width (cm)	$24.54^{b} \pm 0.31$	$25.84^{a} \pm 0.31$	$27.25^{a} \pm 1.22$	0.004
	Daily gain (kg)	$0.33^{c} \pm 0.01$	$0.40^{b} \pm 0.01$	$0.49^{a} \pm 0.04$	0.001
Luxi cattle	Body weight (kg)	$371.67^{b} \pm 16.09$	$428.86^{ab} \pm 26.13$	$491.60^{a} \pm 44.25$	0.043
	Body height (cm)	$129.50^{ab} \pm 2.26$	$133.50^{b} \pm 1.18$	$139.00^{a} \pm 3.42$	0.042
	Body length (cm)	$143.83^{b} \pm 6.05$	$143.33^{b} \pm 18.15$	$160.00^{a} \pm 10.51$	0.049
	Heart girth (cm)	$180.50^{b} \pm 2.39$	$183.67^{b} \pm 4.27$	$197.60^{a} \pm 6.01$	0.036
Ji' an cattle	Body height (cm)	$112.78^{a} \pm 1.82$	$105.87^{b} \pm 0.91$	$98.50^{c} \pm 1.50$	0.001
	Height at hip cross (cm)	$112.67^{a} \pm 1.70$	$108.35^{ab} \pm 0.79$	$104.50^{b} \pm 1.50$	0.021
	Body length (cm)	$126.89^{a} \pm 3.13$	$120.42^{b} \pm 1.06$	$109.00^{b} \pm 1.00$	0.001

Note: values with different superscripts within the same column differ significantly at $P < 0.05$ (a, b, c).

Table 6
Association of bovine *TMEM95* rs41904693 with growth traits of cattle.

Breeds	Traits	Genotypes (Mean ± SD)			*P-values*
		TT	TG	GG	
Ji' an Cattle	Body height (cm)	$104.93^{b} \pm 1.31$	$101.57^{b} \pm 1.02$	$110.30^{a} \pm 2.09$	0.048
	Abdominal girth (cm)	$183.55^{b} \pm 2.52$	$194.38^{b} \pm 2.64$	$198.40^{a} \pm 4.77$	0.004
Jinnan cattle	Body height (cm)	$130.05^{a} \pm 0.75$	$125.22^{b} \pm 1.18$	$130.25^{ab} \pm 1.03$	0.005
	Height at hip cross (cm)	$132.58^{a} \pm 0.80$	$128.48^{b} \pm 1.57$	$130.50^{ab} \pm 1.44$	0.046
	Body length (cm)	$152.27^{ab} \pm 1.23$	$148.04^{b} \pm 2.56$	$162.75^{a} \pm 4.03$	0.043
	Heart girth (cm)	$187.60^{a} \pm 1.58$	$178.78^{b} \pm 2.93$	$192.25^{ab} \pm 4.87$	0.018

Note: values with different superscripts within the same column differ significantly at $P < 0.05$ (a, b, c).

Table 7
The conservation of the rs136174626 and rs41904693 loci in *TMEM95* gene among different species.

Species	Sequence of rs136174626	Sequence of rs41904693
Cow	GAAGGGGTAG**A**GAAAGCCGGG	AGACCCTTAG**T**TCCAGCCCCA
Hybrid - Bos Indicus	GAAGGGGTAG**C**GAAAGCCGGG	AGACCCTTAG**G**TCCAGCCCCA
Hybrid - Bos Taurus	GAAGGGGTAG**A**GAAAGCCGGG	AGACCCTTAG**T**TCCAGCCCCA
Domestic yak	GAAGGGGTAG**C**GAAAGCCGGG	AGACCCTTAG**T**TCCAGCCCCA
Goat	GAAGGGGTAG**C**GAGAGCCAGG	AGACCCTAAG**T**TCCAGCCCCA
Pig	GAAGGGGTAG**C**TCCGGCAAGG	AGCCCCTTAG**T**TCCAGCCCCA
Cat	GAAGGGGTAG**G**TGAGGACGGG	AGCCCCTCAC**T**TCCAATCCCA
Canada lynx	GAAGGGGTAG**G**TGAGGACGGG	AGCCCCTCAC**T**TCCAATCCCA
Lion	GAAGGGGTAG**G**TGAGGACGGG	AGCCCCTCAC**T**TCCAATCCCA
Leopard	GAAGGGGTAG**G**TGAGGACGGG	AGCCCCTCAC**T**TCCAATCCCA

allele of rs136174626 was predicted to be a potential target for bta-miR-2467*, but the A allele could not be targeted by bta-miR-2467*.

4. Discussion

TMEM95 gene was found to be related to bovine reproductive traits, and it could be used in the breeding of cattle. But in the actual breeding, when one trait was improved through one candidate gene, the influence of the modification of the candidate gene on other traits should also be taken into account. For example, when editing and selecting gene related to reproductive traits, the effects of it on growth, body shape, disease resistance, etc., should also be taken into account. Therefore, this study expected to explore whether *TMEM95* had effects on growth and body traits of cattle besides affecting male fertility. The first level of function and molecular mechanism research of a gene is the correlation research, such as GWAS and correlation studies between genetic variations and phenotypes [7,16]. Thus, this study started from the basic correlation analysis to explore the relationship between genetic variations of *TMEM95* gene and growth traits of cattle.

In 2014, Pausch et al. found that *KIF1C*, *PELP1*, *ACADVL*, and *TMEM95* were candidate genes that influenced male reproduction [7]. In the follow-up study, *TMEM95* gene was identified to be associated with male subfertility, and the mutation in *ACADVL* was identified to be associated with growth traits of cattle [7,16]. Reproduction and growth are the two main traits that breeders pay attention to, and numerous genes have been found to affect both these traits, including *SPAG17*, *BMPRIB*, and *GDF9*. *SPAG17* gene was found essential for male germ cell development and fertility [27]. Meantime, a variety of studies pointed that the *SPAG17* was also required for skeletal bone development, and mutations in *SPAG17* gene were associated with body height of human and goat [8,28]. *BMPRIB* and *GDF9* belong to transforming growth factor beta (TGF-β) superfamily, which play crucial roles in tissue development [29]. Furthermore, these two genes are the star genes in reproduction research, due to their mutations play important roles in animal litter size [12,30,31]. Therefore, we speculated that the mutations in *TMEM95* gene may influence the growth and development of cattle.

In the present study, two SNPs in the cattle *TMEM95* gene were detected in 5 Chinese indigenous cattle. The rs136174626 was located in the intron 4 of cattle *TMEM95* gene, which was associated with several growth traits of cattle. Our prediction results showed that the rs136174626 might influence the splicing of intron 4. Although introns would be cut off during mRNA transcription, many introns contain functional elements and participate in the regulation of gene splicing or expression. For instance, the intron 3 G3072A nucleotide substitution in pig *IGF2* occurs in an evolutionarily conserved CpG island, influencing the skeletal muscle growth, subcutaneous fat deposition, and heart size [32]. Subsequently, studies reported the mentioned substitution abrogated the binding site for the transcriptional repressor ZBED6, and this substitution not only regulated the expression of *IGF2*, but also *IGF2 antisense transcript* [32,33]. A C-to-T transition in cattle *CD46* gene intron 8 was noted to be associated with mastitis in Holsteins by retaining a 48-bp sequence from intron 8, which changed the protein sequence of cattle *CD46* gene [34]. Furthermore, the rs2802292 mutation in human *FOXO3* intron 2 was found had enhancer functions, and the G-allele created a new HSE binding site for HSF1, which changed the expression of *FOXO3* gene [35].

In the current research, the rs41904693 was located in the 3′-UTR of cattle *TMEM95* gene, which was associated with the body

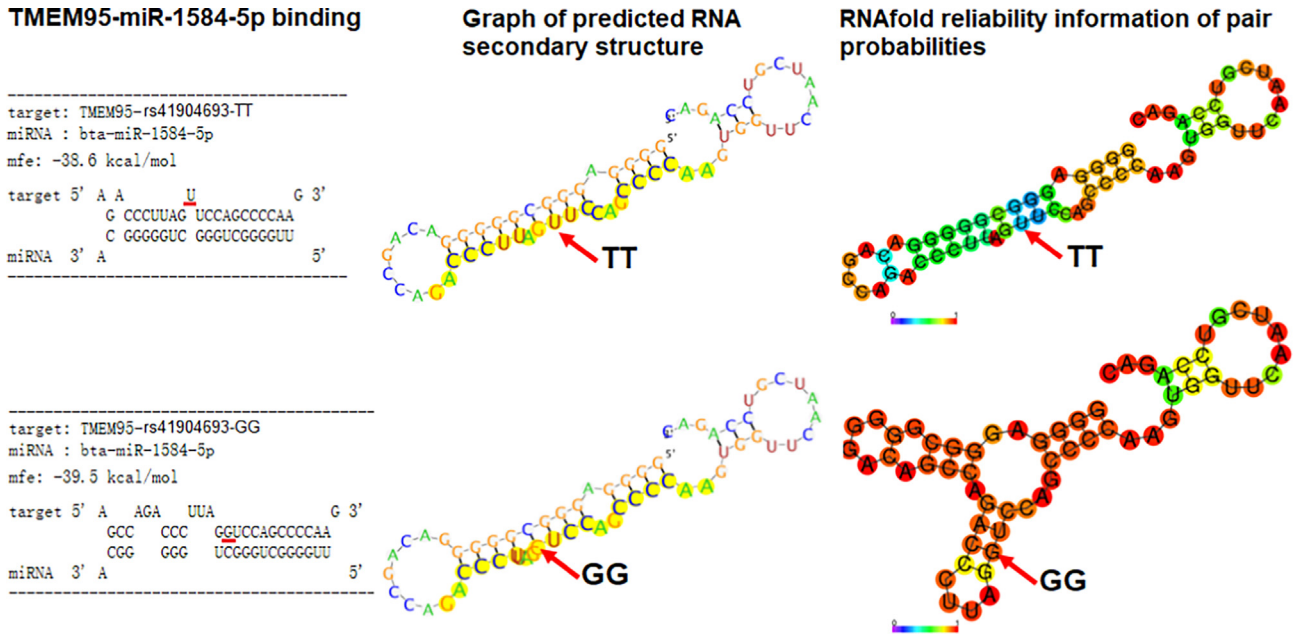

Fig. 4. The influence of rs41904693 mutation in *TMEM95* on miR-1584-5p binding site and mRNA secondary structure.

height of Ji' an and Jinnan cattle. Our prediction results showed that the rs41904693 might influence the binding of bta-miR-1584-5p and cattle *TMEM95* 3′-UTR. It is noteworthy that the function of miR-1584-5p has still remained unknown. The 3′-UTR is the primary regulation region of gene, influencing the expression of gene mainly though binding with miRNAs. Previous studies demonstrated that the SNPs in the 3′-UTR region may affect the binding of miRNAs and target sites, in addition to further influence the expression of the target gene [36]. In goat, a litter size associated SNP, g.173057T>C, in *PRLR* was identified to regulate the binding of miR-302a and *PRLR* 3′-UTR, further influence the expression of PRLR protein [37]. In pig, the expression of *IGF-1* was found to be influenced by the *IGF-1* 3′-UTR SNP (rs34142920) and miR-new14 [38]. In dairy cattle, a SNP (g.4693G>T) in the 3′-UTR of *HSF1*was noted to be associated with the thermo tolerance in Chinese Holstein cattle, and this SNP can affect the expression of *HSF1* by influencing the binding of bta-miR-484 and *HSF1* [39].

5. Conclusions

In summary, two generic variations of cattle *TMEM95* gene were identified in 5 Chinese indigenous cattle breeds. The rs136174626 and rs41904693 were associated with the body growth traits of cattle. For rs136174626, the individuals with CC and AC genotypes were greater than the individuals with AA genotype in Nanyang and Luxi cattle. And for rs41904693, the individuals with GG genotype were greater than the individuals with TT and TG genotypes. The rs136174626 may influence the splicing efficiency of *TMEM95* and rs41904693 may influence the expression of *TMEM95* by affecting the binding efficiency of miR-1584 and *TMEM95* 3′-UTR. These findings would provide a reliable basis for molecular breeding in cattle.

Conflicts of Interest

The authors declare no conflict of interest.

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References

[1] Chen N, Cai Y, Chen Q, et al. Whole-genome resequencing reveals world-wide ancestry and adaptive introgression events of domesticated cattle in East Asia. Nat Commun 2018;9(1). https://doi.org/10.1038/s41467-018-04737-0. PMid: 29904051.
[2] Williams JL. The use of marker-assisted selection in animal breeding and biotechnology. Rev Sci Technol 2005;24(1):379–91. https://doi.org/10.20506/rst.24.1.1571. PMid: 16110903.
[3] Ruan J, Xu J, Chen-Tsai RY, et al. Genome editing in livestock: Are we ready for a revolution in animal breeding industry?. Transgenic Res 2017;26(6):715–26. https://doi.org/10.1007/s11248-017-0049-7. PMid: 29094286.
[4] Strandén I, Kantanen J, Russo IM, et al. Genomic selection strategies for breeding adaptation and production in dairy cattle under climate change. Heredity 2019;123(3):307–17. https://doi.org/10.1038/s41437-019-0207-1. PMid: 30886391.
[5] Dekkers JC. Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. J Anim Sci 2004;82(E-Suppl):E313–28. PMid: 30886391.
[6] Zhang S, Cai H, Yang Q, et al. Identification of novel alternative splicing transcript and expression analysis of bovine TMEM95 gene. Gene 2016;575(2):531–6. https://doi.org/10.1016/j.gene.2015.09.026. PMid: 26385321.
[7] Pausch H, Kölle S, Wurmser C, Schwarzenbacher H, Emmerling R, Jansen S, et al. A nonsense mutation in TMEM95 encoding a nondescript transmembrane protein causes idiopathic male subfertility in cattle. PLoS Genet 2014;10(1):e1004044. PMid: 24391514.
[8] Zhang S, Peng K, Zhang G, et al. Detection of Bovine TMEM95 p.Cys161X mutation in 13 Chinese indigenous cattle breeds. Animals (Basel) 2019;9(7):444. https://doi.org/10.3390/ani9070444. PMid: 31315171.
[9] Fernandez-Fuertes B, Kölle S, Lonergan P. Subfertility in bulls carrying a nonsense mutation in TMEM95 is due to failure to penetrate the zona pellucida. Reprod Fertil Dev 2017;29(1):109–10. https://doi.org/10.1071/RDv29n1Ab4. PMID 28278801.
[10] Fernandez-Fuertes B, Laguna-Barraza R, Fernandez-Gonzalez R, et al. Subfertility in bulls carrying a nonsense mutation in transmembrane protein 95 is due to failure to interact with the oocyte vestments. Biol Reprod 2017;97(1):50–60. https://doi.org/10.1093/biolre/iox065. PMid: 28859280.
[11] Fernandez-Fuertes B, Lonergan P. Sperm Transmembrane Protein 95 (TMEM95) is required for sperm–oocyte interaction and successful fertilization but not mucus penetration in cattle. Reprod Fertil Dev 2018;30(1):209–10. https://doi.org/10.1071/RDv30n1Ab139.
[12] Lamas-Toranzo I, Hamze JG, Bianchi E, et al. TMEM95 is a sperm membrane protein essential for mammalian fertilization. Elife 2020;9:. https://doi.org/10.7554/eLife.53913. PMid: 32484434e53913.
[13] Noda T, Lu Y, Fujihara Y, et al. Sperm proteins SOF1, TMEM95, and SPACA6 are required for sperm-oocyte fusion in mice. Proc Natl Acad Sci U S A 2020;117(21):11493–502. https://doi.org/10.1073/pnas.1922650117. PMid: 32393636.
[14] Wang X, Yang Q, Wang K, et al. A novel 12-bp indel polymorphism within the GDF9 gene is significantly associated with litter size and growth traits in goats. Anim Genet 2017;48(6):735–6. https://doi.org/10.1111/age.12617. PMid: 29023802.
[15] Zhang S, Jiang E, Wang K, et al. Two insertion/Deletion variants within SPAG17 gene are associated with goat body measurement traits. Animals (Basel) 2019;9(6):379. https://doi.org/10.3390/ani9060379. PMid: 31234269.
[16] Zhang S, Dang Y, Zhang Q, et al. Tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) rapidly identified a critical missense mutation (P236T) of bovine ACADVL gene affecting growth traits. Gene 2015;559(2):184–8. https://doi.org/10.1016/j.gene.2015.01.043. PMid: 25620159.
[17] Dang Y, Li M, Yang M, et al. Identification of bovine NPC1 gene cSNPs and their effects on body size traits of Qinchuan cattle. Gene 2014;540(2):153–60. https://doi.org/10.1016/j.gene.2014.03.001. PMid: 24607034.
[18] You FM, Huo N, Gu Y, et al. BatchPrimer3: a high throughput web application for PCR and sequencing primer design. BMC Bioinf 2008;9(1):253. https://doi.org/10.1186/1471-2105-9-253. PMid: 18510760.
[19] Ye S, Dhillon S, Ke X, et al. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 2001;29(17):E88–98. https://doi.org/10.1093/nar/29.17.e88. PMid: 11522844.
[20] Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res 2005;15(2):97–8. https://doi.org/10.1038/sj.cr.7290272. PMid: 15740637.
[21] Cui Y, Yan H, Wang K, et al. Insertion/Deletion within the KDM6A gene is significantly associated with litter size in goat. Front Genet 2018;9:91. https://doi.org/10.3389/fgene.2018.00091. PMid: 29616081.
[22] Zhang S, Kang Z, Sun X, et al. Novel lncRNA lncFAM200B: molecular characteristics and effects of genetic variants on promoter activity and cattle body measurement traits. Front Genet 2019;10:968. https://doi.org/10.3389/fgene.2019.00968. PMid: 31649734.
[23] Kang Z, Jiang E, Wang K, et al. Goat membrane associated ring-CH-type finger 1 (MARCH1) mRNA expression and association with litter size. Theriogenology 2019;128:8–16. https://doi.org/10.1016/j.theriogenology.2019.01.014. PMid: 30711644.
[24] Cartegni L, Wang J, Zhu Z, et al. ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res 2003;31(13):3568–71. https://doi.org/10.1093/nar/gkg616. PMid: 12824367.
[25] Wang X, Zhong J, Gao Y, et al. A SNP in intron 8 of CD46 causes a novel transcript associated with mastitis in Holsteins. BMC Genomics 2014;15(1):630. https://doi.org/10.1186/1471-2164-15-630. PMid: 25070150.
[26] Chang TH, Huang HY, Hsu JB, et al. An enhanced computational platform for investigating the roles of regulatory RNA and for identifying functional RNA motifs. BMC Bioinformatics 2013;14(Suppl 2):S4. https://doi.org/10.1186/1471-2105-14-S2-S4. PMid: 23369107.
[27] Kazarian E, Son H, Sapao P, et al. SPAG17 is required for male germ cell differentiation and fertility. Int J Mol Sci 2018;19(4):1252. https://doi.org/10.3390/ijms19041252. PMid: 29690537.
[28] Teves ME, Sundaresan G, Cohen DJ, et al. Spag17 deficiency results in skeletal malformations and bone abnormalities. PLoS One 2015;10(5):. https://doi.org/10.1371/journal.pone.0125936. PMid: 26017218e0125936.
[29] Yamawaki K, Kondo Y, Okada T, et al. The soluble form of BMPRIB is a novel therapeutic candidate for treating bone related disorders. Sci Rep 2016;6:18849. https://doi.org/10.1038/srep18849. PMid: 26732094.

[30] Wang W, La Y, Zhou X, et al. The genetic polymorphisms of TGFβ superfamily genes are associated with litter size in a Chinese indigenous sheep breed (Hu sheep). Anim Reprod Sci 2018;189:19–29. https://doi.org/10.1016/j.anireprosci.2017.12.003. PMid: 29274749.
[31] Chong Y, Liu G, Jiang X. Effect of BMPRIB gene on litter size of sheep in China: A meta-analysis. Anim Reprod Sci 2019;210:106175. https://doi.org/10.1016/j.anireprosci.2019.106175. PMid: 31635771.
[32] Van Laere AS, Nguyen M, Braunschweig M, et al. A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature 2003;425 (6960):832–6. https://doi.org/10.1038/nature02064. PMid: 14574411.
[33] Braunschweig MH, Van Laere A-S, Buys N, Andersson L, Andersson G. IGF2 antisense transcript expression in porcine postnatal muscle is affected by a quantitative trait nucleotide in intron 3. Genomics 2004;84(6):1021–9. https://doi.org/10.1016/j.ygeno.2004.09.006. PMid: 15533719.
[34] Liu X, Liu H, Wang M, et al. Disruption of the ZBED6 binding site in intron 3 of IGF2 by CRISPR/Cas9 leads to enhanced muscle development in Liang Guang Small Spotted pigs. Transgenic Res 2019;28(1):141–50. https://doi.org/10.1007/s11248-018-0107-9. PMid: 30488155.
[35] Grossi V, Forte G, Sanese P, et al. The longevity SNP rs2802292 uncovered: HSF1 activates stress-dependent expression of FOXO3 through an intronic enhancer. Nucleic Acids Res 2018;46(11):5587–600. https://doi.org/10.1093/nar/gky331. PMid: 29733381.
[36] Clop A, Marcq F, Takeda H, et al. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet 2006;38(7):813–8. https://doi.org/10.1038/ng1810. PMid: 16751773.
[37] An X, Hou J, Gao T, et al. Single-nucleotide polymorphisms g.151435C>T and g.173057T>C in PRLR gene regulated by bta-miR-302a are associated with litter size in goats. Theriogenology 2015;83(9):1477–1483.e1. https://doi.org/10.1016/j.theriogenology.2015.01.030. PMid: 25799469.
[38] Song J, Hao L, Wei W, et al. A SNP in the 3'-UTR of the porcine IGF-1 gene interacts with miR-new14 to affect IGF-1 expression, proliferation and apoptosis of PK-15 cells. Domest Anim Endocrinol 2020;72:106430. https://doi.org/10.1016/j.domaniend.2019.106430. PMid: 32171113.
[39] Li QL, Zhang ZF, Xia P, et al. A SNP in the 3'-UTR of HSF1 in dairy cattle affects binding of target bta-miR-484. Genet Mol Res 2015;14(4):12746–55. https://doi.org/10.4238/2015.October.19.18. PMid: 26505425.