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Exploration of the exonic variations of the iPSC-related *Nanog* gene and their effects on phenotypic traits in cattle

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Abstract. *Nanog* is an important pluripotent transcription regulator transforming somatic cells to induced pluripotent stem cells (iPSCs), and its overexpression leads to a high expression of the growth and differentiation factor 3 (GDF3), which affects animal growth traits. Therefore, the aim of this study was to explore the genetic variations within the *Nanog* gene and their effects on phenotypic traits in cattle. Six novel exonic single nucleotide polymorphisms (SNPs) were found in six cattle breeds. Seven haplotypes were analyzed: TCAACC (0.260), TCAATA (0.039), TCATCC (0.019), TCGACC (0.506), TCGATA (0.137), TCGTCC (0.036), and CTGATA (0.003). There were strong linkage disequilibriums of SNP1 and SNP2 in Jiaxian cattle as well as of SNP5 and SNP6 in both Jiaxian cattle and Nanyang cattle. Moreover, SNP3, SNP4, and SNP5 were associated with phenotypes. The individuals with GG genotype at the SNP3 locus or AA genotype at the SNP4 locus showed better body slanting length and chest circumference or body height and hucklebone width in Nanyang cattle. The superiority of the SNP5-C allele regarding body height and cannon circumference was observed in Jiaxian cattle. The combination of SNP3 and SNP4 (GG–AA) had positive effects on body height, body slanting length, and chest circumference. These findings may indicate that *Nanog*, as a regulator of bovine growth traits, could be a candidate gene for marker-assisted selection (MAS) in breeding and genetics in cattle.

1 Introduction

Induced pluripotent stem cells (iPSCs), derived from transcription-factor reprogramming, redefine our ability to develop the protein production of livestock, to improve disease resistance and production efficiencies, and to develop modeled livestock for research (Liang and Zhang, 2013; Lei el al., 2013). iPSCs are generated directly interacting with Nanog, Klf4, Oct4, Sox2, C-Myc, and other transcription factors. Among them, the iPSC-related transcription factor Klf4 is necessary for adipose differentiation and development (Cervantes-Camacho et al., 2015). Similarly, *Klf7* regulated energy metabolism and affected bovine growth (Ma et al., 2011). Thus, another iPSC-related transcription factor *Nanog* was inferred to affect growth traits in cattle.

As a vital member of iPSC-related genes, *Nanog*, which consists of four exons and three introns in different species, is conservative and blocks the differentiation of pluripotent stem cells (Clark et al., 2004; Sumer et al., 2011). In a comparison and analysis of human, murine, and bovine *Nanog* promoter sequences, the promoters contained highly conserved regions (Rodda et al., 2005). In primordial germ cells (PGCs), *Nanog* was required for germ cell development (Yang et al., 2015). Recently, *Nanog* overexpression promoted the expression of the growth and differentiation factor 3 (GDF3), which is a member of the transforming growth factor beta (TGF-β) family (Park et al., 2012). TGF-β affects differentiation processes during embryonic development and regulates bone formation as well as bone resorption (Shi et al., 2016; McPherron and Lee, 1993). GDF3 is expressed in bone marrow, thymus, spleen, and fat and exhibits a particu-

Table 1. Primers used for detecting mutations of *Nanog* gene in cattle

Primer	Primer sequence (5′–3′)	Product size (bp)	T_m (°C)	Target region
P1	F: TTTCCTTCTCCTTCAACTCA R: TGTGCCTGTGACCATTCTTT	169	55.5	Exon 1
P2	F: CTCCTCTTCCCTCCTCC R: GATGTTCGTCCATGTCAACAA	274	50.0	Exon 2
P3	F: TTTTCCGGTTATCTTTTC R: CCTTATCGTTACCGTAC	113	48.4	Exon 3
P4	F: TCAGTTAATCCATTCTTCTTT R: AACACTGCCGATAACATAC	473	49.3	Exon 4

larly high level of expression in white-fat tissue in mice; this was mainly induced by a high-fat diet and blocking of the fat metabolic pathway (Wang et al., 2004). The presence of *Nanog* inhibits the transcriptional effectors of both the bone morphogenetic protein and NF-kappa B pathways in embryonic stem cells (ESCs) (Li et al., 2016). Moreover, GLI1 and GLI3 bind to *Nanog*, but *Nanog* inhibits GLI transcription and Hedgehog signaling (Li et al., 2016). These proteins and pathways influence the physiological function development of cells and organisms.

The experimental animals used in this study are excellent cattle breeds from different Chinese locations. Qinchuan cattle (QC), Nanyang cattle (NY), Jiaxian cattle (JX), and Jinnan cattle (JN) are the major meat-producing cattle; Angus (AN) and Chinese Holstein (CH) have been introduced from abroad. Based on the previous reports, this study explored the effects of SNPs in the *Nanog* gene on the growth traits of these cattle, which would contribute to cattle breeding and genetics through marker-assisted selection (MAS).

2 Materials and methods

All animal experiments were implemented following relevant laws and institutional guidelines and were approved by the Northwest A&F University Institutional Animal Care and Use Committee.

2.1 DNA samples

A total of 796 cattle belonging to six Chinese breeds were used for this experiment, including Nanyang ($n = 247$), Qinchuan ($n = 144$), Jiaxian ($n = 269$), Jinnan ($n = 28$), Angus ($n = 47$), and Chinese Holstein ($n = 61$). All selected individuals were healthy and unrelated. Records of growth traits and body sizes for different growth periods (postnatal and 6, 12, 18, and 24 months old) were collected for statistical analysis (Pan et al., 2008). Ear tissue and blood samples of individuals were obtained and stored at −80 °C for DNA isolation. Genomic DNA was isolated from ear tissue and leukocytes from blood samples according to the procedure described by Sambrook and Russell (2001) and stored at −20 °C.

2.2 PCR conditions and genotyping

Four pairs of primer were designed for the *Nanog* gene by the Primer Premier 5.0 program (Table 1), covering exons 1–4 based on the available sequence of the bovine *Nanog* gene (GeneBank accession No. AC_000162.1). PCR (polymerase chain reaction) was performed in a 25 µL reaction volume, containing 50 ng genomic DNA, 0.5 µmol L^{-1} of each primer, 1× buffer (including 1.5 mmol L^{-1} $MgCl_2$), 200 µmol L^{-1} dNTPs, and 0.625 unit of TaqDNA polymerase (MBI, Vilnius, Lithuania). The PCR amplification conditions were as follows: initial denaturing at 95 °C for 5 min followed by 36 cycles of 95 °C for 30 s, annealing 30 s, and extending at 72 °C for 30 s, with a final extension at 72 °C for 10 min (Lan et al., 2007).

Aliquots of 5 µL PCR products from different individuals were mixed with 5 µL denaturing solution (95 % formamide, 25 mM EDTA, 0.025 % Xylene cyanole, and 0.025 % bromophenol blue), heated for 10 min at 98 °C, and chilled on ice. Denatured DNA was subjected to PAGE (polyacrylamide gel electrophoresis) (80 × 73 × 0.75 mm) in 1× TBE buffer at constant voltage (200 V) for 2.5–3.0 h. The gel was stained with 0.1 % silver nitrate (Lan et al., 2007). The bands of products on PAGE gels were distinguished and selected for purification and sequencing. Comparing sequencings, we confirmed that two polymorphisms existed in the first exon, one polymorphism in the second exon, and three polymorphisms in the fourth exon in the *Nanog* gene (Fig. 1). To check the result of SSCP (single-stranded conformational polymorphism), we carried out PCR-RFLP (polymerase chain reaction restriction fragment length polymorphism) on SNP1 and SNP5. Aliquots of 20 µL PCR products of the first exon in the *Nanog* gene were digested with 10 U *Mva*I at 37 °C for 6–8 h. The digested products were detected by 2.5–3.5 % agarose gel (Lan et al., 2007). Meanwhile, aliquots of 20 µL PCR products of exon 4 in the *Nanog* gene were digested with 10 U *Mbo*I at 37 °C for 6–8 h. The digested products were detected by 2.5–3.5 % agarose gel (Lan et al., 2007).

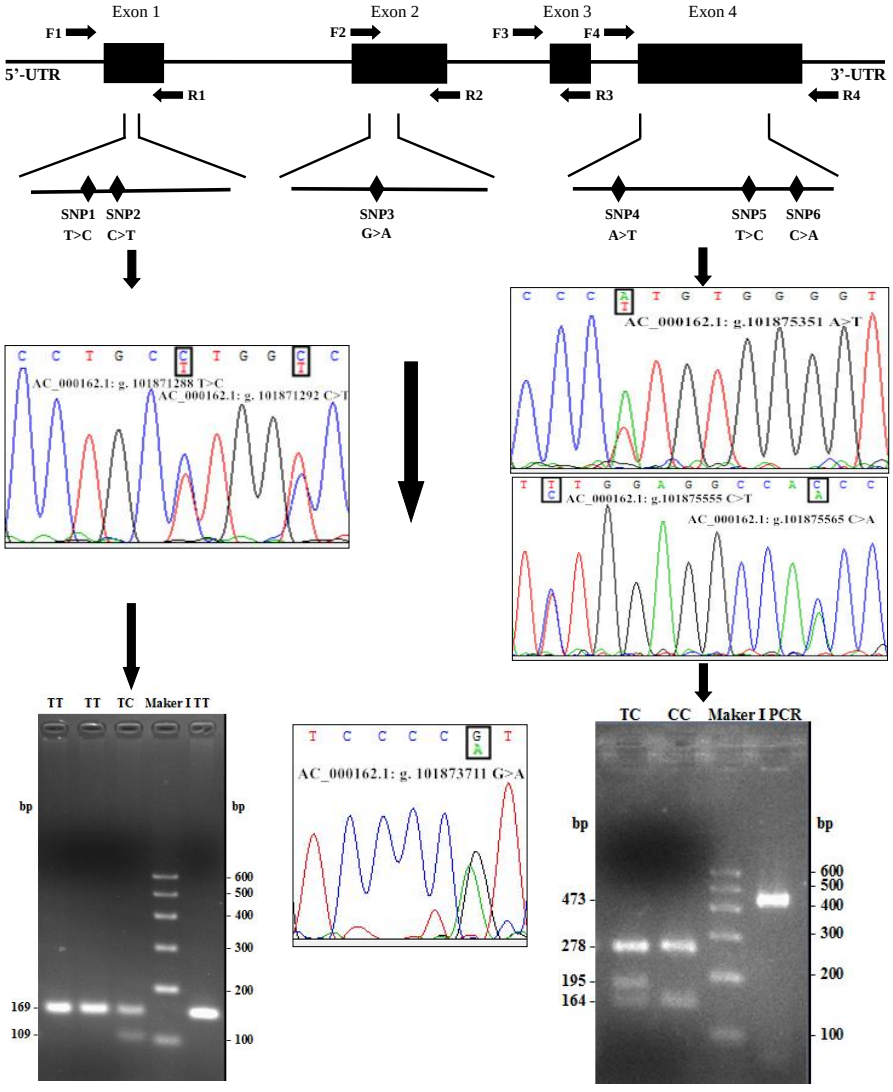

Figure 1. Sequencing of six SNPs and electrophoretograms of SNP1 and SNP5. Note: electrophoresis images show PCR-RFLP on SNP1 and SNP5. At SNP1, TT had 169 bp and TC had 169 bp and 109 bp. At SNP5, CC had 278 + 164 bp and TC had 278 + 195 + 164 bp; total length of the fourth exon: 473 bp.

Table 2. Genotypes of different mutations corresponding to PCR-SSCP types in cattle

Fragments	Variation positions	SNP types	Descriptions
P1	g.101871288 T>C (Exon1_35)	SNP1	12Leu/Pro, missense mutation
P1	g.101871292 C>T (Exon1_39)	SNP2	13Gly/Gly, synonymous mutation
P2	g.101873711 G>A (Exon2_86)	SNP3	74Pro/Pro, synonymous mutation
P4	g.101875351 A>T (Exon4_79)	SNP4	189Met/Leu, missense mutation
P4	g.101875555 C>T (Exon4_283)	SNP5	257Leu/Leu, synonymous mutation
P4	g.101875565 C>A (Exon4_293)	SNP6	260Thr/Asn, missense mutation

Note: descriptions refer to NM_001025344.1 and NP_001020515.1.

Table 3. Genotypic frequencies, allelic frequencies, HWE, and population parameters of the *Nanog* gene in Chinese cattle.

Loci	Breeds	*N*	TT	TC	CC	T	C	*P*	Ho	He	Ne	PIC
SNP1	NY	247	0.996	0.004	0	0.998	0.002	$P > 0.05$	0.996	0.004	1.004	0.004
	QC	144	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	JX	269	0.970	0.030	0	0.985	0.015	$P > 0.05$	0.971	0.029	1.030	0.029
	JN	28	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	Angus	47	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	Holstein	61	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
Loci	Breeds	*N*	CC	CT	TT	C	T	*P*	Ho	He	Ne	PIC
SNP2	NY	247	0.996	0.004	0	0.998	0.002	$P > 0.05$	0.996	0.004	1.004	0.004
	QC	144	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	JX	269	0.970	0.030	0	0.985	0.015	$P > 0.05$	0.971	0.029	1.030	0.029
	JN	28	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	Angus	47	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
	Holstein	61	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0
Loci	Breeds	*N*	GG	GA	AA	G	A	*P*	Ho	He	Ne	PIC
SNP3	NY	151	0.616	0	0.384	0.616	0.384	$P < 0.01$	0.527	0.473	1.898	0.361
	QC	–	–	–	–	–	–	–	–	–	–	–
	JX	74	0.689	0.311	0	0.845	0.155	$P < 0.05$	0.737	0.263	1.356	0.228
	JN	–	–	–	–	–	–	–	–	–	–	–
	Angus	–	–	–	–	–	–	–	–	–	–	–
	Holstein	61	1.000	0	0	1.000	0	$P > 0.05$	1.000	0	1.000	0.000
Loci	Breeds	*N*	AA	AT	TT	A	T	*P*	Ho	He	Ne	PIC
SNP4	NY	101	0.792	0.208	0	0.896	0.104	$P > 0.05$	0.814	0.186	1.229	0.169
	QC	140	0.564	0.436	0	0.782	0.218	$P < 0.01$	0.659	0.341	1.517	0.283
	JX	116	0.940	0.060	0	0.970	0.030	$P > 0.05$	0.941	0.059	1.062	0.057
	JN	–	–	–	–	–	–	–	–	–	–	–
	Angus	–	–	–	–	–	–	–	–	–	–	–
	Holstein	–	–	–	–	–	–	–	–	–	–	–
Loci	Breeds	*N*	CC	CT	TT	C	T	*P*	Ho	He	Ne	PIC
SNP5	NY	101	0.941	0.059	0	0.970	0.030	$P > 0.05$	0.942	0.058	1.061	0.056
	QC	140	0.450	0.550	0	0.725	0.275	$P < 0.01$	0.601	0.399	1.663	0.319
	JX	116	0.276	0.724	0	0.638	0.362	$P < 0.01$	0.538	0.462	1.859	0.355
	JN	–	–	–	–	–	–	–	–	–	–	–
	Angus	–	–	–	–	–	–	–	–	–	–	–
	Holstein	–	–	–	–	–	–	–	–	–	–	–
Loci	Breeds	*N*	CC	CA	AA	C	A	*P*	Ho	He	Ne	PIC
SNP6	NY	101	0.941	0.059	0	0.970	0.030	$P > 0.05$	0.942	0.058	1.061	0.056
	QC	140	0.421	0.550	0.029	0.696	0.304	$P < 0.01$	0.577	0.423	1.733	0.333
	JX	116	0.267	0.724	0.009	0.629	0.371	$P < 0.01$	0.533	0.467	1.875	0.358
	JN	–	–	–	–	–	–	–	–	–	–	–
	Angus	–	–	–	–	–	–	–	–	–	–	–
	Holstein	–	–	–	–	–	–	–	–	–	–	–

Note: HWE – Hardy–Weinberg equilibrium; Ho – homozygosity; He – heterozygosity; Ne – effective allele numbers; PIC – polymorphism information content.

Table 4. Haplotypes and their frequencies of six SNPs in Nanyang cattle and Jiaxian cattle.

HAP (haplotype)	Hap. seq.	Total (no./freq.)	Breeds (no./freq.)	
			NY	JX
Hap1	T C A A C C	80.090/0.260	66.000/0.379	12.510/0.093
Hap2	T C A A T A	11.910/0.039	8.000/0.046	5.490/0.041
Hap3	T C A T C C	6.000/0.019	6.000/0.034	0/0
Hap4	T C G A C C	155.910/0.506	79.000/0.454	78.490/0.586
Hap5	T C G A T A	42.090/0.137	6.000/0.034	34.510/0.258
Hap6	T C G T C C	11.000/0.036	9.000/0.052	2.000/0.015
Hap7	C T G A T A	1.000/0.003	0/0	1.000/0.007

2.3 Statistical analysis

Genotypic and allelic frequencies were calculated by PopGene version 3.2 (Yeh et al., 2000). The Hardy–Weinberg equilibrium (HWE) was analyzed based on a predetermined value of the test statistic (Emigh, 1980). Linkage disequilibrium was performed by the SHEsis online platform (http://analysis.bio-x.cn) (Shi et al., 2005; Li et al., 2009). Population parameters, including gene heterozygosity (He), gene homozygosity (Ho), effective allele numbers (Ne), and polymorphism information content (PIC), were calculated according to Nei's method (Nei and Roychoudhury, 1974). Distribution differences for genotypic and allelic frequencies among different breeds were analyzed using the χ^2 test. The associations between genotypes and growth traits of cattle were analyzed using the statistical software SPSS (Version 22.0, International Business Machines (IBM) Corporation, New York, USA; Pan et al., 2013). The linear model was as follow: $Y_{ijk} = \mu + A_i + G_j + e_{ijk}$, where Y_{ijk} represents the observation of the body measurement traits, μ the overall mean of each trait, A_i the fixed effect of age, G_j the fixed effect of genotype or combined genotype, and e_{ijk} the random residual error (Chen et al., 2016; Zhang et al., 2015). The effect of genotype was a significant variation source, when the P value for the difference was less than 0.05 between the least squares means for each genotype (Lan et al., 2013; Zhao et al., 2013).

3 Results

3.1 Genetic variant identification of the *Nanog* gene

Through the experiment, we found two mutations in exon1 (AC_000162.1: g.101871288T>C, g.101871292C>T), one mutation in exon2 (AC_000162.1: g.101873711G>A), and three mutations in exon4 (AC_000162.1: g.101875351A>T, g.101875555C>T, and g.101875565C>A) in the *Nanog* gene by DNA sequencing and PCR-SSCP, named SNP1–6, respectively (Fig. 1). SNP1, SNP4, and SNP6 were missense mutations corresponding to 12Leu > Pro, 189Met > Leu, and 260Thr > Asn, respectively (Table 2). We performed PCR-RFLP on SNP1 and SNP5 using *Mva*I and *Mbo*I, respectively, and did DNA sequencing on other SNPs (Fig. 1). Frequencies of alleles and genotypes, He, Ho, Ne, and PIC in six cattle breeds are listed in Table 3. The minor allelic frequencies among six breeds of SNP1 and SNP2 ranged from 0.002 to 0.015; those of SNP3 ranged from 0.000 to 0.384, those of SNP4 from 0.030 to 0.218, those of SNP5 from 0.030 to 0.362, and those of SNP6 from 0.030 to 0.371. The χ^2 test showed that SNP1 and SNP2 of all cattle breeds were in Hardy–Weinberg equilibrium (HWE), yet other polymorphisms in several cattle breeds were not in HWE and are shown in Table 3.

3.2 Haplotype analysis and linkage disequilibrium

Seven haplotypes were analyzed in NY and JX cattle breeds, namely Hap1 (TCAACC), Hap2 (TCAATA), Hap3 (TCATCC), Hap4 (TCGACC), Hap5 (TCGATA), Hap6 (TCGTCC), and Hap7 (CTGATA). Frequencies of seven haplotypes of NY were 0.379 (Hap1), 0.046 (Hap2), 0.034 (Hap3), 0.454 (Hap4), 0.034 (Hap5), 0.052 (Hap6), and 0 (Hap7). Haplotype frequencies among JX were 0.093 (Hap1), 0.041 (Hap2), 0 (Hap3), 0.586 (Hap4), 0.258 (Hap5), 0.015 (Hap6), and 0.007 (Hap7). Among them, the frequency of Hap4 was the highest in JX and NY (Table 4). Viewing the linkage disequilibrium of six SNPs (Table 5, Fig. 2), SNP5 and SNP6 had strong linkage ($r^2 > 0.33$) in both NY and JX, and an LD (linkage disequilibrium) between SNP1 and SNP2 was detected in JX.

3.3 Association analysis

SPSS statistics revealed that three mutations (SNP3, SNP4, and SNP5) showed a significant association with the growth traits in NY and JX. At the SNP3 locus, individuals with a GG genotype had a longer body slanting length and chest circumference than those with an AA genotype in 6-month-old NY ($P < 0.05$). NY with an AA genotype at SNP4 showed a longer body length and better hucklebone width than AT heterozygotes at 6 months old ($P < 0.05$). JX that were CC homozygous at SNP5 had greater body height ($P < 0.05$) and a significantly greater cannon circumference ($P < 0.01$) than CT heterozygotes (Table 6). The combined genotype of

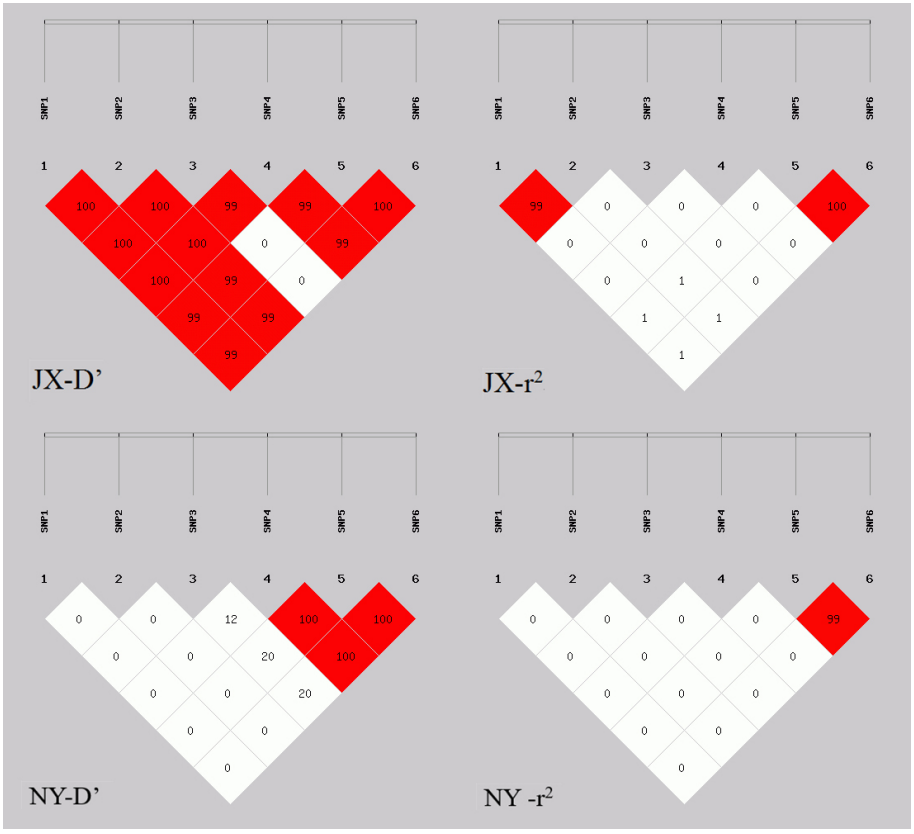

Figure 2. Linkage disequilibrium of *Nanog* gene polymorphisms in Jiaxian and Nanyang cattle. White squares show weak linkage and red squares show strong linkage.

Table 5. Linkage disequilibrium tests for Nanyang cattle and Jiaxian cattle.

NY breed: D′ and r^2: SNP1/SNP2/SNP3/SNP4/SNP5/SNP6							
–	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	D′
SNP6	–	0.000	0.000	0.000	0.000	0.000	SNP1
SNP5	1.000	–	0.000	0.000	0.000	0.000	SNP2
SNP4	0.008	0.008	–	0.130	0.207	0.207	SNP3
SNP3	0.004	0.004	0.001	–	1.000	1.000	SNP4
SNP2	0.000	0.000	0.000	0.000	–	1.000	SNP5
SNP1	0.000	0.000	0.000	0.000	0.000	–	SNP6
r^2	SNP6	SNP5	SNP4	SNP3	SNP2	SNP1	–
JX breed: D′ and r^2: SNP1/SNP2/SNP3/SNP4/SNP5/SNP6							
–	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	D′
SNP6	–	1.000	1.000	1.000	0.996	0.996	SNP1
SNP5	1.000	–	1.000	1.000	0.996	0.996	SNP2
SNP4	0.007	0.007	–	1.000	0.002	0.002	SNP3
SNP3	0.000	0.000	0.002	–	1.000	1.000	SNP4
SNP2	0.017	0.017	0.000	0.001	–	1.000	SNP5
SNP1	0.017	0.017	0.000	0.001	1.000	–	SNP6
r^2	SNP6	SNP5	SNP4	SNP3	SNP2	SNP1	–

Note: values of upper triangular show D' and values of lower triangular represent r^2.

Table 6. The characteristics of three mutations in the *Nanog* gene in cattle. The numbers in the "traits" column indicate age in months.

Traits (NY)	Genotypes (SNP3 G>A)		*P*
	GG	AA	
BSL-6 (cm)	106.79 ± 0.81^{a}	103.25 ± 1.39^{b}	0.022
CC-6 (cm)	130.81 ± 1.17^{a}	126.38 ± 1.62^{b}	0.028
Traits (NY)	Genotypes (SNP4 A>T)		*P*
	AA	AT	
BH-6 (cm)	106.29 ± 0.75^{a}	102.30 ± 1.58^{b}	0.031
HW-18 (cm)	23.30 ± 0.27^{a}	21.95 ± 0.36^{b}	0.035
Traits (JX)	Genotypes (SNP5 C>T)		*P*
	CC	CT	
BH (cm)	126.69 ± 0.75^{a}	124.31 ± 0.60^{b}	0.028
CaC (cm)	17.71 ± 0.15^{A}	16.98 ± 0.13^{B}	0.002

Note: $^{a, b}$ Values with different superscripts in the same row differ significantly at $P < 0.05$. $^{A, B}$ Values with different superscripts in the same row differ significantly at $P < 0.01$. BSL – body slanting length; CC – chest circumference; BH – body height; HW – hucklebone width; CaC – cannon circumference.

Table 7. Relationship between the combined genotypes of two loci and phenotypes in Nanyang cattle. The numbers in the "traits" column indicate age in months.

Traits	SNP3-SNP4				*P*
	GG-AA (31)	GG-AT (5)	AA-AA (16)	AA-AT (5)	
BH-6 (cm)	107.81 ± 0.75^{a}	102.60 ± 2.34^{b}	103.94 ± 1.54^{b}	102.00 ± 2.39^{b}	0.011
BSL-6 (cm)	107.71 ± 0.91^{a}	103.20 ± 3.25^{ab}	103.38 ± 1.84^{b}	101.60 ± 2.38^{b}	0.038
CC-18 (cm)	157.45 ± 1.26^{a}	148.20 ± 4.34^{b}	154.06 ± 1.57^{ab}	153.80 ± 2.60^{ab}	0.041

Note: means ± standard errors of measurement traits of Nanyang cattle with combined genotype (SNP3–SNP4). [a, b] Values with different superscripts in the same row differ significantly at $P < 0.05$. BH – body height; BSL – body slanting length; CC – chest circumference.

SNP3-GG and SNP4-AA was found to have a positive effect on body height and body slanting length in 6-month-old NY. Moreover, the combined genotype GG-AA had better chest circumference in 18-month-old NY (Table 7).

4 Discussion

The familiar function of *Nanog* is that it regulates the pluripotency of ESCs and induces pluripotent stem cell formation (Komatsu et al., 2015). Previous reports showed that iPSC-related *Klf4* affected adipogenesis through interacting with other genes. Equally, *Klf7* also affected bovine growth traits and the quality of milk (Ma et al., 2011). The examples above were evidence to suppose that iPSC-related *Nanog* may influence the phenotypes. Therefore, we checked the effects of six SNPs in the *Nanog* gene on body measurement traits in cattle in this study.

We found six novel exonic SNPs in four exons of the *Nanog* gene. Through the χ^2 test, SNP1 and SNP2 in all cattle breeds were in HWE; SNP1 and SNP2 did not affect growth traits, and artificial selection was not performed among all breeds. SNP3, SNP4, SNP5, and SNP6 were not in HWE in different cattle breeds. The explanation was that artificial selection was performed to acquire better characteristics, and the genetic backgrounds of breeds were different. Although SNP6 was not associated with growth traits, SNP5 and SNP6 were in a strong LD, leading to both of them being selected in artificial selection. The common Hap4 had the highest frequency in both NY and JX, perhaps because Hap4 was present in these breeds for a long time (Zhang et al., 2015). The frequencies of other haplotypes were different, which may be due to variety distinctiveness (Zhang et al., 2015). SNP5 and SNP6 were in a strong LD in NY and JX, and SNP1 and SNP2 showed strong linkage only in JX. NY and JX belong to special Chinese breeds, but they were raised in different environments, which caused their different genetic backgrounds and heritability. SNP4 caused 189Met to be changed into 189Leu so as to affect measurement traits in NY. SNP3 and SNP5 were synonymous mutations, not changing the sequence of amino acids. However, the growth traits of individuals with SNP3 or SNP5 were better than those of the wild type (Table 2). Studies have reported that synonymous codon influenced nucleic acid stability, protein levels, structure, and function without altering the amino acid sequence (Bali and Bebok, 2015). The combined genotype of SNP3-GG and SNP4-AA had a positive effect on body height, body slanting length, and chest circumference. Probably, 6-month-old NY with SNP3-GG and SNP4-AA could be selected to obtain better body height and body slanting length; 18-month-old NY could be selected for better chest circumference. Both SNP3 and SNP4 affected the growth traits in this research.

The association testing showed that the *Nanog* gene had a remarkable effect on growth traits. As far as we know, we are the first to research the relationship between the *Nanog* gene and growth characteristics. For a better comprehension of this study, the possible explanations are listed as follows:

1. The SNP4 was a missense mutation: 189Met > Leu. The missense mutation with the amino acid change could affect protein structure, resulting in a loss of normal function.

2. *Nanog* combines with the binding site of the GDF3 promoter and activates the expression of GDF3 in humans, which belongs to TGF-β family and controls the bone morphogenic protein (McPherron and Lee, 1993; Park et al., 2012). GDF3 is also expressed in spleen, thymus, fat, and bone marrow (Hexige et al., 2005) and increases the adipose accumulation in a high-fat diet in mice, via the receptor ALK7 and ALK4 expressing in adipose tissue (Andersson et al., 2008). We speculated that the mutations of the *Nanog* gene in the coding with a protein structure change possibly regulated GDF3 expression, further impacting growth traits in cattle.

3. *Nanog* also inhibits the transcriptional effectors of both the bone morphogenetic protein and NF-kappa B pathways in ESCs (Li et al., 2016). Maybe the expression of *Nanog* was changed or reduced gradually with age, removing the inhibition of bone morphogenetic protein and NF-kappa B pathways in calves.

4. GLI1 and GLI3 were able to bind to *Nanog*. The presence of *Nanog* inhibited GLI transcription, therefore inhibiting Hedgehog signaling that regulated the homeostasis of several types of adult tissue (Li et al., 2016). Possibly, *Nanog* was also changed or reduced and removed the inhibition of GLI1.

MAS would develop and improve cattle products. Analyzing novel nucleotide variations of the candidate genes and detecting their associations with economic traits are significant for cattle breeding and genetics (Zhang et al., 2015). Novel SNPs of *Nanog* have benefits for implementing MAS in genetics and the breeding of cattle. The material mechanisms of *Nanog* acting on genes and signal paths are unclear and need to be explored further.

5 Conclusions

To summarize, six novel genetic variations (SNP1–6) were found in this study. Among these, SNP3, SNP4, and SNP5 were associated with growth traits in NY and JX which were beneficial to cattle breeding and genetics through MAS.

6 Data availability

The original data are available upon request to the corresponding author.

Appendix A: Abbreviations

GDF3	growth and differentiation factor 3
Klf4	Krüppel-like factor 4
TGF-β	transforming growth factor β
iPSCs	induced pluripotent stem cells
SNP	single nucleotide polymorphism
MAS	marker-assisted selection
bp	base pair
SPSS	statistical product and service solutions
PCR	polymerase chain reaction
SSCP	single-stranded conformational polymorphism
PCR-RFLP	polymerase chain reaction–restriction fragment length polymorphism
HWE	Hardy–Weinberg equilibrium
Ho	homozygosity
He	heterozygosity
Ne	effective allele numbers
PIC	polymorphism information content
LD	linkage disequilibrium
QC	Qinchuan cattle
NY	Nanyang cattle
JN	Jinnan cattle
JX	Jiaxian cattle
BH	body height
BSL	body slanting length
CC	chest circumference
HW	hucklebone width
CaC	cannon circumference

Conflict of interest

All authors have no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author contributions. Meng Zhang conceived the study and wrote the paper. Chuanying Pan performed the experiments. Qin Lin and Shenrong Hu analyzed the data; Ruihua Dang did statistical analysis; Chuzhao Lei and Hong Chen collected the samples; Xianyong Lan edited and reviewed the manuscript.

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