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The novel 22 bp insertion mutation in a promoter region of the *PITX2* gene is associated with litter size and growth traits in goats

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Abstract. The paired-like homeodomain 2 (*PITX2*) gene plays a critical role in regulating development, reproduction, and growth traits in ruminants. Hence, the objective of this study was to explore the polymorphisms of this gene and to evaluate their associations with quantitative traits. Herein, a novel insertion in the promoter region of the *PITX2* gene was reported in Shaanbei white cashmere (SBWC) goats ($n = 1012$). The genotype distributions between mothers of single-kid and multi-kid groups within SBWC goats were significantly different ($P < 0.01$), implying that this indel mutation might affect the litter size. Furthermore, association analysis found that this indel mutation was significantly associated with litter size ($P = 0.001$). Individuals with genotype DD had a significantly smaller litter size than those with other genotypes ($P < 0.01$). Besides, this indel was significantly associated with the body length ($P = 0.042$) and the chest width ($P = 0.031$). Especially, the individuals with genotype DD had a significantly lower body length than those with genotype II ($P < 0.05$), which was consistent with the trend in litter size. These findings suggested that the new 22 bp indel mutation within the *PITX2* gene is significantly associated with litter size and growth traits; this can be utilized as a functional molecular marker in goat breeding.

1 Introduction

The *PITX2* gene was originally identified as the gene associated with human Rieger syndrome, an autosomal dominant disease (Hassed et al., 2017). Subsequently, this gene's additional functions were studied widely. The available studies have shown that the *PITX2* gene is important for asymmetric development of organs in animals (Welsh et al., 2013; Syeda et al., 2017). Thus, this gene is essential for the normal physiological functioning in animals (S. H. Zhang et al., 2018). In addition to Rieger syndrome, mutation or misregulation of *PITX2* can also cause other diseases such as atrial fibrilla-

tion and congenital glaucoma (Yin et al., 2014; Zhou et al., 2015; Chen et al., 2016; Li et al., 2016). Furthermore, reports have also revealed that the *PITX2* gene is important in muscle formation and development. In the mouse model, *PITX2* together with similar upstream regulators, *PAX3* and *PAX7*, participates in gene regulatory networks at different skeletal myogenesis sites (Margaret and Peter, 2014). Likewise, the *PITX2* gene can modulate fetal and adult myogenesis by controlling redox conditions and regulating MyoD, respectively (L'Honoré et al., 2010; Hernandez et al., 2017).

In addition to muscle development, evidence shows that the *PITX2* gene plays irreplaceable roles in signal pathways

such as Wnt/β-catenin and *POU1F1–PROP1*. (1) In Wnt/β-catenin signal pathway, the *PITX2* gene not only activates the canonical pathway by itself and by inducing Wnt ligands but also limits further Wnt activation by down-regulating frizzled receptors. The *PITX2* gene can also mediate mRNA stabilization in the Wnt/β-catenin pathway (Basu et al., 2013). Essential role played by Wnt/β-catenin signal pathway in regulation of progenitor cell fate, development decisions, proliferation during embryonic development, and adult tissue homeostasis is known (Maryam et al., 2018). Consequently, the *PITX2* gene plays a role in diverse functions through Wnt/β-catenin signal pathway. (2) In *POU1F1–PROP1* signal pathway, the *PITX2* gene regulates transcription and expression of genes such as *POU1F1*, *LHX3*, and *PROP1*, which influence the secretion of growth hormone (GH), prolactin (PRL), etc. (Lan et al., 2007; Zhao et al., 2017). These hormones are essential for growth, development, and reproduction of animals; thus, we speculate that the *PITX2* gene may affect production and reproduction in animals.

In terms of reproduction, previous studies have shown that the *PITX2* gene also regulates twinning (Angela et al., 2014), owing to *PITX2* gene function in embryonic axis formation, and influences on embryo polarity. Therefore, *PITX2* is a potential candidate gene for maker-assisted selection (MAS) in breeding and genetics. Currently, there are few studies about this gene and its effects on prolificacy and growth traits in goats.

Shaanbei white cashmere (SBWC) goat is a breed developed by crossing a Liaoning cashmere goat (male parent) and a Shaanbei black goat (female parent) and is characterized by resistance to rough feeding, cold weather, wind and disease; these goats are widely raised in northern Shaanxi province (Yang et al., 2017; Yan et al., 2018; Cui et al., 2018a). However, there are some problems in the breeding process such as thickening of cashmere fiber, decrease of the cashmere yield, and production of single kid, all of which reduce the economic benefit of cashmere goats. This study was conducted to detect possible novel polymorphisms within the *PITX2* gene and to evaluate their effects on litter size and growth traits in SBWC goats.

2 Materials and methods

All experimental procedures were approved by the institutional animal care and use committee (IACUC) of Northwest A&F University (NWAFU). The use of experimental animals was in accordance with the local animal welfare laws, guidelines, and policies.

2.1 DNA samples and related data collection

Ear tissue samples were obtained from 1012 unrelated female SBWC goats (2–5 years old), which were reared on the different farms, under similar managemental and feeding programs, and under normal conditions, in central Yulin,

Shaanxi, China (Wang et al., 2017; Yang et al., 2017; Cui et al., 2018a; Yan et al., 2018). The feeding programs were the following: all ewes were mated with rams at a ratio of 18–24 to 1. All the kids were continuously kept with their dams up to weaning at the age of 3 months (Wang et al., 2018). Furthermore, litter size (LS) at the first birth as well as growth traits including body height (BH), body length (BL), chest width (CW), chest circumference (CC), and chest depth (CD) were measured and recorded (Wang et al., 2017; Yang et al., 2017; Zhao et al., 2017; Yan et al., 2018).

2.2 Genomic DNA isolation and DNA pool construction

All genomic DNA samples isolated from ear tissues, using standard salt–chloroform extraction method (Aljanabi et al., 1997; Sambrook and Russell, 2001; Lan et al., 2007; Zhou et al., 2016; Zhang et al., 2017), were quantified by NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) and then diluted to 50 ng/μL as working solutions (Chen et al., 2016). Fifty DNA samples were randomly selected to construct genomic DNA pool for exploring genetic variation in the *PITX2* gene (Jin et al., 2016).

2.3 Primer design and PCR amplifications

In this work, two indel mutations were obtained from NCBI, which were named rs670348258 (in promoter region) and rs655548222 (in intron). Hence, a total of four primer pairs were designed using Primer Premier 5.0 software (Primer, Canada), based on the reference sequence (GenBank accession number NC_030813.1) (Table 1). Among them, primer pair P1 was used to detect the variations in rs670348258, and the others were used for those in rs655548222 (Table 1). All primer pairs were synthesized by Sangon Biotech (Xian, Shaanxi, China). Polymerase chain reaction (PCR) was performed in 12.5 μL reaction mixture, containing 1.5 μL (10 ng/μL) of genomic DNA, 0.5 μL of each primer, 6.0 μL $2 \times Taq$ Master Mix, and 4.0 μL ddH$_2$O. Two thermal cycling programs were used in this study. The first program involved pre-denaturation for 5 min at 95 °C followed by 34 cycles of denaturation at 94 °C for 30 s; annealing for 30 s at 52 °C, elongation for 15 s at 72 °C; and a final extension at 72 °C for 10 min. The second program, touch-down PCR (TD-PCR), included pre-denaturation for 5 min at 95 °C; followed by 18 cycles of denaturation at 94 °C for 30 s; annealing for 30 s at 68 °C (with a decrease of 1 °C per cycle); elongation for 15 s at 72 °C; and final extension at 72 °C for 10 min (Cui et al., 2018a; Y. H. Zhang et al., 2018). Finally, 4.0 μL PCR products were directly assayed by electrophoresis on 3.0 % agarose gels stained with ethidium bromide.

Table 1. Primers used for detecting goat *PITX2* indel variants.

Indel names	Loci	Location	Primer sequences (5′ to 3′)	Sizes (bp)	Tm
rs670348258	P1	promoter	F1: GCTGGAAGCAGTTCGTCA R1: GGGTTGATAGCCTTGTATGTT	131/153	60.5 °C
	P2	intron	F2: ATCCCACCCTTTTCGGCTTT R2: TTCCTCTTTGCACCCGACAG	140	TD-PCR
rs655548222	P3	intron	F3: GTCTCCGGAACCCTTTGGAA R3: TCCAGCCAAGTGGGGAATGA	131	TD-PCR
	P4	intron	F4: AGCGCAGACCTATGTTCAGG R4: ATACACAAAAGCCGCCGTCT	498	TD-PCR

2.4 Statistical analysis

Genotypic and allelic frequencies were calculated manually. Hardy–Weinberg equilibrium (HWE) analysis was performed using the SHEsis program (http://analysis.bio-x.cn, last access: 6 August 2018) (Yan et al., 2018). The parameters of population genetics are the following: heterozygosity (He), homozygosity (Ho), effective allele number (Ne) and polymorphism information content (PIC) were calculated by PopGene version 1.3.1 (Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, AB, Canada) (Wang et al., 2018).

Distribution differences, for genotypic frequencies, between the mothers of a single kid and multiple kids were analyzed by χ^2 test (Li et al., 2018). The association of indel polymorphism with several traits was measured. These associations were analyzed when the data agreed with the characteristics of normality and homogeneity of variance (ANOVA). The non-parametric Tamhane test was also carried out. ANOVA applied a general linear model (GLM) and the reduced linear model as follows:

$$Y_i = u + G_i + e, \qquad (1)$$

where Y_i is the trait measured for each animal, u the mean for each trait, G_i the fixed effect of genotype or combined genotype, and e the random error (Wang et al., 2018; Yang et al., 2017; Cui et al., 2018a; Yan et al., 2018).

Besides, genetic effects of different genotypes on litter size were also evaluated under different genetic models: codominant, dominant, recessive, and overdominant. The Akaike information criterion (AIC) was used to confirm which genetic effect model was used for the data (EI-Bayomi et al., 2018). The model with the smallest AIC value was probably the best fit based on the evaluation results. All the above analyses were carried out using SPSS software (version 18.0, IBM, USA). The results are expressed as means ± SE. Differences between the means were considered significant at $P < 0.05$ (Ren et al., 2017).

3 Results

3.1 Identification of a novel 22 bp indel variation

A novel 22 bp insertion was uncovered, using DNA pool sequencing (Pak et al., 2002), 967 bp upstream from the first exon of the goat *PITX2* gene, and was described as NC_030813.1: g.15140256-15140257 ins AAGC-CCTTGCTGGAGTAAACAC (Fig. 1). The novel indel sequence was completely different from the sequence registered as NCBI-dbSNP (AAACACAAGCCCTTGCTG-GAGT), and the current position of the indel is 6 bp behind the predicted indel mutation provided by NCBI. Electrophoretic analysis showed that there were three genotypes (Fig. 1).

3.2 Genetic parameter analysis of 22 bp indel

Frequency distributions of the three genotypes and two alleles are summarized in Table 2, in addition to gene homozygosity (H_O), heterozygosity (He), effective allele number (Ne), and the polymorphism information content (PIC). The frequency of the DD genotype (0.425) was the highest in the studied population of SBWC goat. SBWC goat population was not at HWE ($P < 0.05$). The value of PIC (0.358) showed that it had medium genetic diversity ($0.25 < PIC < 0.5$) at the 22 bp indel locus.

3.3 Intra-group analysis of single-kid and multi-kid individuals in SBWC goats

The independent intra-group χ^2 test of 1012 female, goats (Table 3) showed that litter size was significantly correlated with different genotypic frequencies ($\chi^2 = 19.359$, d$f = 2$, $P = 6.70 \times 10^{-7}$).

3.4 Association analysis between indel genotypes and litter size and growth traits in SBWC goats

The association between 22 bp indel in the *PITX2* gene and litter size is shown in Table 4. Individuals with genotype DD had a significantly smaller litter size than those with
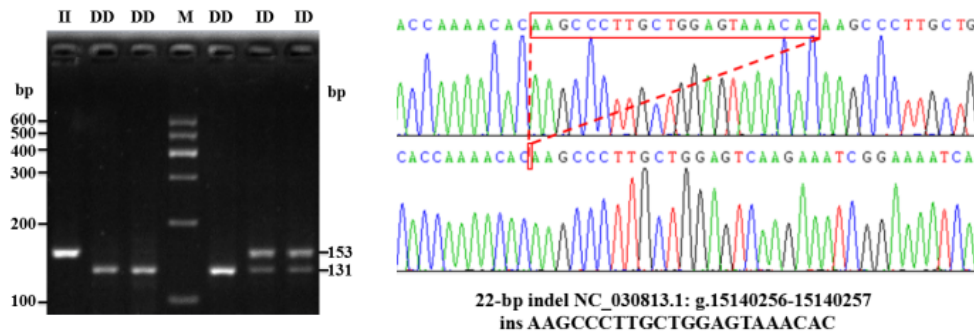
Figure 1. Agarose gel electrophoresis and DNA sequencing of 22 bp indel of the goat *PITX2* gene.

Table 2. Genotypic and allelic frequencies as well as population genetic indices of *PITX2* gene in Shaanbei white cashmere (SBWC) goats.

Genotypic and allelic frequencies					*P* value	Population genetic indexes			
Genotypes (*n* = sample sizes)			Alleles		(HWE)	Ho	He	Ne	PIC
II	ID	DD	I	D	*P* < 0.05	0.532	0.468	1.880	0.358
0.172	0.403	0.425	0.374	0.626					
(*n* = 174)	(*n* = 408)	(*n* = 430)							

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

other genotypes ($P < 0.01$). Likewise, the association of the indel and some growth traits was shown in Table 4. The analysis showed that this indel was significantly associated with the body length ($P = 0.045$) and the chest width ($P = 0.031$). The individuals with genotype DD had a significantly lower body length than the individuals with genotype II ($P < 0.05$), which was consistent with the trend observed in litter size. However, the individuals with genotype DD had a significantly higher chest width than the individuals with genotype II ($P < 0.05$).

3.5 Genetic effects of different genotypes on litter size under different models

According to the EI-Bayomi description (EI-Bayomi et al., 2018), the logistic regression analysis revealed a significant association between the insertion mutation and litter size in the codominant model ($P = 1.5 \times 10^{-5}$), dominant model ($P = 1.9 \times 10^{-5}$), recessive ($P = 0.011$), and overdominant model ($P = 0.019$) (Table 5). In addition, codominant model had the smallest AIC value (AIC $= -1517.742$).

4   Discussion

Indel mutations of the critical genes such as *SPEF2* gene (Chen et al., 2016), *GHR* gene (Yang et al., 2017), *GDF9* gene (Wang et al. 2017), *LHX4* gene (Yan et al., 2018), *KDM5B* gene (Cui et al., 2018b), *KDM6A* gene (Cui et al., 2018a), and *CSN1S1* gene (Wang et al., 2018) are widely known to influence production traits in animals. Several

SNPs in introns and exons within the *PITX2* gene have been reported in goats, but to the best of our knowledge indel polymorphisms of this gene have not been reported until now. According to previous studies, the *PITX2* gene has a significant effect on milk production traits in dairy goats (Zhao et al., 2013). In this study a novel indel within the promoter of the *PITX2* gene in goats was explored. To explore the correlation between the 22 bp indel and some economic traits, a large sample of goats ($n = 1012$) was analyzed. This indel locus showed intermediate polymorphism as shown by the PIC values ($0.25 < PIC < 0.5$). The tested SBWC population was not in accordance with Hardy–Weinberg equilibrium, suggesting that populations were not in dynamic equilibrium probably due to artificial selection, migration, and genetic drift (Zhang et al., 2015).

The influence of the indel on litter size was analyzed in two steps. First, intra-group χ^2 test was utilized to analyze whether the litter size was significantly correlated with different genotypes. Results indicated that the genotypic distribution is associated with litter size because, multi-kid-mother individuals showed a higher frequency of II and ID genotypes than single-kid-mother individuals. Furthermore, association analysis showed that it was not only associated significantly with the litter size ($P = 0.001$) but also with the body length ($P = 0.045$) and the chest width ($P = 0.031$). Together, the individuals with II showed better performance in litter size and body length, which was consistent with our previous reports in the same breed (Wang et al., 2017; Yang et al., 2017; Yan et al., 2018). Interestingly, the lo-

Table 3. Genotypic distribution between mothers of single-kid and multi-kid Shaanbei white cashmere (SBWC) goats.

Types	Sample sizes	Genotypes			Genotypes frequencies			χ^2 (df), P value
		II	ID	DD	II	ID	DD	
Single-kid	661	99	249	313	0.150	0.377	0.473	$\chi^2 = 19.359$ (d$f = 2$),
Multi-kids	351	75	159	117	0.214	0.453	0.333	$P = 6.700 \times 10^{-7}$

Note: single-kid, individuals that give birth to a single kid; multi-kid, individuals that give birth to more than one kid.

Table 4. The relationship between three genotypes of the *PITX2* indel variants and traits in Shaanbei white cashmere (SBWC) goats.

Traits	Different genotypes (mean ± SE)			P values
	II	ID	DD	
LS	1.44 ± 0.04^A	1.40 ± 0.03^A	1.27 ± 0.02^B	$P = 0.001$
BH	57.62 ± 0.37	57.10 ± 0.27	56.54 ± 0.23	$P = 0.236$
BL	68.02 ± 3.47^a	64.35 ± 0.30^{ab}	64.42 ± 0.31^b	$P = 0.045$
CW	17.86 ± 0.22^b	18.05 ± 0.18^{ab}	18.57 ± 0.17^a	$P = 0.031$
CC	84.52 ± 0.67	87.19 ± 1.90	85.84 ± 0.54	$P = 0.395$
CD	27.84 ± 0.23	28.88 ± 0.86	28.11 ± 0.15	$P = 0.362$

Note: BH, body height; BL, body length; CC, chest circumference; CD, chest depth; CW, chest width; LS, litter size. The value with different letters ([a] and [b]) within the same row differ significantly at $P < 0.05$. The value with different letters ([A] and [B]) within the same row differ significantly at $P < 0.01$.

gistic regression analysis uncovered a significant association between the insertion mutation and litter size in different models. The best fit model with smallest AIC value was the codominant model.

The significant effects of the 22 bp indel on reproductive traits might be explained by the function of the *PITX2* gene: first, as a member of *POU1F1–PROP1* pathway, the *PITX2* gene regulates transcription and expression of other genes such as *POU1F1*, *LHX3, PROP1, GH*, and *PRL* (Zhang et al., 2015; Bertko et al., 2017; Zhao et al., 2017). Second, the *PITX2* gene regulates Wnt/β-catenin pathway, affecting embryo implantation (Yusuke et al., 2006; Basu et al., 2013; Yeonsoo et al., 2015); therefore, this might be linked to litter size. Third, the *PITX2* gene also regulates multiplets by influencing embryo polarity and axis formation (Angela et al., 2014). For example, in the chick embryo, the *PITX2* gene is the direct regulator of *Vg1* expression, and localized expression of *Vg1* gene is responsible for formation of embryo poles (Angela et al., 2014). Deletion of *Vg1* gene in zebrafish embryo was reported to cause reproductive failure (Tessa et al., 2017). Finally, our analysis of the goat *PITX2* gene via the Promoter 2.0 Prediction Server showed the location of the indel. In the sequence of I allele, the indel was located 967 bp upstream from the first exon in the promoter region, which includes the 945–1045 bp region upstream from the first exon; however, in the sequence of D allele, the indel was not located in a promoter region (545–645 bp upstream from the first exon). This indicated that the indel may influence

the mechanism of the goat *PITX2* promoter and the hypothesis should be verified by more studies. It is well known that promoters are essential in regulating the level of gene expression and controlling the expression in a spatial and temporal manner (Qiu et al., 2015). Therefore, the indel locus in the promoter region can influence gene expression and, consequently, certain economic traits in many species such as porcine (Cieslak et al., 2013) and cattle (Jin et al., 2016).

Additionally, the significant effect of the 22 bp indel on growth traits can also be explained by the function of the *PITX2* gene: the *PITX2* gene is important in muscle formation and development, and it can influence secretion of hormones such as GH and PRL. Further, the dominant genotypes of some key genes in SBWC goats can improve not only the physical traits but also the litter size. For example, Wang et al. (2017) found that there was a significant relationship between the litter size and body height in the individuals with same genotypes while exploring a 12 bp indel polymorphism within the goat *GDF9* gene. Yang et al. (2017) reported that the dominant genotype of the 14 bp duplicated indel within the *GHR* gene simultaneously and significantly affected litter size and growth traits. Yan et al. (2018) found that a 12 bp deletion within the goat *LHX4* gene significantly affected litter size. Variants of *LHX4* gene were also associated with growth traits (Ren et al., 2010). Therefore, all of the evidence indicates that different alleles have a similar potential advantage in marker-assisted selection (MAS).

Table 5. Different genetic models of *PITX2* indel in mothers of single-kid and multi-kid Shaanbei white cashmere (SBWC) goats.

Inheritance model	B	*P* values	OR (95 % CI)	AIC
Codominant (II / ID / DD)	−0.058	1.5×10^{-5}	0.944 (0.920–0.969)	−1517.742
Dominant (II + ID / DD)	−0.587	1.9×10^{-5}	0.556 (0.425–0.728)	−1517.255
Recessive (ID + DD / II)	0.433	0.011	1.543 (1.106–2.152)	−1505.244
Overdominant (ID / II + DD)	−0.315	0.019	0.730 (0.561–0.949)	−1504.205

Note: AIC: Akaike information criterion; B: regression coefficient; CI: confidence interval; OR: odd ratio.

In summary, the novel indel mutation within the goat *PITX2* gene was significantly associated with litter size and growth traits, which could be used as an effective molecular marker for improving economic traits in the goat industry. The molecular mechanism is limited in this study, and it will be verified by subsequent studies.

Data availability. The original data of the paper are available upon request from the corresponding author.

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