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A novel 12 bp deletion within goat *LHX4* gene significantly affected litter size

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Abstract. The LIM homeobox transcription factor 4 (*LHX4*) gene plays a critical role in regulating the development of the pituitary and the secretion of growth hormone (GH) and prolactin (PRL) associated with reproduction. Thus this gene may affect litter size. Herein, the aim of this study is to detect the novel insertion/deletion (indel) within the *LHX4* gene as well as to test its association with litter size in 1149 Shaanbei white cashmere goats. Herein, a novel 12 bp indel (NC_030823.1:g.60001011_60001022delGGGGAGGAGGGG) was firstly found, which was located in the first intron. Meanwhile, three genotypes were detected in Shaanbei white cashmere goats, and the allelic frequencies of I and D were 0.593 and 0.407, respectively. Interestingly, the genotype distributions between mothers of single-lamb ($n = 895$) and multi-lamb ($n = 254$) groups within Shaanbei white cashmere goats were significantly different, implying that the 12 bp indel might affect the litter size. Furthermore, the association analysis was carried out to find out that the 12 bp indel was significantly associated with litter size in the analyzed goat population ($P < 0.05$). The litter sizes of genotype DD and ID individuals were superior to those of genotype II ($P < 0.05$). These findings suggest that this locus could be considered as a genetic marker for goat breeding, enriching the research category of functional genome of goats.

1 Introduction

Along with the rapid development of "The Belt and Road" policy and improvement of people's living standards, the demand for goat products is increasing in numerous developing countries, especially in China. However, China is experiencing a severe shortage of goat products. Litter size, as one of the most important reproductive and economic traits, is a very critical factor for increasing goat industry. However, it is difficult to improve litter size rapidly using traditional methods because the small size is controlled by multiple genes. Thus using DNA selection via related genes is becoming more and more necessary (Zhang et al., 2015). To date, many important potential molecular markers for goat marker-assisted selection (MAS), including single nucleotide polymorphism (SNP) and insertion/deletion (indel), have been performed by previous studies. For example, the goat *INHA* 651A/G polymorphism significantly affected the litter size in Boer goats (Wu et al., 2009). The polymorphism in the promoter region of the *KISS1* gene has a notable correlation with the litter size (An et al., 2015a). Additionally, FTH1, growth hormone (GH), and serum amyloid A (SAA) genes were significantly associated with high litter size in Jining Grey goats (Feng et al., 2015). Most studies concentrated on SNP related to litter size trait (Li et al., 2008; An et al., 2013a), however, few on indel. For the indel marker, there were several advantages for MAS breeding, such as simple operation, rapid de-

tection, and easy utilization (Jin et al., 2016; M. Zhang et al., 2016). Hence, it is necessary to find the novel indel within the candidate genes associated with litter size in goat industry in the future.

As a member of the LIM-HD gene family, the LIM homeobox transcription factor 4 (*LHX4*) gene plays an important role in regulating the development of the pituitary and nervous system, as well as in participating in the *LHX3–LHX4–PROP1–POU1F1* pathway (Wu et al., 1998; Sloop et al., 2000). Notably, the pituitary is one of the most important endocrine glands of the hypothalamic–pituitary–gonadal (HPG) axis and has a critical effect on reproduction. Thus, it could be indicated that the *LHX4* gene is an excellent candidate gene for reproductive traits in mammals. Previous studies have manifested that the *LHX4* gene regulates the secretion of hormones, such as follicle-stimulating hormone (FSH), GH, luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and prolactin (PRL) by acting on the pituitary gland directly or indirectly. These hormones regulate growth and metabolism, reproductive development, and so on in humans and livestock (Mullena et al., 2007). Deficiency of the *LHX4* and other genes (such as *LHX3*, and *Pitx2*) renders combined pituitary hormone deficiency (CPHD) and pituitary hypoplasia in both humans and mice (Raetzman et al., 2002; Hunter and Rhodes, 2005; Pfaeffle et al., 2008), suggesting that the *LHX4* gene has a significant influence on stimulating the rapid proliferation of undifferentiated pituitary progenitors via activating *LHX3* and maintaining expression of *Pitx2* in mice (Gergics et al., 2015). Moreover, the mutations of the *LHX4* gene are also associated with dominantly inherited GH deficiency. To date, *LHX4*-driven pathway could have influenced the expression of GH (Machinis and Amselem, 2005), POU1F1, PRL, and other genes which are closely related to reproduction of livestock (Wu and Xu, 2000; Lan et al., 2007; Yang et al., 2017). Therefore, the *LHX4* gene was possibly associated with CPHD and reproduction traits in livestock.

To date, the polymorphisms of the bovine *LHX4* gene have been found, and they were associated with growth traits (Ren et al., 2014). A missense mutation within the goat *LHX4* gene was reported, but its function was unknown (Li et al., 2008). Briefly, little information about the *LHX4* gene indel variants and its association with reproduction traits was found. Therefore, in this work, the novel indel mutation of the *LHX4* gene in a Chinese indigenous goat breed was detected, and its association with litter size was analyzed, which would benefit the acquisition of potential useful DNA markers for goat MAS breeding, more so than pushing "one belt and one road" in goat production.

2 Material and methods

All experimental animals in this study were approved by the Institutional Animals Care and Use Committee (IACUC) of Northwest A&F University (NWAFU). Furthermore, the use of experimental animals was in compliance with the local animal welfare laws, guidelines, and policies.

2.1 DNA samples and related data collection

The ear tissue samples from a total of 1149 Shaanbei white cashmere goats were obtained from a farm in central Yulin in Shaanxi Province (Wang et al., 2017; Yang et al., 2017). All the goats were reared on the same farm under normal conditions. Furthermore, the litter size of Shaanbei white cashmere goats in the first birth was recorded.

2.2 DNA extraction and genomic DNA pool construction

DNA samples were isolated from ear tissues using the approach of high salt extraction and diluted to a specific concentration (10 ng µL) (Yang et al., 2017). Fifty DNA samples were randomly selected and mixed into the PCR tube, which could be used as templates to scan the indel mutation in PCR amplification.

2.3 Primer design, PCR amplification, and DNA sequencing

According to the sequence of goat *LHX4* gene (GenBank accession number N_030823.1) in NCBI (www.ncbi.nlm.nih.gov), only one putative indel sequence was provided. Hence, in this work, a pair of primers (F: 5′-AGCGAGGCAAGGCTGAAC-3′; R: 5′-GGGTCCTACATCCCAAGAAA-3′) were designed using Primer Premier 5.0 software (Premier Biosoft International USA) and synthesized by Sangon Biotech (Shanghai, China).

The PCR amplification was performed in a 12.5 µL of reaction volume containing 1.5 µL genomic DNA (10 ng µL), 0.5 µL of each primer, 6 µL 2 × *Taq* Master Mix (BioLinker, Shanghai, China), 4 µL ddH_2O. The Touch-Down PCR (TD-PCR) program was performed as described previously: initially denatured at 95 °C for 5 min; 2 cycles of 94 °C for 30 s; annealing at 68 to 51 °C for 30 s (with a decrease of 3 °C per 2 cycles); extended at 72 °C for 40 s; a final extension at 72 °C for 10 min and cooling to 10 °C (Xu et al., 2017). The amplification product was detected by 2.5 % agarose gel electrophoresis in 1 × TBE with constant voltage (120 V) for about 55 min. At last, agarose gel was stained with ethidium bromide and the PCR product was observed.

2.4 Statistical analysis

Genotypic and allelic frequencies of the goat *LHX4* gene were calculated directly. Hardy–Weinberg equilibrium (HWE) was performed by the SHEsis program (http://analysis.bio-x.cn) (Li et al., 2009). Heterozygosity (He), homozygosity (Ho), effective allele numbers (Ne), and polymorphism information content (PIC) were calculated follow-

Table 1. Genotypic and allelic frequencies and other population indexes in the Shaanbei white cashmere goat *LHX4* gene.

Size	Genotypic frequency			Allelic frequency		*P*(HWE)	Ho	He	Ne	PIC
	II	ID	DD	I	D					
1149	0.345	0.496	0.159	0.593	0.407	$P > 0.05$	0.517	0.483	1.934	0.366

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

Table 2. The genotype distribution between mothers of a single lamb and multiple lambs in Shaanbei white cashmere goats.

Types	Sample sizes	Genotype numbers			Genotype frequencies			Independent χ^2 value, df, P value
		II	ID	DD	II	ID	DD	
Mothers of a single lamb	895	329	435	131	0.368	0.486	0.146	$\chi^2 = 11.242$
Mothers of multiple lambs (≥ 2)	254	67	135	52	0.264	0.531	0.205	df = 4
								$P = 0.036$

ing Nei's method (Nei, 1973) and performed on the program PopGene3.2. Distribution differences for genotypic frequencies between the mothers of a single lamb and multiple lambs were analyzed using the χ^2 test, which was carried out using SPSS software (version 21.0) (IBM Corporation, New York, USA). Meanwhile, a different genotype was considered as an independent variable and litter size was used as the dependent variable using the software program SPSS for correlation analysis. The ANOVA applied to the general liner model (GML) was simplified as follows: $Y_{ij=\mu} + G_i + R_j + P_k + e$, where Y_{ij} is the observation of the litter size, μ is the overall average number of litter size, G_i is the fixed effect of genotype or combined genotype, R_j is the effect of lambing year, P_k is the fixed effect of the parity, and e stands for random residual error (He et al., 2014; Wang et al., 2014; Yang et al., 2016).

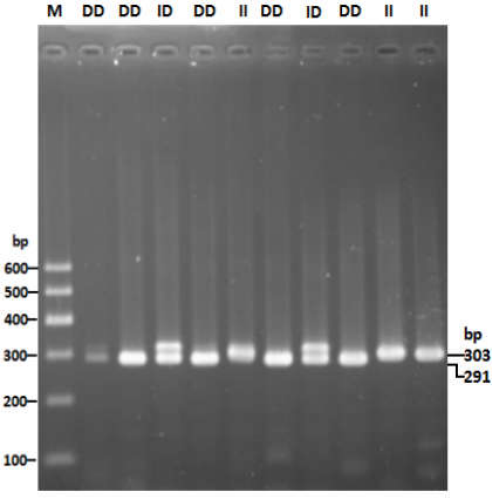

Figure 1. The electrophoresis pattern with 2.5 % agarose gel of the 12 bp indel within goat *LHX4* gene. II = 303 bp; DD = 291 bp; ID = 303 bp + 291 bp; M = DNA marker I.

3 Results

3.1 Identification of a 12 bp indel variation

Agarose gel electrophoresis and PCR production sequencing convinced a 12 bp indel within the *LHX4* gene (Figs. 1; 2). Furthermore, three genotypes (II, ID, and DD) were identified. Genotype II exhibited one band of 303 bp, genotype DD exhibited one band of 291 bp, and heterozygote genotype ID exhibited two bands (303 and 291 bp).

3.2 Genetic parameters analysis

Genotypic frequency, allele frequency, Ho, He, Ne, and PIC of tested goat population were calculated and are shown in Table 1. The frequencies of II and ID genotypes were higher than DD genotype in Shaanbei white cashmere goats. I allele had a higher frequency than D. This indel locus was in accord with Hardy–Weinberg equilibrium (HWE) in tested goat population ($P > 0.05$). Moreover, the genotype distributions between mothers of a single lamb ($n = 895$) and multiple lambs ($n = 254$) in Shaanbei white cashmere goats were significantly different ($P < 0.05$) (Tables 2; 3; Fig. 3).

3.3 Relationship between a 12 bp indel and litter size

The correlation between 12 bp duplication indel of goats *LHX4* gene and litter size was conducted, and this 12 bp indel was revealed to show remarkable association with litter size ($P < 0.05$) (Table 4; Fig. 4). Moreover, the individuals with the genotype DD have the highest average litter size,

Table 3. The genotype distributions among mothers of a single lamb, two lambs, and three lambs in Shaanbei white cashmere goats.

Types	Sample sizes	Genotype numbers			Genotype frequencies			Independent χ^2 value, df, P value
		II	ID	DD	II	ID	DD	
Mothers of a single lamb	895	329	435	131	0.368	0.486	0.146	$\chi^2 = 15.803$
Mothers of two lambs	239	64	129	46	0.268	0.540	0.192	df = 4
Mothers of three lambs	15	3	6	6	0.200	0.400	0.400	$P = 0.003$

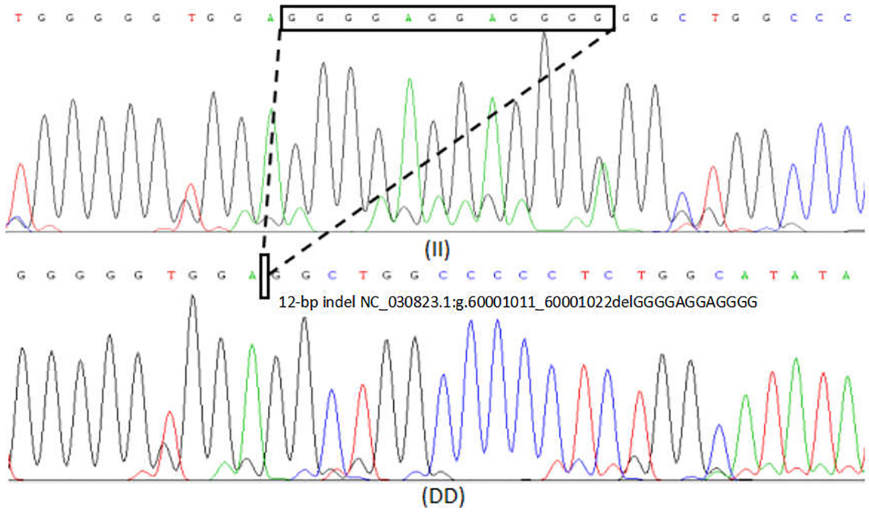

Figure 2. Sequencing graph of a 12 bp indel within goat *LHX4* gene. Panel (II): homozygous insertion genotype II, the sequence with a 12 bp insertion. Panel (DD): homozygous deletion genotype DD.

followed by individuals with heterozygous ID genotype and lowest in II genotype ($P < 0.05$).

4 Discussions

The litter size traits are intricate quantitative traits involving multiple genes and interactions (An et al., 2013a), so it is important to analyze the inner connection of different genes. Previous studies detected that many genetic mutations within candidate genes could affect goat litter size traits. The genetic polymorphism of *KISS1* gene was explored and showed that four SNPs may affect litter size in goats (An et al., 2015a). Polymorphisms of *GNRH1* and *GDF9* genes were identified, and their association with litter size in goats was analyzed (An et al., 2013a). The SNPs of the *PRLR* gene regulated by bta-miR-302a associated with litter size in goats were analyzed (An et al., 2015b). The genetic polymorphisms of *KITLG* gene were explored, and the results indicated that three SNPs may play an important role in litter size (An et al., 2013b; Wang et al., 2017). Otherwise, some candidate genes, such as FTH1, GH, and SAA, were significantly associated with high litter size in Jining Grey goats (Feng et al., 2015). The *LHX4* gene, as a member of the LIM-HD gene family, plays an important role in regulating the development of the pituitary and nervous system and participating in *LHX3–LHX4–PROP1–POU1F1* pathway (Wu et al., 1998; Sloop et al., 2000). On the one hand, *LHX4* gene can stimulate the secretion of FSH and LH by acting on the pituitary. FSH and LH have a direct effect on gonadal development and then affect the litter size. On the other hand, *LHX4* gene could participate in *LHX3–LHX4–PROP1–POU1F1* pathway and then have an influence on POU1F1. POU1F1 can affect the expression of GH, PRL, and ACTH (adrenocorticotropic hormone). GH directly affects the growth and development of organisms. The previous research showed that *LHX4* gene had a notable association with growth traits in livestock (Ren et al., 2014). Meanwhile, the POU1F1 can affect the embryonic development and then have an influence on litter size. Therefore, this work focused on detecting the potential indel variation within the *LHX4* gene and its effects on litter size.

In this study, a novel 12 bp indel was verified. According to the classification of PIC, it was found that this locus owned moderate genetic diversity. Moreover, this indel was in Hardy–Weinberg equilibrium (HWE) ($P > 0.05$), which shows the tested Shaanbei white cashmere goat population was in a state of equilibrium. Through the χ^2 test, the significant genotypic and allelic distribution differences between mothers of a single lamb ($n = 895$) and multiple lambs

Table 4. The relationship between three genotypes of indel variation and litter sizes in Shaanbei white cashmere goats (mean ± SE).

Types	II ($n = 396$)	ID ($n = 570$)	DD ($n = 183$)	P value
Litter size	1.180 ± 0.020^{b}	1.250 ± 0.019^{a}	1.320 ± 0.039^{a}	$P < 0.05$

Note: cells with different letters ($^{a, b}$) mean $P < 0.05$.

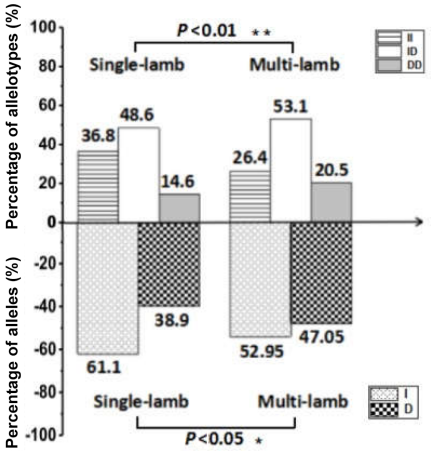

Figure 3. The percentage of allelotypes and alleles of mothers of a single lamb and multiple lambs in Shaanbei white cashmere goats. * $P < 0.05$, ** $P < 0.01$.

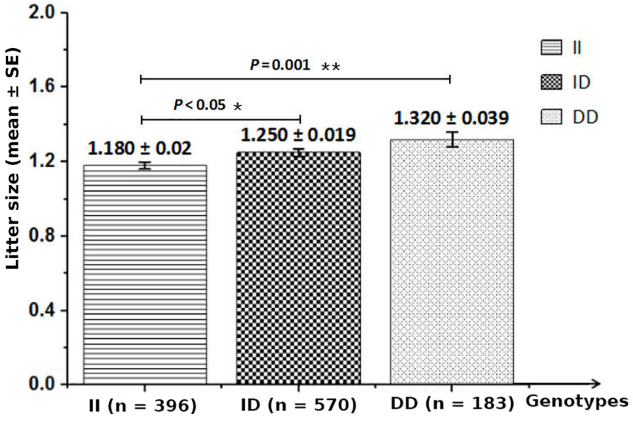

Figure 4. The association of the different genotypes and litter size in Shaanbei white cashmere goats. * $P < 0.05$, ** $P < 0.01$.

($n = 254$) in Shaanbei white cashmere goats were revealed, implying that this indel could affect litter size.

Furthermore, using statistical analysis, this 12 bp indel within *LHX4* gene was significantly associated with litter size in goats. The individuals with DD genotype had higher average litter size than those with II and DD genotypes. Notably, D allele was the positive allele affecting the litter size. A previous study showed litter sizes in sheep and goats were regulated by the hypothalamic–pituitary–gonadal (HPG) axis, which coordinates reproductive behavior with follicular development, ovulation, fertilization, embryogenesis, and parturition (Feng et al., 2015). Although the intron does not appear in the coding region, some conclusions indicated that introns also acted as important gene regulatory elements. Van Laere et al. (2003) discovered a paternally expressed quantitative trait locus (QTL), which can affect muscle growth, fat deposition in pig maps in intron3 of *IGF2*. Studies have shown that introns not only contain many gene expression and regulation elements in relation to gene transcription and mRNA processing especially the alternative splicing but also contain kinds of non-coding RNA (Q. Zhang et al., 2016). Thus, this intronic indel within the *LHX4* gene might affect expression of the cell cycle regulators and then have an influence on litter size in goats.

In summary, a 12 bp deletion of the *LHX4* gene was significantly associated with the litter size in goats, which would extend the indel variations spectrum of the *LHX4* gene and contribute to promising indel markers in goat breeding.

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

Appendix A: Abbreviations

LHX4	LIM homeobox transcription factor 4
Indel	insertion/deletion
II	insertion/insertion
ID	insertion/deletion
DD	deletion/deletion
SNP	single nucleotide polymorphism
MAS	marker-assisted selection
HWE	Hardy–Weinberg equilibrium
Ho	homozygosity
He	heterozygosity
Ne	effective allele numbers
PIC	polymorphism information content
PCR	polymerase chain reaction
HPG	hypothalamic–pituitary–gonadal

Competing interests. The authors declare that they have no conflict of interest.

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