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′-AGCGAGGGAAGCTGAAC-3′; R: 5′-GGGTCTCACATCCCCAGAAA-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 ddH2O. 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.

<table>
<thead>
<tr>
<th>Size</th>
<th>Genotypic frequency</th>
<th>Allelic frequency</th>
<th>( P ) (HWE)</th>
<th>Ho</th>
<th>He</th>
<th>Ne</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>ID</td>
<td>DD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.345</td>
<td>0.496</td>
<td>0.159</td>
<td>0.593</td>
<td>0.407</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.593</td>
<td>0.407</td>
<td>0.159</td>
<td>0.593</td>
<td>0.407</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Types</th>
<th>Sample sizes</th>
<th>Genotype numbers</th>
<th>Genotype frequencies</th>
<th>Independent ( \chi^2 ) value, df, ( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers of a single lamb</td>
<td>895</td>
<td>329, 435, 131</td>
<td>0.368, 0.486, 0.146</td>
<td>( \chi^2 = 11.242 ), df = 4, ( P = 0.036 )</td>
</tr>
<tr>
<td>Mothers of multiple lambs (( \geq 2 ))</td>
<td>254</td>
<td>67, 135, 52</td>
<td>0.264, 0.531, 0.205</td>
<td></td>
</tr>
</tbody>
</table>

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. The 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.

<table>
<thead>
<tr>
<th>Types</th>
<th>Sample sizes</th>
<th>Genotype numbers</th>
<th>Genotype frequencies</th>
<th>Independent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>ID</td>
<td>DD</td>
</tr>
<tr>
<td>Mothers of a single lamb</td>
<td>895</td>
<td>329</td>
<td>435</td>
<td>131</td>
</tr>
<tr>
<td>Mothers of two lambs</td>
<td>239</td>
<td>64</td>
<td>129</td>
<td>46</td>
</tr>
<tr>
<td>Mothers of three lambs</td>
<td>15</td>
<td>3</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

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 $\chi^2$ 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).

<table>
<thead>
<tr>
<th>Types</th>
<th>II (n = 396)</th>
<th>ID (n = 570)</th>
<th>DD (n = 183)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size</td>
<td>1.180 ± 0.020(^b)</td>
<td>1.250 ± 0.019(^a)</td>
<td>1.320 ± 0.030(^a)</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LHX4</td>
<td>LIM homeobox transcription factor 4</td>
</tr>
<tr>
<td>Indel</td>
<td>insertion/deletion</td>
</tr>
<tr>
<td>II</td>
<td>insertion/insertion</td>
</tr>
<tr>
<td>ID</td>
<td>insertion/deletion</td>
</tr>
<tr>
<td>DD</td>
<td>deletion/deletion</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>MAS</td>
<td>marker-assisted selection</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy–Weinberg equilibrium</td>
</tr>
<tr>
<td>Ho</td>
<td>homozygosity</td>
</tr>
<tr>
<td>He</td>
<td>heterozygosity</td>
</tr>
<tr>
<td>Ne</td>
<td>effective allele numbers</td>
</tr>
<tr>
<td>PIC</td>
<td>polymorphism information content</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic–pituitary–gonadal</td>
</tr>
</tbody>
</table>
Competing interests. The authors declare that they have no conflict of interest.

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