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A novel 29 bp insertion/deletion (indel) variant of the *LHX3* gene and its influence on growth traits in four sheep breeds of various fecundity

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Abstract. Belonging to the same LIM homeobox (LHX) family, *LHX3* and *LHX4* are key transcription factors in animal growth and reproduction. Insertion/deletion (indel) is a relatively simple and effective DNA marker. Therefore, four sheep breeds of various fecundity were used to explore the novel indel variants within the sheep *LHX3* and *LHX4* gene, as well as to evaluate their effects on growth traits. Herein, only one novel 29 bp indel (NC_019460.2:g.3107494-3107522delGGCCTGGACTGTGATGGGCACCCTCCGGG) within the sheep *LHX3* gene was found, and three genotypes were detected. Interestingly, the increasing trends of II (insertion/insertion) genotype frequency and I allelic frequency were the same as the growth of the fertility character. Genotypic frequency and allelic frequency distributions were significantly different between the high-fecundity breeds (HS, STHS and LFTS) and low-fecundity breed (TS) based on a χ^2 test ($P < 0.05$). Association analyses showed that body length was significantly different in female TS and STHS and that chest width was significantly different for the female TS and male STHS ($P < 0.05$). These findings suggested that the 29 bp indel could extend the spectrum of genetic variations of the *LHX3* gene in sheep and provide a valuable theoretical basis for the marker-assisted selection (MAS) in sheep breeding and genetics.

1 Introduction

The Tong sheep (TS) is a well-known indigenous sheep breed in China, and it has more than 1200 years of history according to research. The breed is mainly found in Baishui County, Shaanxi Province. TS possess many valuable genetic resources, such as high-quality semi-fine wool, low-odor mutton, large, fat tails and valuable pelts. These genetic resources in the sheep gene bank are valuable not only in China but also globally. However, owing to the characteristics of slow growth and low fecundity, TS have been in danger of becoming extinct in Shaanxi Province.

It is well known that the pituitary gland is the center of the regulating animal growth and reproduction (Hong et al., 2016). Interestingly, there are many prehypophyseal cells resulting from secretion of different hormones to regulate the target organs, including adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), growth hormone (GH), and prolactin (PRL). Most of them had a certain contact with animal growth and reproduction.

Table 1. PCR primer sequences of the sheep *LHX3* and *LHX4* genes.

Name	Primer sequences (5′–3′)	T_m (°C)	Product size(bp)	Notes
LHX3-P1	F: CTCTGAACTGCCAGGACCCA R: ACTCCACGATGCAGCCAAGA	TD-PCR	280	Pool DNA sequencing/ indel classification
LHX3-P2	F: CACTTTCCGGGCGAAGTCAG R: GAAGTAGGAGACGGAGGAGACCC	TD-PCR	299	Pool DNA sequencing
LHX3-P3	F: GCAGACTACGAGACAGCCAAGC R: CTGCTAACTGTCCCCTCCATCTC	TD-PCR	255	Pool DNA sequencing
LHX3-P4	F: CCAAGCCAGCCAGGGACGA R: GCCCAGAGCCTTGAGGGTGAA	TD-PCR	180	Pool DNA sequencing
LHX4-P1	F: CACAACTCCAGGGGCATC R: CCACAGAGTACAACCTTCCAAC	TD-PCR	264	Pool DNA sequencing
LHX4-P2	F: CCCCAACACCTCAAACCTCTT R: TCCGATGTAGGCATGGGAAC	TD-PCR	294	Pool DNA sequencing
LHX4-P3	F: GGAGGAAAGTGTCAACTGGG R: AGCAATAGACGGCGGAGC	TD-PCR	294	Pool DNA sequencing
LHX4-P4	F: GGGCGGTCTCGAAGGACGGA R: GCGCTTCCCAGCCCTTGCTC	TD-PCR	286	Pool DNA sequencing

Notes: TD-PCR, touch-down polymerase chain reaction; *LHX3*, LIM homeobox gene 3; *LHX4*, LIM homeobox gene 4; F, forward primer; R, reverse primer; T_m, melting temperature.

However, some inducing signals and transcription factors play an important role in the development of the pituitary gland itself, such as LIM homeobox gene 3 (*LHX3*) and LIM homeobox gene 4 (*LHX4*) (Park et al., 2013; Voorbij et al., 2015; Yoshida et al., 2016). *LHX3* and *LHX4* are important members of the LIM homeobox family, whose characteristics of the encoded protein include a rich cysteine zinc finger structure. These genes are the most important regulatory factors upstream of the pituitary gland, able to transform expression of *GH* and *PRL* directly, as well as adjust the expression level of the *POU1F1* gene to influence the other regulators (Colvin et al., 2011; Malik and Rhodes, 2014; Seo et al., 2015).

Compared to traditional breeding, molecular breeding has the advantage of saving time and decreasing expenses. During the past decades, insertion/deletion (indel) has become increasingly popular in animal breeding for marker-assisted selection (MAS), and especially exists in eukaryotic genomes (Tian et al., 2008; Yang et al., 2016). In this current study, indel is one of the most important raw materials of evolution and breeding in genomic DNA. According to previous studies, indel accumulation is an important reason for differences in gene expression (Williams and Wernegreen, 2013; Ashkenazy et al., 2014). For the reasons given above, Lanzhou fat-tail Han sheep (LFTS), small-tail Han sheep (STHS) and Hu sheep (HS) were compared with TS to explore the potential indel on *LHX3* and *LHX4* (Song et al., 2012; Zhang et al., 2014; Huang et al., 2015; Miao et al., 2015). In addition, the indel of sheep on the *LHX3* and *LHX4* genes associated with growth traits is limited.

Therefore, the objective of this study was to explore the novel indel variants within the sheep *LHX3* and *LHX4* genes, as well as to evaluate their effects on growth traits in four Chinese indigenous sheep breeds of various fecundity – not only to extend the spectrum of genetic variations of the sheep *LHX3* and *LHX4* genes but also to contribute to implementing MAS in genetics and breeding in sheep.

2 Material and methods

All experiments performed in this study were approved by the International Animal Care and Use Committee of the Northwest A&F University (IACUC-NWAFU). Furthermore, the care and use of animals completely complied with local animal welfare laws, guidelines, and policies.

2.1 DNA samples and data collection

A total of 606 sheep (2–6 years old) were used in this study. These were of four breeds with different fecundity, including Hu sheep (HS, $n = 179$, Mengjin County, Henan Province), small-tail Han sheep (STHS, $n = 195$, Yongjing County, Gansu Province), Lanzhou fat-tail sheep (LFTS, $n = 67$, Yongjing County, Gansu Province) and Tong sheep (TS, $n = 165$, Baishui County, Shaanxi Province). Growth traits for all healthy and unrelated individuals were measured by the same person and using same standard, including body weight (BW), body height (BH), body length (BL), chest circumference (ChC), chest depth (ChD), chest width (ChW), hucklebone width (HuW), hip width (HW) and cannon circumference (CaC); consequently, body length index (BLI), chest circumference index (ChCI), chest width index (ChWI), cannon circumference index (CaCI), hucklebone width index (HuWI) and trunk index (TI) were also cal-

culated on the basis of a related reported description (Lan et al., 2007, 2013; Jia et al., 2015).

2.2 DNA isolation and genomic DNA pool construction

DNA samples were extracted from ear tissue and leukocytes of blood by means of the phenol–chloroform method (Zhang et al., 2015a, b). Quality of DNA samples was assayed with a Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA), diluted to 10 ng μL^{-1}. Considering workloads and that the lower frequency of indel could be found by agarose gel electrophoresis, every 25th sample was used to construct a genomic DNA pool for polymerase chain reaction (PCR) to find the potential indel locus in the sheep *LHX3* and *LHX4* gene (Lan et al., 2013; Chen et al., 2016).

2.3 Primer design and PCR amplification

Based on the single nucleotide polymorphism (SNP) database from NCBI (https://www.ncbi.nlm.nih.gov/snp), eight indel potential sites were found on the sheep *LHX3* and *LHX4* introns, which were designed by Primer Premier software 5.0 (Premier Biosoft International USA) based on the sheep *LHX3* and *LHX4* gene sequence (GenBank NC_019460.1) (Table 1). After touch-down PCR, the products were detected by electrophoresis of 2.5 % agarose gel stained with GelRed (Solarbio Life Science, China); the products were sequenced only when they had different genotypes for each pair of primers (Zhang et al., 2015a; Yang et al., 2016).

2.4 Statistical analyses

Sequences were contrasted and analyzed with BioEdit, using the website www.Msrcall.com to calculate and analyze the genetic data of Hardy–Weinberg equilibrium (HWE), homozygosity (Ho), heterozygosity (He), effective allele numbers (Ne), and polymorphism information content (PIC) (Li et al., 2009). For the χ^2 test between varieties and analysis of variance (ANOVA) in varieties, SPSS software (version 18.0) (IBM, USA) for Windows was used. Statistical testing was carried out on the results (Pan et al., 2013).

3 Results

Through the detection of DNA pools and individuals, no polymorphism was found in the *LHX4* gene, while only one novel indel was found in the *LHX3* gene. Next, using the 2.5 % agarose gel detection, a 29 bp difference in the sheep *LHX3* gene showed three types of bands after 40 min (Fig. 1) – that is, insertion/insertion (II) showed one band (280 bp) and the deletion/deletion (DD) type displayed one band (251 bp), whereas the insertion/deletion (ID) type showed two bands (280, 251 bp). Figure 2 shows that the del portion is "GGCCTGGACTGTGATGGGCACCCTCCGGG" by means of PCR product sequencing, which

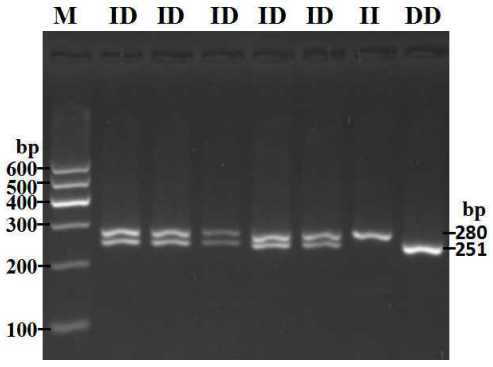

Figure 1. The agarose gel (2.5 %) electrophoresis patterns of the 29 bp indel within the sheep *LHX3* gene. PCR products showed two genotypes at this locus, where the insertion/insertion type (II genotype) consisted of 280 bp, deletion/deletion types (DD genotype) consisted of 251 bp, and the heterozygote type (ID genotype) showed 280 and 251 bp.

mixed the same samples; this result is the same as the prediction of the sheep *LHX3* gene from the NCBI database (NC_019460 g.3107494-3107522).

As can be seen in Table 2, the genotype frequencies and allelic frequencies of 29 bp indel within the sheep *LHX3* gene in four breeds (HS, STHS, LFTS and TS) were evaluated. The four breeds all belong to a moderate polymorphic locus. Homozygosity (Ho) was very close to heterozygosity (He), and effective allele numbers (Ne) were nearly 2. Interestingly, the major II genotype frequency and I allelic frequency increasing trend are the same as fecundity growth (Fig. 3). For current locus, both the HS and TS were at Hardy–Weinberg equilibrium (HWE) ($P > 0.05$).

Genotypic frequency distributions were significantly different between the higher-fertility breeds (HS, STHS, LFTS) and the low-fertility breed (TS) based on a χ^2 test ($P < 0.01$). The results of the χ^2 test on allelic frequency showed that its distribution between the high-fertility breeds (HS, STHS, LFTS) and the low-fertility breed (TS) was the same as the previous conclusion ($P < 0.05$) (Table 3).

The associations between the 29 bp indel and the sheep growth traits were investigated. Significant differences were found between different genotypes and female body length in TS and STHS ($P < 0.05$). Additionally, significant differences were found between different genotypes and chest width in female TS and male STHS ($P < 0.05$) (Table 4). Moreover, the indel loci also have approximately significant effects on some traits such as chest circumference in LFTS ($P = 0.08$) and sacrum height in STHS ($P = 0.07$). In addition, there was no significant relationship between each of the growth traits.

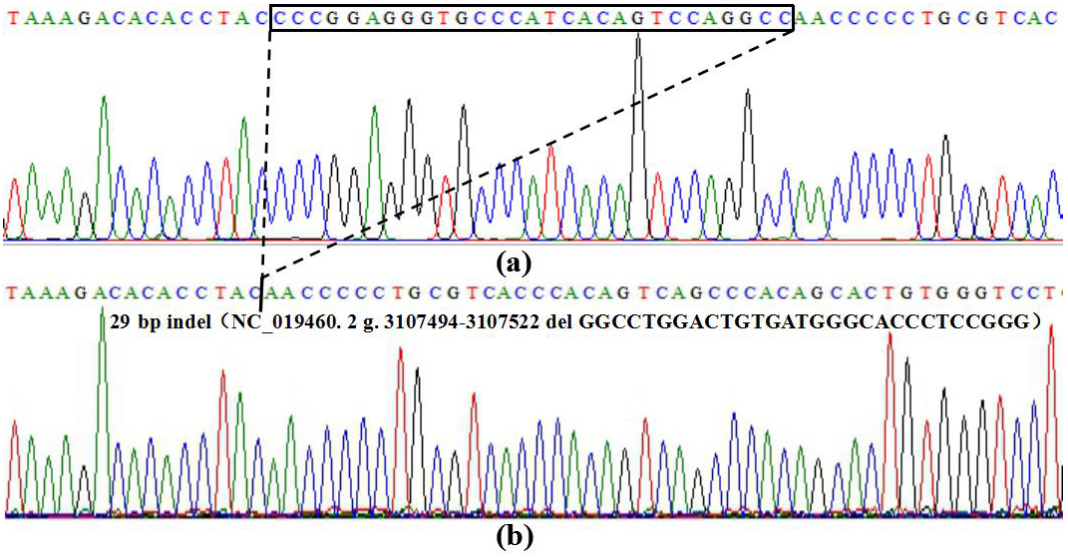

Figure 2. Sequencing maps for the 29 bp indel in the sheep *LHX3* gene. **(a)** Homozygotic insertion type (II); the sequence with the black border is 29 bp deletion. **(b)** Homozygotic deletion type (DD).

Table 2. Genotypes, alleles, He, Ne, and PIC for the novel indel of the sheep *LHX3* gene.

Breeds	Sizes	Genotypic frequencies			Allelic frequencies		HWE	Population parameters			
	N	II	ID	DD	I	D	*P* values	Ho	He	Ne	PIC
HS	179	46	85	48	0.494	0.506	$P > 0.05$	0.500	0.500	2.000	0.375
STHS	195	48	77	70	0.444	0.556	$P < 0.05$	0.506	0.494	1.975	0.372
LFTS	69	13	33	23	0.411	0.589	$P < 0.05$	0.516	0.484	1.939	0.367
TS	163	11	76	76	0.308	0.692	$P > 0.05$	0.574	0.426	1.743	0.335

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

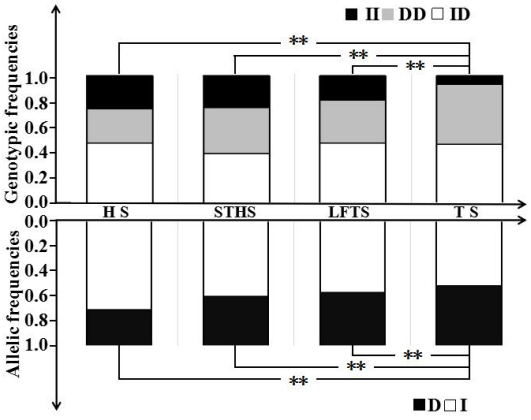

Figure 3. Genotypic and allelic frequencies of the 29 bp indel locus within the *LHX3* gene in the four sheep breeds; ** represents $p < 0.01$.

4 Discussion

Animal growth and development are an extremely complex process, containing a variety of mechanisms with many regulatory factors. It is very important to have great knowledge of the gene structure and function for regulation of growth and development. Recent studies have focused on genetic variations within the *LHX3* gene in bovines and goats, among which a number of SNP sites were found which could impact growth and milk traits (Jing et al., 2008; Liu et al., 2011; Jin et al., 2016; Zhang et al., 2016). Herein, we firstly confirmed a novel 29 bp indel (NC_019460:g.3107494-3107522delGGCCTGGACTGTGATGGGCACCCTCCGGG) within the intron of the sheep *LHX3* gene in four indigenous sheep breeds. This is consistent with what is predicted from NCBI SNP database.

As shown in Fig. 3 and Table 2, it was found that the I allelic and II genotype between the high-fertility breeds (HS, STHS and LFTS) and the low-fertility breed (TS) had significant difference in the χ^2 test. This situation showed remark-

Table 3. χ^2 test of different breeds on novel indel of the sheep *LHX3* gene.

Types	Breeds	HS	STHS	LFTS	TS
Genotypic frequencies	HS	–	$\chi^2 = 3.862$	$\chi^2 = 1.725$	$\chi^2 = 27.629$
	STHS	$p < 0.01$	–	$\chi^2 = 1.681$	$\chi^2 = 20.762$
	LFTS	$p > 0.05$	$p > 0.05$	–	$\chi^2 = 8.874$
	TS	$p < 0.01$	$p < 0.01$	$p < 0.01$	–
Allelic frequencies	HS	–	$\chi^2 = 1.937$	$\chi^2 = 1.786$	$\chi^2 = 26.652$
	STHS	$p > 0.05$	–	$\chi^2 = 0.107$	$\chi^2 = 16.826$
	LFTS	$p > 0.05$	$p > 0.05$	–	$\chi^2 = 7.737$
	TS	$p < 0.01$	$p < 0.01$	$p < 0.01$	–

Notes: HS, Hu sheep; STHS, small-tail Han sheep; LFTS, Lanzhou fat-tail sheep; TS, Tong sheep.

Table 4. Relationship between the novel 29 bp indel of the sheep *LHX3* gene and growth traits in Tong sheep and small-tail Han sheep.

Breeds	Sex	Growth traits	Observed genotypes (LSM$^a \pm$ SE)			
			II	ID	DD	*P* values
TS	Female	Body length	$68.83 \pm 0.83^{a, b}$ ($n = 5$)	67.54 ± 0.96^{b} ($n = 14$)	70.30 ± 0.55^{a} ($n = 6$)	0.02
	Female	Chest width	$31.33 \pm 0.67^{a, b}$ ($n = 3$)	30.31 ± 0.40^{b} ($n = 24$)	31.67 ± 0.38^{a} ($n = 27$)	0.05
STHS	Male	Chest width	18.6 ± 0.48^{b} ($n = 21$)	19.19 ± 0.46^{a} ($n = 45$)	17.23 ± 0.54^{b} ($n = 32$)	0.02
	Female	Body length	60.46 ± 1.45^{a} ($n = 25$)	55.95 ± 1.33^{b} ($n = 32$)	$57.13 \pm 1.13^{a, b}$ ($n = 35$)	0.05

Notes: TS, Tong sheep; STHS, small-tail Han sheep; LSM, least-squares method; SE, standard error; *n*, number. $^{a, b} = p < 0.05$.

able consistency, implying that there is a certain correlation between fertility and this indel locus.

In consideration of the important function of *LHX3* on animal growth and reproduction, the associations between the 29 bp indel and the sheep growth traits were also analyzed. An interesting phenomenon was found in Tong sheep body length and chest width growth traits: the individuals with the II genotype showed superior traits, and the individuals with the homozygote II genotype were significantly better than those with the ID genotype; that is, the II genotype was the most conducive to growth in Tong sheep, which was a potential genetic marker used to improve Tong sheep breeding (Tepaamorndech et al., 2014; Rodrigues et al., 2015). In fact, many studies have also reported that the indel within critical genes was associated with growth traits in livestock. In addition, the best genotypes of chest width within different breeds and sex were not the same; the causes of this phenomenon could be the differences between varieties and sex-specific effects on *LHX3* (Savage et al., 2007). Despite this locus being located in the introns, many studies have demonstrated that the indel could affect the expression of target gene through many channels. For example, a 2 bp indel within the Kruppel-like factor 15 gene (*KLF15*) influences chicken growth and carcass traits (Lyu et al., 2014).

At the same time, according to the classification of polymorphism information content (PIC) and Hardy–Weinberg equilibrium (HWE) in Table 2, the 29 bp indel was identified as mediating polymorphism in all analyzed breeds. That is to say, the 29 bp indel was characterized by abundant genetic diversity, suggesting that this locus could be used for assessing sheep genetic resources. The *P* values of HWE in STHS and LFTS sheep breeds were less than 0.05; the causes of this situation were the lack of population and artificial selection.

Briefly, a novel 29 bp indel within the *LHX3* gene significantly affected growth traits, suggesting that this indel is a potentially useful DNA marker for eliminating or selecting excellent individuals in MAS breeding in relation to growth traits in sheep.

5 Conclusion

Our results confirmed the existence of the 29 bp indel within the intron of the *LHX3* gene in four Chinese indigenous sheep breeds. In addition, they verified significant association with growth traits in four sheep breeds.

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

Author contributions. Xiuzhu Sun and Hongwei Xu designed experiments; Haidong Zhao, Shuai He, Xin Cao, Yong Cai and Renyun Luo collected DNA samples; Haidong Zhao and Shuai He carried out experiments; Haidong Zhao, Shuai He and Yanjiao Zhu analyzed experiments; Xiuzhu Sun and Haidong Zhao wrote the manuscript.

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

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