Three novel single-nucleotide polymorphisms of the bovine *LHX3* gene

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The *LHX3* gene encodes LIM homeodomain class transcription factors that have important roles to play in pituitary and nervous system development. On the one hand, mutations of *LHX3* are associated with deficiencies of growth hormone (GH), prolactin (PRL), luteotrophic hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH); on the other hand, mutations of *LHX3* are also associated with combined pituitary hormone deficiency (CPHD) diseases in human and animal models. To date, few polymorphisms of the bovine *LHX3* gene have been reported. In this study, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing methods were employed to screen the genetic variations within the bovine *LHX3* gene in 802 Chinese indigenous cattle. The results revealed three novel single-nucleotide polymorphisms (SNPs): AY923832: g.7553G>A, 7631C>T and 7668C>G. Among them, a synonymous mutation of exon II was identified: GAG (Glu) >GAA (Glu) at position 72 aa (AY923832:g.7553G>A) of LHX3 (403aa) in the four Chinese bovine breeds. Significant statistical differences in genotypic frequencies for exon II and its flanking region of the *LHX3* gene implied that the polymorphic locus was significantly associated with cattle breeds by the χ^2-test ($\chi^2 = 68.975$, df = 6, $P<0.001$). Hence, the three novel SNPs not only extend the spectrum of genetic variations of the bovine *LHX3* gene, but could also possibly contribute to conducting association analysis and evaluating these as genetic markers in bovine breeding and genetics, and CPHD detection.

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1. Introduction

The LHX3 transcription factor is a member of the LIM homeodomain (LIM-HD) family of gene regulatory proteins (Hunter and Rhodes 2005; Mullen *et al* 2007; Savage *et al* 2007). The *LHX3* gene is an LIM (Lin-11, Isl-1, and Mec-3)-homeobox gene expressed early during pituitary development (Seidah *et al* 1994; Girardin *et al* 1998). With this special structure, LHX3 positively autoregulates its own gene and directly activates the pituitary trophic hormone genes, including the growth hormone (*GH*), prolactin (*PRL*), and thyroid-stimulating hormone beta (*TSHβ*) genes (West *et al* 2004; McGillivray *et al* 2005; Granger *et al* 2006). Hormones released from the pituitary, which are

Keywords. Bovine; genetic variation; *LHX3* gene; PCR-SSCP; single-nucleotide polymorphisms (SNPs)

Abbreviations used: ACTH, adrenocorticotrophic hormone; CPHD, combined pituitary hormone deficiency; CNS, central nervous system; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteotrophic hormone; *LHX3*, LIM homeobox 3 gene; *LHX4*, LIM homeobox 4 gene; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; POU1F1, pituitary-specific transcription factor 1; PRL, prolactin; *PROP1*, paired-like homeodomain factor 1; SNP, single-nucleotide polymorphism; SSCP, single-stranded conformation polymorphism; TBE, tris-borate EDTA; TSH, thyroid-stimulating hormone; TSH*β*, thyroid-stimulating hormone beta

regulated by LHX3, are crucial for physiological processes such as body growth, lactation, metabolism, reproduction, parturition central nervous system (CNS) development, stress response and water homeostasis (Schmitt *et al* 2000). Thus, LHX3 is critical both for early structural events as well as the specification and proliferation of the GH, PRL and TSH*β*-producing pituitary cell lineages (Sheng *et al* 1997; West *et al* 2004; McGillivray *et al* 2005).

The human *LHX3* gene maps to chromosome 9 at 9q34.3 and has seven coding exons (Sheng *et al* 1997; Sloop *et al* 2000–2001; Pfaeffle *et al* 2007). In humans, four novel and recessive mutations have been identified: a deletion of the entire gene (del/del), E173ter and W224ter mutations cause truncated proteins, and A210V mutation causes a substitution in the homeodomain (Pfaeffle *et al* 2007). Human patients with *LHX3* mutations had deficiencies of GH, PRL, TSH, follicle-stimulationg hormone (FSH) and luteotrophic hormone (LH) (Netchine *et al* 2000). Hence, mutations in the *LHX3* gene underlie complex diseases featuring CPHD and, in specific cases, loss of neck rotation considered to result from nervous system abnormalities (Dattani 2005; Savage *et al* 2007). Mutations in the *LHX3* gene are also associated with CPHD diseases in animal models. To our knowledge, GH and PRL play several critical regulatory roles in growth, milk production, reproduction, endocrine and immune diseases (Cui *et al* 2006; Vaclavicek *et al* 2006; Lan *et al* 2007). Moreover, *PRL* gene mutations have been found to be significantly associated with human breast cancer and immune function, mice growth and chicken egg production (Wu and Xu 2000; Cui *et al* 2006; Vaclavicek *et al* 2006). As the *LHX3* gene regulates GH, PRL, TSH, adrenocorticotrophic hormone (ACTH), LH, FSH and pituitary-specific transcription factor 1 (POU1F1) in the *LHX3–LHX4–PROP1–POU1F1* pathway (Wu *et al* 1998; Sloop *et al* 1999–2000), it was thought to be associated with breeding of and CPHD detection in bovine breeds.

To date, few polymorphisms within the bovine *LHX3* gene have been reported. Therefore, analysing the genetic variations of the *LHX3* gene in 802 cattle was a preliminary and interesting study. We are the first to identify the novel genetic variations of the bovine *LHX3* gene by PCR-SSCP and DNA sequencing methods, which will possibly contribute to conducting association analysis and evaluating them as genetic markers for meat production and other functions in animal breeding and genetics.

2 Materials and methods

2.1 *Animal sources*

Four breeds of Chinese cattle (Nanyang; Qinchuan; Jiaxian; Chinese Holstein) were used in this study. The Nanyang animals were from the breeding centre of Nanyang cattle (Nanyang city, Henan Province, P R China); the Jiaxian animals were from the breeding farm of Jiaxian cattle (Jiaxian county, Henan Province, P R China); the Qinchuan animals were from the reserved farm (Weinan city, Shaanxi Province, P R China), the breeding farm of Qinchuan cattle and the fineness breeding centre of Qinchuan cattle (Fufeng county, Shaanxi Province, P R China); and the Chinese Holstein animals were from the breeding farm of milk cattle (in Xi'an city, Shaanxi province, China).

2.2 *DNA samples*

Genomic DNA samples were obtained from 802 bovine cattle belonging to four genetic types: Nanyang bovine (NY, *N*=263), Qingchuan bovine (QC, *N*=302), Jiaxian bovine (JX, *N*=143) and Chinese Hostein bovine (CH, *N*=94) reared in the province of Shaanxi and Henan (China), respectively. DNA samples were extracted from leukocytes according to Mullenbach *et al* (1989).

2.3 *PCR conditions*

According to the bovine sequence of *LHX3* (GenBank accession No. AY923832), one pair of polymerase chain reaction (PCR) primers was designed with Primer 5.0, as follows:

F: 5′-CTGGGAGCTGGGTGGGATGG-3′

R: 5′-TGTTTGGGGAAAAGGAAGGGTG-3′

This was used to amplify a PCR product of about 368 bp for the bovine *LHX3* gene exon II and its flanking region. A 25 μl volume contained 50 ng genomic DNA, 0.5 μM of each primer, 1×buffer (including 1.5 mM $MgCl_2$), 200 μM dNTPs (dATP, dTTP, dCTP and dGTP) and 0.625 units of *Taq* DNA polymerase (MBI). The cycling protocol was 4 min at 95°C, 34 cycles of denaturing at 94°C for 30 s, annealing at 67.5°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 10 min.

2.4 *Single-stranded conformation polymorphism and sequencing*

Aliquots of 5 μl of the PCR products 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 polyacrylamide gel electrophoresis (PAGE) (80×73×0.75 mm) in 1×tris-borate EDTA (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). After the polymorphism was detected, the PCR products of different electrophoresis patterns were sequenced in both directions in an ABI PRIZM 377 DNA sequencer

(Perkin-Elmer) and the sequences analysed with BioXM software (version 2.6).

2.5 *Statistical analysis*

Based on the genotype number of the *LHX3* exon II and its flanking region locus in analysed breeds, genotypic and haplotype frequencies were calculated directly. Differences for genotypic frequencies at the bovine *LHX3* exon II and its flanking region locus among/between the populations studied were analysed using a χ^2-test, which was performed by the SPSS software (version 13.0). Population genetic indices, i.e. gene heterozygosity, gene homozygosity, effective allele numbers, were calculated using the PopGen software (version 3.2), and the polymorphism information content (PIC) was calculated by Botstein's methods (Botstein *et al* 1980).

3. Results and discussion

A human *LHX3* mutation involves a homozygous single-base pair deletion in exon II producing a truncated protein lacking all functional domains and having no predicted function (Bhangoo *et al* 2006; Mullen *et al* 2007). *LHX3* mutations cause an autosomal recessive form of CPHD, creating a clinical phenotype of short stature, secondary hypothyroidism and hypogonadism. Although *LHX3* mutations are rare, the identification of such mutations is important for the clinical management of patients and genetic counselling, and is also instrumental in understanding the mechanism of *LHX3* in development (Pfaeffle *et al* 2007)**.** Based on the contrast of human, we found that the DNA and amino acid sequences had a very high homology between bovine (AY923832) and human (NM_014564) in exon II and its flanking region of the *LHX3* gene. The homology rates of the DNA and amino acid sequences were as high as 95% and 98%, respectively.

In this paper, polymorphisms of the bovine *LHX3* gene were detected by PCR-SSCP and DNA sequencing methods. The results showed that one mutation was located in exon II and the other two mutations in intron II in four Chinese indigenous bovine breeds. The SSCP results showed polymorphic information with three unique SSCP banding patterns observed in four Chinese bovine populations (figure 1). In order to better understand the detailed genetic variation within the Chinese bovine *LHX3* gene, the polymorphic DNA amplification fragments between exon II and its flanking region were sequenced (figure 2). The DNA sequences were deposited in the GenBank database (Accession nos. EU336937, EU372004 and EU340831). Comparison between the nucleotide sequence of the bovine *LHX3* gene (GenBank Accession no. AY923832) and the above sequences revealed three novel SNPs: AY923832: g.7553 G>A (72aa), 7631C>T and 7668 C>G (figures 2 and 3), respectively. In particular, the above SNPs revealed a synonymous mutation: no. AY923832: g.7553 G>A. In detail, the G>A mutation was located in the m.305th (g.7553 G>A) nucleotide position of GenBank Accession no. AY923832 at the *LHX3* locus: GAG (Glu)>GAA (Glu) at position 72 aa of the *LHX3* gene (403 aa) (figure 2). Interestingly, we found a novel mutation: AY923832:g.7631 C>T which had a linkage association with the mutation: AY923832:g.7668 C>G. Three haplotypes were described as: A (G-C-C), B (G-T-G) and C (A-T-G), respectively (figure 2). Accordingly, six genotypes might be described as: AA (G-C-C/G-C-C), BB (G-T-G/G-T-G), CC (A-T-G/A-T-G), AB (G-C-C/G-T-G), AC (G-C-C/A-T-G) and BC (G-T-G/A-T-G). With the sequence data from different individuals, in this study, the three genotypes were conflated and described as: AA, AB, BC. These three genotypes corresponded to three polymorphic patterns found in this study, which were named AA pattern with two bands, AB pattern with three bands and BC pattern with three bands, respectively (figure 1).

The frequencies of genotypes AA, AB and BC were calculated in the four Chinese bovine populations. In the NY population, the frequencies of genotypes AA, AB and BC were 0.4829, 0.3612 and 0.1559, respectively. In the QC population, the frequencies of the genotypes were 0.7715, 0.1159 and 0.1126, respectively. In the JX population, these were 0.6364, 0.2308 and 0.1329, respectively; and in the CH population, they were 0.6702, 0.3192 and 0.0106, respectively. Accordingly, the frequencies of haplotypes A, B and C were calculated in these four Chinese bovine populations. Remarkably, there was only one individual with genotype BC in the CH population. Interestingly, the minimum frequencies of haplotype C (0.0053) and the maximum frequencies of haplotype A (0.8298) were all found in this population (table 1).

With the PopGen software (version 3.2) and according to Botstein's methods, the population genetic indices

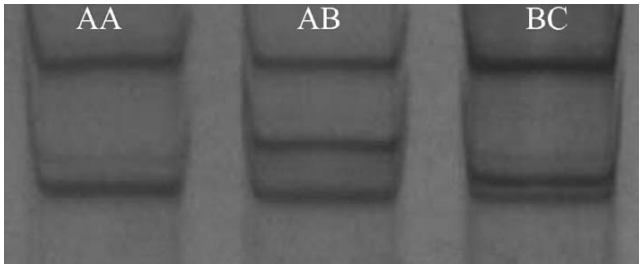

Figure 1. The PCR-SSCP patterns of exon II and its flanking region within the Chinese bovine *LHX3* gene. Three unique SSCP banding patterns. (AA,AB,BC) were observed in four Chinese bovine populations.

Figure 2. SNP loci for exon II and and its flanking region from different haplotypes. *Note:* Haplotype A: G-C-C; Haplotype B: G-T-G; Haplotype C: A-T-G Middle shade: exon II *(ATC is the first codon and TTC is the last codon in this coding region.)* Other shades: forward primer and reverse primer.

Table 1. Genotype distribution and haplotype frequencies at the bovine *LHX3* exon II and its flanking region locus

Breeds	Observed genotypes			Total	Haplotype frequencies		
	AA	AB	BC		A	B	C
Nanyang (NY)	127	95	41	263	0.6635	0.2586	0.0779
Qinchuan (QC)	233	35	34	302	0.8295	0.1142	0.0563
Jiaxian (JX)	91	33	19	143	0.7518	0.1818	0.0664
Chinese Holstein (CH)	63	30	1	94	0.8298	0.1649	0.0053

(i.e. gene homozygosity, gene heterozygosity, effective allele numbers [N_e] and PIC) were calculated (table 2). Hence, gene homozygosity varied from 0.5132 (NY) to 0.7158 (CH) and N_e ranged from 1.3971 (CH) to 1.9487 (NY). The minimum and maximum PIC values were 0.2468 (CH) and 0.4218 (NY). According to the classification of PIC (low polymorphism if PIC value <0.25, moderate polymorphism if PIC value 0.25 to <0.5, and high polymorphism if PIC

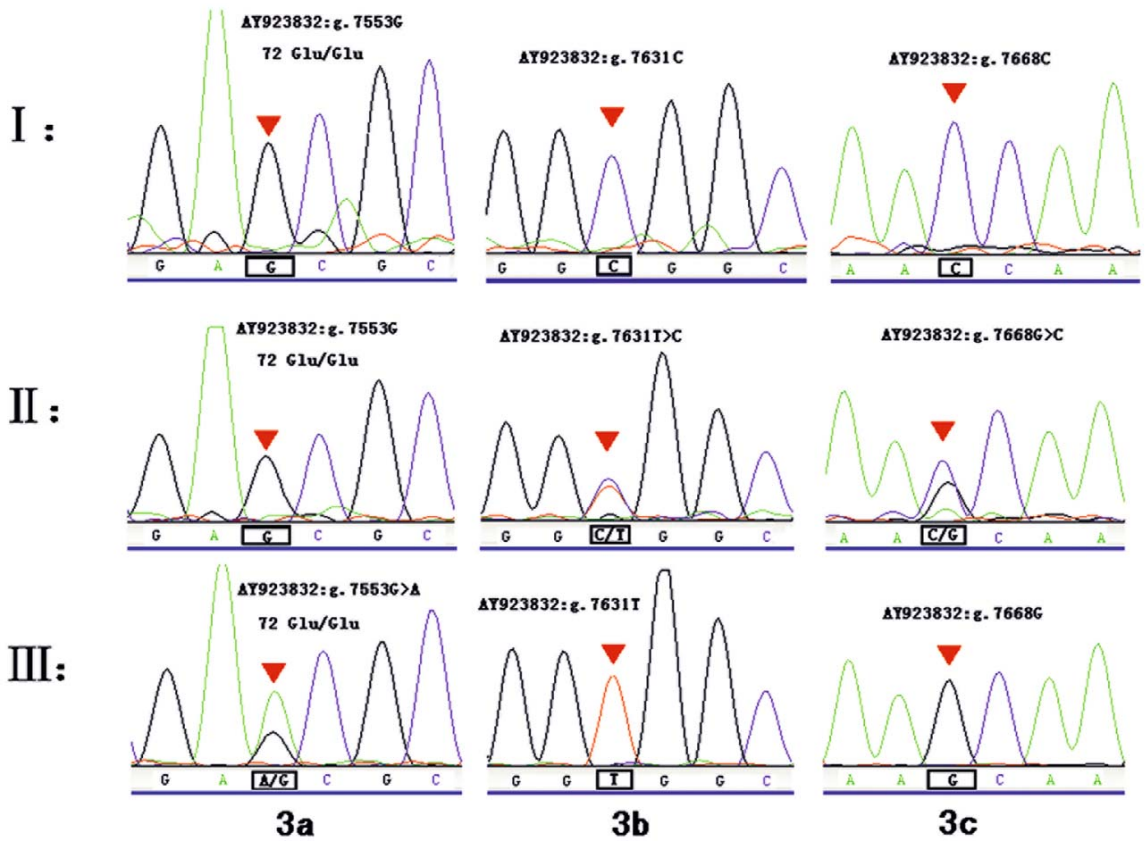

Figure 3. The sequencing maps of three novel SNPs for exon II and its flanking region in the bovine *LHX3* gene. (**a**). Sequencing maps at position AY923832g.7553 (72 aa) from different haplotypes of the bovine *LHX*3 gene. (**b**). Sequencing maps at position AY923832g.7631 from different haplotypes of the bovine *LHX*3 gene. (**c**). Sequencing maps at position AY923832g.7668 from different haplotypes of the bovine *LHX*3 gene. *Note:*I, II and III are the sequences of three different individuals with three unique SSCP banding patterns.

Table 2. Genetic diversity at *LHX3* exon II and its flanking region locus in indigenous Chinese bovine breeds

Breeds	Total number	Types	Gene homozygosity (Ho)	Gene heterozygosity (He)	Effective allele numbers (N_e)	Polymorphic information content (PIC)
Nanyang (NY)	263	Farming and meat	0.5132	0.4868	1.9487	0.4218
Qinchuan (QC)	302	Farming and meat	0.7042	0.2958	1.4200	0.2734
Jiaxian (JX)	143	Farming and meat	0.6026	0.3974	1.6595	0.3548
Chinese Holstein (CH)	94	Milk and meat	0.7158	0.2842	1.3971	0.2468

Table 3. χ^2 and *P* value differences for haplotype frequencies between Chinese bovine breeds in *LHX3* exon II and its flanking region locus

Breeds	Nanyang (NY)	Qinchuan (QC)	Jiaxian (JX)	Chinese Holstein (CH)
Nanyang (NY)		χ^2= 57.137	χ^2= 9.396	χ^2= 17.335
Qinchuan (QC)	*P*<0.001		χ^2=11.151	χ^2=27.456
Jiaxian (JX)	*P*=0.009	*P*=0.004		χ^2=11.808
Chinese Holstein (CH)	*P*<0.001	*P*<0.001	*P*=0.003	

Note χ^2 and *P* value haplotype differences for frequencies between two breeds were shown in this table, respectively (total χ^2 = 68.975, df = 6, $P < 0.001$).

value >0.5), three farming and meat utility breeds (NY, QC and JX) possessed moderate genetic diversity. However, the milk utility breed (CH) had poor genetic diversity. This reflected that the genetic diversity within the Chinese bovine *LHX3* gene in the analysed populations was not very high, which could be explained by the fact that all analysed samples were from healthy individuals without CPHD.

Significant statistical differences in genotypic frequencies for exon II and its flanking region locus of *LHX3* implied that the 72E>E locus was significantly associated with bovine breeds by the χ^2-test (χ^2=68.975, df=6, P<0.001) (table 3). As the analysed breeds represented bovine breeds with different types of utility (farming, meat and milk), the genotypic distribution possibly had significant effects on the utility type and breeds. When compared with the farming and meat utility breeds (NY, QC and JX), the milk breed (CH) possessed higher frequencies of haplotype A (P<0.05 or P<0.01), which implied that haplotype A is possibly associated with milk production. Hence, the bovine *LHX3* gene was considered to possibly have positive effects on milk traits. It implies that there were significant differences in various traits, such as meat and milk production since NY, QC and JX represent meat breeds while CH is a milk breed.

Thus, it is not clear whether there may be any association between the haplotype and the phenotype; however, further research is warranted. The SNPs described (AY923832: g.7553G>A, 7631C>T and 7668 C>G) extend the spectrum of genetic variation of the bovine *LHX3* gene.

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