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TWO NOVEL INSERTION/DELETION VARIANTS OF THE *CRTC3* GENE AND THEIR EFFECTS ON GROWTH TRAITS IN FOUR DIFFERENT CHINESE SHEEP BREEDS

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ABSTRACT

Cyclic adenosine monophosphate response element binding (CREB)-regulated transcription coactivator 3 (*CRTC3*) which is interfere with the metabolism of adipose tissue through distraction catecholamine signaling pathways. On account of its impact on the metabolism, *CRTC3* gene may effect mammalian growth and development. In this study, 605 samples were used to detect the Insertion/Deletion (indel) of *CRTC3* gene in sheep by agarose gel and DNA sequencing. In four varieties of Chinese domestic sheep breeds, 12 bp (NC_019475.2:g.21152662_ 21152663ins CAGGGGCACGTG) and 31bp (NC_019475.2:g.21114157_ 21114187delTTGATGGGTTGCAAAGAGTCAGACATG ACTT) indels polymorphisms of *CRTC3* gene were first evaluated. The two indel loci were significantly associated with sheep growth-related traits including body length, chest depth, hip height, chest circumference and body weight. The allelic frequencies distribution of locus2 were significantly different between the high fecundity breeds (HS) and low fecundity breeds (TS, LFTS) on an χ^2 test (P<0.05). This study demonstrated that the 12bp and 31bp indels could extend the spectrum of genetic variations of *CRTC3* gene in sheep. Both the two loci may be considered as potential functional polymorphisms that may increase the growth traits in sheep.

Key Words: sheep; Cyclic adenosine monophosphate response element binding-regulated transcription coactivator 3 (*CRTC3*); Insertion/Deletion (indel); Growth traits

Abbreviations: **Indel,** insertion/deletion; ***CRTC3*,** Cyclic adenosine monophosphate response element binding-regulated transcription coactivator 3; **PKA,** protein kinase A; **MAS**, marker assisted selected; **HS**, Hu sheep; **TS**, Tong sheep; **LFTS**, Lanzhou Fat-Tail sheep; **STHS**, Small Tail Han Sheep; **TD-PCR**, Touch-Down polymerase chain reaction; **SPSS**, statistical product and service solutions; **bp**, base pair; **HWE**, Hardy-Weinberg equilibrium; **Ho**, homozygosity; **He**, heterozygosity; **Ne**, effective allele numbers; **PIC**, Polymorphism information content; **II**, insertion/insertion; **ID**, insertion/deletion; **DD**, deletion/deletion;

INTRODUCTION

Under the influence of economic globalization, the demand for livestock products is sharply increasing in the world. In particular, there is a dramatic increase in demand for sheep products in countries with a large population, with rapidly growing agricultural and sharply developing textile industries like those in China and India. Especially sheep products face a huge challenge right now. However, lack of effective management and reliance on obsolete breeding pattern has been the two main causes of the decline of germplasm resources in many remote areas of these countries. (Guo and Li 2009). People's eating patterns are inconsistent compared with sheep products that have serious shortcomings. Therefore, improving sheep breeding has become particularly important.

It is well known that fat deposition and muscle formation are direct indicators of animal growth and development. The metabolic activities of adipocytes are related to animals' fattening. Meanwhile changes in muscle cell oxidative capacity have an extraordinary effect on the growth of animals (Deng *et al.* 2010).

Cyclic adenosine monophosphate (cAMP) response element binding (CREB)-regulated transcription coactivator 3 (*CRTC3*) is a member of the CREB-regulated transcription coactivator (CRTC) found in adipocytes. The *CRTC3* proteins were defined as a potent coactivators of CREB (Bittinge *et al.* 2004), affecting cAMP - protein kinase A (PKA) signaling pathway (Liu *et al.* 2017). There has been a research revealing *CRTC3* can interfere with the metabolism of adipose tissue through distracting catecholamine signaling pathways (Prats-Puig *et al.* 2016). It has been reported that the energy consumption in mice whose *CRTC3* gene was knocked out was increased while the adipose tissue content reduced (Song *et al.* 2010). Furthermore, in primary muscle cells, the expression of *PGC-1α* and the mitochondrial oxidative capacity can increase by upregulation of *CRTC3* (Wu *et al.* 2006). This study devoted to explore the relationship between *CRTC3* gene and the growth-related traits in four different Chinese sheep breeds.

The traditional breeding method for improving the growth-related traits of animals requires many generations, while molecular marker breeding can dramatically improve the breeding process of livestock. Genetic polymorphism research has been operating for many years in animal breeding. Previous study has reported that different *BMP8B* variants are associated with bovine growth traits (Cao *et al.* 2013). In recent years, insertion/deletion (indel) has been actived as a marker assisted selected (MAS) in animal breeding (Miedaner and Korzun 2012). But the effect of *CRTC3* gene indel on sheep growth-related traits has not been reported.

This study aims to compare differences between four sheep populations in growth traits and to characterize the growth and development limited factors of *CRTC3* gene in sheep. attempting to provide new material for the MAS method on sheep.

MATERIALS AND METHODS

Experimental animal: Experiments were implemented in accordance with the relevant laws and institutional guidelines and approved by the Institutional Animal Care and Use Committee of Northwest A&F University (NWSUAF). And the use of experimental animals completely observed the local animal welfare laws and policies.

DNA extraction: A total of 605 sheep (2-6 years old) were used in this research. Including 183 female Hu sheep (HS, Mengjin county, Henan province), 161 Tong sheep, 35 male, 126 female (TS, Baishui county, Shaanxi province), 66 Lanzhou Fat-Tail sheep, 11 male, 55 female (LFTS, Yongjing county, Gansu province) and 195 Small Tail Han Sheep, 98 male, 97 female (STHS, Yongjing county, Gansu province). All selected individuals were healthy and unrelated. All DNA were obtained from the ear tissue or blood samples by phenol chloroform (Pang *et al.* 2011).

DNA pool construction: All DNA samples were diluted to working concentration (50 ng/μL) according to previous report by Li *et al* (2013). A total of 100 samples were randomly collected from four varieties. Twenty-five samples were randomly selected from each breed, and 1 μL of each sample was mixed into a tube. After shaking, the mixture was centrifuged to form a DNA pool, which was used as templates for polymerase chain reaction (PCR) amplification to explore indels of *CRTC3* gene (Zhang *et al.* 2015)**.**

Measurement of body size traits: Body size traits for all selected individuals were measured, including body weight (BW), body height (BH), body length (BL), chest circumference (ChC), chest depth (CD), chest width (CW), hucklebone width (HuW), hip width (HW), hip height (HH) and cannon circumference (CaC), according to the method of Gilbert (Gilbert *et al.*, 1993). Consequently, body length index (BLI), chest circumference index (ChCI), cannon circumference index (CaCI), hucklebone width index (HuWI) and trunk index (TI) were also calculated according to the previously reported methods by Fang (Jin *et al.* 2011).

Primer design, PCR protocol and DNA sequencing: Based on the SNP database (https://www.ncbi.nlm.nih.gov/snp) about *CRTC3* gene of sheep from National Center for Biotechnology Information (NCBI), 10 potential indel loci were found on sheep *CRTC3* gene. All of the primers of potential loci were designed. Each pairs of primers were designed to amplify indel fragment using Primer Premier Software (version 5.0) based on the sheep *CRTC3* gene sequence (GenBank Accession NC_019475.2). In subsequent experiments locus 1 and locus 2 were amplified in individuals. Subsequently, to divide different gene varieties, 3.5% and 4% agarose gel were used respectively. The specific PCR reaction procedure Touch-down protocol were used. The Touch-Down PCR program was set to ensure that a sufficient amount of the target fragments were amplified: predegeneration at 95^{o}C for 5 min, followed by 15 cycles of denatured at 95 ^{o}C for 30 s, annealed at 65 ^{o}C to 50 ^{o}C for 30 s (-1^{o}C/cycles), and extended at 72 ^{o}C for 20 s, followed by 24 cycles of denatured at 95 ^{o}C for 30 s, annealed at 53 ^{o}C for 30 s, extended at 72 ^{o}C for 20 s, finally extended at 72 ^{o}C for 10 min.(Zhang *et al.* 2015). Then, the products were sequenced only when each pair of primers showed a single objective band.

Statistical analysis: The genotypic frequencies, allelic frequencies, homozygosity (Ho), heterozygosity (He), effective allele numbers (Ne), polymorphism information content (PIC), *P*-values and Hardy-Weinberg equilibrium (HWE) of four sheep breeds were analyzed using the MSR main program (http://www.msrcall.com/Gdicall.aspx, Klinkenberg *et al.* 2010). Mean while linkage disequilibrium (LD) structure and haplotypes across two loci in four breeds after (Yong and He 2005; Amaral *et al.* 2008). As well as χ^2 test were performed on locus1 and locus 2. The differences in genotype frequencies and allele frequencies were validated in the locus 1. Since the numbers of DD genotype individuals of locus 2 is less than 5, only the differences in allele frequencies were performed. All individuals were tested for association with polymorphism and growth traits. Association analyses were performed using the analysis of variance (ANOVA) procedure in SPSS (version 17.0), when data agreed with the characteristic of normality and homogeneity of variances (Zhao *et al.* 2013). *t* test was applied when only two genotypes were found both in the same breed and same sex.

RESULTS

In this study, two polymorphism loci were selected from 10 potential sites (Table 1). One was a 12 bp indel (NC_019475.2: 21152662_21152663ins CAGGGGCACGTG, locus 1) in intron 13 of the *CRTC3* gene (Figure 1). Another one was a 31 bp indel (NC_019475.2: 21114157_21114187del TTGATGGGTTGCAAAGAGTCAGACATGACTT, locus 2) in intron 12 of the *CRTC3* gene (Figure 2). Both of them have three gene varieties. In locus 1, insertion/insertion (II) genotype showed one band above (168 bp), deletion/deletion (DD) genotype showed one band below (156 bp) and insertion/deletion (ID) genotype showed two bands (168 and 156 bp) (Fig 3). In addition, locus 2 insertion/insertion (II) genotype showed one band in the middle of the fragments (211 bp), deletion/deletion (DD) genotype showed one band below (180 bp) insertion/deletion (ID) genotype showed three bands (211bp, 180bp and heteroduplex A fragment) (Fig 4).

Four sheep breeds were at Hardy-Weinberg equilibrium (HWE) (*P*>0.05) (Table 2). Interestingly, locus 1 exhibited that the D allele has a higher frequency than the I allele, while locus 2 showed opposite. In locus 1, $\chi 2$ test showed that there was no significant difference in genotype frequency and allele frequency among different sheep breeds ($P>0.05$) (Table 3). Surprisingly, allele frequency of locus 2 distribution has great significant difference ($P<0.01$) between LFTS and higher fertility breeds (HS, STHS). Uniformly, allele frequency of locus 2 distribution also has great significant differences between TS and HS ($P<0.01$). Besides, significant differences ($P<0.05$) between TS and LFTS were also reflected in the locus 2 allele comparison (Table 4). The four sheep breeds' PIC values of locus 1 were greater than 0.03. All the sheep breeds' PIC values of locus 2 were less than 0.25 except LFTS. The Ne values of two loci were between 1 and 2 in four sheep breeds (Table 2).

Haplotype frequencies for two indel loci within sheep *CRTC3* gene were analyzed (Table 5). Four haplotypes were found in all breeds. Hap3 (2A) was the primary haplotype in four different sheep breeds, HS (67.1%), TS (60.8%), LFTS (60.3%), and STHS (63.5%). Hap1 (1A) in the four sheep breeds were between 20% and 30%. Furthermore, the linkage disequilibrium of two indel loci were presented (Table 6 and Figure 5). Most of the D' values were high except the D' value of STHS which was 0.451 between locus 1 and locus 2.

Associations between the two loci and the sheep growth-related traits were evaluated, respectively. The different genotypes had significantly associated with BL, demonstrating that the genotype II and ID were superior to DD in male TY in locus 1 ($P<0.05$). The genotype of II had demonstrated significantly superior CD traits than genotype DD in male STHS ($P<0.05$). In addition, different genotypes were found to be significantly associate with HH traits in Female STHS, demonstrating that the genotype II was superior to genotype ID and DD ($P<0.05$). And the different genotypes had great significance in ChC demonstrating that the genotype of II was superior to DD in Female HS ($P<0.01$) (Table 7). In locus 2, BW and ChC of the male LFTS in the II genotype were significantly better than those in the ID genotype ($P<0.05$). Significant difference were found between different genotypes and CD in Female STHS, while the genotype of ID had demonstrated significantly superior CD traits than genotype II ($P<0.05$) (Table 8).

Table 1. PCR primer sequences of the sheep *CRTC3* gene.

Loci	Primer sequences (5'-3')	Tm(°C)	Product size(bp)	Notes
CRTC3-L1	F: TCCAAACAAGCACCTTAGCC R: CGGAAGATTCACTTTCGCAGT	TD-PCR	156	Pool DNA sequencing and Indel classification
CRTC3-L2	F: AGAAGCGGGTGACAGAGGAT R: TGCCCATGAATGAACAGTGAG	TD-PCR	211	Pool DNA sequencing and Indel classification
CRTC3-L3	F: TCACGCAGGCTGAAACACG R: AGGCAGGTCATCAGTCAGGAAT	TD-PCR	161	Pool DNA sequencing
CRTC3-L4	F: CCCTGGTTTCTACGACTTTATG R: CTGGCGTGTTGTTGATTGG	TD-PCR	272	Pool DNA sequencing
CRTC3-L5	F: AGAACAGCCTCAGTATCGTGG R: GTTTGGGATGCTTTGGGT	TD-PCR	295	Pool DNA sequencing
CRTC3-L6	F: AGACTGACAAACACCACCTG R: TGACCTTGCCACTTCTGA	TD-PCR	215	Pool DNA sequencing
CRTC3-L7	F: TCTTCCCTGGTTTCTACGACTT R: CTGGCGTGTTGTTGATTGG	TD-PCR	276	Pool DNA sequencing

CRTC3-L8	F: GTCTTCCAGGTTTCCTATTACAT R: CTGCCATCAGGGTCCATT	TD-PCR	251	Pool DNA sequencing
CRTC3-L9	F: AAACTCGGCATCTCCTGAC R: GGGCTTCCCTGGTGACTAA	TD-PCR	285	Pool DNA sequencing
CRTC3-L10	F: CTTAGAGGAGGGAAGGAACAG R: TCATTTTACTACCCACCAGAA	TD-PCR	317	Pool DNA sequencing

Notes: TD-PCR: Touch-down Polymerase Chain Reaction; *CRTC3*: CREB-regulated transcription coactivator 3; F: Forward primer; R: Reverse primer; Tm: Melting temperature.

Table 2. The genotype frequency, gene frequency, and population index of two indel loci were calculated in four different sheep breeds.

Loci	Breeds	Sizes	Genotypic frequencies			Allelic frequencies		HWE	Population parameters			
		N	DD	ID	II	I	D	*P* values	Ho	He	Ne	PIC
Locus 1	LFTS	66	26	30	10	0.379	0.621	*P* >0.05	0.529	0.471	1.889	0.360
	STHS	195	84	90	21	0.338	0.662	*P* >0.05	0.552	0.448	1.811	0.348
	HS	183	87	73	23	0.325	0.675	*P* >0.05	0.561	0.439	1.782	0.343
	TS	161	65	70	26	0.379	0.621	*P* >0.05	0.529	0.471	1.890	0.360
Locus 2	LFTS	65	1	23	42	0.811	0.189	*P* >0.05	0.693	0.307	1.443	0.260
	STHS	195	1	27	167	0.926	0.074	*P* >0.05	0.862	0.137	1.160	0.128
	HS	183	1	17	165	0.948	0.052	*P* >0.05	0.902	0.098	1.109	0.094
	TS	161	3	29	129	0.891	0.109	*P* >0.05	0.806	0.194	1.240	0.175

Note: HWE, Hardy-Weinberg equilibrium; **Ho**, homozygosity; **He**, heterozygosity; **Ne**, effective allele numbers; **PIC**, Polymorphism information content; **II:** insertion/insertion genotype; **DD:** Deletion / Deletion genotype; **ID:** insertion/ Deletion genotype; LFTS: Lanzhou Fat-Tail sheep; STHS: Small Tail Han sheep; HS: Hu sheep; TS: Tong sheep.

Table 3.χ^2 test of different breeds on novel indel of the locus 1 in the sheep *CRTC3* gene.

Types	Breeds	HS	STHS	LFTS	TS
Genotypic frequencies	HS	—	χ^2=1.54	χ^2=1.32	χ^2=2.03
	STHS	*P*>0.05	—	χ^2=0.96	χ^2=2.23
	LFTS	*p*>0.05	*P*>0.05	—	χ^2=0.08
	TS	*P*>0.05	*P*>0.05	*P*>0.05	—
Allele frequencies	HS	—	χ^2=0.15	χ^2=1.25	χ^2=2.17
	STHS	*P*>0.05	—	χ^2=0.71	χ^2=1.26
	LFTS	*P*>0.05	*P*>0.05	—	χ^2=0.001
	TS	*P*>0.05	*P*>0.05	*P*>0.05	—

Notes: HS: Hu sheep; STHS: Small Tail Han sheep; LFTS: Lanzhou Fat-Tail sheep; TS: Tong sheep.

Table 4.χ2 test of different breeds on novel indel of the locus 2 in the sheep *CRTC3* gene.

Types	Breeds	HS	STHS	LFTS	TS
Allele frequencies	HS	—	χ^2=1.60	χ^2=22.76	χ^2=7.64
	STHS	*P* >0.05	—	χ^2=14.07	χ^2=2.54
	LFTS	*P* <0.01	*P* <0.01	—	χ^2=5.32
	TS	*P* <0.01	*P* >0.05	*P* <0.05	—

Notes: HS: Hu sheep; STHS: Small Tail Han sheep; LFTS: Lanzhou Fat-Tail sheep; TS: Tong sheep. Since the numbers of DD genotype of locus 2 are less than 5, only the differences of the allele frequencies were performed

Table 5. Haplotype frequencies within the *CRTC3* gene in four sheep breeds.

Different Haplotypes	Locus1 & Locus2	Haplotype frequencies			
		HS	TS	LFTS	STHS
Hap1	1A	0.277	0.283	0.207	0.291
Hap2	1B	0.048	0.096	0.179	0.047
Hap3	2A	0.671	0.608	0.603	0.635
Hap4	2B	0.004	0.013	0.01	0.027

Notes: "Hap" represents "haplotype"; The "1" that appears in the column Locus1&Locus2 represents the allele "I" of locus1and "2" represents the allele "D" of locus1; The "A" that appears in the column Locus1&Locus2 represents the allele "I" of locus2 and "B" represents the allele "D" of locus 1; HS: Hu sheep; TS: Tong sheep; LFTS: Lanzhou Fat-Tail sheep; STHS: Small Tail Han sheep.

Table 6. D' and r2 values of pairwise linkage disequilibrium of the *CRTC3* gene in four sheep breeds.

Breed	HS		TS		LZFS		STHS	
	Locus1	Locus2	Locus1	Locus2	Locus1	Locus2	Locus1	Locus2
Locus1		0.899		0.811		0.910		0.451
Locus2	0.092		0.131		0.308		0.032	

Notes: D' and r^2 values of pairwise linkage disequilibrium analysis is shown by the upper right corner and Bottom left corner in the table respectively; HS: Hu sheep; TS: Tong sheep; LFTS: Lanzhou Fat-Tail sheep; STHS: Small Tail Han sheep.

Table 7. Relationship between the novel 12-bp indel of the locus 1 of the sheep *CRTC3* gene and growth traits in Tong sheep, Small Tail Han sheep and Hu sheep.

Locus	Breeds	Sex	Traits	Observed genotypes (LSM±SE)			*p* values
				DD	ID	II	
Locus 1	TS	Male	Body length(cm)	68.50±1.87^{b} (n=5)	73.33±0.86^{a} (n=7)	72.70±1.04^{a} (n=12)	0.027
	STHS	Male	Chest depth (cm)	26.74±0.41^{b} (n=41)	27.85±0.24^{ab} (n=47)	28.35±0.58^{a} (n=10)	0.021
	STHS	Female	Hip height(cm)	60.96±1.17^{b} (n=13)	61.72±0.94^{b} (n=17)	66.40±1.03^{a} (n=13)	0.034
	HS	Female	Chest circumference (cm)	75.77±0.52^{B} (n=87)	77.41±0.48^{AB} (n=74)	79.25±0.67^{A} (n=22)	0.002

Note: (^{a} and ^{b}) = P<0.05; (^{A} and ^{B}) = P<0.01; TS: Tong sheep; STHS: Small Tail Han sheep; HS: Hu sheep.

Table 8. Relationship between the novel 31-bp indel of the locus 2 of the sheep *CRTC3* gene and growth traits in Lanzhou Fat-Tail sheep and Small Tail Han sheep.

Locus	Breeds	Sex	Traits	Observed genotypes (LSM±SE)		*p* values
				ID	II	
Locus 2	LFTS	Male	Body weight(kg)	59.63±2.45^{b} (n=6)	69.88±2.70^{a} (n=5)	0.020
	LFTS	Male	Chest circumference (cm)	96.75±0.57^{b} (n=6)	99.50±1.10^{a} (n=5)	0.044
	STHS	Female	Chest depth (cm)	29.13±0.77^{a} (n=8)	27.14±0.45^{b} (n=27)	0.040

Note: The values with different letters (^{a} and ^{b}) in the same row differ significantly when p<0.05; LSM: Least Square Method; SE: Standard Error; n: Number; LFTS: Lanzhou Fat-Tail sheep; STHS: Small Tail Han sheep.

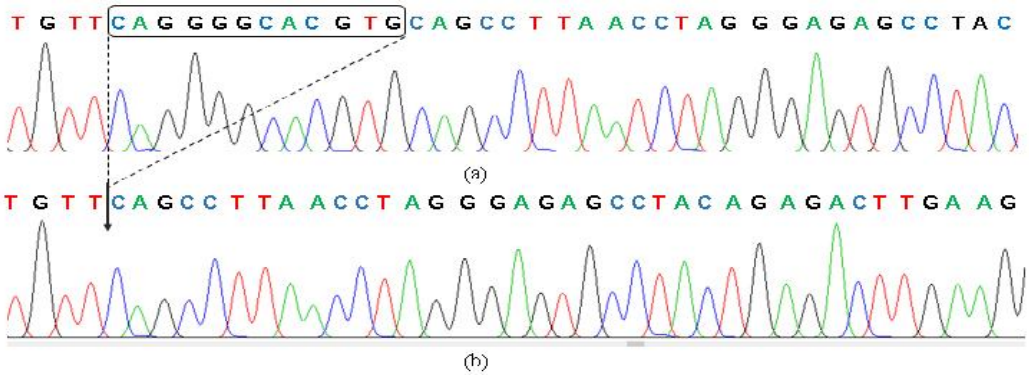

Figure 1. Sequencing maps for the 12 bp indel in the *CRTC3* gene. Panel (a): homozygotic insertion type (II); the sequence with the black border is 12 bp insertion. Panel (b): homozygotic deletion type (DD). Note: the location of the indel locus relates to GenBank No. NC_019475.2.

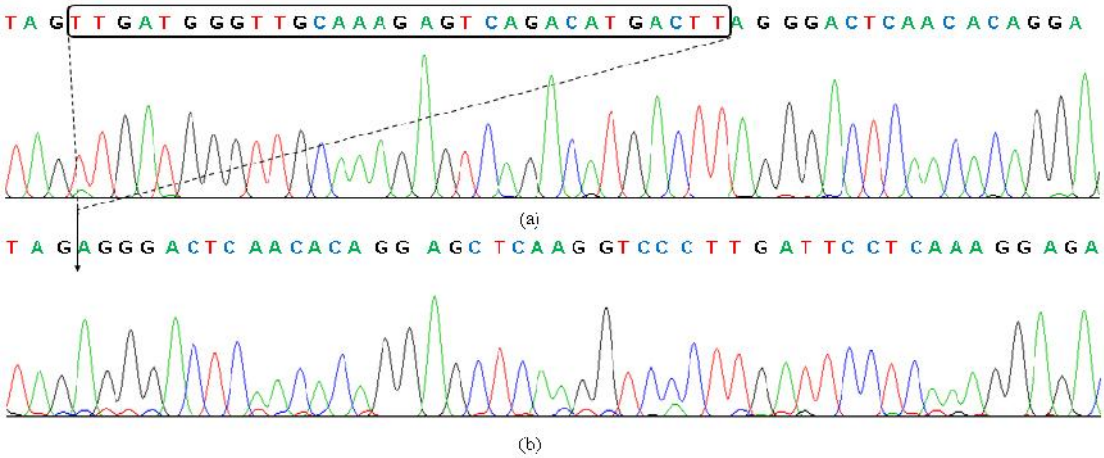

Figure 2. Sequencing maps for the 31 bp indel in the *CRTC3* gene. Panel (a): homozygotic insertion type (II); the sequence with the black border is 31 bp insertion. Panel (b): homozygotic deletion type (DD). Note: the location of the indel locus relates to GenBank No. NC_019475.2.

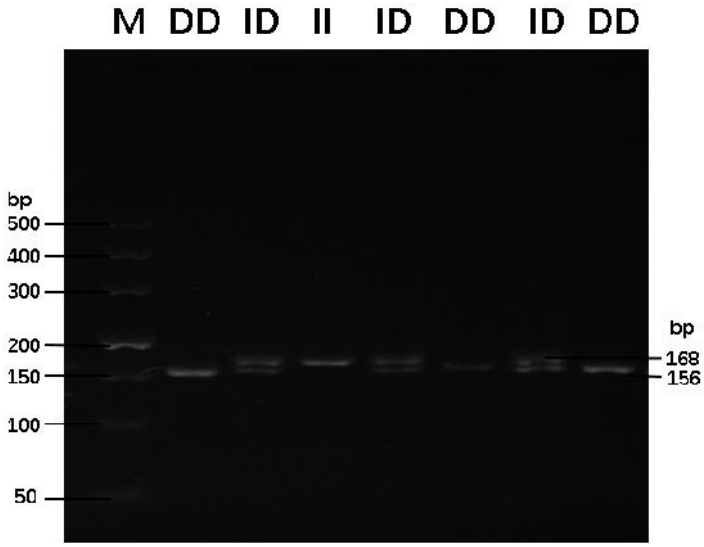

Figure 3. The agarose gel electrophoresis patterns of the 12 bp indel within the locus 1 of *CRTC3* gene. PCR products showed two genotypes at this locus where the insertion/insertion type (II genotype) consisted of 168 bp and deletion/deletion types (DD genotype) consisted of 156 bp. The heterozygote type (ID genotype) showed 168 and 156 bp, which was detected by 3.5% agarose gel electrophoresis.

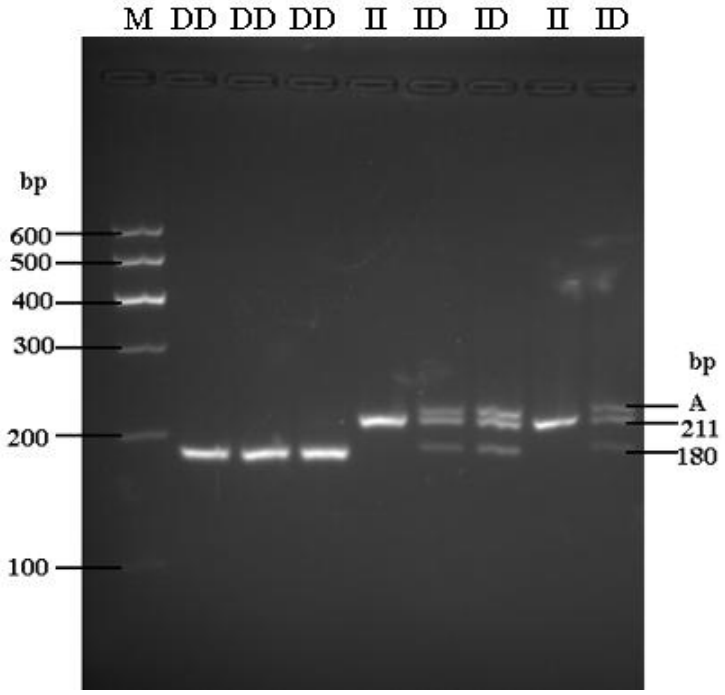

Figure 4. The agarose gel electrophoresis patterns of the 31bp indel within the locus 2 of *CRTC3* gene. PCR products showed two genotypes at this locus where the insertion/insertion type (II genotype) consisted of 211 bp and deletion/deletion types (DD genotype) consisted of 180 bp. The heterozygote type (ID genotype) showed 211bp, 180bp and heteroduplex A fragment, which was detected by 4% agarose gel electrophoresis.

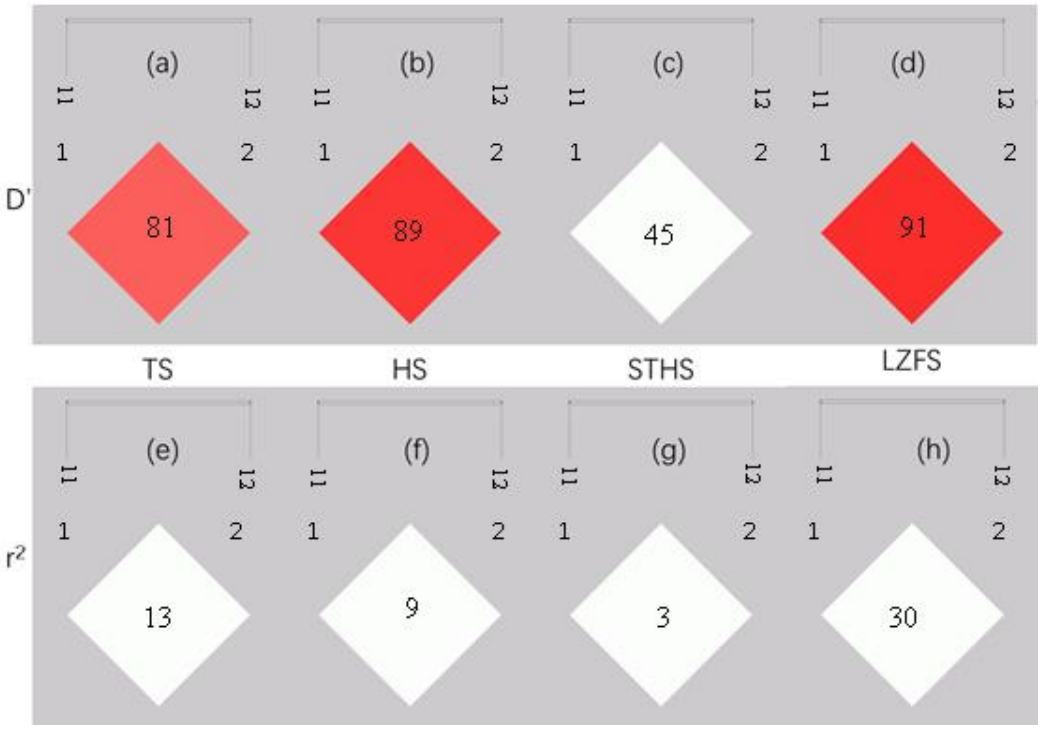

Figure 5. Linkage disequilibrium (LD) plot of CRTC3 gene in four different sheep. HS: Hu sheep; STHS: Small Tail Han sheep; LFTS: Lanzhou Fat-Tail sheep; TS: Tong sheep; Linkage disequilibrium plot of the *CRTC3* gene in TS, HS, STHS and LZFT; (a) D' of TS, (b) D' of HS, (c) D' of STHS, (d) D' of LD, (e) r^2 of TS, (f) r^2 of HS, (g) r^2 of STHS, (h) r^2 of LZFS.

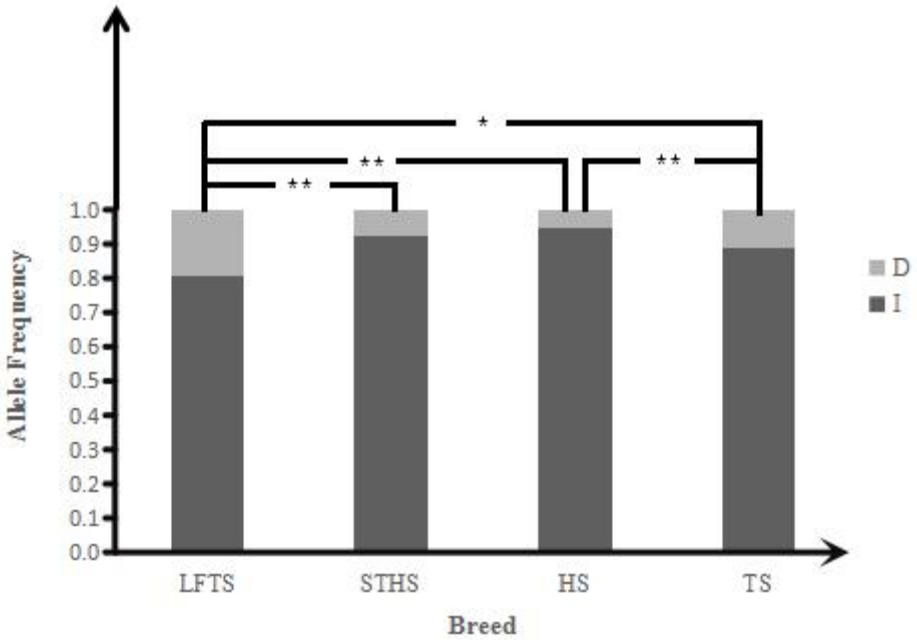

Figure 6. Allele frequencies of the 31 bp indel locus 2 within the *CRTC3* gene in the four sheep breeds. *(p<0.05), **(p<0.01).

DISCUSSION

It is well known that forms of regulation and methods for the animals growth and development are complex. But recent research assessed the correlation between varieties genotypes of *CRTC3* and overweight in Chinese Han population (Ou *et al.* 2014). And the research pointed out there exists significant relativity between variety genotypes of *CRTC3* and overweight. As a member of the CREB regulating transcription coactivators, *CRTC3* always plays an important role in brown adipocyte to effect metabolic activity (Bachman *et al.* 2002). It also influenced mitochondrial respiration and fat activity. All of these evidences suggested that the *CRTC3* gene has a definite relationship to animal energy metabolism. It suggested that *CRTC3* gene maybe affected growth and development traits of livestock. Therefore, this study devoted to identify the relationship between *CRTC3* gene and sheep growth and development.

The association of the two novel indels and growth-related traits were analyzed. In the locus 1, the different genotypes were found to be great significance in the Female ChC in HS; the different genotypes were found to be significantly associated with the male Body length in TY; the different genotypes were found to be significantly associated with the male CD in STHS; the different genotypes were found to be significantly associated with the Female HH in STHS. These significant differences between *CRTC3* gene varieties of locus 1 and diverse growth-related traits were found in three sheep breeds (HS, TY and STHS) (Table 7). And the result displayed the individuals of II genotype were better than individuals of DD genotype. Most of the individuals of ID genotype has same effect but there was no significant difference. It suggested that the allele I of the sheep *CRTC3* gene had positive effects on growth-related traits in these breeds. Analogously, locus 2 genotypes were significant compared to Body weight and Chest circumference in male LFTS. And II genotype was the most conducive to growth-related traits genotype in male LFTS. That is to say that the I allele in the indel loci may serve as potent potential genetic markers to improve the sheep growth traits. Whereas the growth traits of II genotype Female STHS were significantly lower than ID genotype in locus 2, the main presence of excellent traits in different genotypes may be due to different species and sex. Both of locus 1and locus 2 were located at the introns of *CRTC3*, but the gene mutants may affect the structure of the gene, transcription factor binding or alternative splicing, hence affect the expression of genes (Gao *et al.* 2009; Czarnik *et al.* 2011; Kubota *et al.* 2011). It has been reported that 31 bp indel was located at the intron of chicken *PAX7* gene, and the significant associations were found among several body size indices (Zhang *et al.* 2014).

After the χ^2 test, there was no difference among the four sheep breeds (Table 3), regardless of the genotype frequencies or allele frequencies in locus 1. Allele frequency of locus 2 distribution was significantly different between LFTS and three other breeds of sheep (Table 4). Comparing four sheep breeds about the proportion of I allele, HS and STHS were similar, and TS and LFTS were similar (Fig 6). Clearly, TS was

originally the male parent of LFTS. And both of them belong low to fertility breeds. On the contrary, HS and STHS all belong to highly fertility breeds and famous in the world. It suggested that I alleles were one of the potential directions for breeding of sheep with improved growth traits. This study detected haplotypes structure and found the hap3 had an extraordinary high frequency in four sheep breeds compared with others haplotype. It meant that such haplotypes exist for a long time. However, the hap1 (1A) of sheep showed a higher growth traits than the hap3 haplotype (2A), thus provided a reasonable constructive breeding direction to the hap1 direction.

The χ^2 test indicated all breeds were conformed to Hardy-Weinberg (P>0.05). Therefore, all sheep breeds were in dynamic during artificial selected and population migration. Four sheep breeds had medium genetic diversities in locus 1. Since the frequency of the I allele was approximately 2 folds than the D allele frequency and the I allele populations were significantly higher than the D allele in growth traits. It implied that there was more space and potential to further enhance the growth traits of sheep.

Briefly, 12 bp indel and 31 bp indel of *CRTC3* gene were firstly verified in four Chinese domestic sheep breeds. Both of different gene mutations significantly influenced sheep growth traits. And it provides the possibility for them to become a potential genetic marker.

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