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Polymorphisms in the delta-like 2 homolog gene and their association with growth and meat-quality traits in Qinchuan cattle

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ABSTRACT. The delta-like 2 homolog (DLK2) modulates adipogenesis, hematopoiesis, osteogenesis, and other cell-differentiation processes. In the present study, we detected potential polymorphisms in the *DLK2* gene in 604 individuals of Qinchuan cattle by using PCR-RFLP and DNA-sequencing methods. Herein, we identified five novel single-nucleotide polymorphisms (SNPs) (g.888G>A, g.910A>G, g.995G>A, g.4321A>G, g.4850A>G) and analyzed their association with measured traits. Four of the five analyzed polymorphisms were associated with at least one of the following traits: body weight (BW), chest depth (CD), chest circumference (CC), back fat thickness (BT), and rib-eye area (REA). To the best of our knowledge, our research is the first to report the association of *DLK2* gene polymorphisms with growth and meat quality traits in Qinchuan cattle. In summary, the results of our study suggest that the *DLK2* gene can be used as a candidate gene in beef cattle breeding.

Key words: Polymorphisms; Qinchuan cattle; *DLK2* gene; Mutation; Growth and meat traits

INTRODUCTION

The delta-like 2 homolog (*DLK2*) gene encodes the DLK2 protein, also known as epidermal growth factor-like 9 (EGFL9), which is a transmembrane glycoprotein belonging to the epidermal growth factor (EGF)-like-domain family of proteins (Nueda et al., 2007). *DLK2* has a widespread pattern of expression, including in the tissues of lung, brain, adipose, testicles, adult liver, placenta, ovaries, and thymus. DLK2 protein participates in the control of adipogenesis, hematopoiesis, osteogenesis, and other cell-differentiation processes that are also modulated by the homologous protein DLK1 (Nueda et al., 2007). Many of the predicted structural and regulatory features of the DLK2 protein are highly homologous to DLK1, including six EGF-like repeats in its extracellular region, a single transmembrane region, and a short intracellular tail (Nueda et al., 2007). By several ways, DLK2 leads to a reciprocal inhibition for NOTCH signaling in preadipocytes and mouse embryonic fibroblasts (Sanchez-Solana et al., 2011). The NOTCH signaling pathway plays a highly important role in cell differentiation, proliferation, and developmental processes (Demarest et al., 2008), and anomalous NOTCH signaling can lead to tumorigenesis (Wu et al., 2007; Leong and Gao, 2008; Maliekal et al., 2008). It has been proposed that in F2 offspring obtained in breeding, different genotypes of *DLK1* gene are associated with fat deposition and lean muscle mass (Kim et al., 2004). DLK1 may reduce intramuscular fat and inhibit adipogenesis, and the muscular hypertrophies of callipyge sheep, while the *DLK1* gene is highly expressed in Longissimus dorsi or Supraspinatus as determined by immunohistochemistry (Davis et al., 2004). However, the mechanism by which DLK2 modulates adipogenesis of preadipocyte and C3H10T1/2 cells is opposite to that of the DLK1 protein, and interactions between DLK1 and DLK2 may lead to coordinated control of cellular processes (Nueda et al., 2007). These observations suggest that DLK2 may play a role in determining fat deposits and muscular hypertrophy. So far, research on potential polymorphisms in the bovine *DLK2* gene is lacking. Therefore, the objective of this study was to identify DNA sequence variations in the *DLK2* gene in Qinchuan cattle breed and determine possible relationships of any polymorphisms identified in *DLK2* with growth and meat-quality traits in this cattle species.

MATERIALS AND METHODS

Animals, genomic DNA, and data collection

All of the Qinchuan cattle were fed *ad libitum* concentrate and straw up to the adult stage in Shaanxi province. A total of 604 blood samples collected from veins were obtained from unrelated female Qinchuan cattle grown and stratified by age ranging from 12 to 36 months. Blood samples were treated with 2% heparin and stored at -80°C. Genomic DNA was extracted from blood samples using standard procedures (Sambrook et al., 2002). Five traits were measured, including body weight (BW), chest depth (CD), chest circumference (CC), back fat thickness (BT), and rib-eye area (REA) (Gilbert et al., 1993; Rincon et al., 2009).

Primer design, PCR amplification, and DNA sequencing

On the basis of the sequence of the bovine *DLK2* gene (GenBank accession No.

AC_000180.1), ten pairs of polymerase chain reaction (PCR) primers were designed by the Primer Premier 5.0 software to amplify the major part of the coding region of the *DLK2* gene (Table 1). PCR amplifications were performed in 15-µL volumes, containing 50 ng of genomic DNA, 7.5 µL 2X reaction mix (200 µM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, and 2 µL $MgCl_2$), 0.2 µM of each primer, and 0.5 U *Taq* DNA polymerase. The PCR amplification protocol was hold at 95°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at X°C (values for "X" are shown in Table 1) for 30 s, and extension at 72°C for 30 s; a final extension was performed at 72°C for 10 min. The DNA samples ranged in number from 50 to 100 and were randomly chosen for DNA pooling. After pooled PCR amplification, the PCR products were sequenced directly for identification of SNPs.

Restriction fragment length polymorphism (RFLP)

PCR products (10 µL) were mixed with 2 µL 10 X buffer, 10 U enzyme (Fermentas) and 18 µL nuclease-free water, and incubated for 12 h at the optimal reaction temperature as recommended by the supplier. The digested products were detected by electrophoresis on 2.5% agarose gels stained with ethidium bromide.

Statistical analysis

The genotypic frequencies, allelic frequencies, homozygosity (H_O), heterozygosity (H_E), polymorphism information content (PIC), effective number of alleles (N_e), and Hardy–Weinberg equilibrium were calculated according to previous methods (Nei and Roychoudhury, 1974; Nei and Li, 1979). The SHEsis program was used to calculate the LD coefficient (D') and correlation coefficient (r^2) between SNPs (http://analysis.bio-x.cn/myAnalysis.php); linkage disequilibrium (LD) was determined by using D' and r^2 values. The *SPSS* software (13.0 Version) was used to analyze the relationship between different genotypes and measured traits in Qinchuan cattle. The following statistical linear model was used:

$$Y_{ij} = u + G_i + A_i + E_{ijk}$$

where Y_{ij} was the trait measured on the individual cattle, μ was the overall mean for the trait, G_i the effect of the ith SNP genotype, A_i the fixed effect of age, and E_{ijk} the residual error.

RESULTS

Analysis of genetic variation of the *DLK2* gene

Ten pairs of PCR primers were designed to amplify exon and partial intron sequences of the *DLK2* gene (Table 1). Five SNPs were found after analysis of the *DLK2* sequences: one in intron 2 (g.995G>A), two in exon 2 (g.888G>A and g.910A>G), and two in exon 6 (g.4321A>G and g.4850A>G) (Figures 1, 2, 3, 4, and 5). PCR-RFLP methods were adapted to distinguish the genotypes of different individuals. The two mutations

g.888G>A and g.4850A>G resulted in three genotypes named G_1G_1, G_1A_1, and A_1A_1 (Figure 1), and A_4A_4, A_4G_4, and G_4G_4 (Figure 4). The mutations g.995G>A and g.4321A>G resulted in two genotypes named G_2G_2 and G_2A_2 (Figure 2), and A_3A_3 and A_3G_3 (Figure 3). For technical reasons, we were unable to determine genotypes associated with the mutation g.910A<G. The g.888G>A (CC**G**GG-to-CC**A**GG) locus, which is located in the 5' UTR of *DLK2*, is compatible with an *Mva*I endonuclease restriction site. Therefore, after digestion of primer 1-amplified products by *Mva*I, three different patterns were obtained as follows: A 327-bp fragment indicated individuals of G_1G_1 genotype, two 284-bp and 43-bp fragments indicated individuals of A_1A_1 genotype, and three 327-bp, 284-bp, and 43-bp fragments indicated individuals of G_1A_1 genotype (Figure 1). After digestion of the PCR product of primer 3 with *Sma*I, the g.995G>A locus (CCCG**G**G-to-CCCG**A**G), which has a *Sma*I restriction site, could be identified from 2 banding patterns identified as follow: two 156-bp and 74-bp fragments indicated individuals with genotype G_2G_2 and three 230-bp, 156-bp, and 74-bp fragments indicated individuals with genotype G_2A_2 (Figure 2). The locus g.4321A>G (CAC**A**TG-to-CAC**G**TG) showed two banding patterns after digestion of primer 7-PCR product by *Eco*72I. At the g.4321A>G locus, one fragment of 310 bp indicated individuals with A_3A_3 genotype and three fragments (310 bp, 235 bp, and 75 bp in length) indicated individuals with A_3G_3 genotype (Figure 3). The locus g.4850A>G (C**A**CG-to-C**G**CG) showed three banding patterns after *Bsh*1236I digestion of a PCR product amplified with primer 10. At the g.4850A>G locus, one 313-bp fragment indicated individuals of A_4A_4 genotype, two fragments 283 bp and 30 bp long indicated individuals of G_4G_4 genotype, and three fragments of 313 bp, 283 bp, and 30 bp indicated individuals of A_4G_4 genotype (Figure 4). The mutations g.4321A>G and g.4850A>G were both located in exon 6 of the *DLK2* gene. The mutation g.4321A>G did not cause an amino acid change in the DLK2 protein; however, the mutation g.4850A>G caused a change from threonine (ACG) to alanine (GCG) in the translated DLK2 protein (Table 2).

Table 1. Primer sequences for PCR amplification of the *DLK2* gene in Qinchuan cattle.

Primer	Sequence of primer	Sizes (bp)	Tm (°C)	Region (bp) (Ref.AC_000180.1)
P1	F: 5'-TCCTTCACCTCCTTCCCACG-3' R: 5'-GGACGCACAACAGGCAAACG-3'	327	57.6	626-952
P2	F: 5'-GGCCGGCGCTGACCATGCC-3' R: 5'-GGGAGCGCTCACCTATGGCAG-3'	102	60.3	890-991
P3	F: 5'-GGCCTACATCTCGTTTGC-3' R: 5'-TGGGGCAAGGTAGAATAG-3'	230	54.8	923-1152
P4	F: 5'-GCATCTCAGGCAGCCGAAC-3' R: 5'-GCAACCACCGTGCTCCAA-3'	154	58.3	1202-1355
P5	F: 5'-TTGGAAAGGATAGACAGACT-3' R: 5'-GAGGCACTCAATAAATAAGG-3'	429	60.3	2407-2835
P6	F: 5'-CTCCTGACTCCAAATGCAGT-3' R: 5'-GACCCTCAGCCCTAACACTG-3'	382	56.9	3209-3590
P7	F: 5'-GGGTGGTGGGAGCAGGGAT-3' R: 5'-CAGGTTGATGGTGCAGAAGC-3'	310	59.1	4087-4396
P8	F: 5'-CAACCGCTTCTCCTGCCTCT-3' R: 5'-TCGCCGCACCACTTCCTT-3'	313	58.9	4336-4648
P9	F: 5'-GCTGCTGGGCATCTCCGT-3' R: 5'-TGTGGTCTTCCCAGGCTC-3'	288	57.2	4612-4899
P10	F: 5'-CCAGGAGTGTCAGGTTAGC-3' R: 5'-GGACTCCAGGCAGACTTT-3'	313	56.0	4822-5134

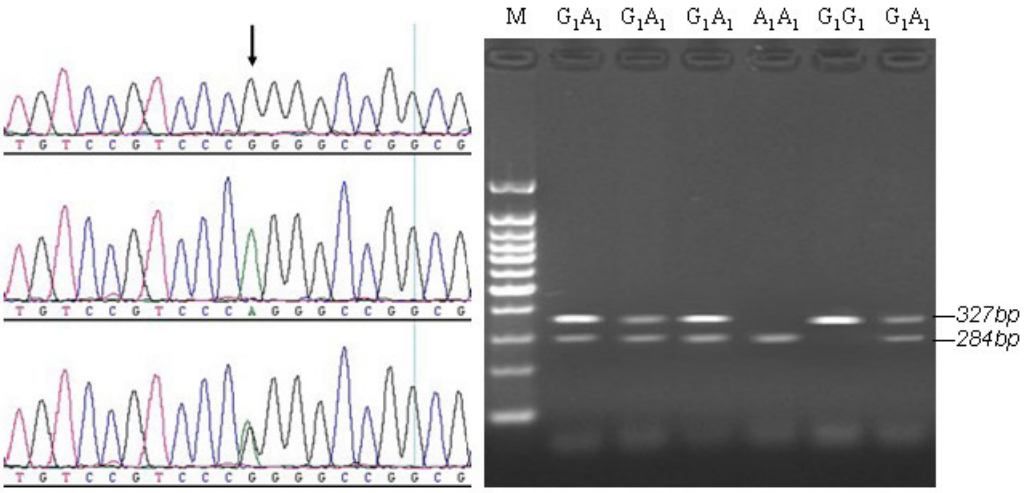

Figure 1. DNA sequencing maps and PCR-RFLP genotypes of the *DLK2* gene g.888G>A locus in Qinchuan cattle.

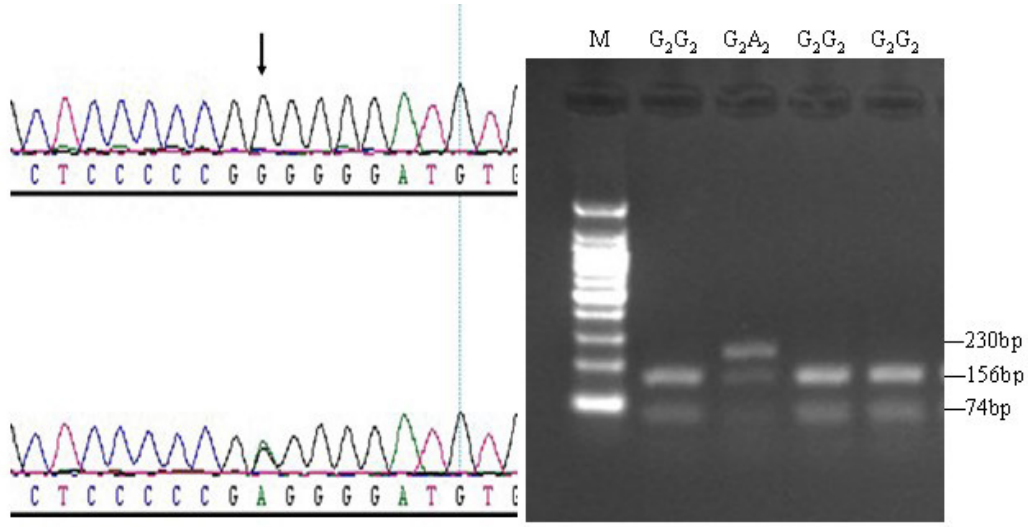

Figure 2. DNA sequencing maps and PCR-RFLP genotypes of the *DLK2* gene g.995G>A locus in Qinchuan cattle.

Analysis of population genetic structure

Table 3 shows the distribution of genotypic and allelic frequencies and of genetic diversity parameters (including H_O, H_E, N_E, and PIC) at the four SNPs loci. At the g.995G>A and g.4321A>G loci, mutant alleles were less frequent than the wild-type allele in Qinchuan cattle, while mutant alleles were almost equally represented at the g.888G>A and g.4850A>G loci. H_E, N_e, and PIC values for the g.995G>A and g.4321A>G loci were lower than the corresponding

values for the other two loci. Considering the value of PIC, the g.888G>A and g.4850A>G loci showed a medium polymorphism, whereas the g.995G>A and g.4321A>G loci showed fewer polymorphisms. According to the Table 3, the loci g.888G>A, g.995G>A, and g.4321A>G were not in Hardy-Weinberg equilibrium ($P < 0.01$); in contrast, the g.4850A>G locus was in Hardy-Weinberg equilibrium ($P > 0.05$).

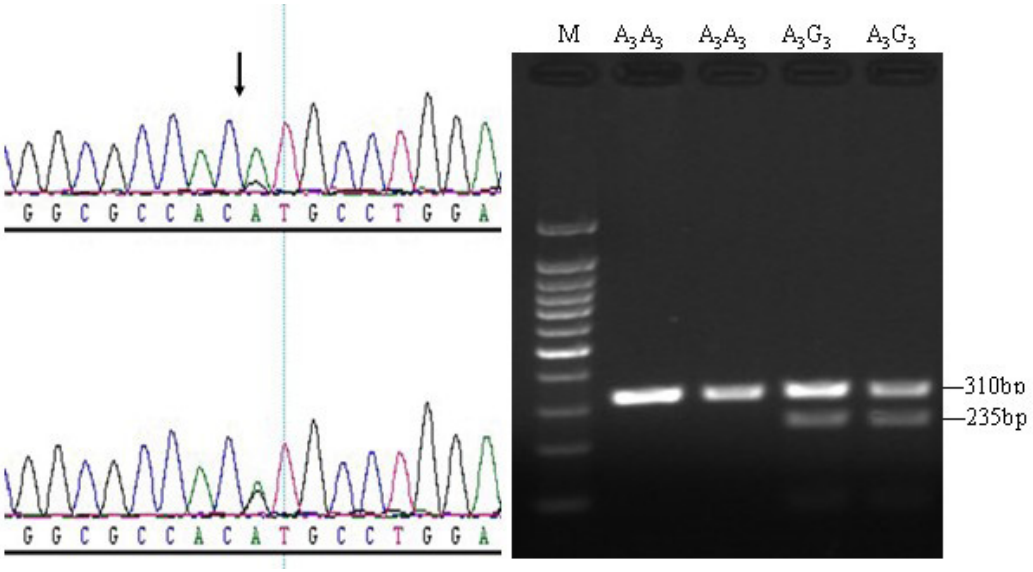

Figure 3. DNA sequencing maps and PCR-RFLP genotypes of the *DLK2* gene g.4321A>G locus in Qinchuan cattle.

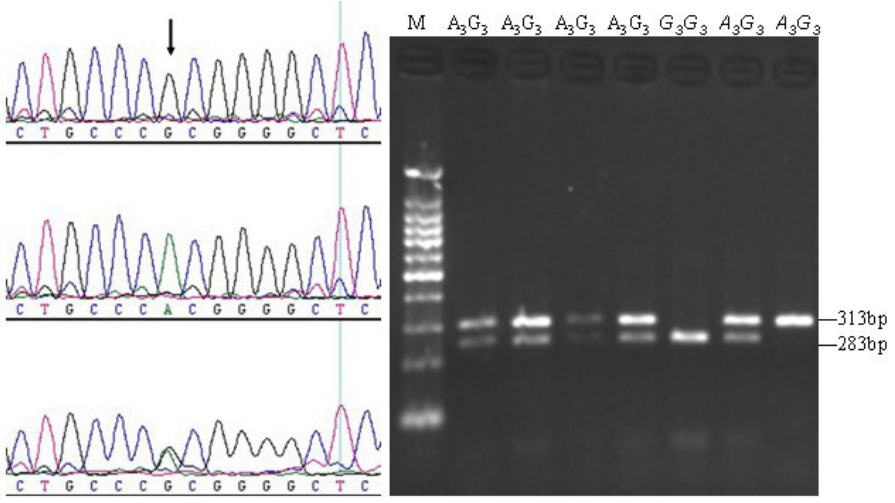

Figure 4. DNA sequencing maps and PCR-RFLP genotypes of the *DLK2* gene g.4850A>G locus in Qinchuan cattle. As the 30-bp fragment was invisible on the 2.5% agarose gel electrophoresis.

Table 2. Variations in the *DLK2* gene of Qinchuan cattle.

Locus	Location	SNP	Codon	Amino acid
g.888G>A	5'-UTR	G/A		
g.910A>G	Exon2	A/G	AGC-to-GGC	Serine-to-Glycine
g.995G>A	Intron2	G/A		
g.4321A>G	Exon6	A/G	ACA-to-ACG	Threonine-to-Threonine
g.4850A>G	Exon6	A/G	ACG-to-GCG	Threonine-to- Alanine

Table 3. Genotype and allelic frequencies and genetic diversity of the *DLK2* gene.

Mutation	Genotype	Frequencies	Allelic frequencies	χ^2	H_O	H_E	N_E	PIC
g.888G>A	G_1G_1	0.234	G (0.528)	20.129**	0.502	0.498	1.994	0.374
	G_1A_1	0.589	A (0.472)					
	A_1A_1	0.177						
g.995A>G	G_2G_2	0.758	G (0.879)	11.416**	0.787	0.213	1.270	0.190
	G_2A_2	0.242	A (0.121)					
g.4321A>G	A_3A_3	0.763	A (0.882)	10.890**	0.791	0.209	1.264	0.187
	A_3G_3	0.237	G (0.118)					
g.4850A>G	A_4A_4	0.295	A (0.521)	5.391	0.501	0.499	1.997	0.375
	A_4G_4	0.452	G (0.479)					
	G_4G_4	0.253						

$\chi^2_{0.05} = 5.99$, $\chi^2_{0.01} = 9.21$, d.f. = 2. ** Polymorphism was not in Hardy-Weinberg Equilibrium ($P < 0.01$). No asterisks in the χ^2 column means the polymorphism was in Hardy-Weinberg Equilibrium ($P > 0.05$).

Analysis of LD for the four SNPs

Haplotypes generally have more informational value than single SNPs. Therefore, we performed LD analysis for the four SNPs in the *DLK2* gene in Qinchuan cattle. The LD structure as estimated by D' and r^2 was calculated (Table 4). The D' value ranged from 0.038 to 0.235, and the r^2 from 0.000 to 0.008. These results indicated that all mutations were in low LD with each other.

Table 4. Estimated values of LD analysis between 4 mutation sites with in the *DLK2* gene.

Mutation	g.888G>A	g.995G>A	g.4321A>G	g.4850A>G
g.888G>A		D' = 0.038	D' = 0.225	D' = 0.065
g.995G>A	$r^2 = 0.000$		D' = 0.071	D' = 0.235
g.4321A>G	$r^2 = 0.006$	$r^2 = 0.005$		D' = 0.190
g.4850A>G	$R^2 = 0.004$	$r^2 = 0.008$	$r^2 = 0.005$	

D' and r^2 above and below the diagonal.

Analysis of the association of *DKL2* SNPs with growth and meat-quality traits

The association of the four mutations in the *DLK2* gene with growth and meat-quality traits (including BW, CD, CC, BT, and REA) in Qinchuan cattle was analyzed (Table 5). At the g.888G>A locus, significant effects on BW, CC, BT, and REA were identified. Individuals of G_1A_1 genotype had larger CC, BT, and REA than individuals with G_1G_1 genotype ($P < 0.05$), heavier BW than individuals with A_1A_1 genotype ($P < 0.05$) and G_1G_1 genotype ($P < 0.01$). At the g.995G>A locus, individuals of G_2G_2 genotype had larger BT than those with G_2A_2 genotype ($P < 0.05$). At the g.4321A>G locus, individuals of A_3A_3 genotype had deeper CD ($P < 0.05$), larger CC ($P < 0.05$) and heavier BW ($P < 0.01$) than those with A_3G_3 genotype. At the

g.4850A>G locus, the individuals of A_4A_4 genotype had larger BT ($P < 0.01$) and larger REA ($P < 0.05$) than those with A_4G_4 genotype and larger BT than those with G_4G_4 genotype ($P < 0.05$).

Table 5. Association analysis of mutation locus of the *DLK2* gene with growth traits in Qinchuan cattle.

Locus	Genotypes	BW (kg)	CD (cm)	CC (cm)	BT (cm)	REA (cm^2)
g.888G>A	G_1G_1	309.72 ± 8.75^B	57.74 ± 0.62	158.92 ± 1.69^b	0.83 ± 0.03^b	43.86 ± 1.32^b
	G_1A_1	334.40 ± 6.35^{aA}	58.87 ± 0.45	162.58 ± 1.23^a	0.89 ± 0.02^a	46.69 ± 0.95^a
	A_1A_1	314.07 ± 9.90^b	57.83 ± 0.71	159.83 ± 1.92	0.83 ± 0.03	43.86 ± 1.48
	P	0.005	0.061	0.031	0.039	0.047
g.995G>A	G_2G_2	321.59 ± 5.68	58.34 ± 0.40	161.09 ± 1.10	0.88 ± 0.02^a	46.03 ± 0.85
	G_2A_2	317.20 ± 8.56	57.95 ± 0.61	159.79 ± 1.66	0.81 ± 0.03^b	44.55 ± 1.28
	P	0.636	0.559	0.469	0.035	0.287
g.4321A>G	A_3A_3	331.44 ± 5.72^A	58.94 ± 0.41^a	162.36 ± 1.11^a	0.88 ± 0.02	45.56 ± 0.86
	A_3G_3	307.35 ± 8.57^B	57.35 ± 0.61^b	158.52 ± 1.66^b	0.83 ± 0.03	45.02 ± 1.29
	P	0.010	0.018	0.034	0.237	0.705
g.4850A>G	A_4A_4	323.56 ± 8.49	58.37 ± 0.61	161.79 ± 1.65	0.90 ± 0.03^{aA}	47.28 ± 1.27^a
	A_4G_4	319.18 ± 6.94	58.28 ± 0.49	160.94 ± 1.34	0.82 ± 0.02^B	44.20 ± 1.04^b
	G_4G_4	315.45 ± 8.55	57.77 ± 0.61	158.60 ± 1.66	0.83 ± 0.03^b	44.39 ± 1.28
	P	0.322	0.312	0.079	0.009	0.022

Data are reported as least square means ± standard errors. Values with different superscript lowercase letters of one locus in the same column differ significant at $P < 0.05$. Values with different superscript capital letters of one locus in the same column differ significant at $P < 0.01$.

DISCUSSION

Previous research on the *DLK2* gene has largely focused on its role in modulating cell processes in humans and mice. Nueda et al. (2007) demonstrated that the *DLK2* gene affected adipogenesis of 3T3-L1 preadipocytes and mesenchymal C3C10T1/2 cells, and C3H10T1/2 distinguish adipocytes, osteocytes, chondrocytes, and myocytes. Therefore, *DLK2* may participate in controlling adipogenesis, hematopoiesis, osteogenesis, and other cell-differentiation processes. To date, no information is available about polymorphisms at the *DLK2* gene and the possible association of these polymorphisms with quantitative trait locus (QTL). Rothschild et al. (1997) have urged that it is important to investigate associations of gene polymorphisms with economical traits in farm animals.

In this study, we report the discovery of five novel SNPs in the bovine delta-like 2 homolog gene *DLK2*, and analyzed the association of four of these SNPs with growth and meat quality traits in Qinchuan cattle. The results showed that the g.995G>A and g.4321G>A loci had only two genotypes and that the frequency of homozygous genotypes was higher than that of heterozygote genotypes. This observation may be the result of an artificial genetic selection. Mutations causing synonymous amino acid exchanges have been reported to affect protein expression by altering or increasing the stability of the mRNA (Capon et al., 2004; Nackley et al., 2006). In addition, ‘‘silent’’ polymorphism changes can affect enzyme substrate specificity (Kimchi-Sarfaty et al., 2007). Additional research has shown that introns can affect transcriptional efficiency of many genes (Greenwood and Kelsoe, 2003; Le Hir et al., 2003). Mutations in the 5’ UTR of a gene can affect mRNA stability and modulate the efficiency of translation of mRNA (Woodman et al., 1996; Stefanovic et al., 1999). The mutation g.888 G>A is located in the 5’ UTR within a CG-rich region (CCCGGGGCCGGCGC) of the *DLK2* gene. This may affect transcription factor-binding sites at a CpG island. The mutation of g.4850A>G led to a threonine-to-alanine amino acid change in the translated DKL2 protein, which may affect its

function. However, we failed to distinguish different banding patterns of the g.910A>G mutation, which, based on the result of DNA fragment sequencing (Figure 5), is predicted to result in the change of a serine to a glycine amino acid. In conclusion, our research showed that differences in genotype of the *DLK2* gene had a significant effect on growth and meat-quality traits in Qinchuan cattle. Therefore, the *DLK2* gene could be used as a candidate gene for Qinchuan beef cattle breeding in China. However, further research is needed to validate our findings.

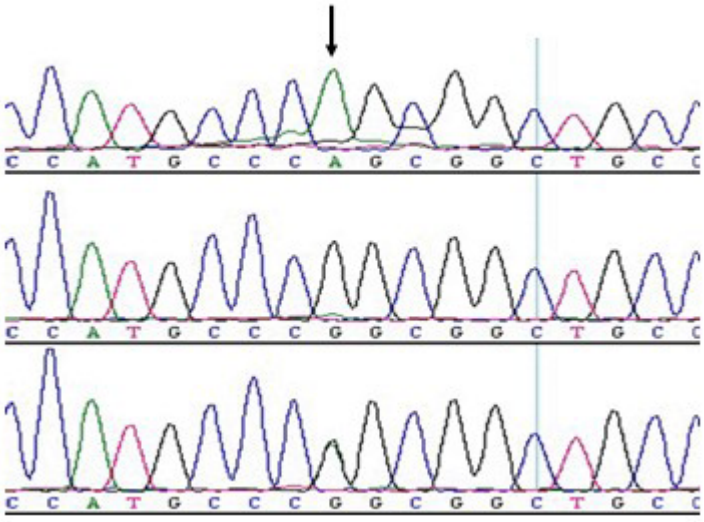

Figure 5. DNA sequencing maps of *DLK2* gene g.910A>G locus in Qinchuan cattle.

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