GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Novel SNPs in the exon region of bovine *DKK4* gene and their association with body measurement traits in Qinchuan cattle

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Genet. Mol. Res. 12 (4): 6664-6672 (2013)
Received July 27, 2012
Accepted November 22, 2012
Published February 28, 2013
DOI http://dx.doi.org/10.4238/2013.February.28.30

ABSTRACT. The aim of this study was to determine whether single nucleotide polymorphisms (SNPs) of bovine Dickkopf homolog 4 (*DKK4*) are associated with body measurement traits in Qinchuan cattle. By using PCR-SSCP technology and DNA sequencing, we discovered 5 *DKK4* SNPs in Qingchuan cattle, including -65G>A and -77G>T in the 5'-untranslated region, 1532C>G and 1533T>C in exon 2, and 2088C>T in exon 3. The sequencing map showed that 1532C>G and 1533T>C were in close linkage disequilibrium and were treated as 1532C>G-1533T>C in this study. Allele frequencies were calculated and analyzed by the chi-square test, which showed that -65G>A and 1532C>G-1533T>C were in Hardy-Weinberg equilibrium ($P > 0.05$), whereas -77G>T and 2088C>T were not in all 633 tested Qinchuan cattle individuals ($P < 0.01$). Gene heterozygosity (H_E), effective allele number (N_E), and polymorphism information content (PIC) were 0.407, 1.686, and 0.324 at -65G>A; 0.472, 1.894, and 0.361 at -77G>T; 0.476, 1.908, and 0.363 at 1532C>G-1533T>C; and

0.218, 1.279, and 0.195 at 2088C>T. We also evaluated the potential association of these SNPs with body measurement traits in all 633 individuals; the results suggest that several SNPs in Qinchuan cattle *DKK4* were significantly associated with body length, hip height, rump length, hip width, heart girth, and pin bone width ($P < 0.05$ and $P < 0.01$). These results suggest that bovine *DKK4* could be used as candidate gene for Qinchuan cattle breeding.

Key words: *DKK4* gene; Qingchuan cattle; SNPs; PCR-SSCP; Body measurement traits

INTRODUCTION

Qinchuan cattle are a major source for animal traction in farming system and beef cattle in China, predominantly in Shaanxi Province. In recent years, agricultural reforms in China have led to development of the beef cattle industry and the use of Qinchuan cattle in this industry has contributed to the economic growth of Shaanxi Province and improved the income of local farmers. However, Qinchuan cattle have some underdeveloped characteristics such as small body size; improving these traits will improve the class of this beef cattle population and improve the national economy.

Marker-assisted selection (MAS), applied to DNA makers for improving body measurement traits, is an efficient and powerful tool (Nkrumah et al., 2004).

The mammalian Dickkopf (*DKK*) family is comprised of extracellular signaling molecules that control embryonic cell growth and bone formation and tissue homeostasis in adults (Glinka et al., 1998; Krupnik et al., 1999; Nie et al., 2005). At the embryonic stage, *DKKs* are involved in a variety of tissues and organs, such as the olfactory epithelia, muscles, bones, and teeth. Similarly, they mainly participate in postnatal bone and muscle tissue regulation (Nie, 2005). Four *DKK* isoforms (*DKK1*, *DKK2*, *DKK3*, and *DKK4*) and a unique DKK-3-related protein named *Soggy* (*Sgy*) have been identified in vertebrates including *Xenopus*, mouse, and human (Krupnik et al., 1999; Monaghan et al., 1999; Kawano and Kypta, 2003). Excepting *DKK3* and *Sgy*, the *DKK* family plays a vital role in modulating the Wnt/β-catenin pathway. *DKK* homolog 4 (*DKK4*), which encodes a novel secreted protein, acts as an inhibitor of the Wnt receptor by combining with LRP5/LRP6 and the transmembrane protein Kremen (Mao et al., 2002; He et al., 2004). Recently, it has been recognized that *DKK4* plays a crucial role in regulation of cell growth. Bazzi et al. (2007) reported *DKK4* acts in a negative feedback loop to attenuate canonical Wnt signaling, and may facilitate a switch to the non-canonical Wnt planar cell polarity pathway involved in cell movement during morphogenesis in mouse. In addition, *DKK4* is a specific regulator of bone formation. *DKK4* inhibits secondary axis formation by Wnt8, leading to decreased bone formation in *Xenopus* (Krupnik et al., 1999; Mao and Niehrs, 2003). To a lesser extent, *DKK4* enhances the osteoclastogenic potency of early osteoprogenitors, so it could facilitate osteoclastogenesis by enhancing RANKL/RANK and M-CSF/c-Fms interactions (Fujita and Janz, 2007). These results demonstrate *DKK4* has an important role in regulating animal body metabolism.

The bovine *DKK4* gene is located on chromosome 27; it has 4 exons and encodes 216 amino acids. Proitsi et al. (2008) described 3 *DKK4* polymorphisms associated with human

schizophrenia. To our knowledge, there have been no published descriptions of bovine *DKK4* gene polymorphisms. Based on its important roles in the regulation of cell growth and bone formation in vertebrates, *DKK4* could be a valuable marker for bovine body measurement traits. We aimed to detect *DKK4* SNPs and evaluate their association with body measurement traits in Qinchuan cattle.

MATERIAL AND METHODS

Biological material

Genomic DNA samples were obtained from 633 Qinchuan heifers stratified into age categories of 18-24 months and randomly selected from purebred populations in Shaanxi Province. Body measurement traits including body length (BL), withers height (WH), hip height (HH), rump length (RL), hip width (HW), chest depth (CD), heart girth (HG), and pin bone width (PBW) were measured as described previously (Gilbert et al., 1993a,b). To minimize systematic error, the same person was appointed to measure 1 of the 8 traits in all animals. According to standard procedures (Sambrook and Russell, 2002), genomic DNA from Qinchuan cattle were isolated from 2% heparin-treated blood samples and stored at -80°C.

Primer design and PCR amplification

Based on the bovine *DKK4* gene sequence (GenBank accession No. NC_007328.4), amplification primers were designed in Primer5.0 (Table 1). PCR amplification was performed in a 15-μL reaction volume containing 10 pmol primers, 0.20 mM dNTPs, 2.0 mM $MgCl_2$, 0.5 U Taq DNA polymerase (TaKaRa, Dalian, China), and 50 ng Qinchuan cattle genomic DNA. The PCR cycling program was 95°C for 5 min followed by 30 cycles of 94°C for 30 s, X°C annealing for 35 s, extension at 72°C for 30 s, followed by a final extension for 10 min at 72°C (X°C was 57.8°, 63.6°, 64.3°, and 54.2°C for primer pairs P1, P2, P3, and P4). The PCR products were electrophoresed on 1.0% agarose gel (containing 150 ng/mL ethidium bromide).

Table 1. Primer sequences, size, melting temperature (Tm), and location of targeted DNA sequence of the bovine *DKK4* gene.

Primers	Primer sequences	Size (bp)	Tm (°C)	Location
P1	F: 5'-GGGAGCAGGGAGAAAGGA-3' R: 5'-CACCCGCAGTGGAGCTTT-3'	163	57.8	5'-flanking and 5'-untranslated region
P2	F: 5'-TGCAGAGCTGCCCAGCTT-3' R: 5'-TCCCTTTCGGGGCGTTAC-3'	180	63.6	5'-untranslated region and intron 1
P3	F: 5'-CTTCCTCCACCTCCTGC-3' R: 5'-GCTGTCTCCGCCTTGTT-3'	288	64.3	Intron 1 and intron 2
P4	F: 5'-TTCTTTGCCTTATGTGAG-3' R: 5'-TGTGGCTTTGTAGTATTTG-3'	288	54.2	Intron 2 and intron 3

Single strand conformation polymorphism (SSCP) and DNA sequencing analysis

Aliquots of the PCR products (6 μL) were mixed with 9 μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene-cyanol, and 0.025% bromophenol blue), heated for

10 min at 98°C, and chilled on ice for 10 min. The denatured DNA solution was run on 12% PAGE (polyacrylamide gel electrophoresis) at constant voltage (120 V) for 16-18 h at room temperature after pre-running at 250 V for 30 min. The gel was stained with 0.1% silver nitrate (Lan et al., 2007) and visualized with 2% NaOH solution (containing 0.1% formaldehyde) (Zhang et al., 2007). After the SNPs were detected, the PCR products were purified with a Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology, China) and sequenced in both directions on an ABI PRIZM 377 (Beijing Aolaibo Biotechnology, China; Applied Biosystems, Foster City, CA, USA). The sequences and sequencing maps were analyzed in DNASTAR5.0.

Statistical analysis

All items including genotypic frequencies, allelic frequencies, Hardy-Weinberg equilibrium (HWE), gene homozygosity (H_O), gene heterozygosity (H_E), effective allele numbers (N_E), and polymorphism information content (PIC) were statistically analyzed as described previously (Nei and Roychoudhury, 1974; Nei and Li, 1979). The association between *DKK4* genotypes and recorded body measurement traits (BL, WH, HH, RL, HW, CD, HG, and PBW) were analyzed by the least-square model applied in the GLM procedure of SPSS16.0 (version16.0, SPSS Inc., USA) according to the statistical linear model:

$$Y_{ijkl} = \mu + A_i + G_j + F_k + \varepsilon_{ijkl},$$

where Y_{ijkl} represents the observed body measurement traits, μ is the overall mean for each trait, A_i is the fixed effect of age, G_j is the fixed effect of genotype, F_k is fixed effect of farm, and ε_{ijkl} is the random error.

RESULTS

SNP identification and PCR-SSCP genotyping of *DKK4*

The novel SNPs identified in this study were -65G>A and -77G>T in the 5'-untranslated region; a missense 1532C>G-1533T>C mutation in exon 2, resulting in a Glu to Arg substitution (75th amino acid); a nonsense 2088C>T mutation in exon 3, replacing Trp with a stop codon (116th amino acid) (Figure 1). All three -65G>A genotypes were identified (Figure 2A); the three -77G>T genotypes were also identified (Figure 2B); the 1532C>G-1533T>C polymorphism appeared with 3 genotypes: CCTT, CGTC, and GGCC (Figure 2C); only two 2088C>T genotypes were identified: CT and TT (Figure 2D).

Genetic diversity of *DKK4*

Allele frequencies of the 5 SNPs were calculated by the chi-square test in the Qinchuan cattle populations in our study (Table 2). The allele frequencies of -65G>A were 0.716(G)/0.284(A); for -77G>T, 0.381(G)/0.619(T); for 1532C>G-1533T>C, CT/GC 0.610(CT)/0.390(GC); and for 2088C>T, 0.125(C)/0.875(T). The chi-square test showed that the genotype distributions were in HWE at -65G>A and 1532C>G-1533T>C ($P > 0.05$); this was not the case at -77G>T and 2088C>T ($P < 0.05$).

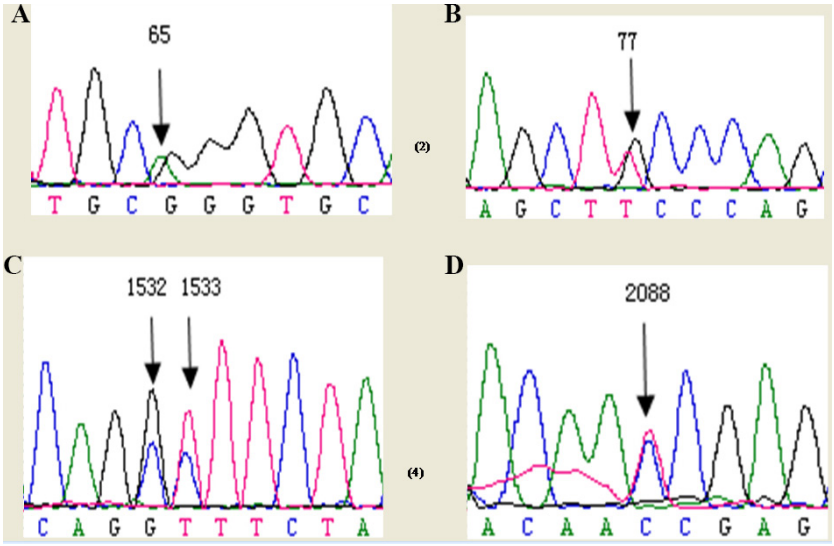

Figure 1. Sequencing maps of *DKK4* gene SNPs in Qinchuan cattle. **A.** -65G>A SNP; **B.** -77G>T SNP; **C.** 1532G>C-1533T>C SNP; **D.** 2088C>T SNP.

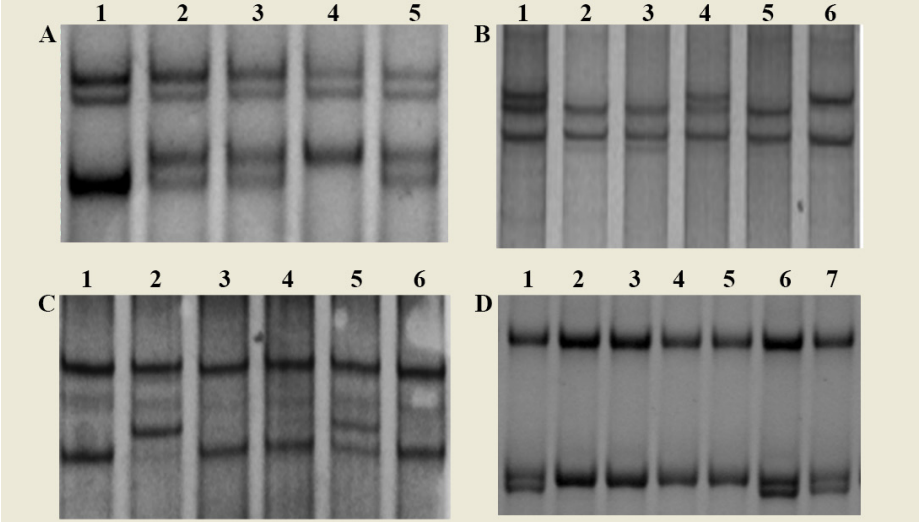

Figure 2. PCR-SSCP electrophoresis patterns of *DKK4* gene SNPs in Qinchuan cattle. **A.** -65G>A SNP. *Lane 1* = GG genotype; *lanes 2*, *3*, and *5* = GA genotype; *lane 4* = AA genotype. **B.** -77G>T SNP. *Lanes 1* and *4* = GT genotype; *lanes 2*, *3*, and *5* = TT genotype; *lane 6* = GG genotype. **C.** 1532C>G-1533T>C SNP. *Lanes 1*, *3*, *4*, and *6* = CCTT genotype; *lane 2* = GGCC genotype; *lane 5* = CGCT genotype. **D.** 2088C>T SNP. *Lanes 1*, *6*, and *7* = CT genotype; *lanes 2*, *3*, *4*, and *5* = TT genotype.

The genetic indexes of each locus included H_E, N_E, and PIC were calculated (Table 3). The PIC classification (low polymorphism if PIC < 0.25, moderate if 0.25 < PIC < 0.5, and

high if PIC > 0.5) indicated the experimental Qinchuan cattle were moderately polymorphic at -65G>A, -77G>T, and 1532C>G-1533T>C, and a low polymorphic frequency at 2088C>T.

Table 2. Genotypic and allelic frequencies (%) of *DKK4* gene SNPs in Qinchuan cattle populations.

SNPs	Observed genotypes (number)	Frequencies		χ^2 (HWE)
		Genotypes	Alleles	
-65G>A	GG (N = 329)	0.519	G = 0.716	0.884
	GA (N = 248)	0.392	A = 0.284	
	AA (N = 56)	0.089		
-77G>T	GG (N = 107)	0.169	G = 0.381	6.268*
	GT (N = 269)	0.425	T = 0.619	
	TT (N = 257)	0.406		
1532C>G-1533T>C	CCTT (N = 227)	0.359	CT = 0.610	1.959
	CGTC (N = 318)	0.502	GC = 0.390	
	GGCC (N = 88)	0.139		
2088C>T	CT (N = 158)	0.249	C = 0.125	12.871**
	TT (N = 475)	0.751	T = 0.875	

HWE = Hardy-Weinberg equilibrium; $\chi^2_{0.05} = 5.991$, $\chi^2_{0.01} = 9.210$. *Polymorphism was not in HWE ($0.01 < P < 0.05$), **polymorphism was highly not in HWE ($P < 0.01$)

Table 3. Genetic indexes of *DKK4* gene SNPs in Qinchuan cattle populations.

SNPs	Gene homozygosity	Gene heterozygosity	Effective allele numbers	Polymorphism information content (PIC)
-65G>A	0.593	0.407	1.686	0.324
-77G>T	0.528	0.472	1.894	0.361
1532C>G-1533T>C	0.524	0.476	1.908	0.363
2088C>T	0.782	0.218	1.279	0.195

PIC > 0.5 means high polymorphism; 0.25 < PIC < 0.5 means moderate polymorphism; PIC < 0.25 means low polymorphism.

Association of *DKK4* variants and body measurement traits

We analyzed 8 Qinchuan cattle body measurement traits for their association with variants in the *DKK4* gene (Tables 4 and 5). Individuals with the -65GA genotype had higher RL than those with the GG genotype ($P < 0.05$); BL, HW, HG, and PBW were also higher in the -65GG group ($P < 0.01$). Meanwhile, individuals with the AA genotype had higher HG, PBW ($P < 0.05$), and BL and HW ($P < 0.01$) than individuals with the GG genotype. There were no significant trait differences between the GA and AA genotypes. A significant difference in BL ($P < 0.01$) was found between individuals with the -77GT and GG genotypes; BL ($P < 0.01$) and HH ($P < 0.05$) significantly differed between individuals with the TT and GG genotypes. There was no significant difference in these traits in GT and TT individuals. At 1532C>G-1533T>C, individuals with the CGTC genotype had higher BL, HH, and HG than individuals with the CCTT genotype ($P < 0.01$) and also higher RL and HW ($P < 0.05$); individuals with the GGCC genotype had higher BL, HH, RL, HW, and HG than individuals with CCTT genotype ($P < 0.01$); these traits did not significantly differ between CGTC and GGCC individuals. Individuals with the 2088TT genotype showed significant differences in RL, HG ($P < 0.01$), and HH ($P < 0.05$) in comparison to individuals with the CT genotype. These results suggest -65A, -77T, 1532G-1533C, and 2088T could be valuable molecular markers for selecting desired body measurement traits in Qinchuan cattle.

Table 4. Association of -65G>A and -77G>T SNP genotypes with body measurement traits.

Traits	Genotypes (cm, means ± SE)					
	-65G>A			-77G>T		
	GG	GA	AA	GG	GT	TT
BL	134.486 ± 0.763^{A}	137.933 ± 0.878^{B}	139.732 ± 1.848^{B}	132.136 ± 1.342^{A}	137.251 ± 0.846^{B}	136.294 ± 0.866^{B}
WH	121.203 ± 0.627	122.958 ± 0.722	123.670 ± 1.520	119.916 ± 1.088	121.578 ± 0.686	121.451 ± 0.702
HH	123.799 ± 0.434	125.030 ± 0.500	125.268 ± 1.052	122.383 ± 0.757^{a}	123.812 ± 0.478^{ab}	124.286 ± 0.489^{b}
RL	42.535 ± 0.274^{a}	43.607 ± 0.316^{b}	43.488 ± 0.664^{ab}	41.645 ± 0.474	42.684 ± 0.299	42.658 ± 0.306
HW	38.416 ± 0.343^{A}	39.794 ± 0.395^{B}	41.804 ± 0.831^{B}	38.084 ± 0.613	38.914 ± 0.386	38.782 ± 0.395
CD	59.794 ± 0.423	60.940 ± 0.487	60.893 ± 1.026	58.421 ± 0.730	59.818 ± 0.461	59.619 ± 0.472
HG	163.605 ± 1.166^{Aa}	169.823 ± 1.308^{B}	169.768 ± 2.752^{b}	163.916 ± 2.007	164.401 ± 1.266	166.148 ± 1.295
PBW	18.751 ± 0.202^{Aa}	19.903 ± 0.233^{B}	19.875 ± 0.491^{b}	18.813 ± 0.353	18.955 ± 0.233	19.089 ± 0.228

^{a,b}Means with different superscripts are significantly different ($P < 0.05$). ^{A,B}Means with different superscripts are significantly different ($P < 0.01$). BL = body length; WH = withers height; HH = hip height; RL = rump length; HW = hip width; CD = chest depth; HG = heart girth; PBW = pin bone width; SE = standard error.

Table 5. Association of 1532C>G-1533T>C and 2088C>T SNP genotypes with body measurement traits.

Traits	Genotypes (cm, means ± SE)				
	1532C>G-1533T>C			2088C>T	
	CCTT	CGTC	GGCC	CT	TT
BL	132.949 ± 0.954^{A}	137.258 ± 0.806^{B}	138.750 ± 1.533^{B}	134.496 ± 0.949	136.832 ± 0.681
WH	120.297 ± 0.720	121.897 ± 0.608	121.864 ± 1.157	120.726 ± 0.611	122.335 ± 0.556
HH	122.524 ± 0.512^{A}	124.868 ± 0.433^{B}	125.875 ± 0.823^{AB}	123.122 ± 0.574^{a}	124.661 ± 0.356^{b}
RL	41.996 ± 0.324^{Aa}	43.075 ± 0.274^{b}	43.636 ± 0.521^{B}	41.892 ± 0.310^{A}	43.101 ± 0.235^{B}
HW	37.868 ± 0.404^{Aa}	39.777 ± 0.324^{b}	39.477 ± 0.649^{B}	38.613 ± 0.388	39.347 ± 0.304
CD	59.654 ± 0.489	60.294 ± 0.413	61.205 ± 0.785	59.712 ± 0.406	60.249 ± 0.351
HG	161.969 ± 1.334^{A}	166.934 ± 21.127^{B}	169.6364 ± 2.143^{B}	161.2014 ± 1.415^{A}	167.245 ± 0.986^{B}
PBW	18.634 ± 0.243	19.208 ± 0.206	19.409 ± 0.391	18.64 5 ± 0.275	19.214 ± 0.169

^{a,b}Means with different superscripts are significantly different ($P < 0.05$). ^{A,B}Means with different superscripts are significantly different ($P < 0.01$). For abbreviations, see legend to Table 4.

DISCUSSION

In this study, PCR-SSCP technology and DNA sequencing methods were used to screen genetic variation among 5 exons of the Qinchuan cattle *DKK4* gene. Our results showed 5 novel SNPs, including 2 non-coding mutations, 1 missense mutation, and 1 nonsense mutation. Recently, the crucial role of non-coding genomic regions has been widely acknowledged. The 5'-UTR plays an important role in the regulation of gene expression because of its effect on the post-transcriptional process, including the initiation and efficiency of translation as well as the stability of mRNA (van der Velden and Thomas, 1999; Wilkie et al., 2003; Pickering and Willis, 2005). It is also indispensable for the initiation of protein synthesis (Conklin et al., 2002). The -65G>A and -77G>T SNPs are located in the 5'-UTR and may thus indirectly affect *DKK4* expression by regulating post-transcription messages and initiation of protein synthesis. Nackley et al. (2006) reported that missense and nonsense SNPs affect protein function by altering the mRNA secondary structure. The 1532C>G-1533T>C and 2088C>T polymorphisms might have a similar effect on *DKK4* mRNA and in turn affect the production traits of Qinchuan cattle.

PCR-SSCP electrophoresis patterns showed 3 genotypes at -65G>A, -77G>T, and 1532C>G-1533T>C, but just 2 genotypes at 2088C>T. No individual with a CC genotype was

found at 2088C>T. This may be due to the lower frequency of the C allele due to random drift, the non-existence of the CC genotype in Qinchuan cattle, or our small sample size.

The chi-square test showed the genotype distributions were in HWE at -65G>A and 1532C>G-1533T>C ($P > 0.05$), but not at -77G>T and 2088C>T ($P < 0.05$). The reasons for this likely include the effects of genetic drift, migration, or isolation, but mainly artificial selection. Following the standard of PIC, we suggest a moderate polymorphic level at -65G>A, -77G>T, and 1532C>G-1533T>C, making these sites valuable markers for further genomic selection, whereas 2088C>T exhibited a low polymorphic frequency and may not be a very valuable marker.

DKK4 is a multifunctional gene whose main role is in negatively modulating the Wnt/β-catenin pathway to regulate cell growth and bone formation in vertebrate development. Proitsi et al. (2008) reported 3 novel SNPs of human *DKK4* are associated with schizophrenia (Proitsi et al., 2008). This result has been supported by other researchers who claimed that *DKK4* SNPs are associated with body height, body weight, and surface area in humans (Ho et al., 1980; Zhang et al., 2011). Our results suggest the bovine *DKK4* gene is associated with body measurement traits including BL, HH, RL, HW, HG, and PBW in Qinchuan cattle ($P < 0.05$ and $P < 0.01$; Tables 4 and 5).

In conclusion, we identified 5 SNPs in the bovine *DKK4* gene in Qinchuan cattle populations. We also demonstrated the *DKK4* gene may influence body measurement traits and could be a major candidate gene for Qinchuan cattle breeding. However, further research is needed in a larger population before its use in MAS. It will also be interesting to screen the whole sequence to investigate the possibility of linkage disequilibrium with other causative mutations.

ACKNOWLEDGMENTS

Research supported by the Program for Changjiang Scholars and Innovative Research Team (#2010IRT0940), the "13115" Scientific and Technological Innovation Program of Shaanxi Province (#2010ZDGC-01), and the "12th Five-Year" National Science and Technology Support Program (#2011BAD28B04-03). The biological material was provided by Qinchuan Beef Cattle Breeding Center, Kingbull Industry Co., Ltd., and Qinchuan Cattle Industry Group of Shaanxi Province (China). We sincerely thank all who contributed to this study and extend special thanks to Professor Zan and Dr. Cheng for their technical guidance.

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