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# Molecular cloning and functional analysis of *MRLC2* in Tianfu, Boer, and Chengdu Ma goats

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**ABSTRACT.** To determine the molecular basis of heterosis in goats, fluorescence quantitative polymerase chain reaction (PCR) was performed to investigate myosin-regulatory light chain 2 (*MRLC2*) gene expression in the longissimus dorsi muscle tissues of the Tianfu goat and its parents, the Boer and Chengdu Ma goats. The goat *MRLC2* gene was differentially expressed in the crossbreed, and the purebred mRNA were isolated and identified using fluorescence quantitative reverse transcription-PCR (RT-PCR). The complete coding sequence of *MRLC2* was obtained using the cDNA method, and the full-length coding sequence consisted of 513 bp encoding 172 amino acids. The EF-hand superfamily domain of the MRLC2 protein is well conserved in caprine and other animals. The deduced amino acid sequence of MRLC2 shared significant identity with MRLC2 from other mammals. Phylogenetic tree analysis revealed that the MRLC2 protein was closely related to MRLC2 in other mammals. Several predicted miRNA target sites were found in the coding sequence of caprine *MRLC2* mRNA. Analysis by RT-PCR showed that *MRLC2* mRNA was present in the

 

heart, stomach, liver, spleen, lung, small intestine, kidney, leg muscle, abdominal muscle, and longissimus dorsi muscles. In particular, the high expression of *MRLC2* mRNA was detected in the longissimus dorsi, leg muscle, abdominal muscle, stomach, and heart, but low levels of expression were also observed in the liver, spleen, lung, small intestine, and kidney. The expression of the *MRLC2* gene was upregulated in the longissimus dorsi muscle of Boer and Tianfu goats, and it was moderately upregulated in Chengdu Ma goats.

**Key words:** Heterosis; Tianfu goat; *MRLC2* gene; Sequence analysis; Expression

## INTRODUCTION

The use of heterosis in different livestock animals has achieved great success and is considered essential for meeting the food needs of the world's population (Duvick, 1999). Heterosis, or hybrid vigor, refers to the phenomenon that the progeny of diverse inbred varieties exhibit greater biomass and speed of development than the better of the two parents; however, the molecular mechanism of heterosis remains to be elucidated (Xu et al., 2005; Xie et al., 2006). The development of quantitative trait locus mapping has facilitated the investigation of heterosis (Syed and Chen, 2005). However, the phenomenon of heterosis is controlled by gene expression and regulation in the heterozygote (Xu et al., 2005; Liu et al., 2005). Thus, isolating the specific genes that are differentially expressed between the crossbreeds and purebreds and elucidating the function of these genes are essential for understanding the molecular mechanism of heterosis in goats.

The head of myosin in skeletal muscles consists of two pairs of light chains: myosin regulatory light chain 2 (MRLC2) and MRLC1 or MRLC3 (Huang et al., 2000; Umeda et al., 2005). MRLC2 is the primary regulatory component of thick filament-linked systems. Phosphorylation of MRLC2 has been proposed as an efficient regulatory mechanism in the cross-bridge cycle, calcium sensitivity, and other parameters strengthening muscle performance (Sweeney et al., 1993; Reddy et al., 2002). Phosphorylation of MRLC2 is likely crucial for generating force in non-muscle cells and smooth muscle cells (Murata-Hori et al., 2000; Luo et al., 2004). Previous studies have shown that phosphorylation of MRLC at Ser19 and Thr18 enhances the actin-activated Mg-ATPase activity of myosin II and promotes the assembly of myosin II filaments *in vitro* (Ikebe, 1989).

Xie et al. (2006) used the SSH technique to analyze gene expression differences of the swine *MRLC2* gene in the longissimus dorsi of Meishan x Yorkshire F1 crossbreeds and Meishan pigs. The Tianfu goat is an emerging breed of goat in China. The Tianfu goat was formed by multispecies crossing technology with a maternal local breed, a Chengdu Ma goat from the Sichuan Province, and paternal imported breeds of Boer goat from Germany. In China, this goat species is characterized by good quality meat, rapid growth, roughage resistance, and strong adaptability (Zheng et al., 2012). In this study, we analyzed the differential expression of the *MRLC2* gene in purebreds and crossbreeds by obtaining the cDNA sequence of *MRLC2* and investigating the function of *MRLC2* in goats. This study provides an important starting point for future functional studies of *MRLC2*.

 

## MATERIAL AND METHODS

### Experimental animals and sample collection

The study on the Tianfu goat was approved by the Sichuan Agricultural University Animal Centre. All goats were bred under standard conditions and slaughtered at the same age. The heart, stomach, liver, spleen, lung, small intestine, kidney, leg muscle, abdominal muscle, and longissimus dorsi muscle samples were harvested and frozen in liquid nitrogen for total RNA extraction. Animal care and use were approved by the Sichuan Agricultural University Animal Care and Use Committee.

### RNA isolation and synthesis of cDNA

Tissue samples were randomly removed from the liquid nitrogen jar and triturated in liquid nitrogen. Total RNA was extracted from the triturated sample using the TRIzol total RNA extraction reagent according to the manufacturer protocol (TaKaRa, Dalian, China). Total RNA was analyzed by electrophoresis of 1 μL RNA on 1.5% agarose gels stained with ethidium bromide. The RNA was dissolved in RNase-free water at a concentration of 1 μg/μL and preserved at -70°C. First strand cDNA was synthesized using 2 μg purified total RNA using a real-time RT-PCR kit (TaKaRa) according to the manufacturer protocol. First strand cDNA was obtained and preserved at -20°C.

### Cloning of the *MRLC2* gene

A pair of homologous primers (Table 1) was designed using the DNAMAN 6.0 software (http://www.lynnon.com) on the basis of conserved regions of the cattle, pig, human, and rat *MRLC2* gene sequences. Using these primers, a cDNA fragment was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using first strand cDNA as the template. The thermocycling program was as follows: pre-denaturation at 95°C for 5 min; 37 cycles at 94°C for 40 s, 55°C for 40 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. The PCR products were separated by 1.5% agarose gel electrophoresis and extracted using a gel extraction kit (Shanghai Biotechnology Co., China). The products were cloned into the pMD19-T vector (TaKaRa) and submitted to LiuHe HuaDa Biotechnology Co. (Beijing, China) for sequencing.

**Table 1.** Primer pairs used to amplify the *MRLC2* gene.

| Primer | Sequence (5'-3') | Fragment length | Application |
|---|---|---|---|
| MR1-f | F: CACCATGTCGAGCAAAAGAGCAAAG | 776 bp | cDNA clone |
| MR1-r | R: TACTGGGGGCGGGGAGGGTA | | |
| MR2-f | F: CCCAGCGTGCGACCTCCAAT | 197 bp | Expression |
| MR2-r | R: TGGGACCTGGAGCCTCATTCATCA | | |
| G-f | F: GCAAGTTCCACGGCACAG | 118 bp | Expression |
| G-r | R: TCAGCACCAGCATCACCC | | |

F = forward, R = reverse sequences.

### Sequence analysis

Sequence analysis of the predicted MRLC2 protein, translated from the caprine

*MRLC2* sequence, was performed by searching against the NCBI database (http://www.ncbi.nlm.nih.gov) and by using the ExPaSy translation tool (http://www.expasy.org). Protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval tool on the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST), and the MRLC2 protein sequences from related species were aligned with the goat sequence using ClustalW (www.empiritag.com/site/align.genome.jp/). Putative signal peptides were predicted using the SignalP 4.0 server (http://cbs.dtu.dk/services/SignalP/). Psort II (http://psort.hgc.jp/) was used to predict protein sorting signals and intracellular localization. The transmembrane domains of the amino acid sequences were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The domain structure of the caprine protein was analyzed using the SMART server (http://smart.embl.de/). The secondary structures of the deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/). Web-based miRNA prediction was used to locate potential conserved miRNA targets (miRBase, http://www.mirbase.org/). The SWISS-MODEL server (http://www.expasy.org/swissmod/SWISSMODEL.html) was used to model the protein 3D conformation, which was then visualized using the Swiss PDB Viewer (Guex and Peitsch, 1997; Schwede et al., 2003).

### Analysis of the *MRLC2* gene

The tissue-specific expression pattern of the *MRLC2* gene was analyzed by RT-PCR by using the constitutively expressed *GAPDH* gene as an internal control. The RT-PCR primers used in the study are listed in Table 1. The amplifications were performed in a 25-μL reaction volume containing 12.5 μL 2X SYBR Premix ExTaq (TaKaRa), 3-5 pmol of each primer, and 2 μL diluted cDNA. The PCR amplification was conducted at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 57°C for 34 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression level of *MRLC2* in each tissue (Livak and Schmittgen, 2001). Three microliters of the RT-PCR products was electrophoresed on a 1.5% agarose gel.

## RESULTS

### Sequence analysis of *MRLC2*

A 776-bp fragment of *MRLC2* was obtained by cloning the cDNA from the longissimus dorsi muscle of caprine as template (GenBank: JN257256). A BLASTP search of the NCBI nucleotide sequence database revealed that the *MRLC2* fragment was remarkably similar to other mammalian *MRLC2* gene sequences. The goat *MRLC2* nucleotide sequence and the deduced amino acid sequence are shown in Figure 1. An open reading frame extending from positions 8 to 527 of the nucleotide sequence encodes a protein of 172 amino acids. The predicted molecular weight is 19.8 kDa, and the predicted pI is 4.65. Nine phosphorylation sites were predicted by neural network analysis (Figure 1). Using the SignalP software, it was predicted that MRLC2 does not contain a putative signal peptide. The TMHMM results indicated that this protein has no obvious transmembrane domain, suggesting that MRLC2 is not an epimembranous acceptor or a transmembrane protein. Subcellular localization analysis by the Psort II software showed 52.2% nuclear, 26.1% cytoplasmic, and 17.4% mitochondrial localization.


**Figure 1.** Nucleotide and deduced amino acid sequence of the MRLC2 from caprine. (*) = stop codon; red indicates initiation codon; 9 phosphorylation sites were highlighted in blue; single underline = putative EF-hand conserved domain sequence.

The putative protein was also analyzed using the CD-search and the SMART software, and two conserved EF-hand domains were identified, the first from amino acids 33 to 61 and the second from amino acids 102 to 130 (Figures 1 and 2). EF-hands are calcium-binding motifs consisting of a 12-residue loop flanked on either side by a 12-residue alpha helix that undergoes a conformational change upon binding of calcium ions. Prediction of the secondary structure indicated that the caprine MRLC2 sequence is composed of α-helix (58.14%), extended strand (2.91%), β-sheet (7.56%), and random coil (31.40%). Thus, the majority of the protein was predicted to be alpha helical (Figure 3).

## Multiple sequence alignment and evolutionary relationship of *MRLC2*

Sequence alignment using ClustalW revealed that the amino acid sequence of caprine MRLC2 was 99.42% similar to cattle MRLC2 and 98.26, 95.95, 98.26, and 98.26% similar to rat, pig, human, and house mouse, respectively (Figure 4). Furthermore, the multiple sequence alignment revealed that caprine and cattle MRLC2 differed by only a single amino acid.

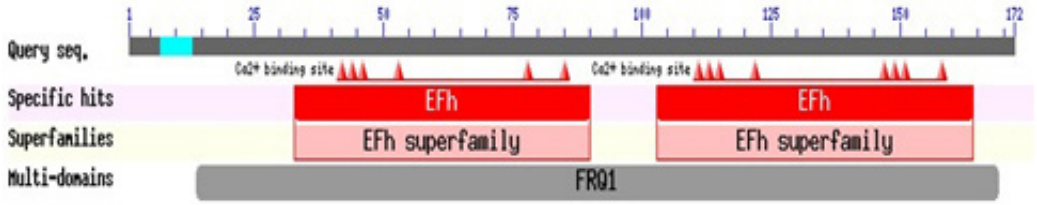


**Figure 2.** Putative conserved domain of protein encoded caprine MRLC2 was also analyzed by the CD-search server.

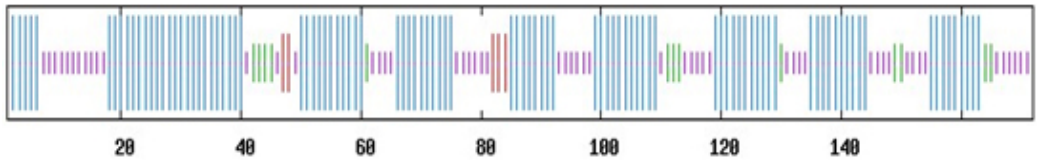


**Figure 3.** Predicted secondary structure of the caprine MRLC2 protein by SOPMA. α-helices, extended strands, β-turn, random coils are indicated, respectively, with the longest, the second longest, the third longest, and the shortest random coils.

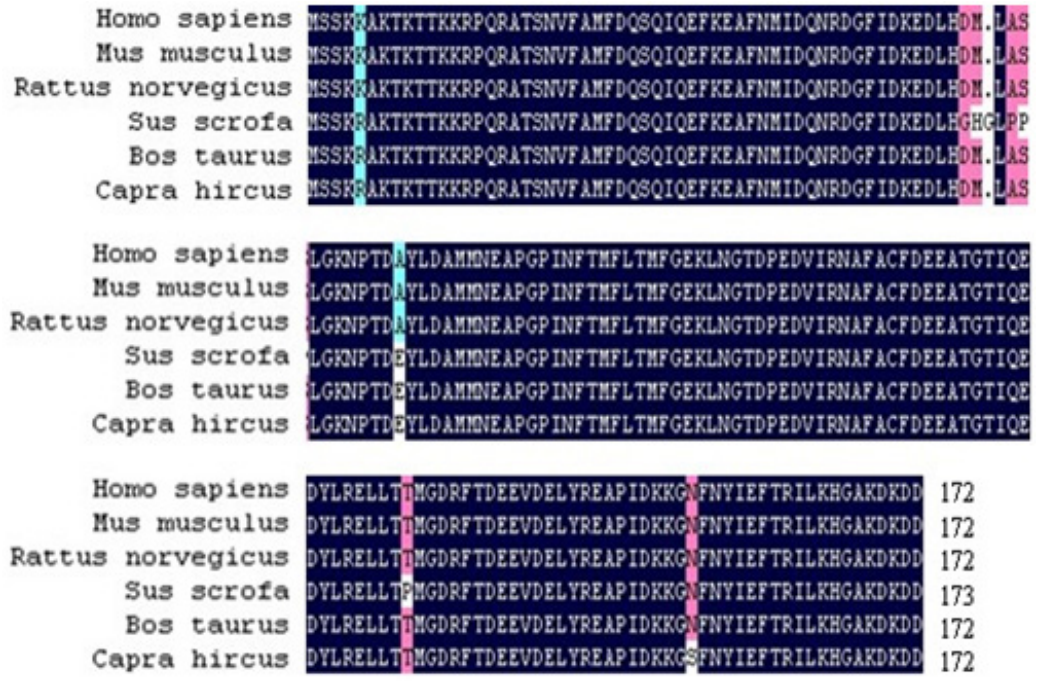


**Figure 4.** Alignments of amino acid sequence of *MRLC2* among caprine and *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Sus scrofa*, and *Bos taurus*.

To evaluate the evolutionary relationship between caprine MRLC2 with other organisms, we constructed a phylogenetic tree based on MRLC2 amino acid sequences by using the MEGA 4.0 software (Figure 5). The amino acids sequences of the nine species were used for the rooted phylogenetic tree, constructed by the neighbor-joining method. From the phylogenetic tree, MRLC2 proteins from different species were divided into four subgroups. *Capra hircus* (Tianfu goat), *Bos taurus*, and *Sus scrofa* were grouped into an early-diverged cluster. *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, and *Macaca mulatta* formed a cluster separated from the early-diverged cluster. The caprine MRLC2 sequence was more similar to the predicted MRLC2 sequences of other mammals than to *Xenopus tropicalis* and *Hydra magnipapillata* (Figure 5).

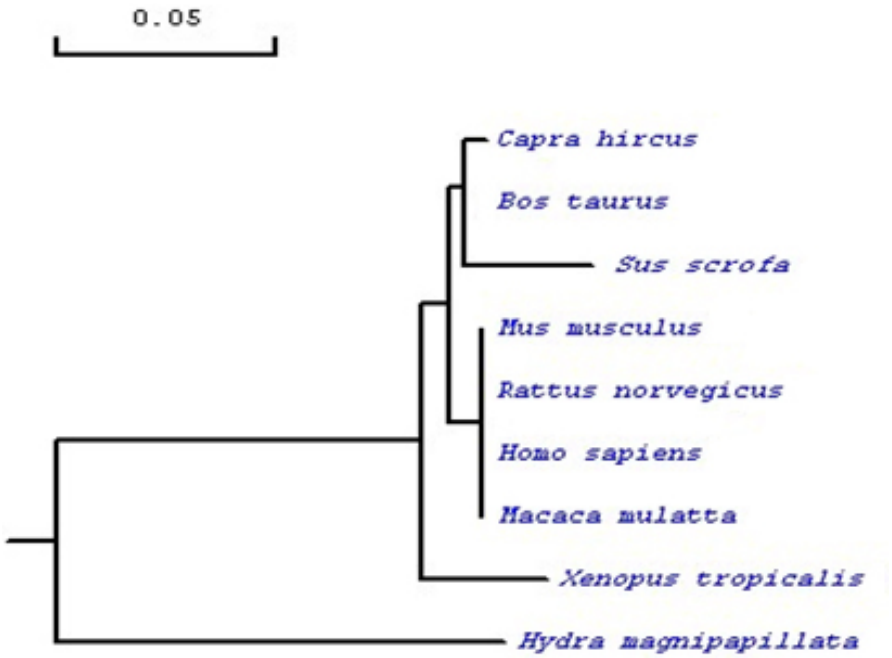


**Figure 5.** Phylogenetic tree of aligned amino acid sequences of the caprine MRLC2 protein and the putative MRLC2 homolog proteins. The tree was obtained by bootstrap analysis with neighbor-joining method; numbers on the branches represent bootstrap values for 1000 replications.

## Homology modeling

To better understand the structure of caprine MRLC2, homology modeling was performed to predict the 3D structure of MRLC2. The 3D structure of MRLC2 (amino acids 1-143) was similar to that of the chick EF-hand domain (3J04E, amino acids 26-168) (Figure 6). As shown in Figure 6B, the MRLC2 EF-hand domain and the chick EF-hand domain structures are very similar; two differences in the domain structure are highlighted in yellow. Prediction of the 3D structure provides a basis to further study the structure-function relationship of MRLC2.

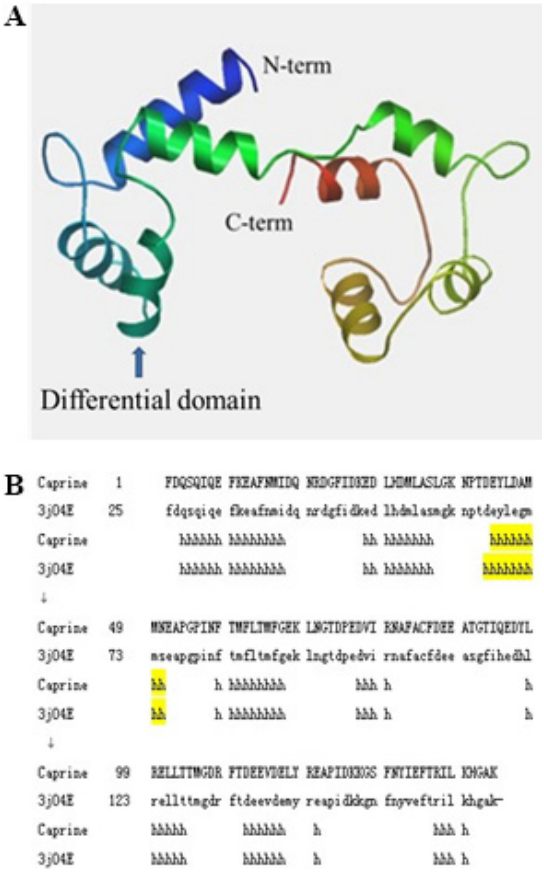


**Figure 6.** Homology modeling of MRLC2 of caprine based on the crystal structure of FE-hand domain (3j04E: 26-168 AA). **A.** 3-D structure of grass carp MRLC2 (1-143AA) constructed by the comparative protein modeling program SWISS-MODEL. **B.** Amino acid sequence alignments: h = amino acid residues responsible for formation of the ‘‘alpha helix’’.

## Location of potential miRNA targets

In this study, one *M. musculus* miRNA (mmu-mir-871) and one *H. sapiens* miRNA (hsa-miR-4786) had target sites in the Tianfu goat MRLC2 coding sequence, 343-actggcaccattc aggaggat-363 and 124-gaccagaacagggatgggttga-145, respectively.

## Analysis of caprine *MRLC2* expression

Fluorescence quantitative RT-PCR was conducted to determine the *MRLC2* gene expression in the longissimus dorsi muscle and other tissues of the Tianfu goat and its parent, the Chengdu Ma goat. The ratio of the target band intensity to the *GAPDH* band intensity was used to represent the relative expression level of the target gene.

The *MRLC2* gene was expressed in 10 of the tissues tested in Tianfu goats. The expression level did vary significantly among tissues, with higher expression observed in the longissimus dorsi muscle, abdominal muscle, and leg muscle, and lower expression observed in the liver, spleen, lung, small intestine, and kidney (Figure 7).

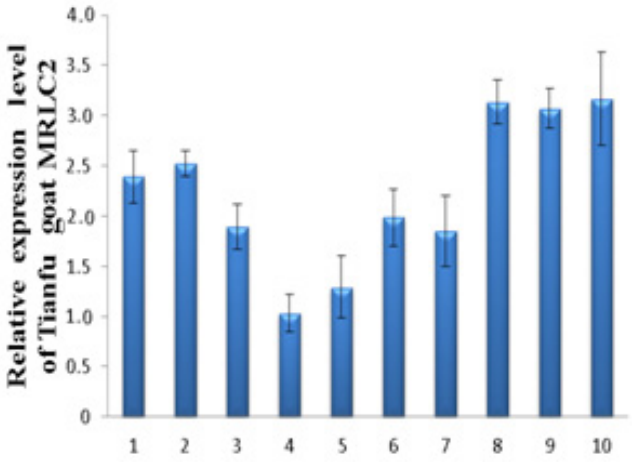


**Figure 7.** Tissue specific expression of the Tianfu goat *MRLC2* gene. Data are reported as means ± standard deviation (N = 3). The samples 1-10 represent heart, stomach, liver, spleen, lung, small intestine, kidney, leg muscle, abdominal muscle, and longissimus dorsi muscle.

The *MRLC2* gene was expressed in Tianfu, Chengdu Ma, and Boer goats, but to a lesser extent in Chengdu goats (Figure 8).

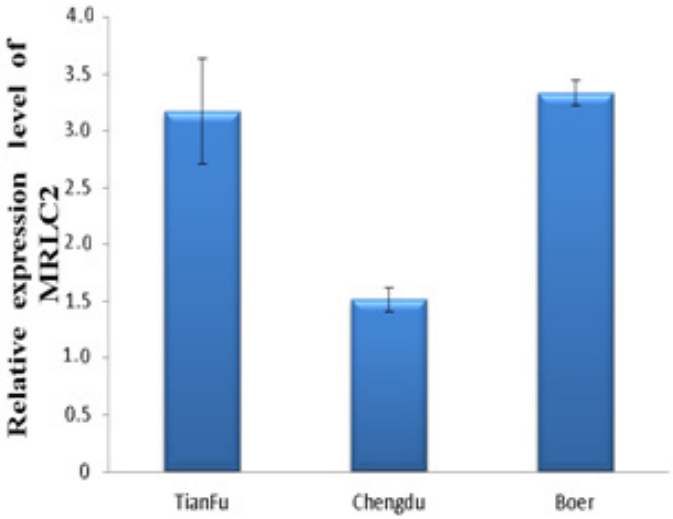


**Figure 8.** Relative expression level of MRLC2 in Tianfu, Chengdu Ma, and Boer goats. Data are reported as means ± SD (N = 5).

## DISCUSSION

Numerous *MRLC2* genes have been isolated from mammals, including human, rat, mouse, dog, pig, and cattle *MRLC2* (Xie et al., 2006). In this study, a 776-bp cDNA sequence corresponding to the *MRLC2* gene was cloned from the Tianfu goat. The cDNA contained a 512-bp coding sequence for 172 amino acids with two predicted calcium-binding motifs (EF-hand domains) and nine putative phosphorylation sites. The protein sequence had no predicted signal peptide or transmembrane region. Many protein functions are regulated by calcium binding and phosphorylation/dephosphorylation, and the caprine MRLC2 protein has two calcium-binding domains and several phosphorylation sites. Phosphorylation of MRLC2 at Thr18 and Ser19 is known to increase the actin-activated Mg-ATPase activity of myosin (Iwasaki et al., 2001). Thus, caprine MRLC2 may exert its function through calcium binding and phosphorylation/dephosphorylation. This study provides a basis for future analysis of caprine *MRLC2*.

To better understand the structure and function of the caprine MRLC2 protein, homology modeling of the caprine MRLC2 protein was performed using the chick EF-hand domain (3J04E) as a template. The results indicated that the conserved domain of goat MRLC2 is an 8-helix bundle, implying that the caprine MRLC2 protein has a function similar to that of the chick MRLC2 protein. The caprine MRLC2 protein was 99.42 and 98.26% identical to cattle and rat MRLC2. Phylogenetic tree analysis revealed that the caprine *MRLC2* gene is closely related to other mammalian *MRLC2* genes. Therefore, the *MRLC2* gene has likely been conserved during evolution.

miRNAs are small noncoding RNAs of 17-24 nucleotides that regulate gene expression by binding to the coding region of target mRNAs (Zeng et al., 2003; Weber et al., 2007). miRNA target prediction showed that one *M. musculus* miRNA (mmu-mir-871) and one *H. sapiens* miRNA (hsa-miR-4786) have target sites in the coding sequence of the Tianfu goat *MRLC2* gene. Further investigation is needed to confirm whether these miRNAs regulate *MRLC2* gene expression in caprine animals.

Quantitative RT-PCR showed that the *MRLC2* gene was not differentially expressed in the 10 tissues analyzed, and this result is consistent with former research on pigs (Xie et al., 2006). However, examination of the *MRLC2* gene expression showed some differences in the expression patterns in muscle tissues compared with non-muscle tissues. For example, the expression level in skeletal muscle was higher than that in non-muscle tissues, perhaps because of the growth and development of muscles. In addition, we found high gene expression in the longissimus dorsi muscle of the Boer and Tianfu goats, but weak expression in the Chengdu Ma goat. Differential gene expression in purebreds *vs* crossbreeds is largely responsible for the heterosis of the crossbreeds (Liu et al., 2005; Xie et al., 2006). The *MRLC2* gene is upregulated in the Boer and Tianfu goats. Elucidation of the intrinsic associations between the differential expression of the goat *MRLC2* gene and the heterosis of goats, for example, roughage resistance, strong adaptability, disease resistance, and rapid growth, requires further study.

In conclusion, we isolated the caprine *MRLC2* gene and used bioinformatic tools to analyze the coding sequence and the predicted protein sequence. We predicted the 3D structure of the MRLC2 protein, investigated temporal mRNA expression differences, and analyzed the differential expression of *MRLC2* in the Tianfu goat and its parents, Boer and Chengdu Ma goats. This information provides an important theoretical basis for further research on molecular-assisted selection during animal breeding.

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