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Full Length Research Paper

The polymorphisms of *κ-casein* gene and their associations with milk production traits and expression analysis in Chinese Holstein cattle

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The polymorphisms of exon 4 and 5 of κ-casein (***CSN3***) **gene and their associations with milk production traits and expression pattern in Chinese Holstein cattle were investigated. Nine mutational sites, of which seven were novel mutational sites, were identified and genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), created restriction site-PCR (CRS-RFLP) and sequencing methods in 398 cows. Linkage disequilibrium analysis showed that SNP-1 (g.10891 T > C rs 43703015, g.10927 C > A rs 43703016, g.10988 G > A ss 256302464 and g.10966 A > T ss 256302465) and SNP-2 (g.12907 A > G ss 256302466, g.12950 G > A ss 256302468, g.12989 C > T ss 256302469 and g.13028 A > G ss 256302470) were completely linked, respectively. Correlation analysis showed that SNP-1, SNP-2 and SNP-3 (g.12980 T > C ss 256302467) markers were closely correlated to the fat content. The SNP-3 marker had a remarkable effect on the protein content (P < 0.05). 16 combined genotypes of the three SNPs were found. Fat and protein content in combinations of genotypes were varied significantly (P < 0.05). Genotypes *BBCCEE* and *ABTCDD* individuals had the highest fat and protein content, respectively, which may be useful for marker assisted selection program in dairy cattle. The expression of *CSN3* mRNA in the mammary tissue was higher than that of in the liver tissue (P < 0.05) and the expression in the spleen of *BB* genotype was higher than that of *AA* genotype in the SNP-1 (P < 0.05) by fluorescent quantitation real-time PCR (Q-PCR) assay.**

Key words: SNPs, *CSN3* gene, combined genotype, Q-PCR, milk production traits.

INTRODUCTION

Milk protein is an important functional nutrient in bovine milk. Its content is also the capital parameter for assessing the quality of milk. Casein occupies 80% of the amount of milk protein and comprises α-, β-, γ- and *CSN3* proteins. *CSN3* protein, the only casein fraction that contains the sulphur amino acids cysteine and methionine, constitutes approximately 13% of milk casein (Farrell et al., 2004). *CSN3* protein plays an important role in the formation, stabilization and aggregation of casein protein micelles, being of great significance in cheese production (Chessa et al., 2003).

The *CSN3* gene is located on bovine BTA6, containing 5 exons and 4 introns, with the total length of about 13 kb (Alexander et al., 1988). Variants of *CSN3* gene have been extensively studied in cattle and 13 alleles have been identified with *A, B* and *E* as the most common (Farrell et al., 2004; Prinzenberg et al., 2008). The amino acids threonine and aspartic acid are encoded by allele *A* at positions 136 and 148; the corresponding amino acids for allele *B* are isoleucine and alanine (Eigel et al., 1984). At position 155, allele *E* encodes the amino acid glycine, while the amino acid serine commonly occurs at this position (Erhardt, 1989). Allele *B* is often associated with milk protein quality and coagulation properties (Bovenhuis et al., 1992; Matějíček et al., 2008).

Several SNPs of *CSN3* gene were reported to be significantly associated with the bovine milk production traits; however, the results of different reports are conflicting. A positive effect of the allele *B* has been found

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Table 1. Genotyping SNPs detection in the bovine *CSN3* gene by PCR, PCR-RFLP and CRS-PCR.

SNP	Primer sequence	AT (%)	SAF (bp)	RE
g.10891 T>C g.10927 C>A g.10988 G>A	5'CGCTGTGAGAAAGATGAAAGATTC3' 5'AGATTCAAGGAGTATACCAATTGTTG3'	58	779	*Taq* I *Hin*d III *Pst* I
g.10996 A>T	5'GAAATCCCTACCATCAATACCAT3' 5'GTCTTCTTTGATGTCTCCTTAGAAT3'	56	186	*Ssp* I
g.12907 A>G	5'AACTACTTGGATACAGACATGAAGC3' 5'CAAAGGAAAGTTTGAAGTAGTGATT3'	59	148	*Hinf* I
g.12950 G>A	5'TTCCTTTGGCCAGTTGTCTGCCTCC3' 5'CATTTGATTGGCTTTATTATGCAGG3'	63	139	*Msp* I
g.12980 T>C g.13028 A>G	5'CGCTGTGAGAAAGATGAAAGATTC3' 5'AGATTCAAGGAGTATACCAATTGTTG3'	55	378	*Bgl* II *Tas* I
g.12989 C>T	5'TACATATCATGAGAACTGTTAGCAC3' 5'AAAGTAAAATGTAAGAGGAGACCAG 3'	57	183	*Eco*R II

*Letters with underlines denote mismatching base in CRS-PCR method; SNP, single nucleotide polymorphism; AT, annealing temperature; SAF, size of amplification fragment; RE, restriction enzyme.

on protein yield (Ng-kwai-hang et al., 1984; Van eenennaam and Medrano, 1991) and protein content (Gonyon et al., 1987; Bovenhuis et al., 1992) in most cases. The allele *B* has been associated with higher (Ng-kwai-hang et al., 1986) and lower (Bovenhuis et al., 1992) milk yield, whereas, other studies have indicated no significant effect (Lundén et al., 1997). The possible reason is that the milk production traits are affected by multi-gene and environmental factors. The effect of one single mutational site may be affected by other mutational sites, so the associations between the combinations of many SNPs and milk production traits were assessed to elevate the accuracy of marker assisted selection (MAS) by several authors (Ikonen et al., 2001; Kamiński et al., 2006).

To have a better coverage of *CSN3 gene* in this study, the relationships between SNPs and their combinations with milk production traits and the *CSN3* gene expression in different tissues by Q-PCR were investigated.

MATERIALS AND METHODS

Animals and traits

A total of 398 Chinese Holstein individuals (ages ranging from 4 to 7 years, including first to fourth parity) were from seven farms in China and milk samples were taken from each cow once a month, in the course of routine control milking, during the whole lactation. Data of milk performance traits (305 d milk yield, protein and fat contents) were collected from the laboratory of dairy herd improvement (DHI) center (OX Biotechnology, Shandong, China) using the milk composition analyser (Foss MilkScan FT 6000, Denmark) for statistical analysis.

Genotyping tests

The genomic DNA was isolated from bovine blood samples by the method described by Gan et al. (2007). Primers (Table 1) of PCR were designed according to GenBank reference sequence (Accession No. NC_007304) and CRS-PCR method with one of the primers containing a nucleotide mismatch, which enables the use of restriction enzymes for discriminating sequence variations (Zhao et al., 2003). A PCR was carried out using 0.5 U of Taq DNA polymerase (TaKaRa, Dalian, China), 2.5 µl 10 × PCR Buffer, 50 ng DNA, 1.8 mmol L^{-1} $MgC1_2$, 0.2 mmol L^{-1} dNTPs and 0.28 µmol L^{-1} of each primer in a total volume of 25 µl. After an initial denaturation for 5 min at 95°C, PCR was performed by 35 cycles of denaturing at 95°C for 45 s, annealing for 45 s, primer extension at 72°C for 45 s (Table 1). The final extension was undertaken at 72°C for 7 min. PCR reactions were performed using a PCR system thermal cycler dice (TaKaRa, Dalian, China). The PCR products were detected by 1% agarose gel electrophoresis. After PCR products were recovered with DNA fragment recovery kit, they were conjugated with pEASY-T3 carrier (TaKaRa, Dalian, China) and transformed into competent cell DH5α. Subsequently, the positive clones were sequenced using an ABI PRISMTM 3730 DNA sequencer (Applied Biosystems) and BigDye terminator v3.1 sequencing kit (Shanghai Sangon, China). Based on the results obtained from the sequence analysis, products were digested by appropriate restriction enzymes as shown in Table 1. The digested products were detected by 2.5% agarose gel or 12% polyacryolamide gel electrophoresis. Genotype was identified by the electrophoretic banding pattern.

Fluorescence quantitative real-time PCR

Total RNA was isolated from the heart, liver, spleen and mammary tissue from 20 Chinese Holstein cattle using TRIzol reagent (Bioteke, Beijing, China) according to the manufacturer's instructions. cDNA was synthesized with the transcriptor first-strand cDNA synthesis kit (TaKaRa, Dalian, China). Real time PCR analysis was performed in a total volume of 20 µl mixture containing 50 ng cDNA, 0.4 µM of sense and anti-sense primers, 6.8 µl of dH_2O,10.0 µl of SYBR® Premix Ex TaqTM (2 ×) and 0.4 µl of ROX reference dye (50 ×) (TaKaRa, Dalian, China). To normalize differences in the amount of total cDNA added to each reaction, *β-actin* gene expression was used as an endogenous control. The reaction mixture was denatured for 30 s at 95°C and incubated for 40 cycles (denaturing for 5 s at 95°C, annealing for 31 s at 61°C).

The primers used in the experiment were as follows: *CSN3*

(Accession No. NM_174294) (sense 5'-AAGAAGACAACGCAGGTCTAGC-3' and antisense 5'-ATGTAAGAGGAGACGAGGAAGG -3') and *β-actin* (Accession No. NM_173979) (sense 5'-GCACAATGAAGATCAAGATCATC-3' and antisense 5'- CTAACAGTCCGCCTAGAAGCA-3'). The PCR was monitored by the ABI PRISM 7000HT fast real-time PCR system. Relative quantification of the *CSN3* gene expression was calculated using the standard curve based method for relative real time PCR (Larionov et al., 2005).

Statistical analysis

The linkage disequilibrium analysis was performed by SHEsis software (Shi and He, 2005). Genotypic and allelic frequencies, polymorphism information contents (*PIC*), heterozygosities (H_e) and effective population of allele (N_e) were calculated. Allele substitution effect was analyzed using the method described by Liu et al. (2004). The associations between SNP marker as well as combined genotypes of *CSN3* gene and milk production traits were analyzed by the least squares as implemented in the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA, 2002) according to the following linear model:

$$Y_{ijkl} = \mu + G_i + S_j + H_k + P_l e_{ijkl}$$

Where, Y_{ijkl}, is the observed value; μ, overall mean; G_i, the fixed effect of genotype or combined genotype; S_j ,the fixed effect of season; H_k, the fixed effect of farm; P_l, the fixed effect of parity and e_{ijkl}, random error. Values of $P < 0.05$ and $P < 0.01$ were regarded as significant. The CSN3 mRNA expression was calculated using the standard curve based method for relative real time PCR.

RESULTS

Polymorphisms of CSN3 gene in Chinese Holstein cattle

Nine mutational sites were identified and genotyped in exon 4 and 5 of *CSN3* gene in 398 Chinese Holstein cattle (Figure 1). The seven novel SNPs were submitted to the National Centre for Biotechnology Information (submitted SNP numbers: g.10988 G > A ss 256302464, g.10966 A > T ss 256302465, g.12907 A > G ss 256302466, g.12950 G > A ss 256302468, g.12989 C > T ss 256302469, g.13028 A > G ss 256302470 and g.12980 T > C ss 256302467). Products were digested and divided into three genotypes. Digested bands below 50 bp were not shown in Figure 1.

Allele and genotype frequencies, values of χ^2, *PIC*, H_e, and N_e of the three SNPs in bovine *CSN3* gene are shown in Table 2. Allele *A*, *D* and *T* in the SNP-1, SNP-2 and SNP-3 were predominant in the population, respectively. The results of χ^2 test indicated that, the three SNPs did not deviate from the Hardy-Weinberg equilibrium ($P > 0.05$).

The linkage disequilibrium between the three SNPs in the population was estimated, which indicated that within the two groups, SNP-1 (g.10891 T > C, g.10927 C > A, g.10988 G > A and g.10966 A > T) and SNP-2 (g.12907A > G, g.12950 G > A, g.12989 C > T and g.13028 A > G), had complete linkage in the ested bovine samples ($r^2 = 1$), respectively.

Association between the SNPs in the *CSN3* and milk production traits

Correlation analysis showed that the SNP-1 marker was significantly correlated with the fat content ($P < 0.05$). As shown in Table 3, the cows with genotype *BB* and *AB* had marked higher fat content than those of genotype *AA*. The cows with genotype *DE* had significantly higher fat content than those of genotype *DD* ($P < 0.01$) in SNP-2. Compared to genotype *TT*, genotype *TC* had significantly higher fat ($P < 0.01$) and protein contents ($P < 0.05$) in SNP-3. However, no significant difference in 305 d milk yield was found among the different genotypes in the three SNPs.

Allele substitution effects are presented in Table 3, indicating that allele *B, E* and *C* corresponding to *A, D* and *T* had a positive effect on the increase of fat and protein contents, respectively. 16 combined genotypes of three SNPs were found in the 398 cows (Table 4). Combined genotype *AATTDD* had the highest frequency (49.5%), whereas, the number of the seven genotypes, *AATTDE* (n = 3), *AATTEE* (n = 3), *AATCEE* (n = 2), *BBTCDD* (n = 2), *ABCCEE* (n = 1), *ABTCEE* (n = 1) and *ABCCDD* (n = 1) were less than 4 samples, not involved in this association analysis. Fat and protein contents in combinations of genotypes were significantly different ($P < 0.05$). No significant difference in 305 d milk yield was observed in the various combinations.

Expression of the bovine CSN3 mRNA

The 20 cows were divided into three groups based on the genotype in SNP-1: AA (n = 7), AB (n = 10) and BB (n = 3). The CSN3 mRNA expression in the heart, liver, spleen and mammary from the 20 cows are shown in Figure 2. The Q-PCR results revealed that, *CSN3* mRNA expression in the spleen of *BB* genotype was higher than that of *AA* genotype in SNP-1 ($P < 0.05$) (Figure 2a). No significant differences in mRNA expression were found in the various genotypes of the other tissues ($P > 0.05$). The expression of *CSN3* mRNA in the mammary tissue was higher than those in the liver tissue ($P < 0.05$) (Figure 2b).

DISCUSSION

The polymorphisms of *CSN3* gene were focused mainly on the exon 4, intron 2 (Damiani et al., 2000), 5' flanking region (Robitaille et al., 2005) and promoter (Keating et al., 2007) in the different cattle breeds. Two common mutational sites (g.10891 T > C and g. 10927 C > A) of

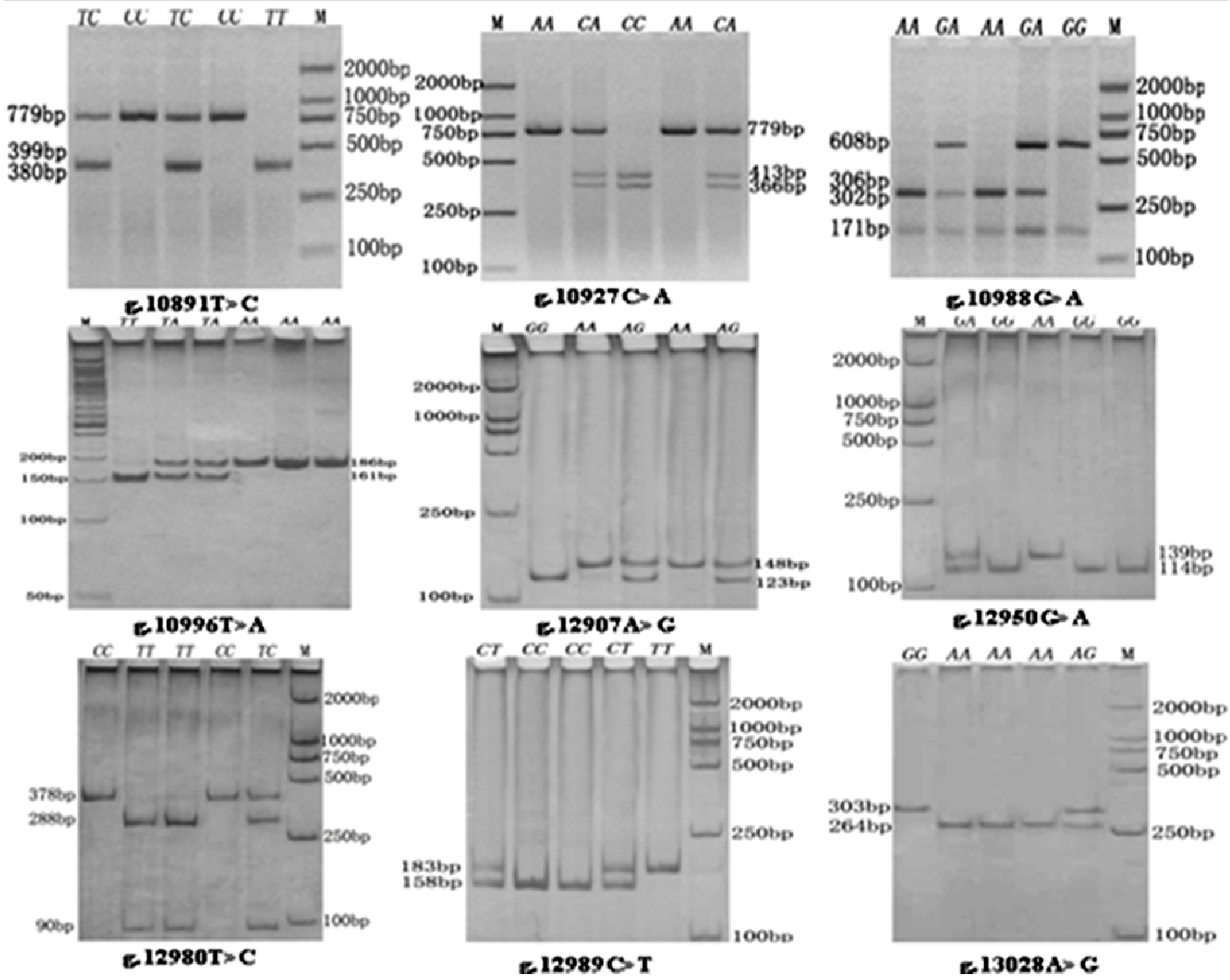

Figure 1. Detections of PCR-RFLP and CRS-PCR on exon 4 and 5 of *CSN3* gene in Chinese Holstein cattle.

Table 2. Genetic parameters of *CSN3* gene in Chinese Holstein cattle (n = 398).

SNP	Genotype	Sample (n)	Genotypic frequency (%)	Allele	Allelic frequency (%)	*PIC*	H_e	N_e	χ^2
SNP-1	*AA*	293	73.71	*A*	86.03	0.21	0.24	1.32	0.33
	AB	98	24.63	*B*	13.97				
	BB	7	1.66						
SNP-2	*DD*	297	74.68	*D*	85.64	0.22	0.25	1.33	5.09
	DE	87	21.91	*E*	14.36				
	EE	14	3.41						
SNP-3	*TT*	208	52.3	*T*	72.0	0.33	0.42	1.72	0
	TC	157	39.4	*C*	28.0				
	CC	33	8.3						

Table 3. Least squares means and standard errors of milk production traits for different *CSN3* genotypes in Chinese Holstein cattle.

SNP	Genotype	Fat content (%)	Protein content (%)	305 d milk yield (kg)
SNP-1	*AA*	3.60 ± 0.10^{b}	3.22 ± 0.05	5439 ± 271.5
	AB	3.81 ± 0.11^{a}	3.27 ± 0.06	5529 ± 296.8
	BB	4.02 ± 0.23^{a}	3.35 ± 0.11	5454 ± 597.4
	α_1	-0.210	-0.054	-66.704
SNP-2	*DD*	3.63 ± 0.10^{B}	3.20 ± 0.05	5457 ± 291.6
	DE	3.86 ± 0.11^{A}	3.27 ± 0.06	5442 ± 322.6
	EE	3.87 ± 0.20^{AB}	3.23 ± 0.10	5088 ± 600.4
	α_2	-0.135	-0.054	11.158
SNP-3	*TT*	3.59 ± 0.11^{B}	3.18 ± 0.05^{b}	5443 ± 301.9
	TC	3.78 ± 0.10^{A}	3.26 ± 0.05^{a}	5457 ± 300.5
	CC	3.68 ± 0.14^{AB}	3.27 ± 0.07^{ab}	5432 ± 400.2
	α_3	-0.155	-0.031	8.070

*Mean values with the different lower case letters (*a, b*) in the same mutational site and column denote significant difference ($P < 0.05$) and those with the different upper case letters (*A, B*) denote significant difference at $P < 0.01$. α_1, α_2 and α_3 are mean gene substitution effects.

Table 4. Least squares mean (LSM) and standard error (SE) for milk yield, fat and protein contents of different combined genotypes of *CSN3* gene in Chinese Holstein cattle.

Genotype	Sample (n)	Genotypic frequency (%)	Fat content (%)	Protein content (%)	305 d milk yield (kg)
AATTDD	197	49.50	3.62 ± 0.11^{Edl}	3.18 ± 0.05^{a}	5465 ± 307.0
AATCDD	62	15.58	3.68 ± 0.12^{hk}	3.24 ± 0.06	5324 ± 331.9
AATCDE	17	4.27	3.75 ± 0.16^{j}	3.27 ± 0.08	5590 ± 464.4
ABTCDE	57	14.32	3.90 ± 0.12^{EFgh}	3.25 ± 0.06	5531 ± 349.3
ABTCDD	16	4.02	3.94 ± 0.18^{Bcd}	3.35 ± 0.09^{a}	5758 ± 508.6
AACCDD	9	2.26	3.45 ± 0.20^{cg}	3.20 ± 0.10	5928 ± 590.2
ABCCDE	16	4.02	3.77 ± 0.17^{i}	3.30 ± 0.09	5176 ± 485.0
BBCCEE	6	1.51	4.00 ± 0.27^{A}	3.31 ± 0.14	4957 ± 758.1
ABTTDD	5	1.26	3.07 ± 0.26^{ABFijkl}	3.20 ± 0.13	5874 ± 799.6
P value			0.0029	0.3192	0.9019

* Mean values with the same lower case letters (*a, b,c,d,g,h,i,j,k,l)* in the same column denote significant difference at $P < 0.05$ and those with the same upper case letters (*A,B,E,F*) in the same column denote significant difference at $P < 0.01$. Means marked with different superscript or without any letters denote no significant difference.

exon 4 in this study corresponded with the previously mutational sites reported by Eigel et al. (1984). Other two mutational sites (g.10988 G > A and g.10966 A > T); synonymous mutation, in exon 4 were also identified. Little is known of the association between the polymorphisms in exon 5 of *CSN3* gene and milk production traits in Chinese Holstein cattle. In this study, two SNPs (synonymous mutation) were identified in exon 5 of *CSN3* gene.

PIC value of the three SNPs was less than 0.5, suggesting low polymorphism in exon 4 and 5 of *CSN3* gene in this population. The *A* allele of *CSN3* gene was dominant in Chinese Holstein cattle with an allelic frequency of 73.71%, that is similar to the result of Irish dairy cattle (Keating et al., 2007) and Russian dairy cattle breeds (Sulimova et al., 2007). This suggests that the frequency of its alleles cannot serve as a breed specific characteristic.

The result of the χ^2 test indicated that, the three SNPs met with the Hardy-Weinberg equilibrium ($P > 0.05$). It implied that the selection pressure on the three SNPs in the population was not too powerful. The linkage disequilibrium analysis revealed firstly that, four mutational sites (g. 10891 T > C, g. 10927 C > A, g. 10988 G > A and g. 10966 A > T) of the exon 4 and another four mutational sites (g.12907 A > G, g.12950G >

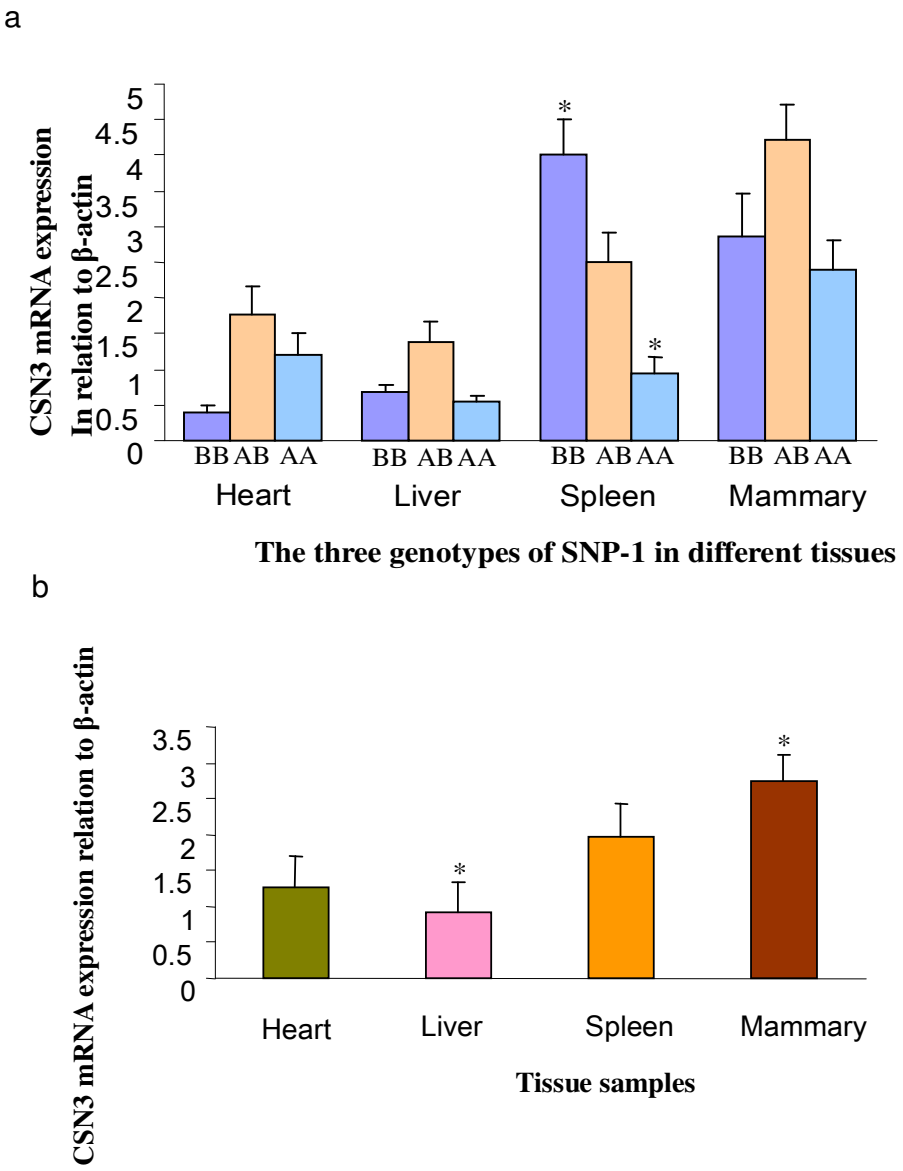

Figure 2. a, Q-PCR analysis of *CSN3* mRNA expression in the three genotypes of SNP-1; **b.** Q-PCR analysis of *CSN3* mRNA expression in the heart, liver, spleen and mammary tissues.

A, g. 12989 C > T and g. 13028 A > G) in the population were completely linked, respectively. Whether it has the same result in other cattle breeds and population or not, further study are needed to confirm it. Similarly, Keating et al. (2007) reported that, the 3 SNPs (Position -514, -426 and -384) of promoter in *CSN3* gene were linked, but they were not linked with the coding sequence halotypes *A* and *B*.

Allele *B* was reported to increase milk protein and milk fat contents, while allele *A* increased milk and protein yields (Kučerová et al., 2006; Matějíček et al., 2008). In this study, when compared with the cows in genotype *AA* of exon 4, genotype *BB* and *AB* had higher protein, fat content and milk yield. Allele substitution effect also indicated that, *B* allele had a positive effect on the milk production traits. The results of this study are not consistent with the result reported by Tsiaras et al. (2005). The difference may have resulted from different breeds, statistical model and so on. Moreover, *E* and *C* alleles of exon 5 of *CSN3* gene had positive effects on fat and protein content, whereas they had slightly negative effects on milk yield.

16 combinations of the three SNPs of *CSN3* gene were observed in the 398 cows, less than the expected 27 genotype combinations. There were two explanations for it. One explanation was that, the sample number was not enough to include the other genotype combinations. The other was that, the effects of selection and inbreeding

had led to fixation of genes in the population. Association analysis showed that different genotype combinations had evident influences on bovine fat content. Out of 16 combinations, genotypes *BBCCEE, ABTCDD* and *AACCDD* had the highest fat content, protein content and milk yield, respectively. While genotypes *ABTTDD*, *AATTDD*, and *BBCCEE* had the lowest values in fat content, protein content and milk yield, respectively. Obviously, the effect of single mutational sites and combined effects on milk production traits of the three SNPs were inconsistent. A possible explanation was that, the three SNPs were not far away from each other. The present effect on milk production traits was not only affected by one single mutational site itself, but also by the interaction of several SNPs. It is preferable to apply the effects of the combined genotypes to assess the genetic improvement of breeds or herds. Meanwhile, the distribution of genotype frequency should be taken into account.

In this study, the differences in *CSN3* gene expression in different tissues were also investigated. Robitaille and Petitclerc (2000) reported that, a differential allele-specific accumulation of *CSN3* mRNA in mammary epithelial cells was not linked to the protein variants of *CSN3* (*CSN3 A* and *CSN3 B*). In *CSN3 AB* cows, the allele *A*-specific *CSN3* gene was expressed with lower efficiency in mRNA (Vachon et al., 2004). The quantitative data implied that, the cow with the allele *B* had the highest fat and protein contents, while no significant difference for the *CSN3* mRNA expression in the mammary tissue was found among the three genotypes in SNP-1. The possible explanation for the result will be that, the *CSN3* proteins had different variants and it is possible that the *CSN3* mRNA was not affected by SNP-1. The SNP-1 may play its role on the milk protein content in cow but not at the mRNA level. Therefore, the *CSN3* variants in the total milk protein needed to be investigated in the further study.

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