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Original Article

DNA Polymorphisms in the *β-lactoglobulin* and *κ–casein* Genes Associated with Milk Production Traits in Dairy Cattle

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ABSTRACT: In recent years, the polymorphism of the milk proteins has been used as marker systems in modern animal breeding programmes. The current study was designed to evaluate significant associations between single nucleotide polymorphisms (SNPs) and milk production traits in Chinese Holstein cows. In this study, we detected the polymorphism of *β-lactoglobulin* and *κ–casein* genes by Matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF) and DNA sequencing methods in 752 individuals from Chinese Holstein cattle breeds. A single nucleotide polymorphism (g.1810C>T) in exon III in β-lactoglobulin gene and two single nucleotide polymorphisms (g.10888T>C and g.12703G>T) in exon IV and intron IV in κ–casein gene were detected. Polymorphism g.10888T>C was predicted to result in an amino acid replacement from threonine (ACC) to isoleucine (ATC) in the κ–casein protein whereas polymorphism g.1810C>T was predicted to silent mutation (asparagines>asparagines) in β-lactoglobulin protein. The results revealed the significant effects (P<0.05) of all identified SNPs of β-lactoglobulin and κ–casein genes on the milk production traits in Chinese Holstein. Our result demonstrated that the β-lactoglobulin and κ–casein genes possibly contributed to conducting association analysis and can be recognized as genetic marker in programs of gene-assisted selection for the genetic improvement of milk production traits in dairy cattle.

KEYWORDS: Single Nucleotide Polymorphism (SNP), β-lactoglobulin, κ–casein, dairy cattle.

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INTRODUCTION

Cow milk normally contains 3%-5% protein, of which 80% is casein and 20% is whey protein. Beta-lactoglobulin is 1 of 2 major whey proteins found in the milk of many species, mainly ruminants. It is absent from the milk of rodents, rabbits, camels and also in human[1].

Caseins are milk proteins secreted by mammary gland cells, constitute about 78-82% of bovine milk proteins and are subdivided into four main groups: αS1-casein, αS2-casein, β-casein, and κ-casein[2] where approximately 12% contribution comes from κ-casein[3]. The casein genes are tightly linked and inherited as a cluster so they have a potential value and can play an important role in marker-assisted selection for milk traits[4]. The association of genetic polymorphism with milk production and composition has stimulated interest in using genetic polymorphism of casein genes in marker assisted selection (MAS) to improve milk performance traits in farm animals[5]. Selection of κ-casein alleles is a part of cattle breeding programs in many developed countries[6].

Polymorphism of the κ-casein gene has been known since 1964[7], and nine alleles are known in all. Two major genetic variants of κ-casein, A and B, have been identified in cattle. Variant A shows Thr (ACC) and Asp (GAT) at positions 136 and 148, respectively, whereas the B variant shows Ile (A*T*C) and Ala (G*C*T) at the same positions[8]. A is associated with higher milk yield but lower protein content, while allele B is linked with higher protein content[9] and higher milk quality[10] but lower milk yield[11]. In general, the B variant of κ-casein has been recognized as superior for milk quality in European cattle breeds; the B allele of κ-casein is associated with shorter renneting time of the milk[12]. Milks of Holstein cows with the κ-casein BB genotype have been shown to contain, on average, 0.13% more protein than those of the other genotypes[13]. Significant differences have been found in the content of both variants A and B of κ-casein in milks of heterozygous AB cows[14]. Usually there is more of the protein variant encoded by allele A than that encoded by allele B. κ-casein gene has been mapped on chromosome 6[15] in cattle[16]. The overall length of the κ-casein gene is close to 13 kb, but most of the coding sequences for the

mature κ-casein protein are contained in the fourth exon. κ-casein is considerably different from other caseins in structure and other properties. It is highly homologous to the γ chain of fibrinogen and fulfills a similar function, while serving as a stabilizing factor during the formation of micelle structure in curdling milk[3].

The β -lactoglobulin gene was detected on bovine chromosome 11 and encodes the main whey protein. β -lactoglobulin (LGB) loci affect the milk production traits and quality of milk. Their polymorphisms explain a part of the genetic variance and improve the estimation of breeding value. Polymorphism of β -lactoglobulin gene was discovered in 1955 and a total of 15 alleles are known[17]. Common alleles are A, B, C and D, with alleles A and B being the most frequent[18]. The bovine β -lactoglobulin A variant differs from B variant by two amino acids only, aspartate-64 and valine-118. These amino acids are substituted by glycine and alanine respectively in the B variant[19]. β-LG protein variants A and B are associated with different amounts of β-LG protein in bovine milk: variant A has a higher β -LG protein concentration than variant B. It is likely that this difference in amount of β-LG protein is not caused by the amino acid substitutions, but rather by different levels of expression of the corresponding A and B alleles of the β -LG gene[20]. β-Lactoglobulin is amphiphatic and an extremely acid stable protein which exists at the normal pH of bovine milk as a dimer with a molecular weight of 36,000 Daltons. It is a single chain polypeptide of 18 kDa comprising of 162 amino acid residues[21]. The biological functions of this protein are still not fully known. It could have a role in metabolism of phosphate in the mammary gland and the transport of retinol and fatty acids in the gut[20].

Chinese Holsteins belongs to dairy cattle, which was cultured through grading cross between native cow (Chinese Yellow cattle) and pure-bred bull of Holstein introduced to China[22]. Continuous import of foreign Holstein bulls, semen and embryos, mainly from USA and a few from Canada and Europe have been implemented, which were directly used in AI or via crosses with Chinese Holstein cows through planned mating to generate breeding bulls. The current selection direction of breeding programmes for the Chinese Holstein is aimed at higher milk yield while retaining no decrease in fat and protein percentage[23]. Breeding for milk protein genotypes has therefore been suggested as a practical way of altering the composition of the milk protein fraction. Genotypes of particularly κ-casein (κ-CN), and whey protein β-lactoglobulin (β-LG), have been shown to have significant effect on milk protein composition in several studies[24-25]. Our goal was rather to draw attention on Chinese Holstein Cattle showing how single nucleotide polymorphisms (SNPs) responsible for milk production traits at genomic level and exploitable in selection programmes.

MATERIALS AND METHODS

Experimental animal

Blood or semen samples for DNA analysis were collected from the 752 Chinese Holstein cows and the 14 sire bulls from 14 Holstein cattle farms in Beijing, including 14 sire families with 5– 153 daughters from each sire. Phenotypic data for five milk production traits (i.e. milk yield, fat yield, protein yield, fat percentage and protein percentage over 305 days) were obtained from the Dairy Data Processing Center of China, Dairy Association of China (DAC). Genomic DNA was extracted from whole blood samples of cows using a commercial kit [TIANamp Blood DNA Kit; Tiangen Biotech (Beijing) Co., Ltd., China] following the manufacturer's instructions and frozen semen of bulls by a standard phenol–chloroform method.

Identification and genotyping of the polymorphisms

DNA pool (50ng/μL/bull) was constructed from the aforementioned 14 sires. Same concentrations of DNA were taken from each sire sample by adjusting volume of the DNA sample. Five sets of primers (exon1-6) for β-lactoglobulin and six sets of primer (exon1-5) for κ–casein were designed for selective amplification of the all exons and their partial flanking introns using as reference sequence of GenBank (Accession No NC_007309 and NC_007304) and Primer3 web Program (v .0.4.0) for β-lactoglobulin and κ–casein genes. The primers were synthesized by Invitrogen (Invitrogen Life technologies, Beijing, China).

For detection of β-lactoglobulin genotypes, a 358 bp DNA fragment with exon III was amplified by polymerase chain reaction (PCR), which was set by adding sense primer (5´-CCC CAG AGG TGA CAG TGA GT-3´) and antisense primer (5´-CAC GGC AGT GTC TTC ATC AC-3´). 589 bp and 501 bp fragments containing exon IV and V with part of intron IV of κ-casein gene were amplified by PCR using forward primer (5´-TCT GCT TCT GCT GCT GCT AA-3´) and reverse primer (5´-GAT CTC AGG TGG GCT CTC AA-3´) for exon IV and forward primer (5´-TGG ACA CAA AAT GAA AAC AAG T-3´)and reverse primer (5´-AGA CGA GGA AGG AGC CAG AT-3´)for exon V with part of intron IV. PCR were performed with a programmable thermal cycler, (Bio-Rad, DNA Engin, Dyad and Tetad 2, Peltier Thermal Circlers, Mexico) in a final reaction volume of 25 μl using PCR reagents from Invitrogen. The PCR reaction mixture consisted of 2 μl containing 50 ng genomic DNA, 1 μl of each primer, 12.5 μl premix (Master mix with Taq) and 8.5 μl ddH_2O. The PCR protocol was 5 min at 94ºC for initial denaturing followed by 35 cycles at 94ºC for 30 s; 56ºC for 30 s; 72ºC for 30 s; a final extension at 72ºC for 7 min for all the primers. Amplification was confirmed by running the PCR products on 2% agarose gels and visualizing under UV rays.

Then, 40 μl of each PCR product was sent to the company (TSINGKE, China) for sequencing. Sequences were generated with both the forward and reverse primers. Chromatographs generated from sequencing were processed using BioEdit Sequence Alignment Editor (version 7.0.9.0) to verify the sequences in the β-lactoglobulin and κ–casein genes. Both forward and reverse primer sequences were then aligned using the ClustalW multiple sequence alignment programs to determine the presence of genetic polymorphism.

Genotyping of SNPs ware performed using Matrix-assisted laser desorption/ ionization time of flight mass spectrometry (*MALDI-TOF)* method [Bio Miao Biological Technology (Beijing) Co., Ltd. China].

Statistical analysis

For the association studies, the traits of interest were analyzed using several statistical programs. Pedigree information of the genotyped animals was traced back for three generations. As a result, the total number of animals included in the analysis reached 2212. The kinship matrix (A-matrix) was calculated using MATLAB version 7.11.0.584. Allelic and genotypic frequencies for the β-lactoglobulin and κ –casein locus, and Hardy–Weinberg equilibrium tests were calculated using population genetic analysis software POPGENE version 1.32. Finally, the effects of genotype on the productive traits were estimated using the mixed procedure of SAS 9.1.0 software. With the following animal model[26], each trait was analysed separately and each polymorphism was also fitted separately:

$$Y=\mu+hys+L+G+\alpha+ei$$

where,

Y - the phenotypic value of cows
μ - the overall mean
hys - a herd-year-season effect
L - fixed effect of lactation
G - a fixed effect corresponding to the genotype of polymorphisms
α - a random polygenic component account for all known pedigree relationships
εi - a random residual

Bonferroni correction was performed for multiple t-testing through dividing the significance level by the number of tests. The additive (a) and dominance (d) and allele substitution (α) effects were estimated according to the equation of Falconer & Mackay[27], i.e.

$$a = (AA - BB)/2$$
$$d = AB - (AA + BB)/2$$
$$\alpha = a + d\,(q - p)$$

where,

AA and BB represent the two homozygous genotypes
AB is heterozygous genotype
p and q is the allele frequency of respective locus

RESULTS AND DISCUSSION

In our study, SNPs in β-lactoglobulin and κ –casein loci were genotyped in Chinese Holstein cattle, and their effects on milk production traits (305 days milk yield, fat yield, protein yield, fat percentage and protein percentage) were estimated. The genotypic and allelic frequencies were summarized in Table 1. Based on a Chi-square test (χ^2), all genotypic frequencies in the population were out of Hardy-Weinberg disequilibrium, demonstrated that selection pressure in this site was not too powerful (Table 1). Effects of the genotyped polymorphisms on milk production traits were shown in Table 2. Results have been discussed for each gene.

K–casein

The *κ–casein* gene has been extensively studied in cattle due to its influence on the manufacturing properties of milk. In current study, we identified two SNPs (Figure 1) that associate with milk production traits (305 day milk yield, fat yield, fat percentage, protein yield and protein percentage) in Chinese Holsteins (Table 2); such associations remained significant even after Bonferroni correction for multiple testing. Out of two SNPs, one (g.10888T>C) was found in exon IV and one (g.12703G>T) was found in intron IV. Polymorphism g.10888T>C was predicted to result in an amino acid replacement from threonine (ACC) to isoleucine (ATC) in the κ-casein protein.

The genotypic and allelic frequencies were summarized in Table 1. At locus g.10888T>C, the frequency of cows with CC genotype (0.7286) was higher than the cows ones with genotype CT (0.2499) and TT (0.0214). The frequencies of genotype GG, GT and TT at locus g.12703G>T, were 0.0199, 0.2428 and 0.7371 respectively, with genotype GG lowest. The C allele at position g.10888T>C was by far the more frequent in the population (0.5595), followed by the T allele (0.4405). The values of Chi-square test (χ^2) showed that all genotypic frequencies in the population were in Hardy-Weinberg equilibrium (P>0.05) (Table 1), indicating that selection pressure on this site in the population was not too powerful.

Table 1. Genotypic and allelic frequencies and Hardy–Weinberg equilibrium χ^2 test of β-lactoglobulin and κ–casein locus.

Polymorphisms	Genotypic frequency			Allelic frequency		Hardy–Weinberg equilibrium χ^2 test
g.1810C>T (β-lg)	CC	CT	TT	C	T	P>0.05
	0.313	0.4929	0.194	0.5595	0.4405	
g.10888T>C (κ–casein)	CC	CT	TT	C	T	P>0.05
	0.7286	0.2499	0.0214	0.8536	0.1464	
g.12703G>T (κ–casein)	GG	GT	TT	G	T	P>0.05
	0.0199	0.2428	0.7371	0.1414	0.8586	

Table 2. Least squares mean (LSM) and standard errors (SE) for milk production traits of different β-lactoglobulin and κ–casein genotypes in Chinese Holsteins.

Locus	Génotypes	Milk yield (kg)	Fat yield (kg)	Fat (%)	Protein yield (kg)	Protein (%)
g.1810C>T (β-lg)	CC	9901.6 ± 79.64^{A}	363.33 ± 3.62^{A}	3.744 ± 0.0353	305.12 ± 2.63^{A}	3.123 ± 0.0113
	CT	10024 ± 74.10^{B}	371.41 ± 3.43^{B}	3.780 ± 0.0335	308.38 ± 2.49^{B}	3.114 ± 0.0105
	TT	10227 ± 97.32^{CA}	380.56 ± 4.01^{CA}	3.771 ± 0.0399	314.36 ± 2.92^{CA}	3.116 ± 0.0132
g.10888T>C (κ–casein)	CC	9920.04 ± 78.508	369.25 ± 3.58	3.752 ± 0.0327	307.37 ± 2.61^{A}	3.112 ± 0.0101^{A}
	CT	9821.08 ± 88.364	369.8 ± 3.92	3.790 ± 0.0364	305.06 ± 2.86^{b}	3.121 ± 0.0118^{b}
	TT	9826.09 ± 222.04	380.34 ± 9.07	3.870 ± 0.0890	314.78 ± 6.61^{Ab}	3.206 ± 0.0319^{Ab}
g.12703G>T (κ–casein)	GG	10283 ± 135.6^{aC}	384.4 ± 5.70^{aC}	3.775 ± 0.0549	320.58 ± 4.15^{A}	3.127 ± 0.0191^{A}
	GT	9967.39 ± 94.38^{b}	372.45 ± 4.15^{b}	3.772 ± 0.0388	312.29 ± 3.03^{B}	3.145 ± 0.0127^{B}
	TT	9857.52 ± 78.93^{C}	371.41 ± 3.60^{c}	3.792 ± 0.0329	306.22 ± 2.62^{AC}	3.118 ± 0.010^{AC}

Notes: Means with the different small letters within the same column and loci differ at P<0.05 and means with different capital letters within the same column and loci differ at P<0.01.

Table 3. Additive effects, dominant effects and allele substitution effects of the 3 SNPs on milk production traits of β-lactoglobulin and κ–casein genotypes in Chinese Holsteins.

Locus	Genetic effects	Milk yield (kg)	Fat yield (kg)	Fat (%)	Protein yield (kg)	Protein (%)
g.1810C>T (β-lg)	Additive (a)	-162.6**	-8.6158**	-0.0137	-4.62**	0.0037
	Dominant (d)	-40.0507	-0.5395	0.0227	-1.36	-0.0055
	Allele substitution (α)	-157.79**	-8.551**	-0.016	-4.46**	0.0044
g.10888T>C (κ–casein)	Additive (a)	46.98	-5.55	-0.059	-3.71*	-0.047**
	Dominant (d)	-51.98	-5.00	-0.022	-6.01	-0.038*
	Allele substitution (α)	83.76	-2.01	-0.044	0.55**	-0.020**
g.12703G>T (κ–casein)	Additive (a)	212.79**	6.49**	-0.009	7.18**	0.0044**
	Dominant (d)	-102.92	-5.46	-0.011	-1.11	0.0227
	Allele substitution (α)	137.59**	2.55**	-0.0167	6.32**	0.020**

Notes: The asterisk (*) means the additive effect or dominance effect or allele substitution effect of the locus indicate differ at P<0.05 and the asterisk (**) means the additive effect or dominance effect or allele substitution effect of the locus indicate differ at P<0.01.

In our study, cows with g.10888T>C -TT genotypes had the highest protein yield and protein percentage (Table 2), which indicated that the allele g.10888T>C -T might be associated with an increase in protein yield and protein rate in the population. Statistical results of the allele substitution analyses (Table 3) showed that g.10888T>C –T allele increased protein yield (0.5487 kg) and decrease protein percentage (0.01968 kg). Statistical result demonstrated that cows with genotype GG at the g.12703G>T locus showed the greatest 305day milk yield, fat yield and protein yield (Table 2), with more than 425.48 kg, 12.99 kg and 14.36 kg respectively compared with TT cows; GT cows were in an intermediate position. There was no effect of g.12703G>T locus on fat percentage, whereas the protein percentage of GT cows was significantly greater than that of GG cows, with a difference of about 0.027 kg. Similarly, significant additive effects and allele substitution effects on 305 day milk yield (p<0.01), fat yield (p<0.01) and protein yield (p<0.01) were also observed (Table 3). Effects of κ-casein polymorphisms on milk production traits have been investigated during the past decades and, in some cases, results are still conflicting. Our findings considered were in agreement with previous reports at g.10888T>C locus on the different cattle population[28-30]. All these studies showed an individual effect of K-Cn gene on milk fat and protein content. However, our results are different from those reported by Ju *et al* (2009)[31] who studied polymorphisms of κ–casein gene at exon 4 and 5 and its association with milk performance traits in Chinese Holstein cattle and found that cows of TC and CC genotypes had significantly (P<0.05) higher protein content than those of genotype TT[31]. Hamza et al. (2010)[30] who studied polymorphisms of κ–casein gene at exon 4 in Chinese Holstein cattle and found that the T allele was more frequent in the population (0.60) whereas frequency of T allele we found 0.4405. Exon IV of kappa-casein gene is very important as it contains most of the sequence coding for its molecule[32]. Our results at g.10888T>C locus also disagree with literature data demonstrated by Strzalkowska et al. (2002)[10], who claimed that K-Cn gene had no significant influence on milk components. Majority of studies in kappa casein genotyping and allele frequency have been considered only A and B variants in exon IV and V; however, no other studies on κ-casein alleles (T and G) in intron IV (g.12703G>T) were found in the literature.

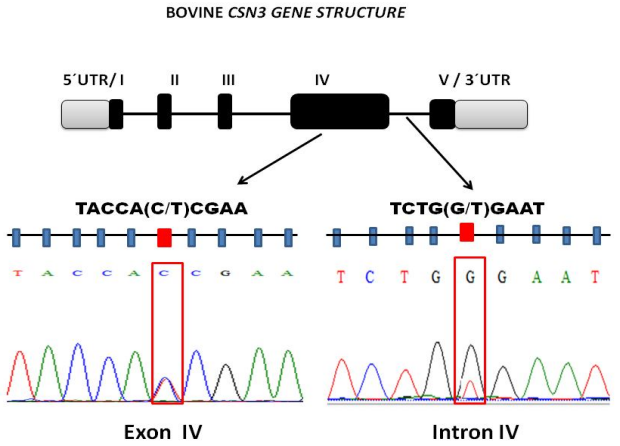

Figure 1. Detection of polymorphic site in bovine κ–casein gene. Black mark indicates the coding sequences, gray mark indicates the 5′-UTR and 3′-UTR and black line indicates the introns.

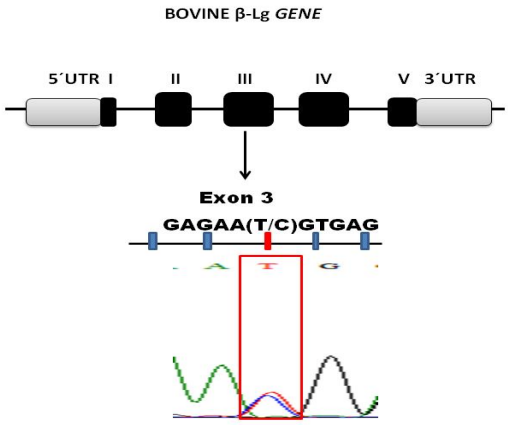

Figure 2. Detection of polymorphic site in bovine β-lactoglobulin gene. Black mark indicates the coding sequences, gray mark indicates the 5_-UTR and 3_-UTR and black line indicates the introns.

β-lactoglobulin

In our study, we identified a single nucleotide polymorphisms (g.1810C>T) in exon3 (Figure 2) of β-lactoglobulin gene (NC_007309) that associates with milk production traits in Chinese Holstein cattle. Polymorphism g.1810C>T was detected in mRNA position 277 (NC_007309) and did not replace amino acid Asparagine (AAC>AAT) in the β-lactoglobulin protein. Our statistical result showed that the frequencies of genotype CC, CT and TT at locus g.1810C>T were 0.313, 0.4929 and 0.194 respectively, with genotype TT lowest. Cows with genotype TT showed the greatest 305day milk

yield, fat yield and protein yield (Table 2), with more than 325.4 kg, 17.23 kg and 9.24 kg respectively compared with CC cows; CT cows were in an intermediate position. The value of Chi-square test (χ^2) was higher than the critical value, demonstrate that the genotype distribution of β-lactoglobulin gene at locus g.1810C>T was significantly deviating from the Hardy–Weinberg equilibrium in the population investigated (Table 1). Similarly, significant additive and allele substitution effects were observed for all of the traits, except fat and protein percentage (Table 3). First published paper by Gani et al (2009) who identified the same polymorphism as ours in Dutch Holstein Friesian cattle, showed that genotypic frequencies were out of Hardy–Weinberg equilibrium[33]. Our result contradicts with this result. This seemingly paradoxical result could be explained by several reasons, e.g., small sample size, the long-term breeding for different purpose and selection history and paternal effect.

In conclusion, we showed effect of SNPs in β-lactoglobulin and κ–casein genes on milk production traits in Chinese Holstein Cattle. This study confirms the results of several studies concerning the analysis of polymorphisms of β-lactoglobulin and κ–casein gene in Chinese Holstein Cattle and it is possible to claim that these polymorphisms could be useful as genetic marker for additional improvements for this dairy breed.

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