Short Communication: New Alleles of the Bovine κ -Casein Gene Revealed by Resequencing and Haplotype Inference Analysis

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ABSTRACT

We tested the hypothesis that extensive undiscovered genetic diversity exists in important functional genes from domestic and wild cattle species (*Bos* spp.). We resequenced 483 bp of a key exon (exon IV) from the kappa (κ)-casein gene (*CSN3*) for a panel of samples of domestic cattle from 8 countries and a close relative species, the gayal (*Bos frontalis*). Six single nucleotide polymorphisms were identified. Haplotype inference revealed 12 haplotypes, of which 8 were newly discovered. Among these 8 new haplotypes, 5 differed by one nonsynonymous mutation and 3 differed by one silent mutation from previously well-characterized *CSN3* alleles. From those, one was shared by the gayal and Zebu, was different from *CSN3*B* at position Ile136Thr, and showed a close phylogenetic relationship with the banteng, gaur, and yak. The other 7 new haplotypes were detected in our panel of worldwide local cattle breeds but were absent from previously reported commercial breeds. These results support the hypothesis that genetic diversity at the coding region of *CSN3* has been underestimated. This study also highlights how important it is to resequence functionally important genes in worldwide local cattle breeds, many of which are threatened by extinction or replacement by commercial breeds.

Key words: κ -casein, single nucleotide polymorphism, haplotype inference

The bovine κ -casein protein plays an important role in cheese production technology (e.g., stabilization of casein micelles), and in physiological processes such as cytotoxic and antibacterial activities important for disease resistance (Dziuba and Minkiewicz, 1996; Matin and Otani, 2002). For example, previous studies demonstrated that genetic variants A and B of this protein

show different biological activities (e.g., Yvon et al., 1994) and are associated with milk production traits (e.g., Ng-Kwai-Hang et al., 1984). Because different variants of this protein might have different functional activities or associations with milk yield traits, it is of longstanding interest to identify and characterize the diversity harbored in the κ -casein gene (*CSN3*) in the bovine species (*Bos* spp.).

Until now, researchers have reported 10 well-characterized genetic variants (A, A¹, B, C, E, F, G, H, I, and J) in domestic cattle (*Bos taurus* and *Bos indicus*) (Prinzenberg et al., 2008) and 4 genetic variants found in the domestic yak (*Bos grunniens*; Bai et al., 2008; Prinzenberg et al., 2008). So far, most of these variants have been genotyped by using PCR-single strand conformation polymorphism analyses (e.g., Prinzenberg et al., 1999, 2008). This routine genotyping system is cost-effective and sufficiently sensitive for both the genotyping of known mutations and the detection of new mutations in DNA fragments. However, its efficiency is poor for detecting sequence variation for larger DNA fragments (e.g., >300 bp; Sheffield et al., 1993; Jaekel et al., 1998).

Characterization of variation in functional genes (such as the milk protein genes) with potentially adaptive variation is increasingly urgent because numerous local breeds and wild relative taxa are being lost at an accelerating rate (Food and Agriculture Organization of the United Nations, 2007). However, most studies on functional genes include only a few selected cattle breeds from Europe (Prinzenberg et al., 1999). Apart from the few studies in the most widely distributed breeds of *B. indicus* (e.g., Sahiwal, Gir), there is a great lack of information on genetic diversity in native Asiatic cattle at functional important genes, such as the *CSN3*. For example, the few existing reports on the genetic diversity of *CSN3* in native Asiatic cattle were limited to the genotyping of the major genetic variants (alleles A and B) by using techniques such as PCR-RFLP, which does not allow detection of new variants (Mitra et al., 1998). Thus, it is important to screen for

Received March 29, 2008.

Accepted May 26, 2008.

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Table 1. Observed genotypes (G01 to G13) with their distribution and inferred haplotypes (H01 to H12) with estimated frequencies (confidence interval probability 0.97 to 1.00)

Item	SNP position ¹							Distribution in countries ² and in gayal								Haplotype frequency, phase	
	n ³	5110	5306	5309	5345	5352	5406	Kaz	Kyr	Mon	Tur	Ind	Yem	Egy	Mor		Gayal
G01	9	C	C	Y	M	R	R				1	3	2	1	1	1	
G02	7	C	Y	Y	M	A	R					4	1	1		1	
G03	6	C	C	C	C	A	G					3				3	
G04	10	C	T	C	A	A	A				1	8				1	
G05	13	C	Y	C	A	R	A					8	4			1	
G06	3	C	C	C	A	G	A					2	1				
G07	1	C	Y	C	A	G	A					1					
G08	2	C	Y	C	A	A	A	1			1						
G09	9	C	C	Y	M	A	R	2			2				5		
G10	4	C	C	T	C	A	G		2		1				1		
G11	5	C	C	C	A	A	A		2		1				2		
G12	1	C	C	Y	M	A	G			1							
G13	1	Y	C	Y	M	A	R								1		
H01		C	C	C	C	A	G					6				6	0.0860
H02		C	C	C	A	A	A	3	4		5				9		0.1536
H03		C	C	C	A	A	G			1							0.0057
H04		C	C	C	A	G	A				1	16	8	1	1	2	0.2024
H05		C	C	T	C	A	G	2	4	1	5	7	3	2	9	2	0.2408
H06		C	T	C	A	A	A	1			3	28	5	1		4	0.2941
H07		C	T	C	A	G	A					1					0.0088
H08		T	C	C	A	A	A								1		0.0029
H09		C	C	T	C	G	G										0.0001
H10		C	C	T	A	A	A										0.0001
H11		C	C	T	A	A	G										0.0013
H12		T	C	T	C	A	G										0.0041

¹The single nucleotide polymorphism (SNP) positions are relative to the reference sequence of the κ-casein gene (X14908). Heterozygous sites are shown by International Union of Pure and Applied Chemistry codes (Y-C/T, M-A/C, and R-A/G).

²The country is represented by an abbreviation. Kaz, Kyr, Mon, Tur, Ind, Yem, Egy, and Mor represent Kazakhstan, Kyrgyzstan, Mongolia, Turkmenistan, India, Yemen, Egypt, and Morocco, respectively. The number of haplotypes is double the number of individuals, because each individual carries 2 haplotypes. H09, H10, H11, and H12 haplotypes potentially exist but were not directly observed in our small panel of samples.

³n indicates the number of observed individuals.

Figure 1. Neighbor-joining tree of new haplotype sequences, known cattle *CSN3* alleles, and sequences from the yak, banteng (AF030324), and gaur (AF030323). The water buffalo (D14370) and goat (D14373) were used as outgroups. Cattle *CSN3**C and *CSN3**J were reconstructed following the method of Prinzenberg et al. (2008). Because of ambiguity of alignment, the 12-bp duplication in the yak sequence (AY095311) was not considered, and this made it the same as our new haplotype H01. Numbers above the nodes indicate bootstrap values from 1,000 bootstrap replications (only values >50 are shown).

genetic diversity at the *CSN3* (as well as in the other milk protein and functional genes) in cattle from Asia to have a more accurate worldwide estimation of the genetic diversity patterns for this gene.

Because very few molecular-based data exist on the genetic origin of cattle from Central and South Asia, it is important first to use a more efficient approach to detect new variants in the *CSN3* gene. For that task, resequencing of the entire exon IV, which harbors all the functionally important mutations, is highly recommended. Indeed, technological advances have contributed to a significant reduction of the resequencing cost; therefore, resequencing has become an affordable

alternative to other techniques such as PCR-single strand conformation polymorphism.

The aim of this study was to use resequencing to search for new *CSN3* alleles in a panel of native Asian and Northern African cattle. Because limited information exists on the genetic diversity in this gene for other species in the *Bos* genus, we also sequenced samples from the supposed Zebu × gaur (*Bos gaurus*) hybrid, the gayal (*Bos frontalis*).

Tissue samples were collected from local cattle in Kazakhstan (*B. taurus*, n = 3), Kyrgyzstan (*B. taurus*, n = 4), Mongolia (*B. taurus*, n = 1), Turkmenistan (*B. taurus* × *B. indicus*, n = 7), India (*B. indicus*, n = 29),

Yemen (*B. indicus*, $n = 8$), Egypt (*B. taurus*, $n = 2$), and Morocco (*B. taurus*, $n = 10$). In addition, 7 samples from Myanmar gayal (*B. frontalis*) were included in this study. Because most of the countries in Central and Southern Asia represent a contact zone between taurine- and indicine-like cattle (Felius, 1995), some individual samples from Kazakhstan, Kyrgyzstan, Yemen, and Turkmenistan, might in fact be crosses between *B. taurus* and *B. indicus*. Therefore, we expected to observe sequence variants originating in both the taurine and indicine lineages. Efforts were made to avoid sampling related individuals. We sampled only from small remote villages and excluded research centers, large cities, and coastal harbors where recent international shipping of cattle was possible. Genomic DNA was extracted by a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

The *CSN3* exon IV was PCR amplified by using primers ExIV#1 and 551#2 (Prinzenberg et al., 1999). Polymerase chain reactions were performed in a 20- μ L volume containing 1 $\times$ PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide 5'-triphosphates, 0.4 μ M each primer, 0.6 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), and approximately 30 ng of genomic DNA. The PCR mixture underwent 10 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 52°C, 45 s at 72°C, and a final 5 min at 72°C on a GeneAmp PCR System 9700 (Applied Biosystems). Polymerase chain reaction products were purified and sequenced in the High-Throughput Genomics Unit, Department of Genome Sciences, University of Washington. Sequence trace files were checked and aligned by using DNASTar 6.0 (DNASTar Inc., Madison, WI). All new haplotype sequences were submitted to GenBank (accession numbers EU595506 to 595513).

A Bayesian model-based algorithm (Phase 2.1; Stephens et al., 2001; Stephens and Scheet, 2005) permitted us to infer haplotypes with a high confidence level (probability 0.97 to 1.00) from unphased genotypes and to estimate their frequencies. Several independent runs were performed to check the consistency between different runs. A neighbor-joining tree of newly inferred haplotypes and known bovine *CSN3* alleles was constructed under uncorrected p distance by using MEGA4 (Tamura et al., 2007).

In our small panel of samples, 13 different genotypes were defined by 6 SNP, including 5 phase-known genotypes (G03, G04, G06, G10, and G11) and 8 unphased genotypes (Table 1). Notably, 5 genotypes (G01 to G05) are shared by gayal samples and domestic cattle. From these 13 genotypes, 12 haplotypes (Table 1) were reconstructed and their frequencies were estimated. Among the 12 haplotypes, H02, H04, H05, and H06 corresponded to the known cattle alleles *CSN3**A,

*CSN3**A¹, *CSN3**B, and *CSN3**H, respectively. The new haplotypes, H08 and H10, differed from *CSN3**A by one nonsynonymous mutation (T5110C–Ser70Pro and T5309C–Ile136Thr), whereas the new haplotypes H01, H11, and H12 were differentiated from *CSN3**B by one nonsynonymous mutation (T5309C–Ile136Thr, A5345C–Ala148Asp, and T5110C–Ser70Pro). Finally, the new haplotypes H03, H07, and H09 were different by one silent mutation from *CSN3**A, *CSN3**H, and *CSN3**B, respectively (Table 1). Interestingly, H01 was observed in 3 gayal and 3 Zebu individuals (Table 1) and had a close phylogenetic relationship with the banteng (*Bos javanicus*), gaur, and yak (Figure 1). Our results support the hypothesis that genetic diversity in exon IV of *CSN3* has been underestimated in domestic cattle; it also highlights the urgent need to resequence functionally important genes in locally adapted cattle populations. This study shows the advantages of reporting the haplotypes instead of single sequences. Notably, a search in GenBank for the number of unique sequences (haplotypes) from cattle *CSN3* exon IV returned only 9 different haplotypes (representing 7 polymorphic sites). This demonstrates that genetic diversity is highly underestimated, because this study alone contributed 8 new haplotypes.

The haplotype information will help us to understand the evolution of this gene as well as the consequences of different combinations of amino acid changes in the protein structure and function. In fact, this short survey (71 individuals) was enough to identify a new nonsynonymous SNP and several new haplotypes absent so far from previously reported commercial cattle breeds (e.g., Prinzenberg et al., 1999). Thus, our study provides a simple but clear example of how important it is to define diversity in the context of livestock genetic resources by using sensitive resequencing techniques and worldwide samples from local breeds and related species.

ACKNOWLEDGMENTS

This work was funded by Fundação para a Ciência e Tecnologia (FCT) project POCI/CVT/56758/2004. SYC and AB-P are supported by FCT grants SFRH/BPD/26802/2006 and SFRH/BPD/17822/2004, respectively. GL was supported by the Portuguese-American Foundation for Development, CIBIO, and Universidade do Porto.

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