Genetic Variation in the κ -Casein Gene (*CSN3*) of Chinese Yak (*Bos grunniens*) and Phylogenetic Analysis of *CSN3* Sequences in the Genus *Bos*

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ABSTRACT

Variants of κ -casein (*CSN3*) have been extensively studied in cattle and 13 alleles have been identified at the protein and DNA levels to date. Evolution of some of these alleles and a possible common ancestor remain unclear. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of *CSN3* exon IV in domesticated yak revealed a 2-allele polymorphism showing migration patterns different from known cattle variants. The PCR products of both yak *CSN3* SSCP alleles were sequenced. All yak had nucleotide sequences coding for Thr in AA position 136 (identical to bovine *CSN3**A) and Ala in position 148 (identical to bovine *CSN3**B). The sequencing results were confirmed by PCR-RFLP analysis using *Hind*III and *Taq*I. A 12-bp insertion in the coding region, representing a repeated nucleotide and AA motif, was found in 1 yak allele. The duplication corresponds to the codons for AA 147 to 150 (Glu-Ala-Ser-Pro) or 148 to 151 (Ala-Ser-Pro-Glu), which are repeated identically. In 21 yak samples genotyped by PCR-SSCP analysis, frequencies for the insertion variant and the short variant were about 68 and 32%, respectively. The loss of the insertion may have led to the ancestral *CSN3* allele from which all currently known variants of *CSN3* in the genus *Bos* evolved. This is the first report of polymorphisms in the yak *CSN3* gene and may be helpful for future studies on genetic variation within and between yak populations or on associated traits.

Key words: yak, κ -casein, *CSN3* gene, polymorphism

INTRODUCTION

Variants of κ -casein (*CSN3*) have been extensively studied for decades. Altogether, 13 alleles have been

identified in the *Bos* genus using either electrophoretic separation of protein variants, protein and DNA sequence analyses, or various DNA mutation detection techniques. These are summarized, including conflicting allele names, by Formaggioni et al. (1999) and Farrell et al. (2004). Up to 9 alleles (A, B, C, E, F, G, H, I, and A^I) have been simultaneously discriminated in *Bos taurus* and *Bos indicus* by PCR-single strand conformation polymorphism (SSCP) analysis so far (Prinzenberg et al., 1999). Evolution of these alleles remains unclear particularly for *CSN3**A and *CSN3**B, with 2 mutations discriminating these alleles. A possible common ancestor and the presence of an intermediate ancestral protein were postulated earlier (Mercier et al., 1976; Gorodetskii and Kaledin, 1987). Udina et al. (1995) identified a *CSN3* variant named *CSN3**G by PCR-RFLP analysis in European bison (*Bison bonasus*), which corresponds to bovine *CSN3**A in codon 136 (Thr) and to *CSN3**B in codon 148 (Ala), thus representing such an intermediate protein. Based on microsatellite data, Ritz et al. (2000) proposed that *Bos taurus*, *Bos indicus*, and yak should be considered as subgenera of the *Bos* genus, whereas *Bison* clearly forms a separate genus. Sulimova et al. (1996) identified in yak a *CSN3* exon IV sequence similar to that found in *Bison* with an additional mutation changing the stop codon from TAA to TGA in yak. Fan et al. (2000) also reported a unique yak *CSN3* exon IV sequence (published in GenBank with accession no. AF194989). A κ -casein “X” protein variant of Nepalese yak (Kawamoto et al., 1992) was never characterized at either the AA or nucleotide sequence level.

In contrast to cattle, β -casein and κ -casein were found to be monomorphic in yak milk (Mao et al., 2004). Some studies about genetic variation of milk proteins within and between yak populations have been performed (Xuebin and Jianlin, 2000; Yucai et al., 2000). They also suggest that genetic variation is very low at the protein level and mainly occurs because of cattle introgression through hybridization. The aim of this study

Received October 2, 2007.

Accepted December 8, 2007.

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was to identify and characterize the genetic variation in Chinese yak *CSN3* at the DNA sequence level and to establish the phylogenetic relationship of *CSN3* variants in the *Bos* genus.

MATERIALS AND METHODS

DNA Samples

Blood samples of 15 male and 6 female yak (*Bos grunniens*/*Poëphagus*) were collected from 4 domesticated yak herds (4 to 6 samples per herd) in Luqu county of Gannan prefecture, Gansu Province, China, by a member of the Gansu Agricultural University staff in 1998. The DNA was extracted using essentially the high salt protocol of Montgomery and Sise (1991).

PCR-SSCP Analysis

The coding region of exon IV of the *CSN3* gene was PCR amplified as described by Prinzenberg et al. (1999) using primers ExIV#1 (5' AGA AAT AAT ACC ATT CTG CAT 3') and 551'2 (5' GTT GAA TTC TTT GAT GTC TCC TTA GAG T 3'). The fragment comprised positions 4,892 to 5,442 of the *CSN3* gene (accession number X14908; Alexander et al., 1988). Aliquots of 4 μ L of each PCR reaction were mixed with 6 μ L of denaturing loading dye (containing 95% wt/vol formamide), heated at 92°C for 2 min, and chilled on ice. Subsequently, 4 μ L of the mixture was loaded on 8.5% polyacrylamide gels (acrylamide:bisacrylamide ratio of 37:1) containing 0.53% glycerol. Fragments were separated for 18 h at 340 V and 5°C constant temperature. For silver staining, the protocol of Bassam et al. (1991) was used with a modified stop solution (0.04 M Na-EDTA at 10°C). Alleles were manually scored on a white, translucent screen using cattle samples with known genotypes, including the alleles *CSN3**A, B, C, E, G, H, and I as reference samples (Prinzenberg et al., 1999).

PCR-RFLP Analyses

For restriction enzyme digestion, 10 to 15 μ L of PCR product of each SSCP type was incubated with 5 U of *Hind*III, *Taq*I, and *Msp*I (MBI Fermentas, St. Leon Roth, Germany) following the manufacturer's guidelines. The fragments were separated by electrophoresis in 1.2% agarose gels and visualized by ethidium bromide staining and UV-fluorescence. Fragments were scored manually by comparison with a 100-bp ladder.

Cloning and Sequencing

The PCR products for cloning and sequencing were prepared from 8 yak (2 of type 11, 3 of type 12, and 3

of type 22) using the same primers and conditions as for the PCR-SSCP and PCR-RFLP analyses except for the use of *Pwo* polymerase with the buffer supplied by the manufacturer (AGS, Heidelberg, Germany). Blunt-ended PCR products were gel purified and cloned into *pCR-Blunt* using the Zero Blunt PCR Cloning kit (Invitrogen, Groningen, the Netherlands). Positive clones were selected by colony PCR and subsequent SSCP analysis of the respective PCR products. Clones with migration patterns different from those of PCR products derived from the genomic DNA were discarded. Plasmid DNA was prepared using a GFX plasmid kit (Amersham Biosciences, Freiburg, Germany) and cycle sequencing was performed with a CycleReader Auto kit (MBI Fermentas). Sequences were run and evaluated on an A.L.F.*express* sequencer (Amersham Biosciences). Long Ranger (Biozym, Hess. Oldendorf, Germany) and ReproGel (Amersham Biosciences), respectively, mixes were used for gel matrix preparation and both gels were subjected to UV light for a uniform polymerization. Additional clones were custom sequenced by SeqLab (Göttingen, Germany). The sequences were submitted to GenBank and assigned accession numbers AY095311 and AY095312.

Sequence and Phylogenetic Analyses

Sequences X14908 (including variant sites for *CSN3* A and B), AF041482, AF123250, AF123251, AF105260, AF121023, AF092513, AJ619772, and AF194989 representing *CSN3* alleles A, B, E, F, G, H, I, A', respectively, an unassigned *Bos taurus* sequence (AJ619772), and a yak sequence (AFA194989) were retrieved from GenBank. Based on the *CSN3**B sequence, DNA sequences for *CSN3**C and *CSN3**J were deduced from the protein sequences, differing in codons 97 (Arg→His; Miranda et al., 1993) and 155 (Ser→Arg; Mahé et al., 1999), which makes single G→A and C→T substitutions most probable. A recognition site search for *Hind*III, *Hinf*I, *Msp*I, *Pst*I, and *Taq*I was done for X14908 (A and B) and the yak sequences AF194989, AY095311, and AY095312 using the pDRAW32 1.0 DNA analysis freeware (AcaClone, 2007). Because of different lengths of the sequences, only partial sequences corresponding to the coding region of exon IV were used for phylogenetic analysis. The Gendoc software (Nicholas and Nicholas, 1997) was used for sequence alignments and cutting. Construction of a DNA nucleotide sequence-based neighbor joining tree using genetic distance P was done with the DAMBE software package (Xia and Xie, 2001). Randomized input order and bootstrapping with 100 data sets were used to obtain a consensus tree. TreeviewX (Page, 2005) was used to redraw the

Figure 1. Polymerase chain reaction-single strand conformation polymorphism analysis of the yak *CSN3* exon IV. Lanes are numbered above the gel; the genotypes are given below each sample lane. Lanes 1 to 7 and 18 to 20 represent cattle samples with known genotypes, lanes 8 to 17 show yak samples with different migration patterns named 1 (slow migration) and 2 (fast migration) and all possible genotypes 11, 12, and 22.

phylogram provided in DAMBE as a rectangular cladogram.

RESULTS AND DISCUSSION

Primers derived from the bovine *CSN3* sequence amplified a fragment in the expected size (about 550 bp in agarose gels) in all yak. Analysis by PCR-SSCP revealed 2 distinct migration patterns in the yak samples that did not correspond to any of the known cattle *CSN3* variants. The 2 migration patterns were preliminarily named type 1 and type 2 in the order of increasing mobility (Figure 1). The lower fragment of both yak variants moved close to the cattle *CSN3***B* fragment. Migration of the upper fragments was more different, with positions similar to *B* (type 1) or below the cattle *CSN3***A* (type 2, Figure 1). Frequencies of the 2 yak *CSN3* PCR-SSCP patterns were 0.32 (type 1) and 0.68 (type 2).

In PCR-RFLP analysis with *Hind*III, *Msp*I, and *Taq*I, there was no clear difference between the 2 PCR-SSCP genotypes in the restriction fragments in the agarose gels. All yak samples showed identical restriction fragments. All PCR products were uncut with *Taq*I and *Msp*I, which indicates Thr₁₃₆ (corresponding to *CSN3***A* in cattle) and excludes the silent mutation in the codon

Pro₁₅₀ present in *CSN3***A*^I. With *Hind*III, all PCR products were cut (about 450 and 100 bp), as would be expected for *CSN3***B* in cattle with Ala₁₄₈. Gel pictures are available in an online-only data supplement (<http://jds.fass.org/content/vol91/issue4/>).

A 12-bp sequence duplication in the coding frame of exon IV in PCR-SSCP type 2 (AY095311) was detected by sequencing. Due to the sequence structure, the position of the inserted motif could not be unequivocally assigned. The duplication corresponded to the codons for AA either from 147 to 150 (Glu-Ala-Ser-Pro) or from 148 to 151 (Ala-Ser-Pro-Glu), which were repeated identically. This has led to a total of 164 AA encoded in the yak type 2 *CSN3* exon IV, whereas the sequence of yak type 1 (AY095312) had the same length as in cattle, encoding 160 of the 169 AA of mature κ -casein. Another difference was noticed in the stop codon, which was TAA for type 2, corresponding to all previously known cattle variants, and TGA for type 1. The deduced AA sequences showed identity with bovine *CSN3***A* in position 136 (Thr) and with bovine *CSN3***B* in position 148 (Ala), which is in accordance with the results from PCR-RFLP analysis. Comparison of AY095311 and AY095312 with yak sequence AF194989 in GenBank showed identical length of the coding region in exon IV and a shared stop codon (TGA) between AY095312 (type

Figure 2. Nucleotide sequence-based neighbor joining consensus tree (random input order, 100 bootstrap datasets) of *CSN3* sequences in the genus *Bos* (*Bos taurus*, *Bos indicus*, and *Bos grunniens*) drawn as ladderized rectangular cladogram. AY095311 was used as outgroup. DNA sequences for *CSN3**C and *J* are not available in GenBank and were reconstructed based on protein sequence data assuming single nucleotide mutations.

1) and the sequence AF194989, but 2 additional mutations (C/T and C/G), both changing the AA sequence (codon 36 Pro/Leu and 130 Pro/Arg) were present in AF194989. The in silico restriction analysis using pDRAW32 confirmed the results obtained in PCR-RFLP analysis. The restriction sites for *HindIII*, *HinfI*, *PstI*, and *TaqI* are indicated in an online-only data supplement (<http://jds.fass.org/content/vol91/issue4/>). With *HindIII*, the inserted motif led to a second recognition site in AY095311 and consequently 12 bp were cut out. This makes the insertion undetectable in agarose gel electrophoresis. Although 2 mutations are present in AF194989, pDRAW restriction analysis revealed identical cutting positions in all 3 yak sequences and bovine *CSN3**B for *HinfI* and *PstI*. Due to the insertion a 12-bp length difference in the largest fragment resulted in AY095311 compared with the other yak sequences with these 2 enzymes, but this will be hard to discriminate in agarose gel electrophoresis. For *TaqI* in all 3 yak sequences no restriction site was present, which corresponds to bovine *CSN3**A. Therefore, discrimination of yak *CSN3* from the bovine *CSN3* variants by PCR-RFLP requires at least 2 enzymes including *TaqI* and one chosen of the group *HindIII*, *HinfI*,

and *PstI*. Reliable discrimination of the yak variants AY095311 and AY095312 by *HinfI* and *PstI* furthermore will require high-resolution agarose or even polyacrylamide gel electrophoresis to make the 12-bp difference clearly visible. *HindIII*, although the most popular enzyme in *CSN3* PCR-RFLP analysis, seems the least suitable choice for yak *CSN3* variant discrimination, because gel electrophoresis in this case must be able to detect a 12-bp fragment aside from possible excess primers.

Because AY095311 seemed to be the most divergent sequence due to the duplication, AY095311 was used as outgroup in the construction of a neighbor joining tree with DAMBE. The resulting consensus tree (Figure 2) shows 3 main branches, 1 with the out-grouped yak sequence and 2 main sequence groups. The nucleotide and AA sequence differences are summarized in Table 1 and provided in full in an online-only data supplement (<http://jds.fass.org/content/vol91/issue4/>). The first group comprises *CSN3**A, *E*, *I*, *F*, *G*, *H*, *A^I*, and the unassigned taurine sequence AJ619772, all being identical to *CSN3**A in codons 136 and 148. The nucleotide sequences of *CSN3**E, *I*, *F*, *H*, *A^I*, and AJ619772 each differ from *CSN3**A in a single mutation, and thus

Table 1. Differences in AA and nucleotide sequences of *CSN3* variants in the genus *Bos*¹

GenBank no. and allele name ²	AA position of mature κ -casein										
	10	36	97	104	130	135	136	148	148a–151a	155	Stop
X14980 A	Arg CGC	Pro CCT	Arg CGT	Ser TCA	Pro CCT	Thr ACC	Thr ACC	Asp GAT		Ser AGC	TAA
X14908 B							Ile ATC	Ala GCT			
Not available C			His CAT				Ile ATC	Ala GCT			
AF041482 E											
AF12350 F	His CAC										
AF123251 G			Cys TGT			Ile ATC					
AF105260 H						Ile ATC					
AF121023 I				Ala GCA							
Not available J							Ile ATC	Ala GCT		Arg AGA	
AF092513 A ^I											
AJ619772 Unassigned			His CAT								
AY095311 Yak type 2							Ala GCT	Ala Ser Pro Glu GCTTCTCCAGAA			
AY095312 Yak type 1							Ala GCT				TGA
AF194989 Yak		Leu CTT			Arg CGT		Ala GCT				TGA

¹Only positions deviating from X14980 (*CSN3**A) are shown. Amino acids are provided in the upper lane, and nucleotide codons in the lower lane of each sequence. Position of the duplication is indicated as 148a–151a, although 147a–150a is also possible.

² κ -Casein allele name.

probably directly evolved from *CSN3**A. Allele *CSN3**G differs from *CSN3**A in 2 positions, 1 identical to *CSN3**H. Thus, *CSN3**G probably evolved from *CSN3**H by a single mutation. The second main branch is subdivided into the 3 bovine sequences corresponding to *CSN3**B, C, and J (group “B”, with *CSN3**C and J developing from *CSN3**B by single mutations) and another branch with the 2 yak sequences AY095312 and AF194989, which both differ from all other sequences by the unique TGA stop codon. Because of this stop codon, the sequence AY095312 is most probably not a common ancestor of *CSN3**A and *CSN3**B, although the deduced protein sequence is exactly the intermediate sequence regarding AA 136 and 148. It is unlikely, however, that an identical mutation in the stop codon has occurred in both branches “A” and “B” after the sequence divergence. The ideal common ancestor of today’s *CSN3**A and *CSN3**B as postulated by Mercier et al. (1976) would correspond to AY095311 without the 12-bp duplication. Such a sequence was not found in samples from the current study, which might be an effect of the low sample number. The sequence originally reported in bison (Udina et al., 1995) might

represent such an ancestral sequence, although data on the stop codon are not reported. As the *Bos* and *Bison* genera diverged before *Bos taurus/indicus* and *Bos grunniens* departed (Ritz et al., 2000), the same ancestral protein might exist in *Bison* and eventually also in *Bos*.

The sequence AY095312, corresponding to PCR-SSCP type 1 in this study, might be identical to the report of Sulimova et al. (1996), including the mutated stop codon in yak. The sequence AY095311, including the 12-bp duplication, is most probably new at the molecular level. However, it cannot be excluded that the resulting protein of AY095311, AY095312, or AF194989 may be identical to the variant “X” described in the early 1990s in yak (Kawamoto et al., 1992).

Two genetic variants of yak *CSN3* were detected by PCR-SSCP analysis simultaneously with bovine *CSN3* variants and show clearly different migration patterns. This technique enables reliable genotyping of yak *CSN3* variants. Analysis by PCR-RFLP using *Hind*III and *Taq*I and agarose gels for fragment separation distinguishes yak and bovine variants, but does not discriminate between the 2 yak variants.

CONCLUSIONS

κ -Casein was found to be polymorphic in domesticated yak at the DNA sequence level. The deduced protein sequences were also different, but in the current study no milk samples were available to check electrophoretic mobility. At least 2 yak-specific sequences are present and can be discriminated by PCR-SSCP or sequence analysis. This can be considered in future studies on the genetic variation within and between yak populations, on the introgression of cattle genes into domesticated yak, and on the influence of *CSN3* polymorphisms on technological or nutritional parameters of yak milk and association with production traits.

ACKNOWLEDGMENTS

The authors thank H. Wagner, M. Uebel, and T. Gans for assistance in cloning and sequencing. H. Jianlin was supported by a grant of the Yak and Camel Foundation in Germany.

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