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Duplication polymorphisms in exon 4 of κ-casein gene in yak breeds/populations

S. Pingcuo[1]*, J. Gao[1]*, Z.R. Jiang[2], S.Y. Jin[1], C.Y. Fu[1], X. Liu[2], L. Huang[1] and Y.C. Zheng[1]

[1]College of Life Science and Technology, Southwest University for Nationalities, Chengdu, China
[2]Institute of Animal Science and Veterinary Medicine of Ganzi Prefecture, Kangding, Sichuan, China

*These authors contributed equally to this study.
Corresponding author: Y.C. Zheng
E-mail: yucaizheng65@hotmail.com

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ABSTRACT. The objective of this study was to compare 12 bp-duplication polymorphisms in exon 4 of the κ-casein gene among 3 breeds/populations of yak (*Bos grunniens*). Genomic DNA was extracted from yak blood or muscle samples (N = 211) and a partial sequence of exon 4 of κ-casein gene was amplified by polymerase chain reaction. A polyacrylamide gel electrophoresis assay of the products (169 bp) revealed 2 variants. These variants differed in a 12-bp duplication of the nucleotide sequence corresponding to amino acids 147-150 (Glu-Ala-Ser-Pro) or 148-151 (Ala-Ser-Pro-Glu). The genotype frequency and gene frequency of the 2 κ-casein variants differed among the 3 yak breeds/populations. The long form of the κ-casein gene was the predominant allele, and the Jiulong yak showed the highest frequency of the short form variant of the κ-casein gene. In addition, 2 nucleotide differences resulting in amino acid substitutions were also identified in

yaks. These results are significant for designing a breeding strategy to improve the genetic makeup of yak herds.

Key words: Gene duplication; Genetic polymorphism; κ-casein; Yak

INTRODUCTION

The κ-casein plays an essential role in stabilizing casein micelles and therefore has significant influence on the manufacturing properties of milk (Creamer et al., 1998). Genetic variants of κ-casein have been extensively studied in cattle at the protein and DNA levels, and numerous alleles have been revealed (Barroso et al., 1998; Prinzenberg et al., 1999; Gallinat et al., 2013). The association between milk coagulation properties and κ-casein variants and other casein variants have been reported previously (Bonfatti et al., 2010; Poulsen et al., 2013), and the genotyping of κ-casein and other caseins are important for determining milk processing properties.

Yak (*Bos grunniens*) milk contains high protein and fat contents and shows good coagulation properties (Zheng YC, Liu WJ and Jin SY, unpublished data). Several studies using the polymerase chain reaction (PCR)-single strand conformation polymorphism method have identified a 12-bp duplication in exon 4 of the yak κ-casein gene (Bai et al., 2008; Prinzenberg et al., 2008). This duplication represents a repeated nucleotide and an amino acid motif (4 amino acids) and accounted for 68% in 1 yak population (N = 21) (Prinzenberg et al., 2008). However, these studies used a small number of yaks. Because of the apparent amino acid differences, the 2 variants of κ-casein may differ in structure and function, thus influencing the coagulation and other processing properties of yak milk. The frequencies of κ-casein variants may differ among yak breeds. Therefore, in this experiment, we developed a PCR protocol followed by simple and rapid polyacrylamide gel electrophoresis separation to identify duplication variants of the κ-casein gene in 2 yak breeds and a yak population in order to investigate the distribution of duplication variants in the yak κ-casein gene. These data will be useful for yak breeding practice.

MATERIAL AND METHODS

Animals and sample collection

Three yak (*B. grunniens*) breeds/populations were studied in this experiment, including the Maiwa yak breed (N = 72), Jiulong yak breed (N = 72), and Changtai yak population (N = 67) in the Sichuan Province of China. Yaks grazed on natural pasture at an altitude of approximately 3500 m. The major distribution areas of the 3 yak breeds/populations were all at a distance of approximately 300 km. A longissimus muscle sample was taken from each individual of Jiulong yaks and Maiwai yaks in slaughter houses, and blood samples were taken from Changtai yaks. All muscle samples were promptly frozen and stored at -80°C. This experiment was conducted according with the guidelines of the Chinese government for the use of experimental animals and EC Directive 86/609/EEC for animal experiments.

Detection of duplication fragment in exon 4 of the yak κ-casein gene

Genomic DNA was isolated from the blood or skeletal muscles of the experimental yaks using the Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Shiga, Japan).

Qualified DNA was stored at -20°C until further analysis.

To amplify the κ-casein gene fragment, a pair of PCR primers were designed based on the DNA sequence of yak κ-casein (GenBank accession No. AY095311) as follows: Yakkcn-169F: 5'-AATCCCTACCATCAATACC-3', Yakkcn-169R: 5'-TTAGACCGCAGTTGAAGTA-3', the expected fragment size was 169 bp. PCR amplifications were performed in standard conditions: denaturation at 94°C for 3 min, then 37 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min. PCR products were evaluated by 1.5% agarose gel electrophoresis and genotyped on 8% polyacrylamide gels using a Mini Protein 3 electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was carried out in 1X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) on ice at 110 V for 110 min. The gels were silver-stained and the images were acquired using a Versa Doc 1000 gel imaging system (Bio-Rad). The PCR products of the 2 variants of the κ-casein gene showed different mobilities on the gels. The Popgene version 1.32 software was used to calculate the allele frequency and genotype frequency of the κ-casein gene for each yak breed/population.

Sequence assay of variants of the yak κ-casein gene

For the homozygous κ-casein gene, the PCR products of 2 yaks carrying different variants of the κ-casein gene were sequenced directly in both directions; for the heterozygous κ-casein gene, the amplified short form fragments (N = 4) of the κ-casein gene were purified from agarose gels and cloned into the pMD 19-T Vector (TaKaRa), followed by transfection into *Escherichia coli* DH5α competent cells. Three to five positive clones were sequenced from both strands. Sequences were analyzed using DNAMAN Version 5.2.10 Demo (Lynnon BioSoft, San Ramon, CA, USA).

RESULTS AND DISCUSSION

PCR amplification of partial sequence of the yak κ-casein gene

An expected 169-bp fragment was amplified using primers derived from the yak κ-casein gene as shown on the agarose gels (Figure 1A). The genotypes of these fragments were further determined by 8% polyacrylamide gel electrophoresis (Figure 1B), and 2 variants (A: long form, B: short form) were observed in all of the 3 yak breeds/populations.

Direct sequencing of the homozygous PCR products and gene cloning confirmed that the 169-bp fragment was a partial sequence of the κ-casein gene. The 2 variants showed a 12-bp duplication difference, representing a repeated nucleotide and amino acid motif (Figure 2). The duplication corresponds to the codons for amino acids 147-150 (Glu-Ala-Ser-Pro) or 148-151 (Ala-Ser-Pro-Glu). The protocol for genotyping the κ-casein gene in this study was simpler than using the PCR-single strand conformation polymorphism method (Bai et al., 2008; Prinzenberg et al., 2008).

Two single-nucleotide polymorphisms of the κ-casein gene were identified among yak individuals (**Figure S1**). The difference at amino acid 148 (Ala and Asp) was previously reported in bovine κ-casein A and B alleles (GenBank accession No. X14908), but had not been identified in yaks (Table 1). Because the 2 amino acids differ significantly in properties such as charge and molecular weight, this substitution may affect the function of κ-casein as well as the κ-casein derived antibacterial peptide (Malkoski et al., 2001). In addition, the

amino acid change at position136 (Thr and Ile) was observed in both yaks (Table 1) and cattle (GenBank accession No. X14908 and AF123251), and Ala at this position appeared to be predominant in most yaks (Table 1), which is different from in cattle (Prinzenberg et al., 2008).

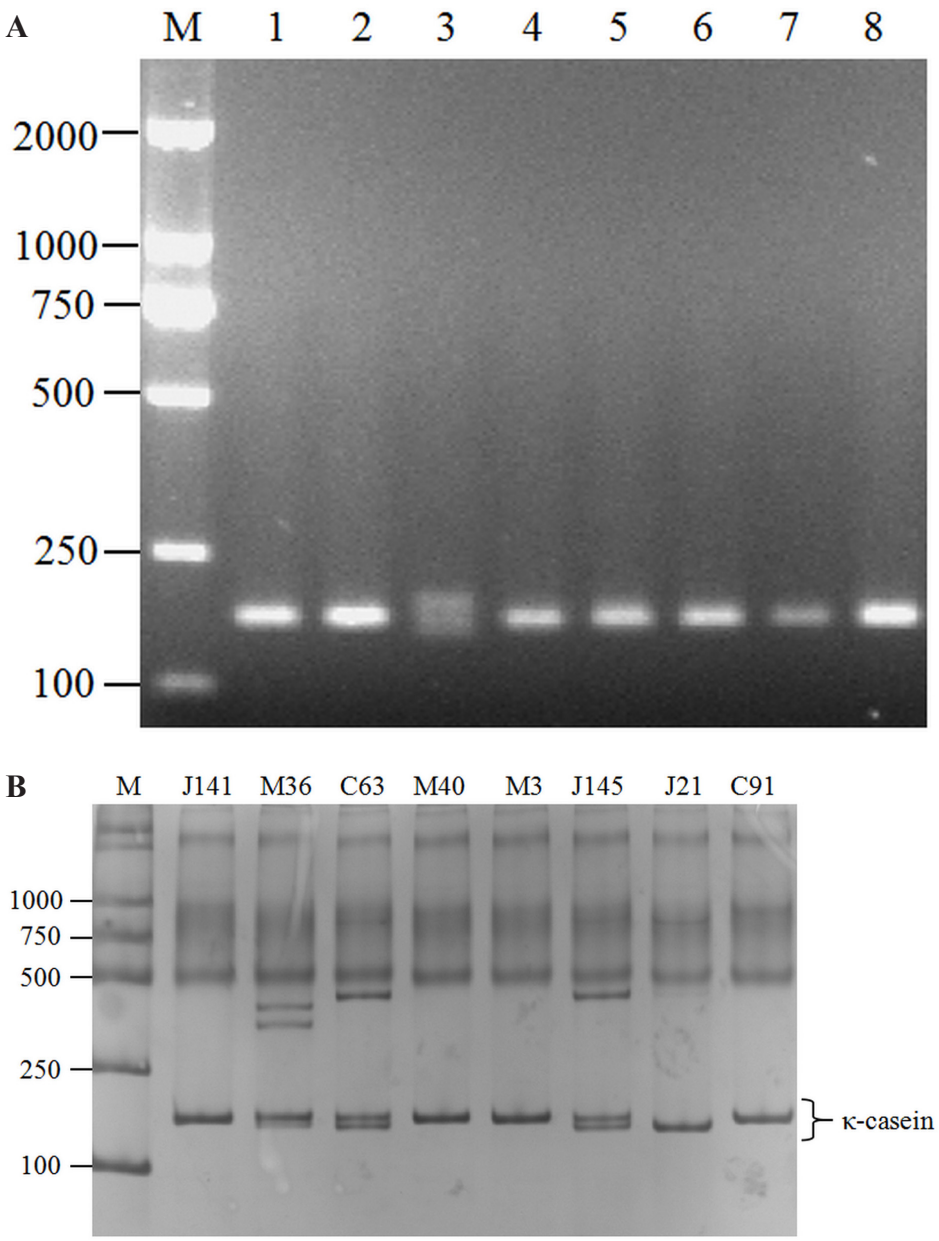

Figure 1. Agarose gel (**A**) and polyacrylamide gel (**B**) electrophoresis of PCR products after amplifying partial sequence in exon 4 of the yak κ-casein gene. *Lane M* = 2000-bp DNA ladder marker (bp); other lanes represent different yak samples.

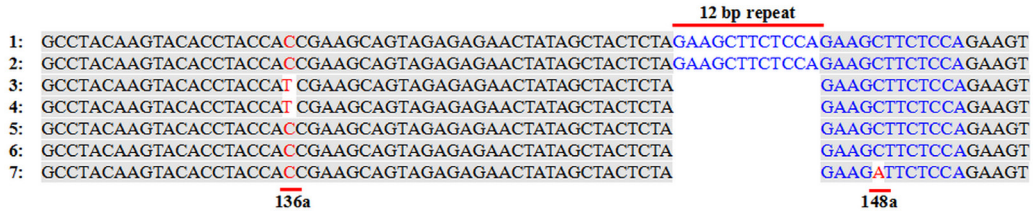

Figure 2. Alignment of partial sequence of the κ-casein gene of yaks. The 2 variants of the κ-casein gene showed a difference of a 12-bp nucleotide duplication (GAA GCT TCT CCA) as shown on the top of the figure, representing a repeated amino acid motif from 147-150 (Glu-Ala-Ser-Pro) or 148-151 (Ala-Ser-Pro-Glu). The 136a and 148a represent the amino acid positions of mature κ-casein encoded by 2 codons, respectively. 1: Yak (GenBank accession No. AY095311), 2: Maiwa-19, 3: Jiulong-22, 4: Jiulong-59, 5: Changtai-23, 6: Changtai-50, 7: Changtai-99.

Table 1. Difference in deduced amino acids in exon 4 of the yak κ-casein gene.

Yak or GenBank accession No.	Amino acid position of k-casein			GenBank submitter
	136	147-150	148	
AH009225.2	Thr (ACC)		Ala (GCT)	Fan et al.
AF030326	Thr	Glu-Ala-Ser-Pro	Ala	Ward et al.
AY095311	Thr	Glu-Ala-Ser-Pro	Ala	Prinzenberg et al.
AY095312	Thr		Ala	Prinzenberg et al.
EF565131	Thr		Ala	Bai et al.
Maiwa-19	Thr	Glu-Ala-Ser-Pro	Ala	Present study
Changtai-23	Thr		Ala	Present study
Jiulong-22	**Ile (ATC)**		Ala	Present study
Changtai-59	Thr		**Asp (GAT)**	Present study

Maiwa-19 to Changtai-59 represent 4 yak individuals of different breeds/populations. Amino acid positions are compared with mature bovine κ-casein. Position of the duplication is indicated as amino acid 147-150, although 148-151 was also possible. The 3 letters in the bracket represent the codons of corresponding amino acids. Bold letters at positions 136 and 148 represent the changed amino acids (codons).

Genotyping of the κ-casein gene in three yak breeds/populations

Based on the results of polyacrylamide gel electrophoresis analysis (Figure 1B), the genotype frequency and gene frequency of the 2 variants of the κ-casein gene differed among the 3 yak breeds/populations (Table 2). The B variant (long form) of the κ-casein gene was the predominant allele, while the Jiulong yaks showed the highest frequency of the A variant (short form). These characteristics suggest that breeding isolation occurred between Jiulong yaks and Maiwa yaks or Changtai yaks, as the immigration of foreign yak breeds was forbidden and pure breeding as a traditional method was used in the breeding practice of Jiulong yaks.

Milk protein composition traits are associated with protein genetic variants (Huang et al., 2012). Yak κ-casein may exist as several variants because of the occurrence of 4 amino acid duplication as well as other amino acid substitutions as shown in Table 1 and other studies (Prinzenberg et al., 2008; Bai et al., 2008). Therefore, it is necessary to compare the structures and functions of different genetic variants of yak κ-casein, particularly the 2 duplication variants. However, the characteristics of yak κ-casein have not been well-documented. Yak milk shows good coagulation following the addition of chymosin, and no individual showed non-coagulating milk (Zheng YC, Liu WJ and Jin SY, unpublished data) as in bovine milk (Okigbo et al., 1985; Poulsen et al., 2013). This is an advantage of yak milk during cheese-making, although the related mechanism remains unclear. The coagulation property of bovine milk is

likely associated with milk protein genetic variants (Jensen et al., 2012a, 2012b). Genotyping of yak κ-casein will be useful for determining its functions.

Although the 12-bp nucleotide sequence duplication has been reported previously in yaks (Prinzenberg et al., 2008), this study surveyed polymorphisms in a large number of yaks from 3 breeds/populations and revealed useful information. It has been speculated that the loss of the 12-bp nucleotide sequence duplication led to the ancestral allele of the κ-casein gene (Prinzenberg et al., 2008). The higher gene frequency of the κ-casein gene A allele (short form) in Jiulong yaks compared to in Maiwa and Changtai yaks (Table 2) suggests that this breed contains the original allele of the κ-casein gene. In fact, Jiulong yak is the largest yak breed in body size (Wiener et al., 2003), and may show lower diversity than other yak breeds (Zheng et al., 2006), which is likely related to their distribution region and breeding practice. In this study, we developed a simple protocol for analyzing the 12 bp-duplication polymorphisms in exon 4 of the κ-casein gene. Analysis of κ-casein gene polymorphisms in 3 yak breeds/populations will allow monitoring of the inbreeding level and can help in designing a breeding strategy for improving the genetic makeup of yak herds. Additionally, our results are important for determining the evolutionary and functional characteristics of the yak κ-casein gene.

Table 2. Genotype and gene frequencies of 2 variants of the yak κ-casein gene.

Breed/population	N	Genotype frequency			Gene frequency	
		AA	AB	BB	A	B
Changtai yak	67	1	11	55	0.097	0.903
Maiwa yak	72	1	16	55	0.125	0.875
Jiulong yak	72	3	32	37	0.264	0.736

A, short form; B, long form with 12-bp nucleotide sequence duplication.

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Supplementary material

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