Short Communication: The β -Casein (*CSN2*) Silent Allele *C1* Is Highly Spread in Goat Breeds

S. Chessa,* D. Rignanese,* J. Küpper,† G. Pagnacco,* G. Erhardt,† and A. Caroli‡

*Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare, Università degli Studi di Milano, Via Trentacoste 2, Milano, 20134 Italy

†Institut für Tierzucht und Haustiergenetik, Justus-Liebig-Universität, Ludwigstr. 21 b, 35390 Gießen, Germany

‡Dipartimento di Scienze Biomediche e Biotecnologie, Università degli Studi di Brescia, Viale Europa 11, Brescia, 25123 Italy

ABSTRACT

Several single nucleotide polymorphisms have been identified in the goat milk casein genes, most of them modifying the amino acid sequence of the coded protein. At least 9 variants have been found in goat β -CN (*CSN2*); 6 of them were characterized at the DNA level (*A*, *A1*, *C*, *E*, *O*, and *O'*), whereas the other 3 variants were described only at the protein level. The recently identified silent *A1* allele is characterized by a C→T transition at the 180th nucleotide of the ninth exon. In the present work, typing results from different breeds (3 Italian, 3 German, and a composite of African breeds for a total of 335 samples) demonstrated that the same mutation is carried by the *CSN2***C* allele. In addition, the T nucleotide at the 180th nucleotide of the ninth exon was always associated with *CSN2***C* in all the breeds analyzed. Thus, another silent allele occurs at goat *CSN2* and can be named *CSN2***C1*. The much wider distribution of *C1* with respect to the *A1* allele indicates that the single nucleotide polymorphisms characterizing the silent mutation originated from *CSN2***C*. A method for the identification of this allele simultaneously with 5 of the 6 DNA-characterized alleles is also proposed. The mutation involved codifies for the same protein of the *C* allele; nevertheless, its location in the 3' untranslated region of the gene might affect the specific casein expression.

Key words: β -casein, goat, genetic polymorphism, 3' untranslated region

A large number of single nucleotide polymorphisms (SNP) have been identified in the goat milk casein genes, most of them modifying the amino acid sequence of the coded protein, thus affecting goat casein qualitative and quantitative variability as well as milk protein composition (Martin et al., 2002). At least 9 alleles have been found in goat β -CN (*CSN2*). Six of these alleles

(*A*, *A1*, *C*, *E*, *O*, and *O'*) were characterized at the DNA level (Rando et al., 1996; Persuy et al., 1999; Chessa et al., 2005; Cosenza et al., 2005), whereas the *CSN2***B* and the *CSN2***D* variants were described only at the protein level (Mahé and Grosclaude, 1993; Galliano et al., 2004). A new variant has been recently found by Chianese et al. (2007) at the protein level, but it is not yet characterized. Different techniques were used to type *CSN2* at the DNA level, but a complete description of *CSN2* variation within the breeds has never been reported. In general, *CSN2***A* and *CSN2***C* were found to be the most common alleles in many breeds, with a predominance of the *CSN2***A* in some African and Indian breeds (Caroli et al., 2007; Chessa et al., 2007), whereas the *CSN2***C* was the most common variant in some Italian and Turkish breeds (Chessa et al., 2005, 2007; Caroli et al., 2006). The *A1* silent allele has been recently identified in an undefined genetic type reared in the province of Naples, with a frequency of 0.23 (Cosenza et al., 2005). It differs from *CSN2***A* for a C→T transition at the 180th nucleotide of the ninth exon, corresponding to the nucleotide position 10562 of the reference *CSN2***A* GenBank No. AJ011018. The presence of C₁₀₅₆₂ might represent the ancestral condition of the gene because it has been found also in other ruminant species (Cosenza et al., 2005).

To understand how this mutation is distributed in different breeds and to elucidate if T₁₀₅₆₂ could be in association with other *CSN2* alleles, a total of 335 DNA samples belonging to different breeds (3 Italian breeds, 3 German breeds, and a composite of African breeds) were analyzed at *CSN2* level both by the PCR-single strand conformation polymorphism (SSCP) described by Chessa et al. (2005) and by the PCR-RFLP described by Cosenza et al. (2005). The PCR-SSCP discriminates the *CSN2***A*, *CSN2***C*, *CSN2***E*, and *CSN2***O'* alleles, whereas the PCR-RFLP allows the detection of the transition C₁₀₅₆₂→T₁₀₅₆₂ identified in *CSN2***A1*. The African samples were randomly chosen from those previously characterized in the casein genes by Caroli et al. (2007).

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¹Corresponding author: stefania.chessa@unimi.it

Table 1. Typing combinations between the single strand conformation polymorphism (SSCP) developed by Chessa et al. (2005), and the PCR-RFLP analysis developed by Cosenza et al. (2005)¹

Breed	SSCP	RFLP (C/T ₁₀₅₆₂)		
	CSN2	CC	CT	TT
Frisa (n ² = 48)	AA	4	0	0
	AC	5	8	0
	CC	3	8	8
	AE	7	0	0
	CE	1	4	0
	EE	0	0	0
Orobica (n = 49)	AA	0	0	0
	AC	0	1	0
	CC	1	10	37
Verzasca (n = 48)	AA	3	0	0
	AC	0	18	0
	CC	0	2	25
Bunte Deutsche Edelziege (n = 49)	AA	6	0	0
	AC	12	6	0
	CC	16	9	0
Thüringer Waldziege (n = 45)	AA	19	0	0
	AC	1	15	0
	CC	1	8	1
Weiße Deutsche Edelziege (n = 46)	AA	14	0	0
	AC	6	17	0
	CC	1	4	4
African breeds (n = 50)	AA	26	0	0
	AC	16	0	0
	CC	7	1	0

¹C = cytosine at 10562 position of the GenBank reference sequence accession no. AJ011018; T = thymine at the same position (as in *CSN2*A1*). Bolded: the double heterozygous combinations AC × CT, possibly carrying the *CSN2*A-T*₁₀₅₆₂ haplotype.

²n = number of samples analyzed.

The results of the genotyping are listed in Table 1. No T₁₀₅₆₂ was found in association with the *CSN2*AA* genotype in all breeds. On the contrary, T₁₀₅₆₂ was found in samples homozygous or heterozygous for *CSN2*C*. Thus, T₁₀₅₆₂ may be likely associated with *CSN2*C* in the breeds analyzed. Only the double heterozygous combinations AC × CT might carry the *CSN2*A-T*₁₀₅₆₂ haplotype, corresponding to the *A1* allele. In particular in the African goats, where *CSN2*A* is predominant (Caroli et al., 2007), only one sample carrying T₁₀₅₆₂ at the heterozygous condition associated to the *CSN2*CC* genotype was observed.

The occurrence of the intragenic haplotype *CSN2*C-T*₁₀₅₆₂ was confirmed by sequencing 6 *CSN2*C* homozygous samples with different RFLP patterns at the nucleotide position 10562 (2 CC, 2 CT, 2 TT). The samples were sequenced by PRIMM Srl (Milano, Italy), using the primers designed for the PCR-RFLP by Cosenza et al. (2005) and confirming the association of T₁₀₅₆₂ with the T nucleotide at codon 135 of the seventh exon (position 8946 of GenBank accession no. AJ011018), characterizing *CSN2*C*. Thus, another silent allele occurs at goat *CSN2*, characterized by T₈₉₄₆ and T₁₀₅₆₂, that can be named *CSN2*C1*. Table 2 summarizes the nucleotide and deduced amino acid exchanges between

the *CSN2* alleles characterized at the DNA level until now. The wide distribution of this silent allele suggests that T₁₀₅₆₂ originated from *CSN2*C*, with the following evolutionary pattern, starting from the *CSN2*A* allele, which is considered the ancestral one (Chessa et al., 2005): *CSN2*A-C*₁₀₅₆₂ → *CSN2*C-C*₁₀₅₆₂ → *CSN2*C-T*₁₀₅₆₂. This evolutionary hypothesis fits also with the ancestral origin of C₁₀₅₆₂ (Cosenza et al., 2005). Most probably the *A1* silent variant identified by the same authors and characterized by *CSN2*A-T*₁₀₅₆₂ haplotype arose from an intragenic recombinant event spread in the population as a consequence of the genetic drift.

To type *CSN2*A1* and *CSN2*C1* simultaneously with *CSN2*A*, *CSN2*C*, *CSN2*E*, and *CSN2*0'* without any further analysis, the PCR-SSCP assay described by Chessa et al. (2005) was adapted. Because the mutations distinguishing these variants are located within exons 7 and 9, a multiplex PCR (**mPCR**) was developed. Primers used for the amplification of the 374-bp fragment of *CSN2* containing part of the exon 7 were the ones employed by Chessa et al. (2005), whereas a new pair of primers was designed to amplify a 325-bp fragment within exon 9. The new primers were CSN2-9C1 Fw (5'-CAGAATTGACTGCGACTGGA-3') and CSN2-9C1 Rv (5'-TGCCTAAGGGTTAATTTATTGAAA-3'),

Table 2. Differences among the β -casein (*CSN2*) alleles characterized in goats

Item						Reference
Nucleotide position	8561	8913	8915	8946	10562	
Amino acid position	58	166	167	177	— ¹	
Exon (codon within exon)	7 (16)	7 (124)	7 (125)	7 (135)	9 (60)	
Allele						
<i>CSN2*A</i>	A	C	C	C	C	Rando, 1998
	Leu	Ser	Gln	Ala	—	
<i>CSN2*A1</i>					T	Cosenza et al., 2005
<i>CSN2*C</i>				T	—	Chessa et al., 2005
<i>CSN2*C1</i>				Val		
				T	T	Present work
				Val	—	
<i>CSN2*E</i>	A	A				Caroli et al., 2006
		Tyr				
<i>CSN2*O'</i>			T			Rando et al., 1996
			Stop ²			
<i>CSN2*O</i>	—					Persuy et al., 1999
	Stop					

¹The single nucleotide polymorphism is located in the 3' untranslated region.²Stop = the nucleotide mutation generates a stop codon.

Figure 1. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis of different β -casein (*CSN2*) genotypes. A) The 374-bp fragment containing exon 7 is fixed in the higher part of the gel at the end of the electrophoretic run. Two bands are visible for each allele. White star = *CSN2*C*, white circle = *CSN2*A*, black triangle = *CSN2*O'*, and black rhombus = *CSN2*E*. B) The 325-bp fragment containing exon 9 is fixed in the lower part of the gel. Two bands are visible for each variant. White square = C_{10562} , black square = T_{10562} . The genotype is given by the combination of the results obtained for the 2 fragments. In the gel a reference sample homozygous for *CSN2*O'* was also used.

designed on the basis of the caprine sequence GenBank accession no. AJ011018, using Primer3 software (Rozen and Skaletsky, 2000).

The PCR was performed in a 25- μ L reaction mixture containing 4 μ L of DNA solution (200 to 300 ng), 1 $\times$ PCR Master Mix (Fermentas, Vilnius, Lithuania) and 10 pmol of each of the 4 primers. The following conditions were used for the mPCR assay: an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 60 s, 56°C for 60 s, 72°C for 60 s, and a final extension step of 72°C for 7 min using a PTC-200 DNA Engine Thermal Cycler (MJ Research Inc., Waltham, MA). The mPCR product was subsequently analyzed using the same SSCP technique described by Chessa et al. (2005). The only modification to this protocol was the duration of the run, which was reduced from 14 to 13 h to not have the fast migrating bands at the far end of the gel (Figure 1). Two series of bands were obtained by the PCR-SSCP analysis, slow (Figure 1, part A) and fast migrating (Figure 1, part B), corresponding to the exon 7 and 9 PCR product, respectively. As for the exon 7 PCR product, the patterns obtained were the same described by Chessa et al. (2005) and Caroli et al. (2007). As for the exon 9 PCR product, 3 different patterns were found. Each pattern was composed of 2 bands, corresponding to the 2 DNA strands. The fastest set of strands corresponded to the T_{10562} polymorphism, the slowest one to C_{10562} . The genotype is given by the combination of the 2 series of bands. The method was validated on all the Italian samples already analyzed by using both the PCR-SSCP and the PCR-RFLP assays. Full agreement was found between the PCR-SSCP assay here developed and the 2 separated

analyses. As expected, no *CSN2**A1** was identified in the analyzed samples; nevertheless, the new method could easily detect this allele, which is not included in Figure 1 due to the nonavailability of a reference sample. To understand the association between nucleotide position 10562 and the *CSN2**O** allele, not found in the breeds analyzed, different reference samples available from previous studies (Sacchi et al., 2005) were also analyzed by the new assay (Figure 1). Only the intragenic haplotype *CSN2**O*-C₁₀₅₆₂* was found in the samples analyzed (data not shown).

The possibility of typing the *CSN2**C1** allele here described simultaneously with 5 of the 6 DNA characterized alleles may provide an opportunity to have a more refined picture of goat *CSN2* variability, which is important given the wide distribution of the new allele. Even if the nucleotide exchange does not result in a different protein variant, particular attention should be given to this SNP because it is located in the 3' untranslated of the gene, possibly affecting the mRNA stability and, consequently, the expression of the specific protein (Xu et al., 1997; Cosenza et al., 2005).

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