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A *Ssp*I PCR-RFLP detecting a silent allele at the goat *CSN2* locus

Gianfranco Cosenza, Alfredo Pauciullo, Daniela Gallo, Dino Di Berardino and Luigi Ramunno*

Dipartimento di Scienze Zootecniche ed Ispezione degli Alimenti, Università degli Studi di Napoli ''Federico II'', Portici, Italia

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The goat calcium-sensitive caseins (α_{s1}, β and α_{s2}) represent, over many years, an excellent model for demonstrating that the major part of the variability observed in the content of these proteins in goat milk is mostly due to the presence of autosomal alleles at single structural loci (*CSN1S1, CSN2* and *CSN1S2* respectively) clustered on a 200 kb segment of chromosome 6; furthermore, *CSN1S1* and *CSN2* are convergently transcribed and are only 12 kb apart (Rijnkels, 2002).

Analogously to what has been observed so far at the *CSN1S1* and *CSN1S2* loci (Martin et al. 1999; Ramunno et al. 2001), a similar quali-quantitative polymorphism concerns the goat *CSN2* locus. Quantitative individual differences in the amount of β-casein in the goat milk are determined by at least six alleles: *CSN2* A (Roberts et al. 1992), B (Mahè & Grosclaude, 1993), C (Neveu et al. 2002) and D (Galliano et al. 2004) associated with a normal and *CSN2* 0 (Persuy et al. 1999) and *CSN2* 01 (Ramunno et al. 1992) associated with a non-detectable amount of this protein in milk.

The goat β-casein encoding gene consists of nine exons ranging in size from 24 (exon 5) to 492 bp (exon 7). This gene encodes for a 1088 nucleotides (nt) mRNA (prior to polyadenylation). Exon 2 contains the translational start signals and encodes for the 15-amino acid signal peptide and the first two residues of the mature protein. Exon 7 encodes for about 82% of the mature protein, while exon 8 encodes only for the last codon (Val) (Roberts et al. 1992).

To date, the molecular events responsible for the formation of the major part of the alleles at such a locus are known. Particularly, the C, 01 alleles are both characterized by a single nucleotide substitution (C→T transition) which took place respectively at 404 (Neveu et al. 2002) and 373 (Rando et al. 1996) positions of the 7th exon, while the 0 allele originated by a single nt deletion (adenine) in a row of four adenines between the nt 16 and 19 of the same exon (Persuy et al. 1999).

The mutations that characterize the two null alleles, 0 and 01, are responsible for the formation of a premature stop codon respectively at the 58 (Persuy et al. 1999) and 182 (Rando et al. 1996) positions, while the substitution that characterizes the C allele is responsible for the amino acid substitution Ala→Val in position 177 of the mature protein (Neveu et al. 2002). The *CSN2* D allele is characterized by the amino acid substitution Val^{207}→Asn (Galliano et al. 2004), while the DNA and protein sequences of the *CSN2* B allele remain undetermined.

Here we report the identification of a silent allele at the goat *CSN2* locus and describe a method based on Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) for its detection.

Materials and Methods

Milk, DNA and RNA samples

Genomic DNA was extracted from leucocytes obtained from individual blood samples of 170 goats belonging to an undefined genetic type reared in the province of Naples (Italy). Total RNA was extracted from somatic cells present in the milk, as described by Martin et al. (1996), of two lactating goats with known genotypes (*CSN2* A/A and *CSN2* 01/01) and at comparable age, type of feed, diet, feeding level and lactation stage.

Reverse transcription and PCR

The reverse transcription of total RNA was performed using the primer cDNA9R (5′-GAAATGACTGGTTAGG-AA-3′, reverse primer corresponding to the complementary nucleotides (nt) 285–302 of the 9th exon, of the sequence of the goat *CSN2* gene allele A (GeneBank Acc. No. AJ011018), and Improm-II™ Reverse Transcriptase (Promega Corporation, Madison WI, USA) in a 20-μl reverse transcriptase assay according to the manufacturer's protocol.

PCR was performed by using the following primers: cDNA9R and cDNA1F (5′CTCCTCTACTTGGAAAA-3′, forward primer). The sequences of the cDNA1F correspond to nt 28–45 of the 1st exon of the sequence of the goat *CSN2* gene (GeneBank Acc. No. AJ011018). The 100-μl PCR reaction mix comprised: the 20 μl of RT reaction

*For correspondence; e-mail: ramunno@unina.it

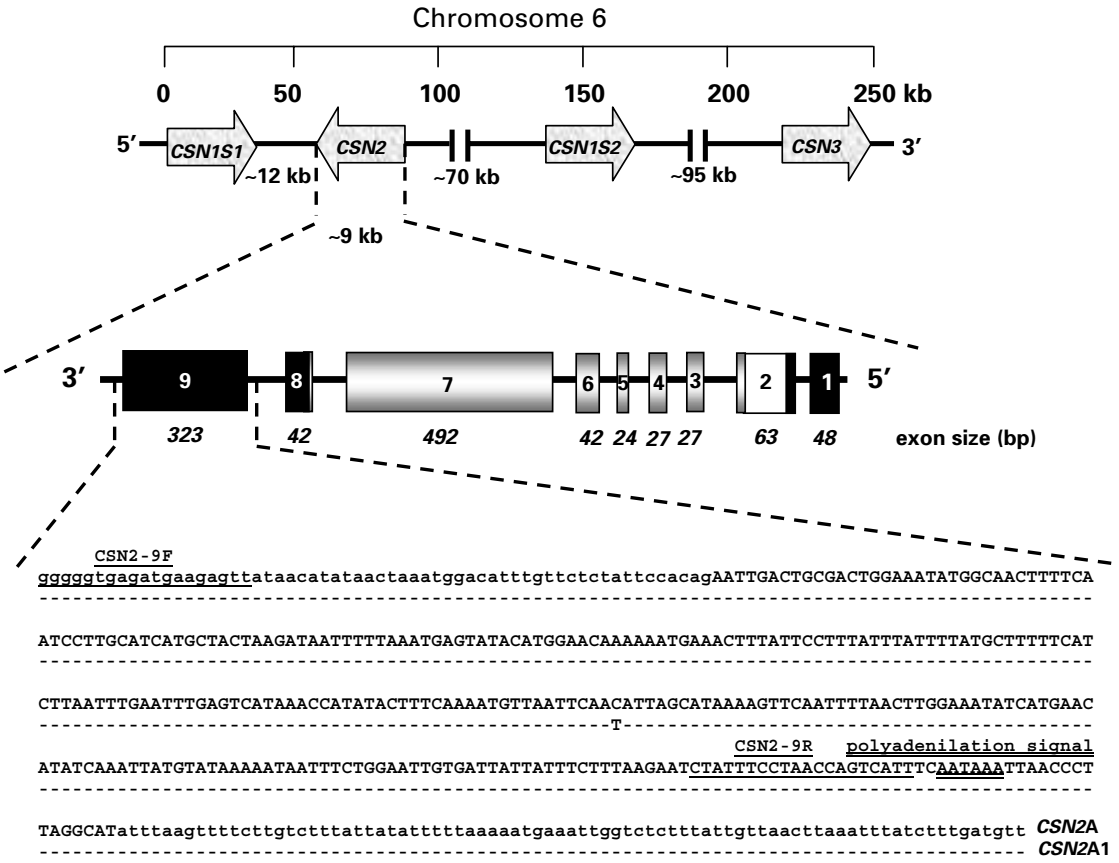

Fig. 1. Physical map of the casein locus in the goat; schematic representation of the *CSN2* gene of the goat and nucleotide sequence relative to the 9th exon (capital letters) and flanking regions (small letters). Primers and polyadenylation signal sequences are underlined and double underlined, respectively. ■ 5′ and 3′ untranslated regions, □ region encoding the signal peptide, ▣ regions encoding the mature protein.

product, 50 mM-KCl, 10 mM-Tris–HCl (pH 9·0), 0·1% Triton X-100, 2 mM-$MgCl_2$, 10 pmol of each primer, dNTP each at 0·2 mM, 5 U of *Taq* DNA Polymerase (Promega).

The amplification protocol consisted of 39 cycles: the first cycle involved a denaturation step at 97 °C for 2 min, an annealing step of 51 °C for 30 s and an extension step at 72 °C for 1 min and 30 s. Each of the next 37 cycles was performed under the following conditions: 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min and 30 s. In the 39th cycle, conditions remained the same for denaturation and annealing steps, but the extension step was carried out at 72 °C for 10 min.

The amplified products were analysed by electrophoresis on 3% agarose gel in 1× TBE buffer.

Genotyping of CSN2 *alleles by means of SspI PCR-RFLP*

A 360 bp fragment spanning part of the 9th exon and flanking regions of the goat *CSN2* gene was amplified by means of PCR carried out by using Gene Amp PCR System 2400 (Perkin Elmer) with the following primers: CSN2-9F, forward: 5′-GGGGGTGAGATGAAGAGTT-3′ (nucleotides 9625–9643); CSN2-9R, reverse: 5′-AATGACTGGTTAGG-AAATAG-3′ (complementary to nucleotides 9965–9984). Numbering is according to the sequence of goat *CSN2* gene (GeneBank Acc. No. AJ011018).

The 50-µl reaction mix comprised: 100 ng of genomic DNA, 50 mM-KCl, 10 mM-Tris–HCl (pH 9·0), 0·1% Triton X-100, 3 mM-$MgCl_2$, 5 pmol of each primer, dNTP each at 400 µM, 2·5 U of *Taq* DNA Polymerase (Promega), 0·04% BSA.

The amplification programmes consisted of 31 cycles. The first one was characterized by a denaturation at 97 °C for 2 min, annealing at 53 °C for 45 s and an extension step at 72 °C for 2 min. The next 30 cycles involved a denaturation step at 94 °C for 45 s, annealing at 53 °C for 45 s and extension at 72 °C for 2 min with the exception that in the last cycle the extension time was 10 min.

Digestion of 17 µl of each PCR amplification was accomplished with 10 U of *Ssp*I endonuclease (Promega) for 5 h at 37 °C following the supplier's directions for buffer conditions. PCR and digestion products were analysed directly by electrophoresis in 1·5% TBE agarose gel (Biorad) in 1× TBE buffer and stained with ethidium bromide.

Table 1. Genotype distributions and allelic frequencies at the goat *CSN2* locus

	Observed Genotypes				Allelic frequencies	
Genetic type	*CSN2* A1/A1	*CSN2* A1/X†	*CSN2* X†/X†	*n*	*CSN2* A1	*CSN2* X†
Neapolitan local population	9	60	101	170	0·23	0·77

CSN2 X† = non A1 allele

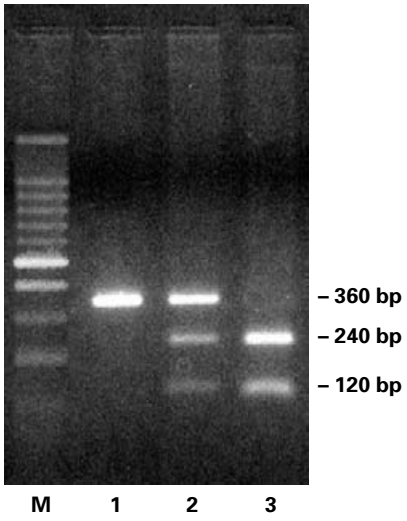

Fig. 2. Observed genotypes after *Ssp*I digestion of fragments obtained by PCR of the DNA region spanning the 9th exon and flanking regions of the goat *CSN2* gene. M = 100 bp DNA ladder (Promega); lane 1: *CSN2* X*/*CSN2* X*; lane 2: *CSN2* A1/*CSN2* X*; lane 3 *CSN2* A1/*CSN2* A1. *CSN2* X* = non *CSN2* A1 allele.

Primer design, DNA sequencing and computer analyses

Primers were designed by means of OLIGO 5.0 software (National Biosciences Inc., Plymouth MN, USA), using as templates the sequences of the goat *CSN2* gene (GeneBank Acc. No. AJ011018). Nucleotide sequencing was carried out according to the dideoxynucleotide chain-termination technique by using a BigDye™ Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) and an ABI PRISM 377-18 (Applied Biosystems, Foster City CA, USA) nucleotide sequencer. All regions were sequenced in both directions. Homology searches, comparison among sequences, and multiple alignments were accomplished by means of DNASIS-Pro software (Hitachi Software Engineering Co., San Bruno CA, USA).

Results and Discussion

The comparison between the cDNA sequence obtained and the published sequences of the goat *CSN2* alleles (GeneBank Acc. No. AF40909, AJ011018, AJ011019, M90562) showed in the sample homozygote for the A allele a new single nucleotide polymorphism (SNP) (transition C→T) at the 180th nucleotide of the ninth exon (Fig. 1). This mutation, which took place at 124 nt from the polyadenylation site, identifies a silent allele at the *CSN2* locus named *CSN2* A1. With the exception of this mutation, no other difference at exon level characterizes the *CSN2* A1 compared with the A allele.

The presence of cytosine at the *CSN2* ninth exon might represent the ancestral condition of the gene because it has been found also in other ruminant species such as cattle (GeneBank Acc. No. M55158), sheep (GeneBank Acc. No. X79703), river buffalo (GeneBank Acc. No. AJ005165) and the domestic yak (GeneBank Acc. No. AF194985).

Since the 9th exon C→T transition creates a *Ssp*I endonuclease restriction site (AAT↓ATT), the *Ssp*I digestion of a PCR product of 360 bp spanning the 9th exon and flanking regions, would allow carriers for the presence of thymine to be identified. As a consequence, the PCR product, uncut in the presence of cytosine, is now restricted into two fragments of 120 bp and 240 bp (Fig. 2).

The allelic frequency of the *CSN2* A1 allele, determined in 170 goats belonging to an undefined genetic type reared in the province of Naples (Italy), was 0·23 and the genotype distributions was in agreement with Hardy-Weinberg equilibrium (Table 1).

It has been observed (Xu et al. 1997) that the sequences in the 3′ untranslated regions (UTR), proximal to the polyadenylation site, can affect the mechanism of mRNA deadenylation and degradation. Therefore, it is reasonable to hypothesize that the C→T transition might, directly or indirectly, influence the stability of the mRNA and, consequently, the amount of protein produced. Further investigations are necessary to determine a possible effect on the expression of the goat *CSN2* gene.

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