Technical Note: Simultaneous Identification of *CSN1S2* A, B, C, and E Alleles in Goats by Polymerase Chain Reaction-Single Strand Conformation Polymorphism

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ABSTRACT

Most variability in goat caseins originates from the high number of genetic polymorphisms often affecting the specific protein expression, with strong effects on milk composition traits and technological properties. At least 7 alleles have been found in the goat α_{S2} -CN gene (*CSN1S2*). Five of them (*CSN1S2**A, *CSN1S2**B, *CSN1S2**C, *CSN1S2**E, and *CSN1S2**F) are widespread in most breeds, whereas the other 2 (*CSN1S2**D and *CSN1S2**0) are rarer alleles. Four different PCR-RFLP tests are needed to detect all of these variants at the DNA level. The objective of this study was to develop and validate a rapid method for typing 4 of the 5 most-common goat *CSN1S2* alleles by means of PCR-single strand conformation polymorphism (SSCP). The method was validated by analyzing 37 goat samples at the protein and DNA level, respectively, by milk isoelectrofocusing and PCR-RFLP methods already described. The genotypes obtained using the PCR-SSCP approach were in full agreement with those obtained by the validation analyses. The newly developed PCR-SSCP approach provides an accurate and inexpensive assay highly suitable for genotyping goat *CSN1S2*.

Key words: α_{S2} -casein, goat, polymerase chain reaction-single strand conformation polymorphism

INTRODUCTION

The qualitative and quantitative variability of goat caseins originates from the high number of genetic polymorphisms and by multiple posttranslation modifications, in addition to environmental effects. The 4 casein genes are located within a 250-kb segment of caprine chromosome 6 (Martin et al., 2002). The polymorphisms, some of which occurred through mutations af-

fecting expression of specific proteins, have strong effects on milk composition traits and technological properties (Martin et al., 2002). The large genetic variation requires several techniques for the alleles to be completely identified. Among the typing methods carried out at the DNA level, PCR-single strand conformation polymorphism (SSCP) was successfully used for the identification of alleles or groups of alleles at goat α_{S1} -CN (*CSN1S1*; Caroli et al., 2007), β -CN (*CSN2*; Chessa et al., 2005), and κ -CN (*CSN3*; Chessa et al., 2003; Prinzenberg et al., 2005). The listed assays also allowed the identification of new variants such as *CSN1S1**B' (Caroli et al., 2007), *CSN2**E (Caroli et al., 2006), *CSN3**C, and *CSN3**M (Chessa et al., 2003; Prinzenberg et al., 2005).

At least 7 alleles have been found for α_{S2} -CN (*CSN1S2*); *CSN1S2**A, *CSN1S2**B (Boulanger et al., 1984), *CSN1S2**C (Bouniol et al., 1994), *CSN1S2**E (Veltri et al., 2000; Lagonigro et al., 2001), and *CSN1S2**F (Ramunno et al., 2001a) code for a normal content of α_{S2} -casein (about 2.5 g/L), whereas *CSN1S2**D and *CSN1S2**0 (Ramunno et al., 2001a,b) are associated with reduction and absence of α_{S2} -CN in milk, respectively. The D allele is characterized by a 106-nucleotide deletion starting from the last 11 nucleotides of exon 11, which causes the loss of the AA Pro₁₂₂, Thr₁₂₃, and Val₁₂₄. The *CSN1S2**0 allele is characterized by 19 single nucleotide polymorphisms (SNP), but the mutation responsible for the null content of this variant seems to be the transition G→A in codon 27 of exon 11 because it changes the codon TGG (coding for Trp₁₁₀) into a TAG stop codon (Ramunno et al., 2001b). The alleles coding for normal α_{S2} -CN content are characterized by the SNP responsible for AA substitutions, as indicated in Table 1.

Four different PCR-RFLP tests are needed to detect all 7 alleles at the DNA level. The *CSN1S2**A, B, and C alleles can be distinguished by means of the PCR-RFLP protocol developed by Ramunno et al. (1999). The method is based on the amplification of a region spanning from exon 17 to exon 18 of the gene and giving

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Table 1. Single nucleotide polymorphisms differentiating goat *CSN1S2**A from the other alleles coding for normal α_{S2} -CN content¹

Allele	Nucleotides involved	Gene region	AA involved	Reference
F	G ₁₀₆ /A ₁₀₆	Exon 3, codon 5	Val ₇ /Ile ₇	Ramunno et al., 2001a
B	G ₂₇₇ /A ₂₇₇	Exon 9, codon 4	Glu ₆₄ /Lys ₆₄	Bouniol et al., 1993
C, E	A ₅₈₇ /T ₅₈₇	Exon 16, codon 2	Lys ₁₆₇ /Ile ₁₆₇	Bouniol et al., 1994
E	C ₆₆₅ /G ₆₆₅	Exon 16, codon 28	Pro ₁₉₃ /Arg ₁₉₃	Veltri et al., 2000

¹The first nucleotide (column 2) and deduced AA (column 4) refer to *CSN1S2**A (GenBank accession no. X65160).

2 invariant 700-bp and 1,700-bp fragments together with a 900-bp fragment for *CSN1S2**A, a 1,300-bp fragment for *CSN1S2**B, and a 950-bp fragment for *CSN1S2**C after digestion with *Pst*I.

Two separate PCR-RFLP protocols allow the detection of the other 2 normal coding α_{S2} -CN alleles. In fact, *CSN1S2**E and *CSN1S2**F can be differentiated from the others because the mutations responsible for these alleles, indicated in Table 1, eliminate a *Nla* III (Veltri et al., 2000) and an *Alw*26 I (Ramunno et al., 2001b) restriction site, respectively. Finally, PCR-RFLP is also used for the simultaneous detection of *CSN1S2**D and *CSN1S2**O (Ramunno et al., 2001a). In the fragment analyzed by this method, the *CSN1S2**O allele is characterized by a mutation eliminating an *Nco* I restriction site, whereas *CSN1S2**D is characterized by a deletion giving 2 fragments smaller than those from the other variants after digestion.

Typing the genetic variability by these PCR-RFLP assays is time-consuming. Moreover, the genotyping for the A, B, and C alleles is made on the basis of polymorphisms not directly involved in the AA exchange characterizing the 3 variants. The objective of this work was to develop a PCR-SSCP protocol for the simultaneous genotyping of *CSN1S2**A, *CSN1S2**B, *CSN1S2**C, and *CSN1S2**E alleles as an alternative to the 2 PCR-RFLP techniques previously described (Ramunno et al., 1999; Veltri et al., 2000).

Because the mutations distinguishing the 4 alleles A, B, C, and E are in different exons (Table 1), 2 PCR assays were necessary. The first PCR amplified a 247-bp fragment of exon 9 containing the mutation distinguishing B allele from A, C, and E. The primers used were B9 Fw (5'-GGACTCTAAATATACTTAATGAATT-3'), and B9 Rv (5'-GCTTATCGTCCACAGTAATCTT-3'). The second PCR amplified a 273-bp fragment of exon 16 and contained the 2 mutations distinguishing C and E from A and B, as well as from each other. The primers used were C16 Fw (5'-CTGTTTGGTATCATTTAGAATTAT-3'), and E16 Rv (5'-CTCTTTTATTACAAAAGACAACCTT-3'). The primers were designed on the basis of the caprine sequences with GenBank accession no. AJ131465, AF096872, and AJ242528. The PCR reaction

was performed in a 25- μ L reaction mixture containing 2 μ L of DNA solution (10 to 100 ng), 10 pmol of each primer, and 1 $\times$ PCR Master Mix (Fermentas, Vilnius, Lithuania). The following conditions were used: an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 1 min 30 s, and a final extension step of 72°C for 5 min using PTC-0200 DNA Engine thermal cycler (MJ Research Inc., Waltham, MA).

The 2 PCR fragments were subsequently analyzed by using the SSCP technique performed on a 13% acrylamide:bisacrylamide gel (37.5:1) with 2% glycerol in 0.5 $\times$ Tris-borate-EDTA buffer. Six microliters of exon 9 PCR product was added to 8 μ L of denaturation solution (0.05% of xilenecyanol, 0.05% of bromophenol blue, 0.02 M EDTA in deionized formamide). After heat denaturation at 95°C for 8 min, the samples were immediately chilled on ice and run at 200 V and 12°C for 1 h in a Penguin TM Dual Gel Water-Cooled Electrophoresis System (OWL Scientific Inc., Woburn, MA). Then, 6 μ L of exon 16 PCR product was added to 8 μ L of the denaturation solution and, after heat denaturation of 95°C for 8 min, they were loaded in the same gel and position of the corresponding exon 9 PCR product of the same individual. The run lasted 16 h at 200 V and 12°C. Bands were visualized by silver staining (Bassam et al., 1991).

Two series of bands were obtained by PCR-SSCP analysis, slow migrating (Figure 1A) and fast migrating (Figure 1B); the first corresponded to exon 16 PCR product and the second to exon 9 PCR product. This correspondence was confirmed after loading the 2 PCR products under the same electrophoretic conditions but in different slots, alternating exon 9 and 16 PCR products of the same samples. For the exon 16 PCR product, 3 different patterns were detected. Each pattern was composed of 4 bands, corresponding to a principal and an additional conformer for both strands of each DNA sample. The additional conformers are differently visible depending on the strand and on the sample, but when visible they can be used to confirm the assigned genotype. The slower set of 4 bands belonged to *CSN1S2**C allele, the intermediate set to *CSN1S2**E,

Figure 1. Polymerase chain reaction-single strand conformation polymorphism analysis of different *CSN1S2* genotypes. A) The 273-bp fragment containing exon 16 is fixed in the higher part of the gel at the end of the electrophoretic run. Three different patterns were detected, each composed of 4 bands. Black diamond = *CSN1S2**C; black star = *CSN1S2**E; white diamond and # = *CSN1S2**A or *CSN1S2**B. B) The 247-bp fragment containing exon 9 is fixed in the lower part of the gel. A set of 2 bands was visible for each allele. Black triangle = *CSN1S2**B; white triangle and \$ = other alleles. The genotype is given by the combination of the results obtained for the 2 fragments.

and the faster set to non-*C*, non-*E* alleles. For exon 9 PCR product, a set of 2 bands was visible for each pattern. The bands corresponding to the *CSN1S2**B were faster, whereas the other alleles comigrated more slowly, having no differences within the amplified fragment. The *CSN1S2**A allele was assigned by exclusion.

The test was validated by screening *CSN1S2* variability in 37 Italian goat samples; DNA was extracted from blood (when available) or milk by standard methods. The DNA samples were analyzed by the novel PCR-SSCP protocol and by 2 PCR-RFLP tests already available, respectively, for the detection of *CSN1S2**A, *CSN1S2**B, and *CSN1S2**C (Ramunno et al., 1999), and for the detection of *CSN1S2**E (Veltri et al., 2000). The milk samples were typed by isoelectric focusing (Caroli et al., 2001; Chiatti et al., 2007) to check the correspondence between the DNA polymorphisms and the phenotypic expression.

Full agreement was found in the typing results obtained by the PCR-SSCP protocol developed, the 2 PCR-

RFLP methods, and the isoelectric focusing analysis (which allows the simultaneous detection of the 4 variants at the protein level). The PCR-SSCP test used in this study may be a more convenient alternative to the 2 PCR-RFLP methods for *CSN1S2* typing at the DNA level, reducing to 3 the number of tests necessary to identify the 7 *CSN1S2* alleles described. The simultaneous identification of different alleles and the possibility of detecting new variants, as already demonstrated for the other casein fractions, make PCR-SSCP a convenient and suitable molecular tool for DNA typing. However, to have a complete picture of the α_{S2} -CN variability in goat, 2 specific DNA tests are necessary to detect *CSN1S2**F, as well as the rare *CSN1S2**0 and *CSN1S2**D alleles. Based on the actual knowledge on goat *CSN1S2* genetic variation, a typing protocol should be followed to minimize the number of analyses required for a complete *CSN1S2* screening. After samples have been typed by the PCR-SSCP assay, the alleles identified as B, C, and E do not require further

investigation. However, both *CSN1S2**A homozygous (A/A) and heterozygous (A/–) animals should be further analyzed, because *F*, *O*, and *D* alleles originated from A in *CSN1S2*, according to the α_{S2} -CN phylogeny proposed by Ramunno et al. (2000). It should be recommended to type A/A and A/– animals first for *CSN1S2**F (Ramunno et al., 2001b), which is rather common in goat breeds (Caroli et al., 2006). Then, animals still classified as A/A or A/– should be further typed for *CSN1S2**O and *D* (Ramunno et al., 2001a). This analysis could be performed on a randomly chosen subset of animals only to exclude the occurrence of these rare alleles in the goat population examined. Work is in progress to include *CSN1S2**F in the PCR-SSCP method developed to further reduce the number of analyses and samples to be tested with more methods.

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