Short Communication: Simultaneous Identification of Five κ -Casein (*CSN3*) Alleles in Domestic Goat by Polymerase Chain Reaction-Single Strand Conformation Polymorphism

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ABSTRACT

Until now, a total of nine polymorphic sites corresponding to six different alleles have been described at the κ -casein (*CSN3*) locus in the domestic goat (*Capra hircus*). A protocol for the rapid and simultaneous genotyping of five goat *CSN3* alleles by using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique was developed. Moreover, the developed test was validated by screening the *CSN3* variability in four Italian breeds, Garganica, Jonica, Maltese, and Camosciata. Seven different patterns were readily identifiable. These corresponded to five known alleles and two newly identified variants. The G/A substitution at nucleotide position 471, which is not identifiable at the protein level but was found to be very frequent in the typed breeds, is easily detectable by the protocol developed. The PCR-SSCP analysis is a powerful tool for the genetic study of *CSN3* variability in domestic goats, allowing both the simultaneous identification of different alleles, and the detection of new variants.

(Key words: goat, κ -casein, genetic polymorphism, single strand conformation polymorphism)

Abbreviation key: *CSN3* = κ -casein locus, **IEF** = isoelectrofocusing, **PCR-SSCP** = polymerase chain reaction-single strand conformation polymorphism.

Investigation of milk protein genetic polymorphism started more than 40 yr ago and is still arousing re-

search interest because of the relationships with milk quality, composition, and technological characteristics (Martin, 1993; Grosclaude et al., 1994). It is well known that CN genes are organized as a cluster (Ferretti et al., 1990; Threadgill and Womack, 1990) including α_{S1} -CN, β -CN, α_{S2} -CN, and κ -CN locus (*CSN3*). Among the CN fractions, κ -CN plays an important role in the formation, stabilization, and aggregation of CN micelles.

Little information was available about genetic variation of caprine *CSN3* until 1990, when two variants were described at the protein level (Di Luccia et al., 1990) and named A and B. The occurrence of the B variant was successively confirmed in different breeds (Budelli et al., 2000). The corresponding allele was characterized at the DNA level (Caroli et al., 2001) and compared with the first goat *CSN3* nucleotide sequence described (Coll et al., 1993).

Further *CSN3* variants were reported by Yahyaoui et al. (2001) and Angiolillo et al. (2002), whereas additional variants have been deposited in GenBank (<http://www.ncbi.nlm.nih.gov>) but not published in literature. Table 1 shows the different alleles found in domestic goats and the conflicting nomenclature, especially as it pertains to the B and D variants. A new nomenclature is now proposed based on the order of submission of sequences to GenBank. Moreover, the new nomenclature proposed is also in agreement with the previous literature about goat *CSN3* identification at the protein level (Di Luccia et al., 1990) and its characterization at the protein level (Budelli et al., 2000). Mercier et al. (1976a, 1976b) observed a polymorphism in the κ -CN goat protein sequence, finding a heterozygous animal at the amino acid position 119 (Val/Ile). The two sequences described correspond to the variants named A and D, respectively, in this study. Nevertheless, the authors

Received March 25, 2003.

Accepted July 17, 2003.

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Table 1. κ -Casein locus (*CSN3*) nucleotide and amino acid differences among domestic goats. Nucleotide positions are compared with GenBank accession no. X60763. The date of GenBank sequence submission, new and old nomenclature, and reference of the published papers are also reported. Synonymous mutations are in italics.

GenBank acc. No.	Date of submission	Nomenclature		Nucleotide position									Published reference
		New	Old	245	247	284	309	385	471	509	583	591	
X60763	02/07/91	A	A	T	A	G	G	A	G	A	C	T	Coll et al., 1993
AY027868	22/02/01	B	B	Tyr	Gln	Leu	Val	Asp	Val	Thr	Ala	Ser	Caroli et al., 2001
AY090465	18/03/02	B	D	<i>C</i>	<i>G</i>		A		A			C	Yahyaoui et al., 2001
AF485341	20/02/02	C	C	<i>Tyr</i>	Arg		A		A			Pro	Yahyaoui et al., 2001
AF434988	16/10/01	D	B	<i>C</i>		A	A		A	G	T	Pro	Yahyaoui et al., 2001
AF485340	20/02/02	D	B	<i>Tyr</i>		Leu	Ile		Ile	Thr	Val	Pro	
AF486523	22/02/02	E	E						A				Yahyaoui et al., 2001
AY090466	18/03/02	* ¹	F					G	Ile				Angiolillo et al., 2002
AY090467	18/03/02	G	G	C			A	Gly	A			C	Yahyaoui et al., 2001
				<i>Tyr</i>			Ile		Ile			Pro	
				<i>C</i>					A			C	
				<i>Tyr</i>					Ile			Pro	

¹Identified only in wild goat (*Capra pyrenaica*).

did not propose any nomenclature for the two variants nor perform studies on their genetic transmission.

The new nomenclature will be used in this note to refer to the different variants. The *F* allele was also described (GenBank accession no. AY090466), but previously it has been found only in wild goat (*Capra pyrenaica*) (Yahyaoui et al., 2001). Thus, referring to the published sequences, a total of nine polymorphic sites and six DNA alleles, corresponding to six different protein variants, have been identified in domestic goats (Table 1). The importance of developing a molecular method for a complete typing of goat *CSN3* variability is evident.

The polymerase chain reaction-single strand conformation polymorphism (**PCR-SSCP**) method was successfully used for the molecular identification of six protein variants and three additional DNA polymorphisms in bovine *CSN3* (Prinzenberg et al., 1999). The same technique was used to distinguish *CSN3**A from *CSN3**B (Caroli et al., 2001) in domestic goat. To improve the PCR-SSCP method for goat *CSN3* genetic analysis, we describe a new PCR-SSCP test and results regarding its application for typing four Italian domestic goat breeds.

Samples. DNA samples sequenced within *CSN3* exon IV, and corresponding to different genotypes (*AB*, *BB*, *BD*, *CD*, *DD*, and *EE*) were used as references to develop the PCR-SSCP protocol. Moreover, blood and individual milk samples were randomly collected from three Southern Italy goat breeds, Garganica (n = 34), Jonica (n = 119), and Maltese (n = 105); and one Northern Italy breed, Camosciata (n = 45), to validate the

developed test. The DNA was extracted from blood by a commercial kit (GFX Genomic Blood DNA Purification Kit, Amersham Biosciences, Piscataway, NJ). The following analyses were performed.

PCR. A 406-bp fragment containing exon IV of the goat *CSN3* gene was amplified by PCR, using primers designed on the basis of the caprine sequence GenBank accession no. AY027868. The PCR reaction was performed in a 25- μ l reaction mixture containing 2 μ l of DNA solution (10 to 100 ng), 7 pmol of the primers KCN I F and KCN I R, and 1 $\times$ PCR Master Mix (Fermentas).

KCN I F (5' GGTATCCTAGTTATGGACTCAAT 3'),
KCN I R (5' GTTGAAGTAACTTGGGCTGTGT 3').

The following conditions were used: an initial denaturation step of 95°C for 2 min was followed by 35 cycles of 94°C for 1 min, 59°C for 40 s, and 72°C for 3 min, and concluded with a final extension step of 72°C for 5 min using a PTC-0200 DNA Engine thermal cycler (MJ Research Inc., Waltham, MA).

SSCP. Six microliters of PCR product were added to 8 μ l of stop mix containing 0.05% of xylene-cyanole, 0.05% of bromophenol blue, and 0.02 M EDTA in deionized formamide. After heat denaturation of 95°C for 7 min, the samples were immediately chilled on ice and then run overnight (16.30 h) on 9.25% polyacrylamide gels with 1% glycerol (Penguin TM Dual Gel Water-Cooled Electrophoresis System, OWL Scientific Inc., Woburn, MA) in 0.5 $\times$ TBE buffer at 275 V and 12°C. Bands were visualized by silver staining (Bassam et al., 1991).

Figure 1. Polymerase chain reaction-single strand conformation polymorphism analysis of different κ -casein locus (*CSN3*) genotypes. Unknown migration patterns X and Y are also shown.

Isoelectrofocusing. Milk samples were analyzed by isoelectrofocusing (IEF) according to Caroli et al. (2001) for a comparison of the molecular results with the protein patterns.

Simultaneous discrimination of *CSN3* alleles A, B, C, D, and E was obtained by PCR-SSCP analysis (Figure 1). Two sets of bands, fast migrating and slow migrating, were present in duplication for each allele and were used for the genotype identification. Besides the five migration patterns related to the known variants, two further polymorphisms were found, preliminarily named X and Y. Studies are in progress to identify the mutations responsible for these different patterns.

Beside *CSN3*B* allele, identifiable at protein level, *CSN3*A*, *CSN3*C*, *CSN3*D*, and *CSN3*E* are clearly separated (Figure 1). By IEF analysis of milk samples, alleles A, C, and D variants overlap. Thus, the molecular approach is necessary to type goats for *CSN3* correctly to recognize mutations that could be related to a particular functional meaning as already demonstrated for *CSN3* variants in cattle (Pagnacco and Caroli, 1987). In particular, the separation between the A and D variant is really important due to the small difference existing between the two variants: a substitution G/A at position 471, resulting in an amino acid exchange (Val/Ile, position 119) not identifiable by IEF at the protein

level. In fact, the monitoring of *CSN3* variability in four domestic goat breeds performed by the technique developed clearly indicates the predominance of the D allele, occurring with a frequency of 0.73, 0.73, 0.70, and 0.63, respectively, in Garganica, Jonica, Maltese, and Camosciata breeds of goat (Table 2). The same variant, named B according to the old nomenclature, was described by Yahyaoui et al. (2001) but could not be distinguished from the A variant by the PCR-RFLP technique used, allowing the detection of the C allele. A PCR-RFLP protocol was described for the detection of the E allele (Angiolillo et al., 2002). In the Italian breeds genotyped in this study, no evidence was found for the C and E variants. More recently, a protocol for rapid simultaneous genotyping of *CSN3* goat variants using the primer extension method has been described (Yahyaoui et al., 2003), and a nomenclature has been proposed still conflicting with the present paper as well as with Jann et al. (2003), who have detected further caprine *CSN3* alleles. An effort has to be made at international level for full agreement about the nomenclature of such a polymorphic locus in goat.

The two further polymorphisms preliminarily named X and Y, found in three of the four analyzed breeds but with a low frequency (less than 2%), are also confounded with the A/D variant group at protein level, whereas the B variant typing corresponded both at the protein and DNA level.

Interestingly, *CSN3*A* and B occurred in different frequencies, ranging from 1.1% (*CSN3*B* in Camosciata) to 35.6% (*CSN3*A* in the same breed). In Garganica and Jonica, the two variants presented balanced frequencies. In a previous study, Caroli et al. (2001) described *CSN3*A* as the most widely distributed allele in domestic goat breeds. However, the allele frequencies were determined at the protein level using IEF, which does not allow the separation of most variants from *CSN3*A*. Moreover, the PCR-SSCP analysis developed in the previous paper, using different primers, did not allow differences between *CSN3*A* and *CSN3*D* to be determined. Therefore, the protocol presented here is indicated for the analysis of goat *CSN3* polymorphisms.

The PCR-SSCP test used in this study is a rapid method to screen goat breeds at *CSN3* locus. The use of this technique gives the opportunity to differentiate variants that are undistinguishable at protein level and to type animals independent of age, sex, and lactation. It also allows the identification of further alleles at the *CSN3* locus. The demonstrated polymorphism at this locus must be carefully taken into consideration in addition to the already known variants at the other casein loci, in order to include casein haplotype information in breeding programs for the genetic improvement of domestic goat breeds.

Table 2. Allele frequencies at κ -casein locus (*CSN3*) locus in the Garganica, Jonica, Maltese, and Camosciata breeds.

<i>CSN3</i> variant	Allele frequency			
	Garganica (n = 34)	Jonica (n = 119)	Maltese (n = 105)	Camosciata (n = 45)
A	0.132	0.136	0.086	0.356
B	0.118	0.106	0.200	0.011
C	—	—	—	—
D	0.735	0.729	0.700	0.633
E	—	—	—	—
X ¹	—	0.017	0.010	—
Y ¹	0.015	0.013	0.005	—

¹Preliminary nomenclature.

ACKNOWLEDGMENTS

We are grateful to MURST contract 2001077279_002 and to VIGONI project for financial support, to Eva-Maria Prinzenberg for valuable comments. We thank Antonella Angiolillo and Josep Maria Folch for *CSN3**E and *CSN3**C reference samples.

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