Characterization of two new alleles at the goat *CSN1S2* locus

L. Ramunno*, G. Cosenza*, M. Pappalardo*, E. Longobardi*, D. Gallo*, N. Pastore*, P. Di Gregorio† and A. Rando†

*Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti, Università degli Studi di Napoli 'Federico II', Napoli, Italy. †Dipartimento di Scienze delle Produzioni Animali, Università degli Studi della Basilicata, Potenza, Italy

Summary

Two novel alleles at the goat *CSN1S2* locus have been identified: $CSN1S2^F$ and $CSN1S2^D$. Sequence analyses revealed that the $CSN1S2^F$ allele is characterized by a G → A transition at the 13th nucleotide in exon 3 changing the seventh amino acid of the mature protein from Val to Ile. The $CSN1S2^D$ allele, apparently associated with a decreased synthesis of αs2-casein, is characterized by a 106-bp deletion, involving the last 11 bp of the exon 11 and the first 95 bp of the following intron. Methods (PCR–RFLP and PCR) for identification of carriers of these alleles have been developed.

Keywords αs2 casein, *Capra hircus*, milk, PCR.

Introduction

The three Ca-sensitive caseins (αs1, β and αs2) of goat exhibit both qualitative and quantitative variation arising from genetic polymorphism in the encoding genes (*CSN1S1*, *CSN2*, *CSN1S2*, respectively) (for a review, see Rando *et al.* 2000).

At present, four alleles at the goat *CSN1S2* locus are known: $CSN1S2^A$, $CSN1S2^B$ and $CSN1S2^C$, associated with about a 2.5-g/l content per allele of αs2-casein (Boulanger *et al.* 1984; Grosclaude *et al.* 1987; Bouniol *et al.* 1994) and $CSN1S2^O$, associated with a 'null' content of such protein (Ramunno *et al.* 2001). The alleles $CSN1S2^B$ and $CSN1S2^C$ differ from the $CSN1S2^A$ allele by single nucleotide substitutions: a G → A transition at the 10th nt of exon 9 and an A → T transversion at the 5th nt of exon 16, respectively. These mutations are responsible for amino acid substitutions: Glu^{64} → Lys and Lys^{167} → Ile, respectively (Bouniol *et al.* 1994). The mutation characterizing the $CSN1S2^O$ allele is a G → A transition at the 80th nt of exon 11. This mutation creates a premature stop codon in position 110 (Ramunno *et al.* 2001). Recently, a new allele at the goat *CSN1S2* locus, $CSN1S2^E$, characterized by a mutation in the 28th codon of exon 16 and responsible for the amino acid substitution Pro (CCA) → Arg (CGA) (Veltri *et al.* 2000) has been identified.

The present paper reports the identification and characterization of two new alleles at the goat *CSN1S2* locus and methods for identification of carriers of these alleles.

Address for correspondence

L. Ramunno, Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti, Università degli Studi di Napoli 'Federico II', Via Università 133, 80055 Portici (Na), Italy.
E-mail: ramunno@unina.it

Accepted for publication 17 July 2001

Materials and methods

Milk and DNA samples were obtained from 182 goats of an undefined genetic type belonging to a population reared in the province of Naples. Skimmed milk samples were analysed by means of sodium dodecyl sulphate (SDS) (Grosclaude *et al.* 1987) and/or UREA (Medrano & Sharrow 1989) polyacrylamide gel electrophoresis (PAGE) at alkaline pH. The DNA samples were extracted from leucocytes according to Goossens & Kan (1981) and analysed by means of polymerase chain reaction (PCR) under the following conditions:

$CSN1S2^F$: Carriers of this allele were identified by means of PCR–RFLP by using the following primers: CASFf, 5′-TCTCTTGCCATCAAAACA-3′ (forward) and CASFr, 5′-TGGTCTTTATTCCTCTCT-3′ (reverse), nt 385–402 and complement of nt 677–694, respectively (EMBL, Accession No. AJ238475). The PCR reaction was carried out in a 50-μl volume containing: 100 ng of genomic DNA, 20 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Promega Corporation, Madison, WI, USA), 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100 (Promega), 3 mM $MgCl_2$, dNTPs each at 200 μM, 0.04% BSA. The amplification protocol consisted of an initial cycle of 97 °C

for 2 min, 53.9 °C for 45 s and 72 °C for 2 min 30 s followed by 31 cycles of 94 °C for 45 s, 53.9 °C for 45 s, 72 °C for 2 min 30 s; in the final cycle the extension step was carried out at 72 °C for 10 min. Aliquots (20 μl) of PCR products were digested with 10 U of *Alw*26I endonuclease for 5 h at 37 °C following the supplier's directions for buffer condition.

CSN1S2^D: The PCR reaction for identification of carriers of this allele was accomplished by using primers CASDf, 5′-GACACATAGAGAAGATTC-3′ (forward, nt 627 to nt 644) and CASDr, 5′-CGTTGGGACATTTTATCT-3′ (reverse, complement to nt 910–927) (EMBL accession No. AJ131370). The PCR was carried out as above except that dNTPs were each at 400 μM. The amplification protocol was as above except that the annealing temperature was 50.6 °C. Aliquots (20 μl) of PCR products were digested with 10 U of *Nco*I endonuclease for 5 h at 37 °C following the supplier's directions for buffer condition.

The PCR and digestion products were analysed by means of electrophoresis in 3% agarose gel stained with ethidium bromide.

Cloning of PCR products in pCR® 2.1 – TOPO plasmid was accomplished by using the TOPO-TA Cloning kit (Invitrogen, Groningen, the Netherlands). Sequencing was accomplished according to Sanger *et al.* (1977) by using the *fmol*™ DNA Sequencing System (Promega). All fragments were sequenced in both directions.

Genotyping at *CSN1S1* and *CSN1S2* loci

Alleles *CSN1S1^E*, *CSN1S1^F* and *CSN1S1^O* were detected by means of PCR (Jansá Pérez *et al.* 1994), *Xmn*I PCR–RFLP (Ramunno *et al.* 2000a) and allele specific-polymerase chain reaction (AS–PCR) (Ramunno *et al.* 2000b), respectively. *CSN1S2^B* and *CSN1S2^C* alleles were detected by means of allele specific multiplex PCR (ASM–PCR) (Ramunno *et al.* 2000b).

Results and discussion

CSN1S2^F: During sequencing of the goat *CSN1S2* gene, a new allele (*CSN1S2^F*), characterized by a G → A transition at the 13th nucleotide of exon 3, was identified. This mutation affects a *Alw*26I restriction site and changes the GTC^{Val} codon in position 7 of the mature protein to an ATC^{Ile} codon (both apolar amino acids). The αs2-Cn F variant shows the same electrophoretic mobility of the αs2-Cn A and C variants in SDS–PAGE.

Digestion of the amplified DNA fragment containing the third exon and part of the flanking regions with *Alw*26I shows an undigested fragment of 310 bp for the *CSN1S2^F* allele and two fragments of 179 bp and 131 bp for the other alleles of this locus (Fig. 1). Frequency of the *CSN1S2^F* allele in the analysed population (182 samples) was 0.261 (Table 1).

CSN1S2^D: The SDS–PAGE analysis of individual milk samples revealed two individuals whose milk samples are characterized by the apparent absence of αs2-casein and the presence of a band with electrophoretic mobility similar to that of αs1-CnB and αs1-CnE variants and lower than that of the m factor band (Grosclaude *et al.* 1987) (Fig. 2, lane 5). The *CSN1S1* genotype of the two goats, determined at protein and DNA level, was *CSN1S1$^{A/F}$*. The UREA–PAGE analysis showed that both samples are characterized by an

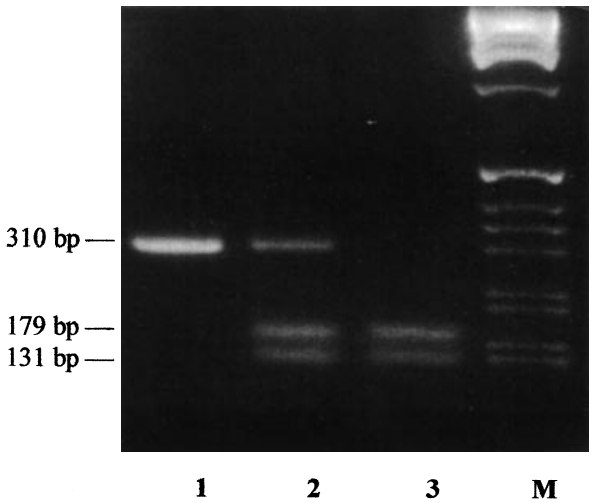

Figure 1 DNA electrophoretic patterns obtained after digestion with *Alw*26I endonuclease of the DNA region containing the 3rd exon of the goat *CSN1S2* gene. Lane 1: *CSN1S2$^{F/F}$*; lane 2: *CSN1S2$^{N*/F}$*; lane 3: *CSN1S2$^{N*/N*}$*; N* = non F allele (A, B, C, D, E or 0); M = Marker 1Kb

Table 1 Observed genotypes and allelic frequencies at *CSN1S2* locus in a goat population reared in the province of Naples.

		CSN1S2[(1)]																
Goat	Observed	AA	AB	AC*	AD	AF	A0	BC*	BF	B0	C*C*	C*F	C*0	DF	D0	FF	F0	00
no. 182	genotypes	41	4	20	3	25	5	2	2	1	17	19	10	2	2	21	5	3
	Allelic	A			B			C*			D			F			0	
	frequencies	0.382			0.025			0.233			0.019			0.261			0.080	

[(1)] *CSN1S2* C* = *CSN1S2* C + E.

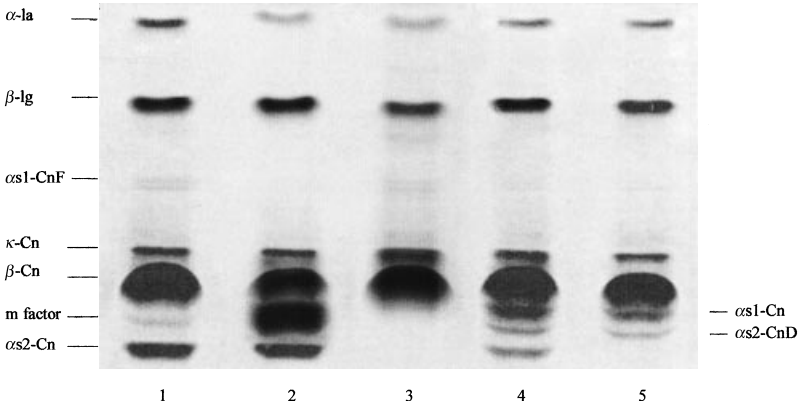

Figure 2 Electrophoretic patterns in SDS-PAGE of individual goat milk samples. Lane 1: *CSN1S2*$^{A*/A*}$, *CSN1S1*$^{F/F}$, lane 2: *CSN1S2*$^{A*/A*}$, *CSN1S1*$^{A/B}$; lane 3: *CSN1S2*$^{0/0}$, *CSN1S1*$^{F/F}$; lane 4: *CSN1S2*$^{A*/D}$, *CSN1S1*$^{A/F}$; lane 5: *CSN1S2*$^{D/0}$, *CSN1S1*$^{A/F}$. *CSN1S2A** = A+F+C, undistinguishable in SDS-PAGE.

electrophoretic band with a mobility different from the migration region of the group of the αs (αs1 + αs2) caseins (Fig. 3, lane 2). A similar pattern was also observed by Chianese *et al.* (1992) by means of disc-PAGE at pH 8.8. The PCR amplification of a 301-bp fragment spanning the exon 11 and part of the flanking introns of the *CSN1S2* gene evidenced, in both samples, another fragment of about 200 bp. The two fragments were cloned and sequenced. Comparison of the nucleotide sequences of the two fragments showed that the shorter one (195 bp) is characterized by a 106 nt deletion spanning the last 11 nt of exon 11 (from nt 113) and the first 95 nt of the following intron (EMBL accession no. AJ238684). Such deletion would be responsible for the lack of at least three codons coding for Pro (CCC^{122}), Thr (ACC^{123}) and Val (GTG^{124}), respectively. Furthermore, the last undeleted nucleotide (A) of exon 11 (the first of codon 121) together with the two nucleotides of the following intron (AT) could give rise to a new codon (AAT^{121}) followed by a new GT dinucleotide splicing donor site. In this case, this variant (called αs2-CnD), would be 205 amino acids long and have Asn rather than Thr in position 121 (Fig. 4).

Sequence of the 301 bp fragment shows the G → A transition characterizing the *CSN1S2*^{0} allele which can be identified through the lack of a *Nco*I endonuclease restriction site in the amplified fragment (Ramunno *et al.* 2001). Therefore, digestion with this endonuclease of the products of amplification of the DNA region spanning exon 11 of the *CSN1S2* gene shows an undigested fragment of 301 bp for *CSN1S2*^{0} allele (Fig. 5, lanes 1 and 2), two fragments of 133 bp + 62 bp for *CSN1S2*^{D} allele (Fig. 5, lanes 2 and 3) and two fragments of 168 bp + 133 bp for the other alleles (Fig. 5, lanes 3 and 4). As a consequence, by using this single typing method it is possible to correctly identify carriers of both *CSN1S2*^{D} and *CSN1S2*^{0} alleles.

Frequency of the *CSN1S2*^{D} allele in the analysed population was 0.019 (Table 1). Informative genotypes show that *CSN1S2*^{D} allele is in *cis* with *CSN1S1*^{A} and *CSN2*^{A} alleles.

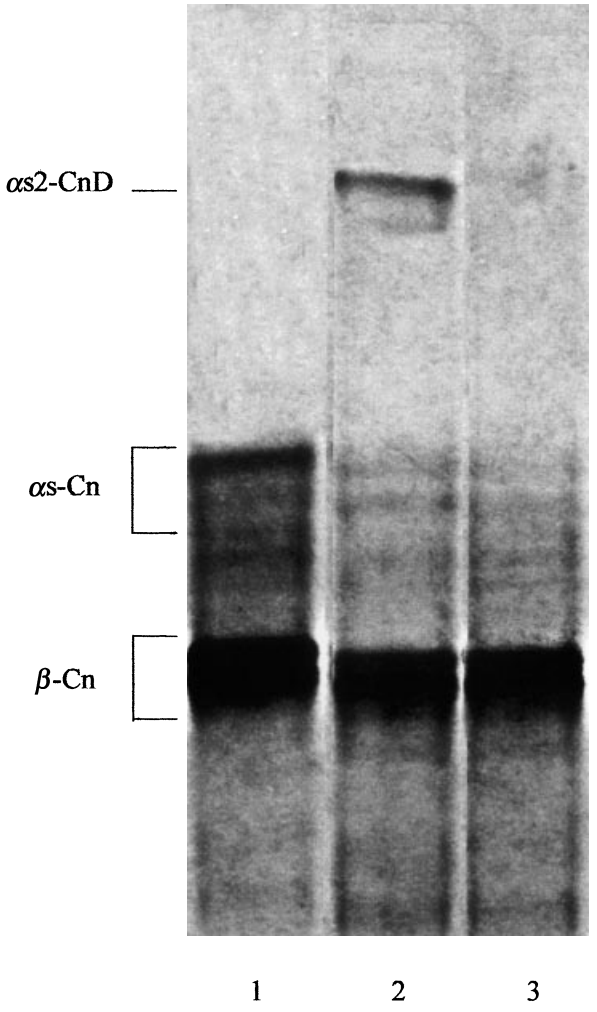

Figure 3 Electrophoretic patterns in UREA-PAGE of individual goat milk samples. Lane 1: *CSN1S2*$^{A*/A*}$; lane 2: *CSN1S2*$^{D/0}$; lane 3: *CSN1S2*$^{0/0}$ A*=A+F+C, undistinguishable in UREA-PAGE.

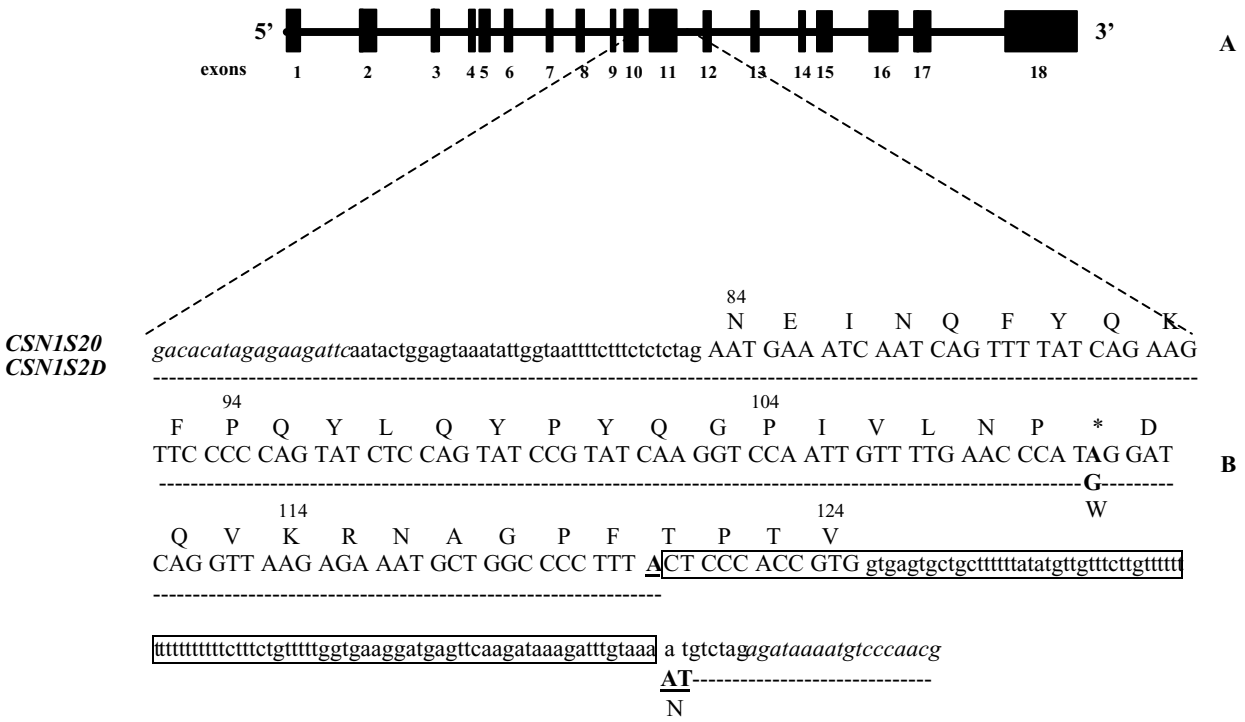

Figure 4 A = Structure of the goat *CSN1S2* gene. B = Partial sequence of the 11th exon and flanking regions of the *CSNIS2*^{O} (EMBL acc.n° AJ131370) and *CSN1S2*^{D} (EMBL acc.n° AJ238684) alleles. Primers are indicated in italics and the deleted DNA region of *CSN1S2*^{D} allele is boxed.

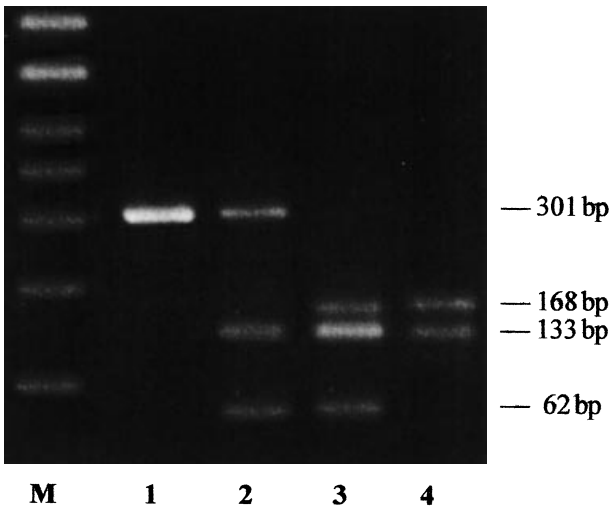

Figure 5 DNA electrophoretic patterns obtained after digestion with NcoI endonuclease of the DNA region containing the 11th exon of the goat CSN1S2 gene. M=Marker 100bp; lane 1: *CSN1S2*$^{O/O}$; lane 2: *CSN1S2*$^{D/O}$; lane 3: *CSN1S2a*$^{N*/D}$; lane 4: *CSN1S2*$^{N*/N*}$. N*=*CSN1S2* A, B, C, D, F or E.

Analysis of SDS–PAGE patterns of individual milk samples evidenced that the band relative to the αs2-Cn D variant shows a clearcut reduction of intensity when compared with the other variants at this locus (Fig. 2, lane 4). According to this result, it is plausible that the *CSN1S2*^{D} allele is associated with a lower than normal (intermediate) level of synthesis. Analyses at both transcription and translation level are in progress.

As a conclusion, we have identified two new alleles, at the goat *CSN1S2* locus. This locus is characterized by seven alleles (*A*, *B*, *C*, *D*, *E*, *F*, and 0) associated with at least three 'quantitative' levels of the corresponding protein: null (*CSN1S2*^{0}), intermediate (*CSN1S2*^{D}) and normal (the remaining five others). This high level of variability is comparable with that observed at the goat *CSN1S1* locus which is characterized by at least 13 alleles associated with four different levels of synthesis: high, medium, low and null (Martin *et al.* 1999). It is known that differences in the primary structure of milk proteins may affect, directly or indirectly, milk quality properties (for a review, see Grosclaude 1988). There is evidence that goats homozygous for alleles associated with a high content of αs1-casein produce milk characterised by a minor diameter of micelles, significantly higher percentage of protein, fat, total calcium and better parameters for curd firming time, curd firmness and cheese yield compared with goats homozygous for alleles associated with a low or intermediate content (Lenoir & Schneid 1987; Remeuf *et al.* 1989; Manfredi *et al.* 1993; Remeuf 1993;

Grosclaude *et al.* 1994). Similar effects may exist for milk obtained from goats with a different *CSN1S2* genotype (for example, $CSN1S2^{O/O}$, $CSN1S2^{D/D}$ and $CSN1S2^{A/A}$).

Acknowledgements

This work was supported by Cofinanziamento Programmi di Rilevanza Nazionale (MURST).

References

Boulanger A., Grosclaude F. & Mahè M.F. (1984) Polymorphisme des caseines αs1 et αs2 de la chèvre (capra hircus). *Génétique Sélection Evolution* **16,** 157–75.

Bouniol C., Brignon G., Mahé M.F. & Printz C. (1994) Biochemical and genetic analysis of variant C of caprine αs2-casein (*Capra hircus*). *Animal Genetics* **25,** 173–7.

Chianese L., Mauriello R., Intorcia N., Moio L. & Addeo F. (1992) New α_{s2}-casein variant from caprine milk. *Journal of Dairy Research* **59,** 299–305.

Goossens M. & Kan Y.W. (1981) DNA analysis in the diagnosis of hemoglobin disorders. *Methods in Enzymolgy* **76,** 805–17.

Grosclaude F. (1988) Le polymophisme genetique des principales lactoproteines bovines. *INRA Prodctions Animales* **1,** 5–17.

Grosclaude F., Mahè M.F., Brignon G., Di Stasio L. & Jeunet R. (1987) A Mendelian polymorphism underlying quantitative variationes of goat αs1-casein. *Génétique Sélection Evololution* **19,** 399–412.

Grosclaude F., Ricordeau G., Martin P., Remuef F., Vassal L. & Bouillon J. (1994) Du gene au fromage: le polymorphisme de la caseine αs1 caprine, ses effets, son evolution. *INRA Prodctions Animales* **7,** 3–19.

Jansá Pérez M., Leroux C., Bonastre A.S. & Martin P. (1994) Occurrence of a line sequence in the 3′ UTR of the goat αs1-casein E-encoding allele associated with reduced protein synthesis level. *Gene* **147,** 179–87.

Lenoir J. & Schneid N. (1987) L'aptitude du lait a la coagulation par la présure. In: *Eck A., 'Le fromage'*, 2ème édition, Tec et Doc Lavoisier, Paris, 139–50.

Manfredi E., Barbieri M.E., Bouillon J., Piacère A., Mahé M.F., Grosclaude F. & Ricordeau G. (1993) Effets des variants de la caséine αs1 sur le performances laitières de chèvres. *Lait* **73,** 567–72.

Martin P., Ollivier-Bousquet M. & Grosclaude F. (1999) Genetic polymorphism of casein: a tool to investigate casein micelle organization. *International Dairy Journal* **9,** 163–71.

Medrano J.F. & Sharrow L. (1989) Milk protein typing of bovine mammary gland tissue used to generate a complementary deoxyribonucleic acid library. *Journal of Dairy Science* **72,** 3190–6.

Ramunno L., Cosenza G., Pappalardo M., Pastore N., Gallo D., Di Gregorio P. & Masina P. (2000a) Identification of the goat $CSN1S1^{F}$ allele by means of PCR–RFLP method. *Animal Genetics* **31,** 342–3.

Ramunno L., Pappalardo M., Cosenza G., Pastore N., Gallo D., Grimaldi G., Rubino R., Calandrelli M. & Rando A. (2000b) Caratterizzazione genetica ai *loci* delle caseine αs1, β e αs2 di una popolazione caprina allevata nella penisola sorrentina. *XIV Congresso Nazionale S.I.P.A.O.C. Vietri Sul Mare 18–21* Ottobre, 321–4.

Ramunno L., Longobardi E., Pappalardo M., Rando A., Di Gregorio P., Cosenza G., Mariani P., Pastore N. & Masina P. (2001) An allele associated with a non-dectable amount of αs_2-casein in goat milk. *Animal Genetics* **32,** 19–26.

Rando A., Ramunno L. & Masina P. (2000) Mutations in casein genes. *Zootecnica E Nutrizione Animale* **26,** 105–14.

Remeuf F., Lenoir J. & Duby C. (1989) Etude des relations entre les caractéristiques physico-chimiques des laits de chèvre et leur aptitude à la coagulation par la présure. *Lait* **69,** 499–518.

Remeuf F. (1993) Influence du polymorphisme genetique de la caseine αs1 caprine sur les caracteristique physico-chimiques et technologiques du lait. *Lait* **73,** 549–57.

Sanger F., Nicklen S. & Coulson A.D. (1977) DNA sequencing with chain terminating inhibitor. *Proceedings of the National Academy of Science of the United State of America* **74,** 5436–67.

Veltri C., Lagonigro R., Pietrollà E., D'Andrea M., Pilla F. & Chianese L. (2000) *Molecular Characterisation of the Goat* αs2-Casein E Allele and its Detection in Goat Breeds of Italy. Seventh International Conference on Goats, Tours, France, p. 727.