Technical Note: Detection of the C Allele of β -Casein (CSN2) in Czech Dairy Goat Breeds Using LightCycler Analysis

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ABSTRACT

A protocol was developed for rapid genotyping of *A* and *C* variants at the *CSN2* locus in goat species (White Shorthaired and Brown Shorthaired goat) by PCR and LightCycler analysis. The LightCycler technique combines rapid and efficient in vitro amplification of DNA in glass capillaries, with melting curve analysis based on fluorescence resonance energy transfer, for the sensitive detection of point mutation. Analysis of the *CSN2* variability in the 2 goat breeds reared in the Czech Republic validated the genotyping test. Monitoring of *CSN2* variability in the goat breeds indicates the predominance of the *C* allele. In both breeds, *CSN2**A and *CSN2**C showed almost similar frequencies. Variant *CSN2**C occurred with a frequency of 0.699 in White Shorthaired goats and 0.570 in Brown Shorthaired goats.

Key words: goat, *CSN2*, genetic polymorphism, LightCycler analysis

INTRODUCTION

The goat *CSN2* encoding gene lies on chromosome 6 and consists of 9 exons ranging in size from 24 (exon 5) to 492 bp (exon 7; Roberts et al., 1992; Hayes et al., 1993; Rijnkels 2002; Cosenza et al., 2005). Currently, genetic polymorphism of the goat *CSN2* casein locus is characterized by extensive variability, which is under the control of at least 8 autosomal alleles: *A*, *A1*, *B*, *C*, *D*, *E*, and 2 null alleles (*0* and *0'*; Mahé and Grosclaude, 1993; Neveu et al., 2002; Galliano et al., 2004; Cosenza et al., 2005; Caroli et al., 2006). The genetic variants *A*, *A1*, *B*, *C*, *D*, and *E* were found to be associated with normal β casein content. Two null alleles (*0* and *0'*) of the *CSN2* gene were identified, both characterized by mutations responsible for premature stop codons in exon 7 (Ramunno et al., 1995; GenBank Accession number AJ011019; Persuy et al., 1999; GenBank Accession number AF172260). Both are associated with

a nondetectable amount of this protein in milk. The mRNA analysis revealed that the transcript product amounts were almost 10 (Ramunno et al., 1995) and 100 (Persuy et al., 1999) times smaller for the null alleles than for the *A* variant.

Genetic variants *A*, *B*, *C*, *D*, *E* and a further variant are not yet characterized (Chianese et al., 2007); however, they have been detected at the protein level using various techniques: isoelectrofocusing (Mahé and Grosclaude, 1993), peptide mass fingerprinting and tandem mass spectrometry (Neveu et al., 2002), reversed phase HPLC/electrospray ionization mass spectrophotometry (Galiano et al., 2004), and immunoelectrophoretic analysis (Chianese et al., 2007). Single-strand conformation polymorphism (Chessa et al., 2005; Caroli et al., 2006), allele-specific-PCR (Ramunno et al., 1995), and PCR-RFLP (Cosenza et al., 2005) have been utilized to identify genetic variants *A*, *A1*, *C*, *E*, and *0* at the DNA level. In regard to the *0* allele, Southern and Northern blotting analyses were used to confirm the variant and an allele-specific-PCR has been implemented to discriminate it.

In population studies to estimate genetic differences among individuals or groups of individuals, the single-strand conformation polymorphism technique is often used. This technique provides a rapid and sensitive way to identify known/unknown mutations in an amplified DNA fragment. Double-stranded DNA is denatured, and the single stranded products are separated by gel electrophoresis (PAGE) under precisely controlled non-denaturing conditions (Orita et al., 1989; Sunnucks et al., 2000).

In the present study, we report the genotyping of the genetic variant *C* at the goat *CSN2* locus. The *C* variant differs from *A* by a single amino acid substitution Ala₁₇₇ → Val₁₇₇ of the mature protein. At DNA level the protein polymorphism is caused by a nucleotide substitution C→T, as can be observed by alignment of the sequence GenBank accession number AF409096 (Wang et al., 2001; direct submission) with AH001195 sequence (Roberts et al., 1992).

We describe a method based on the PCR followed by LightCycler (Roche Applied Science, Indianapolis, IN) analysis for its detection in 2 Czech dairy goat breeds

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Table 1. Oligonucleotide sequence [GenBank accession no. M90560 (exon and intron 7)]

Name	Oligonucleotide sequence	M90560
CSN2 F	5' - ctttccaacgcgtcatgtt - 3'	450–470
CSN2 R	5' - acaaaaaatcaataatgaatgggtc - 3'	715–690
Anchor CSN2	5' - ccctttctcagcccaagttctgcctgt – FL - 3'	489–517
Sensor C Red 640	5' - cccagaaagCagtgccc ¹ - 3'	519–537

¹Point mutation is in bold c → t nucleotide substitution.

(White Shorthaired and Brown Shorthaired), which represent the genetic resources. We also determined the allelic and genotype frequencies performed on basic population data to characterize this small population.

MATERIALS AND METHODS

Samples

Blood samples were collected from 125 White (**WSH**) and 105 Brown (**BSH**) Shorthaired goats randomly chosen from the national flock. Genomic DNA was extracted from blood using ABI PRISM 6100 Analysis (Nucleic Acid Prep. Station, Applied Biosystem Co., Foster City, CA) by standard protocol.

PCR

A 266-bp fragment containing exon 7 of the goat *CSN2* gene was amplified by PCR, using primers designed on the basis of the caprine sequence GenBank accession no. M90560. Polymerase chain reaction was performed in a 15-μL reaction mixture consisting of 2 μL genomic DNA (10 to 100 ng), 0.3 U of Taq DNA polymerase, 30 μM of each dNTP (Top Bio Ltd. Co., Prague, Czech Republic), 0.2 μM of each primer (TIB-MOLBIOL, Berlin, Germany), 2.5 mM MgCl₂ and 1 × PCR buffer (Top Bio Ltd. Co.). Primers were BCN F: 5' - CTTTCTCCAACCGTCATGTTT - 3' and BCN R: 5' - GAACCATTCATTATTGATTTTTTTTGT - 3' (Table 1).

Thermal cycling conditions were an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 45 s, 56°C for 1 min, and 73°C for 75 s (Thermoblock: 050-801-T Gradient 96, Biometra, Goettingen, Germany).

LightCycler Analysis

LightCycler analysis is based on fluorescence resonance energy transfer. The hybridization probe system consists of 2 fluorescently labeled oligonucleotides. The first hybridization probe, labeled with fluorescein isothiocyanate as the donor fluorophore on its 3' end, can hybridize in close proximity to a second hybridization probe labeled with the acceptor fluorophore LightCy-

cler Red 640 (LCRed640) at its 5' end. The fluorescein is stimulated by a light-emitting diode source and transfers energy to the acceptor fluorophore. The acceptor fluorophore then emits light of a long wavelength that can be measured with a photodiode because the intensity of the fluorescence resonance energy transfer signal depends on the amount of the specific PCR product generated. This detection strategy allows monitoring of the amplification process on a per cycle basis. More important, genotyping using 2 hybridization probes is possible with a shorter detection probe that spans the polymorphic site and a longer anchor probe that causes the detection probe to melt off the template at a lower temperature. Polymorphic alleles can thus be distinguished by the melting temperature of the detection probe. Continuous monitoring of the fluorescence as the temperature is raised from annealing to denaturation demonstrates a sharp decrease in fluorescence when the detection probe dissociates from the template (Lay and Wittwer, 1997; Caplin et al., 1999; Loeffler et al., 2000; Hoffman et al., 2007).

Sequence of the used probes is presented in Table 1. The hybridization probes were designed by TIB-MOLBIOL. The mutation probe was designed to bind to a region including the investigated mutation, whereas the anchor probes were designed to bind on one nucleotide away from the mutation probe. Furthermore, the design of the mutation probe was such that its melting temperature was approximately 5 to 10°C lower than that of the anchor probe.

The PCR product (10 μL) was re-pipetted to a glass capillary, and 1.6 μM of each probe (Table 1) was added. The capillaries were closed, centrifuged at room temperature (1 min/1,100 rotation speed), and placed into the LightCycler for analysis. The anchor and sensor sequences were anchor: 5' - TCCCTTTCTCAGC-CCAAAGTTCTGCCTGT - 3' and sensor: 5' - CCCCA-GAAAGCAGTGCCCC - 3' (Table 1).

The LightCycler was programmed to conduct a melting cycle to determine the melting temperature of the amplicon and probe. This consisted of a denaturing step at 95°C for 3 min, followed by cooling down to 40°C for 2 min, after which the temperature was slowly increased again at a rate of 0.1°C s⁻¹ to reach a final value of 95°C. Each assay run included a refer-

Table 2. Number of individuals (n) and genotype and allele frequencies at the *CSN2* locus in White (WSH) and Brown (BSH) Shorthaired goats

<i>CSN2</i> ¹	WSH		BSH	
	n	Frequency	n	Frequency
	125		105	
Genotype				
A ¹ A ¹	16	0.128	20	0.190
A ¹ C	47	0.376	50	0.476
CC	62	0.496	35	0.334
Allele				
A		0.316		0.430
C		0.684		0.570

¹A¹ represents the non-C alleles (A, A1, E, O, and O').

ence sample for each of the 3 genotypes and a negative control containing water instead of sample DNA. All samples returned unequivocal genotyping results with genotypes AA, AC, and CC in both goat breeds.

PowerMarker data analysis software (Liu and Muse, 2005) was used to estimate allele and genotype frequencies and polymorphic information content and to verify the Hardy-Weinberg equilibrium and population differentiation test in WSH and BSH goat breeds.

RESULTS AND DISCUSSION

Six samples with giving 3 different patterns by LightCycler analysis (assumed AA, AC, and CC) were sequenced on a genetic analyzer (ABI PRISM 3130, Applied Biosystems) to confirm the results regarding differences in single nucleotide substitution between genetic variants A (EMBL M90560) and C (AY563136; data not shown). These variants differ by nucleotide substitution C→T, and amino acid exchange Ala (GCA) → Val (GTA) at position 177. At this position, the A1, E, O, and O' alleles are all identical and carry a cytosine. These alleles were therefore considered as a group and referred to as the A allele. In the WSH goats, the most frequent genotype found was CC (0.496), followed by the heterozygous genotype AC (0.376), whereas the homozygous genotype AA (0.128) had a low frequency. Similar results were obtained in BSH goats, where the heterozygous genotype AC (0.476) had the greatest frequency compared with the homozygous genotypes CC (0.334) and AA (0.190) (Table 2). The frequencies were similar to those reported in Italian goat populations (Chessa et al., 2005; Caroli et al., 2006). The WSH and BSH populations were in Hardy-Weinberg equilibrium at the *CSN2* locus. Polymorphic information content (0.339, WSH and 0.369, BSH) showed a low frequency in both goat populations. The *CSN2* C→T genotyping of the reference DNA samples, and all additional DNA samples, led to distinct melting temperatures at 55.6

and 64.3°C for the C and T alleles, respectively (Figure 1). For a correct genotyping, all the animals carrying at least a cytosine should be further analyzed for the A1, D, E, O, and O' variants.

Our study revealed that melting curve analysis via LightCycler could be reliable to detect mutations and single nucleotide polymorphisms (SNP). The goal of SNP studies is to detect different allelic variants of a given SNP, or the identification of polymorphic positions at which previously unknown SNP occur, without determining the allele. Melting curve raw data are generally represented by plotting fluorescence over temperature, with the data resolution and temperature range being higher with high resolution dyes than with labeled probes. To better illustrate results, the negative first derivatives (-dF/dT) are often used, revealing melting temperatures at peaks (Figure 1). Data obtained with homozygous wild types, homozygous mutants, and heterozygous samples can differ regarding peak number, peak position, or a combination of both.

CONCLUSIONS

With LightCycler analysis, processing of samples is simple and rapid compared with other techniques used so far, providing prompt results and the possibility of high-throughput genotyping. The advantage of LightCycler analysis is based on speed, efficiency, and safety (absence of hazardous chemicals). The LightCycler-based assay can be a valuable tool for routine typing of animals independent of single nucleotide polymorphism, sex, age, stadium of lactation, number of animals, and origin, as well as for the rapid screening of clinical isolates in numerous acquired and hereditary diseases in microbiology and clinical chemistry. The assay allows the use of different specific oligonucleotides in one reaction mixture, and amplification/postamplification analysis is performed in closed glass capillaries, thus minimizing the risk of carryover contamination. At present, work is in progress to analyze further polymorphisms within *CSN2* locus to allow the simultaneous detection of all the known genetic variants of the gene and exploit all the potential of the described technique.

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Figure 1. Melting peak for the *A/C* polymorphism in the goat *CSN2* casein gene. *A*¹ represents the non-*C* alleles (*A*, *A*¹, *E*, *0*, and *0*⁺).

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