Screening for K-Casein (*CSN3*) Gene Variation in Carpathian Goat Breed by Isoelectric focusing (IEF) and DNA Sequencing

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Abstract

In goats, k-casein (*CSN3)* locus is highly polymorphic with up to 16 allele currently characterized. They produce 13 protein variants (CSN3) that were classified in two groups (A^{IEF} and B^{IEF}), according to their isoelectric point. Isoelectric focusing (IEF) of milk samples allows the detection of these two CSN3 groups, but for correct identification of *CSN3* alleles DNA based genotyping methods are needed. Therefore the objective of this study was to identify the types of alleles occurring at the *CSN3* locus in Carpathian goat breed by using a combined IEF and DNA sequencing approach. IEF analysis of milk samples collected from two Carpathian goat populations reared in Romania revealed two distinct CSN3 patterns. Amplification and sequencing of *CSN3* cDNA obtained from these goats revealed four polymorphic sites located in the exon 4 that are responsible for amino acids substitutions, as compared with the reference sequence of A allele. By comparative analysis of IEF and cDNA sequencing data obtained from the two populations, we shown that A^{IEF} alleles are represented by B allele, while B^{IEF} alleles are represented by D allele. However, the variation of *CSN3* locus in Carpathian goat breed could be more complex, therefore further studies are needed to characterize it.

Keywords: alleles, Carpathian goat, genetic polymorphism, k-casein

1. Introduction

In goat milk, casein fraction is organized in micelle that contains k-casein (CSN3) and three calcium sensitive caseins: α_{S1}-casein (CSN1S1), β-casein (CSN2) and α_{S2}-casein (CSN1S2). The CSN3 molecule has a hydrophilic part located at the exterior of casein micelle that plays a crucial role in their formation and stabilization [1, 2]. In cattle and goats the k-casein (*CSN3*) gene was mapped on chromosome 6 [3, 4] in the vicinity of other three genes encoding for: α_{S1}-casein (*CSN1S1*), β-casein (*CSN2*) and α_{S2}-casein (*CSN1S2*) that cluster in a 250-kb region [2- 5]. In goats a large genetic variation was described at *CSN3* locus, with up to 16 alleles currently characterized, which produce 13 protein variants that are characterized by point mutations (SNP) causing amino acid substitutions in the peptide chain [6, 7]. However by isoelectric focusing (IEF) of milk samples just two patterns for the 13 CSN3 variants can be distinguished, which were classified in two groups according to their isoelectric point *i.e.* the first group A^{IEF} contains 12 variants (A, B, B′, B″, C, C′, F, G, H, I, J and L), with an IP=5.29 and the second group B^{IEF} contains 4 variants (D, E, K and M), with an IP = 5.66 [7].

The genetic variation at the goat *CSN1S1* locus was convincingly associated in various goat

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breeds with milk quality, rheological properties or cheese yield [8-12].

In goats just few studies were focused on the relationships between the polymorphism of *CSN3* locus and milk traits. It was suggested that CSN3 B^{IEF} variant could be associated with higher casein percentage in milk as compared with A^{IEF} variant [13]. Furthermore, in Orobica goat breed B^{IEF} variant was associated with higher protein and casein percentage [14]. Therefore correct identification of *CSN3* genetic variation in a particular goat breed is an essential step to conceive breeding strategies aiming to improve milk quality.

However, CSN3 IEF phenotypes could be represented by a pool of alleles belonging to the two groups (A^{IEF} and B^{IEF}). Therefore the objective of this study was to identify the alleles occurring at the *CSN3* locus in Carpathian goat breed by using a combined IEF and DNA sequencing approach.

2. Materials and methods

Biological samples (milk and blood) were collected from 84 Carpathian goats from Transylvanian region. Peripheral blood samples (2ml/goat) were collected from jugular veins into tubes containing K_3-EDTA as an anticoagulant, while milk samples (15ml/goat) were collected in sterile tubes by hand milking and stored at -20 ºC.

After defrosting, milk samples were centrifuged at 2000 g for 15 min at 4 ºC. The skimmed milk was transferred into fresh tubes and the resulting cell pellets were saved for whole RNA extraction. Denatured milk samples (diluted in 8 M urea and 3% dithiothreitol) were analyzed by IEF as described before [15] *i.e.* in 4 % ultrathin (0.5 mm) polyacrylamide gels that contained a mixture of three ampholytes: pH=2.5-5, pH=4.2-4.9, pH=5.0-7.0 and 8 M urea (GE Healthcare, Sweden). Electrophoresis was conducted in a Multiphor II Electrophoresis System and subsequently IEF gels were stained with PhastGel Blue R (GE Healthcare, Sweden).

The total RNA was purified from milk somatic cell pellets (10 samples exhibiting different CSN3 IEF patterns), which were saved in previous step, using 1 ml of PureZOL reagent (Bio-Rad, USA) and following manufacturer instructions. The DNA was extracted from 100 µl of whole blood with the Quick-gDNA MiniPrep kit (Zymo Research Corporation, USA) and according to the manufacturer instructions. The DNA and RNA concentrations and purities were determined on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

To synthesize the first cDNA strand the iScript™cDNA Synthesis Kit (Bio-Rad, USA) was used. The reverse transcription reactions were set up at 20 µl final volume that contained the reaction mix and 500 ng of total RNA. The cycling conditions were: 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min.

The amplification of the coding region of the goat *CSN3* cDNA was achieved using specifically designed primers (ChKCZ - F: 5`- GCA ATG ATG AAG AGT TTT TT -3`, ChKCZ - R: 5`- TCC TTA GAG TTT TTA GAC CT -3). The primers were designed with the Primer3 v.0.4.0 software [16], based on the goat *CSN3* mRNA sequence of A allele (GenBank Acc.No. X60763).

Polymerase chain reactions were performed in 25 µl reactions that contained 1X Tissue Green PCR Master Mix (Fermentas, Lithuania), 10 pmol of each primer and 1 µl of each reverse transcription reactions. The amplification consisted of: 1 cycle at 94 ºC for 3 min followed by 35 cycles at 94 ºC for 1 min, 58 ºC for 1 min, 72 ºC for 1 min and a final extension step at 72 ºC for 7 min. The resulting cDNA amplicons were analyzed on a 3 % agarose gel in 1X TBE buffer and 1X SybrSafe (Invitrogen, USA).

Both strands were sequenced with the same PCR primers and using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing products were analyzed on an Applied Biosystems 3730 device (Applied Biosystems, USA).

Nucleotide sequences were analyzed with the BioEdit tool [17] and translated into amino-acid sequences using the ExPASy translate tool [18]. The CLUSTALW2 analysis software [19] was used to align DNA or protein sequences.

3. Results and discussion

Phenotyping of Carpathian goat milk samples by IEF analysis allowed use to discriminate two CSN3 patterns, which are in agreement with the electrophoretic behaviour the two CSN3 groups: A^{IEF} (IP=5.29) and B^{IEF} (IP = 5.66) (Figure 1).

The CSN3 A^{IEF} variants can be distinguished as a band (Figure 1, white arrow), which is located between the 3rd and 4th band of CSN2 A/C variants (Figure 1, white stars).
The CSN3 B^{IEF} variants (Figure 1, white arrow) are much more difficult to distinguish due to their electrophoretic behaviour, which is very similar to CSN1S2 C variant (Figure 1, white square). However, in CSN1S2 AA samples the CSN3 B^{IEF} variants are distinguishable due to the more cathodic behaviour of CSN1S2 A variant (Figure 1, black square).

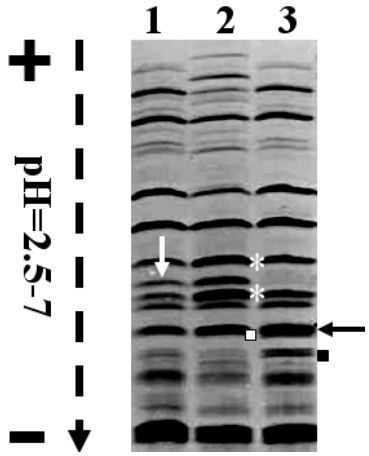

Figure 1. IEF patterns of some Carpathian goat milk samples separated in a pH range of 2.5-7. CSN3 A^{IEF} - white arrow; CSN3 B^{IEF} - black arrow; CSN2 - white stars; CSN1S2 C - white square: CSN1S2 A - black square.

Based on these data the frequencies of the CSN3 IEF variants were calculated: 0.84 for A^{IEF} and 0.16 for B^{IEF}. The B^{IEF} variant was detected with a lower frequency in the two analysed Carpathian goat populations as compared with other goat breeds [14, 20]. They were found in combinations associated with three phenotypes: $A^{IEF}A^{IEF}$ (0.77), $A^{IEF}B^{IEF}$ (0.13) and $B^{IEF}B^{IEF}$ (0.10).
As long as these CSN3 IEF phenotypes could be represented by a pool of alleles belonging to the two groups we further analysed the goat cDNA sequences derived from sequencing of the entire *CSN3* coding region from chosen IEF polymorphic samples.
To assign the nucleotide sequences determined in the course of this research to a certain *CSN3* allele we compared these data with the reference sequence of A allele (GenBank Acc. No. X60763) [21]. Furthermore to denote the *CSN3* alleles found in Carpathian goat, the proposed nomenclature was used [7]. The comparison of our sequencing data derived from the analysed samples revealed two types of *CSN3* sequences that are characterized by three polymorphic sites located in exon 4 causing three amino acid substitutions in the peptide chain: a 247A>G (CAA to CGA), responsible for a p.Gln44Arg, a 309G>A (GTT to ATT), responsible for a p.Val65Ile and a 591T>C (TCT to CCT), responsible for a p.Ser159Pro (Figure 2).

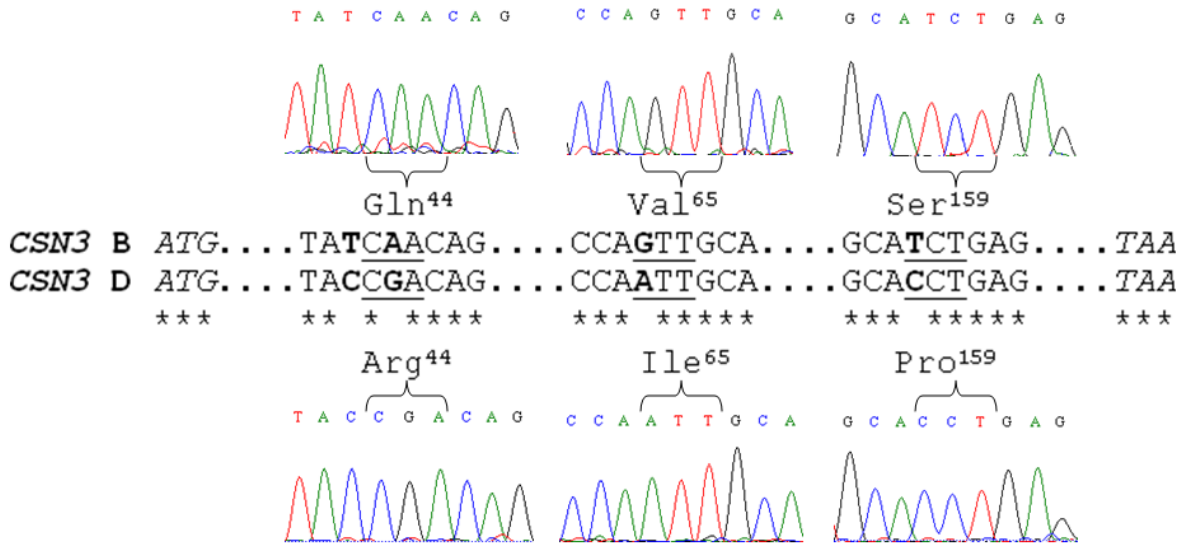

Figure 2. Comparison of partial genomic cDNA sequences of *CSN3* B (A^{IEF}) and D (B^{IEF}) alleles highlighting the main distinctive features. The codons involved in amino acid substitutions are underlined and the substituted nucleotides are marked in capital bold letters. Start and stop codons are marked in capital italic letters.

In fact the p.Gln44Arg substitution is responsible for the IP differences between the CSN3 A^{IEF} and B^{IEF} variants [7, 14]. Both types of nucleotide sequences differ from reference sequence of A allele (GenBank Acc. No. X60763) by a 471G>A mutation (GTC to ATC), which is responsible for a p.Val119Ile substitution in the peptide chain (Table 1).

Table 1. Alleles identified at *CSN3* locus in the analyzed Carpathian goat populations

Nucleotide position variation at *CSN3* locus compared with X60763															*CSN3* alleles
170	245	247	274	284	290	298	309	384	385	471	509	550	583	591	
C	T	A	A	G	C	A	G	G	A	G	A	T	C	T	A (A^{IEF})[1]
-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	B (A^{IEF})[2]
-	C	G	-	-	-	-	A	-	-	A	-	-	-	C	D (B^{IEF})[3]

[1]The reference sequence of A allele GenBank Acc. No. X60763 [21]
[2,3]Alleles identified in present study

Based on the available IEF and sequencing data we concluded that in all analysed samples A^{IEF} profiles (Figure 1, white arrow) contain a single protein variant that is B, while B^{IEF} profiles (Figure 1, black arrow) contain a single protein variant that is D (Table 1).
Further cDNA sequences comparison revealed other polymorphic sites that contain silent mutations, which confirmed the presence of both mentioned *CSN3* alleles (B and D) in the analyzed Carpathian goat population (Table 1).

4. Conclusions

We describe in this paper the identification and characterization at the DNA level of the *CSN3* alleles occurring in two Carpathian goat populations reared in Romania. By comparative analysis of IEF and cDNA sequencing data obtained from the two populations, we shown that A^{IEF} group is represented by B allele, while B^{IEF} group is represented by D allele. To our knowledge this is the first report of *CSN3* locus variation at the DNA level in this breed. However, the variation of *CSN3* locus in Carpathian goat breed could be more complex, therefore further studies are needed to characterize it. Due to positive associations between certain *CSN3* alleles and milk quality in some goat breeds (that is still poorly understood), the knowledge of the entire genetic variation at the *CSN3* locus in Carpathian goat breed is an essential step to conceive breeding strategies aiming to improve this trait.

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References

1. Alexander, L.J., Stewart, A.F., Mackinlay, A.G., Kapelinskaya, T.V., Tkach, T.M., Gorodetsky, S.I., Isolation and characterization of the bovine kappa-casein gene, Journal of Dairy Science, 1988, 178, 395-401.
2. Martin, P., Szymanowska, M., Zwierzchowski, L., Leroux, C., The impact of genetic polymorphisms on the protein composition of ruminant milks. Reproduction Nutrition Development, 2002, 42, 433-459.
3. Threadgill, D.W., Womack, J.E., Genomic analysis of the major bovine casein genes, Nucleic Acids Research, 1990, 18, 6935-6942.
4. Hayes, H., Petit, E., Bouniol, C., Popescu, P., Localisation of the α-S2-casein gene (CASAS2) to the homoeologous cattle, sheep, and goat chromosomes 4 by in situ hybridisation, Cytogenetics and Cell Genetics, 1993, 64, 281-285.
5. Ferretti, L., Leone, P., Sgaramella, V., Long range restriction analysis of the bovine casein genes. Nucleic Acids Research, 1990, 18, 6829-6833.
6. Jann, O.C., Prinzenberg, E.M., Luikart, G., Caroli, A., Erhardt, G., High polymorphism in the k-casein (CSN3) gene from wild and domesticated caprine species revealed by DNA sequencing, Journal of Dairy Research, 2004, 71, 188-195.
7. Prinzenberg, E.M., Gutscher, K., Chessa, S., Caroli, A., Erhardt, G., Caprine κ-casein (CSN3) polymorphism: New developments of the molecular knowledge, Journal of Dairy Science, 2005, 88, 1490-1498.

8. Delacroix-Buchet, A., Degas, C., Lamberet, G., Vassal L., Influence des variants AA et FF de la caséine αs1 caprine sur le rendement fromager et les caractéristiques sensorielles des fromages, Lait, 1996, 76, 217-241.
9. Caravaca, F., Carrizosa, J., Urrutia, B., Baena, F., Jordana, J., Amills, M., Badaoui, B., Sanchez, A., Angiolillo, A., Serradilla, J.M., Short communication: Effect of alphaS1-casein (CSN1S1) and kappa-casein (CSN3) genotypes on milk composition in Murciano-Granadina goats, Journal of Dairy Science, 2009, 92, 2960-2964.
10. Devold, T.G., Nordbø, R., Langsrud, T., Svenning, C., Brovold, M.J., Sørensen, E.S., Christensen, B., Adnøy, T., Vegarud, G.E., Extreme frequencies of the αs1-casein 'null' variant in milk from Norwegian dairy goats-implications for milk composition, micellar size and renneting properties, Dairy Science and Technology, 2010, 91, 39-45.
11. Yue, X.P., Zhang, X.M., Wang, W., Ma, R.N., Deng, C.J., Lan, X.Y., Chen, H., Li, F., Xu, X.R., Ma, Y., Lei, C.Z., The CSN1S1 N and F alleles identified by PCR-SSCP and their associations with milk yield and composition in Chinese dairy goats, Molecular Biology Reports, 2011, 38: 2821-2825.
12. Balteanu, V.A., Serradilla, J. M., Vlaic, A., Carsai, T.C., Amills, M., Miresan, V., Effect of αS1-casein (CSN1S1) genotypes on milk composition and cheese yield in Carpathian goat breed, Proc. XI International Conference on Goats, Spain, 2012, pp. 216.
13. Chianese, L., Portolano, B., Troncone, E., Pizzolongo, F., Ferranti, P., Addeo, F., Alicata, M.L., Pilla, F., Calcagna, G., The quality of Girgentana goat milk, Proc. 7th Int. Conf. on Goats, France, 2000, pp. 946-949
14. Chiatti, F., Chessa, S., Bolla, P., Cigalino, G., Caroli, A., Pagnacco, G., Effect of κ-casein polymorphism on milk composition in the Orobica goat. Journal of Dairy Science, 2007, 90, 1962-1966.
15. Balteanu, V.A., Carsai, T.C., Vlaic, A., Identification of an intronic regulatory mutation at the buffalo α_{S1}-casein gene that triggers the skipping of exon 6, Molecular Biology Reports, 2013, 40, 4311-4316.
16. Rozen, S., Skaletsky, H., Primer3 on the WWW for general users and for biologist programmers, Methods Molecular Biology, 2000, 132, 365-386.
17. Home page address: http://www.mbio.ncsu.edu/bioedit/bioedit.html
18. Home page address: http://www.expasy.org/
19. Home page address: www.ebi.ac.uk/tools/msa/clustalw2/
20. Caroli, A., Chiatti, F., Chessa, S., Rignanese, D., Bolla P., Pagnacco, G., Focusing on the goat casein gene complex. Journal of Dairy Science, 2006, 89, 3178-3187.
21. Coll, A., Folch, J.M., Sanchez. A., Nucleotide sequence of the goat kappa-casein cDNA, Journal of Animal Science, 1993, 71, 2833.