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# RT-PCR–based identification of genetic variants of alpha S1 casein in milking goats

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**Abstract.** Genetic variants of the αS1 casein gene in goats determine the different casein and protein content of milk, variant milk processing quality and cheese flavour. The main seven alleles of αS1 casein belong to four groups: strong alleles, which code for a high αS1 content (3.6 $gL^{-1}$), medium alleles (1.6 $gL^{-1}$), weak alleles (0.6 $gL^{-1}$) and allele 0 (complete lack of αS1 casein). An original method was designed, based on PCR with three specific primers and mRNA isolated from milk somatic cells. The length of PCR products varied due to differences between alleles on cDNA level, since allele *F* has a 111 nt deletion of 9-11 exons, allele *D* is associated with deletion of 36 nt (exon 9), and allele *E* includes a 457 nt retropozon insertion in exon 19. Forty-nine goats coming from four different farms were genotyped. Each of the analysed goats is the carrier of at least one 'strong' allele, which codes for the beneficial high level of αS1 casein.

**Key words**: αS1 casein, cDNA, goats, RT-PCR.

## Introduction

Alpha S1 casein gene polymorphism in goats has a major effect on several milk features crucial for processing, e.g. casein and overall protein content, fat content (BARBIERI et al. 1995), curd strength and cheese flavour (REMEUF 1993, VASSAL et al. 1994, DELACROIX-BUCHET et al. 1996).

There are seven main αS1 casein alleles, divided into four groups according to their expression level. Strong alleles (variants *A*, *B*, *C*) determine a high αS1 content (3.6 g per litre of milk). Weak alleles (*D*, *F*) are associated with 0.6 g of casein per litre. The medium allele (*E*) codes for 1.6 $gL^{-1}$. Allele *0* is associated with lack

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of αS1 casein. Thus the difference in αS1 casein content between strong and weak homozygous goats may reach 3 $gL^{-1}$.

Strong genetic variants of caprine αS1 casein have a beneficial effect on improvement of milk processing features, and since correlation coefficient between the αS1 casein genotype and casein fraction content r = 0.68 (regression coefficient b = 0.64) (KRZYŻEWSKI et al. 1995), identification of the αS1 casein genotype may serve as a selection marker for milk type goats. Two main methods of molecular identification of αS1 casein alleles have been used for genotyping. One is quantitative milk protein electrophoresis (GROSCLAUDE et al. 1987) used for milking goats. The other one is RFLP (restriction fragment length polymorphism), performed on DNA isolated from blood leukocytes, which requires using of at least three restriction endonucleases, but still it is impossible to distinguish one of the weak alleles – *D* (LEROUX et al. 1990). The last method is usually used for buck genotyping or early selection of females.

Importance of the effect of αS1 on milk processing features and necessity of including αS1 casein as a main parameter in selection programmes for milking goats have been frequently reported (KRZYŻEWSKI et al. 1995, MANFREDI et al. 1998, STRZAŁKOWSKA et al. 1998).

The aim of this study was to develop a simple and reliable method of identification of the polymorphism in the αS1 casein gene locus on cDNA (complementary DNA) level in milking goats, which would allow to distinguish the group carrying strong alleles from the groups carrying medium or weak alleles.

## Material and methods

### Material

Mammary gland tissue had been used as a mRNA source at the beginning of the study, but since the biopsy is a highly invasive method and requires anaesthetic usage, somatic cells of milk were finally used as a mRNA source. The method was tested on thirteen goats from the Institute of Genetics and Animal Breeding Polish Academy of Sciences in Jastrzębiec, whose αS1 casein genotypes had been defined earlier, using milk protein electrophoresis (GROSCLAUDE et al. 1987). Subsequently, 36 goats from private farms in Lower Silesia were also genotyped.

### Methods

#### Isolation of mRNA

Approximately 50 mL of milk were collected from each goat. Total RNA was isolated from somatic cells using the Gibco procedure based on TRIzol Reagent (Gibco BRL), which is a modification of a method described by CHOMCZYŃSKI and SACCHI (1987).

**Procedure**. Samples were pelleted by centrifugation. Cells were lysed with TRIzol by repetitive pipetting and incubated for 5 min. at room temperature. Then 0.2 mL of chloroform per each 1 mL of TRIzol was added. After 15 s of shaking the samples were incubated at room temperature for 3 min. and then centrifuged for 15 min. at 3°C (max. 12000 × g). Upper-water phase was moved into a fresh tube, and 0.5 mL of isopropylalcohol was added per each 1 mL of TRIzol Reagent used. The samples were incubated for 10 min. and centrifuged for 10 min. at 5°C (max. 12000 × g). Supernatant was removed, the pellet was washed with 75% ethanol and centrifuged at 5°C for 5 min. (max. 7500 × g). RNA pellet was vacuum-dried for 10 min. Next, RNA was dissolved in deionised water including 2% DEPC and incubated for 5 min. at 60°C.

**Reverse transcription**

After checking the presence of total RNA using agarose electrophoresis, RT-PCR was performed using a RT-PCR Stratagene kit on a PCR 100 cycler (MJ Research).

**Primers**

The sequence of αS1 casein mRNA, to which specific primers were designed, was provided by the GenBank (http://www.ncbi.nlm.nih.gov/).
The designed specific primers:

F1: 5'-gac aac cat gaa act tct cat cc-3',
R1: 5'-gga tta ggg atg tca gag aat ga-3',
F3: 5'-tct tac atg cgt tcc caa aca tg-3',
R3: 5'-ctt aca gga gag gtg att caa ag-3',

each of 23 nucleotides, were synthesised in BIONOVO® (Legnica). The melting temperature (2 × (A+T) + 4 × (G+C)) for all primers was identical (66°C). Two pairs of primers (F1/R3, F3/R3) were used to identify the genetic variant of αS1 casein while another pair (F1/R1) was used to verify the performed genotyping, considering alleles *D* and *F*. Since the weak variant *D* has a 33 bp deletion (lack of exon 9) and the weak variant *F* has a deletion of 111 bp (exons 9-11) (BRIGNON et al. 1990), amplification with primers F1/R3, flanking the αS1 casein gene, gives a PCR product of 1002 bp for strong alleles, 890 bp for *F* and 968 bp for *D*. Allele *E* is associated with a nearly threefold reduction in its transcript amount, which is connected with the existence of a 457 nt retroposon insertion of the LINE (long interpersed nucleotide elements) type in the non-coding exon 19. The insertion causes secondary structure modifications and is supposed to lead to a loss of mRNA stability in the cytoplasm (PÉREZ et at.1994). Thus, to ensure the presence or absence of allele *E*, another pair of primers (F3/R3) was used. Allelospecific primer F3 is complementary exclusively to the LINE insertion of allele *E*, and the existence of F3/R3 PCR products (515 bp) attests the presence of allele *E* in the sample.

Allele *0* has a 8 kb deletion at the 3'end (at genomic DNA level) (MARTIN, ADDEO 1995) and it seems probable that at cDNA level it has an approx. 4 kb deletion at the 3'end. All the designed and used primers were positively tested using program BLAST 2.0 to ensure that they are complementary exclusively to the αS1 casein sequence.

**Amplification**

All PCR reactions were performed in the following conditions: 94°C/120s, 30 × (94°C/60s, 68°C/30s, 72°C/45s), 72°C/420s (cycler PTC 100, MJ Research).

**Agarose electrophoresis**

PCR products were analysed by electrophoresis in 1.5% agarose gels (3/4 Sea Kem® Le Agarose,1/4 NuSieva® GTG® Agarose) in 0.5% TBE; 180 V for 45 minutes. Visualisation was carried out using the GelDoc 2000 system (BioRad).

## Results and discussion

In this study two groups of animals were analysed. The first one, including 13 milking goats, originated from the Institute of Genetics and Animal Breeding Polish Academy of Sciences in Jastrzębiec. For this group identification of genotypes was earlier performed using a biochemical method.

**Amplification F1/R3**. The results of F1/R3 PCR for 12 goats are shown in Figure 1.

Out of 12 goats, all gave a 1002 bp product and 3 (lane 7, 8, 9) gave an additional 890 bp band, characteristic for allele *F*. PCR reaction with 40 cycles (Figure 2) showed that goat 272, which gave a single 1002 bp product in 30 PCR cycles, this time had two bands, 1002 and 890 bp (Figure 2, lane 2). Therefore goat 272 was recognized as a heterozygote *S/F*. Goat 303, not shown in Figure 1, was recognized as a heterozygote *S/F* (Figure 2, lane 3).

Summing up, goats 1-4, 6 and 10-12 (Figure 1) were recognized as strong homozygotes and goats in lanes 5 and 7-9 as heterozygotes *S/F*.

**Amplification F1/R1**. To ensure that the detected 111 bp deletion in PCR product length is associated with the existence of allele *F* in the recognized heterozygous genotypes, another PCR was performed under identical thermal conditions but with primers F1/R1, which flank the 5' part of the αS1 casein gene (exons 1 to 17). The results are shown in Figure 3.

**Amplification F3/R3**. Due to the reported threefold lower amount of E transcript and its instability (PÉREZ et at.1994), a pair of primers F3/R3 was used to detect allele *E* in the analysed samples. Results of amplifications with primers F3 (complementary exclusively to the *E*-associated 457 bp insertion) and R3 are presented in Figure 4.

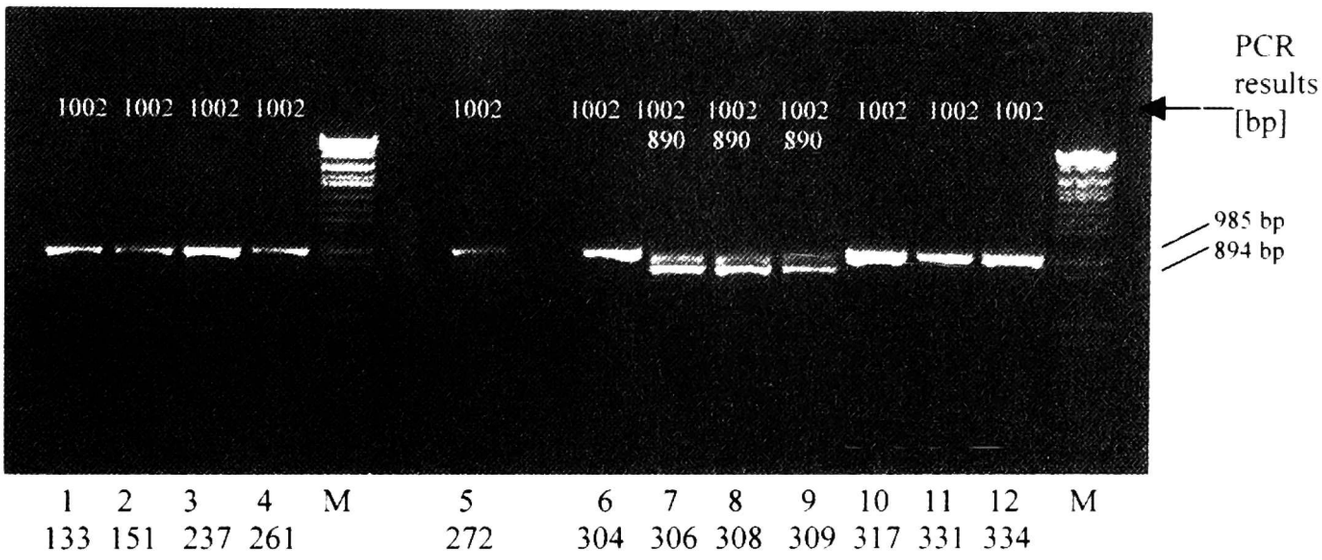



Figure 1. Agarose electrophoresis of F1/R3 PCR (30 cycles) products. Numbers under the lanes are numbers of individual goats. All samples gave a 1002 bp band, characteristic for strong alleles. Goats in lanes 7-9 have an additional 890 bp band, associated with deletion of three exons (9-11) in allele *F*.

Lambda DNA/Eco471 (AvaII) Marker 13 (MBI Fermentas) was used as a size marker in all analyses.

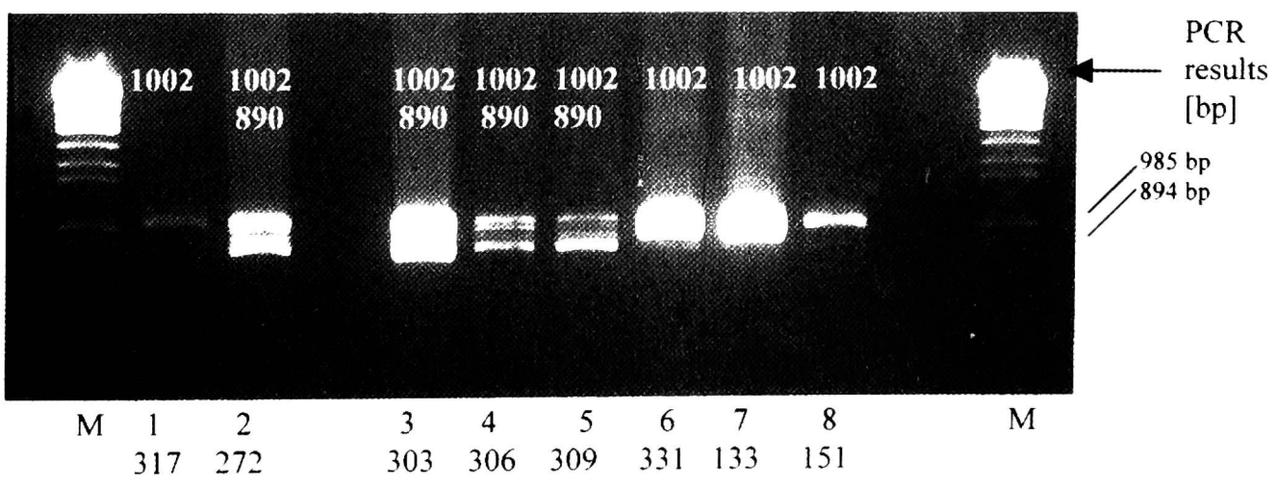
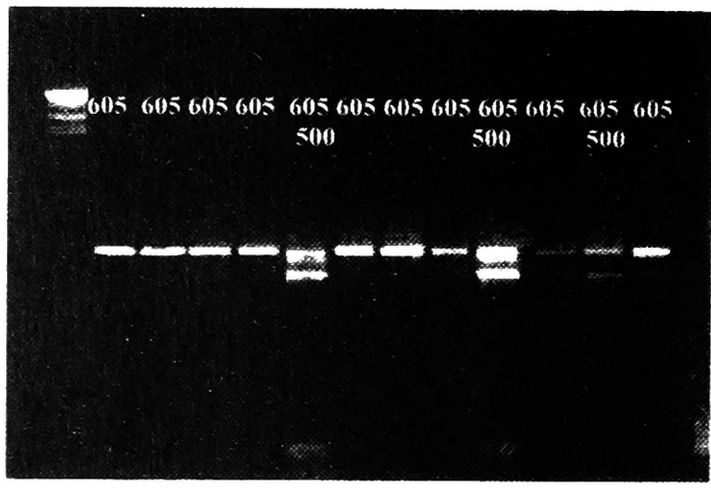


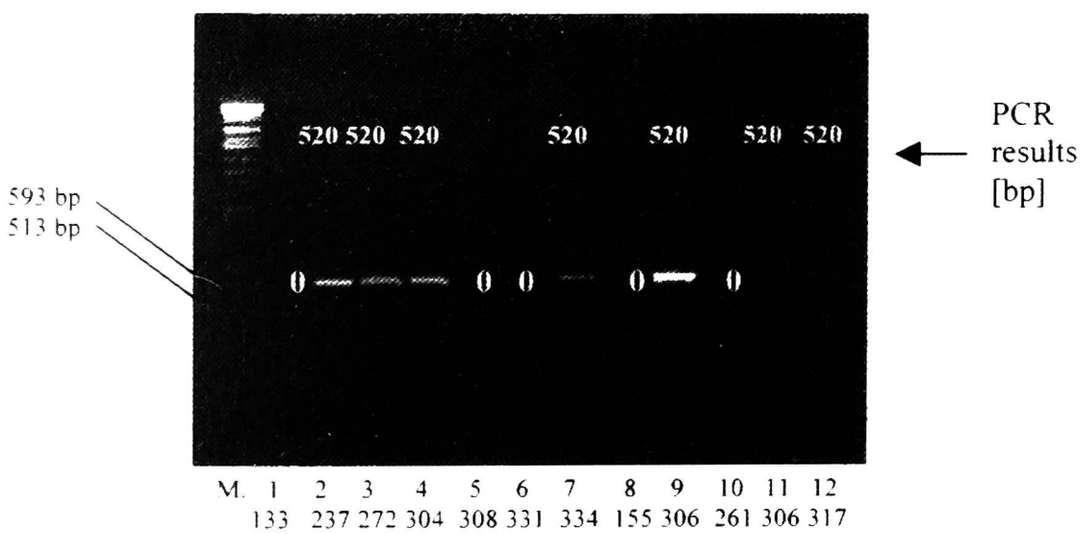

Figure 2. Agarose electrophoresis of F1/R3 PCR (40 cycles) products. Numbers under the lanes are numbers of individual goats. Bands 1002 and 890 bp, which appeared in goat 272, are in lane 2.




M 1 2 3 4 5 6 7 8 9 10 11 12

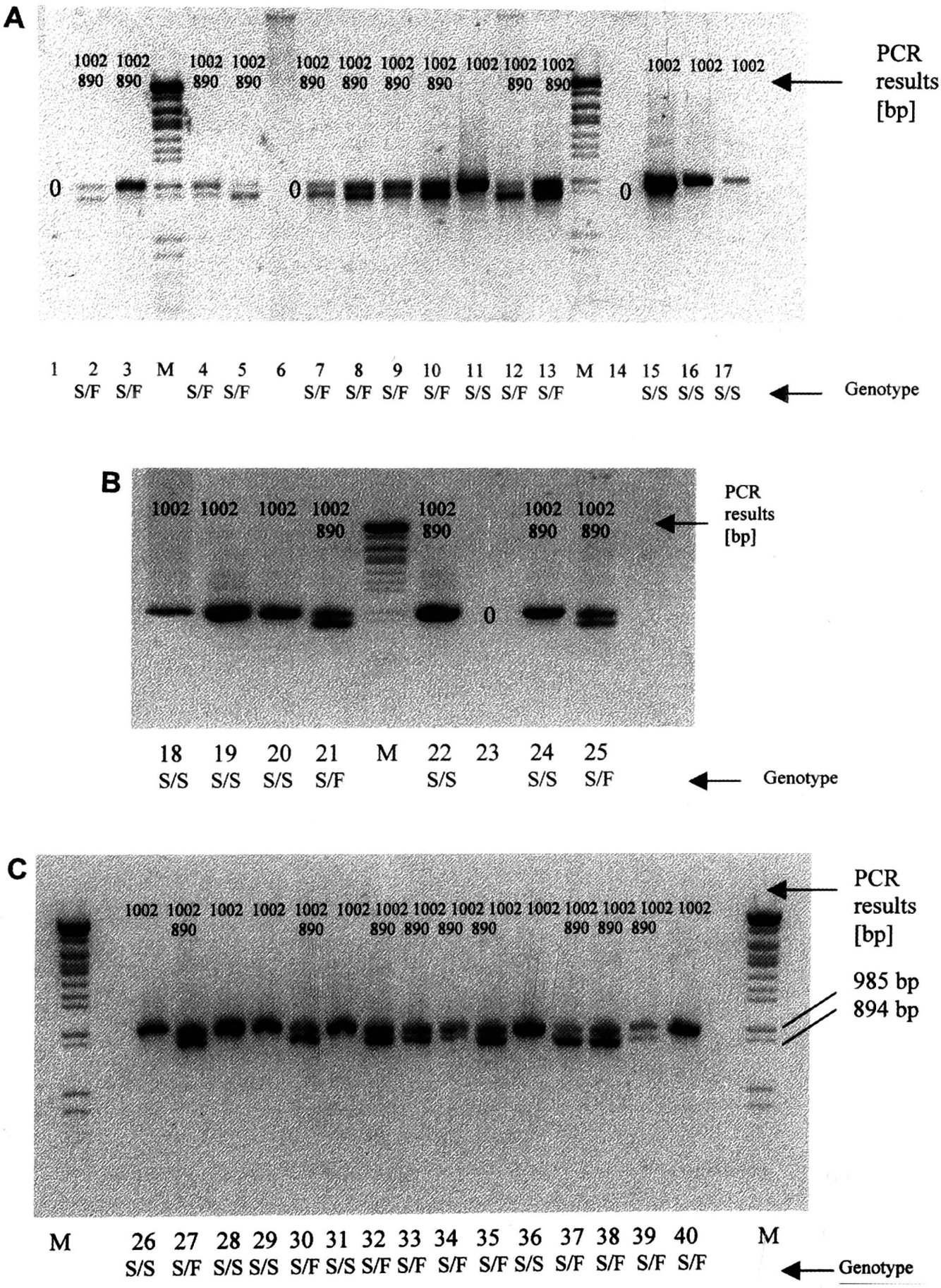
133 272 308 334 306 309
237 304 331 151 261 317

Figure 3. Agarose electrophoresis of F1/R1 PCR products. Numbers under the lanes are numbers of individual goats. All samples gave a 605 bp band, expected for strong alleles. Goats in lanes 5 and 9 have an additionale 500 bp product, expected for allele *F*, associated with deletion of 111 nucleotides.




M. 1 2 3 4 5 6 7 8 9 10 11 12
133 237 272 304 308 331 334 155 306 261 306 317

Figure 4. The recognized genotypes of goats from the Institude of Genetics and Animal Breeding Polish Academy of Sciences in Jastrzębiec, defined using amplification with specific primer pairs (F1/R3, F3/R3) and electrophoretical division analysis.




Figures 5A, B, C. Results of PCR amplifications with primers F1/R3. Under the diagrams are numbers of lanes and proposed genotypes. M is the symbor for a size marker (Lambda DNA/Eco 471 (Avall) Marker 13, MBI Fermentas).

Although seven out of 13 goats (lanes 2, 3, 4, 7, 9, 11, 12) had not shown the presence of allele *E* after PCR with primers F1/R3 and therefore were initially defined as strong homozygotes they gave evident F3/R3 bands and thus were recognized as heterozygotes *S/E*.

Three (!) alleles have been repetitively found in goat 306 and 272 genotypes. The schedule of the project has not allowed to start working out these unusual cases. The reason seems to be alternative splicing or certain transcription disorders, but it still needs to be explained. The list of goats and the verified genotypes is shown in Table 1.

**Table 1**. List of goats and verified genotypes of goats from the Institute of Genetics and Animal Breeding Polish Academy of Sciences in Jastrzębiec

| Genotype, defined using milk protein quantity electrophoresis and goat number | PCR product size (primers F1/R3) | Presence and size of PCR products (primers F3/R3) including E insertion sequence | Genotype verified after PCR analyses |
|---|---|---|---|
| (*AA*) 133 | approx. 1002 bp | lack | *S/S* |
| (*AE*) 151 | approx. 1002 bp | lack | *S/S* |
| (*AA*) 237 | approx. 1002 bp | approx. 520 bp | *S/E* |
| (*AE*) 261 | approx. 1002 bp | approx. 520 bp | *S/E* |
| (*EE*) 272 | approx. 1002 + 890 bp | approx. 520 bp | ? |
| (*EE*) 303 | approx. 1002 + 890 bp | lack | *S/F* |
| (*BB*) 304 | approx. 1002 bp | approx. 520 bp | *S/E* |
| (*BE*) 306 | approx. 1002 + 890 bp | approx. 520 bp | ? |
| (*OO*) 308 | approx. 1002 + 890 bp | lack | *S/F* |
| (*BE*) 309 | approx. 1002 + 890 bp | lack | *S/F* |
| (*AE*) 317 | approx. 1002 bp | lack | *S/S* |
| (*BB*) 331 | approx. 1002 bp | approx. 520 bp | *S/E* |
| (*BB*) 334 | approx. 1002 bp | approx. 520 bp | *S/E* |

In this study, only two of the 13 studied goats from the Institute of Genetics and Animal Breeding, Polish Academy of Sciences in Jastrzębiec confirmed previous genotyping, performed using the protein quantity electrophoresis method. Genotypes of two goats, number 272 and 306, were not identified, due to the above-mentioned presence of three genetic variants of αS1 casein cDNA, which disabled reliable genotyping. In none of the 13 analysed goats, variants *D*, *G* or *0* were detected.

Genetic variants of the αS1 casein gene of 36 goats from three private farms from Lower Silesia were identified. The results are shown in Figures 5A, B, C.

Results of PCR reaction with primers F1/R3 and an analysis of electrophoresis products allow to conclude that genotypes of all the examined goats contain at

**Table 2**. Preliminary identification of genotypes of goats from private farms

| Owner | Gel lane | F1/R3 PCR product sizes | Genotype |
|---|---|---|---|
| Józef Burczak | 2 | 1002 + 890 bp | *S/F* |
| (Wojtkowice) | 3 | 1002 + 890 bp | *S/F* |
| | 4 | 1002 + 890 bp | *S/F* |
| | 5 | 1002 + 890 bp | *S/F* |
| | 7 | 1002 + 890 bp | *S/F* |
| | 8 | 1002 + 890 bp | *S/F* |
| | 9 | 1002 + 890 bp | *S/F* |
| | 10 | 1002 + 890 bp | *S/F* |
| | 11 | 1002 bp | *S/S* |
| | 12 | 1002 + 890 bp | *S/F* |
| | 13 | 1002 + 890 bp | *S/F* |
| | 15 | 1002 bp | *S/S* |
| | 26 | 1002 bp | *S/S* |
| | 27 | 1002 + 890 bp | *S/F* |
| | 28 | 1002 bp | *S/S* |
| | 29 | 1002 bp | *S/S* |
| | 30 | 1002 + 890 bp | *S/F* |
| | 31 | 1002 bp | *S/S* |
| | 32 | 1002 + 890 bp | *S/F* |
| | 33 | 1002 + 890 bp | *S/F* |
| | 34 | 1002 + 890 bp | *S/F* |
| | 35 | 1002 + 890 bp | *S/F* |
| | 36 | 1002 bp | *S/S* |
| | 37 | 1002 + 890 bp | *S/F* |
| | 38 | 1002 + 890 bp | *S/F* |
| | 39 | 1002 + 890 bp | *S/F* |
| | 40 | 1002 bp | *S/S* |
| Maria Stekiel | 16 | 1002 bp | S/*S* |
| (Nowa Wieś | 17 | 1002 bp | *S/S* |
| Goszczańska) | 18 | 1002 bp | *S/S* |
| | 19 | 1002 bp | *S/S* |
| | 20 | 1002 bp | *S/S* |
| | 21 | 1002 + 890 bp | *S/F* |
| | 22 | 1002 bp | *S/S* |
| Małgorzata Bujak | 24 | 1002 bp | *S/S* |
| (Jelcz-Laskowice) | 25 | 1002 + 890 bp | *S/F* |

least one strong allele in the αS1 casein locus. Due to the described difficulties with detection of the weak *E* allele, homozygous *S/S* individuals should be additionally analysed by F3/R3 PCR. General information about the analysed goats and the proposed genotypes is contained in Figure 6. Out of the 36 genotyped goats coming from private farms, 15 were initially diagnosed as strong homozygotes S/S and 21 as heterozygotes S/F.

The developed and presented here original method of identification and differentiation between strong, medium and weak alleles of caprine αS1casein has a comparatively simple procedure, is reliable and repeatable. The method of quantitative electrophoresis of milk proteins (GROSCLAUDE et al. 1987), traditionally used for female genotyping, is based on visual estimation of the amount of αS1casein, which thus may be easily overestimated or underestimated (RYNIEWICZ, personal communication). An a additional doubtless valour of the presented method is using milk as a source of αS1casein gene mRNA, instead of invasive collecting of mammary tissue samples. Another method of molecular identification of αS1casein variants, used especially for buck genotyping, relies on DNA-RLFP (LEROUX et al. 1992), but cannot distinguish the weak variant *D*.

A specific problem with mRNA isolation was observed. During two years of analyses, it turned out to be hardly possible to obtain RT-PCR products out of total RNA isolated from milk samples collected in summer and autumn (June – December). Milk samples collected in winter and spring (January – May) regularly gave relatively stable mRNA (cDNA).

The results obtained confirm difficulties with identification of allele *E*, which were already reported by PÉREZ et al. (1994). Detection of allele *E* using primers common also to other alleles appears very difficult and requires designing of at least one *E*-allelospecific primer.

## Conclusions

An original method of detection of genetic variants of caprine αS1 casein on the mRNA level has been worked out. The method is based on originally designed primers, which allow to distinguish the group of strong alleles (*A*, *B*, *C*) from medium (*E*) and weak alleles (*D*, *F*).

The source of αS1 casein mRNA were somatic cells of milk. Collection of milk samples is simple and non-invasive in contrast to mammary gland tissue biopsy, used heretofore as an exclusive source of αS1 casein mRNA for analyses. No reports describing other casein mRNA sources are available.

The number of analysed goats is not sufficient to estimate the frequency of different αS1 casein alleles and genotypes in the goat population, but each of the analysed animals is the carrier of at least one strong allele.

Application of the presented method associated with appropriate selection tools may lead to a fast and significant increase in the frequency of strong, beneficial for milk processing, alleles of αS1 casein in the goat population.

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