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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

A/C polymorphism in the β-4 defensin gene and its association with phenotypic and breeding values of milk production traits in Polish-Friesian cows*

Emilia Bagnicka, Nina Strzałkowska, Tomasz Szreder, Beata Prusak, Artur Jóźwik, Ewa Kościuczuk, Józef Krzyżewski, Lech Zwierzchowski**

Polish Academy of Sciences Institute of Genetics and Animal Breeding
Jastrzębiec, 05-552 Wólka Kosowska, Poland.

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Two novel single nucleotide polymorphisms (SNPs) were found in the bovine β4-defensin gene intron – A/C transversion at position 1674 and C/T transition at position 1877 – both recognized with *Bsr*I endonuclease. Observed frequencies of the A1674C *AA*, *AC*, *CC* genotypes were 0.65, 0.32 and 0.03, respectively, while those of alleles *A* and *C* – 0.81 and 0.19. For the C1877T polymorphism in the Polish-Friesian (PF) population studied only the *CC* homozygotes (frequency 0.94) and *CT* heterozygotes (frequency 0.06) were found. Associations were evaluated between the A1687C polymorphism and milk production traits of cows. The data set comprised 8814 records of daily milk, fat, protein and ECM yields, fat, protein, lactose and dry matter contents, and somatic cell count (SCC) of milk of 352 cows. Moreover, 897 records were used of milk fat and milk protein yield and content achiewed in the whole and the standard 305-day lactation, as well as estimates of breeding values of these traits in 352 animals from official recording of milk performance (National Breeding Programme). In the whole lactation the β4-defensin gene A1674C polymorphism was found significantly related to protein yield while in the standard lactation to fat and protein contents. Moreover, the polymorphism was found related to the breeding value for protein yield, and for fat and protein content.

KEY WORDS: breeding value / β4-defensin / cattle / gene polymorphism / lactation / milk production traits

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**Corresponding author: e.bagnicka@ighz.pl

Searching for polymorphisms in genes which are expected to determine production or functional traits of livestock is the subject of numerous studies. In particular, single nucleotide polymorphisms (SNPs) are very useful genetic markers for the development of genetic tests for such traits. They are stable through many generations and can provide direct assessment of individual animal's genetic merit if they are in linkage disequilibrium with genetic variation in productive or functional traits [Stone *et al.* 2005]. The marker-assisted selection (MAS) is particularly important for traits with low accuracy, for conventional selection, for traits with low heritability, traits appearing late in life (e.g. disease resistance) or for after-slaughter recording [Meuwissen 2003].

The cluster of defensin genes was found in the bovine chromosome 27 [O'Brien *et al.* 1993], where also QTLs affecting body conformation and dairy traits are located [Ashwell *et al.* 1998]. Therefore, the defensin genes could be attractive candidates for genetic markers of the udder health and milk production traits in cattle, an assumption that was confirmed in our earlier study [Bagnicka *et al.* 2007]. Taking both aspects into consideration we decided to continue our search for novel polymorphisms in the bovine β4-defensin gene and to look for their associations with milk production traits in dairy cows.

In this paper two novel polymorphisms in the bovine β4-defensin gene – the A/C transversion at position 1674 and the C/T transition at position 1877 (according to GenBank AF008307) – are reported, both identified with *Bsr*I endonuclease. A relationship was found between the A1674C polymorphism and milk yield and composition (fat, protein, lactose, energy content), somatic cell count of milk and breeding values of these traits. The relations were analysed for the whole and the standard 305-days lactation.

Material and methods

Animals

The study was carried out on 352 dairy Polish-Friesian (PF) Black-and-White cows, maintained in the loose barn at the Institute of Genetics and Animal Breeding, Jastrzębiec. According to INRA feeding standards the animals were fed the complete total mixed ration (TMR) of corn silage, wilted grass silage and concentrates supplemented with minerals and vitamins, had free access to water and were machine-milked twice a day. The mean annual yield of the herd amounted to 8945 kg milk/cow, with 4.13% fat and 3.43% protein. Individual 10-ml blood samples were withdrawn from the jugular vein to test tubes containing K_2EDTA (1.6 mg/ml blood). All procedures involving animals were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and were approved by the Local Ethics Commission (Permission No. 17/2005).

Detection of β4-defensin gene polymorphism

The isolation of DNA from whole blood was conducted with the method of Kanai *et al.* [1994]. The following pair of PCR-primers was designed using Primer3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), that matched the bovine β4-defensin gene between nucleotide 1569 and 2016 (according to GenBank AF008307):

forward: 5'-GAGGATGCGGAGACTGAGAC-3', and

reverse 5'-GGACGAAATGATG-CATCTGAA-3'.

PCR-amplified was the 446 bp-long DNA fragment, encompassing a part of β4-defensin gene intron. The reaction was performed as follows: initial denaturation at 94°C for 10 min, 34 cycles of denaturation (94°C, 60 s), annealing (55.0°C, 60 s), elongation (72°C, 40 s), final extending (72°C, 10 min); in a TETRAD thermocycler (MJ Research, Inc., Waltham, MA, USA).

DNA sequencing

To detect a nucleotide sequence polymorphism in the bovine β4-defensin gene sequenced were the DNA samples derived from six PF cows. The PCR products, after purification with the GenElute PCR DNA Purification Kit (SIGMA), were sequenced in an ABJ377 sequencer (APPLIED BIOSYSTEM, CA, USA). The sequencing was done at the Polish Academy of Sciences Institute of Biochemistry and Biophysics, Warsaw. Sequences were analysed using the CHROMAS programme (http://www. technelysium. com.au/chromas.html).

Restriction fragment length polymorphism

The 446-bp PCR products were digested for 3 hours with 5 U of *Bsr*I endonuclease (NEW ENGLAND BIOLABS). The restriction DNA fragments were analysed electrophoretically in 2% agarose gel (SIGMA-ALDRICH, Munich, Germany) with ethidium bromide in TBE buffer. DNA bands were visualised and documented using the Molecular Imager System FX (BIO-RAD, CA, USA). In order to confirm the identity of the analysed fragment the PCR products representing the β4-defensin, *AA*, *AC* and *CC* genotypes were sequenced.

Milk sampling and analyses

Milk samples were taken from each cow once a month on test-days, between years 2000 and 2007, in the course of routine control milking, during the whole lactation. The oldest cows were born in 1998, while the youngest in 2005. The cows were between first and eighth lactation. The milk yield was determined as well as fat, protein, lactose and dry matter content, and somatic cell count (SCC) in milk samples by the independent laboratory within the framework of official recording of milk performance (National Breeding Programme). The energy-corrected milk (ECM) was established according to the formula: ECM (kg/day) = milk (kg/day) × [38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 16.54 × lactose (g/kg) +20.7]/3140 [Sjaunja *et al*. 1990].

The phenotypic data about milk fat and protein yield and fat and protein content from whole and 305-days lactation, and pedigree information as well as estimates of breeding values of these traits were also taken from the Polish national data set.

Statistical

A total of 8814 records on daily milk yield and composition were collected from 352 cows sired by 116 bulls. The pedigree file covered the information about 1564 animals back to generation 3. The information about milk, fat and protein yield and fat and protein content in the standard and the whole lactation covered 897 records from 322 cows. The records from lactations shorter than 270 days were ignored. The longest whole lactation lasted 749 days. Also the data about animals' breeding value for milk production traits were used to detect associations between the β4-defensin gene polymorphism, and milk yield and composition. The 2209 records about the breeding values of investigated traits were available for 352 cows.

For the statistical analysis the data were divided into three groups of parity with group III covering the information about the third and/or later lactations. Two seasons of calving were distinguished. The first comprised the information about cows which calved between October and March and the second one – about those that calved between April and September. On this basis 16 classes of year-season of calving interaction were established.

In order to determine the significance of association between the β4-defensin gene polymorphism and milk production traits the one-trait repeatability test-day animal model with DMU package [Madsen and Jensen 2000] was used. Except for the random animal additive genetic and permanent environmental effects the model included the date of test, year-season of calving, parity and defensin genotype as fixed effects. Effects of stage of lactation were modeled using Legendre polynomials nested within parity [Brotherstone *et al.* 2000]. The differences between estimates of genotype effects were verified with Duncan's test, and the differences between observed and expected frequencies of genotypes – with chi-square test. Somatic cell count (SCC) was transformed to the natural logarithm scale (lnSCC).

In order to determine the significance of association between the β4-defensin gene polymorphism and the breeding value of milk, fat and protein yield and fat and protein content of milk in the standard and whole lactation used was the one-trait repeatability animal model with DMU package [Madsen and Jensen 2000].

Results and discussion

The 446-bp DNA fragment was amplified encompassing a part of the bovine β4-defensin gene intron (defensin genes consist of two exons and one intron). After sequencing the PCR-amplified DNA derived from six non-related cows and the alignment of obtained sequences with each other and with that deposited in the GenBank (acc. no. AF008307), a nucleotide substitution was found (A→C transversion

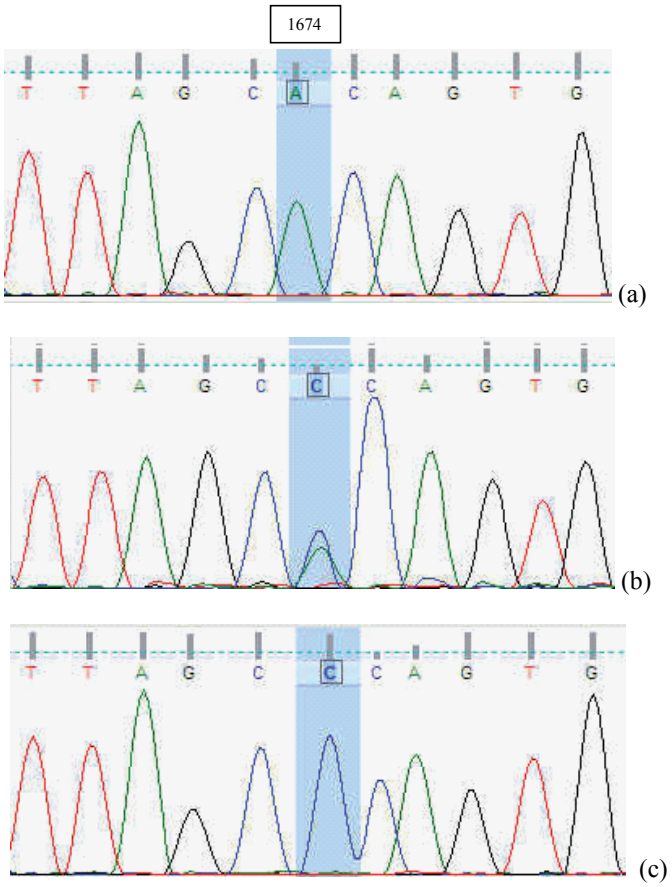

Fig. 1. The sequences of 11-bp fragment of the bovine β4-defensin gene showing the polymorphism found in this study, the A/C transversion at position 1674, according to the GenBank acc. no. AF008307, which creates a new restriction site for *Bsr*I endonuclease; (a) – *AA* homozygote, (b) – *AC* heterozygote, (c) – *CC* homozygote.

at position 1674 – Figure 1), which created a new digestion site for *Bsr*I endonuclease. Digestion of the 446-bp amplicone with *Bsr*I resulted in two restriction fragments (of 342 and 104 bp) for the genotype *CC*, three fragments (of 446, 342, and 104 bp) for genotype *AC*, and uncut amplification product (of 446 bp) for the genotype *AA* (Photo 1). A cohort of 352 PF cows was genotyped with RFLP-*Bsr*I. Observed frequencies of genotypes *AA*, *AC* and *CC* were 0.65, 0.32 and 0.03, respectively, and were not different from the expected. The frequencies of alleles *A* and *C* were 0.81 and 0.19, respectively.

Twenty-two out of 352 DNA samples showed different band patterns after digestion with *Bsr*I endonuclease (Photo 2) indicating the presence of additional

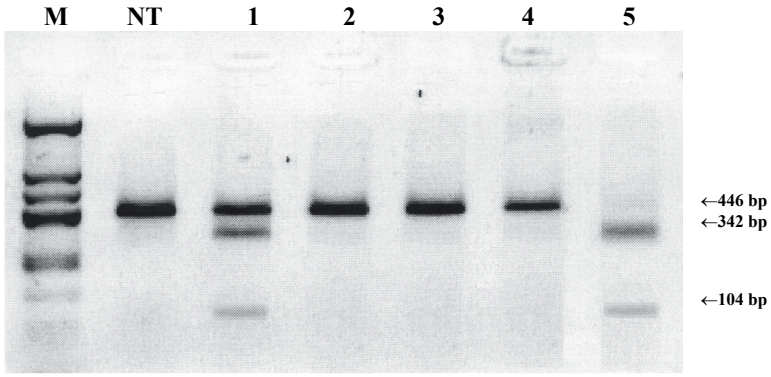

Photo 1. RFLP-*Bsr*I analysis of the A1674C polymorphism in the bovine β4-defensin gene. The 446-bp PCR products were digested with *Bsr*I endonuclease and resolved by electrophoresis in the 2% agarose gels. M – DNA length marker 1444-bp (BTL Łódź); NT – undigested PCR product; β4-defensin genotypes: lane 1 – *AC*; lanes 2, 3, 4 – *AA*, and lane 4 – *CC*. The identity of genotypes was confirmed by DNA sequencing.

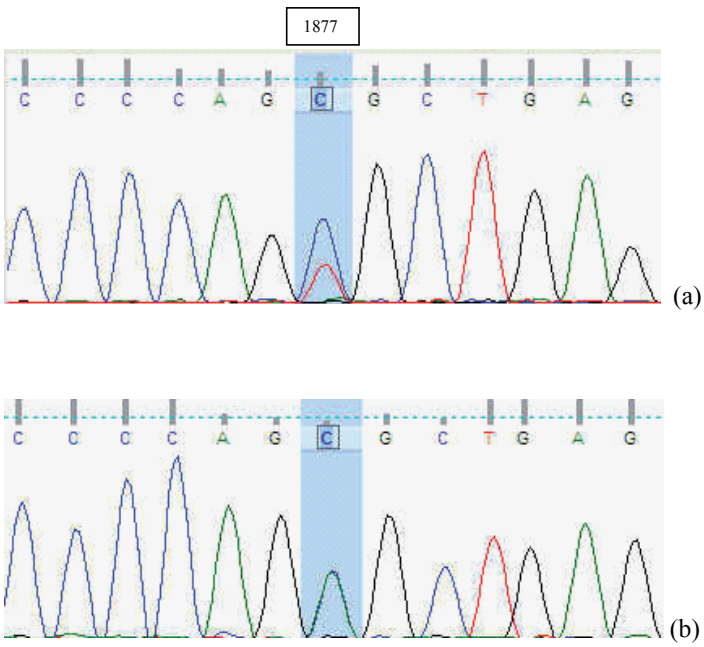

Fig. 2. The sequences of 13-bp fragment of the bovine b4-defensin gene showing another polymorphism found in this study, the C/T transition at position 1877, according to the GenBank acc. no. AF008307, which creates a restriction site for *Bsr*I; (a) – *CT* heterozygote, (b) – *CC* homozygote.

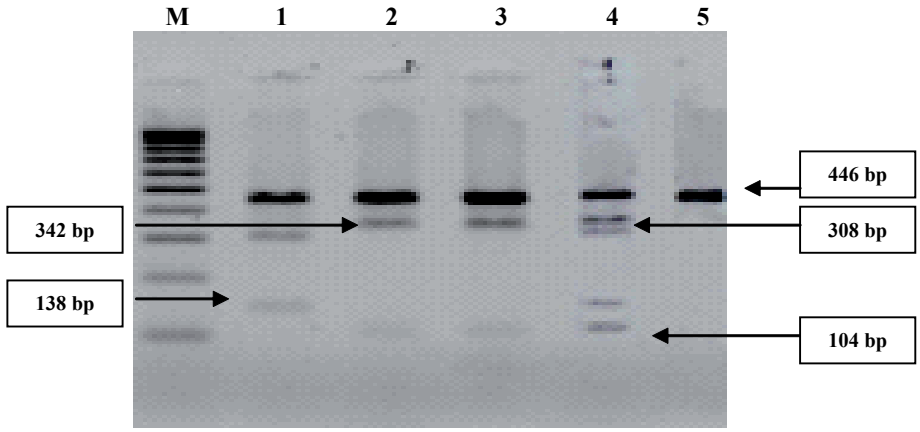

Photo 2. The electrophoresis in the 2% agarose gel with PCR-RFLP analysis of two nucleotide substitutions in the bovine β-defensin gene – the A/C at position 1674 and C/T at position 1877. M – DNA length marker 100-1000-bp (Sigma). The bovine β-defensin genotypes: lane 1 – *AA/CT*; lanes 2, 3 – *AC/CC*; lane 4 – *AC/CT*; lane 5 – *AA/CC* genotypes at 1674/1877 *Bsr*I restriction sites, respectively. The identity of genotypes was confirmed by DNA sequencing.

*Bsr*I digestion site. After sequencing the representative DNAs another nucleotide substitution was found, the C→T transition at position 1877 (according to GenBank; acc. no. AF008307) – Figure 2, Photo 2 – which also created a new *Bsr*I endonuclease digestion site. Due to overlapping SNPs at positions 1674 and 1877, digestion with *Bsr*I resulted in these animals in appearance of the 446, 308 and 138-bp DNA restriction fragments, in addition to those of 342 and 104 bp. For the C1877T polymorphism only *CC* homozygotes and *CT* heterozygotes were found in the population studied. Because of a very low frequency of the *CT* genotype (0.06), animals with the *T* variant of the β4-defensin gene at position 1877 were excluded from further statistical analysis.

The effects of RFLP-*Bsr*I (A1674C) defensin genotypes on production traits in the test-days in whole *vs*. standard lactation as well as on the breeding value of production traits are shown in Tables 1, 2, 3 and 4. The polymorphism was shown to influence significantly the daily milk, fat and protein yield, mean daily fat, protein, lactose, and dry matter contents and ECM daily yield (Tab. 1). The *CC* genotype occurred related to the lowest milk, fat and protein yield, lowest lactose content and lowest ECM, simultaneously being the best one when the fat and protein contents of milk were considered. Because of the highest fat and protein content, the genotype *CC* also presented the highest content of dry matter. The *AC* and *CC* genotypes did not differ significantly. The SCC was not found related to the β4-defensin gene A1674C polymorphism.

As shown in Tables 2 and 3, the relationship between the A1674C polymorphism in the β4-defensin gene and milk, fat and protein yield and fat and protein content differed between the whole and the 305-day lactation. In the whole lactation the *CC*

Table 1. Estimated least squares means (LSM) and their standard errors (SE) in daily tests of investigated traits across the A1674C genotypes (RFLP-*Bsr*I)

Genotype	n		Estimate								
			milk (kg)	fat (kg)	protein (kg)	fat (%)	protein (%)	lactose (%)	SCC (ln)	dry matter (%)	ECM (kg)
AA	5675	LSM	30.52^{A}	1.17^{a}	0.73^{a}	4.43^{A}	3.62^{A}	4.52^{A}	7.54	13.36^{A}	31.70^{A}
		SE	0.90	0.06	0.02	0.11	0.04	0.03	(0.17)	0.14	0.90
AC	2884	LSM	30.43^{A}	1.18^{a}	0.71	4.45^{A}	3.64^{A}	4.52^{A}	7.53	13.40^{A}	31.73^{A}
		SE	0.90	0.06	0.02	0.12	0.05	(0.04)	(0.19)	0.15	0.95
CC	255	LSM	28.26^{B}	1.13^{b}	0.69^{b}	4.61^{B}	3.71^{B}	4.46^{B}	7.55	13.56^{B}	30.10^{B}
		SE	1.58	0.08	0.05	0.18	0.07	0.06	(0.39)	0.24	1.52

$^{ab, AB}$Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

Table 2. Estimated least squares means (LSM) and their standard errors (SE) in the whole lactation across the A1674C genotypes (RFLP-*Bsr*I)

Genotype	n		Estimate					
			milk (kg)	fat (kg)	protein (kg)	fat (%)	protein (%)	ECM (kg)
AA	564	LSM	10152.42	416.57	348.91^{a}	4.36	3.20	12033
		SE	272.92	9.70	6.22	0.08	0.03	425
AC	299	LSM	10064.96	415.79	350.21^{a}	4.38	3.22	12022
		SE	290.88	10.42	7.06	0.09	0.03	439
CC	34	LSM	9713.70	404.17	329.92^{b}	4.41	3.24	11668
		SE	726.99	18.14	14.69	0.15	0.06	557

^{ab}Within columns means bearing different superscripts differ significantly at P≤0.05.

Table 3. Estimated least squares means (LSM) and their standard errors (SE) in the 305-days lactation across the A1674C genotypes (RFLP-*Bsr*I)

Genotype	n		Estimate					
			milk (kg)	fat (kg)	protein (kg)	fat (%)	protein (%)	ECM (kg)
AA	416	LSM	9389.64	373.27	323.69	3.99^{a}	3.38^{A}	10204
		SE	274.43	8.59	9.66	0.08	0.03	301
AC	219	LSM	939.98	375.95	324.56	4.02	3.42^{B}	10175
		SE	287.63	9.00	10.11	0.08	0.03	315
CC	26	LSM	9045.87	365.05	314.40	4.13^{a}	3.40	9949
		SE	458.16	14.92	14.80	0.15	0.06	462

$^{ab, AB}$Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

Table 4. Estimated least squares means (LSM) and their standard errors (SE) for breeding values of investigated traits across the A1674C genotypes (RFLP-*Bsr*I)

Genotype	n		Estimate				
			milk (kg)	fat (kg)	protein (kg)	fat (%)	protein (%)
AA	1427	LSM	76.60	-12.26	-7.95^{A}	-0.100^{a}	-0.043^{A}
		SE	112.41	4.73	3.66	0.03	0.04
AC	728	LSM	77.48	-12.66	-7.89^{A}	-0.106^{a}	-0.036^{B}
		SE	114.79	4.80	3.72	0.04	0.01
CC	54	LSM	38.61	-12.97	-10.46^{B}	-0.071^{b}	-0.052^{C}
		SE	147.75	5.81	4.54	0.06	0.03

$^{ab, AB}$Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

genotype at RFLP-*Bsr*I was significantly associated with lowest protein yield, while in the standard lactation the *CC* animals had a higher mean fat content. Moreover, in the standard lactation the *AA* animals showed the lowest fat and protein contents of milk.

The *AC* genotype cows had the highest breeding value for protein yield and protein content but the lowest for fat content. The opposite results were obtained for the *CC* genotype (Tab. 4). Therefore, differences were found between the phenotypic values of investigated traits and their breeding values.

Defensins are a part of immunological system of humans and animals. Results from investigations conducted over the recent years point to an important defensive role of cell peptides existing in various human and animal organs including the mammary gland epithelium [Exner *et al*. 2000]. Using of antimicrobial peptides, gramicidin S and polymyxin B, which are produced by some bacteria, fungi or actinobacteria, led already to the experience that antimicrobial peptides are the factors in choice to fight against human contagious diseases [Hancock and Chapple 1999]. The polymorphism of β-defensin genes is the subject of intensive studies, almost all of them concerning the human antimicrobial peptides. The best known is the polymorphism of the human

β1-defensin (hBD-1). First hBD-1 polymorphisms, shown by Dork and Stuhrmann in 1998, were four RFLPs in the cDNA sequence, three of them being located in the region encoding 5'-UTR and one in 3'-UTR. The mentioned authors concluded that the SNP variants of the hBD-1 gene might be useful genetic markers in linkage and association studies of respiratory diseases. Other polymorphisms found in the hBD-1 gene were nonsynonymous substitutions in the coding region [Vatta *et al*. 2000, Circo *et al*. 2002, Tesse *et al*. 2008]. These and other polymorphisms in the hBD-1 gene were checked for associations with human diseases.

An information available on polymorphisms of bovine β-defensin genes is limited [Ryniewicz *et al*. 2002, 2003, Wojdak-Maksymiec *et al*. 2006, Bagnicka *et al*. 2007]. All these studies indicated an association between nucleotide sequence polymorphisms in defensin genes and milk production traits and milk SCC.

The novel polymorphism reported here – the A1674C transversion (RFLP-*Bsr*I) located in the intron of the bovine β4-defensin gene – showed no effect on SCC of milk. Thus, it could not be a marker of mammary gland health status. But the *CC* genotype, occurring with the lowest frequency in the studied group of cows, was significantly related to the lowest daily milk, fat, and protein yield, ECM, as well as fat, protein, lactose and dry matter content of milk. However, as to the milk traits in the whole and in the 305-days lactation and their breeding values, the polymorphism identified showed an impact on some traits only (protein yield, fat and protein content). It should be stressed that the breeding values of milk traits were estimated based on data from the National Breeding Programme including the whole dairy cow population in Poland. Therefore, these results are more reliable than those obtained from the phenotypic values in a small animal population, without comparing with other animals.

Recently, SNPs have gained additional importance in animal genetic studies [Vignal *et al*. 2002]. Whole-genome dense SNP marker maps have become particularly important after development of DNA cheap technology in which thousands of SNPs can be applied in projects used for breeding value prediction for genomic selection [de Roos *et al*. 2007]. Therefore, discovering novel SNPs with the proved association with the productive or functional traits is eagerly needed.

Concluding, two novel single nucleotide polymorphisms were found in the bovine β4-defensin gene – the transversion A1674C and transition C1877T (both RFLPs-*Bsr*I). An effect of the A1674C polymorphism on milk production traits was shown in Polish Friesian cows. The polymorphism is located in the β4-defensin gene intron and therefore does not influence the amino acid sequence of the peptide. This suggests that the mutation is not the causative one for the traits under study. The A/C transversion may rather be considered as a genetic marker possibly linked to other polymorphism(s) located closely in the same or in another gene.

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Emilia Bagnicka, Nina Strzałkowska, Tomasz Szreder,
Beata Prusak, Artur Jóźwik, Ewa Kościuczuk,
Józef Krzyżewski, Lech Zwierzchowski

Polimorfizm A/C w genie β-defensyny 4 polskiego bydła fryzyjskiego i związek tego polimorfizmu z cechami produkcyjnymi i ich wartością hodowlaną

S t r e s z c z e n i e

W populacji polskiego czarno-białego bydła fryzyjskiego (pf) liczącej 352 krowy wykryto dwa nowe polimorfizmy w genie β-defensyny 4. Podstawienie nukleotydów A/C stwierdzono w pozycji 1674, a podstawienie C/T w pozycji 1877. Obie mutacje rozpoznawane są przez endonukleazę restrykcyjną *Bsr*I. Dla polimorfizmu A1674C frekwencja genotypów *AA*, *AC* i *CC* wyniosła odpowiednio 0,65, 0,32 i 0,03, natomiast frekwencja alleli *A* i *C* – 0,81 i 0,19. W przypadku polimorfizmu C1877T, w badanej populacji bydła pf wykryto tylko homozygoty *CC* (frekwencja 0.94) i heterozygoty *CT* (frekwencja 0.06). Określono zależności między polimorfizmem A1674C a cechami mleczności krów. Badania przeprowadzono na danych o 8814 udojach dziennych uzyskanych w ramach oficjalnie prowadzonej kontroli użytkowości 352 krów mlecznych rasy pf odmiany czarno-białej. Informacje obejmowały dzienną rzeczywistą, jak również skorygowaną na zawartość energii wydajność mleka, a nadto wydajność tłuszczu i białka oraz zawartość (%) tłuszczu, białka, laktozy i suchej masy oraz liczbę komórek somatycznych w mleku. W analizie statystycznej uwzględniono również wydajność mleka, tłuszczu i białka oraz zawartość tłuszczu i białka w pełnej oraz 305-dniowej laktacji tych krów. Oszacowano także związek wykrytego polimorfizmu z wartością hodowlaną wydajności mleka, tłuszczu i białka oraz zawartości tłuszczu i białka w mleku badanych krów. Wykazano wpływ polimorfizmu A1674C (RFLP-*Bsr*I) na dzienną wydajność mleka, tłuszczu i białka oraz przeciętną zawartość tłuszczu, białka, laktozy i suchej masy. Wykazano związek stwierdzonego polimorfizmu z wydajnością białka w pełnej oraz przeciętną zawartością tłuszczu i białka w 305-dniowej laktacji. Polimorfizm ten wiązał się z wartością hodowlaną wydajności białka oraz zawartości tłuszczu i białka.