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Polymorphism in coding and regulatory sequences of beta-casein gene is associated with milk production traits in Holstein-Friesian cattle*

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Beta-casein gene *(CSN2)* is considered a marker of milk production traits in dairy cattle. Allele A2, the most common in Holstein-Friesian breed, is beneficial for milk protein yield. However, within *CSN2* other mutations occur which could potentially be involved in shaping the variation of milk production traits. In this study a new SNP (C/T) was found located within a regulatory sequence of *CSN2* (position -1578), called enhancer which is thought to play an important role in the amount of the beta-casein mRNA produced. The aim of the study was to verify effects of both SNPs on milk production traits in Polish Holstein-Friesian (HF) dairy cattle. Six-hundred-and-fifty bulls were genotyped with two methods, PCR-ACRS (Amplification-Created Restriction Site, Mph1103 I) and PCR-RFLP (EcoR I), for the A1/A2 polymorphism and the C/T SNP polymorphism (BCE129), respectively. Although both SNPs are located close to each other, their allele frequencies were found significantly different (0.33, 0.67, 0.89 and 0.11 for the A1, A2, C and T, respectively). A mixed linear model was used for testing the association between these polymorphisms and deregressed breeding values for production traits. The analysis revealed that the allele coding the A2 protein variant increases breeding values for milk and milk protein yields while allele C of BCE129 increases the milk fat yield.

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Finding effective genetic markers for economically important traits in farm animals has been a challenge in animal genetics for decades. Bovine chromosome 6 is an object of remarkable interest of many research groups searching for Quantitative Trait Loci (QTL) involved in milk production traits. A meta-analysis approach showed that BTA6 harbours at least 77 QTLs, among which 48 are responsible for milk yield and protein content [Khatkar *et al*. 2003]. Szyda *et al*. [2005] reported a significant QTL on BTA6 affecting the variation in milk production traits (for fat yield the effect of this QTL amounted to 14% of the additive polygenic variance $\hat{\sigma}^2_\alpha$, while 7%$\hat{\sigma}^2_\alpha$ and 11% $\hat{\sigma}^2_\alpha$ for milk and protein yield, respectively). Schopen *et al*. [2009] found that 3.5% of phenotypic variance for protein content of milk may be due to a polymorphism within the beta-casein gene.

Beta-casein is a member of casein cluster and with 13 protein variants known it is the most polymorphic milk protein gene. The variants most common in Holstein-Friesian cattle are A1 and A2, the others (e.g. B, A3, C) being rare [Roginski 2003]. Polymorphism in one of the beta-casein gene codons – CCT $\rightarrow$ CAT – causes a substitution of proline (A2) by histidine (A1, B) in position 67 in the amino-acid sequence. That polymorphism was reported to increase both protein yield and content as well as to decrease milk fat content and yield [Velmala *et al*. 1995, Ikonen *et al*. 1999, 2001, Nilsen *et al*. 2009]. We hypothesize that these somewhat contradictory (in view of a positive genetic correlation between the traits) effects may be caused by two different SNPs located within the beta-casein *locus*. In light of this we undertook an attempt at identifying another SNP within beta-casein 5'-flanking region and comparing its effects to those caused by A1/A2 polymorphism.

Material and methods

The analysis involved 650 Polish Holstein-Friesian (HF) bulls born between 1997 and 2003, chosen randomly from 1520 progeny-tested bulls available in our DNA repository. The only criterion for bulls to be included into the analysis was good accuracy of their estimated breeding values (EBVs) expressed by having at least 100 daughters with milk recording data. Genomic DNA was isolated from the half volume of one commercial semen straw with the use of Wizard Plus Megapreps DNA Purification System (PROMEGA). To identify beta-casein genotype, the PCR-ACRS (Amplification-Created Restriction Site) method was used. Based on the genomic sequence available in GenBank (X14711), two fragments of beta-casein gene were amplified: coding within the exon VII, in which a point mutation was identified (A1/A2) and within the enhancer of the gene (C/T, position -1578), called BCE129 [Myers *et al* 1998]. The following primers were designed by the Primer3 programme (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi):

– CASB forward: 5' GCAGAAT TCTAGTCTATCCCTTCCCTGGACCCATGC 3';
– CASB reverse: 5' ACGGACTGAGGAG GAAACATGACAGTTGGAGGAAG 3';
– BCE129 forward: 5' GGAAGGACATGCTTTC TTTTGAA 3';
– BCE129 reverse: 5'ATATGGCCCAGGA AGTC 3'.

The genomic specificity of the primers was tested using the BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/). In PCR reaction 0.02 µM of primers, 0.8 U of Tfl DNA polymerase, 300 ng of genomic DNA, 50 µM of $MgCl_2$ and 20 µM of dNTPs were used. The amplification programme consisted of an initial DNA denaturation (94°C/3 min), 35 cycles of denaturation (94°C/30 s), annealing (62.5°C/30 s) and elongation (72°C/30 s) and final synthesis (72°C/5 min). To determine the genotype within coding fragment of the beta-casein *loci* restriction enzyme Mph1103 I (Ava III, FERMENTAS) was used.

The point mutation within enhancer of beta-casein gene was detected by a 3-step procedure. First, about 1.5 µl of PCR product was collected for SSCP analysis and mixed with 4.5 µl of denaturation solution (50 mM NaOH, 1 mM EDTA) and 1 µl of loading buffer (30% glicerol, 0.25% bromophenol blue, 0.25% xylene cyanol). Prepared samples were denatured for 14 min at 85°C in a thermoblock, rapidly chilled on ice and then loaded into sample wells of the gel. Electrophoresis was run using precast 15% polyacrylamide gels prepared for separation according to manufacturer's instructions. Electrophoretic separations were carried out with the use of a system for horizontal electrophoresis Multiphor/MultiTemp III at 12°C according to the two following programmes: (1) initial electrophoresis: 200 V, 20 mA, 10 min; and (2) electrophoresis: 375 V, 30 mA, 145 min. Gels were silver-stained. Next, amplicons showing distinct SSCP patterns were sequenced. Finally, point mutations were confirmed by the use of EcoR I (FERMENTAS) restriction enzyme addressed to identify genotypes.

The allelic effect of two considered SNPs on bulls' breeding values was estimated using the following mixed model:

$$y = \mu + Z\alpha + X\beta + \varepsilon,$$

where: y is a vector of deregressed breeding values for milk production trait (milk, fat or protein yield); μ is an overall mean; $\alpha \sim N(0, A\sigma_\alpha^2)$ is a random additive polygenic effect of a bull with A representing a numerator relationship matrix between individuals and σ_α^2 being an additive polygenic variance; β is a vector of fixed additive effects of two considered SNPs; $\varepsilon \sim N(0, D\sigma_\varepsilon^2)$ is a residual effect with D being a diagonal matrix weighted by effective daughter contribution (EDC) for each bull and σ_ε^2 being a residual variance; Z is an incidence matrix for α and X {-1,0,1} (-1 and 1 for two possible homozygous genotypes and 0 for a heterozygous genotype) is a design matrix for effect β.

Parameters of the above model were estimated using dedicated programmes written in R [R Development Core Team 2010] with variance components assumed as known and amounting to $\sigma_\alpha^2 = 0.33 \cdot \sigma_y^2$ and $\sigma_e^2 = 0.67 \cdot \sigma_y^2$ for milk yield and $\sigma_\alpha^2 = 0.29 \cdot \sigma_y^2$ and $\sigma_e^2 = 0.71 \cdot \sigma_y^2$ for fat and protein yields what reflects values estimated for the whole

active population of Polish HF cattle. To evaluate the significance of SNP effects, the Wald test was used as follows:

$$W = \frac{\hat{\beta}}{S(\hat{\beta})} \sim N(0,1)$$

where $S(\hat{\beta})$ – standard error for $\hat{\beta}$.

Bulls' deregressed breeding values and effective daughter contributions corresponded to the national genetic evaluation release from January 2009. Additionally, linkage disequilibrium between two considered SNP markers was estimated using "genetics" package in R [Warnes 2003].

Results and discussion

Photo 1 shows a typical result of CSN2 genotyping. Two, one or three restriction fragments of expected sizes enabled to identify A1A1, A2A2 and A1A2 genotypes, respectively. Among 650 bulls, 79, 294 and 217 with A1A1, A1A2 and A2A2 respective genotypes were determined (Tab.1).

Photo 2, shows a typical result of genotyping at BCE129 *locus*. New polymorphism C/T located at position -1578 was identified with the use of restriction enzyme EcoR I. Two, one or three restriction fragments of expected sizes enabled to identify CC, TT and CT genotypes, respectively. Among 650 bulls, 7, 509 and 134 with TT, CC and CT genotypes, respectively, were determined (Tab. 1).

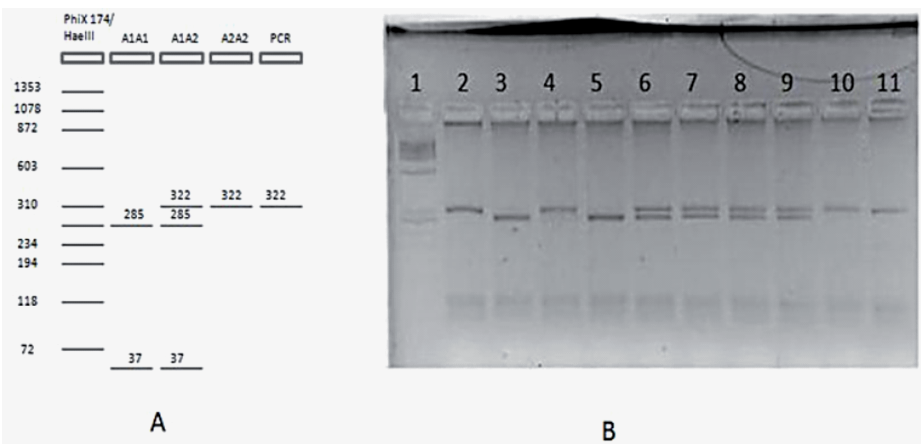

Photo 1. Electrophoregram showing the genotyping of beta-casein A1/A2 *locus*. Theoretical (A) and observed (B) patterns of DNA restriction fragments. Path 1 – DNA size marker ΦX174/HaeIII; paths 2, 4, 10 – homozygote A2A2, paths 3, 5 – homozygote A1A1, paths 6, 7, 8, 9 – heterozygote A1A2, path 11 – PCR product undigested by *Nsi* I. DNA band of 37 bp size diffused out of the gel and is not visible.

Table 1. Frequency of genotypes and alleles within beta-casein exon VII and BCE129 enhancer in a population of 650 Holstein bulls born between 1996 and 2003

Genotype	No of bulls	Genotype frequency	Allele	Allele frequency
Polymorphism in exon VII				
A1A1	79	0.122	A1	0.33
A2A2	294	0.452	A2	0.67
A1A2	277	0.436		
Polymorphism in enhancer (BCE129)				
CC	509	0.783	C	0.89
CT	134	0.206	T	0.11
TT	7	0.011		

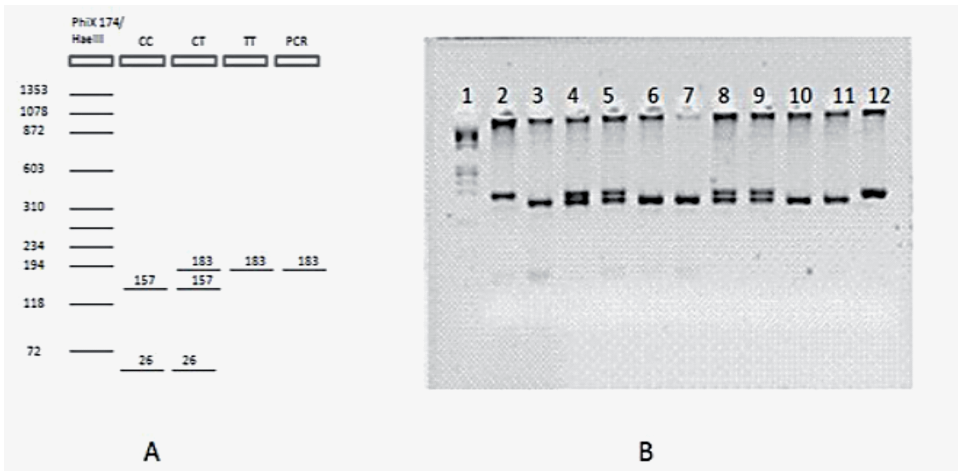

Photo 2. Electrophoregram showing the genotyping of beta-casein BCE129 enhancer. Theoretical (A) and observed (B) patterns of DNA restriction fragments. Path 1 – DNA size marker ΦX174/HaeIII; path 2 – homozygote TT, paths 3, 6, 7, 10, 11 – homozygote CC, paths 4, 5, 8, 9 – heterozygote CT, path 12 – PCR product undigested by Eco RI. DNA band of 26 bp size diffused out of the gel and is not visible.

Table 2. Estimated additive effects of beta-casein exon VII and enhancer BCE129 polymorphisms on bulls' deregressed breeding value for milk production traits

Trait	SNP	Allele	Estimate	SE	WS	P
Milk yield (kg)	CSN2	A2	33.3303	13.7883	2.4172	0.0078
	BCE129	C	-8.6989	22.4432	-0.3875	0.6508
Fat yield (kg)	CSN2	A2	0.3966	0.5090	0.7791	0.4359
	BCE129	C	1.8867	0.8285	2.2773	0.0228
Protein yield (kg)	CSN2	A2	1.9068	0.3502	5.4452	<0.0001
	BCE129	C	0.7586	0.5700	1.3309	0.1832

WS = Wald statistic.
SE = standard error.

Table 2 shows the effects of the examined polymorphism on bulls' breeding values for milk production traits. The A2 allele significantly increased breeding values for milk and protein yield, while allele C, within the enhancer, increased the fat yield. Linkage disequilibrium between the two SNPs amounted to D' = 0.332.

BTA6 is known as containing several QTL with large effects on milk production traits. So far only casein *loci* were indicated as candidate genes responsible for significant portion of trait variation. *CSN2* as a member of casein cluster consisting of 4 genes was indicated as a gene influencing milk performance traits. Polymorphism occurring within exon VII giving rise to A1 and A2 alleles was reported to increase both protein yield and content and simultaneously to decrease the content and yield of milk fat [Velmala *et al*. 1995, Ikonen *et al*. 1999, 2001, Nilsen *et al* 2009]. We hypothesized that these differential effects can be caused by two different SNPs located within beta-casein *locus*. To verify this assumption we screened the 5'flanking region of beta-casein by SSCP followed by sequencing.We found one SNP – the substitution C/T in the position -1578, located within the enhancer – a regulatory sequence thought to be involved in the regulation of gene transcription and the yield of mRNA. For the new point mutation the PCR-RFLP test was developed to genotype 650 AI bulls. Based on deregressed breeding values for milk production traits, the effects of both SNPs located close to each other (separated by 7956 bp) were estimated. Strong linkage disequilibrium between them could have been expected. However, we noted significant difference in genotype and allele frequency between them (Tab. 1); linkage disequilibrium was only moderate, leading to their different effects on milk production traits. Indeed, A2 allele turned to be associated with EBV for both milk and milk protein yield, while allele C of the beta-casein enhancer – with EBV for fat yield. Our present results seem to support those obtained earlier on Finnish Ayrshire by Velmala *et al*. [1995] who identified nine different casein haplotypes, each including 4 polymorphic sites (alpha s1-casein, variants B, C; beta-casein, variants A1, A2; the microsatellite within the kappa-casein gene, variants ms5, ms4 and kappa-casein exon IV, variants A, B, E). In one out of four grandsire families the chromosomal segment characterized by haplotype 3 (B-A2-ms4-A) was associated with decrease in fat content of milk when compared to haplotype 8 (B-A1-ms4-E). The positive effect of A2 variant on both protein and milk yield has also been proved for German Holstein cattle [Freyer *et al*. 1999, Lipkin *et al.* 2008]. Winkelman and Wickham [1997] reported similar effect of A2/A2 genotype on milk yield in Friesian cows. Negative relation of allele A2 on fat content of milk was reported by Ikonen *et al.* [1999] on Finnish Ayrshire cattle. These observations were confirmed later by the same authors [Ikonen *et al*. 2001] who showed that cows with β/κ-casein haplotype A2/A and A2/B yielded more milk and protein, and all haplotypes with A2 allele of beta-casein significantly reduced fat content of milk. Recently, Nilsen *et al*. [2009] analysed casein haplotypes and their associations with milk production traits. One of their conclusions was that A2 allele of beta-casein is a marker of elevated protein and milk yield. The extraordinary effect of allele A2 was evaluated economically by Kearney *et al*. [2005]. Taking into

account the current priorities of dairy cattle selection, which prefer milk protein yield, obtained results indicate that the both polymorphisms located within beta-casein *locus* may be considered as useful in marker-assisted selection.

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