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Association of *Hin*dIII-polymorphism in kappa-casein gene with milk, fat and protein yield in holstein cattle*

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The aim of this paper was to evaluate the effect of genetic polymorphism of kappa-casein on milk production in Holstein cattle. Two hundred and ten Holstein cows were used in this study. We established genotype structure of cattle population and calculated allelic frequencies based on PCR-RFLP analyses. The three genotypes: AA (69.52%), AB (27.62%), and BB (2.86%) were detected. Frequency of allele A was 83.33%, and of allele B 16.67%. The Holstein cattle kept in Slovak Republic exhibit a high value of homozygosity (0.7222) and low values of polymorphism information content (0.2392), effective number of alleles (1.3847) and level of possible variability realization (27.91%). The effect of polymorphism of *CSN3* gene on average breeding values for milk production traits, such as yield of milk, fat and protein expressed in kilograms, as well as percentage content of fat and protein in milk, has been assessed. In our assessment of the observed traits' variability's dependence on *CSN3* gene polymorphism, we detected a statistically significant difference between genotypes only in case of the average breeding value for the percentage of protein in milk.

Key words: Holstein cattle, milk production, *CSN3*, kappa-casein, genetic structure

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Abbreviations: CSN3, kappa-casein; d.f., degrees of freedom; Heobs, experimental heterozygosity; Heexp, theoretical heterozygosity; PIC, polymorphism information content; E, expected homozygosity; ENA, effective number of alleles; V%, level of possible variability realization

INTRODUCTION

Milk and dairy products such as cheese, yoghurt, butter and many others, are a rich source of proteins and minerals and hence constitute an important part of human nutrition. Improvement of milk yield and its composition is a primary goal of animal selection in dairy industry (Caroli *et al.* 2009; Gouda e*t al.*, 2013). The nutritional and technological quality of milk is influenced by breed, dietary factors (Dewhurst *et al.*, 2006; Chilliard *et al.*, 2007; Gálik *et al.*, 2011; Šimko *et al.*, 2014), environment (Vidra *et al.*, 2000; Šťastná & Šťastný, 2016), health (Šťastná & Šťastný, 2015a, 2015b) and genetic background of the cows (Vidra *et al.*, 2001; Stoop *et al.*, 2009; Marchitelli *et al.*, 2013). One way to improve milk, milk fat, and milk protein production is through animal selection based on molecular markers (Riaz *et al.*, 2008). Kumar and coworkers (Kumar *et al.,* 2006) reported that DNA polymorphic markers enable for determination of individual genotypes at many loci and provide information on population parameters such as allelic and genotypic frequencies, and can be used as a tool for improving animal selection. Analysis of the milk protein related polymorphism provides a useful information to both the breeders and the processors of milk. Many research reports have indicated that certain milk protein variants may be associated with milk production, milk composition (Robitaille *et al.,* 2002) and the effectiveness of cheese production (Riaz *et al.*, 2008). Genomic variation in the κ-CN locus has been strongly associated with differences in milk composition, its processing properties and resulting dairy products (Riaz *et al.*, 2008). In cattle, *CSN3* presents two most common alleles A and B. The kappa-casein B allele is related to milk production, and more favourable chemical composition and technological parameters of milk, such as thermal resistance, 10-30% shorter coagulation time, coagulum firmness greater by 20–100%, as well as 5-8% increased efficiency of both fresh and mature cheese production, as compared to allele A (Litwińczuk *et al.*, 2004; Sitkowska *et al.*, 2008; Doosti *et al.*, 2011).

This report aimed to evaluate the effect of genetic polymorphism of kappa-casein on the milk production traits of Holstein cows.

MATERIALS AND METHODS

Animals. A total 210 of Holstein cows were used in this study. Samples of animals' hair came from two farms. Sampling strategy was as follows: in every farm the samples were collected from approximately the same numbers of the top, the average and the worst ranked animals in each herd, based on the Slovak Production Index. Slovak Production Index is the Slovak Selection Index of aggregate genotypes of breeding values for milk, fat and protein yield. Genomic DNA was isolated from the samples of hair roots by using commercial column kit QIAamp® DNA Mini Kit (Qiagen). The concentration and purity of DNA was measured using a spectrophotometer NanoPhotometer™ (Implen GmbH). The DNA samples were stored at –20°C.

Genotyping. For genotyping of A→C *CSN3* gene polymorphism in position 5345 of X14908 sequence (Gen.Bank No.), we used the PCR-RFLP method as described by Schlieben *et al.* (1991).

PCR amplification. The amplification of 443 bp fragment of *CSN3* gene was done using the following specific primers: forward primer 5'-GCTGAGCAGG-TATCCTAGTTAT-3', reverse primer 5'-CTTCTTT-

GATGTCTCCTTAGAG-3'. The PCR reactions were run in a gradient thermocycler C1000 Touch ™ (Biorad). Each reaction mixture had a total volume of 25 µl and contained 50 ng DNA, 1.5 U Taq polymerase (Thermo Scientific), 1X PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 1.5 mM $MgCl_2$, 200 µM dNTP, and 5 pM of each primer. The following amplification parameters were applied: 95°C for 5 minutes followed by 30 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The reaction was completed by the final synthesis: 72°C for 5 minutes.

RFLP analysis. After PCR reaction was finalized, the samples were genotyped using RFLP analysis. The 443 bp PCR products were digested with 5 units of *Hin*dIII restriction enzyme (Thermo Scientific). Restriction digestion fragments were loaded on 2% agarose gel (Invitrogen) containing GelRed™ (Biotium) in 1×SB buffer (Brody & Kern, 2004) at 180 V for 15 minutes. Then the gels were analyzed with the UV rays and a documentary system Olympus C-7070 was used to record the results.

Genetic structure. We determined the genotypic structure of the population studied for *CSN3* gene and estimated the allelic frequencies using molecular genetics analyses. A statistical significance of the differences between experimental and theoretically expected frequencies of genotypes was calculated with the χ^2-test. Effectiveness of allele incidence was evaluated with the following parameters: theoretical heterozygosity (He_{exp}), experimental heterozygosity (He_{obs}), polymorphism information content (PIC), expected homozygosity (E), effective number of alleles (ENA), and level of possible variability realisation (V%).

– Theoretical heterozygosity (He_{exp}) (Nei, 1973)

$$He_{exp}=1-\sum(p^2+q^2)$$

– Polymorphism information content (*PIC*) (Boltstein *et al.,* 1980)

$$PIC=1-\sum\left(p^2+q^2\right)-\left(\sum_{i=1}^{n-1}\sum_{j=i+1}^{n}2p_i^2p_j^2\right)$$

– Expected homozygosity (*E*) (Crow & Kimura, 1970)

$$E=\sum p^2_i$$

– Effective number of alleles (*ENA*) (Crow & Kimura, 1970)

$$ENA=\frac{1}{p^2+q^2}$$

– Level of possible variability realization (*V*%) (Crow & Kimura, 1970)

$$V=\frac{1-E}{1-\frac{1}{N}}\times 100$$

Association studies. For the association study we used five breeding values of the cows (milk, fat and protein yield expressed in kilograms, and milk's fat and protein percentage) as the phenotype values. Breeding values were estimated based on the national Test-Day-Animal model. We analyzed the breeding values already corrected for the other factors (age, number of lactations, season). The results of molecular genetic analysis were used to confirm a relationship between polymorphism of *CSN3* gene and the production traits.

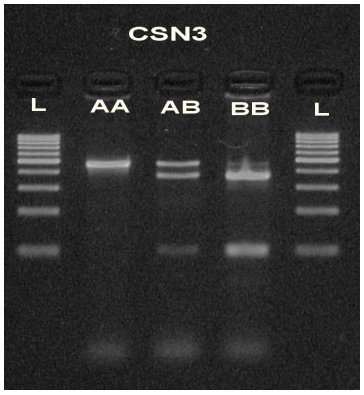

Figure 1. Illustration of CSN3 genotypes on agarose gel.
Genotype AA (443 bp), genotype AB (443 bp, 348 bp, 95 bp), genotype BB (348 bp, 95 bp), L – 100 bp ladder (Thermo Scientific BioScience)

To describe the effect of *CSN3* genotypes on the average breeding values for milk, fat and protein yield expressed in kilograms, as well as fat and protein percentage of milk, a statistical analysis was used. The impact of individual genotypes of *CSN3* gene on the variability of average breeding values was evaluated using a two-way analysis of variance:

$$y=\mu+G_i+S_j+e_{ij}$$

y – breeding value (kg milk, kg protein, kg fat, % protein, % fat); μ – mean value; G_i – fixed effect of genotype, $_i$=1, 2, 3; S_j – fixed effect of herd, $_j$=1, 2; e_{ij} – residual effect.

To test the influence of *CSN3* genotypes on variability of the average breeding values for milk, fat and protein yield, as well as fat and protein percentage of milk, we used SAS package, version 9.3 (SAS Inc., 2011).

RESULTS

The *CSN3* genotypes of Holstein cows were determined using PCR-RFLP, with the following separation of allele-specific fragments in 2% agarose gel (Fig. 1).

Genotype and allele frequencies of Holstein cattle for *CSN3* are shown in Table 1.

Genetic equilibrium of analysed population was evaluated based on the χ^2-test. In the population included in the study, the differences in frequencies of genotypes for *CSN3* gene were not significant. Effectiveness of alleles CSN3 in tested population is shown in Table 2.

During our assessment of the variability of the observed traits dependence on *CSN3* gene polymorphism, we detected a statistically significant difference between genotypes only in case of the average breeding value for the percentage of protein in milk (Fig. 3). For the other breeding values, the impact of individual *CSN3* genotypes on their variability was not observed (Fig. 2 and Fig. 3). The average breeding values for milk production traits of Holstein cows in relation to analyzed *CSN3* genotypes are shown in Table 3.

We found a statistically significant difference between *CSN3* genotypes' effect on the average breeding value for protein content of milk. Statistical analysis confirmed that the AA genotype significantly reduces the average value of the protein content of milk (0.09% on average), compared with genotype BB.

Table 1. Genotype and allele frequencies of Holstein cattle for *CSN3*

Locus	Genotype frequencies			Allelic frequencies		χ2	P
	AA	AB	BB	A	B		
CSN3	0.6952	0.2762	0.0286	0.8333	0.1667	0.007	0.9967

Table 2. Effectiveness of *CSN3* alleles in Holstein cattle population

Locus	Alleles	Heobs	Heexp	PIC	E	ENA	V %
CSN3	A; B	0.2762	0.2778	0.2392	0.7222	1.3847	27.91

Table 3. Average breeding values for milk production traits of Holstein cows with different *CSN3* genotypes

Genotype	Number	Breeding values	Average	Standard error	Minimum	Maximum	*P*-value
AA	146	BVM	850.1650685	560.8474392	–606.6000000	1977.40	n.s.
		BVF	13.7454110	15.5539216	–21.2700000	56.9100000	n.s.
		BVF%	–0.2465068	0.2208966	–0.6100000	0.4800000	n.s.
		BVP	20.0867123	13.9646545	–14.6500000	49.5100000	n.s.
		BVP%	–0.0974658++	0.1390302	–0.3900000	0.3400000	0,002
AB	58	BVM	713.5603448	551.7021949	–595.1000000	2026.50	n.s.
		BVF	12.2413793	16.2296974	–26.7500000	57.1300000	n.s.
		BVF%	–0.2051724	0.1834680	–0.4900000	0.2900000	n.s.
		BVP	19.7758621	16.8919232	–16.7100000	63.8900000	n.s.
		BVP%	–0.0393103	0.1038363	–0.3800000	0.1500000	n.s.
BB	6	BVM	718.7500000	371.9125852	327.1000000	1196.00	n.s.
		BVF	11.5950000	5.3510550	5.4600000	18.5800000	n.s.
		BVF%	–0.2200000	0.1141928	–0.3900000	–0.1100000	n.s.
		BVP	22.1833333	7.7873556	11.3800000	29.7800000	n.s.
		BVP%	–0.0016667	0.0858875	–0.1200000	0.1200000	n.s.

BVM – breeding values for the yield of milk (kg), BVF – breeding values for the yield of fat (kg), BVF% – breeding values for the contents of fat (%), BVP – breeding values for the yield of protein (kg), BVP% – breeding values for the contents of protein (%), $P \leq 0.01$ – statistically highly significant (++), $P \leq 0.05$ – statistically significant (+), $P \geq 0.05$ – statistically non-significant (n.s.)

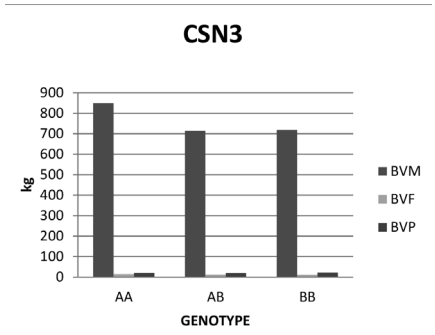

Figure 2. Effect of different *CSN3* genotypes on the average breeding values for the yield of milk, fat and protein shown in kilograms

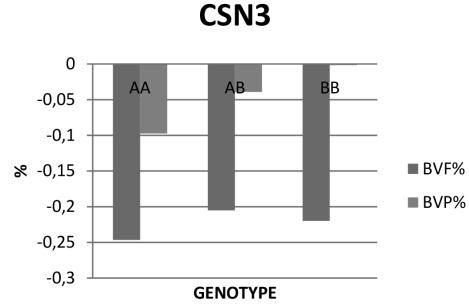

Figure 3. Effect of different *CSN3* genotypes on the average breeding values for fat and protein percentage of milk

DISCUSSION

Genetic structure

Using a molecular-genetic PCR-RFLP method for CSN3 genotyping in Holstein cattle population kept in Slovakia, we detected a predominance of the AA genotype (69.52%); a lower frequency of the AB genotype (27.62%) and the least representation of the BB genotype (2.86%).

The frequency of allele A was very high in the population and amounted to 83.33%. The frequency of allele B was 16.67%. The results of our study are compatible with the results of Botaro and others (Botaro *et al.,* 2009), who found a considerable predominance of the AA genotype in the population of Holstein cattle, with the AA genotype frequency of 66.83%, the AB genotype frequency of 31.84% and the lowest BB genotype frequency of 1.33%. They also detected predominance of the allele A (82.75%) over the allele B (17.25%) in the tested population. Similarly, Sitkowska and others (Sitkowska *et al.,* 2008) observed a high proportion of the AA genotype in the population of Holstein cows, with the AA genotype frequency of 71%, the AB genotype present in 23% of the cattle and the BB genotype present in only 6% of the animals. These data suggested the advantage of the allele A, with its frequency of 83%, while frequency of the B allele was 17%. Gouda and others (Gouda *et al.*, 2013) observed only two genotypes in a population of Holstein cows. The AB genotype was represented by 76% of cows, and the AA genotype was represented by 24% of cows, and the frequencies of the A and B alleles were 62% and 38%, respectively. In accordance with these results, Doosti and others (Doosti *et al.,* 2011) detected only the AA and AB genotypes in the Holstein breed, with a prevalence of AB genotype

(82%) compared to AA genotype (18%). The frequencies of the A and B alleles in this study were 59% and 41%, respectively. The frequencies of the A and B alleles of *CSN3* gene in Holstein cattle population tested by us, and in the population of Holstein cattle tested by other authors, were similar to those reported by Kučerová and others (Kučerová *et al.,* 2006), Bulla and others (Bulla *et al.,* 2007), Brka and others (2010) in Pinzgau cattle. A higher frequency of the allele A in Slovak Pinzgau cattle population was reported Miluchová and others (Miluchová *et al.,* 2009, Miluchová *et al.,* 2014).

Effectiveness of alleles

The loss of genetic variation arising from a limited population size in the captive populations is an important concern. The heterozygosity has been widely used as a descriptor because it is proportional to the amount of genetic variance at a locus, and enables for theoretical consideration of the effect of limited population size on genetic variation (Gautschi *et al*., 2003). In the analysed population of Holstein cattle, the polymorphism of bovine *CSN3* gene showed a high proportion of AA homozygosity (69.52%), as described with the high value of the coefficient of homozygosity (0.7222). Effectiveness of alleles in a population can be described with the effective number of alleles. In a two-allele system, a limit of 2.0 indicates that both alleles are effectively involved in the development of genotypes. In our case, this value was decreased to 1.3847, showing that the effect of alleles A and B is not balanced. The PIC value (0.2392) was substantially lower than a threshold value (0.5), also indicating a low polymorphic level. The low level of polymorphism caused a decrease in a level of possible variability realization (27.91%).

Association studies

The most common gene variants A and B of *CSN3* are shown to be associated with the processing properties of milk (Alipanah *et al*., 2007; Hamza *et al*., 2010). Azevedo and others (Azevedo *et al.,* 2008) reported, that the B allele is associated with thermal resistance, by shorter coagulation time (10–30%), higher yield of fresh and ripe cheeses (5–8%), better curd firmness (20–100%) and micelles of various sizes, necessary for the production of cheese. Kučerová and others (Kučerová *et al.,* 2004) suggest, that the B allele of *CSN3* gene is associated with forming of harder and thicker curd as well as with a higher cheese production. Azevedo and others (Azevedo *et al.,* 2008) also argue that the yield of the curd from milk of cows with the BB genotype is 10% higher compared to cows with AA genotype. Kučerová and others (Kučerová *et al.,* 2004) also present that the AA genotype of *CSN3* gene is mostly associated with a higher yield of milk, proteins and fat, opposite to the BB genotype, which is in turn bound with a higher percentage of protein and fat. In our assessment of the variability of observed traits' co-occurrence with a polymorphism of *CSN3* gene as described with *Hin*dIII restriction analysis of *CSN3* gene fragment, we detected statistically significant difference between genotypes only in an average breeding value for the percentage of protein in milk. The statistical analysis presented in this study confirmed that the AA genotype significantly reduces the average breeding value for the percentage of protein in milk by 0.09%, compared to BB genotype. The study by Ziemiński and others (Ziemiński *et al.,* 2005) previously confirmed the association between CSN3 polymorphism and milk production for a higher percentage of protein in milk of cows with the BB genotype, but, in contrast to our finding, they observed also a higher percentage of fat in the milk of cows with BB genotype. According to Patel and others (Patel *et al.,* 2007), the B variant of *CSN3* gene has a significant effect on milk yield and milk protein yield. Botaro *et al.* (2009) reported that cows with the AB genotype produced milk with a high fat percentage, compared to cows with the AA and BB genotypes. Sitkowska and others (Sitkowska *et al.,* 2008) found that the AA genotype of CSN3 increased the yield of milk fat and protein.

CONCLUSION

The results of our study suggest that *CSN3* gene polymorphism has a measurable effect on the average breeding value for the percentage of protein in milk of Holstein cows. Statistical analysis confirmed that the AA genotype significantly reduced the average breeding value for the percentage of protein in milk.

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