LEPTIN PROMOTER REGION GENOTYPE FREQUENCIES AND ITS VARIABILITY IN THE CZECH FLECKVIEH CATTLE*

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To investigate the differences between the lineages of the Czech Fleckvieh cattle in Leptin promoter SNP C963T, 695 Czech Fleckvieh cows (650 from production herds and 45 from the Genetic Resources Program (GR)) were examined using PCR-RFLP. The *C* and *T* alleles of Leptin promoter were observed with a predominance of *C* allele in both groups. The most frequent genotypes were *CC* (63%) in production herds and *CT* (48%) in the GR population. The present study describes, for the first time, the genetic differences in production herds and GR population in Leptin promoter C963T SNP. Variation within the Czech Fleckvieh population was observed and resulted in an advantage to the GR population. Results presented herein emphasized the importance of the GR program as a reservoir of genetic diversity for indigenous breeds.

single nucleotide polymorphism; cattle breeding; population diversity; leptin; PCR-RFLP

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INTRODUCTION

In the last decade, a large number of quantitative trait loci (QTL) have been identified, particularly for dairy traits (Tellam, 2007). Identifying the genes controlling such traits can provide SNP (single nucleotide polymorphism) markers that can be used for genetic and genomic selection with DNA chip technology. The genotype frequencies vary in the Leptin gene among cattle breeds and some variants can be very rare or even non-polymorphic in particular breeds (Komisarek, Antkowiak, 2007; Komisarek, 2010; Glantz et al., 2012). Therefore, investigation of the SNP frequencies in cattle is very important and can be the first step in their potential implementation into a genomic chip.

Leptin, the 16-kDa protein hormone (146 amino acids), is a product of the obese gene (ob or LEP). It is synthesized mainly by white adipocytes and it is involved in the regulation of feed intake, energy balance, fertility, and metabolism (Frühbeck et al., 1998). In cattle the Leptin gene was mapped to chromosome 4 (Pomp et al., 1997). It consists of three exons, but the first one is not transcribed into protein. Until now, 1165 mutations have been identified in the Leptin gene (www.ensembl.org).

Leptin genetic variants have been associated with milk yield (Chessa et al., 2015), protein, and fat yield (Kulig et al., 2009), carcass traits (Silva et al., 2014), perinatal mortality (Brickell et al., 2010), body condition score (Oikonomou et al., 2011), and fertility (Giblin et al., 2010; Clempson et al., 2011).

The C963T mutation was described in Leptin promotor region by Liefers et al. (2005). This mutation was primarily studied for an association with energy

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status (Liefers et al., 2005) and reproduction traits in cattle (Liefers et al., 2005, Komisarek, 2010). The most frequent reasons for unwilling cow culling are low fertility or leg and udder problems. However, many health risks such as fatty liver, ketosis, disturbed fertility, lameness and infectious diseases, like mastitis and metritis, are correlating with negative energy balance (Martens, 2013; Huzzey et al., 2015). Therefore, markers associated with energy status are very promising for the future animal breading and profitable farming.

Later, the C963T Leptin polymorphism was found to be associated with milk yield and milk content (Giblin et al., 2010; Matteis et al., 2012; Kadlecová et al., 2014). This information suggests the important role of C963T Leptin polymorphism in dairy production. At the moment, no knowledge about genotype distribution of the C963T mutation in Leptin is known in Czech Fleckvieh cattle, or differences in variation between the production population and the population registered in the Genetic Resources Program (GR). The genetic structure of the GR population can be different than in the production population and could be very useful for further breeding.

The aim of this study was to describe genetic variation in the C963T mutation in the Leptin gene between and within the production population and the population registered in the Genetic Resources Program in Czech Fleckvieh cattle. Furthermore, the genotypic frequencies between Czech Fleckvieh cattle and other dairy breeds were compared.

MATERIAL AND METHODS

Animals and samples

The study was conducted on two populations of the Czech Fleckvieh cattle. This local breed has been improved in the 1970s using the Red Holstein sires, and since the 1990s, it has been undergoing continuous upgrading procedures using imported Montbéliarde and German Fleckvieh genetic material. A small population of animals, descendants of the original unimproved Czech Fleckvieh breed, has been bred as a dispersed conservation nucleus within the National Genetic Resources Program (http://efabis.tzv.fal.de). Only animals with at least two generations of ancestors, in both parents' lines, of original pure Czech Fleckvieh are listed in the program.

The first population involved 650 cows from improved production herds, calved between 2003 and 2010 in the Czech Republic from five farms. Each of the 650 cows were daughters of 27 bulls. The second population was presented by 45 cows registered in the GR program. Each of the 45 cows were daughters of 22 bulls.

Genetic and statistical analyses

Genomic DNA for molecular analyses was extracted from peripheral blood using a 6100 Nucleic Acid PrepStation instrument (Applied Biosystems, Foster City, USA) according to the manufacturer's recommended protocol. The C963T (rs109956567) genotypes were determined by PCR-RFLP. The PCR reaction was performed with primers designed according to Komisarek, Antkowiak (2007). Amplified fragments were digested at 37°C for 12 h with 5 U of Dra I restriction endonucleases (Thermo Fisher Scientific, Waltham, USA), and next subjected to electrophoretic separation in 3.5% ethidium bromide-stained agarose gel SeaKem® LE Agarose (Lonza, Basel, Switzerland). The restriction enzyme cut only PCR product with *T* allele resulting in 268 bp and 27 bp fragments. Once genotypes were determined, allele frequencies were estimated and deviations from the Hardy-Weinberg (H-W) equilibrium were tested by χ^2 test using GenAlEx 6.502 software (Peakall, Smouse, 2006, 2012). The approach of allele estimation assumes random mating and no selection in the population, which was not followed in studied animals; therefore the approximate standard errors of allele frequencies were calculated as:

$$P = \sqrt{\frac{1-P}{n}}$$

where:
P = allelic frequency
n = number of cows

To measure the informativeness of a genetic marker, the polymorphic information content (PIC) was evaluated. The PowerMarker V3.25 software (Liu, Muse, 2005) was used to evaluate the gene diversity and PIC. The level of diversity between and within populations was estimated by the analysis of molecular variance (AMOVA) using the Arlequin 3.5.2.1 statistical program (Excoffier, Lischer, 2010). The significance of the AMOVA test was verified by 1023 random permutations. Further descriptive characteristics of the population, e.g. effective number of alleles and fixation effect for the populations, were evaluated by GenAlEx 6.502 (Peakall, Smouse, 2006, 2012).

RESULTS

The laboratory analysis showed three genotypes in Leptin C963T. Genotype and allele frequencies estimated for the locus are shown in Table 1. Genotype *TT* was presented with the lowest frequency in both populations. The most frequent in the production population was *CC* genotype (63%). The highest level of animals with the *CT* genotype was observed in the GR population (48%); meanwhile this genotype was

Table 1. Leptin C963T genotype and allele frequencies in the Czech Fleckvieh cattle

Population	*Genotype frequency (n)*			*Allele frequency*		χ^2
	CC	*CT*	*TT*	*C*	*T*	
Production herds	0.6261 (407)	0.3046 (198)	0.0692 (45)	0.7785	0.2215	8.87**
Genetic resources	0.3467 (13)	0.4841 (27)	0.1690 (5)	0.5888	0.4111	2.57

$\chi 2$ = chi-square test; n = number of observations

**P < 0.01

Table 2. Analysis of molecular variance (AMOVA) in the Czech Fleckvieh cattle

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*
Between population	1	3.025	0.01682	8.67	0.0000**
Among individuals within population	693	133.486	0.16187	7.92	0.01173*
Within individuals	695	112.500	0.19407	83.41	0.00978**

d.f. = degree of freedom

*$P < 0.05$, **$P < 0.01$

second most common in the production population. The estimated frequency of the *C* allele was 78% and 59% for the production and the GR population, respectively. The estimated standard error for the locus was 1.6% in the production population and 7.3% in GR population.

The distributions of genotypes were tested for concordance with expected values according to H-W equilibrium. The production population showed a statistically significant ($P < 0.01$) departure of the equilibrium. According to the fixation effect, the production population presented a lack of heterozygotes of approximately 11% from the expected value (Fig. 1). In contrast, the GR population followed the H-W equilibrium and the fixation effect in the GR population showed the excess of heterozygote of almost 24% relative to Hardy-Weinberg expectations (Fig. 2).

To describe the level of polymorphism and diversity in the studied populations, the genetic diversity and PIC were evaluated. The PIC values of the locus were 0.29 in the production population and 0.37 in the GR population. Also, the level of gene diversity was higher in the GR population than in production herds (0.48 and 0.34, respectively).

The effective number of alleles was used as a supplementary tool to illustrate heterozygosity levels in populations. The GR population presented a higher effective number of alleles (1.93) than the production population (1.52).

AMOVA showed that around 9% of the total genetic variation corresponded to differences between populations (Table 2). Lower variability was among individuals within the population (8%). However, a high level of variation was found among the individu-

Fig. 1. Observed vs expected genotype counts in production herds in the Czech Fleckvieh cattle

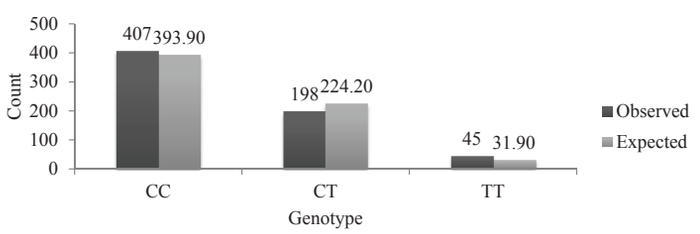

Fig. 2. Observed vs expected genotype counts in Genetic Resources population in the Czech Fleckvieh cattle

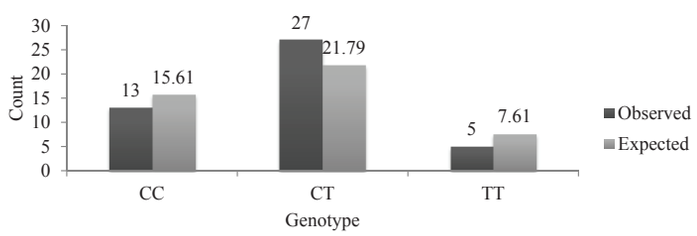

Table 3. Genotypes and allele frequencies within Europe and cattle breeds

Breed	Genotype frequency (%)			Allele frequency (%)		Reference	Country
	CC	CT	TT	C	T		
H	42	45	13	65	35	Banos et al., 2008l	Scotland
H	28	55	17	56	44	Komisarek, 2010	Poland
H	39	47	16	61	39	Glantz et al., 2012	Sweden
H	37	47	16	60	40	Clempson et al., 2011	UK
J	66	34	0	83	17	Komisarek, Antkowiak, 2007	Poland
H	38	40	22	58	42	Kadlecová et al., 2014	Czech Republic

H = Holstein; J = Jersey

als in general (83%). All differences were significant, at least at $P < 0.05$.

DISCUSSION

Differences in genotypic frequencies among Czech Fleckvieh and other cattle breeds were observed. Table 3 shows frequencies of C963T Leptin genotype and allele frequencies over dairy breeds in Europe. These differences can be caused mainly by differences in the breeding goal and also by the production type of breed. Surprisingly, despite the Red Holstein influence on the production Fleckvieh population, the genotypic frequencies were more similar to those of Jersey. The reason for this similarity could be due to a limited use of the Jersey breed to improve local cows during the 1960s. However, in all breeds the *C* allele was the most frequent as well as in the Czech Fleckvieh breed. In C963T Leptin SNP the *C* allele was associated with increased milk yield (Giblin et al., 2010; Matteis et al., 2012) and the dominance of *C* allele is in concordance with the increasing milk yield trend.

Concerning the GR population, a high level of heterozygosity was observed and compared to the production population; a higher level of *TT* genotypes was present. The *TT* genotype was previously associated with higher feed intake (Liefers et al., 2005). We suppose that the higher *TT* genotype frequency in GR could be based on the dual purpose production direction of this breed which was not changed thanks to pure mating.

In numerous studies of Holstein cattle is described as the most common *CT* genotype (Banos et al., 2008; Komisarek, 2010; Clempson et al., 2011; Glantz et al., 2012). This finding is consistent with our results in the GR Czech Fleckvieh population. Similar genotypic frequencies were also observed in Holstein population in the Czech republic (Kadlecová et al., 2014). The description of the C963T Leptin genotype frequencies in the Czech Fleckvieh cattle has not been presented so far, therefore it is not possible to compare our findings with other results in this breed.

Selection favouring certain alleles and decreased number of bulls may lead to departures from the H-W equilibrium. The point that the production population of Czech Fleckvieh did not follow the H-W equilibrium and the high level of *CC* genotype in the population could indicate the previous indirect selection for this SNP. However, if more animals were included in the study, the H-W equilibrium could be achieved. The difference of C963T Leptin genotype frequencies between the production population and the GR population could be caused by the selection pressure for *C* allele in productive herds and by the crossbreeding in the production population.

Furthermore, the genetic variability between the Czech Fleckvieh populations was studied. The PIC values confirmed higher polymorphism level in the GR population compared to the production population. The lower level of PIC in production herds may be explained by the lower number of bulls involved in breeding and also by the selection pressure in this polymorphism. In concordance with the lower level of polymorphism, the production herds showed lower effective numbers of alleles. The negative value of fixation effect, lower level of effective numbers of alleles, and decreased gene diversity can indicate an inbreeding mating in the production population.

AMOVA showed that the total of 83% variance was explained by differences within populations, whereas almost 9% was the result of differences between populations. This is probably caused by the influence of other breeds used to increase the milk yield in the production population. More informative results could be reached if the breeds used for improving the production population would be included in this study of variance.

The differences among Czech Fleckvieh cattle and other cattle breeds were previously studied by Czerneková et al. (2006), though their study was conducted only on production herds and used different markers. They proposed the Czech Fleckvieh cattle as the breed with the highest values of heterozygosity, polymorphic information content, and effective population size; unfortunately no description across the population was done. The genetic distance be-

tween Czech Fleckvieh and other Central European cattle breeds was also studied by Čítek et al. (2006); however to our knowledge the differences between the production population and the GR population have not yet been studied at all. Therefore the results of the present study cannot be compared with previous investigations.

CONCLUSION

Artificial insemination brought a great advantage for animal breeding with a higher emphasis on bull selection. The rigorous selection proved a great genetic progress, but it also decreased animal variability in the population. The existing variability among cattle breeds may cause that efficient approach in one breed may not be ideal for another. Therefore the effort should be made to gain knowledge across the cattle breeds and their variability.

The differences in genotype frequencies between Czech Fleckvieh cattle and other dairy breeds were described, as well as differences between the production population and the GR population in the Czech Republic. The Czech Fleckvieh cattle production population shows different spread of genotypes in comparison to Holstein cattle. These results could suggest a need for customized arrays for a genomic breeding approach in the Czech Fleckvieh cattle. Our results also reveal the need for further studies to discover if the differences between the Czech Fleckvieh cattle and the other breeds are presented also in other loci.

The study of genetic variability between the production population and the GR population showed differences in all the studied parameters in benefit to the GR population. The higher level of heterozygosity and genetic variance in the GR population can indicate that despite the low number of animals, the genetic variability in the studied SNP was preserved. The results also indicate a selection pressure in C963T Leptin SNP in the production population; therefore the importance to save the variability in the GR population is increasing because the loss of variability in more loci can be supposed. The study brought a novel, so-far unstudied knowledge about genetic diversity and variation in Czech Fleckvieh important for further breeding in the Czech Fleckvieh cattle and suggests the significance of the national GR program.

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