UDC 575
DOI: 10.2298/GENSR1403865M
Original scientific paper

ANALYSIS OF GENETIC STRUCTURE IN SLOVAK PINZGAU CATTLE USING FIVE CANDIDATE GENES RELATED TO MILK PRODUCTION TRAITS

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Miluchova M., M. Gabor, and A. Trakovicka (2014*): Analysis of genetic structure in Slovak pinzgau cattle using five candidate genes related to milk production traits*- Genetika, Vol 46, No. 3, 865-875

The goal of the paper was to identify genetic structure of five candidate genes for milk production in Slovak Pinzgau breed. A total of 86 mothers of bulls of Slovak Pinzgau cattle were use in this study. To genotype of cows for candidate genes we used PCR methods (PCR-RFLP, ARMS–PCR, multiplex PCR-RFLP). On the basis of PCR analyses we established genotype structure of cattle population and calculated allelic frequencies. Effectiveness of allele incidence and genetic diversity was evaluated with following parameters: theoretical heterozygosity (*He exp*), experimental heterozygosity (*He obs*), polymorphism information content (*PIC*), expected homozygosity (*E*), effective number of alleles (ENA), level of possible variability realization (*V%*). Slovak Pinzgau cattle exhibit the high values of heterozygosity, polymorphism information content, effective number of alleles and level of possible variability realization for genes CSN2, CSN3 and LALBA. In opposite, for genes CSN1S1 and LGB show high values of homozygosity.

Key words: candidate genes; genetic structure, milk production; Slovak Pinzgau cattle

INTRODUCTION

Pinzgau breed is registered by the UN FAO as threatened with extinction and it is classified as Animal Genetic Resource – AnGR since 1994 (KRUPA *et al.,* 2011). Pinzgau cattle belong to the alpine breeds, and from beginning of exports from Austria they have been allocated to mountains areas of Slovakia (KADLECIK *et al.,* 2004). At present, a Slovak Pinzgau breed represent of dual purpose cattle. The development of the size of cow population in Slovakia is from long-term perspective unfavourable. In the year 2013 the size of the active population of the Pinzgau cows in milk recording system was 3412 heads.

Genetic determinants of protein content in ruminant milk have been the subject of many studies for six decades. Milk proteins were classified in two groups: caseins (alpha *S1 – CSN1S1*, alpha *S2 – CSN1S2*, beta – *CSN2* and kappa – *CSN3*) constitute about 80 % of the

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protein content of milk and are associated with the casein micelle; the remaining about 20 % of milk protein content consist of whey proteins (beta-lactoglobulin – *LGB* and alpha-lactalbumin – *LALBA*) (KAMIŃSKI *et al.,* 2005; BRAUNSCHWEIG, 2008; GÁLIK *et al.,* 2011, ARORA *et al.*, 2010). Analysis of milk proteins polymorphism provides useful information to both the breeders and 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 cheese production (RACHAGANI *et al.*, 2006).

This report aims at analysing Slovak Pinzgau breed to widen the knowledge on their genetic structure for five candidate genes for milk production.

MATERIALS AND METHODS

Animals

A total 86 mothers of bulls of Slovak Pinzgau cattle were use in this study. Bovine genomic DNA was isolated from whole blood by using commercial kit NucleospinBlood (Macherey-Nagel). The concentration and purity of DNA was measured using a spectrophotometer NanoPhotometer™ (Implen GmbH). All DNA samples were stored at -20°C.

Genotyping

For this study were used following five candidate genes for milk production: *CSN1S1*, *CSN2*, *CSN3*, *LGB*, *LALBA*. To genotype of cows for these candidate genes we used PCR methods such as PCR-RFLP, ARMS–PCR, multiplex PCR-RFLP. The PCR reactions were optimized on a thermocycler C1000^{TM} (Biorad) in total volume 25 µl. Primer sequences used in this study and conditions of used methods are shown in Table 1.

Table 1. PCR primers and condition

LOCUS	GENETIC POLYMORPHISM	GENOTYPING METHOD	PRIMER SEQUENCES	Tm	Mg^{2+} (mM)	AMPLICON SIZE	RESTRICTION ENDONUCLEASE	SIZE OF RESTRICTION FRAGMENTS
CSN1S1	1954 A→G GenBank No. X59856	ARMS PCR	FOR inner: 5' - CAT TCC ATT TCC TGT ATA ATG AGG C A - 3' REV inner: 5' - AAT TCT AAG GAG AGT TTA CAA CAA AGA CGC - 3' FOR outer: 5' - TGC ATG TTC TCA TAA TAA CC - 3' REV outer: 5' - GAA GAA GCA GCA AGC TGG - 3' (Kimcen & Medrano 2003)	59 °C	2.5	234 bp (allele B) 130 bp (allele C) 310 bp (from two outer primers)	-	-
CSN2	S101 A→C GenBank No. X14711	PCR-RFLP	FOR: 5' - CCT TCT TTC CAG GAT GAA CTC CAG G- 3' REV: 5' - GAG TAA GAG GAG GGA TGT TTT GTG GGA GGC TCT- 3' (McLachlan 2004)	58 °C	3	121 (PCR product)	DdeI	121 bp (allele A1) 86 bp, 35 bp (allele A2)
	S247 C→G GenBank No. X14711	ARMS PCR	FOR inner: 5' - AAT ATC CAG TTG AGC CCT TTA CTG AAT GC - 3' REV inner: 5' - CAA CAT CAG TGA GAG TCA GGC TCA GC- 3' FOR outer: 5' - AAC ATC CCT CCT CTT ACT CAA ACC CCT G - 3' REV outer: 5' - CTT CTT TGA TGT CTC CTT AGA G - 3' (Kimcen & Medrano 2003)	63 °C	2	217 bp (allele A) 177 bp (allele B) 338 bp (from two outer primers)	-	-
CSN3	5345 A→C GenBank No. X14908	Multiplex PCR-RFLP (CSN3, LGB)	FOR: 5' - GCT GAG CAG GTA TCC TAG TTA T - 3' REV: 5' - CTT CTT TGA TGT CTC CTT AGA G - 3' (Schlee bener et al. 1991)	55 °C	1.5	443 bp (PCR product)	HindIII	443 bp (allele A) 348 bp, 95 bp (allele B)
LGB	5243 C→T GenBank No. X14710		FOR: 5' - TGT GCT GGA CAC CGA CTA C AA AAA G - 3' REV: 5' - GCT CCC GGT ATA TGA CCA CCC TCT - 3' (Medrano & Aguilar-Cordova 1990)			247 bp (PCR product)	HaeIII	148 bp, 99 bp (allele A) 99 bp, 74 bp (allele B)
LALBA	753 A→G GenBank No. X06366	ARMS PCR	FOR inner: 5' - GTG TGG TGA CCC CAT TTC AGA ATC TGG A- 3' REV inner: 5' - GAG ACA AAG GAC ATC ATT TTG GTG ACC ACC- 3' FOR outer: 5' - CTC TTC CTG GAT GTA AGG CTT - 3' REV outer: 5' - AGC CTG GGT GGC ATG GAA TA - 3' (Kimcen & Medrano 2003)	60 °C	3.5	97 bp (allele A) 127 bp (allele B) 144 bp (from two outer primers)	-	-

Statistical Analysis

On the basis of molecular genetics analyses we established genotype structure of cattle population and calculated allelic frequencies. Significance of differences between experimental and theoretically expected frequencies of genotypes we verified with χ^2-test. Effectiveness of allele incidence was evaluated with following parameters: theoretical heterozygosity (*He exp*), experimental heterozygosity (*He obs*), polymorphism information content (*PIC*), expected homozygosity (*E*), effective number of alleles (*ENA*), level of possible variability realisation (*V%*) .

⇒ Experimental heterozygosity (*He obs*) (NEI, 1973)

$$\mathrm{He}_{\exp} = 1 - \sum\left(p^2 + q^2\right)$$

⇒ Polymorphism information content (*PIC*) (BOLTSTEIN *et al.,* 1980)

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

⇒ Expected homozygosity (*E*) (CROW & KIMURA, 1970)

$$\mathrm{E} = \sum p_i^2$$

⇒ Effective number of alleles (*ENA*) (CROW & KIMURA, 1970)

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

⇒ Level of possible variability realization (*V%*) (CROW & KIMURA, 1970)

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

RESULTS AND DISCUSSION

CSN1S1, *CSN2*, *CSN3*, *LGB* and *LALBA* genotypes were determined using 3 % agarose gel (Invitrogen) containing dye GelRed™ (Biotium) in 1 × SB buffer (BRODY & KERN, 2004) at 180 V for 15 minutes and the gel were analyzed in the UV rays and the documentary system Olympus C-7070 were used to record the results (Figure 1).

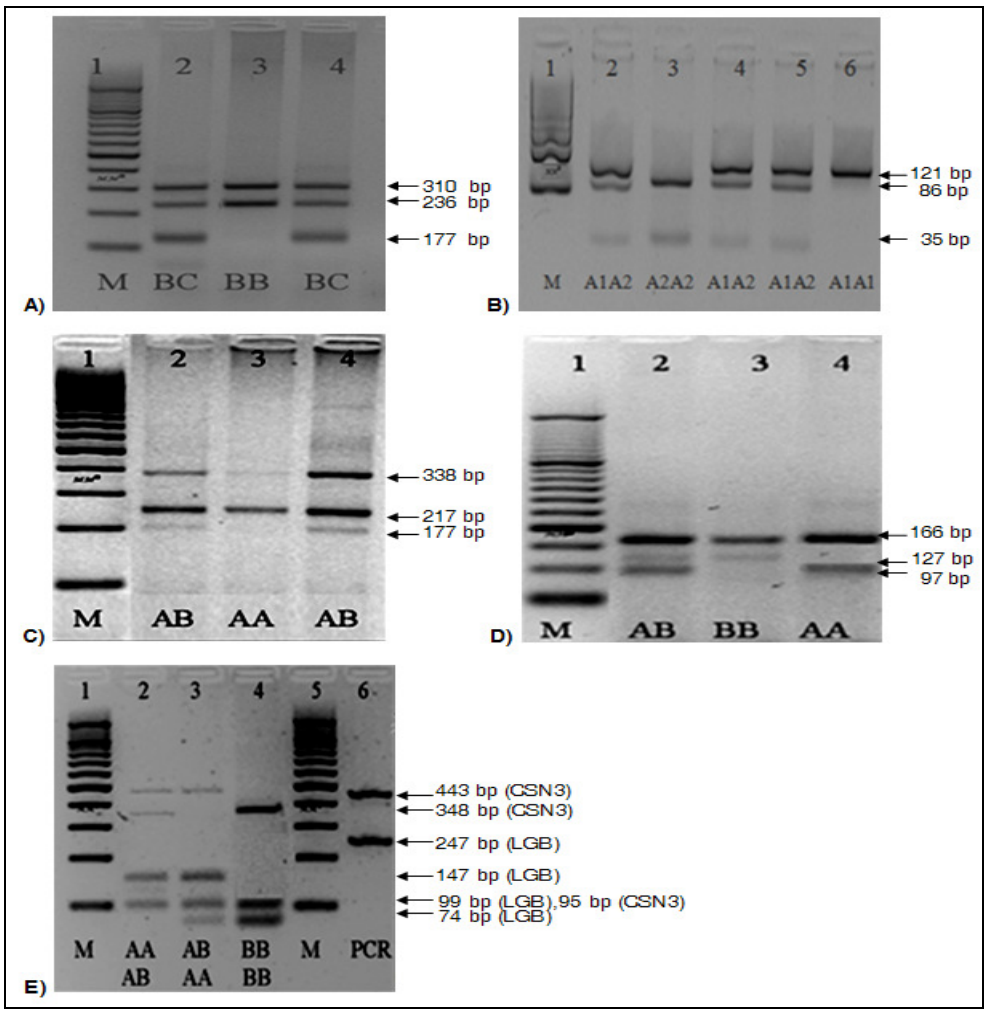

Figure 1. Illustration of CSN1S1, CSN2, CSN3, LGB and LALBA genotypes on agarose gels. A) genotypes of CSN1S1 gene, M – 100 bp DNA Ladder (Fermentas); B) genotype of CSN2 gene (alleles A1, A2), M – 100 bp DNA Ladder (Fermentas); C) genotypes of CSN2 gene (alleles A, B), M – 100 bp DNA Ladder (Fermentas); D) genotypes of LALBA gene, M -50 bp DNA Ladder (Promega); E) genotypes of CSN3 and LGB genes, M – 100 bp DNA Ladder (Fermentas)

Genetic structure of Slovak Pinzgau cattle of five candidate genes for milk production (CSN1S1, CSN2, CSN3, LGB, LALBA) was analysed by using PCR methods. Genotype and allele frequencies of Slovak Pinzgau cattle for these candidate genes are shown in Table 2.

Table 2. Genotype and allele frequencies of Slovak Pinzgau cattle for candidate gene of milk production

LOCUS	GENOTYPE FREQUENCIES			ALLELIC FREQUENCIES		χ^2	P
	BB	BC	CC	B	C		
CSN1S1	0.8605	0.1395	-	0.9302	0.0698	0.0172	0.8955
	A1A1	A1A2	A2A2	A1	A2		
CSN2	0.3023	0.5233	0.1744	0.5640	0.4360	0.3515	0.8388
	AA	**AB**	**BB**	**A**	**B**		
CSN2	0.2209	0.7791	-	0.6105	0.3895	35.01	2.8×10^{-8}
CSN3	0.4535	0.4651	0.0814	0.6860	0.3140	0.5468	0.7608
LGB	0.0465	0.2674	0.6861	0.1802	0.8191	0.0759	0.7829
LALBA	0.0581	0.8023	0.1396	0.4593	0.5407	32.56	8.5×10^{-8}

Genetic equilibrium of analysed population was evaluated on the base χ2- test. In the population included in the study non-significant differences in frequencies of genotypes were found for genes CSN1S1, CSN2 (alleles A1 and A2), CSN3 and LGB. Differences between experimental and theoretically expected frequencies of genotypes for genes CSN2 (alleles A and B) and LALBA were significant.

Effectiveness of alleles incidence were evaluated with following parameters: theoretical heterozygosity (*He exp*), experimental heterozygosity (*He obs*), Polymorphism Information Content (*PIC*), expected homozygosity (*E*), effective number of alleles (*ENA*), level of possible variability realization (*V%*) (Table 3).

The loci for milk proteins have been studied most extensively in cattle. Many research reports have indicated that certain milk protein variants may be associated with milk production.

Table 3. Effectiveness of alleles for milk candidate gene in population Slovak Pinzgau cattle

LOCUS	ALLELES	$He_{(obs)}$	$He_{(exp)}$	PIC	E	ENA	V %
CSN1S1	B; C	0.1395	0.1298	0.1213	0.8702	1.1492	13.13
CSN2	A; B	0.7791	0.4756	0.3625	0.5244	1.9069	48.12
CSN2	A1; A2	0.5233	0.4918	0.3709	0.5082	1.9677	49.76
CSN3	A; B	0.4651	0.4308	0.3380	0.5692	1.7569	43.59
LGB	A; B	0.2674	0.2954	0.2517	0.7046	1.4192	29.89
LALBA	A; B	0.8023	0.4963	0.3730	0.5037	1.9853	50.21

Genetic structure

Alpha S1 casein (*CSN1S1*) genotype significantly influenced milk yield, fat yield, and protein yield with the highest yields obtained for the genotype BB. Protein percentage was influenced by *CSN1S1*, with the genotypes BC and BB, respectively, having the highest percentages (KUČEROVÁ *et al*., 2006; ZAKIZADEH *et al*., 2013). Significantly higher lactation cheese yields were estimated with *CSN1S1* genotype BB. In the total population of cattle homozygotes BB (0.8605) were the most frequent, while BC (0.1395) were the least frequent ones. Genotype CC was not detected. This suggests a superiority of allele B (0.9302). Frequencies of alleles in our population were similar to those of *CSN1S1* gene as reported by KUČEROVÁ *et al.* (2006). CAROLI *et al.* (2008) reported superiority of allele B for Carora cattle. The higher frequency of the allele B in population of Pinzgau cattle reported BEJA-PEREIRA *et al.* (2003). CAROLI *et al.*, (2004) present lower frequency of the allele C in population of Reggiana breed.

The most common forms of beta-casein (*CSN2*) in dairy cattle breeds are A1 and A2, while B is less common. The β-casein A1 variant was associated with the incidence of diabetes mellitus type I, coronary heart disease and autism (LAUGESEN & ELLIOT, 2003). Fat percentage was estimated with *CSN2* genotype A1A1. *CSN2* genotype significantly influenced milk yield, fat yield, and protein yield with the highest yields obtained for the genotype A2A2. The A2 variant reduces serum cholesterol. The A3 variant of bovine *CSN2* is associated with higher milk yield while higher protein, casein yields and fat are associated with the B variant (MICINSKI & KLUPCZYNSKI, 2006). In the population included in the study were detected homozygote genotype AA with frequency 0.2209, heterozygote genotype AB with frequency 0.7791 and homozygote genotype BB has not been observed. This suggests superiority of allele A – 0.6105. We also detected dominance of heterozygous genotype A1A2 (0.5233) above the homozygous genotype A1A1 (0.3023). The homozygous genotype A2A2 was detected in a few isolated cases with frequency 0.1744, therefore allele A1 dominated above the allele A2, with frequencies 0.6105 and 0.3895, respectively. The high frequency of allele A (0.928 and 0.915) in population of Pinzgau cattle reported BEJA-PEREIRA *et al.* (2003) and BLÁHOVÁ *et al.* (2004). In accordance with these results, CAROLI *et al.* (2004) observed superiority of allele A in population Reggiana breed with frequency 0.72. RINCON *et al.* (2006) found in population of Creole breed only allele A. Frequencies of A1 allele in our population were similar to those of *CSN2* gene as reported by MILUCHOVÁ *et al*. (2014) for Pinzgau cattle. HANUSOVÁ *et al.* (2010) reported slight superiority of allele A1 for Holstein bulls. The higher frequency of the allele A2 in population of Pinzgau cattle was reported by BEJA-PEREIRA *et al.* (2003). The predominance of *CSN2* A2 allele (0.764) detected CAROLI *et al.* (2008) in population of Carora cattle and MILUCHOVÁ *et al*. (2013) in population of

Slovak Spotted breed. MANGA *et al.* (2006) presented lower frequency of the allele A1 in population of Czech Spotted and Czech Holstein breed.

The genotype AA of kappa casein (*CSN3*) is mostly associated with higher yield of milk, proteins and fat, opposite to BB genotype which is binded with higher percentage of proteins and fat content in cow milk. The allele B of κ-casein gene (*CSN3*) is associated with better technological properties of the milk such as decreasing of coagulation time, forming of harder and thicker curd, higher cheese production (KUČEROVÁ *et al.,* 2004). In the present study the estimated frequencies of the *CSN3* genotypes AA and AB for the Pinzgau cattle were nearly equal (0.4535 and 0.4651). The frequency of the genotype BB (0.0814) was very low. Results point out that frequency of A allele was high and was represented 0.6860. Frequency of B allele was 0.3140. Our data correspond with several authors. BRKA *et al*. (2010) and DINC *et al.* (2013) in the cattle population detected by the predominance of allele A above allele B. BEJA-PEREIRA *et al.* (2003) indicates the predominance of allele A in the Pinzgau cattle, whose frequency is close to 0.783. High frequency of allele A (0.598) is also consistent with results of authors KUČEROVÁ *et al.* (2006). Similarly, BULLA *et al.* (2007) found prevalence of A allele (0.72).

The β - lactoglobulin (*LGB*) expressed in milk and is important in the evaluation of milk production potential, butterfat and protein content. The AA genotype of *LGB* is associated with higher milk yield, the BB genotype with higher fat and casein content and is more desirable for cheese making (Micinski & Klupczynski, 2006). In case of *LGB* gene we detected dominance of homozygous genotype BB above heterozygous genotype AB with frequencies of occurrence 0.6861 and 0.2674, respectively. The homozygous genotype AA was represented with frequency 0.0465; therefore allele B (0.8191) dominated above the allele A (0.1802). Frequencies of alleles in our population Slovak pinzgau breed were similar to those of *LGB* gene as reported by BULLA *et al.* (2007), who observed superiority of the allele B (0.73) in population of cattle. The higher frequency of the allele B (0.79) in population of cattle reported KLAUZIŃSKA *et al.* (2004) and MILUCHOVÁ *et al.* (2012). In opposite, KUČEROVÁ *et al.* (2006) present higher frequency of the allele A (0.511) in Czech Fleckvieh breed and MILUCHOVÁ *et al.* (2011) in Slovak Spotted breed (0,6071). These data are consistent with the finding DINÇ (2009), who observed higher proportion of A alleles in the Turkish Grey breeds (0.52) and Holstein (0.54).

The alpha-lactoalbumin (*LALBA*) genotype AA is mostly associated with higher yield of milk and fat, opposite to BB genotype which is bind with higher percentage of proteins and fat content in cow milk (KAZMER *et al.,* 2001). Dominance of heterozygous genotype AB (0.8023) for gene *LALBA* in the cattle population was detected. The other to homozygous genotypes AA (0.0581) and BB (0.1396) were represented in lower number. These results suggest that the frequencies of allele A (0.4593) and allele B (0.5407) were nearly equal. Our results disagree with the results of BEJA-PEREIRA *et al.* (2003) and VĂTĂSESCU-BALCAN *et al.* (2008), which detected only genotype BB in population of Pinzgau cattle and Romanian Black Spotted cattle.

Effectiveness of alleles

The loss of genetic variation caused by limited population size in captive populations is an important concern. Heterozygosity has been widely used because it is proportional to the amount of genetic variance at a locus and lends itself readily to theoretical consideration of the effect of limited population size on genetic variation (GAUTSCHI *et al*., 2003).

In the analyzed population was observed a high proportion of homozygous BB (86.05 %) for bovine *CSN1S1* gene, as demonstrated by the high value of the coefficient of homozygosity

(0.8702). Effectiveness of allele in a population, we expressed the effective number of alleles. In two-allele system is a limit of 2.0 indicates the assumption that both alleles are effectively involved in the development of genotypes. In our case, the value decreases to 1.1492, so the effect of alleles B and C is not balanced. At low levels of polymorphic points and PIC value (0.1213), which is compared with a threshold value (0.5), is substantially lower. Low levels of polymorphic caused a decrease of the level of possible variability realization (13.13%).

In the case of *CSN2* gene is relatively equal frequency alleles A1 and A2, like A and B showed the effective number of alleles, which was close to the upper limit of 2.000 (1.9677 and 1.9069, respectively). Level of possible variability realization was implemented to 49.76% and 48.12%, respectively. Observed heterozygosity reaches the highest value (0.5233 and 0.7791, respectively) and confirms the high proportion of heterozygotes in the population.

The expected homozygosity for gene *CSN3* is in the population stated a slight increase in homozygosity (0.5692). This caused a slight decrease in the level of possible variability realization (43.59%), which corresponds to the level of polymorphic locus (1.7569).

In the case of *LGB* gene was observed greater increases homozygosity (0.7046), due to a high proportion of homozygous BB in the population. This resulted in a decrease in the level of possible variability realization (29.89%), which corresponds to the effective number of alleles in the population (1.4192).

High levels of the effective number of alleles (1.9853) for *LALBA* gene have shown a high the level of possible variability realization, which amounted to 50.21%. The higher observed heterozygosity (0.8023) than expected (0.4963) and the average homozygosity (0.5037) indicate sufficient heterozygotes in the population.

It may be concluded that Slovak Pinzgau cattle exhibit the high values of heterozygosity, polymorphism information content, effective number of alleles and level of possible variability realization for genes *CSN2, CSN3* and *LALBA*. A slight heterozygote excess was found in the locus *CSN3* (0.4651). The higher observed heterozygosities were in loci *CSN2* and *LALBA* and they ranged from 0.5233 to 0.8023. The range of *PICs* was between 0.3239 (*CSN3*) and 0.3730 (*LALBA*). Effective number of alleles ranked between 1.7569 (*CSN3*) and 1.9856 (*LALBA*) and values of level of possible variability realization were from 43.59 % to 50.21%.

In opposite, for genes *CSN1S1* and *LGB* show high values of homozygosity (0.8702 and 0.7046, respectively). This may be due to the fact that has been tested small number of animal or intensive selection. However, the number of alleles remaining is important for the long-term response to selection and survival of populations.

ACKNOWLEDGMENT

This work has been supported by the grants: The Slovak Research and Development Agency under the contract No. LPP-0220-09 and No. APVV-0636-11.

Received June 22h, 2014

Accepted Octoberr 12th, 2014

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ANALIZA GENETIČKE STRUKTURE KOD SLOVAČKIH GOVEDA PRIMENOM PET GENA KANDIDATA VEZANIH ZA PROIZVODNJU MLEKA

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Izvod

Cilj istraživanja je bio identifikacija genetičke structure pet gena kandidata za kontrolu proizvodnju mleka u Slovačkoj rasi Pinzgau. Ispitivano je 86 grla – majki bikova rase Pingzau. Genotiping krava na prisustvo gena kandidata su korišćene *PCR* zasnovane metode (PCR-RFLP, ARMS–PCR, i multipleks PCR-RFLP). Evaluacija efektivnosti i genetičke divergentnosti je vršeno preko parametara: teoretska heterozigotnost (*He*) (*He exp*), eksperimentalna heterozigotnost (*He obs*), informacija o sadržaju polimorfizma (*PIC*), očekivana homozigotnost (*E*), efektivan broj alela (*ENA*) i nivo realzacije moguće variabilnosti (*V%*). Slovačka Pinzgau goveda imaju visok nivo heterozigotnosti , visok nivo polimorfizma, efektivan broj alela i alela koji kontrolišu moguću realizaciju gena *CSN2, CSN3* i *LALBA*. Kod gena *CSN1S1* i *LGB* je utvrđen visok nivo homozigotnosti.

Primljeno 22. VI. 2014.
Odobreno 12. X. 2014.