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Polymorphism of LEP, PKM2 and CSN3 genes in Polish cold-blooded horse population

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The aim of the study was to determine frequencies of leptin (LEP), pyruvate kinase M2 (PKM2) and kappa casein (CSN3) genes and genotypes in Polish cold-blooded horse population. The investigation was performed on 194 individuals. Genotyping of the selected genes was performed by PCR-RFLP technique with restriction enzymes *Taq*I (LEP gene), *Apo*I (PKM2), *Pst*I and *BseY*I (CSN3). Frequencies of genes and alleles were as follows: LEP/*Taq*I AA – 0.1443, AB – 0.4897, BB – 0.3660, A – 0.3892, B – 0.6108; PKM2/*Apo*I AA – 0.0000, GA – 0.0979, GG – 0.9021, A – 0.0490, G – 0.9510; CSN3/*Pst*I AA – 0.4948, AG – 0.5000, GG – 0.0052, A – 0.7448, G – 0.2552; CSN3/*BseY*I AA – 0.3866, AC – 0.3247, CC – 0.2887, A – 0.5490 and C – 0.4510. Any statistically significant differences in the observed frequencies of genotypes between mares and stallions' groups were not found. In case of leptin and pyruvate kinase genes, the population was found in Hardy-Weinberg equilibrium.

KEY WORDS: cold-blooded horses / genetic polymorphism / PCR-RFLP

The genes, polymorphism of which may be associated with the results of performance and exterior of animals, inspire the breeders and geneticists' interest. From among various genes, the leptin gene (LEP) raises the expectations for application in practical breeding; the product of the discussed gene is an essential factor, regulating the body weight, fat reserves, food intake and reproductive functions [11, 16, 17, 27]. It refers also to PKM2 gene which codes the M2 type of pyruvate kinase and takes part in the final stage of glycolysis. It undergoes expression in kidneys, adipose tissue, lungs, and all proliferating tissues such as: trophoblast, tissues of embryo or mother cells of adults, as well as the cancer cells [6]. Kappa-casein gene (CSN3) determines lactation possibilities of mammal females – mutations within the scope of CSN3 make breeding the young animals impossible due to destabilisation of other milk protein micelles of mammary gland [9, 10, 21, 22].

The polymorphism of the mentioned genes had already been studied in the populations of various farm animal species, confirming many times the relationship of single genotypes and the level of performance traits [1, 7, 8, 12, 13, 14, 15, 19, 23, 24, 26]. It was found that the incidence of particular polymorphic forms of leptin gene is associated with various

levels of dairy traits of the cows. The animals with BB homozygous genotype of protein are characterised by the highest percentage content of fat in milk, whereas the milk of AB genotype cows has the lowest content of the protein [19]. Liefers et al. [15] obtained the results which proved that AB LEP heterozygous cows revealed higher milk productivity and higher lactose and protein content in milk as compared to the animals of other leptin genotype. In the study of Buchanan et al. [3], the authors proved that the TT leptin homozygous genotype was associated with the increase of milk yield and protein quality. The LEP genotype in cattle, is associated with feeding – AT animals intake much more fodder than the AA homozygous ones [14]. In the research on the relation between the LEP genotype of pigs and the breeding traits, it was revealed that the sows differed significantly in age of the first farrowing – the females of TT genotype were younger than the animals of CC and CT genotypes [14]. Kurył et al. [13] studied the relationship of LEP genotype and the quality of fatness of various pig breed and indicated significant differences in dressing percentage, content of meat in ham, and mass of loin in TT pigs as compared to TC animals. The authors stated also higher weight and meat content in ham, as well as lower weight of pork fat and fat content in ham as for TT genotype of pigs in comparison with the TC genotype. Mikolasova and Urban [17] did not prove essential influence of the single LEP genotype on the researched traits of meat quality, but they stated significant and highly significant relations between the combined LEP*LEPR genotype and pH_{24} and remission. The relationship of PKM2 polymorphism and the performance meat traits was studied in pigs and revealed the dependence of the incidence of TT PKM2 pigs' genotype and the growth of glycolic potential and lactate content in the carcass. The inter-breed differences in the expression level of PKM2 gene were also stated in the muscular tissue [24]. In relation to CSN3 in cattle, its 14 variants were found in various species. In case of domestic cattle, the relation between polymorphism in the scope of the gene and the quantity and technological quality of the produced milk was proved. Tsiaras et al. [26] showed the influence of κ-casein genotype on the quantity and protein content in milk of Holstein cows – the animals of AB genotype produced milk of higher quality in comparison with the AA homozygous ones. It results from the data of other authors [7] that the milk of AA CSN3 homozygous cows gave a lower cheese yield; the cheeses were also of worse quality than the cheeses produced from the milk of cows of other CSN3 genotypes [7]. The studies, carried out on the multi-breed cattle population [8] showed that there was a relationship between the CSN3 genotype and the value of the selected meat traits – the AB heterozygous animals reached the highest average body weight at weaning and the highest average daily gains. The poorest results in respect of meat traits were obtained for BB CSN3 homozygous cows [8]. Kappa-casein gene is polymorphic also for goats and sheep. The dependence between the CSN3 genotype and goat milk content was proved – the milk of AB heterogeneous and BB homozygous animals contained more caseins in comparison with the milk of AA homozygous animals. Also, the total percentage content of protein was the lowest in case of AA CSN3 homozygous goats [5]. The kappa-casein gene is – apart from genes which code other caseins and β-lactoglobulin – studied as a marker of goat and sheep milk quality [18]. In the research on rabbits of INRA line, Bolet et al. [1] obtained the results which confirmed the relation between the kappa-casein genotype of females and the number of the young animals in the litter and the weight of the litter – somewhat higher values of the mentioned indicators were found for the AB heterogeneous females.

Polish population of cold-blooded horses was created on the basis of native female material with the use of West European reproducers. Initially, it was characterised by relatively higher phenotype differentiation. Presently, it is kept as Polish breed of cold-blooded horses and used for the slaughter and draught purposes. The continuous betterment of type and size [20] still remains the objective of genetic improvement.

The aim of the research was to define the frequency of genes and genotypes of leptin (LEP), pyruvate kinase M2 (PKM2) and kappa-casein (CSN3) in the population of Polish cold-blooded horses.

Material and methods

The research was carried out on 194 Polish cold-blooded horses coming from Nowe Jankowice Stud Farm; it included 123 mares and 71 stallions. The blood became the material for molecular analysis; it was collected from the jugular vein for EDTA samples. DNA was isolated with the use of MasterPure™ DNA kit (Epicentre Technologies).

The molecular research was carried out by the PCR-RFLP method. PCR reactions for all genes proceeded in the volume 20 µl and the reaction mixtures were used in the following content: 100 ng genomic DNA, 10 pmol each of the starters, 200 µM of each of dNTP, 1x concentrated buffer for DreamTaq polymerase, 1U (in the case of CSN3 gene – 0,5U) of DreamTaq polymerase (Fermentas).

The molecular analysis of LEP gene was carried out according to Caetano et al. method [4]. They based on the LEP gene GenBank: NC_009147.2 sequence. The intensified gene segment was of the length of 1900 bp, including segments 2 and 3 of exon separated with intron 2. Thermal profile of PCR reaction was following: initial denaturation at 94°C for 5 minutes, 30 proliferating cycles for 40 s at 94°C, 45 s at 58°C, and 2 minutes at 72°C and final extension at 72°C for 10 minutes. The obtained DNA sequence was subject to enzymatic hydrolysis with restrictive enzyme *Taq*I (Fermentas) for 3.5 h at 65°C.

Gene PKM2 genotyping was carried out according to Dall'Olio et al. method [6]. They based on the PKM2 gene GenBank: AM182983.2 and AM182984.2 sequences. The identified mutation was the transition G>A in the position of 37. The gene sequence of the length 231 bp was intensified. The thermal profile of PCR reaction was following: the initial denaturation at 95°C for 5 minutes, 35 proliferating cycles for 30 s at 95°C, 30 s at 65°C and 30 s at 72°C and the final extension at 72°C for 5 minutes. The obtained DNA sequence was subject to etching with restrictive enzyme *Apo*I (Fermentas) for 3.5 h at 37°C.

CSN3 gene genotyping was carried out according to Hobor et al. method [10]. The CSN3 GenBank: AY579426 gene sequence was utilized. The identified mutations were as follows: the transition A>G in position 187 and the transversion C>A in position 217. The gene sequence of the length equal to 237 bp was intensified, including the final part of promotor and exon 1. The thermal profile of PCR reaction was as follows: the initial denaturation at 94°C for 5 minutes, 30 proliferating cycles for 1 minute at 94°C, 30 s at 63°C and 1 minute at 72°C, and the final extension at 72°C for 7 minutes. The obtained DNA sequence was subject to etching in 2 enzymatic systems – with *Pst*I (NEB) and *BseY*I (NEB) for 3.5 h at 37°C.

The obtained restrictive segments were then separated in 2% (LEP) and 3.5% (PKM2, CSN3) agarose gels with the addition of ethidium bromide (0,5 μg/ml), in the presence of DNA markers – for LEP, it was O'RangeRuler100 bp + 500 bp (Fermentas), for PKM2 and CSN3 – pUC19/*Msp*I (Fermentas).

On the basis of the obtained results of molecular analysis, the examined population was characterised and the genes and genotypes' frequencies with consideration of gender and in the total account were determined. In order to verify the significance of differences in the scope of the observed genotypes' frequencies between the horses of different sex and to check the agreement of the distribution of genotype frequency with the Hardy-Weinberg principle, the χ^2 test was employed [25].

Results and discussion

All the studied genes may be characterised by polymorphism. In case of LEP/*Taq*I and CSN3/*Pst*I and CSN3/*BseY*I genes, three genotypes were identified: AA, AB, and BB, AA, AG and GG, and AA, AC, and CC respectively. The restrictive analysis of PKM2 gene with *Apo*I enzyme enabled to find two genotypes: GA and GG. The individuals of AA genotype of pyruvate kinase in the researched horse population were not found. The frequency of gene and genotypes was presented in the table 1a and 1b. In the scope of the analysed genes, the frequencies of alleles in the mares and stallions' groups were quite equal; especially in the case of PKM2/*Apo*I gene, the differences were statistically insignificant. Any statistically significant influence of the sex on the frequency of incidence of particular genotypes of the studied genes (table 1a and 1b) was not found.

Higher allele A LEP/*Taq*I frequency was found in the mares' group (0.4106), in comparison with the stallions, whereas that one of B allele was recorded for the stallions (0.6479). The similar values of gene frequency were found for the whole population (A – 0.3892 and B – 0.6108 respectively) (table 1a). From among 194 examined individuals, AB leptin heterozygous animals (0.4897) were most numerously represented. The remaining genotypes were recorded with the respective frequency: 0.3660 – BB; 0.1443 – AA. In the mares' group, the heterozygous ones were identified with the similar frequency as in the whole population (0.5122), whereas among the stallions, they were less numerous (0.4507) (table 1a).

The obtained results correspond with the results of Kęszka [11], who performed the analysis of LEP/*Taq*I polymorphism in the population of 413 mares of various breeds and revealed the similar percentage participation of leptin AB heterozygous animals (50%) in the group of 88 mares of Polish cold-blooded horses. The participation of AA homozygous ones was lower (12.79%) than that one recorded in the own research, but also in the mentioned studies they were the least numerous in comparison with the remaining leptin genotypes. In other breed groups, including mares' population of other origin and utility type (hot-blooded breeds), analysed in the researches of the mentioned author [11], the greatest representation was recorded for BB LEP/*Taq*I homozygous animals. The exception was the group of Polish Konik horses, among which the mares of homozygous genotypes AA and AB were numerously the same (each 46.15%); however, the frequency of AB heterozygous animals was low (7.69%) [11]. In the population of 117 individuals of Polish cold-blooded horses, as being studied by Borowiec-Chłopek and Pikuła [2], the participation

of A and B leptin variants (A – 0.38, B – 0.62) was similar to that one, obtained in the own studies. Also, the frequencies of individual genotypes, as being observed by the authors, corresponded with the results of the own analysis. Most often, the AB LEP (frequency 0.504) genotype was identified, the frequencies of the remaining genotypes were found on the following level: 0.128 – AA, 0.368 – BB, respectively [2].

In the analysed gene locus PKM2/*Apo*I, the genes' frequencies were very similar in the mares and stallions' groups; the most often identified allele was G (0.9472 for mares and 0.9577 for stallions – table 1a). It seems to be consistent with the results of Dall'Olio et al. [6] who, depending on the researched horse breed, obtained the G allele frequency of the pyruvate kinase on the level from 0.61 to 1.00, whereas A variant of the gene was recorded on the level of 0.00-0.39. In the own research, the high frequency of G PKM2/*Apo*I allele resulted in a substantial participation of GG homozygous animals both in the whole studied population (frequency 0.9021) as well as among mares (0.8943) and stallions (0.9155). In the examined horse population, the occurrence of individuals with AA genotypes in PKM2/*Apo*I (table 1a) locus was not found.

In case of kappa-casein gene in the analysed CSN3/*Pst*I locus, the A allele was characterised by much higher frequency, the frequency which was approximately 3 times higher than that of G gene, both in the whole studied population (0.7448), and in the mares and stallions' groups (frequencies: 0.7520 and 0.7324, respectively – table 1b). In the researched group of 115 horses of various breeds, Hobor et al. [10] also found higher frequency of A allele (equal to 0.87) in comparison with the G variant of kappa-casein (0.13). In the population of Slovenian cold-blooded horses, as being analysed by the authors, the frequency of A allele was found on the level of 0.60. Also, in case of the remaining horse breeds, covered by the studies of Hobor et al. [10], the A gene was more often observed CSN3 variant; in a few breed groups, the existence of G CSN3 allele was not found at all. The results of Selvaggi et al. [21] correspond with the above mentioned ones as well as with the own analysis results; in the population of 45 horses of Murgese breed, the mentioned authors recorded the frequency of A CSN3/*Pst*I gene 4 times higher than that one observed for G gene (0.80 and 0.20, respectively). The frequencies, with which the horses of AA and AG genotypes were identified in the own studies, had the similar values (AA – from 0.4789, AG – from 0.4959). Among the mares, the incidence of GG homozygous individuals in the locus were not found, whereas among the stallions, the frequency of the individuals with the discussed genotype was low (0.0141) – table 1b. The results seem to be consistent with the results of the analysis of Selvaggi et al. [21] who also did not find the participation of GG kappa-casein homozygous animals in the researched population, and the frequencies of the remaining genotypes were: AG 40%, AA 60%. Hobor et al. [10], when analysing the group of 17 cold-blooded horses, estimated also the frequency of occurrence of GG CSN3 homozygous individuals on the lowest level (0.12) in comparison with AG heterozygous (0.58) and AA homozygous animals (0.29). In the remaining breed groups, as being studied by the authors – each of 17 horses of Trotter and Haflinger breeds, the AA kappa-casein homozygous animals were observed with 100% frequency occurrence [10].

In the second examined restrictive place of (CSN3/*BseY*I) kappa-casein gene, the observed frequencies of both varieties, occurring in the gene variety population, were found on

Table 1a
Frequency of genotypes and genes in the examined population of horses

Group	n		Frequency									
			genes		genotypes			genes		genotypes		
			LEP/*Taq*I					PKM2/*Apo*I				
			A	B	AA	AB	BB	A	G	AA	GA	GG
♀	123	observed	0.4106	0.5894	0.1545	0.5122	0.3333	0.0528	0.9472	0.0000	0.1057	0.8943
		expected			0.1686	0.4840	0.3474			0.0028	0.1000	0.8972
		χ^2				0.40					0.30	
♂	71	observed	0.3521	0.6479	0.1268	0.4507	0.4225	0.0423	0.9577	0.0000	0.0845	0.9155
		expected			0.1240	0.4563	0.4198			0.0018	0.0810	0.9172
		χ^2				0.00					0.20	
∑	194	observed	0.3892	0.6108	0.1443	0.4897	0.3660	0.0490	0.9510	0.0000	0.0979	0.9021
		expected			0.1515	0.4754	0.3731			0.0024	0.0932	0.9044
		χ^2				0.00					0.20	

Value χ^2 tab $p \leq 0.05 = 5.99$; $p \leq 0.01 = 9.21$

Table 1b
Frequency of genotypes and genes in the examined population of horses

Group	n		Frequency									
			genes		genotypes			genes		genotypes		
			CSN3/*Pst*I					CSN3/*BseY*I				
			A	G	AA	AG	GG	A	C	AA	AC	CC
		observed	0.7520	0.2480	0.5041	0.4959	0.0000	0.5772	0.4228	0.4146	0.3252	0.2602
♀	123	expected			0.5655	0.3730	0.0615			0.3332	0.4881	0.1788
		χ^2				10.90					11.10	
		observed	0.7324	0.2676	0.4789	0.5070	0.0141	0.5000	0.5000	0.3380	0.3239	0.3380
♂	71	expected			0.5364	0.3920	0.0716			0.2500	0.5000	0.2500
		χ^2				8.60					12.40	
		observed	0.7448	0.2552	0.4948	0.5000	0.0052	0.5490	0.4510	0.3866	0.3247	0.2887
∑	194	expected			0.5547	0.3801	0.0651			0.3014	0.4952	0.2034
		χ^2				9.90					11.90	

Value χ^2 tab $p \leq 0.05 = 5.99$; $p \leq 0.01 = 9.21$

a similar level (in the whole population: A – 0.5490, B – 0.4510, and for mares: A – 0.5772 and B – 0.4228), or even, as in case of the stallions' group, on the same level (A – 0.5000 and B – 0.5000) – table 1b. In the group of cold-blooded horses, covered by the studies of Hobor et al. [9], there was found the occurrence of alleles with similarly equalized frequency – 0.52 for C allele, whereas for A allele it was 0.47. However, the mentioned authors noted different participation of both variants, depending on the analysed breed of the horse group; in the populations of Slovenian hot-blooded horses, Ljutomer trotter and Lipizzan breeds, C kappa-casein was most often observed (with the frequency in breeds: 0.80; 0.80 and 0.97, respectively) [9]. Similar results were obtained by Selvaggi et al. [21] who identified C kappa-casein allele in the researched population as being 3 times more frequent than the A variety of the gene (frequencies: 0.74 and 0.26, respectively). The frequencies of single CSN3/*BseY*I genotypes in the whole population and within both sexes, as obtained in own analysis, were similar and amounted to: AA – from 0.3380 to 0.4146; AC – from 0.3239 to 0.3252 and CC – from 0.2602 to 0.3380, respectively, depending on the group (table 1b). Different results were reported by Hobor et al. [10], who identified CSN3 genotypes with differentiated frequency in all studied breeds. The lowest frequency was characteristic of AA CSN3 homozygous animals (in the population of cold-blooded horses – 0.17, Trotter – 0.06 and Haflinger – 0.12). The highest frequency was noted for AC heterozygous among the horses of cold-blooded and Haflinger breeds (frequencies: 0.47 and 0.58, respectively). CC kappa-casein homozygous animals were recorded in the population of Trotter breed (0.58) [10]. Selvaggi et al. [21], who carried out the research on various horse breeds, stated that they did not identify the horses with AA CSN3/*BseY*I genotype, whereas the frequencies of CA and CC genotypes were: 51.12% and 48.88%, respectively.

When comparing the real frequency of genotypes in respect of LEP and PKM2 genes with the expected frequency, it was stated that the studied population was found in the state of genetic equilibrium, both in the scope of the horses of the particular genders and in the total scope, what may be confirmed by the obtained χ^2 values (table 1a). In case of CSN3 gene, the obtained χ^2 values prove the lack of agreement between the observed genotype distribution and the theoretical one, in the both studied restrictive places (table 1b). The lack of genetic equilibrium, in respect of CSN3 gene, is, most probably, caused by the conducted selection, oriented towards the increase of body size and it may suggest the modification of gene activity by other genes [28].

To sum it up, it should be stated that in the examined population of Polish cold-blooded horses, the analysis of polymorphism of leptin and kappa-casein genes has revealed the occurrence of all possible genotypes, whereas in the case of pyruvate kinase gene of M2 type, two from three predicted genotype forms were identified (the existence of AA PKM2 homozygous horses was not found). The observed genotype distributions of leptin and pyruvate kinase were consistent with the principle of genetic equilibrium. Any statistically significant differences in the frequency of the particular studied genotype forms between mares and stallions were not found.

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