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MC4R AND *PGAM2* GENES POLYMORPHISM ASSOCIATION WITH PRODUCTION TRAITS IN RABBIT (*ORYCTOLAGUS CUNICULUS*)

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ABSTRACT

Mutations of the melanocortin-4 receptor (MC4R) gene are associated with body weight, obesity, and growth. PGAM2 is associated with drip loss, meat colour, fat deposition, lean content, muscle fiber diameter and carcass traits. The present study had been carried out to investigate the association between a single nucleotide polymorphism (SNP) of MC4R (101G>A) and PGAM2 (195C>T) gene and selected production traits in rabbits. Genomic DNA for all analyses was extracted from muscle (*biceps femoris*) using NucleoSpin kit. The c.101G>A (MC4R) and c.195C>T (PGAM2) was genotyped by PCR-RFLP in a total of 44 rabbits. The average allele frequency was 0.63 for allele A and 0.37 for G, resp. 0.66 for allele C and 0.34 for T. The association results revealed the AA genotype of c.101G>A was associated significantly ($P < 0.05$) with greater live body weight. Statistically significant association of the genotypes with other production traits was not observed.

Keywords: SNPs, candidate gene, association analysis, *MC4R*, *PGAM2*, production traits

INTRODUCTION

Direct research on mutations in functional genes and their association with economically important traits, such as body weight, growth characteristics and other carcass traits is the great importance of the rabbit industry. Genes associated with mentioned traits have been identified using single-nucleotid polymorphism (SNPs) **(Óvilo *et al.*, 2006)**. Rabbits produce white, delicate, and tender meat that has good nutritional value because it contains more protein, less cholesterol, less fat, and fewer calories than pork, beef, lamb, or chicken **(Rogel-Gaillard *et al.*, 2008)**.
Mutations of the melanocortin-4 receptor (MC4R) gene are associated with body weight, obesity, and growth in pig **(Kim *et al.*, 2000; Kováčik *et al.*, 2009),** mice **(Huszar *et al.*, 1997)**, chicken **(Qiu *et al.*, 2006; Yan *et al.*, 2013)** and human **(Yeo *et al.*, 1998; Ramachandrappa and Farooqi, 2011)**. The rabbit MC4R gene has been already included on chromosome 9 **(Fontanesi *et al.*, 2013)**. The rabbit MC4R gene is mainly expressed in the hypothalamus, and it has many important functions and effects on body weight **(El-Sabrout and Aggag, 2017)**, the sexual desire behaviour **(El-Sabrout, 2017; El-Sabrout and Soliman, 2017)**. Phosphoglycerate mutase (PGAM) is the glycolytic enzyme that catalyzes the conversion of 3-phosphoglycerate into 2-phosphoglycerate in the glycolytic pathway **(Fontanesi *et al.*, 2008)**. PGAM is a dimer of two distinct 30 kDa subunits, including the ubiquitously expressed brain form - PGAM1 and the muscle form - PGAM2 **(Wu *et al.*, 2015)**. This gene (PGAM2) is associated with drip loss, meat colour, fat deposition, lean content, muscle fiber diameter and carcass traits in pigs **(Dragos-Wendrich *et al.*, 2003; Fontanesi *et al.*, 2008; Ovilo *et al.*, 2002)**.
The present study had been carried out to investigate the association between a single nucleotide polymorphism (SNP) of MC4R and PGAM2 gene and selected production traits in rabbits. Single-nucleotide polymorphism (SNP) is a potent method for detecting polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotid position within locus **(Liu, 2007)**.

MATERIAL AND METHODS

Animals, samples and data collection

In our study were used a total of 44 breed lines 84 post-weaned rabbits (meat line P91 - 18 males, meat line M91 - 26 females). All rabbits were collected from Research Institute for Animal Production in Nitra. Rabbits were fed *ad libitum* by commercial pelleted feed and had free access to water. Animals were maintained under standard conditions of humidity (55 ± 10%), temperature (20 ± 2◦C) and photoperiod (12 h light : 12 h dark). In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by ethical committee. Animal were healthy during all experiments. At the end of the trial the animals were slaughtered, muscle samples were collected, and selected parameters, such as live weight, body weight (after sacrified), weight of skin, carcass weight and weight of thig were measured. All selected traits are expressed as g.

MC4R gene sequencing and mutation detection

Genomic DNA for all analyses was extracted from muscle (*biceps femoris*) using NucleoSpin kit (Macherey-Nagel GmbH & Co., Germany). Primers were designed according to **Fontanesi *et al.* (2013)**. The first fragment was amplified with primers of sequence: forward primer, 5´ CAT GAA CTC CAC CCA CCA C 3´, and reverse primer, 5´ CTC ATA GCA CCC TCC ATC AGA CTA G 3´.
DNA amplification was carried out by PCR in a final volume of 25 µL containing AmplitaqGold360 Buffer 10X, MgCl 25 mM, dNTP 10mM, Primer mix (For+Rev) 10µM, AmplitaqGold360 polymerase 5 U/µl. PCR program included the following steps: 10 min at 95ºC; 35 amplification cycles of 15 seconds at 95ºC, 20 seconds at 59ºC, 30 seconds at 72ºC; a final extension step of 10 min at 72ºC. PCR was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, Hercules, California, USA).
The SNP (101G>A) was genotyped by the PCR-RFLP (*Bcu*I). PCR-RFLP mastermix contains Anza Buffer 10 x, Anza *Bcu*I, PCR product and was digested

by *Bcu*I. DNA bands were visualized using electrophorese in agarose gel. PCR-RFLP patterns were the following: allele A produced one fragment of 100-bp, regarding allele G we expected an undigested fragment of 127-bp (figure 1). The heterozygote has both alleles.

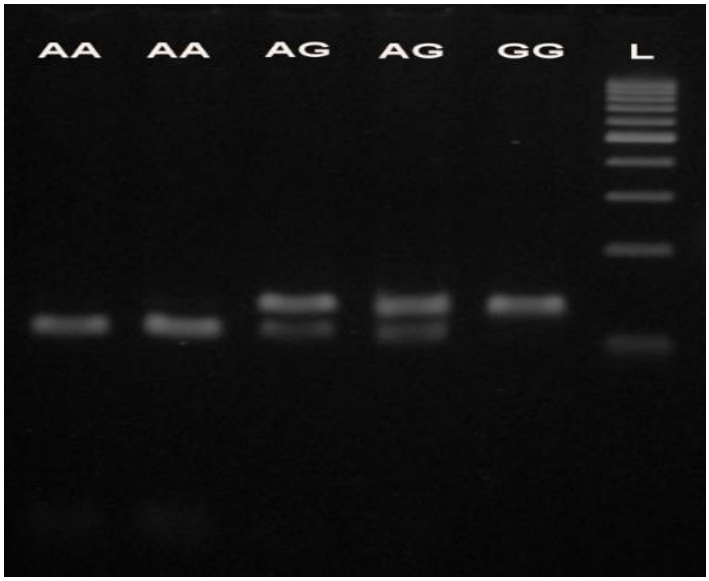

Figure 1 *Bcu*I digestion of PCR product followed by agarose gel electrophoresis. Allele A produced 100-bp fragment, and allele G produced 127-bp fragment.

***PGAM2* gene sequencing and mutation detection**

Genomic DNA for all analyses was extracted from muscle (*biceps femoris*) using NucleoSpin kit (Macherey-Nagel GmbH & Co., Germany). Primers were designed according to **Wu *et al.* (2015)**. The first fragment was amplified with primers of sequence: forward primer, 5´ GAA TGC TGA TTG GCA GTT GGC 3´, and reverse primer, 5´ CCA GTT GTC TGA AAC CCC TGT G 3´.
DNA amplification was carried out by PCR in a final volume of 25 µL containing AmplitaqGold360 Buffer 10X, MgCl 25 mM, dNTP 10mM, Primer mix (For+Rev) 10µM, AmplitaqGold360 polymerase 5 U/µl. PCR program included the following steps: 10 min at 95ºC; 40 amplification cycles of 15 seconds at 95ºC, 20 seconds at 60ºC, 30 seconds at 72ºC; a final extension step of 7 min at 72ºC. PCR was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, Hercules, California, USA).
The SNP (195C>T) was genotyped by the PCR-RFLP (*Csp*6I). PCR-RFLP mastermix contains Anza Buffer 10 x, Anza *Csp*6I, PCR product. DNA bands were visualized using electrophorese in agarose gel. PCR-RFLP patterns were the following: allele C produced two fragments of 613-bp and 242-bp; allele T resulted in three fragments of 309-bp, 304-bp and 242-bp (figure 2).

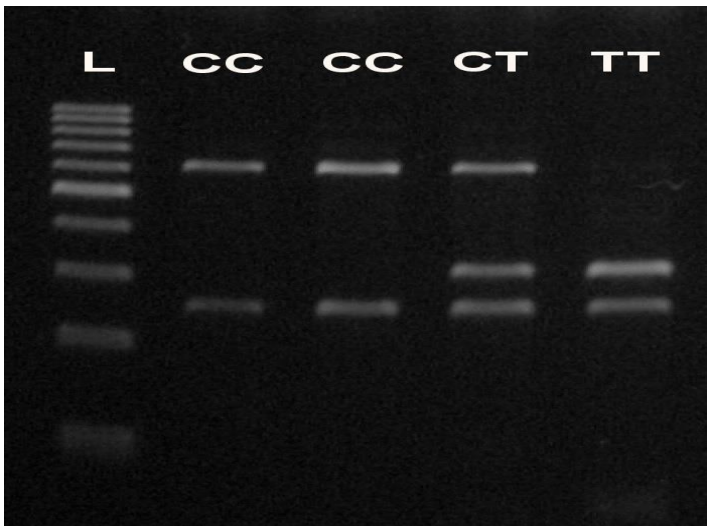

Figure 2 *Csp*6I digestion of PCR product followd by agarose gel electrophoresis. Allele C produced two fragments of 613-bp and 242-bp; allele T resulted in three fragments of 309-bp, 304-bp and 242-bp (fragments 304 and 309 bp appeared as a single band after electrophoresis)

Digestion fragments were screened by electrophoresis using 2.5% agarose gel for MC4R gene and 1.5% agarose gel for PGAM2 gene in 1 X sodium borate electrophoresis buffer containing GelRed dye (Biotium) at 180 V for 15 minutes. The gels were analyzed using UV transilluminator and photographed with a documentation system Olympus C 7070 (Figures 1 and 2).

Statistical analysis

Statistical analysis was performed using statistical software SAS Enterprise Guide 5.1 (SAS Institute Inc., 2009). The results are expressed as mean, standard deviation, minimum and maximum. Statistical differences in individual production traits between the groups were determined by ANOVA followed by Tukey´s HSD test GLM procedure.

RESULTS AND DISCUSSION

This study aimed to detect MC4R polymorphism (101G>A) and PGAM2 (195C>T) polymorphism associated to live weight, body weight, carcass weight, weight of skin, weight of thig **(Blasco *et al.*, 1993)**. We tested 44 rabbits of New Zealand breed using SNP analysis. RFLP analysis of PCR product using *Bcu*I (MC4R) restriction endonuclease produce two fragments of 100-bp and 27-bp; allele G resulted in an undigested fragment of 127-bp (figure 1). The heterozygote has both allele A and G fragments. Secondly we use RFLP analysis of PCR product using *Csp*6I (PGAM2) restriction endonuclease. Allele C produced two fragments of 613-bp and 242-bp; allele T resulted in three fragments of 309-bp, 304-bp and 242-bp as the PCR-RFLP. A *Bcu*I and *Csp*6I PCR-RFLP protocol was applied to genotype mutation on rabbits, and the frequencies observed for the genotypes AA, AG and GG resp. CC, CT and TT are presented in Table 1. Obtained allele frequencies of PGAM2 are comparable with Wu et al. (2015) results, as well as results of MC4R alleles **(Fontanesi *et al.*, 2013)**.

Table 1 Allele and genotype frequencies of SNP c.101G>A of *MC4R* and of SNP c.195C>T of *PGAM2*

Genotype frequency (MC4R) (n)			Allele frequency (MC4R)	
AA	AG	GG	A	G
0.38 (17)	0.48 (21)	0.14 (6)	0.63	0.37
Genotype frequency (PGAM2)			Allele frequency (PGAM2)	
CC	CT	TT	C	T
0.36 (16)	0.59 (26)	0.05 (2)	0.66	0.34

Legend: n – number of individuals

Table 2 reports results of the association study of MC4R and production traits. The results of the analysis of average live weight showed significant differences ($p<0.0.5$) between AA genotype versus GG genotype. Homozygote AA genotype showed also higher values of body weight, carcass weight, weight of skin, weight of thig compared to AG and GG genotypes, however non-significant. The results indicate that A allele is associated with higher live weight, body weight, carcass weight, weight of skin, weight of thig.
The melanocortin-4 receptor (MC4R) is a G-protein coupled, seven-transmembrane receptor which is expressed in the ventromedial nucleus of the hypothalamus, a region of the brain intimately most important in the regulation of feeding behaviour and are associated with body weight **(Mountjoy and Wong, 1997; Yeo *et al.*, 1998)**. **Kim *et al.* (2000)** confirmed significant associations of MC4R genotypes with backfat and growth rate in a number of lines as well as feed intake. On the base of our results we can confirmed only live weight effect.
Jiang *et al.* (2008) tested SNPs in the coding sequence of melanoeortin-4 receptor in five rabbit breeds (Harbin white rabbit, Tianfu black rabbit, Belgian hare, ZIKA rabbit, and California rabbit). Their results showed that the allele A was pre-dominant allele for each of meat rabbit breeds and AA genotype frequency was higher than AG genotype in the five studied rabbit breeds. Authors also confirmed that AG genotype is associated with body weight, eviscerated weight and feed conversion efficiency.
Association analysis between SNP and the recorded traits indicated c.195C>T genotypes are presented in Table 3. Rabbits with genotype TT had a higher levels of all production traits compared to CC and CT genotypes, however this was not statistically significant, which could be caused by small number of individuals with TT genotype. On the other hand authors **Wu *et al.* (2015)** confirmed significant association of SNP c.195C>T of PGAM2 day weight and average daily gain, however they did not confirmed associations with semi-eviscerated weight, semi-eviscerated slaughter percentage, eviscerated weight and eviscerated slaughter percentage. **Fontanesi *et al.* (2008)** by association analysis confirmed effect of PGAM2 for ham weight in pigs, but not confirmed associations for average daily gain, lean cuts and backfat thickness.
Our results could be important for the guidance of breeding plans to improve growth efficiency in commercial meat rabbit populations. MC4R and PGAM2 might be an interesting genes for economically important traits.

Table 2 Associations between *MC4R* SNPs and production traits in rabbit

Genotypes (n)	AA (17)		AG (21)		GG (6)		*P* value
Traits	**Mean ± SD**	**Min-Max**	**Mean ± SD**	**Min-Max**	**Mean ± SD**	**Min-Max**	
live weight	3085.00±398.11[a]	2050.00-3550.00	2993.18±418.39	1780.00-3820.00	2701.67±274.62[a]	2340.00-3120.00	0.049
body weight	2923.13±384.84	1910.00-3420.0	2845.45±363.05	1760.00-3340.00	2608.33±247.18	2270.00-2910.00	n.s.
weight of skin	486.25±81.31	280.00-610.00	465.00±64.64	280.00-580.00	431.67±56.01	340.00-480.00	n.s.
carcass weight	1673.25±245.34	1100.00-1996.00	1622.18±237.99	900.00-1972.00	1480.00±186.44	1256.00-1744.00	n.s.
weight of thig	530.25±71.52	364.00-624.00	518.27±70.266	296.00-612.00	487.33±61.31	416.00-568.00	n.s.

Legend: Values are presented by the means and standard deviation (Mean ± SD); [a] – statistically significant; n.s. – non-significant; n – number of individuals

Table 3 Associations between PGAM2 SNPs and production traits in rabbit

Genotypes (n)	CC (16)		CT (26)		TT (2)		*P* value
Traits	**Mean ± SD**	**Min-Max**	**Mean ± SD**	**Min-Max**	**Mean ± SD**	**Min-Max**	
live weight	3041.25±375.50	2420.00-3820.00	2940.38±436.90	1780.00-3550.00	3155.00±162.63	3040.00-3270.00	n.s.
body weight	2851.88±297.40	1910.00-3340.00	2815.77±411.35	1760.00-3420.00	3090.00±169.71	2970.00-3210.00	n.s.
weight of skin	465.00±51.38	360.00-580.00	467.31±83.02	280.00-610.00	505.00±35.36	480.00-530.00	n.s.
carcass weight	1632.25±203.31	1302.00-1972.00	1602.38±262.46	900.00-1996.00	1781.00±156.98	1670.00-1892.00	n.s.
weight of thig	528.75±62.67	420.00-612.00	508.92±74.98	296.00-624.00	559.00±21.21	544.00-574.00	n.s.

Legend: Values are presented by the means and standard deviation (Mean ± SD); n.s. – non-significant; n – number of individuals

CONCLUSION

To summarize, SNP c.195C>T of PGAM2 and c.101G>A of MC4R genes polymorphism were genotyped in a total of 44 rabbits. Association analysis showed that c.101G>A polymorphism was significantly linked with live weight (P = 0.049). Individuals with AA genotype (MC4R) indicates higher values of measured parameters. Rabbits with genotype TT (PGAM2) had a higher levels of all observed parameters compared to CC and CT genotypes, however this was not statistically significant, which could be caused by small number of individuals with TT genotype.

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