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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Polymorphism in troponin T gene (*TNNT3*) and its effect on gene expression level and production traits in pigs of selected breeds*

Małgorzata Witoń, Marian Różycki**

Department of Genetics and Animal Breeding,
National Research Institute of Animal Production,
Krakowska1, 32-083 Balice, Poland

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The aim of the study was to investigate the T2 fragment of *TNNT3* gene, identify its polymorphism and evaluate the relation between that polymorphism and *TNNT3* expression level and production traits in most common pig breeds in Poland. The study was carried out on a total of 357 unrelated Polish Large White (PLW), Polish Landrace (PL), Pietrain and Duroc gilts. Additionally, 51 Pulawska, 23 Hampshire pigs and 48 wild boars were used in search for *TNNT3* polymorphism. Three fragments of the *TNNT3* gene: Exon_1314, Exon_1415 and Exon_15 were analyzed using the PCR-SSCP method. Two alleles (A and B) were found in fragment Exon_1314 and the sequences were submitted to the GenBank database with accession numbers EF644567 and EF644568, respectively. Fragment Exon_1415 revealed the presence of three alleles (C, D and E).

Expression analysis using Real-Time PCR did not show any relation between mutations found and *TNNT3* expression level.

***TNNT3* polymorphism did not reveal any relation with the shank weigh without skin and backfat, loin eye, meat content of primary cuts, meat content of carcass and meat weight in primary cuts. However, a relation was observed between the polymorphism of *TNNT3* gene and the meat quality expressed by post-slaughter pH_{45} and pH_{24}. The values of daily gain and backfat thickness in PL pigs were related to the genotype of fragment Exon_1314 while in Pietrain pigs backfat thickness was related to the genotype of fragment Exon_1415 fragment.**

KEY WORDS: expression / gene / pigs / polymorphism / tropomyosin / troponin

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**Corresponding author: e-mail: malgorzata.witon@op.pl

The troponin-tropomyosin complex consists of troponin C, troponin I, troponin T and tropomyosin and it is a thin filament regulatory component which makes the muscle contraction possible. It is located at regularly spaced intervals which are determined by the length of tropomyosin. Troponin is a complex composed of three polypeptide chains which differ in their structure and function. Troponin C (TnC-18 kDa) binds calcium ions, troponin I (TnI - 24 kDa) is responsible for the interaction with actin by permitting the troponin complex to physically block the mutual interaction between actin and myosin, and troponin T (TnT-37 kDa), has the capacity to bind tropomyosin [Farah i Reinach 1995, Wrzosek 1997, Malnic 1998, Perry 1998, Gordon 2000].

Gene *TNNT3* in pigs was mapped on the chromosome 2 (2p14-17) by Davoli *et al*. [2002, 2003]. The protein it encodes has a sequence of 271 amino acids. The N-terminus of its polypeptide chain is rich in glutamic acid residues (more than 30% of this protein's glutamic acid residues are found in the segment from the N-terminus to exon 8). Exon 7 is rich in proline and histidine residues and also contains VHE sequence (valine – histidine – glutamic acid) repetitions – Kitamura *et al*. [2006]. Bovine TnT has a similar structure [Muroya *et al*. 2003].

Chymotrypsin and other proteolytic enzymes quickly degrade troponin T into two fragments [Oliveira *et al*. 2000, Grabarek 2001]. The first fragment (T1) is composed of amino acid residues 1 to 158 (159) and the second (T2) of residues 159–259 (160–260) – Perry [1998], Stefancsik [2003]. T1 is responsible for binding the C-terminus of tropomyosin [Oliveira *et al*. 2000], while T2, which is localized on the globular head of the troponin complex, takes part, together with troponin C (TnC) and troponin I (TnI), in the interactions between all of the components of the complex [Gordon *et al.* 2000, Huxley 2004, Chaudhuri *et al*. 2005]. It has been shown that fragment T2 plays a key role in transmitting conformational changes (controlled by calcium ions) in proteins of the thin filament [Liou i Chao, 2007].

This study aimed at investigating fragment T2 of *TNNT3* gene, at analyzing its polymorphism, and identifying the relation between that polymorphism, *TNNT3* expression level and production traits in pigs commonly reared in Poland.

Material and methods

The study was carried out on 357 unrelated gilts including 119 animals of Polish Large White (PLW), 112 of Polish Landrance (PL), 55 of Pietrain (P) and 71 animals of Duroc (D) breed. The animals came from the Polish Pig Progeny Testing Stations in Pawłowice, Mełno and Rossocha. Moreover, 51 Pulawska, 23 Hampshire (H) and 48 wild boars (WB) were used in the search for *TNNT3* polymorphism. The analysis was carried out on representatives of the PLW, PL and Duroc breeds that were free of RYR1 mutations.

Material for DNA analysis came from blood samples or from the *longissimus dorsi* (LD) muscle and was taken after slaughter and stored at -20°C. Material for

RNA analysis consisted of samples of the LD muscle taken up to 15 minutes post slaughter and stored in liquid nitrogen.

DNA was isolated using Wizard Genomic DNA Purification Kit from PROMEGA according to the protocol provided by the manufacturer. *TNNT3* gene polymorphism was determined using PCR-SSCP [Davoli *et al*. 2003]. The analysis was carried out on exons 13, 14 and 15. Because the products of the PCR were later used for single strand conformation polymorphism (SSCP) analysis, the primers were designed in such a way that the fragments produced would not be longer than 300 bp (Tab. 1). The amplification was carried out in three separate PCR reactions: exons 13-14 including intron 13 (Exon_1314), exons 14-15 including intron 14 (Exon_1415) and exon 15 (Exon_15).

Table 1. Primer sequences used for amplification of fragment T2 of *TNNT3* gene

PCR	Primer forward (5'-3')	Primer reverse (5'-3')
Exon_1314	AGAAGGCCCGGCGGGAGG	CACTGAGGTGGTCGATGTTGAG
Exon_1415	CAGACGGCCCGGGAAATGA	TCGTATTTCTGGCGCTTCA
Exon_15	ATTCAACGAAGCCTCACCAC	CTCTGCTGGTGGGTGAAAG

The products obtained from the Exon_1314, Exon_1415 and Exon_15 reactions were then used in PCR-SSCP analysis. After denaturation PCR products were separated in 10% polyacrylamide gel according to Nakajima *et al.* [1996], Fontanesi *et al.* [2001] and Nowak *et al*. [2003]. The gels were stained with silver according to Sambrook and Russel [2001] and Słomski [2004]. PCR products with different SSCP conformer patterns were sequenced at the DNA Sequencing and Oligonucleotide Synthesis Laboratory of the Polish Academy of Science Institute of Biochemistry and Biophysics (IBB PAN) in Warsaw. The obtained sequences were analyzed using BioEdit software, version 7.0.0 (www.mbio.ncsu.edu/bioedit/bioedit.html). To confirm the discovered polymorphisms, the PCR products were treated with restriction enzymes chosen using NEBcutter software, version 2.0 (http://tools.neb.com/NEBcutter2/index.php).

The entire RNA was isolated using TRI Reagent according to a modified Chomczyński and Sacchi [1987] protocol. *TNNT3* expression level in the LD muscle was determined using Real-Time PCR with a TaqMan probe (Tab. 2) on a 7500 Real-Time PCR System from APPLIED BIOSYSTEMS. Thirty animals from 6 different genotype groups were selected for the investigation, with five animals from each group. The samples were analyzed in triplicates and the housekeeping gene was GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

The carcasses dissection was carried out in accordance with standard procedure. The following traits were used to identify the relationship between *TNNT3* genotypes and the animal phenotype: mean daily live weight gain (g), gain during fattening period (g), ham weight without skin and backfat (kg), backfat thickness (mean from

Table 2. Primer sequences and the TaqMan probe used in the study

Gene	Primer sequences (5'-3')	Probe sequence (5'-3')
TNNT3	F: GAGGACGACCTGAAGAAAAAGAAG R: TTGGCCAGGTAGCTGCTGTA	CGCTGTCCTCCATGGGCGC
GAPDH	F: ACCAGGGCTGCTTTTAACTCTG R: TGACAAGCTTCCCGTTCTCC	ACCTCCACTACATGGTCTACATGT TCCAGTATGATT

5 measurements,cm), loin eye area (cm^2), meat content of primary cuts (%), meat content of carcass (%), meat weight of primary cuts (kg), loin pH 45 min. post slaughter (pH_{45}), and loin pH 24 h post slaughter (pH_{24}).

A statistical evaluation was carried out separately for each breed using least squares means method from the Generalized Linear Models (GLM) package (SAS Institute Inc., Cary, NC, USA). Covariance of the weight of right half-carcasses was used for the assessment of slaughter traits.

Results and discussion

A 284bp band was obtained as a result of Exon_1314 amplification. Single strand conformation polymorphism (PCR-SSCP) manifested the presence of two conformers (alleles A and B; Fig. 1) which were then sequenced. The sequencing revealed the presence of three single nucleotide polymorphism (SNP) mutations which distinguished the two conformers from each other: A→G change at position 42, T→C change at position 161 and C→T change at position 194. The sequences of both alleles were submitted to GenBank with accession numbers EF644567 and EF644568. The first and third mutations occurred in exon 13 and 14 respectively, and the second mutation occurred in intron 13. The C→T mutation at position 194 was used in PCR-RFLP applying *BfuC*I enzyme.

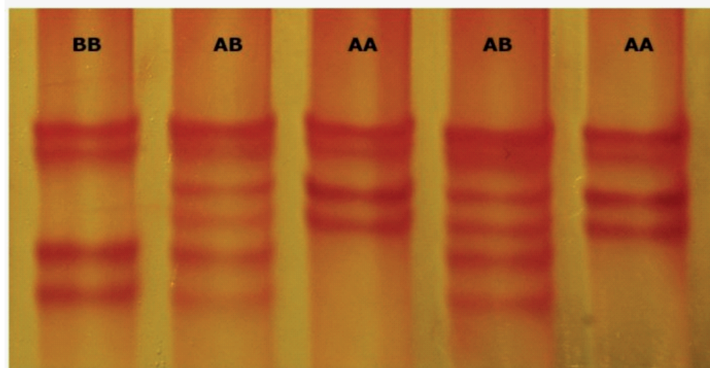

Fig. 1. SSCP analysis of fragment Exon_1314 of gene TNNT3 (10% polyacrylamide gel). AA – homozygote AA; BB – homozygote BB; AB – heterozygote.

An analysis using BioEdit computer software did not reveal any influence of the discovered mutations on the amino acid sequence of the protein.

In breeds studied the alleles occurred with different frequencies (Tab. 3). Allele B dominated in PLW, PL and Duroc while allele A in Pietrain and Hampshire gilts. In Pulawska pigs the two alleles occurred with equal frequency. In WBs only allele B was found.Genotype frequency also differed greatly between breeds (Tab. 4).

Table 3. Allele frequencies across the breeds studied. Bolded are values for more frequent alleles within each breed

Breed \ *Locus*	Exon_1314		
	n	allele A	allele B
Polish Large White	119	0.244	**0.756**
Polish Landrace	110	0.182	**0.818**
Duroc	71	0.303	**0.697**
Pietrain	55	**0.918**	0.082
Puławska Spotted	50	0.460	**0.540**
Hampshire	16	**0.687**	0.313
Wild boar	48	0.000	**1.000**

Table 4. Genotype frequencies across the breeds studied. Bolded are values for more frequent genotypes within each breed

Breed \ *Locus*	Exon_1314		
	AA	BB	AB
Polish Large White	0.101	**0.613**	0.286
Polish Landrace	0.036	**0.673**	0.291
Duroc	0.028	0.423	**0.549**
Pietrain	**0.890**	0.055	0.055
Puławska Spotted	0.320	**0.400**	0.280
Hampshire	**0.500**	0.125	0.375
Wild boar	0.000	**1.000**	0.000

A 272bp band was obtained as a result of the amplification of fragment Exon_1415. PCR-SSCP analysis revealed the existence of three conformers (allele C, D and E) – Figure 2. Samples which gave different pattern on the polyacrylamide gel were sequenced, and the sequencing revealed the presence of two SNP-type mutations and one triple-nucleotide insertion (allele C). All mutations occurred in the intron and showed no effect on the amino acid sequence of the protein. Mutation C→T at position 129 was used for PCR-RFLP applying enzyme *Sma*I which made it possible to distinguish between allele E and alleles C and D. No enzyme was found which could be used to distinguish allele C from allele D. Table 5 and 6 present alleles and genotypes frequencies, respectively.

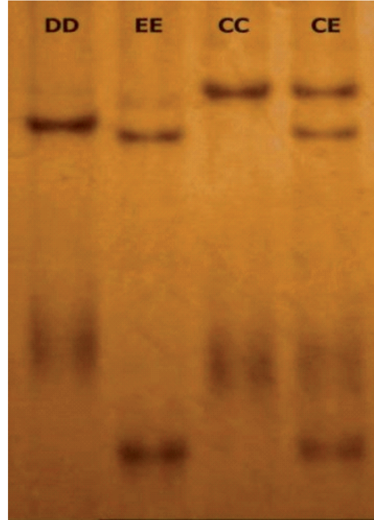

Fig. 2. SSCP analysis of fragment Exon_1415 of gene *TNNT3* (10% polyacrylamide gel). CC – homozygote CC; DD – homozygote DD; EE – homozygote EE; CE – heterozygote.

Table 5. Allele frequencies across the breeds studied. Bolded are values for more frequent genotypes within each breed

Breed \ *Locus*	Exon_1415			
	n	allele C	allele D	allele E
Polish Large White	114	**0.711**	0.254	0.035
Polish Landrace	112	0.406	0.085	**0.509**
Duroc	56	**0.625**	0.232	0.143
Pietrain	48	0.188	**0.812**	0.000
Puławska Spotted	51	**0.520**	0.402	0.078
Hampshire	23	0.261	**0.522**	0.217
Wild boar	42	**0.786**	0.000	0.214

Table 6. Genotype frequencies across the breeds studied. Bolded are values for more frequent genotypes within each breed

Breed \ *Locus*	Exon_1415					
	CC	DD	EE	CD	CE	DE
Polish Large White	**0.553**	0.114	0.000	0.263	0.053	0.017
Polish Landrace	0.107	0.036	0.259	0.098	**0.500**	0.000
Duroc	**0.393**	0.036	0.107	**0.393**	0.071	0.000
Pietrain	0.062	**0.688**	0.000	0.250	0.000	0.000
Puławska Spotted	**0.392**	**0.314**	0.039	0.177	0.078	0.000
Hampshire	0.000	0.304	0.174	**0.435**	0.087	0.000
Wild boar	**0.595**	0.000	0.024	0.000	0.381	0.000

A 176bp band was obtained as a result of the amplification of fragment Exon_15. SSCP did not reveal any differences in the migration of the conformers (Fig. 3) which suggests a lack of polymorphism in this fragment.

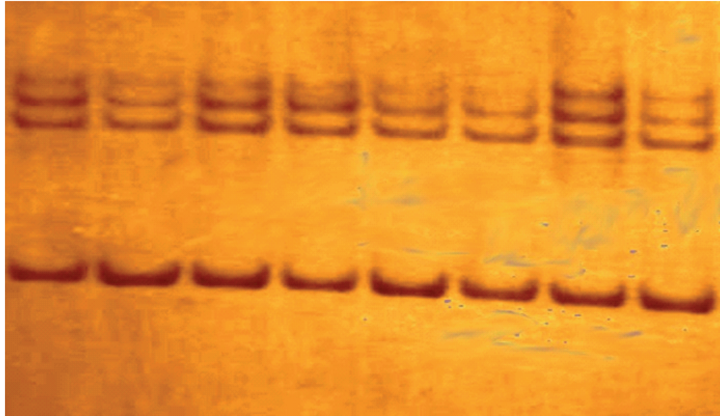

Fig 3. SSCP analysis of fragment Exon_ 15 of gene *TNNT3* (10% polyacrylamide gel).

The PLW and PL breeds exhibit good breeding value and are used as maternal component in cross-breeding. In this study, both showned similar allele distribution frequency in fragment Exon_1314 with a significant dominance of allele B. On the other hand, analysis of fragment Exon_1415 demonstrated a varying frequency of occurrence of the different alleles between these two breeds. PLW gilts exhibited a dominance of allele C. Similar results were obtained for WBs. For the PL gilts a similar frequency of occurrence of alleles C and E was characteristic. Davoli *et al*. [2003] in PLW pigs found a higher frequency of allele C (called allele 1) than of allele D (allele 2) and sporadic occurrences of allele E (allele 3).

Duroc, Pietrain and Hampshire breeds are used in cross-breeding as the paternal component. In the present investigation a dominating occurrence of allele A in fragment Exon_1314 (frequency 0.918 for Pietrain and 0.687 for Hampshire) and of allele D in fragment Exon_1415 (frequency 0.812 for Pietrain and 0.522 for Hampshire) was observed in animals from these breeds. Representatives of the Duroc breed did not follow this rule. In their case the allele frequency was similar to that observed for the PLW breed with allele B (frequencies 0.697 for Duroc and 0.756 for PLW) and allele C (frequency 0.625 for Duroc and 0.711 for PLW) occurring most often. Similar results in the analysis of fragment Exon_1415 in Pietrain and Hampshire breeds were obtained by Davoli *et al.* [2003]. Frequency of allele D, *i.e.* the most frequent allele in these breeds, was 0.80 and 1.00, respectively. On the other hand, Davoli *et al*. [2003] obtained results which differ from these of the present work in the case of the Duroc breed where allele D was the most frequent allele just like in the ather meat breeds (P and H). In a presenr study allele D occurred in only 23.2% of the tested animals.

The lack of alleles A and D in WBs suggests that these alleles resulted from mutations which occurred during breeding carried out to improve the meat quality of pigs. The even distribution of alleles in representatives of the Pulawska pigs both in fragment Exon_1314 and fragment Exon_1415 (Tab. 3 and 5, respectively) confirms that this breed is highly genetically diversified [Babicz *et al.* 2003].

Due to the occurrence of characteristic genotypes for the various breeds, it was not possible to assess the expression levels of all of the known genotypes in each breed. Therefore the expression of gene *TNNT3* was analyzed in animals with the most commonly occurring genotypes only, *i.e* CC/BB and CD/AB in PLW, CE/BB, EE/BB and EE/AB in PL, and DD/AA in Pietrain. The *TNNT3* gene expression was assessed in terms of the influence of the genotypes on each of fragments analysed (Fig. 4 and 5) and in terms of the two fragments pooled (Fig. 6). The following were numbers of animals of each genotype: for Exon_1314 – 5 animals AA, 10 animals AB, 15 animals BB; for Exon_1415 – 5 animals CC, 5 animals CD, 5 animals CE, 10 animals EE, 5 animals DD. The results are presented as the relation between

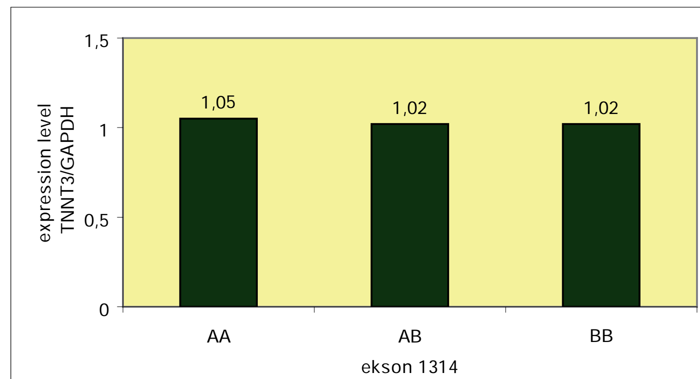

Fig 4. Expression of gene *TNNT3* depending on the genotype of fragment Exon_1314.

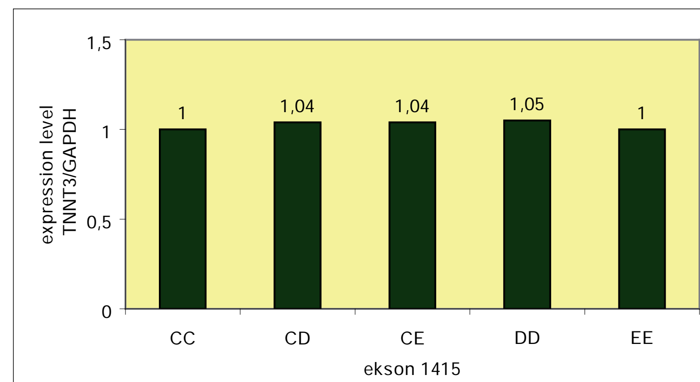

Fig. 5. Expression of gene TNNT3 depending on the genotype of fragment Exon_1415.

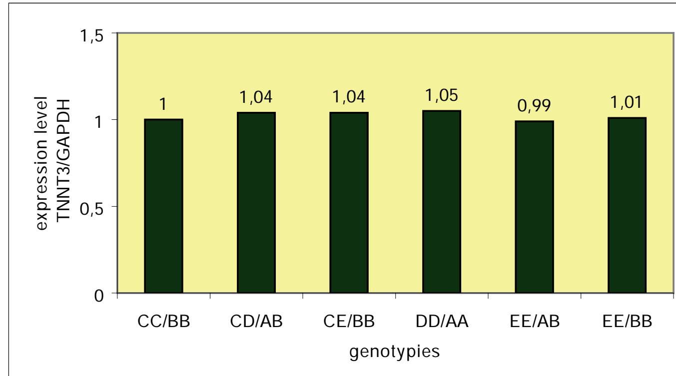

Fig. 6. Expression of gene *TNNT3* depending on the genotype in the TNNT3 locus (Exon_1314 and Exon_1415)

the level of *TNNT3* expression and the constant expression value of *GAPDH*. Changes in expression level between groups were not observed in any of the three analyses.

In this project the *TNNT3* expression level was measured in relation to the discovered mutation in fragments Exon_1314 and Exon_1415, which is a region not subject to alternative splicing. The primers and probe were projected for the conservative regions. None of the mutations were shown to have any influence on *TNNT3* expression in the LD muscle of pigs. However, Lin *et al*. [2006] showed that the mRNA level of heart troponin T decreased by 61% in comparison to a control group in turkeys with dilated cardiomyopathy resulting from a mutation on the gene encoding heart muscle troponin T.

Różycki *et al*. [2008] showed that *TNNT3* expression level in the LD muscle of pigs depends on the animal's age. However, such a relation was not confirm for the *semimembranosus* muscle. In the present study the muscle samples were taken from animals of a similar age (about 210 days) in order to eliminate the possible influence of this factor on the results.

Because of the wide variations in the frequency of different genotypes in each of the breeds studied, only groups of animals with more than five members were chosen for the statistical evaluation. The effect of the genotype of fragment Exon_1314 on the production traits was assessed based on all three genotypes present in the PLW breed and the AB and BB genotypes present in the PL and Duroc breeds. For the Pietrain breed, because of the significant domination of the AA genotype, the effect of the polymorphism of fragment Exon_ 1314 on production traits was not analyzed.

The analysis of production traits due to fragment Exon_1415 polymorphism included representatives of the PLW breed with genotype CC, DD and CD and from the PL breed with genotypes CD, CE and EE. For the Duroc breed, animals with genotypes CC and CD were analyzed and for the Pietrain breed - those with genotype

Table 7. The effect of the genotype at *locus* Ekson 1314 on production traits in pigs

Breed	Trait	Genotype	
		AB	BB
Polish Landrace	daily gain (g)	927[a]	858[a]
	mean backfat thicknes (cm)	1.63[a]	1.43[a]
Polish Large White	pH_{45}	6.60[a]	6.32[a]
Duroc	pH_{24}	5.68[A]	5.34[A]

[aA]Within rows means bearing the same superscripts differ significantly at: small letter – P≤0.05; capitals – P≤0.01.

Table 8. The effect of the genotype at *locus* Exon_1415 on production traits in pigs

Breed	Character	Genotype		
		CC	DD	CD
Polish Landrace	pH_{45}	6.32[a]	6.50	6.63[a]
Duroc	pH_{24}	5.89[A]	-	5.58[A]
Pietrain	pH_{45}	-	6.21[A]	5.58[A]
	mean backfat thicknes (cm)	-	1.18[A]	1.52[A]

[aA]Within rows means bearing the same superscripts differ significantly at: small letter – P≤0.05; capitals – P≤0.01.

DD and CD. Tables 7 and 8 show the values of only those breeds and traits that manifested significant differences between genotypes.

Significant differences in meat pH_{45} were observed in the PLW breed between the AB and the BB genotypes (6.60 and 6.32, respectively). A similar tendency was observed for the Duroc breed where the pH_{24} values differed significantly between genotype AB and BB (5.68 and 5.34, respectively). The values of the other traits for these two breeds did not show a significant difference among the analyzed groups of animals. In the case of the PL breed, faster daily gain and thicker backfat were observed in the AB then in BB genotype. In both cases the differences were significant. No significant differences were identified between the analysed genotypes in the remaining parameters.

Significant difference was observed in pH_{45} of meat between PL animals of CC and CD genotype (6.32 and 6.63, respectively). A contrary tendency occured in the Duroc breed where the pH_{24} values in animals with CC genotype were higher than in those with genotype CD (5.89 and 5.58, respectively). Highly significant differences were noted in the Pietrain breed between the pH_{45} of CD and DD genotypes (5.58 and 6.21). In that breed genotype DD also exhibited a smaller mean backfat thickness than did genotype CD. These differences were highly significant.

The obtained results do not provide a definite answer to the question whether the polymorphism of fragment T2 of *TNNT3* gene has an effect on production traits in

pigs. Taking into account the occurrence of numerous mutations of the human heart muscle troponin T encoding gene and their impact on heart function, the research should be broadened to include the entire *TNNT3* gene in pigs. Interesting results will likely be obtained by including in the analysis a sensory evaluation and assessment of technological properties of meat samples taken from representatives of different genotypes in the *TNNT3 locus*.

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Małgorzata Witoń, Marian Różycki

Polimorfizm genu mięśniowej troponiny T (*TNNT3*) oraz jego związek z ekspresją i cechami użytkowymi świń

S t r e s z c z e n i e

Celem pracy było poznanie fragmentu T2 genu *TNNT3*, analiza jego polimorfizmu oraz określenie związku pomiędzy polimorfizmem, a poziomem jego ekspresji oraz cechami użytkowymi świń hodowanych w kraju.

Badaniami objęto łącznie 357 niespokrewnionych loszek rasy wbp, pbz, pietrain oraz duroc. Do poszukiwania polimorfizmu wykorzystano dodatkowo 51 osobników rasy puławskiej, 23 sztuki zwierząt rasy hampshire i 48 dzików.

Spośród trzech, analizowanych metodą PCR-SSCP fragmentów genu – Exon_1314, Exon_1415 oraz Exon_15, polimorfizm wykazano w dwóch pierwszych. We fragmencie Exon_1314 znaleziono dwa allele: A oraz B, których sekwencje umieszczono w bazie GenBank, pod numerami akcesyjnymi EF644567 oraz EF644568. Fragment Exon_1415 wskazywał na występowanie trzech alleli: C, D oraz E. W obu fragmentach zaobserwowano występowanie pewnych, charakterystycznych dla ras genotypów.

Analiza komputerowa wykrytych mutacji (głównie typu SNP) nie wykazała ich wpływu na sekwencję kodowanego białka.

Analiza ekspresji techniką Real-Time PCR nie wykazała zależności pomiędzy poziomem ekspresji genu *TNNT3,* a mutacjami w obrębie fragmentu T2 tego genu.

Polimorfizm badanego fragmentu nie ukazał związku z cechami rzeźnymi świń, takimi jak: masa szynki zadniej bez skóry i słoniny, powierzchnia oka polędwicy, zawartość mięsa w wyrębach podstawowych, zawartość mięsa w tuszy oraz masa mięsa wyrębów podstawowych. Zaobserwowano natomiast związek pomiędzy polimorfizmem genu *TNNT3*, a jakością mięsa, określaną poubojowym jego odczynem (pH oraz pH24).

Wartości przyrostu dziennego oraz grubości słoniny, u osobników rasy pbz, były zależne od genotypu we fragmencie Exon_1314, natomiast u zwierząt rasy pietrain grubość słoniny zależała od genotypu we fragmencie Exon_1415.