POLYMORPHISMS OF CANDIDATE GENES AND THEIR ASSOCIATION WITH INTRAMUSCULAR FAT IN DUROC PIG

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ABSTRACT

This study aimed to determine single nucleotide polymorphisms (SNPs) in four candidate genes: Fatty Acid Binding Protein 3 (*FABP3*), Adrenoceptor Beta 3 (*ADRB3*), Perilipin 2 (*PLIN2*), and Acyl-CoA Synthetase Long-Chain Family Member 4 (*ACSL4*) as well as anassociation between these SNPs and intramuscular fat (IMF) in Duroc pig population. A total of 200 pigs was collected, and DNA samples were extracted. The PCR-RFLP method was used to analyze five SNPs, including *FABP3-Hinf*I, *FABP3-Bsrf*I, *ADRB3-Taq*I, *PLIN2-Mva1269*I and *ACSL4-Rsa*I.A General Linear Model was used to evaluate the association between SNPs and IMF. Results estimated the frequency of alleles and genotypes for SNPs in these candidate genes. While only two genotypes were found in three SNPs, *FABP3*/*Hinf*I, *PLIN2*/*Mva1269*I, and *ACSL4*/*Rsa*I, SNP at *ADRB3*/*Taq*I indicated three different genotypes, and the SNP at *FABP3*/*Bsrf*I was homozygous. All homozygous genotypes of SNPs accounted for higher frequency except the SNP of *ADRB3*/*Taq*I. However, they had no significant association between the SNPs and the IMF in experimenting with the Duroc pig population.

Keywords: *Duroc pig, Intramuscular fat, single nucleotide polymorphism, genetic marker, FABP3*, *ADRB3*, *PLIN2*, *ACSL4*.

INTRODUCTION

The intramuscular fat (IMF) content is essential for determining the meat quality (Van Wijk et al., 2005). This parameter exhibits a positive correlation with meat tenderness, juiciness, and taste (Fernandez et al., 1999). Several factors are influencing IMF contents, such as genetics and breed, management, and nutrition. This characteristic is so far in concern to improve meat quality aiming to serve customer's needs.

According to several studies assessing genetic markers related to meat quality traits in pig populations, potential candidate genes consist of *FABP3*, *ADRB3*, *PLIN2*, *ACSL4* proved to develop the genetic markers for high IMF deposition selection. *FABP3* or *H-FABP*, the fatty acid-binding protein, is widely expressed in many tissues such as the intestine, liver, and kidney, localized on porcine chromosome 6 (Gerbens et al., 1997). Gene *FABP3* plays a critical role in intracellular long-chain fatty acid uptake and transport by binding lipids and regulating metabolic homeostasis (Chmurzyńska, 2006). *ADRB3*, beta-3-adrenergic receptor gene, is located in chromosome 15q13-14 within quantitative trait loci (QTL) regions for fatness trait (Nowacka-Woszuk et al., 2008). *ADRB3* encodes a significant mediator of lipolytic and thermogenic effects in adipose tissue (Xue et al., 2015a). *PLIN2*, perilipin 2, a cytosolic protein, promotes the formation and stabilization of the intracellular lipid droplets, organelles involved in the storage of lipid depots (Brasaemle et al., 1997). Gene *PLIN2* becomes a biological and positional candidate gene for fat deposition, a polygenic trait affecting carcass and meat quality (Davoli et al., 2011). A gene encodes for long-chain acyl-CoA synthetase 4, *ACSL4*, plays a critical role in lipid biosynthesis and fatty acid degradation (Mercade et al., 2006), which makes this gene a potential candidate gene associated with IMF content. The studies on molecular marker-assisted selection related to economic characteristics areessential to productivity improvement and efficiency increase. Therefore, to

satisfy the practical needs, pig breeds selection for high intramuscular fat content based on the functional potential molecular markers would be necessary. This study determined the SNPs on *FABP3*, *ADRB3*, *PLIN2*, and *ACSL4*, correspondingly, the association between these SNPs with the IMF content in the imported Duroc pig population raising in Dabaco Nucleus Breeding Pig Company Limited.

MATERIAL AND METHODS

Time and location

The experiment was carried out at Key Laboratory of Animal CellBiotecnology, National Institute of Animal Science, Thuy Phuong, Bac Tu Liem, Ha Noi and Dabaco Nucleus Breeding Pig Company, Tien Du, Bac Ninh from June 2019 to December 2020.

Animal and phenotype data

The experimental population includes 200 Duroc pigs, which were generations of imported parents. Pigs were raised in the same condition following the porkerfeeding procedure in Dabaco Nucleus Breeding Pig Company Limited, Tien Du, Bac Ninh.

The IMF content was collected from the experimental population when each individual's body weight reached 95 to 110 kg using ALOKA SSD 550v Ultrasound Console with the UST 5011 12.5cm linear probe 10th rib. The data collected was analyzed on Biosoft Toolbox.

SNPs analysis by PCR-RFLP

The genomic DNA was isolated from the ear samples following the GeneJET Genomic DNA Purification Kit procedure (Thermo Fisher Scientific, Vilnius, Lithuania).

The candidate genes *FABP3*, *ADRB3*, *PLIN2* and *ACSL4* fragments were amplified from the genomic template extracted by PCR by specific primers reported from previous studies (Table 1).

Table 1. Primer sequence and PCR-RFLP conditions for each SNP

Gene	Primer sequence	Tm	Site	Size	RE
FABP3	5'- GGA CCCAAGATGCCTACGCCG -3' 5'- CTGCATCTTTGACCAAGAGG -3' (Gerbens et al., 1997)	57	HM591296 5'-UTR c.-314T>C	693bp	(*Hinf*I)
FABP3	5'- CAGCCCAAGAGTGAGTTTCC -3' 5'- GAATAGGAAGCCCCATG -3' (Schwab et al., 2009)	57	HM591296 5'-UTR c.-158G>T	321bp	(*Bsrf*I)
ADRB3	5'- CGTTCAACCCGCTCATCTACTGC -3' 5'- GGTTCCCTACTCTGTGCCCGTCTT -3' (Cieslak et al., 2009)	63	ENSSSCT 00000017229 Exon 1 c.1192G>A	315bp	*(TaqI)*
PLIN2	5'- AGAAAATTCAAGGCACTCAGG -3' 5'- TTAGCTGCATCCTGTTAGGG -3' (Davoli et al., 2011)	55	GU461315 Intron 7 g.184G>A	317bp	*(Mva1269I)*
ACSL4	5'-CAGAAGATGCTTAAATATTAAGCATGACA-3' 5'-TGTCTAACCTACACAACAATTATGAATCC -3' (Ruść et al., 2011)	61.8	DQ144454 3'-UTR g.2645G>A	181bp	*(RsaI)*

Note: T_m: Annealing temperature; RE: Restriction Enzyme

The amplification conditions were: 94°C for 5 min, 35 cycles at 94°C for 30 s, Tm for 50 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The Tm was specific for each candidate gene in Table 1. The PCR product quality was checked, and identified the target size by electrophoresis on 2% agarose gel.

The RFLP method was used for genotyping the candidate genes. The PCR products were incubated with respective restriction enzymes (*Hinf*I, *Bsrf*I, *Taq*I, *Mva1269*I, *Rsa*I). The digestion reaction was carried out at 65°C for *Taq*I and 37°C for the other enzymes for 16 h.

The digestion PCR products were run on 2-3% agarose gel for identification of 5 SNPs on gene fragments *FABP3* (*Hinf*I), *FABP3* (*Bsrf*I), *ADRB3* (*Taq*I), *PLIN2* (*Mva1269*I), *ACSL4* (*Rsa*I) based on the fragment size.

Statistical analysis

All statistical analyses were performed using Minitab 16 (Minitab, LLC.). The frequency of allele and genotype of each SNP were calculated.

Evaluation of the association between each SNP of *FABP3*, *ADRB3*, *PLIN2,* and *ACSL4* and the IMF content following the model:

$$y_{ijk} = \mu + G_i + Se_j + G_i*S_j + S_k + e_{ijk}$$

Which: y_{ijk} : the observed value of trait IMF content; μ : the least square mean;

G_i : the effect of genotype i (i=3, three genotypes respectively);

Se_j : the effect of sex j (j=2, male or female);

G_i*S_j : the effect of interaction between genotype i and sex j;

S_k : the effect of sire; e_{ijk} : the random error

Tukey's post-hoc tests were performed when significant differences were identified

RESULTS AND DISCUSSION

The SNPs of the candidate genes

The SNP in 3'-UTR of *ACSL4* was found by using the restriction enzyme *Rsa*I. The result of the electrophoresis image (Figure 1) showed that the tested Duroc pig population existed two genotypes consisting of a homogenous AA (135 and 47 bp) and a heterogenous AG (135, 108, 47, and 26 bp).

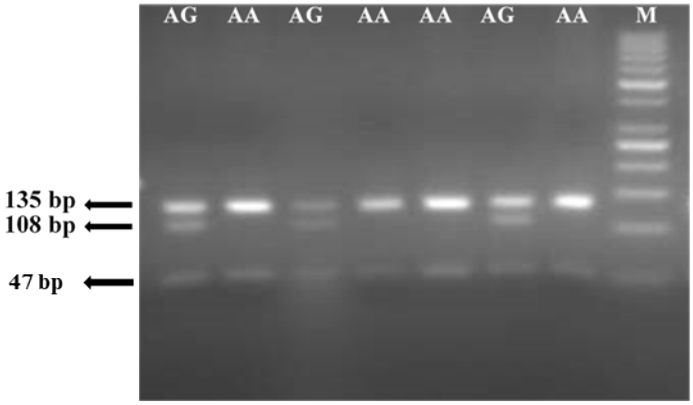

Figure 1. Genotyping of *ACSL4* by PCR-RFLP. M = Marker 50 bp (Thermo Fisher Scientific)

Restriction enzyme *Mva1269*I identified the SNP in intron 7 g.184G/A of *PLIN2*. This SNPhad two genotypes, including a homogenous GG (186 and 131 bp) and a heterogenous AG (317, 186, and 131 bp) (Figure 2).

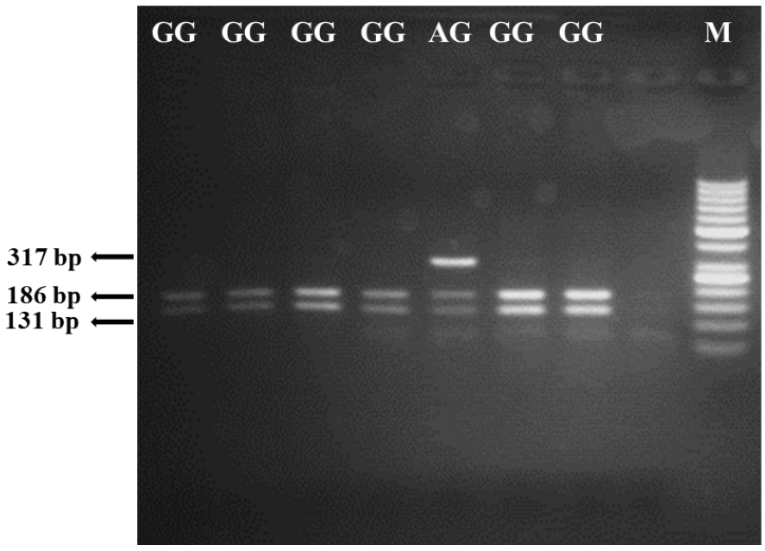

Figure 2. Genotyping of *PLIN2* by PCR-RFLP. M = Marker 50 bp (Thermo Fisher Scientific)

The polymorphism at 5'- Upstream -309 of *FABP3* was found by enzyme *Hinf*I which indicated two genotypes, a homogenous TT (339, 172, 98, 59, and 25 bp) and a heterogenous CT (339, 231, 172, 98, 59, and 25 bp) (Figure 3). This gene's polymorphism was at 5'-UTR (G/T, -158) by enzyme *Bsrf*I. However, in this study, only one homogenous genotypeTT was found with the size of 321 bp (Figure 4), with the allele frequency of T was 1 (Table 2).

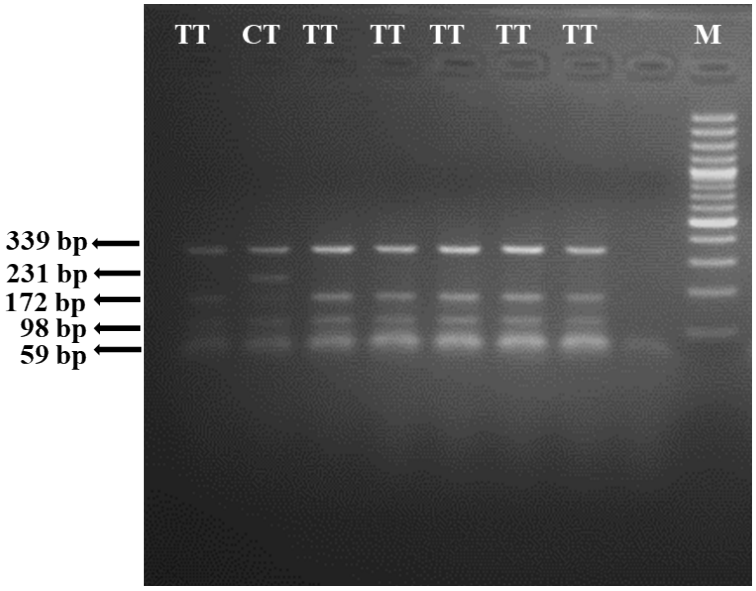

Figure 3. Genotyping of *FABP3* (*Hinf*I) by PCR-RFLP. M = Marker 50 bp (Thermo Fisher Scientific)

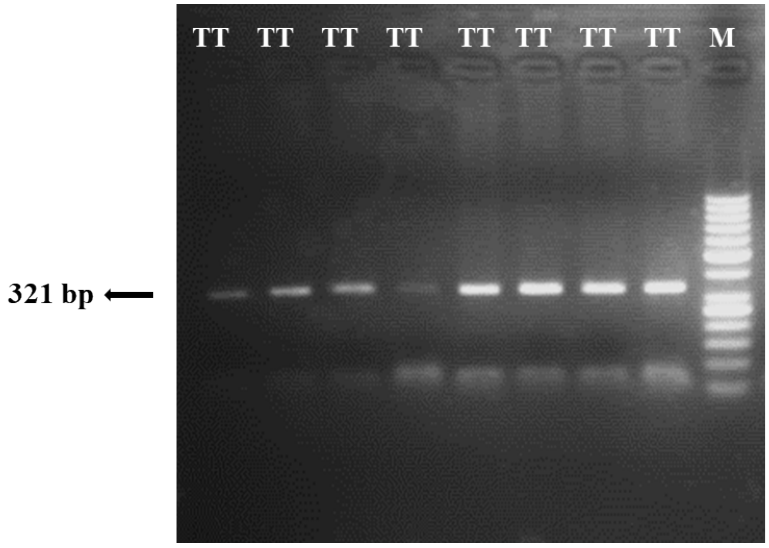

Figure 4. Genotyping of *FABP3* (*Bsrf*I) by PCR-RFLP. M = Marker 50 bp (Thermo Fisher Scientific)

Restriction enzyme *Taq*I was used to determine the SNPs of *ADRB3* in the exon 1 region, in which three genotypes found: a homogenous AA (315 bp), a heterogenous AG (315, 143, and 172 bp), and a homogenous GG (172 and 143 bp) (Figure 5).

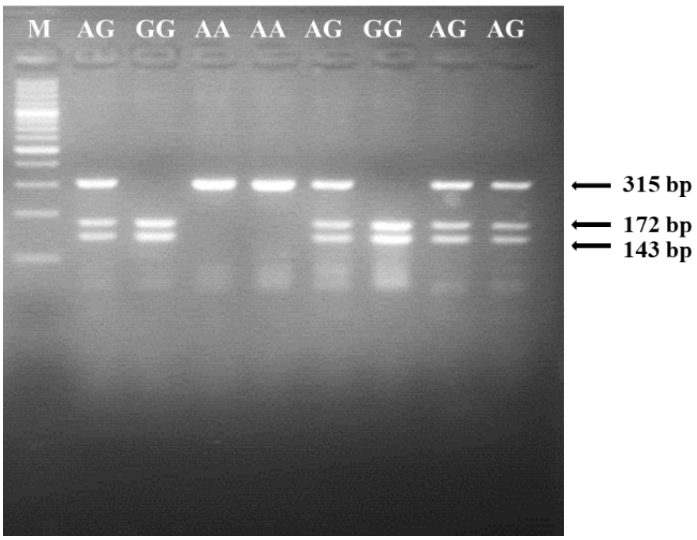

Figure 5. Genotyping of *ADRB3* by PCR-RFLP. M = Marker 100 bp (Thermo Fisher Scientific)

Allele and genotype frequency of the SNPs

Genotype and allele frequencies of SNPs in candidate genes were shown in Table 2. In the *ACSL4* gene, the AA genotype was the highest percentage with 91.5%, and the AG genotype was 8.5%. The *PLIN2* gene consisted of 93.5% GG genotype and 6.5% AG genotype, with 0.03 allele A and 0.97 allele G in the test Duroc pig population. The same result was found in an Italian Duroc pig population with 0.92 allele G and 0.08 allele A at the g.184G/A SNP (Davoli et al., 2011). Gene *FABP3*at5′- Upstream 1125-1817 (-309) SNP had two genotype TT and CT with the genotype frequencies were 0.925 and 0.075, respectively; and the allele frequencies for T and C were 0.96 and 0.04 (Table 2). Schwab's study found that the allele T

frequency was 0.98, and no CC genotype report in the Duroc pig population (Schwab et al., 2009). Gene *ADRB3* had three genotypes AA, AG, and GG, with the highest genotype frequency, which was AG at 0.625. This result was also confirmed in Duroc pig in Poland (Cieslak et al., 2009) and in the crossbred Shanzhu × Duroc pig population (Xue et al., 2015a) with the highest genotype frequency belonged to the heterogeneous genotype AG.

Table 2. The allele and genotype frequency of the SNPs

SNP	Allele frequency	Genotype frequency
ACSL4 (*Rsa*I)	A: 0.95 G: 0.05	AA: 0.92 (183) AG: 0.08 (17) GG: 0 (0)
PLIN2 (*Mva1269*I)	A: 0.03 G: 0.97	AA: 0 (0) AG: 0.07 (13) GG: 0.93 (187)
FABP3 (*Hinf*I)	T: 0.96 C: 0.04	TT: 0.93 (185) CT: 0.07 (15) CC: 0 (0)
FABP3 (*Bsrf*I)	T: 1 G: 0	TT: 1 (200) GT: 0 (0) GG: 0 (0)
ADRB3 (*Taq*I)	A: 0.4 G: 0.6	AA: 0.07 (15) AG: 0.63 (125) GG: 0.30 (60)

The association between the SNPs and the IMF content

Table 3 showed the association analysis between the SNPs of four candidate target genes with the IMF content. The results indicated no association between the SNPs of *FABP3*, *ADRB3*, *PLIN2*, and *ACSL4* and the IMF in the recent study.

Table 3. The association between the SNPs and the IMF content

Gene	Genotype	IMF (%)			
		N	***LSM***	***SE***	***P***
ACSL4	AA	183	2.84	0.05	0.10
	AG	17	2.63	0.13	
	GG	0	0	0	
PLIN2	AA	0	0	0	0.22
	AG	13	2.66	0.14	
	GG	187	2.85	0.05	
FABP3 (*Hinf*I)	TT	185	2.84	0.14	0.10
	CT	15	2.62	0.05	
	CC	0	0	0	
ADRB3	AA	15	2.59	0.16	0.17
	AG	125	2.82	0.05	
	GG	60	2.9	0.07	

The SNP c.-314T>C of *FABP3* was analyzed in several pig breeds such as Yanan, Jinhua, Duroc, Landrace, Yorkshire, and Duroc × (Landrace × Yorkshire). This SNP, along with two other SNP sidentified by restriction enzymes *Msp*I, *Hae*III were proved associated with the IMF content in a hybrid Duroc × (Landrace × Yorkshire) (p <0.01)(Chen et al., 2014). Moreover, this SNP was also found in a crossbred Duroc × Meishan due to their correlation with the fatty acid absorption and consumption function (Binas et al., 1999) andthe expression in adipose tissue (Li et al., 2007). Another SNP was determined in gene *FABP3* at 5′-UTR (G/T, -158) by enzyme *Bsrf*I. It was correlated with backfat thickness in synthetic line 990 (the crossings of Polish Large White, Duroc, Hampshire, and three lines of Landrace) (Chmurzynska et al., 2007). However, these two SNPs of *FABP3*had no association with the IMF content in this study. The same result was shown in a commercial crossbred Shanzhu × Duroc; the SNP*FABP3*/*Hinf*I consisted of two genotypes TT and CC, with no correlation with the IMF content (p<0.05) (Xue et al., 2015a). The SNP*FABP3*/*Bsrf*I was significantly associated with the estimated breeding value for intramuscular fat (IMF-EBV) in the Duroc control line. Still, there was no correlation in the Duroc select line (the result of five generations of selection for increased IMF) (p>0.99) (Schwab et al., 2009).

For gene *ADRB3*, the statistically analyzed result showed no association between the SNP of gene *ADRB3* with the IMF content. In the commercial crossbred Shanzhu × Duroc pig population, the SNP c.1192G>A on exon 1 was associated with the IMF content, monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) (p<0.05) (Xue et al., 2015b). However, this SNP did not correlate with productivity characteristics such as backfat thickness and abdominal fat weight in several breeds, including Yorkshire, Landrace, Duroc, Pietrain, and Hampshire (Cieslak et al., 2009). In the research on investigating the correlation ofthe IMF content and fatty acid components with the mRNA expression level of candidate genes consisting of *AdPLA*, *ADRB3*, *LEPR*, *MC4R*, *PPARγ*, *PPARα*, *LPL*, *PEPCK*, and *SCD* in the commercial crossbred Duroc × Shanzhu pig population, gene *ADRB3* was found no association with the analyzedtraits (Wang et al., 2013).

The SNP of gene *ACSL4* at 3′-UTR did not correlate with the IMF content trait in this study. In a crossbred(Landrace × Yorkshire) × Duroc pig population, gene *ACSL4* was associated with some meat quality parameters such as water and glycogen content, glycolytic potential, and pH (Ruść et al., 2011). Furthermore, the SNP *ACSL4*/*Rsa*I was also determined in Duroc, Landrace, Yorkshire, and the crossbredDuroc × (Landrace × Yorkshire) pig populations, and the association with the IMF content (Chen et al., 2014). This association was statistically significant only in exported pig breeds but not an indigenous Chinese pig population.

No correlation between the SNP of gene *PLIN2* at intron 7 g.184G/Aand the IMF content was shown (Table 3). This SNP was also found in Duroc, Landrace, Pietrain, and Belgian Landrace pig populations by High Resolution Melting method, with the same result of the dominant G allele (>0.8), still, the study showed no association between g.184G/A SNP with the IMF content (Davoli et al., 2011).

While comparing and evaluating this study's results and previous studies on the same selected candidate genes, genetic polymorphisms were similar. The study did not show a significant association between these polymorphisms with the IMF content, as indicated in previous studies. The reason could be related to the difference in breeds, size, the selection process for mating, or random mating of tested populations.

CONCLUSION

Inthe experimental Duroc pig population, the SNPs of four candidate genes *FABP3*, *ADRB3*, *PLIN2*, and *ACSL4* were described by the number of genotypes as well as each SNP's allele and genotype frequency. While only two genotypes were found inthree SNPs, *FABP3*/*HinfI,PLIN2*/*Mva1269*I, and *ACSL4*/*Rsa*I, SNP at *ADRB3*/*Taq*I indicated three different genotypes, and the SNP at *FABP3*/*Bsrf*I was homozygous. Allhomozygous genotypes of SNPs accounted for higher frequency except SNP *ADRB3/TaqI.* Although no significant association between SNPs with the IMF contentin the experimental Duroc pig population was found, further studies on other SNPs of these candidate genes or other pig populations are recommended.

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REFERENCES

Binas, B., Danneberg, H., McWhir, J. I. M., Mullins, L., and Clark, A. J. 1999. Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. The FASEB Journal, 13(8), pp. 805–812.

Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J., and Londos, C. 1997. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. Journal of Lipid Research, 38(11), pp. 2249–2263.

Chen, J. N., Jiang, Y. Z., Cen, W. M., Xing, S. H., Zhu, L., Tang, G. Q., Li, M. Z., Jiang, A. A., Lou, P. E., and Wen, A. X. 2014. Distribution of H-FABP and ACSL4 gene polymorphisms and their associations with intramuscular fat content and backfat thickness in different pig populations. Genet. Mol. Res, 13(3), pp. 6759–6772.

Chmurzyńska, A. 2006. The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. Journal of Applied Genetics, 47(1), pp. 39–48.

Chmurzynska, A., Szydlowski, M., Stachowiak, M., Stankiewicz, M., and Switonski, M. 2007. Association of a new SNP in promoter region of the porcine FABP3 gene with fatness traits in a Polish synthetic line. Animal Biotechnology, 18(1), pp. 37–44.

Cieslak, J., Nowacka-Woszuk, J., Bartz, M., Fijak-Nowak, H., Grzes, M., Szydlowski, M., and Switonski, M. 2009. Association studies on the porcine RETN, UCP1, UCP3 and ADRB3 genes polymorphism with fatness traits. Meat Science, 83(3), 551–554. https://doi.org/10.1016/j.meatsci.2009.07.001

Davoli, R., Gandolfi, G., Braglia, S., Comella, M., Zambonelli, P., Buttazzoni, L., and Russo, V. 2011. New SNP of the porcine Perilipin 2 (PLIN2) gene, association with carcass traits and expression analysis in skeletal muscle. Molecular Biology Reports, 38(3), pp. 1575–1583. https://doi.org/10.1007/s11033-010-0266-0

Fernandez, X., Monin, G., Talmant, A., Mourot, J. and Lebret, B. 1999. Influence of intramuscular fat content on the quality of pig meat—1. Composition of the lipid fraction and sensory characteristics of m. longissimus lumborum. Meat Science, 53(1), pp. 59–65.

Gerbens, F., Rettenberger, G., Lenstra, J. A., Veerkamp, J. H., and Te Pas, M. F. W. 1997. Characterization, chromosomal localization, and genetic variation of the porcine heart fatty acid-binding protein gene. Mammalian Genome, 8(5), pp. 328–332. https://doi.org/10.1007/s003359900433

Li, B., Zerby, H. N. and Lee, K. 2007. Heart fatty acid binding protein is upregulated during porcine adipocyte development. Journal of Animal Science, 85(7), pp. 1651–1659.

Mercade, A., Estelle, J., Pérez-Enciso, M., Varona, L., Silio, L., Noguera, J. L., Sanchez, A. and Folch, J. M. 2006. Characterization of the porcine acyl-CoA synthetase long-chain 4 gene and its association with growth and meat quality traits. Animal Genetics, 37(3), pp. 219–224.

Nowacka-Woszuk, J., Szczerbal, I., Fijak-Nowak, H. and Switonski, M. 2008. Chromosomal localization of 13 candidate genes for human obesity in the pig genome. Journal of Applied Genetics, 49(4), pp. 373–377.

Ruść, A., Sieczkowska, H., Krzecio, E., Antosik, K., Zybert, A., Koćwin-Podsiadła, M. and Kamiński, S. 2011. The association between acyl-CoA synthetase (ACSL4) polymorphism and intramuscular fat content in (Landrace × Yorkshire) × Duroc pigs. Meat Science, 89(4), pp. 440–443. https://doi.org/10.1016/j.meatsci.2011.05.008

Schwab, C. R., Mote, B. E., Du, Z. Q., Amoako, R., Baas, T. J. and Rothschild, M. F. 2009. An evaluation of four candidate genes for use in selection programmes aimed at increased intramuscular fat in Duroc swine. Journal of Animal Breeding and Genetics, 126(3), pp. 228–236. https://doi.org/10.1111/j.1439-0388.2008.00770.x

Van Wijk, H. J., Arts, D. J. G., Matthews, J. O., Webster, M., Ducro, B. J. and Knol, E. F. 2005. Genetic parameters for carcass composition and pork quality estimated in a commercial production chain. Journal of Animal Science, 83(2), pp. 324–333.

Wang, W., Xue, W., Jin, B., Zhang, X., Ma, F. and Xu, X. 2013. Candidate gene expression affects intramuscular fat content and fatty acid composition in pigs. Journal of Applied Genetics, 54(1), pp. 113–118.

Xue, W., Wang, W., Jin, B., Zhang, X. and Xu, X. 2015a. Association of the ADRB3, FABP3, LIPE, and LPL gene polymorphisms with pig intramuscular fat content and fatty acid composition. Czech J Anim Sci, 60, pp. 60–66.

Xue, W., Wang, W., Jin, B., Zhang, X. and Xu, X. 2015b. Polymorphisms with pig intramuscular fat content and fatty acid composition. 2015(164320), pp. 60–66. https://doi.org/10.17221/7975-CJAS

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