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Identification and characterization of novel single nucleotide polymorphism markers for fat deposition in muscle tissue of pigs using amplified fragment length polymorphism

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Objective: This study was conducted to identify and evaluate the effective single nucleotide polymorphism (SNP) markers for fat deposition in the *longissimus dorsi* muscles of pigs using the amplified fragment length polymorphism (AFLP) approach.
Methods: Sixty-four selective primer combinations were used to identify the AFLP markers in the 20 highest- and 20 lowest-intramuscular fat (IMF) content phenotypes. Five AFLP fragments were converted into simple codominant SNP markers. These SNP markers were tested in terms of their association with IMF content and fatty acid (FA) composition traits in 620 commercially crossbred pigs.
Results: The SSC7 g.4937240C>G marker showed an association with IMF content ($p<0.05$). The SSC9 g.5496647_5496662insdel marker showed a significant association with IMF content and arachidonic levels ($p<0.05$). The SSC10 g.71225134G>A marker revealed an association with palmitoleic and ω9 FA levels ($p<0.05$), while the SSC17 g.61976696G>T marker showed a significant association with IMF content and FA levels of palmitoleic, eicosenoic, arachidonic, monounsaturated fatty acids, and ω9 FA levels. However, no significant association of SSC8 g.47338181G>A was observed with any IMF and FA levels in this study.
Conclusion: Four SNP markers (SSC7 g.4937240C>G, SSC9 g.5496647_5496662insdel, SSC10 g.71225134G>A, and SSC17 g.61976696G>T) were found to be associated with IMF and/or FA content traits in commercially crossbred pigs. These findings provide evidence of the novel SNP markers as being potentially useful for selecting pigs with the desirable IMF content and FA composition.

Keywords: Amplified Fragment Length Polymorphism (AFLP); Intramuscular Fat; Fatty Acid; Pig

INTRODUCTION

Increasing levels of intramuscular fat (IMF) content has a positive impact on the meat quality of pork [1]. The fatty acid (FA) composition of pork is an important factor in defining lipid quality due to its implications for human health [2]. Fat deposition and fatty acid composition in pork are very complex traits that are likely to be controlled by a variety of genes [3]. There have been several attempts made to identify the quantitative trait loci (QTL) for IMF content and FA composition in pigs using microsatellite markers [2,4,5]. Recently, advances in the high-density single nucleotide polymorphisms (SNPs) chip approach have allowed for the genotyping of a large number of SNPs throughout the genome [6]. The genome wide association study approach has been carried out to detect QTLs affect on IMF and FA content in the muscle tissue of pigs [7-9]. Empirical evidence of identifying QTL for IMF and FA content traits have been successfully performed using F2 or crossbred experimental populations, e.g. Iberian and Land-

race [7], White Duroc and Erhualian [9], Yorkshire and Korean native pigs [10]. However, QTL segregation must be confirmed in different breeds for successful marker-assisted and genomic selection [5]. Very few research efforts have undertaken the QTL for IMF and FA content traits in the commercial line pig breeds [4,8]. Genome scanning of chromosome regions for IMF and FA content traits in the commercial lines is important for improving meat quality in the pig production process.

Amplified fragment length polymorphism (AFLP) genome scan is an alternative relevant approach in detecting potential genetic markers with specific traits. It is a robust and highly-throughout tool for screening the whole genome and produces a large number of markers that can be converted to simple codominant locus-specific markers without prior knowledge of the specific sequences [11]. The AFLP approach has been used to successfully identify the QTLs for meat quality and carcass traits in both pigs and cattle [11,12]. In this present study, we identified and evaluated the effects of novel SNP markers for IMF and FA composition in the *longissimus dorsi* (LD) muscle tissue of the commercially crossbred pigs using the AFLP approach.

MATERIALS AND METHODS

Animals, DNA isolation, intramuscular fat, and fatty acid content determination

The study protocol was approved by the Animal Ethics Committee of the Faculty of Agriculture, Chiang Mai University, Thailand. A total of 620 commercially crossbred pigs (Duroc and Large White×Landrace) (322 gilts and 298 barrows) were reared under commercial conditions. Animals were slaughtered according to applicable standards at the slaughter-weight of 90 kg. The LD muscles were collected from the 10th rib for DNA extraction and for IMF content measurement. The genomic DNA was extracted according to the standard phenol-chloroform protocol. The IMF content of each LD sample was determined by the ether extraction method and expressed as the percentage of IMF in fresh meat. Fatty acid composition was determined by using a gas chromatography apparatus (SCION 456-GC, Bruker Daltonic Inc, Fremont, CA, USA) with a RT-2560 capillary column (RESTEK, Bellefonte, PA, USA). Fatty acid composition was reported as the percentage of total fatty acids.

Amplified fragment length polymorphism analysis

The AFLP analysis was conducted by selecting the specimens with the 20 highest- and 20 lowest-IMF content from the 620 commercially crossbred pigs. The AFLP procedures were performed according to the previous study [11]. Genomic DNA samples (250 ng) were digested with FastDigest *Eco*RI and subsequently with FastDigest *Taq*I (Fermentas, Hanover, MD, USA) based on the manufacturer's instructions. Restriction fragments were ligated to 10 pmol of *Eco*RI-adapters and 50 pmol of *Taq*I-adapters in 30 μL of the ligation mixture that contained 1 U T4 DNA ligase. The reaction was incubated at 20°C for 2 h and then at 4°C overnight. The ligated DNA fragments were diluted 1:5 with double-distilled water and used as a template for amplification. Pre-selective amplification was performed in 25 μL containing 10 ng of diluted ligation fragments, 1× *Taq* Buffer (20 mM Tris–HCl, pH 8.4, 50 mM $(NH_4)_2SO_4$; Fermentas, USA), 3.0 mM $MgCl_2$, 0.25 mM each of the four dNTPs, 0.25 U *Taq* DNA polymerase (Fermentas, USA), 4 pmol of *Eco*RI-N primer (E-A) and 4 pmol of *Taq*I-N primer (T-C). The polymerase chain reaction (PCR) program was performed as follows: 3 min at 94°C, 20 cycles of 30 s at 94°C, 1 min at 56°C and 1 min at 72°C, followed by 5 min at 72°C, and ended at 4°C. The pre-amplification products were diluted 1:20 with double-distilled water and then used as DNA templates for selective-amplification. The selective amplification was carried out with 64 primer combinations (E-ANN and T-CNN). The reaction was carried out in 12.5 μL containing 2.5 μL of diluted pre-selective amplification products, 1× *Taq* Buffer (20 mM Tris–HCl, pH 8.4, 50 mM $(NH_4)_2SO_4$; Fermentas, USA), 3.0 mM $MgCl_2$, 0.25 mM of each of the four dNTPs, 0.25 U *Taq* DNA polymerase (Fermentas, USA) and 2 pmol of *Eco*RI-NNN primer and 2 pmol of *Taq*I-NNN primer. A touchdown thermal protocol was performed, as follows: 3 min at 94°C, 2 cycles of 30 s at 94°C, 1 min at 62°C and 1 min at 72°C, reduction annealing temperature by 2°C in four steps of three cycles each. The PCR was proceeded with 20 cycles of 30 s at 94°C, 1 min at 52°C and 1 min at 72°C, followed by 5 min at 72°C, and was ended at 4°C. An aliquot of the selective amplification was added 1:10 with formamide-containing loading buffer. Denatured products (10 μL) were loaded on 6% urea-containing polyacrylamide gels and electrophoresis at a constant power of 55 W for 3 h. The gels were visualized using silver staining.

Cloning and sequencing of AFLP fragments

The AFLP fragments of interest were excised from the gel samples and eluted in 20 μL of 1× PCR buffer at 4°C overnight. The solution was boiled at 95°C for 10 min and 5 μL of DNA fragments were reamplified using the same primer for the selective amplification of the PCR conditions. The PCR products were gel purified and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The inserted clones were sequenced using automated sequencer CEQ8000 (Beckman Coulter, Brea, CA, USA). The sequences were analyzed for homology with NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and Ensembl (http://asia.ensembl.org/Multi/blastview) databases.

Conversion of AFLP markers into simple SNP markers and SNP genotyping

In order to discover the polymorphisms in the restriction sites

Table 1. Primer sequences used for genotyping the SNP markers in pigs

SNP marker	Primer sequence (5′ to 3′)	Size (bp)	T_m (°C)	Restriction enzyme
SSC7 g.4937240C>G	F: TGTCTCTCCTCTTGCCCATC R: CAGTCAAGCCTCTTCTTGTC	286	58	*Bsu*RI
SSC8 g.47338181G>A	F: CTGGCATCATCACCCTAAAC R: CCACACCAGTCTTGTGAACT	288	60	*Taq*I
SSC9 g.5496647_5496662insdel	F: GCTATGGCTTTCCCTTCTGC R: AGCTTCAGAGCGTCCACGAA	216/200	58	16-bp Ins/Del
SSC10 g.71225134G>A	F: GCCACCAACCGAGACTACAA R: CTACACGCGGGACAAACAAT	365	58	*Rsa*I
SSC17 g.61976696G>T	F: CTTTCTGGGTGTTCCCTCCA R: CAGGTAACAGACTGGCAGAG	384	58	*Hin*6I

SNP, single nucleotide polymorphism.

and within the AFLP fragments, the PCR fragments obtained from pigs of both extreme phenotypes were comparatively sequenced. Primer sequences were designed by covering the AFLP fragments based on the flanking sequence information acquired from the Ensembl (http://asia.ensembl.org/index.html) database (Table 1). The New England BioLabs cutter (NEBcutter) software (http://nc2.neb.com/NEBcutter2/) was used to identify the specific restriction enzymes for each SNP marker. To identify the SNP genotypes, the PCR reaction was carried out in a final volume of 20 μL containing 50 ng of genomic DNA, 1× *Taq* Buffer (20 mM Tris–HCl, pH 8.4, 50 mM $(NH_4)_2SO_4$; Fermentas, USA), 1.5 mM $MgCl_2$, 0.25 mM each of the dNTPs, 0.2 U *Taq* DNA polymerase (Fermentas, USA) and 2 pmol of each primer (Table 1). A thermal protocol was performed as follows: 3 min at 94°C; 38 cycles of 30 s at 94°C, 30 s at 58°C to 60°C and 30 s at 72°C; and the final step was done for 5 min at 72°C. The amplified fragments were digested with restriction enzymes (Table 1) and separated on 8% polyacrylamide gel electrophoresis. The gels were visualized using silver staining.

Statistical analysis

The genotype and allele frequencies of the SNP markers were calculated. Significance of the AFLP fragment frequencies between the extreme two groups (high- and low-IMF contents) was tested using the chi-square analysis. Association analysis of the simple SNP markers with IMF content and FA composition traits was examined using a general linear model. The following statistical model was used:

$$y_{ijklmn} = \mu + sire_i + dam_j + sex_k + batch_l + marker_m + e_{ijklmn}$$

Where y_{ijklmn} represents the observed value of the phenotype traits, μ is representative of the population mean average of the measurements, $sire_i$ represents the fixed effect of the sires (i = 1 to 5), dam_j represents the fixed effect of the dams (j = 1 to 15), sex_k is a fixed effect of the sexes (k = 1 to 2), $batch_l$ is representative of the fixed effect of the slaughter batch (l = 1 to 12), $marker_m$ is representative of the fixed effect of the marker genotypes (m = 1 to 3), and e_{ijklmn} represents any random error. Significance was detected at the 5% level of all statistical analyzes.

RESULTS

AFLP analysis

Sixty-four selective primer combinations were used to identify the AFLP markers for IMF content. A total of 1,454 markers were observed with an average of 35 amplified fragments per primer combination. The majority of the AFLP fragments ranged in size from 50 to 800 bp. From these, 145 AFLP markers were found to be polymorphic between the two extreme IMF content groups. A representative AFLP marker and the associated fragment profile are shown in Figure 1. Twelve polymorphic fragments revealed the most striking significant differences between the two groups of high- and low-IMF contents ($p<0.05$). These AFLP fragments were obtained from the selective primer combinations as shown in Table 2. From these, the AFLP fragments were cloned, sequenced and used as candidate markers for fat deposition in muscle tissue.

Identification of AFLP fragments and chromosome locations

Five AFLP fragments were successfully sequenced. Homology searching and *in silico* mapping were performed using BLAST and Ensembl databases. All AFLP fragments were homology identified to the genomic DNA sequence of the pigs (Table 3). An AFLP fragment shared significant similarities with a known gene of pigs. The AFLP2 fragment showed a significant level of similarity with the porcine ankyrin repeat domain 16 (*ANKRD16*) gene (GenBank accession no. XM_013980330.1) and was located on chromosome SSC10 at 71.2 Mb. On the other hand, four AFLP fragments (AFLP1, AFLP3, AFLP7, and AFLP10) exhibited sequence identity with porcine DNA sequences (90% to 100%) and were located on chromosome SSC17 at position 61.97 Mb, SSC7 at 4.93 Mb, SSC9 at 5.49 Mb, and SSC8 at 47.33 Mb, respectively (Table 3).

To identify the causal polymorphism of these AFLP markers,

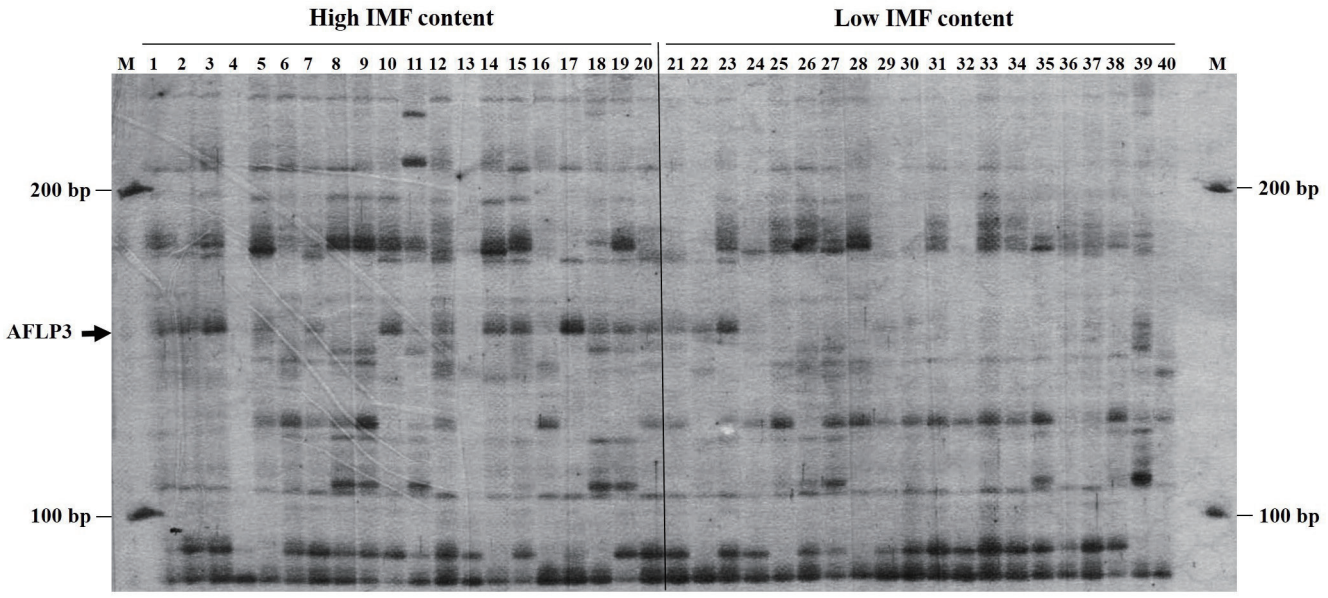

Figure 1. A representative AFLP profile, of two extremely high and low IMF contents, was obtained with the primer combination E-AGC/T-CCA. The arrow indicates the potential AFLP fragment (AFLP3) for IMF content trait that was recovered from gels and was then analyzed further. Lane M = 100 bp DNA ladder. Lanes 1 to 20 represent DNA samples with high IMF content phenotypes and Lanes 21 to 40 represent DNA samples with low IMF content phenotypes. AFLP, amplified fragment length polymorphism; IMF, intramuscular fat.

Table 2. Selected AFLP markers based on fragment frequency between high- and low-IMF contents in muscle tissue of pigs

Marker	Primer combination	Fragment size (bp)	Frequency		p-value (x^2)
			High-IMF	Low-IMF	
AFLP1	E-ACC/T-CCG	223	0.25	0.45	0.0157
AFLP2	E-ACG/T-CTG	71	0.30	0.48	0.0230
AFLP3	E-AGC/T-CCA	167	0.15	0.43	0.0013
AFLP4	E- AGC/T-CTG	136	0.48	0.28	0.0106
AFLP5	E- AGG/T-CAT	84	0.18	0.35	0.0574
AFLP6	E- AGG/T-CCA	188	0.03	0.20	0.0230
AFLP7	E-ATC/T-CGT	79	0.05	0.28	0.0069
AFLP8	E-ATC/T-CGA	176	0.50	0.18	<0.0001
AFLP9	E-ACG/T-CTG	152	0.15	0.35	0.0268
AFLP10	E-AGC/T-CTG	171	0.05	0.23	0.0336
AFLP11	E-ATC/T-CGA	198	0.15	0.45	0.0003
AFLP12	E-ACG/T-CTG	145	0.10	0.28	0.0500

AFLP, amplified fragment length polymorphism; IMF, intramuscular fat.

the primers were designed based on the flanking sequence information that covered the AFLP fragments. The DNA fragments of the pigs that represented the IMF content extremes were amplified and comparatively sequenced. Four fragments (AFLP1, AFLP2, AFLP3, and AFLP10) were identified as SNP at the *Taq*I restriction site, selective sites or within the AFLP fragments and were named as SSC17 g.61976696G>T, SSC10 g.71225134G>A, SSC7 g.4937240C>G, and SSC8 g.47338181G>A, respectively. The other fragment (AFLP7) was identified as insertion/deletion (Ins/Del) in the AFLP fragment and was named as SSC9 g.5496647_5496662insdel (16-bp Ins/Del).

Association of SNP markers with IMF content and FA composition

In order to elucidate the effect of the SNP markers (derived from AFLP fragments) on IMF content and FA composition, the relevant traits were investigated in pigs. Five SNP markers (SSC7 g.4937240C>G, SSC8 g.47338181G>A, SSC9 g.5496647_5496662insdel, SSC10 g.71225134G>A, and SSC17 g.61976696G>T) were genotyped in 620 commercially crossbred pigs. Genotype and allele frequencies of these five SNP markers are shown in Table 4. The results of the association analysis of five SNP markers and the fat deposition traits in the LD muscles are shown in Table 5 to 8. The SSC7 g.4937240C>G marker was found to be significantly associated with IMF content but, showed no

Table 3. Characterization of AFLP markers for fat deposition in muscle tissue of pigs

Marker	Homology	GenBank accession no.	Chromosome location (Mb)	Base pair identity (%)	Mapped close to gene
AFLP1	porcine DNA	NC_010459.4	SSC17 (61.97)	223/223 (100)	*CYP24A1*, *DOK5*
AFLP2	*ANKRD16*	NC_010452.3	SSC10 (71.22)	71/71 (100)	*ANKRD16*
AFLP3	porcine DNA	NC_010449.4	SSC7 (4.93)	167/169 (99)	*RREB1*
AFLP7	porcine DNA	NC_010451.3	SSC9 (5.49)	63/79 (90)	*OR51V1*
AFLP10	porcine DNA	NC_010450.3	SSC8 (47.33)	171/171 (100)	*GUCY1B3*

AFLP, amplified fragment length polymorphism; *ANKRD16*, ankyrin repeat domain 16; *CYP24A1*, cytochrome P450 family 24 subfamily A member 1; *DOK5*, docking protein 5; *GUCY1B3*, guanylate cyclase 1 soluble beta 3; *OR51V1*, olfactory receptor family 51, subfamily V, member 1; *RREB1*, ras responsive element binding protein 1.

Table 4. Genotype and allele frequencies of SNP markers in commercially crossbred pigs

SNP	N	Genotype frequency			Allele frequency	
		AA	AB	BB	A[1)]	B
SSC7 g.4937240C>G	565	0.48	0.36	0.16	0.65	0.35
SSC8 g.47338181G>A	513	0.64	0.24	0.12	0.76	0.24
SSC9 g.5496647_5496662insdel	585	0.28	0.58	0.14	0.57	0.43
SSC10 g.71225134G>A	601	0.81	0.19	0.00	0.90	0.10
SSC17 g.61976696G>T	570	0.52	0.30	0.18	0.67	0.33

SNP, single nucleotide polymorphism.
[1)] Allele A represents major alleles of the SSC7 g.4937240C, SSC8 g.47338181A, SSC9 g.5496647_5496662del, SSC10 g.71225134A and SSC17 g.61976696G loci, respectively and allele B represents minor alleles of the SSC7 g.4937240G, SSC8 g.47338181G, SSC9 g.5496647_5496662ins, SSC10 g.71225134G and SSC17 g.61976696T loci, respectively.

association with any FA levels (Table 5). The SSC9 g.5496647_5496662insdel marker showed a significant association with IMF content and arachidonic levels (Table 6). No association of the SSC10 g.71225134G>A marker with IMF content was observed. However, this marker was significantly associated with palmitoleic and ω9 FA levels (Table 7). The SSC17 g.61976696G>T marker was significantly associated with IMF content and FA levels of palmitoleic, eicosenoic, arachidonic, monounsaturated fatty acids (MUFA), and ω9 FA levels (Table 8). However, no significant association of SSC8 g.47338181G>A with any IMF and FA composition values was observed in this study (data not shown).

DISCUSSION

The AFLP approach is generally considered a powerful method of genome-wide scan technology for the QTL identification of complex traits. Several studies have successfully identified the QTLs and candidate genes for growth, meat quality and carcass traits in various livestock species [11,12]. In the present study, the genome-wide scan for fat deposition in the muscle tissue of pigs was investigated using the AFLP approach. The selective markers were then further elucidated to determine an association with IMF content and FA levels. Four of the five candidate markers showed a significant association with IMF content and/or FA composition.

In this study, the SSC7 g.4937240C>G marker revealed a significant association with IMF content in the crossbred pigs. This result is consistent with those of previous studies in pigs that were used to which identify the QTLs for IMF content on SSC7 at position 2.0 to 11.6 Mb [13]. The SSC7 g.4937240C>G marker obtained from the primer combination E-AGC/T-CCA

Table 5. Association of the SSC7 g.4937240C>G marker with IMF content and fatty acid composition traits in *longissimus dorsi* muscles in commercially crossbred pigs

Traits (%)	Genotypes (Least squares mean±standard error)			p-value
	CC (n = 180)	CG (n = 129)	GG (n = 63)	
IMF	2.628±0.170[b]	2.364±0.177[a]	2.212±0.258[a]	0.0454
C14:0 (Myristic)	1.175±0.062	1.020±0.072	1.074±0.127	0.1241
C16:0 (Palmitic)	13.605±1.049	11.539±1.221	12.498±2.131	0.2944
C18:0 (Stearic)	12.366±0.897	10.827±1.044	10.999±1.823	0.3275
C20:0 (Arachidic)	0.303±0.067	0.278±0.078	0.187±0.136	0.7342
SFA	24.451±1.429	23.666±1.663	25.759±2.903	0.1074
C16:1n-9 (Palmitoleic)	3.957±0.320	3.534±0.373	4.433±0.651	0.4641
C18:1n-9 (Oleic)	32.675±1.924	32.657±2.239	31.358±3.909	0.9581
C20:1n-9 (Eicosenoic)	2.394±0.403	2.237±0.469	1.559±0.820	0.6521
MUFA	39.027±1.960	38.429±2.281	37.351±3.981	0.9093
C18:2n-6 (Linoleic)	24.373±1.592	26.243±1.852	27.504±3.234	0.1572
C18:3n-6 (Linolenic)	0.124±0.044	0.155±0.051	0.145±0.090	0.8318
C20:2n-6 (Eicosadienoic)	1.682±0.251	1.548±0.292	1.925±0.509	0.8332
C20:3n-6 (Homolinolenic)	0.142±0.050	0.274±0.058	0.185±0.102	0.1097
C20:4n-6 (Arachidonic)	0.166±0.119	0.224±0.139	0.232±0.143	0.3448
PUFA	26.489±1.610	28.447±1.873	29.429±2.270	0.1637
ω3 FA	1.311±0.263	1.539±0.306	1.473±0.535	0.2251
ω6 FA	24.993±1.651	27.031±1.921	27.004±1.353	0.1657
ω9 FA	36.633±1.944	36.192±2.261	37.792±3.948	0.9673

IMF, intramuscular fat content; SFA, saturated fatty acids (C14:0+C16:0+C18:0+C20:0); MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9+C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6); ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3); ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6); ω9 fatty acids (C16:1n-9+C18:1n-9).
[a,b] Values in each row with different superscript letters are considered significantly different ($p<0.05$).

Table 6. Association of the SSC9 g.5496647_5496662insdel marker with IMF content and fatty acid composition traits in *longissimus dorsi* muscles in commercially crossbred pigs

Traits (%)	Genotypes (Least squares mean±standard error)			p-value
	InsIns (n = 55)	InsDel (n = 230)	DelDel (n = 105)	
IMF	1.716±0.269[a]	2.648±0.159[a]	2.526±0.304[b]	0.0155
C14:0 (Myristic)	1.150±0.243	1.096±0.073	1.025±0.103	0.7189
C16:0 (Palmitic)	13.729±3.503	13.287±1.054	11.538±1.487	0.4236
C18:0 (Stearic)	10.347±2.861	12.200±0.861	12.427±1.215	0.8258
C20:0 (Arachidic)	0.380±0.213	0.348±0.064	0.326±0.090	0.5580
SFA	25.307±4.809	26.933±1.448	25.316±2.042	0.6706
C16:1n-9 (Palmitoleic)	5.429±1.107	3.837±0.333	3.467±0.470	0.2907
C18:1n-9 (Oleic)	34.674±3.855	34.279±1.763	33.626±2.486	0.9379
C20:1n-9 (Eicosenoic)	1.646±1.075	2.424±0.384	1.829±0.541	0.3757
MUFA	39.224±4.899	40.541±1.776	38.924±2.505	0.7707
C18:2n-6 (Linoleic)	22.838±3.009	22.644±1.508	24.971±2.127	0.3214
C18:3n-6 (Linolenic)	0.158±0.138	0.113±0.041	0.68±0.058	0.5761
C20:2n-6 (Eicosadienoic)	1.267±0.804	1.687±0.242	1.513±0.341	0.7911
C20:3n-6 (Homolinolenic)	0.139±0.168	0.179±0.050	0.243±0.071	0.4402
C20:4n-6 (Arachidonic)	0.145±0.115[a]	0.150±0.105[a]	0.453±0.149[b]	0.0282
PUFA	27.864±3.033	24.774±1.515	27.350±2.137	0.3266
ω3 FA	1.395±0.842	1.134±0.253	1.291±0.357	0.8691
ω6 FA	25.380±3.185	23.250±1.561	25.644±2.201	0.3371
ω9 FA	39.077±4.933	38.116±1.786	37.094±2.519	0.9032

IMF, intramuscular fat content; SFA, saturated fatty acids (C14:0+C16:0+C18:0+C20:0); MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9+C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6); ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3); ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6); ω9 fatty acids (C16:1n-9+C18:1n-9).

[a,b] Values in each row with different superscript letters are considered significantly different ($p < 0.05$).

was located at position 4.93 Mb on porcine chromosome 7 close to the porcine ras responsive element binding protein 1 (*RREB1*, 4.59 Mb) gene. The *RREB1* gene encodes a zinc finger transcription factor [14]. It is involved in regulating the renin-angiotensin system. The *RREB1* variants showed an association with type 2 diabetes and end-stage kidney disease in humans [15]. It has been reported that the *RREB1* was associated with fat distribution and fasting glucose displaying potential effects related to the observed type 2 diabetes association [14,15]. Moreover, the type 2 diabetes locus was identified in the *RREB1* region [16].

The SSC8 g.47338181G>A marker derived from the primer combination E-AGC/T-CTG (AFLP10) was located at position 47.33 Mb on porcine chromosome 8 close to the porcine guanylate cyclase 1 soluble beta 3 (*GUCY1B3*) (46.71 Mb) gene. The *GUCY1B3* gene is a beta subunit of the soluble guanylate cyclase and belongs to the nitric oxide system. It is involved in adipose tissue biology by influencing adipogenesis, insulin-stimulated glucose uptake and lipolysis [17]. Several studies have reported on the importance of the presence of QTL for lipid accretion and IMF content on SSC8 [7,8]. Although the AFLP10 revealed significant differences in frequency between the two groups of high- and low-IMF contents, the SNP detected in this fragment had no effect on the IMF and FA composition traits. The results indicate a lack of linkage disequilibrium between this SNP marker and the causal mutations for the studied traits in these crossbred pigs.

The SSC9 g.5496647_5496662insdel marker secured from the primer combination E-ATC/T-CGT was located at position 5.49 Mb on porcine chromosome 9 close to the porcine olfactory receptor family 51, subfamily V, member 1 (*OR51V1*, 5.46 Mb) gene. The olfactory receptor genes are a member of a large family of G-protein-coupled receptors and are encoded with single coding-exon genes. Olfactory receptors reveal a seven-transmembrane domain structure and are specifically responsible for the recognition and G protein-mediated transduction of odorant signals [18]. However, the molecular basis of the olfactory receptors for controlling fat deposition in animals is poorly understood. It has been indicated that the olfactory receptors may play a role in the sensing and regulation of dietary fat, and may be important with regard to the individual susceptibility of obesity in rats [19]. Moreover, the increasing expression levels of the olfactory receptor genes have been identified in adipose tissue during the development of obesity in mice [20]. In the present study, the SSC9 g.5496647_5496662insdel marker showed a significant association with IMF content and arachidonic levels. This result is consistent with those of previous studies which found that the QTLs for IMF content and fatty acid composition were located on the SSC9 at position 5.9 to 6.0 Mb [21] and 11.1 to 17.8 Mb [5], respectively.

The SSC10 g.71225134G>A marker derived from the primer combination E-ACG/T-CTG showed 99% homology with porcine *ANKRD16* gene and was located at position 71.2 Mb of porcine chromosome 10. In this study, the SSC10 g.71225134G>A

Table 7. Association of the SSC10 g.71225134G>A marker with IMF content and fatty acid composition traits in *longissimus dorsi* muscwles in commercially crossbred pigs

Traits (%)	Genotypes (Least squares mean±standard error)		p-value
	AA (n =198)	AG (n = 57)	
IMF	2.287±0.121	2.397±0.282	0.7155
C14:0 (Myristic)	1.085±0.050	1.208±0.171	0.2614
C16:0 (Palmitic)	13.084±0.811	11.539±2.333	0.5121
C18:0 (Stearic)	11.384±0.646	13.692±1.858	0.2224
C20:0 (Arachidic)	0.263±0.048	0.323±0.140	0.6680
SFA	25.819±1.127	26.834±3.302	0.7210
C16:1n-9 (Palmitoleic)	3.860±0.243[a]	5.323±0.699[b]	0.0440
C18:1n-9 (Oleic)	34.576±0.919	36.785±2.366	0.3480
C20:1n-9 (Eicosenoic)	2.010±0.225	1.581±0.648	0.5126
MUFA	40.160±1.045	44.119±2.692	0.1436
C18:2n-6 (Linoleic)	25.510±1.213	22.110±3.491	0.3364
C18:3n-6 (Linolenic)	0.135±0.031	0.134±0.092	0.9963
C20:2n-6 (Eicosadienoic)	1.689±0.180	1.163±0.517	0.3170
C20:3n-6 (Homolinolenic)	0.181±0.038	0.089±0.110	0.4101
C20:4n-6 (Arachidonic)	0.115±0.089	0.034±0.257	0.7549
PUFA	27.632±1.227	23.532±3.529	0.2531
ω3 FA	1.294±0.177	0.907±0.603	0.5257
ω6 FA	26.155±1.257	23.634±3.617	0.3368
ω9 FA	35.992±1.291[a]	44.322±3.655[b]	0.0284

IMF, intramuscular fat content; SFA, saturated fatty acids (C14:0+C16:0+C18:0+C20:0); MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9+C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6); ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3); ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6); ω9 fatty acids (C16:1n-9+C18:1n-9).

[a,b] Values in each row with different superscript letters are considered significantly different ($p<0.05$).

marker revealed a significant association with palmitoleic and ω9 FA levels. However, this SNP was identified in the non-coding regions of the porcine *ANKRD16* gene. It has been hypothesized that the SSC10 g.71225134G>A might be in linkage disequilibrium with other causal polymorphisms, which may be located in another region of the *ANKRD16* gene. There is a limited amount of information regarding the *ANKRD16* gene and its potential functions relating to fat deposition in pigs. However, a previous study pointed to multiple QTLs in the location of 68.0 to 76.9 Mb on SSC10, and was associated with serum lipid traits in pigs [22].

In this study, the SSC17 g.61976696G>T marker showed a significant association with IMF content and FA composition of palmitoleic, eicosenoic, arachidonic, MUFA, and ω9 FA levels. This marker derived from the primer combination E-ACC/T-CTA was located at 61.9 Mb of porcine chromosome 17 close to the porcine cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*, 61.89 Mb) and docking protein 5 (*DOK5*, 62.40 Mb) genes. The *CYP24A1* is a catabolic vitamin-D-24-hydroxylase enzyme [23] and belongs to a member of the oxygenase cytochrome P450 genes [20]. It is expressed in adipose tissue and showed a relationship with obesity in humans [24]. The trans-regulated *CYP24A1* gene revealed an association with palmitic and saturated FA contents in the muscle tissue of pigs [25]. The *DOK5* gene belongs to the downstream of the kinase family [26]. It is a substrate in the insulin signaling and is highly expressed in skeletal muscles [27]. The *DOK5* exhibited

Table 8. Association of the SSC17g.61976696G>T marker with IMF content and fatty acid composition traits in *longissimus dorsi* muscles in commercially crossbred pigs

Traits (%)	Genotypes (Least squares mean±standard error)			p-value
	GG (n = 175)	GT (n = 127)	TT (n = 52)	
IMF	2.104±0.128[a]	2.594±0.195[b]	2.172±0.335[ab]	0.0451
C14:0 (Myristic)	1.123±0.068	1.020±0.086	1.035±0.127	0.5482
C16:0 (Palmitic)	13.107±1.013	12.797±1.270	11.354±1.875	0.6481
C18:0 (Stearic)	11.768±0.812	12.327±1.019	10.808±1.054	0.6829
C20:0 (Arachidic)	0.301± 0.062	0.291±0.078	0.321±0.115	0.9702
SFA	26.301±1.373	26.437±1.722	23.519±2.542	0.2927
C16:1n-9 (Palmitoleic)	4.250±0.246[b]	3.008±0.309[a]	3.175±0.356[a]	0.0153
C18:1n-9 (Oleic)	37.334±1.145	34.459±1.436	38.881±2.120	0.0967
C20:1n-9 (Eicosenoic)	2.322±0.372[b]	1.726±0.467[a]	1.846±0.489[a]	0.0491
MUFA	43.906±1.278[b]	39.194±1.603[a]	38.904±1.565[a]	0.0255
C18:2n-6 (Linoleic)	22.760±1.523	22.848±1.910	23.422±2.819	0.9722
C18:3n-6 (Linolenic)	0.145±0.039	0.107±0.049	0.210±0.073	0.4165
C20:2n-6 (Eicosadienoic)	1.596±0.224	1.403±0.281	1.242±0.415	0.6608
C20:3n-6 (Homolinolenic)	0.127±0.047	0.210±0.059	0.288±0.088	0.1730
C20:4n-6 (Arachidonic)	0.125±0.014[a]	0.285±0.011[b]	0.120±0.021[a]	0.0432
PUFA	24.655±1.538	24.756±1.929	25.370±2.847	0.9684
ω3 FA	1.164±0.254	1.100±0.319	1.201±0.234	0.7431
ω6 FA	23.297±1.575	23.377±1.975	24.200±2.915	0.9510
ω9 FA	41.584±1.274[b]	37.468±1.598[a]	37.638±1.859[a]	0.0472

IMF, intramuscular fat content; SFA, saturated fatty acids (C14:0+C16:0+C18:0+C20:0); MUFA, monounsaturated fatty acids (C16:1n-9+C18:1n-9+C20:1n-9); PUFA, polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6); ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3); ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6); ω9 fatty acids (C16:1n-9+C18:1n-9).

[a,b] Values in each row with different superscript letters are considered significantly different ($p<0.05$).

a strong association with obesity and type 2 diabetes in humans [26]. Additionally, several QTLs for IMF content and fatty acid composition traits have been identified and located within the regions at position 57.6 to 67.9 Mb on SSC17 [28,29]. These results suggest that the SSC17 g.61976696G>T might be in linkage disequilibrium with *CYP24A1* and *DOK5* genes.

These results highlight the importance of the selected AFLP markers. They can be used to identify the effective SNP markers for IMF content and FA composition in the muscle tissue of pigs. Additionally, the *in silico* mapping showed that the AFLP markers were located on SSC7, SSC8, SSC9, SSC10, and SSC17 and were mapped close to the strong functional candidate genes for fatness traits including, *RREB1*, *GUCY1B3*, *OR51V1*, *ANKRD16*, *CYP24A1*, and *DOK5*, respectively. These findings promote the importance of all genes as the positional candidate genes for fat deposition in the muscles of pigs. Further studies on the single nucleotide polymorphisms of these genes are required in order to identify their association with fat deposition in muscles.

In the current study, we have identified the AFLP markers for IMF content and FA composition. Four novel SNP markers (SSC7 g.4937240C>G, SSC9 g.5496647_5496662insdel, SSC10 g.71225134G>A, and SSC17 g.61976696G>T) were found to be associated with IMF and/or FA content traits in commercially crossbred pigs. We demonstrated the possibility of taking advantage of the AFLP approach with regards to identification of the positional candidate genes for fat deposition in the muscle tissue of pigs.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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