Haplotype analysis within quantitative trait locus affecting intramuscular fat content on porcine chromosome 7

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ABSTRACT: Previous results of fine mapping for quantitative trait loci affecting intramuscular fat content identified a 3.0-Mb chromosome interval on porcine chromosome 7, which contains at least 9 genes, based on the pig genome assembly. Therefore, we proposed these nine genes (*LOC100154481*, *LOC100155711*, *LOC100155276*, *SPATA7*, *PTPN21*, *ZCH14*, *EML5*, *TTC8*, and *FOXN3*) as positional candidate genes. The coding exons of the nine genes were characterized, and 45 polymorphisms were detected in F_2 Duroc × Meishan population. Within the nine genes, 10 non-synonymous substitutions and 1 insertion were genotyped among three European breeds (Landrace, Large White, and Duroc) and 1 Chinese breed (Meishan). Genotyping data was used to perform the haplotype analysis. Polymorphisms were found in all the studied genes, except *ZCH14*. We surveyed the frequency of 33 haplotypes that formed non-synonymous substitutions in four breeds. One of them was distributed widely in the Landrace, Large White, and Meishan breeds, but not in Duroc. Each breed had different major haplotypes.

Keywords: pig; chromosome 7; intramuscular fat; positional candidate genes; polymorphism

Intramuscular fat (IMF) content is one of the economically most important traits affecting meat quality in pigs. A higher level of IMF, or marbling, generally has a positive influence on the sensory experience associated with eating pork (Fortin et al., 2005). Fernandez et al. (1999) reported the following observations: (1) the favourable effect of increased IMF levels on consumer acceptability is observed only if IMF levels remain below approximately 2.5–3.5%. Higher values are associated with a substantial risk of meat rejection by consumers; (2) the favourable effect is only observed with increased IMF levels that are not associated with an increase in the level of intermuscular fat.

Fortin et al. (2005) reported that 1.5% IMF was the minimum level necessary to ensure a pleasing experience based on eating attributes. Therefore, it is important to elucidate the factors controlling IMF deposition and appropriately modulate the IMF content to match consumer preferences.

Several whole-genome scans have been conducted to identify pig chromosomal regions containing quantitative trait loci (QTL) for IMF content or marbling (de Koning et al., 1999; Harlizius et al., 2000; Grindflek et al., 2001; Malek et al., 2001; Ovilo et al., 2002; Kim et al., 2005; Nii et al., 2005; Rohrer et al., 2005; van Wijk et al., 2006; Edwards et al., 2008). We previously identified three IMF

QTL, one located on each of porcine chromosomes 7, 9, and 13, by using a porcine F_2 resource population produced from a cross between a Meishan sow and a Duroc boar (Sato et al., 2003). We reported that one QTL on porcine chromosome 7 was fine-mapped to the interval between DNA markers *SJ169* to *MM70*, which spans approximately 3.0 Mb and contains at least 9 genes, based on the pig genome assembly (Sato et al., 2005). However, the gene or single nucleotide polymorphism associated with porcine IMF has not been identified.

In livestock, despite the large number of QTL that have been found for economically important traits, very few causative polymorphisms have been convincingly identified (Womack, 2005). Hundreds of QTL have been identified in pigs (Rothschild, 2003; Rothschild et al., 2007), but, in this species, only a few causative mutations have been reported so far, including those affecting muscle mass and fat deposition (Jeon et al., 1999; Nezer et al., 1999), glycogen content (Milan et al., 2000), tenderness (Ciobanu et al., 2004), and vertebral number (Mikawa et al., 2011).

The aim of the present study was to search for polymorphisms in the exons of the 9 genes located in a QTL for intramuscular fat on porcine chromosome 7 as well as PCR-restriction fragment length polymorphism (PCR-RFLP) assays for non-synonymous polymorphisms, allele frequencies, and haplotypes occurring in the Landrace, Large White, Duroc, and Meishan breeds.

MATERIAL AND METHODS

DNA samples

A three-generation resource population was created and managed as described by Sato et al. (2003). In brief, the cross of a Meishan granddam and Duroc grandsire produced the F_1 generation; then, F_1 individuals were intercrossed to produce 865 F_2 animals. One hundred and sixty-five males were measured for IMF content and used for locating the IMF QTL on a linkage map. Moreover, tissue samples were collected from four different purebred lines of pigs, including European breeds Duroc (*n* = 41), Landrace (*n* = 97), and Large White (*n* = 49), and the Chinese breed Meishan (*n* = 48), which may be a descendant of a very small founding stock that was experimentally introduced into Japan. Genomic DNA was extracted from tissue or blood samples by a standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

Amplification and sequencing of coding exons

To search for polymorphisms in the coding regions of the 9 candidate genes, 6 individuals from F_2 (2 animals with Duroc haplotypes, 2 animals with Meishan haplotypes, and 2 animals with heterozygous Duroc/Meishan haplotypes) were selected according to genotypes of microsatellite markers in the QTL region. Polymorphisms were identified by comparative sequencing of PCR products amplified from the DNA of these 6 animals. To design PCR primers for amplification of exons within the nine studied genes the following DNA sequences were used for particular genes: *LOC100154481* (CT842122), *LOC100155711* (CT842122), *LOC100155276* (CT757500 and CU407076), *SPATA7* (CT757500), *PTPN21* (CT737288 and CT757500), *ZCH14* (CT737288), *EML5* (CU468512 and CT737288), *TTC8* (CU468512 and CT737406), and *FOXN3* (CT824637, CT737421 and CT737419). To determine quality and quantity for DNA sequencing the PCR products were electrophoresed in 2% agarose. The PCR products were purified using ExoSAP-IT (GE Healthcare UK Ltd., Buckinghamshire, England) and sequenced on an ABI 3130xl DNA sequencer (Applied Biosystems, Foster City, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA).

SNP genotyping and haplotype analysis

To survey the allelic frequencies in European and Chinese pig breeds, we genotyped 10 non-synonymous substitutions and an insertion of a (GGA) motif in exon 12 of the *PTPN21* gene, which is a part of the IMF QTL identified on porcine chromosome 7. Genotyping for 10 non-synonymous substitutions was performed using the PCR-RFLP method in polyacrylamide gel electrophoresis. For the insertion of a (GGA) motif in exon 12 of *PTPN21*, a PCR-based fragment analysis was designed to distinguish between genotypes by using an ABI 3130xl DNA sequencer (Applied Biosystems, Foster City, USA). Primers and restriction enzymes are shown in Table 1. Pairwise comparisons of the means were

Table 1. Genotyping primers and allele frequencies of 10 non-synonymous substitutions and an insertion for four breeds

Gene	Polymorphism		Primer sequence (5´ to 3´)	PCR products (bp)	Restriction enzyme	PCR-RFLP pattern (bp)	Allele	Duroc	Landrace	Large White	Meishan
	c.604A>G	**R202G**	Forward: GAGAGACTACACCCAGGTCTAC	263	*Apa*I	A; 263	A	1.000	0.974	0.620	0.354
			Reverse: GGGGTAAACGGCAAATGTCTG			G; 119 + 144	G	0	0.026	0.380	0.646
SPATA7	c.1432A>G	**I478V**	Forward: CAGGAAACGTCAACAATACCG	124	*Nla*III	A; 22 + 102	A	1.000	0.964	0.628	0.354
			Reverse: CTTCTGAATACTTTGATTTGTACA			G; 124	G	0	0.036	0.372	0.646
	c.1685C>T	**T562I**	Forward: GCAGGTATGTTAGACAGTGTA	185	*Dde*I	C; 21 + 164	C	1.000	0.974	0.967	0.906
			Reverse: GATTTTAAGAGTTGGTCCCTCA			T; 185	T	0	0.026	0.033	0.094
	c.2136 ins[GGA]	**713+E**	Forward: GCAGGAAAGCCTGAGATACG	178, 181			*178*	0.588	0.043	0.635	0.598
			Reverse: GCCTACTCCCAGGAAGCC				*180*	0	0	0	0.022
							181	0.413	0.957	0.365	0.402
PTPN21	c.2188G>C	**G730R**	Forward: GAGCAGGAAAGCCTGAGATACG	180	*Bss*HII	G; 180	G	0.575	0.011	0.244	0
			Reverse: GGCTTCCTGGGGAGTAGGCC			C; 59 + 121	C	0.425	0.989	0.756	1.000
	c.2327G>A	**R776K**	Forward: CATCCTGGAGCCCCAGTCCCAC	188	*Hpy*AV	G; 188	G	1.000	1.000	1.000	0.524
			Reverse: CCGGCCGTTTCTTCAGCGAGTC			A; 48 + 140	A	0	0	0	0.476
	c.2351A>G	**Q784R**	Forward: CATCCTGGAGCCCCAGTCCCAC	188	*Hpy*CH4V	A; 70 + 118	A	0.763	0.021	0.319	0
			Reverse: CCGGCCGTTTCTTCAGCGAGTC			G; 188	G	0.237	0.979	0.681	1.000
EML5	c.2083A>C	**I695L**	Forward: TTATAATCGACAGCAGAACACA	146	*Mbo*II	A; 146	A	0.526	0.036	0.140	0.462
			Reverse: CTAATTTATCTGGCCTGTTGTC			C; 68 + 78	C	0.474	0.964	0.860	0.538
	c.70G>A	**A24T**	Forward: CTGGAGCTATTTTAGGCGCAGG	128	*Hha*I	G; 37 + 91	G	0.525	0.361	0.760	1.000
			Reverse: CAGTCTTATCGCCGGGATCAAC			A; 128	A	0.475	0.639	0.240	0
TTC8	c.1499G>A	**R500E**	Forward: CAGAGAAGCTATGTTGCTGCTC	171	*Ape*KI	G; 85 + 86	G	1.000	0.969	0.551	0.378
			Reverse: ATATACGCATACTACTTGTCCCTTG			A; 36 + 50 + 85	A	0	0.031	0.449	0.622
FOXN3	c.1183T>C	**C395R**	Forward: AGCCACAGTGAGACGGAGGAAGAC	154	*Hpy*CH4V	T; 35 + 119	T	1.000	0.984	0.969	0.354
			Reverse: TCTTTTTGAGGGGCAGCCGTGTC			C; 154	C	0	0.016	0.031	0.646

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statistically analysed by one-way analysis of variance with a general linear model and Tukey's studentized range test using the statistical package R (Ihaka and Gentleman, 1996) to detect significant differences between genotype-phenotype combinations. Haplotypes for each animal were inferred using the PHASE computer program version 2.1 (Stephens et al., 2001).

RESULTS AND DISCUSSION

Detection of polymorphisms in F$_2$ population

In total, by direct sequencing of genomic PCR products, 45 sequence variants, including 10 non-synonymous substitutions, 1 insertion and 34 synonymous substitutions, were identified from 8 candidate genes in the F$_2$ population (Table 2). There was no polymorphism in the *ZCH14* gene. In the *SPATA7* gene, 7 SNPs were found in exons 6 and 12, including 3 SNPs – c.604A>G, c.1432A>G, and c.1685C>T – that caused 3 non-synonymous substitutions R202G, I478V, and T562I, respectively. In *PTPN21*, 10 SNPs and 1 insertion were found in several exons. One insertion (713+E) and 3 non-synonymous substitutions (G730R, R776K, and Q784R) were located in exon 12. In *EML5*, 14 SNPs were found, including c.2083A>C in exon 15, which caused the non-synonymous substitution I695L. In *TTC8*, 4 SNPs were found in several exons. Two of these SNPs caused non-synonymous substitutions A24T and R500E in exons 1 and 15, respectively. In *FOXN3*, we detected 2 SNPs, c.294T>C in exon 2 and c.1183T>C in exon 7. The c.294T>C in exon 2 was silent, but c.1183T>C led to the putative amino acid replacement, C395R, in the predicted protein.

Preliminary association was studied between IMF content and different alleles of 10 non-synonymous substitutions and 1 insertion in the F$_2$ generation of a Duroc × Meishan cross. We previously reported that the Duroc allele of this QTL had an additive positive effect on IMF content (Sato et al., 2003). There was an association ($P < 0.05$) between IMF content and the insertion (713 + E in *PTPN21*) and 3 non-synonymous substitutions, c.2083A>C in *EML5*, c.1499G>A in *TTC8*, and c.1183T>C in *FOXN3*, for which the Meishan granddam was heterozygous, while there was a highly significant association ($P < 0.001$) between IMF content and

another non-synonymous substitution. These results suggested that the insertion and the 3 non-synonymous substitutions might be excluded from the candidates for polymorphisms responsible for IMF content in pigs.

Allele frequencies

Table 1 shows the allele frequencies of candidate genes on IMF QTL for the four breeds. This genotyping revealed the characteristic allele frequencies for each breed. Two SNPs – c.2327G>A in *PTPN21* and c.1183T>C in *FOXN3*, were almost fixed for 1 allele within the three European breeds, but Chinese Meishan had both alleles. In contrast, 3 SNPs – c.2188G>C and c.2351A>G in *PTPN21* and c.70G>A in *TTC8*, were fixed within Meishan, but the European breeds had both alleles. Landrace and Duroc showed higher frequencies of either allele on several SNPs compared with Large White and Meishan. Specifically, 3 SNPs – c.604A>G and c.1432A>G in *SPATA7* and c.1499G>A in *TTC8*, were almost monomorphic in Landrace and Duroc. Genotyping for an insertion within *PTPN21* revealed 3 alleles in the Meishan pig breed. One allele (size 180 bp) seems to be a frame shift of the amino acid sequence. This may be significant because it could change the structural properties of the resulting coded protein and thus alter its function. PTPN21 (also called PTPD1; protein-tyrosine phosphatase D1) binds to and activates tyrosine kinases, such as src, enhancing epidermal growth factor-dependent mitogenic signalling (Cardone et al., 2004). A-kinase anchor protein 121 (AKAP121) inhibits PTPD1-dependent signalling by binding and sequestering the phosphatase to the mitochondria. This complex is an important regulator in mitochondrial metabolism (Livigni et al., 2006) and is relevant to energetic metabolism.

Haplotype analysis

The analysis of DNA from 235 individuals for 10 non-synonymous substitutions and an insertion revealed 33 haplotypes (*Hap_1* to *Hap_33*). Table 3 shows nucleotide sequences of these haplotypes compared to the sequences of reference haplotypes found in the Ensembl pig (Sscrofa9 [April 2009] assembly). The analysis of the distri-

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Table 2. Polymorphisms in the eight candidate genes for porcine intramuscular fat content QTL on porcine chromosome 7 with accession numbers of sequences that were used to specify the positions of the SNPs

Gene symbol	Gene name	GenBank accession number	Exon	Polymorphism	Amino acid
LOC100154481	galactocerebrosidase-like	XM_001924866	14	c.1413C>T	silent
			14	c.1458C>T	silent
			18	c.1914T>C	silent
LOC100155711	psychosine receptor-like	XM_001924675	2	c.87T>G	silent
			2	c.483G>A	silent
			2	c.863T>C	silent
LOC100155276	potassium channel subfamily K number 10-like	XM_001924909	6	c.1062G>A	silent
SPATA7	spermatogenesis associated 7	NM_001177908	6	c.429T>C	silent
			6	c.462A>C	silent
			6	c.604A>G	**R202G**
			12	c.1353C>A	silent
			12	c.1371G>A	silent
			12	c.1432A>G	**I478V**
			12	c.1685C>T	**T562I**
PTPN21	protein tyrosine phosphatase, non-receptor type 21	XM_001926312	1	c.67C>T	silent
			1	c.132T>C	silent
			12	c.1140G>A	silent
			12	c.2019G>C	silent
			12	c.2136 ins[GGA]	**713+E**
			12	c.2188G>C	**G730R**
			12	c.2327G>A	**R776K**
			12	c.2351A>G	**Q784R**
			14	c.2694T>C	silent
			16	c.3015G>A	silent
			16	c.3120G>T	silent
ZCH14	zinc finger CCCH-type containing 14	XM_001926546		none	
EML5	echinoderm microtubule associated protein like 5	XM_001925068	9	c.984G>A	silent
			13	c.1797C>T	silent
			15	c.2083A>C	I695L
			17	c.2295G>A	silent
			18	c.2370T>C	silent
			20	c.2646C>T	silent
			23	c.3009A>G	silent
			24	c.3177C>T	silent
			27	c.3681A>G	silent
			32	c.4062C>T	silent
			35	c.4242A>T	silent
			35	c.4374C>T	silent
			36	c.4440C>T	silent
			37	c.4746T>C	silent
TTC8	tetratricopeptide repeat domain 8	XM_001927081	1	c.70G>A	**A24T**
			3	c.205C>T	silent
			11	c.963C>T	silent
			15	c.1499G>A	**R500E**
FOXN3	forkhead box N3	NM_001044536	2	c.294T>C	silent
			7	c.1183T>C	**C395R**

Non-synonymous substitutions are given in bold

Table 3. Haplotypes segregating among four breeds

Haplo-type	N	Breed				SPATA7 c.604A>G R202G	SPATA7 c.1432A>G I478V	SPATA7 c.1685C>T T562I	PTPN21 c.2188G>C G730R	PTPN21 c.2327G>A R776K	PTPN21 c.2351A>G Q784R	PTPN21 c.2136ins [GGA] 713+E	EML5 c.2083A>C I695L	TTC8 c.70G>A A24T	TTC8 c.1499G>A R500E	FOXN3 c.1183T>C C395R
		L	W	D	M											
Reference sequence in Ensembl – D						A	A	C	G	G	A	178	A	G	G	T
Duroc grandsire – D						A	A	C	G	G	A	178	C	A	G	T
Meishan granddam – M						G	G	T	C	A	G	178/181	A/ C	G	A/ G	C/ T
Hap_1	4			4		1	1	1	1	1	1	1	1	1	1	1
Hap_2	5			5		1	1	1	1	1	1	1	1	2	1	1
Hap_3	5	1		4		1	1	1	1	1	1	1	2	1	1	1
Hap_4	4		4			1	1	1	1	1	1	1	2	1	2	1
Hap_5	52		18	34		1	1	1	1	1	1	1	2	2	1	1
Hap_6	5		5			1	1	1	1	1	1	1	2	2	2	1
Hap_7	18	2		16		1	1	1	1	1	2	2	1	1	1	1
Hap_8	3		2	1		1	1	1	1	1	2	2	2	1	1	1
Hap_9	1	1				1	1	1	2	1	2	1	2	2	1	1
Hap_10	38	3	10	18	7	1	1	1	2	1	2	2	1	1	1	1
Hap_11	103	56	20		27	1	1	1	2	1	2	2	2	1	1	1
Hap_12	1	1				1	1	1	2	1	2	2	2	1	2	1
Hap_13	121	120	1			1	1	1	2	1	2	2	2	2	1	1
Hap_14	3	3				1	1	1	2	1	2	2	2	2	2	1
Hap_15	2		2			1	1	2	2	1	2	2	1	1	1	1
Hap_16	1		1			1	1	2	2	1	2	2	2	1	2	1
Hap_17	1		1			2	2	1	1	1	2	1	2	1	1	1
Hap_18	1		1			2	2	1	1	1	2	1	2	1	2	2
Hap_19	2		2			2	2	1	2	1	2	1	1	1	2	1
Hap_20	4				4	2	2	1	2	1	2	1	1	1	2	2
Hap_21	29		29			2	2	1	2	1	2	1	2	1	2	1
Hap_22	10		2		8	2	2	1	2	1	2	1	2	1	2	1
Hap_23	1				1	2	2	1	2	1	2	3	2	1	2	2
Hap_24	2				2	2	2	1	2	2	2	1	1	1	1	2
Hap_25	21				21	2	2	1	2	2	2	1	1	1	2	2
Hap_26	15				15	2	2	1	2	2	2	1	2	1	2	2
Hap_27	1				1	2	2	1	2	2	2	2	1	1	2	2
Hap_28	1				1	2	2	1	2	2	2	3	2	1	2	2
Hap_29	1	1				2	2	2	1	1	1	2	2	1	1	2
Hap_30	3	2			1	2	2	2	2	1	2	1	1	1	2	2
Hap_31	4	4				2	2	2	2	1	2	1	2	1	1	1
Hap_32	2				2	2	2	2	2	1	2	2	1	1	2	2
Hap_33	6				6	2	2	2	2	2	2	1	1	1	2	2
	470	194	98	82	96											

L = Landrace, W = Large White, D = Duroc, M = Meishan, 1 = allele of reference sequence found in Ensembl Pig (Sscrofa9 [April 2009] assembly), 2,3 = other allele

bution of these 33 haplotypes showed that 24 haplotypes occurred several times, while each of the remaining 9 haplotypes was found only once. A previous report found that linkage disequilibrium (LD) was mostly organized in haploblocks of up to 10 kb in Chinese breeds, while in European breeds, LD haploblocks were up to 400 kb in size, and haplotype diversity within the haploblocks was higher in Chinese breeds (Amaral et al., 2008). Haplotype inference with a wide genomic region (3.0 Mb) was a likely cause of the lowest frequencies of haplotypes such as *Hap_9*, *12*, *16*, *17*, *18*, *23*, *27*, *28*, and *29*.

Landrace had 11 haplotypes, and *Hap_11* and *13* were the major types. Large White had 14 haplotypes, and *Hap_5*, *11*, and *21* were the major types. Duroc had 7 haplotypes, and *Hap_5*, *7*, and *10* were the major types. Meishan had 13 haplotypes, and *Hap_11*, *25*, and *26* were the major types. *Hap_11* was a major haplotype in all breeds studied, except Duroc. Amaral et al. (2008) reported that Chinese breeds had a higher number of haplotypes than wild boar and European breeds, and that smaller haploblocks were found in Chinese breeds, resulting in a larger number of haplotypes. In this study, the number of haplotypes found was almost equal for Chinese Meishan and for the European pig breeds, except for Duroc, which had a substantially lower number. The results of these experiments provide new information on polymorphisms in this genomic region and on the characterization of European breeds and Chinese Meishan, with specific haplotypes for each.

The parental Duroc of our F_2 resource population exhibited a homozygous haplotype of *Hap_5*, and the parental Meishan exhibited a heterozygous haplotype of *Hap_33* and a different type. The high frequency of the *Hap_5* haplotype is possibly associated with high IMF, because Duroc QTL alleles are associated with high IMF content compared with the Meishan alleles in this region of porcine chromosome 7.

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