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Genome-wide study on intramuscular fat in Italian Large White pig breed using the PorcineSNP60 BeadChip

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1 Title page: Intramuscular fat association study in Italian Large White pig breed

2

3 **Genome-wide study on intramuscular fat in Italian Large White pig breed using the**
4 **PorcineSNP60 BeadChip**

5

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Genome-wide study on intramuscular fat in Italian Large White pig breed using the PorcineSNP60 BeadChip

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17 **Summary**

18 Genome-wide association study results are presented for intramuscular fat in Italian Large White pig
19 breed. 886 individuals were genotyped with PorcineSNP60 BeadChip. After quality control
20 performed with Plink software and in R environment, 49,208 markers remained for the association
21 analysis. The GWAS was conducted using linear mixed model implemented in GenABEL. We
22 detected 7 new SNPs of genes till now not found associated to IMF. Three markers map in a wide
23 intergenic region rich of QTL linked to fat traits, one map 388 kb upstream gene *SDK1*, one map
24 inside *PPP3CA* gene, one inside *SCPEP1* gene and the last is not mapped in the porcine genome yet.
25 Associations here presented indicate a moderate effect of these genes on IMF. In particular *PPP3CA*,
26 that is involved in the oxidative metabolism of skeletal muscle, could be considerate as an interesting
27 candidate gene for IMF content in pigs. However further studies are needed to clarify the role of these
28 genes on the physiological processes involved in IMF regulation. These results may be useful to
29 control this trait that is important in terms of nutritional, technological and organoleptic
30 characteristics of fresh meat and processed products.

31

32 **Key Words:** Intramuscular fat deposition, porcine genome, candidate genes, meat quality traits.

33

34 **Introduction**

35 Intramuscular fat (IMF), referred also as marbling, consists of the fat scattered inside a muscle; its
36 content influences important qualitative traits of meat as flavor, juiciness and, tenderness and also
37 technological characteristics so that a muscle with an adequate content of this kind of fat results
38 suitable for the transformation in particular for dry cured products (Bosi and Russo 2004). IMF is a
39 complex quantitative trait difficult to measure and is often not included in the breeding programs,
40 despite its heritability value ranging from 0.21 (Davoli 2015, unpublished data) to 0.86 (Ciobanu *et al.*
41 *al.* 2011), with an approximate average of 0.50 (Ciobanu *et al.* 2011). The genetic basis of IMF is
42 difficult to know because there are several biochemical and metabolic processes influencing fat
43 deposition in muscles. Different authors indicate variations in IMF content among breeds: for
44 example Chinese breeds are fatter than the European ones and among the major European purebreds,
45 Duroc breed is usually the fattest one (Casellas *et al.* 2013; Ciobanu *et al.* 2011; Lo Fiego *et al.* 2010).
46 To date, several quantitative trait loci (QTL) associated to IMF are reported on the pig QTLdb
47 (<http://www.animalgenome.org/cgi-bin/QTLdb/SS/index>). QTL associated with IMF were found on
48 chromosomes 1, 2, 5, 6, 8, 12, 13 and 17 (Ciobanu *et al.* 2011). In particular, a QTL mapping in the
49 region of heart fatty-acid binding protein (*H-FABP*) gene on SSC6 has been reported as responsible
50 for the 15-20% of the IMF variation in different crosses (Iberian x Landrace and Duroc x Pietrain),
51 with Duroc and Iberian variants increasing the trait (Ciobanu *et al.* 2011). In addition to QTL,
52 candidate genes that can be implied in IMF content were also analysed. Putative candidate for IMF
53 deposition are the leptin receptor (*LEPR*), melanocortin 4 receptor (*MC4R*) (Casellas *et al.* 2013).
54 Another important gene reported for its involvement in the IMF deposition is the insuline growth
55 factor 2 (*IGF2*) that contributes to control the lean and fat deposition in muscles and the backfat (BF)
56 thickness (Aslan *et al.* 2012). A genetic variant of the promoter region of this gene is positively
57 associated with a higher IMF content in Large White pig muscles. Thanks to the high throughput
58 genotyping PorcineSNP60 BeadChip (Illumina), it is possible to carry out genome-wide association
59 studies (GWAS) and put in light markers associated to intramuscular fat content. On the whole, the

60 regulation of IMF is a yet poorly understood aspect and the GWAS till now performed did not show
61 yet a complete list of genes influencing it.

62 In the present research a population of Italian Large White (ILW) pigs, the main breed utilized for
63 the PDO dry cured ham production, was genotyped with PorcineSNP60 v2 BeadChip in order to
64 identify markers and genes associated to IMF performing a genome wide association study between
65 SNPs and IMF content.

66

67 **Materials and Methods**

68 *Sampling of animals and analysis workflow*

69 Samples available for this study were 889 ILW pigs , included in the national selection sib test
70 program. These animals were bred at Genetic Test Station of national pig breeders association
71 (ANAS, <http://www.anas.it>) from the weight of about 30 to about 150 kg and were fed quasi ad
72 libitum, meaning that about 60% of the pigs are able to ingest the entire supplied ration. The sib test
73 program is based on triplets of siblings from the same litter, two females and one castrated male that
74 are individually performance tested at the Genetic Test Station for the genetic evaluation of a boar.
75 ILW pigs utilized belong to 380 litters, originated from 86 boars and each boar had from 1 to 60
76 piglets. All ILW pigs were slaughtered at the same average weight (with a difference of 30 days (from
77 222 to 252 days) from the youngest to the oldest slaughtered animals) after electrical stunning in the
78 same commercial abattoir during the year 2012 in 26 different days.

79

80 *Phenotyping and estimated breeding values (EBV)*

81 IMF values of ILW population were determined by extracting with petroleum ether 1 g of fresh
82 *Semimembranosus* muscle by means of a XT15 Ankom apparatus (Macedon, NY, USA) according
83 to Official procedure AOCS Am 5-04 (AOCS, 2005). Since IMF was not normally distributed, the
84 phenotypic values were transformed using the box cox method with MASS package of R statistical
85 environment (<http://www.R-project.org>).

86

87 *Genotyping and quality control*

88 Genomic DNA of ILW pigs was extracted by standard protocols from blood samples in. The
89 PorcineSNP60 v2 BeadChip developed by Illumina, which contains 61,565 SNP markers across
90 whole genome (Ramos *et al.* 2009), was used to genotype all animals.

91 Quality control was first carried out using PLINK (Purcell *et al.* 2007) and then with GenABEL
92 package (Aulchenko 2007) of R environment. Through PLINK filtering, SNP markers were removed
93 when they had genotype missing rate > 0.1 (GENO), minor allele frequencies (MAF) < 0.01, Hardy-
94 Weinberg Equilibrium (HWE) < 0.001 and call rate < 0.90 (MIND). After this filtering, the dataset
95 was composed of 49,662 markers and 889 subjects.

96 Applying GenABEL quality control procedure, SNPs with a call rate < 95%, a minor allele frequency
97 < 0.28%, an identity by state value $\geq 95\%$, and a significant divergence from Hardy-Weinberg
98 equilibrium with a P value lower than $10E-3$ were excluded. This quality control procedure excluded
99 454 markers and 1 pig due to low call rate. Moreover, two additional pigs were omitted because of
100 too high identity by state.

101 At the end of the cleaning procedures 49,208 markers and 886 pigs were used for further analysis.
102 The position of these markers was updated due to the release of the last version of the Sscrofa10.2
103 genome assembly (http://www.ensembl.org/Sus_scrofa/Info/Index).

104

105 *Genome Wide Association Study*

106 The GWAS was conducted using a linear mixed model implemented in GenABEL. The model
107 included a random polygenic effect for which the variance-covariance matrix is proportional to
108 genome-wide IBS and includes sex and age as fixed effects.

109 The model is shown below:

110
$$\mathbf{y} = \mu + \mathbf{X}\mathbf{b} + \mathbf{S}\mathbf{c} + \mathbf{Z}\boldsymbol{\alpha} + \mathbf{e}$$

111 where \mathbf{y} is the vector of IMF of all genotyped pigs measured in *Semimembranosus* muscle, μ is the
112 overall mean, \mathbf{b} is the vector of fixed effects including sex (females and castrated males) and age of
113 the pigs, \mathbf{c} is the vector of SNP effects; α is the vector of random polygenic additive effects calculated
114 as $N(0, G\sigma^2_\infty)$ where G is the genomic kinship matrix and σ^2_∞ is the polygenic additive variance; \mathbf{e} is
115 the vector of the residual error and \mathbf{X} , \mathbf{S} , \mathbf{Z} are the relative incidence matrix for \mathbf{b} , \mathbf{c} and α .

116 To account for relatedness, the variance / covariance matrix was estimated from the genomic kinship
117 matrix, constructed using pair-wise identities by state, calculated for all samples based on all
118 autosomal SNPs, as implemented in the GenABEL package. Then, the association was tested using
119 the mmscore function on the residuals that have been corrected for familiar relatedness using the
120 kinship matrix and thus should be independent of pedigree or prior selection (Chen and Abecasis
121 2007).

122 The influence of population stratification was evaluated by using the genomic control and by
123 examining the distribution of statistic test generated from the thousands of association test and their
124 deviation from the null distribution were assessed in a quantile-quantile (Q-Q) plot performed in R
125 environment. The genome wide significance threshold was considered 5E-5 as proposed by Sanchez
126 *et al.* (2014). These authors proposed to consider three levels of significance as also Teyssedre *et al.*
127 (2012) reports: 5E-6, 5E-5, 5E-4. The first threshold corresponds to an approximation of 10,000
128 independent tests Bonferroni corrected, the second was proposed as the threshold detecting moderate
129 associations and the last was suggested as fair association identifying a QTL effect of the region in
130 which the SNP maps. Linkage disequilibrium (LD) analyses were performed using Haploview 4.2
131 software with default settings (Barrett *et al.* 2005); LD blocks were determined for each chromosome
132 region containing the significant markers identified.

133

134 **Results**

135 GWAS was performed on 886 pigs and 49,208 markers after quality filtering . In Supplementary
136 Table 2 the descriptive statistics of observed and normalized IMF values are indicated. The Q-Q plot

137 that compares the distribution of observed χ^2 statistics with the distribution of those expected under
138 the null hypothesis is shown in Figure 1A. From this plot, appears that no overall systematic bias is
139 present. The deflation factor λ is 1.02, indicating that population stratification was eliminated.
140 (Pearson and Manolio 2008). The Manhattan plot showing GWA results is presented in Figure 1B,
141 while the summary of the SNPs associated with IMF, their map locations and their P -values, corrected
142 for the genomic control, are reported in Table 1. Three of these significant markers map in an
143 intergenic region extending for 1.2 Mb on SSC1. They are part of a linkage block including three
144 additional markers ALGA0119142, DRGA0001750, and DRGA0001747 (Figure 2) that did not
145 result significantly associated with intramuscular fat content on GWAS. Those markers show a very
146 high LD with r^2 included between 0.87-0.95. In the genomic region where the considered markers
147 map, QTL associated with backfat and carcass traits related to fat deposition were identified and
148 reported in pig QTLdb (Supplementary Table 3). Marker ASGA0012975 is located on SSC3 and it
149 is placed 388,535 bp from sidekick cell adhesion molecule 1 (*SDK1*) gene. The marker
150 MARC0059507 is located on SSC8 on first intron of protein phosphatase 3, catalytic subunit, alpha
151 isozyme (*PPP3CA*) gene. ASGA0099478 is localized on the eighth intron of serine carboxypeptidase
152 1 (*SCPEPI*) gene on SSC12. Finally, MARC0114865 marker does not map on the most recent
153 genome assembly, but is located on genomic clone NW_003540371.1.

154

155 **Discussion**

156 The study presented here is a GWAS for IMF content in *Semimembranosus* muscle of ILW pig breed.
157 In this research, we identified 7 new SNPs not yet indicated in previous studies as significantly
158 associated with IMF. In this paper we consider $5E-5$ and $5E-4$ as acceptable significance levels to
159 consider the association of a quantitative trait, according to Sanchez *et al.* (2014) and Teysedre *et*
160 *al.* (2012). Generally, the Bonferroni correction is used to consider GWA results significance, but it
161 is reported that this correction is too stringent, because tests are not independent due to LD. Strucken
162 *et al.* (2014) stated that genotyping large samples is required to enlighten even small effects of

163 markers for quantitative traits as IMF and to indicate significant interactions between markers and
164 trait. It happens because there is not only one causative gene controlling the trait, but several genes
165 implied in the biochemical and metabolic processes determining it (Barendse 2011). Regarding
166 sample size, our data set is one of the widest used for GWAS in pig. Results show the presence of
167 moderate associations of the trait with some regions of porcine genome containing genes/regulatory
168 elements potentially involved in fat deposition.

169 Some significant markers are located on chromosome 1 in an intergenic region of 1.2 Mb that appears
170 devoid of genes. The lack of genes detected in this region may be due to the still incompletely
171 annotated pig genome, or else to an existing gene desert region, defined by Ovcharenko (2005) as
172 long regions (> 500 kb) containing no protein-coding sequences. Some of these gene desert regions
173 have been shown to contain regulatory sequences acting at long distances to control the expression
174 of neighboring genes (Harmston *et al.*, 2013).

175 Marker ASGA0012975, located on SSC3, presents the most significant *P*-value (Table 2). Neither
176 genes nor QTL are described so far in the region around, since it is still poorly studied in pig. On the
177 other hand, on the base of the significance found for marker ASGA0012975, we could hypothesize
178 that the SNP could be included in a likely regulatory element not yet described, since the pig genome
179 is not completely annotated. The gene nearest to this marker indicated by pig genome database is
180 *SDK1* that maps 388 kb downstream the marker. Nguyen *et al.* first described this gene in *Drosophila*
181 *melanogaster* in 1997 as a determinant of retinal photoreceptors destiny. The protein encoded is a
182 cell adhesion molecule that pertains to the immunoglobulin family; this protein is found to guide
183 axonal terminals to specific synapses in developing neurons (Yamagata and Sares 2008). In pig this
184 gene has not been studied yet and nothing is known about protein's functions. The literature does not
185 supply any link between IMF and *SDK1* as it has never been investigated in muscle in any species.
186 Further studies are needed to clarify its role in the IMF pathway at any level.

187 The marker MARC0059507 located on the first intron of *PPP3CA* resulted associated with IMF. The
188 *PPP3CA* gene encodes for a calcium- and calmodulin-dependent protein phosphatase called also

189 calcineurin, belonging to the Serine/threonine phosphatases (PPP) family. Calcineurin is a widely
190 distributed phosphatase and has a role in a variety of physiological pathways, including skeletal
191 muscle development (da Costa *et al.* 2007). In particular, da Costa *et al.* (2007) pointed out that
192 calcineurin is a key enzyme in the muscle fiber differentiation as it participates to down-regulate
193 genes acting in the fast fiber phenotype determination to facilitate the switching to slow oxidative
194 fibers. Differences in structure and metabolic characteristics of skeletal muscle fibers determine meat
195 transformation events in myocytes and are, therefore, of great importance for meat quality.
196 *Semimembranosus* is a white skeletal muscle classified mainly as glycolytic, even if its myofiber
197 composition has been described with a major proportion of type IIA fast twitch oxidative glycolytic
198 myofiber than type IIB fast twitch glycolytic myofiber (Herault *et al.* 2014). However, the metabolic
199 properties of this muscle show higher oxidative capacity compared to other white skeletal muscles
200 like *Longissimus* (Herault *et al.* 2014). In porcine *Semimembranosus* muscle *PPP3CA* gene, that we
201 have found associated to IMF content, could be also involved in the switching and conversion from
202 glycolytic to oxidative fibers. Favorable meat traits such as color, flavor, tenderness and greater IMF
203 value have been found to be closely associated with a higher content of oxidative fibers in muscles
204 (Hocquette *et al.* 2012). Moreover, *PPP3CA* gene has been shown to be involved in the differentiation
205 of perimuscular pre-adipocytes in cattle (Taniguchi *et al.*, 2008).

206 The marker ASGA0099478 maps in the eighth intron of *SCPEP1* gene on porcine chromosome 12.
207 *SCPEP1* gene, called also Retinoid-inducible serine carboxypeptidase, encodes for a novel protease
208 containing the putative catalytic triad (Ser-Asp-His) common to all members of the serine protease
209 family based upon homology with many other serine carboxypeptidase (López-Otín and Bond 2008).
210 Genes encoding the carboxypeptidases are considered candidate genes for traits related to meat
211 quality due to an important role in the regulation of the body fat content (Shin and Chung 2007).
212 *SCPEP1* gene was originally identified in rat aortic smooth muscle cells by screening for retinoid
213 inducible genes (Chen *et al.* 2001). Lee *et al.* (2009) study demonstrates a role for *SCPEP1* activity
214 in modulating smooth muscle proliferation, migration, and vascular remodeling. Nothing is known

215 about *SCPEPI* gene and function on skeletal muscle but further studies are needed to clarify the role
216 of this serine carboxypeptidase in this specific tissue. From the present study, appears that the marker
217 ASGA0099478, located on *SCPEPI* gene, is associated with IMF and that the allele G is related to
218 a greater IMF deposition, in ILW pigs. Our results and *SCPEPI* location in porcine genome suggest
219 studying this gene more in deep to understand its functional role on muscle fat deposition.

220 The obtained results for GWAS for intramuscular fat in ILW breed identified new genomic regions
221 and genes associated to IMF content in porcine genome. In particular, *PPP3CA*, *SCPEPI* and *SDK1*
222 were never found linked to IMF content in previous studies. Identification of several genomic regions
223 and putative positional genes associated with lipid metabolism reported here should contribute to the
224 better knowledge of the genetic basis of IMF content.

225

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230

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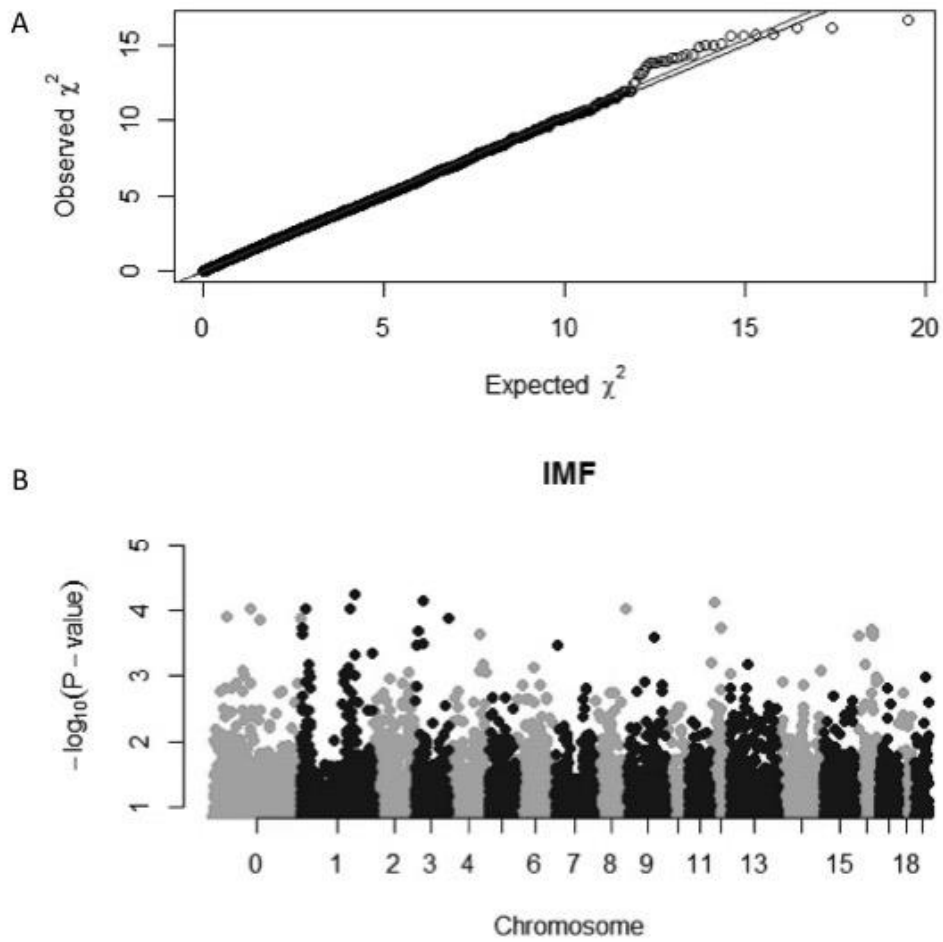
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306 **Figure Captions**

307

308 **Figure 1. A.** Q–Q plot of observed against expected P -values for IMF trait. **B.** Manhattan plot

309 showing the significance of association between 49208 SNPs and IMF content.



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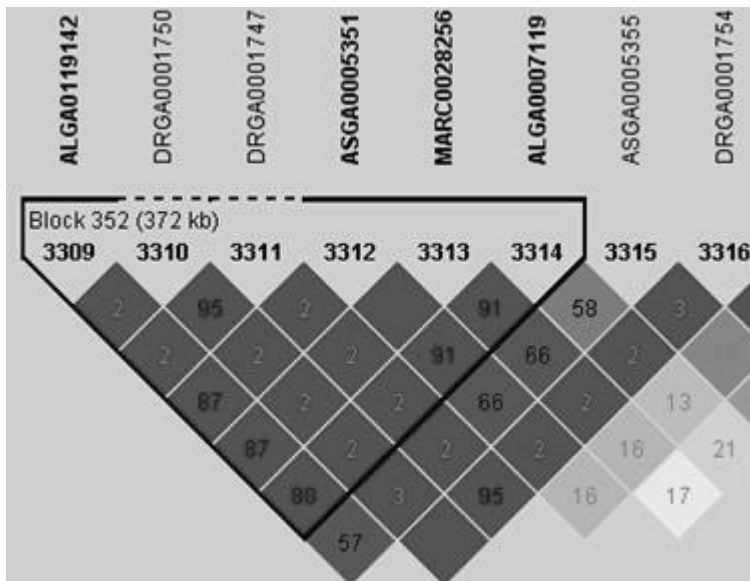
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318 **Figure 2.** Linkage disequilibrium plot of the region of 372 kb where markers ALGA0007119,
 319 ASGA0005351 and MARC0028256 are localized.



320

321

322 **Table 1.** Summary of the identified SNPs, their map locations and their *P*-values obtained in GWAS.

| SNP | SSC ¹ | Position ² | <i>P</i> -value ³ | Gene |
|-------------|-----------------------------|-----------------------|------------------------------|--------------------------|
| ALGA0007119 | 1 | 199,414,449 | 5.65E-05 | |
| ASGA0005351 | 1 | 199,407,859 | 9.18E-05 | |
| MARC0028256 | 1 | 199,375,236 | 9.18E-05 | |
| ASGA0012975 | 3 | 3,082,378 | 7.08E-05 | <i>SDK1</i> ⁵ |
| MARC0059507 | 8 | 128,498,159 | 9.53E-05 | <i>PPP3CA</i> |
| ASGA0099478 | 12 | 34,002,369 | 7.32E-05 | <i>SCPEP1</i> |
| MARC0114865 | NW_003540371.1 ⁴ | 1,763 | 9.53E-05 | |

323

324 ¹SNPs chromosome location as mapped on *Sus scrofa* Build 10.2 assembly, annotation release 104.

325 ²SNP position derived from *Sus scrofa* Build 10.2 assembly, annotation release 104.

326 ³*P*-value corrected for genomic control.

327 ⁴Genomic clone.

328 ⁵The marker is not inside the gene, but 388,535 bp upstream.

329

330 **Supplementary information**

331 **Supplementary Table 1.** Summary description of IMF% values, considering all ILW pigs available.

332

| | IMF | Normalized IMF |
|----------|-------|----------------|
| Mean | 2.042 | 0.522 |
| Minimum | 0.59 | -0.564 |
| Maximum | 8.64 | 1.667 |
| SD | 1.11 | 0.418 |
| Variance | 1.23 | 0.175 |

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334

335 **Supplementary Table 2.** QTL list reported for the region of SSC1 where the markers ALGA0007119, ASGA0005351 and MARC0028256 map.

336

| QTL | Description | Start ¹ (bp) | Stop ¹ (bp) | Size (bp) |
|---------|---------------------------------|-------------------------|------------------------|-------------|
| FP | Fat ratio (percentage) | 16,114,132 | 288,774,146 | 272,660,014 |
| LRIBF | Backfat at last rib | 16,114,132 | 288,774,146 | 272,660,014 |
| TOTLIP | Total lipid | 16,114,132 | 288,774,146 | 272,660,014 |
| LUMBBF | Backfat at last lumbar | 133,275,468 | 247,820,871 | 114,545,403 |
| FEEDIN | Feed Intake | 167,140,441 | 226,764,196 | 59,623,755 |
| ADIPDI | Adipocyte diameter | 167,140,441 | 226,764,196 | 59,623,755 |
| HCWT | Carcass weight (hot) | 167,140,441 | 265,323,706 | 98,183,265 |
| BFT | Average backfat thickness | 167,140,441 | 265,323,706 | 98,183,265 |
| LEAI | Loin Eye Area Intercept | 169,149,638 | 280,337,982 | 111,188,344 |
| LEAI13W | Loin eye area (13 weeks of age) | 169,149,638 | 280,337,982 | 111,188,344 |

337

338 ¹QTL position derived from *Sus scrofa* Build 10.2 assembly, Annotation release 104.

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