Journal of
Animal Breeding and Genetics

J. Anim. Breed. Genet. ISSN 0931-2668

ORIGINAL ARTICLE

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

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Keywords
Candidate genes; Intramuscular fat deposition; meat quality traits; porcine genome.

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Received: 1 April 2015;
accepted: 3 September 2015

[Correction added on 22 February 2016, after first online publication: The first name of the 6th author, A. Serra, was previously misspelt and this is now corrected in this version.]

Summary

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

Introduction

Intramuscular fat (IMF), referred also as marbling, consists of the fat scattered inside a muscle; its content influences important qualitative traits of meat as flavour, juiciness and tenderness and also technological characteristics so that a muscle with an adequate content of this kind of fat results suitable for the transformation in particular for dry-cured products (Bosi and Russo 2004). IMF is a complex quantitative trait difficult to measure and is often not included in the breeding programmes, despite its heritability value ranging from 0.21 (Davoli 2015, unpublished data) to 0.86 (Ciobanu *et al.* 2011), with an approximate average of 0.50 (Ciobanu *et al.* 2011). The genetic basis of IMF is difficult to know because there are several biochemical and metabolic processes influencing fat deposition in muscles. Different authors indicate variations in IMF content among breeds: for example, Chinese breeds are fatter than the European ones and among the major European purebreds, Duroc breed is usually the fattest one (Lo Fiego *et al.* 2010; Ciobanu *et al.* 2011; Casellas *et al.* 2013). To date, several quantitative trait loci (QTL) associated to IMF are reported on the pig QTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/SS/index). QTL associated with IMF were found on chromosomes 1, 2, 5, 6, 8, 12, 13 and 17 (Ciobanu *et al.* 2011). In particular, a QTL mapping in the region of heart fatty-acid binding protein (*H-FABP*) gene on SSC6 has been reported as responsible for the 15–20% of the IMF variation in different crosses (Iberian × Landrace and Duroc × Pietrain),

  doi:10.1111/jbg.12189

with Duroc and Iberian variants increasing the trait (Ciobanu *et al.* 2011). In addition to QTL, candidate genes that can be implied in IMF content were also analysed. Putative candidate for IMF deposition is the leptin receptor (*LEPR*), melanocortin 4 receptor (*MC4R*) (Casellas *et al.* 2013). Another important gene reported for its involvement in the IMF deposition is the insulin growth factor 2 (*IGF2*) that contributes to control the lean and fat deposition in muscles and the backfat (BF) thickness (Aslan *et al.* 2012). A genetic variant of the promoter region of this gene is positively associated with a higher IMF content in Large White pig muscles. Thanks to the high throughput genotyping PorcineSNP60 BeadChip (Illumina Italy SRL, Milan, Italy), it is possible to carry out genome-wide association studies (GWAS) and put in light markers associated to intramuscular fat content.

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

Materials and methods

Sampling of animals and analysis workflow

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

Phenotyping and estimated breeding values

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

Genotyping and quality control

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

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

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

At the end of the cleaning procedures, 49 208 markers and 886 pigs were used for further analysis. The position of these markers was updated due to the release of the last version of the SSCROFA10.2 genome assembly (http://www.ensembl.org/Sus_scrofa/Info/Index).

Genome-wide Association Study

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

The model is shown below:

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

where $\mathbf{y}$ is the vector of IMF of all genotyped pigs measured in *Semimembranosus* muscle, μ is the overall mean, b is the vector of fixed effects including sex (females and castrated males) and age of the pigs, c is

the vector of SNP effects; α is the vector of random polygenic additive effects calculated as $N(0, G\sigma^2_\infty)$ where G is the genomic kinship matrix, and σ^2_∞ is the polygenic additive variance; e is the vector of the residual error, and **X**, **S** and **Z** are the relative incidence matrix for *b*, *c* and α.

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

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

Results

Genome-wide association studies was performed on 886 pigs and 49 208 markers after quality filtering. In Table S1, the descriptive statistics of observed and normalized IMF values are indicated. The Q–Q plot that compares the distribution of observed chi-square statistics with the distribution of those expected under the null hypothesis is shown in Fig. 1a. From this plot, it is observed that no overall systematic bias is present. The deflation factor λ is 1.02, indicating that population stratification was eliminated (Pearson & Manolio 2008). The Manahattan plot showing GWA results is presented in Fig. 1b, while the summary of the SNPs associated with IMF, their map locations and

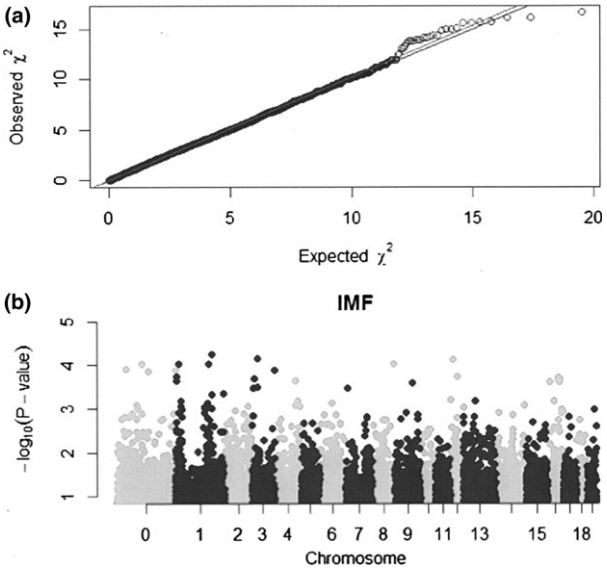

Figure 1 (a) Q–Q plot of observed against expected p-values for IMF trait. (b) Manhattan plot showing the significance of association between 49 208 SNPs and IMF content.

their p-values, corrected for the genomic control, are reported in Table 1. Three of these significant markers map in an intergenic region extending for 1.2 Mb on SSC1. They are part of a linkage block including three additional markers ALGA0119142, DRGA0001750 and DRGA0001747 (Fig. 2) that did not result significantly associated with intramuscular fat content on GWAS. Those markers show a very high LD with r^2 included between 0.87 and 0.95. In the genomic region where the considered markers map, QTL associated with backfat and carcass traits related to fat deposition were identified and reported in pig QTLdb (Table S2). Marker ASGA0012975 is located on SSC3, and it is placed 388 535 bp from sidekick cell adhesion molecule 1 (*SDK1*) gene. The marker MARC0059507 is located on SSC8 on first intron of protein phosphatase 3, catalytic subunit, alpha isozyme (*PPP3CA*) gene. ASGA0099478 is localized on the eighth intron of serine carboxypeptidase 1 (*SCPEP1*) gene on SSC12. Finally, MARC0114865 marker does not map on the most recent genome assembly, but is located on genomic clone NW_003540371.1.

Discussion

The study presented here is a GWAS for IMF content in *Semimembranosus* muscle of ILW pig breed. In this research, we identified seven new SNPs not yet indicated in previous studies as significantly

SNP	SSC[1]	Position[2]	p-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	*SDK1*[4]
MARC0059507	8	128 498 159	9.53E-05	*PPP3CA*
ASGA0099478	12	34 002 369	7.32E-05	*SCPEP1*
MARC0114865	NW_003540371.1[5]	1763	9.53E-05	

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

[1]SNPs chromosome location as mapped on *Sus scrofa* Build 10.2 assembly, annotation release 104.
[2]SNP position derived from *Sus scrofa* Build 10.2 assembly, annotation release 104.
[3]*P*-value corrected for genomic control.
[4]The marker is not inside the gene, but 388 535 bp upstream.
[5]Genomic clone.

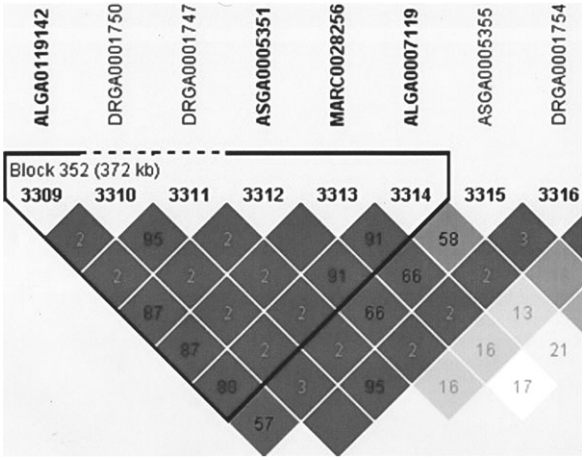

Figure 2 Linkage disequilibrium plot of the region of 372 kb where markers ALGA0007119, ASGA0005351 and MARC0028256 are localized.

associated with IMF. In this study, we consider 5E-5 and 5E-4 as acceptable significance levels to consider the association of a quantitative trait, according to Sanchez *et al.* (2014) and Teyssedre *et al.* (2012). Generally, the Bonferroni correction is used to consider GWA results significance, but it is reported that this correction is too stringent, because tests are not independent due to LD. Strucken *et al.* (2014) stated that genotyping large samples is required to enlighten even small effects of markers for quantitative traits as IMF and to indicate significant interactions between markers and trait. It happens because there is not only one causative gene controlling the trait, but also several genes implied in the biochemical and metabolic processes determining it (Barendse 2011). Regarding sample size, our data set is one of the widest used for GWAS in pig. Results show the presence of moderate associations of the trait with some regions of porcine genome containing genes/regulatory elements potentially involved in fat deposition.

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

Marker ASGA0012975, located on SSC3, presents the most significant p-value (Table 1). Neither genes nor QTL are described so far in the region around, because it is still poorly studied in pig. On the other hand, on the base of the significance found for marker ASGA0012975, we could hypothesize that the SNP could be included in a likely regulatory element not yet described, because the pig genome is not completely annotated. The gene nearest to this marker indicated by pig genome database is *SDK*1 that maps 388 kb downstream the marker. Nguyen *et al.* first described this gene in *Drosophila melanogaster* in 1997; as a determinant of retinal photoreceptors destiny. The protein encoded is a cell adhesion molecule that pertains to the immunoglobulin family; this protein is found to guide axonal terminals to specific synapses in developing neurons (Yamagata & Sanes 2008). In pig, this gene has not been studied yet and nothing is known about protein's functions. The literature does not supply any link between IMF and *SDK1* as it has never been investigated in muscle in any species. Further studies are

needed to clarify its role in the IMF pathway at any level.

The marker MARC0059507 located on the first intron of *PPP3CA* resulted associated with IMF. The *PPP3CA* gene encodes for a calcium- and calmodulin-dependent protein phosphatase called also calcineurin, belonging to the serine/threonine phosphatases (PPP) family. Calcineurin is a widely distributed phosphatase and has a role in a variety of physiological pathways, including skeletal muscle development (da Costa *et al.* 2007). In particular, da Costa *et al.* (2007) pointed out that calcineurin is a key enzyme in the muscle fibre differentiation as it participates to downregulate genes acting in the fast fibre phenotype determination to facilitate the switching to slow oxidative fibres. Differences in structure and metabolic characteristics of skeletal muscle fibres determine meat transformation events in myocites and are, therefore, of great importance for meat quality. *Semimembranosus* is a white skeletal muscle classified mainly as glycolytic, even if its myofibre composition has been described with a major proportion of type IIA fast twitch oxidative glycolytic myofibre than type IIB fast twitch glycolytic myofibre (Herault *et al.* 2014). However, the metabolic properties of this muscle show higher oxidative capacity compared to other white skeletal muscles like *Longissimus* (Herault *et al.* 2014). In porcine, S*emimembranosus* muscle *PPP3CA* gene, that we found associated to IMF content, could be also involved in the switching and conversion from glycolytic to oxidative fibres. Favourable meat traits such as colour, flavour, tenderness and greater IMF value have been found to be closely associated with a higher content of oxidative fibres in muscles (Hocquette *et al.* 2012). Moreover, *PP3CA* gene has been shown to be involved in the differentiation of perimuscular pre-adipocytes in cattle (Taniguchi *et al.* 2008).

The marker ASGA0099478 maps in the eighth intron of *SCPEP1* gene on porcine chromosome 12. *SCPEP1* gene, called also retinoid-inducible serine carboxypeptidase, encodes for a novel protease containing the putative catalytic triad (Ser-Asp-His) common to all members of the serine protease family based upon homology with many other serine carboxypeptidase (López-Otín & Bond 2008). Genes encoding the carboxypeptidases are considered candidate genes for traits related to meat quality due to an important role in the regulation of the body fat content (Shin & Chung 2007). *SCPEP1* gene was originally identified in rat aortic smooth muscle cells by screening for retinoid-inducible genes (Chen *et al.* 2001). Lee *et al.* (2009) study demonstrates a role for *SCPEP1* activity in modulating smooth muscle proliferation, migration and vascular remodelling. Nothing is known about *SCPEP1* gene and function on skeletal muscle but further studies are needed to clarify the role of this serine carboxypeptidase in this specific tissue. From the present study, it appears that the marker ASGA0099478, located on *SCPEP1* gene, is associated with IMF and that the allele G is related to a greater IMF deposition, in ILW pigs. Our results and *SCPEP1* location in porcine genome suggest studying this gene more in deep to understand its functional role on muscle fat deposition.

The obtained results for GWAS for intramuscular fat in ILW breed identified new genomic regions and genes associated to IMF content in porcine genome. In particular, *PP3CA, SCPEP1* and *SDK1* were never found linked to IMF content in previous studies. Identification of several genomic regions and putative positional genes associated with lipid metabolism reported here should contribute to the better knowledge of the genetic basis of IMF content.

Acknowledgements

This work was supported by Progetto 'AGER – Agroalimentare e ricerca': Advanced research in genomics and processing technologies for the Italian heavy pig production – Hepiget (Grant N. 2011- 0279).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary description of IMF% values, considering all ILW pigs available.

Table S2. QTL list reported for the region of SSC1 where the markers ALGA0007119, ASGA0005351 and MARC0028256 map.

