

1 Identification of marbling gene loci in commercial pigs in Canadian herds.

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5 Abstract.

6 We examined the amount of marbling and tested the genome of boars from 5 breeds of Duroc, Iberian,
7 Lacombe, Berkshire and Pietrian that were commercially available for a swine herd in Canada. The
8 marbling was ranked according to the amount of intramuscular fat % obtained in loin chops consisting of
9 the *longissimus dorsi* muscle. The genetics were analysed by genome wide association study using
10 80,000 single nuclear polymorphism (SNP) microarrays. Our samples had pork that achieved > 7 % IMF
11 from 110kg animals. Meta-analysis revealed SNP markers that were associated with the highest marbled
12 pork chops on chromosomes 5, 7, and 16. Using the susScr 11.1 map, we determined that the nearest
13 genes were SSNP, Rh glycoprotein and EGFLAM. We tested a sub-population of Duroc sired animals and
14 found a different set of markers close to GRLB and KCNJ3 on chromosomes 8 and 15. Based on our
15 sample, we can achieve pork with good marbling from animals conventionally raised to standard market
16 weights of 110kg. The choice of a good marbling line of pig is not necessarily breed specific.

17 **Keywords:** pigs, genetics, marbling, IMF %, GWAS

19 Introduction.

20 A genome wide association study (GWAS) was performed on commercial boars and sows and their
21 offspring to find the quantitative trait loci (QTLs) that best matched up with the degree of intramuscular
22 fat percentage (IMF %) found in the *longissimus dorsi* muscle at the time of slaughter. The IMF % is also
23 called marbling and is associated with better palatability of the cooked pork chops. Marbling or IMF % is
24 the fat in the muscle fibres of loin pork chop that gives flavour, texture, and moisture and some
25 tenderness, to the cooked meat. It is visually assessed as small white fat deposit by spectro-analysis,
26 ultrasound or chemical analysis (Cheng et al. 2015). Chemical analysis can be objectively scored as the
27 degree of fat obtained from lean muscle and measured by petroleum ether extraction (Soxtec) or by
28 nuclear magnetic resonance (NMR) (Keeton et al. 2003) with a Smart Trac analyzer. Most pork produced
29 in Canada contains on average 1.5% IMF in lean longissimus muscle. Sensory taste panels have
30 determined that the ideal minimal amount of fat should be 3% (Eikelenboom et al. 1996) (Fortin et al.
31 2005). Low IMF pork has problems with the lack of taste, moisture and tenderness. Beef has set their
32 minimal amount of marbling fat at 7% IMF in a fresh loin steak (Cameron et al. 1994), which maintains a
33 premium grade of AAA or prime. In recognition of the value of IMF in pork loin muscle, the 2017 USDA
34 (Docket no. AMS-LPS-17-0046) now proposes a grade system be set up for rewarding pork which
35 contains >3% IMF. <https://regulations.justia.com/regulations/fedreg/2017/10/23/2017-22934.html>

IMF % is determined by the animal's genetics and environment. Heritability of IMF% has been estimated between 0.39 and 0.65 and is co-dependent upon other measures such as back fat, sex, age, diet and final slaughter weight (Schwab et al. 2010). The correlation between IMF and other fat deposit such as the subcutaneous back fat ($r = 0.53$, $P < 0.01$) or belly seam fat ($r = 0.18$) have some relationship (Eusebi et al. 2017; Solanes et al. 2009) but they are not directly linked. The overt concern with lean meat yield, which has a negative correlation with IMF ($r = -0.55$, $P < 0.01$) (Sellier et al. 2010) and minimal back fat has adversely led to the loss of IMF in typical pork carcasses (Knapp et al. 1997). Selection for pigs with good marbling is being developed but the current system can only perform IMF % measures *post mortem* (Cheng et al. 2015). The application of sound genetics will assist in the Canadian industry efforts to meet a minimal level of IMF.

Materials and Methods.

Animals

The selection of various genotypes of commercial pigs was performed at the Lacombe piggery. For the first round of breeding, sires of Duroc, Berkshire, Peitrain, Iberian and Lacombe were selected from commercial artificial insemination stock from Magnum Swine Genetics (Fort Macleod, AB) or Ontario Swine Improvement (Innerkip, ON) and bred to in-house sows of Large Whites. Their > 600 F1 offspring were raised to a market weight of 110 kg. Animals were fed standard diets of barley and wheat according to the National Research Council requirements (NRC 2012) and are care for according to Canadian Council for Animal Care guidelines (CCAC 2009). Upon reaching market weight of ~ 110kg in ~180 days, the animals were slaughtered and their carcasses were processed prior to a 24 h post-slaughtered chilling at +4°C. The 24h *longissimus dorsi* muscle was cut into 4 cm thick chops and the 12th to 13th rib site chop was saved for further processing by visual assessment of marbling and colour grading. The grading was performed initially, by the collection of 50g of lean meat sample which were ground and prepared for analysis of moisture by microwaves analysis and IMF % by nuclear magnetic resolution (NMR) on the Smart trac analyzer 2 (CEM Corp, Mathews, NC, USA). (Figure 1)

Genotyping

The genomic DNA from the pigs were collected from the blood or from the tissue and purified with the Sigma genomic kits. The selected samples ($n = 96$) were run on the Illumina Neogen, Geneseek Genomic Profiler (GGP) porcine array (Illumina, Markham, ON, Canada) containing 80000 SNPs with an average marker spacing of ~42 kb by Delta Genomics DNA (Edmonton, CA). The animals ($n = 600$) were selected to represent the top 10% and the bottom 10% of their breed's IMF% in the male castrated barrows. The results from the GGP porcine chips were filtered by Illumina Genome Studio v.2 for markers to remove those markers with low minor allele frequency ($MAF < 0.05$) and significant deviations from Hardy-Weinberg equilibrium ($P < 10^{-6}$).

Genome wide association study (GWAS)

The phenotypic IMF was standardized according to results of 3 samplings and the average mean was used as the main determination. Individual genotypes were loaded into file by use of R-code (GNU general public licence v.3.4.4) and matching IMF values were assigned to the database which was then analyzed by SVS variation suite from Golden Helix (Bozeman, MT, USA: http://goldenhelix.com/resources/SNP_Variation/index.html). The individual genotypes were then assigned the Illumina GGP porcine 80K map to generate a physical location and allow us to create Manhattan plots of a meta-analysis GWAS of marbling. The chromosomal locations of the SNPs markers were used in the SVS variation suite program to also give a proximity to nearby genes. We used the University of California Santa Clara (UCSC) genome browser <https://genome.ucsc.edu/> of the Pig Genome SusScr11 assembly to help identify and map the position of the genes and link it to known SNPs of the genes by using the NCBI Gene of the RefSeq (Kent et al. 2002). The GWAS was also subdivided into the various breeds of the sire to see if the markers were universal or breed specific concerning the individual markers.

Single SNP association analysis.

The SNPs above genome wide significance ($P > 4 \times 10^{-8}$) location of markers were placed onto the Sscrofa 11.1/susScr 11 map (UCSC genome browser: <http://genome.ucsc.edu/>). The 63,714 SNPs were adjusted for the degree of IMF % measured on the animals LD muscle and compared across the population of animals ($n = 80$). The SVS variation suite was used to generate Manhattan and Q-Q plots of the P-values of the single-nucleotide polymorphism (SNP)-based associated with IMF % and was set at a threshold of ($P < 4.5 \times 10^{-8}$) to identify the markers for genome wide significance. The markers position on the susScr11 map and the nearest genes were identified according to the position. Most of the markers were found in the repeat mask elements but were close to nearby genes. Many of the proximate genes contained additional SNPs that were identified by reference dbSNP databank. The refSNP of the nearest genes were extrapolated by their Gene bank number within National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) data banks (Table 1 and 2).

Results and Discussion.

We conducted a GWA analysis on the intramuscular fat percentage (IMF %) from the longissimus dorsi muscle in pigs. Pig sires were selected from commercial breeding companies and represent the 5 major breeds available in Canada. The LD muscle was selected because it represents the major meat found in a typical pork loin chop cut. The IMF % was performed on the LD muscle and is commonly referred to as marbling. The average marbling was between Duroc at 2.9 ± 0.7 % and Pietrain at 2.0 ± 0.4 % between the five breeds, representing > 600 samples (Figure 1). The ideal marbling is determined to be > 3%,

1 according to past taste panel reviews (Eikelenboom et al. 1996). We selected the top 10% and bottom
2 10% of IMF% from LD muscle of the 5 breeds, to investigate if certain genes could be associated with
3 superior marbling. The genomic microarray investigation revealed a set of single nucleotide polymorphic
4 markers that significantly correlated with the appearance of IMF %. These SNPs were located on
5 chromosomes 5, 7 and 16, in the meta-analysis of IMF% in the loin muscle of pigs. The markers were
6 located near the porcine genes sarcospan (SSPN) (Genbank# AK349478) on chromosome 5, Rh
7 glycoprotein (RHAG)(Genbank# XM_003128440) on chromosome 7 and EGF-like fibronectin and laminin
8 G domains protein (ELGFAM) (Genbank# AK396237) on chromosome 16, which have their unique SNPs
9 listed in the refSNP report, accordingly (Table 1).

10 We also tried a GWAS analysis on just the Duroc sired pigs. The number of animals (n = 40) was still
11 enough to get a significant correlation between the IMF % and markers as seen in the Manhattan plot
12 and QQ-plot data. Significant correlation with genetic markers was found to be unique in the Duroc sired
13 pigs as opposed to the meta-analysis of all the pig breeds. The best markers were found on
14 chromosomes 8 and 15 which were mapped close to gene, glycine receptor beta (GLRB) (Genbank#
15 AJ715855) and prostate expressed FAM198B (Genbank# AK396463) on 8 and potassium inwardly-
16 rectifying channel 1 (KCNJ3) (Genbank# AF540391) on 15 (Table 2). The unique map identification was
17 due to the reduced phenotypic number of samples and to the fact that different sires have each their
18 own unique marbling genes. The subpopulation of Durocs had individuals with high IMF % values
19 approaching 5% to 7.1 %. Part of this study was to see if we had in Canada a genetic line with extreme
20 marbling, similar to the Bono Brown pigs reported in Japan (Mikawa and Yoshioka 2012). The Bono
21 brown are a Duroc line that can achieve an average IMF of $6.3\% \pm 1.9\%$ in barrows with a 2 cm average
22 back fat, on a special diet. The Bono pork line was loosely mapped by 125 microsatellite markers to
23 regions on chromosome SSC7 and SSC14. In this project, some of the 68,000 markers used in our meta-
24 genome study were uniquely significant towards the region of chromosomes 7 but not chromosome 14.
25 We had approximately 20 sires that had their barrow offspring with IMF % values above 4.0% but it
26 included Berkshire sires too. It will be interesting to see if the > 6% IMF can be maintained in the
27 extremely marbled F1 offspring.

28 This research was part of larger goal to find a simple genetic test that would guarantee
29 adequate marbling in pork. The use of GWAS and microarrays are useful method to check useful
30 genotypes but it is still a bit expensive and labour intensive. There are a lot of claims of superior
31 marbling in various genetic lines but these claims must be backed up with verifiable genetic test that can
32 be performed by a third party. Ideally this would be in the form of a simple genetic test, similar to RyR1
33 DNA test for swine halothane susceptibility (Brenig and Brem 1992) or the PRKAG3 DNA test (Meadus et
34 al. 2002) for the Rendement Napole *RN* defect in pig processing. Unfortunately, the genes that control
35 marbling in pigs appear to be multifactorial (Hausman et al. 2014; Sato et al. 2016; Won et al. 2018).
36 There are a number of publications that report on genes linked to IMF % and marbling which are all
37 valid, depending on the background genetics and the environmental effects. On a final note, although
38 the Duroc sired pigs were among the best marbled, there were offspring from Duroc that gave poor
39 marbling with IMF% below 1.5% but still gave good meat yield. Under our current system, these Duroc
40 pigs would be index highly for their carcass value but the meat will be considered poor.

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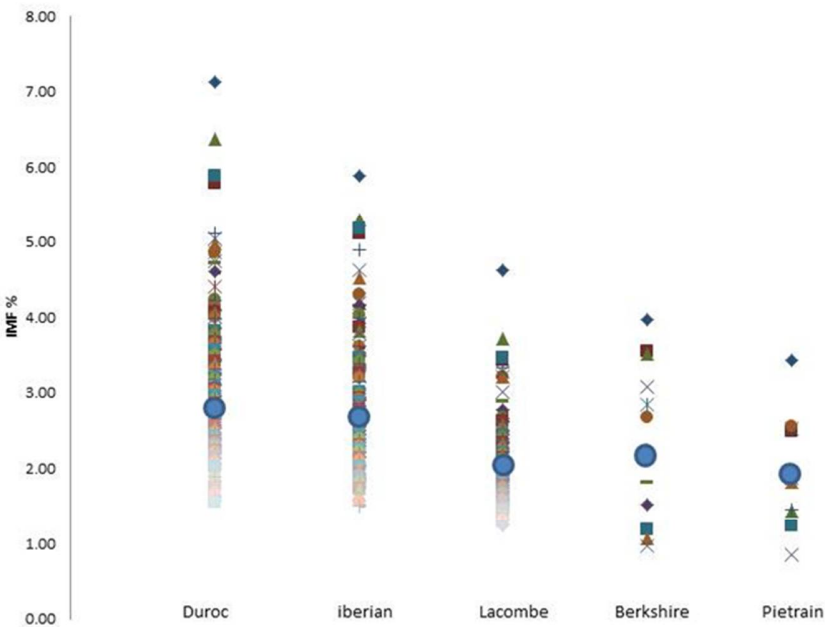
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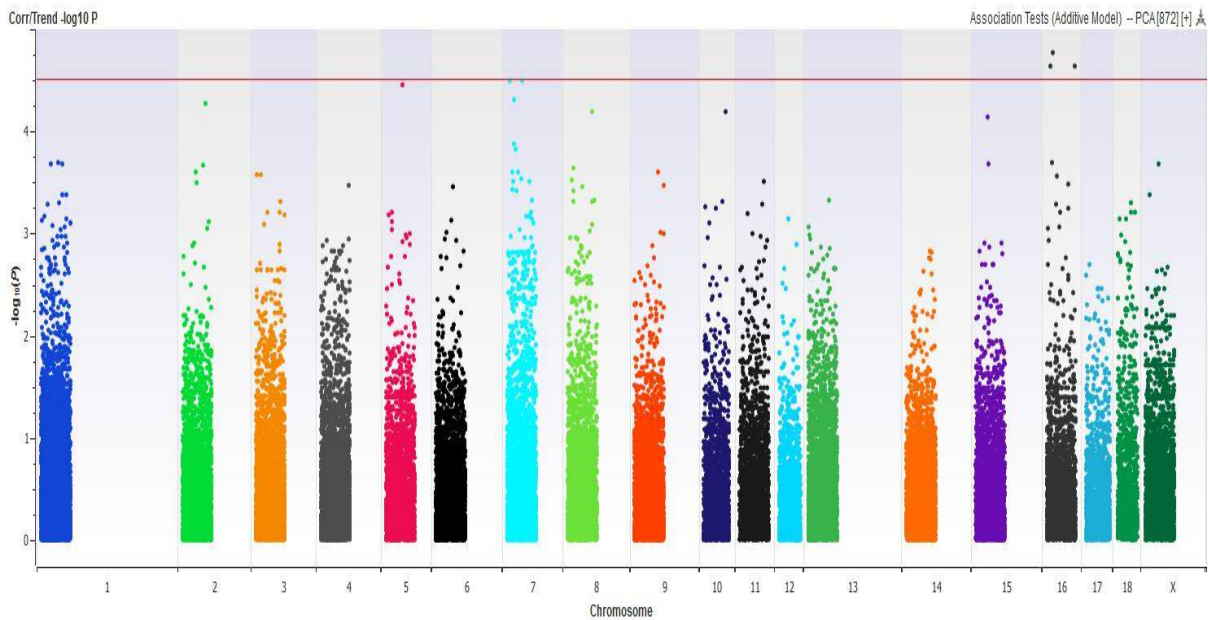
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Figure 1. The average IMF% in the longissimus dorsi muscle of the five cross breeds. The IMF% was calculated on the lean muscle by use of the NMR analyzer. The overall average mean ($\mu \pm \text{std dev}$) is represented by the blue dot within each breed sire subgroup. Duroc 2.9 ± 0.7 , Iberian 2.8 ± 0.6 , Lacombe 2.0 ± 0.4 , Berkshire 2.4 ± 0.8 and Pietrain 2.0 ± 0.6 .

Meta-analysis all Pigs.

(A)



(B)

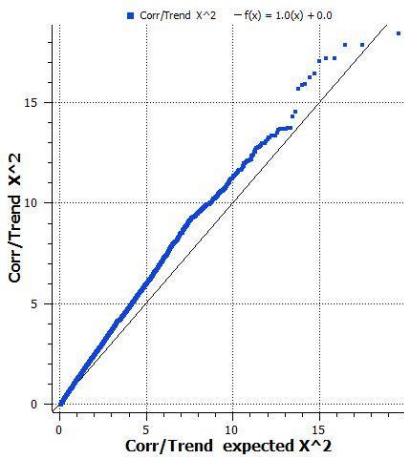


Figure 2. Manhattan plot (A) and QQ (B) of P-values of the single-nucleotide polymorphism (SNP) based association meta-analysis against the intramuscular fat content percentage (IMF %) in pigs. The red line indicates the threshold for genome wide significance ($P < 4.5 \times 10^{-8}$) for 68,529 SNPs adjusted for sex and MAF < 0.01.

Chromosome	Position	Marker	Base	Nearest Gene	Gene	refSNP	SNP#
5	47606484	WU_10.2_5_64313075	A/G	AK349478	SSPN	rs322211582	8
7	16758370	ALGA0044073	A/G	XR_002346095	ncRNA	n/a	n/a
7	43707458	ASGA0035629	T/C	XM_003128440	RhGlycoprotein	rs698040624	25
16	18742425	ALGA0116333	T/C	AK343791	Aveolar macrophage	n/a	n/a
16	23611505	ALGA0092214	T/C	AK396237	EGFLAM	rs690289509	54
16	73722502	ASGA0097252	A/G	AK240470	Uterus	n/a	n/a

Table 1. Location of significant markers and the nearest genes, identified by meta-analysis of GWAS related to the IMF% in commercial pig sires. The chromosome and position of the markers were generated by the Manhattan plot and the nearest gene given with the GenBank number and their reference SNP cluster report (refSNP) were extrapolated by mapping them onto the *Sus scrofa* Ssc/Scr map 11.1. The refSNP was chosen out of the existing SNP databank based on the position from the multiple targets of individual SNPs, when available.

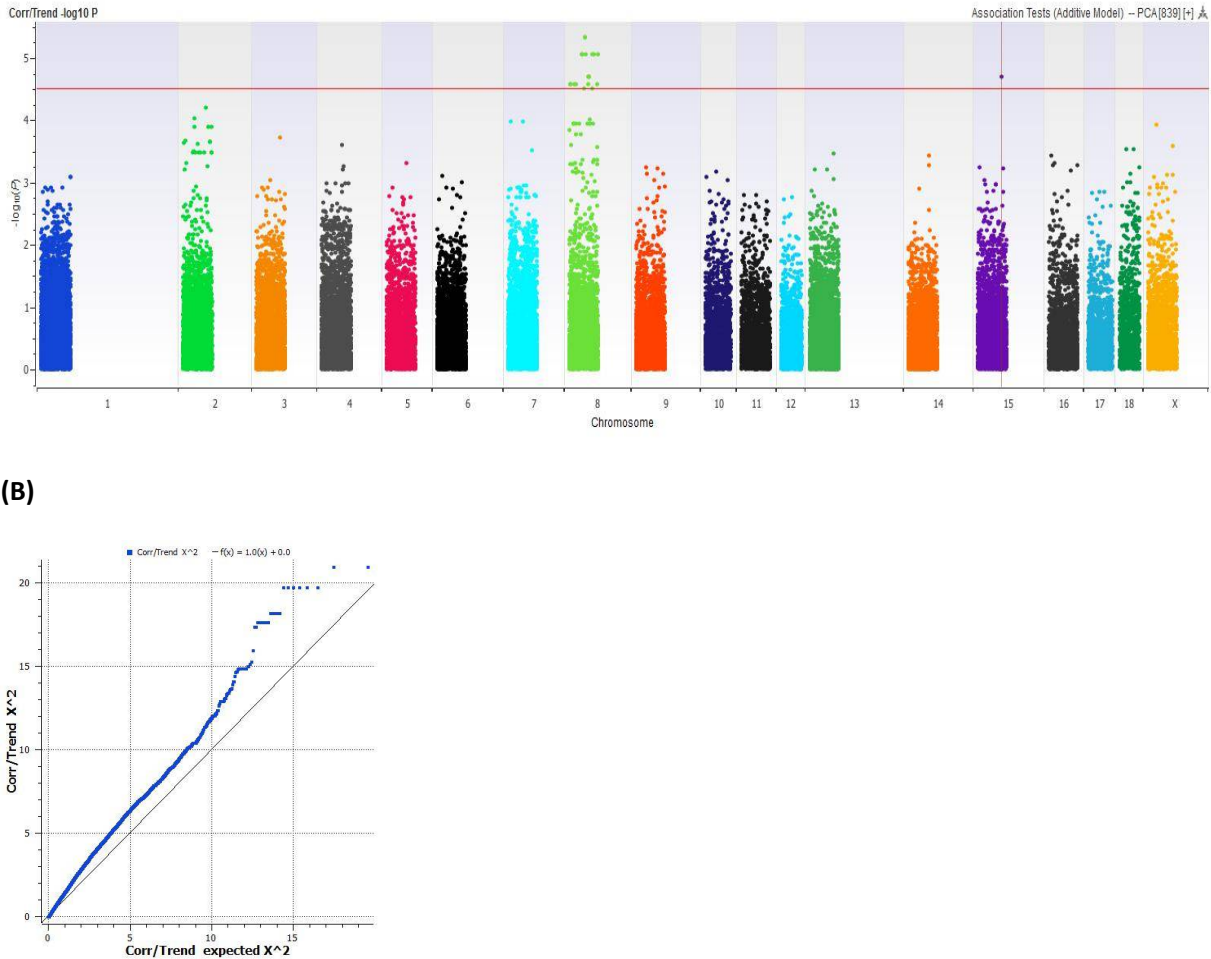


Figure 3. Manhattan (A) and QQ plot (B) of the GWAS analysis of IMF% in the (n = 40) Duroc sired pigs. The red line indicates the threshold for genome wide significance ($P < 4.5 \times 10^{-8}$) for 68,529 SNPs adjusted for sex and MAF < 0.01.

Chromosome	Position	Marker	Base	Nearest Gene	Gene	refSNP	SNP#
8	46622432	H3GA0025835	A/G	AJ715855	GLRB	n/a	n/a
8	46677501	MARC0004983	T/C	AK396463	FAM198B	rs705641356	24
15	62778504	CASI0009038	A/C	AF540391	KCNJ3	rs711844269	6

Table 2. Location of significant markers and nearest genes for Duroc GWAS related to the IMF%. The chromosome and position of the markers were generated by the Manhattan plot and the nearest gene and their reference SNP cluster report (refSNP) were extrapolated by mapping them onto the Sus scrofa Ssc/Scr map 11.1. The refSNP was chosen out of the existing SNP databank based on the position from the multiple targets of unique SNPs, when available.

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