University of São Paulo
"Luiz de Queiroz" College of Agriculture

Genome-wide association studies reveal genomic regions and positional candidate genes for fat deposition in chickens

Gabriel Costa Monteiro Moreira

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

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2018

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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1. Herdabilidade genômica 2. QTL 3. Gordura abdominal 4. Peso da pele 5. Conteúdo de gordura na carcaça 6. Armazenamento de gordura. I. Título

DEDICATION

I dedicate this work to my two main inspirations in life: my lovely mother and sweet and dedicated father. Their support and love means everything to me.

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It's time have a cup of coffee. With big smile and a good story to share, I invite you to come with me!

It's summer outside the windows of my lab and I almost finished my Doctorate. Four years of hard work, smiles, surprises, partnerships and learning. Four years of science, joy and happiness.

On my way, a lot of good people that I want to express all my gratitute. Let's start the whole story!

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I already finished my cup of coffee! It's time to keep this amazing taste in me, inside my memories. It was a big pleasure to share this coffee and my stories with you!

EPIGRAPH

"the limits of my language are
the limits of my world"

Ludwig Wittgenstein
(engineer, mathematician philosopher)

SUMMARY

RESUMO

Estudos de associação genômica ampla revelam regiões genômicas e genes candidatos posicionais para deposição de gordura em galinhas.

O excesso de deposição de gordura é um fator negativo para a produção de aves, o que afeta a eficiência alimentar e consequentemente os custos da produção de carne. A incorporação das ferramentas genômicas em programas de melhoramento de aves pode ajudar a acelerar a seleção para aumentar a eficiência da produção. Neste contexto, genotipamos cerca de 2.000 aves de 42 dias de duas populações diferentes (população F_2 experimental brasileira e população de corte referência TT) usando um chip de SNPs de alta densidade (600K, Affymetrix) para estimar a herdabilidade genômica de características relacionadas à deposição de gordura, para identificar regiões genômicas e genes candidatos posicionais (PCGs) associados a essas características. Realizamos análises de associação genômica ampla (GWAS) usando o programa GenSel (abordagem Bayesiana) para identificar janelas genômicas de 1 Mb associadas com características de gordura abdominal, pele e conteúdo de gordura na carcaça. A busca por PCGs foi feita dentro de cada janela genômica associada, considerando os *Gene Ontology (GO) terms* e também a informação da literatura. Integramos neste estudo NGS-SNPs identificados em animais parentais de ambas as populações, e além disso, regiões de assinaturas de seleção identificadas na população F_2 experimental brasileira para refinar a lista de PCGs. Os valores de herdabilidade genômica para as características relacionadas à gordura foram de moderado a alto (maior que 0,30). Identificamos QTL para características de gordura abdominal, pele e conteúdo de gordura na carcaça contendo PCGs envolvidos em processos biológicos de deposição de gordura. Identificamos vários NGS-SNPs anotados em regiões potencialmente funcionais em nossos PCGs e alguns desses foram preditos como mutações deletérias e de alto impacto. Além disso, alguns genes se sobrepuseram com regiões de assinatura de seleção na população F_2 experimental brasileira. Foram identificados importantes genes candidatos para a deposição de gordura, fornecendo novos *insights* para alcançar uma melhor compreensão do controle genético da deposição de gordura em frangos.

Palavras-chave: 1. Herdabilidade genômica 2. QTL 3. Gordura abdominal 4. Peso da pele 5. Conteúdo de gordura na carcaça 6. Armazenamento de gordura

ABSTRACT

Genome-wide association studies reveal genomic regions and positional candidate genes for fat deposition in chickens

Excess of fat deposition is a negative factor for poultry production, which affects feed efficiency and consequently the costs of meat production. The incorporation of genomic tools in poultry breeding programs may help to accelerate the selection for increased production efficiency. In this context, we genotyped approximately 2,000 42 days-old chickens from two different populations (Brazilian F2 Chicken Resource population and TT broiler Reference Population) using a high-density SNP array (600K, Affymetrix) to estimate genomic heritability of fatness-related traits, to identify genomic regions and positional candidate genes (PCGs) associated with these traits. We performed genome-wide association (GWAS) analysis using GenSel software (Bayesian approach) to identify 1 Mb genomic windows associated with abdominal fat, skin and carcass fat content traits. The search for PCGs were made within each genomic windows associated considering their Gene Ontology (GO) terms and also the literature information. We also integrated into this study NGS-SNPs data from both populations and selection signature regions identified in Brazilian F2 Chicken Resource population to refine the list of PCGs. The genomic heritability values for fatness-related traits were from moderate to high (greater than 0.30). We identified quantitative trait loci (QTL) for abdominal fat, skin and carcass fat content traits harboring several PCGs involved in biological processes of fat deposition. We identified several NGS-SNPs annotated in potential functional regions in our PCGs and some of those were predicted as deleterious and high impact mutations. Besides that, some genes overlapped with selection signature regions in Brazilian F2 Chicken Resource population. Important candidate genes for fat deposition were identified, providing new insights to achieve a better understanding of the genetic control of fat deposition in chickens.

Keywords: 1. Genomic heritability 2. QTL 3. Abdominal fat 4. Skin weight 5. Carcass fat content 6. Fat storage

1. INTRODUCTION

Poultry breeding programs have been developed to improve performance and carcass traits. More specifically, the breeding programs of broiler chickens aim to increase growth rate and improve body condition (performance traits) (BERRI et al., 2001). However, animals selected for rapid growth exhibit higher fat deposition in carcass (BAEZA AND BIHAN-DUVAL, 2013). The excess of fat deposition in broiler chickens is a negative factor for poultry industry because it decreases feed efficiency and also impacts the lean meat yield (ABASHT & LAMONT, 2007; JENNEN et al. 2004, ZHOU et al. 2007, BAÉZA & LE BIHAN-DUVAL, 2013).

Chicken is considered an important model for genomic studies because it has a social structure that allows large social groups of males with females, easy to establish crosses between the generations, sexual precocity, limited agility and adaptation to a variety of environments (CROOIJMANS, 1996), and also the ability to obtain DNA from nucleated red blood cells. Additionally, the chicken was the first domesticated animal to have the genome sequenced (HILLER et al., 2004). The genome sequenced was from a single female of the domestic herd's wild ancestor, Red Jungle Fowl (ABPLANALP, 1992; DODGSON, 2011).

The high rate of recombination found in domestic chicken enables the development of experimental lines for QTL mapping studies (SIEGEL et al., 2006). Studies with experimental lines allow the identification of the genetic aspects involved in phenotype expression, with a focus on the identification of quantitative traits locus (QTLs), candidate genes and mutations.

Based on CHICKEN QTLDB, RELEASE 33 (2017), a total of 424 QTLs were mapped for fatness traits: 139 for abdominal fat percentage; 200 for abdominal fat weight; six for carcass fat content; five for carcass fat content on dry matter basis; five for cingular fat width; four for fat distribution; 23 for intramuscular fat; 14 for skin fat weight; eight for subcutaneous fat thickness; seven for subcutaneous neck fat weight; seven for total white fat weight and six for visceral fat weight. The two chromosomes that exhibit the greater number of QTLs mapped for fatness traits are GGA1 and GGA5 with 45 and 28 QTLs, respectively.

In Brazil, in 1999 a collaboration was established between EMBRAPA Swine and Poultry and ESALQ / USP to conduct research on chicken genomics, which enabled the mapping of several QTLs and the identification of genes associated with traits of economic interest, mainly for performance and carcass yield. For these researches an F2 population obtained from reciprocal crosses between non-endogamic broiler lines (TT) and a layer line (CC) was used. The animals of this F2 population were grouped in CTCT and TCTC (ROSÁRIO et al., 2009). In TCTC, several QTLs were mapped on GGA1, 2, 3, 10, 12, 15 and 27 for abdominal fat weight and percentage (NONES et al., 2005; CAMPOS et al., 2009; BOSCHIERO et al., 2013) and on GGA1, 15 and 27 for carcass fat content and carcass fat content on dry matter basis (NONES et al., 2012). In CTCT population, QTLs were associated with abdominal fat weight in GGA5 (SILVA et al., 2011).

The Brazilian broiler TT population was another population developed in Brazil, from the expansion of a broiler parental line (called TT) belonging to the Chicken Breeding Program of EMBRAPA Swine and Poultry, to validade genomic studies. (FORNARI et al., 2014). Chickens of the parental broiler parental line TT underwent selection since 1992 over many generations for several traits such as body weight, feed conversion, carcass and cuts yield, viability, fertility, hatchability and to reduce abdominal fat (NONES et al., 2012; PÉRTILLE et al., 2015). In this population, QTLs were mapped for abdominal fat weight and percentage on GGA2 (FORNARI et al., 2014).

The QTL mapping is first step in the identification of candidate genes and mutations for fatness regulation in chickens. In this context, few genome-wide association studies (GWAS) were performed aiming to identify candidate genes and SNPs associated with abdominal fat weight and percentage in chickens (SUN et al., 2013; LIU et al., 2013; WANG et al., 2016), however, most of these using lower than 100,000 genetic markers. The use of a high density of SNPs may improve the power and resolution to identify QTLs helping also in the search of candidate genes and mutations for fatness regulation in chickens.

Thus, in this study, the use of a higher density of SNPs to perform GWAS for fatness traits in chicken populations allowed the identification of novel QTLs for fatness traits and also a better resolution in the QTL mapping. We selected putative candidate genes encompassed in the detected QTLs and we also selected few potentially candidate mutations identified by sequencing. This thesis generated novel results that can contribute to a better understanding of fat deposition in chickens and further studies may be performed to validate our findings. Our results may be useful for chicken selection studies aiming to reduce the excessive fat depots.

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2. GENOMIC REGIONS AND CANDIDATE GENES ASSOCIATED WITH FATNESS IN A F_2 CHICKEN POPULATION

ABSTRACT

Excessive fatness is a negative factor for poultry production, which reduces feed efficiency and increases the costs of meat production. We used a high-density SNP array (600K) to genotype 497 birds in a Brazilian F2 Chicken Resource Population representing a cross between broiler and layer lines, to estimate genomic heritability of fatness-related traits, to identify genomic regions and positional candidate genes (PCGs) associated with these traits. Whole genome sequence data of the founders from this population were used to identify regions with selection signatures to refine the list of PCGs, and to identify potentially causative SNPs. The genomic heritability values for fatness-related traits were high (0.43 – 0.56) in our population. We identified 22 unique quantitative trait loci (QTL) for abdominal fat and carcass fat content that harbored 26 PCGs that were involved in biological processes of fat deposition. Five of these 26 genes overlapped with selection signature regions identified using sequence data in the founders. We identified 5,164 SNPs in founder animals that were annotated in functional regions of the PCGs and, from those, 12 were predicted to be deleterious mutations. The identified QTLs, PCGs and potentially causative SNPs provide new insights into the genetic control of fatness.

2.1. Introduction

In the past, slow growth rates were a challenge in poultry production systems; consequently, intensive selection on this trait in elite great grand parent lines has dramatically increased poultry productivity. Modern commercial broiler chickens are produced from crosses that have been simultaneously selected for rapid growth, increased meat-production, and improved carcass yield[1,2]. However, chickens selected for higher body weight might exhibit increased appetite and excessive energy consumption, which may lead to excessive fat accumulation[3–5].

Excessive fat deposition in chickens is a negative factor for meat production because it reduces feed efficiency and the value of the carcass[6]. Therefore, understanding the genetic architecture, uncovering genomic regions, and finding positional candidate genes (PCGs) associated with fatness related traits could be helpful in breeding programs. In chickens, the main fat deposits are in the skin, on the coatings of organs, in the abdomen portion (abdominal plate)[7], and in the carcass during the later phase of development.

A total of 200 quantitative trait loci (QTL) have been reported for abdominal fat weight, 139 for abdominal fat percentage, and 11 for carcass fat content[8]. Previous QTL studies performed in the same population evaluated in this study (Embrapa F_2 Chicken Resource Population) using a total of 128 microsatellite markers for 22 autosomal chromosomes mapped QTLs for abdominal fat traits[9–11] and carcass fat traits[10] but the genomic locations of these QTL had large confidence intervals.

The genotyping of animals using high-density marker arrays should enable the identification of genomic regions with smaller intervals[12], which, in turn, facilitates the identification of PCGs. Thus, the aims of this study were to estimate genomic heritability for different fatness-related traits and to identify genomic regions and PCGs associated with these traits in an F_2 cross between a broiler and a layer line. We also integrated these results with selection signature information previously obtained using a large dataset of SNPs identified by whole genome sequencing from the parental generation of the F_2 population evaluated in this study.

2.2. Materials and Methods

All experimental protocols related to animal experimentation in this study were performed in agreement with the resolution number 010/2012 approved by the Embrapa Swine and Poultry Ethics Committee on Animal Utilization to ensure compliance with international guidelines for animal welfare.

2.2.1. Animals and Population

A total of 529 chickens from the Embrapa F_2 Chicken Resource Population were genotyped (28 parental chickens from layer and broiler lines; 5 chickens from F_1 and 496 chickens from the F_2-TCTC generations). This F_2 population is the result of crosses between two closed parental lines: broiler line (called TT) and a layer line (called CC). The TT line was selected for higher body weight, feed conversion, carcass and breast yield, viability, fertility, hatchability, reduction of abdominal fat weight and metabolic syndromes. The CC line was selected for egg production, egg weight, feed conversion, viability, sexual maturity, fertility, hatchability, egg quality and lowest body weight. More details about the Embrapa F_2 Chicken Resource Population were described by Rosário et al.[13].

This F_2 chicken population was previously used to map numerous QTLs for performance, carcass and chemical components traits[9–11,14–16] using microsatellite markers. For this study, the selection of progenies for genotyping and GWAS was based on those sires that appeared to be heterozygous for QTL effects reported in those previous studies.

2.2.2. Phenotype measurement

The total of 502 chickens from the F2 population were slaughtered and eviscerated at 42 days of age. The body weight at 42 days of age (BW42) was measured after 6 h of fasting. The carcass was stored in 4º C for 6 h, and then the carcass and parts were weighed. Abdominal fat was removed from chilled carcass to weighing (abdominal fat weight, ABF); abdominal fat percentage (ABFP) was calculated based on BW42[11].

Fat (ether extract) was measured by near-infrared reflectance spectroscopy (NIRS), and estimated as percentage according to the weight of each sample (250g of ground and homogenized carcass). Carcass fat content weight (CFC) was estimated multiplying the percentage in the sample by BW42. Carcass fat content on dry matter basis (CFCDM) was estimated dividing sample fat by carcass dry matter content and multiplying by 100. More details about traits measurement can be found at Campos et al.[11], Nunes et al.[3] and Nones et al.[10].

2.2.3. DNA extraction, genotyping and quality control

Genomic DNA was extracted from blood samples with DNAzol® protocol. After extraction, DNA integrity was evaluated on agarose gel (1%), quantified in spectrophotometer NanoDrop® (Thermo Fisher Scientific), and diluted to the final concentration of 20 $ng.\mu L^{-1}$. Diluted genomic DNA was prepared for genotyping following recommended Affymetrix protocols, and genotyped with the 600 K Axiom Chicken Genotyping Array. This array comprises SNPs segregating for different chicken lines[17].

Quality control analysis and genotype calling were performed with Affymetrix Power Tools v1.17.0 (APT). Samples that exhibited DishQC $\geq$ 0.82 and call rate $\geq$ 90% were kept for further analysis. Considering kept samples, the most accurate and polymorphic markers (*Polymorphic High Resolution SNPs*), and from those, SNPs with call rate $\geq$ 98% and minor allele frequency (MAF) $\geq$ 2% were kept for further analysis. In this step, R scripts from the package SNPolisher were used. SNPs located in the sex chromosomes, unmapped linkage groups, those without genomic annotation and those that were monomorphic were removed from the analysis. SNP annotation was based on the most recent chicken assembly (Gallus_gallus-5.0, NCBI). Missing genotypes were replaced by their average covariate value at that locus[18].

2.2.4. Descriptive statistics and heritability

The mean and the standard deviation of each phenotype were calculated using in-house scripts in R software (http://www.r-project.org/). The estimation of variance components was performed using

a Bayes C model in GenSel software[19]. The resultant posterior means of the variance components were used as *priors* in a Bayes B model to estimate genomic heritability for each trait, also using GenSel.

2.2.5. Genome-wide association analysis (GWAS)

The SNPs retained after quality control were used in the GWAS analysis with a Bayesian approach, performed with the GenSel software[19]. In the first step, a Bayes C model was used to estimate the genetic and residual variances and these values were used to run a Bayes B model, as performed by Cesar et al.[18]. The Bayes B model samples the effects of SNPs assuming that some fraction of their effects are zero and with unequal variance of each effect[20]. The mathematical model presented below was used in the association analysis:

$$\boldsymbol{y} = \boldsymbol{Xb} + \sum_{j=1}^{k} \boldsymbol{a}_j \beta_j \delta_j + \boldsymbol{e},$$

In this model, $\boldsymbol{y}$ represents the vector of phenotypic values, $\boldsymbol{X}$ is the incidence matrix for fixed effects, $\boldsymbol{b}$ is the vector of fixed effects, k is the number of SNPs, a_j is the column vector representing the SNP as a covariate in locus j coded with the number of B alleles, βj is the random substitution effect for locus j assumed to be normally distributed $N(0, \sigma^2_{\beta i})$ when $\delta j = 1$ but $\beta j = 0$ when $\delta j = 0$, with δj being a random variable 0/1 indicating the absence (with probability π) or presence (with probability 1-π) of the locus j in the model, and $\boldsymbol{e}$ is the vector of residual effects assumed to be normally distributed $N(0, \sigma^2_e)$. Sex and hatch were included as fixed effects in the model and BW42 was used as a fixed covariate for ABF and CFC.

We assumed $\pi = 0.9988$ in the Bayes B models and obtained 41,000 Markov Chain Monte Carlo (MCMC) samples with the first 1,000 samples being discarded. A map file was used to position the SNPs into 943 non-overlapping windows of 1 Mb. Each window is expected to explain 0.1060% of the genetic variance (100%/943) based on an infinitesimal model[21], and windows that explained five times more than the expected value (0.53%) were considered to be biologically significant. Thus, we selected only biologically significant windows to characterize and identify PCGs. Additionally, within each significant window (QTLs), we selected the SNP most frequently included in the model.

2.2.6. Overlapping with QTLs previously mapped

We checked the overlaps of all genomic windows detected, with QTLs previously reported as being mapped in chickens[8], using Chicken QTLdb - release 33, accessed in September, 2017. We used the BED file from the Animal QTL database with the QTL coordinates updated to the most recent chicken

genome assembly (Gallus_gallus-5.0, NCBI), and checked the overlaps using an in-house script in R. Some previously mapped QTLs were reported by their respective QTL ID numbers. The genomic windows that did not overlap with previously annotated QTL regions were considered novel discoveries.

2.2.7. Identification of candidate genes, overlap with selection signature regions and SNP screening

The list of annotated genes within each associated genomic window were searched using NCBI and OMIM databases, and also BioMart tool and literature to find GO terms and biological processes related to abdominal fat, lipid metabolism, fat content, and fat deposition. For all the analyses, we considered the gene annotation from *Ensembl Genes 90 Database*, and the last chicken genome assembly (Gallus_gallus-5.0, NCBI).

To refine the list of candidate genes, we compared our list of PCGs against selection signature regions identified in a previous study with 28 parental chickens from the two lines that generated the F_2 population analyzed in our study[22]. This study used whole genome sequence to identify SNPs and the Fst method[22,23] to estimate the divergence between populations and identify regions under selection (TT *vs.* CC lines). We used the CrossMap tool (http://crossmap.sourceforge.net/) to convert selection signatures coordinates to the most recent chicken genome assembly (Gallus_gallus-5.0, NCBI).

Additionally, to identify potential candidate genetic variants for fat deposition in chickens, we performed a screening of SNPs located in PCGs genes, using the same dataset of SNPs used to detect the selection signature regions (all the 13 million SNPs from NGS data identified were submitted to dbSNP-NCBI with the handle "LBA_ESALQ"). In order to refine our list of potential candidate genetic variants, we searched for genetic variants predicted as deleterious and also, genetic variants predicted as high impact.

To predict whether SNPs in coding regions are deleterious or not (may affect the protein function), we calculated the SIFT (sorting intolerant from tolerant) score. This score is an assessment of the level of conservation in homologous protein sequences[24] implemented by the VEP tool version 86[25]. SIFT scores were calculated for all the non-synonymous and stop codon (gained/lost) variants located in the PCGs.

The prediction of high impact SNPs was also performed using VEP tool[25] that provides an estimation of the putative impact of the variant classified as high impact, i.e. annotating all the mutations annotated as transcript ablation, splice acceptor, splice donor, stop gained, frameshift, stop loss, start lost and transcript amplification, mutations that may cause protein truncation, loss of function or trigger nonsense mediated decay (http://www.ensembl.org/info/genome/variation/predicted_data.html).

2.3. Results

2.3.1. Genotyping and quality control

From the 529 genotyped chickens, 40 were removed before the GWAS analysis. Among them, 12 were removed because they did not pass quality control (DishQC ≥ 0.82 and call rate ≥ 90% filter) and 28 did not have complete phenotypic data. As a result, 489 F_2 chickens were used for the association analysis.

From the 580,961 SNPs, originally available on the chicken SNP array, 399,693 SNPs segregating in the F_2 population were kept for further analysis. A total of 4,304 were removed due to low minor allele frequency (MAF) (≤ 0.02), while 23,603 SNPs located in sex chromosomes and unmapped linkage groups were also removed. After these filtering criteria, 371,786 SNPs from the autosomal chromosomes (GGA1-28) remained for the GWAS analysis. The average genotype density per chromosome was 541 SNPs/Mbp, with the lowest density being observed on GGA2 (297 SNPs/Mbp), and the highest density on GGA21 (816 SNPs/Mbp).

2.3.2. Descriptive statistics

The number of animals, mean and standard deviation, variance components and estimated genomic heritabilities are in Table 1. Genomic heritability values ranged from 0.43 for Carcass fat content (CFC) to 0.56 for abdominal fat percentage (ABFP).

Table 1 - Descriptive statistics, variance components and genomic heritability.

Trait	N	Average ± SD[1]	Genetic variance	Residual variance	Total variance	Genomic heritability[2]
ABF	476	15.60 ± 7.26	11.481	10.301	21.782	0.47
ABFP	476	1.56 ± 0.60	0.133	0.169	0.302	0.56
CFC	451	145.35 ± 40.52	212.237	159.809	372.047	0.43
CFCDM	451	39.75 ± 4.63	8.404	9.859	18.263	0.54

ABF: abdominal fat weight in grams; ABFP: abdominal fat percentage; CFC: carcass fat content in grams; CFCDM: carcass fat content on dry matter basis.
[1] Means and standard errors.
[2] Genomic heritability estimated with a Bayes B model.

2.3.3. GWAS

The genomic windows associated with fat traits are detailed in Table 2. A total of 22 significant unique 1 Mb windows (based on genome position) were identified and represented GGA1, 2, 7, 15, 20, 27 and 28. The posterior probability of association (PPA), as described by

Onteru et al.[26], ranged from 0.41 to 0.85 for these regions and the percentage of genetic variance explained by the windows ranged from 0.53 to 1.71.

Table 2 - Characterization of 1 Mb genomic windows associated with abdominal fat and carcass fat content traits in the Embrapa F_2 Chicken Resource Population.

Trait	GGA_Mb[1]	Genome interval (start – end position)[1]	N° of SNP/ window	% genetic variance explained	PPA[2]	SNP ID[3]	Model frequency
ABF	1_52	52,000,127 – 52,998,004	387	1.23	0.56	rs313050579	0.0108
	1_53	53,002,697 – 53,997,943	282	1.32	0.56	rs312317108	0.0158
	1_54	54,001,671 – 54,998,619	257	0.75	0.42	rs15271198	0.0173
	1_179	179,001,074 – 179,999,169	411	0.67	0.54	rs13557213	0.0111
	2_30	30,004,050 – 30,999,519	315	0.63	0.47	rs317553502	0.0138
	2_61	61,003,805 – 61,992,322	290	0.94	0.52	rs13619262	0.0527
	2_62	62,001,908 – 62,998,786	307	1.23	0.56	rs314667858	0.0253
	27_3	3,000,222 – 3,996,811	820	0.84	0.77	rs315719114	0.0097
	28_4	4,004,758 – 4,964,406	629	0.53	0.68	rs314073448	0.0038
ABFP	2_61	61,003,805 – 61,992,322	290	0.71	0.57	rs13619262	0.0272
	2_62	62,001,908 – 62,998,786	307	0.66	0.47	rs14193698	0.0193
	7_35	35,001,761 – 35,996,723	386	0.58	0.58	rs312894632	0.0106
	28_0	23,942 – 999,295	829	1.09	0.80	rs316394502	0.0512
	28_3	3,000,142 – 3,988,940	621	0.82	0.73	rs15251024	0.0146
CFC	1_53	53,002,697 – 53,997,943	282	0.91	0.51	rs314857319	0.0083
	1_168	168,005,668 – 168,997,872	318	0.56	0.41	rs312378109	0.0093
	1_169	169,001,420 – 169,999,438	346	0.82	0.44	rs315077363	0.0109
	1_170	170,002,808 – 170,999,129	446	0.98	0.48	rs13973557	0.0223
	1_171	171,000,120 – 171,999,874	407	0.91	0.52	rs315852521	0.0142
	1_175	175,003,078 – 175,996,880	405	1.28	0.52	rs313574684	0.0246
	7_35	35,001,761 – 35,996,723	386	0.73	0.52	rs314947533	0.0125
	7_36	36,000,235 – 36,898,384	257	0.58	0.44	rs312848275	0.0158
	15_9	9,002,743 – 9,999,015	639	0.82	0.64	rs316091564	0.0637
	15_10	10,001,717 – 10,999,147	577	0.71	0.63	rs13528818	0.0156
	28_4	4,004,758 – 4,964,406	629	0.82	0.75	rs314212680	0.0069
CFCDM	1_105	105,000,541 – 105,997,476	383	1.16	0.60	rs13916775	0.0154
	1_175	175,003,078 – 175,996,880	405	1.49	0.60	rs313574684	0.0206
	1_179	179,001,074 – 179,999,169	411	0.89	0.53	rs317863254	0.0121
	7_35	35,001,761 – 35,996,723	386	0.95	0.58	rs16614131	0.0170
	7_36	36,000,235 – 36,898,384	257	1.23	0.57	rs312848275	0.0257
	20_12	12,000,087 – 12,998,691	562	0.54	0.67	rs739732531	0.0204
	28_3	3,000,142 – 3,988,940	621	0.76	0.62	rs315921612	0.0176
	28_4	4,004,758 – 4,964,406	629	1.71	0.85	rs313086976	0.0181

ABF: abdominal fat weight in grams; ABFP: abdominal fat percentage; CFC: carcass fat content in grams; CFCDM: carcass fat content on dry matter basis.

[1] Map position based on Gallus_gallus-5.0, NCBI assembly.
[2] Posterior probability of association (PPA) as described by Onteru et al.[26].
[3] SNP within the window with the highest model frequency.

Carcass fat content had the highest number of significant QTLs (Figure 1), followed by ABF, CFCDM and ABFP (APPENDIX A, B and C). For CFC, the region that explained the largest amount of genetic variation was on GGA1 (175 Mb), with 1.28% of the genetic variance. For ABF, the region was on GGA1 (53 Mb), with 1.32% of the genetic variance, for CFCDM it was on GGA28 (4 Mb), with 1.71% of the genetic variance, and for ABFP it was on GGA28 (0 Mb), with 1.09% of the genetic variance.

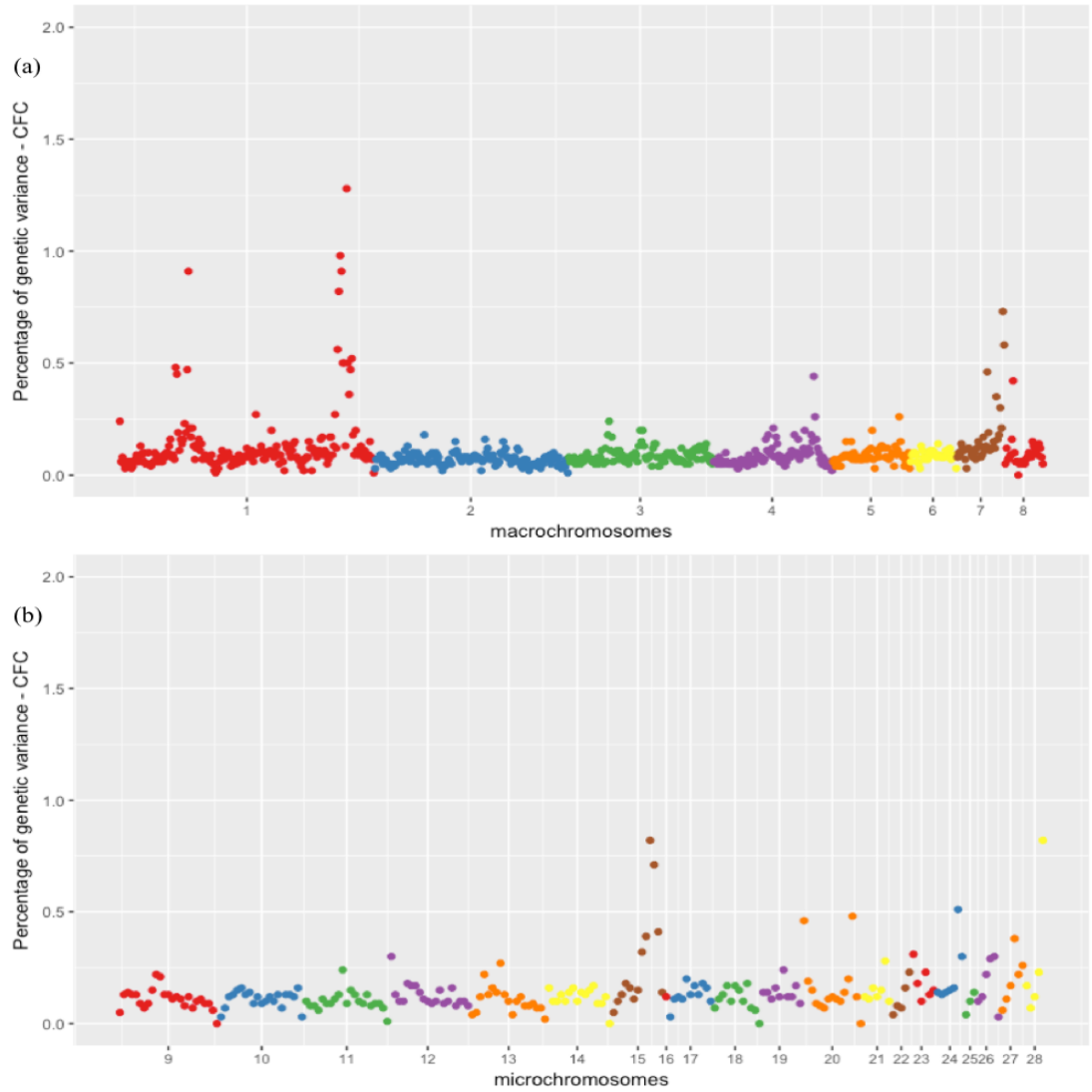

Figure 1 – Manhattan plot of the posterior means of the percentage of genetic variance explained by each 1 Mb SNP window across the 28 autosomal chromosomes for CFC (carcass fat content in grams): (A) genomic windows located on macrochromosomes, and (B) genomic windows located on microchromosomes.

Adjacent significant windows were considered as representing the same QTL and their respective proportions of genetic variance explained were summed up. For abdominal fat traits, three

adjacent windows on GGA1 (52-54 Mb) were identified that cumulatively accounted for 3.3% of the genetic variance for ABF and two adjacent windows on GGA2 (61-62 Mb) cumulatively accounted for 2.17% and 1.37% of the genetic variance for ABF and ABFP, respectively (Table 2).

For carcass fat content traits, four adjacent windows on GGA1 (168 -171 Mb) were identified that cumulatively accounted for 3.27% of the genetic variance. There were two adjacent windows on GGA7 (35-36 Mb) that cumulatively accounted for 2.18% and 1.31% of the genetic variance for CFCDM and CFC, respectively; two adjacent windows on GGA15 (9-10 Mb) that cumulatively accounted for 1.53% of the genetic variance for CFC, and two adjacent windows on GGA28 (3-4 Mb) that cumulatively accounted for 2.47% of the genetic variance for CFCDM (Table 2).

Co-located QTLs (the same QTL associated with abdominal fat and carcass fat content traits) were identified on GGA1 at 53 Mb, GGA1 at 179 Mb, GGA7 at 35 Mb and on GGA28 (3-4 Mb).

2.3.4. Overlapping with previously reported QTLs

One hundred and nineteen previously published QTLs for fatness traits mapped in different populations overlapped with the 21 QTLs identified in our study. The QTL on GGA28 at 0 Mb was novel (Figure 2).

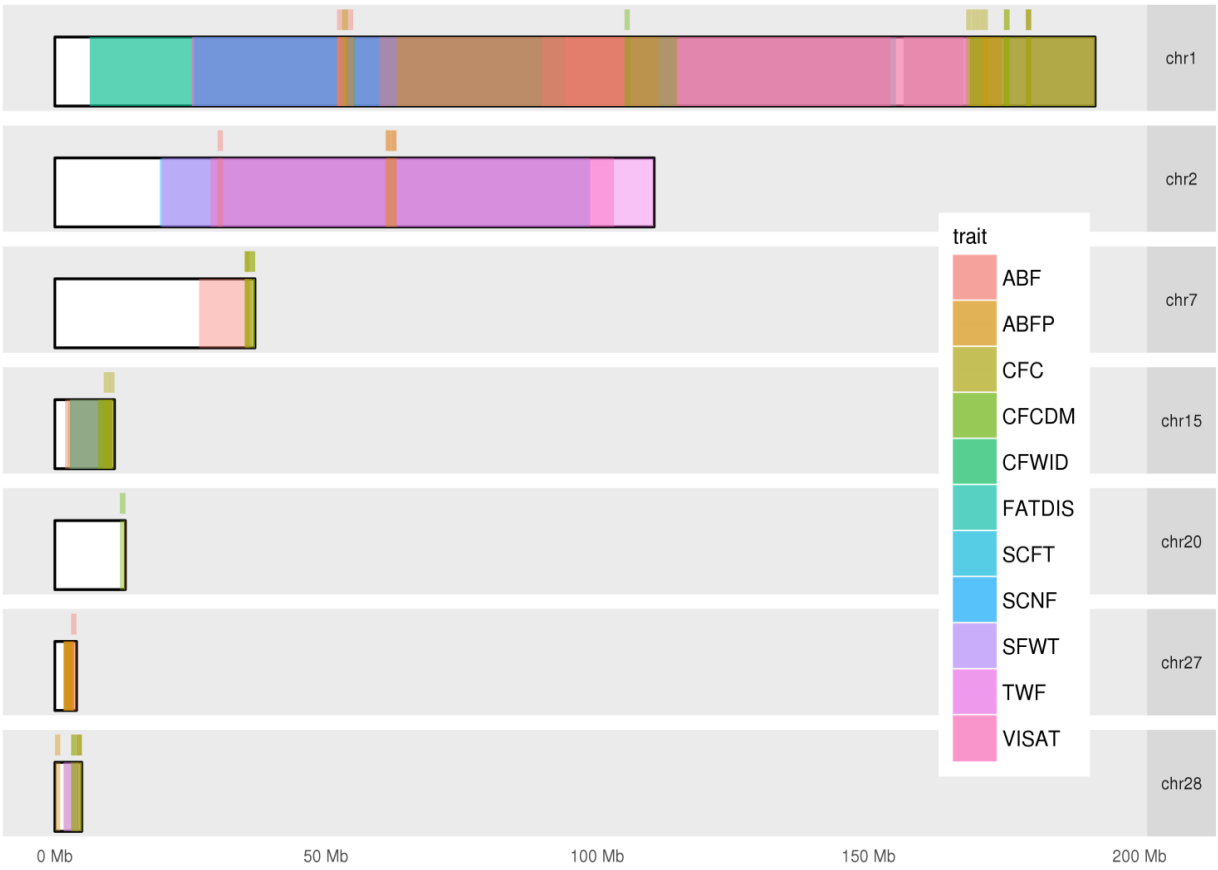

Figure 2 –Distribution of previously mapped QTLs identified as associated with fatness traits (from Animal QTLdb[8]) on autosomal chromosomes 1, 2, 7, 15, 20, 27 and 28 (in black boxes), and those detected in our study (above the black boxes). The colors represent the traits: ABF is abdominal fat weight; ABFP is abdominal fat percentage; CFC is carcass fat content; CFCDM is carcass fat content on dry matter basis; CFWID is Cingular fat width; FATDIS is fat distribution; SCFT is subcutaneous fat thickness (fat thickness under skin); SCNF is subcutaneous neck fat weight (subcutaneous neck adipose tissue); SFWT is skin fat weight; TWF is total white fat weight (total white adipose tissue); VISAT is visceral fat weight (visceral adipose tissue weight). The colors change or get denser according to the number of QTL previously mapped superimposing each other.

The QTLs on GGA1 (52-54 Mb), GGA1 (168-171 Mb), GGA1 at 179 Mb, GGA15 (9-10 Mb), GGA27 at 3 Mb and GGA28 (3-4 Mb) overlapped with previously published QTLs associated with the same traits: ABF (QTL #6806[27]; QTL #12478[28]; QTL #6869[29]; QTL #9665[30]; QTL #7010[31]; QTL #66072[32]; QTL #11817 and QTL #11809[11]; QTL #2417 and QTL #12632[33]); for CFC (QTL #17129 and QTL #17122[10]); for CFCDM (QTL #17130 and QTL #17120[10]).

Additionally, the known QTLs for ABF (#11817, #11809), CFC(#17129, #17122), and for CFCDM (#17130 and #17120) previously reported were mapped in the same F_2 population using microsatellite markers[10,11] covering 1.9 Mb, 1.9 Mb, 23.1 Mb, 2.7 Mb, 23.1 Mb and 23.1 Mb, respectively.

2.3.5. Positional candidate genes, selection signature regions and SNP screening

From the 22 QTLs identified, 14 contained PCGs annotated for fat deposition. Among these, four PCGs overlapped with selection signature regions (Table 3) identified in a previous study from our group. In this study, we reported a genome-wide characterization of genetic variations and regions under selection based on the Fst method applied to the sequence variants of 14 broilers and 14 layers (founders from Embrapa F_2 Chicken Resource Population)[22]. Additionally, one PCG (*CRY1*) was located 1.5 kb distant from a selection signature region (Table 3, APPENDIX D).

Table 3 – Genomic windows associated with fatness traits that harbor positional candidate genes.

GGA (Mb)	PCG[1]	Ensembl gene ID[2]	Number of SNPs[3]	SNP density (SNPs/kb)[4]
1 (52)	*MB*	ENSGALG00000012541	221	60
1 (53)[5]	*CRY1*[7]	ENSGALG00000012638	675	20
1 (54)[5]	*CHST11*[6]	ENSGALG00000030607	2593	17
1 (168)	*HTR2A*	ENSGALG00000016992	535	21
	RB1	ENSGALG00000016997	1673	20
1 (170)	*FOXO1*	ENSGALG00000017034	835	13
1 (175)	*SLC7A1*	ENSGALG00000017085	559	30
2 (30)	*IL6*	ENSGALG00000010915	161	60
7 (36)[5]	*NR4A2*[6]	ENSGALG00000012538	196	27
	GPD2[6]	ENSGALG00000012543	628	15
15 (9)	*PLA2G1B*	ENSGALG00000020989	145	88
	SIRT4	ENSGALG00000007244	314	71
15 (10)	*SELM*	ENSGALG00000025972	166	82
20 (12)	*DOK5*	ENSGALG00000007786	663	19
28 (0)	*SLC1A6*	ENSGALG00000000558	554	25
	ANGPTL4	ENSGALG00000000619	305	43
	RAB11B	ENSGALG00000000613	384	30
28 (3)	*STK11*	ENSGALG00000040008	738	22
	GDF3	ENSGALG00000003161	103	49
	TM6SF2	ENSGALG00000029015	214	65
	SLC25A42	ENSGALG00000002621	239	35
	SLC5A5	ENSGALG00000041932	244	54
	SLC39A3	ENSGALG00000020582	170	116
28 (4)[5]	*PIK3R2*	ENSGALG00000003428	275	15
	INSR[6]	ENSGALG00000040758	578	17
	SLC35E1	ENSGALG00000003794	103	22

[1] Positional candidate genes.
[2] Ensembl gene ID based on Galgal5 (*Ensembl Genes 90 Database*).
[3] Number of NGS-SNPs annotated on the PCG.
[4] SNP density in the respective PCG.
[5] Genomic windows that overlapped with signature selection regions[22].
[6] Positional candidate genes annotated within selection signature regions.
[7] Located 1.5 kb distant from one signature selection region.

Using the data generated from the whole genome sequence of the founders of this population, we observed that 13,271 SNPs were located in the 26 PCGs. The annotation of these SNPs are shown on Figure 3.

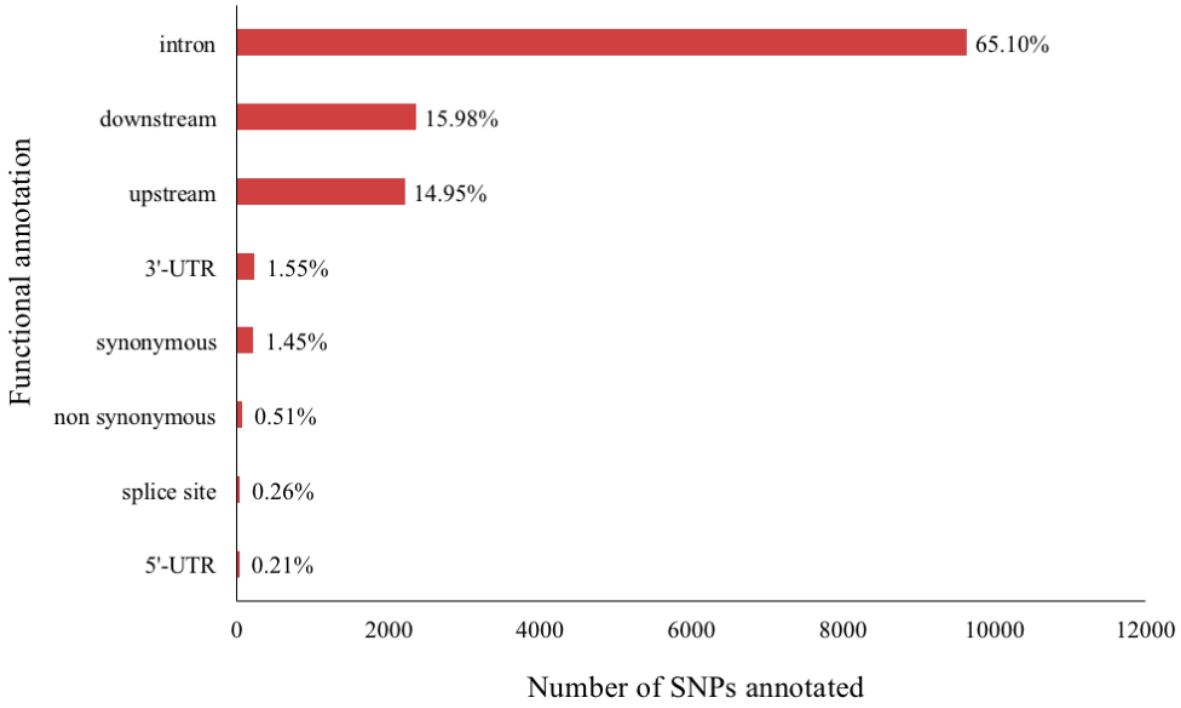

Figure 3 –Functional annotation of unique NGS-SNPs identified in 26 PCGs. The percentage was estimated based on the total number of SNPs annotated in 26 PCGs.

We also looked for potentially deleterious and high impact SNPs, which may affect gene expression and/or function. Twelve potentially deleterious SNPs were identified in eight PCGs (Table 4) but none of the mutations in our PCGs were annotated as having high impact.

Table 4 – Characterization of deleterious NGS-SNPs identified in our PCGs.

Associated Gene Name	SNP ID	GGA	Genome position[1]	SIFT score[2]	Amino acid changed
FOXO1	g.170581941>C/T	1	170,581,941	deleterious low confidence[3] (0.01)	Pro/Leu
	g.36224286>C/T		36,224,286	deleterious (0)	Val/Met
NR4A2	g.36225242>G/T	7	36,225,242	deleterious (0)	Arg/Ser
	g.36225278>C/T		36,225,278	deleterious (0.01)	Val/Met
SIRT4	rs316192467	15	9,435,175	deleterious (0.02)	Ala/Thr
DOK5	g.12473540>C/A	20	12,473,540	deleterious (0.02)	Lys/Asn
ANGPTL4	g.846035>G/A	28	846,035	deleterious (0.03)	Ser/Phe
TM6SF2	g.3553753>T/C	28	3,553,753	deleterious low confidence[3] (0.01)	Leu/Pro
	rs315426765		3,554,427	deleterious (0.05)	Leu/Phe
	rs741325985		3,554,836	deleterious (0.04)	Val/Met
SLC39A3	rs316529053	28	3,285,533	deleterious (0)	Arg/His
SLC35E1	g.4327417>G/A	28	4,327,417	deleterious (0.05)	Gly/Arg

[1] Position based on *Gallus_gallus* 5.0 assembly.
[2] SIFT (Sorting Intolerant From Tolerant) score.
[3] Deleterious low confidence: little sequence diversity in this position affecting the substitution model and consequently, means of conservation value and the confidence of the prediction.

2.4. Discussion

2.4.1. Genomic heritability

Genomic heritability was estimated using relationships inferred from high-density SNP panel genotypes instead of pedigree-based relationships. The use of close relatives and a higher density of SNPs may lead to a better genomic prediction with less bias[34,35].

For the evaluated traits, only pedigree-based heritability estimates were found in the literature. In a study with 1,069 purebred full-sib male chickens, Chen et al.[36] reported heritability estimates for ABF and ABFP of 0.62 and 0.24, respectively. Zerehdaran et al.[37] reported heritability estimates for ABF and ABFP of 0.62±0.09 and 0.71±0.09, respectively, using 3,278 chickens from a meat-type population after 9 generations of intercrossing. A higher value was reported for ABF (0.82) using the records of 300 chickens from a commercial female grandparent stock[38] and could be explained by the fact that female broiler chickens generally deposit more abdominal fat then male broiler chickens[4,39].

In the same population (Embrapa F_2 Chicken Resource Population), pedigree-based heritability for ABF was estimated at 0.33±0.19 in the F_2-CTCT population (layer males crossed with broiler females)

and 0.82±0.3 in the F_2-TCTC population (broiler males crossed with layer females)[40]. Considering the carcass fat content traits, heritability estimates for CFC expressed in percentage of wet carcass was equal to 0.53±0.10, and the heritability for fat percentage in dry-matter basis (CFCDM) was 0.55±0.10, using records of 3,422 chickens[3].

Our genomic heritability estimates (Table 1) for fatness-related traits were high (greater than 0.43), similar to the pedigree-based heritability reported for the same traits in the same F_2 population.

2.4.2. GWAS

We observed that genetic variation explained by each 1Mb genomic windows ranged from 0.53 to 3.3% depending on the trait analyzed. Cumulatively, the significant windows associated with ABF, ABFP, CFC and CFCDM explained 8.14%, 3.28%, 9.12% and 8.73% of the genetic variance, respectively. Most genomic windows explained <0.53% for the traits analyzed and were not considered. Due that, only a small proportion of the genetic variance were explained by our QTLs detected.

Almost all the QTLs detected overlapped with previously reported QTLs associated with fatness traits in this or in other populations, showing a greater reliability in our findings. Approximately 60% of the QTLs detected exhibited overlaps for exactly the same traits while the others were deemed novel for the traits analyzed. The QTL located in the first bases of GGA28 was totally novel and can be a population specific QTL.

Considering the QTL span of those previously mapped in the same population (#11817, #11809, #17129, #17122, #17130 and #17120), we refined these regions achieving a reduction in the size estimated around 47% for ABF QTLs, 68% for CFC QTLs and 95% for CFCDM QTLs. These six previously known QTLs were mapped using 128 microsatellite markers to cover 22 chromosomes and interval mapping approach to detect[10,11]. Our approach using a high density of SNPs and genomic prediction methodology to associate 1 Mb non-overlapping windows provided us with better resolution.

The QTLs observed on GGA1 at 53 Mb, GGA1 at 179 Mb, GGA7 at 35 Mb and on GGA28 (3-4 Mb) were associated with both abdominal fat and carcass fat content traits suggesting that they can be pleiotropic QTLs or even multiple QTLs. The discovery of PCGs within these regions may give insight to understanding the complex biological processes involved in abdominal fat and carcass fat content regulation in chickens.

Additionally, we also checked the overlap of our QTLs associated with fatness-related traits and previsiouly mapped QTLs for body weight at 35 (BW35) and also 41 (BW41) days-of-age mapped in the same population[9,14,41,42]. From the 22 QTLs detected, 17 did not overlap QTLs for body weight traits as expected since we included BW42 as covariate in our analysis.

2.4.3. Positional candidate genes for fat deposition

We selected PCGs based on their Gene Ontologies (GO) terms and literature information for each gene. From the 26 PCGs selected, 17 had GO terms for fat cell differentiation, insulin and triglycerides levels among other processes involved in fat deposition (Table 5).

Positional candidate genes were selected based on their associated Gene Ontology (GO) terms and literature information. From the 26 PCGs selected, 17 had GO terms for fat cell differentiation, insulin and triglycerides levels among other process involved in fat deposition (Table 5).

Table 5 - List of PCGs that exhibited GO terms related to lipid metabolic processes.

Gene	Gene Ontology terms
CRY1	response to insulin, lipid storage, glucose homeostasis
HTR2A	positive regulation of fat cell differentiation
RB1	regulation of lipid kinase activity
FOXO1	cellular response to insulin stimulus, insulin receptor signaling pathway, negative regulation of fat cell differentiation, glucose homeostasis
IL6	positive regulation of B cell activation
NR4A2	fat cell differentiation
GPD2	oxidation-reduction process, gluconeogenesis, glycerol-3-phosphate dehydrogenase activity
PLA2G1B	phospholipid metabolic process, lipid catabolic process, lipid metabolic process
SIRT4	negative regulation of insulin secretion, positive regulation of lipid biosynthetic process
SELM	adipose tissue development
DOK5	insulin receptor binding
ANGPTL4	triglyceride homeostasis, negative regulation of lipoprotein lipase activity
RAB11B	insulin secretion involved in cellular response to glucose stimulus
STK11	negative regulation of lipid biosynthetic process, glucose homeostasis
TM6SF2	regulation of lipid metabolic process
PIK3R2	cellular response to insulin stimulus, insulin receptor signaling pathway, cellular glucose homeostasis
INSR	cellular response to insulin stimulus, insulin binding, insulin-activated receptor activity, insulin-like growth factor receptor binding, insulin-like growth factor I binding, insulin-like growth factor II binding, insulin receptor substrate binding, insulin receptor signaling pathway, insulin receptor complex, glucose homeostasis, positive regulation of glucose import

From the genes described in Table 5, related to lipid metabolic processes, eight were annotated with GO terms for insulin synthesis, secretion and regulation, including the *IL6* gene that exhibited a GO term for positive regulation of B cell activation (responsible for synthesizing and secreting insulin[43]). Increases in insulin levels in chickens, may affect the expression of few genes related glucose and lipid metabolism[44], consequently affecting fat accumulation. Thus, the genes annotated with GO terms related to insulin are PCGs for lipid metabolism and fat deposition regulation in chickens.

Several genes, namely *PLA2G1B, SELM, DOK5, HTR2A* and *GDF3*, have been previously reported to be involved in obesity. The *PLA2G1B* gene harbored a SNP associated with fat accumulation and distribution in humans[45]. In the mouse, the knock-out of *SELM* gene resulted in elevated white adipose tissue deposition[46]. In the *DOK5* gene, genetic variants were associated with obesity in North Indian patients[47]. In the *HTR2A* gene, polymorphisms were associated with central adiposity in a study with humans[48]. Mice that were *GDF3* deficient exhibited a modest reduction in adiposity[49]. These studies corroborate the selection of these genes as PCGs for fat deposition.

Four genes were within selection signatures regions identified by comparing the sequence from founders of the F_2 population used in this study: *CHST11*, *NR4A2*, *GPD2* and *INSR*. The *CHST11* gene is associated with lipid metabolism, and its expression affects lipid accumulation in adipocytes[50]. This gene overlapped with a selection signature region (APPENDIX D), indicating that SNPs in these genes exhibit frequency differences between the parental lines, and may be associated with lipid accumulation.

The *NR4A2* gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. NR4A receptors regulate hepatic glucose[51], and consequently, lipid metabolism. Additionally, this gene influences retinoid signaling[51–53], and although the mechanisms has still not been clearly elucidated, it is known that retinoid plays an important role in lipid metabolism[54].

The *GPD2* gene is a mitochondrial dehydrogenase, and its expression may affect gluconeogenesis and glucose homeostasis[55]. In a study with mice, Brown et al.[56] reported a reduction of 40% in the weight of white adipose tissue in the individuals with knocked-out *GPD2*. *NR4A2* and *GPD2* genes overlapped with selection signatures regions (APPENDIX D), indicating that selection possibly affected the frequency of SNPs in both genes. These SNPs may be associated with glucose homeostasis and lipid metabolism.

The *INSR* gene plays an important role in insulin signaling[57] and, as mentioned before, insulin levels affect lipogenesis and consequently lipid accumulation. Additionally, the *INSR* gene overlapped with a selection signature region (APPENDIX D) with genetic variants mainly fixed in the broiler line[22]. These findings indicate that selection affected the frequency of SNPs in this particular gene in the broiler line.

The *CRY1* gene is located 1.5 kb distant from a selection signature region (APPENDIX D) and genetic variants located in this gene may have their frequencies affected by selection[58]. The *CRY1* was expressed in subcutaneous abdominal and visceral fat depots[59] in a study with humans.

Besides the *CHST11* gene, seven other genes were not annotated with GO terms related to fat deposition: *MB*, *SLC7A1*, *SLC1A6*, *SLC25A42*, *SLC5A5*, *SLC39A3* and *SLC35E1*. However, there was

some evidence in the literature to support them as PCGs. The Myoglobin (*MB)* expression was associated with fatty acid metabolism in a study with mice[60]. Six PCGs identified are members of the solute carrier (SLC) family that encodes proteins located on the cell membrane. The superfamily of SLC genes encodes membrane-bound transporters[61], and one of these has been associated with obesity in humans[62,63]. The SLC1 family regulates glutamate transport and, in liver cells, this amino acid is a precursor of fatty acid biosynthesis[64]. In a study with rats, mice and rabbits, Collin et al.[65] demonstrated that glutamate transporters activity might regulate energy balance. Energy balance is directly related with fatty acids biosynthesis and consequently, fat storage.

2.4.4. NGS-SNPs annotated in PCGs

The density of SNPs in our PCGs ranged from 13 to 116 SNPs/kb. Previous studies in chickens reported density of SNPs across the entire genome ranging from 5 to 78 SNPs/kb[17,57], corroborating our findings for most of the PCGs. The top three genes with the highest SNP density were *SELM, PLA2G1B and SLC39A3* (Table 3). These genes should be thoroughly investigated since, this high variability may be affecting fat deposition in this F_2 Chicken Resource Population.

Genetic variants in PCGs that overlapped with selection signature regions may exhibit polymorphisms responsible for phenotypic variation[66–68]. Thus, the genetic variants annotated in potentially functional gene regions from *CRY1*, *CHST11*, *NR4A2*, *GPD2* and *INSR* are important candidates for further association and functional studies.

Approximately 65% of the SNPs detected in our PCGs belonged to intronic regions (Figure 3). SNPs located in intron can affect gene expression and consequently affecting the phenotype[69,70], however, they are commonly deemed as potentially neutral. The other 35% of the SNPs detected were annotated in functional gene regions: up/downstream from the gene, 3'and 5'-UTRs, exons (synonymous and non-synonymous), and splice sites (Figure 3).

Variants in coding regions can be related to phenotypic variation and, more specifically, non-synonymous variants imply in amino acid changes[71]. Changes in amino acids can potentially affect protein function. To predict whether SNPs in coding regions are deleterious or not (may affect the protein function), we calculated the SIFT score as described in Methods section.

Twelve potentially deleterious NGS-SNPs were identified in eight PCGs (Table 4) and most of them are located in PCGs involved in lipogenesis, triglycerides levels and obesity. Moreover, two of these genes exhibited more than one deleterious mutation: *NR4A2,* involved in the regulation of hepatic glucose affecting lipid metabolism, and *TM6SF2,* involved in the regulation of triglycerides levels in the liver. Changes in the function of these genes may affect fat deposition in chickens. Thus, all the deleterious mutations are important candidates for further association and functional studies.

From the 12 potentially deleterious SNPs, only rs315426765 on the *TM6SF2* gene was common to both datasets (whole genome sequence SNPs and SNPs from the Affymetrix genotyping array). However, that SNP was removed after genotyping quality control. The search for potential candidate SNPs in whole genome sequence data allowed us to identify a great number of SNPs, which are not present in the Affymetrix genotyping array.

The GWAS using a high density of SNPs allowed us to map QTLs with better resolution than had been found in the past using microsatellites. The PCGs identified in our study, especially those that overlapped with selection signatures and harboring genetic variants located on potentially functional gene regions, can be targets in poultry breeding to reduce excessive fat deposition, thus improving feed efficiency.

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3. A GENOME-WIDE ASSOCIATION STUDY REVEALS NOVEL GENOMIC REGIONS AND POSITIONAL CANDIDATE GENES OR FAT DEPOSITION N MEAT-TYPE CHICKENS

ABSTRACT

Excess fat content in chickens has a negative impact on poultry production. The discovery of QTL associated with fat deposition in the carcass allows the identification of positional candidate genes (PCGs) that might regulate fat deposition and be useful for selection against the excess of fat content in chicken's carcass. This study aimed to estimate genomic heritability coefficients and to identify QTLs and PCGs for abdominal fat (ABF) and skin (SKIN) traits in a broiler chicken population. ABF and SKIN are moderately heritable traits in our broiler population with estimates ranging from 0.23 to 0.33. Using a high density SNP panel (355,027 informative SNPs), we detected nine unique QTLs that were associated with these fat traits. Among these, four QTL were novel, while five have been previously reported in the literature. Thirteen PCGs were identified that might regulate fat deposition in these QTL regions: *JDP2*, *PLCG1*, *HNF4A*, *FITM2*, *ADIPOR1*, *PTPN11*, *MVK*, *APOA1*, *APOA4, APOA5,* ENSGALG00000000477, ENSGALG00000000483, and ENSGALG00000005043. We used sequence information from founder animals to detect 4,843 SNPs in the 13 PCGs. Among those, two were classified as potentially deleterious and two as high impact SNPs. This study generated novel results that can contribute to a better understanding of fat deposition in chickens. The use of high density array of SNPs increases genome coverage and improves QTL resolution than would have been achieved with low density. The identified PCGs were involved in many biological processes that regulate lipid storage. The SNPs identified in the PCGs, especially those predicted as potentially deleterious and high impact, may affect fat deposition. Validation should be undertaken before using these SNPs for selection against carcass fat accumulation and to improve feed efficiency in broiler chicken production.

3.1. Introduction

The chicken was the first domesticated animal species that was whole-genome sequenced and it has emerged as an excellent model for genomic studies in agriculture, developmental biology, fatness and leanness [1]. The main fat deposits in chicken are located in the skin (including subcutaneous fat) and within the abdominal cavity (abdominal plate) [2–4]. Excess fat deposition in broiler chickens is a negative factor for the poultry industry because it decreases feed efficiency and reduces the nutritional value of

carcass parts and, consequently, their commercial value [5–7].

Broiler chicken lines have been selected for rapid growth, and carcass yield [7,8]. Rapid growth results in increased fat deposition within the carcass [8] and commercial chickens exhibit higher fat deposition compared with unselected chickens [7]. The selection of chickens for rapid growth and reduced carcass fat deposition is challenging because these two traits have a positive genetic correlation [8].

Some studies have been conducted to map genomic quantitative trait loci (QTLs) associated with variation in abdominal fat [5,9–13] and skin traits [9,14]. However, most previously published QTLs were detected using low density of markers (ranging from 102 to 410 markers), and the detected intervals spanned tens of centimorgans (cM) [15].

Previous genome-wide association studies (GWAS) have been performed for abdominal fat weight and fat percentage in an F2 chicken population's using a 60 K SNP chip (Illumina) [16,17] and in a local population of a local Chinese breed using approximately 90,000 SNPs [18]. To the best of our knowledge, no GWAS was reported for fatness traits in a meat-type population using the high-density SNP chip (600 K) from Affymetrix [19].

Fat deposition is an economically-relevant trait in fast-growing chickens, and knowledge about the genetic regulation of this trait is essential for breeding programs. Based on this fact, the main goal of this study was to perform GWAS analysis using a high-density SNP panel (600K) to identify QTL and positional candidate genes (PCGs) and possibly candidate mutations for fat deposition in broiler chickens.

3.2. Materials and Methods

All experimental protocols related to animal experimentation in this study were performed in agreement with resolution number 010/2012 approved by the Embrapa Swine and Poultry Ethics Committee on Animal Utilization to ensure compliance with international guidelines for animal welfare.

3.2.1. Chicken population

This study was conducted with a paternal broiler line (TT) belonging to the Chicken Breeding Program of EMBRAPA Swine and Poultry National Research Center, in Concordia, Santa Catarina State – South of Brazil. This line, originated from the White Plymouth Rock and White Cornish breeds, has been under multiple trait selection since 1992 mainly for body weight, feed conversion, carcass and cuts yield, viability, fertility, hatchability and reduced abdominal fat [13, 20-23]. The TT Reference Population evaluated in this study was founded in 2008 and consisted of 1,430 chickens (652 males and 778 females)

generated in five hatches from 20 males and 92 females (1:5). Previous genomic studies were performed in this population, and more details can be found in [20-23].

3.2.2. Phenotype measurement

Chickens were slaughtered at 42 days of age after six hours of fasting when the body weight at 42 days of age was recorded (BW42). In this step, a blood sample from each chicken was immediately collected for subsequent DNA extraction then, the carcass was cooled. After 4 h of cooling, the weights of the carcass, skin covering each carcass part (thigh, drumstick, and breast) and abdominal fat (abdominal fat pad) were measured. The percentage of each trait was calculated dividing the weigh by BW42 and multiplying by 100. Total skin weight and percentage were used as indicators of subcutaneous fat, as discussed by Zerehdaran et al. [3]. More details about the rearing conditions and phenotypes measurements are available in Fornari et al. [22].

3.2.3. DNA extraction, genotyping and quality control

Genomic DNA from 1,430 blood samples were extracted using the PureLink® Genomic DNA (Invitrogen, Carlsbad, CA, USA) kit and were quantified using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). After extraction, DNA integrity was evaluated on agarose gel (1%) and diluted to 10 ng/μL. Diluted genomic DNA was prepared following recommended Affymetrix protocols in order to perform the genotyping analysis using 600 K Affymetrix Axiom Genotyping Array (Affymetrix, Inc. Santa Clara, CA, USA). This genotyping array comprises segregating SNPs for different chicken populations, including four commercial broilers (broiler chicken lines), as detailed by Kranis et al. [19].

Initially, Axiom™ Analysis Suite (Affymetrix®) software was used to filter genotypes based on the DishQC parameter, after which PLINK v.1.9 [24] software was used to perform quality control analysis and for genotype calling. Only samples that exhibited DishQC $\geq$ 0.82 and call rate $\geq$ 90% were kept for further analysis. Considering these retained samples, in order to select markers with high quality, and to avoid potential genotyping errors or even DNA contamination, further edits were undertaken based on literature recommendations [25], to remove single nucleotide polymorphisms with a call rate $\leq$ 98%, minor allele frequency (MAF) $\leq$ 2% or significant deviations from HWE (p-value < 0.000001). Single nucleotide polymorphisms located in the sex chromosomes, and those SNPs not mapped in the chicken assembly (Gallus_gallus-5.0, NCBI) were excluded from the analysis. Only the SNPs annotated to autosomal chromosomes from GGA1 to GGA28 were used in statistical analyses. After all the filtering

steps, the few remained missing genotypes were replaced by the average of covariate values at that particular locus, as described by Cesar et al. [26].

From a total of 1,430 genotyped chickens, 22 samples were removed from the analysis after applying the DishQC criteria, and a filter on sample call rate ≥ 90% loci. From the total of 580,961 SNPs available on the SNP array, 355,027 informative polymorphic SNPs on the autosomal chromosomes (GGA1-28) were kept after filtering. The average density of SNPs was 520 SNPs/Mbp, with the lowest chromosome-wise density observed on GGA2 (268 SNPs/Mbp), and the highest chromosome-wise density on GGA21 (898 SNPs/Mbp) (APPENDIX E).

3.2.4. Descriptive statistics and heritability

The mean and the standard deviation of each phenotype were calculated using R scripts. The estimation of variance components (genetic variance, residual variance, and total variance) was performed using a Bayes C model in GenSel software [27] using the samples and SNPs remained after genotyping and filtering. The resultant posterior means of the variance components were used as *priors* in subsequent Bayes B models to estimate genomic heritability for each trait.

3.2.5. GWAS

The SNPs that passed the quality control filters were used in the GWAS analysis using genomic prediction methodology with a Bayesian approach in GenSel software [27]. In the first step, a Bayes C model was used to estimate the genetic and residual variances for each trait and these values were then used as *priors* to run a Bayes B model as performed by Cesar et al. [26]. The Bayes B models sample the effects of SNPs assuming some fraction of the effects are zero and with unequal variance of each effect [28]. The mathematical model was:

$$\boldsymbol{y} = \boldsymbol{X}\boldsymbol{b} + \sum_{j=1}^{k} \boldsymbol{a}_j \beta_j \delta_j + \boldsymbol{e},$$

In this model, $\boldsymbol{y}$ represents the vector of phenotypic values; $\boldsymbol{X}$ is the incidence matrix for fixed effects; $\boldsymbol{b}$ is the vector of fixed effects; K is the number of SNPs; a_j is the column vector representing the SNP as a covariate in locus $_j$ coded with the number of B alleles; βj is the random substitution effect for locus j assumed to be normally distributed $N(0, \sigma^2_{\beta i})$ when $\delta j = 1$ but $\beta j = 0$ when $\delta j = 0$, with δj being a random variable 0/1 indicating the absence (with probability π) or presence (with probability 1-π) of the locus j in the model, and $\boldsymbol{e}$ is the residual associated with the analysis. Sex and hatch were included as fixed effects in the model and BW42 (slaughter age) as a fixed covariate for ABF and SKINW.

We assumed $\pi = 0.9970$ in the BayesB models and obtained 41,000 Markov chain Monte Carlo (MCMC) samples with the first 1,000 samples being discarded. A map file was used to position the markers into 947 non-overlapping 1 Mb windows. The windows that had the marker with higher model frequency in the MCMC interactions had their effect predicted as mentioned by Van Goor et al. [29]. Each window is expected to explain 0.1054% of the genetic variance (100%/947) based on an infinitesimal model [30, 31], and windows that explained five times more than the expected (0.53%) were considered significant. Thus, we selected only significant windows to characterize and identify PCGs.

3.2.6. Overlapping with known QTLs

The overlap of our genomic windows with previously mapped QTLs for fat-related traits in chickens was determined using the information available at Chicken QTLdb - release 33, accessed in September, 2017 [32]. We used the available BED file with the QTL coordinates according to the last chicken genome assembly (Gallus_gallus-5.0, NCBI) to check the overlaps using in-house R scripts. The genomic windows that did not overlap with previously annotated QTLs for fat traits were considered to be novel discoveries. All the previously mapped QTLs were reported by QTL ID numbers, available at Chicken QTLdb [32].

3.2.7. Identification of positional candidate genes

A list of annotated genes within each QTL (genomic window) and their respective GO terms and biological processes were obtained using Ensembl BioMart tool [33, 34]. Genes that had GO terms and a biological process related to fat deposition were initially selected. Next, two different databases (NCBI, OMIM) were searched to identify literature to support/refute the positional candidate genes identified.

Enrichment analysis of gene list was performed with the Functional Annotation Tool (FAT) in Database for Annotation, Visualization and Integrated Discovery software (DAVID bioinformatics resources v.6.8, [35, 36]) to identify possible enriched clusters of genes. To select a gene cluster as enriched, we considered an enrichment score > 1.00 and within the cluster, GO terms for biological process (BP) with a raw p-value < 0.05 and p-value adjusted for multiple testing by Benjamini & Hochberg [37] method < 0.1.

3.2.8. Screening for SNPs from NGS data in the candidate genes

To refine our list of candidate genes, we screened our PCGs for genetic variants using a dataset of SNPs identified in the parental generation from our genotyped population, which were generated by next generation sequencing (NGS) of 14 parental males (from 112) with approximately 13 X of coverage performed by HiScanSQ (Illumina) with a read length of 101 bp. Further details about library preparation and sequencing are available in Moreira et al. [38,39].

SNP calling was performed using the most recent chicken genome assembly Gallus-gallus-5.0 (UCSC) with SAMtools software v.1.2 [40], with mapping and base qualities (Phred score) ≥ 20. The filtering criteria and further details about SNP calling are available in Boschiero et al. [41]. After the filtering of variants, the dataset of SNPs was annotated using Variant Effect Predictor (VEP) tool version 86 [42].

After functional annotation, we selected only the SNPs annotated in our 13 PCGs for further characterization, and to identify genetic variants potentially affecting gene expression and/or function. Considering all the SNPs annotated in each PCG and its gene length, we estimated the SNP density.

Variants located in coding regions can lead to phenotypic changes [38,39,43]. To predict whether SNPs that caused changes in amino acids were tolerant or not (may affect the function of the gene product), we calculated the SIFT (sorting intolerant from tolerant) score. This score is an assessment of the level of conservation in homologous protein sequences using the SIFT algorithm [44] implemented by the VEP tool version 86 [42]. SIFT scores were calculated for all the non-synonymous and stop codon (gained/lost) variants located in the PCGs.

High impact SNPs were also evaluated in the candidate genes. The VEP tool [42] provides an estimation of the putative impact of the variant classified as high impact, i.e. annotating all the mutations annotated as transcript ablation, splice acceptor, splice donor, stop gained, frameshift, stop loss, start lost and transcript amplification, mutations that may cause protein truncation, loss of function or trigger nonsense mediated decay [43].

3.3. Results

3.3.1. Descriptive statistics and genomic heritability

The number of animals, averages and standard errors, variance components and estimated genomic heritability from the Bayes B model are given in Table 6 for ABF, ABFP, SKINW and SKINP. We estimated genomic heritability values to be moderate for all traits evaluated; ABF traits exhibited higher genomic heritability compared to SKIN traits.

Table 6 - Descriptive statistics, variance components and genomic heritability for body weight at 42 days of age, abdominal fat and skin weights and percentages in the TT Reference Population.

Trait	N	Average ± SD[1]	Genetic variance	Residual Variance	Total variance	Genomic heritability[2]
BW42	1,311	2220.30 ± 258.86	12378.000	25423.100	37801.100	0.33
ABF	1,287	47.10 ± 14.03	46.599	96.079	142.677	0.33
ABFP	1,287	2.13 ± 0.62	0.094	0.205	0.299	0.31
SKINW	1,303	94.55 ± 16.12	29.936	96.443	126.379	0.23
SKINP	1,303	4.25 ± 0.56	0.063	0.203	0.266	0.23

BW42: body weight at 42 days of age in grams; ABF: abdominal fat weight in grams; ABFP: abdominal fat percentage; SKINW: skin weight in grams; SKINP: skin percentage.
[1] Standard deviation of the mean.
[2] Genomic heritability estimated by Bayes B model.

3.3.2. GWAS

The QTLs (significant genomic windows) associated with fat deposition are described in Table 7. Nine unique significant 1 Mb windows (with different unique positions) were identified on GGA 5, 9, 10, 13, 15, 20, 24, 26, and 27. The posterior probability of association (PPA), as described by Onteru et al. [31], ranged from 0.82 to 0.95 for each region, and the proportion of genetic variance explained by the window ranged from 0.54 to 1.49.

Table 7 - Characterization of 1 Mb significant genomic windows for abdominal fat and skin traits in the TT Reference Population.

Trait	GGA (Mb)[1]	SNP window (first – last position)[1]	Number of SNP/ window	Number of genes/ window[2]	Proportion of genetic variance explained by the SNP window	PPA[3]
ABF	5 (38)	38,000,437 – 38,996,916	396	31	0.92	0.84
	10 (7)	7,000,336 – 7,998,549	592	21	0.58	0.93
	13 (3)	3,002,617 – 3,998,616	460	16	1.45	0.88
	20 (5)	5,000,651 – 5,999,452	492	53	0.94	0.88
	26 (1)	1,002,598 – 1,999,851	662	74	1.06	0.95
ABFP	5 (38)	38,000,437 – 38,996,916	396	31	0.64	0.82
	10 (7)	7,000,336 – 7,998,549	592	21	0.61	0.90
	13 (3)	3,002,617 – 3,998,616	460	16	1.49	0.89
	26 (1)	1,002,598 – 1,999,851	662	74	0.54	0.92
SKINW	15 (6)	6,000,311 – 6,999,944	544	62	0.73	0.89
	24 (5)	5,000,105 – 5,999,010	778	60	0.56	0.91
	27 (3)	3,000,222 – 3,997,124	933	52	0.60	0.94
SKINP	9 (4)	4,000,836 – 4,999,336	482	50	0.73	0.83
	15 (6)	6,000,311 – 6,999,944	544	62	0.71	0.91
	27 (3)	3,000,222 – 3,997,124	933	52	0.57	0.95

ABF: abdominal fat weight; ABFP: abdominal fat percentage; SKINW: skin weight; SKINP: skin percentage.
[1] Map position based on Gallus_gallus-5.0 assembly (NCBI).
[2] Number of genes annotated within the genomic window based on *Ensembl Genes 90 Database.*
[3] Posterior probability of association (PPA) as described by Onteru et al. [31].

The Manhattan plot of the posterior means of the proportion of genetic variance explained by each SNP window across the 28 autosomal chromosomes for ABF are presented in Figure 4. The Manhattan plots for ABFP, SKINW and SKINP are in APPENDIX F, G and H, respectively.

Figure 4 – Manhattan plot of the posterior means of the proportion of genetic variance explained by each 1-Mb SNP window across the 28 autosomal chromosomes for abdominal fat weight (ABF): (A) genomic windows located on macrochromosomes, and (B) windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis. Red lines indicate the threshold to deem significant SNP windows.

In order to support our findings, we checked the effect of the markers within the associated genomic windows. Manhattan plots of the SNP effect distribution within each significant SNP window for ABF are colored by chromosome and presented in Figure 5. The Manhattan plots for ABFP, SKINW and SKINP are in APPENDIX I, J and K, respectively.

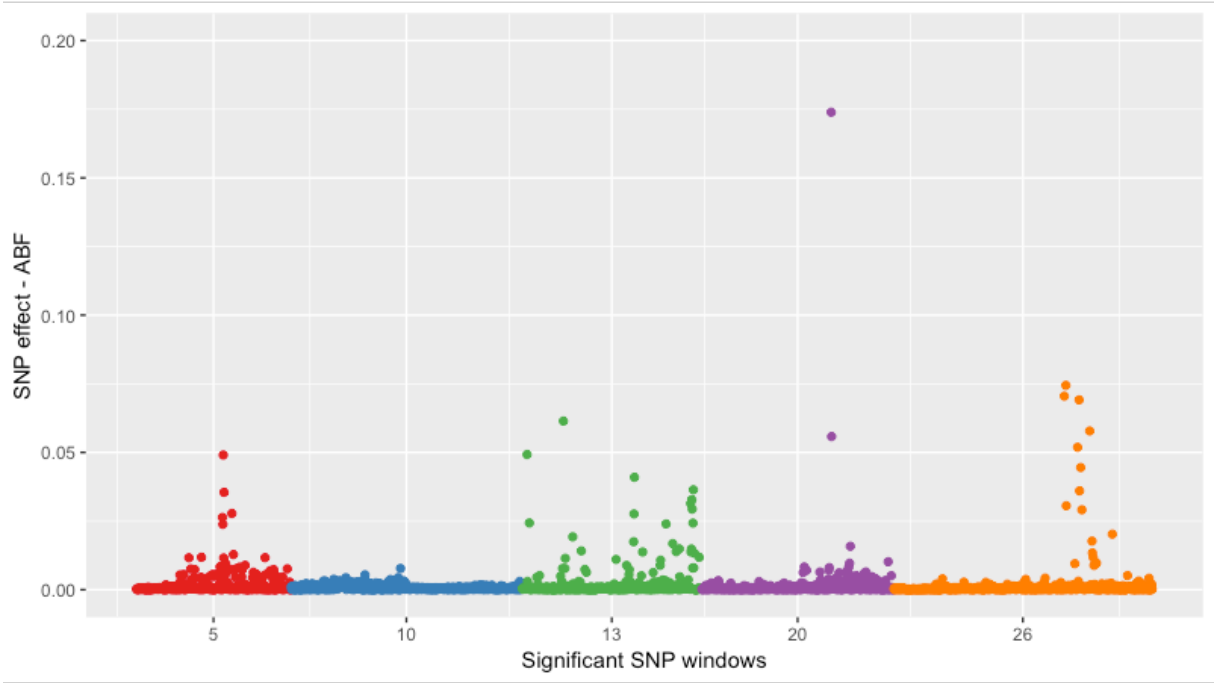

Figure 5 – Manhattan plot of the SNP effect distribution within each significant window for abdominal fat weight (ABF). The X-axis represents the significant SNP window represented by the number of the respective chromosome and Y-axis shows the SNP effect from Bayes B analysis.

3.3.3. Overlap with previously known QTLs

Twenty-seven previously published QTLs for fat traits overlapped with five of the QTLs identified in our study. The QTL located on GGA5 at 38 Mb, associated with ABF and ABFP overlapped with three known QTLs: two QTLs associated with for ABF (QTL #3321, [45]; QTL #9432, [46]) and one associated with ABFP (QTL #9433, [46]).

The QTL, which was located on GGA15 at 6 Mb and associated with SKINW and SKINP, overlapped with 11 QTLs previously associated with fat traits: four were associated with for ABF (QTL #2337, [5]; QTL #9451, [46]; QTL #2347, QTL #12631, [9]), three were associated with ABFP (QTL #2339, QTL #2340, [5]; QTL #9450, [46]), one associated with fat distribution (total weight of skin fat analyzed with ABF as covariate) (QTL #12645, [9]), one QTL associated with subcutaneous neck fat weight (QTL #17331, [2]), one QTL associated with total white fat weight (QTL #17337, [2]), and one associated with visceral fat weight (QTL #17323, [2]).

The QTL located on GGA24 at 5 Mb that was associated with SKINW, overlapped with one QTL previously reported to be associated with ABF (QTL #9405, [47]). While, the QTL identified on GGA26 at 1 Mb that was associated with ABF and ABFP, overlapped with two QTLs: one associated

with visceral fat weight (QTL #17324, [2]), and one associated with total white fat weight (QTL #17338, [2]).

The QTL identified on GGA27 at 3 Mb that was associated with SKINW and SKINP, overlapped with 10 QTLs previously reported to be associated with fat traits: three associated with ABF (QTL #66072, [48]; QTL #11817, QTL #11809, [49]), three associated with ABFP (QTL #11820, [49]; QTL #3354, [50]; QTL #11934, [51]), two associated with carcass fat content (QTL #17135, QTL #17126, [13]), one associated with carcass fat content on a dry matter basis (QTL #17125, [13]), and one associated with intramuscular fat (QTL #3360, [50]).

No previously reported QTL overlapped with the QTL identified on GGA9 at 4 Mb, GGA10 at 7 Mb, GGA13 at 3 Mb, and GGA20 at 5 Mb.

3.3.4. Positional candidate genes

We identified 419 genes in the nine QTL genomic windows. Further analysis against gene ontology terms and the literature revealed 13 candidate genes for fat deposition (Table 8).

Table 8 – List of candidate genes within the genomic windows associated with abdominal fat and skin traits that exhibited GO terms related to lipid metabolic processes in the TT Reference Population.

(continues)

GGA (location, Mb)	Trait associated	Gene Name	Ensembl Gene ID	GO Term (GO Accession)
5 (38)	ABF, ABFP	*JDP2*	ENSGALG00000010322	negative regulation of fat cell differentiation (GO:0045599)
15 (6)	SKINW, SKINP	*PTPN11*	ENSGALG00000004821	lipid metabolic process (GO:0006629) triglyceride metabolic process (GO:0006641)
		MVK	ENSGALG00000013848	lipid metabolic process (GO:0006629) cholesterol metabolic process (GO:0008203)
		Novel gene	ENSGALG00000005043	fatty acid biosynthetic process (GO:0006633) acetyl-CoA carboxylase activity (GO:0003989)

Table 8 – List of candidate genes within the genomic windows associated with abdominal fat and skin traits that exhibited GO terms related to lipid metabolic processes in the TT Reference Population.

(conclusion)

GGA (location, Mb)	Trait associated	Gene Name	Ensembl Gene ID	GO Term (GO Accession)
20 (5)	ABF	*FITM2*	ENSGALG00000026285	lipid storage (GO:0019915) lipid particle organization (GO:0034389)
		PLCG1	ENSGALG00000003750	lipid metabolic process (GO:0006629) lipid catabolic process (GO:0016042)
		HNF4A	ENSGALG00000004285	lipid metabolic process (GO:0006629) regulation of lipid metabolic process (GO:0019216) lipid homeostasis (GO:0055088) fatty acid binding (GO:0005504)
24 (5)	SKINW	*APOA4*	ENSGALG00000007109	lipid homeostasis (GO:0055088) multicellular organismal lipid catabolic process (GO:0044240) positive regulation of triglyceride catabolic process (GO:0010898) cholesterol homeostasis (GO:0042632) cholesterol metabolic process (GO:0008203) positive regulation of fatty acid biosynthetic process (GO:0045723) triglyceride homeostasis (GO:0070328)
		APOA5	ENSGALG00000014368	positive regulation of lipid catabolic process (GO:0050996) positive regulation of fatty acid biosynthetic process (GO:0045723) lipid transport (GO:0006869)
		APOA1	ENSGALG00000007114	lipid metabolic process (GO:0006629) lipid storage (GO:0019915) cholesterol homeostasis (GO:0042632)
26 (1)	ABF, ABFP	Novel gene	ENSGALG00000000477	lipid metabolic process (GO:0006629) lipid catabolic process (GO:0016042) lipid particle (GO:0005811) lipid homeostasis (GO:0055088)
		Novel gene	ENSGALG00000000483	lipid catabolic process (GO:0016042) lipid particle (GO:0005811) lipid homeostasis (GO:0055088) triglyceride lipase activity (GO:0004806) triglyceride catabolic process (GO:0019433)
		ADIPOR1	ENSGALG00000000094	regulation of lipid metabolic process (GO:0019216) fatty acid oxidation (GO:0019395)

[1] All the GO terms accession numbers and names were obtained from BioMart (*Ensembl Genes 90 Database*).

Additionally, all 419 genes located within the detected QTLs were used to perform enrichment analysis. One cluster was enriched (enrichment score of 2.62) and within this cluster, there were

enrichment in four GO terms: regulation of intestinal cholesterol absorption; high-density lipoprotein particle assembly, lipoprotein metabolic process and positive regulation of fatty acid biosynthetic process (raw p-value < 0.05 and p-value adjusted for multiple testing by Benjamini & Hochberg [37] method < 0.1). These GO terms were annotated for the same genes: *APOA1*, *APOA4*, and *APOA5*.

3.3.5. NGS-SNPs in candidate genes

A previous study has been performed using sequencing data to identify and characterize genome-wide SNPs, INDELs, putative regions under selection and also find putative pathways under selection in two Brazilian chicken lines [41] but neither was based on the TT broiler reference population.

We used a dataset of high quality SNPs from NGS data identified in 14 parental chickens from TT Reference Population in order to screen for SNPs potentially affecting gene expression and/or function and identified 3,639 SNPs located within the 13 PCG. SNP density (SNPs/kb) within each PCG and the functional annotation of the SNPs are presented in Figure 6. The PCG that had the greatest density of SNPs was *FITM2* (84 SNPs/kb).

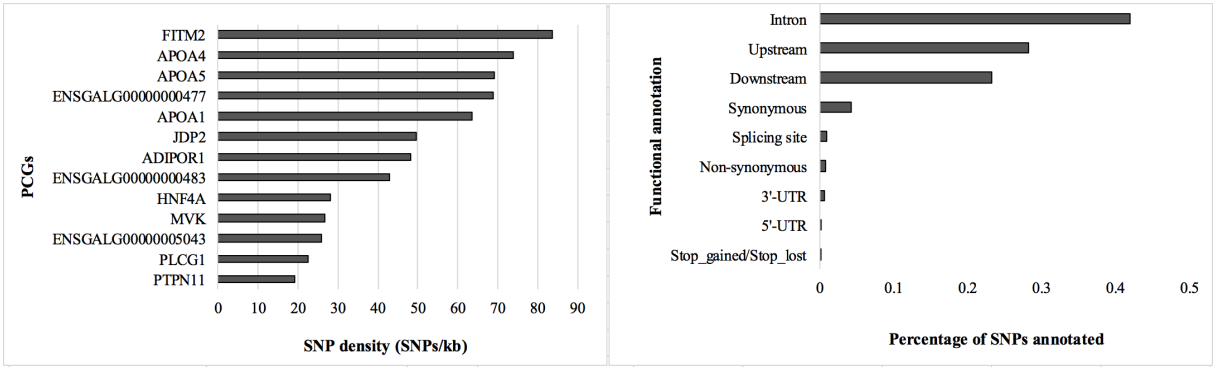

Figure 6 – (a) Plot of NGS-SNP density (SNPs/kb) for each PCG. (b) Plot with the functional annotation of unique NGS-SNPs identified in our 13 PCGs.

Single nucleotide polymorphisms were evaluated for potentially deleterious and high impact mutation annotation, which may potentially affect gene expression and/or function. Two high impact variants were identified: one located within *FITM2* and another located in ENSGALG00000000483 gene. In addition, two potentially deleterious variants were identified: one located in *PLCG1* and another in ENSGALG00000000477 gene (Table 9). The non-synonymous SNP located in the *PLCG1* gene is novel.

Table 9 – Characterization of potentially deleterious and high impact NGS-SNPs identified in the 13 PCGs.

Associated Gene Name	Ensembl Gene ID	SNP ID	Position[1]	Consequence
FITM2	ENSGALG00000026285	rs315805239	5,614,711	Stop gained
Novel gene	ENSGALG00000000483	rs740555722	1,514,268	Stop loss
PLCG1	ENSGALG00000003750	g.5072909A>T	5,072,909	Non-synonymous
Novel gene	ENSGALG00000000477	rs737351616	1,509,828	Non-synonymous

[1] Position based on *Gallus_gallus* 5.0 assembly.

3.4. Discussion

3.4.1. Genomic heritability

Genomic heritability estimates for abdominal fat and skin traits in a broiler chicken population characterized by close relatives (full and half-sibs) were obtained using genotypes from a high-density SNP panel. Close relatives may have long chromosome segments in common, thereby sharing alleles and QTLs in the same pattern, which may lead to less bias and consequently, higher prediction accuracy for genomic heritability [52].

Heritabilities estimates for ABF and ABFP have been reported to be 0.62 and higher, while the heritability of skin traits is between 0.24 and 0.28 [3, 53]. The number of generations of artificial selection and/or the genetic background may differ for each chicken population thus, different heritability estimates may be observed. The TT broiler line, used to obtain the TT Reference population, have been under multi-trait selection, with emphasis on body weight, which have a strong positive genetic correlation with abdominal fat and feed conversion thus, changes in fat deposition in our population is mainly due to the response to selection. Therefore, artificial selection may also affect the genetic variance and may reduce heritability over the generations [54]. Comparisons between heritabilities reported in the literature should be interpreted with caution.

Using the same population reported here, Fornari et al. [22] observed similar pedigree-based heritability estimates to those we obtained using genomic information, namely for abdominal fat weight (0.33) and two skin related traits: drumstick skin weight (0.17) and thigh skin weight (0.28). The existence of moderate genomic heritabilities for the analyzed phenotypes indicates that a reasonable proportion of the total variance for these traits can be explained by a set of markers [52]. Therefore, selection against fat deposition in this population may be successful.

3.4.2. QTL discovery and resolution

Bayesian approaches have been commonly used in genomic prediction and selection studies [55,56] as well as for GWAS [29,30,57,58] in chickens. The main advantage of this approach is that genotypes are simultaneously fitted in the model, accounting for population structure, and the use of high-density markers does not reduce the power to detect association [59]. Thus, we decided to use genomic prediction methodology to perform GWAS.

Five out of the nine QTLs detected were previously identified in different populations, corroborating our results and indicating that these QTLs probably originated from the founder lines used to generate the broiler TT line used in this study.

The novel QTLs identified could be false positives, exclusive to our population or a consequence of the number of animals and the higher SNP density compared to other QTL mapping studies for abdominal fat and skin traits in chickens [5,8-14]. The PPA (ranging from 0.83 to 0.93) and the proportion of the genetic variance explained by the novel QTLs (ranging from 0.58 to 1.49) suggests that they are not false positives. Besides that, the Manhattan plots of the SNP effects within the QTLs detected showed few peaks indicating that some markers exhibit higher effects within the QTL and, the direction of the effects provides helpful information for further studies aiming to fine-map these QTLs.

In general, we observed small effects for the markers fitted simultaneously within the QTLs detected. This could be due to not enough power to capture the genetic variability in our population or, due to artificial selection. As mentioned before, the artificial selection can lead to the fixation of several loci affecting the the phenotypic and genetic variability [54,60]. Thus, SNPs with higher effect on fatness may have been fixed over the generations and can not have their effect estimated in our GWAS; we selected only SNPs that are not fixed in our population (MAF<2%).

In contrast to the QTL mapped on GGA5 at 38 Mb, previously reported QTLs for the same trait were larger than 1 Mb [45,46]. The use of a higher density of markers (600 k) may help to explain the improved resolution observed in QTL mapping.

The QTL mapped on GGA27 at 3 Mb overlapped with some known QTLs for fatness related traits, mapped in a Brazilian F2 population established by crossing a broiler male line (TT) and a layer line (CC), and these known QTLs are segregating in different families from the Brazilian F2 population [13,49]. Furthermore, the broiler male line (TT) used in the crossing to establish this population, is the same line used to obtain the TT Reference Population [23] thus, we should expect this QTL segregating in our broiler population, corroborating our findings.

We identified only a few QTLs associated with fat traits in this population. For quantitative traits, a greater number of alleles are expected to present a small effect [61] and the number of samples used in this study may not have been sufficiently large enough to identify these small effect QTLs. Despite this, novel QTLs for fat traits in broiler were identified. These QTLs should be considered as novel QTLs may be population-specific.

3.4.3. Positional candidate genes for fat deposition

We identified 13 PCG in five of the nine QTLs identified (Table 8). In the QTL on GGA5 at 38 Mb we identified Jun dimerization protein 2 (*JDP2*) gene. This gene regulates lipid accumulation in adipose tissue acting as a repressor of adipocyte differentiation [62,63].

We identified *PLCG1*, *HNF4A* and *FITM2* in the QTL on GGA20 at 5-Mb. In human primary adipocytes, phospholipase C gamma 1 (*PLCG1*) gene is involved in the calcium signaling pathway. The expression of *PLCG1* has been show to affect adipocyte triglyceride content [64]. Hepatocyte nuclear factor-4α (*HNF4A*) controls insulin metabolism and triglycerides level [65]. Triglycerides are the main lipid stored in avian fat cells. Thus, different levels of plasma triglyceride may affect fat deposition [66]. The fat storage inducing transmembrane protein 2 (*FITM2*/*FIT2*) gene belongs to a family of proteins that play a role in fat storage [67]. In a study with humans, the *FITM2*/*FIT2* was reported to be associated with lipid droplets biogenesis and accumulation [68] which consequently impacts lipid storage.

In the QTL on GGA26 at 1 Mb, we found *ADIPOR1*, ENSGALG00000000477 and ENSGALG00000000483 genes. In chickens, the adiponectin receptor 1 (*ADIPOR1*) gene is expressed in fat, liver, and muscle, and this gene affect adipocyte differenciation [69]. *ADIPOR1* is the main receptor of adiponectin. It is negatively correlated with fat deposition [69], and is involved in lipid-induced mitochondrial biogenesis in chicken adipocytes [70]. ENSGALG00000000477 and ENSGALG00000000483 code for uncharacterized proteins, but their gene ontologies are related to lipid metabolism and storage (Table 8). Further studies with those novel genes may help elucidate their role in fat deposition.

In the QTL on GGA15 at 6 Mb, we found *PTPN11, MVK* and ENSGALG00000005043 genes. The protein tyrosine phosphatase, non-receptor 11 (*PTPN11*) encodes for a Src homology-2 domain-containing protein tyrosine phosphatase 2 (*SHP2*). Its expression has been reported to affect energy balance and lipid and glucose metabolisms [71]. Additionally, in a study with mice, *SHP2* was reported to be associated with obesity [72]. Mevalonate kinase (*MVK*) encodes for a mevalonate kinase enzyme that plays an important role at the beginning of cholesterol biosynthesis [73]. Changes in the cholesterol biosynthesis, and consequently cholesterol levels, may affect hepatic lipid metabolism [74]. ENSGALG00000005043 is a novel gene that has been annotated with GO term related to the fatty acid biosynthetic process and Acetyl-CoA carboxylase activity (Table 8). Further studies with these genes may help to better understand their role in fat deposition.

In the QTL located on GGA24 at 5 Mb we found *APOA1*, *APOA4* and *APOA5* genes. These three genes belong to a gene family (Apolipoproteins – APO) that encodes important regulators of lipid biosynthesis and metabolism [75]. Additionally, these three positional genes were annotated with four enriched GO terms: regulation of intestinal cholesterol absorption, high-density lipoprotein particle assembly, lipoprotein metabolic process and positive regulation of the fatty acid biosynthetic process. Apolipoprotein A1 (*APOA1*) is involved with cholesterol transport [75]. While, Apolipoprotein A-IV (*APOA4*) and Apolipoprotein V (*APOA5*) are involved with triglycerides metabolism [75]. Additionally, the *APOA4* was also reported as a regulator of triglycerides metabolism in mice [76].

Corroborating our findings, no overlapping between our positional candidate genes and genes under selective pressure reported in a previous study with the same dataset from TT Broiler Reference Population [23] was observed.

Additionally, comparing a dataset of SNPs and INDELs identified in Brazilian broiler and layer lines, our group recently identified regions under selection [41], and genes under selective pressure related to fat deposition were reported. No overlapping was observed between our PCGs for fat deposition and genes under selective pressure reported in this study; with the exception of the *APOA1* gene, annotated in one regions under selection identified using INDELs. One possible explanations for the no overlaps observed are because different chicken lines were compared in these studies, and because in our current study SNPs fixed were removed, and as consequence, it decreased the chance to identify genes under selection.

3.4.4. Potential causative SNPs in PCGs

We observed a large number of SNPs annotated in intronic regions of the PCGs (approximately 42% of the SNPs; Figure 6b). According to the literature, introns can play a role in the regulation of alternative splicing, gene expression, and may be associated with mRNA transport [77]. As an example, in a study with humans, SNPs located in an intronic region of *FTO* gene affected primary transcript levels, and were associated with body mass index and type II diabetes [78]. Thus, even the SNPs annotated in introns can play a role in the regulation of any trait, including fat deposition in chickens.

Approximately 58% of the total of SNPs found in the 13 PCG were located in potentially functional regions such as up/downstream, 3'and 5'-UTRs, exons (synonymous and non-synonymous), splicing site and stop codon (gained/lost; Figure 6b). Genetic variants within non-coding regions (3'and 5'-UTRs), may control gene expression by modulating transcription or mRNA turnover [79]. We observed 36 NGS-SNPs in 3/5'-UTR regions. Two SNPs were classified as potentially deleterious and were located in *PLCG1* and ENSGALG00000000477 genes (Table 9), PCGs for fat deposition regulation. Potentially deleterious SNPs in this genes could be causative mutations.

Two high impact SNPs were annotated in *FITM2* and ENSGALG00000000483 genes (Table 4). High impact SNPs in these genes may affect lipid metabolism and storage (fat deposition) in chickens.

From the four SNPs (Table 9), one is novel (g.5072909A>T) and the other existing are not included on the Affymetrix SNP array. Thus, the integration of GWAS and NGS-SNPs brought additional information in the search for potential causative mutations, not included on the SNP array. Further studies are necessary to achieve a better understanding of the role of these SNPs in fat deposition.

Additionally, compared to the other PCGs, *FITM2* (84 SNPs/kb) exhibited a higher SNPs density (Figure 6a). This observation needs to be further investigated, since it could be related to fat deposition in our population.

The GWAS results using the high-density panel improved the precision of mapping QTLs and the accuracy of effect estimates for small and medium-sized QTL. The validation of putative candidate genes identified in this study that regulate fat deposition, could lead to the use of markers in those genes to improve the accuracy of early selection against carcass fat accumulation and this would improve the efficiency of broiler production. Further, the use of NGS data from the same population evaluated for GWAS, can bring additional information for the elucidation of the functions of the candidate genes identified for fat related traits.

3.5. Conclusions

This study confirmed previously published QTLs and discovered novel ones, thus contributing to a better understanding of fat deposition in chickens. The use of a high-density SNPs panel in our GWAS analyses provided a better resolution in QTL detection. The PCGs identified in the QTL are involved in many biological processes that regulate lipid storage. We found NGS-SNPs located in the PCGs providing additional information in the search for potential causative mutations and further validation studies could be helpful to understand their role in fat deposition regulation. Our findings can be potentially applied to improve the accuracy of early selection against carcass fat accumulation and improve feed efficiency in broiler chicken production.

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Figure 1 – Manhattan plot of the posterior means of the percentage of genetic variance explained by each 1 Mb SNP window across the 28 autosomal chromosomes for CFC (carcass fat content in grams): (A) genomic windows located on macrochromosomes, and (B) genomic windows located on microchromosomes.

Figure 2 – Distribution of previously mapped QTLs identified as associated with fatness traits (from Animal QTLdb[8]) on autosomal chromosomes 1, 2, 7, 15, 20, 27 and 28 (in black boxes), and those detected in our study (above the black boxes). The colors represent the traits: ABF is abdominal fat weight; ABFP is abdominal fat percentage; CFC is carcass fat content; CFCDM is carcass fat content on dry matter basis; CFWID is Cingular fat width; FATDIS is fat distribution; SCFT is subcutaneous fat thickness (fat thickness under skin); SCNF is subcutaneous neck fat weight (subcutaneous neck adipose tissue); SFWT is skin fat weight; TWF is total white fat weight (total white adipose tissue); VISAT is visceral fat weight (visceral adipose tissue weight). The colors change or get denser according to the number of QTL previously mapped superimposing each other.

Figure 3 – Functional annotation of unique NGS-SNPs identified in 26 PCGs. The percentage was estimated based on the total number of SNPs annotated in 26 PCGs.

Figure 4 – Manhattan plot of the posterior means of the proportion of genetic variance explained by each 1-Mb SNP window across the 28 autosomal chromosomes for abdominal fat weight (ABF): (A) genomic windows located on macrochromosomes, and (B) windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis. Red lines indicate the threshold to deem significant SNP windows.

Figure 5 – Manhattan plot of the SNP effect distribution within each significant window for abdominal fat weight (ABF). The X-axis represents the significant SNP window represented by the number of the respective chromosome and Y-axis shows the SNP effect from Bayes B analysis.

Figure 6 - (a) Plot of NGS-SNP density (SNPs/kb) for each PCG. (b) Plot with the functional annotation of unique NGS-SNPs identified in our 13 PCGs.

Table 1 - Descriptive statistics, variance components and genomic heritability.

Table 2 - Characterization of 1 Mb genomic windows associated with abdominal fat and carcass fat content traits in the Embrapa F_2 Chicken Resource Population.

Table 3 – Genomic windows associated with fatness traits that harbor positional candidate genes.

Table 4 - Characterization of deleterious NGS-SNPs identified in our PCGs.

Table 5 - List of PCGs that exhibited GO terms related to lipid metabolic processes.

Table 6 - Descriptive statistics, variance components and genomic heritability for body weight at 42 days of age, abdominal fat and skin weights and percentages in the TT Reference Population.

Table 7 - Characterization of 1 Mb significant genomic windows for abdominal fat and skin traits in the TT Reference Population.

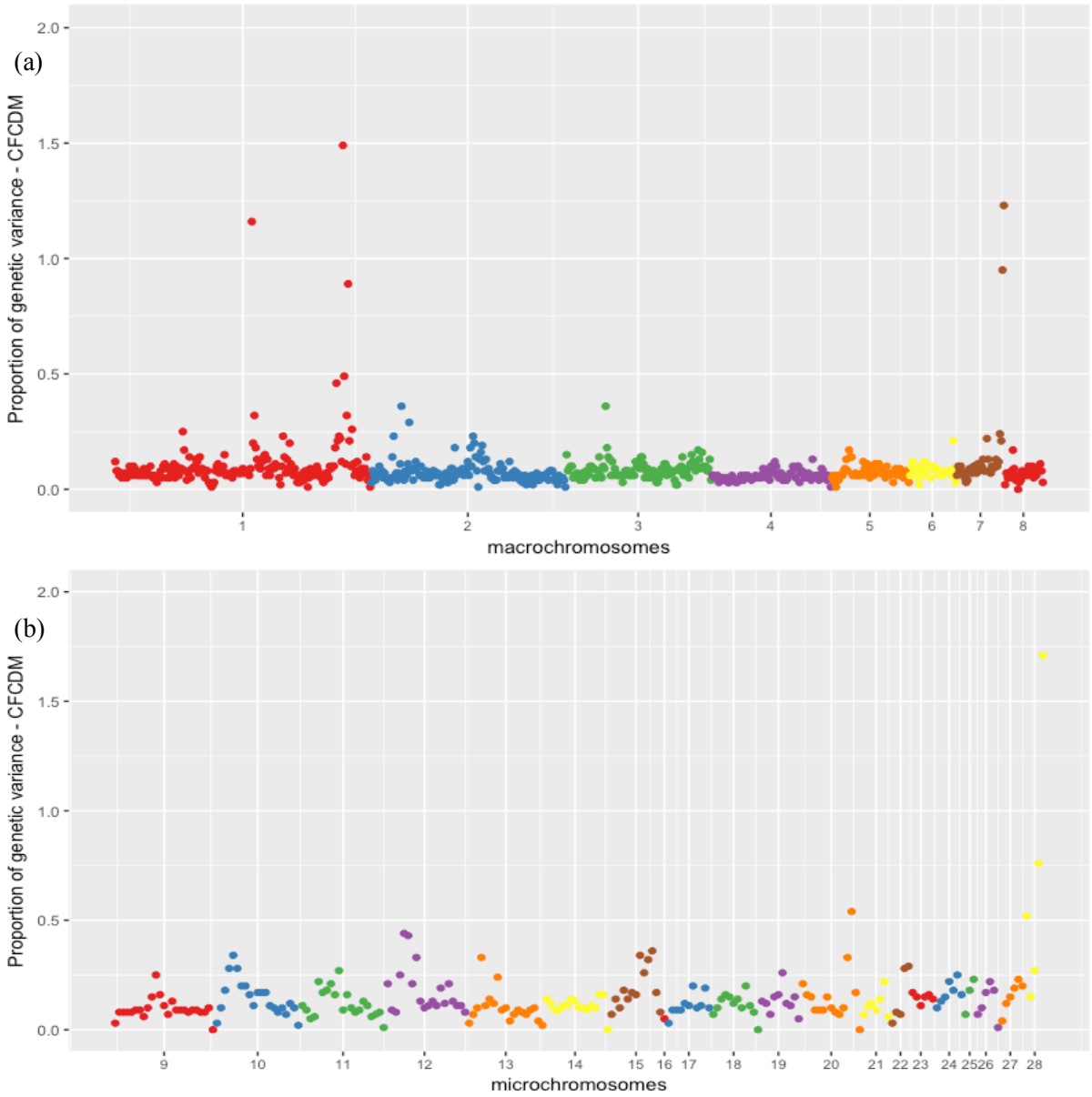
Table 8 – List of candidate genes within the genomic windows associated with abdominal fat and skin traits that exhibited GO terms related to lipid metabolic processes in the TT Reference Population.

Table 9 - Characterization of potentially deleterious and high impact NGS-SNPs identified in the 13 PCGs.

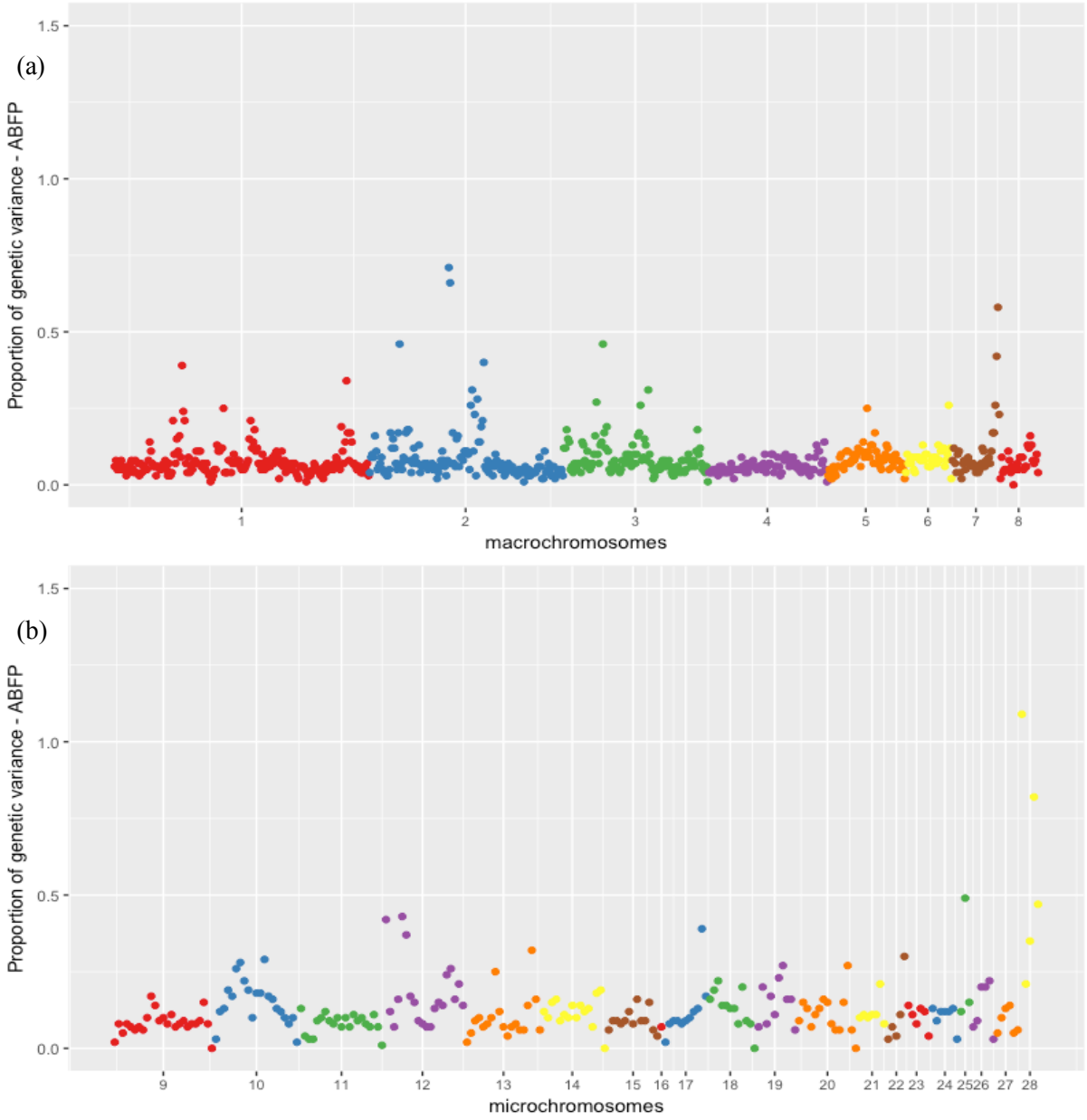
APPENDIX

APPENDIX A. Manhattan plot of the proportion of genetic variance explained by each SNP window across the 28 autosomal chromosomes for ABF trait. (a) SNP windows located on macrochromosomes, and (b) SNP windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis.

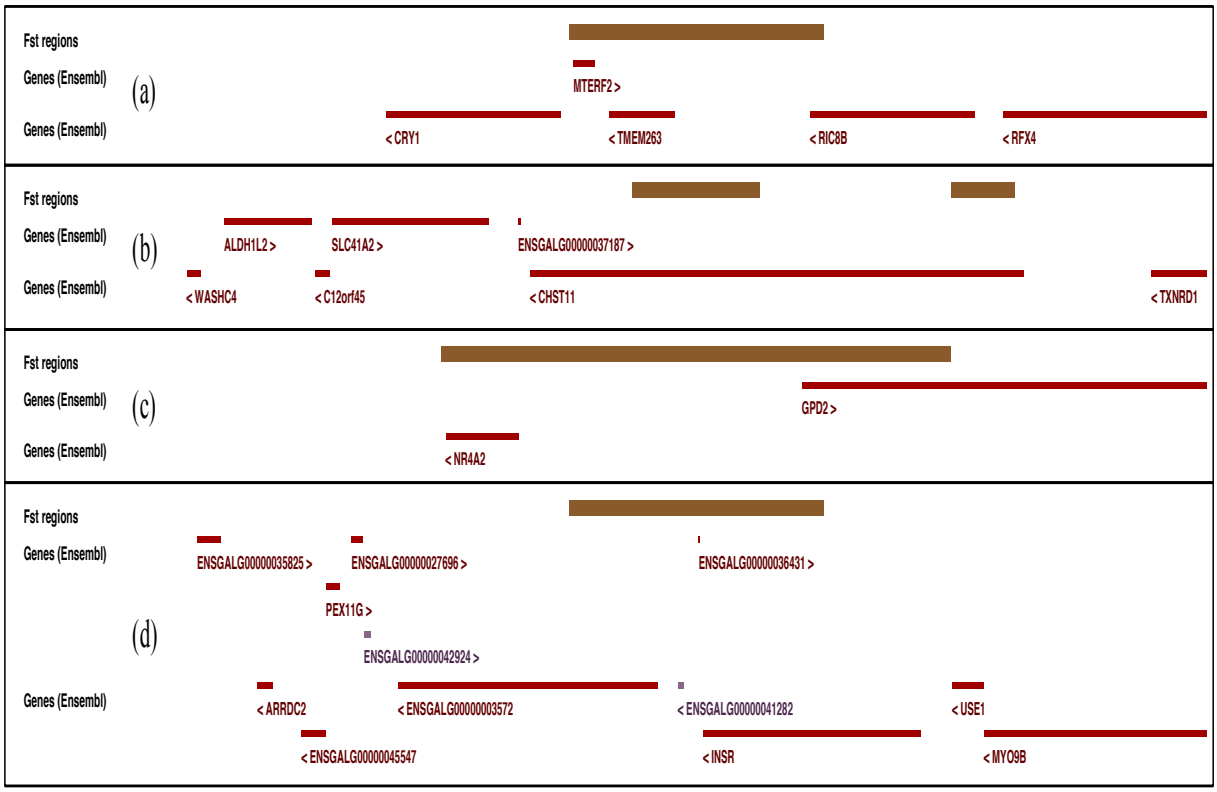
APPENDIX B. Manhattan plot of the proportion of genetic variance explained by each SNP window across the 28 autosomal chromosomes for CFCDM trait. (a) SNP windows located on macrochromosomes, and (b) SNP windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis.



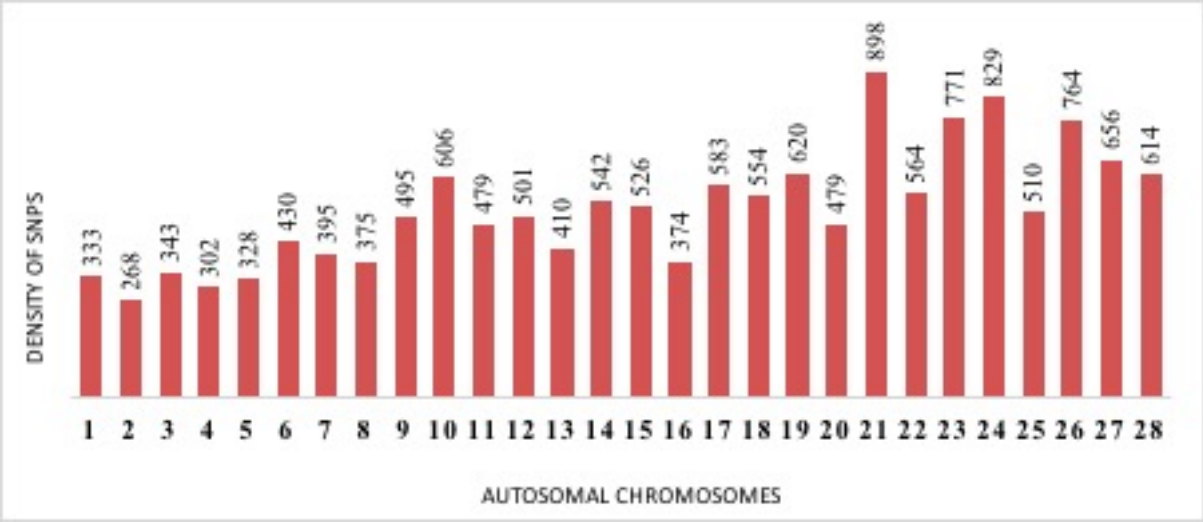
APPENDIX C. Manhattan plot of the proportion of genetic variance explained by each SNP window across the 28 autosomal chromosomes for ABFP trait. (a) SNP windows located on macrochromosomes, and (b) SNP windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis.



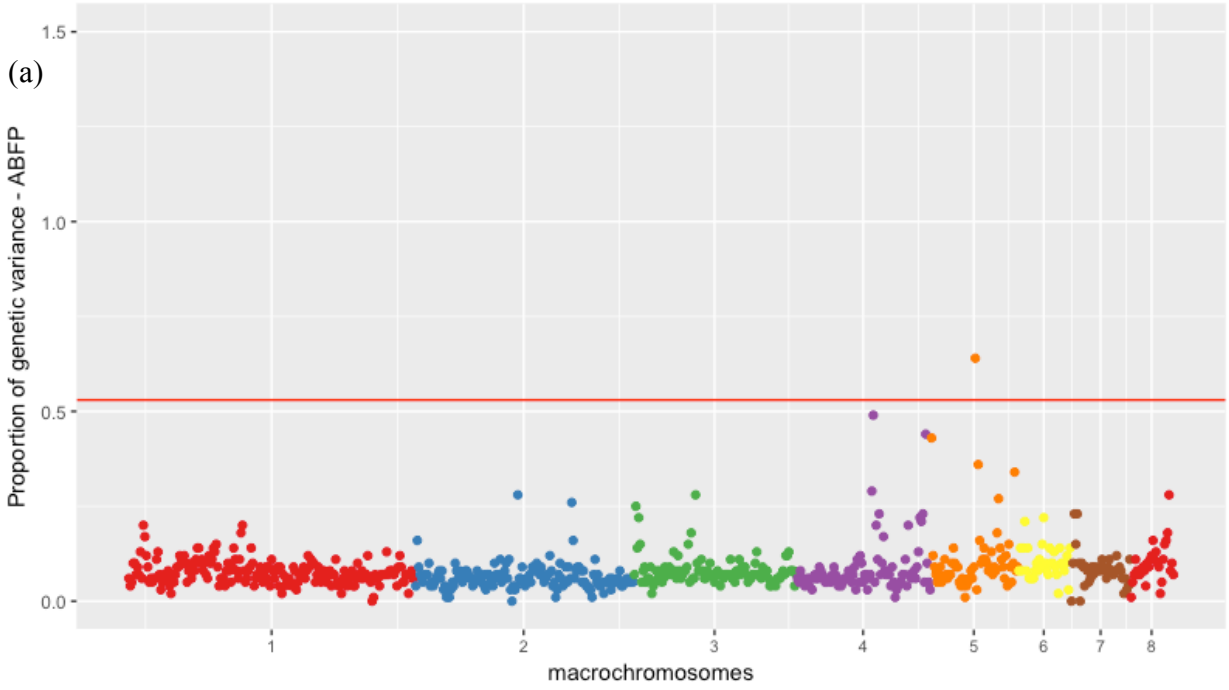
APPENDIX D. Plot showing the overlapping of the selection signature regions and PCGs. Fst regions means selection signature regions. (a) Plot of the region around CRY1 gene (GGA1, 53,603,247-53,803,224 bp) showing the selection signature region nearby CRY1 gene. (b) Plot of the region around CHST11 gene (GGA1, 53,603,247-53,803,224 bp) showing the overlap of two selection signature regions. (c) Plot of the region around NR4A2 and GPD2 genes (GGA7, 36,195,323-36,295,960 bp) showing the overlap of one selection signature region. (d) Plot of the region around INSR gene (GGA28, 4,051,975-4,211,970 bp) showing the overlap of one selection signature region. The genome positions of selection signatures regions are: GGA1 from 53,678,235 to 53,728,228, from 54,470,039 to 54,510,038, from 54,570,039 to 54,590,038; on GGA7 from 36,220,480 to 36,270,797; on GGA28 from 4,111,972 to 4,151,970.

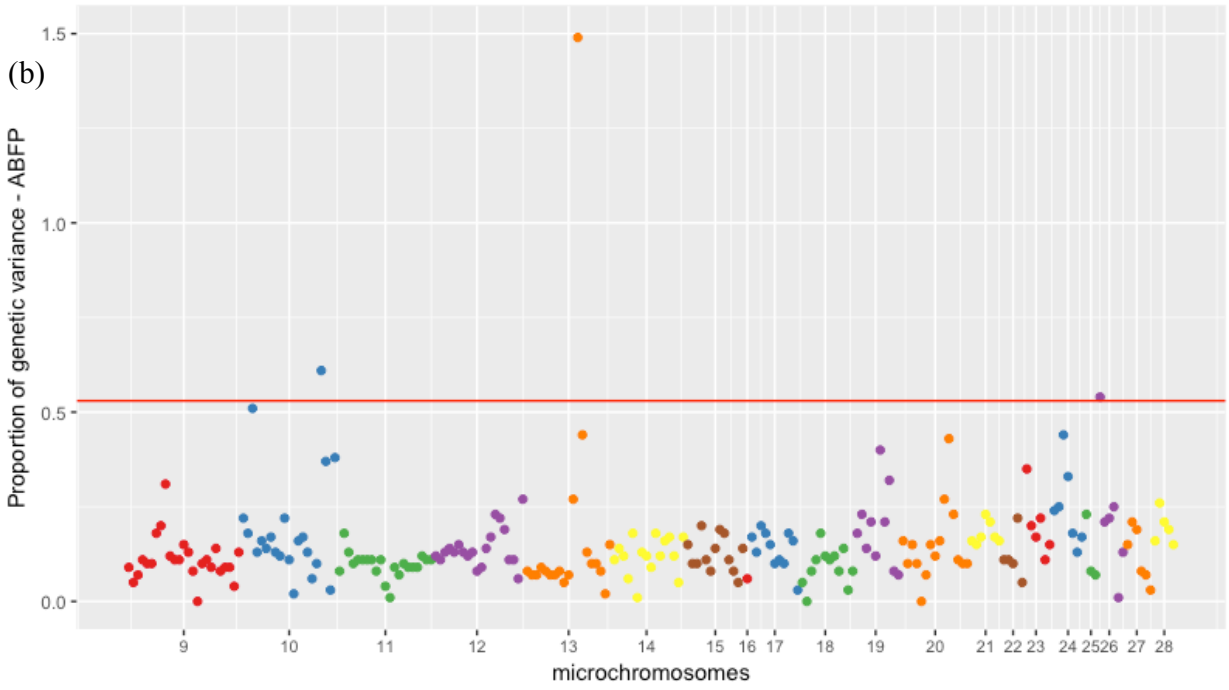


APPENDIX E. Plot of the density of SNPs per Mbp in each autosomal chromosome after filtration.



APPENDIX F. Manhattan plot of the posterior means of the proportion of genetic variance explained by each 1-Mb SNP window across the 28 autosomal chromosomes for abdominal fat percentage (ABFP): (A) genomic windows located on macrochromosomes, and (B) windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis. Red lines indicate the threshold to deem significant SNP windows.

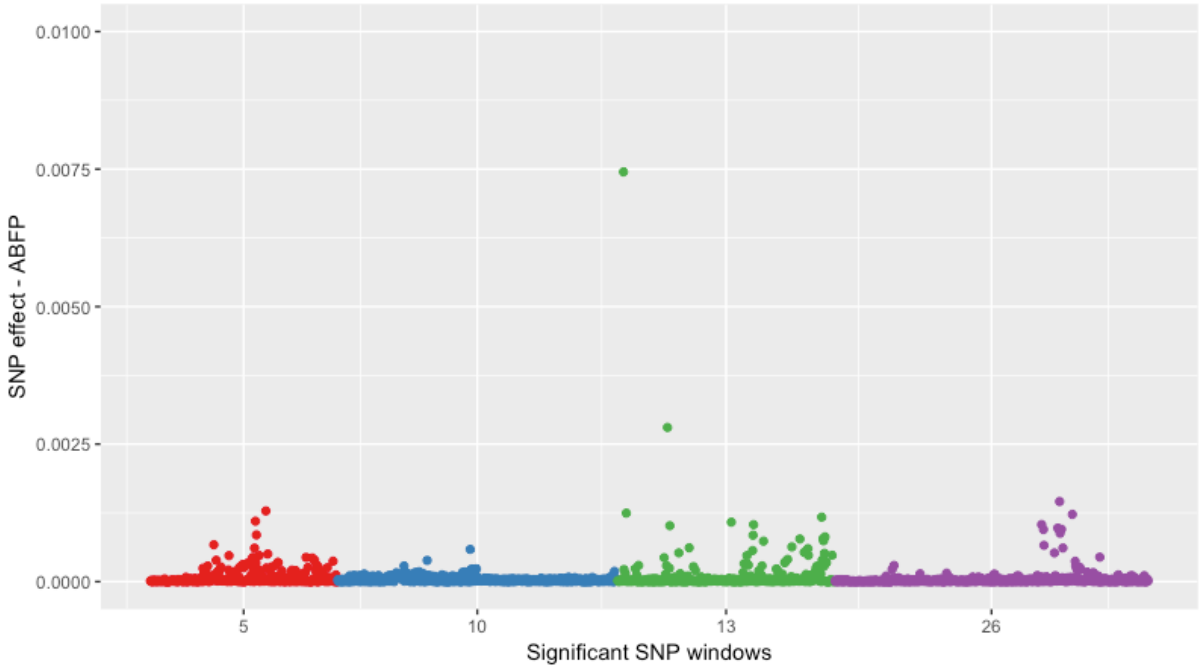




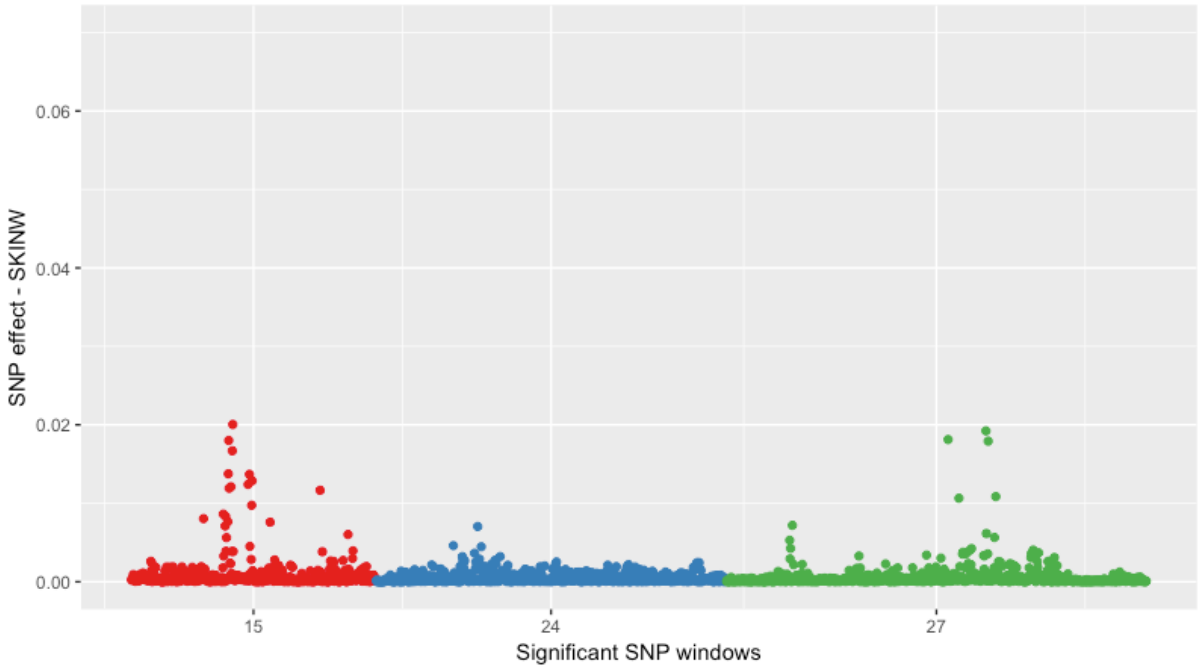
APPENDIX G. Manhattan plot of the posterior means of the proportion of genetic variance explained by each 1-Mb SNP window across the 28 autosomal chromosomes for skin weight (SKINW): (A) genomic windows located on macrochromosomes, and (B) windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis. Red lines indicate the threshold to deem significant SNP windows.

APPENDIX H. Manhattan plot of the posterior means of the proportion of genetic variance explained by each 1-Mb SNP window across the 28 autosomal chromosomes for skin percentage (SKINP): (A) genomic windows located on macrochromosomes, and (B) windows located on microchromosomes. The X-axis represents the chromosomes and Y-axis shows the proportion of genetic variance explained by each window from Bayes B analysis. Red lines indicate the threshold to deem significant SNP windows.

APPENDIX I. Manhattan plot of the SNP effect distribution within each significant window for abdominal fat percentage (ABFP). The X-axis represents the significant SNP window represented by the number of the respective chromosome and Y-axis shows the SNP effect from Bayes B analysis.



APPENDIX J. Manhattan plot of the SNP effect distribution within each significant window for skin weight (SKINW). The X-axis represents the significant SNP window represented by the number of the respective chromosome and Y-axis shows the SNP effect from Bayes B analysis.



APPENDIX K. Manhattan plot of the SNP effect distribution within each significant window for skin percentage (SKINP). The X-axis represents the significant SNP window represented by the number of the respective chromosome and Y-axis shows the SNP effect from Bayes B analysis.