Genome-Wide Detection of Allele Frequency Change and Quantitative Trait Loci for Respiratory Disease and Immune-Related Traits in Landrace Pigs Selected for Mycoplasmal Pneumonia of Swine Resistance

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Abstract

Background: The genetic improvement of disease resistance in pig has been well-received. Identification of a quantitative trait locus (QTL) related to a chronic respiratory disease such as Mycoplasmal pneumonia of swine (MPS) and immune-related traits is important for understanding the genomic background of disease resistance and to apply marker-assisted selection. The objective of this study was to understand the influence of genomic factors on respiratory disease and immune-related traits in MPS-selected pigs.

Results: A total of 874 Landrace purebred pigs, which were selected based on MPS resistance, were genotyped using the Illumina PorcineSNP60 BeadChip, and were then used for genomic analyses. First, we performed genome-wide association studies (GWAS) to detect a novel QTL for a total of 22 performance, respiratory disease, and immune-related traits using additive and nonadditive genetic effects. Second, we evaluated the changes in allele frequency due to selection for MPS resistance and compared the putative selected regions with the detected QTL. GWAS detected a total of 11 genome-wide significant single nucleotide polymorphisms (SNPs) with an additive effect in five traits and a total of three significant SNPs with a nonadditive effect in three traits. Most of these detected QTL regions were novel regions with some candidate genes located in them. With regard to a pleiotropic region among traits, only five of these detected QTL regions overlapped among traits. Changes in allele frequencies at the many putative selected regions were spread across the whole genome and overlapped with the detected QTL. Some of these selected regions were the ones that contained the detected QTL for MPS score and other traits.

Conclusion: These results suggest that a closed-line breeding population is a useful target population to refine and confirm QTL regions by integrating the results of GWAS and

allele frequency changes. The study provides new insights into the genomic factors that affect respiratory disease and immune-related traits in pigs.

Keywords: additive and nonadditive effects, immune capacity, Landrace pigs, peripheral blood cytokines, respiratory disease

Background

Mycoplasma hyopneumoniae (Mhp) is considered to play a primary role in porcine respiratory disease complex and Mycoplasmal pneumonia of swine (MPS) is a chronic respiratory disease caused by Mhp [1]. Chronic respiratory diseases caused by Mhp are associated with serious economic losses in the pig industry as they may result in decreased growth performance and feed efficiency and, sometimes, increased mortality rates [2]. Therefore, it is important to prevent Mhp infection and maintain animal health in the pig industry. Strategies for the control of MPS include improving housing conditions and providing antimicrobial medication and vaccination [2]. However, the complicated mechanisms of Mhp infection and its co-infection with other respiratory pathogens make it difficult to maintain high performance by controlling the spread of MPS in the pig industry.

Recently, the genetic improvement of disease resistance - genetic selection for resistance to disease by selection of a disease-resistance indicator such as infection rate or immune response - has been well-received. For example, Kadowaki et al. [3] reported a selection-based closed-line breeding experiment carried out in Landrace purebred pigs over five generations to improve MPS resistance using the aggregate breeding value of their production traits and the MPS lesion score (MPS score). The MPS-selected Landrace line showed significantly lower MPS score than that of a non-selected Landrace line [4], which confirmed the effectiveness of genetic selection based on infection rate such as the MPS score. In addition, Okamura et al. [5] and Sato et al. [6] also evaluated the correlated responses of immune capacity traits and peripheral blood cytokines in MPS-selected pigs, and observed the indirect selection of these immune-related traits due to the genetic decrease in the MPS score. These authors suggested that, not only the infection rate, but

115 also immune-related traits can be useful indicators for genetic improvement of disease
116 resistance in pigs.

117 Identification of quantitative trait loci (QTL) related to respiratory disease and
118 immune-related traits is important for understanding the genomic background of disease
119 resistance and to apply marker-assisted selection as a selection indicator. A close-line
120 breeding population is useful as a target population for detecting significant QTL because
121 changes in allele frequencies associated with selection traits can occur in this population
122 [7,8,9]. Okamura et al. [10] reported several significant QTL for respiratory disease and
123 immune-capacity traits via linkage-based analysis using microsatellite markers in MPS-
124 selected pigs.

125 A high-density single nucleotide polymorphism (SNP) array has recently made
126 it possible to evaluate genetic diversity for specific regions of the genome and to
127 effectively detect significant QTL using genome-wide association studies (GWAS).
128 Genome-wide analysis using high-density SNP arrays helps to detect observed changes
129 in allele frequency that are attributed to selection on the genome in a closed-line breeding
130 population. Sato et al. [9] reported that, in a simulation analysis in a close-line breeding
131 population, the power of SNP-based GWAS was greater than that of haplotype-based
132 GWAS - a linkage-based method wherein haplotypes are constructed based on pedigree
133 and linkage disequilibrium (LD) information. Therefore, a novel significant QTL for
134 respiratory disease and immune-related traits could be detected by SNP-based GWAS in
135 such populations. In addition, it is normally assumed in GWAS that each QTL has an
136 additive effect. However, GWAS that account for nonadditive effects are also important
137 in a trait related to disease resistance as resistance to disease is a fitness trait [11,12].

138 The objective of this study was to understand the influence of genomic factors on

139 respiratory disease and immune-related traits in MPS-selected pigs by (a) performing
140 GWAS to detect a novel QTL for respiratory disease and immune-related traits by using
141 models that accounted for additive and nonadditive effects and (b) evaluating the changes
142 in allele frequencies due to selection for resistance to MPS and compared the putative
143 selected regions with the detected QTL.
144
145
146 **Materials and Methods**
147 ***Experimental animals and phenotyping***
148 A complete description of the experimental population was previously reported by
149 Kadowaki et al. [3], Okamura et al. [5,10], and Sato et al. [6]. In brief, a total of 874
150 Landrace purebred pigs, selected over five generations from 2002 to 2008 at the Miyagi
151 Prefecture Livestock Experimental Station, Japan, were used for SNP genotyping. Table
152 1 shows the number of animals per generation. This population was selected based on an
153 average daily gain (DG105) from 30 to 105 kg of body weight (BW), ultrasound backfat
154 thickness at 105 kg BW (BF), MPS score, and plasma concentrations of cortisol at 105
155 kg BW (CORT_105). The pigs were infected with respiratory diseases under natural
156 conditions. The detailed selection method and procedure for measuring traits has been
157 described by Kadowaki et al. [3].
158 A total of 22 traits were used in this study and are listed in Table 2. The details
159 of the measurement methods are described by Kadowaki et al. [3], Okamura et al. [5,10],
160 and Sato et al. [6]. In brief, average daily gain from birth to 105 kg BW (TDG) and from
161 birth to 30 kg BW (DG30), DG105, and BF were measured for production traits. For
162 respiratory disease traits, the atrophic rhinitis score (AR score) based on a scale of 0 to 4

163 [10] and the MPS score based on a scale of 0 to 100 % [13] were measured in sib-tested
164 pigs slaughtered at 105 kg BW. For immune-related traits, whole blood was collected
165 from the cranial vena cava of the pigs under anesthesia at 7 weeks of age and 105 kg BW.
166 Phagocytic activity at 105 kg BW (PA_105) and at 7 weeks (PA_7w) was determined in
167 heparinized blood using chemiluminescence analysis. The total number of white blood
168 cells at 105 kg BW (WBC_105) and 7 weeks off age (WBC_7w) was measured in
169 ethylenediaminetetraacetic acid (EDTA)-treated whole blood. The ratio of granular
170 leucocytes to lymphatic cells at 105 kg BW (RGL_105) and at 7 weeks of age (RGL_7w)
171 in heparinized blood was measured. The complement alternative pathway activity in
172 serum at 105 kg BW (CAPA_105) and at 7 weeks of age (CAPA_7w) was measured as
173 the change in light-scattering properties of rabbit erythrocytes upon lysis. CORT_105 and
174 plasma concentration of cortisol at 7 weeks of age (CORT_7w) were also measured.
175 Antibody production at 105 kg BW (AP) was determined by measuring the titer of IgG
176 antibodies against sheep red blood cells (SRBC) after two inoculations with SRBC at 70
177 kg BW and at 100 kg BW. Cytokine concentrations of interleukin (IL)-10, IL-13, IL-17,
178 tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in the peripheral blood serum were
179 measured in sib-tested pigs slaughtered at 105 kg BW.

180 As the distributions of the phenotypic values were highly skewed [6,10] for
181 detecting the MPS score and immune-related traits, these phenotypic values were
182 transformed to the natural logarithmic scale using the formula $\log_e(x)$ for immune-
183 related traits and the formula $\log_e\left(\frac{x+0.5}{100-x+0.5}\right)$ [14] for the MPS score, where x is a
184 phenotypic value. Phenotypic values within a mean ± 3 standard deviation (SD) were
185 used in this study and the descriptive statistics of these traits are shown in Table 2.
186

187 ***SNP genotyping***

188 Genomic DNA was extracted from ear tissue, as previously described by Okamura et al.
189 [10]. Sample DNA was quantified and genotyped using the Illumina PorcineSNP60
190 BeadChip (v1 and v2; Illumina, San Diego, CA, USA) according to the manufacturer's
191 protocol. Image data were analyzed with the iScan (Illumina, San Diego, CA, USA)
192 system and the genotype data were then called using the genotyping module contained in
193 the GenomeStudio software (Illumina, San Diego, CA, USA). All SNP positions were
194 updated according to the SNPchiMp v.3 database [15] and the Sscrofa 11.1 reference
195 sequence assembly downloaded from Ensembl (release 97)
196 (http://ftp.ensembl.org/pub/release-97/variation/vcf/sus_scrofa/). SNP quality control
197 was assessed using the PLINK 1.9 software [16]. The exclusion criteria for SNPs were
198 minor allele frequency (MAF) < 0.05, call rate < 0.95, and Hardy-Weinberg equilibrium
199 test with p-value < 0.001. The exclusion criterion for pigs was a call rate < 0.95. After
200 quality control, a total of 874 pigs genotyped at 37,299 SNPs on autosomal chromosomes
201 were available for genomic analysis.
202
203 ***LD information***
204 The LD coefficient (r^2) values, which are a measure of LD, were calculated for all pairs
205 of SNPs that were less than 10 Mbp apart using the PLINK 1.9 software [16]. Average r^2
206 values for a given intermarker distance, with marker distances grouped in 2 kbp bins,
207 were estimated for each autosome and the average r^2 values among chromosomes were
208 then calculated.
209
210 ***GWAS account for additive and nonadditive effects***

We performed a GWAS to detect significant SNPs that accounted for the additive and nonadditive effects. As for the SNP genotypes in association analysis, the additive and nonadditive effects were assumed and tested in this study. The basic concept of SNP genotype for additive and nonadditive effects was shown by Tsepilov et al. [17] and Nicolini et al. [18]. In brief, for a single SNP locus with two alleles (A and B), the three possible genotypes, AA, AB, and BB, can be coded as 0, 1, and 2 for the additive effect; 0, 1, and 1 for the dominance effect; 0, 0, and 1 for the recessive effect; and 0, 1, and 0 for the overdominance effect, respectively.

The adjusted phenotypes were first obtained using the single-trait animal model as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{Wc} + \mathbf{e}, \qquad (1)$$

where **y** is a vector of the observations; **X**, **Z**, and **W** are the known design matrices relating observations to fixed and random effects. **b** is a vector of fixed effects due to sex (three classes: boar, barrow, and gilt), the generation (five classes), and the rearing environment (three classes, only included in the traits at 105 kg BW). **u** is a vector of breeding values ($\mathbf{u} \sim N(\mathbf{0}, \mathbf{A}\sigma_u^2)$), where **A** and σ_u^2 are the additive relationship matrix and the additive genetic variance, respectively. **c** is a vector of common litter environmental effects (only included in the traits of 7-week-old-pigs and DG30) of dam ($\mathbf{c} \sim N(\mathbf{0}, \mathbf{I}\sigma_c^2)$), where **I** and σ_c^2 are the identity matrix and the common litter environmental variance, respectively. **e** is a vector of residual effects ($\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\sigma_e^2)$), where σ_e^2 is the residual variance. A total of 1395 pigs, six generations from the base generation (G0) to the fifth generation (G5), were used as the pedigree information. The ASReml 4.1 software [19] was used to estimate the genetic variance, phenotypic variance, and heritability, and the estimated values are shown in Table 2. The random effects were

also predicted and the adjusted phenotypes ($\mathbf{y}_{adj}$) were then derived by

$$\mathbf{y}_{adj} = \hat{\mathbf{u}} + \hat{\mathbf{e}},$$

where $\hat{\mathbf{u}}$ and $\hat{\mathbf{e}}$ are the predicted values of the breeding value and the residual value obtained in model (1), respectively.

The adjusted phenotypes were used as the dependent traits in a linear mixed model approach for each SNP:

$$\mathbf{y}_{adj} = \beta_i \mathbf{w}_i + \mathbf{a} + \boldsymbol{\varepsilon}, \qquad (2)$$

where β_i is the allele substitution effect at the *i*-th SNP and $\mathbf{w}_i$ is a vector of SNP genotypes at the *i*-th SNP, and the SNP genotypes for additive and nonadditive effects (dominance, recessive, and overdominance effects) were assumed in the elements of $\mathbf{w}_i$. $\mathbf{a}$ is a vector of additive genetic effects ($\mathbf{a} \sim N(\mathbf{0}, \mathbf{G}\sigma_a^2)$), where $\mathbf{G}$ and σ_a^2 are the genomic relationship matrix proposed by VanRaden [20] and the SNP genetic variance, respectively. $\boldsymbol{\varepsilon}$ is a vector of residual effects ($\boldsymbol{\varepsilon} \sim N(\mathbf{0}, \mathbf{I}\sigma_\varepsilon^2)$), where σ_ε^2 is the residual variance. The regression coefficient and *p* values tested by the Wald test were obtained using the genome-wide mixed-model association (GEMMA) software [21].

For the results of GWAS with an additive effect, the proportion of phenotypic variance explained by the *i*-th SNP effects was calculated using the formula [12]:

$$\text{Proportion}_i = \frac{2p_i(1-p_i)\hat{\beta}_i^2}{\hat{\sigma}_p^2},$$

where p_i is the MAF of the *i*-th SNP, $\hat{\beta}_i$ the estimated allele substitution effect of the *i*-th SNP obtained in model (2), and $\hat{\sigma}_p^2$ the estimated phenotypic variance obtained in model (1) (Table 2).

Multi-GWAS

To detect the results of GWAS with an additive effect, a multiple trait meta-analysis was also performed using the approximate multi-trait test statistic described by Bolormaa et al. [22] in R software (http://www.r-project.org) as follows:

$$\chi^2_{df=22} = \mathbf{t}_i' \mathbf{V}^{-1} \mathbf{t}_i,$$

where $\mathbf{t}_i$ is a vector of signed t-value at the *i*-th SNP for the 22 traits, and the element of $\mathbf{t}_i$ is $\beta_{ij}/se(\beta_{ij})$ (β_{ij} is the allele substitution effect at the *i*-th SNP for the *j*-th trait and $se(\beta_{ij})$ is the corresponding standard error), and $\mathbf{V}^{-1}$ is the inverse of the 22 × 22 correlation matrix between traits calculated from these signed t-values. These allele substitution effects and their standard errors were obtained from the results of 22 single-trait GWAS with an additive effect.

Changes in allele frequency

Changes in allele frequency from the first generation (G1) to G5 in each SNP were calculated as

$$\Delta p = |p_{G5} - p_{G1}|,$$

where p_{G5} and p_{G1} are the allele frequencies in G5 and G1, respectively. To identify the region on the genome attributed to selection in MPS-selected pigs, the observed Δp were compared to the expected Δp under random genetic drift obtained by gene dropping in the R software (http://www.r-project.org) [23,24]. A single SNP genotype was randomly assigned to the pigs in G1 and subsequently the alleles were dropped to the pigs in G5 through the pedigree based on Mendelian sampling. A total of 885 pigs, 874 genotyped and 11 non-genotyped, comprising a family from G1 to G5, were used as pedigree information. The expected p_{G1} was set an increment of 0.01 in the range of 0.01-0.50 (50 MAF-classes), and p_{G5} and Δp frequencies per MAF-class in G1 were

obtained. By replicating this process 100,000 times the distribution of the expected Δp distribution per MAF-class was calculated. The observed p_{G1} in each SNP was classified into the 50 MAF classes, and the observed Δp above the 99.9 % and 99.99 % threshold of the expected Δp distribution was regarded as the suggestive and significant allele frequency change due to selection, respectively.

Gene annotation of target SNPs

For the results of GWAS, Bonferroni correction was applied to determine the 5 % genome-wide significance thresholds (p-value = 1.34×10^{-6}). The extent of LD in this population was approximately 400 kbp (see Results), and the genome-wide significance thresholds defined by the Bonferroni correction were too conservative. Therefore, the genome-wide suggestive threshold was also defined as p-value = 5.0×10^{-5}. The positional candidate genes within the range of the significant and suggestive SNPs ± 200 kbp region were annotated using the Ensembl database (release 97) (http://ftp.ensembl.org/pub/release-97/gff3/sus_scrofa/). In addition, SNPs with suggestive and significant allele frequency changes were also annotated with SNPs ± 200 kbp.

Results

LD in Landrace pigs

Average r^2 values plotted against intermarker distance are shown in Figure 1. The results showed that moderate LD ($r^2 = 0.20$) extended to about 400 kbp in this population.

306 ***GWAS for additive QTL***

307 We performed GWAS for a total of 22 performance, respiratory disease, and immune-
308 related traits in MPS-selected pigs by accounting for their additive and nonadditive effects.
309 The values of an inflation factor were less than 1.1 in all results, and thus the results
310 successfully accounted for population stratification.

311 Genome-wide plots of *p* values with genome-wide significant SNPs for additive
312 QTL in MPS-selected pigs are shown in Figure 2, and the details of the genome-wide
313 significant and suggestive SNPs for the traits are shown in Table 3 and Addition file 1:
314 Table S1, respectively. A total of 11 genome-wide significant SNPs were detected in 5
315 traits and 108 genome-wide suggestive SNPs were detected in 18 traits. For production
316 traits, a genome-wide significant SNP (rs80975749) for BF was detected on SSC1. No
317 significant SNPs were detected in this study for respiratory disease traits. For immune-
318 related traits, one genome-wide significant SNP was detected on SSC7 for RGL_105
319 (rs80902125) and on SSC10 for CORT_7w (rs81236875), three SNPs for CAPA_105
320 (rs81229756, rs81312964, and rs81379304) were detected within a 60 kbp region of
321 SSC3, and four SNPs for CORT_105 (rs80996428, rs80918930, rs80966458, and
322 rs80953170) were detected within a 57 kbp region of SSC7.

323 A multi-trait meta-analysis was performed for the 22 traits using the results of
324 GWAS with an additive model to evaluate the presence of a pleiotropic QTL for these
325 traits. The multi-trait meta-analysis detected no genome-wide significant region and five
326 genome-wide suggestive regions were detected on SSC2, 3, 7, and 10 (Addition file 1:
327 Table S1). Only two of the five regions, those on SSC2 (for WBC_105 and WBC_7w)
328 and SSC7 (for MPS score and CORT_105), overlapped with the regions obtained from
329 multiple traits.

330

331 ***GWAS for nonadditive QTL***

332 To evaluate the relevance of nonadditive effects on the traits, we performed GWAS for
333 testing dominance, recessive, and overdominance effects on these traits. Genome-wide
334 significant SNPs with a non-significant additive effect were extracted under the condition
335 that the number of pigs per genotype was more than 50 in each SNP. A total of three
336 significant SNPs were detected in three traits as shown in Figure 3 and Table 4. The
337 rs80819903 SNP on SSC15 had a genome-wide significant recessive effect on TDG. The
338 rs80992257 SNP on SSC2 and the rs341122035 SNP on SSC14 had a genome-wide
339 significant dominance effect on WBC_105 and TNF-α, respectively. The distributions of
340 an adjusted phenotype for each significant SNP genotype are shown in Figure 4. These
341 box plots clearly show that the AG and GG genotypes in WCG_105 and the AC and CC
342 genotypes in TNF-α have a lower phenotype than the AA genotype in the dominance
343 effect, and AG and AA genotypes have a lower phenotype than the GG genotype in the
344 recessive effect.

345

346 ***Changes in allele frequencies***

347 The observed Δp values were compared with the expected Δp values obtained by gene
348 dropping, and the relationship between the observed Δp and the 99.9 % threshold and
349 the 99.99 % threshold of the expected Δp distribution per MAF-class in G1 is shown in
350 Figure 5. A total of 96 and 14 SNPs had higher Δp than the 99.9 % threshold and the
351 99.99 % threshold, respectively. The SNPs above the 99.9 % threshold and the 99.99 %
352 threshold across the genome are shown in Figure 6 and Addition file 2: Table S2, and
353 these SNPs were spread across the whole genome.

354

355 ***Overlapping regions across studies***

356 We summarized the regions associated with traits for additive and nonadditive effects and
357 the putative regions attributed to selection across the genome in Figure 7. With regard to
358 the pleiotropic region among traits, only five of these genome-wide significant and
359 suggestive regions overlapped among traits (the region on SSC2 for WBC_105 and
360 WBC_7w; the region on SSC7 for MPS score and CORT_105; the region on SSC9 for
361 DG30, MPS score, and CORT_105; the region on SSC12 for IL13, IL17, and IFN-γ; and
362 the region on SSC14 for TDG and TNF-α) in the results of additive QTL. For the
363 nonadditive effect, the significant QTL for WBC_105 and TNF-α also had an additive
364 effect with suggestive level, and overlapped with the QTL for other traits (WBC_7w and
365 TDG, respectively).

366 In the putative selected regions, many regions, which were on SSC2, 3, 4, 5, 6,
367 7, 9, 12, 13, 14, 17, and 18 overlapped with the detected QTL for the production,
368 respiratory disease, and immune-related traits. In addition, three of those selected regions
369 on SSC2, SSC7, and SSC18 were the same regions with the suggestive QTL for MPS
370 score.

371

372

373 **Discussion**

374 It is important to understand the genomic background of the relationship among
375 production, respiratory disease, and immune-related traits by detecting the QTL for these
376 traits to increase animal productivity through genetic selection for disease resistance.
377 Several GWAS studies have reported significant QTL for immune-related traits such as

hematological [25,26,27], T lymphocyte subpopulations [28,29], and cytokine levels [30,31] in different purebred and crossbred pigs. However, there are few genomic regions that overlap among reports, and this strongly depends on the genetic background of the studies. Only a few studies have reported GWAS in respiratory disease [32], and the details of the genomic relationship among these traits have not yet been reported. Therefore, we performed GWAS for a total of 22 performance, respiratory disease, and immune-related traits in MPS-selected pigs. In addition, the combination of results derived from several genome-wide analyses was able to refine and confirm QTL regions, and thus the changes in observed allele frequencies were compared to the expected changes under random genetic drift in the MPS-selected pigs.

Additive QTL analysis detected a total of six genome-wide significant regions in five traits and some of the genome-wide suggestive regions in 18 traits. For the results of relationships among traits, only five regions were associated with multiple traits, and most of the regions did not overlap among traits. In addition, multi-GWAS was performed to evaluate the possibility of a pleiotropic QTL among these traits, and no genome-wide significant region was detected. For genetic correlation among production, respiratory disease, and immune-related traits, Clapperton et al. [33] reported that several of the peripheral blood mononuclear leukocyte subsets were negatively genetically correlated with daily gain in Large White pigs. Flori et al. [34] also reported that genetic correlations among immune-related traits were weak, except for a few traits that mostly include cell subsets in Large White pigs. In our population, there were low genetic correlations between production and respiratory disease traits [3,5], low-to-moderate genetic correlations between production and immune-related traits [5,6], and low-to-moderate genetic correlations among immune-related traits [5,6]. As a result of the detected QTL

in this study, many regions independently affected the traits. Thus, a polygenic effect could contribute to the genetic correlation among the traits when the genetic correlations between traits are moderate.

Resistance to disease is thought to be a fitness trait, and favorable genes are fixed, and unfavorable genes are eliminated under natural selection [12,35]. Thus, it is possible that not only an additive effect but also a nonadditive effect could exist in a fitness trait. However, GWAS have mainly focused on genetic variants with additive effects, whereas nonadditive effects have received much less attention. In fact, Nicolini et al. [18] reported significant nonadditive QTL in male fertility, which is a fitness trait in Holstein cattle. Therefore, we evaluated the relevance of nonadditive effects on the traits. Genome-wide significant SNPs with nonadditive effects were detected for TDG, WBC_105, and TNF-α in this study. Also, a non-fitness trait, such as TDG, also had a significant QTL with nonadditive effects in this population. Thus, nonadditive QTL still exist within a genetic variance and nonadditive effects may be genetically affected not only for fitness traits but also for non-fitness traits.

Artificial selection changes allele frequency attributed to selection traits, and detecting selected loci is important to understand genomic mechanisms controlling phenotypes under selection. Some studies have reported that selection based on the best linear unbiased prediction (BLUP) under an infinitesimal model is able to increase allele frequencies of a few major genes associated with selection traits in cattle [24] and pigs [36,37]. The MPS-selected pigs were selected based on the aggregated breeding value of DG105, BF, MPS score, and CORT_105. Thus, significant changes in allele frequency associated with the selection traits could be observed compared to the expected changes under random genetic drift. Our study detected some suggestive and significant selective

regions on a whole genome, and some of the selected regions overlapped with the detected QTL for not only the selection traits such as MPS score but also other production, respiratory disease, and immune-related traits. The changes in allele frequency in these regions are considered to play an important role in genetic selection for resistance to MPS and increase in daily gain. These results suggest that a closed-line breeding population is a useful target population to refine and confirm QTL regions by integrating the results of GWAS and allele frequency changes.

Okamura et al. [10] performed linkage-based QTL analysis to detect QTL for respiratory disease and immune-capacity traits in the same population as our study. They detected significant QTL for WBC_7w and WBC_105 in the region on SSC2, which was the same region as that detected in our study. They also detected a significant QTL for CORT_105 on SSC7 with the highest significance, on which the most significant SNPs were also detected in our study. Although some of these regions were similar to those reported by Okamura et al. [10], novel QTL were detected in several genomic regions in our study. However, a significant QTL for MPS score was not detected in our study, whereas a significant QTL for MPS score was detected in the upper region on SSC2 by Okamura et al. [10]. Linkage-based QTL analysis considers only the association between DNA markers and QTL in the larger LD region, which is defined only by within-family recombination, and GWAS consider the association in the LD region across the entire population [38]. Thus, it is considered that the frequency of a specific haplotype, which is not related to the MPS score and is the extent for longer distance, changed from G1 to G5 by selection for MPS resistance, and thus linkage analysis may show false positives. However, the suggestive change in allele frequency was observed in the upper region of SSC2 in our study, which is the same region as that described by Okamura et al. [10].

Some of the selected regions were the same as those with the suggestive QTL for MPS score in this study, and thus the selected region on SSC2 might be associated with the MPS score. Thus, further study is needed to detect the association of the variant around the region on SSC2 with MPS score.

GWAS detected a total of six significant additive QTL and three significant nonadditive QTL, as well as a positional candidate gene by genome-wide QTL mapping, which was previously only reported in the SSC7 region of CORT_105 specimens in different breeds [39,40,41]. The significant additive QTL for CORT_105 was detected on SSC7, and the *corticosteroid-binding globulin* (*CBG*, also known as *SERPINA6*) gene was regarded as a positional candidate gene in the QTL region. The *SERPINA6* gene encodes CBG affecting cortisol-binding capacity and SNPs at the *SERPINA6* locus influence plasma cortisol levels in humans [42] and pigs [39,40,41]. Our results supported the finding that the *SERPINA6* gene would be a positional candidate gene for CORT_105.

The other regions detected in our study were not detected by previous genome-wide QTL mapping and were novel regions. No candidate gene was located on the significant additive QTL for RGL_105 and the significant dominance QTL for TNF-α. However, some candidate genes were located in the QTL regions. For production traits, the significant additive QTL for BF was detected on SSC1, and *vacuolar protein sorting-associated protein 4B* (*VPS4B*) gene was the candidate gene in the QTL region. The SNPs near the *VPS4B* gene were associated with abdominal fat in chicken, and a significantly different expression of the *VPS4B* gene was reported in cohorts of chickens with the highest and lowest abdominal fat content [43]. A significant recessive QTL for TDG was detected on SSC15 and the *G protein-coupled receptor 55* (*GPR55*) gene was the candidate gene in the QTL region. GPR55 is an atypical cannabinoid receptor and the

474 *GPR55* gene is expressed in various brain regions and peripheral tissues and regulates
475 energy expenditure by modulating physical activity [44]. The *GPR55* gene has been
476 shown to be associated with eating disorders such as anorexia nervosa in humans [45].
477 As for a candidate gene of immune-related traits, the significant additive QTL
478 for CAPA_105 was detected on SSC3 and the *Synaptotagmin-17* (*SYT17*) gene was the
479 candidate gene in the QTL region. SYT17 is increased in the exosomal fraction of urine,
480 and this increase is associated with activation of the IL-6 amplifier in humans [46].
481 Complement component 3 (C3), which plays a vital role in CAPA, is an acute-phase
482 protein whose expression is regulated by cytokines such as IL-6 [47]. Thus, the *SYT17*
483 gene could be indirectly associated with CAPA_105. The significant additive QTL for
484 CORT_105 was detected on SSC5, and the *5′-Nucleotidase Domain Containing 3*
485 (*NT5DC3*) gene was the candidate gene in the QTL regions. NT5DC3 is a mitochondrion-
486 related protein, and a difference in gene expression between high- and low-stress
487 reactivity in mouse lines was observed in the *NT5DC3* gene [48]. The significant additive
488 QTL for CORT_7w was detected on SSC10 and the *GATA binding protein 3 (GATA3)*
489 gene was the candidate gene in the QTL region. GATA3 is upregulated during Th2 cell
490 differentiation and glucocorticoids such as cortisol increase Th2 activity [49], and thus
491 the expression of *GATA3* might indirectly affect CORT_7w. A significant dominance QTL
492 for WBC_105 was detected on SSC2, and *RAS protein activator like 3 (RASAL3)* gene
493 was the candidate gene in the QTL region. RASAL3 regulates the number and functions
494 of natural killer T cells [50]. RASAL3 is significantly upregulated in *Mycoplasma*
495 *pneumoniae* pneumonia (MPP) children, and NK cells are involved in the pathogenesis
496 of MPP [51]. These candidate genes could be associated with the traits, and further study
497 is needed to understand the details of genomic mechanisms.

498

499 ***Conclusion***

500 In this study, we performed GWAS for a total of 22 production, respiratory disease, and
501 immune-related traits in MPS-selected pigs by accounting for additive and nonadditive
502 effects. We also evaluated the changes in allele frequency due to selection for resistance
503 to MPS in the population. Our GWAS results showed a total of six significant additive
504 QTL and three significant nonadditive QTL. The detected regions except for the region
505 on SSC7 for CORT_105 were not detected by previous genome-wide QTL mapping, and
506 these regions were novel regions with some candidate genes. With regard to a pleiotropic
507 region among traits, only a few detected QTL regions overlapped among traits. However,
508 many putative selected regions overlapped with the detected QTL for not only the
509 selection traits such as MPS score but also other production, respiratory disease, and
510 immune-related traits. These results suggest that a closed-line breeding population is a
511 useful target population to refine and confirm QTL regions by integrating the results of
512 GWAS and allele frequency changes. The study provided new insights into the genomic
513 factors affecting respiratory disease and immune-related traits in pigs.

514

515

516 **Availability of data and materials**

517 All information supporting the conclusions of this article are included within the article
518 and its additional files. The raw datasets used in the current study are available from the
519 authors upon reasonable request and with permission of Miyagi Prefectural Government.

520

521 **Abbreviations**

AR: atrophic rhinitis; BF: backfat thickness; BW: body weight; C3: complement component 3; CAPA: complement alternative pathway activity in serum; CBG: corticosteroid-binding globulin; CORT: plasma concentrations of cortisol; DG: average daily gain; EDTA: ethylenediaminetetraacetic acid; GATA3: GATA binding protein 3; GEMMA: genome-wide mixed-model association; GPR55: G protein-coupled receptor 55; GWAS: genome-wide association study; IFN-γ: interferon γ; IL: interleukin; LD: linkage disequilibrium; MAF: minor allele frequency; Mhp: Mycoplasma hyopneumoniae; MPP: Mycoplasma pneumoniae pneumonia; MPS: Mycoplasmal pneumonia of swine; NT5DC3: 5'-Nucleotidase Domain Containing 3; PA: phagocytic activity; QTL: quantitative trait locus; RASAL3: RAS protein activator like 3; RGL: ratio of granular leucocytes to lymphatic cells; SSC: sus scrofa chromosome; SD: standard deviation; SNP: single nucleotide polymorphism; SRBC: sheep red blood cells; SYT17: Synaptotagmin-17; TNF-α: tumor necrosis factor α; VPS4B: vacuolar protein sorting-associated protein 4B; WBC: total number of white blood cells

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720 ***Contributions***

721 YU designed the experiment, performed the statistical analysis, and contributed to writing
722 and improving the manuscript. KI performed DNA extraction and statistical analysis, and
723 also contributed toward writing and improving the manuscript. TM performed DNA
724 extraction and performed SNP genotyping. NO, HT, HK, CKS, and ES designed the
725 resource population and collected samples and phenotypes. TO collected samples and
726 phenotypes and performed DNA extraction. HA, HK, MS, HU, and KS designed the
727 experiment, managed the entire project, and contributed to writing and improving the
728 manuscript. All authors have read and approved the final manuscript.

729

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733

734 **Ethics declarations**

735 ***Ethics approval and consent to participate***

736 Animal Care and Use Committee approval was not obtained for this study because the
737 phenotype and pedigree data were obtained from an existing database [3,5,6] and DNA
738 samples for DNA [10] were used for SNP genotyping.

739

740 ***Consent for publication***

741 Not applicable.

742

743 ***Competing interests***

744 The authors declare that the research was conducted in the absence of any commercial or

745 financial relationships that could be construed as a potential conflict of interest.

746

747

748 **Additional information**

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754 **Supplementary Information**

755 **Additional file 1: Table S1.** The genome-wide suggestive SNPs associated with

756 production, respiratory disease, and immune-related traits.

757

758 **Additional file 2: Table S2.** The SNPs above the 99.9-threshold and the 99.99-threshold

759 based on changes in allele frequency from the first generation (G1) to the fifth generation

760 (G5).

761

762 Table 1. Population number by generation.

Generation	All	Sib-tested
1	182	124
2	188	135
3	180	126
4	177	119
5	147	118
Total	874	622

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765

766 Table 2. Descriptive statistics and estimated genetic parameter of the study subjects.

Traits[1]	Abbreviation	Unit[2]	Descriptive statistics							Estimated genetic parameter								
			N	Mean	SD	Min	Max	Skewness	Kurtosis	Genetic variance			Phenotypic variance			Heritability		
Production traits																		
Average daily gain from birth to 105kg body weight (BW)	TDG	g/day	857	650	71	459	857	0.13	2.74	1522	±	266	2849	±	179	0.53	±	0.07
Average daily gain from birth to 30kg BW	DG30	g/day	479	451	36	343	549	-0.21	2.98	235	±	123	1118	±	87	0.21	±	0.10
Average daily gain from 30 to 105kg BW	DG105	g/day	483	858	118	530	1180	0.09	2.69	4607	±	1080	8649	±	716	0.53	±	0.09
Ultrasound backfat thickness	BF	mm	468	22.76	3.91	13.50	34.80	0.31	2.68	7.51	±	1.48	11.97	±	1.02	0.63	±	0.08
Respiratory disease traits																		
Atrophic rhinitis score	AR score	-	618	0.93	0.81	0.00	3.00	0.40	2.39	0.18	±	0.06	0.64	±	0.04	0.28	±	0.09
Lesion score of mycoplasma pneumonia of swine	MPS score	%	620	-1.76	0.51	-2.30	-0.31	0.33	2.02	0.076	±	0.03	0.218	±	0.01	0.35	±	0.10
Immune-related traits																		
Phagocytic activity at 105kg BW	PA_105	10^6 RLU	848	0.52	0.33	-0.49	1.36	-0.49	3.18	0.016	±	0.01	0.084	±	0.00	0.19	±	0.06
Phagocytic activity at 7-week old	PA_7w	10^6 RLU	857	0.54	0.32	-0.46	1.25	-0.48	2.88	0.018	±	0.01	0.093	±	0.01	0.19	±	0.09
Complement alternative pathway activity at 105kg BW	CAPA_105	OD_{413}	846	-0.48	0.34	-1.47	0.12	-0.95	2.78	0.002	±	0.00	0.077	±	0.00	0.03	±	0.03
Complement alternative pathway activity at 7-week old	CAPA_7w	OD_{413}	842	-0.50	0.29	-1.44	-0.08	-1.20	4.05	0.007	±	0.00	0.071	±	0.00	0.09	±	0.07
Total number of white blood cells at 105kg BW	WBC_105	$\times 10^4$/mL	839	0.27	0.08	0.02	0.51	0.04	3.24	0.001	±	0.00	0.006	±	0.00	0.25	±	0.07
Total number of white blood cells at 7-week old	WBC_7w	$\times 10^4$/mL	846	0.28	0.10	-0.03	0.59	-0.02	2.92	0.003	±	0.00	0.011	±	0.00	0.30	±	0.10
Ratio of granular leucocyte to lymph cells at 105kg BW	RGL_105	-	850	-0.16	0.26	-0.91	0.57	0.57	3.02	0.003	±	0.00	0.030	±	0.00	0.10	±	0.05
Ratio of granular leucocyte to lymph cells at 7-week old	RGL_7w	-	682	-0.17	0.19	-0.68	0.37	-0.04	2.91	0.001	±	0.00	0.035	±	0.00	0.03	±	0.07
Plasma concentrations of cortisol at 105kg BW	CORT_105	μg/dL	764	-0.01	0.35	-1.00	0.91	-0.08	2.87	0.010	±	0.01	0.112	±	0.01	0.09	±	0.05
Plasma concentrations of cortisol at 7-week old	CORT_7w	μg/dL	468	0.32	0.30	-0.67	0.97	-0.57	3.13	0.017	±	0.01	0.081	±	0.01	0.21	±	0.11
Antibody production at 105kg BW	AP	Titre	840	1.68	0.39	0.64	2.67	0.27	2.56	0.041	±	0.01	0.130	±	0.01	0.32	±	0.07
Serum concentration of interleukin 10 at 105kg BW	IL-10	pg/mL	550	0.81	0.73	-1.17	2.98	0.07	2.94	0.107	±	0.05	0.536	±	0.03	0.20	±	0.09
Serum concentration of interleukin 13 at 105kg BW	IL-13	pg/mL	517	2.96	0.58	1.19	4.65	-0.21	3.02	0.027	±	0.02	0.324	±	0.02	0.08	±	0.07
Serum concentration of interleukin 17 at 105kg BW	IL-17	pg/mL	554	1.44	0.65	-0.63	3.25	0.04	3.22	0.112	±	0.04	0.416	±	0.03	0.27	±	0.10
Serum concentration of interferon γ at 105kg BW	IFN-γ	pg/mL	346	1.56	0.90	-0.97	4.28	-0.25	2.64	0.00000002	±	0.00	0.460	±	0.04	0.00	±	0.00
Serum concentration of tumor necrosis factor α at 105kg BW	TNF-α	pg/mL	258	1.89	0.44	0.72	3.10	0.03	2.87	0.047	±	0.03	0.197	±	0.02	0.24	±	0.16

767 [1]MPS and immune-related traits were transformed to the natural logarithmic scale and the descriptive statistics of the transformed values are

768 shown.

769 [2]Titer unit is equivalent to 104/dilution degrees of sample serum.

770

771 Table 3. The genome-wide significant SNPs associated with production and immune-related traits.

Traits[1]		SNP information[2] SSC	Position(bp)	refSNP varidation ID	EA	EAF	SNP effect β			Proportion[3]	P-value	Gene symbol within the SNP ±200 kbp region
Production traits												
	BF	1	158,309,021	rs80975749	A	0.46	-1.43	±	0.28	0.08	4.23E-07	SERPINB13,SERPINB12,SERPINB5,VPS4B, KDSR
Immune-related traits												
	CAPA_105	3	26,434,892	rs81229756	A	0.09	-0.13	±	0.03	0.04	6.08E-07	TMC5,TMC7,COQ7,ITPRIPL2,SYT17
	CAPA_105	3	26,439,289	rs81312964	A	0.09	-0.13	±	0.03	0.04	6.08E-07	TMC5,TMC7,COQ7,ITPRIPL2,SYT17
	CAPA_105	3	27,030,167	rs81379304	A	0.09	-0.13	±	0.03	0.04	3.92E-07	XYLT1
	RGL_105	7	18,210,546	rs80902125	G	0.12	-0.06	±	0.01	0.03	2.63E-07	-
	CORT_105	5	80,808,190	rs81326027	G	0.48	0.10	±	0.02	0.04	1.67E-07	NT5DC3
	CORT_105	7	115,114,488	rs80996428	A	0.27	0.10	±	0.02	0.04	9.14E-07	PRIMA1,ASB2,CCDC197,OTUB2
	CORT_105	7	115,550,783	rs80918930	C	0.40	0.11	±	0.02	0.06	7.35E-11	ISG12(A),PPP4R4,SERPINA6,SERPINA1,SERPINA11,UABP-2,SERPINA12
	CORT_105	7	115,571,143	rs80966458	G	0.13	0.14	±	0.03	0.04	2.72E-07	PPP4R4,SERPINA6,SERPINA1,SERPINA11, UABP-2,SERPINA12
	CORT_105	7	115,679,840	rs80953170	G	0.29	0.10	±	0.02	0.04	2.74E-07	PPP4R4,SERPINA6,SERPINA1,SERPINA11, UABP-2,SERPINA12,SERPINA4,SERPINA5
	CORT_7w	10	63,410,258	rs81236875	A	0.05	0.19	±	0.04	0.04	3.13E-07	GATA3,TAF3,ATP5F1C,KIN

772 [1]Abbreviations of traits are shown in Table 2.

773 [2]SSC: Sus Scrofa chromosome, EA: Effect allele, EAF: Effect allele frequency.

774 [3]The proportion of adjusted phenotypic variance explained by the SNP effects.

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777 Table 4. The genome-wide significant SNPs with non-additive effect.

Traits[1]	SSC[2]	Position(bp)	refSNP varidation ID	MAF[3]	Allele A	Allele B	Number of animals AA	Number of animals AB	Number of animals BB	Non-additive effect Effect[4]	β			P-value	P-value(Add)[5]	Gene symbol within the SNP ±200 kbp region
TDG	15	131,605,148	rs80819903	0.27	A	G	444	337	60	Recessive	33.84	±	6.81	8.05E-07	1.33E-02	CAB39,ITM2C,GPR55,SPATA3,C2orf72
WBC_105	2	62,246,385	rs80992257	0.30	A	G	404	360	73	Dominance	-0.03	±	0.01	9.92E-07	2.79E-06	CYP4F22,PGLYRP2,RASAL3,AKAP8L,AKAP8,BRD4,EPHX3,NOTCH3,ILVBL,SYDE1
TNF-α	14	17,600,242	rs341122035	0.47	A	C	65	142	51	Dominance	-0.32	±	0.06	1.74E-07	2.40E-06	-

778 [1]Abbreviations of traits are shown in Table 2.

779 [2]SSC: Sus Scrofa chromosome.

780 [3]MAF: Minor allele frequency

781 [4]Dominance: The genotype is represented as AA(0), AB(1), and BB(1). Recessive: The genotype is represented as AA(0), AB(0), and BB(1).

782 [5]P-value of additive model.

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785

Figure legends

Figure 1. Average linkage disequilibrium coefficient (r^2) values plotted against intermarker distance for all autosomal chromosomes in Landrace pigs. The *x*-axis indicates the distance between single nucleotide polymorphisms (SNPs) and the *y*-axis indicates the r^2 values between SNPs.

Figure 2. Manhattan plots representing the genome-wide significant association of additive effect with production and immune-related traits in Landrace pigs. Abbreviations of the traits for BF (a), CAPA_105 (b), RGL_105 (c), CORT_105 (d), and CORT_7w (e) are shown in Table 2. The x-axis indicates the chromosome number and the y-axis indicates $-\log_{10}$(*p*-value).

Figure 3. Manhattan plots representing the genome-wide significant association of nonadditive effect with production and immune-related traits in Landrace pigs. Abbreviations of the traits for TDG (a), WBC_105 (b), and TNF-α (c) are shown in Table 2. The x-axis indicates the chromosome number and the y-axis indicates $-\log_{10}$(*p*-value). The genotype of the dominance effect is represented as AA(0), AB(1), and BB(1), and the genotype of the recessive effect is represented as AA(0), AB(0), and BB(1).

Figure 4. Box plot for the genome-wide significant loci with nonadditive effects. Abbreviations of the traits for TDG (a), WBC_105 (b), and TNF-α (c) are shown in Table 2. The box plots show the distribution of adjusted phenotypes among the different genotypes, and the bold line is the median of the trait's per-genotype group.

Each dot represents a unique pig.

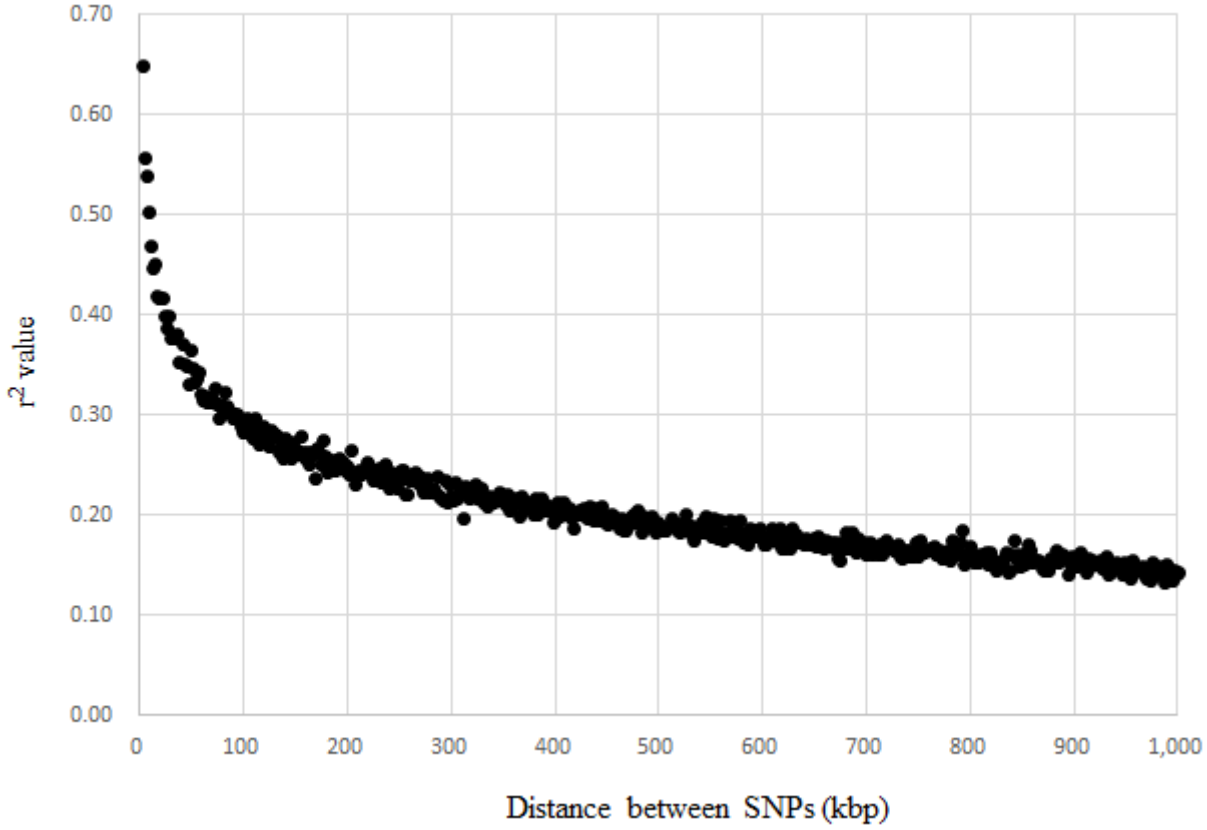
Figure 5. Plot for the relationship between minor allele frequency (MAF) in the first generation (G1) and the absolute values of changes in allele frequency from G1 to the fifth generation (Δp). The observed Δp were plotted per MAF-class in G1, and the orange and the red lines represent the 99.9 % and 99.99 % threshold of the expected Δp distribution (100,000 replicates), respectively.

Figure 6. Manhattan plots representing the suggestive and significant allele frequency changes due to selection in Landrace pigs. The x-axis indicates the chromosome number and the y-axis indicates the absolute values of changes in allele frequency from the first generation to the fifth generation (Δp). The single nucleotide polymorphisms (SNPs) in orange (n = 96) and red (n = 14) have the observed Δp above the 99.9 % threshold and the 99.99 % threshold of the expected Δp distribution (100,000 replicates), respectively.

Figure 7. The summary regions associated with traits and putative selected regions across genome. Each row represents the results of genome-wide association study (GWAS) in each trait accounted for an additive and a nonadditive effects, and the results of allele frequency changes. Each column represents a genomic region containing single nucleotide polymorphisms (SNPs) with suggestive and significant levels in each result. For the results of GWAS, and *p*-value = 5.0×10^{-5} and *p*-value = 1.34×10^{-6} were regarded as genome-wide suggestive and significant associations with a trait, respectively. For putative selected region, the observed allele frequency changes

above the 99.9 threshold and the 99.99 threshold of the expected Δp distribution (100,000 replicates) were regarded as suggestive and significant changes, respectively. Only traits with at least one associated SNP and SNPs associated with at least one trait are shown. Abbreviations of the traits are shown in Table 2.

Figures



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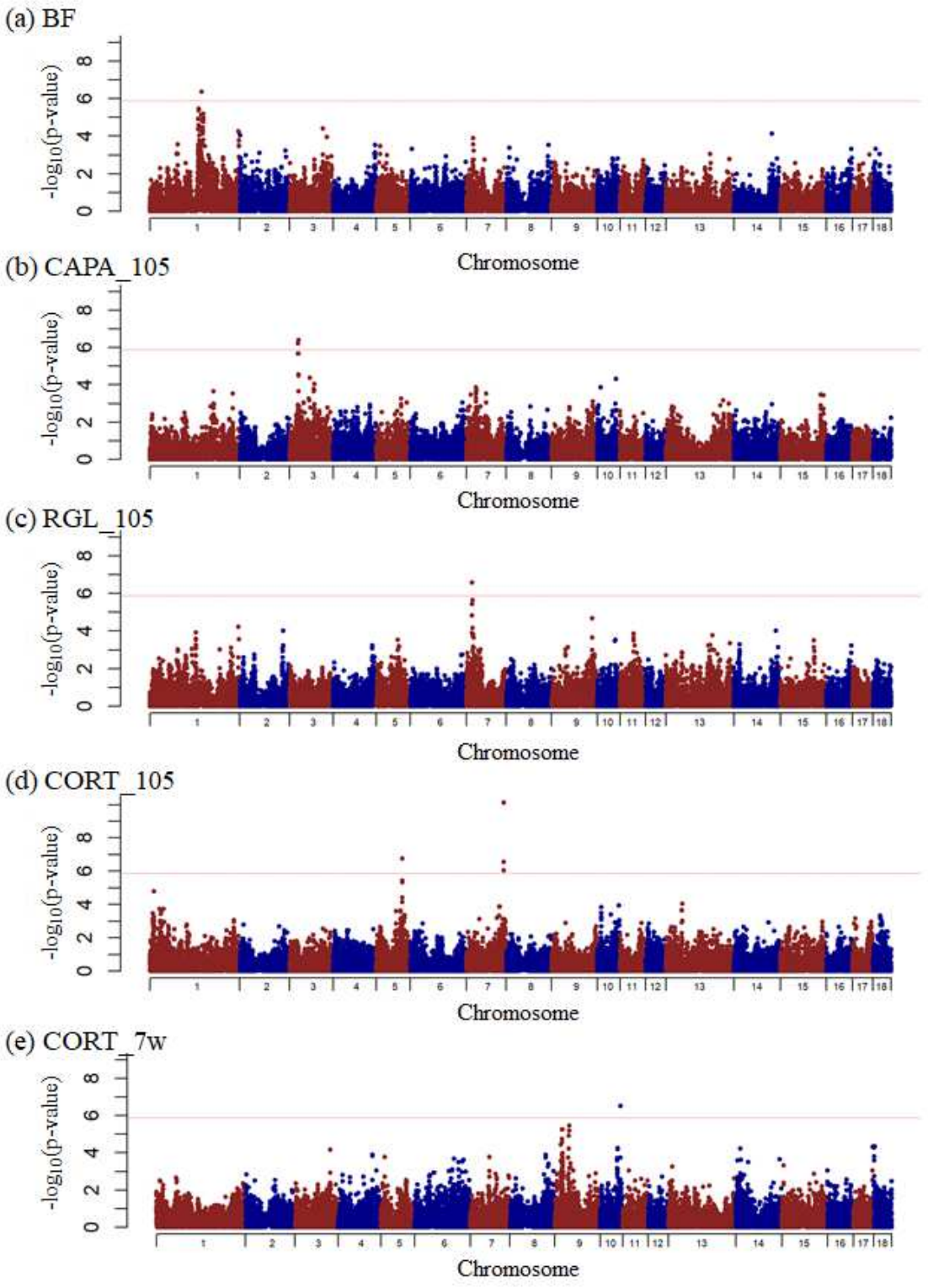

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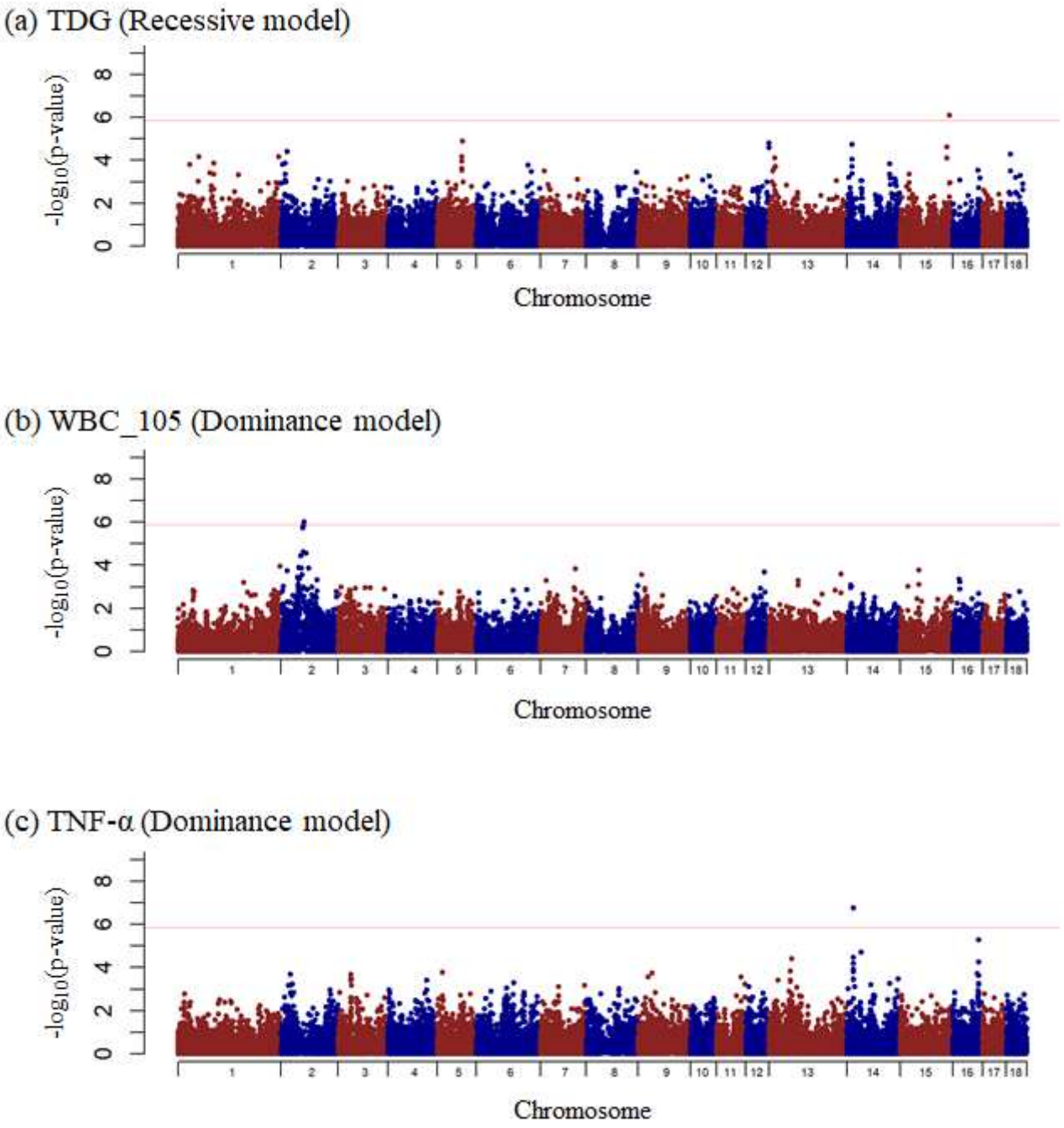

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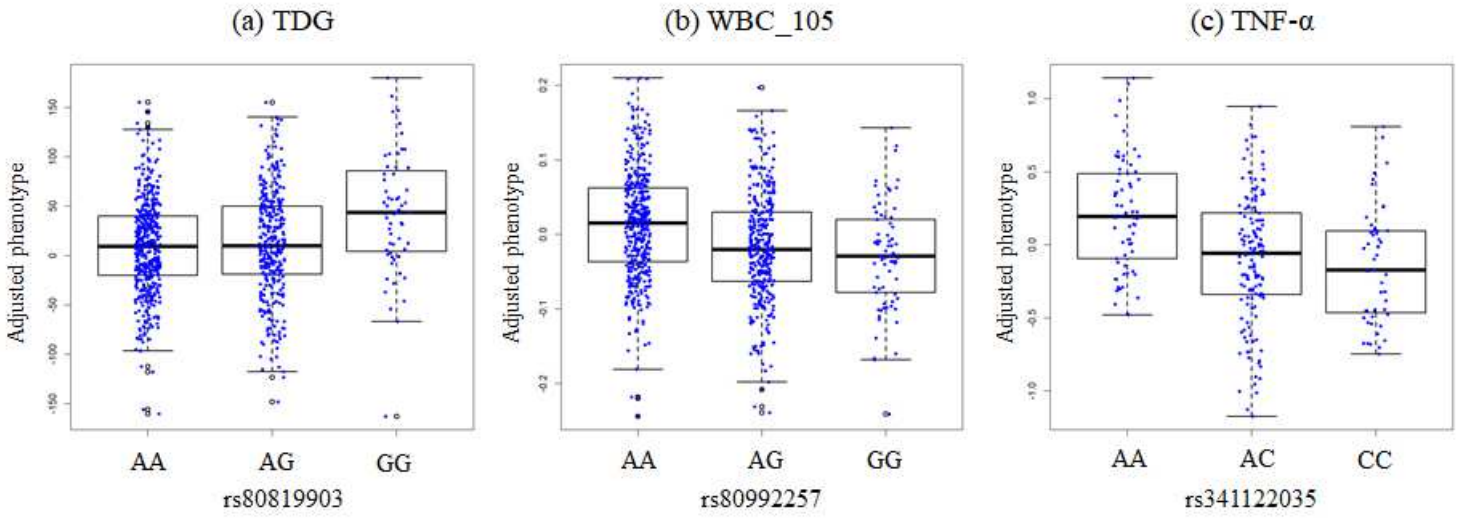

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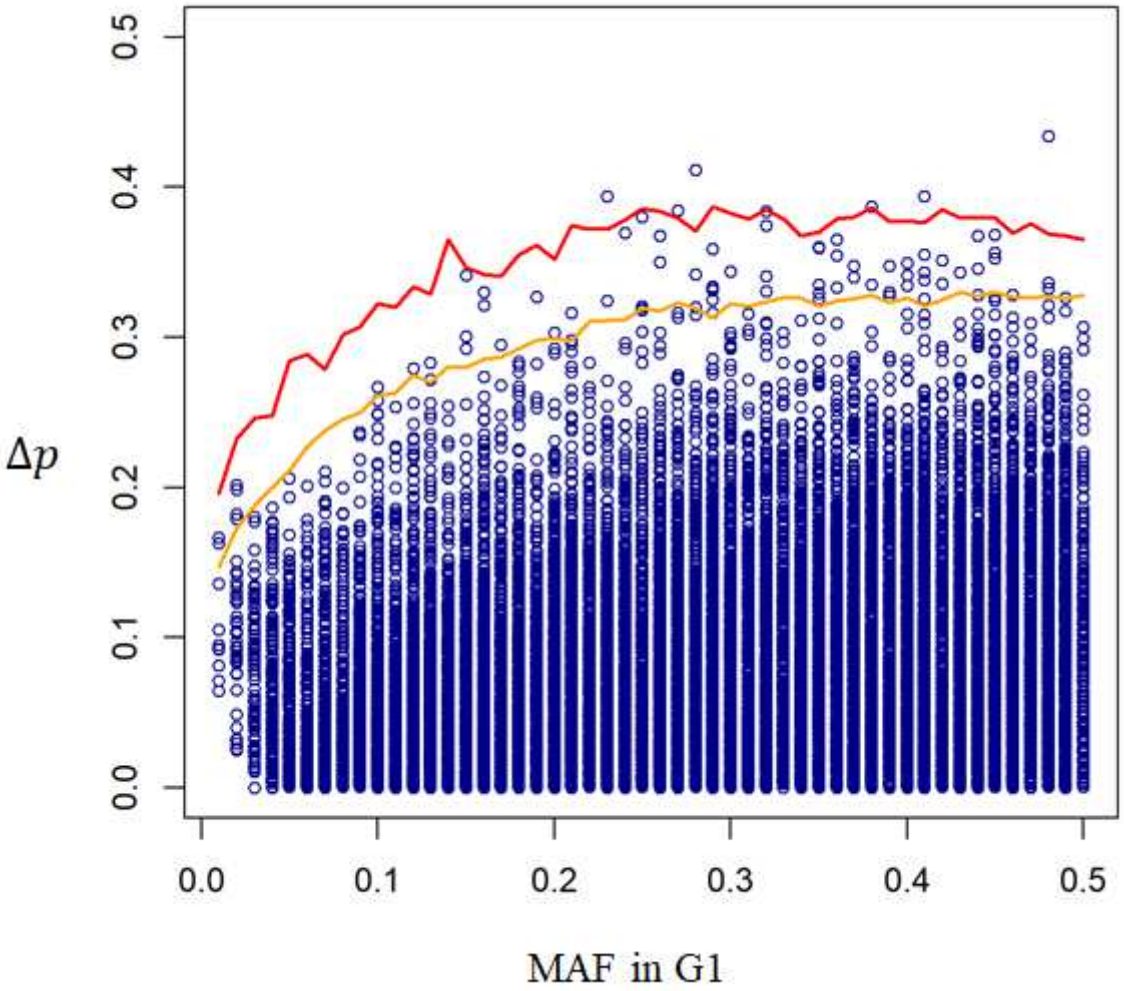

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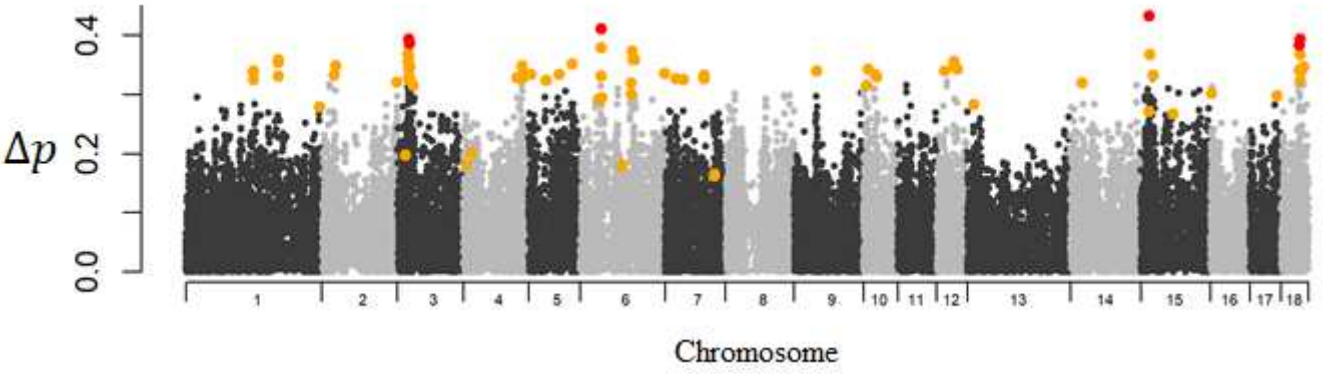

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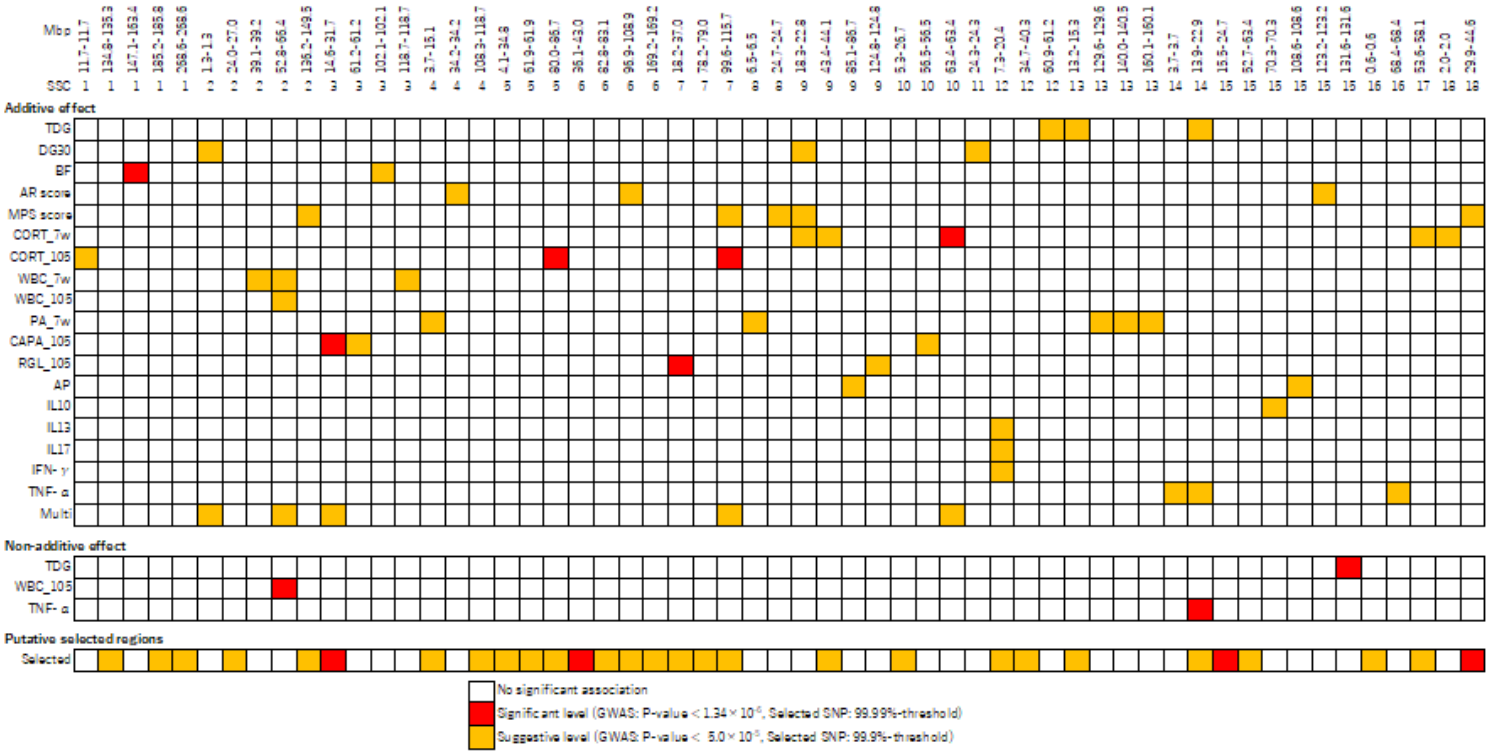

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1.pdf
- Additionalfile2TableS2.pdf