Rare and population-specific functional variation across pig lines

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Abstract

Background

It is expected that missense and loss-of-function (LOF) variants are responsible for phenotypic differences among breeds, genetic lines and varieties of livestock and crop species that have undergone diverse selection histories. However, there is still limited knowledge about the existing missense and LOF variation in livestock commercial populations, in particular regarding population-specific variation.

Methods

We performed whole-genome re-sequencing of 7,848 individuals from nine commercial pig breeding lines (average coverage: 4.1x) and imputed genotypes for 440,610 pedigree-related individuals. The variants were categorized according to predicted functional annotation (from loss-of-function to intergenic) and prevalence level (number of lines in which the variant segregated; from private to widespread). Variants in each category were examined in terms of distribution along the genome, minor allele frequency, Wright’s fixation index (FST), individual load, and association to production traits.

Results

Of the 46 million called variants, 28% were private (i.e., called in only one line) and 21% were widespread (i.e., called in all nine lines). Genomic regions with low recombination rate were enriched with private variants. Low-prevalence variants were enriched for lower allele frequencies, lower FST, and putatively functional and regulatory variants (including loss-of-function and deleterious missense). Only a small subset of low-prevalence variants was found at intermediate allele frequencies and had large estimated effects on production traits. Individuals on average carried...
less private deleterious missense alleles than expected compared to other predicted
consequence types. A small subset of low-prevalence variants with intermediate allele
frequencies and higher $F_{ST}$ were detected as significantly associated to the production
traits and explained small fractions of phenotypic variance (up to 3.2%). These
associations were tagged by other more widespread variants, including intergenic
variants.

**Conclusions**

Most low-prevalence variants are kept at very low allele frequency and only a small
subset contributed detectable fractions of phenotypic variance. Low-prevalence
variants are therefore unlikely to hinder across-breed analyses, in particular when
predicting genomic breeding values using reference populations of a different genetic
background.
Introduction

Genetic variation is the basis of selective breeding in livestock and crop species. From a molecular point of view, genetic variants that result in either altered protein structures or altered gene expressions are believed to be responsible for much of the existing variation in production traits. Missense variants change one amino acid of the encoded protein. Loss-of-function variants (LOF) are predicted to disrupt protein-coding transcripts in a way that they will not be translated into proteins or that they will be translated into non-functional proteins. Loss-of-function variants may change one amino acid codon into a premature stop codon (nonsense variants), change the reading frame during translation (frameshift indels) or change mRNA splicing (splicing variants). As such, potentially functional variants in protein-coding regions are assumed to be easier to detect (e.g., by association analyses) than variants that moderate gene expression. Thus, missense and LOF variants are typically prioritised as putative causal variants for the traits of interest (e.g., [1–4]).

Missense and LOF mutations can be pathogenic. For instance, missense and nonsense variants account for 57% of the entries in the Human Gene Mutation Database [5] (accessed on 30 April 2021), while small indels account for 22% and splicing variants account for another 9%. Similarly, in livestock species many missense and LOF variants have been described as causal of genetic diseases ([6,7]; Online Mendelian Inheritance in Animals [8], accessed on 30 April 2021), embryonic lethality [9,10] or product defects [11,12]. Deleterious missense and LOF variants are subject to purifying selection and are more likely to be rare, as they are related to disease risk or reduced fertility.

However, some missense and LOF mutations can be beneficial too [13]. Moreover, some alleles that would be detrimental in the wild may be preferred in
artificial selection settings. The artificial selection performed in livestock and plant breeding programs is expected to increase the frequency of alleles that favourably affect the traits included in the selection objectives. Therefore, it is also expected that missense and LOF variants are responsible for differences among breeds, genetic lines and varieties of livestock and crop species that have undergone diverse selection histories. Identification of such functional variants would have direct applications in gene-assisted and genomic selection [14–16]. Furthermore, strategies based on genome editing have been theorized to either promote favourable alleles [17] or remove deleterious alleles [18] in selection candidates. Nevertheless, there is still limited knowledge about the existing missense and LOF variation in livestock commercial populations, in particular regarding population-specific variation, often referred to as ‘private’.

Next-generation sequencing has great potential for animal breeding. One of its main benefits is the power to detect large amounts of variants, many of which will be specific to the population under study. Sequencing a large number of individuals is necessary to achieve high variant discovery rates, particularly for low-frequency variants [19,20]. There are several sequencing studies that profile the genomic variation in pigs [21–23], cattle [24,25], or chicken [26]. These studies involved the sequencing of a low number of individuals (up to a few hundreds) at intermediate or high sequencing coverage. Alternatively, exploring lower coverage allows affordable sequencing of a much larger number of individuals, which would enable the identification of a much larger number of variants.

The objective of our study was to characterize the genetic variants detected in nine intensely selected pig lines with diverse genetic backgrounds. Particular emphasis was given to quantifying rare and population-specific functional variants, as
well as the number of missense and LOF variants that an average individual carried.  

We also assessed the contribution of population-specific functional variants to the variance of production traits.

Materials and Methods

Populations and sequencing strategy

We performed whole-genome re-sequencing of 7,848 individuals from nine commercial pig lines (Genus PIC, Hendersonville, TN) with a total coverage of approximately 32,114x. Sequencing effort in each of the nine lines was proportional to population size. Approximately 1.5% (0.9-2.1%) of the pigs in each line were sequenced. Most pigs were sequenced at low coverage, with target coverage of 1 or 2x. A subset of pigs in each line was sequenced at higher coverage of 5, 15, or 30x. Thus, the average individual coverage was 4.1x, but the median coverage was 1.5x. The number of pigs sequenced at different coverage levels for each line is summarized in Table 1.

The sequenced pigs and their coverage were selected following a three-part sequencing strategy with the objective of representing the haplotype diversity in each line. First (1), top sires and dams with the largest number of genotyped progeny were sequenced at 2x and 1x, respectively. Sires were sequenced at greater coverage because they individually contributed more progeny than dams. Then (2), the individuals with the greatest genetic footprint on the population (i.e., those that carry more of the most common haplotypes) and their immediate ancestors were sequenced at a coverage between 1x and 30x (AlphaSeqOpt part 1; [27]). The target sequencing
coverage was assigned by an algorithm that maximises the expected phasing accuracy of the common haplotypes from the cumulated family information. Finally (3), pigs that carried haplotypes with low cumulated coverage (below 10x) were sequenced at 1x (AlphaSeqOpt part 2; [28]). Sets (2) and (3) were based on haplotypes inferred from marker array genotypes (GGP-Porcine HD BeadChip; GeneSeek, Lincoln, NE), which were phased and imputed using AlphaPhase [29] and AlphaImpute [30].

Most sequenced pigs, as well as pedigree-related pigs in those lines, were also genotyped either at low density (15k markers) using the GGP-Porcine LD BeadChip (GeneSeek) or at high density (80k markers) using the GGP-Porcine HD BeadChip (GeneSeek).

**Sequencing and data processing**

Tissue samples were collected from ear punches or tail clippings. Genomic DNA was extracted using Qiagen DNeasy 96 Blood & Tissue kits (Qiagen Ltd., Mississauga, ON, Canada). Paired-end library preparation was conducted using the TruSeq DNA PCR-free protocol (Illumina, San Diego, CA). Libraries for resequencing at low coverage (1 to 5x) were produced with an average insert size of 350 bp and sequenced on a HiSeq 4000 instrument (Illumina). Libraries for resequencing at high coverage (15 or 30x) were produced with an average insert size of 550 bp and sequenced on a HiSeq X instrument (Illumina). All libraries were sequenced at Edinburgh Genomics (Edinburgh Genomics, University of Edinburgh, Edinburgh, UK).

DNA sequence reads were pre-processed using Trimmomatic [31] to remove adapter sequences. The reads were then aligned to the reference genome *Sscrofa11.1*.
GenBank accession: GCA_000003025.6) using the BWA-MEM algorithm [32].
Duplicates were marked with Picard (http://broadinstitute.github.io/picard). Single nucleotide polymorphisms (SNPs) and short insertions and deletions (indels) were identified with GATK HaplotypeCaller (GATK 3.8.0) [33,34] using default settings.
Variant discovery was performed separately for each individual and then a joint variant set for each population was obtained by extracting the variant positions from all the individuals in it. Between 20 and 30 million variants were discovered in each population.

We extracted the read counts supporting each allele directly from the aligned reads stored in the BAM files using a pile-up function. This approach was set to avoid biases towards the reference allele introduced by GATK when applied on low-coverage whole-genome sequence data [35]. That pipeline uses the tool pysam (version 0.13.0; https://github.com/pysam-developers/pysam), which is a wrapper around htslib and the samtools package [36]. We extracted the read counts for all biallelic SNP positions, after filtering variants in potential repetitive regions with VCFtools [37]. Such variants were here defined as variants that had mean depth values 3 times greater than the average realized coverage.

**Genotype imputation**
Genotypes were jointly called, phased and imputed for a total of 537,257 pedigree-related individuals using the ‘hybrid peeling’ method implemented in AlphaPeel [38–40], which used all available marker array and whole-genome sequence data. Imputation was performed separately for each line using its complete multi-generational pedigree, which encompassed from 15,495 to 122,753 individuals each (Table 1). We have previously published reports on the accuracy of imputation
in the same populations using this method [39]. The estimated average individual-wise dosage correlation was 0.94 (median: 0.97). Individuals with low predicted imputation accuracy were removed before further analyses. An individual was predicted to have low imputation accuracy if itself or all of its grandparents were not genotyped with a marker array or if it had a low degree of connectedness to the rest of the population. These criteria were based on the analysis of simulated and real data on imputation accuracy [39]. A total of 440,610 individuals remained, from 5,247 to 104,661 individuals for each line (Table 1). The expected average individual-wise dosage correlation of the remaining individuals was 0.97 (median: 0.98) according to our previous estimates. We accounted for the whole minor allele frequency spectrum for our analyses. However, variants with a minor allele frequency lower than 0.023 had an estimated variant-wise dosage correlations lower than 0.90 [39].

**Variant predicted consequence types**

The frequency of the alternative allele was calculated based on the imputed genotypes. We defined the ‘prevalence level’ of each variant as the number of lines in which the variant segregated. To distinguish between allele frequency and prevalence level we used the terms ‘rare’ and ‘common’ to refer to variants in terms of allele frequency and ‘private’ and ‘widespread’ in terms of prevalence level, where private variants were those called only in one line and widespread variants those called in all studied lines. We calculated Wright’s fixation statistic (FST) [41] for each variant among the lines where the variant segregated as $F_{ST} = (H_T - H_S)/H_T$, where $H_T$ is the expected heterozygosity across the combined lines assuming Hardy-Weinberg equilibrium and $H_S$ is the average heterozygosity within lines assuming Hardy-Weinberg equilibrium.
Variants were annotated using Ensembl Variant Effect Predictor (Ensembl VEP; version 97, July 2019) [42] using both Ensembl and RefSeq transcript databases. For variants with multiple predicted consequence types (e.g., in case of multiple transcripts), the most severe predicted consequence type for each variant was retrieved. Stop-gain, start-loss, stop-loss, splice donor, and splice acceptor variants were classified as LOF variants. While frameshift indels are typically included in the LOF category, we considered them as a separate category in order to be able to quantify their impact separately. The SIFT scores [43] for missense variants were retrieved from the Ensembl transcript database. Such variants were classified either as ‘deleterious’ when their SIFT score were less than 0.05, or ‘tolerated’ otherwise. Missense variants according to the RefSeq transcript database were left unclassified.

For further analyses we considered the following nine predicted consequence types: LOF, frameshift indels, deleterious missense, tolerated missense, unclassified missense, synonymous, variants in the 5’ and 3’ untranslated regions (UTR), intronic, and intergenic variants. We considered the predicted consequence types of LOF, frameshift indel, deleterious missense, and tolerated missense as putatively functional variants; the predicted consequence types of synonymous and UTR as putatively regulatory variants; and the predicted consequence types of intronic and intergenic as putatively neutral variants.

**Load of putatively functional alleles**

We used the imputed genotypes to estimate the number of alleles of each predicted consequence type and prevalence level that an average individual carried. For the most common predicted consequence types, that number was estimated from 50,000 variants sampled randomly. For tolerated missense variants, we used the...
50,000 variants with the highest SIFT scores. To account for the different number of variants within each predicted consequence type and prevalence level category, we calculated ‘heterozygosity’ as the percentage of variants of each category that an individual carried in heterozygosis, and the ‘homozygosity for the alternative allele’ as the percentage of variants of each category that an individual carried in homozygosis for the alternative allele.

**Association to production traits**

To further explore the association of variants by prevalence level and functional annotation to selected traits, we performed genome-wide association studies (GWAS) for the three largest lines. For each line, we performed GWAS for three complex traits with moderate to high heritability. Most pigs with records were born during the 2008–2020 period. Breeding values were estimated by line with a linear mixed model that included polygenic and non-genetic (as relevant for each trait) effects. Deregressed breeding values (dEBV) were obtained following the method by VanRaden and Wiggans [44]. Only individuals in which the trait was directly measured were retained for further analyses. We fitted a univariate linear mixed model that accounted for the genomic relationship matrix as:

\[
y = x_i \beta_i + u + e,
\]

where \(y\) is the vector of preadjusted phenotypes, \(x_i\) is the vector of genotypes for the \(i\)th SNP coded as 0 and 2 if homozygous for either allele or 1 if heterozygous, \(\beta_i\) is the additive effect of the \(i\)th SNP on the trait, \(u \sim N(0, \sigma_u^2K)\) is the vector of polygenic effects with the covariance matrix equal to the product of the polygenic additive variance \(\sigma_u^2\) and the genomic relationship matrix \(K\), and \(e\) is a vector of uncorrelated
residuals. Due to computational limitations, the genomic relationship matrix $\mathbf{K}$ was calculated using only imputed SNP genotypes for the high-density marker array and its single-value decomposition was taken. We used the FastLMM software [45, 46] to fit the model.

We considered the associations with a p-value equal or smaller than $10^{-6}$ as significant. We calculated an enrichment score for each predicted consequence type and prevalence level category as:

$$\log\left(\frac{n_{\text{SignCategory}}/n_{\text{NotSignCategory}}}{n_{\text{SignTotal}}/n_{\text{NotSignTotal}}}\right),$$

where $n_{\text{SignCategory}}$ was the number of variants with significant association (with at least one trait in one of the three lines) in a given predicted consequence type and prevalence level category, $n_{\text{NotSignCategory}}$ was the number of variants with no significant association in the same category, and $n_{\text{SignTotal}}$ and $n_{\text{NotSignTotal}}$ were the total numbers of variants with and without significant association, respectively.

Linkage disequilibrium is pervasive between nearby significant variants due to the extremely high variant density of whole-genome sequence data. To account for this, we defined haplotype blocks so that only a single variant per haplotype block was considered as the putative driver of the association detected in that region. We defined the haplotype blocks for each line separately using the --blocks function in Plink 1.9 [47, 48]. Pairs of variants within 5 Mb of each other were considered. Two variants were considered to be in strong linkage disequilibrium if the bottom of the 90% confidence interval of $D'$ was greater than 0.7 and the top of the confidence interval was at least 0.9. If the top of the confidence interval was smaller than 0.7, it was considered as strong evidence for historical recombination between the two variants. The remaining pairs of variants were considered uninformative. Regions where at least 90% of the informative pairs showed strong linkage disequilibrium
were defined as a haplotype block. Within each haplotype block, we selected one
‘candidate variant’ as the variant with the most severe predicted consequence type. If
there was more than one variant with the same predicted consequence type, the one
with the lowest p-value was selected. This process was performed separately for each
trait and line. Establishing which of the variants in linkage disequilibrium is the most
likely to be causal remains one of the greatest challenges for GWAS. Nevertheless,
keeping the most severe variant in a haplotype block is a common assumption made
by researchers for prioritisation of variants as candidate.

We calculated the additive variance explained by each variant as $2pq a^2$, where
$p$ and $q$ were the allele frequencies and $a$ was the estimated allele effect of the variant.
We expressed the additive variance explained as a proportion of the phenotypic
variance of each trait. We calculated the maximum percentage of phenotypic variance
explained by an individual variant in each predicted consequence type and prevalence
level category. Finally, we calculated the median $F_{ST}$ of the candidate variants within
each predicted consequence type and prevalence level category. We compared the
median $F_{ST}$ of the candidate variants to the median $F_{ST}$ of the same category as the
logarithm of the ratio of the former to the latter.

Results

Prevalence of variants

A large number of variants were widespread across all nine lines. Private
variants represented a much smaller proportion of the variants called within each line.
However, when counted across lines, they cumulatively predominated over the
prevalent ones. Most variants were neither private nor widespread. A total of 46,344,624 biallelic variants were called and passed quality control criteria across all lines. The distribution of these variants by line is shown in Table 2. Most variants (38,642,777) were SNPs, of which 10,595,681 were called in a single line (27%; 366,486 to 2,743,965 within each line) and 8,377,578 (22%) were called in all nine lines. The remaining 7,701,847 variants were indels, of which 2,436,674 were called in a single line (32%; 121,525 to 506,149 in each line) and 1,560,353 (20%) were called in all nine lines.

Initially, 24,394,763 additional variant positions were called across all nine lines (adding up to 70,739,387 unique variant positions) but failed to meet quality control criteria. Of these, 148,825 variants were discarded because they had mean depth values 3 times greater than the average realized coverage, 1,927,221 were multiallelic within line, and 1,673,219 were biallelic within line but multiallelic when all lines were considered. The remaining variants were imputed for all pedigreed individuals, but 20,645,588 of them were fixed for the reference allele in the imputed individuals that passed our accuracy quality control. This affected mostly variants that had been called in only one line and indicated that the alternative allele segregated at very low frequency. Although this could also be caused by false positives in variant calling, this hypothesis seems unlikely to be the main cause as for more than 99% of these variants we read the alternative allele in at least two individuals. Additionally, we previously confirmed with high-coverage data 96.9% of the variants that were called using low-coverage data [35].
Distribution of variants and relationship with recombination rate

The number of variants by chromosome was strongly correlated with chromosome length ($r=0.98$, $P<0.05$; Table S1). Due to the recombination landscape of each pig chromosome [49], the average variant density by chromosome was negatively correlated with chromosome length ($r=-0.87$, $P<0.05$; Table S1). The distribution of variants within chromosomes was positively correlated to recombination rate ($r=0.65$, $P<0.05$, between variant density and recombination rate in 1-Mb non-overlapping windows [49]; Figure 1a). For example, within line A, there was on average one variant every 81 bp, but in the 5% 1-Mb windows with the lowest and highest recombination rates there was on average one variant every 152 and 54 bp, respectively (2.8-fold more variants in windows with high recombination rate).

Across all lines, there was one variant every 49 bp on average, but in the 5% 1-Mb windows with the lowest and highest recombination rates there was on average one variant every 79 and 34 bp, respectively (2.3-fold more variants in windows with high recombination rate).

The distribution of private and widespread variants along the genome also differed. The distribution of widespread variants was more correlated with recombination rate than that of private variants (Figures 1b and 1c). As a consequence, private variants represented a larger proportion of the variation in regions with low recombination rate, which were depleted of widespread variants. Across all lines, in the 5% 1-Mb windows with the highest recombination rates there was on average one private variant every 167 bp and one widespread every 148 bp (1.1-fold more private variants relative to widespread). In the 5% 1-Mb windows with the lowest recombination rates there was on average one private variant every 260 bp and one widespread every 531 bp (2.0-fold more private variants relative to widespread).
There were no genomic regions that were enriched for private variants across lines (Figure S1).

**Frequency and fixation index**

The prevalence level and alternative allele frequency were related, in a way that less prevalent variants had also lower allele frequency (Figure 2) and lower $F_{ST}$ (Figure 3). Private variants had an average alternative allele frequency of 0.03 (SD 0.09) as opposed to widespread variants, which had an average alternative allele frequency of 0.50 (SD 0.25). As a consequence of the less prevalent variants generally having low frequencies in the lines where they segregated, these variants showed a small degree of differentiation between the lines in which they segregated ($F_{ST}$=0.04, SD=0.07). In contrast, the widespread variants allowed for the largest degree of differentiation between lines ($F_{ST}$=0.21, SD=0.11).

**Prevalence and frequency of putatively functional variants**

The predicted consequence types of the variants are shown in Table 3. Half (50.1%) of the variants were called in intergenic regions and another 47.0% of the variants were called in intronic regions. Only 2.0% of the variants were called in the 5’ and 3’ untranslated regions. The coding variants comprised 0.9% of the total variants, of which more than half were missense (45.5%), frameshift indels (3.1%) or LOF (3.7%). The variant density of putatively functional variants was only weakly correlated to recombination rate (Figures 1d).

The low-prevalence variants (i.e., the variants that were identified in one or few lines) were enriched with missense and LOF variants, as well as potentially regulatory variants such as those located in 5’ and 3’ untranslated regions and other
intronic variants. On the other hand, the high-prevalence variants (i.e., the variants that were identified in many or all lines) were enriched with frameshift indels, synonymous (non-significant correlation) and intergenic variants. Frameshift indels are typically included in the LOF category. However, our results show that the LOF category is very heterogeneous and the frameshift indels presented opposite patterns to other LOF variants. Therefore, we studied them as a separate category.

Whereas the LOF variants had lower allele frequency than the intergenic variants in low-prevalence levels, they had similar allele frequencies in high-prevalence levels (Table 4). Thus, there was a set of LOF variants that were prevalent across lines and also had particularly high frequencies within lines. The missense variants, especially those classified as deleterious, had lower allele frequencies than the intergenic variants for all prevalence levels. The low-prevalence missense variants were enriched with a larger fraction of deleterious variants and lower SIFT scores (Figure 4). As opposed to LOF and missense variants, the frameshift indels had intermediate allele frequencies that were much higher than those of the intergenic variants, which indicated that in many cases the minor allele was the reference one. Within prevalence level, the LOF and deleterious missense variants had lower $F_{ST}$ than the intergenic variants (Table 5), probably because they were kept at low allele frequencies due to negative selection pressure. The frameshift indels also had lower $F_{ST}$ despite their intermediate allele frequencies.

**Load of putatively functional alleles by prevalence level**

Most of the missense deleterious and LOF variants that an individual carried in homozygosis for the alternative allele were high-prevalence variants. Only a small proportion of these variants were private. An average individual carried 1,048 (SD 57)
LOF variants in homozygosis for the alternative allele, of which 713 (SD 36) were widespread across all nine lines and only 20 (SD 7) were private. An average individual carried 1,379 (SD 165) deleterious missense variants in homozygosis for the alternative allele, of which 1,012 (SD 79) were widespread and only 4 (SD 3) were private. An average individual carried 1,080 (SD 89) LOF and 2,632 (SD 235) deleterious missense variants in heterozygosis.

We found signals of negative selection against deleterious missense variants, in particular the private ones. Individuals proportionally carried less deleterious missense variants in homozygosis for the alternative allele than variants of other predicted consequence types, regardless of prevalence level (Figure 5). Individuals also carried a proportionally lower number of private tolerated missense, synonymous and LOF variants in homozygosis for the alternative allele than expected, but not in heterozygosis.

Association of low-prevalence variants to production traits

Significant variants were enriched with putatively functional and regulatory variants of different prevalence levels, and depleted of intergenic variants. A total of 108,109 variants were significantly associated to at least one trait in one line. Figures 6a and 6b summarise the enrichment scores for all significant variants. The predicted consequence types that reached the greatest enrichment scores were LOF, frameshift indels and unclassified missense variants, with various prevalence levels. Variants with intermediate prevalence levels were amongst the most enriched. These trends were accentuated after selecting candidate variants from haplotype blocks. In each line we defined from 1,554 to 2,118 haplotype blocks. A total of 6,692 candidate variants remained after accounting for linkage disequilibrium within each haplotype
block for all lines and traits. Figures 6c and 6d summarise the enrichment scores for
the candidate variants. The enrichment scores based on the candidate variants
revealed a stronger depletion of intergenic variants, as well as intronic (with the
exception of high-prevalence), and a much stronger enrichment for LOF, frameshift
indels and missense variants. For putatively functional variants, there were no clear
trends of their enrichment scores across prevalence levels.

In general, the lower allele frequency of low-prevalence variants hindered the
detection of significant associations for these markers. Candidate variants with low
prevalence that were detected as significantly associated to the production traits
actually had intermediate allele frequencies that were greater than expected for their
prevalence level. Another consequence of this was that low-prevalence variants in
general explained low percentages of variance (Figure 7), although there were some
instances of low-prevalence variants that explained up to 3.2% of phenotypic
variance. Significant variants had higher $F_{ST}$ than other variants of the same predicted
consequence type and prevalence level (Figure 8). This enrichment was especially
strong for low-prevalence variants, which in some instances reached $F_{ST}$ estimates
around 0.15.

**Discussion**

Our results contextualize the importance of population-specific and low-
prevalence genetic variants. Next, we will discuss: (1) the distribution and functional
annotation of low-prevalence variants, (2) the load of putatively functional alleles by
prevalence level, and (3) the association of low-prevalence variants to production
traits.
Distribution and functional annotation of low-prevalence variants

The main difficulty for the study of low-prevalence genetic variants is that the prevalence of a variant across several lines is strongly related to its allele frequency, in a way that the low-prevalence variants are also rare within the lines where they occur. This is possibly because low-prevalence variants are relatively recent and are constrained by negative selection.

On one hand, the distribution of private variants was only weakly correlated to recombination rate and, therefore, regions with low recombination rate were enriched for private variants. Although the interplay between recurring sweeps, background selection and other phenomena at play is not fully understood yet, it is generally accepted that selection on linked variants leads to loss of variation in regions with low recombination rates [50]. Our observation that regions with low recombination rate were enriched for private variants suggests that private variants may have been less affected by selective sweeps than widespread variants. This would be consistent with previous observations of the younger age of rare and low-prevalence variants [51], and suggests that many private variants arose more recently in time than widespread variants, likely after line differentiation, and accumulated in low-recombining regions due to the reduced efficacy of purifying selection in those regions [52,53].

On the other hand, the low-prevalence variants were enriched for putatively functional variants. Variants that affect performance traits or that cause a detrimental condition are under the action of directional selection and are therefore driven towards low allele frequencies or fixation [54,55]. The low \( F_{ST} \) estimates for the low-prevalence variants indicated that selection pressure keeps these variants at low minor allele frequency even when they occur in several lines, especially if they are putatively functional [56]. This could be caused by natural selection or similar
selection objectives across livestock populations. These observations were also consistent with previous reports showing that most putatively functional variants were private to single cattle breeds [24], that putatively functional variants were less likely to have high frequency of the alternative allele across multiple chicken lines [26], and that population-specific variants in non-African humans were enriched with putatively functional variants [57].

The relationship between variant prevalence across lines and allele frequency highlighted the suitability of using a low-coverage sequencing approach to study this fraction of genetic variation. Nonetheless, bioinformatics pipelines for calling, genotyping and even imputing such variants should account for the increased uncertainty associated to their low allele frequency. We decided on using a very relaxed variant calling strategy with little filtering to account for as many rare variants as possible, but a sizeable fraction of these rare variants were discarded after imputation because they were fixed for the imputed individuals that passed quality control. Low-coverage sequencing is also unsuitable for other types of genetic variants, such as structural variations (CNVs, tandem duplications and inversions), which could also be putatively functional and population-specific [58]. Of course, the number of called variants and the proportion that were private or widespread depends of the number of sequenced lines [23,26] as well as the sequencing effort in each line.

Our results also suggest that what is typically grouped as LOF is actually a heterogeneous category. In particular, frameshift indels showed patterns that did not conform to the other predicted consequence types.
Load of putatively functional alleles by prevalence level

We found that an average individual carried a larger number of LOF and missense deleterious than previously reported in other livestock species or in humans. However, there is not yet a clear consensus on the number of LOF and deleterious missense alleles that are present in the genome of an average individual. In humans, it has been estimated that an average individual carries 100-150 LOF alleles [54,59–61] and around 800 weakly deleterious mutations [62], most of which are rare. In domestic livestock populations, the number of LOF and deleterious alleles carried on average by individuals has been reported to be greater than in wild populations [63], including estimates of 100 to 300 deleterious variants in domestic pigs [64], over 400 deleterious variants in domestic chicken [64], and 1,200-1,500 deleterious variants in domestic yak [65]. Similar magnitudes have been reported in dogs [66], rice [67], and sunflower [53].

It has been debated why healthy individuals carry a larger number of LOF variants in homozygosis than expected [68,69]. These could be driven by the fact that not all predicted LOF variants are detrimental and their functional impact should be validated before being considered as such. Many predicted LOF variants are in fact neutral, advantageous, or even may arise simply because of sequencing and annotation errors [68]. This claim is supported by the large proportion of LOF observed in homozygosis for the alternative allele compared to the other consequence types, which casts doubt on the real impact of those variants. On the contrary, individuals carried a lower proportion of alleles predicted to be deleterious missense in homozygous state, which supports that variants predicted as such may have a real impact on genetic variation of production traits and, therefore, be subjected to selection pressure.
These observations have implications for the identification of variants to be used for genomic prediction or subjected to genomic edition strategies such as PAGE [17] or RAGE [18]. Efforts to promote or remove alleles should target variants that make a substantial contribution to traits of interest, namely functional variants. However, it is hard to computationally predict and statistically estimate the effects of such variants, especially if they have low allele frequency. The number of LOF variants in homozygosis for the alternative allele suggests that predicted loss of function is not a good indicator that a variant is strongly deleterious in the context of animal breeding. Similarly, bioinformatics predictors of missense variant effects appear to be not very accurate [70,71]. High-throughput fine-mapping and variant screening would be needed to ascertain variant causality and disentangle causality from linkage disequilibrium.

**Association of low-prevalence variants to production traits**

Genome-wide association studies on three polygenic traits of economical importance in the three largest lines revealed that the significant markers were enriched for putatively functional variants, such as LOF, frameshift indels and missense variants, and depleted of intergenic variants. This pattern of enrichment was similar to previous reports from human datasets [72]. However, only a few of the population-specific and low-prevalence variants were significantly associated to the traits, even after accounting for linkage disequilibrium. Most of the significant variants showed intermediate or high prevalence levels. These observations are consistent with previous meta-analyses in cattle that showed that significant variants are often common variants [73]. This could be explained by either the fact that real quantitative trait nucleotides have intermediate or high allele frequencies or the fact...
that most studies are underpowered to map rare causal variants. The latter scenario still seems more likely given that the significant private and low-prevalence variants had intermediate allele frequencies. This also supports that these significant variants have biological functions that contributed to trait phenotypic variance rather than they reached intermediate allele frequencies by drift or by hitchhiking with linked variants under selection [74]. However, these amounted to a small number of variants that explained small fractions of variance. Other more widespread variants, including intergenic variants, successfully acted as tag variants for them and captured much larger fractions of trait variance. This makes them more suitable for applications in animal breeding, as is already the case with marker arrays. A similar result was found in cattle, where splice site and synonymous variants explained the largest proportions of trait variance, while missense variants explained almost null variance [75].

It can be hypothesized that some of the low-prevalence variants with low allele frequency have non-negligible marker effects for traits of interest. Despite the large amount of individuals included in this study, the large volume of variants and the pervasiveness of linkage disequilibrium among them still make it very challenging to disentangle their contribution to trait variance. While genome-wide association studies involving more than one breed typically find multiple breed-specific associations (e.g., [76]), based on our results it seems unlikely that breed-specific associations arise from the low-prevalence variants. They would instead stem from differences in allele frequency, linkage disequilibrium structure or genetic background that affect the power to detect the effect of prevalent variants across different populations. Significant variants were enriched with higher $F_{ST}$ estimates than non-significant variants, which is also consistent with previous reports [73]. Although the enrichment was greater for low-prevalence variants, it remains unclear to which
degree these variants could relate to selection history or explain differences among
lines for the studied traits.

Conclusion

Low-prevalence variants are enriched for putatively functional variants,
including LOF and deleterious missense variants. However, most low-prevalence
variants are kept at very low allele frequency and are therefore unlikely to hinder
across-breed analyses. This is particularly relevant when predicting genomic breeding
values using reference populations of a different genetic background. Only a small
subset of low-prevalence variants was found at intermediate allele frequencies and
had large estimated effects on production traits.

Ethics approval and consent to participate

The samples used in this study were derived from the routine breeding activities of
PIC.

Consent for publication

Not applicable.

Availability of data and material

The software packages AlphaSeqOpt, AlphaPhase, AlphaImpute and AlphaPeel are
available from the AlphaGenes website (http://www.alphagenes.roslin.ed.ac.uk). The
datasets generated and analysed in this study are derived from the PIC breeding
programme and not publicly available.
Competing interests

The authors declare that they have no competing interests. BDV, CYC, and WOH are employees of Genus PIC.

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Authors' contributions

RRF, MJ and JMH designed the study; RRF, MJ and GG performed the analyses; RRF and MJ wrote the first draft; BDV, CYC, WHO, GG and JMH contributed to the interpretation of the results and provided comments on the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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References


QTL discovery and genomic prediction of complex traits. BMC Genomics. 2016;17:144.


Koufariotis LT, Chen Y-PP, Stothard P, Hayes BJ. Variance explained by whole genome sequence variants in coding and regulatory genome annotations for six dairy traits. BMC Genomics. 2018;19.

**Figures**

**Figure 1.** Variant density in line A (black and grey bars) and recombination rate (red line). The correlation (r) between variant density and recombination rate in 1-Mb non-overlapping windows is reported.
Figure 2. Frequency of the alternative allele by prevalence level. Red dots indicate means. In blue, values greater than 1.5 times the interquartile range.
Figure 3. Wright’s fixation statistic ($F_{ST}$) by prevalence level. Red dots indicate means. In blue, values greater than 1.5 times the interquartile range.
Figure 4. Classification of the missense variants and median SIFT score by prevalence level.
Figure 5. Percentage of variants in homozygosis for the alternative allele or in heterozygosis in an average individual by predicted consequence type, including frameshift indels, and prevalence level. LOF: loss-of-function; UTR: untranslated regions.
Figure 6. Enrichment scores for the significant variants in the genome-wide association study by variant prevalence level and predicted consequence type. Either all significant variants (panels a and b) or only the candidate variants after accounting for linkage disequilibrium (panels c and d) were used. Prevalence level was considered across all 9 lines (panels a and c) or only across the 3 lines included in the genome-wide association study (panels b and d).
Figure 7. Maximum percentage of phenotypic variance explained by the individual candidate variants within each prevalence level and predicted consequence type. Only the candidate variants after accounting for linkage disequilibrium were used. Prevalence level was considered across all 9 lines (panel a) or only across the 3 lines included in the genome-wide association study (panel b).
**Figure 8.** Enrichment scores for the F\textsubscript{ST} median of the candidate variants within each prevalence level and predicted consequence type. Only the candidate variants after accounting for linkage disequilibrium were used. Prevalence level was considered across all 9 lines.
## Tables

### Table 1. Number of sequenced and analysed pigs.

<table>
<thead>
<tr>
<th>Line</th>
<th>Individuals sequenced</th>
<th>Individuals sequenced by coverage</th>
<th>Individuals used in analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1x</td>
<td>2x</td>
</tr>
<tr>
<td>A</td>
<td>1,856</td>
<td>1,044</td>
<td>649</td>
</tr>
<tr>
<td>B</td>
<td>1,491</td>
<td>628</td>
<td>728</td>
</tr>
<tr>
<td>C</td>
<td>1,366</td>
<td>685</td>
<td>545</td>
</tr>
<tr>
<td>D</td>
<td>760</td>
<td>394</td>
<td>274</td>
</tr>
<tr>
<td>E</td>
<td>731</td>
<td>362</td>
<td>311</td>
</tr>
<tr>
<td>F</td>
<td>701</td>
<td>351</td>
<td>255</td>
</tr>
<tr>
<td>G</td>
<td>445</td>
<td>217</td>
<td>176</td>
</tr>
<tr>
<td>H</td>
<td>381</td>
<td>193</td>
<td>137</td>
</tr>
<tr>
<td>I</td>
<td>321</td>
<td>111</td>
<td>158</td>
</tr>
</tbody>
</table>
## Table 2. Number of variants by line.

<table>
<thead>
<tr>
<th>Line</th>
<th>Biallelic variant sites (M)</th>
<th>SNPs All biallelic (M)</th>
<th>Private (M)</th>
<th>Widespread (M)</th>
<th>Indels All biallelic (M)</th>
<th>Private (M)</th>
<th>Widespread (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28.83</td>
<td>24.38</td>
<td>1.56</td>
<td>8.38</td>
<td>4.44</td>
<td>0.39</td>
<td>1.56</td>
</tr>
<tr>
<td>B</td>
<td>28.57</td>
<td>24.32</td>
<td>2.74</td>
<td>8.38</td>
<td>4.24</td>
<td>0.51</td>
<td>1.56</td>
</tr>
<tr>
<td>C</td>
<td>28.88</td>
<td>24.60</td>
<td>2.51</td>
<td>8.38</td>
<td>4.28</td>
<td>0.44</td>
<td>1.56</td>
</tr>
<tr>
<td>D</td>
<td>21.44</td>
<td>17.94</td>
<td>1.23</td>
<td>8.38</td>
<td>3.50</td>
<td>0.32</td>
<td>1.56</td>
</tr>
<tr>
<td>E</td>
<td>19.06</td>
<td>15.71</td>
<td>0.51</td>
<td>8.38</td>
<td>3.35</td>
<td>0.22</td>
<td>1.56</td>
</tr>
<tr>
<td>F</td>
<td>20.21</td>
<td>16.86</td>
<td>0.42</td>
<td>8.38</td>
<td>3.35</td>
<td>0.16</td>
<td>1.56</td>
</tr>
<tr>
<td>G</td>
<td>23.38</td>
<td>19.64</td>
<td>0.50</td>
<td>8.38</td>
<td>3.74</td>
<td>0.16</td>
<td>1.56</td>
</tr>
<tr>
<td>H</td>
<td>22.32</td>
<td>18.78</td>
<td>0.37</td>
<td>8.38</td>
<td>3.55</td>
<td>0.12</td>
<td>1.56</td>
</tr>
<tr>
<td>I</td>
<td>24.59</td>
<td>20.82</td>
<td>0.76</td>
<td>8.38</td>
<td>3.77</td>
<td>0.13</td>
<td>1.56</td>
</tr>
<tr>
<td>Total</td>
<td>46.30</td>
<td>38.64</td>
<td>10.60</td>
<td>8.38</td>
<td>7.70</td>
<td>2.44</td>
<td>1.56</td>
</tr>
</tbody>
</table>
Table 3. Predicted consequence types of the variants by prevalence level. The most severe consequence of each variant was used. The main Sequence Ontology (SO) terms are shown in order of severity (more severe to less severe) as estimated by Ensembl Variant Effect Predictor. The correlation (r) between the percentage of variants of each consequence type and prevalence is reported. In bold, categories that will be analysed in the next sections.

<table>
<thead>
<tr>
<th>Consequence type</th>
<th>Percentage of variants (%) by prevalence level</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Loss-of-function¹</td>
<td>0.061</td>
<td>0.035</td>
</tr>
<tr>
<td>Splice acceptor/donor</td>
<td>0.038</td>
<td>0.023</td>
</tr>
<tr>
<td>Stop-gain</td>
<td>0.015</td>
<td>0.009</td>
</tr>
<tr>
<td>Stop-loss</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>Start-loss</td>
<td>0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>Frameshift indel</td>
<td>0.014</td>
<td>0.017</td>
</tr>
<tr>
<td>In-frame indel</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>Missense</td>
<td>0.557</td>
<td>0.38</td>
</tr>
<tr>
<td>Deleterious</td>
<td>0.201</td>
<td>0.092</td>
</tr>
<tr>
<td>Tolerated</td>
<td>0.223</td>
<td>0.171</td>
</tr>
<tr>
<td>Splice region</td>
<td>0.105</td>
<td>0.098</td>
</tr>
<tr>
<td>Synonymous</td>
<td>0.240</td>
<td>0.313</td>
</tr>
<tr>
<td>Untranslated regions</td>
<td>2.068</td>
<td>2.034</td>
</tr>
<tr>
<td>5’ UTR</td>
<td>0.633</td>
<td>0.594</td>
</tr>
<tr>
<td>3’ UTR</td>
<td>1.435</td>
<td>1.440</td>
</tr>
<tr>
<td>Non-coding transcript exon</td>
<td>0.107</td>
<td>0.116</td>
</tr>
<tr>
<td>Intronic</td>
<td>47.743</td>
<td>47.570</td>
</tr>
<tr>
<td>Downstream gene</td>
<td>2.660</td>
<td>2.679</td>
</tr>
<tr>
<td>Intergenic</td>
<td>43.147</td>
<td>43.467</td>
</tr>
</tbody>
</table>

¹If frameshift indels were included in this category: r = -.06 (P>.05)

Significant correlation (P<.05)
Table 4. Frequency of the alternative allele by predicted consequence type and prevalence level. Values are medians.

<table>
<thead>
<tr>
<th>Consequence type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss-of-function</td>
<td>.0010</td>
<td>.017</td>
<td>.048</td>
<td>.061</td>
<td>.089</td>
<td>.114</td>
<td>.151</td>
<td>.225</td>
<td>.489</td>
<td>.020</td>
</tr>
<tr>
<td>Frameshift indel</td>
<td>.4847</td>
<td>.758</td>
<td>.757</td>
<td>.420</td>
<td>.304</td>
<td>.260</td>
<td>.339</td>
<td>.456</td>
<td>.693</td>
<td>.634</td>
</tr>
<tr>
<td>Deleterious missense</td>
<td>.0006</td>
<td>.018</td>
<td>.043</td>
<td>.061</td>
<td>.078</td>
<td>.092</td>
<td>.125</td>
<td>.170</td>
<td>.350</td>
<td>.010</td>
</tr>
<tr>
<td>Tolerated missense</td>
<td>.0011</td>
<td>.027</td>
<td>.047</td>
<td>.066</td>
<td>.083</td>
<td>.106</td>
<td>.143</td>
<td>.202</td>
<td>.443</td>
<td>.074</td>
</tr>
<tr>
<td>Synonymous</td>
<td>.0037</td>
<td>.032</td>
<td>.049</td>
<td>.066</td>
<td>.086</td>
<td>.107</td>
<td>.151</td>
<td>.205</td>
<td>.447</td>
<td>.110</td>
</tr>
<tr>
<td>Untranslated regions</td>
<td>.0018</td>
<td>.034</td>
<td>.059</td>
<td>.077</td>
<td>.099</td>
<td>.122</td>
<td>.167</td>
<td>.226</td>
<td>.476</td>
<td>.102</td>
</tr>
<tr>
<td>Intronic</td>
<td>.0015</td>
<td>.035</td>
<td>.059</td>
<td>.080</td>
<td>.102</td>
<td>.126</td>
<td>.171</td>
<td>.235</td>
<td>.485</td>
<td>.110</td>
</tr>
<tr>
<td>Intergenic</td>
<td>.0015</td>
<td>.033</td>
<td>.058</td>
<td>.080</td>
<td>.105</td>
<td>.129</td>
<td>.173</td>
<td>.237</td>
<td>.483</td>
<td>.116</td>
</tr>
</tbody>
</table>
Table 5. Wright’s fixation statistic (FST) by predicted consequence type and prevalence level. Values are medians.

<table>
<thead>
<tr>
<th>Consequence type</th>
<th>FST by prevalence level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Loss-of-function</td>
<td>.003</td>
</tr>
<tr>
<td>Frameshift indel</td>
<td>.010</td>
</tr>
<tr>
<td>Deleterious missense</td>
<td>.005</td>
</tr>
<tr>
<td>Tolerated missense</td>
<td>.009</td>
</tr>
<tr>
<td>Synonymous</td>
<td>.013</td>
</tr>
<tr>
<td>Untranslated regions</td>
<td>.009</td>
</tr>
<tr>
<td>Intronic</td>
<td>.009</td>
</tr>
<tr>
<td>Intergenic</td>
<td>.009</td>
</tr>
</tbody>
</table>
### Table S1. Number of analysed variants by chromosome.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Length (Mb)</th>
<th>SNPs (M)</th>
<th>Indels (M)</th>
<th>Variant density (thousands/Mb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>274.3</td>
<td>3.77</td>
<td>0.76</td>
<td>16.5</td>
</tr>
<tr>
<td>2</td>
<td>151.9</td>
<td>2.60</td>
<td>0.52</td>
<td>20.5</td>
</tr>
<tr>
<td>3</td>
<td>132.8</td>
<td>2.35</td>
<td>0.44</td>
<td>21.0</td>
</tr>
<tr>
<td>4</td>
<td>130.9</td>
<td>2.21</td>
<td>0.43</td>
<td>20.2</td>
</tr>
<tr>
<td>5</td>
<td>104.5</td>
<td>1.95</td>
<td>0.39</td>
<td>22.4</td>
</tr>
<tr>
<td>6</td>
<td>170.8</td>
<td>2.80</td>
<td>0.55</td>
<td>19.6</td>
</tr>
<tr>
<td>7</td>
<td>121.8</td>
<td>2.20</td>
<td>0.43</td>
<td>21.6</td>
</tr>
<tr>
<td>8</td>
<td>139.0</td>
<td>2.37</td>
<td>0.50</td>
<td>20.6</td>
</tr>
<tr>
<td>9</td>
<td>139.5</td>
<td>2.47</td>
<td>0.48</td>
<td>21.1</td>
</tr>
<tr>
<td>10</td>
<td>69.4</td>
<td>1.60</td>
<td>0.31</td>
<td>27.5</td>
</tr>
<tr>
<td>11</td>
<td>79.2</td>
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<td>0.31</td>
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</tr>
<tr>
<td>12</td>
<td>61.6</td>
<td>1.35</td>
<td>0.25</td>
<td>26.0</td>
</tr>
<tr>
<td>13</td>
<td>208.3</td>
<td>2.97</td>
<td>0.64</td>
<td>17.3</td>
</tr>
<tr>
<td>14</td>
<td>141.8</td>
<td>2.38</td>
<td>0.48</td>
<td>20.2</td>
</tr>
<tr>
<td>15</td>
<td>140.4</td>
<td>2.20</td>
<td>0.46</td>
<td>18.9</td>
</tr>
<tr>
<td>16</td>
<td>79.9</td>
<td>1.50</td>
<td>0.30</td>
<td>22.5</td>
</tr>
<tr>
<td>17</td>
<td>63.5</td>
<td>1.32</td>
<td>0.25</td>
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<td>18</td>
<td>56.0</td>
<td>1.04</td>
<td>0.19</td>
<td>22.0</td>
</tr>
<tr>
<td>Total</td>
<td>2,501.9</td>
<td>38.64</td>
<td>7.70</td>
<td>18.5</td>
</tr>
</tbody>
</table>
**Figure S1.** Variant density for the private variants in each line.