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Short title: CNV in European pig breeds

Summary

In this study we identified copy number variants (CNVs) in 19 European autochthonous pig breeds and in two commercial breeds (Italian Large White and Italian Duroc) that represent important genetic resources for this species. The genome of 725 pigs was sequenced using a breed specific DNA pooling approach (30-35 animals per pool) obtaining an average depth per pool of 42×. This approach maximized CNV discovery as well as the related copy number states characterizing, on average, the analysed breeds. By mining more than 17.5 billion reads, we identified a total of 9592 CNVs (~683 CNVs per breed) and 3710 CNV regions (CNVRs; 1.15% of the reference pig genome), with an average of 77 CNVRs per breed that was considered as private. A few CNVRs were analysed in more details, together with other information derived from sequencing data. For example, the CNVR encompassing the *KIT* gene was associated with coat colour phenotypes in the analysed breeds, confirming the role of the multiple copies in determining breed specific coat colours. The CNVR covering the *MSRB3* gene was associated with ear size in most breeds. The CNVRs affecting the *ELOV6* and *ZNF622* genes were private feature observed in the Lithuanian Indigenous Wattle and in the Turopolje pig breeds, respectively. Overall, genome variability here unravelled can explain part of the genetic diversity among breeds and might contribute to explain their origin, history and adaptation to a variety of production systems.

Keywords: CNV; *ELOV6*; Genetic resource; *KIT*; *MSRB3*; Next generation sequencing; *Sus scrofa*; *ZNF622*.

Introduction

Livestock genomes have been shaped by natural and artificial selection, leading to the accumulation of a broad range of phenotypic and genetic variability that have largely contributed to differentiate populations and constitute modern breeds. As a result, livestock populations and breeds represent a reservoir of genetic diversity, harbouring genetic variants that span from single nucleotide polymorphisms (SNPs) to more complex structural variants, some of which with small to large phenotypic effects on a variety of exterior and economically relevant traits (Andersen *et al*. 2011). Copy number variants (CNVs) are a type of structural variants in the form of large DNA segments, usually more than 1kb of length, which are present in a variable copy number within a species as compared to its reference genome (Feuk *et al*. 2006).

CNVs represent an important source of genetic variability, by influencing phenotypes through a variety of molecular mechanisms such as gene dosage effect, disruption or alteration of coding and regulatory regions among several other modifications (Redon *et al*. 2006, Zhang *et al*. 2006, Bickhart & Liu 2014). Detection of CNVs is technically challenging when applied on genome-wide scale and different technologies have been applied to this aim. Among them, the most commonly used are array comparative genome hybridization (aCGH), high density SNP chip high-throughput sequencing (HTS) platforms (Winchester *et al*. 2009; Alkan *et al*. 2011; Pirooznia *et al*. 2015; Pollard *et al*. 2018). However, due to the decreased cost of HTS analyses and the advantage that this approach has to obtain more precise information on CNVs, whole genome resequencing is becoming a standard approach to discover and characterize CNVs in complex genomes.

Genetic diversity described by CNVs and CNV regions (CNVRs; i.e. CNVs present in different individuals in the same or overlapping genome regions) has been extensively studied in livestock, including, for example, cattle (Fadista *et al*. 2010; Bickhart *et al*. 2012), sheep (Fontanesi *et al*. 2011; Yang *et al*. 2018), goats (Fontanesi *et al*. 2010b; Liu *et al*. 2019a), rabbits (Fontanesi *et al*. 2012) and chickens (Yi *et al*. 2014), among other species. Several studies investigating CNVs and CNVRs have been also reported in pigs, including also an interspecies survey within the genus *Sus* (Paudel *et al*.

2015). Studies have been focused on the main commercial European breeds (i.e. Duroc, Landrace, Large White, Hampshire, Yorkshire, Piétrain) (e.g. Fadista *et al*. 2008; Li *et al*. 2012; Chen *et al*. 2012; Fowler *et al*. 2013; Wang *et al*. 2014, 2015a, c, 2019b; Jiang *et al*. 2014; Wiedmann *et al*. 2015; Revay *et al*. 2015; Long *et al*. 2016; Revilla *et al*. 2017; Stafuzza *et al*. 2019) and Asian breeds (Meishan, Erhualian) (Wang *et al*. 2012, 2014, 2015b, c; Li *et al*. 2012; Chen *et al*. 2012; Jiang *et al*. 2014). Other studies screened commercial pig populations in the attempt to capture part of the missing heritability (expected to be explained by CNVs) on economically important traits, including number of piglets born alive (Stafuzza *et al*. 2019), fertility (Revay *et al*. 2015), meat quality traits (Wang *et al*. 2015c), fatty acid composition and growth traits (Revilla *et al*. 2017), fat deposition (Fowler *et al*. 2013; Schiavo *et al*. 2014), among other traits.

Although the modern pig industry relies on few commercial pig breeds, autochthonous pig populations subsist in many different regions, mainly associated with local and traditional niche markets (Čandek-Potokar and Nieto 2019). These breeds represent genetic resources adapted to local agro-climatic and environmental conditions. Up to date, the genome architecture of CNVs has been studied mainly in Asian autochthonous populations/breeds (Li *et al*. 2012; Wang *et al*. 2014, 2015b, 2019a; Jiang *et al*. 2014; Dong *et al*. 2015; Xie *et al*. 2016). European autochthonous pig breeds have been mainly investigated by exploring their genetic variability using SNP data (e.g. Ovilo *et al*. 2002; Tomás *et al*. 2011; Wilkinson *et al*. 2013; Silió *et al*. 2016; Yang *et al*. 2017; Muñoz *et al*. 2018, 2019; Schiavo *et al*. 2018, 2019, 2020a, b; Ribani *et al*. 2019). A few studies, using SNP arrays, analysed CNVs in European autochthonous pig breeds (e.g. Iberian, Swallow-Bellied Mangalitsa) (Ramayo-Caldas *et al*. 2010; Fernández *et al*. 2014; Molnár *et al*. 2014).

Results of CNV studies in pigs showed a limited degree of agreement in terms of CNVRs number and size ranges. Even if part of these discrepancies may be attributed to breed-specific genome features, the remaining discrepancies may derive from the different technologies and algorithms used to unravel CNVs, which mainly used aCGH and SNP arrays. Few other studies analysed CNV and CNVRs in the pig genome using HTS platforms (e.g. Rubin *et al*. 2012; Jiang *et* *al*. 2014; Paudel *et al*. 2015; Wang *et al*. 2015c, 2019b; Long *et al*. 2016; Revilla *et al*. 2017; Keel *et al*. 2019).

In this study, we provide a detailed survey of CNVs and CNVRs in the pig genome by whole genome resequencing of DNA pools constituted from 21 European pig breeds: 19 autochthonous breeds and belonging to nine different countries and two Italian commercial breeds. These breeds, some of them untapped, stem from different production systems and breeding programmes in Europe. Therefore, dissection of their genome architecture at the level of CNVs could provide new insights into their histories, origin, potential selection signatures and adaptation to different local agro-climatic and environmental conditions.

Materials and methods

Animals

Blood samples were collected from a total of 30 or 35 animals from each of the 21 pig breeds included in the study, distributed in nine European countries (from West to East and then North; Fig. 1): Portugal (Alentejana and Bísara); Spain (Majorcan Black); France (Basque and Gascon); Italy (autochthonous: Apulo-Calabrese, Casertana, Cinta Senese, Mora Romagnola, Nero Siciliano and Sarda; and commercial breeds: Italian Large White and Italian Duroc); Slovenia (Krškopolje pig, hereafter indicated as Krškopolje); Croatia (Black Slavonian and Turopolje); Serbia (Moravka and Swallow-Bellied Mangalitsa); Germany (Schwäbisch-Hällisches Schwein); and Lithuania (Lithuanian indigenous wattle and Lithuanian White old type). Selection of individuals for sampling was performed by avoiding highly related animals (no full- or half-sibs), balancing between sexes, and prioritizing adult individuals or at least animals with adult morphology. All animals were registered to their respective Herd Books and presented standard breed characteristics. Details on the analysed animals and investigated breeds, including geographical distribution and phenotypic description, are reported in Table S1.

DNA samples and sequencing

Genomic DNA was extracted from 8–15 mL of peripheral blood for each pig, collected in Vacutainer tubes containing 10% 0.5 M EDTA (ethylenediaminetetraacetic acid, disodium dihydrate salt) at pH 8.0. The extraction was performed using either a standardized phenol-chloroform (Sambrook *et al*. 1989) or the NucleoSpin® Tissue commercial kit (Macherey-Nagel, Düren, Germany). A total of 21 DNA pools were constructed, including in each pool 30 or 35 individual DNA samples pooled at equimolar concentration (Table S2).

A sequencing library was generated for each DNA pool by using the Truseq® Nano DNA HT Sample preparation Kit (Illumina, CA, USA), following the manufacturer's recommendations. Briefly, DNA was randomly sheared to obtain 350 bp fragments which were end polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing with further PCR amplification. PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent 2100 Bioanalyzer and quantified using real-time PCR. The qualified libraries were then fed into an Illumina Hi-Seq sequencer for paired-end sequencing, obtaining 150 bp length reads.

Quality controls and sequence alignment

Obtained reads underwent several cleaning and filtering steps including removal of (i) adapters, (ii) reads containing more than 10% unknown bases (N) and (iii) reads containing low quality bases ($Q \leq 5$) over 50% of the total sequenced bases. FASTQ files were sub-sequentially inspected with FASTQC v.0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) that highlighted very high-quality reads.

Reads were mapped on the latest version of the *Sus scrofa* reference genome (Sscrofa11.1) with BWA tool 0.7.17 (Li & Durbin, 2009) (function: MEM) and the parameters for paired-end data. Picard v.2.1.1 (https://broadinstitute.github.io/picard/) was used to remove duplicated reads. Whole genome sequencing data statistics are reported in Table S2.

Detection of CNVs and CNVRs from sequencing data

The cn.Mops v.1.32 tool (Klambauer *et al*. 2012) was used to identify autosomal CNVs. cn.Mops was run with default parameters except for the window size that was lowered to 750 bp. Since three consecutive genome windows positive for copy number are required by cn.Mops to assert the presence of a CNV, the minimum size of a detected CNV was 2250 bp. The 750 bp window size allowed us to detect short CNVs (CNV ≥ 3 kbp with default parameters) with a length fitting the definition of CNV (usually more than 1 kbp). Smaller window sizes were tested resulting in longer computational times without any specific indication on their reliability. CNVs identified in the different breeds were merged into CNVRs with Bedtools v.2.17.0 (Quinlan & Hall 2010) (function: merge) whenever overlapping genome windows, constituting the different CNVs, were encountered.

CNVRs were then compared with previous studies. The comparison was carried out remapping CNVRs on the Sscrofa11.1 using the NCBI genome remapping tool (https://www.ncbi.nlm.nih.gov/genome/tools/remap) looking for CNVRs sharing at least one nucleotide, as proposed by Keel *et al.* (2019).

Cluster analysis of breeds based on CNVRs

Pig breeds were clustered based on the read count ratio of each genome window covered by a CNVR. This ratio was defined as $\frac{\mathrm{RC}}{RC_g}$, where *RC* and RC_g indicate the exact number and the average number of reads in a genome window for a specific pig breed, respectively. Hierarchical clustering was computed in R v.3.6 (R Core Team, 2018) (function: hclust) using the Ward.D2 distance (we excluded genome windows presenting a ratio ≥ 50 in at least one pig breed).

Genomic analysis of repeated elements in CNVs/CNVRs and flaking regions

The GFF file reporting the location of repeated elements interspersed in the *S. scrofa* genome was downloaded from the UCSC Genome Browser (https://genome.ucsc.edu/). For CNVs/CNVRs and the related 1-kb flanking regions, we counted the number of bases overlapping each repeated element (Bedtools; function: intersect), assessing their enrichment via Fisher's exact test as implemented in Python 2.6 (Scipy library; function: stats.fisher_exact; alternative hypothesis: greater). We considered statistically enriched classes of repeated elements presenting a *P* < 0.05, Bonferroni corrected.

Annotation of CNVRs

Annotated genes overlapping the identified CNVRs were retrieved from the Sscrofa11.1 NCBI's GFF file by using Bedtools (function: intersect). Functional analysis was carried out with PANTHER (Mi *et al*. 2019) via Fisher's exact test. Analyses were run over a subset of the Gene Ontology – Biological Process resource (PANTHER GO-slim v.14.1; release 2019-03-12; no. = 2004 biological processes) and the Reactome database (Reactome v.65; release 2019-03-12; no. = 1569 pathways). We made use of pig specific gene annotations. We considered statistically enriched terms presenting a *P* < 0.05, FDR corrected.

The presence of QTLs in CNVRs was evaluated and tested via Fisher's exact test. QTLs were downloaded from the Pig Quantitative Trait Locus Database (Pig QTLdb; release 39) (Hu *et al*. 2019) and checked. Distribution of QTL size pointed out a fraction of long QTLs (> 2 Mbp) probably due lack of resolution derived by the information retrieved from several QTL studies. These QTLs were discarded. We noted that for a given QTL class (i.e. trait) several DNA markers, defining the QTL in different breeds, were close to each other. Thus, QTLs that were less than 500 kbp of distance were merged with Bedtools (function: merge) to obtain QTL regions. The final dataset presented a total of 295 traits and 1978 QTL regions. For each trait, the fraction of CNVR nucleotides overlapping QTLs was retrieved with Bedtools (function: intersect). Fisher's exact test was run in Python, retrieving statistically enriched traits presenting a *P* < 0.05, Bonferroni corrected.

Results

Sequenced reads and genome wide identification of CNVs

About 17.5 billion reads were produced from the sequencing of the 21 pig DNA pools. On average, each DNA pool presented about 417.7 million of mapped reads spanning 98.5% of the *S. scrofa* reference genome, with an average read depth of about 42×. Summary statistics of sequencing data are reported in Table S2.

Using cn.Mops we identified a total of 9592 CNVs (14344 events) across the 21 analysed breeds. On average, each pig breed had 683 CNVs (median = 601; min. = 209, Sarda; max. = 1440 Turopolje) covering 0.18% (s.d. = 0.09%) of the reference genome, with the smallest fraction in Sarda (0.04%) and the largest coverage in Turopolje (0.40%), reflecting the lowest and highest number of CNVs, respectively (Table 1). For each pig breed, CNVs were divided in losses (copy number < 2, as inferred by cn.Mops) and gains (copy number > 2, as inferred by cn.Mops) that represented the most frequent copy number (CN) state characterizing the animals analysed in the pools. On the whole, we identified a total of 3492 losses, 5012 gains and 638 showing a mix of copy number loss and gain. The losses/gains ratio was around 0.79. Stratified by chromosome, this value ranged from 0.57 to 1.02, for SSC12 and SSC1, respectively (Table S3). Considering the CNVs detected in each breed, the number of losses and gains strongly correlated (r = 0.93). CNV length ranged from 2250 to 560250 bp. The longest CNV (560250 Mbp) was detected on SSC8 in the Italian Large White and Lithuanian White Old Type pig breeds (Table 1). The number of CNVs and the chromosome length had a medium-high Pearson's correlation coefficient (r = 0.69; P < 0.05).

Identification of CNVRs

CNVs were merged across breeds resulting in a total of 3710 CNVRs (Table S4). The distribution of CNVRs along each chromosome is presented in Fig. 2. SSC1, SSC2 and SSC3 had the largest number of detected CNVRs (no. = 359, no. = 361 and no. = 307, respectively; Table 2). The number of CNVRs and the chromosome length highly correlated (r = 0.87; P < 0.05). Positive correlation (r = 0.92, P < 0.05) was observed also between the number of CNVRs and their total length. On average, each pig breed had 586 CNVRs (min. = 180 in Sarda; max. = 1257 in Turopolje; Table S5). Among the 3710 CNVRs, 1615 (43.5%) were breed specific (and indicated as private CNVRs; Table S5). Size of CNVRs ranged from 2250 bp up to 560250 bp (the same of CNVs), with an average length of 7038 bp and a median value of 3750 bp (Table 2). Distribution of CNVR size showed a decrease in CNVR counts while increasing their size. CNVRs occupied a total of 26.1 Mbp, equal to 1.15% of the Sscrofa11.1 reference genome. Among the CNVRs, based on the copy number state (i.e. the number of copies; CN state) provided by cn.MOPS, 1305 (35.2%) had only copy number gains (duplication), 1323 (35.6%) had only copy number losses (deletion), and 1082 (29.2%) showed a mix of copy number losses and gains from different pig breeds.

The 3710 detected CNVRs encompassed a total of 34821 genome windows. After filtering, the read count ratio of each genome window was used to cluster pig breeds (Fig. 3), which grouped breeds in agreement to their main specific phenotypes or their geographic origin. A first group encompassed breeds that have a coat colour with white background or white patterns (Lithuanian Indigenous Wattle, Italian Large White, Krškopolje, Bísara and Lithuanian White Old Type). This may be due to the strong signals of genome windows encompassing the *KIT* gene, that accounts for ~15% of the total positive windows for CNVs. The two reddish brown coloured breeds (Mora Romagnola and Italian Duroc) were on the same branch. Three autochthonous Italian breeds (Casertana, Nero Siciliano and Sarda) constituted a cluster whereas one Portuguese and one Spanish breed (Alentejana and Majorcan Black, respectively) constituted another cluster. The Turopolje pig breed was the only one that clustered apart from all other breeds.

Repeated elements within and flanking CNVs and CNVRs

Highly repetitive sequences were investigated for their co-occurrence with CNVs and CNVRs (Table S6). The following classes of repeated elements were statistically over-represented within CNVs: long interspersed nuclear elements (LINE), long terminal repeats (LTR), satellites, rolling-circle (RC/Helitron) and pseudogenes (tRNAs, snRNAs, srpRNAs, and rRNAs). Additionally, CNV flanking regions (1-kbp per side) were enriched for the following classes: short interspersed nuclear elements (SINE), simple repeat and low complexity. CNVRs differed for the absence of RC elements and the absence of SINE and srpRNAs in the 1-kbp flanking regions. However, SINE were over-represented when the flanking region size was extended to 10-kbp.

QTLs in CNVRs

A total of 1978 QTL regions, associated to 554 phenotypic traits, were retrieved from the pig QTL database. CNVRs overlapped a total of 336 QTL regions representing 295 phenotypic traits. Enrichment analysis identified 126 traits (~ 43%) significantly over-represented ($P < 0.05$, Bonferroni corrected). These traits spanned different classes, including meat quality, body shape and conformation, reproduction, disease susceptibility, haematological and metabolism related traits (Table S7).

Functional annotation of CNVRs and detailed analysis of selected genes

A total of 1571 genes overlapped the identified CNVRs, including 1296 protein coding genes, 261 lncRNAs, 3 miRNAs and 11 tRNAs. The number of overlapped genes correlated with the number of CNVRs ($r = 0.99$). A total of 993 protein-coding genes were annotated by PANTHER and used for functional enrichment over the GO slim Biological process resource. A total of 17 terms were over-represented (Table S8), encompassing different biological processes such as sensory perception, nervous system process, fatty acid metabolic process, gene expression and biological adhesion. Over the Reactome database, PANTHER over-represented the olfactory signalling pathway and the related mechanism of transduction mediated by G protein-coupled receptors (Table S8). Analysis of genes located in private CNVRs did not identify any over-represented process/pathway.

The *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (*KIT*) and the *methionine sulfoxide reductase B3* (*MSRB3*) genes were two important genes presenting variable copies among breeds. CNVs affecting the *KIT* gene are responsible for different coat colour phenotypes (Johansson

Moller *et al*. 1996; Marklund *et al*. 1998; Johansson *et al*. 2005; Rubin *et al*. 2012) whereas variable copies of the *MSRB3* have been associated with ear size in pigs (Chen *et al*. 2018).

The detailed analysis of the *KIT* gene indicated the presence of the four duplicated regions (DUP1-4; Fig. 4a) previously described by Rubin *et al.* (2012). Structural variants as well as the presence of the splice mutation at the first base in intron 17 (g.41486012G>A, rs345599765) are all required for manifesting a solid white coat colour (Marklund *et al.* 1998). Using sequence data, we estimated the allele frequencies of this SNP (Fig. 4a; Table S9) to complement CNV results. Pools from colored pigs did not show any CNV and the splice mutation. White pigs (Italian Large White and Lithuanian White Old Type) had DUP1-4 and the splice mutation (allele A). However, allele frequencies were divergent (Table S9) suggesting a different structure of the CNV (different gene copies with the the A or G nucleotides). The Sarda (not fixed for any coat colour and including many spotted animals) and Lithuanian Indigenous Wattle breeds presented DUP1, did not have DUP2-4 and had allele A (the only two other breeds having the splice mutation). Bísara, another spotted breed, had also DUP2-3. The piebald breed Basque and the belted breed Cinta Senese had DUP2-4, whereas the other two belted breeds (Krškopolje and Schwäbisch-Hällisches Schwein) had only DUP2 and DUP4.

The detailed analysis of the *MSRB3* gene region revealed the presence of the 38.4-kbp duplication (SSC5:29826981-29865653; Fig. 4b) previously described by Chen *et al.* (2018). Copy number gains encompassing the *MSRB3* exons 6 and 7 have been associated with large ear size in Chinese pig breeds and with half-floppy ears in Landrace pigs (Chen *et al.* 2018). Alentejana, Cinta Senese, Mora Romagnola, Italian Duroc and Italian Large White that are breeds characterized by small/medium ear size, had a normal copy number state (that means no gain of copies). The remaining pig breeds showed variable copy number which seems to be correlated to ear size (Fig. 4c). Regression analysis between the average CN state and the ear size (coded as follows: small = 1, medium = 1.5, medium/large = 1.75 and large = 2) resulted in a positive association (P = 0.0001). However, other breeds characterized by small ears (i.e. Nero Siciliano and Sarda) had variable copy numbers. Variability in ear size was also analysed by estimating the allele frequency of two SNP in the 5' flanking region (g.29695369C>T; rs340841870) and in the 3'-untranslated region (g.29862412C>T; rs326411202) of the *MSRB3* gene, that Zhang *et al.* (2015) reported to be associated with ear size. These SNP positions are not included in the CNVR of this gene. For each SNP, the regression analysis pointed out a significant association between allele frequencies and ear size ($P < 0.0001$). Additionally, allele frequencies of these two SNPs (Table S9) correlated with the average CN state ($|r| > 0.8$; $P < 0.0001$; Fig. 4d).

We further explored genomic regions harbouring private information on CNVRs. Among them, we identified two interesting examples. The first one, characterizing Lithuanian Indigenous Wattle pigs, encompassed the intron 10 of the *ELOVL fatty acid elongase 6* (*ELOVL6*) gene (Fig. 5a). Variants in this gene has been associated with fatty acid composition in pigs (Corominas *et al.* 2013). The second one, characterizing Turopolje pigs, was the *Zinc finger protein 622* (*ZNF622*) gene, a regulator of early embryonic development (Hasegawa *et al.* 2015). The CNV affecting this gene was quite complex. Copy number gains were in the correspondence of the exonic regions but also included the complete intron 1, intron 2 and intron 5. Most of introns 3 and 4 were not affected by CN gains (only small and contiguous intronic segments to the exonic regions were included in the CN gains) (Fig. 5b). The regions with CN gains were clearly evidenced in all breeds except Turopolje, which did not have any copy number and, in part, in Krškopolje and Italian Duroc, that had CN higher than that of Turopolje but lower than that of all other breeds (Fig. 5b).

Comparison with other studies

The positions of CNVRs we detected were compared with the CNVRs reported by previous studies, which analysed different pig breeds and other species of the *Sus* genus using whole genome sequencing. A total of five datasets, which investigated Asian pig breeds, commercial and European pig breeds, and five species of the genus *Sus*, were considered for this comparison (Table S4). The overlap ranged from about 10 to 25% (Table S10). Overall, a total of 595 CNVRs detected in our work (16%) overlapped with CNVRs reported by the considered studies (Table S4).

Discussion

In this study we carried out a genome-wide CNV/CNVR analysis in 19 European autochthonous and two Italian commercial pig breeds. Breeds were analysed by using a whole genome sequencing strategy from breed specific DNA pools to maximize CNV discovery. CNVs were detected via cn.MOPS, a tool that implements a Bayesian approach that models depth of coverage across samples by decomposing its variability in a part coming from copy numbers and the remaining part due to noise, in order to reduce false discoveries (Klambauer *et al.* 2012). Other software based on different assumptions have been also developed and used for CNV detection from HTS datasets. However, there is no consensus in the literature on the strategy and methodology that might be applied for this purpose.

As our study was based on DNA pools from a large number of populations, we maximized the power of cn.MOPS in reducing the false discovery rate, as this tool is specifically designed to deal with multiple samples.

Even if this design could not precisely define the exact number of copy gains or losses for all animals in the sequenced pools, the obtained results made it possible to capture within breed averaged states. This was supported by the agreement among the different coat colour phenotypes and the expected CN states, rightly detected at the *KIT* locus which indirectly confirmed and validated CNV calls from cn.MOPS. This approach demonstrated that CNVs detected using whole genome sequencing can be useful to identify breed specific features (including in this definition the most frequent breed features) and describe genetic diversity across pig breeds, complementing SNP based studies.

We confirmed a high correspondence between CNV data detected from sequenced DNA pools and SNP information using Pearson's correlation calculated considering the fraction of the pig genome covered by CNVRs detected for each breed (Table 1) and SNP based diversity measured on the same animals genotyped with the GeneSeek ® GGP Porcine HD Genomic Profiler (Muñoz *et al*. 2019). Among these SNP averaged variability parameters, correlation with the above mentioned CNVR parameter was highly negative with both the minor allele frequency (MAF; *r* = -0.90) and expected heterozygosity (*r* = -0.90), whereas highly positive correlation with the Fixation Index (F_{ST}; *r* = 0.96) values. These correlations mean that when within breed variability was low, it increased the possibility to identify losses/gains at variable CN state and that the fraction of the genome covered by CNVRs detected in DNA pools is a good indicator of the diversity among breeds.

With few differences, these breeds were clustered resembling the relationships that we already reported using array SNP datasets obtained from individually genotyped pigs and SNPs detected from whole genome sequencing (Muñoz *et al*. 2018, 2019; Bovo *et al*, in preparation). Geographical and some major morphological features (i.e. coat colour) mainly determined breed clusters obtained from CN states. Turopolje, the breed that accounted for the largest number of CNVs (with the largest fraction of the genome covered by CNVRs), was clustered apart, as also reported with SNP data (Muñoz *et al*. 2018, 2019; Bovo *et al*, in preparation).

Some CNVRs were considered as breed specific or identified in a few breeds, suggesting that this variability might contribute to determine several phenotypic characteristics that distinguish autochthonous and commercial European breeds. In addition, considering the whole patterns of CNVRs that we detected, a quite high frequency of these events was classified as mixed CNVs (including both gains and losses). This indicates that despite breeds share genome regions affected by CNV, the single breed carries a gain or a loss specific for the breed itself.

In the current study, an average of 77 CNVRs (~16% of all breed reported CNVRs) was considered as private for each analysed breed, highlighting the power of the DNA pooling strategy in capturing distinctive breed features. However, as the sequencing depth is not so high, for a given private CNVR we cannot completely exclude the possibility that few animals of the other investigated breeds could carry the same alleles in these regions. The remaining CNVRs were shared among two or more breeds, indicating that admixture and crossbreeding events or a common origin might have contributed to spread this variability. However, further studies are needed to clarify their allelic status or their common origin, as in our first survey we did not characterize into detail the precise breakdown positions and structure of all identified CNVRs.

CNVRs we detected overlapped genes involved in different biological processes including nervous system and sensory perception such as olfactory signalling. Brain functions control several behaviours, including feeding, habitat selection, reproduction and social interaction that strongly depend on the genetics architecture of an individual (Bendesky & Bargmann 2011). Several studies in mammals including pigs reported CNVs in genes involved in the olfactory signalling pathway, linking gene variability to food foraging and mate recognition abilities (Paudel *et al*. 2015; Keel *et al*. 2019). In addition, considering the overlapping of CNVRs and QTL regions, the main traits associated with changes in CN state were meat quality, body shape and conformation, reproduction and metabolism. Variability in chromosome regions harbouring functionally relevant genes or QTL may reflect the adaptation of these breeds to different production systems and environments.

The impact of this type of variability on exterior characteristics of the pigs has been already demonstrated for the CNVs in the *KIT* gene region affecting coat colours and patterns, which characterize the *Dominant white* phenotype (Rubin *et al*. 2012). Other evidences came for the CNVs in the *MS3B3* gene region, involved in ear size as mainly reported in Chinese breeds (Chen *et al*. 2018). These CNVRs were also detected in our study with some interesting new information for some of the analysed breeds.

The complexity of the *Dominant white KIT* locus has been explained by the presence of six main allele groups (in addition to a few other potential variants; Fontanesi & Russo 2013): (i) a recessive wild-type allele *i* (that is carried by wild boar and coloured pigs), (ii) the *Patch* allele I^P (determining spotted patterns), (iii) the *Belt* allele I^{Be} (determining the belted phenotype), (iv) the *Roan/Gray* allele I^{Rn} or I^d (causing the grey-roan phenotype), (v) the dominant white alleles *I*, comprising several forms (e.g. I^1, I^2 and I^3) and causing the white solid phenotype that mainly characterize Large White and Landrace breeds and (vi) the *I*^{L} allele, a null and lethal allele (Johansson Moller *et al*. 1996; Marklund *et al*. 1998; Johansson *et al*. 2005; Rubin *et al*. 2012). Variants in this chromosome region are mainly associated with a 450-kbp duplication encompassing the entire *KIT* gene (DUP1; the only CN of the *I*^{P} allele), including also another 4.3-kbp duplication (DUP2) located ~100 kbp upstream of *KIT* gene, and a 23-kbp duplication (DUP3) ~100 kbp downstream from *KIT*, which in turn resulted to contain another 4.3-kbp duplication (DUP4; Rubin *et al*. 2012). The *I* alleles presented variable copy numbers of DUP1/2/3/4, whereas DUP2/3/4 were identified in pigs with the *I*^{Be} allele (Rubin *et al*. 2012). Moreover, a recent whole genome resequencing study uncovered new *KIT* alleles conferring different coat colour phenotypes (Wu *et al*. 2019). The CN state states that we identified in our study encompassed all four duplicated regions, describing for the first time the structure of the *KIT* gene in several autochthonous pig breeds (Fig. 4a).

In addition, analysis of sequencing data let us to estimate the frequency of the splice mutation g.41486012G>A (rs345599765) that distinguish the CN state of the *I*^{p} from the *I Dominant white* allele series (Marklund *et al*. 1998). As expected, all breeds that did not show any duplicated regions are characterized by solid coat colours and did not have the splice mutation. They are considered to carry only the *i* wild-type at the *Dominat white* locus. Sarda, which is a breed not fixed for any coat colours and that includes also white and white spotted pigs, showed the presence of DUP1, with some faint signs at the DUP4 position (with a low frequency of the splice mutation). Several alleles at the *KIT* gene might be present in this breed, including *I*^{P}, *I* variants and *I*^{Be} forms. A similar pattern was observed in the Lithuanian Indigenous Wattle breed, which includes mainly spotted pigs. According to the CN state observed in this breed, *I*^{P} might be the most frequent allele, even if other and *I*^{Be} and *I* forms (including also DUP4) might be present. A more marked copy number pattern was evidenced for the Bísara breed (which has mainly heterogeneous coats: grey or black and white or spotted) that reported DUP1 copy number status similar to Sarda and Lithuanian Indigenous Wattle) in addition to DUP2-3 (without signals indicating the presence of DUP4).

The analysis of the *KIT* gene region in breeds characterized by a belted phenotype, even if not homogeneous, indicated that more alleles at this locus might produce belted pigs even if with some different phenotypic effects. Cinta Senese and Basque had equal CN state at DUP2-3 but differed in DUP4 (higher in Basque and lower in Cinta Senese). Cinta Senese is a classical belted breed whereas Basque pigs are usually black and white with heterogenous patterns but usually with black head and rump. Other breeds having white belts of varying size and shape (Krškopolje and Schwäbisch-Hällisches Schwein) showed only DUP2 and DUP4. The connection between the two breeds might be derived by ancestral origins (not clearly defined), that preserved the same structure at the *Dominant white* locus. Wu *et al*. (2019) observed that the presence of DUP2 together with DUP4 can produce a belted phenotype in Duroc × (Landrace × Large White) hybrid pigs. The presence of multiple alleles conferring a belted phenotype is also confirmed by the results of the analysis of the rs328592739 SNP in the *KIT* gene that was associated with the belted pattern in Cinta Senese pigs (Fontanesi *et al*. 2016) but not in Krškopolje and Schwäbisch-Hällisches Schwein pigs (Ogorevc *et al*. 2017).

White breeds (Italian Large White and Lithuanian White Old Type) had a classical copy number pattern in DUP1-4 and the splice mutation already described for completely white pigs carrying *I* alleles (Fontanesi *et al.* 2010a). Heterogeneity on the presence of the splice mutation suggested that *Dominant white* alleles having different G/A ratios at this position. In Lithuanian White Old Type, gene copies at this position carried G only in 1 out of 5 copies (as estimated from its 0.20 frequency). In Italian Large White, about 2 out of 3 gene copies carried the G nucleotide (G = 0.68), suggesting that the CNV structure in this breed might be determined by different *Dominant white* alleles than those frequently present in the Lithuanian White Old Type breed.

Interesting copy number patterns were also observed in the region of SSC5 encompassing the last exons of the *MSRB3* gene (Fig. 4b), which is associated with ear size (Chen *et al*. 2018). These authors proposed that large ear size is due to the increased CN state in this region, which affects the expression of the nearby miR-584-5p that in turn inhibits the expression of its target gene *MSRB3*. Our CNV analysis for the *MSRB3* gene across autochthonous European pig breeds indicated, with the exception of some breeds, a significant correlation between ear size and the average CN state (Fig. 4c). The latter also correlated with allele frequencies estimated for the rs340841870 and rs326411202 SNPs (outside this CNVR), which suggested the presence of linkage between these two types of variants: allele C at both positions is associated with a normal copy state whereas the alternative allele at both sides (T) is associated with the presence of 5 or 6 copies (of the linked multiple copy region), as estimated from the sequencing data in the CNVR. Even if pigs of the studied breeds were in general described to have breed-specific traits, heterogeneity for ear size has been already reported in some breeds which might not actually have fixed ear size shape (Schiavo *et al.* 2019). Therefore, correlation between CN state and ear size might not precisely estimated by the DNA pooling approach (Fig. 4b). It is also worth mentioning that ear size and position have been already shown to be under polygenic control with a few major genes affecting these traits (e.g. Wei *et al.* 2007; Ma *et al.* 2009; Ren *et al.* 2011). Thus, other genomic regions and polymorphisms could be responsible for the ear size phenotype in some of the analysed breeds.

The CNV in the *ELOVL6* gene might interesting to explain economically relevant traits, considering the role of this gene in affecting fatty acid composition in pigs (Corominas *et al.* 2013). Other studies reported that variability in this gene or variability in its expression level might explain, at least in part, differences of intramuscular fat accumulation and lipid metabolism among breeds, which are relevant for meat quality, considering also genotype-feeding interactions to design appropriate fatty-acid diets in pigs to maximize this aspect (e.g. Benítez *et al.* 2016; Muñoz *et al.* 2018; Revilla *et al.* 2018). Association studies and functional analysis of the CNV in this gene are needed to understand if this variability could be involved in affecting meat quality traits in pigs. Targeted analyses are also needed to detect with more precision if this variability segregates within the analysed breeds as well as in other breeds in which meat quality parameters are important factors determining the quality of their products.

Detailed analyses of CN states of some chromosome regions can also identify (or suppose) the occurrence of other or more complex mutational events that might not be properly considered as derived by CNVs. The case of the *ZNF622* gene that reported three distinct copy number gains (mainly in the correspondence of exonic regions) might raise a few hypotheses on the occurrence of this strange pattern. The three divided copy number gains might be due to the presence of a pseudogene derived by the *ZNF622* gene (inserted somewhere into the genome) or that the duplication of the gene subsequently underwent other mutational events that eliminated most of the sequence of introns 3 and 4 (Fig. 5b). Other studies are needed to clarify these hypotheses. After a preliminary analysis, CN states reported in the correspondence of this gene appeared to produce a private condition in the Turopolje breed that did not have any copy number gain (common in all other breeds). Inspection of the clustering analysis for the CN at this gene in all breeds, indicated that two other breeds (Krškopolje and Italian Duroc) might not have fixed copy number gains, mainly in the correspondence of the annotated exons of the *ZNF622* gene.

On the whole, our survey on European pig breeds reported that CNVRs occupy 26.1 Mbp, representing 1.15% of the reference genome size. Compared to other whole genome sequencing based studies, this genome fraction is similar to what was reported by Paudel *et al*. (2015) and Keel *et al*. (2019) (17.83 and 22.9 Mbp, respectively). Other two studies (Paudel *et al*. 2013; Jiang *et al*. 2014) identified larger fractions of the pig genome covered by CNVRs (39.2 and 102.8 Mbp, respectively). Although this divergence could be attributed in part to the algorithms used to detect CNVs and the sequencing approaches (single pigs *vs* pools of individuals), it might be also due to differences among the studied pig populations. Distribution of CNVR sizes showed a decrease in CNVR counts while increasing their size, as also described by Jiang *et al*. (2014). Differences among breeds were also clearly shown in our study, as detailed above. Some of the CNVRs we detected in our study overlapped with CNV events reported by the other whole genome sequencing mentioned studies (on average, ~13% of overlap), pointing out that they could exist also in other breeds that we did not survey. However, they represent just fraction a small fraction, strengthening the evidence that CNV are breed-specific genome features. Additional studies are needed to obtain a global overview of CNVs segregating in the *Sus scrofa* species, by comparing more breeds and populations.

As CNVs mutate about 2-3 times faster than SNPs, some of the CNVRs that we detected across several breeds could eventually also be derived from recurrent mutational events through nonallelic homologous recombination, potentially driven by the presence of repeated regions within or in flanking positions (Liu *et al.* 2012). Analyses of CNVRs and their flanking regions identified enrichments of different classes of repeated elements, confirming what other studies reported this species (e.g. Paudel *et al*. 2013; Wang *et al*. 2015b). This further suggest that these sequence features might contribute to chromosome instability and mutational mechanisms promoting these structural changes also in *Sus scrofa.*

Our study investigated CNVs in the porcine genome over a large number of pig breeds that represent important European genetic resources for this species. This variability can explain part of the genetic diversity among breeds and might contribute to explain their origin, history and adaptation to a variety of production systems. Further studies are needed to better understand how CNVs could be considered in defining conservation programmes of these autochthonous genetic resources.

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Availability of data

Sequence data generated and analysed in the current study are available in the EMBL-EBI European Nucleotide Archive (ENA) repository (http://www.ebi.ac.uk/ena), under the study accession

PRJEB36830. CNVRs are available as Supplementary Table S4 and from the corresponding author on reasonable request.

Competing interests

The authors declare they do not have any competing interests.

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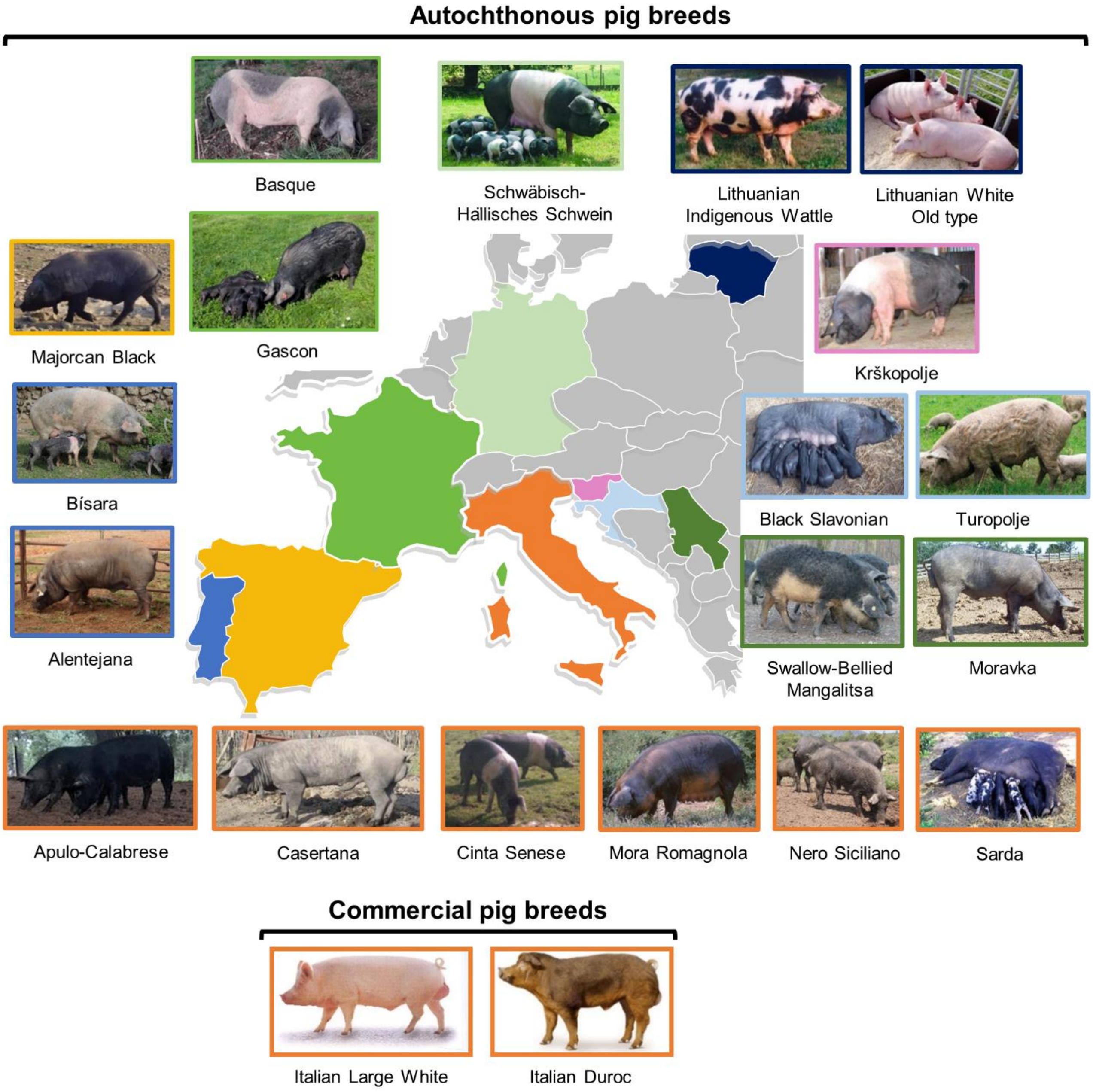
835 **Figures**

836 **Fig. 1.** Phenotypes and geographical origin of the 21 analysed pig breeds.



837

Fig. 2. Distribution of CNVRs along each autosomal chromosome.

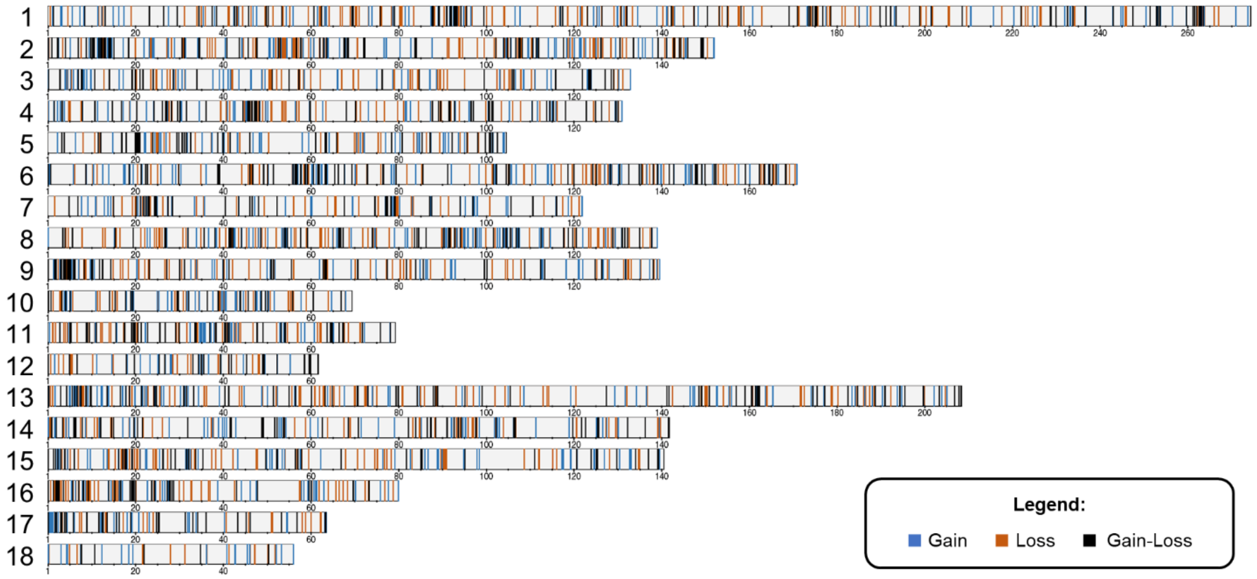


Fig. 3. Dendrogram representing the hierarchical clustering of the copy number state. Acronyms of the breed name are explained in Table 1.

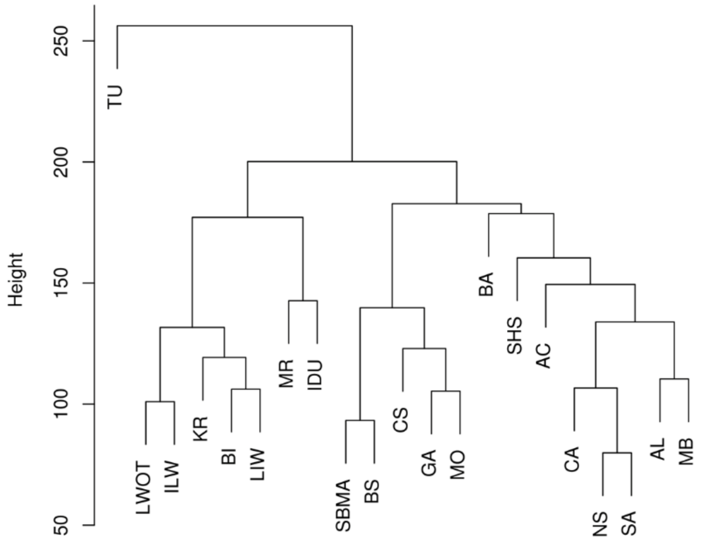


Fig. 4. (**a**) Heatmap of the read count ratios over the *KIT* gene. Coat colour reported in the correspondence of the breeds indicates breed main characteristics. SA (Sarda) has heterogeneous and not-fixed patterns. It was included among the spotted based on the frequency of this phenotype in the breed and according to the copy number (CN) state at this locus. Basque (BA) has spotted/belted heterogeneous patterns but was included among the belted breeds according to the CN state at this locus – (see text and Table S1 for details). KIT-G: frequency of the allele G of the single nucleotide polymorphism (SNP) rs345599765 (splice mutation of the intron 17; Marklund *et al.* 1998). (**b**) Heatmap of the read count ratios over the *MSRB3* gene. Ear size indicated in (b): L = large; M = medium; S = small (see text and Table S1 for details). The light-dark blue bar at the top of (a) and (b) indicates the CN ratio (1 = normal state without any gain or loss). For each breed, the read count ratio was computed in 750-bp consecutive genome windows. Acronyms of the breed name are explained in Table 1. (**c**) Average CN state of the *MSRB3* gene in relation to ear size. (**d**) Relationship between the average CN state of the *MSRB3* gene and the SNPs rs340841870 (green) and rs326411202 (blue). Pearson's correlation coefficient (*r*) are reported.

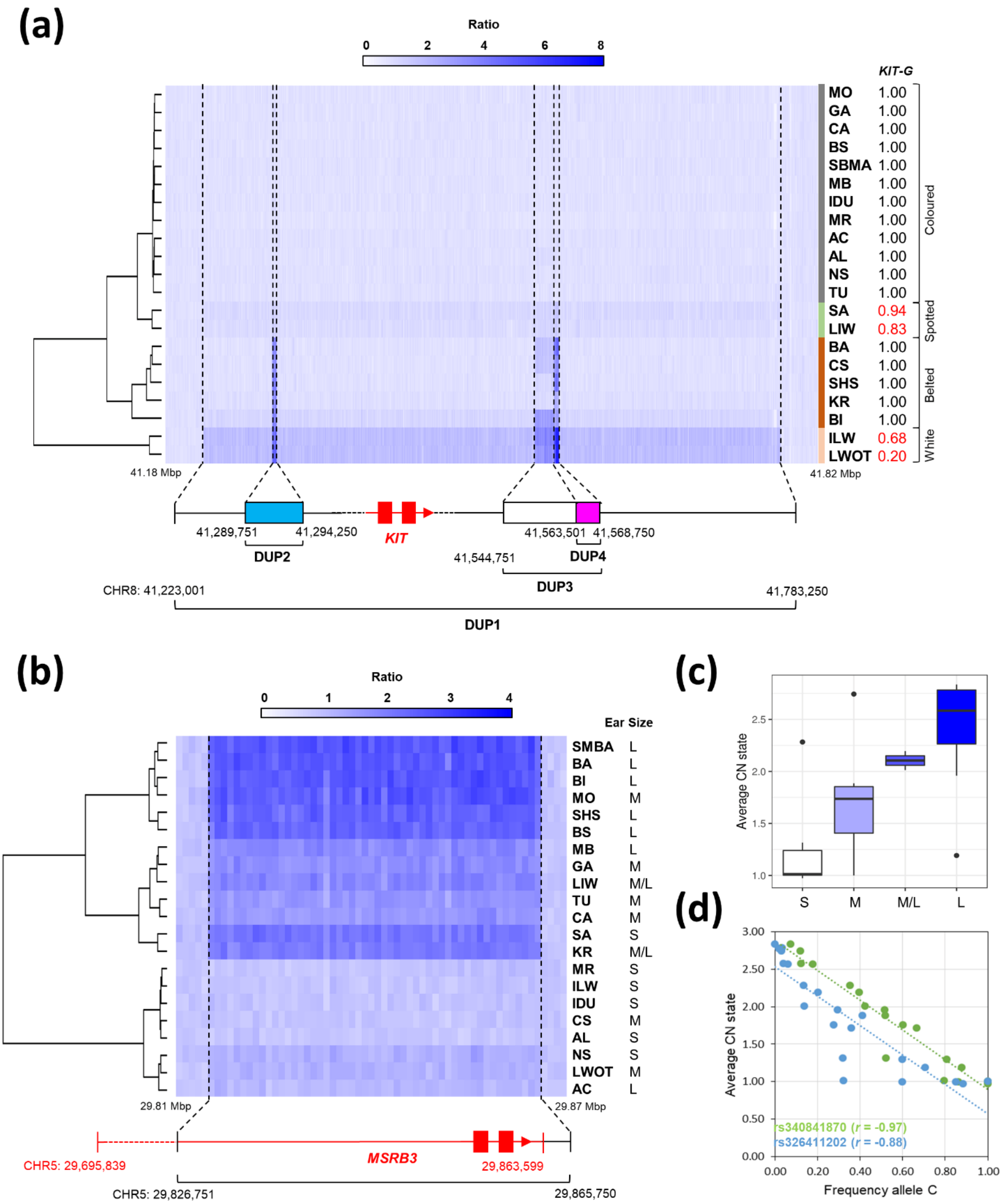
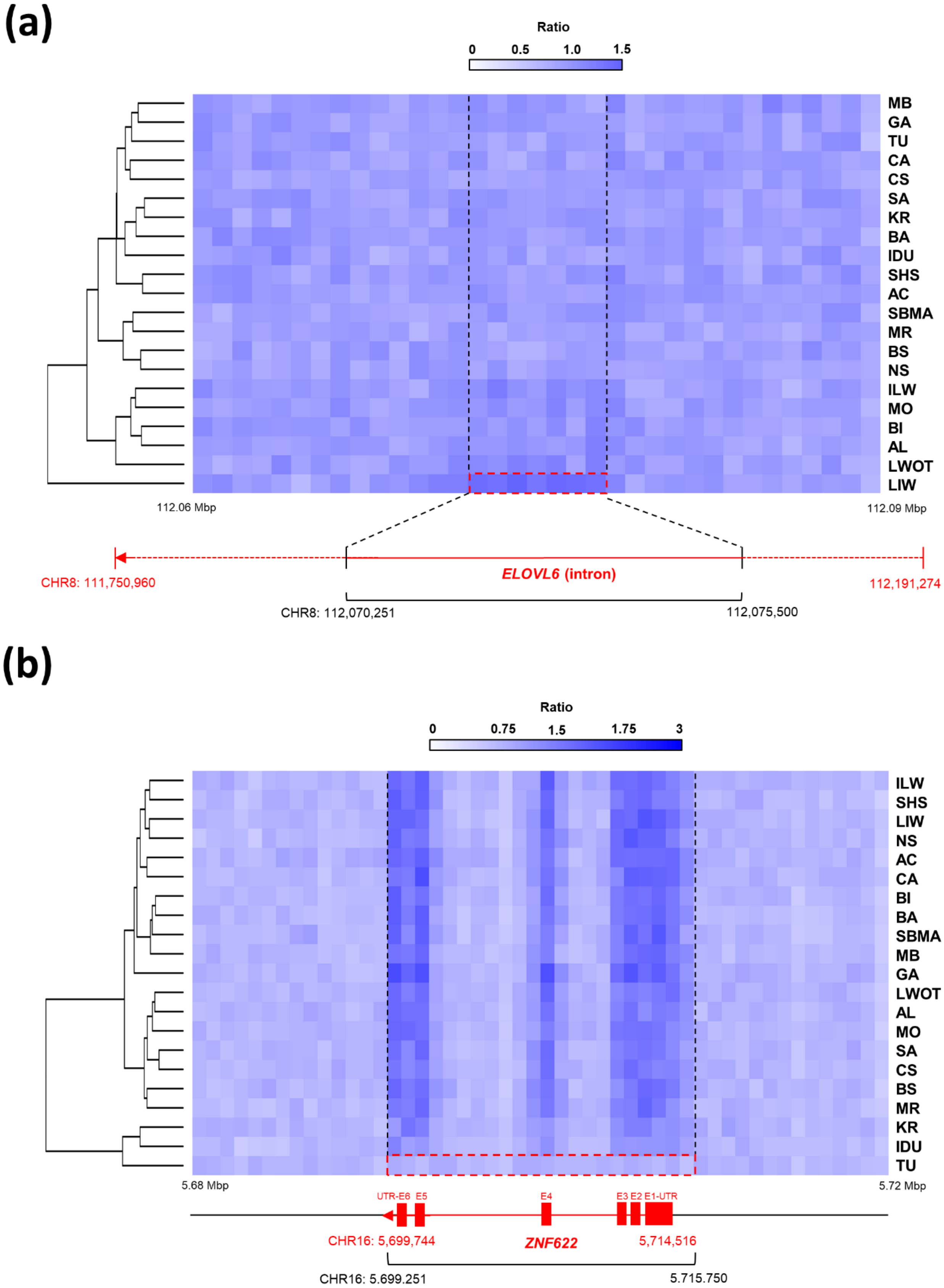

(a)
Ratio
0 2 4 6 8
KIT-G
MO 1.00
GA 1.00
CA 1.00
BS 1.00
SBMA 1.00
MB 1.00
IDU 1.00
MR 1.00
AC 1.00
AL 1.00
NS 1.00
TU 1.00
SA 0.94
LIW 0.83
BA 1.00
CS 1.00
SHS 1.00
KR 1.00
BI 1.00
ILW 0.68
LWOT 0.20
Coloured
Spotted
Belted
White
41.18 Mbp
41.82 Mbp
41,289,751
41,294,250
KIT
DUP2
41,563,501
41,568,750
41,544,751
DUP4
DUP3
CHR8: 41,223,001
41,783,250
DUP1
(b)
Ratio
0 1 2 3 4
Ear Size
SMBA L
BA L
BI L
MO M
SHS L
BS L
MB L
GA M
LIW M/L
TU M
CA M
SA S
KR M/L
MR S
ILW S
IDU S
CS M
AL S
NS S
LWOT M
AC L
29.81 Mbp
29.87 Mbp
CHR5: 29,695,839
MSRB3
29,863,599
CHR5: 29,826,751
29,865,750
(c)
Average CN state
2.5
2.0
1.5
1.0
S
M
M/L
L
(d)
Average CN state
3.00
2.50
2.00
1.50
1.00
0.50
0.00
rs340841870 (r = -0.97)
rs326411202 (r = -0.88)
0.00
0.20
0.40
0.60
0.80
1.00
Frequency allele C

Fig. 5. Heatmap of the read count ratios over the *ELOVL6* (**a**) and *ZNF622* (**b**) genes. Exons below the heatmap for the *ZNF622* gene are numbered (E1-E6) according to the annotation in the Sscrofa11.1 genome version. Untranslated regions (UTR) are also reported. The light-dark blue bar at the top of (A) and (B) indicates the copy number (CN) ratio (1 = normal state without any gain or loss). For each breed, the read count ratio was computed in 750-bp consecutive genome windows. Acronyms of the breed name are explained in Table 1.


(a)
Ratio
0
0.5
1.0
1.5
MB
GA
TU
CA
CS
SA
KR
BA
IDU
SHS
AC
SBMA
MR
BS
NS
ILW
MO
BI
AL
LWOT
LIW
112.06 Mbp
112.09 Mbp
CHR8: 111,750,960
ELOVL6 (intron)
112,191,274
CHR8: 112,070,251
112,075,500
(b)
Ratio
0
0.75
1.5
1.75
3
ILW
SHS
LIW
NS
AC
CA
BI
BA
SBMA
MB
GA
LWOT
AL
MO
SA
CS
BS
MR
KR
IDU
TU
5.68 Mbp
5.72 Mbp
UTR-E6
E5
E4
E3
E2
E1-UTR
CHR16: 5,699,744
ZNF622
5,714,516
CHR16: 5.699.251
5.715.750

Tables

Table 1. Summary of CNVs of the 21 analysed pig breeds. Data are stratified by breed.

Breed	Short name	CNV[1]	CNL[2]	CNG[3]	Length$_{\text{Min}}$[4]	Length$_{\text{Max}}$[5]	Length$_{\text{Median}}$[6]	% length in CNV[7]
Autochthonous								
Alentejana	AL	601	345	256	2250	69750	3000	0.17
Apulo-Calabrese	AC	676	313	363	2250	142500	3000	0.18
Basque	BA	1122	626	496	2250	99750	3750	0.29
Bísara	BI	437	162	275	2250	63000	3000	0.11
Black Slavonian	BS	504	225	279	2250	142500	3000	0.13
Casertana	CA	596	272	324	2250	113250	3000	0.16
Cinta Senese	CS	662	352	310	2250	89250	3750	0.19
Gascon	GA	781	379	402	2250	126000	3000	0.20
Krškopolje	KR	510	152	358	2250	101250	3000	0.13
Lithuanian Indigenous Wattle	LIW	710	295	415	2250	90750	3750	0.19
Lithuanian White Old Type	LWOT	711	308	403	2250	560250	3750	0.21
Majorcan Black	MB	546	328	218	2250	101250	3750	0.15
Mora Romagnola	MR	1255	647	608	2250	137250	3000	0.34
Moravka	MO	391	159	232	2250	100500	3000	0.10
Nero Siciliano	NS	298	149	149	2250	42750	3000	0.07
Sarda	SA	209	72	137	2250	38250	3000	0.04
Schwäbisch-Hällisches Schwein	SHS	576	277	299	2250	147000	3000	0.15
Swallow-Bellied Mangalitsa	SBMA	757	433	324	2250	121500	3000	0.22
Turopolje	TU	1440	845	595	2250	99750	3750	0.40
Commercial								
Italian Duroc	IDU	1111	249	862	2250	116250	3000	0.28
Italian Large White	ILW	451	148	303	2250	560250	3000	0.14

[1] Total no. of copy number variants; [2] Total no. of copy number losses; [3] Total no. of copy number gains; [4] Minimum length (bp) of CNVs; [5] Maximum length (bp) of CNVs; [6] Median length (bp) of CNVs; [7] Percentage of the *S. scrofa* genome occupied by CNVs.

Table 2. Summary of CNVRs of the 21 analysed pig breeds stratified by chromosome.

Chromosome	**CNVR**[1]	**Length$_{Min}$**[2]	**Length$_{Max}$**[3]	**Length$_{Median}$**[4]	**% length in CNVR**[5]
SSC1	359	2250	137250	3760	0.88
SSC2	361	2250	43500	3760	1.54
SSC3	162	2250	147750	3010	0.82
SSC4	227	2250	81000	3760	0.99
SSC5	215	2250	46500	3760	1.45
SSC6	302	2250	120750	3760	1.07
SSC7	167	2250	96750	3760	1.16
SSC8	244	2250	560250	3760	1.37
SSC9	259	2250	159000	3760	1.86
SSC10	114	2250	85500	3010	0.96
SSC11	159	2250	153750	3760	1.54
SSC12	110	2250	108000	3385	1.33
SSC13	307	2250	91500	3760	1.05
SSC14	212	2250	195750	3760	1.34
SSC15	196	2250	63000	3760	0.86
SSC16	138	2250	41250	3010	0.88
SSC17	132	2250	80250	3010	1.27
SSC18	46	2250	16500	3010	0.35

[1] Total no. of copy number variant regions; [2] Minimum length (bp) of CNVs; [3] Maximum length (bp) of CNVs; [4] Median length (bp) of CNVs; [5] Percentage of the chromosome occupied by CNVRs.

Supporting information

Table S1. Details on the analysed animals and investigated breeds, including geographical distribution and phenotypic description.

Table S2. Summary statistics of whole-genome sequencing.

Table S3. Summary statistics of detected CNVs stratified by chromosome.

Table S4. CNVRs detected over all analysed breeds.

Table S5. Summary statistics of detected CNVRs, stratified by pig breed.

Table S6. Over-represented repeated element classes.

Table S7. Within CNVRs over-represented QTLs.

Table S8. Within CNVRs over-represented biological functions.

Table S9. Allele frequency of the single nucleotide polymorphisms at the *KIT* and *MSRB*3 genes estimated from sequencing data.

Table S10. Summary statistics of CNVRs previously identified in other studies.