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Genome-wide association analyses for several exterior traits in the autochthonous Casertana pig breed

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1    **Genome-wide association analyses for several exterior traits in the autochthonous Casertana**  
2    **pig breed**

3

4    Giuseppina Schiavo<sup>a</sup>, Samuele Bovo<sup>a</sup>, Silvia Tinarelli<sup>a,b</sup>, Francesca Bertolini<sup>c</sup>, Stefania Dall'Olio<sup>a</sup>,  
5    Maurizio Gallo<sup>b</sup> and Luca Fontanesi<sup>a,\*</sup>

6

7    <sup>a</sup>Department of Agricultural and Food Sciences, Division of Animal Sciences, University of Bologna,  
8    Viale Fanin 46, 40127 Bologna, Italy

9    <sup>b</sup> Associazione Nazionale Allevatori Suini, Via L. Spallanzani 4, 00161 Roma, Italy

10    <sup>c</sup> National Institute of Aquatic Resources, Technical University of Denmark, 2800 Kongens Lyngby,  
11    Denmark

12

13    \* Corresponding author

14    E-mail address: [luca.fontanesi@unibo.it](mailto:luca.fontanesi@unibo.it) (L. Fontanesi)

15

16    **Running title:** Mining the genome of Casertana pigs

17    **Highlights**

- 18        • Casertana is an autochthonous pig genetic resource reared in Central-South of Italy.
- 19        • Genome-wide association studies and  $F_{ST}$  analyses were carried out in this breed.
- 20        • Two ear conformation traits, presence/absence of wattles and coat colours were studied.
- 21        • Markers associated with the presence of wattles were identified on SSC1 and SSC11.
- 22        • Markers associated with coat colours were identified on SSC6, SSC8, SSC14 and SSC15.

23   **Abstract**

24           Casertana is an autochthonous pig breed reared in the Central-South of Italy, mainly in  
25   extensive or semi-extensive production systems. A small number of animals are registered to the  
26   breed herd-book. The population of this breed is not completely fixed for several exterior traits,  
27   including ear size (small vs large), ear bearing (forward vs floppy), presence or absence of wattles  
28   and coat colour (black vs slate-grey) that we recorded in a total of 101 pigs from six different farms.  
29   All these pigs were also genotyped with the Illumina PorcineSNP60 BeadChip. Then, with the aim  
30   to identify DNA markers associated with these traits, we run four genome-wide association studies  
31   (GWAS) comparing pigs having different phenotypes for each of the four external traits. Genome-  
32   wide fixation index ( $F_{ST}$ ) analyses were run to confirm the results. GEMMA software was used in the  
33   GWAS. Single nucleotide polymorphisms (SNPs) associated to the wattle trait were identified on  
34   porcine chromosome (SSC) 1 and SSC11. Markers associated to the coat colour trait were located on  
35   SSC6, SSC8, SSC14 and SSC15. These chromosome regions were confirmed by the independent  $F_{ST}$   
36   analyses.  $F_{ST}$  did not confirm the signals detected for the two ear conformation traits. Candidate genes  
37   were identified according to the function of the annotated genes in the detected genome regions.  
38   Despite the small number of pigs that could be investigated, this study showed that autochthonous  
39   animal genetic resources might be useful to disclose genetic factors affecting traits that are not fixed  
40   in a population.

41

42   **Keywords:** Animal genetic resource; coat colour; ear conformation; single nucleotide  
43   polymorphism; *Sus scrofa*; wattle.

## 44    **1. Introduction**

45            Autochthonous breeds are important animal reservoirs of genetic diversity. These genetic  
46 resources are usually characterized by associated inheritable phenotypes, in several cases not  
47 completely fixed, that define the uniqueness of these populations and that could be explored to  
48 understand their adaptation to different production systems and their history and origin (Leroy et al.,  
49 2016).

50            Casertana is a local pig breed mainly raised in Central-South regions of the Italian peninsula.  
51 This breed is considered an endangered pig breed as its herd book currently accounts only for about  
52 100 registered boars and sows (ANAS, 2019). Casertana pigs are regarded as the descendant of the  
53 ancient Neapolitan pigs that were used to shape the genetic pool of the British pig populations over  
54 the 19<sup>th</sup> century (Porter, 1993). Animals of the Casertana breed have a characteristic slate-grey or  
55 black coat colour, curly tail, wrinkled skin, forward-bending ears of generally medium-size and a  
56 typical hairless phenotype. Pigs could have wattles, their head is usually of medium size with a  
57 truncated conical-shape and a rectilinear or slightly concave profile with long and thin snout (Bozzi  
58 et al., 2019). However, Casertana population is not completely fixed for most of these traits, thus  
59 phenotypic variability exists within the breed. This is probably due i) to recent introgression from  
60 European wild boars, which are in close contacts to this domesticated population, usually raised in  
61 extensive or semi-extensive production systems and ii) to crossbreeding with other pig breeds, which  
62 occurred in the past to recover this small population.

63            Variability related to a few morphological traits in Casertana made it possible to design  
64 effective genome wide association studies (GWAS) that already identified gene markers associated  
65 with the targeted traits. Schiavo et al. (2018) carried out a GWAS in this breed to compare the genome  
66 of pigs without hairs (the majority) against that of few pigs with hairs that were identified in this  
67 population. Bertolini et al. (2018) did a similar GWAS in this breed comparing genotyping data of  
68 animals with curly tail vs animals of straight tail and identified a single nucleotide polymorphism  
69 (SNP) associated with the shape of the tail in pigs.

Studies in other pig populations have already contributed to understand the genetic factors affecting a few other morphological traits which are variable in Casertana. For example, QTL and GWAS and selection signature analyses identified chromosome regions and gene markers associated to ear morphology in several pig breeds and crossbred reference families (Wei et al., 2007; Ma et al., 2009, 2015; Ren et al., 2011; Li et al., 2012; Wilkinson et al., 2013; Zhang et al., 2014; Zhang et al., 2015; Chen et al., 2018; Liang et al., 2019). The presence of wattles in pigs, which are defined as appendages suspended from the mandibular contiguous region of the neck, has been studied by few authors so far. These works proposed that in pigs this phenotype could be controlled by a single locus, with a dominant mode of inheritance based on two alleles ( $Wa^W$ , dominant for the presence;  $Wa^+$ , for the absence; Kronacher, 1924; Roberts and Morrill, 1944; Sabbioni et al., 2011).

The genetics of coat colours has been largely studied in pigs (reviewed in Fontanesi and Russo, 2013). The black coat colour (a morph allowed in Casertana) is mainly determined by dominant alleles at the *Extension* locus, which is encoded by the melanocortin 1 receptor (*MC1R*) gene (Kijas et al., 1998). Casertana is not fixed at this locus as recently reported by Muñoz et al. (2018) and Ribani et al. (2019) and at least four *MC1R* alleles segregate in this breed. The genetic determinism of the other colour morph allowed in Casertana (slate grey) is not well defined in pigs yet. A few studies reported the effect of *KIT* gene alleles on roan or grey phenotypes and QTL studies suggested the presence of a few chromosome regions that might contribute to define greyish coat colours in *Sus scrofa* (Hirooka et al., 2002; Fontanesi et al., 2010).

In this study, we took advantage from the phenotypic variability of several exterior traits (two ear morphological traits, presence or absence of wattles and different coat colours) that we recorded in a Casertana pig population to carry out GWAS and comparative genome-wide  $F_{ST}$  analyses with the final objectives to identify genomic regions that could affect the observed heterogeneity in this local pig breed.

## 2. Materials and methods

## 2.1. *Animals and traits*

This study did not have any ethical implications as animals were not treated in any way. Operations on the animals were carried out under routine veterinary inspections. All animals were raised according to national and European legislation and not ethical permit was therefore needed.

A total of 101 Casertana pigs (of about 7 to 20 months old) from six different farms were included in this study. Photographic records and subjective evaluations of each animal were obtained to capture information on several traits: ear size (small or large); ear bending (forward or floppy); presence or absence of wattles. Ear size was defined according to an approximation based on the ratio between the ear length (EL) and the snout length (SL): small, when  $EL/SL \leq 1/3$ ; large when  $EL/SL \geq 2/3$ . A few pigs with intermediate EL/SL ratio were not considered in this study that attempted to capture extreme differences considering the small population size that was analysed. Ear size was based on a relative parameter calculated from photographic records, as precise measures could not be obtained due to the difficulties to immobilize the head of the animals in the routine operating conditions of the extensive production systems in which animals were kept. In addition, this relative measure could overcome the problem derived by the age and size effect of the animals that could not be determined. Table 1 summaries the number of animals that were included in the different classes for the four mentioned traits. Figure 1 reports a few examples of the recorded phenotype variability in Casertana pigs.

## 2.2. *Genotyping of single nucleotide polymorphisms*

Hair roots, collected from the studied Casertana pigs, were used for DNA extraction that was obtained with the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. The Illumina PorcineSNP60 BeadChip v.2 (Illumina, Inc., San Diego, CA, USA) was used for the genotyping of 61,565 single nucleotide polymorphisms (SNPs). The positions of the markers were assigned by applying the mapping method described in Fontanesi et al. (2012) and using the Sscrofa11.1 genome version as reference.

122 Genotyping data were filtered using PLINK 1.9 software (Chang et al., 2015) using the following  
123 criteria already used in similar studies (Bertolini et al., 2018; Schiavo et al., 2018): genotyping call  
124 rate >0.9, minor allele frequency (MAF) >0.01 and Hardy-Weinberg equilibrium  $P>0.001$ . After  
125 filtering, the number of SNPs in the GWAS for ear size, ear bearing, presence/absence of wattles and  
126 coat colours was 39,732, 41,027, 38,423 and 41,230, respectively.

127

### 128 **2.3. Genome-wide analyses**

129 Multidimensional scaling (MDS) plots were obtained with the PLINK 1.9 software (Chang et  
130 al., 2015) to evaluate distance relationships among the animals of the investigated cohort included in  
131 the different comparative genome-wide analyses for the considered exterior traits. In another work,  
132 aimed to the selection of a SNP panel for breed classification (Schiavo et al., 2020), the same  
133 individuals have been correctly assigned to Casertana breed without any classification errors to other  
134 breeds. This indicates that the eventual clusters in MDS plot are only due to within breed stratification  
135 problems. Genome-wide association studies were carried out by applying the univariate mixed model  
136 of GEMMA (Zhou and Stephens, 2012) that can accommodate the centered relatedness matrix  
137 calculated from SNP genotypes to correct for population stratification in a case and control analysis.  
138 The model applied for the different exterior traits included the farm and the sex as fixed effects. To  
139 be able to identify markers affecting exterior traits in this experiment that could include a low number  
140 of animals and considering the fact that the used SNP chip might have an ascertain bias (derived by  
141 the fact that local breeds were not used for the selection of the informative markers), the significant  
142 threshold was defined at the  $P_{nominal\ value}<5.00E-05$  level, as already applied in several other GWAS  
143 in livestock (e.g. Fontanesi et al., 2012; Sanchez et al., 2014) and in similar analyses for the Casertana  
144 breed (Bertolini et al., 2018; Schiavo et al., 2018). GenABEL (Aulchenko et al., 2007) was used to  
145 obtain genomic inflation factors ( $\lambda$ ) and quantile–quantile (Q–Q) plots for the GWAS.

146 To confirm the results of the GWAS,  $F_{ST}$  analyses were performed on the same dataset and for  
147 each trait using PLINK 1.9 software (Chang et al., 2015), by comparing single markers between the



148 groups of pigs with alternative phenotypes for each analysed exterior trait. Relevant  $F_{ST}$  differences  
149 were defined considering the SNPs over the 99.9th percentile distribution.

150 Gene annotation of the genomic regions identified by both genome-wide association and  
151 genome-wide  $F_{ST}$  analyses was retrieved from the Sscrofa11.1 genome version available at the  
152 Ensembl database ([http://www.ensembl.org/Sus\\_scrofa/Info/Index](http://www.ensembl.org/Sus_scrofa/Info/Index)), release 97 (June 2019),  
153 considering 500 kbp from both sides of the significant markers.

154

### 155 3. Results and discussion

156 A recent phenotypic characterization of the autochthonous Casertana pig population that we  
157 carried out evidenced several morphological differences, including the presence or absence of hairs  
158 and the shape of the tail, among the animals of this local breed. Despite the number of animals  
159 involved was limited, this diversity made it possible to successfully identify SNPs associated to these  
160 exterior traits using genome-wide analyses and, in turn, to identify candidate genes based on the  
161 annotations in the corresponding genome regions (Bertolini et al., 2018; Schiavo et al., 2018).

162 In this phenotypic survey of the Casertana population, we also reported differences for two ear  
163 phenotypes (size and position), presence or absence of wattles and coat colours (Figure 1; Table 1),  
164 that we explored in genome-wide analyses to identify markers associated with the observed  
165 phenotypic variability.

166 Figure 2 shows the Manhattan plots obtained in the GWAS and genome-wide  $F_{ST}$  analyses for  
167 the four investigated exterior traits. Q-Q plots obtained for the GWAS are reported in Figure S1.  
168 Table 2 lists the markers that were confirmed by the two genome-wide approaches, as they passed  
169 the thresholds in both analyses for the different considered traits.

170 Ear size was classified into two categories (small and large, 51% and 49% of the pigs,  
171 respectively). Phenotypic characterization of ear size was opportunistic for practical reasons.  
172 Therefore, only a total of 37 animals that could be classified for this phenotype were included in the  
173 genome-wide analyses. Multidimensional scaling plot based on these two ear size categories

174 identified potential substructures in the analysed sub-populations, defined according to this  
175 approximated phenotype (Figure S2). The GWAS, that according to the  $\lambda$  value (1.044) was able to  
176 correct most of the observed stratification in this small sample of pigs, identified two significant  
177 markers ( $P=2.52E-6$ ) on porcine chromosome (SSC) 9 (MARC0047658, at position 130,362,191;  
178 ASGA0085666, at position 130,366,477). This result was however not confirmed by the  $F_{ST}$  analysis  
179 that identified an outlier signal on SSC9 (ALGA0118777, at position 125,868,947), about 5 Mbp  
180 apart from the GWAS significant SNPs. Using an F2 population established by crossing European  
181 Large White with Chinese Meishan pigs, Wei et al. (2007) reported a large QTL region for ear size  
182 on SSC9 that might also include the positions we identified in Casertana pigs.

183 Ear bearing included two clearly distinguished conformations in Casertana pigs, forward (28%)  
184 and floppy (72%; Table 1). The MDS plot of the animals classified with these two ear morphological  
185 classes (Figure S2) indicated some potential stratification in the analysed population that was possible  
186 to partially correct in the GWAS that had a  $\lambda$  equal to 1.124. The  $F_{ST}$  analysis indicated several signals  
187 in different chromosomes (Figure 2) that however were not supported by any results in the GWAS,  
188 which did not identify any significant markers.

189 Weak or absence of significant and confirmed genomic regions for the two ear conformation  
190 traits might be due to the quantitative genetic architecture that might control these traits in pigs (Wei  
191 et al., 2007; Ma et al., 2009) and that could not be dissected by the low number of analysed animals.  
192 Simplified phenotypic categories were also used for ear size that might not completely explain the  
193 phenotypic variability for this ear trait. Previous studies that investigated ear size and shape in pigs  
194 included hundreds of phenotyped animals and the most significant markers identified on a few  
195 chromosomes (i.e. SSC5 and SSC7) explained just a fraction of the total genetic variability (Wei et  
196 al., 2007; Ma et al., 2009). Therefore, the absence of meaningful results obtained in our study may  
197 mean that also in Casertana these ear conformation traits cannot be explained by simple Mendelian  
198 inherited genes.

199 About 23% of the Casertana pigs considered in this study had wattles. Animals with one (n. =  
 200 1) or two wattles (n. = 10) were considered as having wattles without any distinction, according to  
 201 previous studies that defined the genetic transmission model of this morphological trait in pigs  
 202 (Kronacher, 1924; Roberts and Morrill, 1944; Sabbioni et al., 2011). The remaining animals for which  
 203 this phenotype was collected (77%) did not have any appendages suspended from the mandibular  
 204 contiguous region of the neck (Table 1; Figure 1). The MDS plot of the pigs with or without wattles  
 205 showed some potential sub-structures of the analysed population (Figure S2) that however were  
 206 corrected in the GWAS, that had a genomic inflation factor equal to 1.005. This association study  
 207 identified two significant markers on SSC11 (MARC0021800, at position 14,148,820,  $P = 1.40E-06$ ;  
 208 and DRGA0010900, at position 14,357,170,  $P = 3.27E-05$ ) and two markers on SSC1 (H3GA005056,  
 209 at position 268,698,293,  $P = 3.27E-05$ ; and INRA0007668, at position 268,102,753,  $P = 4.51E-05$ )  
 210 that were confirmed by the  $F_{ST}$  analysis (Figure 2 and Table 2). The SSC11 region including the  
 211 significant markers harbors seven annotated genes (Table S1). Among them, the FRAS1 related  
 212 extracellular matrix protein 2 (*FREM2*) gene (positions 13,959,865-14,154,754) seems an interesting  
 213 candidate for this phenotype. In humans, mutations in this gene cause the Fraser syndrome, which is  
 214 a multisystem disorder characterized by epidermal blistering, syndactyly and a range of other  
 215 developmental abnormalities (Jadeja et al., 2005; Smyth and Scambler, 2005). The other two  
 216 significant markers located on SSC1 identified a region harbouring 35 annotated genes. This SSC1  
 217 gene rich region includes the nuclear receptor subfamily 6 group A member 1 (*NR6A1*) gene,  
 218 encoding for a DNA-binding transcription factor involved in embryonal developmental stages and  
 219 affecting vertebral numbers in pigs (Mikawa et al., 2007; Duan et al., 2018). The causative mutation  
 220 having an effect on the number of vertebrae (p.P192L; Mikawa et al., 2007) was genotyped in these  
 221 Casertana pigs by Ribani et al. (2019) but no significant association could be observed between this  
 222 *NR6A1* missense mutation and the presence of wattles (data not shown). Therefore, other mutations  
 223 in this gene (or other close genes) could affect, at least in part, the wattle formation in some animals.  
 224 Partial penetrance or oligogenic models should be also considered to explain the genetic architecture

225 of this trait, considering that we identified significant markers in at least two different chromosomes,  
226 despite classical genetic studies indicated that only one locus might be involved in pigs to determine  
227 the presence or absence of wattles (Kronacher, 1924; Roberts and Morrill, 1944; Sabbioni et al.,  
228 2011).

229 Two coat colours were reported in the investigated Casertana population. About 17.5% of the  
230 pigs were black whereas 82.5% had a solid slate grey coat colour (Table 1; Figure 1). Stratification  
231 of the population was present when these two groups of pigs were plotted in an MDS representation  
232 (Figure S2). Genome-wide association analysis, that was not completely able to correct this bias ( $\lambda =$   
233 1.235), identified several significant SNPs, some of which (most on SSC6, one on SSC8, one on  
234 SSC14 and two on SSC15; Table 2) were confirmed in the  $F_{ST}$  analysis. The confirmed SNPs of SSC6  
235 were not located in the *MC1R* region, excluding variability in this gene as being the main driver of  
236 this coat colour diversity. Other positions on SSC6 harbored confirmed SNPs, namely regions at  
237 about 80-81 Mbp, 86.5 Mbp, 125-127 Mbp and 136-137 Mbp in which no obvious candidate genes  
238 could be located (Table S1). The same might be applied for the signals on SSC14 and SSC15, in  
239 which no candidate genes could be identified according to the functions and roles of the annotated  
240 genes in these regions (Table S1). Among the annotated genes around the signal on SSC8, the  
241 fibroblast growth factor receptor 3 (*FGFR3*) gene (positions 879,207-895,912) plays different roles  
242 in several skin processes, which might be relevant for the pigmentation in the hairless Casertana pigs.  
243 In humans, mutations in this gene cause acanthosis nigricans, a pigmentary skin disorder associated  
244 with obesity (Fukuchi et al., 2018), that also might match the high adipogenic potential of the  
245 Casertana pigs.

246 Taking together all results reported in this study for the four considered morphological traits  
247 and what we previously provided in the same Casertana population for the hairless and tail shape  
248 phenotypes (Bertolini et al., 2018; Schiavo et al., 2018), it is interesting to note that in most cases it  
249 was possible to obtain hints that could explain the genetic mechanisms controlling the expression of  
250 simple traits, despite the limits that derived by the small population size. As expected, complex

quantitative traits could not be clearly dissected in this simple experimental design and local pig populations might not be useful for these purposes.

## **Conclusion**

The experimental design, based on a small number of phenotyped and genotyped pigs, posed a few challenging questions that we tried to solve by combining two different genome-wide approaches and by adding a candidate gene analysis based on the functions of the genes located in the genomic regions that were confirmed by the two genomic methods (i.e. GWAS and genome-wide  $F_{ST}$  approaches).

Critically evaluating the obtained results, the applied strategy made it possible to identify candidate genes that could be involved in affecting expected monogenic (or oligogenic) traits, like presence of wattles or coat colours. Potential stratifications that could be in some cases not solved might have introduced false positive signals (i.e. for the coat colour GWAS) that, in part, could be disentangled considering the function of the underlying annotated genes and could be improved by additional annotation and functional characterization of these genes. It is also clear that it would be important to support these results by increasing the number of involved pigs, that however, in the case of this local pig breed, is a challenging solution, considering the low number of available Casertana pigs and the difficulties in collecting data and biological materials in extensive systems. The parallel addition to the study of animals of other breeds (in a multibreed analysis) with similar phenotypes might strengthen the power in detecting genomic regions associated with peculiar exterior (potentially monogenic) traits. This strategy could provide additional values to local and untapped animal genetic resources that might be useful to dissect phenotypic traits that cannot be genetically characterized using commercial or cosmopolitan populations.

## **Declaration of competing interests**

The authors declare no conflict of interest

277

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286

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409 **Table 1.** Phenotypic descriptions and numbers of the Casertana pigs included in this study.

| Trait       | Condition/description | N. of males | N. of females | Total number <sup>†</sup> |
|-------------|-----------------------|-------------|---------------|---------------------------|
| Ear size    | Small                 | 4           | 15            | 19                        |
|             | Large                 | 10          | 8             | 18                        |
| Ear bearing | Forward               | 10          | 14            | 24                        |
|             | Floppy                | 32          | 29            | 61                        |
| Wattles     | Presence              | 5           | 6             | 11                        |
|             | Absence               | 13          | 24            | 37                        |
| Coat colour | Black                 | 5           | 12            | 17                        |
|             | Slate-grey            | 31          | 49            | 80                        |

410

411 <sup>†</sup> The phenotypic description of a few animal/trait combinations was not obtained.

**Table 2.** List of single nucleotide polymorphisms (SNPs) associated with the investigated traits that were confirmed by the  $F_{ST}$  analysis. Annotation of the different chromosome regions is reported in Table S1.

| Traits                      | SSC <sup>†</sup> | Position <sup>‡</sup> | SNP         | P-value GWAS | F <sub>ST</sub> value | Alleles <sup>§</sup> | AF <sup>¶</sup> |
|-----------------------------|------------------|-----------------------|-------------|--------------|-----------------------|----------------------|-----------------|
| Presence/absence of wattles | 1                | 268102753             | INRA0007668 | 4.51E-05     | 0.325                 | G/A                  | 0.932           |
|                             | 1                | 268698293             | H3GA0005056 | 3.27E-05     | 0.340                 | A/C                  | 0.450           |
|                             | 11               | 14148820              | MARC0021800 | 1.40E-06     | 0.397                 | G/A                  | 0.920           |
|                             | 11               | 14357170              | DRGA0010900 | 3.27E-05     | 0.340                 | G/A                  | 0.955           |
| Coat colours                | 6                | 79999211              | MARC0056786 | 6.56E-07     | 0.266                 | C/A                  | 0.219           |
|                             | 6                | 80108499              | MARC0028970 | 1.59E-06     | 0.235                 | A/G                  | 0.214           |
|                             | 6                | 80241553              | ASGA0028642 | 1.59E-06     | 0.235                 | A/G                  | 0.214           |
|                             | 6                | 80459834              | ASGA0093075 | 1.59E-06     | 0.235                 | A/G                  | 0.214           |
|                             | 6                | 80491095              | MARC0045097 | 4.53E-06     | 0.237                 | A/G                  | 0.672           |
|                             | 6                | 80778880              | ASGA0028671 | 1.59E-06     | 0.235                 | A/G                  | 0.214           |
|                             | 6                | 80873393              | H3GA0018314 | 1.59E-06     | 0.235                 | G/A                  | 0.214           |
|                             | 6                | 81130094              | ASGA0101946 | 1.59E-06     | 0.235                 | G/A                  | 0.214           |
|                             | 6                | 81326625              | ASGA0099275 | 1.59E-06     | 0.235                 | A/G                  | 0.214           |
|                             | 6                | 86543912              | ALGA0108236 | 6.76E-07     | 0.246                 | G/A                  | 0.755           |
|                             | 6                | 125700603             | ALGA0036595 | 2.15E-06     | 0.331                 | A/G                  | 0.359           |
|                             | 6                | 126122455             | ALGA0036616 | 2.47E-06     | 0.331                 | A/C                  | 0.359           |
|                             | 6                | 127514190             | ALGA0011111 | 1.71E-06     | 0.340                 | A/C                  | 0.354           |
|                             | 6                | 136414674             | ASGA0029542 | 3.20E-06     | 0.283                 | A/G                  | 0.344           |
|                             | 6                | 136634162             | MARC0070940 | 5.31E-06     | 0.267                 | C/A                  | 0.604           |
|                             | 6                | 136701217             | CASI0009826 | 9.55E-07     | 0.323                 | G/A                  | 0.323           |
|                             | 6                | 136921345             | ASGA0029563 | 1.20E-06     | 0.312                 | A/G                  | 0.651           |
|                             | 8                | 1186987               | ALGA0046044 | 1.88E-05     | 0.259                 | G/A                  | 0.802           |
|                             | 14               | 72379700              | ALGA0079568 | 5.12E-06     | 0.302                 | A/G                  | 0.333           |
|                             | 15               | 27126842              | ASGA0069071 | 3.02E-05     | 0.262                 | A/C                  | 0.505           |
|                             | 15               | 29094014              | MARC0105811 | 5.34E-06     | 0.292                 | G/A                  | 0.661           |

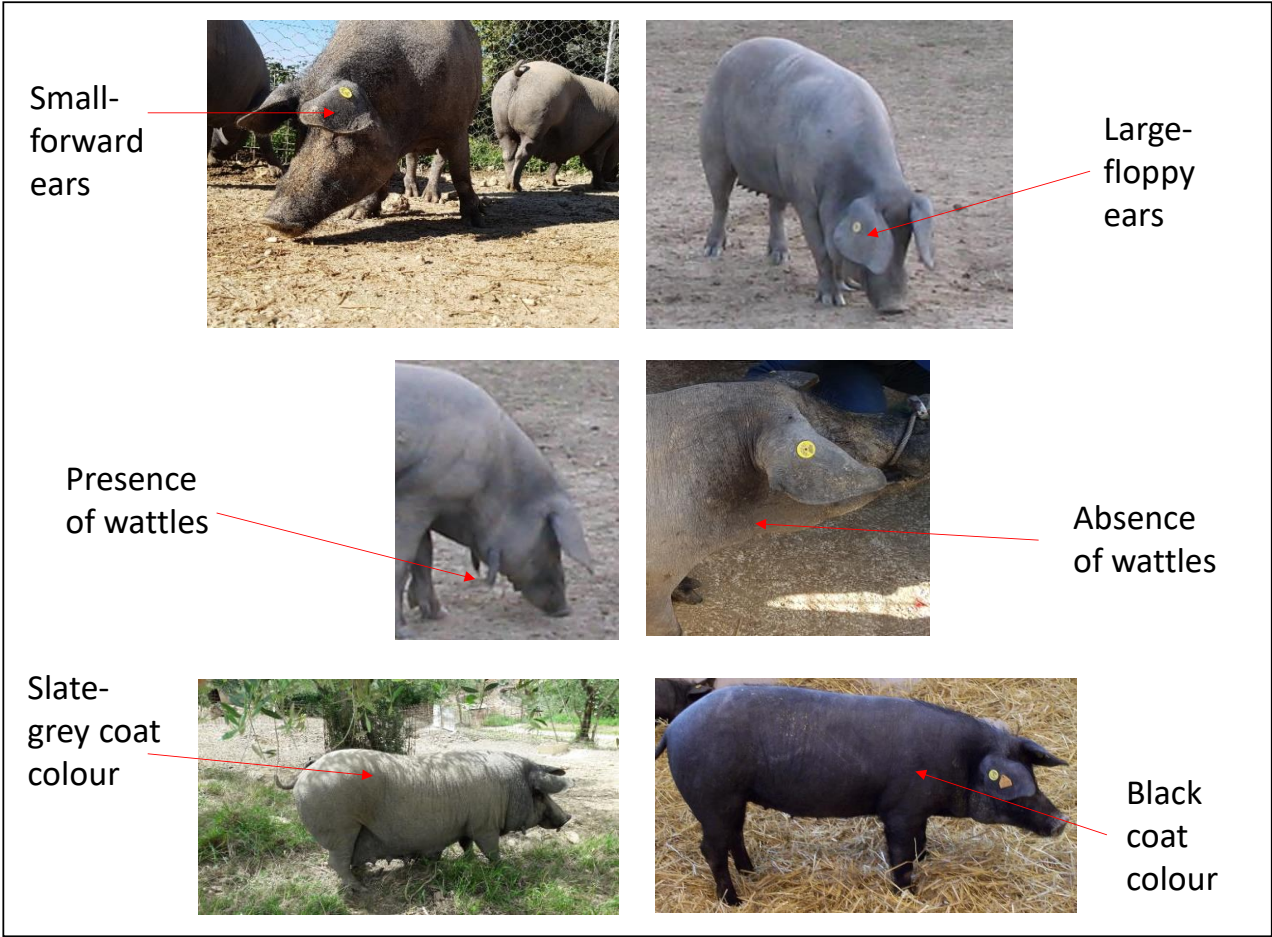
<sup>†</sup> *Sus scrofa* chromosome.

<sup>‡</sup> Position on the indicated chromosome retrieved from Sscrofa11.1 genome version.

416   § Alleles. The first nucleotide is that of the reference genome.

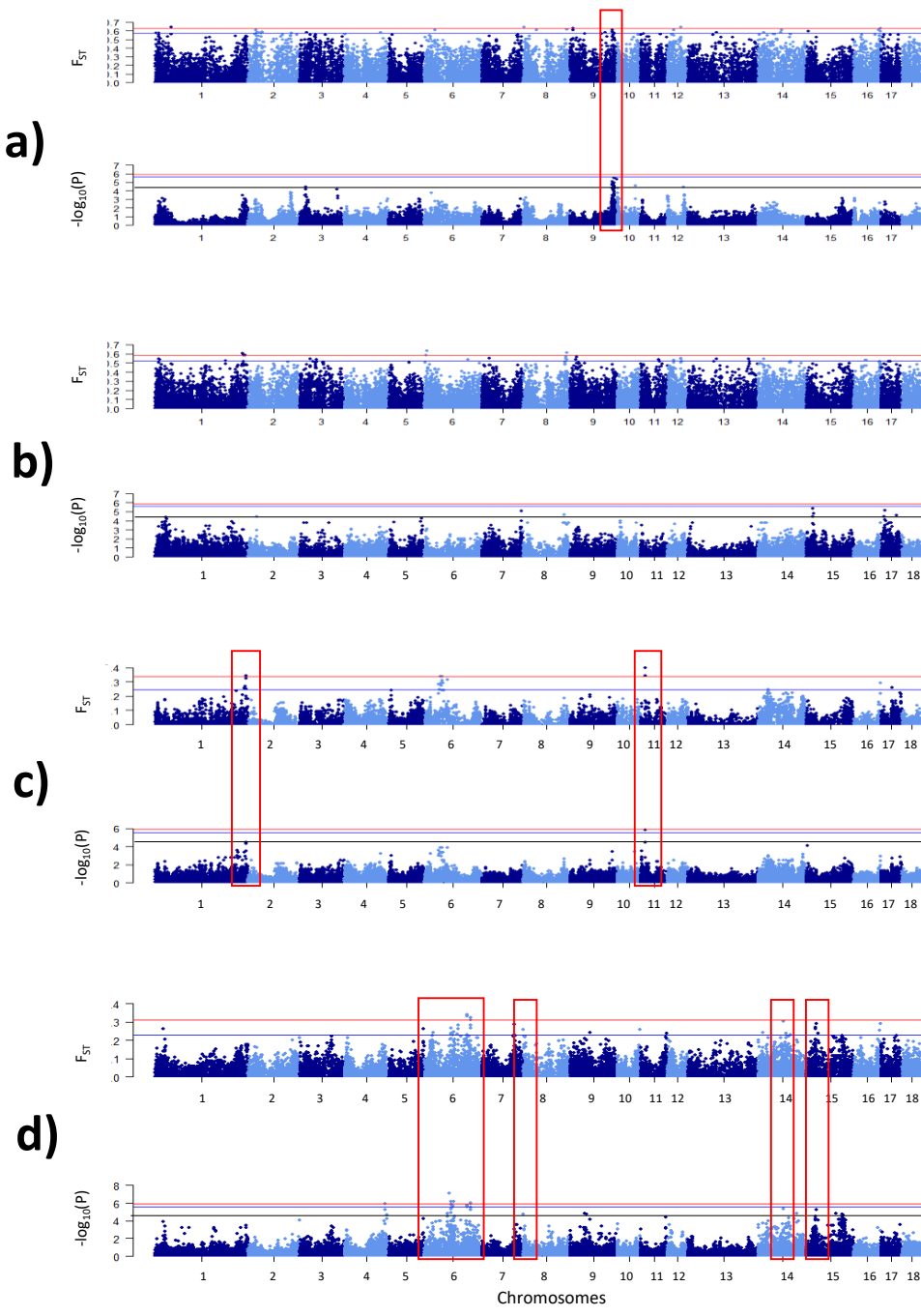
417   ¶ Frequency of the first reported nucleotide in the “allele” column.

418 **Figure 1.** Examples of Casertana pigs with different exterior traits such as ear size (small or large),  
419 ear bearing (forward or floppy), presence or absence of wattles and coat colour (slate-grey or  
420 black).



421

422 **Figure 2.** Manhattan plots of the genome-wide  $F_{ST}$  analyses and genome-wide association studies  
 423 for the four investigated exterior traits: a) ear size; b) ear bearing; c) presence or absence of wattles;  
 424 d) coat colours. Thresholds in the  $F_{ST}$  plots are for the 99.99th percentile (red line) or for the 99.9th  
 425 percentile (blue line). Thresholds in the genome-wide association studies are for p-value = 0.05  
 426 Bonferroni corrected (red line), 0.10 Bonferroni corrected (blue line) or for p-value = 5.0E-05  
 427 (black line). Significant single nucleotide polymorphism regions described in the text are evidenced  
 428 in red.





430 **Supplementary material**

431

432 **Table S1.** Genes included in the chromosome regions harboring single nucleotide polymorphisms (SNPs) associated with the presence or absence  
 433 of wattle and coat colours (slate-grey or black) in Casertana pigs. Chromosome regions discussed in the text were defined considering contiguous  
 434 SNPs and  $\pm 500$  kbp from the first and last SNPs in the detected regions.

| Trait/Chromosome region <sup>†</sup> | SSC <sup>‡</sup> | Gene start (bp) <sup>§</sup> | Gene end (bp) <sup>¶</sup> | Strand | Gene symbol       | Gene Ensembl ID     |
|--------------------------------------|------------------|------------------------------|----------------------------|--------|-------------------|---------------------|
| Wattle/SSC1_region1                  | 1                | 267480768                    | 267770444                  | 1      | <i>RALGPS1</i>    | ENSSSCG00000005607  |
|                                      | 1                | 267639638                    | 267672923                  | -1     | <i>ANGPTL2</i>    | ENSSSCG00000005608  |
|                                      | 1                | 267771636                    | 267923390                  | 1      | <i>GARNL3</i>     | ENSSSCG00000005609  |
|                                      | 1                | 267833052                    | 267833154                  | -1     | <i>RF00026</i>    | ENSSSCG000000020000 |
|                                      | 1                | 267925189                    | 267934094                  | 1      | <i>SLC2A8</i>     | ENSSSCG00000005610  |
|                                      | 1                | 267941953                    | 267959747                  | 1      | <i>ZNF79</i>      | ENSSSCG00000005611  |
|                                      | 1                | 267958456                    | 267965555                  | -1     |                   | ENSSSCG000000033019 |
|                                      | 1                | 267962230                    | 267962359                  | -1     | <i>RF00302</i>    | ENSSSCG000000018580 |
|                                      | 1                | 267964929                    | 268008830                  | 1      | <i>LRSAM1</i>     | ENSSSCG00000005613  |
|                                      | 1                | 268010307                    | 268066016                  | -1     | <i>FAM129B</i>    | ENSSSCG00000005614  |
|                                      | 1                | 268116124                    | 268205801                  | 1      | <i>STXBP1</i>     | ENSSSCG00000005617  |
|                                      | 1                | 268209443                    | 268213894                  | 1      | <i>CFAP157</i>    | ENSSSCG00000005618  |
|                                      | 1                | 268213686                    | 268215523                  | -1     | <i>PTRH1</i>      | ENSSSCG00000005615  |
|                                      | 1                | 268216049                    | 268237839                  | 1      | <i>TTC16</i>      | ENSSSCG00000005616  |
|                                      | 1                | 268233661                    | 268237404                  | -1     | <i>TOR2A</i>      | ENSSSCG00000005619  |
|                                      | 1                | 268237679                    | 268270361                  | -1     | <i>SH2D3C</i>     | ENSSSCG00000005620  |
|                                      | 1                | 268276841                    | 268283860                  | 1      | <i>CDK9</i>       | ENSSSCG00000005621  |
|                                      | 1                | 268287729                    | 268309406                  | 1      | <i>FPGS</i>       | ENSSSCG00000005626  |
|                                      | 1                | 268306120                    | 268343936                  | -1     | <i>ENG</i>        | ENSSSCG00000005625  |
|                                      | 1                | 268355035                    | 268365598                  | -1     | <i>AK1</i>        | ENSSSCG00000005627  |
|                                      | 1                | 268372867                    | 268383544                  | -1     |                   | ENSSSCG00000005623  |
|                                      | 1                | 268389483                    | 268402517                  | -1     | <i>ST6GALNAC4</i> | ENSSSCG00000005628  |
|                                      | 1                | 268408129                    | 268439109                  | 1      |                   | ENSSSCG000000031500 |

|   |           |           |    |                 |                    |
|---|-----------|-----------|----|-----------------|--------------------|
| 1 | 268409449 | 268417562 | -1 | <i>PIP5KL1</i>  | ENSSSCG00000005629 |
| 1 | 268418445 | 268423560 | -1 | <i>DPM2</i>     | ENSSSCG00000032481 |
| 1 | 268427708 | 268465802 | -1 | <i>FAM102A</i>  | ENSSSCG00000005631 |
| 1 | 268527327 | 268530563 | -1 | <i>NAIF1</i>    | ENSSSCG00000005630 |
| 1 | 268531752 | 268576994 | 1  | <i>SLC25A25</i> | ENSSSCG00000005636 |
| 1 | 268581835 | 268590836 | -1 | <i>PTGES2</i>   | ENSSSCG00000022655 |
| 1 | 268609633 | 268621997 | 1  | <i>LCN2</i>     | ENSSSCG00000005638 |
| 1 | 268616034 | 268619637 | 1  |                 | ENSSSCG00000022295 |
| 1 | 268620965 | 268640934 | -1 | <i>CIZ1</i>     | ENSSSCG00000005640 |
| 1 | 268653174 | 268701674 | 1  | <i>DNM1</i>     | ENSSSCG00000005641 |
| 1 | 268691502 | 268691610 | -1 | <i>MIR199B</i>  | ENSSSCG00000019744 |
| 1 | 268701511 | 268721176 | -1 |                 | ENSSSCG00000005643 |
| 1 | 268720306 | 268727670 | 1  |                 | ENSSSCG00000032748 |
| 1 | 268746329 | 268757458 | -1 | <i>TRUB2</i>    | ENSSSCG00000005645 |
| 1 | 268756129 | 268767931 | 1  | <i>COQ4</i>     | ENSSSCG00000005646 |
| 1 | 268773861 | 268788207 | 1  | <i>SLC27A4</i>  | ENSSSCG00000005648 |
| 1 | 268798425 | 268816348 | 1  | <i>URM1</i>     | ENSSSCG00000005649 |
| 1 | 268816961 | 268817057 | -1 | <i>MIR219B</i>  | ENSSSCG00000027037 |
| 1 | 268827445 | 268827664 | 1  | <i>RF00012</i>  | ENSSSCG00000019510 |
| 1 | 268838055 | 268847233 | 1  | <i>CERCAM</i>   | ENSSSCG00000005650 |
| 1 | 268856766 | 268894210 | 1  | <i>ODF2</i>     | ENSSSCG00000005651 |
| 1 | 268894276 | 268922750 | 1  | <i>GLE1</i>     | ENSSSCG00000005652 |
| 1 | 268924948 | 268927232 | 1  |                 | ENSSSCG00000035449 |
| 1 | 268936026 | 269003036 | 1  | <i>SPTAN1</i>   | ENSSSCG00000005654 |
| 1 | 269002245 | 269030719 | -1 | <i>WDR34</i>    | ENSSSCG00000005655 |
| 1 | 269008363 | 269009987 | 1  |                 | ENSSSCG00000033804 |
| 1 | 269056717 | 269064634 | 1  |                 | ENSSSCG00000005656 |
| 1 | 269069388 | 269081016 | 1  | <i>PKN3</i>     | ENSSSCG00000005657 |
| 1 | 269081272 | 269084849 | -1 |                 | ENSSSCG00000005658 |
| 1 | 269090333 | 269122156 | -1 | <i>ZER1</i>     | ENSSSCG00000005659 |
| 1 | 269134527 | 269153066 | 1  | <i>TBC1D13</i>  | ENSSSCG00000005660 |
| 1 | 269154090 | 269157788 | 1  | <i>ENDOG</i>    | ENSSSCG00000005661 |
| 1 | 269154639 | 269167196 | -1 | <i>SPOUT1</i>   | ENSSSCG00000005662 |
| 1 | 269172628 | 269209494 | -1 |                 | ENSSSCG00000005663 |

|                          |    |          |          |    |                |                    |
|--------------------------|----|----------|----------|----|----------------|--------------------|
| Wattle/SSC11_region1     | 11 | 13765501 | 13774791 | 1  | <i>UFM1</i>    | ENSSSCG00000031224 |
|                          | 11 | 13959865 | 14154754 | 1  | <i>FREM2</i>   | ENSSSCG00000009364 |
|                          | 11 | 14131211 | 14147122 | -1 | <i>STOML3</i>  | ENSSSCG00000009366 |
|                          | 11 | 14151151 | 14183851 | -1 | <i>PROSER1</i> | ENSSSCG00000040697 |
|                          | 11 | 14184134 | 14370915 | 1  | <i>NHLRC3</i>  | ENSSSCG00000037746 |
|                          | 11 | 14319425 | 14576135 | -1 | <i>LHFPL6</i>  | ENSSSCG00000029855 |
|                          | 11 | 14576214 | 14723157 | 1  | <i>COG6</i>    | ENSSSCG00000009369 |
| Coat colour/SSC6_region1 | 6  | 79589691 | 79649644 | 1  | <i>ALPL</i>    | ENSSSCG00000032607 |
|                          | 6  | 79662137 | 79735361 | -1 | <i>RAP1GAP</i> | ENSSSCG00000022029 |
|                          | 6  | 79744936 | 79810724 | -1 | <i>USP48</i>   | ENSSSCG00000024197 |
|                          | 6  | 79850757 | 79921097 | -1 | <i>HSPG2</i>   | ENSSSCG00000003514 |
|                          | 6  | 79968501 | 79972061 | -1 |                | ENSSSCG00000033553 |
|                          | 6  | 79995339 | 80002723 | 1  |                | ENSSSCG00000037400 |
|                          | 6  | 80012212 | 80017669 | 1  |                | ENSSSCG00000030015 |
|                          | 6  | 80035048 | 80090934 | 1  |                | ENSSSCG00000003520 |
|                          | 6  | 80068259 | 80088564 | -1 |                | ENSSSCG00000035899 |
|                          | 6  | 80112277 | 80139670 | -1 |                | ENSSSCG00000003521 |
|                          | 6  | 80419826 | 80485378 | 1  | <i>ZBTB40</i>  | ENSSSCG00000026276 |
|                          | 6  | 80521572 | 80548696 | 1  | <i>EPHA8</i>   | ENSSSCG00000003523 |
|                          | 6  | 80586183 | 80591269 | 1  | <i>C1QA</i>    | ENSSSCG00000003524 |
|                          | 6  | 80592198 | 80599440 | 1  | <i>C1QC</i>    | ENSSSCG00000038706 |
|                          | 6  | 80601303 | 80608011 | 1  | <i>C1QB</i>    | ENSSSCG00000033512 |
|                          | 6  | 80649143 | 80843567 | 1  | <i>EPHB2</i>   | ENSSSCG00000003527 |
|                          | 6  | 80870866 | 80882391 | -1 | <i>LACTBL1</i> | ENSSSCG00000003529 |
|                          | 6  | 80896656 | 80898939 | -1 | <i>TEX46</i>   | ENSSSCG00000003530 |
|                          | 6  | 80903358 | 80973632 | 1  | <i>KDM1A</i>   | ENSSSCG00000003531 |
|                          | 6  | 80931318 | 80931385 | 1  | <i>MIR3115</i> | ENSSSCG00000029242 |
|                          | 6  | 80973261 | 81112327 | -1 | <i>LUZP1</i>   | ENSSSCG00000003532 |
|                          | 6  | 81089662 | 81112347 | -1 |                | ENSSSCG00000025500 |
|                          | 6  | 81196062 | 81239387 | -1 | <i>HNRNPR</i>  | ENSSSCG00000023761 |
|                          | 6  | 81250123 | 81261853 | -1 | <i>ZNF436</i>  | ENSSSCG00000035332 |
|                          | 6  | 81268514 | 81304581 | -1 | <i>TCEA3</i>   | ENSSSCG00000032674 |
|                          | 6  | 81309748 | 81360248 | -1 | <i>ASAP3</i>   | ENSSSCG00000028108 |
|                          | 6  | 81376652 | 81398346 | -1 | <i>E2F2</i>    | ENSSSCG00000032710 |

|                          |   |           |           |    |                   |                     |
|--------------------------|---|-----------|-----------|----|-------------------|---------------------|
|                          | 6 | 81422546  | 81424169  | -1 | <i>ID3</i>        | ENSSSCG00000039514  |
|                          | 6 | 81509567  | 81519072  | 1  |                   | ENSSSCG00000038035  |
|                          | 6 | 81522760  | 81526880  | 1  | <i>RPL11</i>      | ENSSSCG00000024260  |
|                          | 6 | 81566801  | 81582028  | 1  | <i>ELOA</i>       | ENSSSCG00000025440  |
|                          | 6 | 81590951  | 81598872  | 1  | <i>PITHD1</i>     | ENSSSCG00000023495  |
|                          | 6 | 81601486  | 81605912  | 1  | <i>LYPLA2</i>     | ENSSSCG00000023191  |
|                          | 6 | 81605918  | 81630365  | -1 | <i>GALE</i>       | ENSSSCG00000027827  |
|                          | 6 | 81610427  | 81630470  | -1 | <i>HMGCL</i>      | ENSSSCG00000026025  |
|                          | 6 | 81637698  | 81723231  | -1 | <i>FUCA1</i>      | ENSSSCG00000027659  |
|                          | 6 | 81659186  | 81692550  | -1 | <i>CNR2</i>       | ENSSSCG00000027849  |
|                          | 6 | 81706663  | 81741526  | -1 | <i>SRSF10</i>     | ENSSSCG00000036293  |
|                          | 6 | 81722026  | 81726489  | 1  | <i>PNRC2</i>      | ENSSSCG00000023326  |
|                          | 6 | 81780869  | 81780977  | 1  | <i>RF00026</i>    | ENSSSCG00000024834  |
|                          | 6 | 81799570  | 81903971  | -1 | <i>MYOM3</i>      | ENSSSCG00000036534  |
| Coat colour/SSC6_region2 | 6 | 85891364  | 86131721  | -1 | <i>SRSF4</i>      | ENSSSCG00000003589  |
|                          | 6 | 86085281  | 86085423  | -1 | <i>RF02271</i>    | ENSSSCG000000033491 |
|                          | 6 | 86130111  | 86130217  | -1 | <i>RF00026</i>    | ENSSSCG000000031401 |
|                          | 6 | 86137754  | 86228145  | 1  | <i>PTPRU</i>      | ENSSSCG00000003590  |
| Coat colour/SSC6_region3 | 6 | 125354757 | 125493965 | -1 |                   | ENSSSCG000000038382 |
|                          | 6 | 125890532 | 126043141 | 1  | <i>PIK3C3</i>     | ENSSSCG00000003749  |
|                          | 6 | 126083379 | 126083698 | -1 |                   | ENSSSCG000000037233 |
|                          | 6 | 127382248 | 127392164 | -1 | <i>SYT4</i>       | ENSSSCG000000037079 |
|                          | 6 | 127866246 | 127890915 | 1  | <i>KCNG2</i>      | ENSSSCG00000005780  |
|                          | 6 | 127881306 | 127952556 | -1 | <i>PQLC1</i>      | ENSSSCG000000023171 |
|                          | 6 | 127954766 | 127991170 | -1 | <i>TXNL4A</i>     | ENSSSCG00000005777  |
|                          | 6 | 127960266 | 127973191 | 1  | <i>HSBP1L1</i>    | ENSSSCG00000005778  |
|                          | 6 | 128004528 | 128030296 | 1  |                   | ENSSSCG000000038475 |
| Coat colour/SSC6_region4 | 6 | 136695884 | 136696193 | -1 | <i>RF00100</i>    | ENSSSCG000000030678 |
|                          | 6 | 137263585 | 137263683 | 1  | <i>RF00026</i>    | ENSSSCG000000024120 |
|                          | 6 | 137410919 | 137453959 | -1 |                   | ENSSSCG000000003774 |
|                          | 6 | 136633437 | 137201213 | -1 | <i>ST6GALNAC3</i> | ENSSSCG000000024494 |
|                          | 6 | 136057959 | 136176334 | 1  | <i>PIGK</i>       | ENSSSCG000000038329 |
|                          | 6 | 135745879 | 136020581 | -1 | <i>AK5</i>        | ENSSSCG000000003773 |
|                          | 6 | 136203309 | 136391034 | -1 | <i>ST6GALNAC5</i> | ENSSSCG000000033425 |

|                           |    |           |           |    |                |                    |
|---------------------------|----|-----------|-----------|----|----------------|--------------------|
| Coat colour/SSC8_region1  | 6  | 137383193 | 137400783 | 1  | <i>ASB17</i>   | ENSSSCG00000031522 |
|                           | 8  | 796389    | 773556    | -1 | <i>FAM53A</i>  | ENSSSCG00000032633 |
|                           | 8  | 818217    | 802940    | -1 | <i>SLBP</i>    | ENSSSCG00000008676 |
|                           | 8  | 825182    | 818588    | -1 | <i>TMEM129</i> | ENSSSCG00000008678 |
|                           | 8  | 837322    | 825339    | 1  |                | ENSSSCG00000008677 |
|                           | 8  | 895912    | 879207    | 1  | <i>FGFR3</i>   | ENSSSCG00000030827 |
|                           | 8  | 928019    | 898181    | -1 | <i>LETM1</i>   | ENSSSCG00000008675 |
|                           | 8  | 1019326   | 964893    | 1  | <i>NSD2</i>    | ENSSSCG00000008682 |
|                           | 8  | 1013071   | 1012948   | 1  | <i>RF00427</i> | ENSSSCG00000020173 |
|                           | 8  | 1038993   | 1018910   | -1 | <i>NELFA</i>   | ENSSSCG00000008681 |
|                           | 8  | 1058148   | 1056750   | 1  | <i>C4orf48</i> | ENSSSCG00000008686 |
| Coat colour/SSC14_region1 | 14 | 71827631  | 71894754  | 1  | <i>CCAR1</i>   | ENSSSCG00000010243 |
|                           | 14 | 71928395  | 71983778  | 1  | <i>STOX1</i>   | ENSSSCG00000010245 |
|                           | 14 | 71970814  | 71970946  | -1 | <i>RF00156</i> | ENSSSCG00000019527 |
|                           | 14 | 71989533  | 72030993  | 1  | <i>DDX50</i>   | ENSSSCG00000010246 |
|                           | 14 | 72040083  | 72061392  | 1  | <i>DDX21</i>   | ENSSSCG00000010247 |
|                           | 14 | 72071874  | 72099801  | 1  | <i>KIF1BP</i>  | ENSSSCG00000010248 |
|                           | 14 | 72183363  | 72199259  | 1  | <i>SRGN</i>    | ENSSSCG00000023374 |
|                           | 14 | 72210483  | 72241657  | 1  | <i>VPS26A</i>  | ENSSSCG00000010250 |
|                           | 14 | 72245532  | 72274827  | 1  | <i>SUPV3L1</i> | ENSSSCG00000010251 |
|                           | 14 | 72282016  | 72336533  | 1  | <i>HKDC1</i>   | ENSSSCG00000010252 |
|                           | 14 | 72382912  | 72460924  | 1  | <i>HK1</i>     | ENSSSCG00000010253 |
|                           | 14 | 72462004  | 72475662  | -1 | <i>TACR2</i>   | ENSSSCG00000010254 |
|                           | 14 | 72501289  | 72555081  | 1  | <i>TSPAN15</i> | ENSSSCG00000036152 |
|                           | 14 | 72604468  | 72606363  | -1 | <i>NEUROG3</i> | ENSSSCG00000034488 |
|                           | 14 | 72637075  | 72649799  | 1  | <i>FAM241B</i> | ENSSSCG00000040498 |
|                           | 14 | 72787602  | 72953095  | 1  | <i>COL13A1</i> | ENSSSCG00000010256 |
| Coat colour/SSC15_region1 | 15 | 26882562  | 27362814  | -1 | <i>CNTNAP5</i> | ENSSSCG00000015726 |
|                           | 15 | 26998479  | 27356862  | 1  |                | ENSSSCG00000036368 |
|                           | 15 | 28530166  | 28530733  | -1 |                | ENSSSCG00000030079 |
|                           | 15 | 28834319  | 28837216  | -1 |                | ENSSSCG00000039209 |

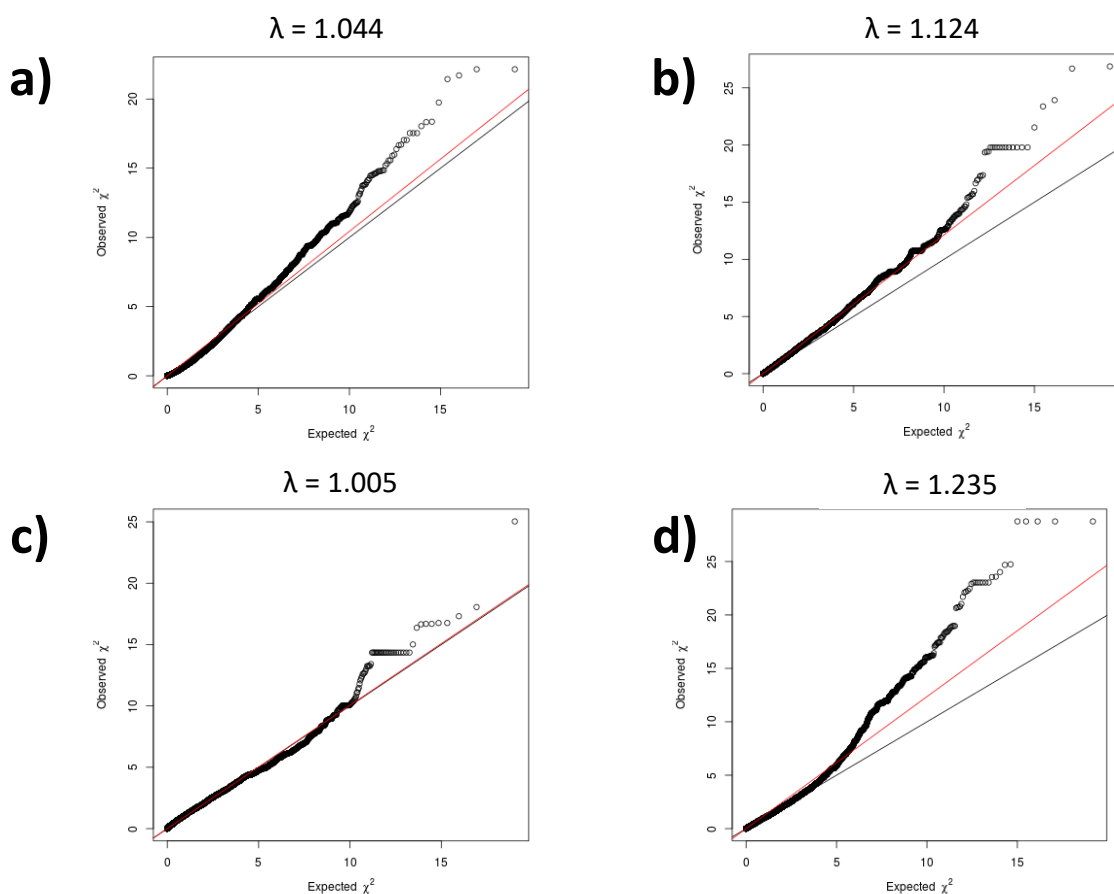
436 † Chromosome regions associated with the presence or absence of wattles (wattles) and slate-grey or black colours (coat colours).

437 ‡ *Sus scrofa* chromosome.

438 § Starting position of the annotated gene on Sscrofa11.1 genome version.

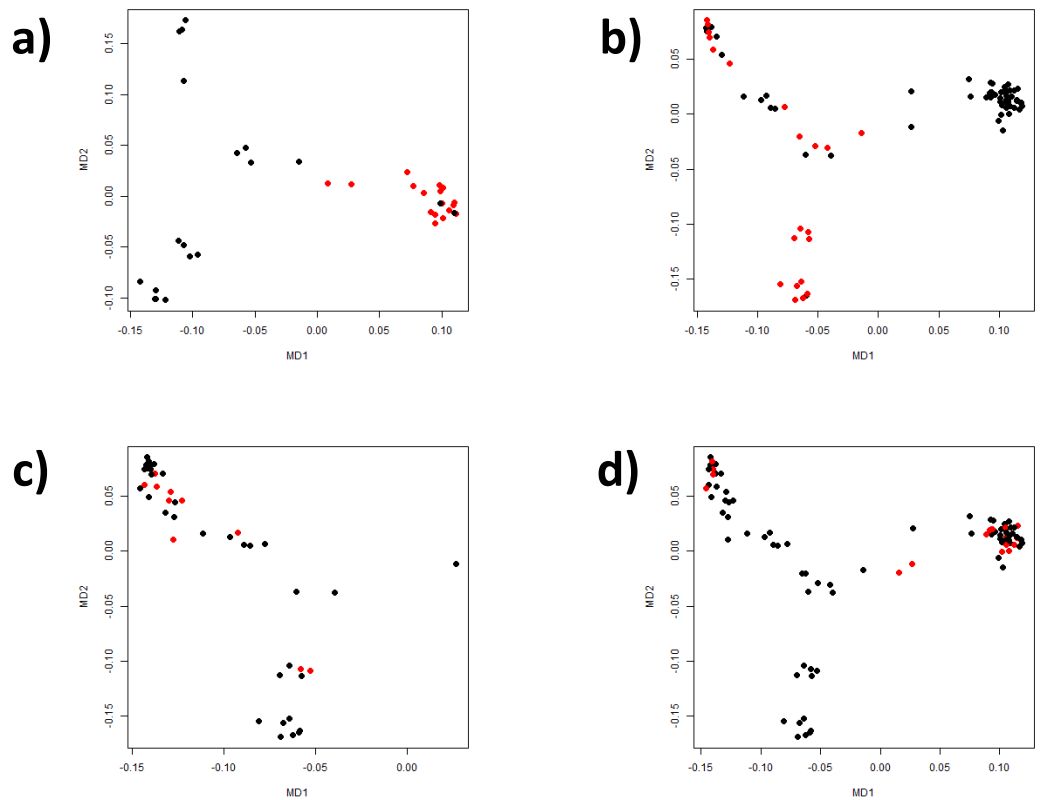
439 ¶ End position of the annotated gene on Sscrofa11.1 genome version.

440 **Figure S1.** Genomic inflation factors ( $\lambda$ ) and quantile–quantile (Q–Q) plots for the genome-wide  
 441 association studies applied for the four exterior traits investigated: a) ear size (39,732 filtered SNPs);  
 442 b) ear bearing (41,027 filtered SNPs; c) presence or absence of wattles (38,423 filtered SNPs); d) coat  
 443 colours (41,230 filtered SNPs).



445

446 **Figure S2.** Multidimensional scaling (MDS) plots obtained for the pigs classified according to the  
447 alternative phenotypes for the investigated traits: a) ear size (small, black dots; large, red dots); b) ear  
448 bearing (floppy, black dots; forward, red dots); c) absence (black dots) or presence (red dots) of  
449 wattles ; d) coat colours (slate-grey, black dots; black, red dots). For each trait, the first (MD1) and  
450 the second (MD2) coordinates are reported.



451