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INNOVATIVE EXPLOITATION OF PIG GENETIC RESOURCES

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Abstract

The recent possibility of using new strategies for genome study, through DNA sequencing and the use of high-density SNP chip, reenforced the already known knowledges about molecular markers that control some important domestic traits in pig breeds, useful to explain the evolutionary process of *Sus scrofa*. Furthermore, information of pig DNA markers affecting different morphological variability is fundamental to evaluate new strategies to preserve animal genetic resources and autochthonous pig breeds biodiversity.

This thesis is the results of a research activity performed during the Industrial Ph.D. program with the National Pig Breeders Association (ANAS). The first study takes advantage of already available knowledges related to molecular marker affecting coat color (MC1R gene) together with knowledge of genetic markers that control vertebrae number variability (NR6A1 p.P192L) in European domestic pigs and in Mora Romagnola breed. We investigated polymorphisms at these two genes to implements the use of these DNA markers and redefine the Mora Romagnola Herd Book breed standard. Furthermore, these DNA markers could be used for food traceability and authentication, improving economic sustainability of low-productivity breeds, whose products are often subject to frauds. The other two parts of the thesis take advantage from the morphological heterogeneity of Casertana breed to study phenotypic traits that cannot be genetically characterized using cosmopolitan pig breeds populations. In the second study of this thesis, we considered Casertana different ears conformation (size and position) and phenotypic variability of other exterior traits (wattles and coat colours) to perform genome wide association study (GWAS) and a genome F_{ST} analyses. The study provides preliminary information about candidate genes involved in effecting monogenic traits not yet fixed in this population. In the third study of this thesis, we considered the important variability of the tail shape in Casertana breed. We run a GWAS comparing the genome of curly-tailed and strait-tailed animals in order to identify genomic regions associated with the tail shape phenotype. Considering the potential relationship between tail shape and pig's behavior and tail biting damages, the results of this study could help to develop further studies aimed at responding to animal welfare. This theme is a current topic in pig breeding, and it is considered also in ANAS new breeding programs for improve sustainability of Italian pig breeds for PDO and PGI productions.

Taken together, the results of this thesis could provide an innovative exploitation of genomic resources in the genetic Italian breeding programs for the Italian pig breeds of the Herd Book managed by the National Pig Breeder Association (ANAS), improving sustainable conservation of local pig breeds and foreseeing development of further studies on behavior and welfare.

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Chapter 1.

Sus scrofa evolution

1.1 Evolution and domestication

In human history the domestication of species has been a fundamental event, as well as for the history of domesticated breeds. Domestication, which should not be confused with the action of learning non-hereditable mechanical behaviors, is the process that has allowed man to choose animals to breed, monitoring their reproduction, feeding and breeding (Price, 1997; Diamond, 2002). This vision, according to population genetics, classifies domestication as a separation process where groups of animals were isolated and domesticated in captivity, reducing their natural ability to respond to new selective pressures. Therefore, domestication is often associated with a reduction in the genetic variability of domesticated population. The first domestication events occurred between the late Pleistocene and the beginning of the Holocene (12000-8200 B.P.), in rather distinct geographical areas. Human choice to breed animals with advantageous phenotypes pointed the basis of domestication process and led to artificial selection. As results, human artificial selection led to the distinction of groups of animals characterized by heritable phenotypic traits and anatomical, behavioral (docility) and genetic modifications (Larson and Burger, 2013; Larson et al. 2014; Zeder, 2017).

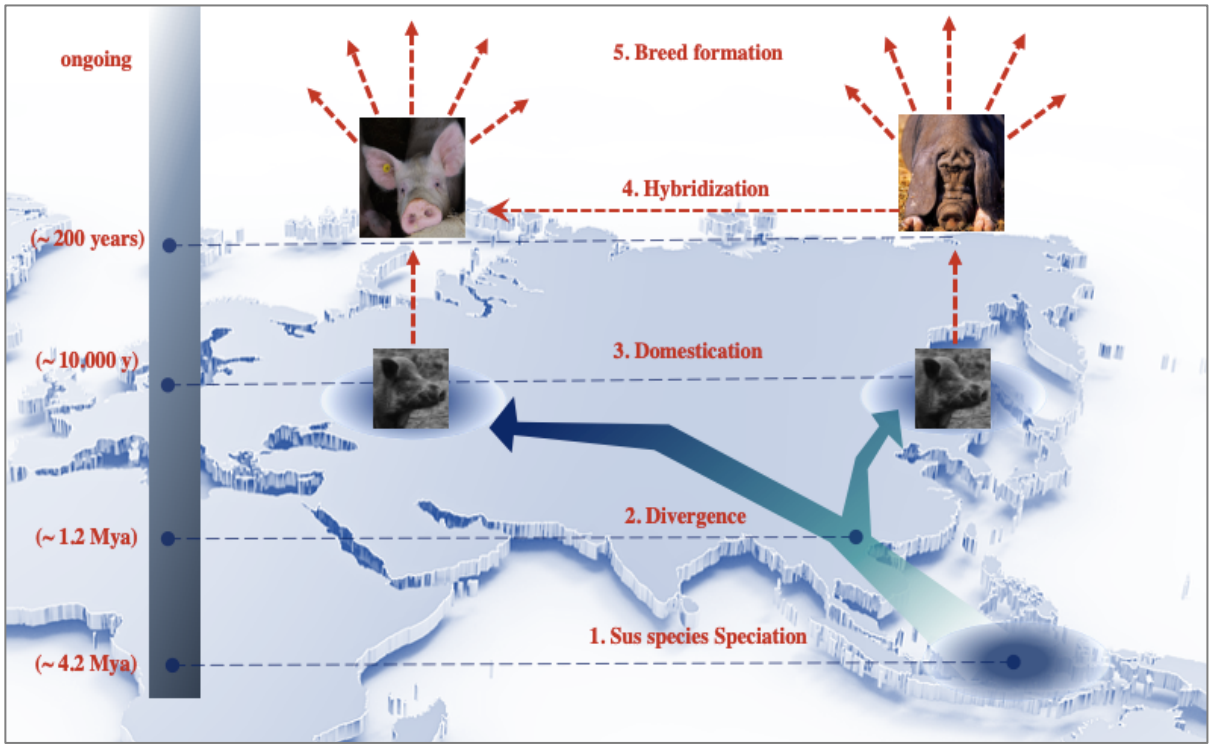
The evolutionary history of pigs started almost within the same time in Anatolia, Europe and in East Asia: the domestic pig has been originated from the wild boar, *Sus scrofa*, by numerous domestication independent process. Interestingly, the wild boar seems to have been the unique *suid* that was domesticated by humans. Mitochondrial analysis of pig genomes and recent data of sequencing of pig genome confirmed the domestication process, which had taken many millennia of years and led to mixing between different gene flows (Ramos-Onsins et al., 2014; Groenen, 2016).

The evolutionary history and domestication of pigs was signed by five major events (Figure 1), which determined the distribution of genetic variation between modern pig's genomes:

1) Speciation of Sus species in Island South-East Asia (ISEA) (~ 4.2 Mya);
2) Divergence between European and Asian lineages (~ 1.2 Mya);
3) Independent domestication leading to separate domesticated clades in Europe and Asia (~ 10000 years);
4) Hybridization between domesticated pigs from Asia and Europe (~ 200 years);

5) Breed formation (nowadays) (Bosse, 2019).

Figure 1: Sus scrofa evolutionary and domestication (reworked image from Bosse,2019)



There is a general opinion that Eurasian wild boar (*Sus scrofa*) and other sister species, such as *Sus celebensis* (Celebes warty pig), *Sus verrucosus* (Java warty pig), *Sus cebifrons* (Visayan warty pig), *Sus philippensis* (Philippine warty pig) and *Sus barbatus* (Bornean bearded pig), emerged in Southeast Asia in the early Pliocene. Almost ~4 Million years ago -Mya- wild *Sus scrofa* has been specificized, and over the past one million years, slowly colonized and spread in almost the entire Eurasian mainland and North Africa (Larson et al., 2005; Groenen et al., 2012; Ramos-Onsins et al., 2014; Groenen, 2016; Bosse, 2019). During the Calabiran stage, a long geographic isolation had determined the constitution of two differentiated *Sus scrofa* gene pools: the Western-European (Europe, Near East and North Africa) and the Eastern-Asian (Asia) (Giuffra et al. 2000; Groenen et al., 2012; Ramos-Onsins et al. 2014; Bosse, 2019). These divergences appeared also within the population size and within the demographic history. The strong climate condition of mid-Pleistocene, around 0.8 Mya ago, led to the extinction of some *Sus scrofa* populations and to the migration and isolation of some others across Eurasia. Furthermore, during the Last Glacial Maximum (LGM ~ 20000 years ago) the geographical distribution of wild boars was remodeled, and population size of both European and Asian wild boars suffered a reduction (a population bottleneck), particularly in Europe. As a consequence, also genetic variability was compromised, causing the lower genetic variability of the modern

European Wild boar compared to Asian *Sus scrofa* (Groenen et al., 2012; Wang et al., 2014; Bosse, 2019).

The domestication of pigs, started around 10000 years ago, is not to be considered a single event but a gradual process with recurrent admixture between wild populations and strongly influenced by the interaction between pigs and humans (Rubin et al., 2012; Groenen, 2016; Ramos-Onsins et al., 2014). Man-to-pig relationship began with the approach of wild boars to human settlements, where food was easily retrieved. Only after millennia, human actually started pig domestication, building fences and shelters within their settlements. This phenomenon occurred at different times and with different methods in China and Europe. In particular, in Europe, until the late Middle Ages, pigs roamed freely in the forests as domesticated herds, while in China pigs had been early confined to enclosures within the settlements. This condition led to a faster approach to selection in Asia and pigs were reared by human for some specific production traits such as early maturation, rapid growth and increased prolificacy. In the 18th century, contextually with the Industrial Revolution, the demand of food and meat pork increased as a consequence of higher Europe urbanization and farmers started to apply new breeding strategies, in order to obtain more productive animals, better adapted to the changed environment (reduction of forest and rising of smaller and strict organized breeding). Therefore, breeders, in particular from the UK, started to hybridize European pigs with the Asian ones, already characterized by many of the production traits (high prolificity and grow rate) (Wang et al., 2017). The adaptive introgression of Asian genetic material into European population is thought to be a widespread phenomenon that had caused a high variability in pig's genome, confirmed by a series of different genetic markers (Ramos-Onsins et al., 2014). Several studies have been shown the crucial role of Asian genes in contributing to increase fertility and fatness in commercial Large White pigs (Giuffra et al., 2000; Wilkinson et al., 2013; Bosse et al., 2014; 2015). The introduction and selection of novel alleles into a population has led to morphological and physiological changes, such as coat color, muscle composition, early maturity, growth rate, body size, reproduction, behavior, and the creation of different pig breeds. As a result, the current domesticated pig breeds of Europe and Asia used for agricultural proposes exhibit different characteristics in anatomic, behavior and physiological phenotypic and are divided in new distinct pig breeds (Laval et al., 2000; Wilkinson et al., 2013).

Today European breeds, such as Italian ones, are classified according to the productive attitude. They are divided in commercial breed, principally from British heritage background (i.e., Large White, Landrace, Pietrain and Duroc) and local or autochthonous breeds, which are considered

in danger of extinction due to their low genetic variability and the small number of sows and boars. Local breeds, mainly reared in semi extensive or extensive way, are genetically closer to wild boars because have not undergone genetic improvement caused by introgression of Asian pig's genome. Several study on microsatellites confirmed this aspect and the diversity between industrial and commercial breeds and local ones (Laval et al., 2000). The industrial breeds are standardized for specific traits (i.e., carcass high quality, high reproductive performances), are reared in intensive farms and follow a genetic breeding program to improve all these productive traits. In this case, animals are chosen for their estimated breeding value (EBV): only small number of boars with high EBV are used in breeding program for the fixation of favorable alleles in new generation, which could cause also in this case a possible loss of genetic variability (Ramirez et al., 2009; Bosse et al., 2019). The clear distinction between production orientation (adaptability to production systems-genetic improvement) and breeding methods between local and commercial breeds influenced their role in breeds distinction and conservation of biodiversity. In particular, local breeds should have priority for breed diversity, while commercial lines have a crucial role in genetic variability in diversity within breeds (Ollivier et al., 2005).

1.2 Genomic of domestication

Identification of genes and molecular mechanisms which underly morphological changes in pigs has been possible considering both changes of early domestication processes and the ones caused by humans' selection or during recent breeds development. It is important to be aware that the genetic components underlying some morphological changes in domestic animals are not caused by recent mutations occurred during domestication or selection events, rather by mutations that already existed before the beginning of these processes. The comparison with other domestic species, that present morphological and behavioral changes similar to pigs, suggests that morphological changes are due to mutations occurred on the same genes within pathways that influence different biological aspects (Groenen, 2016; Larson and Burger, 2013).

In the last twenty years several methods had been applied to reach this purpose, supported by the phylogenetic analyses of wild species and their domesticated relatives.

The first molecular knowledges about pig domestication mainly derive from the mitochondrial DNA (mtDNA) analysis of wild and domestic pigs from Asia and Europe. Mitochondrial DNA, differently from the nuclear DNA, has some characteristics such as lack of recombination, high mutation rate and multiple copies, that identify it as suitable for the study of evolutionary processes. Despite this, mitochondrial markers have a small effective size, are strongly influenced by genetic drift and, therefore, are poor predictors of genetic variation of whole genome. Results from mtDNA provide a maternal prospective of the candidate progenitors involved in domestication process, but to provide greater details about the origins of domesticated species, it is fundamental to integrate information of nuclear DNA of Y chromosome genetic markers (Giuffra et al., 2000; Greminger et al., 2010; Ramos-Onsins et al., 2014; Wang et al., 2014).

Another genomic approach for phylogenetic study during the evolutionary, also in Suinae subfamily, is the identification of nuclear mitochondrial DNA (mtDNA) into nuclear genome of eukaryotic organisms. The horizontal transfer of coding and non-coding regions of mtDNA fragments into the nuclear genome produces nuclear DNA sequences of mitochondrial origin, called *numt*. Polymorphisms in *numts* in different pig breeds provides information about evolutionary events between *Sus* species and could help to explain domestication and pig breeds formation (Schiavo et al., 2017).

Phylogenetic studies have found more effective tools to analyze the evolution of domestication processes with the application of the information acquired through the study of nuclear DNA, starting using microsatellites and single-nucleotide polymorphism (SNPs). The availability of

numerous SNPs and the creation of high-density chip, with relatively low cost of production, led the application of Next Generation Sequencing (NGS) technology (Ramos et al., 2009). This innovative approach has revolutionized genetic studies and has been applied also for investigating domestication and selection process of animal species.

Using NGS technologies allowed the implementation of mapping approaches to identify the genetic variants that underlie phenotypic diversity in domestic animals. These approaches involved scanning the genome (genome-wide scans) at different levels of differentiation and in different populations. Several studies of genome-wide scans revealed candidate genes or genomic region related to morphological variation such as body size, skeletal formation, coat patterns and traits such as muscle conformation in domestic animals (Chen et al., 2007; Wilkinson et al. 2013). In example, Rubin et al. (2012) used pig draft genome sequence (Sscrofa10.2) and whole-genome resequencing to reveal loci associated to morphological traits (body structure) potential target of pig domestication (Rubin et al., 2012). Recently, whole-genome sequencing data of local and commercial European breeds give new tools for contributing to explain origin, evolutionary history and adaptation to production system of different European and Italian pig breeds (Muñoz et al. 2019; Bovo et al.; 2020a).

Investigations on domestication and selection process using NGS technology have been supported by data of phylogenetic, phytogeography and paleogenomics. This latter branch of genomic analyzes the ancient DNA (aDNA) from archeological subfossils and help to understand the genetic and genomic signatures of domestication, giving an insight on natural selection and proofs of admixture between early domestic animal populations and their wild congeners (MacHugh et al., 2017).

1.3 Domesticated traits

As introduced in previous pages, domestication process and selective breeding of pigs have caused a genetic adaptation to different environmental conditions and significant phenotypic changes between pig breeds. To analyze the genetic basis of phenotypic differences in pigs, studies have mainly investigated some important traits for the evolutionary process of the breeds with the aim to detect the possible chromosomal regions or candidate genes and subsequently identifying the underlying causal mutations and allelic frequencies between breeds. Interestingly, a number of these genes being selected in European breeds have an Asian origin (Andersson and Georges, 2004; Wilkinson et al., 2013).

Coat color is considered one of the first morphological traits that has been strongly influenced by domestication and during selection process and, for this reason, it has been largely studied in pigs (reviewed in Fontanesi and Russo, 2013). It is assumed that one of the first evidence of domestication in animal species is the coat color diversity from the wild one. Like in other domestic species, also domestic pig breeds present an enormous variety of coat colors and clearly differ from his progenitor Wild boar, that mainly presents brown coat color, useful for disguising from predators. Several studies deeply investigated on this morphologic variability determining that coat colors also in pig breeds is associated to variations at two important genes, *Melanocortin receptor 1* (MC1R) and *v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog* (KIT, Dominant White locus).

The MC1R gene plays a key role in melanogenesis, regulates synthesis of eumelanin (black/brown) and phaeomelanin (yellow/red) and it is responsible of wild type coat color. Coat color variability in pigs derives from five different allelic forms of this gene: the wild type alleles (E^+), typical of wild board, the dominant black alleles (ED^1 with Asian origin and ED^2 with European origin), the black spotted alleles (E^P) and the recessive red "e" allele. This last mutation, in homozygote animals, determines production of phaeomelanin and consequently red coat color in pigs (Fang et al., 2009; Fontanesi and Russo, 2013; Ramos-Onsins et al., 2014).

Furthermore, variations in number of KIT copies or polymorphism in this gene are responsible of the white coat color, belted and spotted coat color phenotypes. In particular, Dominant White coat color is determined by allele I^1, characterized by the duplication of the KIT gene (copy number variation) and by the presence of a splice mutation in intron 17 in one of the duplicated copies, while Belted phenotype of the Hampshire and Cinta sense pigs is caused by I^B allele, a single copy of KIT gene with a regulatory mutation. This gene has a functional role in melanogenesis process, influencing the melanocyte migration from the neural crest along the

dorsolateral pathway to colonize the final destination in the skin (Rubin et al., 2012; Fontanesi et al, 2010a; Groenen, 2016).

Not only coat color could be defined as a domestic trait. Others important phenotypic traits influenced during domestication and selection processes are vertebrae, ears and tail (Trut et al., 2009). Principally, these traits influenced body structure (i.e, body length) and help distinguish diversity between pig breeds or between wild boar and the domestic pig. These traits differ between wild boar and domestic pigs and several studies investigate on morphological variability between the breeds. Furthermore, these traits have a potentially impact for pigs breeding and their variability in some cases, could be associated to other morphological traits interesting for livestock sector.

1.3.1 Vertebrae number variability

The number of vertebrae is a phenotypical trait of significant interest for the livestock sector. Although the number of vertebrae in mammals is generally preserved, in some species has been observed a significant variability between wild, domestic animals and breeds of different origin. The *Sus scrofa* is one of these species: in different breeds a significative variability in the number of thoracic, lumbar and thoracic lumbar vertebrae has been found (Berge, 1948; King and Roberts, 1960; Mikawa et al., 2007; Rohrer et at.2015). The vertebrae of pigs are divided into five parts: cervical, thoracic, lumbar, sacral and caudal. The lumbar and thoracic vertebrae mainly constitute the backbone and they are subject to a numerical variability: the former vary from 5 to 7, while the number of thoracic vertebrae varies from 13 to 17 in today's commercial breeds (King and Roberts, 1960; Borchers et al., 2004). The other types of vertebrae (cervical, sacral and caudal) have a fixed number, respectively of 7, 4 and 5 vertebrae (Galis, 1999). Wild boars and some Asian pigs (i.e., Meishan) seem to have a number of vertebrae similar to other mammalian species, as they have a tronco-lumbar trait composed of 19-20 vertebrae. Some studies have demonstrated the economic importance of this traits variability because it affects the body length (and consequently of carcass) and the production of meat in different breeds of pigs. This trait is highly heritable, between of 0.60 to 0.62 in industrial pigs (Borchers et al., 2004; Van Son et al., 2019). Body weight and length are economically relevant for meat production: carcasses with longer loins and a higher percentage of lean meat have a higher economic value. Has been estimated that one more vertebra determines a lengthening of the carcass up to 80 mm (King and Roberts, 1960; Fredeen and Newman, 1962). In the past European and Asian breeders made a strong selection for this economic trait which still

nowadays are considered in all the breeding programme for European and Asian pigs. For this reason, Western commercial breeds, including Large White, Duroc, and Landrace, have higher number of thoracic-lumbar vertebrae (n = 21 to 23) in comparison with Chinese indigenous breeds and their ancestor Wild boar.

Due to its high hereditability, a lot of studies had been carried out to identify the specific causative mutations associated with the variability of this phenotypic trait. Chromosomic regions harbour Quantitative Trait Loci (QTLs) or genomic mutation (SNPs - insertions) associated with this morphological variability have been detected mainly on SSC1 and SSC7 chromosomes. In addition, several other genetic markers have been indicated as potential genes controlling vertebrae number for their crucial role in regulation of embryonic development or stature in other domestic animals.

First studies identified QTLs associated to the vertebrae number principally on SSC1 and SSC7 chromosomes using the different swine linkage maps with more than a thousand genetic markers. In a F3 resource population obtained by the cross of Gottingen miniature pig and Meishan pigs a genome scan for QTL showed the presence of a significant QTL of vertebrae number on SSC1qter (terminal band of the q arm of SSC1), despite the small dimension of pigs. Also Sato et al. (2003), in a F2 resource population (Meishan sow x Duroc boar), showed evidence of significant QTLs for vertebra number on SSC1 and SSC7 (Wada et al., 2000; Sato et al., 2003).

Later in 2005, a significant study was performed on Asian, European and miniature pigs by Mikawa et al. (2005). A genome scan analysis showed a QTL region on SSC1qter (around SW705) and another around SW252 on SSC7. These two QTLs have not an epistatic effect, acted independently and each of them had mainly an additive effect (approximately 0.55 and 0.60 per allele, respectively). The presence of the favourable alleles for both QTLs seemed to determine two extra vertebrae in pigs (Mikawa et al., 2005). Through a map-based study of the QTL on SSC1 has been identified a significant region of 300-kb almost fixed in European commercial breeds. This region contained two nuclear receptors genes NR5A1 (*adrenal 4-binding protein*) and NR6A1 (*germ cell nuclear factor*). Interesting, a missense mutation (c.575 T > C) on NR6A1 determines a proline to leucine substitution at codon 192 at nucleotide 748 and is coincident with SSC1 QTL. The mutate allele (T) altered the binding affinity of NR6A1 to its coreceptors (NCOR1 and RAP80) and was detected only in Western commercial breed (Asian pigs had leucine). This polymorphism alters the normal expression of the protein and

for this reason, c.575 T > C has been considered a possible causative mutation for QTL on SSC1 increasing number of both thoracic and lumbar vertebrae in European breeds (Mikawa et al., 2007).

Deeply investigation on the role of SSC7 QTL in European commercial breed were performed in a reference population of Large White inbred families × 11 European breeds. In a refined QTL of 41kb (SJ7121-SJ7114) has been observed a new gene encoding an unknown protein which seems to determine the heterogeneity of vertebrae number. This gene has been called *Vertin* (VRTN). On the intron 1 of this gene, 9 different polymorphisms have been noticed and a 291bp SINE insertion of a PRE1 element has been considered the causative mutation in the QTL. The presence of insertion ("Q" allele) increases the thoracic vertebrae number, while the wild type (WT) allele, without insertion, seemingly reduces vertebrae number in commercial breeds (Mikawa et al., 2011).

Subsequently, few studies performed using a whole genome scan, confirmed the reported QTLs for vertebra number on SSC1 and SSC7 in different reference population and the phenotypic correlation between number of vertebrae and productive traits (carcass weight, length, live weight) (Ren et al., 2012). In particular, Rubin et al. (2012) using pig draft genome sequence (Sscrofa10.2) and whole-genome resequencing revealed loci associated to morphological traits, including number of vertebrae. The results of their study supported the hypothesis of Mikawa et al. (2007) and showed the crucial role of candidate mutation (Pro192Leu) on NR6A1 gene on SSC1 in affecting the number of vertebrae in European domestic pigs (Rubin et al., 2012).

Further investigations on SSC7 QTL were performed by Fan et al. (2013). Using 60K SNP chip data information, a genome wide association study (GWAS) in three experimental populations of Chinese and Western pigs identified loci for number of thoracic vertebrae on SSC7 in all the three population in a region of 947-Kb. This region was refined to a 100-Kb segment where only two genes including VRTN have been detected. The most causative variants underlying the QTL on SSC7 was a SINE insertion g.20311_20312ins291 in intron 1 and g.19034A>C SNP in the promoter region of VRTN gene. Unexpectedly this QTL effects in both Chinese and Western pigs and suggested that favourable allele probably derived from Chinese pig population and not from the European as hypothesized by previous studies (Fan et al., 2013).

Not only VRTN and NR6A1 gene have been suggested as candidate gene for this trait variability. Ren et al. (2012), using a haplotype analysis defined a 900-kb region on SSC7, which harbour VRTN gene for vertebrae number variation and two other positional candidate

genes, *Prospero Homeobox 2* (PROX2) and *Finkel-Biskis-Jinkins murine osteosarcoma viral oncogene homologue* (FOS), which could cause the QTL effects in both Western and Chinese pigs (Ren et al., 2012). Furthermore, also PLAG1 and LCORL genes seemed to potentially explain the genetic basis for an elongated back length and increased number of vertebrae because their crucial role in controlling stature in other domestic animals and in humans (Rubin et al., 2012). Besides, QTLs controlling vertebrae seemed to be located in the Hox gene (*Hox A,B family*). These QTLs seems to increase number of lumbar and tronco-lumbar vertebrae (HoxB cluster) and thoracic (Hox A cluster) because regulate embryonic development (Rohrer et al., 2015). Also the *Latent Transforming Growth Factor Binding Protein 2* (LTBP2) gene seemed to be associated with vertebrae variability, for the presence of the missense mutation (c.4481 A > C) (Park et al., 2017). Interesting, Zhang et al.(2015a) a GWAS study suggested NR6A1 on SSC1 as the candidate for variation of lumbar vertebra number, but indicated VRTN, PROX2, FOS, TGFB3 as candidate genes affecting variability in thoracic vertebrae number of pigs (Zhang et al., 2015a). In a further study, in the same reference population, GWAS revealed 13 and 23 significant SNPs in both SSC1(6.04 Mb) and SSC7 (7,17 Mb), including variants at NR6A1 and VRTN genes. Only SNPs on SSC7 QTL showed an association with vertebrae phenotypic variability. A new gene (TMED10) close to significant SNPs, has been considered a candidate gene because of its expression during a critical period of vertebrae differentiation in mice embryos. Besides, the possible crucial role of TGFβ3 gene has been valuated. This gene has been detected in a refined region of 3Mb on SSC7 and a causative mutation TGFβ3 c.1749 G>A seemed to affect vertebra number ("G" allele increases number of vertebrae in F0 LW population) (Zhang et al., 2017a). Recently, the study performed by Liu et al. (2020) in a reference F2 population of Large White × Minzhu, has shown the crucial role of variants in FOS gene on SSC7 in controlling thoracic vertebrae number. Furthermore, the study has identified on SSC14 two SNPs near to the *bone morphogenetic protein receptor type 1A* (BMPR1A) gene with a significant genome wide association and a dominant effect for the number of thoracic vertebrae. This gene is fundamental for BMP (Bone morphogenetic protein) signalling and it is involved in vertebral specification during somite differentiation (Liu et al., 2020).

The central role of VRTN gene in affecting vertebrae was apparently clear and genetic variability has been detected both in Asian and Western pigs. The possibly introgression of VRTN g.20311_20312ins29 mutation from Chinese pigs into Western pigs has been confirmed by Yang et al. (2016). Vertnin insertion g.20311_20312ins29 ("ins" allele) variants have been

investigated in Chinese Erhualian pig to evaluate segregation of this mutation in Asian population (0,07% “ins” allele frequency) and its association with vertebrae number modification. The GWAS study showed a significant association with thoracic vertebrae number: heterozygous Erhualian pig (ins/del) have higher number of vertebrae, confirming the Asiatic origin of VRTN mutation (Yang et al., 2016).

Despite that, the biological function of this gene still remained unknown until the study performed by Duan et al (2018). Firstly, they confirmed VRTN causative mutations (g.19034A>C and g.20311_20312ins291) and his additive effect in the regulation of thoracic vertebrae number in a reference population (Duroc x Landrace x Large White). Secondly, the study evidenced that *Vertnin* gene is a novel DNA-binding transcription factor because of his location exclusively in the cell-nucleus. His functional role for maturation of thoracic vertebrae has been explained considering embryo development of transgenic mice (Vrtn-knockout mice, Vrtn-/-). VRTN is essential for mice embryo’s development of thoracic somites (at Theiler stage 14) and thus, for determination of thoracic vertebrae number (Vrtn+/- mice have less thoracic vertebrae and ribs than Vrtn+/+mice, wild type). Furthermore, VRTN variants (g.19034A>C and g.20311_20312ins291) together influences the Notch pathway, modulating somite segmentation: pigs homozygous for both variations (QQ) have one more thoracic vertebra, but each thoracic vertebra is shorter because “QQ” pigs are only 1,5 cm longer (half size of normal vertebra) than wild type “qq” animals (Duan et al., 2018).

Recently, Van Son et al. (2019), provide new information about vertebra column development, using a large data set: they combined large scale genotype and phenotype data about number of teats, vertebrae and ribs in three commercial pig breeds (LW; L; D) and reconsidered the hereditability value of number of vertebrae trait. Furthermore, combining medium and high-density SNP data with whole-genome sequence (WGS), showed that effect of VRTN functional mutation on thoracic vertebrae is strictly connected to the genetic background of the breeds. VRTN variant allele frequencies, size of the effect and accuracy of the morphological expression differ between breeds, influencing genetic and phenotypic variance. Furthermore, the study evidenced the presence of two missense mutations in the *ATP binding cassette subfamily D member 4* (ABCD4) gene, located in the upstream of VRTN only in Landrace “wt/wt” animals, that should be more investigated because could alter gene expression and consequently the development of the spine (Van Son et al., 2019). More recently, two new SINE insertion in intron of pig VRTN gene have been revealed by a comparative genomic alignment in eight different pig breeds. These new structural variation in VRTN gene were

never been reported and further studies need to be play in order to investigate their functional role. Allele frequencies for these two new insertion (VRTN-sRTIP2 and VRTN-sRTIP3) have been estimated, showing a *Hardly-Weinberg equilibrium* distribution for almost all pig breed investigated, except Eurhualian and Chinase pig (Zheng et al., 2020).

The scientific relevance of study vertebrae number variability not only concerns investigation on structural body conformation and their embryonic evolution, rather the possible morphological and genetic association with other important livestock traits. Several studies have shown that increasing of vertebrae number seemed to be associated with morphological variety of several carcass traits, including carcass length (King and Roberts, 1960; Yang et al., 2016), daily gain (Borchers et al., 2004), reduction of intramuscular fat content in loin (Hirose et al., 2013), and carcass weight (Burgos et al., 2015). This latter study found that VRTN Ins-allele increased both vertebrae number and carcass weight (Burgos et al., 2015). These results seem discredit by Huang et al. (2017), which showed a negative effect of increased thoracic number on carcass weight in PIC pigs (Huang et al., 2017). Furthermore, Fontanesi et al. (2014a) also investigated the association between VRTNins allele and meat and carcass quality and production traits in Italian Large White reference population, highlighting a negative correlation between "Q" VRTNins allele (increase of vertebrae) and ham weight (Fontanesi et al., 2014a). Besides, other studies have proved the genetic correlation between genes controlling number of vertebrae number and the number of ribs. This anatomical carcass cut of high economic interest, in particular during specific periods of year (Burgos et al., 2015; Van Son et al., 2019; Jiang et al., 2020). Finally, several recent studies on morphological traits influencing reproduction efficiency, in particular on teats number, have shown the strict correlation between QTLs affecting vertebrae number and reproductive traits. VRTN gene has been suggested as the most favourable candidate gene involved in teats variability (Duijvesteijn et al. 2014; Yang et al., 2016; Rohrer et al., 2015; Dall'Olio et al., 2018; Van Son et al., 2019). A positive correlation (r=0.32) between vertebrae and teats number has been shown by Yangh et al. (2016) in Chinese Erhualian, Western commercial purebreeds (Large White, Landrace, Duroc) and F2 population, confirming the pleiotropic effect of VRTNins mutation for higher number of vertebrae on teat numbers (Yang et al., 2016). This association has been evidenced also in a reference population of 793 Large white Italian pig (Dall'Olio et al., 2018). Vertebrae and teats genetic correlation is extremely interesting from pig breeds selection, especially for dam lines (i.e., IL, ILW) with a high maternal aptitude. Sows with a higher number of functional teats can wean more piglets and for these reasons breeding programs focus on both these traits

(Rohrer et al., 2015).

1.3.2 Ear size and conformation

Ear's size and conformation play an important role in breed distinctiveness. For their selective process, Asian and European pigs strongly phenotypically differ, also in ears conformation and dimension. Many Chinese indigenous pig breeds, such as Erhualian and Meishan, two strains of the Taihu breed, have large and floppy ears. In contrast, Wild boars and European commercial breeds (i.e., White Duroc and Large White pigs) show small and half- or fully-pricked ears (Chen et al., 2018). Ear conformation and dimension are interesting morphological traits to focus on, mainly because ear defects could be observed also in other species, including humans. The auditory system includes the external ear which plays a crucial role in collecting sound as the first step of hearing (Ren et al., 2011). Despite the several differences in mechanisms determining ear-size diversity, pig can also be considered an important biomedical model to investigate human's ears external developments and their abnormalities (Zhang et al., 2014; Chen et al., 2018). Pig ears dimension could be also considered a morphological trait with an economic relevance. In some countries, ears are used as food for humans or for pets, therefore ear's dimension and weight are phenotypic trait with significant economic relance (Ma et al, 2009).

For all these reasons, the genetic basis of pig's ears dimension and development have been investigated. Firstly, the major QTLs on specific Sus Scrofa chromosomes have been detected using linkage maps. More recently, the application of different molecular technologies let to identify SNPs or genomic regions, as copy number variant (CNV), involved in the modification of development of ear's components. The external ear is composed of skin, fat, connective tissue and principally of cartilage and, for this reason, genes with a crucial role in skin homeostasis, cartilage development, fat metabolism or involved in the Wnt/b-catenin pathway have been considered as possible candidate genes for ear conformation, disposition and dimension (Zhang et al., 2014).

The first study to detect QTL for ear shape has been conducted by Guo et al (2004). The information of ear shape (pricky, middle, floppy) has been collected on a commercial pig population. Using linkage map, only on SCC6 was found a QTL for ear shape at 1% genome wise level at the end (between Sw1881 and Sw322) (Guo et al. 2004). Later, Wei et al. (2007) investigated on the ear size of Meishan and of European Large White pig breed. Using a linkage maps with 152 markers, identified significant QTLs in different chromosomes (SSC1,5,7,9).

The main QTLs effecting both ear size and erectness were located on SSC5 and SSC7 and the suggested candidate gene for these QTLs were respectively *the growth differentiation factor 11* (GDF11) and *bone morphogenetic protein 5* (BMP5) genes (Wei et al. 2007). Later in 2009, the results on ear dimension (weight and area) obtained by Ma et al. (2009) in a F2 population of pigs confirmed all QTLs detected by previous studies, excluding QTL on SSC6 and 9. Also in this study the two most significant QTLs were confirmed on SSC7 and SSC5, but new QTLs for ear size and erectness, weight and area (i.e. on SSC4) were discovered. In particular, two QTLs on SSC8 (at37 cM and 89 cM) were close to FGF2 and the *fibroblast growth factor receptor 3* (FGFR3) and for these reasons has been suggested as good targets for fine mapping and identification of the causative mutations controlling ears development (Ma et al. 2009).

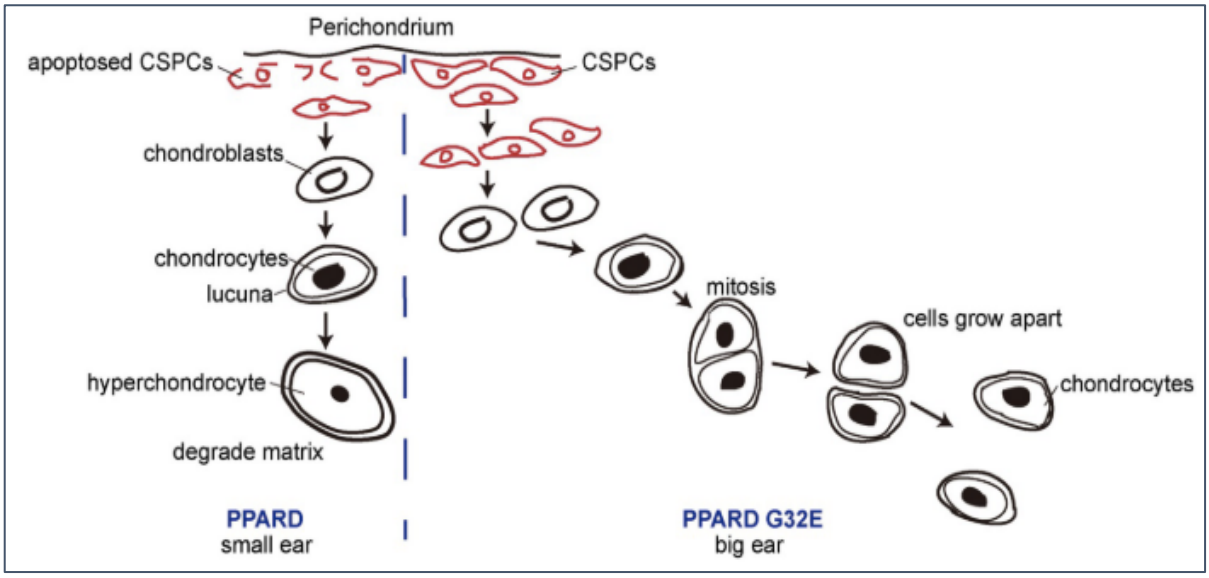
Despite the first studies have identified several QTLs on different chromosomes, the major candidate chromosome regions related to ear morphological variation were localized on *Sus Scrofa* chromosomes 7 and 5. Different studies have been performed and several potential genes have been identified.

Ren et. al. (2011) confirmed the central role of the SSC7 QTL (58 cM) by the identification of a nonconservative missense mutation (G32E) on *peroxisome proliferator-activated receptor delta* (PPARD), an important gene which influences different biological process, such as modulation of keratinocytes and sebocytes of skin or regulation of Wnt/ β -catenin pathway. In particular, PPARD gene was detected in a critical region of 630-kb and has been indicated as a possible positional candidate gene for the major QTL on SSC7. Moreover, sequencing PPARD entire coding region, the missense mutation (G>A) (G32E) has been detected in a conserved functionally important domain. This mutation has been suggested as a causative mutation for enlarged ears. It mediates (inhibits) down-regulation of β-catenin (essential in chondrocyte proliferation and differentiation and it is in a complete concordance (100%) with the QTL genotypes of founder animals (White Duroc; Erhualian). Interesting, this mutation seems to have unique origin in Chinese large-eared pigs (allele frequency >0,80) after domestication and is a signal of selection in Erhualian pigs (Ren et al., 2011).

The real effect of the causative mutation was described by Duan et. al (2013) which clearly showed that G32E is a functional variation that reduces the post-transcriptional activity of the PPARD gene and consequently the ß-catenin expression and its target genes (Duan et al., 2013). Moreover, Zhang et al. (2017b) deeply investigated the role of PPARD variant in influencing cell differentiation in the external cartilage development of the ear. At the transcription level, PPARD gene delays development of auricular cartilage, accelerating apoptosis of cartilage

stem/progenitor cells (CSPCs), extracellular matrix degradation and chondroblast differentiation. Zhang et al. (2017b) identified specific target genes of PPARD, among which the *peroxisome proliferator activated receptor gamma* (PPARG), known to be involved in cartilage development. PPARD gene represses the expression of PPARG and subsequently upregulates the expression of the critical genes inhibiting cartilage growth. In particular, compared to wild type PPARD animal, G32E mutant up-regulates the expression of PPARG causing a downregulation of critical genes that inhibit cartilage growth. Pigs with G23E mutation, like Erhualian, has bigger ear compared to wild-type (Zhang et al., 2017b) (Figure 2).

Figure 2: Effect of the mutation G32E (PPARD) on the differentiation of chondroblasts (Zhang et al. 2017b).



On the other and, several studies have been performed to investigate the chromosomic region on SSC5 which influence ear size and dimension. Due to the large dimension of SSC5 QTL, the identification of potential candidate genes was not an easy undertaking. Li et al., in 2012 refined the SSC5 QTL 11-cM interval to an 8.7-cM interval and suggested some genes (SOX5, HMGA2 and PTHLH) expressed in ear tissue as new positional candidate gene according with their functional role (chondrogenesis regulation, chondrocyte proliferation and differentiation). The study showed an association of HMGA2 g.2836 A > G polymorphism with ear size (G allele is responsible of bigger-large and floppy ear). This gene is the closest to SSC5 QTL and could be considered as potential gene effecting this QTL (Li et al., 2012). Further, whit the first GWAS for ear size, new SNPs in both the two QTLs were described and new candidate gene were suggested to influence ear dimension. In particular were detected 35 SNPs within a 10.78-Mb (30.14–40.92 Mb) region on SSC5. Using a combined approach, the QTL on SSC5 was

refined to about at 450-kb region encompassing *LEM domain containing 3* (LEMD3) and *WNT inhibitory factor 1* (WIF1) genes. These genes could indirectly regulate skin homeostasis, cartilage development, and fat metabolism because they have a crucial role in inhibiting the activity of the TGF-b and Wnt/ β -catenin pathway and for these reasons have been considered as good candidates for ear size (Zhang et al., 2014). In order to investigate transcription tissue expression and polymorphisms of the possible candidate gene for this QTL, molecular cloning studies have been performed. The *methionine sulfoxide reductase B3* gene (MSRB3) is particular near to a SNP (H3GA0016181) reported on SSC5 in previous study (Zhang et al., 2014) and has a crucial role in regulation of cell cycle progression and cell proliferation. Three SNPs has been detected on MSRB3 and among these, SNPs c.-735 C> T (TT genotype) on flanking region, and c.2571 T > C (CC genotype) in the 3'-UTR were significantly associated with larger ear size in a F2 population (LWxMinzhu) (Zhang et al., 2015b). Besides, Liang et al. 2016 cloned and characterized for the first time the candidate gene LEMD3. The study pointed out the presence of different SNPs significantly associated with ear size in Large White × Minzhu F2 population (Liang et al., 2016).

More recently, the mRNA's and protein expression of WIF1, LEMD3, HMGA2, and MSRB3 genes were evaluated by Zhang et al. (2017c) on Erhualian (large ears) and Large White (small ears) pigs. In particular, at the protein level WIF1 mRNA and protein expression levels were significantly higher in Large White than in Erhualian pigs, one of the breeds with largest ears. The results confirmed the role of WIF1 gene, suggesting it as the prime candidate gene. A low expression of this gene modifies Wnt/β-catenin pathway, causing an increment of cartilage cell proliferation during ear development and, consequently, a larger ears dimension (i.e., Erhualian pigs) (Zhang. et al., 2017c). Supporting this result, Liang et al (2019) deeply investigated the role of two HMGA2 and WIF1. By a molecular cloning, SNPs analysis and the tissue expression profiles they confirmed the crucial role of these two gene in influencing ear dimension. In particular, they cloned full-length 2338-bp WIF1 and 2998-bp HMGA2 cDNAs and, using a quantitative real-time PCR, revealed higher expression of WIF1 gene in ear cartilage than all other tissue. Furthermore, the study showed the presence of a missense mutation in the conserved EGF domain of mammalian WIF1 gene (c.1167C>G) and GWAS analysis confirmed the association of G allele with larger ear size in reference population (Liang et al., 2019)

The results obtained by Chen et al. (2018) valorised the role of MSRB3 gene as a potential candidate, observing a new potential causative mutation on this gene. The study reduced the

QTL region to 137.85-kb, including only MSRB3 gene and identified a 38.7kb copy number variant (CNV) whose presence seems to enlarge porcine ear size. In particular, the 38.7kb CNV (starting at 349,577 bp and ending at 388,246 bp) covered the last two exons 6 and 7 of the MSRB3 gene and it was in complete concordance with genotype at the QTL of the F_0 animals in a white Duroc × Erhualian F_2 intercross. Furthermore, CNV has a strongest association with ear and has high frequencies size in Chinese Pig or Landrace pigs (half-floppy ears). The biological mechanism which explains CNV implication in enlarge ears is connected to the increasing expression of miR-584-5p that hinders the expression of MSRB3 gene (Chen et al. 2018).

According with these results, recent studies aim to identify DNA markers associated with ear morphology also in some Italian local and commercial breeds. Investigation in 19 European autochthonous and two Italian commercial pig breeds has been recently carried out by Bovo et al. (2020b). A genome-wide CNV/CNVR analysis has been carried out and a total of 9592 CNVs (~683 CNVs per breed) and 3710 CNV regions (CNVRs; 1.15% of the reference pig genome) had been detected, with an average of 77 CNVRs per breed that were considered as private. Among these, on the last exons of the MSRB3 gene on SSC5, has been detected the 38.4 kbp (SSC5:29826981– 29865653) CNVR previously described by Chen et al. (2018). Excluding some breeds, the presence of CNVR has a significative correlation to ear size (no gain of CN causes smaller or medium ears). Analysis of the two SNPs (not included in the CNVR) in the 50 flanking region (rs340841870 C>T) and in the 3'-UTR region (rs326411202 C>T) reported by that Zhang et al. (2015b) further explained ear size variability in European breed. For each SNP, the regression analysis pointed out a significant association between allele frequencies and ear size ($P < 0.0001$) and with presence/absence of average CN state. In particular C allele for both SNPs seems to be associated with a normal copy, while T allele with five or six copies (Bovo et al., 2020b).

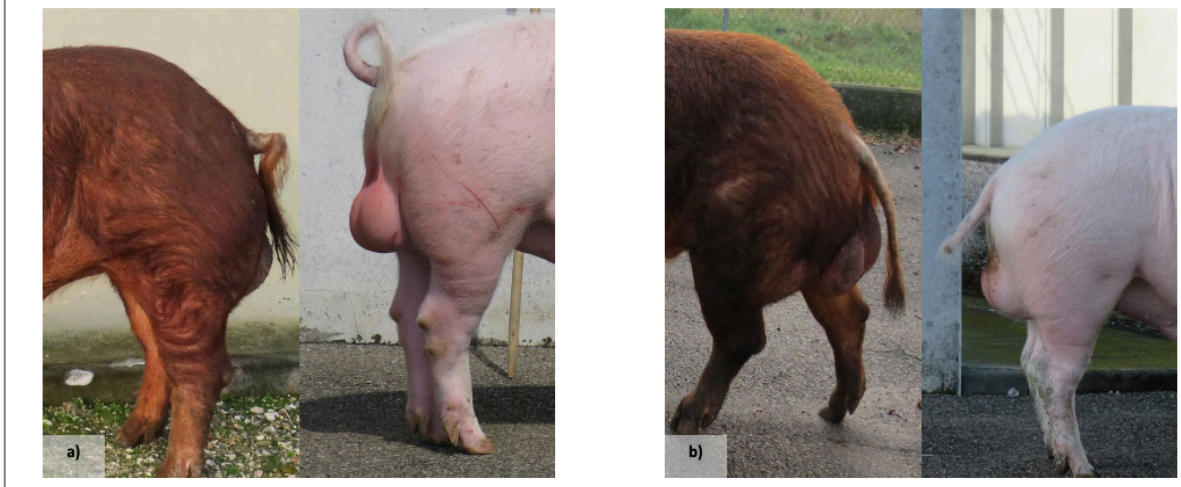
The study of this phenotypic traits helps to explain the genetic basis of morphological difference between Asian, European and Italian breeds and provide information about evolutionary process on the pig breeds developments, highlighting signature of selection or introgression from Asian pigs into European breeds (Ren et al. 2011). For example, Wilkinson et al. (2013), suggested that region on SSC5 of flat-eared breeds came from Asian pigs (Wilkinson et al. 2013). The candidate genes for this trait, such as PPARD gene which regulates some important biological process, such as regulation of fat metabolism, could be potentially interesting for other livestock study or in biomedical field, contributing to reveal genetic bases affecting the

ears diseases in human.

1.3.3 Tail

Tail could be defined as another morphological trait interesting for the study of evolutionary, domestication processes and behaviour of pig breeds. Tail is an extension of the vertebral column usually composed of vertebrae of specific shape. Domestic pigs, differently of other Suidae, has a curled tail (Camerlink and Ursinus, et.al., 2020). Tail position and shape can be variable in pigs (Figure 3). Three of the possible tail postures in different emotional state are a "curled tail" (good pig welfare posture), a "hanging tail" (neutral or sing of fear or submission posture) and a "wagging tail" (Reimert et al., 2013; Camerlink and Ursinus, 2020).

Figure 3: Different tail shape in Italian Boars (ID, IL) a) curly tail; b) straight tail (Personal archive).



Tail shape and the possible morphological diversity is difficult to reveal in pigs reared in intensive farms. In these breeding condition, one of the usual practices is tail docking in the first days of life of piglets despite exist the European Directive 2008/120 (Council Directive, 2009) that prohibits it when practiced routinely. This practice, which consists in the amputation of the distal part of the tail, allows to limit damages related to a behavioural problem of "tail-biting". This problem has been partially reduced with tail docking, without however avoiding its manifestation (Paoli et al., 2016). Tail-biting behaviour, whereby pigs bite and cannibalize tails, occurs unpredictably when pigs are grouped and has negatively effects on animal welfare and on the profitability of the farm (Camerlink and Ursinus, 2020).

Although the difficulty in detecting morphological diversity of phenotypic tail shape, some

studies have shown that its conformation is closely related to pig welfare conditions. For example, in a study of Zonderland et al. (2009) it was found a positive correlation between curled tail shape and the absence of tail biting (Zonderland et al., 2009). In addition, further studies have shown that in a wellness situation for pigs, the tail presents curly shape. For this reason, this trait has been defined as the most important indicator of welfare as it is linked to a positive social status of pigs (Spoolder et al., 2011; Paoli et al., 2016). More recently, curled tail has been shown to be associated with absent tails lesion in enriched habitat (Czycholl et al., 2020). From the point of view of pig breeds evolutionary process, tail shape could help to explain morphological changes related to domestication in pigs. Indeed, among other phenotypic trait, tail curliness has been associated with domestication in mammals (Trut et al., 2009). Different tail shape does not occur exclusively in pigs: dogs breeds, for example, could present tails with different conformations (curly, straight).

For this reason, it is interesting to hypothesize that the morphological phenotypic variability of tail in mammals could be regulated by a genetic variability. Using a single nucleotide polymorphisms (SNPs) chip data to compare genome of dogs having straight tail with a genome of dog breeds with curly tail, a genomic region on chromosome 1 was detected to be responsible of tail shapes (Vaysse et al., 2011). Regarding pig breeds, studies were performed only to investigate the genomic bases of the «kinky tail», a defect of the tail that derives from an irregular fusion of two or more caudal vertebrae which causes rigid angles in tail. These studies did not show the correlation between this defect and the classic curly shape of domestic pigs, rather a possible correlation with other defects such as urogenital disorders (Ollivier and Sellier, 1982).

Considering the different aspects of interest for the livestock sector welfare, behaviour and domestication study), studies on genetic basis could help to develop further research that could be useful for several aspects, including phenotypic characterization of a trait related to domestication processes. In addition, the identification of genetic molecular bases that regulate this trait could have an extreme relevance in swine selection programs in term of welfare of pigs. Molecular information about tail shape could contribute to study and find a solution for one of the pig behavioural problem (tail biting) that most impact on pig's breeding industry.

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Chapter 2.

Italian pig breeds of Herd Book and breeding programs

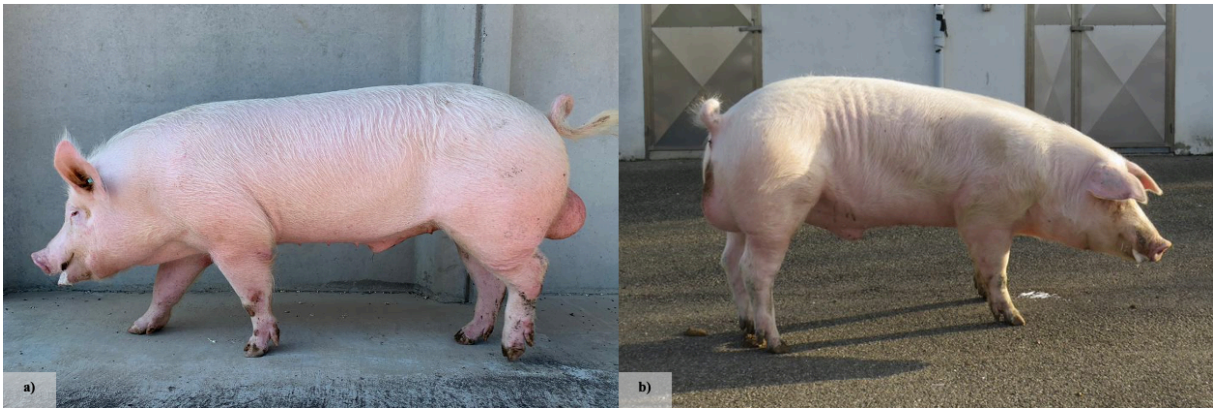
According to EU Regulation 2016/1012, pigs come from different breeds should be registered in the Herd book. The registration of a breed in the Herd Book is a fundamental element for the breed identification and its certification. Therefore, breed name can be attributed to animals and to their products only if they are registered in the Herd Book EU (2016/1012).
The National Pig Breeders Association (ANAS) has always been at the forefront in the defense of quality policies concerning both intensive breeding for Protected Denomination of Origin (PDO) productions and extensive farming for the conservation of local breeds. ANAS is the recognized Italian Pig Breed Society and it supervise the several authorized genetic programs of pure breeds on the entire territory of the Italian Republic. In particular, ANAS manages the breeding programs of the Italian Large White, Landrace and Duroc breeds, which are the reference breeds for PDO productions and for several Protected Geographical Indication (PGI) pig meats processed products. Furthermore, ANAS regulates the conservation programs of the local breeds and other minor breeds. Therefore, the National Pig Breeders Association is responsible of Italian pig biodiversity and it contributes to the characterization of the PDO and PGI final products.

2.1 Italian pig breeds for Protected Denomination of Origin (PDO) productions

Italian pig breeding is oriented to typical productions, mainly for PDO cured hams and for several typical cured hams and salami (PDO and PGI) productions. These types of productions require breeding of heavy pigs (slaughtered at 160- 170 kg live weigh a minimum age of nine months), with carcasses and meat distinguishable for their high-quality characteristics. According to EUROSTAT data, Italian pig farming place at the seventh position in Europe for number of pigs produced (EUROSTAT, 2020). During 2019, in Italy has been produced 10740000 pigs (ANAS estimates based on ISTAT and BDN data) among which, almost 8 million destined for the PDO hams and other PDO and or PGI processed products (~80% of the total) (ANAS, 2020a). PDO productions require meat with specific characteristic typical of Italian Large White, Italian Landrace and Italian Duroc breeds. These breeds derive from the English and North American breeds, but they have been selected for many decades for quality meat for Italian typical pig productions. The genetic breeding programs have clearly

differentiated these breeds from original strains. The Italian Large White (Figure 4a) derives from English Yorkshire. It is a cosmopolitan breed, introduced in Italy in 1872 principally in Padania area. Subsequently, Large White sows and boars belonging to different European selections (Great Britain, Holland, France, Denmark) were introduced in the same area. The Herd Book program for the breed started in December 1970, and the specific breeding goals have formed in the years a distinctive Italian Large White population. Italian Large White animals have a white coat color with pink skin and present straight and pricked ears. This breed is appreciated both for its high prolificacy and a remarkable aptitude for meat production. The Italian Landrace (IL) (Figure 4b) comes from the Danish Landrace, a population of pigs which derive from local pig populations (Celtic) and English Large White. Before the constitution of IL breed, several Landrace breeding animals belonging to different European selections (Denmark, Holland, France, Germany) were introduced in Italy. The Italian Herd Book activities for the breed began simultaneously with the ones of ILW breed and similarly the specific selection goals led to the distinction of Italian Landrace population. Animals have a pink skin, a white coat color with white hairs, forward-floppy ears and a very long backbone. Characteristic of the breed is its maternal attitude with high prolificacy in term of piglets born and farrow, and also a good growth-rate.

Figure 4: a) Italian Large White boar; b) Italian Landrace boar (Personal archive).



The Italian Duroc (Figure 5) derives from a population of pig of United States of America, which was obtained by Iberian pigs, African pigs from Guinea and Berkshire. The first Duroc pigs were introduced in Italy the 1970s. The Herd-book activities began later than for the other Italian breeds, in January 1980. The Italian population is derived from pigs (boars and sows) mainly with North America and Denmark origins. The first ones had carcasses strongly infiltrated with visible fat, while the second ones had leaner carcasses and carried out a negative allele for meat quality *Ryanodine receptor 1* (RYR1), responsible of Pale Soft and Exudative (PSE) meat. Since 2003, the Herd Book selection program led to the identification of an Italian

population, eradicating this negative gene and reducing fat infiltrations. The ID, characterized by a reddish coat with more or less dark shades, is the only strain in the world that also provides a white coat variety completely equivalent to the original-colored breed. The "white" variant of the breed (Figure 5b) was defined in 2007 after an introgression program of white coat genes from the Italian Large White into the Italian Duroc population (ANAS, 2019; Bigi and Zanon, 2020).

Figure 5: Italian Duroc boars. a) traditional variety with reddish coat color; b) ID white variety (Personal archive).

In 2019, a total of 82 farms (42 ILW, 27 IL, 13 ID) were registred in the Italian Herd book. Farms are located mainly in Central and Northern regions of Italy, more suitable for intensive pig farming. In Table.1 is reported the demographic situation of the three breeds (ANAS, 2019)

Table.1: Consistencies of Italian commercial breeds for heavy pig 2019 (ANAS, 2019)

Breeds	Farms	Sows	Boars	Young females	Young males
Italian Large White (ILW)	42	4706	105	5155	529
Italian Landrace (IL)	27	2199	65	1942	152
Italian Duroc (ID)	13	448	101	1060	828

The Herd Book selection for the heavy pig is based on crossbreeding of the F1 Italian dam line (ILW sows x IL boar) with the Italian Duroc boars (sire line). Pig meat derived from ANAS selection is distinguishable because of the balance between lean and fat parts and for a minor loss of water during curing (Bosi and Russo, 2004). The result is a uniform product, with an appropriate fat coverage on ham and grater technological and organoleptic yields.

The selection for Italian breeds aims to produce in a cost-effective way (efficient feed conversion, losses reduction in farms, reduction of slaughterhouse carcass defects) the Italian heavy pigs for the PDO and PGI productions. ANAS breeding programs is based on the

collateral genetic evaluation (SIB TEST) in the ANAS central station, on identification of the best 16,5% of boars tested and on the distribution of their seminal material in Herd Book farms. SIB TEST program led to the evaluation of the genetic traits (transmissible to new generation) of the young boars and their progenitors considering data collected on their siblings. Data associated to a set of traits (performance and quality of the carcass) are processed with statistical models, *Best Linear Unbiased Prediction* (BLUP) *Animal Model Multiple Trait*, to estimate genetic index for each trait and above all for all of them (ANAS 2020a). In particular are considered productive traits, such as average daily gain (ADG) and feed conversion ratio (FRC), carcass traits (weight of lean cuts, LC; ham weight, HW and backfat thickness, BFT) and quality trait (visible intermuscular fat among muscular, VIF and ham weight loss of first salting, HWLFS). These latter quality traits have been introduced in Italian genetic program to assure the dry-cured ham quality. The negative genetic correlations between LC and BFT traits and the different heritability values make the selection for dry-cured ham and carcass quality an ambitious challenge. Selection of the Italian breeds for the heavy pig is complex and represents a "*unicum*", also recognized by the European Union legislation EU (2016/1012). ANAS selection increases efficiency and lean cuts content of the carcass without compromising the traditional ham quality (ANAS, 2016).

In addition, the genetic evaluation for ILW and IL breeds is completed by estimation of prolificacy and longevity genetic quantitative index. Prolificacy of sow is estimated considering number of piglets born alive at the first farrow, while the Longevity index represents the number of sow's farrow during its career (ANAS, 2020a).

2.2 Autochthonous and reconstructed Italian pig breeds

Figure 6: Geographical distribution of autochthonous and reconstructed Italian pig breeds.

The particular and unusual geographical conformation of Italy helped to preserve the biodiversity among autochthonous and local pig breeds which in the past were not selected for performance or productive traits. In Italy, six different autochthonous breeds are reared (Apulo Calabrese, Casertana, Cinta Senese, Mora Romagnola, Nero Siciliano and Sarda) and two minor reconstructed breeds named Nero di Parma and Nero di Lomellina (until 2016 and 2020 respectively). The animals of these two last

populations have been obtained by several crossbreeds in order to recreate phenotypes of ancient breeds that were extinct (Figure 6). The survival of these breeds, considered at the risk of extinction before the beginning of conservation programs of ANAS, was firstly merit of local farmers, which tried to preserve the ancients breeds because of quality of their meat and their robustness in free range and extensive farming. The breeding programs of autochthonous and reconstructed Italian breeds are recognized by Italian Ministry of Agriculture and the breeding animals of different breeds are registered to the Herd book. Since 1997, ANAS carried out the genetic conservation program for Italian autochthonous pig breeds. This program, which aim to preserve the characteristic rusticity of Italian autochthonous pigs, also through the management of mating to control inbreeding, has been fundamental for breeds. ANAS activity is focused on the correct animal's identification, the reliable registration of pedigree information and on the compliance verification of each pig with the breed standard. The correct manage of pedigrees is essential for monitoring inbreeding and to carry out population studies able to monitor genetic variability between and for each breed. Moreover, the availability of complete pedigree information allows to a correct estimation of the inbreeding of each pig (Nanni Costa et al., 2008). Inbreeding monitoring improves productive and reproductive performance under extensive and semi-extensive breeding conditions.

Population consistencies for each breed, updated to 2019, are shown in Table.2 (ANAS, 2019).

Table 2: Consistencies of autochthonous and local Italian pig breeds (ANAS, 2019)

Breeds	Farms	Sows	Boars	Young females	Young males
Apulo Calabrese (AC)	47	546	78	2220	1320
Cinta Senese (CS)	91	719	112	913	288
Casertana (CT)	15	120	22	408	163
Mora Romagnola (MR)	29	297	60	964	155
Nero Siciliano (NS)	76	528	42	2187	2195
Sarda (SR)	12	60	6	119	87
Nero di Parma (NP)	10	99	16	380	18

From morphological point of view, autochthonous and local breeds are clearly distinguishable between each other, even though they are often generally named "black breeds". Cinta Senese (Figure 7a) has a clear distinctive phenotype. The identifying trait of the breed is a black coat color with the presence of a continuous white belt which include front limbs and trunk at shoulder level. This breed is mainly widespread throughout Tuscany and, exclusively for animals born, reared and slaughtered in this region, could be used the PDO certification on fresh meet. The first evidence of the presence animals similar to this breed is date to 1340s. Also

Casertana breed has a characteristic phenotype (Figure 7b). Animals are small in size and have light skeleton and the skin color could be black or gray. The breed is characterized by almost the total absence of hairs and could present wattles. The breeding is traditionally linked to the Campania region, where the presence of glabrous pigs, similar to Asian pigs, is already documented in Roman times. Mora Romagnola breed (Figure 7c) is mainly present in Emilia Romagna region, principally in the east where pig breeding has very ancient origins. Animals have a big size, black or dark gray skin color in all parts of the boy, excluding the abdomen and in the internal faces of anterior and posterior limbs. The coat is reddish-brown until the six months of age, and later the coat color varies to black with lighter reddish abdomen. Characteristic of the breed is the presence of a kind of mane, named "Sparta line" all along the back line.

Figure 7: a) Cinta Senese sows; b) Casertana sows; c) Mora Romagnola piglets (Personal archive).

Apulo Calabrese (Figure 8a) are small to medium size pigs, with black skin and black hairs particularly on the back line. This breed has spread with transhumance along all the peninsula and today has the widest geographical distribution. Several farms of Apulo Calabrese are located in different Italian regions such as Lazio, Abruzzo, Puglia, Basilicata and Calabria. The wide diffusion of the breed has allowed the development of different strains and relative local denominations. Nero Siciliano breeding in Sicily (Figure 8b) dates back to the Carthaginian period and nowadays farms are located principally near to Nebrodi Mountains, in the province of Messina. As the name of the breed suggest, Nero Siciliano pigs principally have black coat

color, with black skin and black hair. In some cases, some white patters could appear on coat, especially on extreme portions of legs or upon the face. Sarda pigs (Figure 8c) have small size and coats presents different colors (black, white, red, gray, reddish or spotted). Distinctive traits of the breed are "cavallina tail", similar to horse tail and a tuft of hair in the lumbar position. The wide genetic variability of the breed has been maintained thanks to the island nature of Sardinia and the extensive breeding of these pigs in inaccessible and isolated areas. Finally, Nero di Parma (Figure 8d) presents a completely black coat, obtained from crossbreeds of some autochthonous breeds (Cinta Senese, Mora Romagnola, Casertana) and Large Black breed animals, whereas Nero di Lomellina (Figure 8e) is characterized by pigs with a black coat with a white frontal list and white-footed legs. Populations are located near to the province of Parma and in the province of Pavia or in Piedmont (Nero di Lomellina) (ANAS, 2020c).

Figure 8: a) Apulo Calabrese boars; b) Nero Siciliano boar; c) Sarda pigs, d) Nero di Parma piglets; d) Nero di Lomellina pigs

The autochthonous Italian breeds represents the identity of geographical region, the agricultural system and the maintenance of local tradition, in particular of gastronomy and landscape. ANAS breeding programs for conversation of these breeds also supports various activities for promotion of breeds and of their products. Mono-breed products from semi extensive or extensive reality contributed to the consolidation and preservation of these breeds. Furthermore, ANAS objective to maintain biodiversity of local breeds is crucial to enhance their resilience and allows the study of genetic markers those could be useful in the future for the breeds of intensive farming.

2.3 Genomic information in Italian pig breeds

ANAS genetic selection for heavy pigs has always used the BLUP Animal Model for genetic estimation of boars and sows, reaching a significant genetic progress in all three Italian breeds. Nevertheless, ANAS has exploited genomic knowledge to improve the efficacy of the breeding programs of the Italian breeds for typical heavy pig and those of the autochthonous and local breeds.

One of first exploitation of genomic resources in breeding program has been the application of a Marker Assisted Selection (MAS) on Italian boars and sows for RYR1 gene. The aim of this selection approach was first the reduction of the allele frequency in Italian population and later the elimination of the causative polymorphism (RYR1 c.1843C> T) responsible of the PSE meet, which causes the stress syndrome in pigs (Fujii et al. 1991). Animals carrying the unfavorable allele for this gene has been excluded from the Herd Book as indicated in breed regulations. Currently, the same analysis is applied also in some autochthonous pig breed (Nero Siciliano and Apulo Calabrese) in order to investigate eventually crossbreeding with commercial European breeds those still carried out the unfavorable allele, such as the Pietrain breed.

Besides, genomic information has been exploited to verify how much the traditional quantitative selection based on estimation of the genetic value with the BLUP Animal Model (SIB TEST) could have modified the allele frequencies of some genes associated with the increase of lean cuts, average daily gain and backfat thickness (IGF2), back fat thickness (MC4R) vertebrae number and lean cuts (VRTN), fat deposition (FTO) and meat quality (PRKAAG3). The study was performed on a group of boars born between 1992-2002 and whit an estimated breeding value reliability > 0.85. The results showed both a positive variation for allele frequencies for genes associated whit higher lean cuts, average daily gain, food conversion rate and a constant allele frequency for PRKAAG3 gene for meat quality during the 20th years of selection. These results confirmed ANAS selection to improve pig's performances preserving meat quality (Fontanesi et al., 2015).

Genomic tools have been used to identify genetic markers or QTLs associated with breeding performance, meat and ham quality in the ILW, IL, ID breeds. A study on IGF2 gene (*insulin-like growth factor 2*) was conducted in order to evaluate his effect on a group of pigs from IL and ILW breeds. Allele expression of this gene shows the imprinting phenomenon with paternal expression and the mutated allele (A), inherited from the paternal line, influences lean meat

deposition and productive breeding performance and, at the same time, reduces reproductive performances (Fontanesi et al., 2010b). Moreover, a Genome Wide Association study (GWA) on a group of 1365 ILW was performed to evaluate the genomic region controlling "drip loss at first salting" traits. This parameter has a quite high heritability (0.30-0.61) and is strictly connect to cure-ham quality. The study identified 44 SNPs and 29 QTLs associated with this trait (Fontanesi et al., 2017). More recently, new markers (EXOSC1, PYGL) associated with meat quality but not with back fat thickness have been identified (Dall'Olio et al., 2020).

In addition, some important morphological productive traits have been considered for genetic investigations. Few studies on vertebrae (VRTN,g .20311_20312ins29) and for teats number and shape was performed on ILW and IL pigs to evaluate the maternal aptitude of the female ANAS breeds and the negative correlation of VRTN "Q" allele with a lower ham weight. The results of the studies confirmed the association between genomic information and phenotype (Fontanesi et al., 2014a; Dall'Olio et al. 2018).

Several studies have been conducted also for the genetic characterization of autochthonous Italian pig breeds. Principally, the studies estimate allele frequencies of some already known genetic markers involved in coat color variation with the aim to identify mono-breed genetic markers (Fontanesi et al., 2010a). An important exploitation of genomic information about coat color is its applicability for authentication mono-breed products. Fontanesi et al. (2016) identified a SNP of KIT gene (C>T) in Cinta Senese breed associated to the typical white belt of Italian Cinta Senese breed which could help to distinguish Cinta Senese meat to industrial white pigs' meat. This information has been patented and nowadays could be exploited for mono-breed pork product authentication (Fontanesi et al. 2016; ANAS, 2017). Furthermore, Schiavo et.al (2018) investigated on the particular hairless phenotype of Casertana breeds, identifying significant QTLs and potential candidate genes on SSC7 and SSC15 which could explain this particular phenotype of the breed (Schiavo et al. 2018).

Recently, new studies opened the possibility to investigate intermediate indicators of the physiological/health status and the genomic inbreeding of Italian pig breeds. Hematological and clinical-biochemical parameters have been used by Bovo et al. (2016) as intermediate phenotypes to associate physiological aspects to genetic marker which could be involved in diseases resistance and resilience (Bovo et al., 2016; Bovo et al.; 2019). Furthermore, SNP genotyping tools let to estimate animal's inbreeding value directly measuring the portion of autosomal genome covered by Run of Homozygosity (ROH), which is an uninterrupted and continuous chromosome portion with homozygosity at all loci. Recently Schiavo et al. (2020)

evaluated the genomic parameters and ROH islands in Italian cosmopolitan and local pigs (Schiavo et al., 2020).

Finally, ANAS also evaluated the applicability of genomic selection (GS) in heavy pigs. This approach consists in the prediction of the breeding value considering genomic information (GEBV). The results of the study of Samorè et al. (2015) showed GS approach does not significantly improve selection compared to traditional quantitative model for medium/high heritability traits (SIB TEST traits) (Samorè et al., 2015). However, GS may be advantageous for traits with a low heritability, such a maternal and reproductive trait. The best approach for Italian pig genomic selection is "*Single Step Model*". In this model all available information on pigs (genomic-pedigree-productive) are considered together for estimation of genetic/genomic index for all pigs (Samorè et al., 2016). Using this approach, ANAS have recently developed a genomic prolificacy index (GEBV) for ILW and IL boars, sows and for the new pig generation (ANAS 2020b).

Aim

The aim of this final thesis is the evaluation of an innovative exploitation of genomic resources in Italian pig breeding programs for a sustainable conservation of autochthonous Italian pig breeds. This work has been performed during an Industrial Ph.D. program with the National Pig Breeders Association (ANAS).

In the first work of this thesis, we completed the genetic characterization for MC1R and NR6A1 polymorphisms of almost all sows and boars of Mora Romagnola breed. This works aims to implement the use of these DNA markers as genetic descriptors in the Mora Romagnola breed Herd Book. These markers can be linked to the genetic authentication of mono-breed productions and could contribute to fight frauds in Mora Romagnola mono-breed products, labelled for their breed origins and usually sold with high price. The aim of the study is driving a sustainable conservation of this local pig breed.

The aims of the other two work of this thesis are the study the genetic diversity associated with heritable phenotypic traits of Casertana breed and the identification of chromosomal regions useful for the genetic characterization of the breed and for the study of biological mechanisms regulating different phenotypes of few domestic traits. In particular, the second study of this thesis investigates on genomic regions that could affect the observed heterogeneity of some domestic traits (ears, wattles and coat color) in Casertana local pig breed. Finally, the principal aim of the third study of this work is the identification of genomic regions associated with the tail shape phenotype in *Sus scrofa* in order to recover information that might have potential impacts in commercial populations.

Taken together, the results from this thesis should allow to implement the genetic breeding programs for the Italian pig breeds of the Herd Book managed by the National Pig Breeder Association (ANAS) and to foresee the development of further studies on behavior and welfare for commercial Italian pig breeds.

Chapter 3.

Research activity

The research activity of this thesis is divided in three different parts and it is preceded by an extensive bibliographic research on genomic molecular bases that regulate domestic traits (vertebrae, ears and tail) that have never been previously reviewed. Until now, several studies revealed genomic regions affecting these traits and their association with other interesting livestock traits; moreover, they helped to explain differences between Asian and European pig breeds.

The first study of this thesis takes advantage of already available knowledges related to molecular markers affecting coat color (reviewed in Fontanesi and Russo, 2013) together with knowledges of genetic markers that control vertebrae number variability in European domestic pigs and in Mora Romagnola breed. The study investigated polymorphisms at the MC1R (coat color) at NR6A1 polymorphism (p.P192L). Polymorphisms at these genes could be considered a diagnostic mutation that help to distinguish domestic pigs from wild boars (Mikawa et al., 2007; Rubin et al., 2012; Ribani et al., 2019). Moreover, MC1R gene has been used to authenticate pork from domestic pig breeds or wild boars (Kijas et al., 1998; D'Alessandro et al., 2007; Fontanesi et al., 2014b). For these reasons, in this study we considered these polymorphisms in order to implement the use of these DNA markers as genetic descriptors in Mora Romagnola breed Herd Book and to improve economic sustainability of low-productivity of this breed by genetic authentication of its mono-breed products.

The other two parts of the thesis take advantage of the morphological heterogeneity of Casertana pig breed. Despite the small size of its population, Casertana pigs are not completely fixed for some domestic traits. Thus, animals of the breed could present a phenotypic diversity. For this reason, we investigated on the genetic component regulating this variability to obtain hints that could explain the genetic mechanisms controlling the expression of simple traits.
In particular, in the second study of this thesis we took advantage from the different ear conformation (size and position) and phenotypic variability of other exterior traits, such as presence or absence of wattles and different coat colours, to carry out GWA study and a genome F_{ST} analyses.
Finally, in the third study of this thesis, we considered the important variability of the tail shape in the Casertana pig population. This domestic trait has an important relevance in livestock sector because it is linked to welfare and pig's behaviour but, until now, genomic regions

affecting tail shape has never been reported. For these reasons, in this study we run a genome wide association study (GWAS) comparing the genome of curly-tailed and strait-tailed animals in order to identify potential genomic regions associated with the tail shape phenotype in Casertana breed. Molecular information about tail shape could contribute to study and find a solution for one of the pig behavioural problem (tail biting) that most impact on pig breeding industry.

3.1 Redefinition of the Mora Romagnola pig breed Herd Book standard based on DNA markers useful to authenticate its "mono-breed" products: an example of sustainable conservation of a livestock genetic resource

Introduction

A sustainable strategy for the conservation of animal genetic resources (i.e. autochthonous and usually less efficient breeds compared to cosmopolitan breeds/lines) is based on the marketing of "mono-breed" meat or dairy products, properly labelled for their breed of origin (Fontanesi, 2009; 2017). These products are usually sold at a higher price compared to undifferentiated ones contributing to assure profitability to the farmers who should be economically interested in raising these less productive animals. The premium price is obtained as the consumers consider positively the link between these breeds and the perceived quality and attributes of their products. On the other hand, this market added value attracts fraudsters that unscrupulously see an economic advantage by selling mis-labelled products to obtain an unjustified additional economic gain. This behavior is considered one of the most critical problems for a sustainable development of mono-breed production chains, as it produces consumer distrust and undermines the commercial added value of many local and niche animal production chains. Therefore, the development of methods that can authenticate mono-breed products is a key issue for a successful construction of a mono-breed production chain that is able to monitor and defend the integrity of its business model and, in turn, its conservation program (Fontanesi, 2009; Hoffmann, 2011).

Autochthonous breeds are usually small populations due to the low number of females and males in the breeding nuclei and the very low effective population size (*Ne*), mainly due to their genetic histories and subsequent management over the last generations (Meuwissen et al., 1994; Groeneveld et al., 2010). Some breeds might not be completely fixed for typical breed specific

features and activities should be oriented to maintain their distinctiveness at the phenotypic and genetic levels (Anderson, 2003; Hoffmann, 2010; Leroy et al., 2017).

In Italy, six autochthonous pig breeds (Apulo-Calabrese, Casertana, Cinta Senese, Mora Romagnola, Nero Siciliano and Sarda) are recognized and managed for their conservation by the National Pig Breeders Association (ANAS). Italian local pig breed products are becoming quite important not only for niche markets driven by local consumption and agritourism activities, but also for the interest of large retailers. For example, Cinta Senese meat has obtained in 2011 the Protected Denomination of Origin (PDO) label that has contributed to the visibility of its products and to the added value of the meat of this breed, favoring the conservation program of Cinta Senese pigs. On the other hand, this aspect evidenced the need to defend its production chain from fraudsters. For this purpose, a DNA based method (that uses a polymorphism in a coat colour gene) useful for the authentication of the meat obtained from this breed has been developed and used as a key tool to defend its production chain (Fontanesi et al., 2016).

Mora Romagnola is another autochthonous pig breed, raised mainly in the eastern side of the Emilia-Romagna region (i.e. Romagna), in the North of Italy. Mora Romagnola pigs are recognized as components of the agricultural traditions of this geographical area. At the beginning of the last century, local pig populations in this area (referred with several local names: e.g. Forlivese, Faentina, Riminese) accounted for more than 300,000 heads. The name ‘’Mora’’, adopted in 1942, refers to the dark (almost black) coat colour (with dark red-brown colour of the abdomen) that is a characteristic trait of these pigs that could be referred as black and tan. After the Second World War, only about 20,000 pigs were estimated to belong to this population that risked the extinction at the end of the 1980s due to the substitution of this breed with more productive breeds and lines, when only 18 Mora Romagnola pigs were recovered in one farm. Since then, a preliminary conservation program was established, that, according to historical records, included some crossbreeding with wild boars and Duroc pigs that contributed to shape, at least in part, the current breed genetic background. Finally, the Heard Book of the Mora Romagnola breed was officially established in 2001 and the breed entered in the conservation program managed by ANAS. Estimated *Ne* of the breed based on molecular markers indicated a very low value, reflecting its genetic history that passed through a strong bottleneck (Muñoz et al., 2019; Schiavo et al., 2021**)**. Mora Romagnola pigs are small-medium sized animals, with ears bent forward and parallel to the muzzle, with a dark skin that along the lumbar region hosts black bristles forming a sort of mane (“*Linea Sparta*”) which is the other

characteristic trait of the breed (Fig. 1). Mono-breed pork products derived from Mora Romagnola pigs are part of an important niche value chain mainly based on a voluntary labelling system that is intrinsically linked to the conservation of this local genetic resources that can only survive due to the added value that its products can obtain at the market.

Black to red coat colours in *Sus scrofa* are mainly determined by alleles at the *Extension* locus, encoded by the melanocortin 1 receptor (*MC1R*) gene (Kijas et al., 1998, 2001). The wild type allele (E^+, indicated also as allele 0101; Fang et al., 2009) is the typical form in European wild boars whereas several other alleles are considered the domestic alleles: alleles E^{D1} (indicated as alleles 0201, 0202 and 0203 by Fang et al., 2009) and E^{D2} (or allele 0301; Fang et al., 2009) that determines the dominant black coat colour (of Asian and European origin, respectively; Kijas et al., 1998; Fang et al., 2009; Muñoz et al., 2018; Ribani et al. 2019]; allele E^P (identified also as alleles 0501, 0502 and 0503; Kijas et al., 2001; Fang et al., 2009) that is usually reported in spotted and completely white pigs; allele *e* (or allele 0401; Fang et al., 2009), that is the recessive allele determining the dark-red coat colour of the Duroc breed (Kijas et al., 1998; Fontanesi and Russo, 2013). DNA markers in the *MC1R* gene have been already proposed to authenticate pork from domestic pig breeds or wild boars (Kijas et al., 1998; D'Alessandro et al., 2007; Fontanesi et al., 2014). Additional genes affecting coat colour have been described in pigs and some of which might contribute to determine the classical colour observed in the Mora Romagnola pigs (Fontanesi and Russo, 2013; Bovo et al., 2020a).

Another diagnostic mutation that could distinguish domestic pigs from wild boars is in the nuclear receptor subfamily 6 group A member 1 (*NR6A1*) gene. A missense mutation (g.299084751C>T), that causes the p.P192L amino acid substitution, fixed in commercial breeds for the domestic allele, is associated with an increased number of vertebrae compared to pure wild boars (21-23 vs 19 vertebrae; Mikawa et al., 2007). Wild boars are fixed or almost fixed for the wild type allele (Rubin et al., 2012; Fontanesi et al., 2014; Ribani et al., 2019).

Applied research activities focused on the sustainable conservation of animal genetic resources have been recently focused on the characterization of European autochthonous pig breeds including phenotypic and genotypic descriptions of local populations, starting from small groups of pigs that might be representative of the whole breed population (Fontanesi et al., 2016; Munoz et al., 2018, 2019; Čandek-Potokar et al., 2019; Bovo et al., 2020a, 2020b). As far as we know, however, no specific attempts have been addressed to obtain a complete characterization of a whole pig breed, in order to establish an efficient and direct monitoring approach that might be able to better implement conservation activities.

In this study we i) monitored phenotypically the Mora Romagnola population to evaluate the compliance to the breed standard, ii) characterized this breed population for polymorphisms in the *MC1R* and *NR6A1* genes by genotyping almost all sows and boars registered to the Herd Book, iii) included information of these DNA markers in the Mora Romagnala breed Herd Book and iv) described how these markers can be linked to the genetic authentication system that was designed to combat frauds that undermine the Mora Romagnola mono-breed production system. These activities have been specifically carried out to lead this local pig breed towards a sustainable conservation of its genetic uniqueness by involving direct actions that included almost the whole breeding population.

Materials and Methods

Mora Romagnola population over the years

Information on the number of pigs of the Mora Romagnola breed registered to the Herd Book from 2001 (the year of official constitution of the breed) to 2019 was retrieved from ANAS records, available in the Mora Romagnola Herd Book database (ANAS, 2020)**.** Animals were classified in breeding pigs (boars and sows) and young pigs, that, according to the breed Herd Book definition, are males that had less than 8 months of age or females until the first farrow (their final destination was not established at the date of the recording: they could become breeding animals, i.e. boars or sows, or they could be slaughtered). Final recording date for statistics and destination of the animals was after the end of each corresponding year, when the collection of the information from the registered farms was completed. Average age of the registered boars and sows was calculated from the records available in the Herd Book database.

Phenotyped animals

Phenotyping of the pigs was carried out in the years 2017-2019. Complete photographic records and/or detailed morphological descriptions based on three breed specific traits [i.e.: coat colour, "*Linea sparta*" and ear shape/position (Fig. 1)] were obtained in this study for 826 Mora Romagnola pigs raised in 27 farms (Table S2), located in different administrative geographic units (provinces (Fig. S1). Phenotyping classes were defined according to the presence or absence of three breed specific trait standards: i) for the coat colour in adult (black and tan) or in young pigs (black and tan or self-red, that changes in adults to become black and tan); ii) for the position of the ears in both adult or young pigs (ears bent forward and parallel to the muzzle as standard trait or carried in other positions, not considered a part of the morphological standard); iii) for the "*Linea sparta*" in both adult and young pigs. The Herd Book of the breed

reports these three traits as descriptors of breed specific features. The adult breeding pigs of this group (357 pigs: 110 boars and 247 sows) were also genotyped, as described below. The young pigs (n. = 469) were only phenotyped.

Genotyping of MC1R and NR6A1 gene markers

The genotyped population included 110 boars and 247 sows registered in the Herd Book as components of the breeding nucleus that were also part of the 826 phenotyped Mora Romagnola pigs. Considering that generations overlapped over the years, the genotyped boars and sows constituted 98% of all active males and 92% of all active females of the breeding nucleus of the whole breed.

To compare genotyping data (see below) between the Mora Romagnola breed and other breeds and populations, this study included a total of other 861 animals belonging to commercial pig breeds or populations (n. 49 Italian Large White; n. 44 Italian Landrace; n. 30 Italian Duroc n. 26 Pietrain; n. 31 Belgian Landrace; n. 18 Hampshire), other Italian local pig breeds (n. 101 Apulo-Calabrese; n. 161 Casertana; n. 122 Cinta Senese; n. 108 Nero Siciliano; n. 58 Sarda) and Italian wild boars (n. 113). Genotyping information of these animals were already included in other studies (Fontanesi et al. 2010, 2014; Munoz et al., 2018; Ribani et al., 2019). In addition, genotyping results from another population of 74 Mora Romagnola pigs, sampled in the years 2010-2014 and described by Ribani et al. (2019) were compared to those obtained in this study from the same breed.

Hair roots were collected from all Mora Romagnola pigs. No ethical permit was needed for this study as biological specimens were collected as part of the routine work of the Mora Romagnola herd book. DNA extraction was carried out using the Wizard (R) Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) or using a standard phenol-chloroform method.

Five autosomal polymorphisms were genotyped: three single nucleotide polymorphisms (SNPs) and one insertion/deletion (indel) in the *MC1R* gene that, on the whole, can distinguish all major alleles at the *Extension* locus (E^+, E^{D1}, E^{D2}, E^P and *e*) described by Kijas et al. (1998, 2001); one missense mutation in the *NR6A1* gene (g.299084751 C>T or p.P192L) that is the causative mutation of the QTL for number of vertebrae, identified on porcine chromosome 1 (Mikawa et al., 2007). PCR conditions and primers were already reported (D'Alessandro et al., 2007; Fontanesi et al., 2010; Ribani et al., 2019). Three fragments were amplified for the *MC1R* gene. One amplicon was 196 bp long and included SNP c.367G>A which differentiates the wild allele E^+ from alleles E^{D2} and E^P. This SNP was analysed by PCR-RFLP using restriction

enzyme *Bsp*HI (recognized sequence: TCATGA) which cuts E^{D2} and E^{P} but not E^{+}. The second *MC1R* amplicon (154 bp long) included two SNPs (c.727G>A and c.729G>A) that were analysed by PCR-RFLP. The first SNP (c.727G>A), that differentiates allele *e* from all other alleles, was genotyped digesting the amplified fragment with restriction enzymes *Hha*I (recognised sequence: GCGC) that cuts all alleles except allele *e*. The second SNP (c.729G>A) that distinguishes all alleles from allele *e* and E^{D1} was analysed by digesting the obtained amplicon with restriction enzyme *Bst*UI (recognized sequence: CGCG) that cuts all alleles except alleles *e* and E^{D1}. The third *MC1R* amplicon included the indel that discriminates allele E^{P} from allele E^{D2}. The amplified DNA was analysed by fragment length analysis on a capillary sequencer (ABI PRISM 3100 Avant Genetic Analyzer, Applied Biosystems): allele E^{D2} was of 168 bp whereas allele E^{P} was of 170 bp due to an insertion of CC at position 67 of the coding region. The *NR6A1* polymorphism (g.299084751C>T) was genotyped by PCR-RFLP from a PCR product of 203 bp using restriction enzyme *Msp*I that cuts the amplified fragment when the wild type allele is present. To confirm the genotyping results, 3-4 fragments, obtained from each primer pairs and showing different genotypes at the PCR-RFLP or fragment analyses, were sequenced using a Sanger sequencing approach, as previously described (Fontanesi et al., 2010, 2014).

Data analyses

Allele and genotype frequencies were calculated for each breed/population at the two investigated loci. Hardy–Weinberg equilibrium was evaluated with the HWE software program (Linkage Utility Programs, Rockefeller University, New York, NY).

Multidimensional scaling (MDS) was used to evaluate the relationships among the analyzed breeds. Briefly, MDS was carried out in R v.3.4.4 previa computation of a dissimilarity matrix D (in which each value represents the Euclidean distance d between two populations) based on allele frequencies. The first two MDS components (C1 and C2) were evaluated.

GENEPOP software version 4.0.7 (Rousset, 2008) was used to calculate population pairwise F_{st} genetic distance and G genic differentiation for each population pair (exact G test) that is a modification of the Fisher's exact probability test (Rousset, 2008). Markov chain parameters used were: Dememorisation: 10000; Batches: 100; Iterations per batch: 5000) including the two gene markers.

Probability to incorrectly assign an unknown meat sample to populations different from Mora Romagnola or crossbred products of this breed versus all other populations (error rate: ER) was calculated using the following formulas (Fontanesi et al.,2016), reported for both loci:

$$ER(MC1R) = 1 - |\delta_{MC1R}| \quad (1)$$

where $|\delta_{MC1R}|$ is the absolute allele frequency difference between the sum of allele frequency of the two Mora Romagnola *MC1R* alleles (E^+ and *e*) observed in the other breeds respect to what observed in Mora Romagnola breed [i.e.: $f(E^+) + f(e) = 1$];

$$ER(NR6A1) = 1 - |\delta_{NR6A1}| \quad (2)$$

where $|\delta_{NR6A1}|$ is the absolute allele frequency difference at the *NR6A1* gene between Mora Romagnola and the other breeds;

$$ERc = ER(MC1R) \times ER(NR6A1) \quad (3)$$

is the combined error rate derived by the two loci.

Vice versa, the probability to correctly assign an unknown meat sample to Mora Romagnola (PMR) was calculated using the following formulas, defined following (Fontanesi et al.,2014; 2016):

$$PMR(MC1R) = 1 - [f(E^+/E^+) + f(E^+/e) + f(e/e)] \quad (4)$$

where $f(E^+/E^+)$, $f(E^+/e)$, $f(e/e)$ are the frequency of occurrence of pigs with *MC1R* genotype E^+/E^+, E^+/e or e/e in the other populations;

$$PMR(NR6A1) = 1 - [f(T/T)] \quad (5)$$

where $f(T/T)$ is the frequency of pigs with *NR6A1* genotype *T/T* in the other populations.

Results

Phenotypic characterization of the Mora Romagnola population

Figure 2 summarizes the number of farms raising Mora Romagnola pigs and the number of boars and sows registered to the breed Herd Book since its constitution. Table S1 reports these numbers as well as the number of young pigs of the breed. The total number of registered reproducers has been increasing since the constitution of the breed Herd Book in 2001, mainly due to an increase on the number of registered sows and young pigs. Therefore, a recurrent phenotyping monitoring is needed for the maintenance of the breed standard and the distinctive characteristics of the Mora Romagnola pig population.

The average age of the sows that were registered in the years 2017-2019 (the years of the phenotyping activities) and that delivered in this period was 2.3 years whereas the average age of the boars in the same period was 2.2 years. Thus, Mora Romagnola can be considered a small population where generations overlap.

The phenotypic characterization that occurred over the same period was based on a total of 826 pigs (357 adult breeding animals and 469 young pigs; Table S3). All adult pigs (male and female breeding pigs) had the standard coat colour except one sow (0.3% of the adult pigs) that had red coat colour. Despite the non-standard coat colour, this animal was still listed in the Herd Book due to a delay in the update of the information available in the database, considering that young pigs (less than 6 months of age) can be registered even if they have this colour that, however, is not tolerated in the breeding animals. Red coat colour was recorded in about 8.9% of the young pigs. For the other two traits (ears position and presence/absence of "*Linea sparta*"), all adult pigs had the standard phenotype, whereas phenotypic variability was observed in the young pigs. About 11% of these animals had half-hanging or raised ears and about 9% of this group did not have "*Linea sparta*".

MC1R and NR6A1 allele and genotype frequencies in Mora Romagnola and breed standard genotypes

The observed genotypes at the *MC1R* and *NR6A1* genes and their frequencies in the genotyped Mora Romagnola breeding pigs are reported in Table 1. The two genes were not completely fixed for one allele. Two major alleles were identified at the *MC1R* gene: *e* (frequency of 0.790) and E^+ (frequency of 0.192). Therefore, alleles *e* and E^+ could be considered the breed specific alleles for the following reasons: 1) the genetic history of the Mora Romagnola breed that experienced, at the beginning of its recovery, crossbreeding with Duroc pigs and wild boars, from which these two alleles may have been introgressed; 2) the observed high frequency of alleles *e* and E^+ in the Mora Romagnola population. Other two alleles were observed at this gene but with very low frequency (E^{D2} = 0.017; E^{D1} = 0.001). Pigs carrying the dominant E^{D1} and E^{D2} alleles appeared a little bit darker than the animals carrying the other *MC1R* alleles, but they could not be clearly phenotypically distinguished from all other pigs of this breed.

Only two pigs carried the wild type allele at the *NR6A1* polymorphic site (one in homozygous and one in heterozygous state). The frequency of the wild type allele (allele g.299084751C) was therefore very low (0.004). Thus, the breed characteristic allele could be considered the

domestic allele (g.299084751T), as already reported in many other domestic breeds (e.g. Fontanesi et al., 2014; Ribani et al., 2019).

The pigs that carried the breed non-specific alleles at the *MC1R* gene were 11 sows and one boar, raised in six different farms: three farms had more than one of these pigs: one had four sows, one had three sows and another one had two sows listed in this group. The boar with genotype E^{D2}/e was raised in a small farm in the province of Ravenna where all other sows carried alleles E^{+} and/or *e*. *NR6A1* allele C was carried by two sows raised in two different farms. All these animals were excluded from the breed Herd Book and were subsequently slaughtered.

After this genotyping activity and subsequent actions, the breed could be virtually considered free from other alleles that are not the breed-specific alleles (i.e., *e* and E^{+} for the *MC1R* gene and T for the *NR6A1* gene). Based on these results, the standard of the Mora Romagnola Herd Book was modified including within the description of the breed the allowed genotypes at the *MC1R* gene (*e*/*e*, E^{+}/e and E^{+}/E^{+}) and at the *NR6A1* gene (T/T), in addition to the other breed-specific phenotypic descriptors.

MC1R and NR6A1 allele frequencies in Mora Romagnola and other pig breeds and in wild boars

Genotyping data at the *MC1R* gene in the Mora Romagnola breed respected the Hardy–Weinberg equilibrium ($P>0.10$). A comparison of the allele frequencies at the *MC1R* and *NR6A1* genes between Mora Romagnola and several other pig breeds and wild boars is summarized in Table 2. Mora Romagnola allele frequencies have been re-calculated on a total of 342 breeding pigs that were maintained in the Herd Book after the phenotyping and genotyping analyses that excluded 15 animals (one after the phenotyping and 14 after the genotyping activities). Genotyping data from all other breeds (six cosmopolitan breeds and five Italian local breeds) and from an Italian wild boar population sampled in the Emilia Romagna region have been compiled from our previous works (Fontanesi et al., 2010, 2014; Munoz et al., 2018; Ribani et al., 2019). Figure 3 shows the MDS derived by allele frequencies at these two genes in Mora Romagnola and 11 other pig breeds and in wild boars.

Mora Romagnola had $|\delta_{MC1R}| > 0.80$ with four out of five local breeds and five out of six commercial breeds. Sarda had $|\delta_{MC1R}| = 0.638$ and Duroc, that is fixed for allele *e*, had $|\delta_{MC1R}| = 0.191$. Wild boars had both E^{+} and *e* alleles but with opposite extreme frequencies than those observed in Mora Romagnola, i.e. allele E^{+} was the most frequent allele, 0.925, whereas allele

e was the less frequent variant observed in this population). The highest $|\delta_{NR6A1}|$ was observed against the wild boar population, that was almost fixed for the wild type allele (allele C). This allele was also observed in some of the local breeds. All cosmopolitan breeds were fixed for allele T that is the same allele fixed in Mora Romagnola.

Allele frequencies in the 2010-2014 Mora Romagnola population, derived by previous studies, matched the results reported in the current larger investigation. Two *MC1R* alleles were identified at that time: alleles E^+ and *e* had frequencies of 0.176 and 0.824 (versus 0.192 and 0.809 in the 2017-2019 sample). Only allele T was detected at the *NR6A1* gene (Ribani et al., 2019).

Pairwise F_{st} measures indicated that all comparisons of Mora Romagnola breed against all other breeds and populations were significant (P<0.001) (Table 3). *MC1R* was informative in all comparisons, whereas *NR6A1*, that was fixed or almost fixed for the T allele in most breeds, had a limited informativeness. The F_{st} value was quite low against the Italian Duroc breed that had also the lowest $|\delta_{MC1R}|$ value. Genic differentiation for each population pair (exact G test) was highly significant in all pairwise analyses except against the 2010-2014 Mora Romagnola data, as also confirmed by the negative and close to zero F_{st} value, further indicating that there were no differences between the two Mora Romagnola populations sampled in different time windows.

Usefulness of MC1R and NR6A1 to differentiate Mora Romagnola meat

Based on the genotyping data obtained in the different pig populations, we evaluated if *MC1R* and *NR6A1* gene markers could be useful to differentiate Mora Romagnola meat from meat of other pig breeds and from wild boars (Table 4). In this first analysis, we have excluded the Italian Duroc breed as the F_{st} estimate indicated that it was the closest breed to Mora Romagnola.

MC1R was very informative against all breeds. The unique allele frequency distribution at this locus in the Mora Romagnola breed made it possible to estimate a very low error rate defined as the probability to incorrectly assign an unknown meat sample to populations different from Mora Romagnola. The error rate was reduced when combined with that derived from *NR6A1*, particularly against wild boars that, on the contrary had a lower effectiveness from the *MC1R* gene due to the presence in high frequency of the E^+ allele. Sarda breed gave the combined highest error rate, due to the quite heterogeneity at the two analysed loci and high frequencies of both E^+ and *e*, compared to all other breeds (Table 4).

On the other hand, the probability to correctly assign an unknown meat sample to Mora Romagnola breed considering one or the other locus was equal to 1 against eight breeds/populations (Casertana, Cinta Senese, wild boars, Italian Large White, Italian Landrace, Pietrain, Belgian Landrace and Hampshire) and >0.90 against Apulo Calabrese, Nero Siciliano and Sarda (Table 4).

Italian Duroc was the breed that determined the highest error rate (0.809). Based on the *MC1R* and *NR6A1* loci, meat derived from Italian Duroc or Mora Romagnola could not be distinguished.

Discussion

Mora Romagnola pig breed is nowadays considered an icon of the traditional agricultural sector of the Emilia Romagna region. Its products fill an important local niche of the market where quality and tradition are considered an added value that makes it possible the sustainable conservation of this local breed. However, this value chain has been affected by several frauds due to mis-labelling products not derived from Mora Romagnola pigs. This type of frauds is considered one of the main problems for the economical maintenance of Mora Romagnola farms and processors.

Since the constitution of the breed Herd Book, Mora Romagnola population has been increasing in terms of number of heads even if the total number of animals is still quite limited (Fig. 2). The relatively low number of heads, the increasing market demand of Mora Romagnola products and the low performances of the Mora Romagnola pigs in terms of growth rate, feed and reproduction efficiencies have also driven some farmers on the use of more performing animals in crossbreeding plans, as it also happened in several similar situations in different local breeds (e.g.Porter,1993).

Based on these critical conditions, in order to preserve the phenotypic and genetic uniqueness of the breed, monitoring and genotyping programs have been started with the final aim to provide information and tools to support the Mora Romagnola value chain, starting from the breeding nucleus of its population. Within this program, almost all Mora Romagnola farms were inspected and animals have been phenotyped to preliminarily evaluate the level of potential admixture (even if based on morphological analyses and on only two DNA markers) from other breeding stocks. This activity did not identify any specific problems or drifts towards incomplete morphological correspondence to the Herd Book standard. The breeding nucleus (boars and sows) was clearly in compliance with the Herd Book standard even if the routine

phenotyping system of morphological traits of the breed does not record all details that we included in this study. That means that few out-of-type animals, that could eventually appear sometimes, are then efficiently identified, culled and not admitted to the breeding nucleus. Only one sow, still listed among the breeding animals had red coat colour that is not allowed by the breed standard. Young pigs can have red/reddish colour that, after a few months usually changes to the classical black and tan standard colour (Fig. 1). The mechanisms by which only some of the young pigs can have red colour, that in most cases changes to the standard colour after a few months, are not known and need to be investigated. The *agouti* locus might play a role in determining the standard colour of the adult animals and, potentially, allele(s) inherited from the wild boars could contribute to the peculiar coat colour phenotype of this breed. However, whole genome resequencing data obtained for this breed (Bovo et al., 2020a, 2020b) did not evidence any clear signature of selection patterns in the correspondence of the agouti signaling protein (*ASIP*) gene that is the genetic determinant of the *agouti* locus. Other genes could contribute to determine the Mora Romagnola characteristic coat colour phenotype. Another phenotypic trait, used to identify the animals of this breed, is the so called "*Linea sparta*", that is a sort of mane of hairs on the back of the pigs. The genetic determinant(s) of this specific trait is (are) not known yet and other studies are needed to clarify this peculiar phenotype. Another trait that is considered to be breed-specific is the position of the ears. Young Mora Romagnola pigs could have more frequently half hanging ears that, when the animals grow up, could change position towards the standard phenotype.

The results obtained by the genotyping of markers in the *MC1R* and *NR6A1* genes confirm, to some extent, the genetic isolation of the breed. Two *MC1R* alleles can be considered the breed specific alleles: E^+, that is also common in wild boars and *e*, that might contribute to the reddish phenotype (in part evident in the young and adult Mora Romagnola animals) and that is fixed in Duroc pigs. The high frequency of both alleles in Mora Romagnola breed confirms its genetic history where wild boars and Duroc blood were probably introduced in the early breed population. The genetic closeness between Mora Romagnola and Italian Duroc breeds has been already reported by whole genome sequencing results (Bovo et al., 2020a, 2020b). Allele frequency observed in the animals sampled in 2017-2019 did not differ from the allele frequencies at the same two loci obtained in past generations, indicating that the population is quite stable and there are no specific pressures against one or the allele at the *MC1R* gene. It is however not completely clear what could be the precise role of these alleles in determining the coat colour of the Mora Romagnola animals. Animals that have the three different genotypes at

this locus (*e*/*e*, E^+/e and E^+/E^+) do not have a clear distinct coat colour in adult pigs. It could be possible that different *MC1R* genotypes are associated with some pigmentation differences that are observed in young pigs. Further studies should clarify this question.

Genotyping of the *MC1R* and *NR6A1* markers identified only few animals (14 out of 357 breeding pigs) that did not carry alleles that were expected in the breed and that were considered breed-specific alleles. Two other *MC1R* alleles have been detected: E^{D2} and E^{D1}, with a very low frequency (Table 1). Their effect on coat colour might be masked by other genes that determine or that contribute to the standard coat colour of the adult animals. These alleles could be derived by the use of some other black pigs in few crossbreeding cases that, according to these genetic evidences, it might be infrequent. Two other pigs carried the wild type allele at the *NR6A1* gene. This allele is considered the original form in wild boars. It was surprising to note that despite the wild type allele at the *MC1R* gene had a relatively high frequency, the same was not the case for the wild type allele at the other locus. As the *NR6A1* T allele (the domestic allele) is associated with an increased number of vertebrae (and indirectly with an increased number of teats; Mikawa et al., 2007), it could be possible that selection towards domesticated traits, related to improved performances, could have contributed to indirectly eliminate the wild-type form from the breed.

Based on the results obtained in this study, ANAS redefined the Herd Book standard of the breed including, as additional descriptors, the genotypes at the *MC1R* and *NR6A1* genes: pigs belong to the Mora Romagnola breed if, in addition to the three main breed-specific morphological traits (black and tan coat colour of the adult pigs, hanging ears and "*Linea sparta*"), have only the *MC1R* E^+/E^+, E^+/e or *e*/*e* genotype and the *NR6A1* T/T genotype. The fixation of these two loci did not create any problems to the reduction of genetic variability of the breed as just 3.9% of the breeding animals were excluded. The "genetic cost" however has been compensated by the fact that, starting from the clear definition of the Mora Romagnola breeding nucleus, it was possible to establish a genetic link useful for the authentication of the Mora Romagnola products. A DNA test can be easily implemented to detect the *MC1R* and *NR6A1* genotypes. Only meat products that can have the allowed genotypes at these two loci can be considered to be obtained from Mora Romagnola pigs. A similar approach has been already described in another Italian local pig breed, Cinta Senese (mainly raised in Tuscany), that was characterized for another DNA markers associated with its belted phenotype (Fontanesi et al., 2016). The Herd Book of Cinta Senese breed has been modified following the same strategy described in the Mora Romagnola breed, even if the larger size of the Tuscany

breed could make it possible to genotype only a smaller fraction of the breeding population (Fontanesi et al., 2016).

DNA based authentication systems of mono-breed products are mainly based on the distribution and frequency of informative alleles and genotypes in the different animal populations that needs to be distinguished and separated by the targeted breed. The level of fixation of specific markers in the targeted population is also very relevant for the practical and successful implementation of the system. A few examples that used coat colour gene markers have been reported in other livestock species, e.g. cattle and sheep (Russo et al., 2007; Fontanesi et al., 2011). One of the advantages of these systems is that they can be implemented in practice and that they can discourage frauds, considering that fraudsters, at these levels, cannot usually compete against DNA authentication methods (Fontanesi, 2009).

The method designed for the Mora Romagnola breed based on *MC1R* and *NR6A1* relies on the related allele frequency distribution in many pig breeds and populations. Even if in theory, the error rate that could be expected from the combined *MC1R* and *NR6A1* genotyping approach is not zero and the probability to correctly assign an unknown meat sample to Mora Romagnola is not 100%, in practice the system can be useful to identify the major potential frauds due to the mis-labelling of non-Mora Romagnola products. It is also clear that potential frauds would not be originated by the substitution of meat coming from other local breeds (that, according to their genetic structure could give a quite high error rate) but only using cheaper meat originated from commercial populations or cosmopolitan breeds that carry the E^P or E^{D2} alleles at the *MC1R* gene, that are not present or allowed in the Mora Romagnola breed. One problem could be due to the Duroc breed whose products cannot be distinguished by those of Mora Romagnola origin using this genotyping system. It is however well known that Duroc pigs are only used as breeding animals (mainly as sires) in commercial crossbreeding plans and the number of pigs of this breed that are slaughtered and commercialized is very limited. Hybrid commercial pigs could eventually carry only one copy of allele *e* if the animal is originated by crossing a Duroc with another commercial line, that might be derived from cosmopolitan breeds that carry E^P or E^{D2}.

This system, currently based on just two genes, could be further refined adding other markers if it will be demonstrated their discrimination potential to distinguish Mora Romagnola pigs, and in turn Mora Romagnola derived products, from all other breeds or commercial lines. For example, if polymorphisms associated with "*Linea Sparta*" would be identified, these markers could be the right candidates to be included in this system as they would also strengthen the

phenotypic uniqueness of this breed. DNA markers in SNP chip panels could also provide information useful for the authenticity of mono-breed products (e.g. Bertolini et al., 2015; Schiavo et al., 2020), including Mora Romagnola products, but it would not be practical the use of their genotyping information to discriminate the pigs that could be registered to the Herd Book.

Conclusions

To our knowledge this is one of the first study that involved almost the complete breeding population of a breed with the final aim to develop a system able to link the Herd Book and the authentication of mono-breed products. Two molecular markers had useful features for this purpose in the Mora Romagnola pig breed and were used as breed-specific descriptors. The final aim was to combat frauds in the meat derived niche value chain. The results of genotyping activities showed that it was possible to fix *MC1R* and *NR6A1* alleles in this breed population without affecting too much its genetic variability. The activities that have been carried out in Mora Romagnola can represent an important case study for similar approaches in other small breeds, that should be adapted according to the genetic structure and potential sources of other genetic materials used by fraudsters. The monitoring of the meat products labelled as being derived from Mora Romagnola pigs and sold in niche markets is currently under way.

Supplementary Materials: Figure S1: Geographic distribution of Mora Romagnola farms in the different administrative units (Provinces) of the North of Italy, Emilia Romagna Region: red dots indicate the geographic localization of the farms in which pigs were phenotyped and sampled in the years 2017-2019. Table S1: Number of Mora Romagnola Herd Book registered farms, boars, sows and young pigs per year (from 2001 to 2019; ANAS, 2020). Table S2: Number of Mora Romagnola pigs that were phenotyped in the years 2017-2019, distributed in different farms and provinces. The breeding animals were also genotyped. Table S3: Number of Mora Romagnola pigs having different phenotypes for three recorded exterior traits (coat colour, ear position and "*Linea sparta*").

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Figure 1. Mora Romagnola pigs: **a)** Piglets with the reddish coat colour; **b)** Sow with black coat color and reddish abdomen (black and tan) and with ears bent forward and parallel to the muzzle; **c)** A detail of the bristle back line known as "*Linea Sparta*" (Sparta line).

Figure 2. Number of Mora Romagnola Herd Book registered farms, boars and sows (y axis) from 2001 to 2019 (x axis). Detailed information is reported in Table S1.

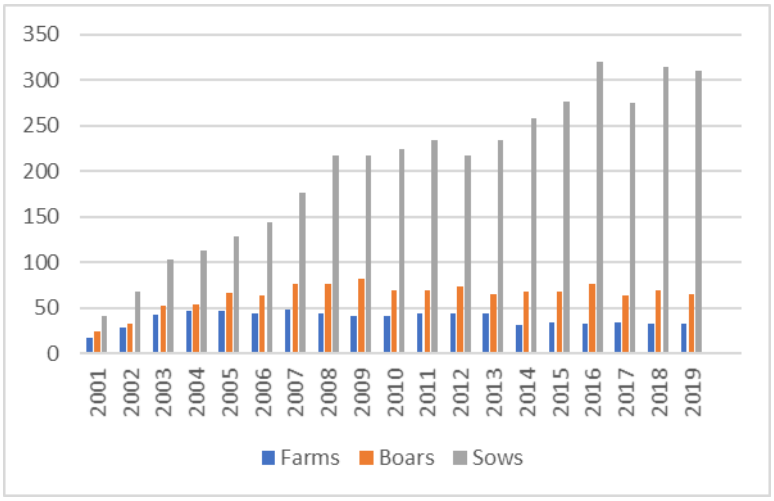

Figure 3. Multidimensional scaling (MDS) plot derived by the allele frequencies at the *MC1R* and *NR6A1* genes in Mora Romagnola and other 11 pig breeds and wild boars analysed pig breeds. Components 1 and 2 (C1 and C2) are represented. The cosmopolitan breeds having the same allele frequencies (Belgian Landrace, Italian Large White, Italian Landrace and Pietrain) at the two genes are grouped in just one point.

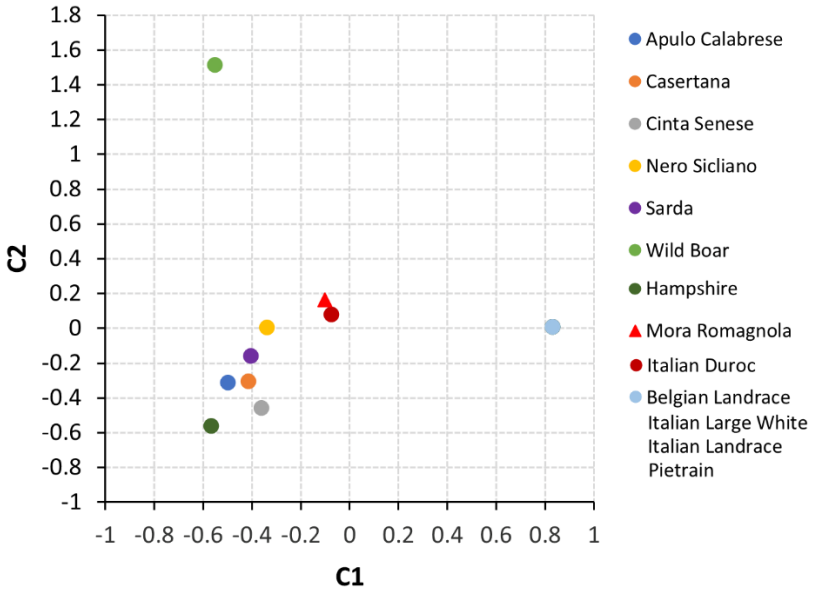

Table 1. Genotypes at the *MC1R* and *NR6A1* genes observed in the analysed Mora Romagnola pigs.

***MC1R* genotypes**	**No. of pigs**	**Genotype frequency**
e/e	225[1]	0.630
E^+/e	109	0.305
E^+/E^+	11	0.031
E^+/E^{D2}	5	0.014
E^{D2}/e	5	0.014
E^+/E^{D1}	1	0.003
E^{D1}/E^{D2}	1	0.003
***NR6A1* genotypes**	**No. of pigs**	**Genotype frequency**
T/T	355[1]	0.994
T/C	1	0.003
C/C	1	0.003

[1] The sow that had red coat colour that was described in the previous paragraph (Phenotyping characterization) had genotype *e*/*e* and T/T at the *MC1R* and *NR6A1* genes, respectively.

Table 2. Allele frequencies at the *MC1R* and *NR6A1* genes in different breeds and populations

Breeds/ populations	No. of pigs	*MC1R* alleles						*NR6A1* alleles		
		E^+	*e*	E^{D1}	E^{D2}	E^P	$\|\delta_{MC1R}\|$[1]	T	C	$\|\delta_{NR6A1}\|$[1]
Mora Romagnola	342[2]	0.192	0.809	0.000	0.000	0.000	-	1.000	0.000	-
Apulo Calabrese	101	0.040	0.035	0.000	0.856	0.069	0.925	0.856	0.144	0.144
Casertana	161	0.124	0.019	0.000	0.767	0.090	0.857	0.938	0.062	0.062
Cinta Senese	122	0.004	0.041	0.004	0.820	0.131	0.955	1.000	0.000	0.000
Nero Sicliano	108	0.120	0.005	0.056	0.630	0.190	0.875	0.718	0.282	0.282
Sarda	58	0.302	0.060	0.060	0.578	0.000	0.638	0.991	0.009	0.009
Wild Boar	113	0.925	0.022	0.000	0.004	0.049	0.733-0.787 (0.053)	0.018	0.982	0.982
Italian Large White	49	0.000	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000
Italian Landrace	44	0.000	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000
Italian Duroc	30	0.000	1.000	0.000	0.000	0.000	0.191	1.000	0.000	0.000
Pietrain	26	0.000	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000
Belgian Landrace	31	0.000	0.000	0.000	0.000	1.000	1.000	1.000	0.000	0.000
Hampshire	18	0.000	0.000	0.000	1.000	0.000	1.000	1.000	0.000	0.000

[1]Absolute delta |δ| allele frequency differential of the alleles between Mora Romagnola breed (sampled in 2017-2019) and all other breeds and populations. This parameter was calculated for the two loci separately: $|\delta_{MC1R}|$ and $|\delta_{NR6A1}|$. $|\delta_{MC1R}|$: for *MC1R*, two alleles were considered to be representative for the Mora Romagnola breed, therefore the comparison was made combining the allele frequency of E^+ and *e* in the other breeds. In the wild boar comparison, $|\delta_{MC1R}|$ was obtained calculating the allele frequency differential for the two alleles separately. In parenthesis is also reported $|\delta_{MC1R}|$ in the combined analysis. [2] Number of breeding pigs that were maintained in the Herd Book after the phenotyping and genotyping analyses.

Table 3. Pairwise F_{st} and genic differentiation (exact G test) comparing Mora Romagnola data (derived by the 2017-2019) sampling versus all other investigated breeds and populations.

Breeds/populations	F_{st}[2]	P value of the G test
Mora Romagnola (2010-2014)[1]	-0.003[3]	0.621
Apulo Calabrese	0.663 (0.694; 0.267)	<0.0001
Casertana	0.623 (0.646; 0.090)	<0.0001
Cinta Senese	0.679	<0.0001
Nero Sicliano	0.572 (0.596; 0.449)	<0.0001
Sarda	0.564 (0.565; 0.024)	<0.0001
Wild Boar	0.848 (0.683; 0.991)	<0.0001
Italian Large White	0.756	<0.0001
Italian Landrace	0.754	<0.0001
Italian Duroc	0.107	<0.0001
Pietrain	0.745	<0.0001
Belgian Landrace	0.747	<0.0001
Hampshire	0.740	<0.0001

[1]Pairwise analyses between the Mora Romagnola genotyping data obtained from the 2017-2019 samples were also carried out against the Mora Romagnola data obtained from the 2010-2014 population. 2 Fst values were estimated including the two genes: the estimates obtained with the MC1R and NR6A1 genes are reported in brackets when both loci were informative. When only MC1R was informative, only one value was reported. 3Fst comparison was not significant (the negative value does not have any biological meaning and should be considered as equal to zero). In all other pairwise analyses, the test was significant (P<0.001)

Table 4. Probability to incorrectly assign an unknown meat sample to populations different from Mora Romagnola (error rate) and probability to correctly assign an unknown meat sample to Mora Romagnola in comparison to all other breeds and population investigated as determined by genotyping *MC1R* and *NR6A1* gene markers.

Breeds	ER(MC1R)[1]	ER(NR6A1)[2]	ERc[3]	PMR(MC1R)[4]	PMR(NR6A1)[5]
Apulo Calabrese	0.075	0.856	0.064	0.990	0.277
Casertana	0.143	0.938	0.134	1.000	0.081
Cinta Senese	0.045	1.000	0.045	1.000	0.000
Nero Sicliano	0.125	0.718	0.090	0.963	0.435
Sarda	0.362	0.991	0.359	0.948	0.017
Wild Boar	0.240 (0.947)	0.018	0.004 (0.017)	0.106	1.000
Italian Large White	0.000	1.000	0.000	1.000	0.000
Italian Landrace	0.000	1.000	0.000	1.000	0.000
Italian Duroc	0.809	1.000	0.809	0.000	0.000
Pietrain	0.000	1.000	0.000	1.000	0.000
Belgian Landrace	0.000	1.000	0.000	1.000	0.000
Hampshire	0.000	1.000	0.000	1.000	0.000

[1] Error rate calculated from the *MC1R* genotyping data. In the comparison with wild boar data, |δ| obtained from the alleles E^+ and *e* was averaged (Table 2). The result obtained considering the combined |δ| derived by summing E^+ and *e* allele frequencies in wild boars is reported in parenthesis. [2] Error rate calculated from the *NR6A1* genotyping data. [3] Combined error rate from the two loci. [4] The probability to correctly assign an unknown meat sample to Mora Romagnola based on the *MC1R* genotyping data. [5] The probability to correctly assign an unknown meat sample to Mora Romagnola based on the *NR6A1* genotyping data.

Supplementary Material

Figure S1. Geographic distribution of Mora Romagnola farms in the different administrative units (Provinces) of the North of Italy, Emilia Romagna Region: red dots indicate the geographic localization of the farms in which pigs were phenotyped and sampled in the years 2017-2019.

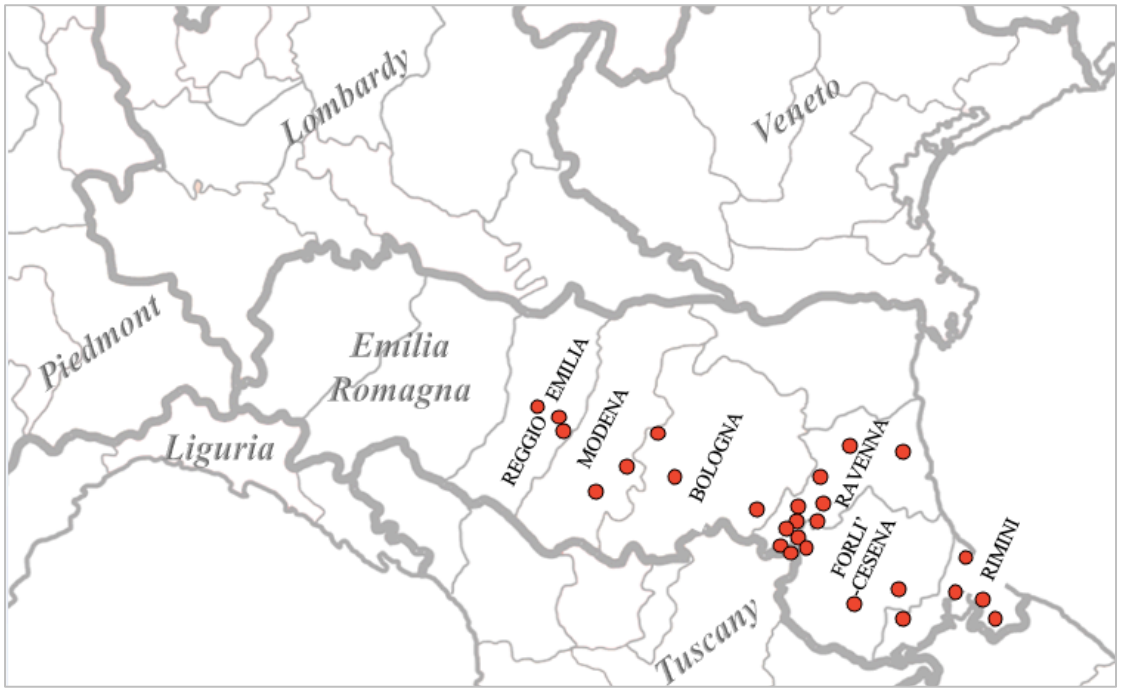

Table S1. Number of Mora Romagnola Herd Book registered farms, boars, sows and young pigs per year (from 2001 to 2019; [25]).

Years	Farms	Boars	Sows	Young pigs[1]
2001	17	24	41	155
2002	29	33	68	233
2003	43	52	103	411
2004	47	54	113	501
2005	47	67	129	684
2006	44	64	144	803
2007	48	77	177	989
2008	44	77	218	924
2009	42	82	218	660
2010	41	69	225	654
2011	44	69	234	675
2012	44	74	218	754
2013	44	66	234	658
2014	32	68	259	829
2015	34	68	277	1000
2016	33	77	320	1151
2017	34	64	275	1393
2018	33	70	314	1596
2019	33	66	311	1410

[1] Males that had less than 8 months of age or females until the first farrow.

Table S2. Number of Mora Romagnola pigs that were phenotyped in the years 2017-2019, distributed in different farms and provinces. The breeding animals were also genotyped.

Province[1]	**Farms**[2]	**Pigs per province**	**Breeding animals**[3]	**Young pigs**[4]
Reggio Emilia	3	25	25 (9 + 16)	0
Modena	2	74	19 (4 + 15)	55
Bologna	3	25	16 (6 + 10)	9
Ravenna	12	530	222 (71 + 151)	308
Forlì Cesena	2	19	18 (3 + 15)	1
Rimini	5	153	57 (17 + 40)	96
Totals	27	826	357 (110 +247)	469

[1] Geographic distribution of the farms in the administrative units referred as provinces. Provinces are listed from west to east (see also Fig. S1). [2] Number of farms where pigs were phenotyped and sampled. [3] Total number of breeding pigs that were phenotyped and sampled for the subsequent genotyping. The first and second number in parenthesis indicates the boars and the sows. [4] Pigs with less than 6 months of age that were only phenotyped.

Table S3. Number of Mora Romagnola pigs having different phenotypes for three recorded exterior traits (coat colour, ear position and "*Linea sparta*").

Province[1]	No. of farms	Pigs per province	Coat colour[2]			Ears position[4]			*Linea sparta*[5]	
			Standard	Red[3]	Other	Hanging	Half-hanging	Raised	Present	Absent
Reggio Emilia	3	25	25	0	0	25	0	0	25	0
Modena	2	74	55	19	0	73	0	1	70	4
Bologna	3	25	24	1	0	24	1	0	23	2
Ravenna	12	530	507	22	1	483	17	30	501	29
Forlì Cesena	2	19	19	0	0	19	0	0	19	0
Rimini	5	153	116	37 (1)	0	151	2	0	146	7
Totals	27	826	746	79	1	775	20	31	784	42

[1] Geographic distribution of the farms in the administrative units referred as provinces. Provinces are listed from west to east (see also Fig. S1). [2]Recorded coat colour: Standard (black and tan in both adult and young pigs); Red (dark red over the whole body in adult or young pigs); Other coat colours in adult or young pigs (e.g. spotted patterns). [3] Red is considered a standard colour in the young pigs that have less than 6 months of age, according to the Herd Book of the breed; the red coat colour in the young pigs usually change to the black and tan colour in the adult age; the number of adult pigs out of all indicated pigs that still showed a red coat colour is reported within brackets (not considered the standard of the breed; adult animals with red coat colour are excluded from the Herd Book). [4]Hanging ears, ears that are bent forward and parallel to the muzzle constitute the standard of the breed; half-hanging and raised define other positions of the ears (half-hanging is intermediate between hanging and raised) that, in this study, have been observed only in young pigs. In adult animals, ears usually become hanging. If not, animals are excluded from the Herd Book of the breed. [5] In this study, the absence of "*Linea sparta*" was observed only in young pigs. Only pigs having this trait can be registered to the Herd Book of the breed.

3.2 Mining genomic information in a local animal genetic resource: genome wide association analyses for several exterior traits in the autochthonous Casertana pig breed

This work has been accepted by the Livestock Science, but the current text is still not in the final format owned by the journal.

Introduction

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

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

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

Materials and methods

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.

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). These markers were assigned to the Sscrofa11.1 genome version, as described in Fontanesi et al. (2012). Genotyping data were filtered using PLINK 1.9 software (Chang et al., 2015) using the following criteria already used in similar studies (Bertolini et al., 2018; Schiavo et al., 2018): genotyping call rate >0.9, minor allele frequency (MAF) >0.01 and Hardy-Weinberg equilibrium $P>0.001$.

Genome wide analyses

Multidimensional scaling (MDS) plots were obtained with the PLINK 1.9 software (Chang et al., 2015) to evaluate distance relationships among the animals of the investigated cohort included in the different comparative genome wide analyses for the considered exterior traits. Genome wide association (GWA) studies were carried out by applying the univariate mixed model of GEMMA (Zhou and Stephens, 2012) that can accommodate the centered relatedness matrix calculated from SNP genotypes to correct for population stratification in a case and control analysis. The model applied for the different exterior traits included the farm and the

sex as fixed effects. To be able to identify markers affecting exterior traits in this experiment that could include a low number of animals and considering the fact that the used SNP chip might have an ascertain bias (derived by the fact that local breeds were not used for the selection of the informative markers), the significant threshold was defined at the $P_{nominal\ value}$<5.00E-05 level, as already applied in several other GWA studies in livestock (e.g. Fontanesi et al., 2012; Sanchez et al., 2014) and in similar analyses for the Casertana breed (Bertolini et al., 2018; Schiavo et al., 2018). The stringent thresholds for declaring significant markers were the following: 0.05 and 0.10 Bonferroni corrected P values that corresponded to a nominal p-value equal to $1.26E^{-06}$ and $2.52E^{-06}$ for the first GWAS, to a nominal p-value equal to $1.22E^{-06}$ and $2.44E^{-06}$ for the second GWAS, to a nominal p-value equal to $1.30E^{-06}$ and $2.60E^{-06}$ for the third GWAS and to a nominal p-value equal to $1.21E^{-06}$ and $2.43E^{-06}$ for the fourth GWAS.

GenABEL (Aulchenko et al., 2007) was used to obtain genomic inflation factors (λ) and quantile–quantile (Q–Q) plots for the GWA studies.

To confirm the results of the GWA studies, F_{ST} analyses were performed on the same dataset and for each trait using PLINK 1.9 software (Chang et al., 2015), by comparing single markers between the groups of pigs with alternative phenotypes for each analysed exterior trait. Relevant F_{ST} differences were defined considering the SNPs over the 99.9th percentile distribution.

Gene annotation of the genomic regions identified by both GWA and genome wide F_{ST} analyses was retrieved from the Sscrofa11.1 genome version available at the Ensembl database (), release 97 (June 2019), considering 500 kbp from both sides of the significant markers.

Results and discussion

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

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

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

Ear size was classified into two categories (small and large, 51% and 49% of the pigs, respectively). Phenotypic characterization of ear size was opportunistic for practical reasons. Therefore, only a total of 37 animals that could be classified for this phenotype were included in the genome wide analyses. Multidimensional scaling plot based on these two ear size categories identified potential substructures in the analysed sub-populations, defined according to this approximated phenotype (Figure S2). The genome wide association study, that according to the λ value (1.044) was able to correct most of the observed stratification in this small sample of pigs, identified two significant markers (P=2.52E-6) on porcine chromosome (SSC) 9 (MARC0047658, at position 130,362,191; ASGA0085666, at position 130,366,477). This result was however not confirmed by the F_{ST} analysis that identified an outlier signal on SSC9 (ALGA0118777, at position 125,868,947), about 5 Mbp apart from the GWA study significant SNPs. Using an F2 population established by crossing European Large White with Chinese Meishan pigs, Wei et al. (2007) reported a large QTL region for ear size on SSC9 that might also include the positions we identified in Casertana pigs.

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

Weak or absence of significant and confirmed genomic regions for the two ear conformation traits might be due to the quantitative genetic architecture that might control these traits in pigs (Wei et al., 2007; Ma et al., 2009) and that could not be dissected by the low number of analysed animals. Simplified phenotypic categories were also used for ear size that might not completely explain the phenotypic variability for this ear trait. Previous studies that investigated ear size and shape in pigs included hundreds of phenotyped animals and the most significant markers identified on a few chromosomes (i.e. SSC5 and SSC7) explained just a fraction of the total genetic variability (Wei et al., 2007; Ma et al., 2009). Therefore, the absence of meaningful

results obtained in our study may mean that also in Casertana these ear conformation traits cannot be explained by simple Mendelian inherited genes.

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

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

Taking together all results reported in this study for the four considered morphological traits and what we previously provided in the same Casertana population for the hairless and tail shape phenotypes (Bertolini et al., 2018; Schiavo et al., 2018), it is interesting to note that in most cases it was possible to obtain hints that could explain the genetic mechanisms controlling the expression of 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

This study investigated several exterior traits in a small population. 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. GWA studies and genome wide F_{ST} approaches).

Critically evaluating the obtained results, it seems that 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 colors. Potential stratifications that could be in some cases not solved might have introduced false positive signals (i.e. for the coat colour GWA study) that, in part, could be disentangled considering the function of the underlying annotated genes. Improvement on the annotation of these genomic regions and additional functional characterization of these genes could shed additional light and the candidate genes affecting the studied traits. 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 another challenging solution, considering the low number of Casertana pigs that are available. In addition, the extensive systems in which these animals are raised creates other difficulties in collecting data and biological materials for DNA analyses. 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, reducing the problems derived by the stratification of the populations and by the high level of linkage disequilibrium that is usually present in small and inbred populations. 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.

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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**[†]
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

[†] 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[†]	Position[‡]	SNP	P-value GWA study	F_{ST} value	Alleles[§]	AF[¶]
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

† *Sus scrofa* chromosome.

‡ Position on the indicated chromosome retrieved from Sscrofa11.1 genome version.

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

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

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

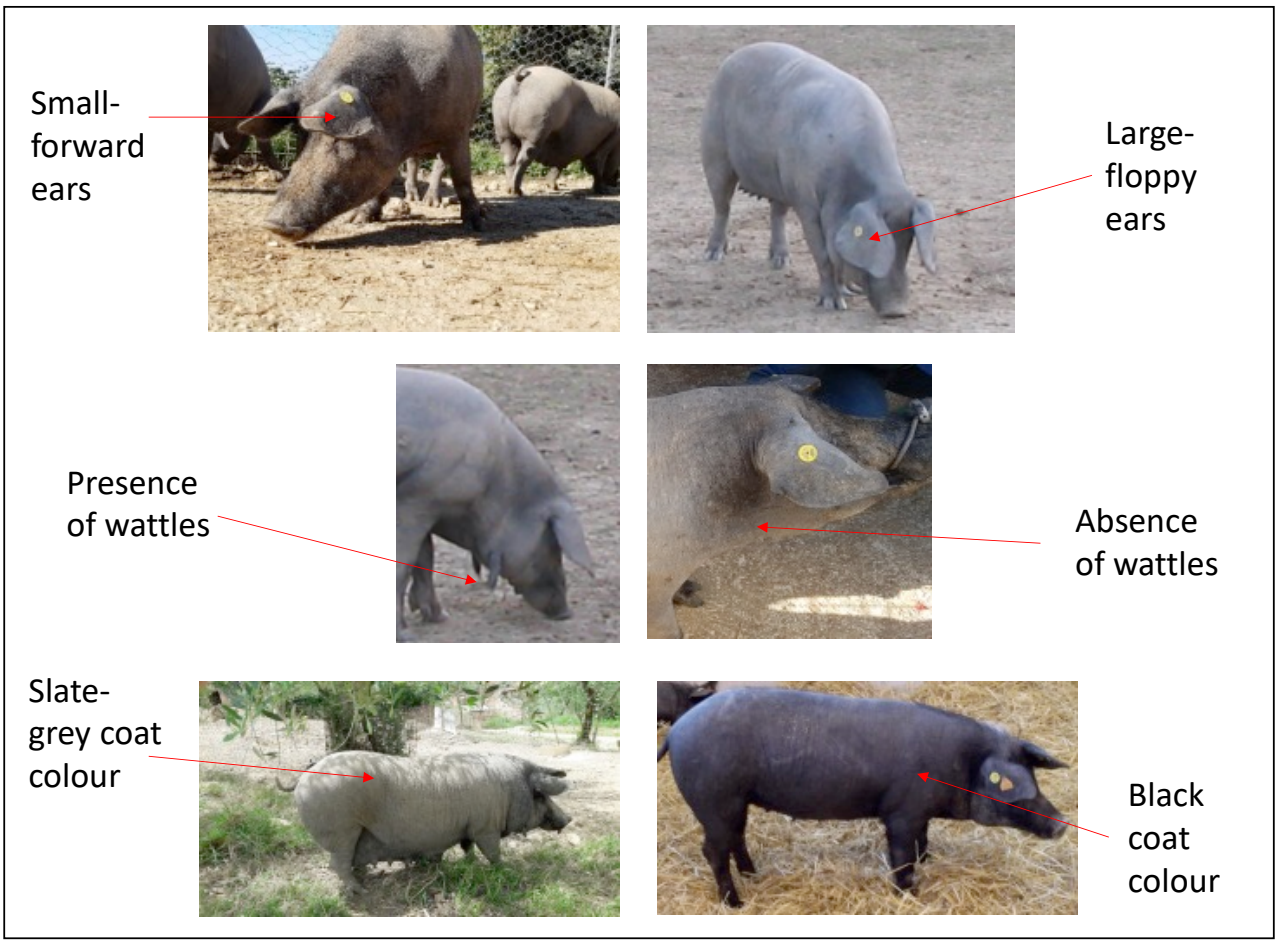
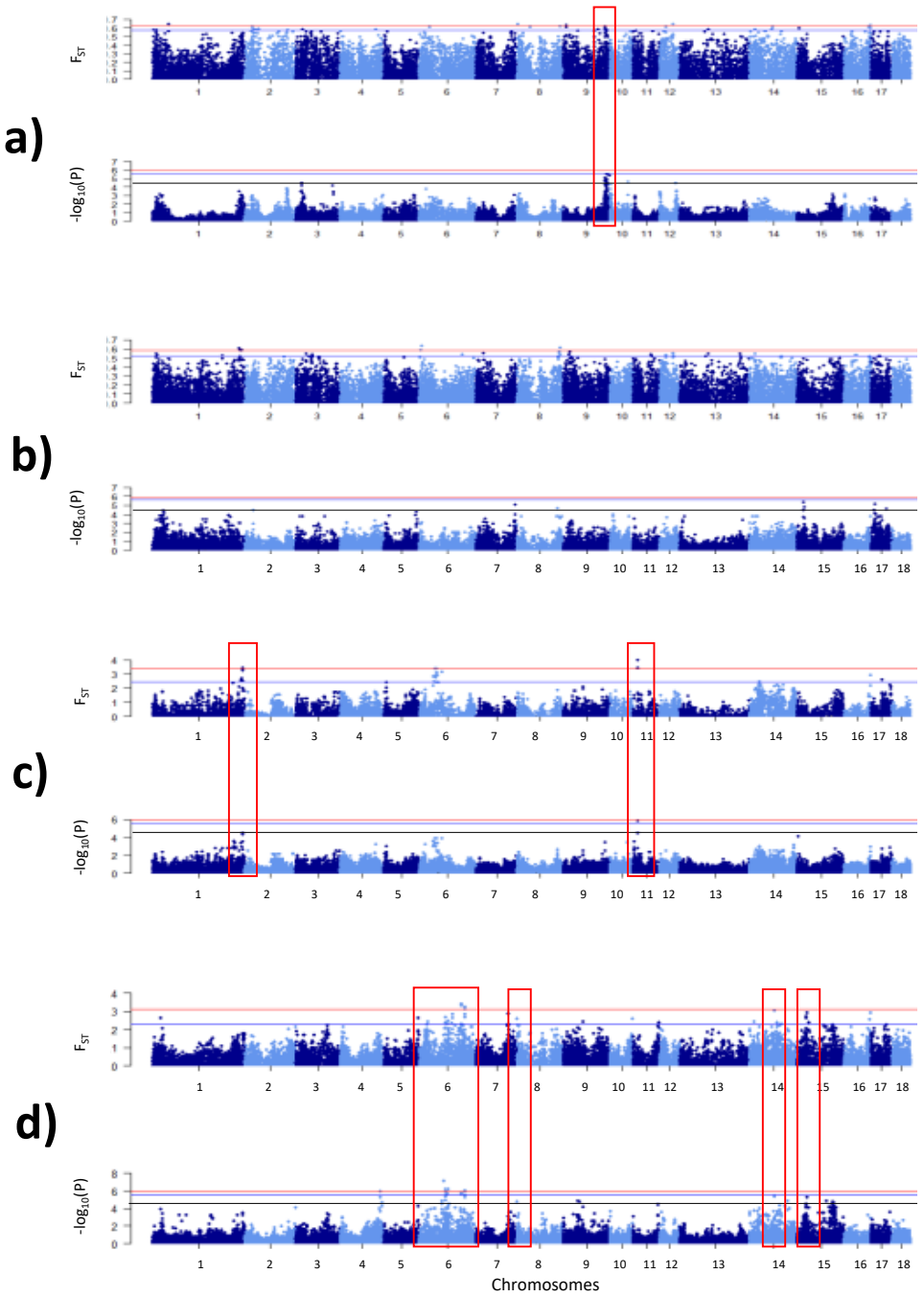


Figure 2. Manhattan plots of the genome wide F_{ST} analyses and genome wide association studies for the four investigated exterior traits: a) ear size; b) ear bearing; c) presence or absence of wattles; d) coat colours. Thresholds in the F_{ST} plots are for the 99.99th percentile (red line) or for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



Supplementary material

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

Trait/Chromosome region[†]	SSC[‡]	Gene start (bp) [§]	Gene end (bp)[¶]	Strand	Gene symbol	Gene Ensembl ID
Wattle/SSC1_region1	1	267480768	267770444	1	*RALGPS1*	ENSSSCG00000005607
	1	267639638	267672923	-1	*ANGPTL2*	ENSSSCG00000005608
	1	267771636	267923390	1	*GARNL3*	ENSSSCG00000005609
	1	267833052	267833154	-1	*RF00026*	ENSSSCG00000020000
	1	267925189	267934094	1	*SLC2A8*	ENSSSCG00000005610
	1	267941953	267959747	1	*ZNF79*	ENSSSCG00000005611
	1	267958456	267965555	-1		ENSSSCG00000033019
	1	267962230	267962359	-1	*RF00302*	ENSSSCG00000018580
	1	267964929	268008830	1	*LRSAM1*	ENSSSCG00000005613
	1	268010307	268066016	-1	*FAM129B*	ENSSSCG00000005614
	1	268116124	268205801	1	*STXBP1*	ENSSSCG00000005617
	1	268209443	268213894	1	*CFAP157*	ENSSSCG00000005618
	1	268213686	268215523	-1	*PTRH1*	ENSSSCG00000005615
	1	268216049	268237839	1	*TTC16*	ENSSSCG00000005616
	1	268233661	268237404	-1	*TOR2A*	ENSSSCG00000005619
	1	268237679	268270361	-1	*SH2D3C*	ENSSSCG00000005620
	1	268276841	268283860	1	*CDK9*	ENSSSCG00000005621

1	268287729	268309406	1	*FPGS*	ENSSSCG00000005626
1	268306120	268343936	-1	*ENG*	ENSSSCG00000005625
1	268355035	268365598	-1	*AK1*	ENSSSCG00000005627
1	268372867	268383544	-1		ENSSSCG00000005623
1	268389483	268402517	-1	*ST6GALNAC4*	ENSSSCG00000005628
1	268408129	268439109	1		ENSSSCG00000031500
1	268409449	268417562	-1	*PIP5KL1*	ENSSSCG00000005629
1	268418445	268423560	-1	*DPM2*	ENSSSCG00000032481
1	268427708	268465802	-1	*FAM102A*	ENSSSCG00000005631
1	268527327	268530563	-1	*NAIF1*	ENSSSCG00000005630
1	268531752	268576994	1	*SLC25A25*	ENSSSCG00000005636
1	268581835	268590836	-1	*PTGES2*	ENSSSCG00000022655
1	268609633	268621997	1	*LCN2*	ENSSSCG00000005638
1	268616034	268619637	1		ENSSSCG00000022295
1	268620965	268640934	-1	*CIZ1*	ENSSSCG00000005640
1	268653174	268701674	1	*DNM1*	ENSSSCG00000005641
1	268691502	268691610	-1	*MIR199B*	ENSSSCG00000019744
1	268701511	268721176	-1		ENSSSCG00000005643
1	268720306	268727670	1		ENSSSCG00000032748
1	268746329	268757458	-1	*TRUB2*	ENSSSCG00000005645
1	268756129	268767931	1	*COQ4*	ENSSSCG00000005646
1	268773861	268788207	1	*SLC27A4*	ENSSSCG00000005648

	1	268798425	268816348	1	*URM1*	ENSSSCG00000005649
	1	268816961	268817057	-1	*MIR219B*	ENSSSCG00000027037
	1	268827445	268827664	1	*RF00012*	ENSSSCG00000019510
	1	268838055	268847233	1	*CERCAM*	ENSSSCG00000005650
	1	268856766	268894210	1	*ODF2*	ENSSSCG00000005651
	1	268894276	268922750	1	*GLE1*	ENSSSCG00000005652
	1	268924948	268927232	1		ENSSSCG00000035449
	1	268936026	269003036	1	*SPTAN1*	ENSSSCG00000005654
	1	269002245	269030719	-1	*WDR34*	ENSSSCG00000005655
	1	269008363	269009987	1		ENSSSCG00000033804
	1	269056717	269064634	1		ENSSSCG00000005656
	1	269069388	269081016	1	*PKN3*	ENSSSCG00000005657
	1	269081272	269084849	-1		ENSSSCG00000005658
	1	269090333	269122156	-1	*ZER1*	ENSSSCG00000005659
	1	269134527	269153066	1	*TBC1D13*	ENSSSCG00000005660
	1	269154090	269157788	1	*ENDOG*	ENSSSCG00000005661
	1	269154639	269167196	-1	*SPOUT1*	ENSSSCG00000005662
	1	269172628	269209494	-1		ENSSSCG00000005663
Wattle/SSC11_region1	11	13765501	13774791	1	*UFM1*	ENSSSCG00000031224
	11	13959865	14154754	1	*FREM2*	ENSSSCG00000009364
	11	14131211	14147122	-1	*STOML3*	ENSSSCG00000009366
	11	14151151	14183851	-1	*PROSER1*	ENSSSCG00000040697

	11	14184134	14370915	1	*NHLRC3*	ENSSSCG00000037746
	11	14319425	14576135	-1	*LHFPL6*	ENSSSCG00000029855
	11	14576214	14723157	1	*COG6*	ENSSSCG00000009369
Coat colour/SSC6_region1	6	79589691	79649644	1	*ALPL*	ENSSSCG00000032607
	6	79662137	79735361	-1	*RAP1GAP*	ENSSSCG00000022029
	6	79744936	79810724	-1	*USP48*	ENSSSCG00000024197
	6	79850757	79921097	-1	*HSPG2*	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	*ZBTB40*	ENSSSCG00000026276
	6	80521572	80548696	1	*EPHA8*	ENSSSCG00000003523
	6	80586183	80591269	1	*C1QA*	ENSSSCG00000003524
	6	80592198	80599440	1	*C1QC*	ENSSSCG00000038706
	6	80601303	80608011	1	*C1QB*	ENSSSCG00000033512
	6	80649143	80843567	1	*EPHB2*	ENSSSCG00000003527
	6	80870866	80882391	-1	*LACTBL1*	ENSSSCG00000003529
	6	80896656	80898939	-1	*TEX46*	ENSSSCG00000003530
	6	80903358	80973632	1	*KDM1A*	ENSSSCG00000003531

6	80931318	80931385	1	*MIR3115*	ENSSSCG00000029242
6	80973261	81112327	-1	*LUZP1*	ENSSSCG00000003532
6	81089662	81112347	-1		ENSSSCG00000025500
6	81196062	81239387	-1	*HNRNPR*	ENSSSCG00000023761
6	81250123	81261853	-1	*ZNF436*	ENSSSCG00000035332
6	81268514	81304581	-1	*TCEA3*	ENSSSCG00000032674
6	81309748	81360248	-1	*ASAP3*	ENSSSCG00000028108
6	81376652	81398346	-1	*E2F2*	ENSSSCG00000032710
6	81422546	81424169	-1	*ID3*	ENSSSCG00000039514
6	81509567	81519072	1		ENSSSCG00000038035
6	81522760	81526880	1	*RPL11*	ENSSSCG00000024260
6	81566801	81582028	1	*ELOA*	ENSSSCG00000025440
6	81590951	81598872	1	*PITHD1*	ENSSSCG00000023495
6	81601486	81605912	1	*LYPLA2*	ENSSSCG00000023191
6	81605918	81630365	-1	*GALE*	ENSSSCG00000027827
6	81610427	81630470	-1	*HMGCL*	ENSSSCG00000026025
6	81637698	81723231	-1	*FUCA1*	ENSSSCG00000027659
6	81659186	81692550	-1	*CNR2*	ENSSSCG00000027849
6	81706663	81741526	-1	*SRSF10*	ENSSSCG00000036293
6	81722026	81726489	1	*PNRC2*	ENSSSCG00000023326
6	81780869	81780977	1	*RF00026*	ENSSSCG00000024834
6	81799570	81903971	-1	*MYOM3*	ENSSSCG00000036534

Coat colour/SSC6_region2	6	85891364	86131721	-1	*SRSF4*	ENSSSCG00000003589
	6	86085281	86085423	-1	*RF02271*	ENSSSCG00000033491
	6	86130111	86130217	-1	*RF00026*	ENSSSCG00000031401
	6	86137754	86228145	1	*PTPRU*	ENSSSCG00000003590
Coat colour/SSC6_region3	6	125354757	125493965	-1		ENSSSCG00000038382
	6	125890532	126043141	1	*PIK3C3*	ENSSSCG00000003749
	6	126083379	126083698	-1		ENSSSCG00000037233
	6	127382248	127392164	-1	*SYT4*	ENSSSCG00000037079
	6	127866246	127890915	1	*KCNG2*	ENSSSCG00000005780
	6	127881306	127952556	-1	*PQLC1*	ENSSSCG00000023171
	6	127954766	127991170	-1	*TXNL4A*	ENSSSCG00000005777
	6	127960266	127973191	1	*HSBP1L1*	ENSSSCG00000005778
	6	128004528	128030296	1		ENSSSCG00000038475
Coat colour/SSC6_region4	6	136695884	136696193	-1	*RF00100*	ENSSSCG00000030678
	6	137263585	137263683	1	*RF00026*	ENSSSCG00000024120
	6	137410919	137453959	-1		ENSSSCG00000003774
	6	136633437	137201213	-1	*ST6GALNAC3*	ENSSSCG00000024494
	6	136057959	136176334	1	*PIGK*	ENSSSCG00000038329
	6	135745879	136020581	-1	*AK5*	ENSSSCG00000003773
	6	136203309	136391034	-1	*ST6GALNAC5*	ENSSSCG00000033425
	6	137383193	137400783	1	*ASB17*	ENSSSCG00000031522
Coat colour/SSC8_region1	8	796389	773556	-1	*FAM53A*	ENSSSCG00000032633

	8	818217	802940	-1	*SLBP*	ENSSSCG00000008676
	8	825182	818588	-1	*TMEM129*	ENSSSCG00000008678
	8	837322	825339	1		ENSSSCG00000008677
	8	895912	879207	1	*FGFR3*	ENSSSCG00000030827
	8	928019	898181	-1	*LETM1*	ENSSSCG00000008675
	8	1019326	964893	1	*NSD2*	ENSSSCG00000008682
	8	1013071	1012948	1	*RF00427*	ENSSSCG00000020173
	8	1038993	1018910	-1	*NELFA*	ENSSSCG00000008681
	8	1058148	1056750	1	*C4orf48*	ENSSSCG00000008686
Coat colour/SSC14_region1	14	71827631	71894754	1	*CCAR1*	ENSSSCG00000010243
	14	71928395	71983778	1	*STOX1*	ENSSSCG00000010245
	14	71970814	71970946	-1	*RF00156*	ENSSSCG00000019527
	14	71989533	72030993	1	*DDX50*	ENSSSCG00000010246
	14	72040083	72061392	1	*DDX21*	ENSSSCG00000010247
	14	72071874	72099801	1	*KIF1BP*	ENSSSCG00000010248
	14	72183363	72199259	1	*SRGN*	ENSSSCG00000023374
	14	72210483	72241657	1	*VPS26A*	ENSSSCG00000010250
	14	72245532	72274827	1	*SUPV3L1*	ENSSSCG00000010251
	14	72282016	72336533	1	*HKDC1*	ENSSSCG00000010252
	14	72382912	72460924	1	*HK1*	ENSSSCG00000010253
	14	72462004	72475662	-1	*TACR2*	ENSSSCG00000010254
	14	72501289	72555081	1	*TSPAN15*	ENSSSCG00000036152

	14	72604468	72606363	-1	*NEUROG3*	ENSSSCG00000034488
	14	72637075	72649799	1	*FAM241B*	ENSSSCG00000040498
	14	72787602	72953095	1	*COL13A1*	ENSSSCG00000010256
Coat colour/SSC15_region1	15	26882562	27362814	-1	*CNTNAP5*	ENSSSCG00000015726
	15	26998479	27356862	1		ENSSSCG00000036368
	15	28530166	28530733	-1		ENSSSCG00000030079
	15	28834319	28837216	-1		ENSSSCG00000039209

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

‡ *Sus scrofa* chromosome.

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

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

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

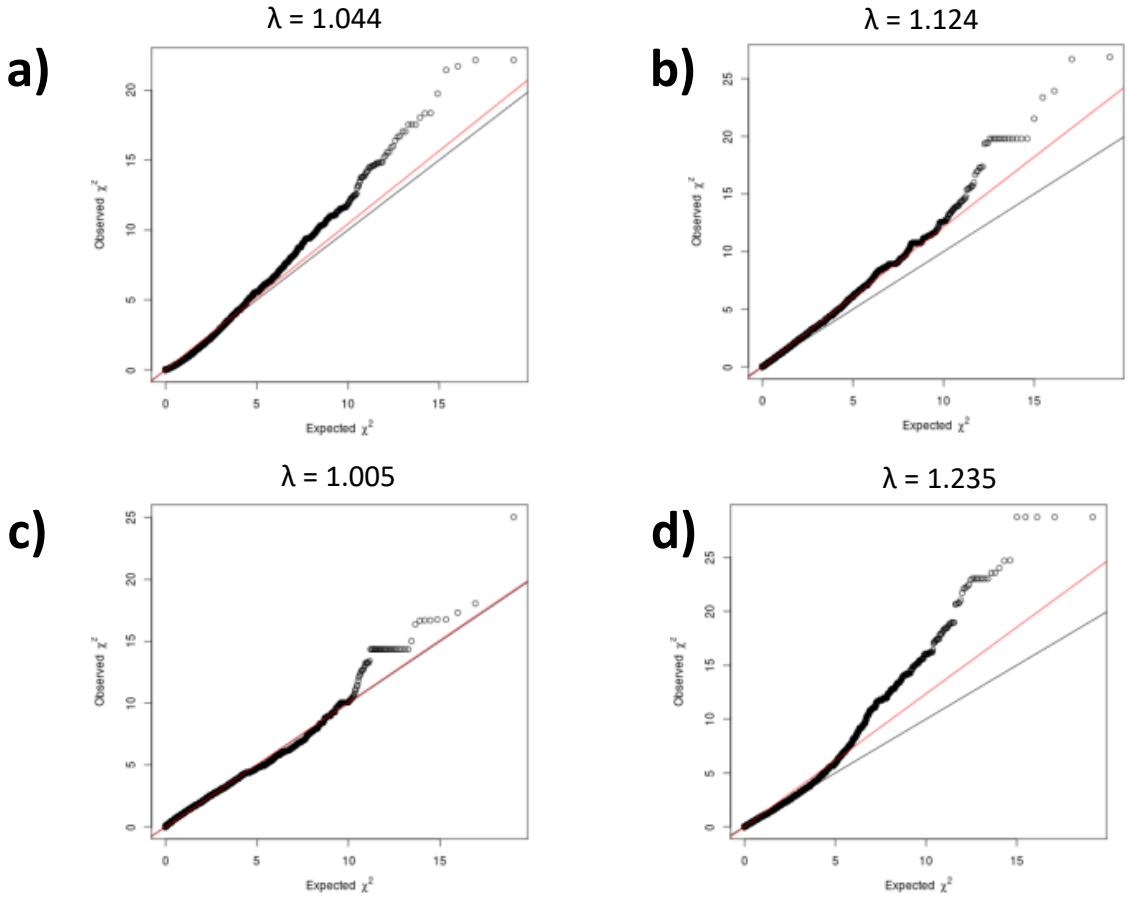
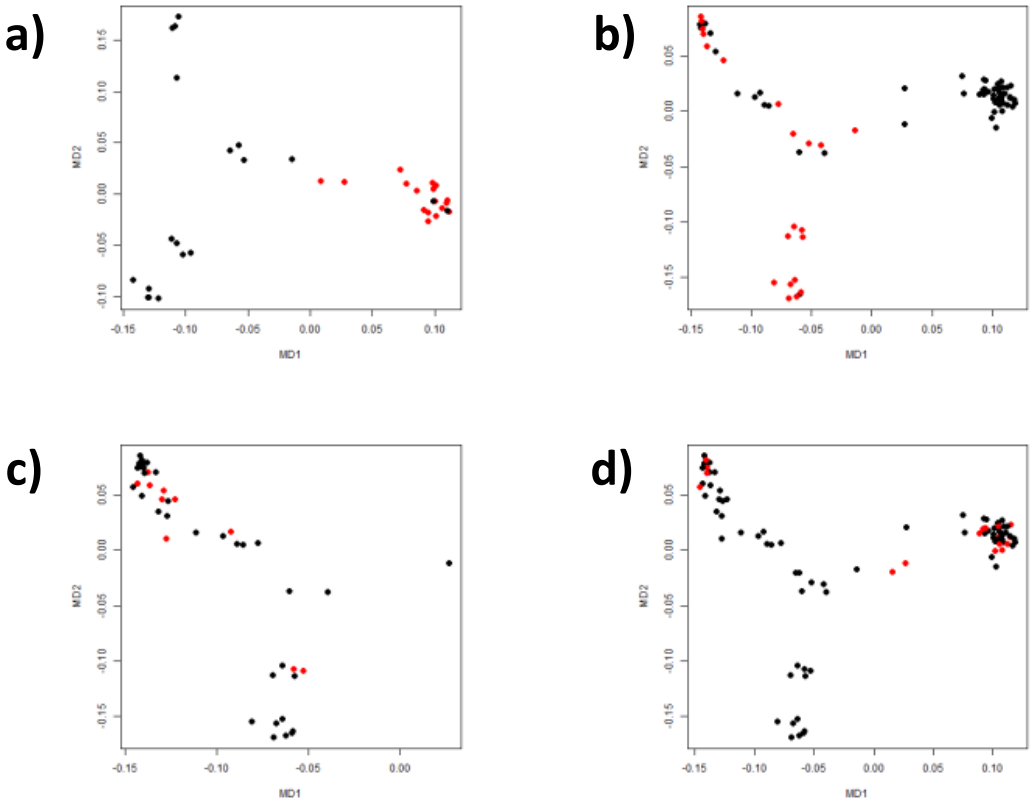


Figure S2. Multidimensional scaling (MDS) plots obtained for the pigs classified according to the alternative phenotypes for the investigated traits: a) ear size (small, black dots; large, red dots); b) ear bearing (floppy, black dots; forward, red dots); c) absence (black dots) or presence (red dots)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



3.3 Exploiting phenotype diversity in a local animal genetic resource: identification of a single nucleotide polymorphism associated with the tail shape phenotype in the autochthonous Casertana pig breed

This work has been accepted by the Livestock Science, but the current text is still not in the final format owned by the journal.

Introduction

Conservation of animal genetic resources is mainly aimed to preserve genetic diversity and associated inheritable phenotypes characterizing different populations that might be interesting for current or future purposes, including potential use in breeding programs. These resources can be also useful to understand biological mechanisms determining unique phenotypes derived by diversity in selection pressures or as result of adaptation to environmental and production conditions (Leroy et al., 2016).

Casertana pigs constitute a local breed mainly raised in Central-South regions of Italy. Pigs of this breed are considered the descendants of the ancient Neapolitan pig population that largely influenced the constitution of the modern commercial pig breeds through introgression of blood into British pig populations during the 19th century (Porter, 1993). Neapolitan pigs were, in turn, influenced by Asian blood in the late 18th century (Porter, 1993). Casertana is enlisted among the endangered animal genetic resources as the Herd Bookof this breed accounts for about 100 boars and sows currently registered (ANAS, 2016). Animals are mainly raised in extensive or semi-extensive production systems with possible contacts and crossbreeding with European wild boars that could have contributed, at least in part, to shape their morphological characteristics. Casertana pigs have a black or grey coat colour, wrinkled skin, forward ears, and usually a typical hairless phenotype. The pigs of this breed are usually curly-tailed, like several other domestic pig populations. However, Casertana population shows some variability for this trait, including animals having straight and wavy tail as in a few other pig breeds and in wild boars.

Domestication in mammals has been a complex and continuous process associated with a series of changes in the domesticated animals compared to the wild counterparts, derived by selective breeding of animals showing favourable production and reproduction performances, and increased docility that indirectly shaped the genome of domesticated populations (Wiener and Wilkinson, 2011; Larson and Burger, 2013; Carneiro et al., 2014; Wang et al., 2014; Wilkins et al., 2014). Several morphological features have been also directly or indirectly selected and,

in most cases, fixed in domesticated populations as result of the domestication process (Darwin, 1868). Coat colour is one of the most common phenotypic traits that has been modified as result of reduced selective pressure against colours with low fitness in the wild and of aesthetic preferences of the breeders, sometimes associated with higher production performances (Clutton-Brock, 1999). Among several other morphological characters, curliness of the tail and shape has been associated with domestication in mammals (Trut et al., 2009).

The tail is considered an extension of the spinal column usually composed of specifically shaped vertebrae. Spontaneous curly tail phenotypes in mice have been the matter of studies that investigated the role of embryonic development in this morphological anomaly (Copp et al., 1988; van Straaten and Copp, 2001; Ohnishi et al., 2017). Curly tail is also commonly observed in many dog breeds. Vaysse et al. (2011) compared the genome of dog breeds having curly tails with that of breeds with straight tails using single nucleotide polymorphisms (SNPs) chip data and identified a genomic region on chromosome 1 significantly associated with these alternative tail shapes.

In pigs, few studies have been reported on the genetic factors affecting tail shape. A putative recessive genetic defect known as kinky tail (or flexed or screw tail), derived by fused caudal vertebrae associated in some cases with other defects, has been described in the mid of the last century (Nordby, 1934; Donald, 1949; Brooksbank, 1958). It is not known if this defect could be, in some way, related or not to the normal curling of the tail that is common in domestic pigs. This signature of domestication, however, seems not fixed in all pig breeds (Porter, 1993) but no systematic study has been conducted so far, probably because the difficulties in retrieving phenotype information due to the usual practice of tail docking in most herds.

In this study, we took advantage from the variability of the shape of the tail that we recorded in the Casertana pig population and run a genome wide association study (GWAS) comparing the genome of curly-tailed and strait-tailed animals to identify genomic regions associated with the tail shape phenotype in *Sus scrofa*.

Materials and methods

Animals

A total of 101 Casertana pigs (of about 7 to 20 months old) from six different farms were evaluated. Photographic records of each animal were obtained to capture information on the tail shape in standardized restraining conditions (including a direct evaluation of the personnel on this phenotype during this phase for the biological sampling) for all animals and after release

(Figure 1). Pigs were classified as follows: 53 (25 males and 28 females) showed the curly tail phenotype; 19 (five males and 14 females) showed the strait-tail phenotype; 29 were not classified and excluded from the study as tail docking, that was practised by the farmers as routine before weaning of the piglets, prevented the recording of any tail phenotype.

Genotyping

Hairs (with roots) were collected from the investigated pigs. DNA extraction was carried out using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. Genotyping of the extracted DNA was obtained with the Illumina PorcineSNP60 BeadChip v.2 (Illumina, Inc., San Diego, CA, USA) that interrogated 61,565 SNPs. Single nucleotide polymorphisms were assigned to the Sscrofa11.1 genome version, as previously described (Fontanesi et al., 2012). PLINK 1.9 software (Chang et al., 2015) was used to filter SNPs and genotyping data using the following criteria already used in a similar study (Schiavo et al., 2018): genotyping call rate >0.9, minor allele frequency >0.01 and Hardy-Weinberg equilibrium P>0.001.

Data analysis and genome wide association

To evaluate distance relationships among the animals of the investigated cohort, multidimensional scaling (MDS) was obtained with the PLINK 1.9 software (Chang et al., 2015). Genome wide association study was carried out by applying the univariate mixed model of GEMMA (Zhou and Stephens, 2012) that can accommodate the centered relatedness matrix calculated from SNP genotypes to correct for population stratification in a case and control analysis. The model also included the farm and the sex as fixed effects. To be able to identify associated markers in this experiment that included a low number of animals (derived by the fact that the analysed pigs were almost a complete representation of the whole population of the Casertana breed) and that used a SNP chip that might originally have an ascertain bias (as local breeds were not used for the selection of the informative SNPs), the significant threshold was defined at the $P_{nominal\ value}$<5.00E-05 level, according to the Wellcome Trust Case Control Consortium (2007) and as also applied in several other GWAS in livestock (e.g. Fontanesi et al., 2012; Sanchez et al., 2014). Genomic inflation factor (λ) and quantile–quantile (Q–Q) plot were obtained with GenABEL (Aulchenko et al., 2007). Gene annotation information was retrieved from the Sscrofa11.1 genome version available at the Ensembl database (http://www.ensembl.org/Sus_scrofa/Info/Index), release 91.

Results and discussion

A recent phenotypic characterization of the endangered Casertana pig population that we carried out noted several morphological differences among distinct animals of this autochthonous breed (data not shown). For example, in addition to the hairless or hypotrichotic condition (that is the characteristic phenotype of the Casertana animals), we already described the presence of haired pigs in this population and this morphological variability was used for a GWAS that we have recently reported (Schiavo et al. 2018). Despite a limited number of animals was included in that study, we were able to identify genomic regions associated with the hairless phenotype, demonstrating that local animal genetic resources can be used to genetically describe phenotypic variability of simple traits (Schiavo et al., 2018).

Another morphological trait that is not fixed in this breed is the shape of the tail (Figure 1). Of the animals for which we could record this phenotype, 26% (19 out of 72) showed a strait tail without any curls, similarly to the usual shape of wild boars. This shape was clearly different from the curly tails reported in the remaining investigated pigs (74%). There was no effect of the age and the sex did not exclusively explain the observed phenotype as both sexes were included in the two phenotype groups. In addition, we could exclude the possible effect of the behavioral change of tail posture on this phenotype (Zonderland et al., 2009). The recording system was based on standardized conditions and subsequent photographic records of the animals confirmed their assignment to one or to the other group of tail shape phenotype. The two groups were observed in animals from all six farms. Limited pedigree record prevented the possibility to evaluate any potential founder effect.

A total of 36,533 autosomal SNPs, mapped to a unique position in the Sscrofa11.1 genome version, was used for MDS. The obtained MDS plot showed some structures not well defined in the analysed pigs that however did not clearly separate the curly and strait tailed Casertana pigs (Figure 2). A stratified sample could be a critical point in GWAS in a very small population where, to some extent, all animals might be related. Figure S1 reports the genomic inflation factor (λ) and Q–Q plot that did not show any biased test statistic distribution, suggesting that the investigated cohort was corrected for a possible stratification effect.

Figure 3 reports the Manhattan plot obtained in this GWAS. One significant SNP (P=2.3E-05) was identified on porcine chromosome 12 (SSC12). This marker indicated as ALGA0064877 (rs81439488) is located at position 10,301,075 of this chromosome.

One of the closest annotated gene in this desert chromosome region is the *SRY-box 9* (*SOX9*) gene (positions 8,641,629-8,647,764, encoded by the -1 strand), that, according to its function, might be the most plausible candidate gene, explaining the recorded phenotypic variability. It is well established that the expression of this gene at the embryonal level marks the onset of cartilage differentiation (Wright et al., 1995; Healy et al., 1996). *SOX9* encodes for a transcription factor that is required during sequential steps of the chondrocyte differentiation pathway, notochord maintenance and skeletogenesis (Akiyama et al., 2002; Barrionuevo et al., 2006; Montero et al., 2017). Continued expression of *Sox9* in differentiated chondrocytes is essential for subsequent hypertrophy and sustains chondrocyte-specific survival mechanisms (Ikegami et al., 2011). Heterozygous mutations within and around human *SOX9* cause campomelic dysplasia that is a malformation syndrome characterized by cartilage derived skeletal structure defects (Foster et al., 1994; Wagner et al., 1994). These mutations, most of which reduce the level of expression of this gene, are located upstream spanning a large region (from 50 kb to more than 1 Mb) in which regulatory elements are present (Wunderle et al., 1998; Bagheri-Fam et al., 2006). Close upstream mutations produce more severe defects whereas far upstream mutations cause mild defects (Pfeifer et al., 1999; Velagaleti et al., 2005; Leipoldt et al., 2007).

Based on these studies in other species it is tempting to suggest a possible regulatory mechanism affecting *SOX9* expression in porcine developing chondrocytes that would, in turn, produce a mild cartilage/skeletal effect determining the shape of the tail. This hypothesis might be worth of further investigation starting from a precise characterization of the structure and morphology of the pig tail with different shapes for which, at present, there is no detailed investigation. Our phenotype records were based only on an external morphological evaluation of the shape of the tail. Furthermore, analysis of gene expression of *SOX9* at different developmental stages should be also carried out to evaluate the role of this gene in the phenotype observed in pigs.

The results we obtained might have broader impacts than those that would be limited to a simple morphological characterization. The shape of the tail could be important in relation to the problem of tail biting in pigs. Tail biting is a widespread behavioral vice with significant animal welfare implications and economic losses in commercial pig farms (Bracke et al., 2004). A few studies have established correlations between tail posture and tail biting incidence suggesting limited damages and related welfare complications with behaviors of the pigs that tended to have a tail posture up that those with tail posture down (Zonderland et al., 2009; Lahrmann et al., 2017). It would be interesting to evaluate if pigs with genetically determined curly tails (as

a possible adaptation derived by the domestication process) are less affected by tail biting damages than pigs with strait tails.

Conclusions

This work demonstrated that autochthonous animal genetic resources, even constituted by very small populations, might be used to disclose genetic factors affecting peculiar traits by exploiting segregating phenotypes and genetic variability. To our knowledge, this is the first study that reported a frequency distribution of the tail shape phenotype in a pig population. Our results indicated that this morphological trait is associated with a marker close to an important gene involved in embryonic development, opening other hypothesis, worth of further investigations. It will be important to validate the results we obtained in this GWAS in other breeds and populations, including a more precise anatomical characterization of this trait, to further extend the impact of the results reported in Casertana pigs. It would be however first needed to know if diversity for this morphological characteristic is common in commercial pig populations as at present, there is not information on this aspect, mainly due to the usual practice of tail docking that prevents the recording of this phenotype. Considering the potential relationship between tail shape and tail biting damages (that, however, remains to be formally demonstrated), it could be possible to envisage practical applications of the identified marker in selection programs aimed to respond to animal welfare issues. Our study represents one of the few examples of exploitation of animal genetic resources to recover information that might have potential impacts in commercial populations.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figure 1. Tail shape of Casertana pigs: a) curly tail; b) strait tail.

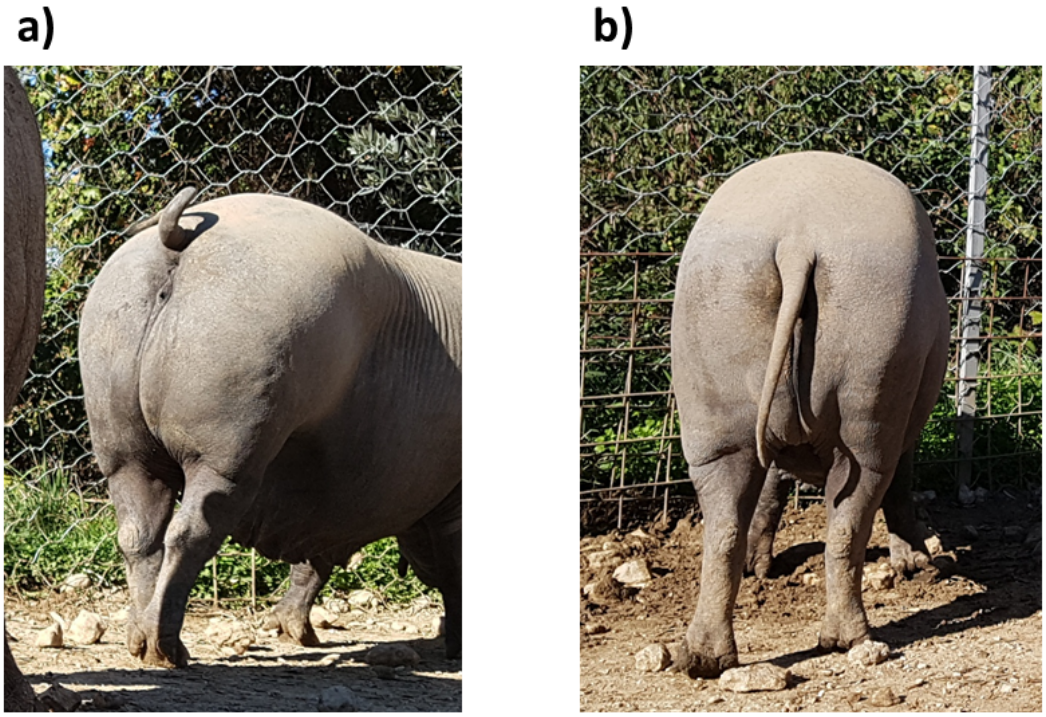


Figure 2. Multidimensional scaling (MDS) with represented the pigs (dots) included in this study divided in the two groups of tail shape.

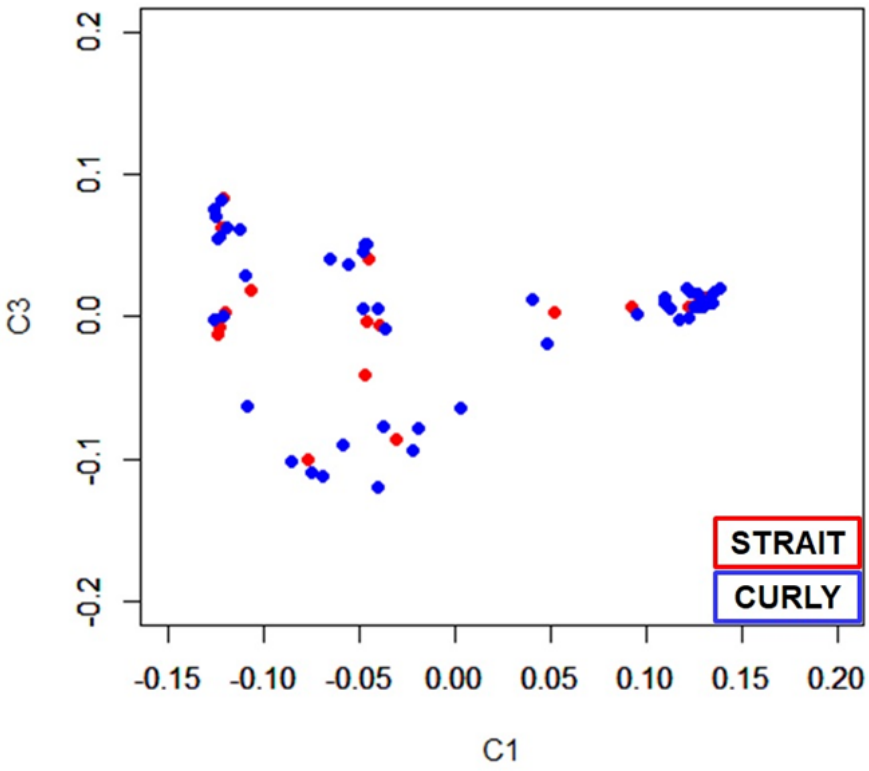
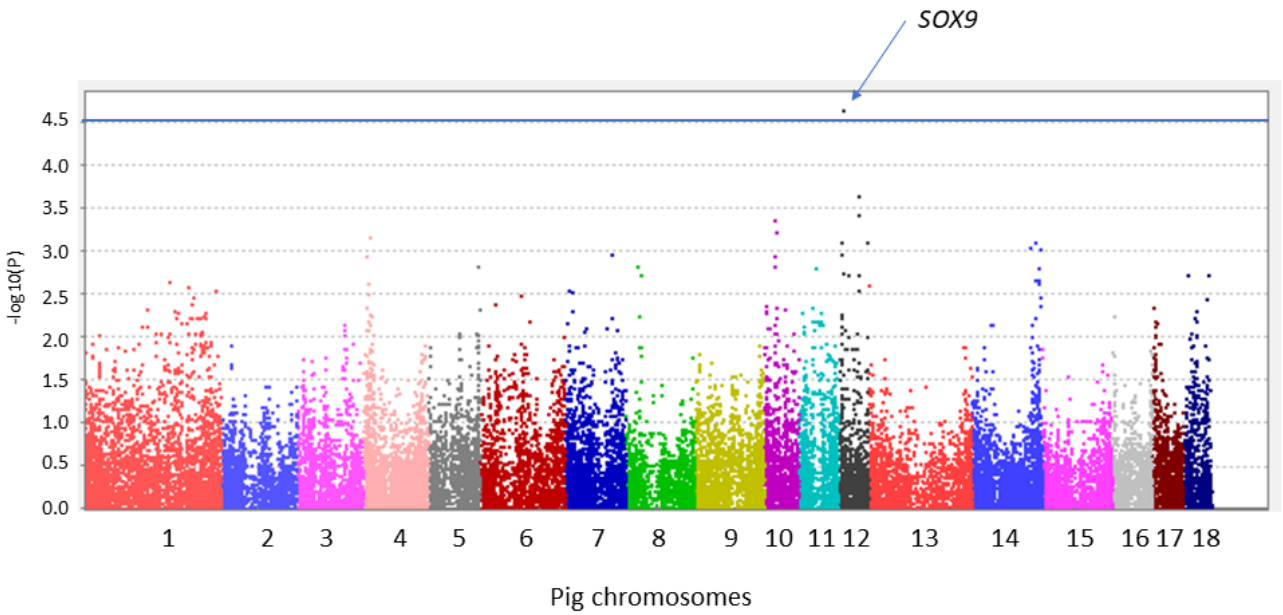
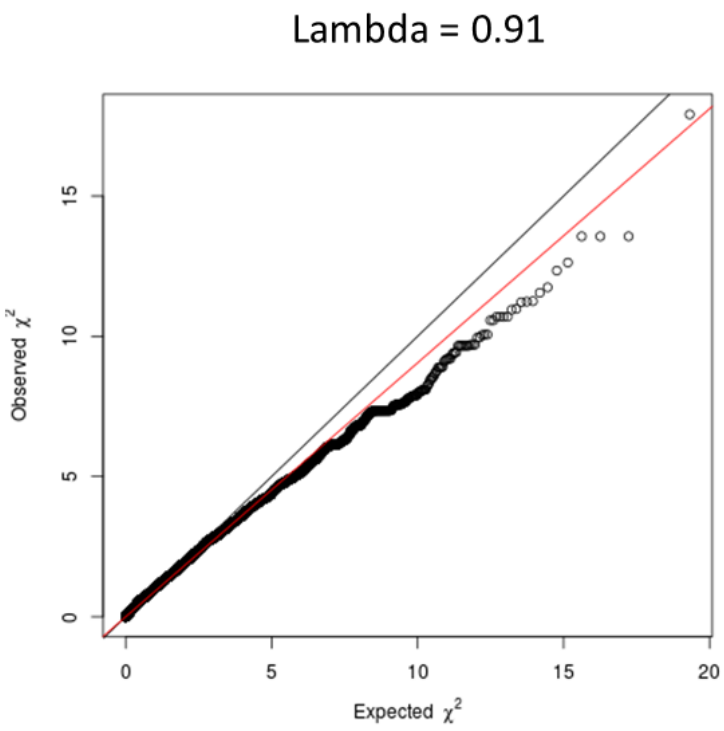


Figure 3. Manhattan plot obtained for the genome wide association study.



Supplementary material

Figure S1. Quantile–quantile (Q–Q) plot obtained from the genome wide association analysis.



General conclusion

The results from this thesis could provide an innovative exploitation of genomic resources in the genetic breeding programs for the Italian pig breeds of the Herd Book managed by the National Pig Breeder Association (ANAS). This thesis is the result of a research activity performed during the Industrial Ph.D. program; thus, the primary aim of the project has been the practical application of the obtained results in the Italian pig sector.

One of the principal ANAS aims is the innovation for the sustainability of autochthonous breeds for their economic exploitation and conservation. The results of the first study of this thesis will help to improve the sustainable conservation of one of these local pig breeds. Through the genetic characterization for polymorphisms in the MC1R and NR6A1 genes of almost all sows and boars registered to the Herd Book, this work implements the use of these DNA markers as genetic descriptors in the Mora Romagnola breed standard. Thus, new strategies in conservation programs for breed management will take into account the genotypes of each individual animal and will help to preserve genetic resources of this breed. In addition, the application of mono-breed marker for traceability and food authentication improves economic sustainability of this low-productivity breed, whose products are often subject to fraud. To our knowledges this study is one of the first example of redefinition of Herd Book standard starting from DNA markers (the genotype of the animals) with a possible practical application for authentication of mono-breed products.

The second study of this thesis investigated several external traits in the Casertana breed, providing preliminary information about candidate genes involved in effecting monogenic traits not yet fixed in this population. This information, together with the ones already available for the breed, could reenforce the genetic identity of the breed and could find, in the future, a practical application in conservation program for the breed. Furthermore, the results of this study exploited animal genetic resources of this autochthonous pig breed and suggested that could be useful dissect phenotypic traits that cannot be genetically characterized using commercial or cosmopolitan populations.

The third study of the thesis confirms the importance of autochthonous animal genetic resources for the definition of molecular basis responsible of the phenotypic variability of some traits difficult to detect in commercial populations, such as tail. To our knowledges, this is the first study that reported a frequency distribution of the tail shape phenotype in a pig population. This study represents another example of exploitation of animal genetic resources to recover

information that might have potential impacts in commercial populations. Considering the potential relationship between tail shape and pig's behavior and tail biting damages, the results of this study could help to develop further studies aimed at responding to animal welfare. Furthermore, according with the results of this study, it could be possible to envisage practical applications of the identified marker in selection programs aimed to respond to animal welfare issues. This theme, in accordance with the guidelines of the Farm to Fork strategy of the European Union, is a current topic in pig breeding and it is considered also in ANAS new breeding programs to improve sustainability of Italian pig breeds for PDO and PGI productions.

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