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MINING THE GENOME OF LIVESTOCK SPECIES TO IDENTIFY MARKERS ASSOCIATED WITH ECONOMICALLY RELEVANT MORPHOLOGICAL TRAITS AND BREED-SPECIFIC FEATURES

Presentata da: Hamed Kazemi

Coordinatore Dottorato

Massimiliano Petracci

Supervisore

Luca Fontanesi

Co-supervisore

Stefania Dall'Olio

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Abstract

Morphological traits in livestock breeding especially the ones that are associated with production or reproduction efficiency are of great importance since they could affect in some aspects the profitability of the farm animals. Over the last decade, the progress in the field of cattle and pig genomics and exploiting the next-generation sequencing methods and bioinformatics pipelines have made it possible to better understand and discover the genetic basis controlling many different traits. Many investigations have been performed in order to identify the main candidate genes and mutations underlying different morphological, productive, and reproductive traits in cattle and pig breeds. The current thesis aimed to better investigate the genomic features and gene markers associated with some important morphological traits in the Reggiana dairy cattle including coat and muzzle colour, stature, presence of the supernumerary nipple, and horn shape and also teat-related parameters encompassing the number of teats, their asymmetry patterns, and the presence of the extra teat in Italian Large White pigs.

With regard to the economic values of the Reggiana that is a dual-purpose autochthonous cattle breed and mainly used to produce a mono-breed branded Parmigiano-Reggiano cheese, we have conducted different genome-wide association studies (GWAS) for different morphological traits. GWASs for stature showed a significant peak on *Bos taurus* chromosome (BTA) 6, with the single nucleotide polymorphisms (SNPs) located on *LCORL-NCAPG* genes and another suggestively significant SNP on BTA14 and *PLAG1* gene which is in a cluster with *CHCHD7*. For muzzle colour including all possible comparisons between pink, grey and black muzzle, GWASs highlighted significant SNPs on BTA18, in a genome region harbouring the *MC1R* gene. Considering the genome scan for presence or absence of supernumerary nipples, results showed two peaks on BTA17 including *TBX3* and *TBX5* genes as the known genes associated with this phenotype and BTA10 with *MCC* gene. GWAS for horn shape identified only a suggestive significant marker on BTA19. Considering the importance of the number of teats in pigs that influences the mothering ability of the sows and their reproduction performances, we carried out GWASs for the total number of teats and other 12 related parameters. For four investigated parameters (total number of teats, number of teats of the left and right lines, and maximum number of teats comparing the two sides), significant markers were identified on SSC7, in the region of the vertnin (*VRTN*) gene. Significant markers for the number of posterior teats and the absolute difference between anterior and posterior teat numbers were consistently identified on SSC6 with the most significant SNP that was an intron variant in the TOX high mobility group box family member 3 (*TOX3*) gene. For the other four parameters (absolute difference between the two sides; anterior teats; the ratio between posterior and anterior number of teats; and the absence or presence of extra teats) only suggestively significant markers were identified on several other chromosomes.

Altogether we dissected the morphological traits in cattle and pigs that might have several implications. The GWASs highlighted genomic regions potentially affecting the biological mechanisms (also support results of previous studies) controlling the developmental programme of morphological features in the Reggiana cattle and Italian Large White pig populations.

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General introduction

1.1 Genomics in dairy cattle and pig breeding industries

Application of Genomic Selection (GS) results in providing more accurate estimates for breeding values earlier in the life of breeding animals, more selection accuracy, and decreasing generation intervals. Genomic selection can potentially double the rate of genetic gain especially in dairy cattle in which the important breeding objective traits can only be measured in females and only after they have reproduced (Van der Werf, 2013).

The effectiveness of selection is dependent on the increase in reliability of genetic evaluations, and this eventually causes an increase in selection intensities and a decrease in generation intervals if available at a much younger age of the animal (Garrick, 2017). Instead of relying on the parent average breeding values, selection decisions could currently be taken with greater precision on young animals in dairy cattle breeding. The fundamental outcomes of this procedure for designing breeding programs are reflected by the fact that a young bull for example, could be used as a sire without progeny rather than waiting until a bull has daughters with phenotypic records (often takes 5-6 years) (Pryce and Daetwyler, 2012). There are also other factors that can improve the effectiveness of GS in livestock breeding encompassing the level and pattern of linkage disequilibrium (LD) in target populations, efficiency rate of imputation techniques, pyramidal structure, and the systematic exploitation of crossing and heterosis (Ibáñez-Escriche et al., 2014).

The genome-wide association analysis (GWAS) has recently been proposed as an effective and powerful technique for detecting a huge amount of subtle genetic variants underlying phenotypic variation of complex polygenic traits in population-based samples, in order to improve the efficiency of GS and quantitative trait loci (QTL) studies, especially in small populations (Jiang, 2013). Generally, after a phenotype of interest is identified, GWAS could act as the potential component in order to provide insights into the genetic architecture of the specific trait, informed parents selection for QTL analyses, and proposing candidates for mutagenesis and transgenics. Therefore, it can be concluded that GWAS and QTL mapping are complementary since they mitigate their limitations when conducted together (Brachi et al., 2010; Zhao et al., 2007). Following the advances in sequencing technologies and subsequent increase in the numbers of animals that will be sequenced in the future, more accurate detection of genetic associations could be provided as a result of the increase in sequenced-based GWASs (Höglund et al., 2014; Mao et al., 2016). In addition, GWAS conducted on local breeds might provide valuable insights into the genetic determinism of the production-related traits by capturing genetic variants that are no longer detectable in cosmopolitan breeds (Sorbolini et al., 2017). Furthermore, the advances in the fields of metabolomics and phenomics especially in pigs could introduce the potential source of new interesting traits related to the quality and level of production, health, and well-being of animals (Samore and Fontanesi, 2016).

Although the resolution of the performed studies was limited by the available marker density for the QTL region, thousands of associated genetic variants have been reported (Mao et al., 2016). GWAS studies have already suggested myostatin, *DGAT1*, and leptin receptor as the main candidate genes through the detected SNPs of these genes significantly associated with several beef and meat quality traits (Jiang et al., 2009). Detection of selection signatures by a combined method as integrated haplotype score (iHS) and global fixation index (FST) in dairy and beef male animals from seven cattle breeds (Angus, Belgian Blue, Charolais, Hereford, Holstein-Friesian, Limousin, and Simmental) and subsequent genome annotation has highlighted several important candidate genes including *DGAT1*, *ABCG2*, *MSTN*, *CAPN3*, *FABP3*, *CHCHD7*, *PLAG1*, *JAZF1*, *PRKG2*, *ACTC1*, *TBC1D1*, *GHR*, *BMP2*, *TSG1*, *LYN*, *KIT* and *MC1R* that have basic roles in milk production, reproduction, body size, muscle formation, and coat colour, respectively (Zhao et al., 2015). It has also been reported that integrating sequence-based GWAS and RNA-Seq could provide novel insights into the genetic basis of mastitis resistance, milk production, and other complex traits in dairy cattle populations (Fang et al., 2017). Also, many GWAS have been carried out in many species including humans, plants, and livestock, identifying thousands of genome-wide significant associations (McCarthy et al., 2008; Bergelson and Roux, 2010; Bermingham et al., 2014). Many of these associations were observed to be across multiple traits in the same category, or even distinct traits, and these associations were described as cross-phenotype (CP) associations (Solovieff et al., 2013).

GS has gained great interest in the pig breeding industry and the application of genomic information for the prediction of breeding values has been evident as a result of the progress in the development of molecular techniques (Garrick, 2017). Compared to dairy cattle, the accuracy of the breeding values for some targeted traits is generally low in pigs, but this could be increased through the application of GS which in turn preserves the generation interval and control of inbreeding (Lillehammer et al., 2011). Implementation of GS seems to be the key factor in the concept of pig selection programs for meat quality and performance, and reproduction traits. Also, this can be applied for several other traits which indirectly have effects on the production efficiency and welfare-related parameters (Samore and Fontanesi, 2016). The sequence analysis from a recent GWAS in Duroc and Landrace pig breeds identified two functional mutations (7: 97615880 and 7: 97614602) of the Vertnin (*VRTN*) gene on SSC7 as one of the most important candidate genes which could significantly influence the number of thoracic vertebrae (ribs) and also the number of teats in pig (Van Son et al., 2019). To give other examples, GWASs considering meat quality and carcass traits in pigs found the most significant SNPs on SSC1 that was included in the largest QTL region and was located within the glutamate ionotropic receptor kainate type subunit 2 (*GRIK2*) gene (Fontanesi et al., 2017), and within the TBC1 domain family, member 1 (*TBC1D1*) gene on SSC8 (Dall'Olio et al., 2020). GWAS carried out for some exterior traits in Casertana pig breed revealed SNPs associated with the coat colour trait were located mostly on SSC6 and also on SSC8, SSC14, and SSC15, confirmed through the independent F_{ST} analyses. Among all the annotated genes, the fibroblast growth factor receptor 3 (*FGFR3*) gene on SSC8

(with different roles in skin processes), was suggested to have a contribution to the pigmentation in the hairless Casertana pigs (Schiavo et al., 2019).

In accordance with the improvement of GS technologies, new versions of cattle, pig, and other ruminant genome assemblies have been released. The new reference genome for cattle as ARS-UCD1.2, made through applying a combination of modern technologies in a de novo assembly, increasing its continuity, accuracy, and completeness with more contigs than the previous version. For the annotation of this assembly, supporting RNA-based data has been greatly expanded that resulted in the identification of the higher number of total genes (30,396 total genes, 21,039 protein-coding) (Rosen et al., 2020). Compared to the previous versions, the new improved pig reference genome assembly (Sscrofa11.1) is highly contiguous and includes also the annotation of a further 11 short-read assemblies by the recent long-read technologies and a whole-genome shotgun strategy which delivers a unique and more detailed overview of the genetic structure of this important model species in agricultural and biomedical studies (Warr et al., 2020). Moreover, the availability of high-throughput SNP platforms (with reduced genotyping cost and a large number of animals) for several livestock species has revitalized the search for DNA markers associated with phenotypic variation in complex traits of economic importance (Bush and Moore, 2012). Next-generation sequencing and related technologies have enabled the identification of breed structure and composition, parent verification, genome diversity, and genome-wide selection sweeps and disclose the importance of genomics in dairy cattle and pig genetic improvement programs (Garrick, 2017; Mrode et al., 2019). The emergence of the new commercially available BovineSNP150k (cattle) and PorcineSNP70k (pig) Beadchips with higher allele frequencies in most pure lines and crosses reflects their effectiveness in providing enough markers to be used for genomic selection in all groups.

As a newly developed molecular technique, the genome editing approach is obviously effective in order to overcome and solve the limits of introgression technique, representing new approaches for genetic improvement, and control of inbreeding rate in livestock populations (Garrick, 2017). On the one hand, this approach could be utilized in functional genomics in order to better elucidate gene function and causal mutations underlying the monogenic traits of interest. On the other hand, there is another use of this approach to introduce useful genetic variations into specialized breeding programs aiming for genetic improvement of livestock, which could contribute to the fixation of some genetic defect, inactivation of undesired genes, and transfer of the beneficial alleles and haplotypes between different breeds (Bishop and Van Eenennaam, 2020).

Altogether, all these advances provide genomic information that could be used in detecting potential candidate genes and their functions in order to have a great understanding of the genetic and biological mechanisms affecting production, reproduction, and morphological traits in cattle and pig breeding industries.

1.2 Introduction to the breeds (Reggiana cow, Italian Large White pig)

Reggiana dairy cattle

Reggiana is a local dairy cattle breed reared mainly in the North of Italy, in the province of Reggio Emilia (Figure 1). The population of cows of this breed was about 40,000 animals in the 1940s; this number decreased progressively till the 1980s when it reached about 500 cows; subsequently it increased, reaching the current number of about 2000 cows. The breed is characterized by the red coat colour, good size, long trunk, solid plant skeleton, and quite long head always very distinct. Compared to the Holstein cattle breed, milk from the Reggiana breed has higher milk solids and particularly well-suited for cheese production because of its high percentage of casein and superb properties for rennet coagulation (Mariani and Pecorari, 1987). Considering the mean milk yield production, the Reggiana breed produces milk about 30% less than that of the Holstein breed (Gandini et al., 2007), which brought up an important concern for the conservation of the Reggiana breed related to profitability (Fontanesi et al., 2015). After that, a consortium of dairy producers has developed a new brand of Parmigiano Reggiano cheese made exclusively of Reggiana milk in order to overcome production limits for this breed. There is a trend for the Parmigiano Reggiano of only Reggiana milk to be sold at around twice the price of the common Parmigiano Reggiano cheese and for this reason, an authentication system has been developed which altogether are revitalizing interest towards Reggiana cows (Russo et al., 2007; Fontanesi et al., 2015).

Figure 1. Reggiana dairy cattle (*https://www.animalgeneticresources.net/index.php/country/italy/*)

Italian Large White heavy pig

The Italian Large White pig is the Italian breed and the most numerous pig breed in Italy which is the strain of the British Large White or Yorkshire pig breeds (Bigi and Zanon, 2008). Based on the Ministero delle Politiche Agricole Alimentari e Forestali (the Italian ministry of agriculture and forestry), this breed is recognised as one of the seven pig breeds of foreign origin and also one of the main four pig breeds for which there is a genealogical herdbook managed by the Associazione Nazionale Allevatori Suini (the Italian national association of pig breeders). The coat is white with white bristles and pink skin. With about 10-12 piglets per farrowing and no less than 14 nipples, the sows have a very good maternal and dairy disposition (Figure 2). Important

features including large size, strong legs, and rapid growth of the Large White pig made it an ideal breed for intensive breeding of the heavy pigs in order to produce prosciutto crudo especially Prosciutto di Parma and Prosciutto di San Daniele and other traditional Italian meat products. One of the main goals of the Italian pig breeding industry is the production of high-quality Protected Designation of Origin (PDO) dry-cured hams for which pigs are raised until they reach about 160 kg live weight (9 months age) with the appropriate fat coverage of the hams (Russo and Nanni Costa, 1995). As one of the important objectives, maintaining fat coverage measured as backfat thickness (BFT) has shaped the genetic pool of Italian heavy pig breeds for a few decades (Fontanesi et al., 2012). This is also necessary to consider two main criteria for the production of PDO dry-cured hams such as obtaining hams of the right weight (12-14 kg) and muscles of the right maturity (Fontanesi et al., 2017).

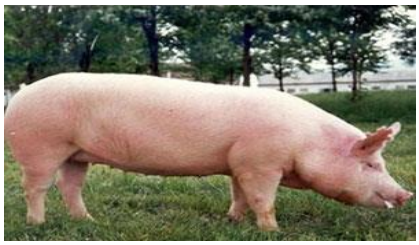

Figure 2. Italian Large White pig breed (*http://www.gransuinoitaliano.it/la-filiera/i-suini/le-razze/*)

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2.1 Overview of mammary glands and milk secretion components in livestock

In the early part of post-fetal life, the capability of mammals to nurture their young by milk secretion through the system of mammary glands has provided survival benefits for these animals. The mammary glands system of mammals is a key factor for the efficient nurturing of their young as their reproductive strategy includes the production of fewer young in comparison with birds, amphibians, and reptiles (Klein, 2013).

Regarding the anatomical features, the milk-secreting cells of the mammary gland grow into hollow structures called Alveoli, the fundamental milk-secreting units of the mammary gland, as a result of the proliferation of Epithelium (epithelial cells that originate from the primary mammary cord) (Figure 1). The mammary glands are developed from embryonic ectoderm. The first representation of the mammary ectoderm is through the parallel linear thickenings on the ventral belly wall. The formed ridge is divided into a suitable number of mammary buds which are the source of the functional components of the mammary gland. Then, the nipple which is an enlarged area of epithelium and the external link to the internal milk-secreting system develops on the surface following this development. In males, although nipples often develop, the primary mammary cord does not differentiate into substantial glandular tissue (Klein, 2013).

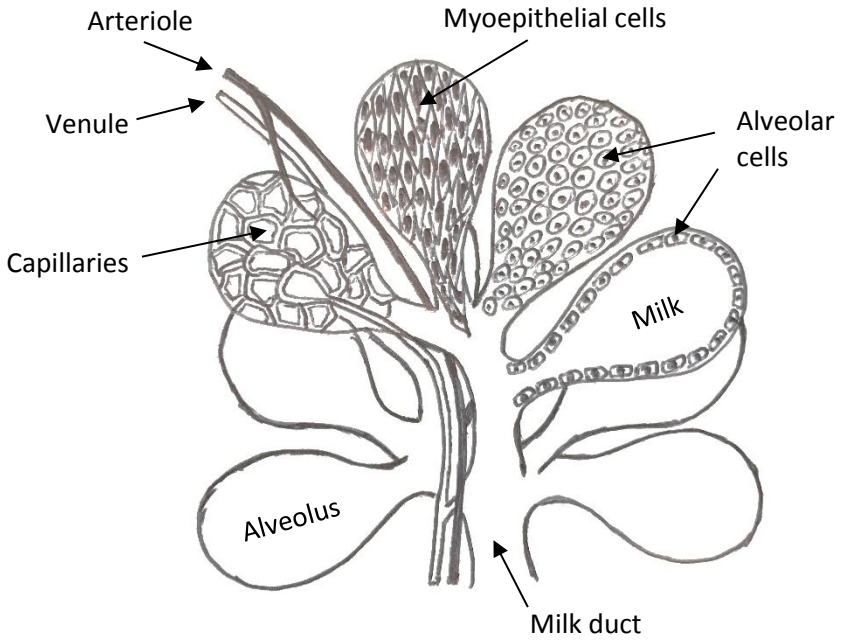

Figure 1. Schematic representation of the cluster of alveoli in the mammary gland of a goat (drawn based on what was presented by Austin and Short, 1984).

The alveoli store much of the milk that accumulates prior to suckling or milking. They connect to the nipple (teat) through the duct systems that enable the process of milk flow from the formation area to the area for secretion and delivery (nipple). All these ducts finally form one main duct per gland with only one opening through the nipple which is observed in sheep, goats, and

cattle. The number of main ducts and their related openings varies among species. In the mare and sow, there are two main ducts and associated openings, while the cat and dog may have 10 or more openings in the nipple, each opening representing a separate gland (Figure 2). There are specialized milk storage areas for both the cow and doe (goat), called cisterns, which are found in the ventral part of the gland and into which all main ducts empty (Figure 2). As a result, these species especially cows have an advantageous ability to synthesize and store greater amounts of milk. Regardless of this adaptation, it is necessary to note that much of the milk present at milking is retained in the duct system of the mammary glands (Figure 3) (Klein, 2013).

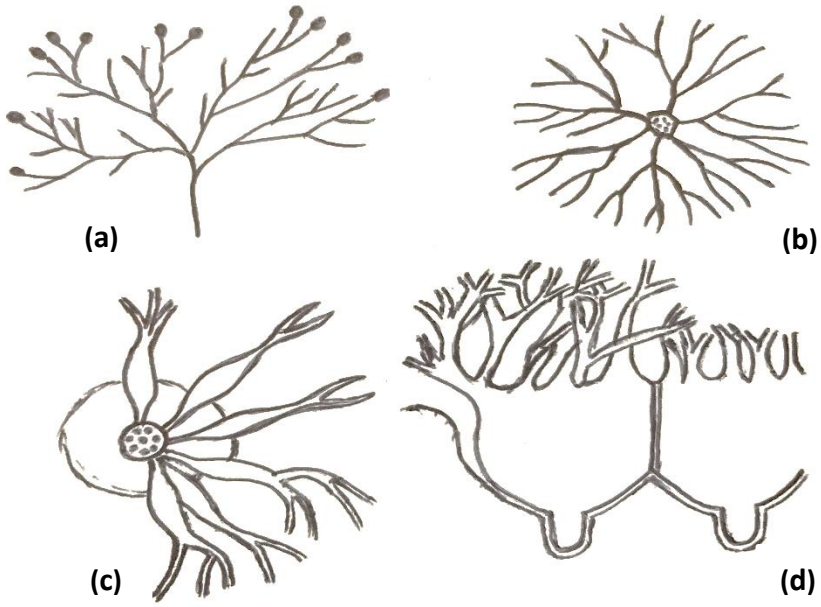

Figure 2. Schematic representation of the mammary duct system. Cow, goat, sheep (a), horse and pig (b), cat and dog (c), cow and goat cistern (d) (drawn based on what was presented by Austin and Short, 1984).

The development of mammary glands occurs usually as paired structures. There is variation in the number of these pairs including one in horses, sheep, and goats, two in cattle, and seven to nine in the sow. Moreover, the location of mammary glands differs among animals being inguinal in cattle, goats, and horses and thoracic in primates with extending the length of the thorax and abdomen in pigs, cats, and dogs (Figure 4). The mammary gland pairs are closely related to each other in some domestic animals such as cattle, goats, horses, and sheep (Klein, 2013). Both genetic and endocrine controls regulate the fetal development of the mammary gland. The primary development of the mammary bud is influenced by the embryonic mesenchyme (connective tissue). The transplantation of mammary mesenchyme to another site will result in the formation of mammary bud at that specific area of transplantation. While the different aspects of fetal mammary development still need to be elucidated, it is not believed to be driven by hormones. However, the exogenous administration of certain hormones to the mother causes the mammary glands to be actively secreting which might be present at birth (Klein, 2013).

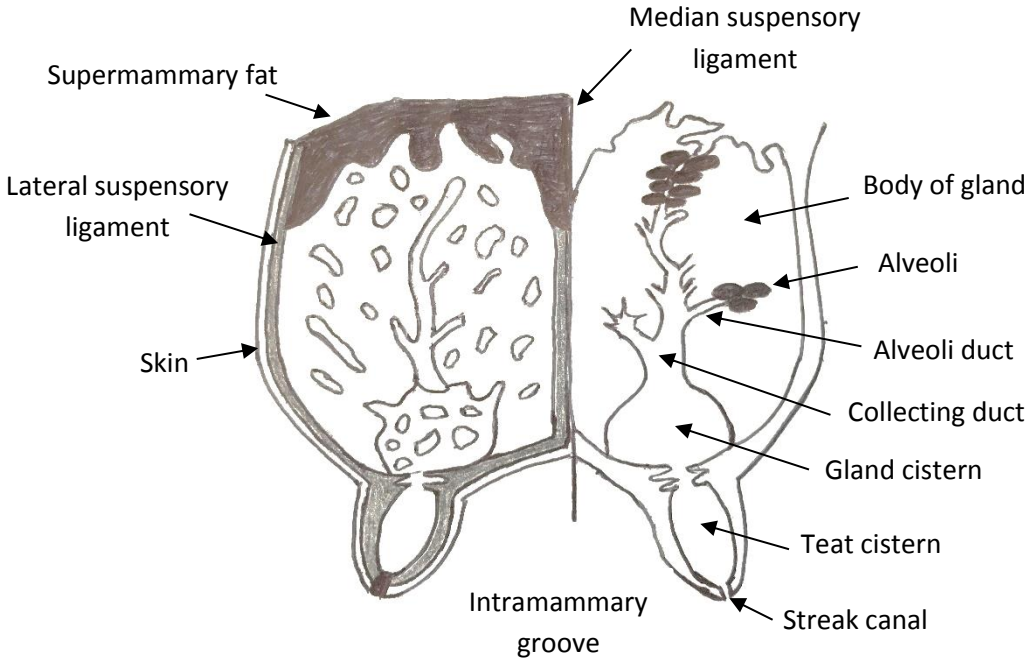

Figure 3. Schematic representation of different components of the bovine udder and teats (drawn based on what was presented by Alany et al. 2013).

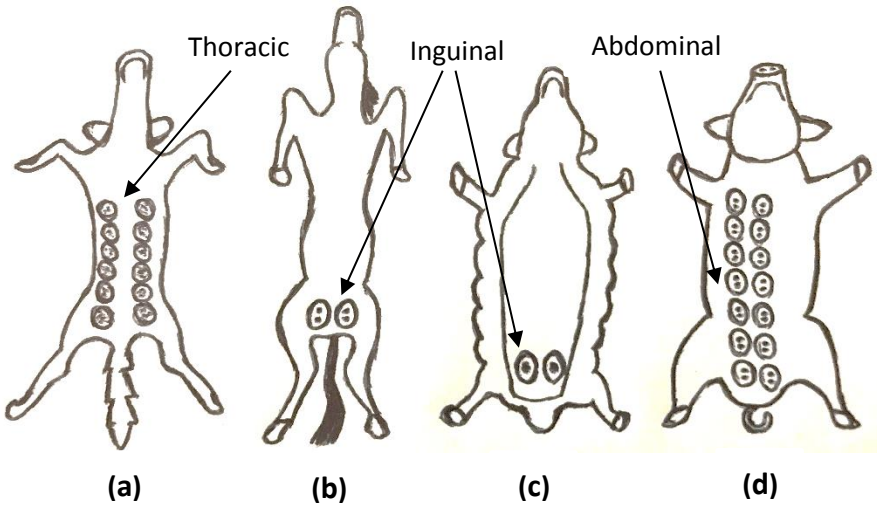

Figure 4. Location of mammary glands in different species, being thoracic in dog (a), inguinal in horse (b) and sheep (c) and abdominal in pig (d).

Mammary gland development in the post-fetal life period typically begins along with puberty. Cyclical ovarian physiological activity induces the production of estrogen and progesterone. The proliferation of the duct system is positively regulated by estrogen along with growth hormone and adrenal steroids. Also, the addition of progesterone and prolactin stimulates the development of alveoli from the terminal ends of the ducts (Figure 5) (Klein, 2013).

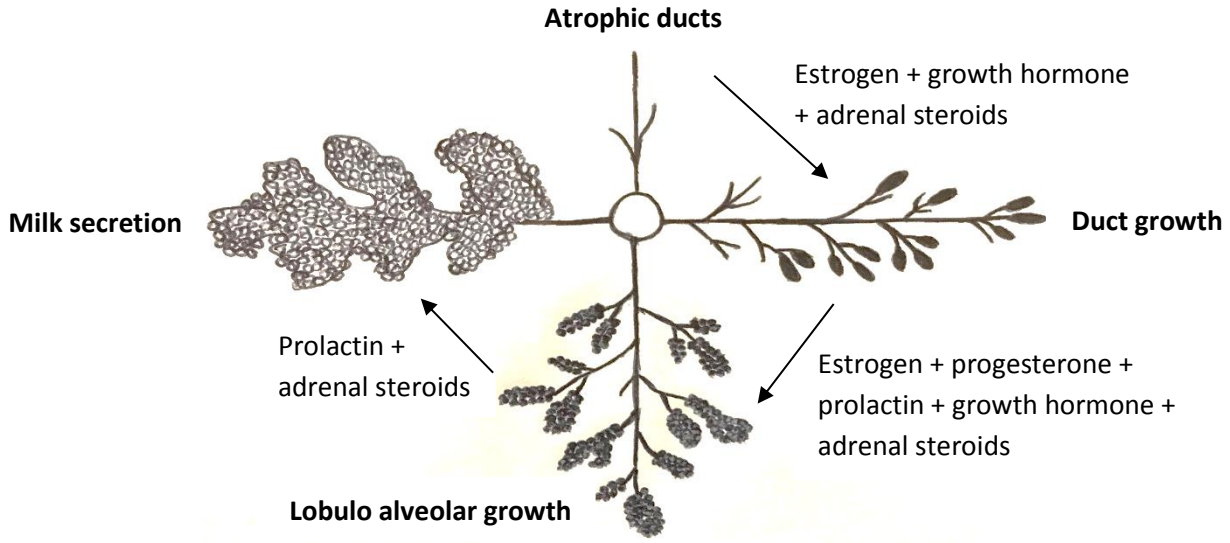

Figure 5. Schematic representation of hormones involved in the growth of the mammary gland and in the initiation of milk secretion (drawn based on what was presented by Lyons, 1958; Austin and Short, 1984)

2.2 Overview of teat morphology and functions in livestock

A teat is the extension of mammalian mammary glands from which milk flows or is expelled to feed their young. There is a difference among mammalian species considering the number and position of teats which corresponds to that animal's average litter size. The establishment of the size and placement of teats may be the criteria for determining the quality of some domesticated animals (Fails and Magee, 2018). The protruding teats and glands that surround them could be located anywhere along the two milk lines. The development of mammary glands happens usually in pairs across these lines with a number that approximately corresponds to the number of young animals at birth. The teat number ranged from 2 in most primates to 18 in pigs that is listed in Table 1 (Lombardi, 2012).

Table 1. The number and position of teats in some mammalian species (Nickerson and Akers, 2011).

Species	Anterior teats (thoracic)	Intermediate teats (abdominal)	Posterior teats (inguinal)	Total teats	Opening per teat
Goat and sheep	0	0	2	2	1
Cattle	0	0	4	4	1
Cat	2	6	0	8	3-7
Dog	2	6	2	10	8-14
Mouse	4	2	4	10	1
Rat	4	4	4	12	1
Rabbit	4	4	2	10	8-10
Pig	6	6	6	10-16	2
Horse	0	0	2	2	2
Human, primates	2	0	0	2	15-25

The major physiological characteristics of the teat structures are the smooth muscle and elastic tissue, as well as the well-developed blood vessels. The teat is spread throughout arteries and veins (large with muscular walls) networks that are connected by arteriovenous anastomoses. As known, estrogens stimulate teat growth which subsequently can affect one teat in case of applying locally. They also cause teat skin thickening and increased pigmentation (Linzell, 1959).

Characteristics of teat in different animals

The teat of farm animals is an important component of the udder, onto which a milking cluster is attached and plays important functions for instance a valve regulating the outflow of milk and a natural barrier for exogenous infections (Hamann and Mein, 1988). Teat morphology shows variation among different breeds, between breeds, and also quarters (cattle) within a specific breed (Zwertvaegher et al., 2012). Teat length (defined as a distance from the base of the teat to the tip of the teat), teat diameter (measured in the mid of the teat barrel), and especially in cattle the teat-tip to floor distance (the distance of the teat-tip from the floor forming an angle of 90º with the floor) are among the most important parameters (Singh et al., 2014). The teat canal is viewed as another fundamental part of the teat morphology in dairy animals. The increase in milk pressure as a result of the filling of the intracisternal region with alveolar milk causes some changes in the morphology of the teat canal. This pre-stimulation guarantees the opening of the teat closure above the teat canal and subsequently milk flow (Ambord et al., 2010). However, these responses to pre-milking stimulation might be different among species and also animal to animal.

The female pig has numerous mammary glands without cisternae and gives birth to a large number of litter (Ellendroff et al., 1982). Pigs develop a well-defined teat order or teat preference. The higher total milk production by the sow comes from their strong teat order, larger litter size, and more glands that are maintained in a milk-secreting state which are different in their milk production ability based on the teat position (Dyck et al., 1987). The teat order influences the performance of growing animals particularly in pigs that develops within the first few days of the neonatal period due to the specificity of each pig's teat (Rosillon-Warnier and Paquay, 1984). However, the teat order specifically in pigs, from a different standpoint could be considered as a competitive order for some of the piglets that is disadvantageous because for these piglets teat fidelity causes some of them to suckle continually from less productive teats resulting in having lower growth rate compared to their littermates (De Passille et al., 1988). Also, inherent differences in milk yield between different teats of the pig (Fraser, 1984), and heavier weight at slaughter with fewer lung and liver lesions for the male castrated pigs suckled anterior teats (Hoy and Puppe, 1992). It was demonstrated that pigs sucked regularly from teats 1, 4, or 6 (anterior to posterior) had similar average daily gain and body weight during the production period (Stull et al., 1999). There was a tendency towards initial suckling from the teats located near to the abdominal midline in piglets, for instance in Large White×Landrace sows, the teats located in the upper row in anterior and posterior parts were first suckled by piglets (Balzani et al., 2016). Teat length and diameter are other important characteristics of the teat in pigs which have been shown to be

increased with parity number and affected by the specific position of the teat in Duroc and Landrace pigs (Ocepek et al., 2016).

It is well-documented that the anatomical and functional characteristics of the mammary glands and teat have fundamental impacts on the milking performance of individual quarter of cow and machine milking process. In dairy cattle, the udder and teat conformation traits which are moderately heritable, have a close relationship with both mastitis resistance and milk somatic cell count (SCC). Differences in teat shape, teat size, and the morphology of teat-end are the main factors for mastitis development (Bardakcioglu et al., 2011). It was shown that Jersey crossbred cows with the pendulous udder, long and thick teat and flat or inverted teat-end were more susceptible to intra-mammary infection followed by the significant effect of teat-end shape on SCC level with highest SCC for the cows with flat teat-end and lowest for pointed teat-end (Bharti et al., 2015). Having a longer teat canal and higher milk yield were observed in Gir cows with slower milk flow in comparison to cows with fast milk flow. There was also evidence of the relationship between the teat-end to floor distance and SCC level (Porcionato et al., 2010). As another teat-related characteristic, teat canal length was found to be negatively associated with milk peak flow rate (PFR) (which is often higher in the rear than in the front quarter) in dairy cattle (Baxter et al., 1950). Differences considering teat position and diameter found in Brown Swiss×German Braunvieh crossbred cattle with longer teats with a smaller diameter in the front quarter when compared to teats in the rear quarter (Weiss et al., 2004).

The internal part of the teat in cattle composed of the papillary duct, Furstenberg's rosette, and distal part of the teat cistern (Vesterinen et al., 2015). Figure 6 shows the position and measurement of some parts of the inner teat morphology. Variation in size and number of mucosal folds was observed in Furstenberg's rosette in dairy cattle through the 3D imaging technique of teat morphology characterization. These variations are quite independent of milking parameters which might affect the teat canal and teat external surface (Vesterinen et al., 2015). It was shown that there is a negative association between teat length and herd size in dairy cattle when a high proportion of short, flat, and round teats observed in large size herds such as Holstein in comparison with short size herds (Gouvea et al., 2020). The average length, diameter, and volume of teat differed among the cattle breeds with the highest average observed in the Brown Swiss, and lowest in the Simmental cattle (Genc et al., 2018). Teat-end hyperkeratosis observed in dairy cattle is relatively associated with the physical characteristics of the teat including shape and position which causes the risk of infection in the mammary gland. It occurs mostly in the front teats and also the teats with sharp and round shape than the flat or inverted teats (Cerqueira et al., 2011).

Regarding he sensitivity of teats and udder in terms of exogenous infections, there are some important factors which contribute to the defensive reaction against the entrance of microorganisms and preventing milk leakage encompassing teat canal, teat length and shape, udder depth, and fore udder attachment (Szencziova et al., 2013). For example, the Holstein dairy cows that had larger distal teat canal perimeters and distal teat canal surfaces demonstrated bacterial

growth in their teat (Martin et al., 2018). The process of opening and closure of the teat canal orifice during milking is done by a smooth muscle sphincter as the most important part of the teat canal against pathogens, and incidence of milk leakage and clinical mastitis (Rovai et al., 2007).

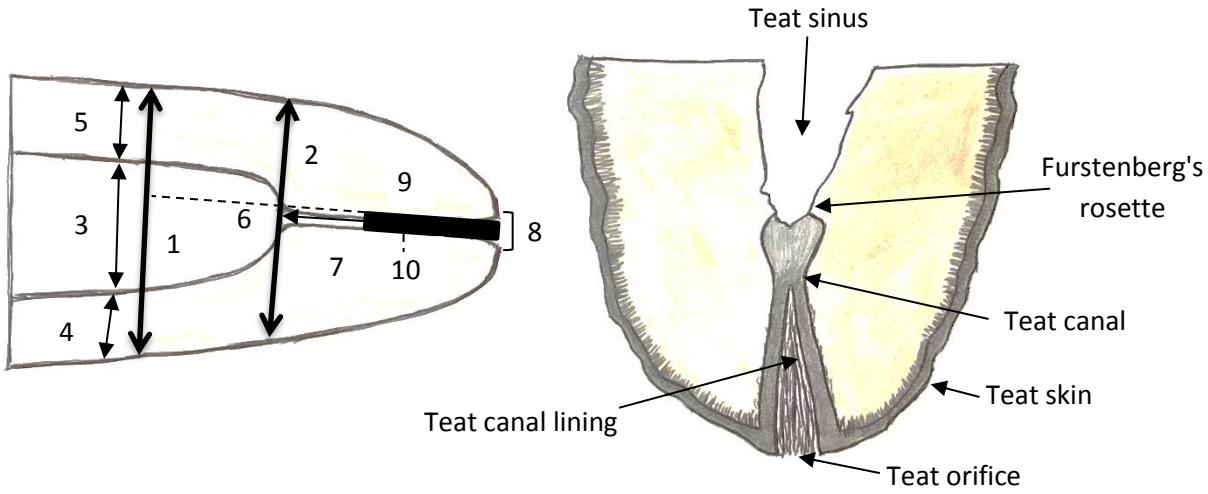

Figure 6. Schematic representation of the inner teat morphology and measurements. 1= width (mm) of the teat proximal to the rosette of Furstenberg; 2= width of the teat end at the rosette of Furstenberg; 3= width of the teat cistern proximal to the rosette of Furstenberg; 4= diameter (mm) of the lower teat wall proximal to the rosette of Furstenberg; 5= diameter of the upper teat wall proximal to the rosette of Furstenberg; 6= length of the teat canal (mm); 7= diameter of the teat canal distal to the rosette of Furstenberg; 8= width of the teat canal distal orifice; 9= perimeter (mm) of the distal teat canal beginning distal to the rosette of Furstenberg until the distal orifice; 10= surface (mm2) of the distal teat canal referring to the perimeter of the distal teat canal (drawn based on what was presented by Martin et al., 2018; Smolenski, 2018).

Compared to cattle, teats in buffalo have fewer muscles and more collagen bundles which cause the teat closure in buffalo much tighter than other livestock (Ambord et al., 2010). Similar to the results in dairy cattle, in buffalo, the longer and wider teats that were located nearer to the floor were found to have more associations with the presence of subclinical mastitis and SCC (Kaur et al., 2018). The morphological characteristics of the teat in sheep include being symmetrical in size in two quarters, cone-shaped, and uniformly smaller than those of the goat (Paramasivan et al., 2013). Analysis in Awassi sheep affected with clinical mastitis revealed a significant relationship between teat wall thickness (thicker and hyperechoic) and somatic cell linear scores (LnSCC) (Ismail et al., 2016). Considering also the dairy camels, they have relatively different teat and udder morphology and udder's milk partitioning in comparison with other dairy livestock. Camels have a specifically larger teat diameter than dairy cattle and buffalo but very limited cistern cavity (Atigui et al., 2016).

2.3 Mammary gland, teat characteristics and supernumerary nipples in cattle

Mammary gland development initiates during the embryogenesis period with the establishment of a specific number of mammary primordia on each flank of the embryo. They are the most characteristic feature of all mammals. As already known, there is a wide variation in the number and location of mammary glands between mammals (Voutilainen et al., 2015). The development of a functionally mature mammary gland is required for the milk synthesis and secretion and survival of offspring regardless of the distinct arrangement or the number of mammary glands for a specific mammal. The unique anatomy of the udder deserves special attention, even though the basics of mammary development are generally similar among species. Phenotypes for udder conformation traits are viewed as a great source of information since mammary gland morphology has a strong association with mastitis susceptibility and productive life span in dairy cattle (Berry et al., 2004; Vollema and Groen, 1997). The mammary glands in the ruminant are clustered together into groups of two (in goat and sheep) or four (in cattle) mammary glands to form the udder. The vascular system, nerve supply, and suspensory apparatus of each udder half are independent of another half in dairy cattle (Nickerson and Akers, 2011). The shape of the mammary buds in cows is more ovoid-shaped which are carried next to the apex of the epidermis resulting in relatively more pointed-shape teats in comparison with males. In males, mammary buds are circular and embedded by the growth of their surrounded mesenchyme which subsequently differentiate to the broad teats with flat ends (Rawson et al., 2012).

Teats vary in shape and especially in length, and there is not any relationship between these two features of a teat and the shape or size of the udder (Nickerson and Akers, 2011). Also, the average length and diameter of the teats in the fore and rear parts of the udder are different in cattle with higher values for the teats of fore udder (Hurley, 2010). The poor and imperfect structure of the teat and udder results in production inefficiencies due to the increased mastitis frequency and early culling of cows. In addition, calf survival and growth rate might be affected because of the delayed teat finding (inappropriate suckling) and reduced milk yield quality related to mastitis (Devani et al., 2019). Regarding various teat shapes observed in dairy cattle, they are generally categorized into three main shapes including cylindrical, funnel, and bottle-shaped teats (Figure 7).

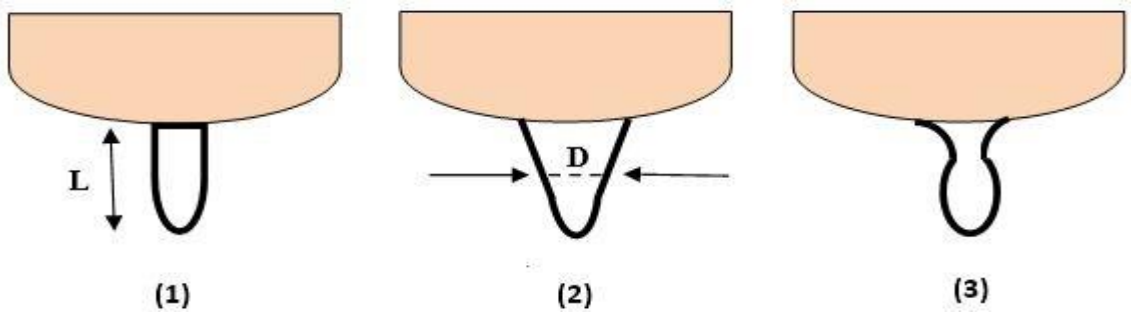

Figure 7. Schematic representation of three main teat shapes in dairy cattle including Cylindrical (1), Funnel (2) and Bottle (3). L= teat length, D= teat diameter (drawn based on what was presented by Hichman, 1964).

These teat shapes differ in the level of functionality and occur in different locations of the udder. It was reported by Brodauf (1963) that the bottle-shaped teat demonstrates a simple autosomal recessive mode of inheritance when they observed the incidence of these teats in front quarters of 6 out of 12 cows sired by the same bull, but only in one of their daughters sired by a different bull. Therefore, teat shape deserves to be specifically considered in the dams of future breeding bulls due to the fact that sires are also of great importance in the transmission of specific teat shape to the next generation (Lojda et al., 1982). The susceptibility frequency to mastitis was observed to be lower for Holstein and Ayrshire dairy cattle possessing funnel-shaped teats with also small diameter on their udder in comparison to the cows with cylindrical-shaped teats. The superiority for the level of production was also attributed to cows with short teats (Hickman, 1964). Furthermore, the funnel-shaped teats are likely to have lower SCC as a result of complete milking than the cylindrical or bottle-shaped teats, in which there is residual milk because of incomplete milk ejection (Okano et al., 2015). Based on these studies, it seems essential to consider this phenotype when breeding programs aiming to improve milk production and decreasing mastitis incidence.

Supernumerary nipples in cattle

There are reports of some developmental and congenital defects particularly in the mammary glands that cause the abnormal teat patterning phenotype in several species, including livestock (Howard and Gusterson, 2000). The known examples of these abnormal teat patterns could be the presence of additional teats (supernumerary/hyperthelia as the most prevalent defect), the absence of teats (athelia/hypothelia), and abnormal locations of teats (Ihara et al., 2007).

Supernumerary nipples are congenital accessory structures and are considered as additional to the main four functional teats in cattle. They might appear in different positions along the milk line (Hardwick et al., 2020). The inheritance pattern of supernumerary nipples is more complex in cattle with some evidence of the oligo or polygenic nature and the incomplete penetrance mode (Brka et al., 2000). The presence of extramammary buds results in the incidence of supernumerary nipples with the approximate frequency of 40-50% in female at the time of birth. The most typical location of supernumerary nipples is in the posterior part and behind the rear teats (Rowson et al., 2012). Supernumerary nipples can negatively affect the dairy cattle profitability through being an incubator for bacteria which could infect the whole udder (a risk factor for new intra-mammary infections), and interference with the machine milking system (decreasing efficiency due to inappropriate positioning). These matters highlight the importance of supernumerary nipples in dairy cattle breeding strategies (Butty et al., 2017). Supernumerary nipples are classified into three main types based on the position including caudal, ramal, and intercalary teats (Skjervorld, 1960). Figure 8 demonstrates all these different teats.

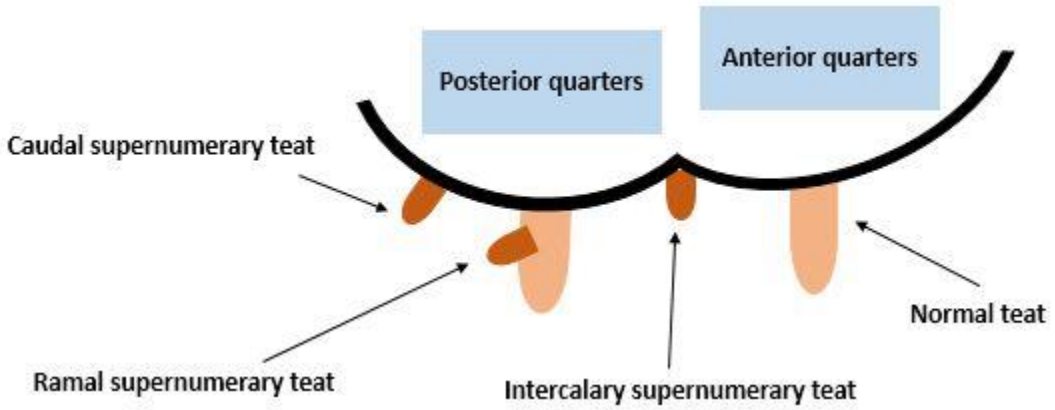

Figure 8. Schematic representation of different types of supernumerary nipples from the side view in cattle. Caudal teats are in the rear, ramal teats are attached to another teat, and intercalary teats are found between two regular teats.

The supernumerary nipples also vary based on the developmental stage. In some cases, they may connect to the sinus of another teat or have their own supernumerary gland (live), but mostly they have no separate mammary gland or have an abortive one (blind). Thus, they are not functional but in rare cases can be functional in terms of milk emission (Gifford, 1934; Pausch et al., 2012; Joerg et al., 2014).

As explained, the most prevalent type of supernumerary nipple in cattle is caudal which locates normally behind the rear teats (on the right, left or both sides) (Gifford, 1934; Rowson et al., 2012). Considering the teat placement on the udder, the caudal supernumerary nipples were found to demonstrate strong bilateral symmetry and observed mostly on the left (Wiener, 1962). The intercalary supernumerary nipples might locate at different positions in line and between the two normal teats on each half of the udder (Gifford, 1934). In the association study by Wiener (1962), no significant association was observed between the number of supernumerary nipples and some production parameters such as age at first calving or milk yield in dairy cattle.

From the biological point of view, it seems that different signaling pathways influence the developmental phases of supernumerary nipples. Hence, multiple genomic regions might be involved in the presence of supernumerary nipples in livestock. Exploiting this information in genomic prediction could enhance the accuracy of selection against supernumerary in order to decrease their prevalence frequency in the udder of dairy animals, and also present an alternative genetic solution instead of surgical removal (Butty et al., 2017). Several GWAS carried out in order to find the causative genes for the presence of supernumerary nipples and mammary gland morphology in cattle and other species (Table 2). The most probable QTL regions and mutations were found on BTA5, BTA20, and particularly BTA17 in different cattle breeds. The identified genes include leucine-rich repeat containing G protein-coupled receptor 5 (*LGR5*) on BTA5 in Brown Swiss (Butty et al., 2017), t-box transcription factor 5 (*TBX5*), and RNA binding motif protein 19 (*RBM19*) on BTA17 in Brown Swiss and Flekvieh cattle (Pausch et al., 2012, 2016;

Fang and Pausch, 2019), and relaxin family peptide receptor 3 (*RXFP3*) and solute carrier family 45 member 2 (*SLC45A2*) on BTA20 in Holstein cattle (Joerg et al., 2014).

Table 2. The identified genomic regions associated with the presence of supernumerary nipples in cattle

Gene	Chr	SNP	Breed	Reference
LOC783893, LOC783966, LOC782348	BTA5	ARS-BFGL-NGS26008	Dual purpose Flekvieh	Pausch et al. (2012)
EXOC6B	BTA11	BTA-16600-no-rs		
TBX3, TBX5, RBM19	BTA17	Hapmap49912-BTA21169		
C6, PLCXD3	BTA20	20: 33244101 - rs41618278	Holstein (affecting caudal supernumerary nipples)	Joerg et al. (2014)
RXFP3		20: 39838584 - rs41946124		
CDH6, DROSHA		20: 42133960 - rs41581110		
SLC45A2, AMACR, ADAMTS12		20: 39810772 - rs42680543		
LGR5, LOC783893	BTA5	5: 1094585 - rs383391542	Brown Swiss (association with udder clearance and the presence of supernumerary mammary gland tissue with a supernumerary nipple)	Butty et al. (2017)
TBX3, TBX5	BTA17	17: 60357076 - rs109396313		
TBX5, RBM19	BTA17	17: 60431658 - rs137563207	Brown Swiss (association with mammary gland morphology)	Fang and Pausch, (2019)
TBX5, RBM19	BTA17	17: 60427991 - rs109134926	German Flekvieh (association with mammary gland morphology)	Pausch et al. (2016)

2.4 Teat number, functionality and disorders in pig

The teats are generally classified into three main categories including functional (desirable), inverted, and supernumerary (extra) teats. A teat is considered as functional when it has a well-developed predominant sphincter and the body of the teat is clearly distinct while the teat with a teat-end turned inward (invisible sphincter or hidden sphincter in the teat body) is called an inverted teat. Compared to the functional (normal) teats, the small teat with the shorter size is recognized as the supernumerary or extra teat (Uzzaman et al., 2018). A basic understanding of the anatomy of the teat seems crucial if good functional teats are to be selected. Figure 9 demonstrates a pattern for different teat conformation in the pig.

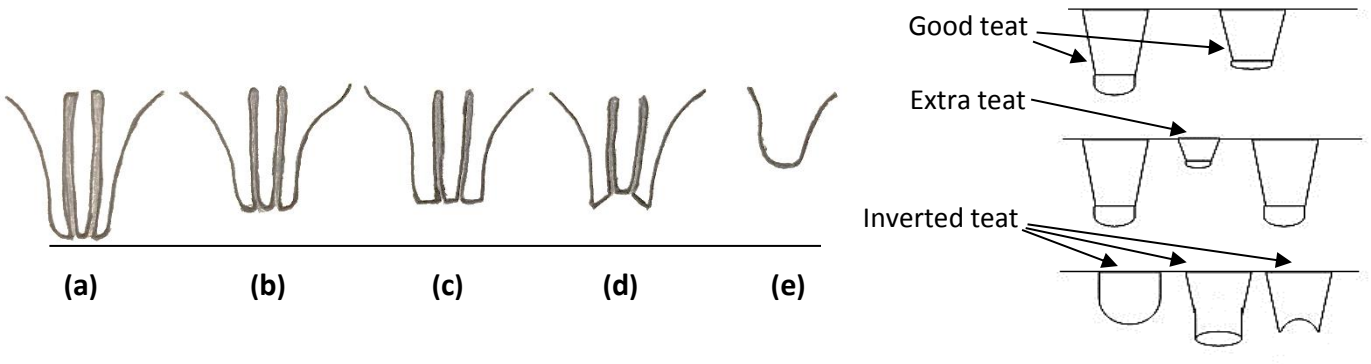

Figure 9. Schematic representation of teat conformation in pig. The normal and well-developed teat with two opening canals (Functional) (a). The teat is not so elongated, but the end of the teat protrudes down

properly (Functional) (b). This is considered as the cut-off point for selection where the teat sphincter (usually seen as a black dot) could still be observed at eye level (Functional) (c). Inverted teat with not visible teat sphingter and short teat canal that turns inward (Non-functional) (d). Teat necrosis as the teat might be rubbed off in the first 48 hours of birth (Non-functional) (e) (drawn based on what was presented by Muirhead and Alexander, 1997).

The maximum, minimum, and average number of functional teats on the breeding gilt is a debatable point and shows the variation in different pig breeds. In general, it is expected that the optimum number of teats should be at least 12 good teats out of 14-16 total teats. As an example, the number of teats ranged normally between 14 to 20 among Italian Large White pigs with an average of 14.87 ± 0.90 (Dall'Olio et al., 2018), which was higher than the average number observed in Swedish Yorkshire sows (14.5 ± 0.9; Lundeheim et al., 2013) and Duroc populations (13.73 ± 1.13; Arakawa et al., 2015; 10.72 ± 1.72; Tan et al., 2017) and lower than Landrace pigs (15.61 ± 1.05; Lopes et al., 2014). In addition to teat conformation, the position pattern of teats on the udder is fundamental in terms of pig production. If the abnormal teat position pattern leads to the poor accessibility of teats for piglets at birth, it does not make sense for a pig to have almost 14 perfect teats. A suitable teat position pattern includes the teats that are equally spaced without any supernumerary (extra) teats between them and being in two parallel lines with symmetry form (Figure 10). However, during the selection process, it is also essential to consider the placement of good teats on the boar which is used to produce breeding females.

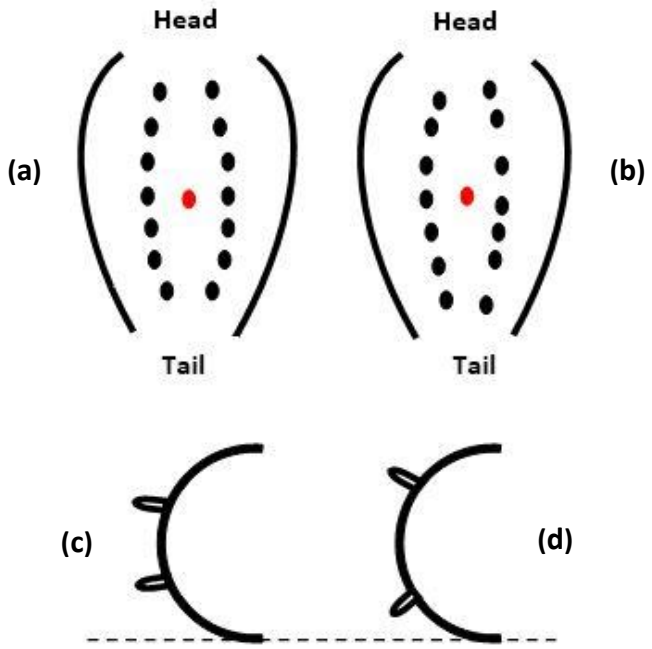

Figure 10. Schematic representation of teat placement patterns. Good placement of teats in which teats are parallel to each other and are in symmetry pattern (a), and bad placement of teats in which teats are not parallel to each other and are in asymmetry pattern (b). Teats are located on the body with a suitable angel based on the floor (side-view of the pig body) (c), and teats are located on the body with an unsuitable angel based on the floor (d).

Functionality and disorders of teats in pig

The presence of a proper number of functional mammary glands and functional teats reflect the fitness level of the sow which is regarded as the key factor for a successful initial rearing period of the piglets (Chalkias et al., 2013). The number of functional teats might have a positive genetic correlation (0.82) with the total number of teats in pigs suggesting that breeding programs based on the total number of teats would affect significantly the possible increase of the functional teats (Lundeheim et al., 2013). The functionality level (with regards to milk proteins yield) was found to be greater for the teats located in the first and second rows in the anterior part and third to fifth rows in the middle part with secretion of higher volumes of colostrum (containing higher concentrations of immunoglobulin A and G) in comparison with the posterior teats located in the sixth and seventh rows (Ogawa et al., 2014).

The non-functional teats such as inverted, blind, and extra teats should be taken into account and to be focused on when considering breeding programs for improving the number of functional teats and reproductive traits of pigs. There is one main issue that these teats have a wide variety of morphological features and shapes which makes errors when some of the non-functional teats missed mistakenly at recording particularly in males. The famous example, in this case, is the extra teats locating near the umbilicus and between two normal teats in right or left lines that might be missed (Chalkias et al., 2013). The inverted teat which inherited as a recessive trait (Chomwisarutkun et al., 2012), is the most frequent inherited mammary gland disorder in pigs which causes non-functional teats that are impossible be suckled by the offspring (Tetzlaff et al., 2009). The main features of inverted teats include the failure of the teat to protrude from the surface and an inward teat canal that forms a small crater preventing the normal milk flow, and eventually increasing the risk of mastitis and diminishing the rearing capacity of pigs (Jonas et al., 2008). They appear most frequently in the umbilical and anterior parts (Clyton et al., 1981), and sometimes are difficult to be distinguished from the normal functional teats in the phenotypic selection of young gilt (Chalkias et al., 2013). A proportion of inverted teats will be drawn out by the piglet at suckling, but at least 50% of them will remain blind. The probable cause of the inverted teat occurrence could be the disturbed mesenchymal-epithelial interactions at the teat ground during all nonrecurring developmental stages from fetus to puberty (Chomwisarutkun et al., 2012).

Teat number in pig

The number of teats is considered as the fundamental trait in the pig breeding industry influencing both the welfare of piglets and the level of production in pig farms, which also shows the relationship with other economically important performance traits. In fact, the coordination of different biological and signaling pathways is required for the embryonic mammary gland development and subsequent proper development of teats (Verdado et al., 2016). The improvement of teat number as a polygenic trait could be achieved through both classical methodology (the BLUP under an animal model) and a molecular approach (Rekiel et al., 2019). Despite the numerous achievements of breeding programs in improving the litter size in pigs, the number of

teats is still not the same as the number of piglets and is often lower than that number which is a key factor for the mothering ability of the sows and survival rate of the piglets (Rodriguez et al., 2005). Hence, association studies for the number of teats in different pig populations have been favored by the researchers. Furthermore, the findings from such studies and analyses may have great importance in order to understand the genetic diversity between different populations (Guo et al., 2008). There is a high risk of mortality rate before weaning when the number of teats is low and thus the suckling competition increases between piglets. Therefore, a higher number of teats and well-developed mammary glands are necessary for pigs to reach their optimum level of production and for piglets to receive a sufficient amount of nutrition (specifically colostrum for proper immune protection) during the lactation period, and a better understanding of the genetic architecture underlying this trait (also type, location, and symmetry status of teats) guarantees more efficient selection process (Guimaraes et al., 2014; Zhou et al., 2019). Another concept that needs to be considered in pig breeding schemes is the teat pair distance which is of great importance for teat use by piglets in almost all the breeds due to its effect on ensuring the suitable colostrum intake by piglets. It was claimed that the greater distance between teat pair might result in more nonfunctional teats regardless of teat location (Ocepek et al., 2016).

The genetic inheritance of the dam is one of the known factors affecting the teat number of pigs. Teat number is also affected by the number of the fetus and the sex ratio (Rekiel et al., 2019). The identification and investigation of the main factors influencing teat number are crucial as it is one of the most important traits used to evaluate gilts whether to maintain them for breeding stock. Based on the previous studies, a higher number of teats in dam followed by a lower proportion of males in litter caused the presence of a greater number of teats in gilt, implying the possible relationship between the number of teats in female pigs and the proportion of males in the litter (Drickamer et al., 1999). Also, the higher number of functional teats in sows was found to have a low relationship with having bigger and heavier piglets at weaning but no significant association with fertility rate. Also, the teats are suckled differently by piglets depending on their specific location along the milk line in the anterior, middle, and posterior parts of the body (with varied milk yield based on the corresponding mammary gland in that specific part) (Rekiel et al., 2014; 2019). In fact, the importance of this trait is not confined only to females where teats are matter but has also been considered in the selection procedure of male breeding stock. In this case, it is important for teat number trait to have at least a moderate genetic variation in males when justifying selection for this trait in males which subsequently will be indicated in their female progeny (Enfield and Rempel, 1961).

The relatively high genetic correlation (0.62 to 0.78) between teat number and some reproductive traits in Duroc pigs including the number of born and alive and litter size at weaning revealed the significant effect of this trait for improving the reproductive traits in pig herds (Ohnishi and Satoh, 2014). Initial attempts to identify quantitative trait loci (QTL) underlying the teat number found QTLs on chromosomes 2, 10, and 12 in Meishan×Dutch pig crosses, of which QTLs on chromosomes 2 and 12 showed imprinting. Only of the QTLs on chromosomes 10 and

12 considering the estimated additive effects, a positive effect of the Meishan allele on the teat number was revealed (Hirooka et al., 2001). Plus chromosome 12, three other significant QTLs on chromosomes 3, 7, and 8 were found for teat number trait through nonparametric interval mapping in a Meishan×Duroc F2 resource population (Sato et al., 2006). Accordingly, a genome-wide association study (GWAS) in Meishan×Large White F2 pigs highlighted QTLs for teat number on similar chromosomes as 3, 7, 8 and also two other chromosomes as 16 and 17, suggesting the fact that teat number and reproductive traits in pig are influenced by various numbers of loci with a range of low to moderate effects (Bidanel et al., 2008). Based on the recent investigations it is perfectly confirmed that chromosome 7 contains the main QTLs which affects significantly teat number in pigs (Rekiel et al., 2019).

The prospero homeobox 2 (*PROX2*) gene on pig chromosome 7 (SSC7) was suggested to have an association with teat number in Large White pigs (Guimaraes et al., 2014). This gene is part of the homeobox gene family and known as one of the important transcriptional regulators for embryonic development (Pistocchi et al., 2008), and has also been suggested as one of the candidate genes underlying the number of vertebrae trait in White Duroc×Chinese Erhualian intercross population (Ren et al., 2012). There was evidence of the gene interactions that were in line with the known mammal's breast biology and transcription factors identified through the network analysis within and across Landrace and Large White pig breeds. The results figured out several sets of putative candidate genes for teat number particularly for each line including *EFNB2*, *KIF6*, *SUZ12*, *VRTN*, and *SYNDIG1L* for Large White and *WBP11*, *MDC1*, *TRPM8*, *DHX16*, *CDH13*, *EVX1*, and *GDF7* for Landrace (Verdado et al., 2016).

The vertnin (vertebrae development associated or *VRTN*) has been found to be the main causative gene and the most important QTLs for the number of teat trait on SSC7 of several pig breeds (Dall'Olio et al., 2018; Rohrer and Nonneman, 2017; Tang et al., 2017). A putative DNA binding factor encoded by the *VRTN* gene was suggested to be one of the fundamental regulators of the embryo development stage (similar to *PROX2*) in several species (Mikawa et al., 2011). Genome-wide association studies have highlighted significant associations between different single nucleotide polymorphisms (SNPs) of this gene and teat number with the widest consensus on SSC7 (Tan et al., 2017). Furthermore, as mentioned earlier, the total number of teat traits may also have a direct relationship with the number of vertebrae that determines the length of the body in sow (Ren et al., 2012). The scientific evidence for this relationship comes from a sequence analysis performed in Duroc pigs which indicated two functional mutations of the *VRTN* gene significantly increased the number of thoracic vertebrae (Van Son et al., 2019).

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3.1 Genetics of coat colour and muzzle colour in cattle breeds

Introduction

The identification of genetic variation underlying important morphological traits in livestock gives an opportunity to get insight into the speciation and population divergence (Theron et al., 2001). Signature of selections, as the particular patterns of genetic variation, are developed by genomic regions due to the phenotypic selection (Randhawa et al., 2015). In order to reveal causal effects of selection, it is vitally important to identify the trait-specific genomic regions which itself necessitates the broad phenotypic and genomic data of large populations (Decker et al., 2012). Variation in coat colour in domestic animals can be a suitable model for investigating the genetic structure of phenotypic diversity in domestic and wild animals, characterisation of different breeds, adaptation and evolutionary processes along with domestication and also studying the genetic architecture of complex traits, presenting the close relationship between phenotype and genotype (Cieslak et al., 2011; Protas and Patel, 2008). In cattle breeds, specific pigmentation patterns play an important role in the value of animals based on the uniformity in coat colour and involve in breeding standards (Drogemuller et al., 2009). Dark/brown coat colours are desirable in the natural environment while for economic objectives, pale coats including white and yellow are preferable. Typically, wild species demonstrate uniformity in phenotype associating with specific colours and patterns. Contradictory, the higher extent of variation in coat colour is observed in domesticated animals in comparison with their wild ancestors (Cieslak et al., 2011). Coat colour associated genes have been shown to be the most important candidates for breeds traceability and authentication. For instance, the authentication of dairy products obtained from local breeds especially Parmigiano Reggiano cheese from Reggiana cattle milk which was based on the detected polymorphisms at the *e* allele of *MC1R* gene on *Bos taurus* chromosome 18 (BTA18) (Russo et al., 2007). Also, the phenotypic selection and fixation of coat colour could indirectly result in the selection and fixation of causative alleles of underlying genes that control this trait (Fontanesi et al., 2010). There is a wide variety of coat colour patterns in cattle including one solid colour, one coat colour with another different colour in some specific parts of the body, mix of different colours and coat colour with other different characteristics like spotted, brindle or belted patterns.

Although the coat colour genetics has been thoroughly studied in dairy cattle, the genetic mechanisms and causative genes resulting in different muzzle colour phenotypes in cattle and other livestock still need to be investigated and current information seems insufficient and limited (Kim et al., 2014). The main three muzzle colours observed in cattle consist of black, grey, and pink but sometimes different patterns such as spotting (often black dots) could be appeared in grey and pink muzzles (Bekge, 1961). The coat and muzzle colours in mammals are determined by the relative distribution of pheomelanin and eumelanin. It is also believed that the bovine coat and muzzle colours are regulated in the same way, however, the molecular mechanism underlying pigment deposition in the dark muzzle has yet to be elucidated. There is evidence suggesting a possible relationship between coat and muzzle colours as it was found that one allele of the *MC1R* gene on

BTA18 (the most famous coat colour gene) could influence also the muzzle colour in cattle (Lee et al., 2002; Park et al., 2012).

Basics of pigmentation

The pigmentation pattern is basically regulated by the two main melanins including eumelanin (black/brown) and phaeomelanin (yellow/red), and their related controlling genetic loci encompass the Extension (*E*) and Agouti (*A*), respectively (Searle, 1968). Referring to the pigmentation process, after the synthesis of melanins (eumelanin and pheomelanin), they are stored in the form of melanosomes as the large organelles composed of melanocytes. Melanocyte is the fundamental factor in the pigmentation mechanism that present not only in skin but also in eyes, ears, heart, brain, lungs, and adipose tissues (Bellone, 2010). Melanoblasts which are the melanocyte precursor cells, originate from the neural crest and then distribute to skin, follicles, eye, inner ear, and other organs. The disruption of this process in cattle will result in having a white hair coat due to the lack of melanocytes in some areas of skin. Therefore, the differences in melanocyte features including size, shape, and transportation to specific areas of skin as well as the variation in amount and type of synthesized melanins are the main factors for emerging different pigmentation patterns observed in cattle breeds (Hirboe, 2011). Also, the regulation of the melanogenesis process plus these factors is done by a number of genes mostly showing pleiotropic effects (Charon and Lipka, 2015). In addition, during the early developmental stages of the pigmentation process, the protein MITF (microphthalmia transcription factor) plays an important role in melanocyte survival. Phosphorylated MITF has an active role in melanocyte biology through its increased transcriptional activity (Seo et al., 2007).

Main genes for determining coat colour patterns

Hitherto, at least eight different genes and their related alleles have been detected to control the coat colour patterns in cattle breeds consisting of *MC1R* (extension - *E*), *ASIP* (agouti - *A*), *TYR* (albinism - *C*), *TYRP1* (brown - *B*), *KIT* (colour sideness, dominant white), *KITLG* (roan - *R*), *MITF* (white spotting) and *PMEL* (dilution - *D*). Other observed patterns such as color sided (*Cs*), blaze (*Bl*), belting (*Bt*), brindle (*Br*), and brockling (*Bc*) are the result of interaction with spotted locus (*S*/*W*) or other underlying genes (Barsh, 2001; Olson, 1999). Proper skin pigmentation in cattle crucially requires the optimal proportion of functional melanocytes. It was revealed that the Piebaldism in cattle which is a coat colour phenotype resulting from a combination of pigmented and non-pigmented areas is due to a disruption in the natural migration of the functional melanocytes (Smith, 2014). Also, coat colour dilution could be occurred either in the primary pathway inducing the dilution of both phaeomelanin and eumelanin pigmentation or in further phases of this process that influences one or other pigments. The dilution pattern of coat colour in cattle composed of a different mixture of white, dun, cream, yellow, gold grey, pale red, and brown colours and some specific names have been proposed to refer to different coat colour patterns in different breeds (Berryere et al., 2003).

Identification of mutations for coat colour associated genes in different cattle breeds

MC1R (*E, extension*)

The *MC1R* gene is located on BTA18 and includes three main alleles E^D, E^+, and *e* that cause the presence of black, red, or reddish-brown and red coat colours in cattle, respectively (Seo et al., 2007). Variation of *MC1R* gene was investigated in some French and commercial cattle breeds, and identified alleles included wild type *E* allele which predominantly observed in Normande cattle, the dominant E^D allele (c.296T>C) mostly in black Holstein and the recessive *e* allele mainly in homozygous animals with a relatively red coat colour (Blonde d'Aquitaine, Charolais, Limousine and Salers). Moreover, E^1 as a new allele (c.655dup) was identified in Gasconne and Aubrac breeds in both homozygous (E^1/E^1) and heterozygous (E^1/E) forms causing fawn to brown and grey coat colours (Rouzaud et al., 2000). It was found in the local Norwegian and Icelandic cattle that the animals carrying E^D and *e*/*e* alleles had basically black and red coat colours, respectively, while heterozygous E^+/e or homozygous E^+/E^+ animals demonstrated a range of black, brown, and red coat colours (Klungland et al., 1995). Also, a strange coat colour phenotype was found in this breed that cows carrying E^D allele show a red coat color with white spotting and a half black area in the head, proposing the reversion of an inactive E^D allele that could affect melanocyte migration and causing this pigmentation pattern (Klungland and Vage, 1999). Jin et al. (2011) suggested that the E^+ allele is the possible allele underlying black coat colour in Korean cattle as Korean black cattle mainly had E^+/e genotypes and E^+/E^+ or E^+/e genotypes were observed in Korean brindle cattle. The identified signal of selection for the *MC1R* gene was shown to cause the red coat colour in Reggiana dairy cattle (Bertolini et al., 2020).

Graphodatskaya et al. (2002) also detected the recessive red allele *e* (c.311del) in Red Holstein and Simmental and the dominant black allele E^D in Holstein that did not respond to a wide range of a-MSH concentrations while two observed alleles in Brown Swiss E^{d1} (c.667C>T), E^{d2} (c.652G>A) and one allele in Simmental e^f (c.890T>C) were found to be responsive to stimulation by a-MSH. There were evidence of associations between *e* allele and a synonymous variant (c.27G>C) at *MC1R* gene with the yellowish-red coat colour and the total amount of melanin and eumelanin in Hanwoo cattle, respectively (Do et al., 2007; Mohanty et al., 2008). The result from another study on coat colour of two Italian cattle breeds including Modicana and Sardo-Modicana showed the role of *E+* and E^1 alleles instead of the *e* allele at homozygote status for the red coat colour in these breeds like other red European cattle breeds (Guastella et al., 2011). With retrieving *MC1R* gene tree, protein domains, and genetic variation of cattle, single nucleotide polymorphisms (c.296T>C, c.583T>C, and c.663C>T) were revealed in CDS with high genetic variability and showed to be associated with the presence and controlling black and white coat colours in Bos taurus cattle breeds (Goud et al., 2020).

Investigating *MC1R* genotypes of Kumamoto sub-breed of Japanese Brown cattle has revealed c.296T>C and c.310G>- as already identified, plus another mutation as c.871G>A. The coat colour for this sub-breed was derived from the *e* allele of the *MC1R* gene as most of the

animals had *e*/*e* genotype. Animals with *E*/*e* genotype showed theoretically black coat colour but some of them with *E*/*e* genotype possessed A/A genotype of c.871G>A, whereas the remaining had G/A genotype. It was demonstrated that the c.871G>A allele may be a loss of function mutation, causing damages to MC1R protein function or structure, and then prevents eumelanin from being produced by melanocytes, resulting in Kumamoto's brown coat colour (Matsumoto et al., 2020). The increase in melanogenesis shown in Korean brindle cattle was due to MC1R and MARK signaling and the cell cycle active mechanism, which resulted in coat colour variation. Also, expression pattern analysis of epigenetic genes in coat colour indicated that the brindle colour formation in calves is due to the changes in the MARK-related and melanogenesis associated major factors (Jung et al., 2020). Considering the eye colour subject, the decrease of melanin pigment in the anterior border layer and iridal stroma was shown to be the main reason for the bilateral iridal hypopigmentation (bicolored iris with a central ring of silver-blue and a peripheral ring of brown-gray) in Holstein-Friesian cattle which was associated with a specific region on BTA8, with A allele at the significant SNP for the increase of iridal hypopigmentation incidence (Hollman et al., 2017).

***ASIP* (*A, agouti*)**

ASIP is known as *A* or agouti locus on BTA13 which is responsible for agouti-signalling protein (ASP) and influences the expression of wild-type pattern. Identified alleles at this locus include A^{+}, *a*, A^{Br}, and a^{i}. Its interaction with Extension locus (*MC1R, E*) determines coat colour in cattle. The mutant a^{i} at this locus has been considered to be the cause of the lighter belly observed in Limousin and Jersey cattle (Seo et al., 2007). The results from a study in Icelandic cattle revealed the mutual effect of Extension (*MC1R, E*) and Agouti (*ASIP, A*) loci in determining coat colour. Although in *A* locus, A^{+} and *a* produce brown and recessive black (nonagouti) colours, they could express their effects only when accompanied with E^{+}/- genotypes and in the homozygous form (Adalsteinsson et al., 1995). The specific coat colour of Normande cattle which is a variable presence of black (eumelanin) hair over a red/brown background, was discovered to be a result of the full-length Bos taurus LINE element inserted in the 5'-genomic sequence of the Agouti gene that triggers over-expression of alternative transcripts. This new Agouti allele in the Normande breed has been named A^{br} (Girardot et al., 2006). Through another investigation on the effects of *MC1R* and *ASIP* genes on the coat colour of Korean Hanwoo and Jeju black cattle (JBC), the *MC1R* - *e*/*e* genotype was not present in JBC while observed predominantly in Hanwoo cattle producing red or yellowish-red coat colour. The cattle from both breeds carrying *ASIP* - A^{Br} genotype did not demonstrate the agouti-like brindle pigmentation patterns and the Extension locus (*MC1R*) was basically related to observed yellowish-brown (*e*/*e*) and dark-brown or black (E^{+}/*e*) coat colour phenotypes (Han et al., 2011).

***KIT* (*Cs, colour sideness and spotting*)**

KIT gene on BTA6 is responsible for color-sided and white spotting coat colour phenotypes in cattle breeds. Four alleles were detected for this gene including S^{+} (non-spotting), S^{H}

(homozygous), S^{P}, and *s* (recessive spotting). It was proposed that there is a link between *KIT*, *MITF*, and *TWIST2* genes with locus heterogeneity for non-syndromic forms of white-spotted coat colour in cattle (Fleck et al., 2016). Reinsch et al. (1999) reported the *KIT* gene and *s* allele as the main candidate gene responsible for the degree of spotting in German Simmental and German Holstein cattle. The identified SNP (g.70214244C>T) in exon 5 of the *KIT* gene in Hereford cattle proposed a key role of this gene and determined the S^{H} allele (homozygous) affecting the degree of spotting in their coat colour pattern (Fontanesi et al., 2010). Durkin et al. (2012) reported in Belgian Blue and Brown Swiss cattle breeds that color sidedness (with different levels of white spotting) is affected by other two alleles including one allele on BTA29 (Cs_{29}) resulted from the translocation of a 492-kilobase BTA6 segment within the *KIT* gene to BTA29, and the second allele on BTA6 (Cs_{6}) arising from the first allele through repatriation of fused 575-kilobase BTA6 and BTA29 sequences to the *KIT* gene. Interestingly, the coat colour patterns in White Galloway and White Park cattle breeds encompassing homozygous animals (Cs_{29}/Cs_{29}) demonstrated a mismarked pattern and heterozygous animals (Cs_{29}/Wt_{29}) showed a range of strongly marked to fully black patterns which were found to be affected by the *KIT* gene on BTA29 through duplication and aberrant insertion (Brenig et al., 2013). GWAS and haplotype analyses revealed a strong signal on BTA6 at *KIT* gene followed by a critical interval of 122kb downstream of the coding region and KIT^{PINZ} variant (S^{P} allele) was shown to be responsible for the presence of a specific white spotting phenotype called line-backed spotting in Pinzgauer cattle which is an autosomal incompletely dominant trait (Kuttel et al., 2019). Subsequently, investigation of the unusual colour-sided pattern of Gloucester cattle (in which the spot does not extend to the head from the tail) uncovered a complex structural variant downstream of the *KIT* gene, which overlaps the regions involved in Cs_{6} and Cs_{29}. The variant is a 310 kb duplication resulting from intron 1 of the *KCND2* gene on BTA4, which has been translocated to BTA6. This variant plus the approximately 9.4 kb deletion on BTA6 cause this phenotype. The previously found KIT^{PINZ} allele as well as the Cs_{6} and Cs_{29} alleles all within a region downstream of *KIT*, affect *KIT* regulation by disrupting a number of putative regulatory elements (Artesi et al., 2020). Mészáros et al. (2015) also reported a strong association of the *KIT* gene with the highest signal on BTA6 and inhibition of circumocular pigmentation and pigmented spots on the cheek in Flekvieh cattle. Whole-genome sequencing of Brown Swiss cattle demonstrated a heterozygous variant affecting the coding sequence of the *KIT* gene on BTA6. The variant is a 40bp deletion in exon 9 (c.1390_1429del) and results in a frameshift that causes a novel 50 amino acid-long C-terminus, including the functionally important intracellular tyrosine kinase domain (p.Asn464AlafsTer50). This loss-of-function *KIT* variant represented probably causative de novo germline mutation for the novel dominant white-spotted phenotype with the variable extent in mother (white-spotted) and son (depigmented or piebald pattern) (Hafliger et al., 2020). Also recently one region under peculiar selection was identified on BTA6 for the *KIT* gene in the Reggiana cattle breed, emphasizing the important role of this gene in coat colour pattern distribution (Bertolini et al., 2020).

***MITF* (*white spotting, piebaldism*)**

This gene is located on BTA22 and is well known for its contribution in white spotting and piebaldism phenotypes of coat colour in cattle. The importance of the *MITF* gene on pigmentation in cattle was revealed when the presence of a strange phenotype in Holstein calf including lack of pigmentation in hair and skin, reduction in eye size, and possibly bone growth deficiency was associated with the lack of function and heterozygous deletion of *MITF* gene (Wiedemar and Drögemüller, 2014). Liu et al. (2009), however, mentioned evidence from QTL analyses indicating the relationship between the *MITF* gene on BTA22 and the white-spotting pattern of coat colour in dairy cattle breeds. Also, GWAS results from a study on the Chinese Holstein population found a SNP harboring chromosome 22 including the *MITF* gene that was associated with teat colour, and proposed this gene as one of the candidate genes affecting pigmentation traits (Fan et al., 2014). The difference in allele frequencies of one SNP in *MITF* gene (g.31650821T>A) between spotted and non-spotted cattle breeds suggested the variability of this gene resulting in different coat colour phenotypes as it was shown to be associated with Piebaldism in spotted breeds consisting of Holstein and Simmental (Fontanesi et al., 2012). In Brown Swiss cattle, one SNP (g.31790063G>A, rs722765315) located in the intron 1 region of the *MITF* gene was suggested as the causative mutation for the presence of white spotting phenotype (Hofstetter et al., 2019). Moreover, the German Flekvieh cattle syndrome characterised with hypopigmentation, heterochromia irides, colobomatous eyes, and bilateral hearing loss was identified as a result of a missense mutation (R210I) in exon 7 of the *MITF* gene causing a negatively dominant effect and also probable mutations of SOX10 and PAX3 genes in their promoter binding sites (Philipp et al., 2011). GWAS results and network and functional annotation clustering analyses detected a new variant of *MITF* gene within intron 2 (g.31651707G>A) in two Ethiopian cattle breeds consisting of Begait and Fogera that suggested a determining role of *MITF* gene and also its mutual interaction with other genes such as KIT in producing spotting patterns in these two breeds (Edea et al., 2017). Recently, GWAS for white spotting phenotype in New Zealand Holstein Friesian dairy cattle highlighted intronic variants in intron 4 of *KIT* on BTA6 (g.70210094A>C) and intron 2 of *MITF* on BTA22 (g.31651379A>G) and a new variation in exon 8 of *PAX3* gene on BTA2 (g.110371724G>A, p.Thr424Met), highlighting the key roles of these three genes as the regulators of melanocyte development, migration and differentiation and modulating pigmentation in animals as already implicated in diverse species (Jivanji et al., 2019).

***PMEL* (*D, dilution*)**

PMEL gene mapped to BTA5 and is responsible for dilution coat colour in cattle based on its main alleles including D_C, d_C^+, D_C^D, D_C^d, +, and *del*. A specific region on BTA5 contained *PMEL* gene was shown to affect the coat colour pattern observed in Charolais×Holstein crossbred population including a partially diluted red and black (*Dc*/dc^+) and completely diluted (*Dc*/*Dc*) coat colour as a result of a missense mutation (c.64G>A) in exon 1 of *PMEL* gene (Gutierrez-Gil et al., 2007). Kuehn and Weikard (2007) also reported different dilution patterns in Charolais×German Holstein cattle including especially pigmented skin from a heterozygous

diluted (Dc^{D}/Dc^{d}) and creamy white skin from a homozygous diluted (Dc^{D}/Dc^{D}) which were associated with variation in *PMEL* gene on BTA5 containing exon 5 to exon 7 regions. In Highland cattle, the presence of different coat colours mainly diluted-colored phenotype was found to be related to a leucine deletion (p.Leu19del) in *PMEL* gene (+ and *del* alleles) in interaction with *MC1R* alleles (E^{D} and *e*) in a semi-dominant manner. The observed coat colours included black (E^{D}/-, +/+), yellow (*e*/*e*, +/*del*), red (*e*/*e*, +/+), white creamy (*e*/*e*, *del*/*del*), dun (E^{D}/-, +/*del*) and silver dun (E^{D}/-, *del*/*del*). The data suggested this deletion as the effective factor on this pigmentation pattern in Highland cattle as the same as the c.64G>A mutation effect that was already observed in Charolais×Holstein crossbred cattle (Schmutz and Dreger, 2013).

KITLG* (*R, roan*) *and MLPH

This gene has the same location as *PMEL* on BTA5 and causes roan coat colour in cattle breeds. The finding of an investigation revealed the main effect of a missense mutation (p.Ala223Glu) in the *KITLG* gene within BTA5 on creating the roan coat colour phenotype (with intermingled colored and white hairs) observed in Belgian blue and Shorthorn cattle breeds (Seitz et al., 1999). It was also indicated that the presence of different coat colour patterns in Belgian blue could be associated with other mutations harboring BTA5 (p.Ala227Asp) and BTA3 (c.87_96del) resulted from the epistasis effect of *KITLG* with *MLPH* gene, respectively, and their interaction in order to produce cool gray dark (*del*/*del* ; *Ala*/*Ala*), cool gray (*del*/*del* ; *Ala*/*Asp*) and roan blue (+/+ ; *Ala*/*Asp*) coat colour phenotypes (Li et al., 2016). Additionally, investigating the genetic cause underlying Larson Blue phenotype in the Holstein cattle population suggested causative missense mutations (p.Met113Val, p.His131Asn, and p.His131Arg) occurred in *MLPH* gene on BTA3 that were considered to be linked to this coat colour. However, no effect of this phenotype was observed on vaginal temperature regulation and milk yield variation during hot seasons compared to Holstein with normal coat colour (Dikmen et al., 2017).

Other coat colour associated genes

There was an indication that the variants in *TYRP1* (B, brown) gene on BTA8 including p.His434Tyr and p.Asp408= and their interaction with *MC1R* alleles in Dexter cattle are responsible for causing dun brown (E^{D}/- ; *b*/*b*) and red (E^{+}/E^{+} ; *B*/*b*) coat colours, respectively (Berryere et al., 2003). Another candidate gene effective in cattle coat colour is *TYR* (C, albinism) on BTA29 that was associated with the presence of Albinism in Braunvieh calf due to a frameshift mutation causing a premature stop codon at residue 316 in the homozygous form (Schmutz et al., 2004). *COPA* gene locating on BTA3 was also suggested to cause Dominant Red phenotype (DR^{DR}/DR^{+}; $MC1R^{D}$/-) in Holstein cattle through a missense mutation (c.478C>T, p.Arg160cys) disrupting *COPA* gene activity and then synthesis of eumelanin via inhibiting *MC1R* signaling pathway. The results suggested the *COPA* gene mutation as the fundamental factor to figure out the potential correlation between *MC1R* signaling and coated vesicle transport (Dorshorst et al., 2015).

Incorporating GWAS data and biological functions of the genes for some pigmentation traits in Chinese Holstein cattle such as the proportion of black colour and teat colour, proposed these traits to be affected by the SNPs in *IGFBP7* and *PDGFRA* genes on BTA6 and *WNT16* and *ING3* genes on BTA4, but significant SNP was found only in intron 1 of *IGFBP7* gene (g.74140844C>T) (Fan et al., 2014). The SNPs in two new genes were also detected in Flekvieh cattle for instance *ERBB3* on BTA5 that was supposed to have a determining role in light intensity of coat colour pigmentation and *AP3B2* on BTA21 suggesting to be a probable candidate for yellow/blue coat colour pattern as well as colour intensity (Mészáros et al., 2015). The Belted phenotype of coat colour which characterised by a lack of melanocytes in a stretch of skin around the midsection, was mapped to a 922kb interval on BTA3 in Brown Swiss cattle. Later on, analysis of belted locus in belted Galloway and Dutch belted (Lakenvelder) showed the largest shared haplotype block between breeds encompasses nine consecutive SNPs in a 336 kb interval on BTA3. Results confirmed that the belted phenotype is caused by the same mutation on BTA3 in these breeds and the Brown Swiss breed probably through an alteration of melanocyte development and/or migration in the midsection of the developing fetus, and the *HES6* gene was proposed as the possible candidate gene. However, no significant association was found with the belted phenotype (Drogemuller et al., 2009).

Mapping BTA3 for discovering other causative variants effective in cattle coat colour revealed *TWIST2* gene involving in the presence of belted coat colour pattern in belted Brown Swiss, Galloway and Lakenvelder due to a belted-associated variant (3'-UTR variant, g.118102940G>A) of *TWIST2* gene (Awasthi Mishra et al., 2017). Subsequently, it was confirmed by Rothammer et al. (2018) that *TWIST2* is the closest gene to the candidate causal mutation (16kb distal) at position 118,029,119-118,034,893 bp on BTA3 in belted Galloway and Dutch belted cattle. From another side, the fact that a gene interaction network performed with GeneMANIA makes a link between *TWIST2* with *KIT* and *ADAMTS20* genes, which are the causal genes for belted phenotypes in pigs and mice, could strengthen the probability of *TWIST2* being as a causative candidate for the belted phenotype in cattle. Recently, a SNP (intergenic variant) on BTA20 (ARS-BFGL-NGS-55928 - rs110452481) was reported to be significantly associated with the colour sideness in Cinisara cattle (a white band along the spine, from the head to the tail, and on the ventral line observed also in Belgian Blue, Brown Swiss) and the only gene that was annotated near the associated SNP in a window of ±200 kb was *PLK2*. This gene encodes a protein belongs to the polo-like kinases, the same family of several known coat colour candidate genes (Mastranglo et al., 2019).

Some diseases associated with coat colour genes

As another important application of coat colour genes in domestic animals, they have an association with several disorders. Such disorders must be considered in the breeding industry due to their negative impacts on suffering or even death of affected animals and also economic losses. Some of them show negative pleiotropic effects and are associated with some hereditary diseases, often of a lethal character. For instance, *KIT* gene dosage with hereditary gonadal hypoplasia,

KITLG gene in which R allele linked to White Heifer Disease (missing or underdeveloped vagina, cervix or uterus) in Belgian Blue and Shorthorn, *MITF* gene with German White Flekvieh syndrome (dominant white phenotype and bilateral deafness), *PMEL* gene with Hypotrichosis (congenital deficiency of hair) in Simmental and Hereford, *TYR* with Albinism in Braunvieh calf and *LYST* gene with Chediak-Higashi syndrome (abnormal granules in leukocytes, partial cutaneous albinism and increased bleeding tendency) in Japanese Black cattle which corresponded to human inheritable disorder. Most of these diseases also have their counterparts in humans (Charon and Lipka, 2015).

Responsible genes and mechanisms for muzzle pigmentation

Unlike coat colour, there is limited information about the genetics underlying muzzle colour phenotype in cattle breeds. The degree of darkness of the muzzle in cattle is specified by the number of black spots in the muzzle, based on the method developed by Lee et al. (2002). Moreover, genetic variation in the *MC1R* gene on BTA18 has been found to be consistent with muzzle colour (Lee et al., 2011; Park et al., 2012). In the dark and light-colored muzzle tissues, the expression of genes involved in mitogen-activated protein kinase (MAPK) and Wnt signaling pathways is uniquely regulated. MAPK pathway in the dark muzzle cattle stimulates eumelanin synthesis through the activation of cAMP response element-binding protein (CREB) and tyrosinase (*TYR*), while in the light muzzle cattle activation of Wnt signaling counteracts this process and increases the amount of pheomelanin (Kim et al., 2014). Two novel genes including *LIN37* (GenBank No. NM-001076026) and *LCN1* (GenBank No. XM-588439) have been found with higher expression in black muzzle than light muzzle cows which could provide an intriguing insight into the muzzle pigmentation mechanisms. Also, high expression of the Adenylate cyclase 1 family genes that interact with *MC1R* might influence CREB expression (Saito et al., 2003), resulting in over-expression of *PRKACB* and *CAMK2a* which are the melanogenesis-associated genes (Busca and Balloti, 2003). In particular, the *PRKACB* gene which controls the expression of CREB, *TYR*, and calcium/calmodulin-dependent protein kinase II alpha (CAMK2a), demonstrated a high degree of expression in cattle with dark muzzle compared to light muzzle ones. On the other hand, in dark muzzle cows the expression of the frizzled family gene (*FZD*) which is the upstream regulator of the Wnt signaling system, was lower than in light muzzle cows (Kim et al., 2014). Additionally, increased expression of genes related to tyrosine metabolism, such as *TYR*, is probably responsible to influence eumelanin synthesis in dark muzzle cows (Berryere et al., 2003). The activation of *MITF* downstream of *MC1R* through MAPK signaling tends to contribute to increased eumelanin synthesis in dark muzzle cows. Wnt signaling takes the lead in the regulation of melanogenesis in light muzzle cows, resulting in decreased *MITF* production and enrichment of pheomelanin (Kim et al., 2014). Activation of tyrosine metabolism is done through inducing *MITF* production (which in turn activates eumelanin synthesis) and also through regulating CREB expression (Cheli et al., 2010). As mentioned before, the lower expression of *FZD* in dark muzzle cows activates Wnt signaling that leads to inhibit GSK-3b, and, as a result, MAPK signaling is

quenched. This causes MITF and TYR synthesis to be decreased and thus, the relative concentration of pheomelanin will exceed that of eumelanin (Kim et al., 2014).

Conclusion

Although the coat colour genes could affect health and performance as two key factors in the dairy cattle industry, studies still seem inadequate in this case. Therefore, the crucial link between different coat colour phenotypes and the incidence of some diseases in the dairy cattle population should be considered due to the presence of disease risks or lethal genes with detrimental impacts on offspring. As one of the main criteria in distinguishing breeds, coat and muzzle colour patterns recognition and their inheritance in cattle breeds are essential as in the traditional market, uniformity in pigmentation is desirable for breeders and these traits can be utilized in breeding and crossing programs intending to introduce cattle that are suitable for specific breeding goal. The effectiveness of coat colour studies is more evident in cattle breeds of tropical and subtropical regions because it is well documented that these breeds have more longevity and productivity due to the darker skin colour, hair coat colour, and specific characteristics in body temperature regulation compared to other breeds. It deserves further investigations in order to find genomic regions affecting both coat and muzzle colour as described there is a link between them in cattle. Hence, having great knowledge about the mechanism of pigmentation patterns in different breeds and their underlying genetics will help to select suitable breeds for breeding programs in a specific environment or desired production traits.

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3.2 Genetics of stature in cattle breeds

Stature

Stature, as one of the important classical quantitative traits, which is defined as the height of the cow at hips, has recently gained more attention from geneticists (Figure 1). Deciphering the molecular basis of inter-individual variations in this trait may not only improve the knowledge about the underlying genetic architecture of this trait and other related complex traits but also provide an opportunity to get valuable insights into the functional mechanisms responsible for organismal growth (Karim et al., 2011). It is almost rare for a high-ranking sire with very good records to have a negative proof for stature referring to the strong and significant correlation between stature and other type traits. For instance, positive relationships between stature and body confirmation (which subsequently implies to the fact that selection for conformation will result in having taller cows), also with the mammary system, feet and legs conditions and only slightly positive associations with production yields that are virtually negligible. Therefore in cattle, optimal height has been focused on selection schemes instead of extreme sizes, following by selection on a wide range of different traits resulting in genetic variation identified for stature in cattle through large meta-analysis more comparable to human height. Following this study, 163 significant DNA variants with an effect on cattle stature were detected, explaining less than 14% of the genetic differences discovered within cattle breeds (Bouwman et al., 2018). It has been shown during the past twenty years that the linkage studies trying to map the QTLs influencing weight, growth, and stature traits in cattle (Li et al., 2004; Maltecca et al., 2009; Cole et al., 2011).

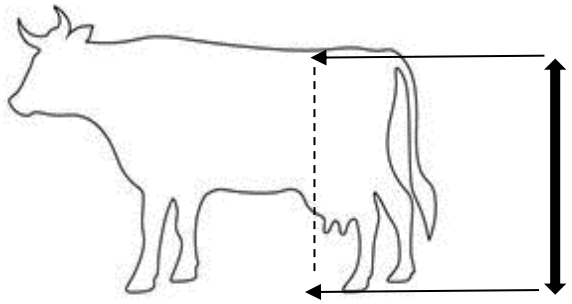

Figure 1. Schematic representation of stature from the hips (cm) in cattle

The process of mapping loci affecting complex traits has been accelerated through the release of the bovine reference genome (Elsik et al., 2009), detection of common SNPs (Matukumalli et al., 2009), and feasibility of high-throughput microarrays. Subsequently, many population-based investigations considering the mutual relationships between main phenotypic traits such as weight, growth, and specifically stature combined with GWAS variants have been performed in different cattle breeds (Snelling et al., 2010; Pryce et al., 2011; Nishimura et al., 2012). Stature is considered to have an impact on predisposition to specific disorders and has been shown to be associated with

productivity in farm animals. Stature usually demonstrates highly heritable behavior (also in humans almost %85) despite the fact that it is sensitive to environmental factors. This is also evidenced that the stature was one of the primary traits influenced by the domestication process of cattle. The extinct Auroch (Bos primigenius), with a shoulder height of around 2 metres, had probably much more height size than its domesticated descendants namely the modern cattle with a shoulder height between 1.1 to 1.5 metres (Nelsen et al., 1986; Northcutt and Wilson, 1993). In order to detect the genes with major effects over the genome landscape of multiple species, genetic architecture underlying height has been thoroughly studied, despite that, the leading model was the human genome (Lettre et al., 2008; Lanktree et al., 2011). The performed genome-wide association studies have reported only a few genes associated with stature entailing body size-related traits (Li et al., 2013; Hoshiba et al., 2013). It has also been reported that there is a genetic correlation between stature and gestation length in cattle (Pozveh et al., 2009). It is considered as a key factor to having a great understanding of the mutual genetic relationship between the early-life traits such as size at birth and mature stature that could strengthen the interpretation of selection systems for stature, subsequently promoting calf size and calving ease (Gutiérrez-Gil et al., 2009; Kneeland et al., 2004). It should be noticed seriously that the selection for increased stature may therefore result in higher weight at birth and then, more calving problems, increasing the risk of higher calf mortality (Fogh et al., 2004). Therefore, detection of multiple signatures of selection and causative genes for stature would improve breeding programs for this trait as selection for this trait may affect also other economic traits (Randhawa et al., 2015).

Genetics of stature

The result of the GWAS study on Brown Swiss cattle population found a pleiotropic locus on BTA25 named *IGFALS* (Bos taurus insulin-like growth factor binding protein acid-labile subunit) affecting stature, milk yield, fat yield, protein yield, lactating cow's ability to recycle after calving and body depth (Gou et al., 2012). This gene is a serum protein and can bind to insulin-like growth factor binding protein (*IGFs*) that has fundamental roles in growth regulation, development, and physiological mechanisms. Interacting with growth hormone (*GH*), *IGFALS* has a positive effect on increasing *GH* half-life and also its vascular localization (Leong et al., 1992). A putative functional mutation (g.23326588C>G, rs109231213) has been detected near *PLAG1* gene on BTA14 which was associated with stature in beef cattle from Bos taurus, Bos indicus, and Tropical composite, representing that the C allele of rs109231213 significantly increased hip height in Bos indicus cattle but hump height in tropical composites (Fortes et al., 2013). The introgression of the Bos taurus allele at the *PLAG1* gene into the Brahman breed was shown to increase the stature and high frequency of this allele revealed the strong selection for this trait (Koufariotis et al., 2018). Also, Karim et al. (2011) in a study on Holstein-Fresian×Jersey line cross, proposed that the probable mutation causing differences in height could be a SNP in the 3′ UTR of the *PLAG1* gene identified as rs109231213 (downstream gene variant). The selection signatures were identified within the region of *PLAG1*, *LCORL*, and *NCAPG* genes on BTA14, associated with stature, conformation, and carcass traits in Reggiana cattle (Bertolini et al., 2020).

In Chinese cattle, the identified Q allele caused by the SNP rs109815800 in *PLAG1* significantly increased body height, and the cows with qq genotype demonstrated shorter height than those with the other two genotypes (Hou et al., 2020).

In a study in which orthologous positions of 55 genes associated with height in four human populations were located on the bovine genome (to test if these loci were controlling stature in cattle), the SNPs were tested for their effect on bovine stature in two cattle populations including Holstein dairy cattle and Brahman beef cattle. The results of this study revealed significant SNPs in both dairy and beef cattle in two regions containing the gene *NCAPG* and a cluster on chromosome 14 (*PLAG1*, *CHCHD7*, and *RDHE2* (*SDR16C6*)), following by SNPs in *FBP2*, *PAPPA*, and *CABLES1* genes specific to dairy cattle and *HMGA2*, *PTCH1*, and *GPR126* in beef cattle where the strongest signal was found for a SNP close to *HMGA2* in the beef data set. This gene has consistently been shown to be associated with stature in human studies, making it more interesting that it seems likely to have a conserved role in cattle. The findings suggest that these genes could contribute to the stature of mammalian species (Pryce et al., 2011).

Also, in Japanese Black cattle, targeted resequencing followed by association analysis highlighted the quantitative trait nucleotides (QTNs) for bovine stature in the *PLAG1-CHCHD7* intergenic region (Nishimura et al., 2012). Additionally, a significant association was observed between the Sirtuin1 (*SIRT1*) promoter region on BTA28 (-274C>G) and growth traits including body height in Nanyang cattle, suggesting that the increase of bovine fat mass and body size may be due to the abnormal transcription factor-based repression of *SIRT1* (Li et al., 2013). Through a GWAS study on Brazilian Nellore cattle (Bos primigenius indicus), the surrounding 1 Mb region presented high identity with human, pig, and mouse autosomes 8, 4, and 4, respectively, and contains the orthologous height-related genes such as *PLAG1* and *CHCHD7*. The region also overlapped 28 quantitative trait loci (QTLs) for mature stature by linkage mapping studies in cattle. Therefore, two hypotheses may arise from the evidence of associated variants with growth and stature traits within BTA14 in both taurine and zebu cattle that first explain introgression of these variants into Nellore cattle through historical admixture with taurine Creole cattle as the maternal line that was maintained in the breed despite the several generations of backcrossing, and the second one assumes theses variants as ancient polymorphisms segregating in the source population of wild Aurochs (Bos primigenius) prior to subspecies formation (Utsunomiya et al., 2013).

Associations recently detected between three SNPs of the *PLAG1* gene including a 19bp novel indel at intron 3 (g.3747444-3747462del), exon 3 (g.44966G>A), and 3'-UTR (14:g.23327346G>A) with some growth traits particularly body height and hip height in Chinese cattle population. The identified mutations of this gene could be considered as the possible candidate molecular markers to be exploited in breeding programs aiming to increase genetic improvement in growth-related traits in cattle breeds (Xu et al., 2018; Li et al., 2019; Zhong et al., 2019). Investigating the evidence of selective sweeps for stature in 9 breeds of European Bos taurus has revealed two strong selective sweeps were detected at loci that cover *UQCC-GDF5* and *PLAG1-CHCHD7* gene pairs on chromosome 13 and 14, respectively (Randhawa et al., 2013).

Another evidence is a multi-trait meta-analysis that detected pleiotropic polymorphism of *PLAG1* gene on BTA14 for stature in beef cattle (Bolormaa et al., 2014).

With applying the composite selection signals (CSS) method to investigate evidence of positive selection in a complex polygenic trait by examining stature in phenotypically diverse cattle comprising European and African Bos taurus breeds and then utilizing comparative mapping information on human height, 30 candidate genes mapped at 12 selection regions (on 8 autosomes) that could be linked to bovine stature diversity. Of these 12 candidate gene regions, three regions contained known genes *NCAPG* (non-SMC condensin I complex subunit G), *LCORL* (ligand dependent nuclear receptor corepressor like), *FBP2* (fructose-bisphosphatase 2), *PTCH1* (patched 1), *PLAG1* and *CHCHD7* related to bovine stature, and nine were not previously described in cattle (five in European and four in African cohorts) (Randhawa et al., 2015). Particularly, *PLAG1* and *CHCHD7* genes on BTA14 have already been reported to be associated with height in humans and stature in cattle (Allen et al., 2010; Karim et al., 2011). In the Nordic Red cattle, however, detected QTLs were found to have large effects on stature and calf size, representing strong evidence for the variants on BTA6 consisting of an intronic variant in the intron 1 of *LCORL* gene (g.37522580C>T) and a missense mutation in the exon 9 of *NCAPG* gene (g.37343379T>G, p.Ile442Met) influencing mature stature and calf size at birth (Sahana et al., 2015).

Previously, new genes have been detected namely *KCNJ12* which was considered as a fundamental candidate gene for economical traits due to its important roles in myoblast development, located on BTA19 that was proposed as one of the possible candidate genes for cattle stature following a missense mutation at exon 3 (g.35362046T>C, p.Cys210Arg) and second, a copy number variation (CNV) of *APOL3* gene on BTA5 which has important roles in cholesterol transport, cellular processes such as modulating gene transcription and signal transduction, observed to be associated significantly with adult stature both found in the Chinese cattle population (Cheng et al., 2019; Peng et al., 2019). Furthermore, researchers proposed that the genetically regulated differences in expression level of the *MATN3* gene which encodes a protein involved in bone development, underlie the bovine stature QTL identified on BTA11 with the significant variant (rs475277351), implicating its contribution as one of the possible candidate genes to cattle growth genetics with subsequent fundamental applications in both dairy and beef production and management (Lopdell and Littlejohn, 2018).

In the analysis of *MEF2C* gene as another candidate for bovine stature in a population of cows from two Chinese native breeds, four SNPs including two novel SNPs in intron 3 (g.88349079C>A, g.88349167G>A), one in intron 4 (g.88329335A>G) and another upstream gene variant (g.88407785T>G) were identified that could demonstrate a strong association of different components of bovine stature with *MEF2C* genotypes (Cao et al., 2016). The significant effects of the bovine *PLAG1* mutation (rs109231213) on body size, body weight, and reproduction, proposed it as the strong candidate since it influenced a highly conserved nucleotide at the 3′-UTR region (Utsunomiya et al., 2017). The identification of relatively similar genetic architecture and significant overlaps in loci of cattle stature compared to human height suggests the probability that

a set of common genes regulate body size in mammals. The *PLAG1* allele is almost fixed in tall cattle breeds such as Limousin, Charolais, and Holstein (Bouwman et al., 2018). Bos taurus insulin-like growth factor 2 (*IGF2*) as a mitogenic hormone influencing the growth and development rate of fetal, its transcription is activated by *PLAG1* that has been indicated to have a key role in genetic variation of stature trait in cattle (Pryce et al., 2011; Fortes et al., 2013).

Final point

As in livestock genetics, genomic loci affecting desirable traits such as stature may be followed by the unfavourable character, therefore it is crucially important to consider the fact that unfavourable characters might be caused by the causative variation for stature or by other close mutations. There is a correlation between stature and live weight in dairy cattle which is regarded as one of the main economic traits and breeding targets in genetic improvement schemes. Hence, selection programs based on the stature trait will subsequently affect live weight which further causes genetic and economic gain due to the improved feed conversion efficiency (FCE). Thus, seeking for genetic markers associated with stature and other related traits will result in unraveling the underlying QTLs that affect these traits and consequently taking advantage of them in performing breeding programs aimed to raise productivity as well as genetic improvement of dairy cattle through genomic selection. Also, the effectiveness of fine-mapping of causal variants that control stature trait in cattle breeds could be improved by merging the data from GWAS and selection signature analyses. In the end, a suitable strategy considering an average optimum height for dairy cattle with which the proper production rate will be guaranteed and undesirable effects will be eliminated seems necessary.

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3.3 Genetics of horn development in cattle breeds

Horn phenotype in cattle

Management problems arising from breeding horned cattle threaten the dairy industry due to imposing veterinary expenses and other related negative consequences such as injuries and infections because of housing and transportation (Prayaga et al., 2007). As an alternative solution to prevent and overcome these issues, breeding the naturally hornless cattle could be beneficial, however, dehorning and disbudding were being commonly used to defeat these problems (Gaspa et al., 2015). Furthermore, another advantage of breeding hornless animals is that they can be accommodated in the same space during transport and also in feedlots, usually are easier to handle in crushes, and quieter compared with horned cattle (Prayaga et al., 2007). There are three main phenotypes considering the presence or absence of horn in cattle including normally fixed horn attached to the skull as horned phenotype, small loosely attached horns as scurs phenotype, and the absence of any horn as polled phenotype (Figure 1) (Mariasegaram et al., 2010). Based on a single gene with the polled allele (*P*) being dominant over horned allele (*p*), polledness genetic location has been mapped to the centromeric region of BTA1 (Georges et al., 1993; Schmutz et al., 1995; Seichter et al., 2012), despite the fact that its actual causative mutation is still unclear (Gaspa et al., 2015). In the modern dairy and beef industries, polledness is considered a potential trait and has the imperative role to meet the demands of animal welfare and also safety for workers at the farm (Glatzer et al., 2013).

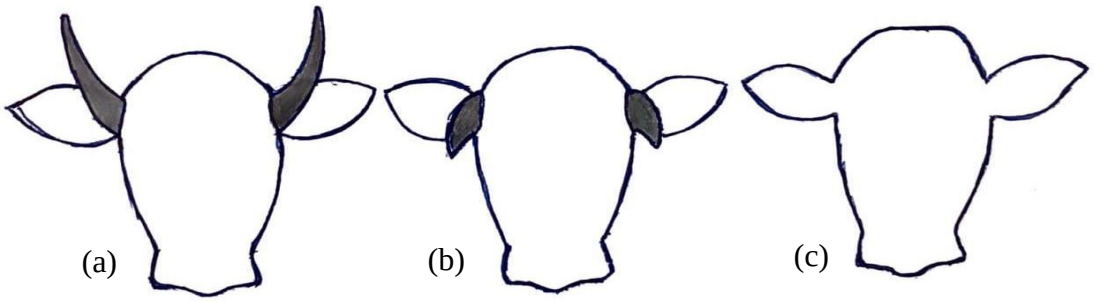

Figure 1. Schematic representation of three main horn phenotypes in cattle including horned (a), scurs (b) and polled (c).

As previously said, breeding polled cattle in order to produce hornless animal is the non-invasive, sustainable, and advantageous practice to all of the other methods used until now and furthermore, no significant differences were found in production and other economic traits between polled and horned cattle based on the previous investigations (Goonewardene et al., 1999). Although recent advances in molecular genetics have promoted the scientific practices to refine the location of polled locus, there is still a large scope to be carried out in this field for identifying a better understanding of probable relationships and confounding effects which are present between polled, scurs and African horn (with *Ha* (horned) and *ha* (polled)) genes, and

improving developed genetic tests that have the ability to detect and identify homozygous/heterozygous animals for these loci (Prayaga et al., 2007), and therefore, proceeding to increase the introgression of the polled condition in cattle herds (Chamberlin, 2017). The application of new genomic tools and breeding schemes for introgression of polled allele has already been started for important horned dairy cattle breeds namely Holstein, Brown Swiss, and Fleckvieh, as well as for the beef breeds such as Charolais (Schafberg and Swalve, 2015). Scurs, as the second locus affecting horn development in cattle, has been stated to grow based on the sex and polled phenotype factors observed in Angus and Galloway cattle breeds (Long and Gregory, 1978). For homozygous polled animals to demonstrate the scurs phenotype, it is needed to carry the *Sc* (scurs) mutation in a homozygous form. Scurs is also observed in heterozygous polled females when they carry *Sc* mutation but in homozygous form and the presence of one or two *Sc* alleles will result in scurs phenotype in heterozygous polled males (Table 1) (Long and Gregory, 1978). However, epistasis indication of polled and scurs loci found by Wiedemar et al. (2014) based on finding 207 scurred animals that were heterozygous for one of the polled mutations does not support these associations. Since in that research 191 homozygous polled cattle did not demonstrate any signs of scurs, it was concluded that polled is epistatic over scurs and scurs phenotype cannot be expressed in homozygous polled individuals (Wiedemar et al., 2014). Therefore, a definitive gene test is fundamentally required for distinguishing horned, polled, and scurred animals in order to proceed with accurate and suitable breeding programs as the scurs mode of inheritance and the expression of the phenotype is affected by age of animal that itself could make it difficult to study the inheritance based on phenotypes (Chamberlin, 2017).

Table 1. Understanding horned, polled and scurs conditions in bulls and cows (Ward, 2015)

Genotype	**Phenotype - Bulls**	**Phenotype - Cows**	**Note**
PP SC/SC	Smooth Polled	Smooth Polled	In all of these cattle the genes for polling appear to be dominant so the cattle appear smooth polled
PP SC/sc	Smooth Polled	Smooth Polled	
PP sc/sc	Smooth Polled	Smooth Polled	
Pp SC/SC	Scurs	Scurs	The presence of two copies of the *SC* gene and a copy of the *p* gene cause scurs in both both bulls and cows
Pp SC/sc	Scurs	Smooth Polled	The single copy of the *SC* and *p* genes lead to scurs in the bulls but not in the cows where a further scurring gene would be needed
Pp sc/sc	Smooth Polled	Smooth Polled	Both bulls and cows appear smoothly polled as only the recessive forms of the scurring genes are present
pp SC/SC	Horned	Horned	In all these cattle the genes for horn mask the effect of genes that would cause scurs
pp SC/sc	Horned	Horned	
pp sc/sc	Horned	Horned	

The horn buds development starts during the first two months of age in the calf. The corium which is the area of cells placed at the junction of the skin and horn is considered to be the section

where the horn is produced (Figure 2). The horn will continue growing if the horn, but not the corium, is removed. Until the two months of age, the horn bud is free-floating in the skin layer above the skull. The horn bud connects to the skull as the calf gets older, more clearly to the periosteum of the frontal bone overlying the frontal sinus.

Subsequently, a small horn starts to grow. When the horn bud is connected to the skull, the horn core becomes a bony part of the skull, and the hollow center of the horn core extends directly into the frontal sinuses of the skull at the age of 7-8 months. Anatomically, except for hornless breeds, bovine horns develop from the lateral angle of the frontal bone. The length, shape, and robustness of the horns differ between breeds and also demonstrate a high individual variability.

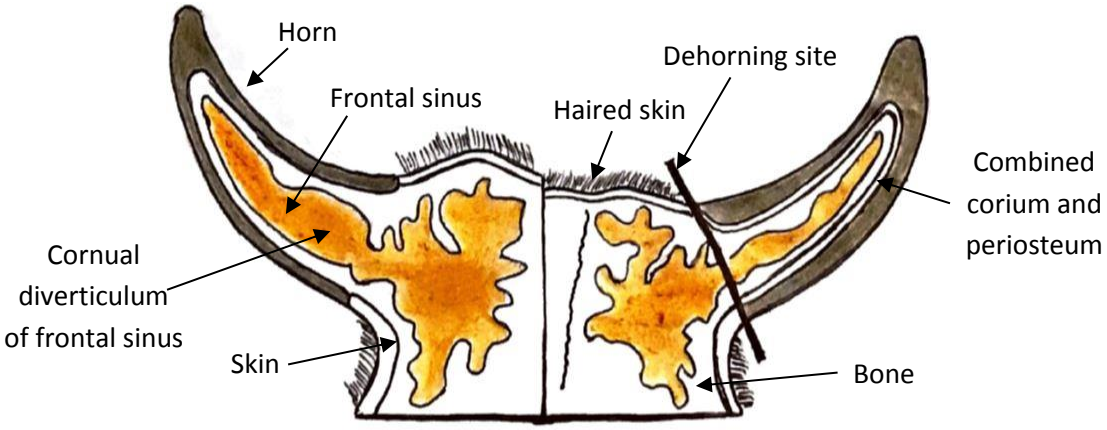

Figure 2. The anatomy of horn and its different components in cattle

Lacking horns in polled animals has made them valuable in the cattle industry because of its subsequent positive economic impacts (Capitan et al., 2009). Identified variants found through genetic analyses are normally associated with the polled trait in beef and also rarely in dairy cattle breeds (Carlson et al., 2016). However, it seems crucial to consider that the different criteria of horn including size, shape, and orientation could be influenced by many genes, each of them with minor effects similar to other quantitative traits (Legates and Warwick, 1979). Therefore, when considering polled calves production as the main breading goal, there will be important factors encompassing the proportion of cows to be bred with polled bulls, mating decisions, and the vital role of polled bulls in the total genetic merit of the population (Spurlock et al., 2014). In this case, problems will arise when exploiting conventional breeding methods for decreasing the frequency of horned allele that increases the rate of inbreeding and finally will result in slow genetic improvement (Mueller et al., 2019). Findings from simulation studies have highlighted the effectiveness of gene editing application both in diminishing the frequency of recessive alleles (e.g., horned) and subsequently reducing their negative impacts on inbreeding and genetic merit (Cole, 2017; Bastiaansen et al., 2018). Preventing and decreasing the risk of injuries, physiological

damages, carcass bruising, and possible infections are the positive outcomes of the introgression of polled condition into cattle breeds which could be performed through Marker-Assisted-Selection (MAS) following genetic markers closely linked to the polled gene (Harzilius et al., 1997). It was suggested through many studies that there are no significant phenotypic differences between polled and horned cattle for traits related to growth, carcass, and reproduction (Frisch et al., 1980; Lange et al., 1990), as Stookey and Goonewardene (1996) did not find any significant differences in average daily weight, weight per day of age and scrotal circumference traits between polled and horned bulls. The development of scurs occurs on the frontal bone similar to horns and because of that the expression of scurs phenotype is masked by the presence of horns (Prayaga et al., 2007). With the identification of scurs in descending order in flat, rounded, peaked, and extremely peaked polls, researchers concluded that the presence of scurs could not be independent of skull shape (Long and Gregory, 1978). The fact that animals that are classified as having scurs phenotype at weaning (6-9 months of age) may develop horns at a later stage in life, adds more complexity in determining accurate phenotype as Brennemen et al. (1996) revealed that through skull dissection observation. As stated, accurate identification of the heterozygous/homozygous phenotypes at polled, scurs, and African horn seems crucial because of their influential impact on reducing the proportion of horn alleles in the breeding population despite keeping a tab on the masked scurs phenotype (due to the lack of expression of the scurs gene in horned animals even if being in the dominant homozygous form). Distinguishing between heterozygous and homozygous polled bulls has made it more difficult to propagate the polled gene in purebred herds, but fortunately, the application of molecular genetics approaches will play a considerable role in repelling this problem (Prayaga et al., 2007).

Genetics of horn presence in cattle

Horn growth is considered as a genetically heritable autosomal recessive trait and polled cattle result from an autosomal dominant pattern of inheritance that has been identified to be based on allelic heterogeneity at polled locus (Medugorac et al., 2012). Due to the strong genetic background of the status of horns (presence or absence) in animals, mating programs could be led to the direction of enhancing the incidence of polled alleles. The horn inheritance pattern is determined by the main three loci affecting horn phenotype in cattle including the polled locus with *P* (polled) allele dominant to *p* (horned) allele, the scurs locus with *Sc* (development of scurs) and *sc* (absence of scurs) alleles and the African horn locus with *Ha* (horned) and *ha* (polled) alleles (Stafuzza et al., 2018). The African horn locus has probably the independent segregation and epistatic effect on the polled locus (Long and Gregory, 1978). This epistatic effect of African horn locus following the sex-influenced effect of the scurs locus may prevent the modification of horn shape in horned animals (Georges et al., 1993). There are three candidate polled mutations that have been detected in Bos taurus cattle on BTA1 (Medugorac et al., 2012, 2017) including an 80,128 bp duplication of Friesian origin in the region of *IFNGR2* locus on BTA1 (P_F) (Rothammer et al., 2014), an allele of Celtic origin (P_C) corresponding to duplication of 212 bp in place of a 10 bp deletion and a third allele, a complex 219 bp duplication-insertion (P219ID) following a 7 bp

deletion and 6 bp insertion (P1ID) (Medugorac et al., 2017). Moreover, the probable existence of additional variants was suggested by Chen et al. (2017) in Chinese cattle (P202ID mutation that was dominant) and by Stafuzza et al. (2018) and Utsunomiya et al. (2019) in Nellore cattle. The 110-kb duplication variant of the polled locus reported in Nellore cattle (Guarani polled allele; P_G) (Utsunomiya et al., 2019), has been shown not to occur in coding sequences, a splice site, or any known regulatory regions (Falomir-Lockhart et al., 2019). The 202 bp InDel (referred to as P202ID) located between *IFNAR2* and *OLIG1* genes was found through identifying polled locus in some British breeds including South Devon, Belgian Blue, and Shorthorn (Chamberlin, 2017).

High-density SNP genotyping confirmed the presence of two different polled associated haplotypes in Simmental and Holstein cattle co-localized on BTA 1, refined the critical region of the Simmental polled mutation to 212 kb, and identified an overlapping region of 932 kb containing the Holstein polled mutation (Wiedemar et al., 2014). Also, the 212 bp insertion-deletion variant (indel) was found to be associated with polledness in beef and dual-purpose cattle breeds and for the Holstein polled mutation (Medugorac et al., 2012; Allias-Bonnet et al., 2013; Wiedemar et al., 2014). Georges et al. (1993) demonstrated linkage with two microsatellite markers, GMPOLL1 and GMPOLL2, 13 cM from the polled locus. As these markers were too far away to be used in a MAS approach, it necessitated efforts to identify closer markers. The complete linkage was observed between each of the microsatellite markers including TGLA49 (DIS14), BM6438 (DIS24), and the polled gene in a Canadian population of the Charolais breed (Schmutz et al., 1995).In addition, Brenneman et al. (1996) localized the polled locus 4.9 cM from TGLA49 in mutual backcross and F2 families from an Angus×Brahman cross. Also, BM6438 and SOD1Micro markers showed complete linkage with polled locus in Simmental cattle (Brockmann et al., 2002). Similarly, TGLA49, SOD1MICRO2, BM6438 markers have been shown to have a tight linkage with the polled phenotype, and it was highly probable that the polled gene is located within the contig of 1 Mb region on bovine Chromosome 1q12 (Drogemuller et al., 2005). These markers have been mapped to the proximal part of BTA1 (Bishop et al., 1994). As the closest genes to this region, the bovine interferon receptor (*IFNAR*) gene was mapped 2 cM from the TGLA49 marker, while the KRTAP8 (KAP8) marker was mapped 5 cM from *IFNAR* (Harlizius et al., 1995; Barendse et al., 1997). It was revealed through the examination of the imputed haplotypes that a complete association exists between an allele at CSAFG29 microsatellite marker and the polled condition while this specific allele was identified to be absent in horned animals (Prayaga et al., 2004). Identification of 13 SNPs as concordant with the polled trait in Holstein cattle showed that only three of them located in a gene coding or regulatory (promoter or UTR) regions, entailing bSYNJ1_C3981T polymorphism located in the 3'UTR of the *SYNJ1* gene and bC2159_C-193T and bC2159_T372C located in the 5'UTR and coding region of the *C21orf59* gene, respectively. It was suggested to be advantageous to perform further investigations to determine if the *SYNJ1* 3'UTR SNP may have a functional effect on the polled trait in Holsteins (Cargill et al., 2008). However, the possibility of the functional role of the bSYNJ1_C3981T SNP for polledness was ruled out as the distribution of the genotypes resulted from this mutation could not confirm an association with the polled phenotype investigated in German Holstein, Limousin, Charolais, and

Pinzgauer cattle breeds (Wohlke et al., 2010). The *SOD1* gene and ALGA17 marker (as adjacent to functional genes) were also suggested to influence polled/horned conditions in the northern Eurasian cattle population (Li et al., 2010), following by the identification of a 303-bp allele of the new microsatellite, CSAFG29, which demonstrated a significant association with the polled allele in Brahman cattle (Mariasegaram et al., 2012). The involvement of *OLIG2*, *FOXL2*, and *RXFP2* genes in horn bud differentiation and development was confirmed in bovine, ovine and caprine polled loci (Allias-Bonnet et al., 2013; Wiedemar et al., 2014). In a study with 31 taurine cattle breeds, a 202-bp-indel located between *IFNAR2* and *OLIG1* genes and one haplotype including *IL10RB*, *IFNAR2*, *OLIG1*, *C1H21orf62*, and *GCFC1* were found and proposed as responsible for polledness in several taurine breeds (Medugorac et al., 2012). In addition to *OLIG1* and *C1H21orf62* genes, the *HIST1H4C* gene was also identified in a 381-kb interval on BTA1 that altogether showed association with polled phenotype in taurine cattle breeds (Seichter et al., 2012). The identified SNP (AC000158: g.1390292G>A) within intron 3 of the *IFNGR2* gene showed a perfect co-segregation with the polled trait in Holstein cattle which could eventually guarantee reliable genotyping of horned, heterozygous, and homozygous polled Holsteins (Glatzer et al., 2013). However, results from another Holstein population rejected this association, stating that the 80-kb duplication as the only remaining variant within the shortened Friesian haplotype is the most likely causal mutation for the polled phenotype of Friesian origin (Rothammer et al., 2014). There was also evidence of the detection of several genes harboring a 3.11 Mb region in BTA1 encompassing *IFNAR1*, *IFNAR2*, *IFNGR2*, *KRTAP11-1*, *MIS18A*, *OLIG1*, *OLIG2*, and *SOD1* that seemed to act together in determining the polled/horned phenotype in Nelore cattle, suggesting the association of this region with polledness trait in this population (Stafuzza et al., 2018). Lately, through a PCR-based screening of the Celtic mutation carried out by Grobler et al. (2018), it was stated that the polled phenotype observed in the South African Bonsmara, Drakensberger, and Hereford is genetically affected by the Celtic allele (P_C). Therefore, the polled Celtic variant was validated as the causative mutation of polledness in three South African beef cattle breeds, suggesting to be the efficient diagnostic tool for polledness. Additionally, a group of researchers could successfully edit the genome of dairy cattle in order to make them hornless using the transcription activator-like effector nucleases DNA editing technique, introducing a natural allele linked to hornlessness into dairy cow embryos with which five healthy calves were born, all without horns (Carlson et al., 2016). The identified collection of five SNPs for accurate prediction of the main P_C (rs383143898 (P202ID)) and P_F (rs801127025, rs799403053, rs210350155, and rs797088784) alleles was referred to as the Optimized Poll Test (OPT). This test could determine 6 potential genotypes considering phenotype-genotype concordance including HH (100% horned), P_CP_C, P_CP_F or P_FP_F (100% polled), HP_F (100% polled), and HP_C (59.9% scurred and 40.1% polled) (Randhawa et al., 2020).

Scurs are small bony growths on the head of cattle that develop in the same area as horns but are not firmly attached to the skull, whereas the bony core of the horn is continuous with the frontal bone of the skull. The two main shapes of scurs phenotype include small and scab-like and large and horn-like, although scurs generally do not develop as large as horns. As scurs has now been

mapped to bovine chromosome 19 which is different from the location of the polled locus (centromeric end of BTA1), therefore these two morphological traits are not linked in Bos taurus (Asai et al., 2004). In the French Charolais breed, it was shown that the inheritance pattern of the scurs phenotype is autosomal recessive with complete penetrance in both sexes that was different from already reported results in other breeds. This could mention to the modified expression level of scurs locus in double heterozygous Hereford and Angus males based on the interference of other unidentified genetic factors and referring to the importance of studying this locus in order to accurately map this gene and uncover the molecular architecture responsible for the development of horns in cattle (Capitan et al., 2009). The results from a study supported the existence of a scurs locus in polled Simmental cattle locating on BTA19 as proposed earlier but clearly pointed to the genetic heterogeneity in this trait as previously reported also in the Charolais breed (Tetens et al., 2015). Mariasegaram et al. (2010) also described for the first time the network of genes involved in horn and scurs development through transcription profiling especially identification of different expression levels of *DHRS7C* gene in horned, scurs, and polled phenotypes in cattle. Recently, the position of scurs locus has been shown to be localized between the gene *DHRS7C* (g.29594018G>C) and microsatellite BP20 through multipoint linkage analysis in Canadian scurred cattle which further supported mapping on BTA19 between BMS2142 and IDVGA46 markers. This study showed epistatic interaction between scurred and polled loci and the need for a scurred DNA test to assist producers of purebred beef in the eradication of the scurs trait (Ketel and Asai-Coakwell, 2020). However, further studies seem necessary to prove the identified loci and discovering genetic loci and causative mutation underlying the scurs phenotype.

Final points

The development of DNA tests will allow homozygous polled animals to be identified for future breeding programs and therefore successful introgression of the polled gene into the population. It is also necessary to undertake simultaneous research and strategies in order to achieve significant progress in replacing the practice of dehorning by breeding polled animals in different breeds. Distinguishing the scurs and horned phenotype could be confusing which is a major problem. Obviously, if the inheritance mode and genetic location of the scurs locus were better characterized, it would be easier to eliminate the scurs trait and map the polled locus, particularly in populations with animals having scurs phenotype with high frequencies.

Due to the lack of a comparable model, it is difficult to identify the exact functional candidate genes responsible for the presence of polled and scurs phenotypes that suggests the exploitation of the application of high-throughput sequencing of the entire candidate region for the final identification of the causal mutations. Future studies into the molecular mechanisms underlying the inhibition of horn development in cattle will therefore expand our knowledge not only of the mechanisms responsible for the differentiation of bovine horn buds but also of ectopic expression in mammals in particular. Despite the lower breeding values of polled bulls, the difference between polled and horned bulls is decreasing, and thus more homozygous polled bulls with high genetic merit are becoming available. Nevertheless, it should be crucially considered to prevent the

probable unnecessary increase in the rate of inbreeding. The parallel increase in *P* allele frequency and polled animal breeding values through genomic selection will result in lower inbreeding levels in comparison with conventional EBV breeding schemes.

While conventional breeding results in long-term genetic progress, if gene editing approach could be utilized for the introgression of the P_C allele into elite dairy genetics, the expected negative impacts on inbreeding and genetic merit would be significantly reduced. Gene editing technology which incorporates the polled genetic variation into dairy cattle genome has been stated by researchers to boost animal welfare through eliminating different methods of physical dehorning and bringing the most beneficial mutations together within a single generation. Moreover, the technology itself did not modify the genome but a limited and specific target region where there has already been a natural genetic variation in cattle populations. Despite the fact that these cattle's regulatory status is undoubtedly unclear, the future advantageous outcomes of these methods appear to be obvious in the dairy cattle production industry.

Considering the polled cattle as an example, the outcome of multi-generational breeding vs. genome editing would be basically the same. Depending on the technologies used to produce them, it would not be feasible to distinguish the two polled dairy animals, emphasizing the fundamental role of genome editing as the contemporary extension of current efforts targeting the genetic development of livestock. It was also widely argued by scientists that livestock farming will benefit potentially from the application of gene editing technology which leads to having healthier and more profitable farm animals. Although it is still early to say, these genome-edited animals might be needed in order to overcome the main future challenges that the livestock production system is facing with.

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Aims

The aim of this thesis was to describe specifically the important morphological traits in the Reggiana dairy cattle and Italian Large White pig at the genome level and to provide new insights into their genetic architecture and relevant mutations. In fact, the importance of evaluating these morphological traits could be linked firstly to Parmigiano Reggiana cheese production of Reggiana dairy cattle as a result of the pigmentation patterns variation and its productive performance, and secondly to the improvement in piglet survival and production through the increase in the teat number in Italian Large White pig.

In the next two chapters of this thesis, we tried to mine the cattle and pig genomes in order to detect gene markers associated with morphological traits including muzzle colour, stature, horn shape and the presence of supernumerary nipples in Reggiana dairy cattle breed, and different teat-related parameters encompassing the number of teats (based on the left and right sides and anterior and posterior parts of the body) and the asymmetry patterns of the teats (based on the presence of the extra teats between teat lines) in Italian Large White pig population.

Research activities took advantage of the availability of the latest version of the *Bost taurus* and *Sus scrofa* genomes and applied different genomic approaches combined with specifically designed experimental works, including next generation sequencing, bioinformatic analyses, targeted SNP genotyping, association analyses, coupled with traditional and novel phenotyping strategies. The obtained results opened new opportunities to design breeding plans in local populations and define new management strategies for autochthonous cattle and pig breeds.

4.1 Association between important morphological traits and genetic markers in cattle

The identification of causal genes and exploiting the beneficial mutations underlying the important morphological, production, and reproduction traits have been the main objectives of livestock genomics in order to improve the livestock breeding industry (Cheng et al., 2019). The great potential of GWASs and whole-genome sequencing technologies in the discovery of numerous candidate genes in livestock breeding has perfectly achieved these goals in recent years (Li et al., 2019).

The classification of breeds based on the phenotype particularly the pattern of coat colour and its variation is one of the most important factors for breed determination in livestock and securing genetic resources worldwide (Jung et al., 2020). The coat colour in cattle includes a wide range of colours also with different combinations which is specific to that breed (Girardot et al., 2006). The investigations for discovering the main molecular mechanisms, biological pathways and causative genes responsible for making different colors in coat, skin, and eye in vertebrates have significantly increased during recent years (Charon and Lipka, 2015). Considering all coloration types, variations in coat colour genes (especially melanocortin 1 receptor (*MC1R*)) have made it possible to study adaptive selection which has affected the genetic variability of coat colour in livestock and other mammalian genomes (Goud et al., 2020). In Reggiana cattle, investigations for coat colour gene polymorphisms have been done in order to find useful markers for the authentication of Reggiana branded Parmigiano-Reggiano cheese (Russo et al., 2007), and to study the relevant genetic mechanisms that differentiate the solid-colored phenotype (Reggiana) from other different coloration patterns, for example, spotted patterns in cattle breeds (Fontanesi et al., 2010, 2012). It has already been revealed that the selective sweeps located on BTA18 (*MC1R* gene) affect coat colour in Reggiana cattle (Bertolini et al., 2020).

As an important body-size related criteria, the stature or height of cattle is another key trait that seems to positively influence meat yield and beef production in the cattle breeding industry (Karim et al., 2011). The stature and live weight are two classical quantitative genetic traits that demonstrate relatively high heritabilities (0.60-0.65) and are widely recorded in cattle (Banos and Coffey, 2012). The high genetic correlation between these two measures suggests that they might be regulated by the same genetic signals (Lopdell and Littlejohn, 2018). To date, BTA14 (with PLAG1 zinc finger (*PLAG1*) and coiled-coil-helix-coiled-coil-helix domain containing 7 (*CHCHD7*) candidate genes) and BTA6 (with ligand dependent nuclear receptor corepressor like (*LCORL*) and non-SMC condensin I complex subunit G (*NCAPG*) candidate genes) were found to be the fundamental chromosomal regions affecting stature and body length in different cattle breeds around the world (Randhawa et al., 2015; Nishimura et al., 2012). All together with these evidence, it is worth to study deeply the genetic basis of stature and consider this trait in the breeding programs for genetic improvement in cattle due to its possible relationship with several production or reproduction traits.

The domestication of cattle (Bos taurus and Bos indicus) highlighted the presence of horn (shape and size) as the prominent morphological feature and another source of phenotypic diversity between cattle breeds (Ajmone-Marsan et al., 2010). Horn pheontype which is considered as a quantitative trait has been mapped to BTA1 (Georges et al., 1993), however, the exact responsible genes and mutations affecting different horn phenotypes including horned, scurs (very small and loosely attached horns), and polled (absence of horns) cattle have not been completely identified yet (Randhawa et al., 2020). The horn growth is generally determined during the prenatal development at embryogenesis through the interaction between tissues originating from the ectoderm and mesoderm (Allias-Bonnet et al., 2013), but it is often difficult to distinguish precisely the scurs phenotype (with sex-influenced effect) from polled cattle in the postnatal development period as they are not easily detectable until one year old or sometimes even older (Wiener et al., 2015). Based on the current tendency of the modern cattle breeding industry towards breeding polled animals, therefore, it seems essential to elucidate the main molecular mechanisms regulating horn growth and to characterize the genetic architecture underlying scurs and polled phenotypes which altogether lead to the better detection of these different phenotypes (Ketel and Asai-Coakwell, 2020).

In livestock, the optimum offspring growth rate and female production and reproduction efficiency underly having a suitable number of well-developed functional teats (or nipples) which is varied among different species. In addition to the main four teats, cows can be born with other extra teats called supernumerary nipples with variation in their size, position, and functionality (Brka et al., 2002). As mentioned in previous chapters, these teats are not desirable in cattle breeding because of their negative impacts on machine milking and susceptibility to mastitis and other udder infections (Pausch et al. 2012). The inheritance pattern of the supernumerary nipple is currently considered as polygenic or oligogenic in cattle (Wiener, 1961; Joerg et al., 2014). Although some genomic regions on cattle chromosomes including BTA5, BTA11, BTA14, BTA17, BTA20, and BTA27 have been proposed to affect the presence of different types of supernumerary nipples (blind and live) (Butty et al., 2017), the main chromosomal region, causative genes and mutations still need to be identified. To solve the issues related to these teats, the practice of supernumerary nipple removal (surgical elimination) is now being implemented in most of the dairy farms around the world but it has been proved as a major risk factor for calf welfare (Vasseur et al., 2010). Therefore, breeding animals without supernumerary nipples through the application of current genomic technologies to elucidate the most probable responsible genomic regions could be the best alternative solution instead of teat removal in order to minimize its prevalence as much as possible in the dairy cattle industry.

4.2 Genome-wide association studies for several morphological traits in Reggiana cattle

Introduction

Reggiana is considered as one of the most important indigenous cattle breeds of northern Italy and is characterised by a red coat colour (almost fixed for e allele of *MC1R* gene) (Russo et

al., 2007). Despite considered as a dual-purpose breed, Reggiana is mainly utilized to produce a mono-breed branded Parmigiano-Reggiano cheese, a Protected Designation of Origin (PDO) dairy product that is famous and known worldwide. Given the economic value of this dairy product, a selection programme in Reggiana started in 1956 with the constitution of the National Association of Reggiana Cattle Breeders (ANABORARE), which officially could be considered for the recognition of the Reggiana breed (ANABORARE, 2019). A few studies investigated polymorphisms in candidate genes to identify genetic markers associated with milk production traits or that could be useful for authentication of Parmigiano-Reggiano cheese obtained from the milk of this breed (Fontanesi et al., 2010, 2015). Selection signatures in the Reggiana genome were recently reported by Bertolini et al. (2020). Understanding the main mechanisms that control variation in behavioural and morphological traits opens new insights into speciation and population divergence procedures (Theron et al., 2001). Genetic dissection of phenotypic traits of this breed is essential to better characterise and preserve this local resource.

Stature (defined as the height from the hips) is an important quantitative trait for cattle performance, which influences the productivity of the herd and has mutual relationships with other physiological features (Hou et al., 2020). As a complex trait with a known history of selective pressure (both negative and positive) over time, stature is robustly affected by the domestication process and under polygenic control with a high degree of heritability in most of the mammalian species encompassing cattle (Karim et al., 2011; Kemper and Goddard, 2012). Hence, in cattle breeds, the identification of multiple signatures of selection and their related candidate genes will unmask the polygenic nature of stature which seems an important trait as selection for this trait may affect other economic traits (Randhawa et al., 2015). Studies in cattle have reported only a few major genes such as *LCORL* and *PLAG1* significantly associated with body size measurements, growth traits, and stature in cattle (Bouwman et al., 2018). A study on Holstein×Jersey line cross proposed that the probable mutation causing variation in height could be one SNP on BTA14 located in the 3′-UTR region of the *PLAG1* gene identified as rs109231213 (Karim et al., 2011). Recently in Reggiana cattle, the region on BTA14 including the *PLAG1* gene which is associated with conformation traits, showed a selection signature for stature trait as well as *NCAPG* and *LCORL* genes on BTA6 (Bertolini et al., 2020). The targeted resequencing followed by association analysis highlighted the quantitative trait nucleotides (QTNs) for bovine stature in the *PLAG1-CHCHD7* intergenic region in Japanese black cattle (Nishimura et al., 2012). Also, a GWAS study on Brazilian Nellore cattle (Bos primigenius indicus) highlighted the surrounding 1 Mb region of BTA14 showing high identity with human, pig, and mouse autosomes 8, 4, and 4, respectively, and contains the orthologous height-related genes such as *PLAG1* and *CHCHD7* associated with mature body height and stature (Utsunomiya et al., 2013). Both *LCORL* and *PLAG1* genes as the recognized adult human height locus, have also been highlighted for their strong associations with body height in cattle and horses, and their selective sweeps in pigs and dogs (Takasuga, 2016). In cattle, *PLAG1* and *LCORL* as the major loci were clarified to contribute to more than a third of the genetic variation in a breed (Saatchi et al., 2014).

Coat colour (hair and skin) is another important exterior trait that characterises many cattle breeds. There are some important economic reasons for the importance of cattle pigmentation genetics such as adaptability to different environments, traceability, and identification of breeds. Therefore, a deep understanding of the major genes and related molecular mechanisms that contribute to the regulation and controlling pigmentation in cattle breeds is favourable for researchers (Hanna et al., 2014). Due to the fact that the muzzle colour shows variation among cows with the same coat pigmentation, this could probably involve different pathways than those affecting hair colour. Two main categories of muzzle pigmentation include flesh-colored muzzle (pinkish-white) which has a similar colour as the rest of the skin and slate or black muzzle because of almost blackish-blue colour. In addition, there is another phenotype that dots of both colours create the muzzle color (pink with black dots) which sometimes is difficult to distinguish. As the underlying skin colour varies from muzzle colour in red cattle breeds, they demonstrate segregation between slate-colored and flesh-colored muzzle (Bekge, 1961). Compared to skin colour, the nature of pigment deposition in the muzzle and associated genes has not been thoroughly investigated and information is limited, although this could also be a marker reflecting genomic diversity (Kim et al., 2014). It seems that the pigmentation patterns of cattle muzzle colour could be affected by some of the genes responsible for determining the coat colour. The development of black spots in the muzzle has been shown to be associated with the E^{+} allele of the *MC1R* gene in cattle (Lee et al., 2002; Park et al., 2012).

Considering the mammary conformation in cattle, supernumerary nipples or teats (hyperthelia, SNT) are viewed as extra to the four main functional nipples, and may develop at any location along the milk line (Hardwick et al., 2020). In cattle, there are three types of SNTs based on their position including caudal (at the rear udder) as the most frequent type, intercalary (between the normal rear and front nipples), and ramal (as the appendix of normal nipples) (Wiener, 1962). The SNTs are mostly blind, don't have a streak canal or attachment to mammary tissue, and normally are not capable of producing milk (Joerg et al., 2014). They are derived from extramammary buds and are present at birth in almost 40-50% of females (Rowson et al., 2012). The value of investigating this trait could also be related to other affecting factors including the difference in the occurrence of SNTs among different cattle breeds, position, size, and their possible connectivity to the mammary gland and normal nipples (Brka et al., 2002). The frequency of SNTs presence is different in cattle breed populations ranging from 15% in Holstein, 31% in Brown Swiss, 40-44% in German Flekvieh, and 69% in German Simmental (Joerg et al., 2014). The increase in the number of parity of dams might result in having cows with a higher incidence of SNTs than the cows born in first parity (Brka et al., 2002), although it has not been reported any significant associations between the number of SNT and milk production traits in cattle. Considering genetics of SNTs, the genes leucine rich repeat containing G protein-coupled receptor 5 (*LGR5*) on BTA5 and T-box transcription factor 5 (*TBX5*) on BTA17 were suggested as the possible candidate genes for the udder clearance and the presence of supernumerary mammary gland tissue with a SNT, respectively, in Brown Swiss cattle (Butty et al., 2017). Findings also revealed that a quantitative trait loci (QTL) on BTA20 (*C6*, *PLCXD3*, *RXFP3*, *ADAMTS12*,

SLC45A2, *DROSHA*, *CDH6*, and *AMACR*) could affect the occurrence of caudal SNTs without mammary gland in Holstein cattle (Joerg et al., 2014). The SNPs from candidate genes identified in the QTL region on BTA17 including T-box transcription factor 3 (*TBX3*), *TBX5*, and RNA binding motif protein 19 (*RBM19*) showed association with udder clearance and mammary gland morphology in dual-purpose Flekvieh and Brown Swiss cattle (Pausch et al., 2012, 2016; Fang et al., 2019). As *TBX3* and *TBX5* are regulated in the highly conserved Wnt/ß-catenin signalling pathway, this pathway has been suggested as the major determinant for the development of SNTs in cattle (Pausch et al., 2012). It was also reported that the genomic region on BTA17 encompassing *TBX3* showed association with the absent nipples in Japanese black cattle (Ihara et al., 2007).

Horn shape could describe another breed-characteristic trait which could, in turn, be important for the management of the animals. Variability in the horn shape is usually present in different cattle breeds (Allais-Bonnet et al., 2013), and horn direction should also be considered in breeding strategies that would minimize dehorning practice for welfare reasons in breeds in which polled genes are not present. Normally fixed horns attached to the skull and another variation which is regarded as small loosely attached horns called Scurs are two types of horn shapes observed in cattle and the cattle that naturally lacking horns show Polled phenotype (Mariasegaram et al., 2010). It was suggested through many studies that there are no significant phenotypic differences between polled and horned cattle for traits related to growth, carcass, and reproduction (Stookey and Goonewardene, 1996). Three most commonly accepted loci for horn inheritance include the Polled locus with P (polled) allele dominant to p (horned) allele, the Scurs locus with Sc (development of scurs) and sc (absence of scurs) alleles, and the African horn locus with Ha (horned) and ha (polled) alleles (Stafuzza et al., 2018). In the concept of horn bud differentiation in cattle, evidence found for the involvement of three genes including *OLIG2*, *FOXL2*, and *RXFP2*, representing the first link between bovine, ovine, and caprine (Wiedemar et al., 2014). Because scurs has now been mapped to bovine chromosome 19 which is different from the location of the polled locus (centromeric end of BTA1), therefore these two morphological traits are not linked in Bos taurus (Asai et al., 2004). Hence, these findings add to the importance of studying horn genetics in order to accurately map the causative genes and uncover the molecular architecture responsible for the shape and development of horns in cattle. In this study, we have conducted several genome-wide association studies for different morphological traits in Reggiana cattle breed including stature, muzzle colour (pink, grey, and black), supernumerary nipples (presence or absence) and horn shape (upward, forward, and downward).

Materials and methods

Animals, phenotypes and genotype data

All animals used in this study were not raised or treated in any way for the aim of this study. A total of 1776 Reggiana cattle, born during the years 2002-2018 were included in this study.

Morphological traits were recorded by the visual inspection of trained personnel who recorded the following traits: i) stature from the hips (Figure 3), ii) muzzle colour (pink, grey and black) (Figure 4), iii) presence (1, 2, 3) or absence (0) of supernumerary nipples (Figure 5) and iv) horn shape (upward, forward and downward) (Figure 6). Blood samples were collected and DNA was extracted using standard protocols. All animals were genotyped with the GeneSeek GGP Bovine 150K following the manufacturer's protocol. All the SNPs were interrogated and further mapped over the latest assembly of the bovine genome (ARS-UCD1.2; GCA_002263795.2). PLINK 1.9 software was used for quality check (Chang et al., 2015). Samples with a genotype missing rate > 0.9 were discarded whereas SNPs were discarded if they presented a call rate < 0.9, a Hardy-Weinberg equilibrium (HWE) *P*-value < 0.001, and a minor allele frequency (MAF) < 0.01. Only SNPs located on autosomal chromosomes were retained. After filtering, the dataset consisted of a total of 1776 animals and 130,365 SNPs. The number of analyzed animals and the total number of analyzed SNPs for each trait is shown in Table 1.

Genome-wide association analyses

To dissect the potential different genetic mechanisms affecting the recorded traits, genome-wide association studies (GWAS) were carried out including distinct groups of cattle and considering different traits as detailed below: i) stature from the hips, ii) muzzle colour [pink vs. grey vs. black; pink vs. grey and black; pink and grey vs. black; pink vs. grey; pink vs. black; grey vs. black], iii) presence or absence of supernumerary nipples and iv) horn shape (upward, forward and downward). Table 1 shows the different classes of animals involved in the studied traits. Genome-wide association analyses were carried out following a linear mixed effect model:

$$\boldsymbol{y} = \boldsymbol{W\alpha} + \boldsymbol{x\beta} + \boldsymbol{u} + \boldsymbol{e}$$

where $\boldsymbol{y}$ ($n \times 1$) is a vector containing parameter for n^{th} animal, $\boldsymbol{W}$ ($n \times k$) is a matrix of covariates with $k = 5$ (a column of 1s, three columns coding the four different operators involved in the animal phenotyping and a column with the age of the animals quantified in days between the birthday and the day of phenotype recording), $\boldsymbol{\alpha}$ is the k-dimensional vector of covariates effects, $\boldsymbol{x}$ ($n \times 1$) is the vector containing genotypes for the i^{th} SNP (coded as 0, 1, 2, according to the number of copies of the minor allele), $\boldsymbol{\beta}$ is the additive fixed effect of the i^{th} SNP on the trait, $\mathbf{u} \sim N(\mathbf{0}, \sigma^2_u \mathbf{K})$ is a multivariate Gaussian polygenic effect, with covariance matrix proportional to the relatedness matrix $\mathbf{K}$ ($n \times n$) and $\mathbf{e} \sim N(\mathbf{0}, \sigma^2_e \mathbf{I})$ is a multivariate Gaussian vector of uncorrelated residuals.

The assessment of the association between each SNP and trait was obtained by testing the null hypothesis H_0: $\beta = 0$. Significance was tested using the Wald test. All analyses were performed using GEMMA v. 0.96 (Zhou and Stephens, 2012) after computing the relatedness matrix **G** as a centred genomic matrix controlling the population structure. A Bonferroni's corrected threshold equal to a nominal value of 0.05 was used to define significant markers (*P*-value = 3.92×10-07). Markers presenting a p-value < 5.00×10^{-05} were considered suggestively associated.

Q-Q plots and Manhattan plots were generated in R v. 3.5.1 by using the "qqman" package while the genomic inflation factors (λ) were computed with the function "estlambda" within the "GenABEL" package. Figures 1, 2, and 8 report the corresponding Manhattan plots and the corresponding QQplots, respectively. Genes annotated in the ARS-UCD1.2 genome version spanning a region of ± 500 kbp around all significant SNPs were retrieved using Ensembl Biomart tool (http://www.ensembl.org/biomart/martview/) and then considered relevant in affecting the associated phenotypes according to a detailed analysis of the literature. For running GWASs analyzes, some covariates were used including age of the animal and operator for stature phenotype and operator covariate for muzzle colour and supernumerary nipple phenotypes, plus the effect of *MC1R* (Figure S1) and *LCORL* genotype as another covariates for the related phenotypes.

Results and discussion

Several genome-wide association studies were performed to identify markers associated with the analysed traits in the Reggiana breed, as shown in Figure 1 and Table 2.

GWAS for stature from the hips identified significant peaks on BTA6, on a region harbouring the genes *LCORL-NCAPG* with the most significant marker as BovinHD0600010697 (6: 37211057) (Tables 2 and S1). Another suggestively significant SNP was found on BTA14 and a QTL region containing *PLAG1* gene (BovinHD1400007268; 14: 23379474) which is closely linked to *CHCHD7* gene. Polymorphisms in the *LCORL* gene are associated with measures of skeletal frame size and adult height. A genomic region on BTA6 containing *LCORL* and *NCAPG* gene has been reported to be associated with variation in body size, height, and growth-related traits in several species such as humans, mice, rats, and livestock including stature in cattle (Bouwman et al., 2018) and withers height in some horse breeds (Metzger et al., 2013). It has also been documented that the *LCORL* gene affects the human height and fetal growth (Weedon and Frayling, 2008), and its large effect on growth-related traits could support the hypothesis that the fetal growth and adult stature might be controlled by a conserved locus across species (Sahana et al., 2015). In addition to *PLAG1-CHCHD7*, there is evidence that *LCORL-NCAPG* on BTA6 has shown a strong signature of selection in multiple European cattle breeds and is significantly linked to bovine stature diversity (Randhawa et al., 2015). In the Nordic Red cattle, however, detected QTLs were found to have large effects on stature and calf size, representing strong evidence for the variants on BTA6 consisting of an intronic variant in the intron 1 of *LCORL* gene (g.37522580C>T) influencing mature stature and calf size at birth (Sahana et al., 2015). Moreover, selection for stature has been demonstrated in five Bos taurus cattle breeds for *LCORL* and in ten breeds for *PLAG1* (Bouwman et al., 2018). Considering the functional roles of the *LCORL* gene in body size and stature in several species, therefore, it could be concluded that the *LCORL* gene is one of the strong candidate genes affecting the body size-related trait especially stature in cattle (Chen et al., 2020). Based on our GWAS analysis, we also found the strong peaks on BTA6 including *LCORL-NCAPG* genes with the most significant SNPs affecting stature in Reggiana cattle population.

PLAG1-CHCHD7 in a close cluster plays fundamental roles in gland morphogenesis, multicellular organism growth, and body height. These genes are known to be linked to the growth traits such as body size, body weight, and stature in different livestock including cattle (Utsunomiya et al., 2013; Karim et al., 2011). Introgression of Bos taurus allele at the *PLAG1* gene on BTA14 into Brahman cattle breed resulted in increased stature (Koufariotis et al., 2018). Orthologous positions of genes associated with height in human populations located on the bovine genome (to test if these loci were controlling stature in cattle), revealed significant SNPs in a cluster on BTA14 including *PLAG1-CHCHD7* genes in Holstein and Brahman cattle breeds. The findings suggest that these genes could generally contribute to the stature of mammalian species (Pryce et al., 2011). A recently found downstream gene variant of *PLAG1* gene (14:g23327346G>A - rs210941459) in Chinese cattle associated with some growth traits particularly body height and hip height that could be as the possible candidate molecular marker in breeding programs aiming to increase genetic improvement in growth-related traits in cattle breeds (Zhong et al., 2019). The QTL region on BTA14 demonstrated high overlapping especially for *PLAG1-CHCHD7* with mature stature by linkage mapping studies in cattle. (Utsunomiya et al., 2013). Identification of a specific *PLAG1* mutation (rs109231213) through haplotype analysis which has major effects on body size and growth in cattle, adds to the importance of this gene as a strong candidate gene through affecting a highly conserved nucleotide at the 3′-UTR region of the *PLAG1* gene. Consequently, variation at the 3′-UTR of a transcript may affect its interaction with regulatory molecules including MicroRNAs (miRNA), which results in influencing the levels of translation (Utsunomiya et al., 2017). The probability that the body size in mammals is regulated by a set of common genes has been strengthened through a meta-analysis study for stature in 17 cattle populations that suggested stature is highly polygenic, has a relatively similar genetic architecture, and significant overlap in loci in cattle compared to the human. The tall cattle breeds such as Limousin, Charolais, and Holstein demonstrate almost fixed allele at *PLAG1-CHCHD7* while in breeds with shorter stature for example Jersey, Brown Swiss, Angus, Montbeliarde, and Fleckvieh the degree of fixation is varied (Bouwman et al., 2018). Investigating the evidence of selective sweeps for stature in 9 breeds of European Bos taurus has revealed a strong selective sweep that covers 1.0 Mb region at *PLAG1-CHCHD7* gene pairs on BTA14 in cattle genome (Randhawa et al., 2013). Also, the composite selection signals (CSS) method to investigate evidence of positive selection in stature of phenotypically diverse cattle including European and African Bos taurus breeds and comparative mapping information on human height, revealed one specific region contained *PLAG1* and *CHCHD7* genes particularly were related to bovine stature (Randhawa et al., 2015). As one of the most important SNPs for the *PLAG1* gene (rs109815800), it could affect bovine stature in Bos taurus cattle breeds (Bouwman et al., 2018) and Chinese cattle in which the Q allele significantly increases body height and cows with shorter height carries qq genotype (Hou et al., 2020). Considering all these previous finding in different cattle breeds, our GWAS result for stature in Reggiana cattle similarly pointed out to the significant effect of *PLAG1-CHCHD7* genes SNP on BTA14.

In GWAS for muzzle colour, we found significant SNPs on BTA18, in a genome region harbouring the *MC1R* gene (18: 14705686) (Tables 2 and S3). Considering different comparisons based on different muzzle colours, GWASs highlighted the peaks on BTA18 and the most significant SNPs of *MC1R* as the responsible gene affecting muzzle colour in Reggiana cattle (pink vs. grey vs. black, pink vs. grey and black, pink vs. black, pink and grey vs. black - MC1R373_3; grey vs. black - MC1R373_2). The only comparison in which no significant result of *MC1R* was found includes GWAS for pink vs. grey muzzle colours. Based on our result, the cows with dark muzzle carrying alleles for dark pigmentation will not be suitable individuals for Parmigiano Reggiano cheese production since only the cows with *ee* genotype (typical red coat colour and light muzzle) are preferred. In addition to *MC1R*, several other peaks were observed on other chromosomal regions through different GWASs for muzzle colour (different comparisons) including BTA2, BTA3, BTA5, BTA12, BTA20, BTA22, and BTA24 but except for BTA3 and BTA20, the identified genes in other chromosomal regions were not relatively relevant to coat and muzzle coloration or any other pigmentation-related mechanisms. A significant region was detected on BTA20 when comparing pink vs. black muzzle class (ARS-BFGL-NGS-93618; 20: 67654736) which contains the ADAM metallopeptidase with thrombospondin type 1 motif 16 (*ADAMTS16*) gene whose function has not been completely characterized yet. A few other genes of the ADAMTS family are however involved in pigmentation defects or in melanoblast survival (Rao et al., 2003; Silver et al., 2008). Another significant signal that was identified comparing the pink vs. gray muzzle class was observed on BTA3 (ARS-BFGL-NGS-38423; 3: 12671675). This SNP is an intron variant of the Fc receptor-like 3 (*FCRL3*) gene and marks a region that also includes two genes [(Fc receptor-like 1 (*FCRL1*) and CD5 molecule like (*CD5L*); positioned at nucleotides 12577541-12610184 and 12508921-12526879, respectively] that have been associated with iris heterochromicity in humans (Jonnalagadda et al., 2019).

The *MC1R* gene is well-known to be the fundamental part of the melanogenesis process and pigmentation of the skin, hair, and eyes in many species. The degree of darkness of the muzzle in cattle is specified by the number of black spots in the muzzle, based on the method developed by Lee et al. (2002). Variability at the *MC1R* gene has been previously associated with different muzzle colours (Lee et al., 2002). One of the main applications of this gene in livestock is related to breed traceability of the animal productions through its genetic variation between different breeds. Red coat in other local Italian cattle breeds such as Modicana and Sardo-Modicana has been shown to be genetically determined by E^{+} and E^{1} alleles instead of *e* allele which was almost fixed in Reggiana (Gaustella et al., 2011). Also, there is evidence that E^{D} and E^{+} alleles cause the black pigment synthesis for coat colour in cattle (Gan et al, 2007; Han et al., 2011; Niemi et al., 2016). For the *MC1R* gene which is located on BTA18, three main known alleles including E^{D}, E^{+}, and *e* cause black, red, or reddish-brown and red coat colours in cattle (Seo et al., 2007). In Korean Hanwoo cattle, there was an association between *e* allele at *MC1R* gene with the yellowish-red coat colour (Do et al., 2007) and also one *MC1R* synonymous variant (c.27G>C) was observed to be associated with the total amount of melanin and eumelanin (Mohanty et al., 2008). On BTA18, a signal of selection included the *MC1R* gene has been detected that causes the red coat

colour in Reggiana dairy cattle (Bertolini et al., 2020). In the dark and light muzzle tissues, it has been shown that *MITF* and *MC1R* are biologically linked and the activation of *MITF* downstream of *MC1R* through in mitogen-activated protein kinase (MAPK) signaling pathway appeared to lead the increased eumelanin synthesis in dark-muzzle cows. This process is done through the activation of cAMP response element-binding protein (CREB) and tyrosinase (TYR), while in the light muzzle cattle activation of Wnt signaling counteracts this process and increases the amount of pheomelanin (Kim et al., 2014). Muzzle phenotype patterns in Hanwoo cattle include black, yellowish-brown, and black-spotted brown. It was indicated that the amount of total melanin in melanocytes of the black muzzle and eumelanin production in brown muzzle cows are significantly increased by α-MSH and nitric oxide while L-cysteine decreases eumelanin production in black muzzle but increases in brown muzzle cows. These effects could be resulted from the simultaneous upregulation of TYR by nitric oxide and α-MSH and downregulation of *TYR, TYRP-2* and *MC1R* genes by L-cysteine observed in muzzle melanocyte (Amna et al., 2012). It highlights the roles of α-MSH and nitric oxide in hyper-pigmentation through enhancing eumelanogenesis and L-cysteine in pheomelanin production in muzzle melanocytes causing different muzzle colour phenotypes in cattle. Studies in yak suggest the probable association of several SNPs of a haplotype of *MC1R* gene including one synonymous (g.14705750C>T - c.375C>T), one missense (g.14706246G>A - c.871G>A, p.Ala291Thr - rs135181132) and one 3'UTR variant (g.14706711G>A - c.482A>G) with black muzzle colour. This *MC1R* haplotype has also demonstrated similarity to cattle haplotypes. These findings could strengthen the fact that the *MC1R* gene could be the dominant determinant of muzzle pigmentation in cattle (Petersen et al, 2019). The results obtained in our study in Reggiana cattle population directly indicate the significant effect of *MC1R* mutation causing different muzzle colour phenotypes that is fundamental (the same as coat colour) for the identification of animals in this breed considering the Parmiginao Reggiano cheese production.

Results of the GWAS for presence or absence of supernumerary nipples indicated suggestively significant markers on BTA 17 (BovinHD1700017901; 17: 60441944) including *TBX3* and *TBX5* genes (Tables 2 and S2). In addition, another significant marker was detected on BTA10 (BovinHD1000000240; 10: 894156) and the genes harbouring this region contained *MCC, DCP2, REEP5, APC*, and *SRP19*. From this chromosomal region, only MCC regulator of WNT signaling pathway (*MCC*) gene was found to probably have some relationships with this phenotype. This gene encodes a tumor suppressor factor which is thought to negatively regulate cell cycle progression. Regarding the supernumerary nipple position, we could only observe the caudal and intercalary supernumerary nipples in the Reggiana cattle population.

TBX3 gene in a close accompany with *TBX2* gene are frequent in various types of human cancers such as breast cancer and considered as the important developmental regulators affecting mammary tissue development. *TBX3* is expressed in the mesenchyme and then in epithelial tissue involving in mammary gland duct morphogenesis and mammary placode formation during the breast development of mice (Fischer and Pflugfelder, 2015). *TBX5* gene is closely linked to *TBX3*

and its encoded protein might contribute to heart development and specification of limb identity. The Holt-Oram syndrome that is known by the developmental disorder affecting the heart and upper limbs is associated with the mutations in the *TBX5* gene (Basson et al., 1999). Also, the ulnar-mammary syndrome (UMS) which is characterized by the severe hypoplasia of the breast (both supernumerary and aplastic nipples and mammary glands are characteristic) was found to be associated with a mutation in *TBX3* resulting in haploinsufficiency of the *TBX3* gene product in humans and mice. Actually, the disruption in the function (loss of function mutation) of *TBX3* and also *TBX5* which is in its close proximity induce the ulnar-mammary syndrome in humans (Borozdin et al., 2006). The homozygous mice for this mutation demonstrated the absence of mammary glands from the primary stages of embryogenesis but the decreased ductal branching was observed in heterozygous adults. These facts could strengthen the evidence of the key role of *TBX3* in the growth and development of mammary epithelial cells (Platonova et al., 2007). Also, the presence of a combined phenotype including Holt-Oram syndrome and supernumerary mammary glands was found to be the result of a heterozygous duplication encompassing the whole coding region of both *TBX5* and *TBX3* genes (Brugues, 2012). The regulation of *TBX3* is a downstream target of Wnt signalling (Eblaghie et al. 2004). The *MCC* candidate gene that we identified in the significant region detected in Reggiana on BTA10 is also involved in the non-canonical Wnt signaling pathway (Young et al., 2014; Renard et al., 2007). Having a fundamental role in initiating the development of the embryonic mammary gland (Turashvili et al., 2006), Wnt signaling also stimulates the formation of mammary placodes through the *TBX3* gene expression (Hens and Wysolmerski, 2005). Studies in cattle demonstrate the main QTL for the development of supernumerary nipples with their own supernumerary mammary gland tissue in Brown Swiss cattle was identified on BTA17 harbouring the *TBX5* and *TBX3* genes (genes lay within 100 kilobases of the SNP) that are located close to each other (Butty et al., 2017). The abnormal teat pattern phenotype (ATPP) which is classified by the absence of one or two teats has been reported in some cattle breeds. Implementing an allele-sharing non-parametric linkage strategy identified significant associations between the *TBX3* gene on BTA17 and severe ATPP in Japanese Black cattle (Ihara et al., 2007). Another evidence refers to the significant effects of *TBX3* and *TBX5* on BTA17 on the teat malformation and mammary gland morphology in dual-purpose Flekvieh cattle. These candidate genes identified in the mentioned QTL region demonstrate the highly conserved Wnt/ß-catenin signalling pathway that could be the main determining factor for the presence of the supernumerary nipple in cattle breeds (Pausch et al., 2012; Pausch et al., 2016).

All these findings support our obtained GWAS result for the presence of supernumerary nipple in Reggiana cattle on BTA17, despite that we could report the SNPs on *TBX3* and *TBX5* genes as suggestively significant affecting this phenotype. The most significant peak that was observed on BTA10 with *MCC* gene could be related (or having some physiological contribution) to this phenotype in cattle. Therefore, we consider BTA10 and BTA17 as the possible candidate regions for the supernumerary nipple presence in Reggiana dairy cattle.

Considering GWAS for horn shape, analyses demonstrated a suggestive marker on BTA19, located near the noggin (*NOG*) gene (BovinHD1900002056; 19: 7485353) (Table 2 and S4, Figure S2). The observed horn phenotypes in Reggiana cattle population as already explained includes the upward, forward, and downward. Since most of the farmers perfom the dehorning of the calves it was difficult to distinguish if the animal is really dehorned or showing the Scurs or Polled phenotypes. Therefore, we consider only the horn growth direction. Horn growth is considered as a genetically heritable autosomal recessive trait (Medugorac et al., 2012). In the concept of horn bud differentiation in cattle, evidence found for the involvement of three genes including *OLIG2*, *FOXL2*, and *RXFP2*, representing the first link between bovine, ovine, and caprine horned/polled loci. Also, horn bud agenesis is proposed to be due to the expression of a lincRNA in P_C/p horn buds (Allias-Bonnet et al., 2013). Studies confirmed the potential roles of *RXFP2* and *FOXL2* genes in ruminant horn development based on a quantitative RT-PCR of skin and horn bud biopsies from different fetal stages, which could highlight the importance of the ruminant specific transcript LOC100848215 for horn bud formation in cattle (Wiedemar et al., 2014). *SOD1* gene and *ALGA17* marker (as adjacent to functional genes) were also proposed to be linked with polled/horned conditions in the northern Eurasian cattle population (Li et al., 2010). No other studies in cattle breeds identified this region associated with horn growth direction, although studies in the human discovered a substantial role of *NOG* gene in the development of bones, representing that *NOG* mutations are linked to several skeletal diseases (Seeman et al., 2009) and bone growth abnormalities, for instance, Tarsal-carpal coalition (fusion of the bones in the wrist, ankles, fingers, and toes) (Dixon et al., 2001), and Brachydactyly type B (incomplete development or absence of the outermost bones of the fingers, toes, and nails) syndromes (Lehmann et al., 2007). *NOG* codes for a secreted polypeptide that binds to and inactivates members of the TGF-beta superfamily signaling proteins particularly bone morphogenetic proteins 2 and 4 (BMP2 and BMP4), regulates BMP signaling and disrupts their binding to the cognate receptors (Gong et al., 1999). It was suggested that the *NOG* gene is critical for normal bone and joint development, also having roles in muscle development, osteoblast differentiation, cartilage development, and chondrogenesis (Takano et al., 2016). Osteoblast cells (large cell responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodeling) also produce and secrete noggin that its expression is necessary for optimum skeletal development (Bassit et al., 2015). During the differentiation process from mesenchymal stem cells to osteoblasts, multiple BMPs and NOG regulate each other's expression. *NOG* demonstrates higher expression significantly during spinal fusion in mice, referring to its antagonist role in posterior spinal fusion development (Klineberg et al., 2014). *NOG* knockout mice showed excessive bone and cartilage formation with thickened long bones (excessive BMP activity), joint abnormalities, skeletal malformation, and osteopenia (Bassit et al., 2015). Moreover, the reduced subchondral bone remodeling is due to the intra-articular injection of Noggin protein in rats (Chien et al., 2020). In cattle, scurs phenotype of horn growth has also been mapped to BTA19 (the same as *NOG*)which is the small bony growth on the head that develops in the same area as horns but is not firmly attached to the skull whereas the bony core of the horn is continuous with the frontal bone of the skull. Various shapes of scurs

phenotype include small scab-like and large and horn-like despite that scurs generally do not develop as large as horns (Asai et al., 2004). The epistatic effect of African horn locus following the sex-influenced effect of scurs locus may prevent the modification of horn shape in horned animals (Georges et al., 1993). It was shown in cattle that the inheritance pattern of the scurs phenotype is autosomal recessive with complete penetrance in both sexes, being dominant in males and recessive in females (Capitan et al., 2009). Considering the different genetic expression, inheritance pattern, and genetic heterogeneity of scurs compared to polled (BTA1), polled animals could have the scur gene, supported by the existence of a scurs locus in polled Simmental (Tetens et al., 2015). As horned is recessive to polled, no horned cattle carry the polled allele, but they may also carry scurs (Allison, 1996). Further analyses are needed to explain better the role of the *NOG* gene in horn growth in Reggiana cattle.

Conclusion

In summary, in this study we dissected the morphological traits in cattle that might have several implications. The genome-wide association analyses highlighted genome regions harbouring genes related to the investigated phenotypes in Reggiana breed representing a potential novel tool for designing optimized breeding schemes in the Reggiana cattle breed. Overall, this study depicts a first step towards investigating this autochthonous breed and further studies are necessary to disentangle genome complexity of this breed and confirm these findings.

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Table 1. The number of animals, SNPs, covariates, pve, and se used for the GWASs for each phenotype

Phenotype	**Total number of animals**	**Number of analyzed animals**[6]	**Number of covariates**[7]	**Total number of SNP/var**	**Number of analyzed SNPs/var**[8]	**pve**[9] **estimate in the null model**	**se**[10] **(pve) in the null model**
Stature	1776	1249	3	130365	130307	0.330746	0.055773
Supernumerary nipple[1] (0 vs. 1 vs. 2 vs. 3)	1776	1112	1	130365	130272	0.292446	0.054947
Muzzle colour 0[2] vs. 1[3] vs. 2[4]	1776	1014	3	130365	130194	0.376111	0.062651
Muzzle colour 0 vs. 1 and 2	1776	1014	3	130365	130194	0.278545	0.0588672
Muzzle colour 0 and 1 vs. 2	1776	1014	3	130365	130194	0.458589	0.0698325
Muzzle colour 0 vs. 1	1776	951	3	130365	130033	0.199499	0.0597798
Muzzle colour 0 vs. 2	1776	848	3	130365	130205	0.522941	0.0740709
Muzzle colour 1 vs. 2	1776	229	3	130365	130160	0.604005	0.142566
Muzzle colour 0 and 1 vs. 2, plus MC1R[5]	1776	1014	5	130365	130190	0.16017	0.0744689
Muzzle colour 0 vs. 2, plus MC1R	1776	848	5	130365	130202	0.148847	0.0723665
Horn shape	804	788	1	134055	124553		

[1] The number of supernumerary nipple observed in different parts of the udder as absence (0) and presence (1, 2, 3)

[2] 0 refers to the pink muzzle phenotype

[3] 1 refers to the grey muzzle phenotype

[4] 2 refers to the black muzzle phenotype

[5] MC1R refers to the MC1R genotype as covariate

[6] Number of animals with phenotype

[7] Number of covariates used for each phenotype

[8] Number of used SNPs for the analysis

[9] Genomic heritability

[10] Standard error of genomic heritability

Table 2. List of significant or suggestive single nucleotide polymorphisms (SNPs) identified in the genome-wide association studies

Phenotype	**BTA**[1]	**Marker (SNP**[2]**)**	**Position**[3]	**p-value**	**lambda**[4]	**Candidate genes**[5]
Stature	6	BovinHD0600010697	37211057	3.82E-14	0.99	*LCORL, NCAPG*
	6	BovinHD0600010698	37214068	3.82E-14		
	6	BovinHD0600010701	37221776	3.82E-14		
	6	BovinHD0600010704	37232142	1.88E-12		
	6	6_38668893	37234954	1.88E-12		
	14	BovinHD1400007268	23379474	4.50E-06	1.00	*PLAG1*, *CHCHD7*
Supernumerary nipple	10	BovinHD1000000240	894156	1.21E-07	1.00	*MCC*
	17	BovinHD1700017901	60441944	8.20E-07	0.99	*TBX3, TBX5*
Muzzle colour (pink vs. grey vs. black)	18	MC1R373_3	14705686	1.41E-29	1.00	*MC1R*
Muzzle colour (pink vs. grey and black)	18	MC1R373_3	14705686	5.29E-12	1.01	
Muzzle colour (pink vs. black)	18	MC1R373_3	14705686	3.18E-51	1.02	
Muzzle colour (pink and grey vs. black)	18	MC1R373_3	14705686	2.03E-53	1.02	
Muzzle colour (grey vs. black)	18	MC1R373_2	14705686	1.15E-21	1.02	
Muzzle colour (pink vs. black)	20	ARS-BFGL-NGS-93618	67654736	9.60E-08	1.02	*ADAMTS16*
Muzzle colour (pink vs. grey)	3	ARS-BFGL-NGS-38423	12671675	3.97E-07	1.01	*FCLR3*, *FCLR1*, *CD5L*
Horn shape	19	BovinHD1900002056	7485353	3.40E-05	1.01	*NOG*

[1] Bovine chromosome (*Bos taurus*)

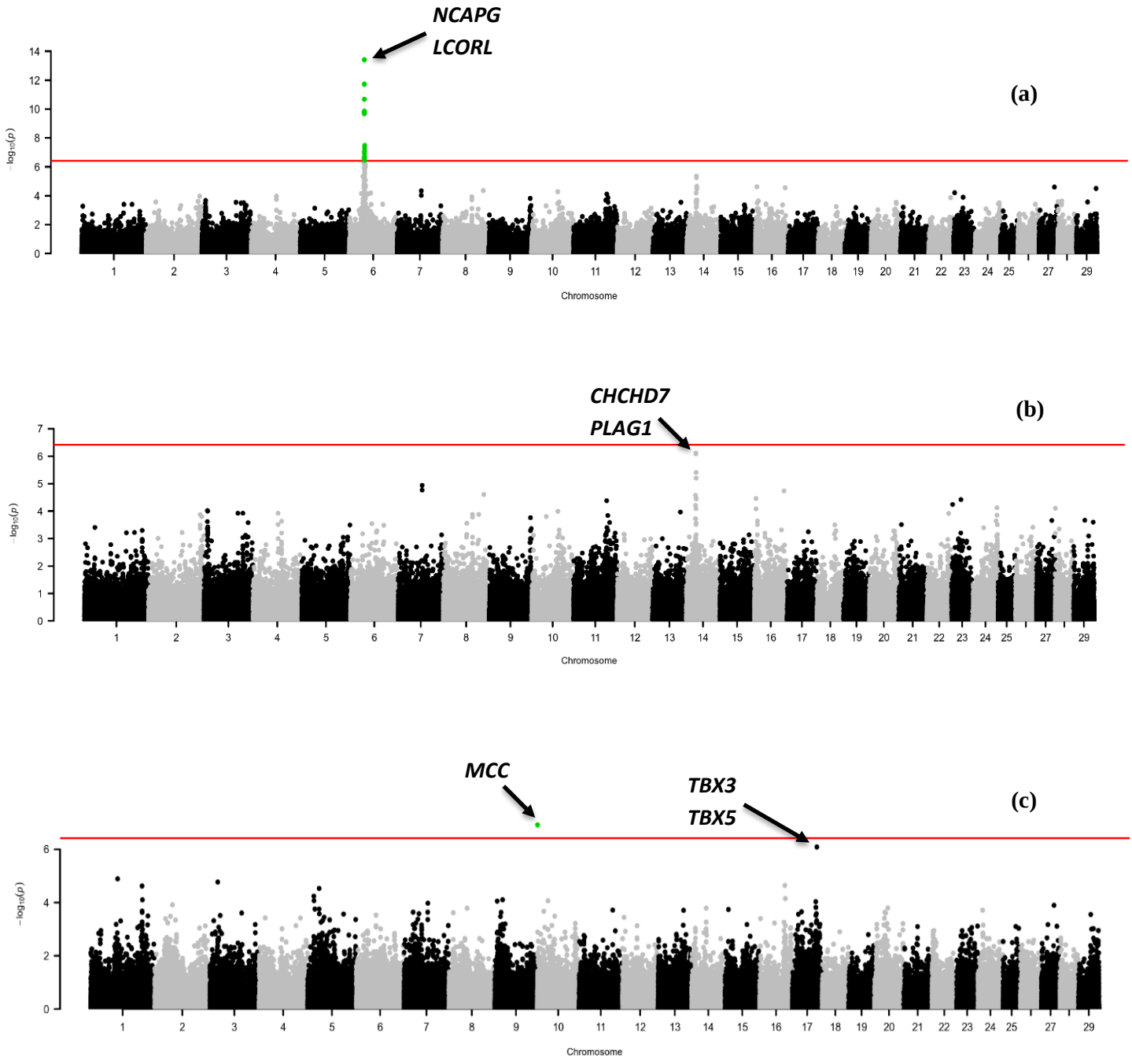
[2] Single nucleotide polymorphism

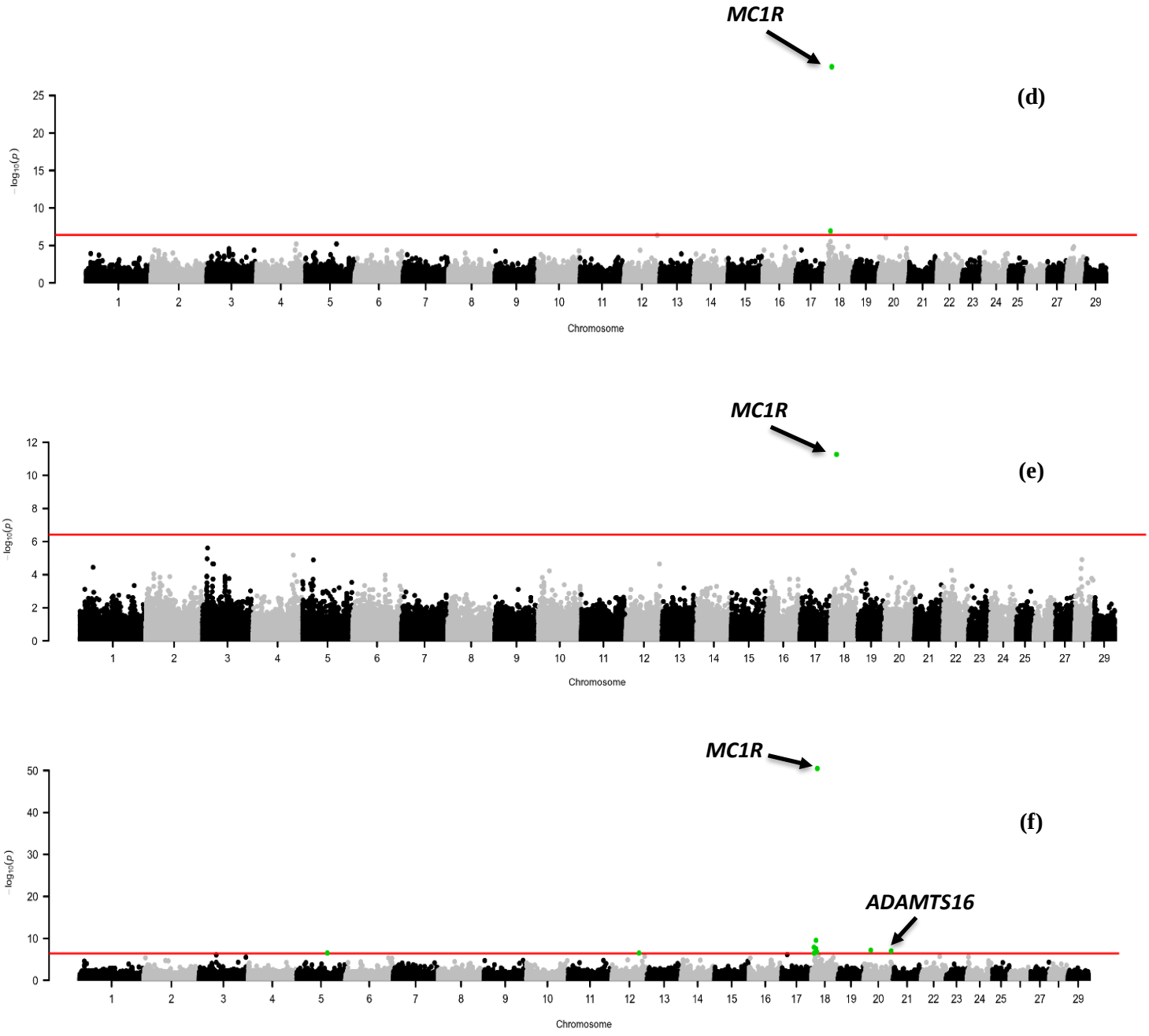
[3] The position of the marker on the corresponding chromosome in the ARS-UCD1.2 genome version

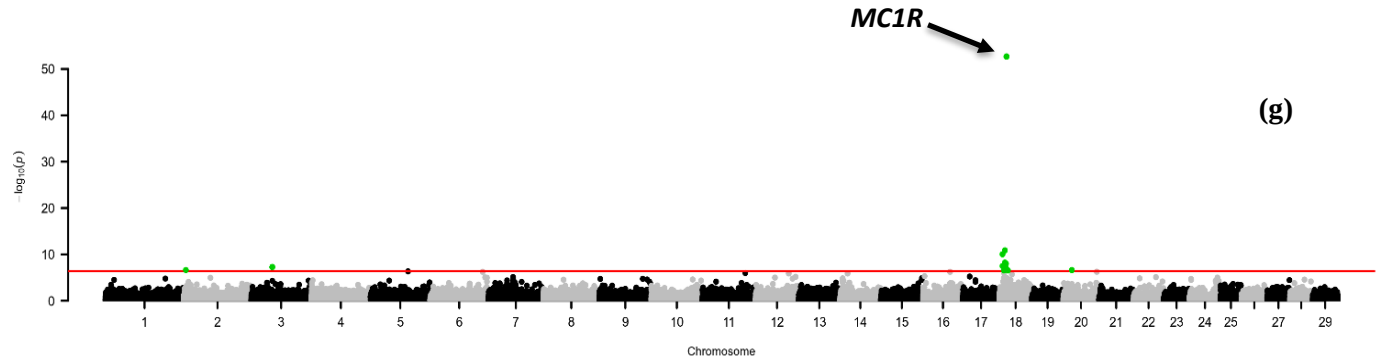
[4] Genomic inflation factor (λ)

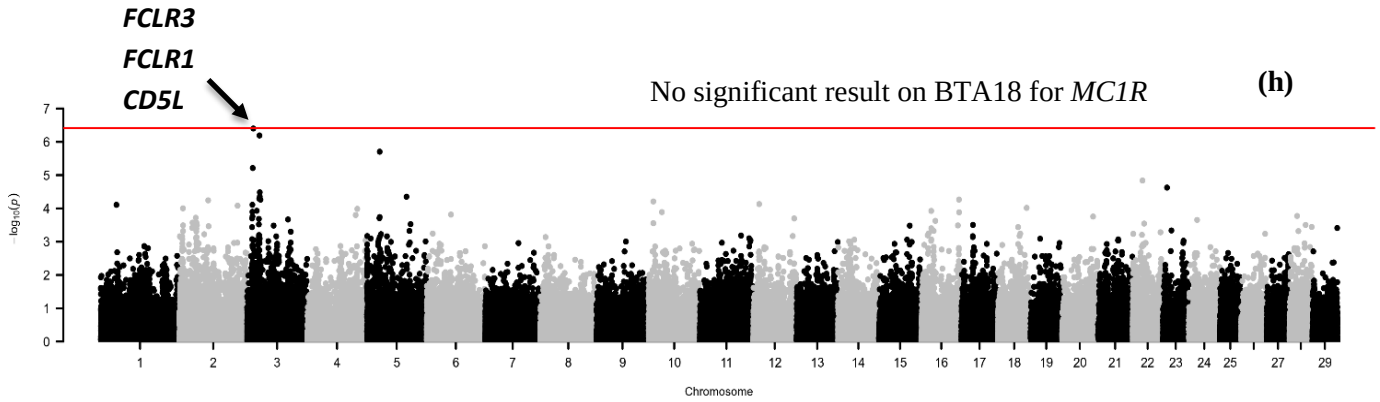
[5] Closest candidate gene identified in the significant SNP region according to its functional and potential role in the analyzed phenotype

Figure 1. Manhattan plots showing the results of the genome-wide association studies. GWAS for stature: age and operator as covariates (a), and age, operator and genotype of *LCORL* as covariates (b), GWAS for supernumerary nipple: operator as covariate (c), and GWAS for muzzle colour, operator as covariate: pink vs. grey vs. black (d), pink vs. grey and black (e), pink vs. black (f), pink and grey vs. black (g), pink vs. grey (h), grey vs. black (i)




MC1R
(d)
−log10(p)
Chromosome
MC1R
(e)
−log10(p)
Chromosome
MC1R
(f)
ADAMTS16
−log10(p)
Chromosome


MC1R
(g)
Chromosome


FCLR3
FCLR1
CD5L
No significant result on BTA18 for MC1R
(h)
Chromosome

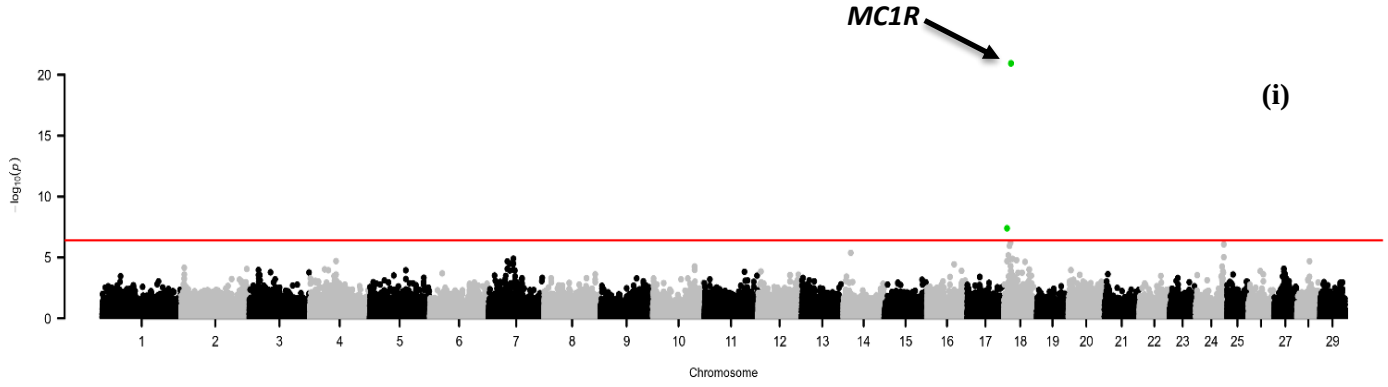

MC1R
(i)
Chromosome

Figure 2. Quantile-quantile plots of the genome-wide association studies described in Figure 1. Q-Q plots for stature: age and operator as covariates (a), and age, operator and genotype of *LCORL* as covariates (b), for supernumerary nipple: operator as covariate (c), and for muzzle colour, operator as covariate: pink vs. grey vs. black (d), pink vs. grey and black (e), pink vs. black (f), pink and grey vs. black (g), pink vs. grey (h), grey vs. black (i)

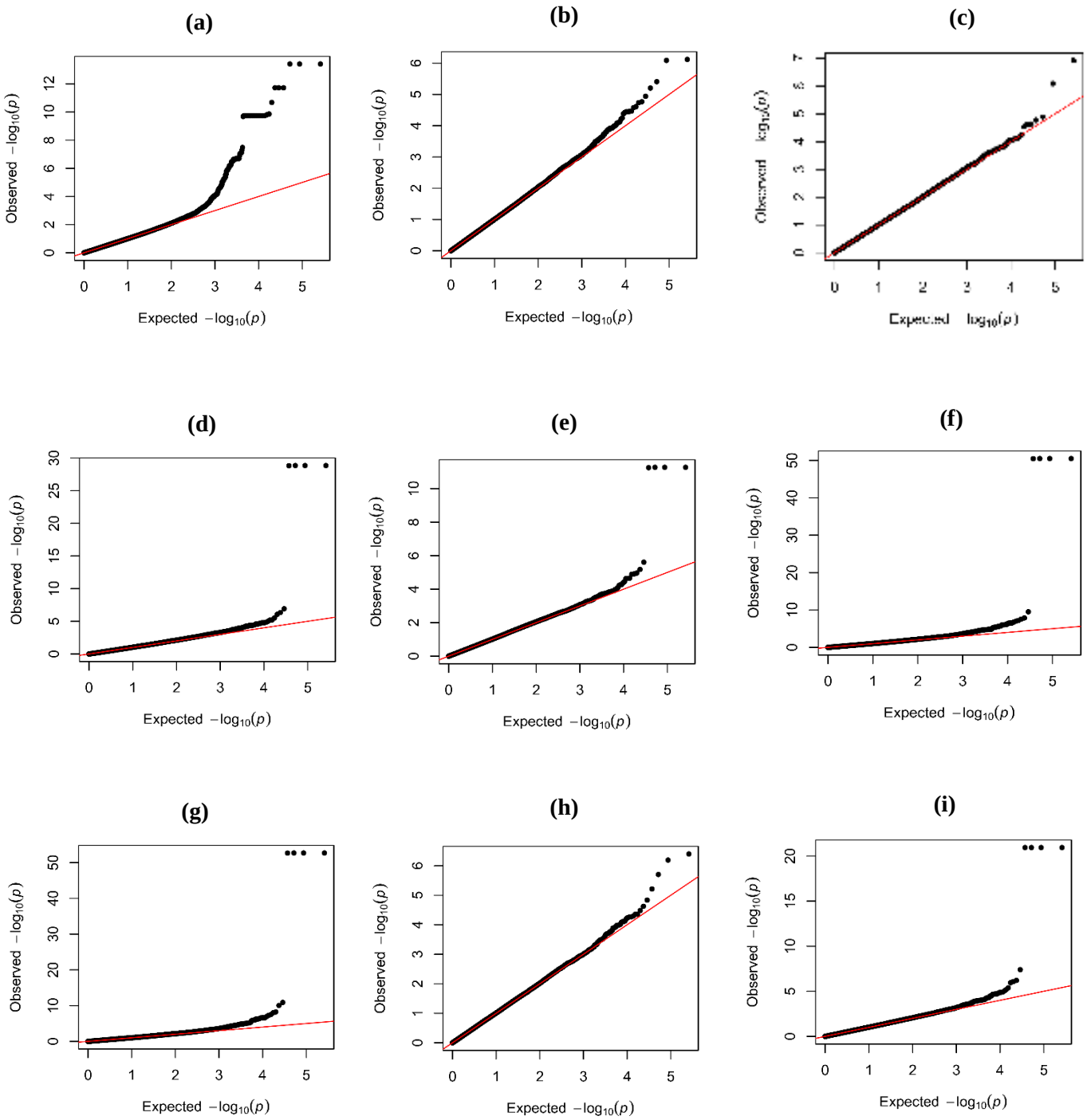
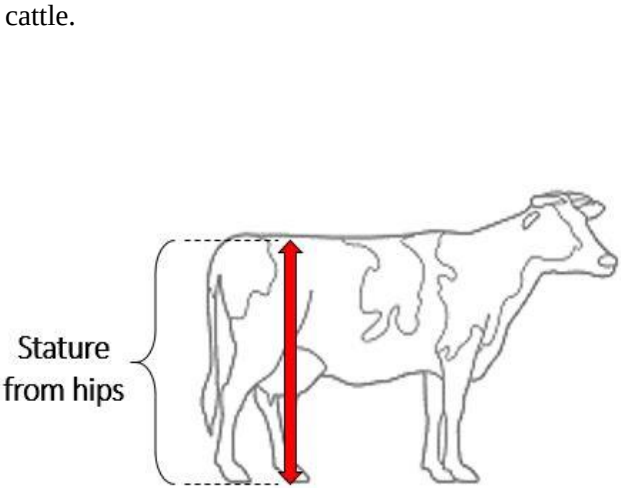


Figure 3. The schematic representation and the recorded phenotype of stature from the hips in Reggiana cattle.



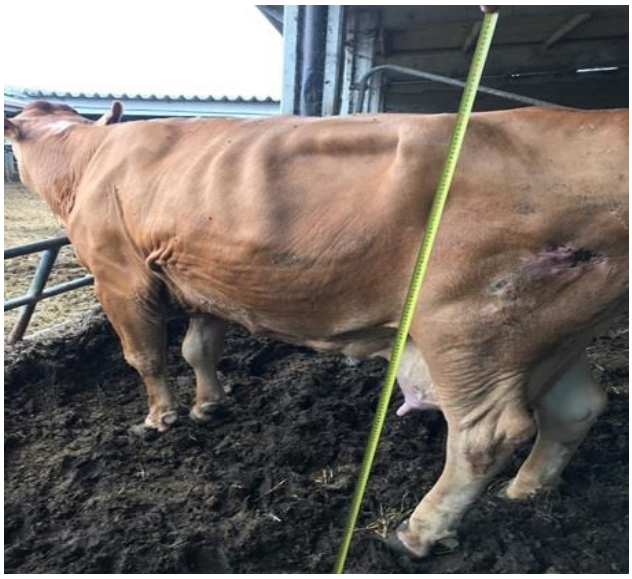


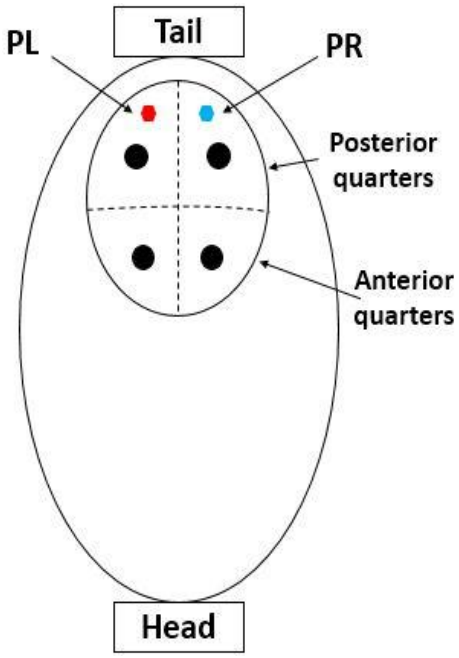
Figure 4. Different muzzle colour phenotype observed in Reggiana cattle. Pink muzzle (a), grey muzzle (pink with black dots) (b) and black muzzle (c)

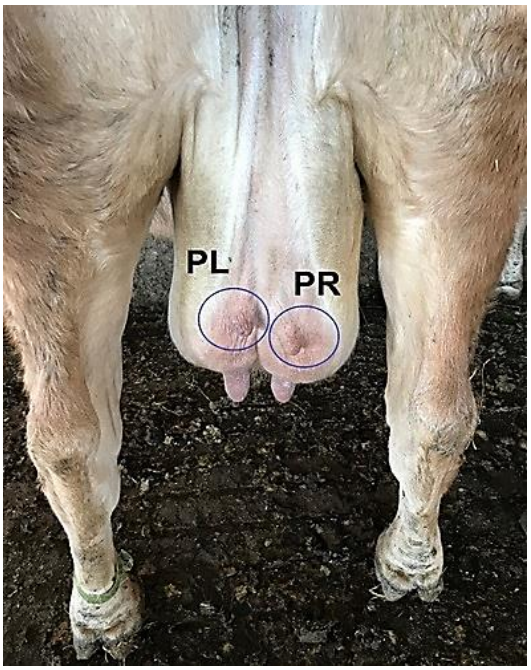
(a)

(b)

(c)

Figure 5. The schematic representation and the recorded supernumerary nipples based on different types and positions observed in Reggiana cattle. Caudal in the right (PR), caudal in the left (PR), intercalary in the right (MR) and intercalary in the left (ML)





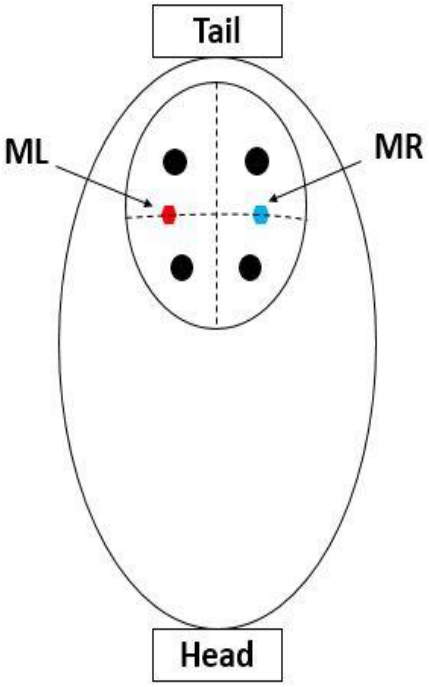
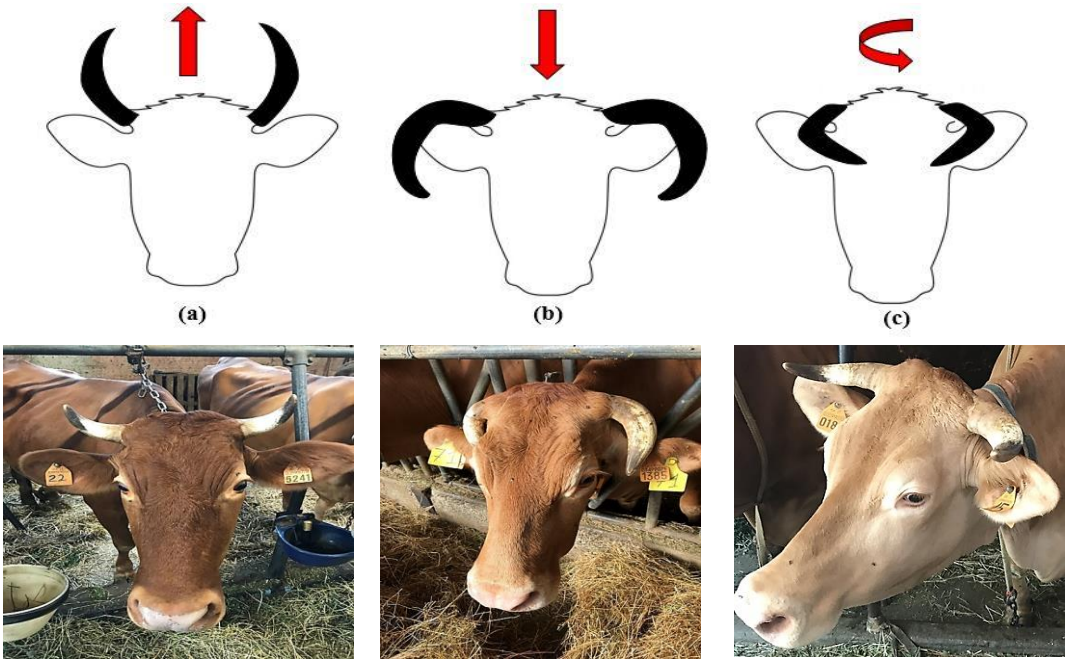


Figure 6. The schematic representation and the recorded horn shape phenotypes observed in Reggiana cattle. Upward horns (a), downward horns (b) and forward horns (c)



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Supplementray materials

Table S1. The complete list of significant or suggestive single nucleotide polymorphisms (SNPs) identified in the genome-wide association studies for stature

Chromosome[1]	Marker	Position[2]	No. miss[3]	Allele 1[4]	Allele 0[5]	MAF[6]	SE[7]	P-value
6	BovineHD0600010697	37211057	0	A	G	0.137	2.59E-01	3.82E-14
6	BovineHD0600010698	37214068	0	A	G	0.137	2.59E-01	3.82E-14
6	BovineHD0600010701	37221776	0	A	G	0.137	2.59E-01	3.82E-14
6	BovineHD0600010704	37232142	0	A	G	0.123	2.74E-01	1.88E-12
6	6_38668893	37234954	0	A	G	0.123	2.74E-01	1.88E-12
6	BovineHD0600010705	37234954	0	A	G	0.123	2.74E-01	1.88E-12
6	BovineHD0600010665	37138139	0	G	A	0.136	2.62E-01	2.07E-11
6	BovineHD0600010675	37159718	1	A	C	0.131	2.65E-01	1.38E-10
6	BovineHD0600010685	37182427	1	A	G	0.131	2.65E-01	1.57E-10
6	BovineHD0600010666	37140287	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010667	37145522	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010668	37147544	0	A	C	0.131	2.65E-01	1.82E-10
6	BovineHD0600010669	37149945	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010670	37150579	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010671	37153831	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010672	37155597	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010673	37156677	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010674	37158384	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010676	37161408	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010677	37163934	0	A	G	0.131	2.65E-01	1.82E-10
6	MS-rs110839532	37165831	0	A	C	0.131	2.65E-01	1.82E-10
6	MS-rs109241256	37166028	0	A	G	0.131	2.65E-01	1.82E-10
6	MS-rs41255599	37166157	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010678	37168914	0	A	C	0.131	2.65E-01	1.82E-10
6	BovineHD0600010679	37172063	0	G	A	0.131	2.65E-01	1.82E-10
6	BovineHD0600010680	37174847	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010683	37178722	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010684	37180966	0	A	G	0.131	2.65E-01	1.82E-10
6	BovineHD0600010686	37184577	0	C	A	0.131	2.65E-01	1.82E-10
6	MS-rs43702361	37165836	1	G	A	0.131	2.65E-01	2.10E-10
6	BovineHD4100004637	38107020	0	C	A	0.034	4.27E-01	3.28E-08
6	BovineHD4100004616	37920305	0	G	A	0.103	2.89E-01	5.12E-08
6	BovineHD0600010798	37776670	0	G	A	0.062	3.52E-01	7.99E-08
6	BovineHD0600010711	37245497	0	G	A	0.094	3.04E-01	8.26E-08
6	BovineHD0600010713	37252978	22	G	A	0.203	2.32E-01	8.60E-08
6	BovineHD0600010716	37270930	0	C	A	0.209	2.30E-01	1.21E-07
6	BovineHD0600010761	37483537	0	A	C	0.084	3.27E-01	1.99E-07
6	BovineHD0600034280	37497517	0	A	G	0.084	3.27E-01	1.99E-07
6	BovineHD0600010766	37528626	0	A	C	0.084	3.27E-01	1.99E-07
6	BovineHD0600010767	37533790	0	G	A	0.084	3.27E-01	1.99E-07
6	BovineHD0600010769	37540368	0	G	A	0.084	3.27E-01	1.99E-07
6	BovineHD0600010770	37546311	0	C	A	0.084	3.27E-01	1.99E-07
6	BovineHD0600010771	37550562	0	A	G	0.084	3.27E-01	1.99E-07
6	BovineHD0600010760	37466356	1	G	A	0.084	3.27E-01	2.08E-07
6	Hapmap26308-BTC-057761	37142174	0	G	A	0.261	2.06E-01	2.25E-07
6	BovineHD4100004621	37931903	0	A	G	0.102	2.88E-01	2.25E-07
6	Hapmap33170-BTC-071249	37933250	0	A	G	0.102	2.88E-01	2.25E-07
6	BovineHD0600010828	37953254	0	A	G	0.102	2.88E-01	2.25E-07

6	BovineHD0600010699	37218423	0	A	C	0.109	2.75E-01	2.31E-07
6	BovineHD0600010700	37219372	0	A	C	0.109	2.75E-01	2.31E-07
6	BovineHD0600010718	37277931	1	G	A	0.077	3.23E-01	3.07E-07
6	BovineHD0600010692	37195504	0	A	C	0.171	2.41E-01	3.19E-07
6	BovineHD0600010693	37196103	0	A	G	0.171	2.41E-01	3.19E-07
6	BovineHD0600010833	37972173	0	G	A	0.104	2.85E-01	3.44E-07
6	BovineHD0600010834	37973508	0	G	A	0.104	2.85E-01	3.44E-07
6	BovineHD0600010799	37777797	0	A	C	0.102	2.84E-01	5.20E-07
6	BovineHD4100004496	36132072	0	A	C	0.114	2.70E-01	5.34E-07
6	Hapmap24292-BTC-070983	38116481	0	A	C	0.038	4.10E-01	6.33E-07
6	BovineHD0600010772	37557764	0	A	G	0.074	3.49E-01	6.46E-07
6	BovineHD0600010774	37573159	0	C	A	0.074	3.49E-01	6.46E-07
6	BovineHD0600010775	37579521	0	A	G	0.074	3.49E-01	6.46E-07
6	BovineHD4100004753	39900932	0	A	C	0.122	2.60E-01	7.64E-07
6	BovineHD4100004684	38400569	0	A	G	0.084	3.24E-01	8.69E-07
6	BovineHD0600011207	39877393	0	G	A	0.121	2.61E-01	9.83E-07
6	BovineHD0600010827	37934272	0	A	G	0.225	2.23E-01	1.00E-06
6	BovineHD0600010652	37049538	0	G	A	0.17	2.41E-01	1.14E-06
6	BovineHD0600010780	37620283	0	C	A	0.073	3.51E-01	1.31E-06
6	BovineHD0600010781	37624676	0	G	A	0.073	3.51E-01	1.31E-06
6	ARS-BFGL-NGS-2946	37634657	0	A	G	0.073	3.51E-01	1.31E-06
6	Hapmap33628-BTC-041023	37505093	1	A	G	0.206	2.29E-01	1.47E-06
6	BovineHD4100004586	37521235	0	A	C	0.206	2.29E-01	1.57E-06
6	BovineHD4100004638	38114717	0	C	A	0.036	4.18E-01	1.62E-06
6	BovineHD0600010856	38066094	0	G	A	0.036	4.30E-01	2.58E-06
6	BovineHD0600010923	38420494	0	C	A	0.084	3.26E-01	2.73E-06
6	BovineHD0600010784	37652729	0	A	G	0.204	2.34E-01	2.87E-06
6	BovineHD0600010689	37191966	0	A	G	0.144	2.55E-01	4.26E-06
14	BovineHD1400007268	23379474	0	G	A	0.185	2.25E-01	4.50E-06
14	BovineHD1400007268	23379474	0	G	A	0.185	2.25E-01	4.50E-06
6	Hapmap40585-BTA-75775	33530543	0	A	G	0.09	3.11E-01	4.60E-06
6	BovineHD0600010717	37273774	0	G	A	0.075	3.46E-01	4.94E-06
14	BovineHD1400007267	23377643	0	G	A	0.188	2.23E-01	5.54E-06
6	BovineHD0600010708	37243159	0	A	G	0.096	2.95E-01	5.58E-06
6	BovineHD0600010709	37243677	0	G	A	0.096	2.95E-01	5.58E-06
6	MS-rs109570900	37343379	0	A	C	0.196	2.34E-01	6.48E-06
6	BovineHD4100004580	37418164	0	A	G	0.196	2.34E-01	6.48E-06
6	BovineHD0600010661	37123023	5	G	A	0.062	3.48E-01	7.89E-06
6	Hapmap26845-BTC-037406	39825526	0	G	A	0.178	2.30E-01	8.25E-06
6	BovineHD0600010655	37104774	0	G	A	0.064	3.45E-01	8.92E-06
6	BovineHD0600010660	37115970	0	G	A	0.064	3.45E-01	8.92E-06
6	BovineHD0600010840	37991751	0	G	A	0.225	2.23E-01	9.29E-06
6	BovineHD0600010697	37211057	0	A	G	0.137	2.59E-01	3.82E-14

[1] Bovine chromosome (Bos taurus)
[2] The position of the marker on the corresponding chromosome in the ARS-UCD1.2 genome version
[3] Number of missed animals in the analysis
[4, 5] Minor and major alleles
[6] Minor allele frequency
[7] Standard error

Table S2. The complete list of significant or suggestive single nucleotide polymorphisms (SNPs) identified in the genome-wide association studies for supernumerary nipple

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
10	BovineHD1000000240	894156	0	G	A	0.144	4.62E-02	1.21E-07
17	BovineHD1700017901	60441944	0	A	C	0.407	3.36E-02	8.20E-07

Table S3. The complete list of significant or suggestive single nucleotide polymorphisms (SNPs) identified in the genome-wide association studies for muzzle colour

pink vs. grey vs. black

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
18	MC1R373_3	14705686	0	C	A	0.05	5.79E-02	1.41E-29
18	MC1R373_4	14705686	0	C	A	0.05	5.79E-02	1.41E-29
18	MC1R373_2	14705686	1	I	D	0.05	5.79E-02	1.52E-29
18	ARS-BFGL-NGS-62316	11637587	0	G	A	0.037	7.21E-02	1.19E-07
12	ARS-BFGL-NGS-74771	81248933	1	G	A	0.033	7.18E-02	4.47E-07
20	BovineHD2000005251	17399506	0	G	A	0.146	3.89E-02	8.58E-07
18	ARS-BFGL-NGS-78423	11582091	3	A	G	0.028	8.39E-02	3.01E-06
5	BovineHD0500021691	75683960	0	G	A	0.029	7.92E-02	6.21E-06
4	BovineHD4100003163	97412705	0	G	A	0.282	3.10E-02	6.33E-06
18	BovineHD1800002320	6680701	0	A	G	0.068	5.43E-02	9.80E-06

pink vs. grey and black

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
18	MC1R373_3	14705686	0	C	A	0.05	4.43E-02	5.29E-12
18	MC1R373_4	14705686	0	C	A	0.05	4.43E-02	5.29E-12
18	MC1R373_2	14705686	1	I	D	0.05	4.43E-02	5.58E-12
3	ARS-BFGL-NGS-38423	12671675	0	A	G	0.449	2.01E-02	2.45E-06
4	BovineHD4100003163	97412705	0	G	A	0.282	2.25E-02	6.59E-06

pink vs. black

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
18	MC1R373_3	14705686	0	C	A	0.053	2.51E-02	3.18E-51
18	MC1R373_4	14705686	0	C	A	0.053	2.51E-02	3.18E-51
18	MC1R373_2	14705686	1	I	D	0.053	2.52E-02	3.46E-51
18	ARS-BFGL-NGS-62316	11637587	0	G	A	0.039	3.48E-02	3.02E-10
18	BovineHD1800002320	6680701	0	A	G	0.074	2.58E-02	1.31E-08
18	ARS-BFGL-NGS-78423	11582091	3	A	G	0.029	4.10E-02	2.88E-08
20	BovineHD2000005251	17399506	0	G	A	0.142	1.93E-02	6.44E-08
20	ARS-BFGL-NGS-93618	67654736	0	A	G	0.043	3.24E-02	9.60E-08
18	BovineHD1800004561	13862514	0	A	G	0.16	1.86E-02	1.76E-07
5	BovineHD0500021691	75683960	0	G	A	0.028	3.88E-02	3.07E-07
12	BovineHD1200028153	67352576	0	A	G	0.018	4.84E-02	3.12E-07
18	BovineHD1800002325	6714498	0	G	A	0.071	2.58E-02	3.30E-07
18	BovineHD4100013431	9393342	5	A	G	0.046	3.15E-02	6.84E-07
17	ARS-BFGL-NGS-22135	13621970	0	G	C	0.013	5.48E-02	7.50E-07

3	BovineHD0300013017	42620262	0	A	G	0.019	4.72E-02	8.78E-07
18	ARS-BFGL-NGS-23632	13929188	0	A	G	0.042	3.42E-02	9.42E-07
18	BovineHD1800005492	17611480	2	A	G	0.262	1.48E-02	9.78E-07
12	ARS-BFGL-NGS-74771	81248933	1	G	A	0.03	3.66E-02	1.87E-06
22	BTA-54618-no-rs	45194185	0	A	C	0.011	6.29E-02	2.05E-06
24	BovineHD2400000943	3219544	0	A	G	0.017	4.85E-02	2.63E-06
18	BovineHD1800003490	9816530	0	G	A	0.045	3.08E-02	2.68E-06
3	ARS-BFGL-NGS-104832	115783015	0	A	G	0.041	3.21E-02	3.13E-06
18	BovineHD1800016032	54274829	0	A	G	0.012	5.54E-02	3.61E-06
6	BovineHD0600031397	106192480	0	A	G	0.016	4.90E-02	4.18E-06
2	BovineHD0200001215	4417762	0	G	A	0.022	4.27E-02	4.25E-06
16	ARS-BFGL-NGS-112337	3428776	0	G	A	0.067	2.54E-02	4.58E-06
6	ARS-BFGL-NGS-112900	114812953	0	A	G	0.261	1.55E-02	5.44E-06
18	BovineHD1800003241	9243644	0	A	G	0.174	1.77E-02	7.33E-06

pink and grey vs. black

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
18	MC1R373_3	14705686	0	C	A	0.05	2.25E-02	2.03E-53
18	MC1R373_4	14705686	0	C	A	0.05	2.25E-02	2.03E-53
18	MC1R373_2	14705686	1	I	D	0.05	2.25E-02	2.15E-53
18	ARS-BFGL-NGS-62316	11637587	0	G	A	0.037	3.03E-02	1.28E-11
18	BovineHD1800002320	6680701	0	A	G	0.068	2.26E-02	9.00E-11
18	ARS-BFGL-NGS-78423	11582091	3	A	G	0.028	3.55E-02	5.92E-09
18	BovineHD1800004561	13862514	0	A	G	0.152	1.62E-02	1.00E-08
18	BovineHD1800002325	6714498	0	G	A	0.068	2.27E-02	3.22E-08
3	BovineHD0300013017	42620262	0	A	G	0.018	4.19E-02	5.24E-08
18	ARS-BFGL-NGS-23632	13929188	0	A	G	0.041	2.94E-02	1.06E-07
20	BovineHD2000005251	17399506	0	G	A	0.146	1.65E-02	2.33E-07
2	BovineHD0200001215	4417762	0	G	A	0.02	3.73E-02	2.34E-07
18	BovineHD4100013431	9393342	5	A	G	0.043	2.76E-02	2.57E-07
18	BovineHD1800005492	17611480	2	A	G	0.264	1.28E-02	2.74E-07
5	BovineHD0500021691	75683960	0	G	A	0.029	3.34E-02	4.44E-07
20	ARS-BFGL-NGS-93618	67654736	0	A	G	0.045	2.71E-02	5.43E-07
6	BovineHD0600031397	106192480	0	A	G	0.015	4.31E-02	5.64E-07
16	ARS-BFGL-NGS-27527	54827505	0	A	G	0.02	4.03E-02	5.71E-07
11	BovineHD1100024887	86719277	4	A	G	0.011	5.37E-02	1.01E-06
18	BovineHD1800003490	9816530	0	G	A	0.044	2.72E-02	1.01E-06
12	BovineHD1200028153	67352576	0	A	G	0.018	4.21E-02	1.26E-06
14	Hapmap60993-rs29025756	15423896	1	A	C	0.046	2.64E-02	1.28E-06
18	BovineHD1800007911	25653647	0	A	G	0.166	1.55E-02	1.88E-06
16	ARS-BFGL-NGS-112337	3428776	0	G	A	0.066	2.22E-02	4.85E-06
17	ARS-BFGL-NGS-22135	13621970	0	G	C	0.014	4.51E-02	5.79E-06
12	ARS-BFGL-NGS-74771	81248933	1	G	A	0.033	3.03E-02	6.64E-06
18	BovineHD1800003688	10331012	0	G	A	0.133	1.68E-02	7.40E-06
7	BovineHD0700014916	49672488	0	A	C	0.064	2.34E-02	7.55E-06
22	BTA-54618-no-rs	45194185	0	A	C	0.012	5.37E-02	7.83E-06
6	BovineHD0600032919	111164942	0	G	A	0.021	3.79E-02	8.97E-06
12	Hapmap43991-BTA-102559	39476523	0	A	G	0.036	2.96E-02	9.93E-06

pink vs. grey

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
3	ARS-BFGL-NGS-38423	12671675	0	A	G	0.444	1.87E-02	3.97E-07
3	Hapmap54955-rs29010328	25013373	0	G	A	0.301	2.01E-02	6.42E-07
5	BovineHD0500007614	25905905	0	A	G	0.137	2.61E-02	1.98E-06
3	BovineHD0300003829	11479692	0	A	G	0.275	2.14E-02	6.11E-06

grey vs. black

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
18	MC1R373_2	14705686	0	I	D	0.127	5.00E-02	1.15E-21
18	MC1R373_3	14705686	0	C	A	0.127	5.00E-02	1.15E-21
18	MC1R373_4	14705686	0	C	A	0.127	5.00E-02	1.15E-21
18	BovineHD1800002320	6680701	0	A	G	0.094	6.57E-02	3.98E-08
18	BovineHD1800004561	13862514	0	A	G	0.177	5.16E-02	6.10E-07
24	Hapmap38685-BTA-58652	56161438	0	C	A	0.164	5.64E-02	8.38E-07
18	ARS-BFGL-NGS-62316	11637587	0	G	A	0.072	8.10E-02	1.11E-06
14	Hapmap60993-rs29025756	15423896	0	A	C	0.07	7.64E-02	4.12E-06
18	BovineHD4100013431	9393342	1	A	G	0.068	8.50E-02	6.66E-06
24	BovineHD2400016180	56145989	0	G	A	0.031	1.17E-01	9.37E-06

pink vs. black, (operator and MC1R genotype as covariates)

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
24	BovineHD2400000943	3219544	0	A	G	0.017	4.12E-02	4.12E-10
3	ARS-BFGL-NGS-19108	96661927	0	A	G	0.015	4.44E-02	5.62E-09
22	BovineHD2200004241	15629756	1	A	C	0.053	2.26E-02	2.92E-08
3	BovineHD0300013017	42620262	0	A	G	0.019	3.99E-02	7.04E-08
5	BovineHD0500021691	75683960	0	G	A	0.028	3.28E-02	9.90E-08
17	ARS-BFGL-NGS-22135	13621970	0	G	C	0.013	4.63E-02	1.03E-07
20	ARS-BFGL-NGS-93618	67654736	0	A	G	0.043	2.66E-02	2.32E-07
22	BTA-54618-no-rs	45194185	0	A	C	0.011	5.29E-02	2.96E-07
6	ARS-BFGL-NGS-100768	114116280	7	C	A	0.026	3.54E-02	3.03E-07
6	BovineHD0600001731	6573422	0	A	G	0.013	4.84E-02	4.56E-07
1	BTA-55326-no-rs	12248784	0	C	A	0.031	3.31E-02	4.75E-07
7	BovineHD0700006500	22427287	0	A	C	0.04	2.93E-02	9.76E-07
16	BovineHD1600015642	54801952	0	G	A	0.038	2.97E-02	1.54E-06
12	ARS-BFGL-NGS-74771	81248933	1	G	A	0.03	3.18E-02	1.96E-06
6	ARS-BFGL-NGS-112900	114812953	0	A	G	0.261	1.29E-02	2.23E-06
20	BovineHD2000005251	17399506	0	G	A	0.142	1.62E-02	2.40E-06
17	BovineHD1700007224	25248800	0	A	G	0.027	3.45E-02	3.77E-06
3	BovineHD0300013018	42626776	0	A	G	0.021	3.89E-02	4.05E-06
17	BovineHD1700007201	25189243	5	A	G	0.028	3.42E-02	5.96E-06
22	Hapmap39760-BTA-55284	3004263	0	A	C	0.025	3.38E-02	6.36E-06
6	BovineHD0600029957	114802363	1	A	G	0.281	1.24E-02	8.44E-06
13	BTA-59658-no-rs	76013752	0	G	A	0.028	3.32E-02	8.69E-06
18	BovineHD1800010935	36118407	0	G	A	0.014	4.98E-02	9.80E-06
18	BovineHD1800010942	36146813	0	A	G	0.014	4.98E-02	9.80E-06

pink and grey vs. black, (operator and MC1R genotype as covariates)

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
3	ARS-BFGL-NGS-19108	96661927	0	A	G	0.017	3.63E-02	7.13E-09
3	BovineHD0300013017	42620262	0	A	G	0.018	3.62E-02	4.61E-08
24	BovineHD2400000943	3219544	0	A	G	0.018	3.49E-02	1.41E-07
5	BovineHD0500021691	75683960	0	G	A	0.029	2.82E-02	3.69E-07
16	BovineHD1600015642	54801952	0	G	A	0.038	2.49E-02	4.21E-07
16	ARS-BFGL-NGS-27527	54827505	0	A	G	0.02	3.47E-02	7.16E-07
6	BovineHD0600001731	6573422	0	A	G	0.014	4.00E-02	1.10E-06
17	ARS-BFGL-NGS-22135	13621970	0	G	C	0.014	3.88E-02	1.53E-06
7	BovineHD0700006500	22427287	0	A	C	0.04	2.54E-02	1.67E-06
6	ARS-BFGL-NGS-112900	114812953	0	A	G	0.264	1.11E-02	2.40E-06
20	BovineHD2000005251	17399506	0	G	A	0.146	1.41E-02	3.32E-06
26	ARS-BFGL-NGS-119524	45853143	0	A	C	0.016	3.69E-02	3.59E-06
22	Hapmap39760-BTA-55284	3004263	0	A	C	0.025	2.95E-02	4.38E-06
14	Hapmap60993-rs29025756	15423896	1	A	C	0.046	2.28E-02	5.02E-06
20	ARS-BFGL-NGS-93618	67654736	0	A	G	0.045	2.28E-02	5.23E-06
6	BovineHD0600029957	114802363	1	A	G	0.285	1.07E-02	7.37E-06
22	BovineHD2200004241	15629756	1	A	C	0.061	1.96E-02	8.09E-06
12	BTB-01365357	1497417	0	A	C	0.021	3.30E-02	9.59E-06
26	Hapmap57526-rs29013535	45429575	0	A	G	0.025	2.99E-02	9.89E-06

Figure S1. Manhattan and Q-Q plots of the GWAS for muzzle colour considering the effect of MC1R genotype and operator as covariates: pink vs. black (a), pink and grey vs. black (b)

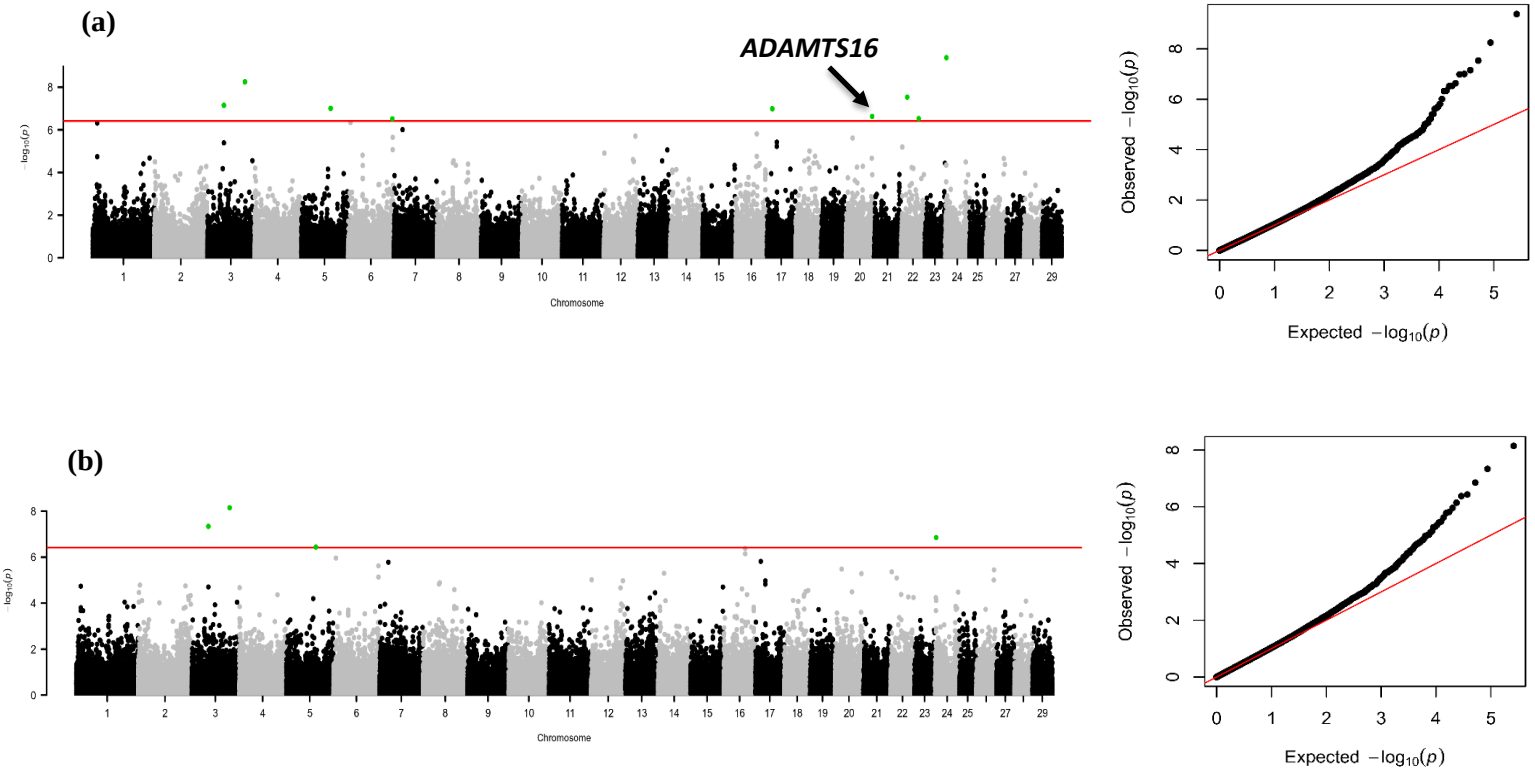
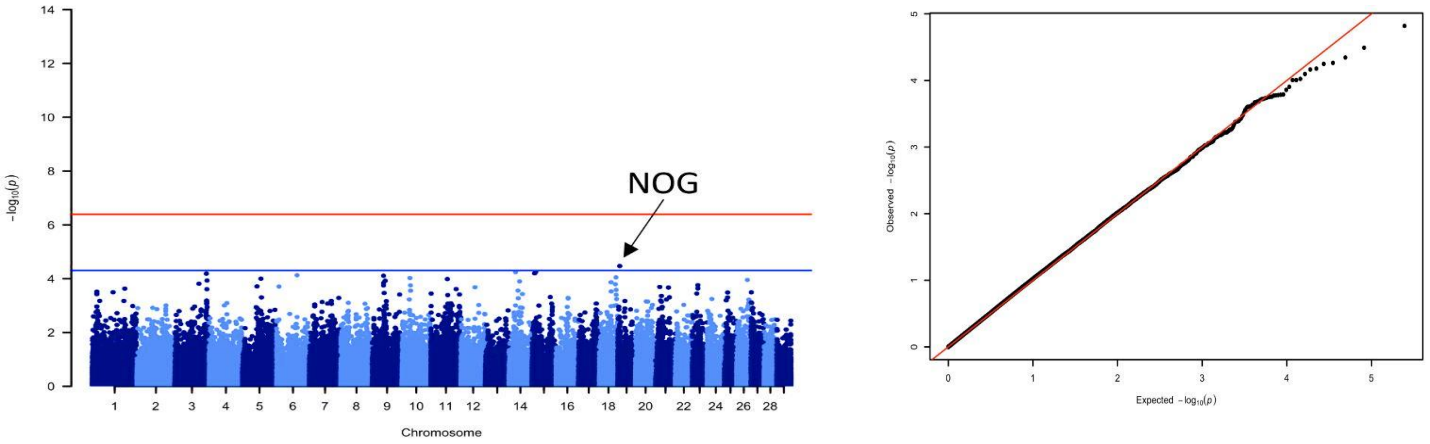


Table S4. The complete list of significant or suggestive single nucleotide polymorphisms (SNPs) identified in the genome-wide association studies for horn shape

Chromosome	Marker	Position	No. miss	Allele 1	Allele 0	MAF	SE	P-value
19	BovineHD1900002056	7485353	0	G	A	0.485	3.56E+04	3.40E-05
15	BTB-01922500	13222719	0	A	C	0.421	3.97E+04	5.60E-05
14	Hapmap54618-rs29021334	23937950	0	G	A	0.197	4.88E+04	5.70E-05
15	BovineHD1500002507	9360609	0	G	A	0.120	5.67E+04	6.33E-05
3	ARS-BFGL-NGS-74948	111526170	0	C	A	0.264	4.34E+04	6.50E-05
6	BovineHD0600020857	73302270	0	A	G	0.107	6.27E+04	7.50E-05
9	BovineHD0900011062	39491253	0	A	G	0.281	3.94E+04	7.82E-05
18	BovineHD1800017434	60098278	1	G	A	0.259	4.22E+04	8.97E-05
10	BovineHD1000009417	28654013	0	C	A	0.410	3.70E+04	9.45E-05

Figure S2. Manhattan and Q-Q plots showing the results of the genome-wide association studies for horn shape in Reggiana cattle (suggestive marker on BTA19)



5.1 Genomics and improved productivity of pigs through increasing teat number

Reproductive performance as a complex quantitative trait is affected by multiple genes, biological processes and regulatory pathways, and environmental factors. Hence, the identification of high-density markers seems essential in order to map the complex traits in livestock (Li et al., 2020; Yang et al., 2019). In the pig breeding industry, improved sow productivity has been a success story, and all the signs for the future point to further increases in the number of piglets born and weaned per litter. Therefore, this can bring a challenge for sow's milk production and proper accessibility of piglets particularly with the bigger size to teats, referring to the great advantage of the pigs having more teats on the udder (Uzzaman et al., 2018; Duijvesteijn et al., 2014).

Based on these facts, the successful pig breeding program underlies the improvement of two fundamental traits as the number of teats and also the number of vertebrae which together can influence directly both production and reproduction efficiency especially the number of weaned piglets (Hirooka et al., 2001). It has been hypothesized that the teat number is the result of natural and human-driven artificial selection as there is variation in the number of teats between and also within different pig breeds (Zhuang et al., 2020). The importance of having an optimum number of teats for pigs could be reflected by the fact that the mortality rate of piglets might increase when the sow has an insufficient number of teats or any other disorders in her mammary gland (Alexopoulos et al., 2018). Therefore, the identification of major QTL regions and candidate genes involved in teat number and also body conformation traits at birth is of scientific and economic importance in terms of providing efficient markers for genetic improvement in pigs (Zhou et al., 2019).

Considering the low heritability rate and sex-limited expression, most of the reproduction traits including the number of teats are difficult to be improved through quantitative selection methods (Clayton et al., 1981; McKay and Rahnefeld, 1990). Over the last 10 years, the use of molecular genetic maps in livestock production has facilitated the discovery of genomic regions that lead to the genetic variation of quantitative traits (Hong et al., 2020). The application of genomic technologies especially GWASs in searching for main chromosomal regions underlying genetic architecture of teat number, and optimized selection techniques have resulted in more rapid genetic progress in terms of productivity including the increase in the number of teats accompanied with the increase of live-born piglets (Uzzaman et al., 2018).

From another point of view, the functionality of teats should be considered as part of selection objectives because the number of functional teats might directly influence the production efficiency in pigs. In addition to the number of teats, the number of functional teats could also be genetically improved due to its sufficient genetic variation. Thus, one possible approach for improvement of teat quality could be the selection of only good functional teats and ignoring any other defected teat phenotypes such as inverted or extra teats both in sows and boars (Marios and Larochelle, 2008), which is different from the selection for the total number of teats.

It has been investigated by many studies that there is a positive relationship between the number of teats and the number of vertebrae traits in the pig. There is a relationship between the basic factors for the developmental procedure of mammary gland and skeletal development and the quantitative genetic parameters that can explain in which way the biological factors influence parameters for practical breeding value estimation (Van Son et al., 2019). Also, the significant SNPs identified for *VRTN* as the main candidate gene on SSC7 have been shown to affect positively both teat number and also the number of vertebrae that could eventually increase the number of piglets born alive. Based on this, it seems that common genetic mechanisms control these traits (number of teats and vertebrae) (Duijvesteijn et al., 2014).

The vertebral number is an important trait in pig production with high heritability (0.60-0.62) and is associated with the length of body (Borchers et al., 2004). The number of thoracic and lumbar vertebrae varies between pig breeds ranging from 19 thoracic-lumbar vertebrae in wild boards and indigenous pigs, and 20-23 in commercial breeds including Large White, Landrace, and Duroc (Huang et al., 2017). This increase in the number of vertebrae in commercial breeds could be the consequence of the long-time intense selection for body size improvement (Fan et al., 2013). There are also some evidence of the relationship between vertebrae number and carcass conformation and meat quality traits in pigs. Therefore, as already mentioned, selection for the increased the number of teats (due to the variability in *VRTN* gene) may result in increased vertebrae numbers and carcass length which eventually could affect other productive and reproductive traits in pigs (Fan et al., 2013; Fontanesi et al., 2014b). Thus, having a broad knowledge about the genetic basis of teat and vertebrae numbers in pigs will help to not only better understand different mechanisms involved in the mammary gland and vertebral developments but also provide potential genetic makers to be used for selection in breeding programs (Zhang et al., 2015).

5.2 Genome-wide association studies for the number of teats and teat asymmetry patterns in Large White pigs

Introduction

The number of teats is an important morphological trait that largely influences the mothering ability of the sows and thus their reproduction performances (Pumfrey et al., 1980; Kim et al., 2005). The number of teats in pigs is considered a quantitative trait with discrete and countable values, with a medium/high level of heritability, showing a considerable variability among breeds as well as within breeds and lines (e.g. Willham and Whatley, 1963; McKay and Rahnefeld, 1990; Borchers et al., 2004; Chalkias et al., 2013; Felleki and Lundeheim, 2015; Rohrer and Nonneman, 2017; Dall'Olio et al., 2018).

Other related parameters that are not usually recorded are: (i) the number of teats divided between the two sides and their differences, which can identify directional and fluctuating

asymmetry (bilateral variation); (ii) the number of anterior and posterior teats (having the navel as the dividing line), which can provide information on their longitudinal body distribution; and (iii) the number of extra or additional teats (i.e. teats not placed in parallel between the two sides). Only a few studies have investigated these phenotypes in pigs, which might provide information on developmental patterns and developmental stability and instability (Palmer and Markow, 1994; Fernandez et al., 2004). Estimations of heritability for some of these teat-related parameters have reported lower values than the heritability for the total number of teats (Willham and Whatley, 1963; McKay and Rahnefeld, 1990; Borchers et al., 2004; Fernandez et al., 2004; Rohrer and Nonneman, 2017).

Several QTL studies for the total number of teats carried out in reference populations, obtained by crossing different pig breeds (including highly prolific Chinese breeds), have shown that this parameter is affected by variants in several chromosome regions, confirming that a complex polygenic influence contributes to determine its variability (Hu et al., 2019). Then, GWASs within breeds and lines added other regions to the list of loci affecting this trait (e.g. Duijvesteijn et al., 2014; Verardo et al., 2015; Rohrer and Nonneman, 2017; Van Son et al., 2019). A major QTL located on porcine chromosome 7 (SSC7), having pleiotropic effects including both the number of teats and the number of vertebrae, has been shown to segregate in several pig populations and breeds (Mikawa et al., 2005; Duijvesteijn et al., 2014; Verardo et al., 2015; Yang et al., 2016; Rohrer and Nonneman, 2017; Dall'Olio et al., 2018; Van Son et al., 2019). Recently another QTL on SSC7 and ATP binding cassette subfamily D member 4 (*ABCD4*) gene was found for teat number in Canadian Duroc pigs using single-locus and meta-analysis of GWAS and the top SNP (rs692640845) demonstrated 8.68% phenotypic variance of teat number (Zhuang et al., 2020).

Mikawa et al. (2011) fine-mapped the number of vertebrae QTL and identified a new gene (vertnin, also known as vertebrae development associated or *VRTN*), encoding for a DNA binding factor required for the development of thoracic vertebrae in mammals (Duan et al., 2018), as the gene affecting this trait. A 291 bp short interspersed nuclear element (SINE) insertion in *VRTN* gene on porcine chromosome 7 (g.20311_ 20312ins291) was shown to affect the vertebral number and several production traits with the allele Q (with the insertion) increases vertebral number compared to the wild type allele (WT, without insertion) (Fontanesi et al., 2014b). Other studies also reported the association between the genotypes of *VRTN* gene with intramuscular fat content in purebred Duroc pigs with the positive effect of *Wt* allele and higher mean for *Wt*/*Wt* individuals (Hirose et al., 2013), and the SNP (g.20311_20312ins291) with the number of ribs, carcass diagonal length and canon bone circumference in a Chinese pig breed (Jiang et al., 2020). The aim of this study was to characterize the responsible genes and SNPs for different traits related to teat number (in the left and right sides and posterior and anterior parts) and also the asymmetry pattern of teats through several GWASs in the Italian Large White pig breed.

Materials and methods

In this study, we carried out GWASs for the total number of teats (TN) and several related parameters (including several asymmetry patterns of teat number and disposition on the ventral side) in 843 Italian Large White heavy pigs (278 castrated males and 565 gilts, obtained from 86 boars and 377 litters). These animals were included in the sib-testing programme of the Italian Large White pig population (Fontanesi et al., 2014a).

A total of 13 teat-related parameters were obtained after slaughtering the pigs, as described below. The ventral side of the carcasses was photographed in the vertical position, before evisceration and dissection. Three independent persons visually inspected all obtained photographs and counted: (i) the number of teats of the left line (LTN) and (ii) the number of teats of the right line (RTN); then (iii) the total number of teats was calculated as the sum of LTN and RTN; and (iv) the number of anterior teats (NAT) and (v) the number of posterior teats (NPT), having the navel as dividing longitudinal line (McKay and Rahnefeld, 1990).

A few other parameters were also calculated based on these records: (vi) the maximum number of teats between the two sides (MAX, obtained comparing the LTN and the RTN and choosing the highest value between the two), which could be considered the best indicator of the genetic potential for proliferation of initial mammary buds (Rohrer and Nonneman, 2017); (vii) the signed difference between the two sides (SDIFF = RTN – LTN), which describes the directional asymmetry; and (viii) the absolute difference between the two sides (ADIFF = |RTN – LTN|), which identifies the fluctuating asymmetry (Palmer and Markow, 1994). The same measures were calculated between anterior and posterior teats: (ix) the maximum number of teats between the two parts (MAX-AP); (x) the signed difference between anterior and posterior teats (SDIFF-AP = NAT – NPT); and (xi) the absolute difference between anterior and posterior teats (ADIFF-AP = |NAT – NPT|).

Additionally, another two parameters were computed: (xii) the ratio between the number of posterior and the number of anterior teats (NPT/NAT); and (xiii) the absence or the presence of extra teats (EX-T, recorded as n = 0, 1, 2), i.e. a different number of teats between the two sides (right and left) because of the extra teat between the regularly spaced teats. Figure S1 shows a schematic representation of all basic teat parameters considered in this study from which calculated parameters were then obtained. Table S1 summarizes statistics for all considered traits. Functional and non-functional teats could not be clearly distinguished by inspecting available photographic records; therefore this trait was not considered in this study.

All pigs were genotyped with the Illumina PorcineSNP60 BeadChip (version 1 or 2; Illumina Co., San Diego, CA, USA). The assignment of the SNPs to the reference pig genome (Sus scrofa 11.1) was obtained as previously described (Fontanesi et al., 2012, 2014a). Genotyping data were filtered using PLINK software 1.9 (Chang et al., 2015) adopting the following criteria: SNPs were retained if located on autosomes and their call rates were > 0.9, MAF > 0.02, and if they did not

deviate from HWE (considering a *P*-value > 0.0001); animals with call rate > 0.90 were used for the analyses. After filtering, the dataset was composed of 821 animals and 50069 autosomal variants. GWASs were carried out following a linear mixed effect model:

$$\boldsymbol{y} = \boldsymbol{W\alpha} + \boldsymbol{x\beta} + \boldsymbol{u} + \boldsymbol{e}$$

where $\boldsymbol{y}$ ($n \times 1$) is a vector containing parameter for the nth animal, $\boldsymbol{W}$ ($n \times k$) is a matrix of covariates (fixed effects) with $k = 2$ (a column of 1s, sex), $\boldsymbol{\alpha}$ is the k-dimensional vector of covariates effects, $\boldsymbol{x}$ ($n \times 1$) is the vector containing genotypes for the ith SNP (coded as 0, 1, 2, according to the number of copies of the minor allele), $\boldsymbol{\beta}$ is the additive effect of the ith SNP on the trait, $\boldsymbol{u} = N(\mathbf{0}, \sigma^2_u \mathbf{K})$ is a multivariate Gaussian polygenic effect, with covariance matrix proportional to the relatedness matrix $\mathbf{K}$ ($n \times n$), and $\boldsymbol{e} = N(\mathbf{0}, \sigma^2_e \mathbf{I})$ is a multivariate Gaussian vector of uncorrelated residuals.

For each trait, the effect of the top associated SNP was evaluated by including in the model, as fixed effect, the genotype of each tested individual. The genotype was coded with dummy variables that led to increase the size of covariate matrix k from 2 to 4 (three genotypes can be coded with $N - 1$ dummy variables). Moreover, for each trait we evaluated the effect of the *VRTN* polymorphism by including it, as fixed effect, in the model. The assessment of the association between each SNP and trait was obtained by testing the null hypothesis H_0: $\beta = 0$. Significance was tested using the Wald test. All analyses were performed using GEMMA version 0.96 (Zhou and Stephens, 2012) after computing the relatedness matrix **G** as a centered genomic matrix controlling the population structure.

A Bonferroni corrected threshold equal to a nominal value of 0.05 was used to define significant markers (*P*-value = 0.05/50069 = 9.9×10^{-7}). To take into consideration also moderate associations and balance the risk of Type I and Type II errors, in our analyses we considered a suggestively significance threshold of *P*-value = 5.0×10^{-5}, as widely adopted in GWAS in farm animals (e.g. Fontanesi et al., 2012; Sanchez et al., 2014; Bovo et al., 2019). For each trait, GEMMA estimated from the whole set of available genotypes the chip heritability (or SNP heritability; h^2_{SNP}; Table S1). Quantile-quantile (QQ) plots and Manhattan plots were generated in R version 3.5.1 (R Core Team 2018) using the 'qqman' package, whereas the genomic inflation factors (k) were computed with the function 'estlambda' (method: 'regression') within the 'GenABEL' package (Aulchenko et al., 2007). Associated peaks were annotated with Biomart (http://www.ensembl.org/biomart/martview/) and the pig QTL database (Hu et al., 2019; Table S2).

Results and discussion

Among the 13 considered teat parameters, those that were based on total or sided counted teats (TN, RTN, MAX, and LTN) had the highest h^2_{SNP} values (0.360, 0.258, 0.261, and 0.158 respectively). These estimates were in line with what was previously reported, even if these values were a little higher than those provided by Rohrer and Nonneman (2017) for the same traits. All

other parameters had low or virtually null h^2_{SNP} values, suggesting that additive genetic factors might have low or negligible effects on most asymmetric patterns, as already reported by other authors (Fernandez et al., 2004; Rohrer and Nonneman, 2017).

The results of the GWAS are summarized in Table 1, which lists significant SNPs for six parameters (TN, LTN, RTN, MAX, NPT, and ADIFF-AP). Figure 1 reports the Manhattan plots for these six parameters. QQ-plots for these GWASs are reported in Figure S2. For the other four parameters (ADIFF, NAT, NPT/NAT, and EX-T) only suggestively significant markers were identified (Figure S3). All suggestively significant markers are reported in Table S2. Figure S4 includes the Manhattan plots and QQ-plots for the remaining traits (SDIFF, MAX-AP, and SDIFF-AP).

For TN, LTN, RTN, and MAX a major significant peak was observed on SSC7, in the region of the *VRTN* gene (located at nucleotide positions from 97614707 to 97624273), with the most significant SNP (MARC0038565 or rs80894106, located at position 97652632) for all four parameters. To confirm that the identified QTL could be attributed to the *VRTN* gene, we genotyped in 821 pigs the indel determined by the insertion of a short interspersed nuclear element PRE1 of 291 bp (AB554652:g.20311_20312ins291), considered a causative mutation for the number of vertebrae/number of teats QTL (Mikawa et al., 2011), using the protocols already described (Fontanesi et al., 2014b).

This marker was included in the GWASs and results were significant (TN and MAX) or suggestively significant (LTN and RTN) and in the same QTL peak as already observed (Table 1 and Table S2). When this marker or the MARC0038565 SNP were conditionally included in the model of the GWAS, all effects for these four parameters were erased, further supporting the presence of only one segregating QTL in this chromosome region.

Significant markers for NPT and ADIFF-AP, and suggestively associated markers for NAT, were consistently identified on SSC6 (Table 1 and Table S2; Figure 1 and Figure S5). The most significant SNP for NPT and ADIFF-AP was ASGA0100698 (rs81476132), an intron variant at position 32528964 within the TOX high mobility group box family member 3 (*TOX3*) gene. This gene encodes a protein with a high-mobility-group motif that modifies chromatin structure by bending or unwinding DNA. It is involved in mediating calcium-dependent transcription and interacts with the cAMP response element-binding protein (Yuan et al., 2009). Mutations in this gene have been implicated in high breast cancer risks (Easton et al., 2007).

Highly conserved genomic structures across mammals might be involved in defining the function of this developmental regulator gene (Harmston et al., 2017), which has also been suggested to be involved in determining asymmetry patterns in embryonic development (Wilting and Hagedorn, 2011). Even if the role and function of *TOX3* are still far from being completely understood, what is currently known might support its candidacy for a role in the teat asymmetry parameter (anterior-posterior numbers) measured in pigs. This parameter is derived by the relative

position of the navel and could describe the effects of biological mechanisms controlling the developmental programme of morphological features in pigs (i.e. the navel position or anterior/posterior shifts of the two teat lines).

Other suggestively significant markers in regions not related to the two previous QTL (Table S2) were identified on SSC1 (RTN and MAX), SSC3 (TN, RTN, MAX, and EX-T), SSC4 (EX-T), SSC5 (ADIFF), SSC9 (NPT/NAT), SSC11 (ADIFF, NPT/NAT, and EX-T), SSC14 (NPT/NAT), SSC16 (ADIFF and NPT/NAT) and SSC17 (NPT and ADIFF-AP). Some of these markers are located in regions where other studies have reported QTL for teat number-related traits or other potentially related morphological traits (Table S2). No other QTL peak emerged in all GWASs that included MARC0038565 or *VRTN* markers as fixed effects in the models.

Conclusion

In conclusion, this study further supports the role of the *VRTN* gene region in affecting the recorded variability in the number of teats in the Italian Large White pig population (Dall'Olio et al., 2018). It seems that this chromosome region (SSC7) harbors one of the few major QTL for the number of teats for which alleles are still segregating in this breed. We already observed an increasing trend of the favorable allele frequency of this gene over the past few decades of directional selection toward higher number of teats in this breed (Fontanesi et al., 2015) that might have also acted in fixing other QTL for this important trait. It will be interesting to understand if epigenetic mechanisms or other factors could explain the observed heterogeneity and variability for most of the other teat-related measures, which could provide information on developmental patterns and instability.

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Table 1. Significant markers identified in the GWASs.

Parameter	SSC[1]	Marker position[2]	Marker	Alleles[3]	MAF[4]	*P*-value[5]	Candidate gene[6]
TN	7	97652632	MARC0038565	G/A	0.494	1.62×10^{-10}	*VRTN* (28359)
	7	97619490	VRTN[7]	Ins/-	0.393	3.32×10^{-07}	*VRTN* (0)
LTN	7	97652632	MARC0038565	G/A	0.494	4.04×10^{-07}	*VRTN* (28359)
RTN	7	97652632	MARC0038565	G/A	0.494	1.11×10^{-09}	*VRTN* (28359)
	7	97881096	H3GA0022659	A/G	0.464	9.91×10^{-07}	*VRTN* (256823)
MAX	7	97652632	MARC0038565	G/A	0.494	1.16×10^{-11}	*VRTN* (28359)
	7	97881096	H3GA0022659	A/G	0.464	$1.05 \times 9\ 10^{-07}$	*VRTN* (256823)
	7	97619490	VRTN[7]	Ins/-	0.393	2.08×10^{-07}	*VRTN* (0)
	7	97795647	M1GA0010653	A/G	0.482	3.70×10^{-07}	*VRTN* (171374)
NPT	6	32528964	ASGA0100698	A/G	0.209	5.55×10^{-08}	*TOX3* (0)
	6	32678775	ALGA0035080	G/A	0.275	5.66×10^{-07}	*TOX3* (68847)
ADIFF-AP	6	32528964	ASGA0100698	A/G	0.209	4.18×10^{-07}	*TOX3* (0)

ADIFF-AP, Absolute difference between anterior and posterior teats (|NAT – NPT|); LTN, number of left line teats; MAX, maximum number of teats between the two sides; NP, number of posterior teats; RTN, number of right line teats; TN, total number of teats.

[1]Porcine chromosome.

[2]Position of the marker on the corresponding chromosome in the Sus scrofa 11.1 genome version.

[3]Minor and major alleles.

[4]Minor allele frequency.

[5]*P*-value from GEMMA (Wald test). Only single nucleotide polymorphisms (SNPs) significantly associated after Bonferroni correction are reported.

[6]Candidate genes identified in the significant SNP region (500 kbp) according to their function and potential role in the analysed phenotypes, in brackets is reported the distance of the marker to the gene in bp. Zero means that the marker was within the gene.

[7]In house genotyped AB554652:g.20311_20312ins291 *VRTN* related polymorphism (Fontanesi et al., 2014b).“Ins” = insertion; ‘‘-’’ = no insertion.

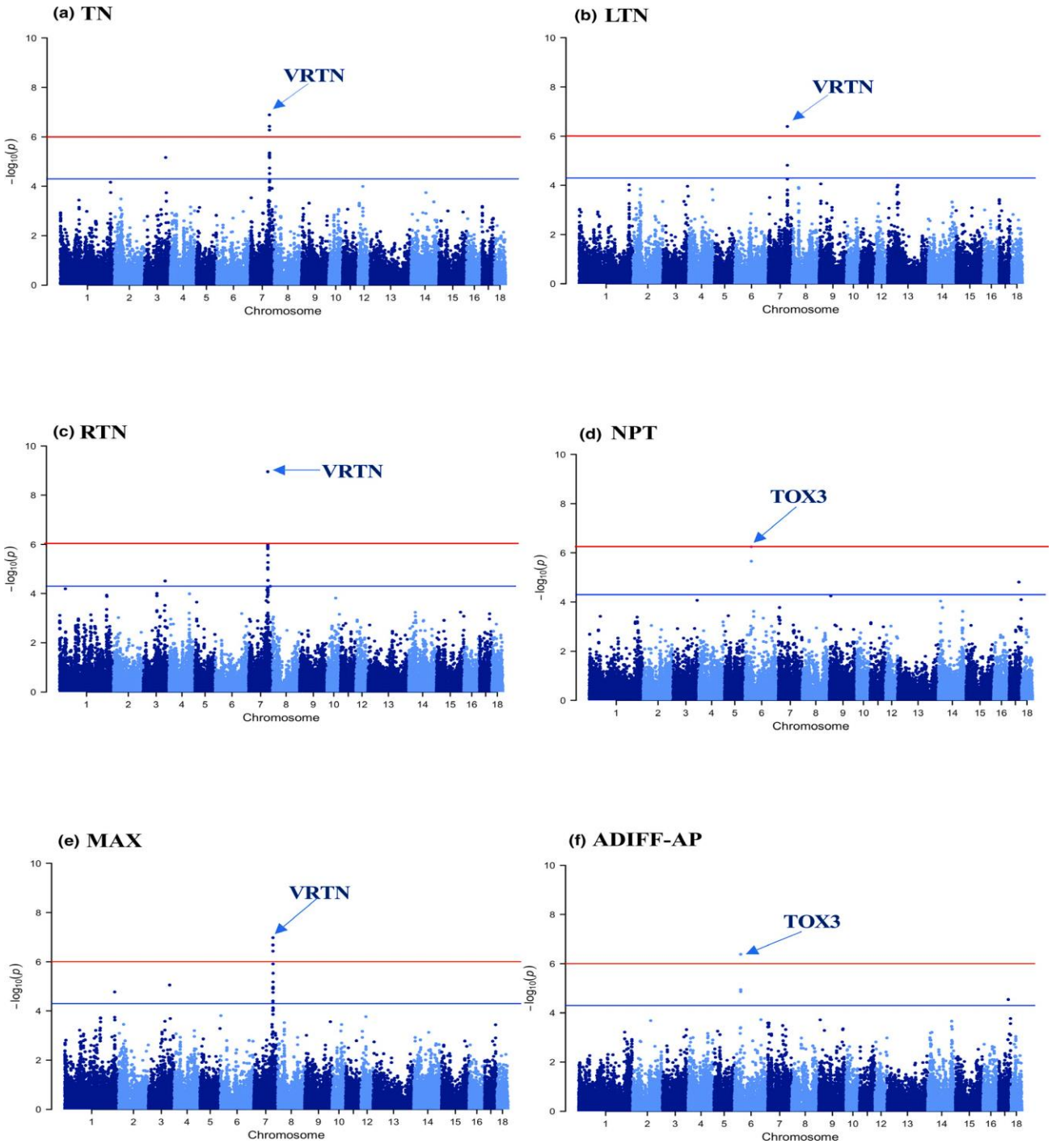

Figure 1. Manhattan plots obtained in the GWAS with teat number-related parameters. The plots are reported only for the six parameters which had significant markers. The red line identifies the threshold for statistical significance; the blue line indicates the suggestively significance threshold. (a) TN, total number of teats; (b) LTN, number of left line teats; (c) RTN, number of right line teats; (d) NPT, number of posterior teats; (e) MAX, the maximum number of teats between the two sides; and (f) ADIFF-AP, absolute difference between anterior and posterior teats (|NAT – NPT|).

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Supplementary materials

Table S1. Summary of basic statistics on the number of teats and related parameters included in this study observed in Italian Large White population. Single nucleotide polymorphism heritability (h^2_{SNP}) is reported together with the genomic inflation factor value (λ) obtained in the corresponding genome-wide association studies.

Parameters	**Acronym**	**No. of pigs**[1]	**Mean**[2]	**s.d.**[3]	**Median**[4]	$\boldsymbol{h^2_{SNP}}$	**s.e.**[5] **of** $\boldsymbol{h^2_{SNP}}$	**λ**
Total number of teats	TN	821	14.79	0.96	15	0.360	0.072	1.019
Total number of left line teats	LTN	821	7.34	0.58	7	0.158	0.055	1.021
Total number of right line teats	RTN	821	7.45	0.60	7	0.258	0.070	1.025
Total number of anterior teats	NAT	667	6.66	0.85	7	<0.001	0.036	0.966
Total number of posterior teats	NPT	667	8.13	0.62	8	0.034	0.036	1.012
Max number of teats comparing the two sides	MAX	821	7.61	0.59	8	0.261	0.068	1.026
Signed difference between RTN and LTN (RTN - LTN)	SDIFF	821	0.10	0.69	0	<0.001	0.037	0.963
Absolute difference between RTN and LTN (|RTN – LTN|)	ADIFF	821	0.44	0.55	0	0.051	0.041	1.023
Max number of teats between anterior and posterior parts	MAX-AP	667	8.18	0.58	8	<0.001	0.036	0.966
Signed difference between anterior and posterior teats (NAT - NPT)	SDIFF-AP	667	-1.47	1.18	-2	<0.001	0.045	0.916
Absolute difference between anterior and posterior teats (|NAT – NPT|)	ADIFF-AP	667	1.56	1.06	2	0.030	0.033	1.007
Ratio between posterior and anterior teats	NPT/NAT	667	1.24	0.22	1.28	0.033	0.039	1.064
Extra teats	EX-T	821	0.43	0.54	0	<0.001	0.187	0.982

[1]Number of pigs with the phenotype information and that were included in the genome-wide association studies: for some parameters it was not possible to obtain the information for all animals. Particularly, the position of the navel was not always possible to clearly distinguish from the available pictures.

[2]Mean: average of the number of teats.

[3]s.d.: standard deviation of the number of teats.

[4]Median: median of the number of teats.

[5]s.e. of h^2_{SNP}: standard error of the chip heritability.

Table S2. Suggestively associated markers identified in the genome-wide association studies.

Parameter[1]	SSC[2]	Marker position[3]	Marker	Alleles[4]	MAF[5]	*p*-value[6]	Closest gene[7]	QTL[8]
TN	7	97795647	M1GA0010653	A/G	0.482	1.10×10^{-06}	*VRTN* (171374)	TVN
	7	97881096	H3GA0022659	A/G	0.464	1.66×10^{-06}	*VRTN* (256823)	TVN
	3	108957096	ALGA0020825	A/C	0.032	8.47×10^{-06}	*LCLAT1* (75074)	-
	7	97147161	H3GA0022644	G/A	0.297	1.49×10^{-05}	*ELMSAN1* (24195)	TVN
	7	98155866	M1GA0010658	C/A	0.393	1.53×10^{-05}	*ENSSSCG00000050616* (0)	VN, TVN
	7	97752476	DIAS0000795	G/A	0.439	1.59×10^{-05}	*LTBP2* (0)	TVN
	7	97732109	ASGA0035500	A/G	0.438	1.84×10^{-05}	*NPC2*(0)	TVN
	7	98595714	ALGA0122954	G/A	0.402	2.24×10^{-05}	*JDP2* (0)	VN, TVN, RTN
LTN	7	97619490	VRTN*	A/G	0.393	1.52×10^{-05}	*VRTN* (0)	NNF, TVN
RTN	7	97619490	VRTN*	A/G	0.393	1.16×10^{-06}	*VRTN* (0)	NNF, TVN
	7	98155866	M1GA0010658	C/A	0.393	1.32×10^{-06}	*ENSSSCG00000050616* (0)	VN, TVN
	7	97795647	M1GA0010653	A/G	0.482	1.53×10^{-06}	*VRTN* (171374)	TVN
	7	98264173	ASGA0035536	C/A	0.477	2.75×10^{-06}	*ACYP1* (0)	VN, TVN
	7	98595714	ALGA0122954	G/A	0.402	5.45×10^{-06}	*JDP2* (0)	VN, TVN, RTN
	7	97752476	DIAS0000795	G/A	0.439	8.87×10^{-06}	*LTBP2* (0)	TVN
	7	97732109	ASGA0035500	A/G	0.438	1.01×10^{-05}	*NPC2*(0)	TVN
	7	98566049	ALGA0112470	G/A	0.485	2.93×10^{-05}	*JDP2* (8149)	VN, TVN, RTN
	3	112227667	ALGA0020993	A/G	0.468	3.08×10^{-05}	*DPYSL5* (0)	TVN
	1	159785087	ASGA0101718	A/G	0.151	4.65×10^{-05}	*CDH20* (32470)	TN
	7	110378434	DRGA0008163	A/G	0.363	5.03×10^{-05}	*PTPN21* (0)	TN
NAT	6	32502689	ASGA0027999	A/G	0.303	5.25×10^{-05}	*TOX3* (0)	TN
NPT	6	32604025	ALGA0035073	G/A	0.239	2.19×10^{-06}	*TOX3* (0)	TN
	6	32529342	ASGA0100674	A/G	0.170	1.42×10^{-05}	*TOX3* (0)	TN
	17	49368877	ASGA0077390	A/G	0.070	1.53×10^{-05}	*ZMYND8* (0)	LVN, THVN
MAX	7	98155866	M1GA0010658	C/A	0.393	1.23×10^{-06}	*ENSSSCG00000050616* (0)	VN, TVN
	7	98595714	ALGA0122954	G/A	0.402	2.92×10^{-06}	*JDP2* (0)	VN, TVN, RTN
	7	98264173	ASGA0035536	C/A	0.477	6.63×10^{-06}	*ACYP1* (0)	VN, TVN
	7	95984726	ALGA0109584	A/G	0.222	6.91×10^{-06}	*DPF3* (0)	TN
	3	108957096	ALGA0020825	A/C	0.032	8.79×10^{-06}	*LCLAT1* (75074)	TN

	7	98440639	MARC0050386	A/G	0.476	1.06×10^{-05}	-	VN, TVN
	7	98066911	H3GA0022664	A/G	0.463	1.11×10^{-05}	*PROX2* (0)	TVN
	7	97347282	INRA0027622	A/G	0.358	1.20×10^{-05}	*BBOF1* (0)	TVN
	7	98089286	ASGA0035527	G/A	0.466	1.24×10^{-05}	*DLST* (1837)	TVN
	1	254612941	ALGA0009455	G/A	0.386	1.69×10^{-05}	*ZNF618* (0)	TN
	7	98116120	DIAS0001088	G/A	0.464	1.76×10^{-05}	*RPS6KL1* (0)	TVN
	7	97752476	DIAS0000795	G/A	0.439	3.86×10^{-05}	*LTBP2* (0)	TVN
	7	97732109	ASGA0035500	A/G	0.438	4.67×10^{-05}	*NPC2*(0)	TVN
ADIFF	11	11961785	ALGA0060867	A/G	0.277	2.17×10^{-06}	*DCLK1* (0)	TN
	5	102805263	M1GA0008203	C/A	0.086	4.38×10^{-05}	*NAV3* (208535)	TVN
	16	70736511	MARC0039548	A/G	0.224	5.26×10^{-05}	*NMUR2* (60195)	TN
ADIFF-AP	6	32604025	ALGA0035073	G/A	0.239	1.13×10^{-05}	*TOX3* (0)	TN
	6	32678775	ALGA0035080	G/A	0.275	1.37×10^{-05}	*TOX3* (68847)	TN
	17	49368877	ASGA0077390	A/G	0.070	2.87×10^{-05}	Z*MYND8* (0)	LVN, THVN
NPT/NAT	16	76153481	ASGA0099992	G/A	0.295	1.28×10^{-05}	-	TN
	11	9046973	H3GA0031250	G/A	0.264	1.30×10^{-05}	*PDS5E* (0)	TN
	9	5519984	ASGA0041143	A/G	0.242	3.12×10^{-05}	-	-
	14	132709906	DBWU0000067	G/A	0.070	3.81×10^{-05}	*ATP5PB* (0)	-
	9	3184033	MARC0002885	A/G	0.298	4.01×10^{-05}	*RRP8* (21421)	-
EX-T	4	23524316	ALGA0024067	G/A	0.399	8.06×10^{-06}	*TRPS1* (365547)	TN
	11	72970630	CASI0009242	G/A	0.074	1.20×10^{-05}	-	-
	3	124103822	H3GA0010881	G/A	0.167	1.33×10^{-05}	*TRIB2* (66855)	TN
	4	23121547	INRA0013304	A/G	0.279	3.32×10^{-05}	*TRPS1* (0)	TN

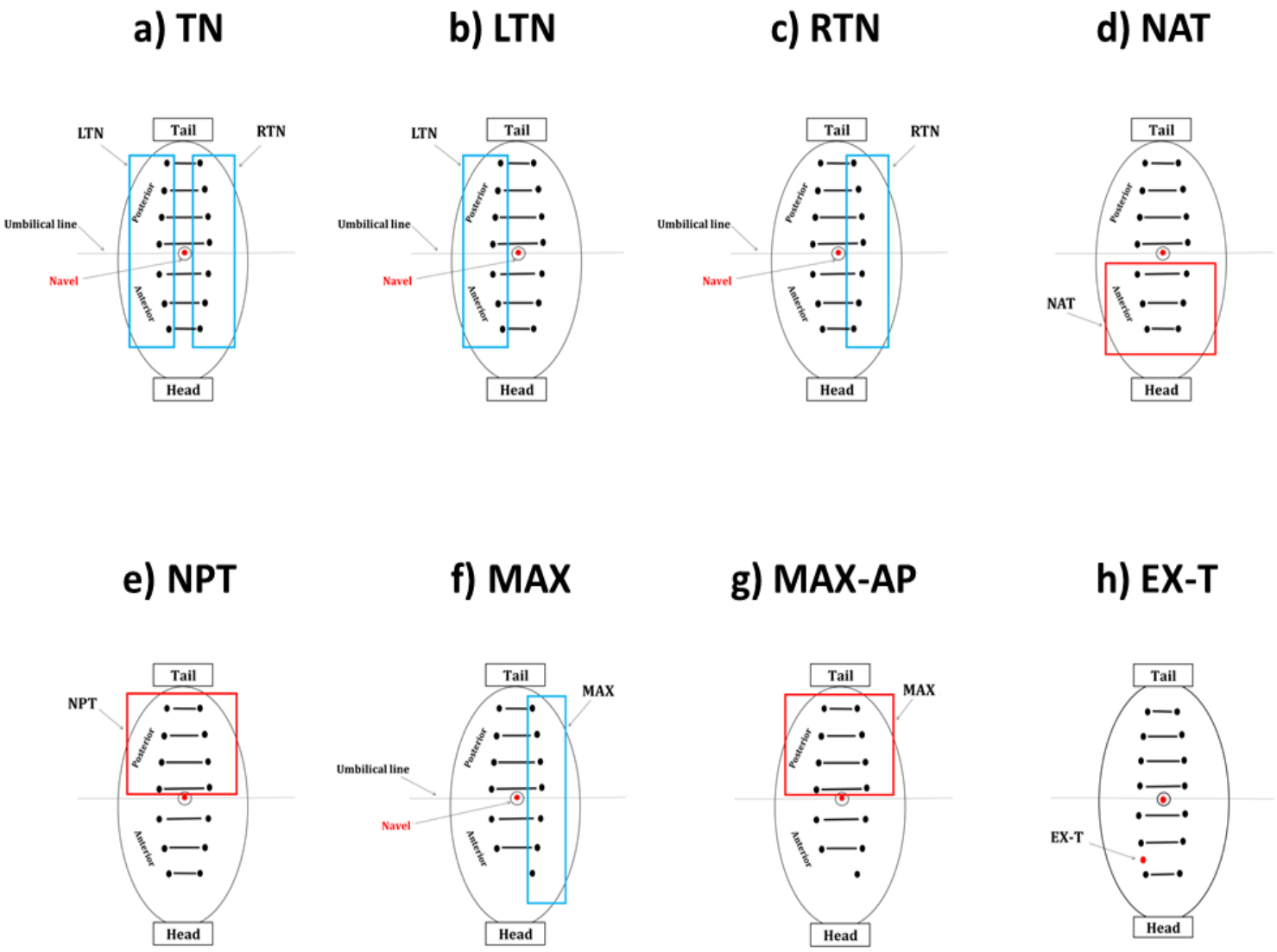
[1] TN, total number of teats; LTN, number of left line teats; RTN, number of right line teats; NAT, number of anterior teats; NPT, number of posterior teats; MAX, maximum number of teats between the two sides; ADIFF, absolute difference between RTN and LTN (|RTN – LTN|); ADIFF-AP, absolute difference between anterior and posterior teats (|NAT – NPT|); NPT/NAT, ratio between the number of poster and the number of anterior teats; EX-T, extra teats. Suggestively significant markers were also identified for all traits for which significant markers were reported in Table 1.

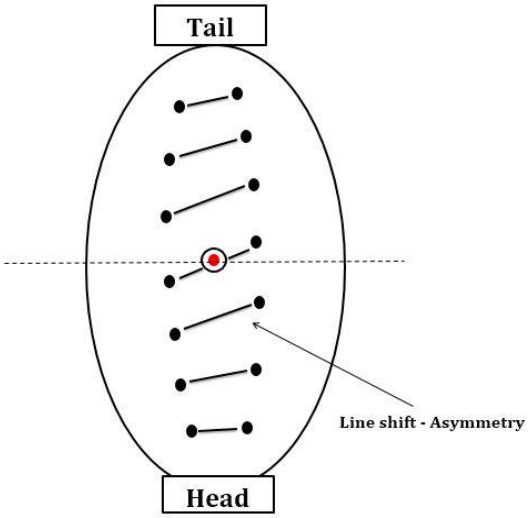
[2] Porcine chromosome.
[3] Position of the marker on the corresponding chromosome in the Sus scrofa11.1 genome version.
[4] Minor and major alleles.
[5] Minor allele frequency.
[6] *p*-value from GEMMA (Wald test).

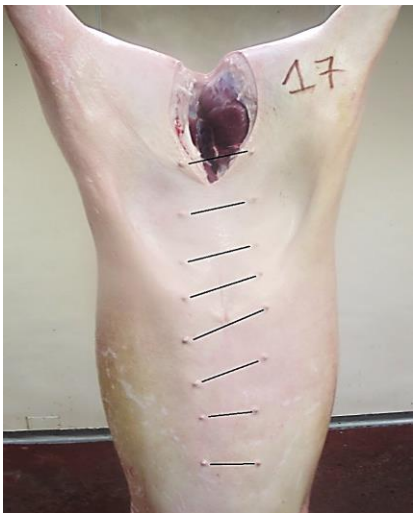
[7] The closest gene to the indicated marker in a region of ± 500 kbp. The distance of the marker to the gene is indicated in brackets (bp). Zero means that the marker was within the gene.

[8] QTL identified in a region of ± 200 kbp with what reported in PigQTL db, related to morphological traits that might be related/correlated to the investigated teat parameters. Short names for QTL are: TVN, Thoracic vertebra number; NNF, Number of non-viable fetuses; VN, Vertebra number; RTN, Right teat number; THVN, Thoracolumbar vertebra number; LVN, lumbar vertebra number; TN, teat number.
*In house genotyped AB554652:g.20311_20312ins291 VRTN related polymorphism (Fontanesi et al., 2014b).

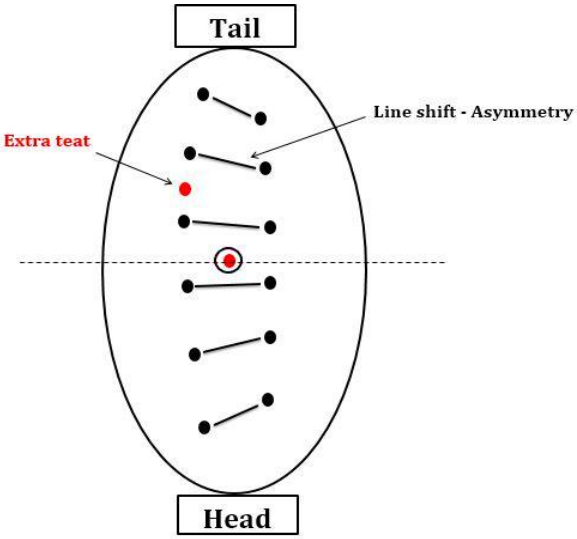
Figure S1. Schematic representation of the eight basic teat number related parameters included in the study: a) TN, total number of teats; b) LTN, number of left line teats; c) RTN, number of right line teats; d) NAT, number of anterior teats; e) NPT, number of posterior teats; f) MAX, maximum number of teats comparing the two sides; g) MAX-AP, maximum number of teats between anterior and posterior parts; h) EX-T, extra teats. The other parameters are derived from these basic parameters: SDIFF, signed difference between RTN and LTN (RTN - LTN); ADIFF: absolute difference between RTN and LTN (|RTL – LTN|); SDIFF-AP, signed difference between NAT and NPT (NAT - NPT); ADIFF-AP, absolute difference between NAT and NPT (|NAT - NPT|); ratio between posterior and anterior teats (NPT/NAT).

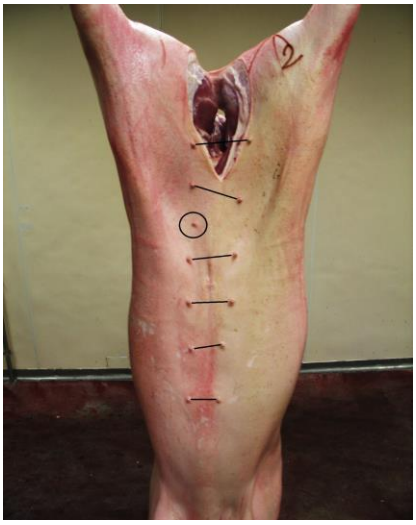




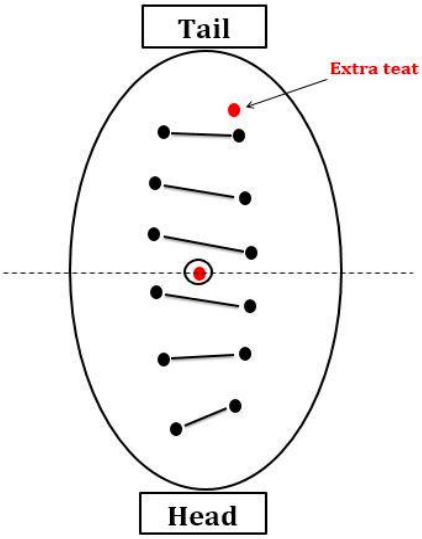


Asymmetry pattern. The same number of teats in two sides (right and left) and in asymmetry form

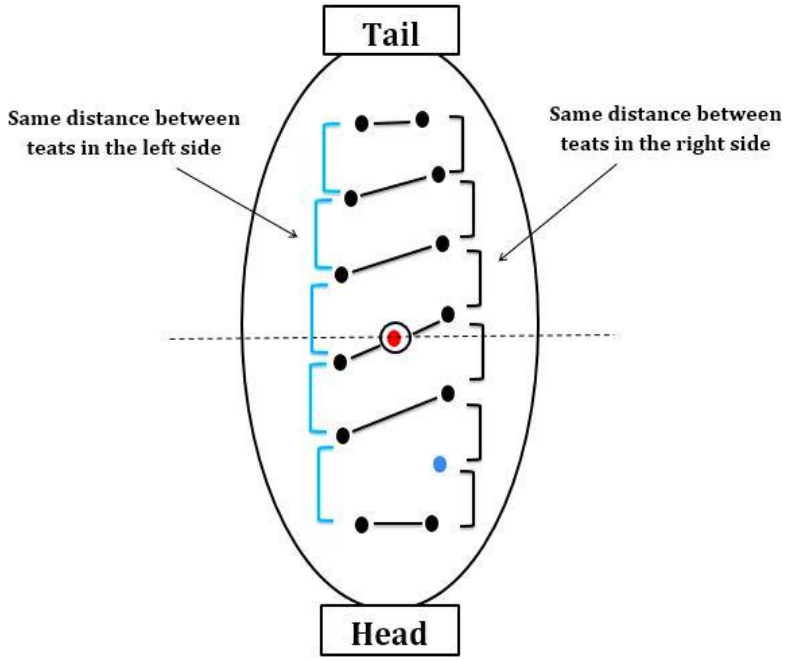


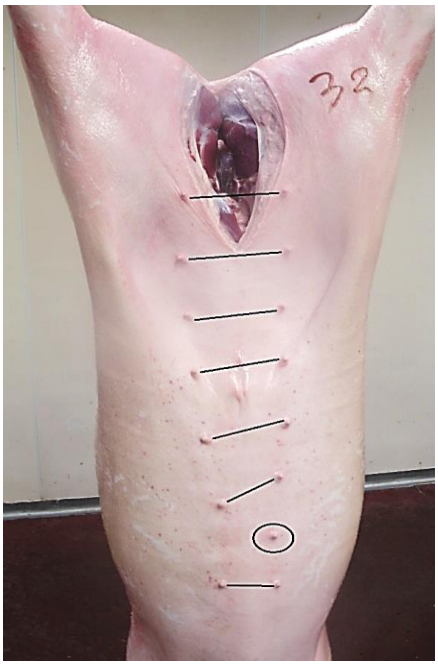


Asymmetry pattern. Different number of teats in two sides (right and left) because of the extra teat between teats inside the lines but in asymmetry form

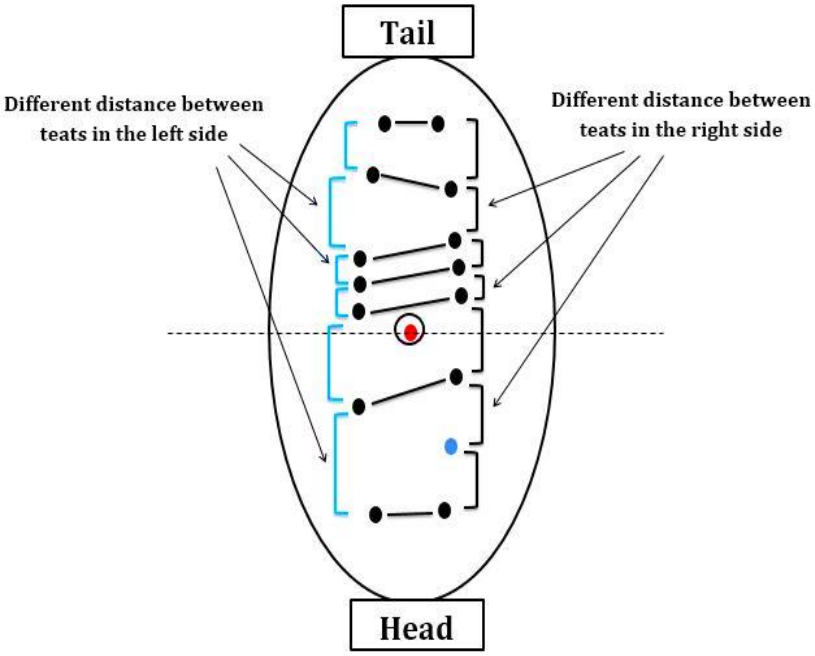


Asymmetry pattern. Different number of teats in two sides (right and left) because of the extra teat outside the teat lines near the tail but in asymmetry form





Asymmetry pattern. Different number of teats in two sides (right and left) because of the extra teat inside the teat lines but in asymmetry form and the distance between the teats in left and right sides is the same for each side



Asymmetry pattern. Different number of teats in two sides (right and left) because of the extra teat inside the teat lines but in asymmetry form and the distance between the teats and their position in left and right sides are different for each side

Figure S2. Quantile-quantile plots of the six genome-wide association studies described in Table 1: a) TN, total number of teats; b) LTN, number of left line teats; c) RTN, number of right line teats; d) NPT, number of posterior teats; e) MAX, the maximum number of teats between the two sides; f) ADIFF-AP, absolute difference between anterior and posterior teats (|NAT – NPT|).

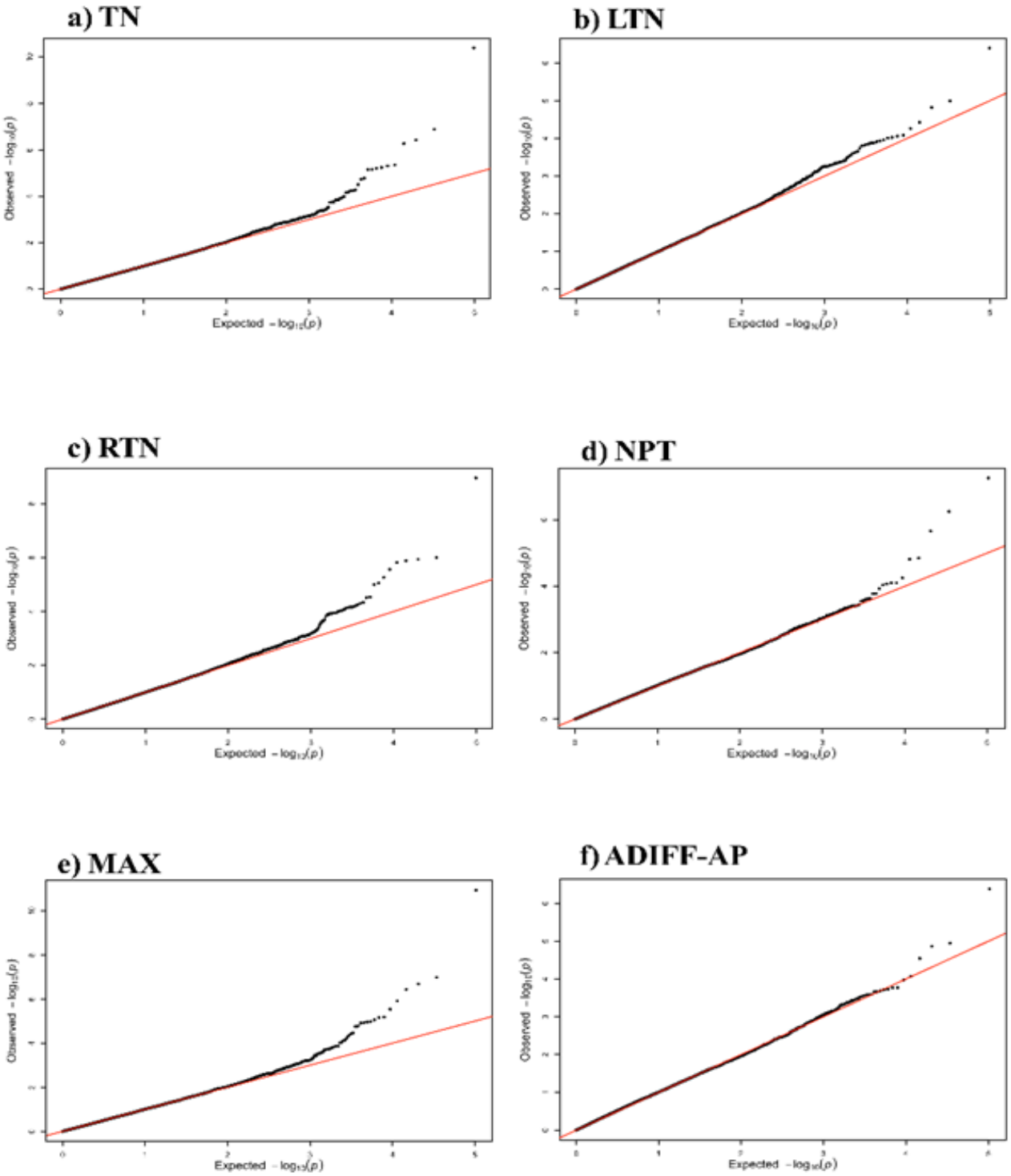


Figure S3. Manhattan plots (left) and quantile-quantile plots (right) of the four genome-wide association studies for which suggestively significant markers were identified: a) ADIFF, absolute difference between RTN and LTN (|RTL – LTN|); b) NAT, number of anterior teats; c) NPT/NAT, ratio between posterior and anterior teats; d) EX-T, extra teats.

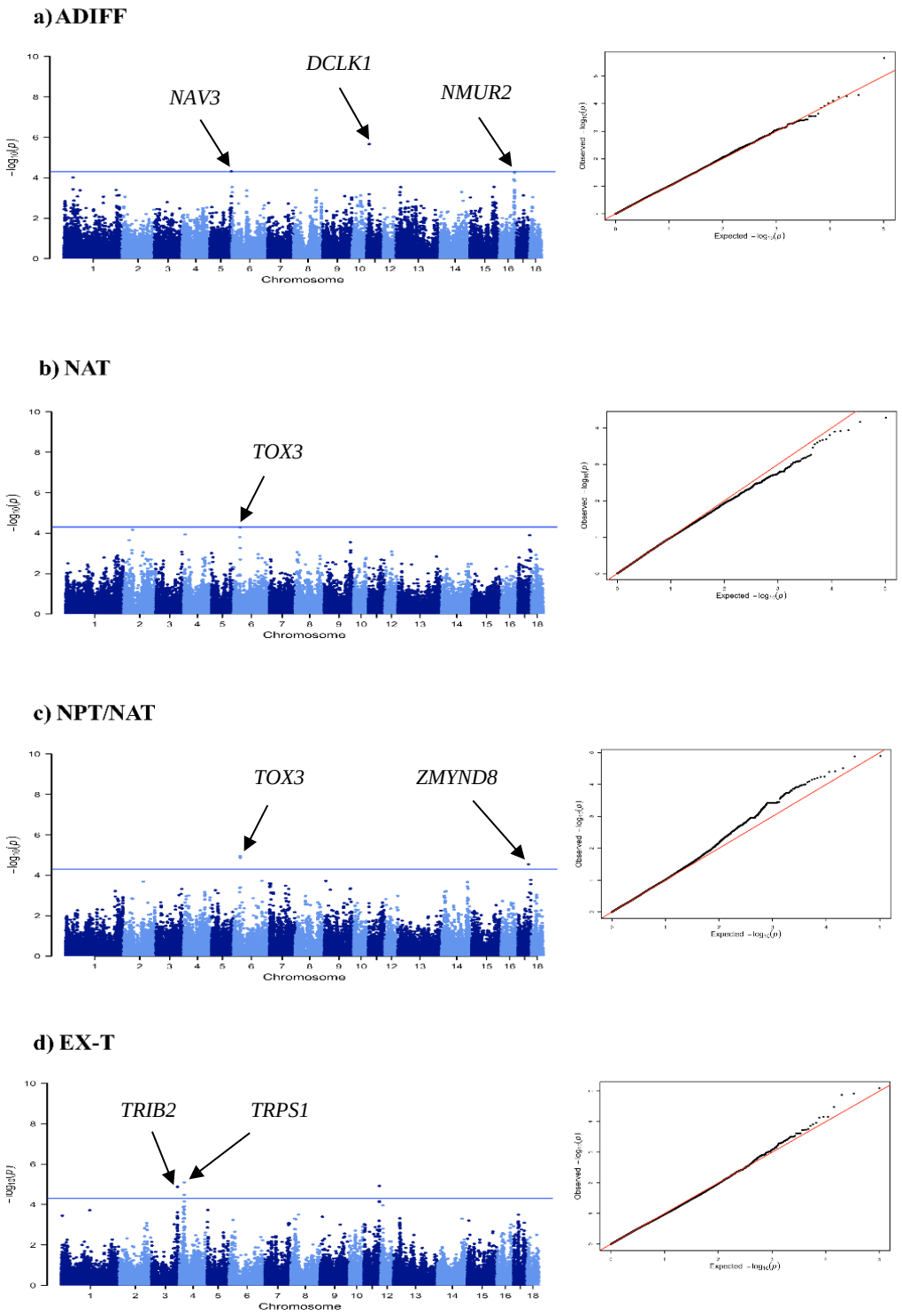
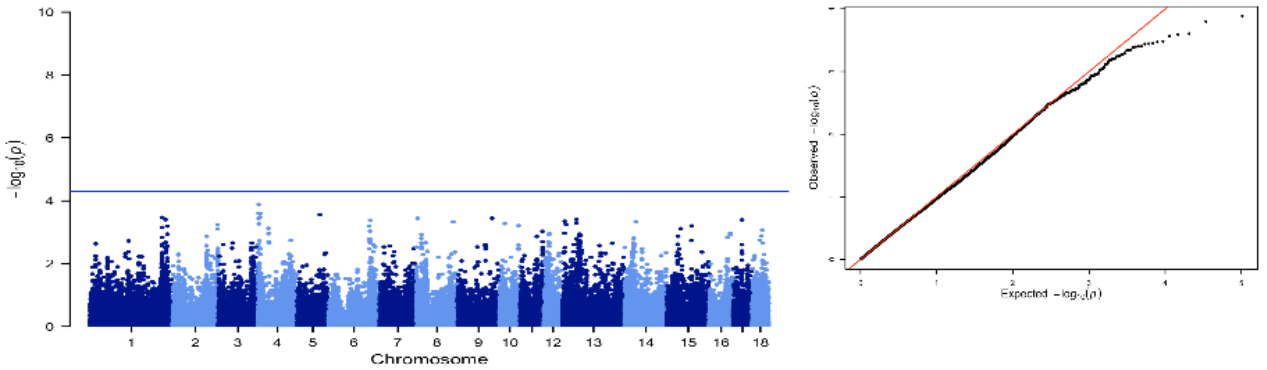
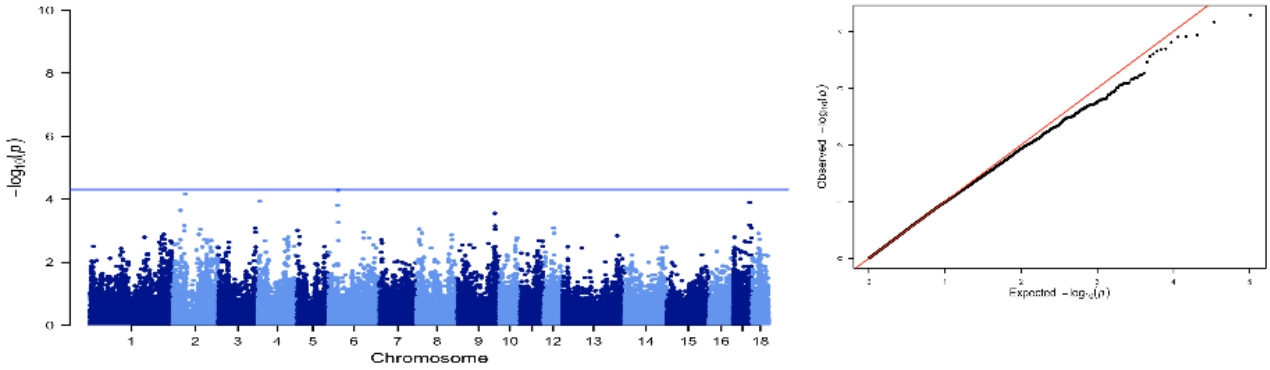


Figure S4. Manhattan plots (left) and quantile-quantile plots (right) of the three genome-wide association studies for which no significant or suggestively significant markers were identified: a) SDIFF, signed difference between RTN and LTN (RTN - LTN); b) MAX-AP, maximum number of teats between anterior and posterior parts; c) SDIFF-AP, signed difference between anterior and posterior teats (NAT - NPT).

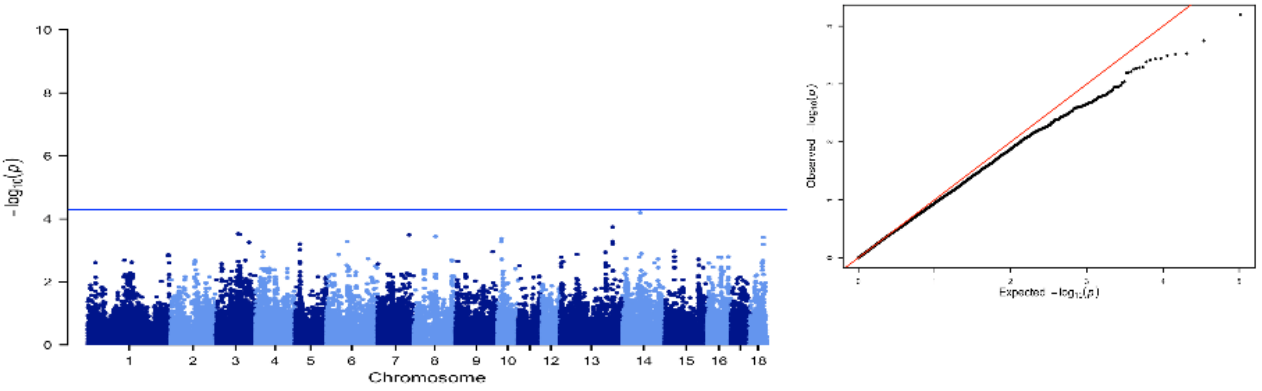
a) SDIFF



b) MAX-AP



c) SDIFF-AP



Final conclusions

This thesis provided some insights into the genetic architecture of the Reggina as one of the important local cattle breeds of Italy as well as the Italian Large White pig breed related to several morphological traits that might be important to define breed-specific traits and economical relevant aspects. In this study, the detected chromosomal regions and mutations showed associations with the studied morphological traits in the Reggiana cattle and Italian Large White pig.

In the Reggiana cattle, the potential practical applications of the obtained results could be related to the gene markers associated with some important morphological traits including i) especially muzzle colour which is useful for breed identification (cows carrying *e* allele at *MC1R* locus and with *ee* genotype causing the typical red coat colour and light-colored muzzle, the most suitable for the production of original Parmigiano Reggiano cheese), authentication of Parmigiano Reggiano cheese obtained from only Reggiana milk through genetic test, and refining breed standards, ii) stature that might influence some reproductive traits, and iii) the presence of the supernumerary nipples that in some cases are considered as a negative feature for udder and machine milking profitability.

Regarding the need to increase the number of teats in Italian Large White pigs, our study could identify gene markers associated with: i) the total number of teats as *VRTN* which in turn could affect the number of vertebrae in pigs, and teat number difference based on different parts of the body and suggestively teat asymmetry pattern as *TOX3*, and altogether, this increase can be fundamental for piglet production and survival rate since it guarantees that the born piglets access properly to the teats and ii) associated suggestively with the presence of the extra teats in different locations that could be problematic when suckled by piglets and disrupts the normal order of teats.

The obtained results could be the potential tools in terms of production system, management of genetic resources, and to pass on ideal traits to the next generation. Taken together, our results can be considered a basis for the use of genetic variability within and among cattle and pig populations. At the same time, the large amount of produced data represents a profitable source of information for comparative purposes and it opens the path to further research aiming to better describe the genetic potential of the local cattle and pig breeds. Another outcome of these analyses could be the identification of breed-specific genomic features for the development of DNA-based tools for traceability and authentication of mono-breed products which would be needed for sustainable conservation of these genetic resources. Mining at the genome level the variability segregating in the local cattle and pig populations could provide additional information to understand the genetic basis of complex and economically relevant traits.

Additional studies with the higher number of animals need to be performed to further refine the obtained results and discover the causative mutations especially of *LCORL* on BTA6 for stature and *TBX3* and *TBX5* on BTA17 for the presence of supernumerary nipple in cattle.