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The genomics behind beauty: disentangling the complex coat color genetic architecture of gir breed

Marcelo José Böck
Magister Scientiae

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Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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Assent:

Marcelo José Böck
Author

Simone Eliza Facioni Guimaraes
Adviser

To my parents, for their constant support and for always believing in me and in my potential. To my brother, sister-in-law, and nephew, as well as to my other family members and friends, for their encouragement and for inspiring me to persevere and continually strive for growth. I dedicate this dissertation.

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“When I am afraid, I put my trust in You.”
— Psalm 56:3

ABSTRACT

BÖCK, Marcelo José, M.Sc., Universidade Federal de Viçosa, February, 2026. **The genomics behind beauty: disentangling the complex coat color genetic architecture of gir breed**. Adviser: Simone Eliza Facioni Guimaraes.

Coat color in cattle is a phenotypic trait of high biological, zootechnical, and economic relevance, having historically been used as a visual criterion in selection programs and as a marker associated with adaptive and productive processes. In zebu breeds such as Gir (Bos indicus), this trait exhibits wide phenotypic variability, constituting an important component of breed identity and attracting scientific interest in tropical dairy production systems. Pigmentation results from the activity of melanocytes, which are responsible for melanin synthesis within melanosomes and is regulated by molecular networks that control the production of eumelanin and pheomelanin through specific signaling pathways. Within the context of population genomics, parameters such as linkage disequilibrium (LD) and effective population size (Ne) allow the characterization of population genetic structure, whereas genome-wide association studies (GWAS) enable the investigation of the genetic architecture underlying coat color. In this study, phenotypic records from 50,341 animals classified into 25 coat color categories were used, along with genomic data from 55,154 Gir cattle. After quality control, 389,172 SNPs were retained for the analyses. Heritability estimates ranged from 0.16 to 0.75, indicating moderate to high genetic control. The average LD (r^2) was 0.064 at 50 kb, while effective population size declined from 53.9 individuals in generation 50 to 13.8 individuals in generation 5. Genomic windows of 0.5 Mb explained up to 24% of the additive genetic variance, enabling the identification of 52 candidate genes associated with coat color variation. The identified genes are related to processes such as melanocyte migration and development, cellular differentiation, cytoskeleton organization, signal transduction, and melanogenesis pathways. Taken together, the results indicate that coat color variation in the Gir breed is controlled by a complex and polygenic genetic architecture, involving multiple genes with pleiotropic effects, with implications for genetic improvement programs, conservation strategies, and the valorization of the breed in tropical production systems.

Keywords: pigmentation; genetic variability; coat color.

RESUMO

BÖCK, Marcelo José, M.Sc., Universidade Federal de Viçosa, fevereiro de 2026. **A Genômica por Trás da Beleza: Desvendando a Arquitetura Genética Complexa da Cor da Pelagem da Raça Gir.**. Orientadora: Simone Eliza Facioni Guimaraes.

A cor de pelagem em bovinos constitui um caráter fenotípico de elevada relevância biológica, zootécnica e econômica, sendo historicamente empregada como critério visual em programas de seleção e como marcador associado a processos adaptativos e produtivos. Em raças zebuínas, como a Gir (Bos indicus), essa característica apresenta ampla variabilidade fenotípica, compondo parte importante da identidade da raça e despertando interesse científico na pecuária leiteira tropical. A pigmentação resulta da atividade dos melanócitos, responsáveis pela síntese de melanina nos melanossomos, regulada por redes moleculares que controlam a produção de eumelanina e feomelanina por vias de sinalização específicas. No contexto da genômica de populações, parâmetros como o desequilíbrio de ligação (LD) e o tamanho efetivo populacional (Ne) permitem caracterizar a estrutura genética da população, enquanto os estudos de associação genômica ampla (GWAS) possibilitam investigar a arquitetura genética da cor de pelagem. Neste estudo, foram utilizados registros fenotípicos de 50.341 animais classificados em 25 categorias de coloração, juntamente com dados genômicos de 55.154 indivíduos da raça Gir. Após o controle de qualidade, 389.172 SNPs foram mantidos para as análises. As estimativas de herdabilidade variaram de 0,16 a 0,75, indicando controle genético moderado a elevado. O valor médio de LD (r^2) foi de 0,064 a 50 kb, enquanto o tamanho efetivo populacional apresentou declínio de 53,9 indivíduos na geração 50 para 13,8 indivíduos na geração 5. As janelas genômicas de 0,5 Mb explicaram até 24% da variância genética aditiva, permitindo a identificação de 52 genes candidatos associados à variação de cor de pelagem. Os genes identificados estão associados a processos como migração e desenvolvimento de melanócitos, diferenciação celular, organização do citoesqueleto, transdução de sinais e vias da melanogênese. Em conjunto, os resultados indicam que a variação da cor de pelagem na raça Gir é controlada por arquitetura genética complexa e poligênica, envolvendo múltiplos genes com efeitos pleiotrópicos, com implicações para programas de melhoramento genético, conservação e valorização da raça em sistemas de produção tropical.

Palavras-chave: pigmentação; variabilidade genética; cor de pelagem

SUMMARY

CHAPTER 1 –GENERAL CONSIDERATIONS

1 INTRODUCTION

The visual appearance of animals is one of the oldest and most widely used traits in selection. Color diversity plays a crucial role in adaptation and natural selection, attracting considerable interest in evolutionary biology (SENCZUK *et al.*, 2020). As observed by Mendel, color variation often follows Mendelian inheritance patterns, with specific genes influencing phenotypes through the regulation of pigment synthesis or the activity of melanocytes, the cells responsible for pigment production (SPONENBERG, 2009).

Pigment patterning in mammals involves the development of melanocytes, cells that function intrinsically within the epidermis (JACKSON, 1994). The distribution and density of melanin depend on the interaction of multiple genes regulating eumelanin and pheomelanin synthesis (CARO; MALLARINO, 2020). The expression of these genes is closely linked to individual fitness, being essential for sexual signaling, camouflage, physiological processes, protection against UV radiation, and defense against parasites (ESPMARK; AMUNDSEN; ROSENQVIST, 2000; KENNAH et al., 2023; OLI et al., 2023).

Coat color attracts considerable interest among breeders, either for aesthetic reasons, as in horses (RAUDSEPP *et al.*, 2019) or for economic purposes, as in cattle (KASSIE; ABDULAI; WOLLNY, 2011), sheep (TERFA *et al.*, 2013) and rabbits (HU *et al.*, 2019). While many horse breeders value coat variation, other livestock species show lower socio-economic appeal, such as certain cattle breeds (SENCZUK *et al.*, 2020). Some *Bos taurus* breeds, including Jersey (ACGJB, 2024), Holstein (ABCBRH, 2024) and Brown Swiss (ACGJB, 2024), display well-defined coat patterns, whereas *Bos indicus* breeds, such as Gir, exhibit high color diversity. This chromatic variability results from crossbreeding in its native region of Gujarat, India, of animals with different coat patterns, giving rise to the modern Gir breed.

Qualitative traits, particularly coat color, may exhibit pleiotropic effects, which explains the caution of breed associations and private companies in identifying genes related to this trait. Adverse effects have been observed in dogs (MARSDEN *et al.*, 2016) and horses (BELLONE, R. R., 2010) and some evidence suggests that certain genes associated with coat color in cattle may influence embryonic development. These findings support the theory of FALCONER & MACKAY, (1996), which states that considering only quantitative traits as selection criteria is limited, since not all traits show positive correlations with each other.

Although coat color is an easily observable phenotype, the genetic mechanisms underlying the extensive variability of colors and patterns in the Gir breed remain poorly

understood, particularly in genotyped animals. Identifying genes associated with this diversity can add value to breeder catalogs, enable correlations with production traits such as longevity, productivity, and resilience, and support the standardization of commercial herds. Accordingly, this chapter of the dissertation reviews general aspects of the Gir breed, linkage disequilibrium (LD) analyses, genome-wide association studies (GWAS), effective population size, as well as melanocyte development and the main genes associated with pigmentation, including modifying genes and post-GWAS approaches.

2 LITERATURE REVIEW

2.1 Gir history

Brazil has the largest commercial cattle population in the world, exceeding 224 million heads in 2023 (FAOSTAT, 2023; IBGE, 2023). It is estimated that approximately 80% of this population consists of Zebu cattle (*Bos indicus*) (ABCZ, 2024). During the 19th and 20th centuries, cattle breeds from India were imported to several countries, with around 7,000 indicine animals introduced into Brazil (SANTIAGO, A. A., 1987). This introduction was facilitated by Spanish and Portuguese colonizers, who brought both Zebu (*Bos indicus*) and taurine (*Bos taurus*) cattle (WILKINS, 1984).

Although the first cattle raised and selected in Brazil were taurine, brought by Portuguese ships, the contribution of Zebu breeds of Indian origin to Brazilian livestock has been significant (EMBRAPA GADO DE CORTE, 2024). The extensive presence of herds composed of Zebu cattle and their crosses has driven the development of both meat and milk production (VILELA, D *et al.*, 2017). In dairy production, European breeds such as Holstein and Jersey stand out for their high productivity under favorable conditions (HERMAS; YOUNG; RUST, 1987; ORJALES *et al.*, 2019; VILELA, DUARTE *et al.*, 2006). However, Brazil's vast territory, combined with challenging climatic conditions, requires more resilient and productive animals, such as the Gir breed, widely used in crossbreeding with specialized taurine breeds for dairy production (FREITAS *et al.*, 2001).

The Gir breed is widely recognized for its adaptability to Brazil's tropical conditions, enjoying strong acceptance among farmers and expanding across all regions of the country (SANTANA et al., 2014). In addition to its remarkable thermoregulation capacity, the Gir Dairy is considered the highest milk-producing Zebu in tropical climates. Its robustness and distinctive characteristics have attracted considerable interest, driving its growth in global dairy production (ABCGIL, 2024).

2.2 Genetic markers

Genetic markers can be classified into three main categories: morphological, biochemical, and molecular. Morphological markers refer to observable phenotypic traits, such as coat color. Biochemical markers, in turn, identify variations in proteins or metabolites, including isoenzymes. Molecular markers, widely recognized and used in scientific literature, detect polymorphisms in DNA, offering high specificity and applicability in genetic studies. This classification has been widely adopted due to its effectiveness across different research contexts (AIKEN; ALDERMAN, 2024).

In other words, genetic markers indicate any genetic polymorphism between two or more individuals. It is common for genetic or genomic markers to be located within introns or in fragments with no known expression. Their main function is to identify (mark) a specific site or region of a chromosome. In animal breeding, genetic markers are the most widely used, since traits of economic interest are typically polygenic and are rarely detected by morphological markers. This is because approximately 90% of DNA (deoxyribonucleic acid) consists of non-coding regions, and polymorphisms in these areas tend to be stable, not resulting in phenotypic changes except in specific cases (STAFUZZA; NUNES, 2012).

The RFLP (Restriction Fragment Length Polymorphism) technique was the first to employ restriction enzymes, which recognize and cleave specific regions of DNA composed of palindromic sequences of 4 to 8 base pairs. This approach detects variations in DNA that arise from the presence or absence of restriction sites, often caused by mutations or genetic rearrangements. These alterations result in fragments of different lengths, which are used as genetic markers (GRIFFITHS et al., 2006).

Other types of markers, such as microsatellites also known as SSR (Simple Sequence Repeats) or STR (Short Tandem Repeats) markers consist of tandemly repeated sequences of 1 to 6 nucleotides, found in various regions of eukaryotic genomes. These markers are analyzed through the polymerase chain reaction (PCR) technique and are more polymorphic than RFLPs, being widely used in paternity testing and traceability of animal-derived products, among other applications. However, a limitation of this approach is its restricted genomic coverage, which may reduce the scope of analyses (TÓTH; GÁSPÁRI; JURKA, 2000).

Finally, SNPs (single nucleotide polymorphisms) are genetic variations that involve the alteration of a single nucleotide in the DNA sequence, resulting from mutations and inherited as allelic variants with a minimum frequency of 1% in the population (KARKI *et al.*, 2015).

These polymorphisms are identified through DNA sequencing by comparing samples with a reference sequence of the species in question. SNPs offer several advantages, such as greater heritable stability than microsatellites, broad genomic coverage, and the ability to directly affect phenotypic variation. In addition, they are suitable for DNA chip technology (CAETANO, 2009).

The choice of the most appropriate molecular marker depends on several factors, including cost, practicality, and the feasibility of automation. The increasing use of tools such as genotyping has been progressively adopted by companies and breeding associations (AZOVTSEVA *et al.*, 2024). The application of high-throughput genetic markers, particularly SNPs, enables the generation of large volumes of data at reduced costs, making it possible to conduct association studies between genotypes and phenotypes. This approach holds great potential for advancing genetic research and improving desirable traits in animal breeding (SURAVAJHALA; KOGELMAN; KADARMIDEEN, 2016).

2.3 Linkage disequilibrium (LD)

Statistical associations between mutations, collectively known as linkage disequilibrium (LD), play a fundamental role in modern evolutionary genetics (SLATKIN, 2008). Correlations between mutations enable genome-wide association studies and related methods that map the genetic basis of diseases (VISSCHER *et al.*, 2017) as well as other traits, such as those observed in animal production (ATASHI; WILMOT; GENGLER, 2022; EL HOU *et al.*, 2021). Genetic correlations are also important for evolutionary dynamics, as combinations of linked mutations provide the raw material upon which natural selection and other evolutionary forces can act.

LD provides important information about historical genetic recombination processes (MCVEAN *et al.*, 2004; ROSEN *et al.*, 2015), natural selection (GARUD *et al.*, 2015; SABETI *et al.*, 2002) and demographic changes (LI; DURBIN, 2011; RAGSDALE; GRAVEL, 2019) that occur within a population. The strength of linkage disequilibrium (LD) is assessed using various metrics, such as D, D', r^2, and p^2, which measure the intensity of association between alleles at genetic loci. These measures range from the simple calculation of D to standardized versions such as D' and r^2, which provide a more precise understanding of the genetic correlation between loci, with r^2 being particularly popular because it reflects the proportion of variation at one locus explained by another (HILL; ROBERTSON, 1966).

The value of linkage disequilibrium (LD) decreases as the distance between loci increases, due to the higher probability of recombination during crossing-over, which occurs more frequently over greater distances. The recombination rate is the primary factor responsible for the breakdown of LD, but other factors, such as species, inbreeding, and effective population size (Ne), also influence this decay (PÉREZ O'BRIEN et al., 2014).

2.4 Effective population size (NE)

The concept of effective population size (Ne) was proposed by (WRIGHT, 1931) based on a theoretical model of an ideal panmictic population, in which genetic drift was considered the only factor responsible for changes in allele frequencies over generations. With advances in population genetics and evolutionary studies, this concept was expanded to include other evolutionary forces that influence Ne in real populations, such as natural selection, migration, and fluctuations in population size over time (MANUNZA *et al.*, 2025). Following this expansion, various methods have been developed to estimate Ne at different spatial and temporal scales, accounting for demographic and genetic variation (WANG, J; SANTIAGO; CABALLERO, 2016).

Effective population size is widely recognized as a fundamental parameter in evolutionary biology, genetic conservation, and animal breeding programs (WAPLES, 2024, 2025). It reflects the intensity of genetic drift and inbreeding within a population and is essential for understanding the maintenance of genetic variability and the potential response to selection. Estimates of Ne can be obtained from demographic data, pedigree information, or genomic data, depending on data availability and type (MANUNZA *et al.*, 2025).

When based on demographic data, estimates typically consider the expected change in the inbreeding coefficient per generation (ΔF), accounting for the number of male and female breeders and the average family size (WAPLES, 2025). In pedigree-based analyses, Ne is calculated similarly but using the observed increase in the inbreeding coefficient across generations (LEROY *et al.*, 2013). The use of genomic data has enabled more precise estimation of Ne, particularly in populations where genealogical information is incomplete or unavailable (WANG, J; SANTIAGO; CABALLERO, 2016).

Among the main genomic methods employed are: (i) the temporal method, based on changes in the inbreeding coefficient (ΔF), which reflects the rate of genetic drift; (ii) the coancestry rate, which measures the increase in average genetic relatedness among individuals

over time; and (iii) the method based on linkage disequilibrium (LD), which uses correlations between neutral loci to infer both historical and contemporary effective population sizes (BEICHMAN; HUERTA-SANCHEZ; LOHMUELLER, 2018; NOVO et al., 2023; SANTIAGO et al., 2024).

In addition to its relevance for Ne estimation, LD is also widely used to identify functional variants associated with complex traits. The decay of LD over time known as *LD decay* is particularly important in genome-wide association studies (GWAS), as it directly affects the ability to detect causal loci (HAPMAP CONSORTIUM., 2005). As LD dissipates among markers, the association signal between a marker and a causal variant may be reduced, limiting the statistical power of analyses (VISSCHER *et al.*, 2012). Therefore, the degree of LD between markers is a critical parameter for determining the minimum marker density required in high-resolution genomic studies (GURGUL *et al.*, 2014) .

2.5 Genome-wide association studies (GWAS)

GWAS can identify both quantitative trait loci (QTLs) and single nucleotide polymorphisms (SNPs). GWAS analyses can identify key genes and haplotypes along with their regulatory mechanisms, which can then be incorporated into genomic selection programs to improve selection efficiency in herds (SCHÖPKE; SWALVE, 2016). There is a vast amount of research related to GWAS, including studies on milk production (SERMYAGIN, 2020), resistance to specific diseases (CAI *et al.*, 2018) reproductive traits (CRISPIM *et al.*, 2015), growth performance (BOLORMAA *et al.*, 2011), and meat quality traits (DANG *et al.*, 2014). In addition, GWAS is an excellent tool for identifying genes related to coat color (MACIEL et al., 2024).

Many of the genetic variants previously described in the literature exhibit linkage disequilibrium (LD) with functional mutations. The degree of LD can affect the accuracy of QTL detection in dairy cattle populations, as high LD levels can cause even distant SNPs to appear associated with the analyzed phenotypes. The use of haplotypes composed of SNPs can increase the accuracy and power of GWAS, particularly in situations where the QTL is located between two individual markers, which is especially useful when the QTL is rare (HÖGLUND *et al.*, 2014; PRYCE *et al.*, 2010).

2.6 Post-GWAS studies

After performing GWAS, which serves only as a starting point for identifying causal variants, additional studies are required to confirm and validate the findings. The use of databases, enrichment of genetic sets, transcription factor analysis, and gene network approaches provides a powerful framework for identifying candidate genes related to the detected QTLs (OTTO et al., 2019). This process is crucial because GWAS alone does not account for the complex interactions among genes within biological networks, which in turn influence the traits of interest (VISONÀ et al., 2024). Therefore, integrating information from gene networks can significantly enhance the understanding of the biological mechanisms underlying the studied traits (DADOUSIS et al., 2017).

2.7 Analyzed Traits

2.7.1 Coat calor origin

From the 18th century onwards, Chinese and Japanese geneticists began collecting mice with unusual coat colors, expanding the possibilities for studying phenotypic variation. Thus, pigmentation phenotypes in mice, and later in rats and other model organisms, became readily available for genetic research (BARSH, 1996; BOWLING, 1987; MARKLUND *et al.*, 1996). Different forms of coat coloration are desirable traits and are often subject to selection in domestic species, with diverse variants being preserved and recorded. In laboratory animals such as mice and rats, genetic studies on coat color are highly relevant due to the rapid development and physiological maturation of these species. In production animals, coat color and patterns have always been distinctive traits, as observed in cattle (ADALSTEINSSON et al., 1995; RIFFERT, 2015).

Although Mendelian inheritance was initially described based on simple patterns of allele segregation, subsequent studies identified more complex genetic mechanisms, such as epistasis and pleiotropy, which involve interactions among multiple genes (CASTLE, 1903; LITTLE, 1917). The multifunctional interaction of genes affecting coat color represents a complex set of traits, including interactions and epistasis among loci, which can be inherited through dominance, recessivity, codominance, or quantitative inheritance processes (SCHMUTZ, 2012). This diversity of spots, shades, and colors provides a wide phenotypic variability, making it highly attractive for genetic studies (BELLONE, REBECCA R. et al., 2013).

Color is synthesized by melanin, produced by melanocytes, specialized pigment-synthesizing cells present in various tissues, including the epidermis, hair follicles, inner ear, and

choroid of the eye (STEINGRÍMSSON; COPELAND; JENKINS, 2004). These melanocytes originate from unpigmented melanoblasts derived from the neural crest during embryogenesis. The process of melanoblast migration and differentiation into melanocytes is complex and not yet fully elucidated. It is known that melanin synthesis is intrinsically linked to embryogenesis, specifically to the neurulation stage, which follows gastrulation and involves the formation of the neural tube and, consequently, the neural crest (MURGIANO et al., 2016). The neural crest serves as the link between melanocytes and enteric ganglia, allowing melanocytes to migrate to the organism's epidermal surface (METALLINOS; BOWLING, 1998). Migration to the surface occurs in a sagittal plane, where melanoblasts, or melanocyte precursor cells, derive from both sides of the neural crest (BROOKS; BAILEY, 2005). These cells migrate from the neural crest toward distal extremities, before colonizing the dermis and ultimately localizing in the epidermis (MAYER, 1973).

Research indicates that pigmentation in animals is controlled by a variety of genes that regulate both the type and the amount of melanin produced in the skin and hair (TANAKA *et al.*, 2019). It is estimated that more than 300 genes are involved in mammalian pigmentation (BAXTER *et al.*, 2019), affecting base color, intensity, and pigment distribution. The two main types of melanin are eumelanin, responsible for brown and black pigments, and pheomelanin, which produces red and yellow tones. The resulting phenotype is determined by the interaction and distribution of these pigments (BROOKS; BAILEY, 2005; MAYER, 1973).

2.8 Main genes

2.8.1 Melanocortin 1 Receptor (MC1R)

The MC1R gene has been the subject of numerous studies across various mammalian species, including humans (VALVERDE *et al.*, 1995), pigs (KIJAS *et al.*, 1998), felids (EIZIRIK *et al.*, 2003), mice (ROBBINS, 1993), sheep (VÅGE *et al.*, 1999), dogs (EVERTS; ROTHUIZEN; VAN OOST, 2000), foxes (VÅGE *et al.*, 1997), bears (RITLAND; NEWTON; MARSHALL, 2001), horses (MARKLUND *et al.*, 1996) and cattle, where the presence of a functional mutation has been associated with black or brown coat color, whereas the absence of mutation at the MC1R locus results in red, yellow, or white colors (JOERG et al., 1996; ROUZAUD et al., 2000).

Coat color determination in mammals is largely influenced by the action of the MC1R gene, which is located on chromosome 18 in cattle (KLUNGLAND *et al.*, 1995). MC1R

encodes a receptor that regulates the activity of the enzyme tyrosinase, responsible for melanin synthesis, thereby determining the levels of dark or light pigments produced (TANAKA *et al.*, 2019). Polymorphisms in this gene exert a strong influence on coloration, depending on the combination of alleles present in the genotype. In cattle, for example, alleles at the E locus are key in determining coat color, influencing black, red, or wild-type variants (KASPRZAK-FILIPEK et al., 2020).

Black, brown, and red colors in cattle are determined by the presence of different alleles at the E and A loci, which influence receptor activity and melanin production (VÅGE et al., 1999). The dominant allele (E) results in high receptor activity, maintaining elevated tyrosinase levels, which promotes eumelanin production, responsible for black and brown coloration in both homozygotes and heterozygotes. The recessive allele (e) produces red color due to reduced receptor activity, caused by a mutation generating a premature stop codon, leading to low tyrosinase levels and pheomelanin production. The red phenotype only appears in homozygous recessives, although heterozygotes can transmit this allele. The wild-type allele (E+) allows expression of both eumelanin and pheomelanin, resulting in color variations in different body parts, such as the head and ears. The general allelic dominance order is: E > E+ > e.

Genetic determinants associated with the MC1R gene, responsible for phenotypic coat color traits, have been extensively characterized in the Holstein breed. This gene also influences coat color in various other cattle breeds, such as Angus, Simmental, Brown Swiss, Limousin, and Normande, among others (HOFSTETTER et al., 2019). Recent evidence indicates that structural variations in the MC1R gene can lead to marked phenotypic changes, as exemplified by a single-nucleotide deletion (thymine) at position 311 which, from the mating of two gray-coated Guzerat animals, resulted in the birth of a single, red-coated individual (SANTANA, CAROLAINE JESUS SILVA et al., 2021). Although the MC1R receptor is one of the main genes involved in color determination, it does not act in isolation. Its effect can be masked by other genes, such as the Coatomer Protein Complex, Subunit Alpha (COPA), which interferes with red coat determination. Furthermore, the Agouti gene, located at the A locus, also plays a key role in regulating the expression of black or brown pigments (FONTANESI, LUCA et al., 2009). This gene is responsible for whitish coloration patterns, allowing differentiation of colors in specific body regions and influencing the appearance of uniform or patterned coats (OLLMANN et al., 1998).

2.8.2 Agouti Signaling Protein (AGOUTI/ASIP)

The Agouti signaling protein (ASIP) is a secreted protein of 132–133 amino acids expressed in humans, cattle, and other species. Its expression varies across tissues, including adipose tissue, heart, liver, kidney, and ovary (ALBRECHT et al., 2012). Studies in mice first characterized Agouti as a pigmentation regulator, functioning as an antagonist of MC1R signaling, causing a switch from eumelanin to pheomelanin (LU et al., 1994). In addition to several members of the MCR family, ASIP also binds the attractin receptor (ATRN) with low affinity and is thought to act as a coreceptor for MCR signaling (HE et al., 2003).

The agouti locus (ASIP), as demonstrated in murine models, acts on melanocytes present both in the skin and in hair follicles, influencing pigment production by antagonizing MC1R (ALBRECHT et al., 2012). Its activity is modulated by melanocyte-stimulating hormone (α-MSH), which, by decreasing cyclic adenosine monophosphate (cAMP) levels, inhibits melanocortin signaling (LU et al., 1994). Consequently, the production of black-brown pigment (eumelanin) is shifted toward yellow-red pigment (pheomelanin) (CONE et al., 1996). This general mechanism is involved in pigmentation processes underlying coat coloration in mammals, as well as in avian feather coloration (GIRARDOT et al., 2005; HIRAGAKI et al., 2008).

In cattle, the ASIP gene presents multiple mRNA species that share the same coding region but differ in their 5′ untranslated regions (ALBRECHT et al., 2012). In certain bovine species, insertion of a long interspersed element (LINE) in the 5′ genomic sequence of the gene results in ASIP expression in multiple tissues (GIRARDOT et al., 2005, 2006). This gene is widely associated with coat color variation in animals, and its expression can impact developmental aspects. While some coat color–related genes can induce embryonic lethality, ASIP has been linked to this effect only in homozygous mice (MICHAUD et al., 1993). In these cases, the presence of a fusion transcript between ASIP and a noncoding exon of the adjacent RALY gene, which encodes a heterogeneous nuclear ribonucleoprotein involved in RNA processing, causes embryonic lethality. In other species, so far, ASIP is not associated with embryonic death.

Beyond its role in color determination, ASIP also influences metabolic traits, making it relevant for studying economically important attributes, such as fat deposition in domestic animals (FONTANESI, LUCA et al., 2010). A recent study by XIE et al., (2022) highlighted ASIP's crucial role in regulating lipid metabolism in cattle. Gene knockout experiments in mammary epithelial cells showed negative regulation of genes controlling fatty acid synthesis, such as fatty acid synthase (FAS), along with changes in cellular fatty acid profiles. These

findings emphasize ASIP's potential as a target in genetic improvement studies aimed at optimizing productive traits and meat quality. Despite its role in regulating fat deposition and lipid metabolism in adipose tissue, no studies have focused on its effects on milk lipid synthesis and metabolism in cattle.

2.9 Modifying genes

As mentioned in the preceding paragraphs, the pigmentation pathways driven by the MC1R gene or its antagonist ASIP establish the foundation for the most readily observable color phenotypes. However, the fine differentiation of spotting patterns, sizes, distribution, and qualitative coat characteristics results from the complex interplay of hundreds of modifier genes, many of which remain uncharacterized (EIZIRIK; TRINDADE, 2021; HOFREITER; SCHÖNEBERG, 2010). The polygenic nature of this trait accounts for the extensive phenotypic variation observed, even within the same primary genetic background. While most studies have focused on mice and horses, recent work in cattle breeding has begun to reveal specific candidates. This section will briefly discuss three of the most well-known and extensively studied modifier genes: Premelanosome Protein (PMEL/SILV), Tyrosinase-Related Protein 1 (TYRP1), and Membrane-Associated Transporter Protein (MATP).

2.9.1 Premelanosome Protein (PMEL/SILV)

The PMEL protein, also known as premelanosome protein 17 (PMEL17) or SILV, is essential for the formation and maturation of melanosomes, specialized organelles responsible for the synthesis and storage of melanin in melanocytes (BERSON et al., 2001). Melanosomes are membrane-bound organelles located in melanocytes and are divided into four developmental stages, each with distinct functions (OHBAYASHI; FUKUDA, 2020). In Stages I and II, known as premelanosomes, melanocytes do not yet produce melanin. At these stages, melanosomes have a fibrillar and elliptical structure, where fibers are synthesized to determine their shape and prepare the matrix required for melanin production (HELLSTRÖM et al., 2011; KNAUST et al., 2020). Melanin production begins only in Stage III, when tyrosinases (such as TYRP1) and ion transporter proteins (ATP7A, TPC2) enter the melanosome and initiate the pigmentation process (BENNETT; LAMOREUX, 2003; HEARING, 2000; SCHIAFFINO, 2010).

The PMEL gene is associated with various pigmentation dilution patterns across species. In mice, it contributes to color reduction (KWON et al., 1994). In chickens, mutations in

this gene result in white plumage, which may appear uniform or mottled (KERJE et al., 2004). In horses, PMEL is responsible for the silver coat color (BRUNBERG et al., 2006). In dogs, a small insertion of a short interspersed nuclear element (SINE) in intron 10 of PMEL is linked to the merle phenotype, producing a marbled appearance with areas of diluted color (CLARK et al., 2006). In cattle, the PMEL gene is associated with white coat color in breeds such as Charolais (KUEHN; WEIKARD, 2007) and is currently being investigated as a candidate gene that, in addition to influencing coat color, may have selective effects on birth weight in the Simmental breed (WANG, JING et al., 2023).

2.9.2 Tyrosinase-Related Protein 1 (TYRP1)

Coat color depends exclusively on the processes involving pheomelanin and eumelanin; however, the different positioning of colors is influenced by the presence of diluter genes such as TYRP1, found in Dexter cattle (BERRYERE et al., 2003). This gene was initially characterized in mice (JACKSON, 1994) and later associated with pigmentary disorders in humans (JACKSON, 1994b) and later associated with pigmentary disorders in humans (BOISSY; NORDLUND, 1997). In mice, three distinct mutations in the TYRP1 gene have been described, altering black pigments to various shades of brown, ranging from pale brown to dark reddish-brown. According to ZDARSKY; FAVOR; JACKSON, these changes are caused by mutations that produce a nonfunctional protein, often due to a nucleotide change in exon 2, resulting in brown coat coloration.

Additionally, (JACKSON et al., 1990) described a recessive mutation that significantly reduces TYRP1 mRNA levels, leading to reddish-brown pigmentation. Finally, (JAVERZAT; JACKSON, 1998) reported a third mutation, characterized by an inversion of exons 1–3 of the gene, resulting in a whitish-brown coat color, with hairs exhibiting bleaching near the body, giving a general pale-brown appearance. These findings illustrate the genetic complexity involved in animal pigmentation patterns.

In cattle, the TYRP1 gene exhibits recessive alleles inherited in an autosomal recessive manner and is associated with color dilution phenotypes. This dilution is frequently compared to the Dun condition in horses, although in equines TYRP1 is responsible for lighter coat aspects. In cattle, as observed in the Dexter breed, TYRP1 also influences coat coloration, resulting in lighter shades (GUTIÉRREZ-GIL; WIENER; WILLIAMS, 2007). The gene acts through encoding the tyrosinase-related protein 1, being a major contributor to dilution phenotypes in

other breeds (BERRYERE et al., 2003). According to the Canadian Galloway Association (CGA, 2003), the pale coat color termed Dun refers to a uniform shade distributed across the entire body. This distinguishes it from other descriptions of bovine coat coloration, such as localized lightening in certain areas OLSON, (1999), or the "cutia" effect described by BERGE, (1949) in some Norwegian breeds, where hair color changes along the shaft. The CGA definition emphasizes the uniformity of Dun, making it distinct from localized or variable patterns found in other breeds.

2.9.3 Membrane-Associated Transporter Protein (MATP)

The MATP gene, also known as SLC45A2, encodes a transporter protein involved in melanin synthesis (DING et al., 2022). This protein plays an essential role in deacidifying mature melanosomes, enabling them to sustain melanogenesis during the later stages of maturation (LE et al., 2020). When highly expressed in melanoma cell lines, MATP can lead to various pigmentary variations across vertebrates (PÉREZ OLIVA et al., 2009) including humans (SOEJIMA; KODA, 2006), zebrafish (DOOLEY et al., 2013) and horses (MARIAT; TAOURIT; GUÉRIN, 2003).

Mutations in the SLC45A2 gene are associated with oculocutaneous albinism type 4 (NEWTON et al., 2001), and polymorphisms within this gene have also been linked to variations in skin and hair pigmentation across several species. In sheep, variations in this gene influence coat coloration sheep, variations in this gene influence coat coloration (WANG, HAIDONG *et al.*, 2016), while in Japanese quails, pigmentary changes have been related to SLC45A2 mutations (GUNNARSSON *et al.*, 2007). In horses, the CCr genotype at the Cream locus acts codominantly, diluting pheomelanin and eumelan (SEVANE; SANZ; DUNNER, 2019). In homozygous CrCr individuals, the genotype dilutes all basic color phenotypes, resulting in animals with pink skin, light blue eyes, and cream-colored coats that appear nearly white.

In cattle, SLC45A2 has been studied in breeds such as Braunvieh, Angus, and Chinese indigenous cattle (DING *et al.*, 2022; ROTHAMMER *et al.*, 2017). Recently, a missense mutation (c.1543A>G, p.Ser515Gly) in this gene was associated with heat tolerance, suggesting its potential adaptive role under harsh environmental conditions.

2.10 Genes associeted with white patterns

In addition to the primary pigmentation pathways represented by MC1R and ASIP and the modulatory effects of modifier genes on coat color intensity and patterning, the occurrence of white spotting and depigmented regions represents another major dimension of phenotypic diversity in cattle. White patterns arise predominantly from alterations in melanocyte development, migration, survival, and differentiation during embryogenesis, rather than from changes in melanin synthesis itself (MORT; JACKSON; PATTON, 2015). Disruptions in these cellular processes lead to the absence or reduced density of melanocytes in specific skin regions, resulting in characteristic white markings of varying size, distribution, and symmetry (BUDAIR, 2024).

These phenotypes are genetically heterogeneous and are controlled by a limited number of key regulatory genes with pleiotropic effects, often interacting with each other and with background genetic architecture (JIVANJI *et al.*, 2019). Although the molecular basis of white spotting has been extensively investigated in model organisms and several domestic species, studies in cattle have increasingly identified conserved genetic mechanisms underlying these patterns. This section will briefly address three of the most relevant and well-characterized genes associated with white patterning in mammals and cattle: the KIT proto-oncogene receptor tyrosine kinase (KIT), the Microphthalmia-Associated Transcription Factor (MITF), and the Endothelin Receptor Type B (EDNRB).

2.10.1 KIT proto-oncogene receptor tyrosine kinase (KIT)

It is well established that the precursor cells of melanocytes, known as melanoblasts, originate from both sides of the neural crest and migrate to the extremities, where they colonize the dermis and later the epidermis (MAYER, 1973).he extremities, where they colonize the dermis and later the epidermis (MAYER, 1973). The differentiation of melanoblasts into mature melanocytes is controlled by several factors, and the migration, proliferation, and survival of these cells are tightly regulated during development (ITO *et al.*, 1999; MACKENZIE *et al.*, 1997; PETERS *et al.*, 2002). KIT encodes a receptor essential for the stem cell growth factor and mast cells, belonging to the tyrosine kinase family. This receptor comprises three main parts: an extracellular immunoglobulin-like domain that detects signaling molecules; a transmembrane region that connects the interior and exterior of the cell; and two intracellular domains responsible for activating (SOEJIMA; KODA, 2006).

Variations in the KIT gene include single nucleotide polymorphisms (SNPs) (YAN *et al.*, 2014), deletions (DÜRIG *et al.*, 2017) and more complex structural variants. One example of such variants is the duplication of the KIT gene, which has been associated with the characteristic coat color pattern of the Hereford breed (WHITACRE, 2014). In addition, chromosomal translocations involving the KIT gene have been reported, resulting in lateralized coat color patterns, such as the phenotype known as *"witrik"* in cattle. In this case, a translocation to chromosome 29 is associated with the lateral coloration observed in breeds such as Brown Swiss, Belgian Blue, and Pustertaler Sprinzen, among others, highlighting the crucial role of KIT in determining coat color patterns in cattle (DURKIN et al., 2012).

In Holstein cattle, (HAYES *et al.*, 2009) reported that 24% of the variation in the proportion of black coat pigmentation could be explained by the combined effect of the KIT gene, the MITF locus, and a region on chromosome 8 near the PAX5 gene. The remaining 76% of variance was attributed to small effects distributed across multiple genes, illustrating the complex genetic architecture of this phenotype. Furthermore, the KIT gene has been associated not only with black pigmentation but also with periorbital pigmentation and cheek spots, as observed by (PETERSEN *et al.*, 2020) reinforcing its importance in multiple coat color patterns in cattle. Altogether, these findings underscore the pivotal role of KIT in the phenotypic diversity of coat coloration, establishing it as a key gene in pigmentation genetics.

2.10.2 Microphthalmia-Associated Transcription Factor (MITF)

Across different coat color patterns, it is common to observe interactions between genes that influence pigmentation expression in the animal's epidermis. One example is the association between the KIT gene and the MITF gene, which encodes a transcription factor essential for melanocyte induction, suggesting heterogeneity in the proportion of white coat in Holstein cattle (HAYES et al., 2009). In addition, yet unidentified genetic variants such as a single intronic nucleotide mutation in the MITF gene have been associated with differences between spotted and non-spotted phenotypes in both Holstein and Simmental cattle (FONTANESI; SCOTTI; RUSSO, 2012; JANSEN et al., 2013).

In other species, such as dogs and horses, non-coding variants of MITF have been linked to white spotting patterns on the head and body (HAUSWIRTH et al., 2012; KÖRBERG et al., 2014; NEGRO et al., 2017). In cattle, coding variants in the MITF gene may lead to white coat coloration associated with ocular malformations, such as microphthalmia (WIEDEMAR;

DRÖGEMÜLLER, 2014) or with bilateral deafness (PHILIPP et al., 2011). Similar traits are also found in humans, where MITF mutations are linked to Waardenburg syndrome type 2A and Tietz syndrome (TIETZ, 1963), which cause deafness due to the absence of melanocytes in the inner ear, as well as pigmentation disorders affecting the skin, hair, and iris (LIU; NEWTON; READ, 1995; SHIBAHARA et al., 2001).

Recently, a 3-bp deletion in the MITF gene was identified in the Angus breed (PETERSEN; SIECK; STEFFEN, 2023). Although this mutation was unique to the affected calf, it is identical to the delR217 variant previously reported in humans and murine models with Waardenburg syndrome type 2A and Tietz syndrome. These syndromes are associated with pigmentation defects and deafness resulting from impaired melanocyte development, underscoring the relevance of this mutation for understanding genetic conditions that affect both animals and humans.

2.10.3 Endothelin Receptor Type B (EDNRB)

The lesser-known EDNRB gene, a member of the endothelin family, plays crucial roles in several physiological processes, including smooth muscle contraction (KOBAYASHI et al., 2016), calcium signaling (VANNUCCI *et al.*, 2018), vascular relaxation (NG *et al.*, 2019) and coat color development (MENZI *et al.*, 2016). Studies investigating genes involved in coat pigmentation have identified three key loci responsible for pigment synthesis, as well as for the survival, migration, and proliferation of melanocytes (VAN RAAMSDONK et al., 2004).

Mutations in the EDNRB gene markedly impair the development of melanocytes and enteric neurons, two cell populations derived from the neural crest, leading to congenital white spotting of the skin and hair (BENNETT; LAMOREUX, 2003). During neural crest development, endothelin receptor type B functions as a G protein–coupled transmembrane receptor and plays a central role in endothelin-mediated signaling pathways, with endothelin-3 acting as its main 21–amino acid ligand (BRAASCH; VOLFF; SCHARTL, 2009). This pathway is functionally illustrated by overo lethal white syndrome (OLWS), a hereditary condition observed in foals from American Paint Horse lineages, in which severe depigmentation is accompanied by intestinal aganglionosis and early postnatal mortality (SANTSCHI *et al.*, 1998). Comparable neurocristopathies reported in humans and rodent models carrying EDNRB mutations further reinforce the conserved role of this receptor in the regulation of neural crest–derived melanocytes and enteric neurons (AURICCHIO, 1996; GARIEPY *et al.*, 1998).

Taken together, these findings underscore the central importance of the endothelin signaling pathway in neural crest development, suggesting that not only the receptor (EDNRB) but also its ligand play critical and complementary roles in this biological process. In this context, genetic alterations in endothelin-3 (EDN3) have been associated with Hirschsprung's disease in humans and lethal white spotting in mice, further highlighting the relevance of the EDN3–EDNRB axis in normal development. Both EDNRB and EDN3 are indispensable for the formation of enteric ganglia and melanocytes, two lineages originating from the neural crest (BAYNASH et al., 1994). Although the involvement of EDNRB in bovine coat color remains poorly explored, recent evidence suggests that this gene may also influence other phenotypic traits, such as skeletal support, as reported in cases of dorsal weakness in the Ayrshire breed from Scotland (AZOVTSEVA et al., 2024).

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The Genomics behind Beauty: Disentangling the Complex Coat Color Genetic Architecture of Gir Breed

Marcelo José Böck1*; Mateus Guimarães dos Santos1; Fabrício Pilonetto3, Renata de Fátima Bretanha Rocha1; Pamela Itajara Otto4; Marcos Vinícius Gualberto Barbosa da Silva2; Simone Eliza Facioni Guimarães1*

[1]Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil, 36570-900

[2]Embrapa Dairy Cattle Research Center, Juiz de Fora, MG, Brazil, 36038-330

[3]Brazilian Association of Girolando Breeders, Uberaba, MG, Brazil, 38040-280

[4]Department of Animal Science, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil; 97105-900

*Corresponding author: sfacioni@ufv.br

ABSTRACT

Coat color in cattle is a visible trait with biological, adaptive, and economic significance, reflecting both natural and artificial selection processes. This study aimed to identify genomic regions and candidate genes associated with coat color variation in the Gir breed (*Bos indicus*). Phenotypic records of 50,341 animals, classified into 25 coat color categories and genotypes from 55,154 animals were provided by EMBRAPA Dairy Cattle. After quality control, 389,172 SNPs were retained for analysis. Linkage disequilibrium (LD) and effective population size (Ne) were estimated using PLINK 1.9, and single-step genome-wide association analyses (ssGWAS) were performed with BLUPF90+ software. Post GWAS gene annotation was conducted based on the *Bos taurus* ARS-UCD2.0 genome and functional databases (GO,

KEGG, GeneCards, and VarElect). Heritability estimates ranged from 0.16 to 0.75, indicating a strong genetic component for coat color. The mean LD (r^2) between markers was 0.064 at 50 Kb, with Ne declining from 53.94 animals in generation 50 to 13.79 animals in generation 5, suggesting reduced genetic diversity in recent generations. The top 10 genomic windows (0.5 Mb) explained up to 24% of the additive genetic variance, revealing 52 candidate genes. These genes are involved in melanocyte migration (e.g., *KIT*, *SPIRE2*), cell differentiation (*LEF1, FRAS1*), cytoskeletal organization (*TUBB3*, *MARCKS*), signal transduction (*WNT6*, *TYK2*), and melanogenesis (*MC1R*). In conclusion, coat color variation in the Gir breed results from complex polygenic regulation involving genes that also participate in embryogenesis, melanocyte biology, and cell signaling, providing new insights into pigmentation mechanisms and breed diversification.

Key words: cell migration, cell differentiation, dairy cattle, melanocyte

INTRODUCTION

Increasing environmental concern, combined with factors such as higher rainfall, rising temperatures, microclimate alterations, and drug resistance, has compromised the efficiency of animal production (Dopelt et al., 2019; Pandey et al., 2024). In this context, studying color diversity in livestock can provide valuable insights from an evolutionary biology perspective, particularly regarding mechanisms of adaptation and natural selection (Senczuk et al., 2020).

In addition to its adaptive role, coat color variation also reflects domestication and artificial selection processes. Like what has been observed in other domesticated species, such as horses, cats, and dogs, coat color variants in cattle have arisen and become stable within populations through both natural and human selection, with gene flow and introgression contributing to complex pigmentation patterns (VonHoldt et al., 2021). The Angus breed, for

instance, is partly characterized by its predominantly black coat. Due to intense selection for this phenotype, most Angus individuals are homozygous for the dominant black variant (ED) of the MC1R gene (Klungland et al., 1995; Matukumalli et al., 2009). Animals whose coat color does not meet the breed standard often have lower market value, highlighting the economic impact of selection for coat color (Kassie et al., 2011).

Beyond economic and selective aspects associated with coat color, its standardization in cattle has a well-defined biological basis. Coat coloration results from continuous activity of melanocytes, cells derived from the embryonic neural crest (NCC) (Bruno et al., 2024). The functions of these cells in target tissues go far beyond the simple synthesis of melanin (Zhang et al., 2025). In cattle, pigment patterning involves coordinated processes of migration, differentiation, and regulation of melanocytes, which act directly in epidermis (Jackson, 1994). Distribution and density of melanin pigments depend on the interaction of multiple genes involved in the synthesis of eumelanin and pheomelanin (Caro & Mallarino, 2020).

In this context, expression of these genes not only affects the individual fitness (Maia et al., 2005), but is also associated with traits of interest in livestock, such as meat quality (Parish et al., 2018), increased milk production (Illa et al., 2021) and enhanced thermoregulation and adaptability, as observed in predominantly black-coated animals with white hairs (Maibam et al., 2018; Silva et al., 2001). However, melanocyte development can also lead to negative pleiotropic effects, including night blindness and leopard-spotted coat patterns in horses (Bellone, 2010), reproductive system malformations in cattle (Fathoni et al., 2025), genetic syndromes in different species (Kunieda et al., 1999) and deafness in dogs (Philipp et al., 2011).

Thus, considering that pigmentation is associated with traits of interest in livestock and undesirable pleiotropic effects, it becomes clear that coat color variation should be analyzed not only as an aesthetic feature but also as a functional one. In this context, some *Bos taurus* breeds, such as Jersey (Huson et al., 2020), Holstein (Dorshorst et al., 2015) and Brown Swiss

(Hofstetter et al., 2019) exhibit well-defined coat color patterns. In contrast, other breeds, such as *Bos indicus*, exemplified by the Gir breed, do not have a fixed pattern but rather display broad color diversity and different patterns of coat pigmentation. This variation results from the crossbreeding of animals with different coat patterns in its region of origin, Gujarat, India, giving rise to the modern Gir (Gaur et al., 2003).

Although coat color is an easily observable phenotype, its genetic architecture in the Gir breed remains largely unexplored. Understanding the genes underlying this unique diversity can provide insights into the mechanisms controlling not only the exquisite pigmentation patterns in this breed but also disentangle the basic molecular mechanisms of coat color development in livestock. Therefore, the present study aims to identify candidate genes and genomic regions associated with various coat color patterns in the Gir breed, encompassing processes from embryogenesis and central nervous system development to melanocyte differentiation, migration, and proliferation.

MATERIALS AND METHODS

Animals and data

A dataset containing coat color records of 50,341 Gir animals was provided by EMBRAPA Dairy Cattle. The dataset included 25 coat color categories, as defined by the Brazilian Association of Zebu Breeders (ABCZ, 2024), in Portuguese: Associação Brasileira dos Criadores de Zebu. Coat color was determined through visual evaluation by trained technicians from Associação Brasileira dos Criadores de Gir Leiteiro (ABCGIL). A visual representation of all 25 evaluated phenotypes is provided in **Figure S1**. Due to the heterogeneity in sample size among coat colors, different analytical scenarios were established. Coat color phenotypes were grouped according to the degree of phenotypic variation, starting from more solid and similar colors **Table 1**.

< Table 1 >

Genotype and quality control

The genotype dataset consisted of approximately 56,098 animals genotyped using chips of different densities, including Illumina BovineSNP50 BeadChip v2 (50K), Illumina BovineHD BeadChip (777K), GGP Indicus (34K), ZChip (30K), and GGP Indicus (50K). The Illumina BovineHD (777K) chip was chosen as the reference, and SNPs from the other chips were extracted or imputed to match the 777K panel. The imputation process was performed by EMBRAPA Dairy Cattle before handling the genotype dataset. Analyses were initiated with a total of 420,561 Single Nucleotide Polymorphisms (SNPs) before quality control. The quality control of genomic data was performed using the default values of the BLUPF90+ family programs, which includes: a minimum allele frequency (MAF) of 0.05, a minimum call rate for markers of 0.90, and a minimum call rate for animals of 0.90. Additionally, SNPs significantly deviating from Hardy–Weinberg Equilibrium (HWE) were removed using a threshold of $p < 1 \times 10^{-6}$. After quality control, 55,154 genotyped animals were retained, with 389,172 remaining SNPs, all located on autosomal chromosomes (BTA1 to 29).

Linkage disequilibrium (LD) and LD decay

To evaluate the extent of LD, we used the r^2 statistic, as it is a robust measure that is not strongly influenced by allele frequency or effective population size (Terwilliger, 2002; Zhao et al., 2007). Linkage disequilibrium was computed as the correlation between allele frequencies (r^2_{ij}) following Hill & Robertson, (1968). Pairwise LD between SNPs was estimated using PLINK 1.9 software (Chang et al., 2015) considering a genomic distance window of 1 Mb. The calculation was performed with the following command: ./plink --bfile gir_plink --chr-set 29 --r2 --ld-window 5 --ld-window-kb 1000 --ld-window-r2 0 --out outname.

$$r_{ij}^2 = \frac{(p_{ij} - p_i q_j)^2}{p_1 p_2 q_1 q_2}$$

where p_{ij} is the probability of the marker allele pair *i* and *j*, p_i and q_{ij} are marginal allelic frequencies at *i* and *j*. and p_1 p_2 q_1 q_2 are the products of four allele frequencies at both loci.

The LD decay was assessed by regression in the distance between pairs of markers based on the nonlinear parametric model described by (Sved, 1971a):

$$LDij = \left(\left[1 + 4\beta d_{ij}\right] + e_{ij}\right)^{-1}$$

where LD_{ij} is the r_{ij}^2 observed between SNPs *i* and *j*; d_{ij} is the distance Kb between SNPs *i* and *j*; β is the distance coefficient that describes LD decay; and e_{ij} is the random residue defined as $e_{ij} \sim N(0, \sigma^2)$.

Effective population size

Effective population size (N_e) was estimated based on relationship between N_e and LD without mutation and recombination rate (c) proposed by (Sved, 1971), using the equation (Hayes et al., 2003; Ospina et al., 2019)

$$N_T = \left(\frac{1}{4c}\right) X \left(\left(\frac{1}{r^2}\right) - 1\right)$$

where *c* represents the map distance in Morgans, assuming 1 cM is equal to 1 Mb, r^2 is LD value, and N_T is effective population size in the T^{th} generation, and T is $^1/_{2c}$. From the estimated r^2, the average values were grouped into four ranges of distances (<0.01 Mb, 0.011–0.03 Mb, 0.031–0.05 Mb, and >0.05 Mb) and used to determine the N_e in 50, 17, 10, and 5 generations, respectively.

Genome-wide Association Analyses (GWAS)

The methodology for the GWAS analyses, including comparisons and total number of animals per analysis, is presented in **Table 2.** After data quality control, a single-step GWAS

approach was employed using the BLUPF90+ family programs (Lourenco et al., 2022). Variance components and genomic estimated breeding values were estimated using the EM-REMLF90 option. The animal model adopted for coat color analysis in Gir breed was as follows:

$$y = X\beta + Zu + e \qquad (1)$$

where y is the vector of observations for the scenarios; β is the vector of fixed effects (overall mean); u is the vector of random additive genetic effects attributed to the animals; e is the vector of residuals; X and Z are incidence matrices relating observations to the fixed and additive genetic effects, respectively.

It is assumed that $u\ N(0; H\sigma_u^2)$ and $eN(0; I\sigma_e^2)$, where H is the relationship matrix that combines pedigree and genomic information (single-step), as described by (Aguilar et al., 2010); I is the identity matrix; and σ_u^2 and σ_e^2 are the additive and residual variances, respectively.

The effects of markers were estimated using the postGSf90 software (http://nce.ads.uga.edu/). GWAS results were expressed as the proportion of additive genetic variance explained by overlapping 0.5-Megabase (Mb) windows (Otto et al., 2020). The percentage of additive genetic variance explained by each window was calculated as described by (Wang et al., 2014). To visualize genomic regions with the greatest impact on the trait, Manhattan plots were constructed using the R software via mhtplot function (R Core TeamEAM, 2019). The variance explained by adjacent windows was calculated as described by (Fragomeni et al., 2014).

< Table 2 >

Post-GWAS analyses

Within the top 10 regions (windows) explaining the largest proportion of additive genetic variance, putative candidate genes were identified based on the initial and final coordinates of each selected window, the *Bos taurus* reference genome ARS-UCD2.0 (GCA_002263795.4), available in the Ensembl database (https://www.ensembl.org/Bos_taurus/Info/Index), was used as reference. Filters by species, chromosome, and window position were applied to refine gene identification (O'Leary et al., 2024). The selection of the top 10 windows explaining the highest percentages of additive genetic variance is a widely used approach in association studies to evaluate various traits across different animal species (Sevillano et al., 2019; Souza et al., 2025).

Different functional databases, including Gene Ontology (Aleksander et al., 2023), KEGG (Kanehisa et al., 2017), GeneCards (Stelzer, Rosen, et al., 2016) and VarElect (Stelzer, Plaschkes, et al., 2016) were used to define functional gene sets. Genes linked to a common functional annotation were considered part of a gene set, characterized by shared biological attributes and typically involved in related molecular mechanisms or biological pathways.

RESULTS

Estimation of heritability (h^2)

Across all analyzed scenarios, the estimated heritabilities (h^2) were moderate to high (**Table 2).** The highest value ($h^2 = 0.75$) was observed in the 11^{th} comparison, involving animals from scenario 1 (solid coat color pattern: red and yellow). The lowest estimate ($h^2 = 0.16$) was found in the 2^{nd} comparison, between scenario 1 (solid color pattern, yellow or red) and scenario 2 (red choker or yellow choker).

Linkage disequilibrium and effective population size

The LD decay pattern in the Gir breed for 55,439 genotyped animals was constructed based on the estimated r^2 values(Sved, 1971a) (**Fig. 1)**.

< Figure 1 >

Using Sved's nonlinear model, the LD decay parameter (β) estimated for the Gir population was 2.311×10^{-5}, which is slightly lower than the value reported by (Verardo et al., 2021) (2.544×10^{-5}). When expressed on the same bp scale, this comparison indicates a somewhat lower LD decay in our dataset, likely reflecting differences in population structure, marker density, and sample size. The estimated r^2 value between pairs of markers was 0.196 ± 0.010 at 40-50 Kb. The mean observed value at distances below 10-20 Kb was 0.363 ± 0.347. Values decreased with increasing distance, as did variability, with mean estimates progressively declining **(Table 3)**. The highest mean observed value was 0.431 ± 0.369 in the 0–5 Kb range, whereas the lowest estimated value was 0.097 ± 0.096 in the 900–1000 Kb range.

< Table 3 >

The estimated effective population size (Ne) of the Gir cattle for the past 50 generations is illustrated in **Fig. 2.** Ne was highest in generation 50, with 53.94 animals, and decreased drastically over subsequent generations, with the most recent generations (5) showing 13.79 animals.

< Figure 2 >

Genome-wide Association Analyses (GWAS) and post-GWAS

The percentage of genetic variance explained by each window of 0.5 Mb is displayed in Manhattan plots for all scenarios together (AS) and scenario comparisons (**Fig. 3)**. The total variance explained by the 10 genomic windows with highest proportion of additive genetic variance for AS was 24% **(Table 4).** For the remaining comparisons, the proportion of variance explained ranged from 1.34% (S1 vs S5) to 22.99% (S1 vs S9), with intermediate values observed in the other scenarios (S1 vs S2: 1.41%; S1 vs S3: 1.36%; S1 vs S4: 1.60%; S1 vs S6: 1.47%; S1 vs S7: 1.40%; S1 vs S8: 7.10%; S1 vs S10: 9.32% and S1: 4.1%).

< Figure 3 >

< Table 4 >

The use of genome-wide association studies (GWAS) with variance windows allowed the identification of 52 candidate genes potentially involved in mechanisms associated with coat color determination **(Supplementary Table 1; Figure 4)**. Of these, 5 genes are related to melanocyte migration (*SPIRE2, LAMA4, CDH26, RNF5*, and *KIT*); 14 to cell differentiation (*TCF25, GSC, SCHIP1, GRIN2B, PATJ, NOTCH4, MIR375, HDAC2, FRAS1, VCAN, HAPLN1, LEF1*, *VANGL1 and AEBP1*); 1 to melanogenesis (*MC1R*); 10 to cytoskeleton regulation (*TUBB3, CRACD, PRKD1, CSTPP1, FNBP4, HIP1R, CLIP1, MARCKS, VAV3*, and *WDR1*); 14 to signal transduction (*DBNDD1, DDB2, PRKAG3, WNT6, WNT10A, TYK2, DUSP12, HEG1, VSTM4, CORIN, CNGA1, TXK, TEC*, and *NGF*); 1 to ciliary function (*GAS8*); 3 to cell proliferation (*SHCBP1*, *SLC12A8 and WSB2*); 2 to cell migration (*ATP8A1 and CNTN4*); and 2 to angiogenesis (*KDR* and *EDIL3*).

DISCUSSION

Estimation of heritability (h^2)

Although coat color may seem like a simple trait due to its eventual control by a few major genes, complex polygenic interactions involving these and other metabolic pathways have already been reported (Gutiérrez-Gil et al., 2007a; Voß et al., 2022). In the present study, heritability estimates were moderate to high, which is consistent with findings in other bovine populations. For example, in animals of the same breed analyzed here, Maciel et al., (2024) reported h^2 values ranging from 0.60 to 0.75, indicating a strong genetic effect. A similar pattern was observed in another *Bos indicus* breed, the Boran cattle, which showed $h^2 = 0.52 \pm 0.11$ (Kirui et al., 2023).

Despite numerical differences among the studied Gir populations, some results are comparable. In Maciel et al., (2024), the heritability estimate considering all phenotypes simultaneously was $h^2 = 0.60$, matching the value found in the present study. For animals with solid coat colors (yellow and red) versus white spots on the dewlap, the heritability was $h^2 = 0.33$, compared to $h^2 = 0.69$ in the cited study. Animals with few white spots on the body (red calico and yellow calico) showed $h^2 = 0.46$, compared to $h^2 = 0.75$, while those with many spots (calico red and calico yellow) had $h^2 = 0.69$, close to $h^2 = 0.76$. These results suggest that differences in phenotypic grouping may influence the magnitude of heritability estimates across coat color categories.

In addition, the highest heritability estimate observed in this study ($h^2 = 0.75$) occurred in the 11th comparison, which focused on animals with solid coat colors (red and yellow). This finding indicates that reducing phenotypic heterogeneity enhances the detection of genetic effects underlying coat color expression in Gir cattle. Similar patterns were reported by Gutiérrez-Gil et al., (2007b), who demonstrated that more uniform coat color phenotypes in cattle tend to reflect the action of major genetic loci, with limited environmental influence. In their study involving Charolais × Holstein populations, phenotypes with diluted and

homogeneous pigmentation, such as the white coat in pure Charolais animals, were primarily associated with key loci related to eumelanin dilution, enabling more robust genotype–phenotype associations. In this context, the grouping of solid red and yellow animals in the present study likely reduced environmental variation and visual classification bias, allowing a clearer partitioning of additive genetic variance. However, the interpretation of these biological patterns should be integrated with methodological aspects that influence heritability estimation across studies.

Despite the overall agreement between our heritability estimates and those reported by Maciel et al., (2024), key methodological differences help explain the numerical variation across phenotypes. Our study analyzed a larger animal population, a more detailed phenotypic structure (25 categories), and a different SNP density, all of which affect variance component estimation. The definition of the control group also differed: Maciel et al., (2024) used only red-coated animals, whereas ours included both solid red and solid yellow individuals; both colors coming from the pheomelanin pathway. In addition, we incorporated unique phenotypes such as yellow choker, yellow calico, calico yellow, light roan, and several gray-based calico and choker categories not assessed in the previous study. These distinctions in sample composition, phenotype definition, and SNP density likely contributed to the differences observed, while still yielding comparable heritability estimates.

When placed in a broader context, our results are also consistent with studies conducted in other cattle populations. For example, a study involving crossbred beef cattle composed of British, Sanga, Zebu, and continental breeds adapted to tropical climates reported moderate heritability ($h^2 = 0.26$; Prayaga & Henshall, 2005). Differences across studies may arise from several factors, including sample size, analytical methodology, and phenotype misclassification. In the absence of standardized procedures for coat color evaluation, visual

assessment criteria remain subjective and population-specific, making direct comparisons across breeds difficult and, in some cases, uninformative.

More precise and less subjective tools have also been explored, such as the use of portable spectrophotometers applying the CIELab scale in Slovak Pinzgau cattle, which reported heritabilities of $h^2 = 0.24$, 0.57, and 0.17 for lightness, hue, and saturation, respectively (Kasarda et al., 2016). These findings reinforce the strong genetic influence of coat color on selection decisions and the prediction of genetic gain while also underscoring a persistent bottleneck in the technological assessment of observable phenotypes. This highlights the need for more objective, standardized, and reproducible methods to support future breeding decisions.

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Linkage disequilibrium and effective population size

The analysis of linkage disequilibrium (LD) provides valuable insights into evolutionary history and effective population size (Ne) in livestock (Robinson & Moyer, 2013; Slatkin, 2008). In the present study, the mean r^2 between markers separated by 0.196 at 40-50 Kb. was lower than that reported by Garcia et al., (2023) with a r^2 value of 0.208, likely reflecting differences in sample size, marker density, and population demographics (Manunza

et al., 2025; Novo et al., 2022; H. Singh et al., 2021).The lower LD values observed here indicate more historical recombination and a higher ancestral Ne, suggesting that the Gir population retained greater genetic diversity in earlier generations.

A rapid decay of LD was observed, from a mean r^2 of 0.431 at short inter-marker distances (0–5 Kb) to 0.097 at 900–1000 Kb, consistent with previous studies in indicine breeds (O'Brien et al., 2014; Porto-Neto et al., 2014). The higher Ne estimated in older generations (53.94 in generation 50) compared to the most recent ones (13.79 in generation 5) supports this pattern, indicating a historical population with broader genetic variability that has gradually declined due to genetic drift, selective breeding, and the limited use of sires. These results reinforce the dynamic nature of Ne across generations, shaped by both historical and recent demographic events.

When compared to pedigree-based analyses, which reported higher Ne values for the Gir breed (Malhado et al., 2010; Reis Filho et al., 2010; Santana et al., 2014), the genomic estimates presented here are notably lower. This discrepancy highlights the greater sensitivity of molecular data in detecting recent reductions in Ne and increased reproductive concentration (Kelleher et al., 2017). Taken together, these findings suggest that although the ancestral Gir population maintained considerable genetic diversity, current breeding strategies may be intensifying genetic bottlenecks, underscoring the need to monitor genetic variability to ensure the long-term sustainability of the breed. In line with other molecular studies, our results are also consistent with those reported by Garcia et al., (2023) in the study "Pedigree reconstruction and population structure using SNP markers in Gir cattle", as well as with (Verardo et al., 2021)**,** who investigated the genetic architecture and candidate genes of Indicine, Taurine, synthetic crossbred, and locally adapted cattle populations. Both studies reported similar patterns of linkage disequilibrium (LD) and effective population size (Ne), reinforcing the robustness and consistency of the estimates obtained in the present study.

Genome-Wide Association Studies (GWAS)

This study provides evidence regarding the role of several genes associated with different coat color patterns in the Gir breed, demonstrating that these genes also participate in other relevant biological processes, such as embryonic development and melanocyte differentiation. 52 candidate genes potentially involved in coat pigmentation mechanisms were identified through genome-wide association studies (GWAS) based on variance windows. Among the scenarios analyzed, genomic regions were included at: BTA1, BTA2, BTA3, BTA4, BTA5, BTA6, BTA7, BTA9, BTA15, BTA17, BTA18, BTA21, BTA22, BTA23, and BTA28. In a similar study on Gir cattle Maciel et al., (2024), reported associations on chromosomes BTA2, BTA6, BTA9, BTA10, BTA16, BTA18, BTA21, and BTA26. Shared genomic regions between both studies were found only on BTA6, encompassing genes such as *CORIN, KIT*, and *KDR*, which are associated with signaling pathways involved respectively in pigmentation, melanocyte development, and angiogenesis.

Several studies on bovine coat color have explored QTL expression influencing phenotypes of interest in animal production (Senczuk et al., 2020; J. Wang et al., 2023), or genes associated with deleterious diseases, as observed in the Angus breed (Petersen et al., 2023). Others have described pigment polarization patterns in breeds such as Belgian Blue and Brown Swiss (Durkin et al., 2012). The distinctive feature of the present research lies not only in the representative number of sampled animals, but also in its integrative approach, aiming to understand the biological process of pigmentation from its earliest stages since embryogenesis to melanocyte differentiation and migration. Although there is extensive literature on pigmentation in humans (Liu et al., 2015) and mice (Cohen et al., 2016), investigations in cattle that address genes involved in the primary formation of melanocytes and in the events preceding their differentiation are still limited.

Several genes identified in this study participate in the early stages of embryonic development, during which the cellular and molecular foundations originating neural crest–derived melanocytes are established (Adameyko et al., 2009; Graham, 2009). Regarding signal transduction process, *WNT6* and *WNT10A* which belong to the Wnt/β-catenin pathway, are essential for the differentiation of neural crest cells into melanocytes (Patapoutian & Reichardt, 2000). Their roles in melanocyte expansion and maturation are well characterized in murine models (Dunn et al., 2000). Other genes related to signal transduction perform distinct functions: some, such as *DBNDD1*, are associated with melanoma susceptibility and may influence pigmentation in humans (Huang et al., 2025), while others, like *TYK2,* a non-receptor tyrosine kinase involved in the modulation of immune and inflammatory responses have a more indirect impact on coat coloration, as observed in mice (Muromoto et al., 2022).

The migration and differentiation of melanocytes also depend on fundamental cellular processes. During neurulation, cytoskeletal regulation is essential for tissue remodeling and for the release of neural crest cells (Kho et al., 2023; Salinas et al., 2025). Among the genes identified in this study, ten are related to cytoskeletal formation and dynamics (*TUBB3, CRACD, PRKD1, CSTPP1, FNBP4, HIP1R, CLIP1, MARCKS, VAV3,* and *WDR1*), which may play a central role in the organization, adhesion, and motility of melanocytes. The cytoskeleton acts as a key structure in maintaining cell shape and facilitating intracellular transport, being indispensable for melanocyte dispersion throughout embryonic development (Gordon-Weeks & Fournier, 2014). Alterations in genes associated with actin, microtubules, and anchoring proteins can affect cell migration and attachment (Strudwick & Cowin, 2012). In melanocytes, such modifications directly influence the transport and distribution of melanosomes to skin and to hair shafts, thereby affecting pigmentation patterns observed among individuals (Cichorek et al., 2013; Schallreuter et al., 1998).

TUBB3 (beta-tubulin 3), in humans, is described as essential for cell proliferation and migration, with reduced expression impairing the organization and invasion of melanocytes in the skin (Orfanidis et al., (2017). This finding corroborates the results of the present study, in which TUBB3 and several other cytoskeleton-related genes were identified across different comparisons: CRACD and PRKD1 in the AS (All Scenarios together) framework; CSTPP1 and FNBP4 in the S1 vs. S2 comparison; HIP1R and CLIP1 in S1 vs. S6; MARCKS and VAV3 in S1 vs. S7; and WDR1 in S1 vs. S10. Together, our results suggest that variation in these genes may be associated with the wide diversity of coat color patterns observed in our scenarios. Thus, the results reinforce that genes involved in cytoskeletal dynamics may contribute to diverse and unique Gir breed coat color by modulating melanocyte migration and spoting during embryonic development.

After neural tube closure (Videira et al., 2013), neural crest cells migrate to different regions of the body rising several lineages, including melanoblasts, the precursors of melanocytes, as well as cells of the peripheral nervous system, cranial bones and cartilages, and the eye choroid (Borovanský J. and Wiley I., 2011; Bonaventure et al., 2013). From this differentiation process melanogenesis begins; a mechanism regulated by a complex signaling network that controls cell maturation and pigment synthesis (D'Mello et al., 2016). In this study, one gene associated with these biological processes was identified: *MC1R,* which is considered one of the main modulators of melanogenesis. Activation of this gene through binding to the α-MSH hormone triggers the cAMP signaling pathway, promoting the production of eumelanin, which is associated with darker coat colors, in contrast to pheomelanin, which is related to lighter tones (Ito & Wakamatsu, 2011).

Through the interplay between *AGOUTI* (also known as *ASIP* in cattle), α-MSH, and the *MC1R* receptor, mammals express yellow, red, and intermediate coat colors (Barsh, 1996). At the cellular level, in the absence of *AGOUTI*, α-MSH readily binds to MC1R, leading to

intracellular accumulation of cAMP. This increase in cAMP activates tyrosinase (Tyr), ultimately driving the synthesis of eumelanin (Le Pape et al., 2008; Ollmann et al., 1998; Walker & Gunn, 2010). In contrast, when *AGOUTI* is present, it binds to MC1R and reduces intracellular cAMP levels, downregulating Tyr expression and shifting melanocyte activity from eumelanin to pheomelanin production. Moreover, natural variation in mammalian pigmentation arises from genetic changes not only in *AGOUTI* and *MC1R*, but also in later steps of the melanogenic pathway, which directly influence the biochemical reactions that determine whether eumelanin or pheomelanin are produced (Kaelin & Barsh, 2013).

Biochemically, melanin synthesis begins with the amino acid tyrosine, which is converted by tyrosinase into DOPA and subsequently oxidized into DOPA-quinone. At this branching point, the presence of cysteine diverts DOPA-quinone toward the pheomelanin pathway, producing yellow- to red-toned pigmentation. In the absence of cysteine, DOPA-quinone undergoes a series of oxidation steps that generate intermediate molecules such as leuco-DOPAchrome and DOPAchrome, which can follow two major routes: one leading to eumelanin without a carboxyl group resulting in darker black pigmentation and another retaining the carboxyl group, producing brown eumelanin. It is important to emphasize that the gene associated with increasing and decreasing cAMP levels (*AGOUTI*) was not identified in the present study, nor in the work of (Maciel et al., 2024) conducted within the same breed. A plausible explanation is that the modulation of this pathway may depend on other factors involved in alternative regulatory mechanisms in *Bos indicus* populations, therefore more research is needed to understand the role of *AGOUTI* gene in indicine breeds.

Building upon this biochemical framework, previous studies have identified *LEF1* as a key regulator of pigmentation, and notably one of the most divergent loci among *Bos indicus* populations with contrasting coat colors (Chen et al., 2023; Guenther et al., 2014). In our analysis, this gene and the *FRAS1* gene was also detected when contrasting yellow and red

animals with the different shades of gray (S1 vs. S9). This finding offers a plausible explanation for the presence of the gray phenotype in Gir cattle, as mutations in *LEF1* have been shown in mice to cause locally impaired melanogenesis in mutant hair follicles, resulting in lighter coat coloration (van Genderen et al., 1994). Moreover, *LEF1* is expressed during both the development and regeneration of hair follicles, a pattern that may contribute to diluted or grayish tones (DasGupta & Fuchs, 1999; Zhou et al., 1995). Taken together, these observations reinforce *LEF1* as a strong candidate acting as a regulatory enhancer influencing melanogenic gene activity (Visser et al., 2012).

Another noteworthy finding emerged in the comparison between S1 and S10, where *CORIN* stood out among a broader set of 14 genes associated with signal transduction (*DBNDD1, DDB2, PRKAG3, WNT6, WNT10A, TYK2, DUSP12, HEG1, VSTM4, CORIN, CNGA1, TXK, TEC, KIT*, and *NGF*). Within this group, *CORIN* is of particular interest because it encodes a serine peptidase expressed in hair follicles and operates downstream of ASIP as a suppressor of pheomelanin production (Enshell-Seijffers et al., 2010). According to Sinding et al., (2021), an haplotype segment of *CORIN* present in modern cattle traces back to the kouprey (*Bos* sp.), an extinct wild bovine characterized by dark coat pigmentation, and this introgressed haplotype has been reported in Leiqiong cattle. However, loss-of-function variants in *CORIN* have been associated with increased pheomelanin banding and lighter coat coloration in several species, including tigers (Xu et al., 2017) and mice (Enshell-Seijffers et al., 2008), supporting its functional relevance in modulating pigmentation patterns.

Among the mechanisms associated with coat color variation, this study identified five genes involved in melanocyte migration: *SPIRE2, LAMA4, CDH26, RNF5*, and *KIT*. Among them, *KIT* is particularly notable, being detected in the contrast involving 12 animals with predominantly white coats (S1 vs. S10), suggesting a specific role in the expression of this phenotype. In Holstein cattle, similar associations have been reported, as demonstrated by

Jivanji et al., (2025), who identified two major loci underlying the characteristic white spotting pattern of the breed. In contrast, in Gir cattle (Maciel et al., 2024) also detected *KIT*, but the variants were associated with distinct coat color patterns, including red phenotypes, “red choker,” “calico clear,” and darker tonalities.

In other species, such as the Tianzhu White yak, fully white coats have been associated with KIT-related alleles, including chromosomal translocations between chromosomes 6 and 29 and structural duplications, suggesting a genetic mechanism shared with taurine cattle (F. Zhang et al., 2023). Conversely, other coat patterns that include white areas such as spotted or roan phenotypes do not appear to depend on *KIT*, as observed in the remaining contrasts evaluated in this study. In Nguni cattle, for example, specific patterns such as white head striping or lateral patches may involve additional genes and signaling pathways, indicating that *KIT* plays a predominant role in extensive depigmentation phenotypes, whereas more subtle variations likely result from the interaction of multiple modifier genes (Kunene et al., 2022).

Among modern cattle breeds, coat phenotype differentiation includes well-recognized examples such as the belted pattern of Galloways, the characteristic white face of Herefords and Simmentals (Awasthi Mishra et al., 2017), and the lateral pigmentation observed in White Parks (Milia et al., 2025). In Holsteins, however, the white spotting pattern is particularly prominent (Jivanji et al., 2025). Although the alleles underlying most of these phenotypes have already been identified, the genetic basis of the Holstein spotting pattern was one of the last to be elucidated at the molecular level (Georges et al., 2019). This characteristic appears to be associated, at least in part, with oligogenic architecture. Rather than being determined by a single Mendelian major-effect variant, the extent of depigmentation in the coat seems to result from the combined influence of several quantitative alleles of moderate effect, along with many others of smaller contribution (Hayes et al., 2010; Jivanji et al., 2019).

A polygenic component was also evident on BTA6, where, in addition to *KIT* widely reported in the literature other genes associated with coat color variation were identified. In Maciel et al., (2024), different phenotypic classification strategies demonstrated the contribution of multiple additional loci on the same chromosome. According to these authors, in scenarios 1 and 2, defined by predominantly red phenotypes or combinations of red and yellow, the association of *PCDH7* was observed. In scenarios 3, 6 and 8, which encompassed more specific coat patterns such as red variants, calico-like spotting, darker tonalities or lighter coats, associations with *CORIM*, *KIT, KDR* and *PDGFRA* were detected. Additionally, scenario 6 included contributions from *ADAMTS3, ALB, AREG, GC* and *RASSF6*, while scenario 8 also demonstrated associations with *CDS1* and *MAPK10*. Together, these results reinforce that BTA6 harbors a diverse set of genes which, acting in a complementary manner, modulate distinct coat color manifestations in Gir cattle, evidencing a substantial polygenic component beyond the major loci already recognized.

Compared to the previous study, three of the genes identified here were also identified by the authors on BTA: *CORIM, KIT*, and *KDR*. Additional associated loci on BTA 6 identified in our current study included *CRACD* (AS), *FRAS1* (S1 vs S8), and *LEF1, TEC, ATP8A1*, and *WDR1* (S1 vs S10). The differences in the genes detected between studies may be partially explained not only by differences in sample size (Mostafavi et al., 2023), but mainly by the population structure and the different scenarios designed in both studies. Nevertheless, although the scenarios analyzed in our study varied widely in their phenotypic characterization and in number of individuals ranging from small subsets to larger phenotypic groups, rigorous and consistent phenotyping made by trained technicians can reduce within-group noise and strengthen the biological signal, helping explain the detection of genes genuinely associated with specific coat patterns.

Our results reinforce the principle that greater phenotypic homogeneity tends to reduce genetic heterogeneity and, consequently, increase statistical power for the detection of significant effects. In this context, the contrast involving the 12 white-coated animals, in which an association with the *KIT* gene was detected, illustrates how relatively small populations, when well-structured and subjected to rigorous phenotyping, may be sufficient to reveal robust and biologically meaningful genetic signals (Dahl et al., 2023; Manchia et al., 2013).

Having identified these loci, it is also important to consider how the genes identified here may functionally contribute to the observed pigmentation phenotypes. Beyond the central role of *KIT* in melanocyte migration and differentiation, several other genes identified in this study may influence the establishment, maintenance, and performance of pigment cells in the epidermis. Among them, *GAS8* is linked to ciliary function and cellular orientation (Lewis et al., 2016); *SHCBP1*, *SLC12A8 and WSB2* to cell proliferation during development (Buckley et al., 2014; Y. Zhang et al., 2019); *ATP8A1* and *CNTN4* to later migratory processes (Kato et al., 2013; Mercati et al., 2013); and *KDR* and *EDIL3* to angiogenesis (Niu et al., 2023), providing essential vascular support for melanocyte survival.

Differential regulation of these genes suggests that pigmentation maintenance depends not only on melanin synthesis but also on mechanisms ensuring presence, distribution, and longevity of pigment cells. Furthermore, these genetic variations may influence not only visible coat coloration but also contribute to physiological adaptation in tropical environments such as enhanced UV protection, improved thermal regulation, and resilience to environmental stressors (Asmarasari et al., 2023; Islam et al., 2023), indicating possible pleiotropic effects of evolutionary and livestock relevance.

Recent discussions have questioned the utility of GWAS based on its limited ability to identify causal variants under formal structural causal inference frameworks (Cantet & Jensen, 2024). However, in livestock populations characterized by structured pedigrees and extended

linkage disequilibrium, GWAS is designed to detect genomic regions in marker–Quantitative Trait Loci to capture additive genetic variance, rather than to isolate individual functional mutations (Dominguez-Castaño et al., 2024; Qanbari, 2020).

Therefore, genomic dissection strategies such as GWAS enable a comprehensive analysis of the genetic underpinnings of complex traits, such as the unique beauty behind coat color pattern in Gir. On the top of that, validation of genes and variants relies on the success of GWAS, which in turn rely on the number of relevant loci, genetic architecture of each trait, sample size, variant panel, and the trait's heterogeneity (Visscher et al., 2017). In line with this statement, validation process should take previous studies into account and as far as we know, only another study has investigated the association of genes and coat color in Gir, which used a low number of animals and a different classification of the color patterns Maciel et al., (2024).

Hence, our study is of relevance for livestock genomics, as it advances our understanding about genetic architecture of coat color patterns. To date, a limited number of studies have investigated this trait in zebu cattle, underscoring the novelty of the present research. In this way, our research presents major evidences about the genomic regions to be explored for coat color patterns in Gir cattle. The results presented here will serve as a basis for further in-depth studies not only aiming functional validation of coat color genes but also uncovering putative associations between the genetics of color with production traits.

CONCLUSIONS

The analysis of linkage disequilibrium (LD) and effective population size (Ne) revealed high historical recombination and a recent reduction in genetic diversity in the Gir breed, reflecting the effects of selection and the concentrated use of sires. Based on this population framework, the GWAS investigation identified 52 candidate genes involved in various coat color mechanisms, including melanogenesis, melanocyte migration and differentiation,

embryonic development, and cytoskeletal dynamics, with particular emphasis on *MC1R, CORIN, KIT,* and *KDR*, which have been widely reported in previous studies. These findings contribute to the understanding of the poligenic architeture and phenotypic variation in the unique Gir breed coat color, providing valuable insights for breeding strategies that consider both visible traits and the maintenance of genetic diversity. For the next steps, studies correlating coat color and production traits are advised.

NOTES

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Data availability

The data sets analyzed during the current study are not publicly available because databases belong to private commercial farms.

Ethics approval statement

Ethics committee approval was not necessary, as we received the database ready for analysis.

Conflict of interest statement

The authors have not stated any conflicts of interest.

ORCIDS

Marcelo José Böck https://orcid.org/0000-0002-1644-3629

Mateus Guimarães dos Santos https://orcid.org/0000-0002-6475-9951

Fabrício Pilonetto https://orcid.org/0000-0002-2161-0831

Renata de Fátima Bretanha Rocha https://orcid.org/0000-0002-0552-7042

Pamela Itajara Otto https://orcid.org/0000-0003-1702-4626

Marcos Vinícius Barbosa da Silva https://orcid.org/0000-0001-5449-1413

Simone Eliza Facioni Guimarães https://orcid.org/0000-0003-3704-8131

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TABLES

Table 1. Grouping of Gir animals according to the distribution of coat color phenotypes in scenarios and the corresponding total number of animals per group.

Scenario	Phenotypes	Total Animals
S1	[a] yellow (n = 81), red (n = 3,739)	3.820
S2	red choker (n = 3,718), yellow choker (n = 38)	3.756
S3	red calico (n = 21,699), yellow calico (n = 221)	21.920
S4	calico red (n = 13,814), calico yellow (n = 185)	13.999
S5	calico clear (n = 4,386)	4.386
S6	light brown (n = 1,587)	1.587
S7	red brown (n = 406), dark brown (n = 206)	612
S8	light roan (n = 73)	73
S9	yellow calico gray (n = 6), yellow gray (n = 7), red gray (n = 7), gray (n = 2), gray yellow (n = 27), gray calico (n = 22), gray calico yellow (n = 17), gray calico red (n = 16), gray choker (n = 1), gray red (n = 50), calico gray (n = 21)	176
S10	white (n = 12)	12
Total		**50.341**

[a] Scenario 1 was used as the reference group. Representative illustrations of all phenotypes are available in Figure S1 (Supplementary Material).

Table 2. GWAS analyses performed for all scenarios together (AS) and scenarios (S1 to S10) comparison, including the total number of animals per analysis and heritability results.

Analyse	Scenario comparison	Total Animals	Heritability (h^2) ± SD

1	AS	50.341	0.58 ± 0.00
2	S1 vs S2	7.576	0.16 ± 0.01
3	S1 vs S3	25.740	0.35 ± 0.01
4	S1 vs S4	17.819	0.66 ± 0.01
5	S1 vs S5	8.206	0.73 ± 0.01
6	S1 vs S6	5.407	0.71 ± 0.01
7	S1 vs S7	4.432	0.22 ± 0.01
8	S1 vs S8	3.893	0.24 ± 0.01
9	S1 vs S9	3.996	0.26 ± 0.01
10	S1 vs S10	3.832	0.27 ± 0.01
11	S1*	3.820	0.75 ± 0.02

* Indicates a comparison between red and yellow Gir animals.

Table 3. Observed and estimated Linkage disequilibrium (r^2), and number of SNP pairs for each distance classes in kilobases (kb).

Distance classes (kb)	**Observed r^2 (mean ± SD)**	**Estimated r^2 (mean ± SD)**
0–5	0.431 ± 0.369	0.804 ± 0.077
5–10	0.388 ± 0.356	0.599 ± 0.047
10–20	0.363 ± 0.347	0.434 ± 0.048
20–30	0.345 ± 0.340	0.309 ± 0.024
30–40	0.330 ± 0.335	0.240 ± 0.014
40–50	0.318 ± 0.330	0.196 ± 0.010
50–100	0.302 ± 0.324	0.144 ± 0.022
100–200	0.278 ± 0.314	0.079 ± 0.012

200–300	0.280 ± 0.319	0.044 ± 0.004
300–400	0.274 ± 0.328	0.031 ± 0.002
400–500	0.308 ± 0.319	0.023± 0.001
500–600	0.344 ± 0.386	0.019 ± 0.001
600–700	0.551 ± 0.403	0.016 ± 0.001
700–800	0.317 ± 0.468	0.014 ± 0.001
800–900	0.255 ± 0.383	0.012 ± 0.001
900–1000	0.097 ± 0.096	0.011 ± 0.001

Table 4. Proportion of additive genetic variance explained by the top 10 genomic windows identified in 0.5 Mb for each scenario comparison.

Scenario comparison	**Variance Explained in the top 10 windows (%)**
AS	24
S1 vs S2	1.41
S1 vs S3	1.36
S1 vs S4	1.60
S1 vs S5	1.34
S1 vs S6	1.47
S1 vs S7	1.40
S1 vs S8	7.10
S1 vs S9	22.99
S1 vs S10	9.32
S1	4.1

AS= All Scenario together; S= Scenario

FIGURES

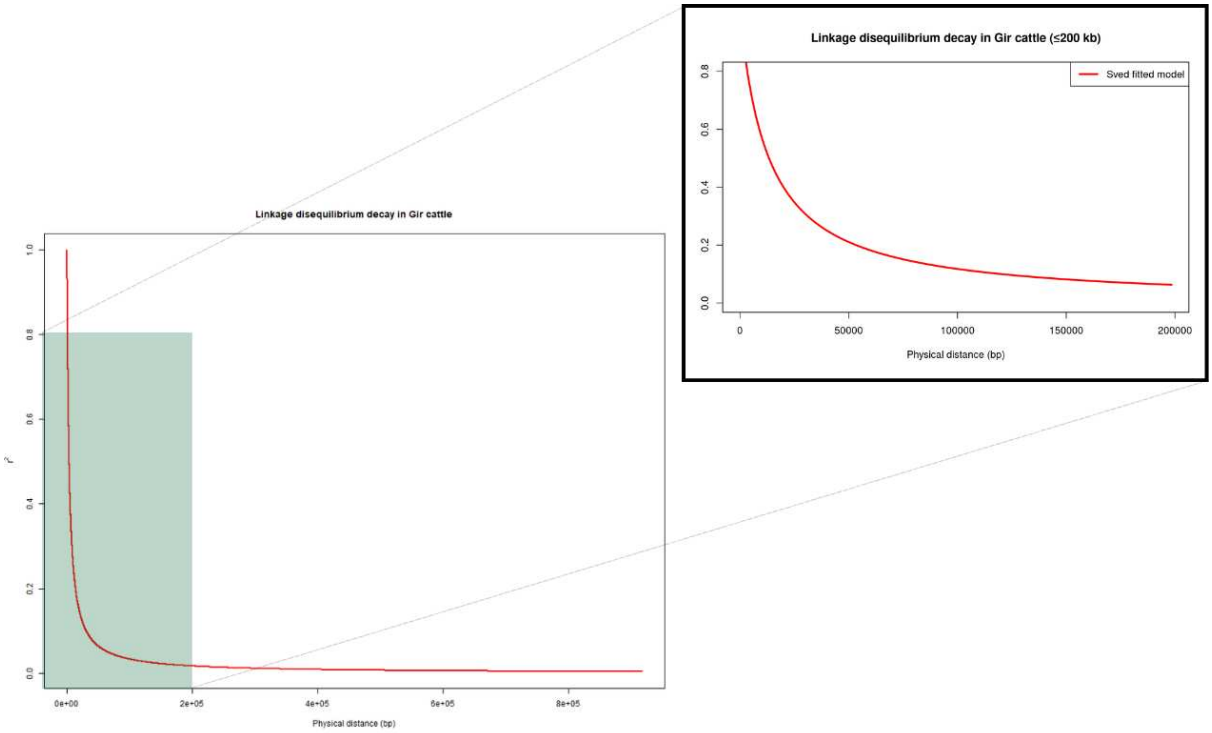

Figure 1. Linkage disequilibrium decay in Gir cattle

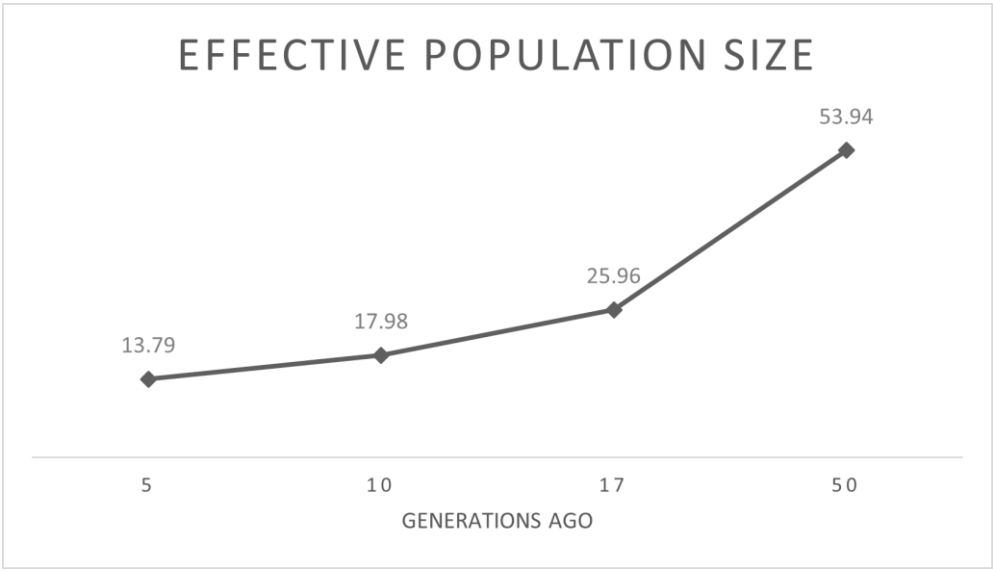

Figure 2. Estimated effective population size based on LD in Gir cattle in the last fifty generations

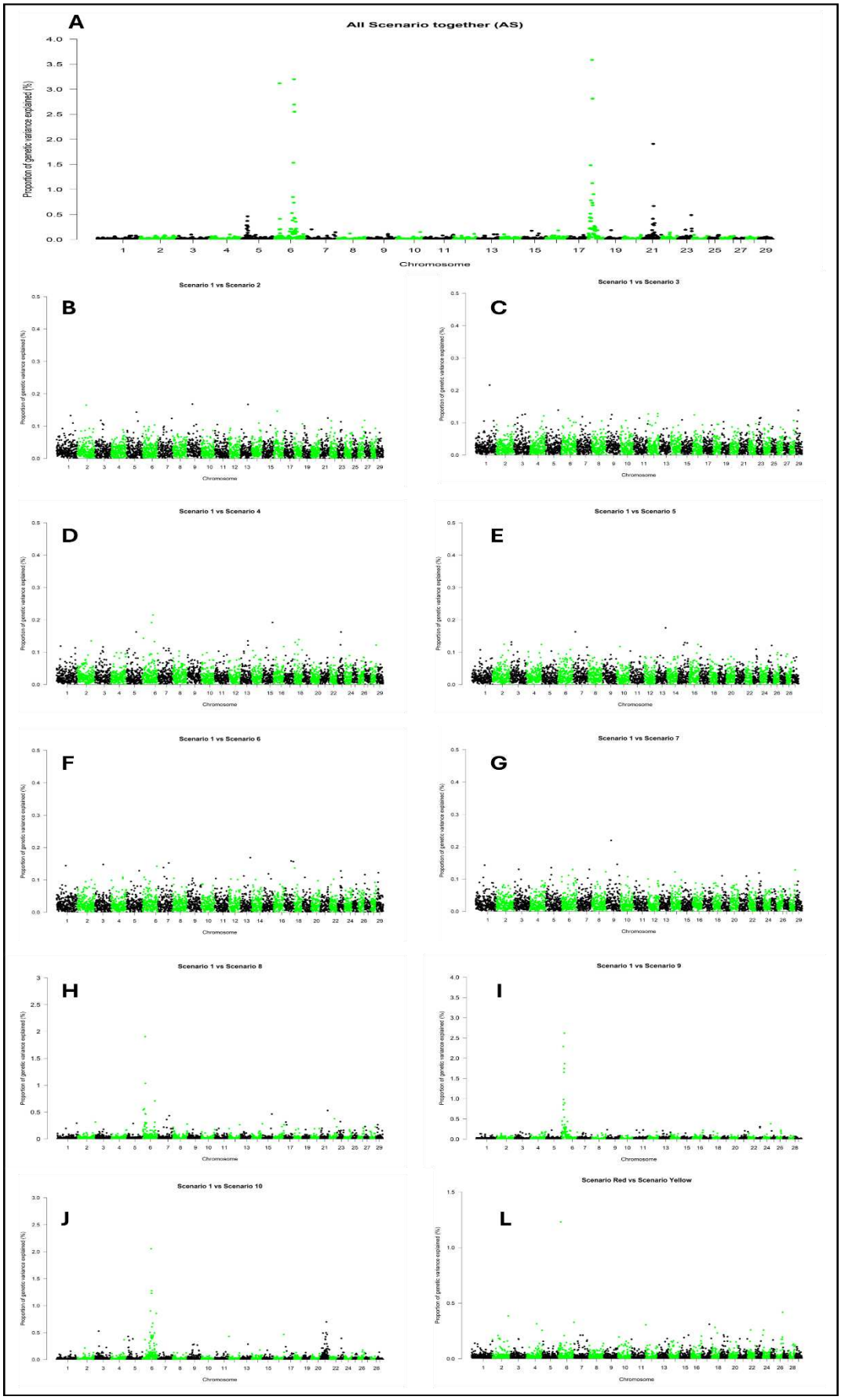
A
All Scenario together (AS)
Proportion of genetic variance explained (%)
Chromosome
B
Scenario 1 vs Scenario 2
C
Scenario 1 vs Scenario 3
D
Scenario 1 vs Scenario 4
E
Scenario 1 vs Scenario 5
F
Scenario 1 vs Scenario 6
G
Scenario 1 vs Scenario 7
H
Scenario 1 vs Scenario 8
I
Scenario 1 vs Scenario 9
J
Scenario 1 vs Scenario 10
L
Scenario Red vs Scenario Yellow

Figure 3. Manhattan plots for (A) All scenarios together (AS); (B) Scenario 1 vs. Scenario 2; (C) Scenario 1 vs. Scenario 3; (D) Scenario 1 vs. Scenario 4; (E) Scenario 1 vs. Scenario 5; (F) Scenario 1 vs. Scenario 6; (G) Scenario 1 vs. Scenario 7; (H) Scenario 1 vs. Scenario 8; (I) Scenario 1 vs. Scenario 9; (J) Scenario 1 vs. Scenario 10 and (L) Scenario Red vs Scenario Yellow.

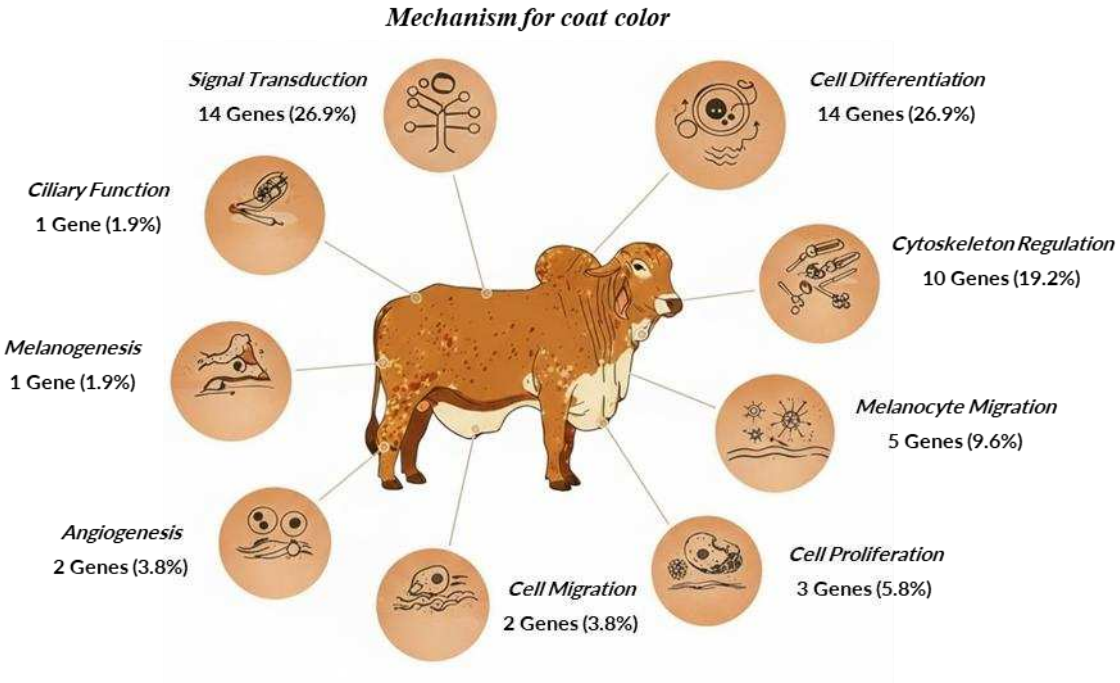

Figure 4. Illustration of the nine main biological mechanisms related to coat color in Gir cattle, displaying gene frequency and percentage distribution per functional category.

Supplementary material:

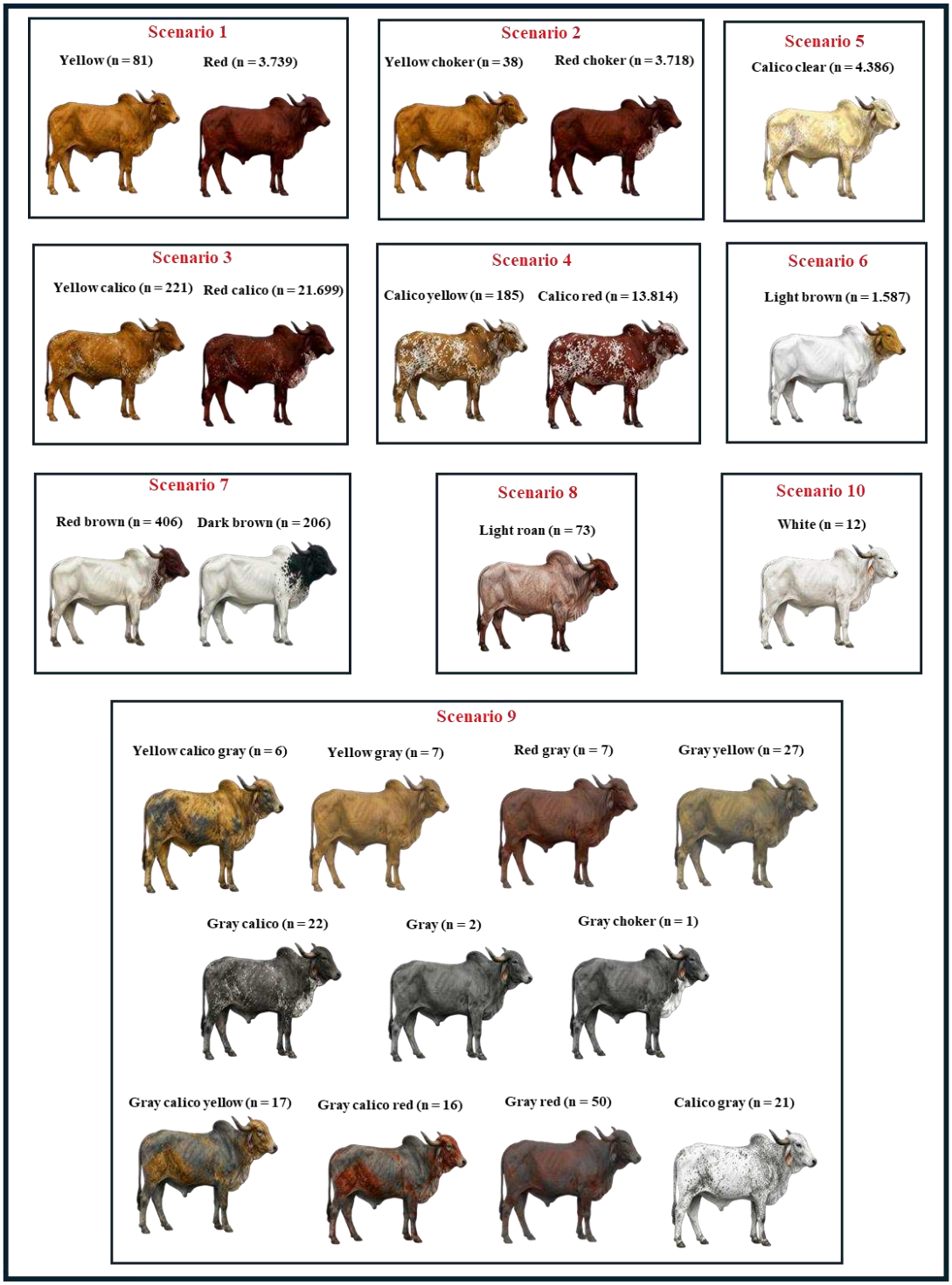

Figure S1. Visual representation of the 25 coat color phenotypes of Gir cattle, organized across the 10 analytical scenarios defined in this study. The images are synthetic representations generated by artificial intelligence (AI) and are intended solely for illustrative purposes to demonstrate the diversity of colors and patterns analyzed.

Supplementary material:

Gene	Chromosome position	Genomic window	Mechanism for coat color	Scenarios
…	BTA18: 14652325-14679679	chr18: 14635894-15117159	Melanocyte migration	AS
TCF25	BTA18: 14681409-14702855	chr18: 14635894-15117159	Cell differentiation	AS
MC1R	BTA18: 14704921-14706836	chr18: 14635894-15117159	Melanogenesis	AS
TUBB3	BTA18: 14708701-14717351	chr18: 14635894-15117159	Cytoskeleton regulation	AS
DBNDD1	BTA18: 14764643-14772638	chr18: 14635894-15117159	Signal transduction	AS
GAS8	BTA18: 14772717-14793471	chr18: 14635894-15117159	Cilia function	AS
SHCBP1	BTA18: 14881869-14921740	chr18: 14635894-15117159	Cell proliferation	AS
KDR	BTA6: 70567551-70612407	chr6: 70444339-70944134	Angiogenesis	AS
CRACD	BTA6: 71456715-71731124	chr6: 70976106-71476002	Cytoskeleton regulation	AS
PRKD1	BTA21: 40113587-40461720	chr21: 39911981-40408803	Cytoskeleton regulation	AS
LAMA4	BTA9: 38176374-38331035	chr9: 37928684-38427192	Melanocyte migration	S1 vs S2
CDH26	BTA13: 56543952-56591024	chr13:56215537-56675942	Melanocyte migration	S1 vs S2
GSC	BTA21: 59627719-59629640	chr21:59156531-59656243	Cell differentiation	S1 vs S2
SCHIP1	BTA1: 107552331-107700466	chr1: 107471401-107967755	Cell differentiation	S1 vs S3
GRIN2B	BTA5: 95878616-96314867	chr5:96128475-96625241	Cell differentiation	S1 vs S3
PATJ	BTA3: 83535662-83936155	chr3: 83850660-84345909	Cell differentiation	S1 vs S3
CSTPP1	BTA15: 76852599-77063226	chr15:77040247-77538313	Cytoskeleton regulation	S1 vs S4
DDB2	BTA15: 77097834-77118219	chr15:77040247-77538313	Signal transduction	S1 vs S4
FNBP4	BTA15: 77482662-77513417	chr15:77040247-77538313	Cytoskeleton regulation	S1 vs S4
NOTCH4	BTA23: 27171379-27196526	chr23:26913683-27413323	Cell differentiation	S1 vs S4
RNF5	BTA23: 27209389-27211704	chr23:26913683-27413323	Melanocyte migration	S1 vs S4
PRKAG3	BTA2: 106786779-106794309	chr2:106568058-107067411	Signal transduction	S1 vs S4
WNT6	BTA2: 106822096-106835109	chr2:106568058-107067411	Signal transduction	S1 vs S4
WNT10A	BTA2: 106841006-106855958	chr2:106568058-107067411	Signal transduction	S1 vs S4
MIR375	BTA2: 106945879-106945957	chr2:106568058-107067411	Cell differentiation	S1 vs S4
TYK2	BTA7: 14876130-14904370	chr7:14713432-15213296	Signal transduction	S1 vs S5

DUSP12	BTA3: 7852461-7860682	chr3:7553170-8051799	Signal transduction	S1 vs S5
HIP1R	BTA17: 52670416-52697383	chr17:52606632-53104143	Cytoskeleton regulation	S1 vs S6
CLIP1	BTA17: 52998901-53116294	chr17:52606632-53104143	Cytoskeleton regulation	S1 vs S6
HEG1	BTA1: 69421853-69501770	chr1:69461843-69958810	Signal transduction	S1 vs S6
SLC12A8	BTA1: 69520941-69670973	chr1:69461843-69958810	Cell proliferation	S1 vs S6
HDAC2	BTA9: 36449497-36498028	chr9:36256957-36756473	Cell differentiation	S1 vs S7
MARCKS	BTA9: 36554305-36558473	chr9:36256957-36756473	Cytoskeleton regulation	S1 vs S7
VAV3	BTA3: 35311303-35744307	chr3:35655214-36144427	Cytoskeleton regulation	S1 vs S7
VSTM4	BTA28: 43287483-43376898	chr28:43252010-43743676	Signal transduction	S1 vs S7
FRAS1	BTA6: 92761853-93295484	chr6:92584159-93083217	Cell differentiation	S1 vs S8
VCAN	BTA7: 83356422-83476757	chr7:83441451-83940138	Cell differentiation	S1 vs S8
HAPLN1	BTA7: 83538398-83616831	chr7:83441451-83940138	Cell differentiation	S1 vs S8
EDIL3	BTA7: 83845453-84467512	chr7:83441451-83940138	Angiogenesis	S1 vs S8
LEF1	BTA6: 17083395-17200675	chr6:16707313-17203853	Cell differentiation	S1 vs S9
CORIN	BTA6: 66273891-66582006	chr6:66492082-66991013	Signal transduction	S1 vs S10
CNGA1	BTA6: 66668903-66706869	chr6:66492082-66991013	Signal transduction	S1 vs S10
TXK	BTA6: 66757241-66817720	chr6:66492082-66991013	Signal transduction	S1 vs S10
TEC	BTA6: 66819043-66989142	chr6:66492082-66991013	Signal transduction	S1 vs S10
KIT	BTA6: 70166682-70254046	chr6:69907488-70407474	Melanocyte migration	S1 vs S10
ATP8A1	BTA6: 61243693-61477856	chr6:61141636-61640981	Cell migration	S1 vs S10
WDR1	BTA6: 105290993-105332781	chr6:105284538-105780909	Cytoskeleton regulation	S1 vs S10
VANGL1	BTA3: 27623362-27677387	chr3:27624802-28124322	Cell differentiation	S1 vs S10
NGF	BTA3: 27925796-28007117	chr3:27624802-28124322	Signal transduction	S1 vs S10
AEBP1	BTA4: 77200630-77212311	chr4:76856202-77352212	Cell differentiation	S1*
WSB2	BTA17: 57115953-57184300	chr17:57076823-57575462	Cell proliferation	S1*
CNTN4	BTA22: 23254010-24279951	chr22:23185282-23682224	Cell migration	S1*

FINAL CONSIDERATIONS

Coat color can be understood as a trait of zootechnical interest, and not merely as an aesthetic criterion for breed standardization

The wide diversity of coat colors observed in the Gir breed represents an important biological resource, with potential links to adaptation, hardiness, and responses to environmental stress, especially in tropical production systems.

Moderate to high heritability estimates indicate strong genetic control of coat color, reinforcing its potential response to selection. Although classified as a qualitative trait, its expression reflects a complex genetic architecture, involving the joint action of multiple genes and pleiotropic effects.

The pattern of linkage disequilibrium (LD) decay and the estimates of effective population size (Ne) indicate high historical recombination, associated with a recent reduction in genetic variability, possibly due to intensified selection and the concentrated use of a limited number of sires.

The complexity of the genetic mechanisms involved in coat color determination, particularly the role of MC1R, ASIP, and modifier genes associated with white patterns, highlights the polygenic nature of the phenotype and expands the possibilities for investigating its relationships with productive traits.

The recurrent identification of genomic regions on chromosome 6, previously reported in the literature as being associated with productive traits in the Gir breed, suggests potential connections between genetic pathways related to pigmentation and productive performance, opening new perspectives for integrative studies in animal breeding.