# RESEARCH





# Selection signatures and inbreeding: exploring genetic diversity in five native horse breeds

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# Abstract

Horses have undergone extensive natural and artificial selection, shaping the diversity of breeds observed today. Native Italian breeds present unique traits influenced by natural selection, such as adaptation to harsh climates. or hoof strength, but face challenges due to population declines and the reduction of their original breeding purpose. This study focuses on five local Italian breeds: Bardigiano, Haflinger, Maremmano, Murgese, and Italian Heavy Draught Horse, to understand how selection has shaped their populations. A total of 1620 individuals were genotyped with a medium-density SNP chip and remapped to EquCab3. After guality control, where data were filtered based on missing genotypes per SNP (>0.10) and missing SNPs per sample (>0.10), 1498 horses and 54,825 SNPs remained for analysis. Population structure and runs of homozygosity (ROH) were identified, and genomic inbreeding coefficients were calculated based on ROH coverage of autosomal SNPs. ROH islands shared by  $\geq$  70% of horses were identified as selection signatures, and candidate genes within these regions were annotated. The inbreeding coefficient (FROH) ranged from 0.15 to 0.23, with Bardigiano and Haflinger showing the highest values probably due to selective breeding, while Maremmano, Murgese, and Italian Heavy Draught Horse displayed lower FROH, reflecting a broader diversity. ROH islands were identified on 12 chromosomes, with 23 islands distributed among breeds. Cold-blooded breeds (Bardigiano, Haflinger, and Italian Heavy Draught Horse) showed the majority, particularly on Equine Chromosome 3 (ECA3). These islands overlapped with 83 quantitative trait loci (QTLs) and 76 genes associated with morphology and health. Health-related traits such as osteochondrosis and hoof health were linked to ROH patterns, particularly in Bardigiano and Haflinger, highlighting selection for disease resistance. Signature of selections were found in the proximity of MC1R and ASIP genes likely due to their role for coat color; especially in the Haflinger and Italian Heavy Draught Horse the genotype frequency of the BIEC2 816499 SNP which is in the vicinity of the causative mutation for chestnut coat color is due to linkage disequilibrium between the two. In conclusion, this study offered valuable insights that breeders could utilize to make sound decisions.

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This issue would ensure the maintenance of breed genetic diversity, and the preservation and improvement of the breed's distinct traits and health standards.

Keywords Genotype, Conservation, Health, Horse

# Background

Both natural and artificial selection have played key roles in shaping horse breeds to become as we know them today [1]. If natural selection [2, 3] drives species to adapt to changing environments and climates, artificial selection has played a fundamental role in shaping horse conformation and behavior from the domestication process onwards. Differently from other livestock species that have been selected mainly for production traits like milk, meat and wool, horses have historically been bred for their use in agriculture, transportation, warfare, for aesthetic qualities and performance [4, 5], as well as for sports-related traits. Nowadays the range of target traits is wide [6] and depends on breeds, including body shape [7], size [8], color, gaits, and endurance [9].

The institution of studbooks and the modernization of breeding methods in the second half of the 19th century increased the selective pressure for desirable traits in horses, resulting in the creation of over 1,000 horse breeds worldwide [10], each with its own unique characteristics and phenotypes [11]. In recent years, differences among breeds have been accentuated even more since breeding programs of several breeds (typically called "warm-blooded") have shifted their focus to increase horse rideability and improve athletic performances [12], driven by the modern market's demand for sport horses [13]. In other breeds (mainly known as "cold-blooded"), the original heavy draught aptitude, widely used in agriculture, was partially replaced by the meat production aptitude during the second half of the 20th century. Nowadays, there is a renewed interest in the draught aptitude, but for use on holiday farms and leisure [14].

On top of that, there are also several horse populations, called native or local breeds, mainly shaped by decades of natural selection with minimal or no human intervention. Those breeds are generally well adapted to the environmental conditions of their native areas and are part of the historical heritage of specific territories. They are mostly used in agriculture and a part also for meat production; the Italian Heavy Draught Horse or the Murgese horse are two examples. However, with a current decline in meat consumption [15] and advancements in agricultural technology, these breeds have lost most of their purposes, resulting in a reduction in population size, a decrease in genetic diversity, and an increase in inbreeding. The selection process, whether natural or artificial, tends to cause changes in the patterns of variation among selected loci. These genomic footprints left by selection are known as selection signatures and can be used to identify loci that have been subjected to selection [16].

A focus on the Italian peninsula reveals the existence of many native breeds exhibiting a diverse range of forms and aptitudes. This setting provides a valuable opportunity to investigate the presence of selection signatures in horse populations. Italy is home to over 30 horse breeds, more than 20 considered native or autochthonous. These breeds fall into two main categories: (a) cold-blooded, primarily brachymorphic horses suited for heavy work; (b) warm-blooded, which are dolichomorphic and favored for sports and athletic activities [17]. Unfortunately, many Italian native breeds are declining in population size and usage as they struggle to compete with highly specialized transboundary breeds. Natural selection still plays an important role in shaping these breeds, as most are bred semi-extensively, such as the Bardigiano and the Haflinger. This allows the horses to adapt to their environment, making them robust and resistant [18].

While research on equine genomics began later than in other species, advancements in genomic tools have significantly improved our ability to comprehend the genetic background of traits, inbreeding, and genes associated with economic and performance traits [19].

In recent years, population-based approaches utilizing the loss of heterozygosity in the genome have successfully identified signals of selection in various domestic species also known as "runs of homozygosity" or ROH [20].

The study of selection signatures in Italian horses is crucial, as the complexity and polygenic effect of selected traits in horses are often challenging to capture using approaches such as genome-wide association studies [21]. Therefore, the identification of signatures of selection might be valuable for breeding organizations to understand further which genomic regions might have a role in traits of interest, thereby increasing the value of these breeds in the market. Recent studies on selection signatures in horses have primarily focused on highly specialized breeds [20, 21], highlighting potential regions under selection that could affect reproduction, sports performance, body size, type, and gaits.

By combining the study of genetic diversity, through the assessment of inbreeding from genotype data (FROH), with the analysis of selection signatures in Italian local breeds, we can gain valuable insights into how selection is shaping the population. This, in turn, can provide actionable recommendations for managing breeding programs and further valorizing peculiar aspects of each breed to ensure the preservation of these unique horse breeds.

In this study, we aim to understand how selection has shaped the local Italian horse breeds. To achieve this, we specifically focused on five different Italian horse breeds, chosen for their consistency, attitude, morphological type and geographical distribution throughout the entire Italian peninsula (Fig. 1): Bardigiano (BAR), Haflinger (HAF), Maremmano (MAR), Murgese (MUR), Italian Heavy Draught Horse or Cavallo Agricolo Italiano da Tiro Pesante Rapido (TPR).

# Results

# **Quality control**

The data filtering process allowed us to identify 54,825 common SNPs across the entire samples of 1498 horses. The mean inter-marker distance was 41.36 kb. The PCA plot, highlighting the genetic distance among the breeds, is presented in Fig. 2.

#### **ROH detection**

The genome length covered by ROH ranged from 347.5 Mb in MAR to 522.9 Mb in BAR. The other breeds had the following average values: HAF (494.9 Mb), MUR (372.3 Mb), and TPR (394.2 Mb). In Fig. 3, the average length (in kb) and number of ROH per breed are represented together through a scatter plot.

A total of 436,922 ROH were identified, with an average of 10,989 ROH detected per horse. For all the breeds, the majority of ROH was found in the shortest class (< 2 Mb),

with 116,323 (83%) in BAR, 72,092 (83%) in HAF, 64,344 (87%) in MAR, 19,669 (87%) in MUR, and 102,286 (90%) in TPR. Long ROH (>16 Mb) were observed in 83% of BAR horses, 60% of HAF, 47% of MAR, 50% of MUR, and 41% of the TPR.

#### Inbreeding

The level of inbreeding, as indicated by FROH, was assessed for each breed (Fig. 4). BAR and HAF breeds exhibited the highest levels of average inbreeding, specifically 0.23 and 0.22, while MAR, MUR, and TPR breeds showed lower levels of 0.15, 0.16, and 0.18, respectively. The breed with the widest range in FROH was BAR, with a minimum of 0.09 and a maximum of 0.41, while the breed with the narrowest range was MUR, with a minimum of 0.09 and a maximum of 0.14.

To differentiate between ancient and recent inbreeding, FROH was calculated based on six ROH length classes as shown in Table 1.

Across all breeds, the highest level of inbreeding was found due to short ROH, with an average of 0.05, suggesting that most inbreeding events occurred in the past. The average inbreeding due to longer ROH (> 16 Mb) was 0.02, with BAR horse exhibiting the highest value (0.03) (Fig. 5).

Furthermore, the analysis of inbreeding per chromosome revealed significant variation among chromosomes in terms of both average values and the distribution of inbreeding levels per individual. ECA23, ECA15, ECA4, ECA11, and ECA28 exhibited the highest average



Fig. 1 Pictures of the five Italian horse breeds evaluated in the study and their geographical place of origin



Fig. 2 Principal component analysis (PCA) plot of the five evaluated Italian horse breeds



Fig. 3 Scatter plot of the average length and number of ROH per breed



Fig. 4 Violin plots of the inbreeding based on ROH in the five Italian horse breeds. In red are presented the boxplot where the red horizontal line showed the median value

 Table 1
 Average number of ROH per each length in the five

 Italian horse breeds, normalized per number of horses

	,				
ROH length (Mb)	BAR	HAF	MAR	MUR	TPR
0.5-1	183	190	160	177	207
1–2	63	70	57	54	72
2–4	22	30	18	18	20
4–8	16	17	9	11	8
8–16	8	6	4	4	3
>16	3	1	1	1	1

inbreeding for BAR (0.29), HAF (0.28), MAR (0.18), MUR (0.20), and TPR (0.21), respectively. On the other hand, the lowest average inbreeding was observed on ECA25 for BAR (0.17), ECA27 for HAF (mean = 0.16), and ECA12 for MAR (0.10), MUR (0.12) and TPR (0.12) (Fig. 6).

#### Signature of selection

The presence of a ROH island, shared by at least 70% of individuals within a specific breed, was considered an indicator of selection. ROH islands were not uniformly distributed across the genome; in fact, 12 chromosomes out of the 31 contained ROH islands, with the following distribution: ECA2 (BAR), ECA3 (BAR, HAF, MUR, TPR), ECA6-7 (HAF), ECA8 (BAR, HAF), ECA10 (BAR, HAF), ECA11 (HAF), ECA13 (BAR), ECA15 (BAR, TPR), ECA16 (HAF), and ECA29 (BAR, TPR). ECA3 was the chromosome with the highest number of ROH islands, with all breeds except MAR having up to 3 ROH islands in this region (Supplementary Table 1).

A total of 23 ROH islands were identified, with varying numbers among horse breeds. HAF had the highest number of ROH islands (n = 10), followed by BAR (n = 7) and TPR (n = 4). The MAR and MUR breeds had only one ROH island each. Out of these identified ROH islands, 11 were found to be private to a single breed, while the remaining ones were shared by two breeds each.

Most of the shared ROH islands were among coldblooded breeds (BAR, HAF, TPR), except for one shared by MUR and TPR on ECA3 (Fig. 7).

A total of 83 QTLs were identified in the regions covered by ROH islands, 48 of which were related to exterior measurements such as wither height, white markings, body size, and other body measurements. The remaining 35 QTLs were related to health traits and diseases such as insect bite hypersensitivity, osteochondrosis, and guttural pouch tympany. Additionally, 76 genes were found in the same regions.

## Focus on coat color and height at withers

Allele frequencies for the SNPs closest to *MC1R (Melanocortin 1 Receptor)* and *ASIP (Agouti Signaling Protein)* genes were analyzed across all horse breeds to investigate their potential role in coat color variation. Only non monomorphic loci are presented as results. The results, presented in Fig. 8, revealed significant differences in genotype frequencies among breeds. Specifically, for the SNP BIEC2\_816499 closest to the *MC1R* locus, a high prevalence of the homozygous genotype AA was observed in HAF (100%) and TPR (95%). In contrast, SNP BIEC2\_618993 near the *ASIP* gene exhibited a different trend, revealing a higher incidence of the homozygous AA genotype in dark-coated horses; 97% of MUR



# Distribution of Inbreeding Coefficients by Class and Breed

Fig. 5 Inbreeding based on ROH per each ROH class, divided per each breed



Fig. 6 Inbreeding based on ROH per each chromosome divided per each breed

and 98% of BAR with a known black coat color presented this genotype.

To investigate whether the SNP closest to the *LCORL* gene, located within a ROH island in MUR and TPR,

might play a role in height at withers, we analyzed genotype frequencies across all breeds and then specifically in the BAR breed, where measured height at withers was available (Fig. 9).



Fig. 7 Veen diagram to represent shared and private ROH islands among breeds





Fig. 8 Genotype frequency for SNPs in the proximity of the Extension and Aguti loci to evaluate their potential to be predictive of coat colors in horses. A) Frequency of BIEC2\_816499 per breed, B) Frequency of BIEC\_618993 per breed, C) Frequency of BIEC2\_618993 per known coat color in Bardigiano horse breed



Fig. 9 Genotype frequency for SNPs in the proximity of the *LCORL* loci to evaluate their potential to be predictive of height at withers in horses. A) Frequency of BIEC2\_99523 per breed, B) Average Height by year category in the Bardigiano horse breed, C) Percentage of BIEC2\_99523 by year of birth class in the Bardigiano horse breed

Genotype GG was found to have a frequency of 6% in BAR and 13% in the HAF. In comparison, amongst three breeds classified as horses, 93% of MAR, 95% of MUR, and 98% of TPR exhibited the AA genotype. Within BAR, the increasing height at the withers (+2 cm) measured from the available phenotypic data corresponded to the rising frequency of the GG genotype across different birth cohorts, starting from 0% in horses born before 2009, increasing to 5.2% for those born between 2007 and 2015, and at 6.7% for horses born after 2015. The GG BAR horses exhibited a higher height at withers than AA or GA BAR horses (respectively+0.1 and +0.16 SD, Fig. 10).

# Discussion

# **ROH Estimation**

Based on the average length and number of ROH per breed, it is possible to visualize two main clusters: one including the cold-blooded breeds: BAR, HAF, and TPR, and the other one with the warm-blooded ones: MAR and MUR. Therefore, it seems that based on ROH length and number, it is possible to differentiate between types, probably due to different breeding strategies.

In particular, cold-blooded breeds exhibited a higher average number of ROH than warm-blooded oriented breeds. This fact could be attributed to the genetic introgression from transboundary breeds into Italian warmblooded breeds to enhance their sport performances. This might, therefore, have increased the level of diversity and subsequently resulted in a reduction in the presence of ROH. Indeed, MAR horses have undergone introgression with external breeds [22], primarily thoroughbreds, which accounted for 20% of their bloodline [23]. Similarly, MUR is historically connected to Arabian and Apulian horses [24]. Additionally, the average length of ROH followed a similar trend in numerosity across breeds, except for TPR, which exhibited a shorter lenght of ROH compared to other cold-blooded breeds. This could be due to a higher selective pressure experienced by TPR in the past for draft or meat production compared to recent years. Indeed, TPR is morphologically distant from the modern type of sport horses, and no selective pressure is ongoing to convert this breed towards the current sport horse type demands.



Fig. 10 Gardner-Altman three groups estimation plot shows the comparison in height at withers in AA, GA, and GG BAR horses code-colored by three birth year cohorts

#### Inbreeding

From previous studies [17] the studied breeds showed a specific identity and unique genetic setting if compared between them and with international breeds. This unicity is a sign of a limited excange of genetic material that could have contributed to an increase of inbreeding level in the population.

The values of FROH exhibited by cold-blooded breeds are generally higher than what is found in warm-blooded. This finding aligns with the results from ROH numbers and length, where cold-blooded breeds presented a higher number of ROH paired with a longer length, resulting in an increased inbreeding level. BAR and HAF are slightly higher than previous estimates but fall within the expected range. For instance, BAR inbreeding was previously reported to be 17% based on genotype data [25], while in HAF it was estimated to be 14% [26]. In both cases, these estimates were higher than those derived from pedigree data, suggesting the potential for hidden relatedness not captured by traditional pedigree records [27].

As mentioned the highest inbreeding in BAR and HAF can be attributed to the higher presence of longer ROH than in the other breeds, indicating recent inbreeding events. Furthermore, their significant differentiation from other Italian breeds [17] suggests a lack of significant introgression from external breeds, along with high selective breeding pressures. Among cold-blooded breeds, the TPR presented a lower inbreeding level due to the lower number of ROH, the value found is consistent with previous studies [14]. This is the first study assessing ROH in MUR and MAR breeds, for which only pedigree analysis were available [28].

The majority of ROH in all breeds were found in the shortest length class. This may be attributed to the limited original population size and genetic pool of local breeds. The lowest percentage of ROH was observed in the longest class (ROH>16 Mb), indicating a relatively small reduction in genetic variability for HAF, MAR, MUR, and TPR in recent generations. However, the highest level of inbreeding due to the long ROH class observed in BAR horses suggests recent inbreeding and potentially reflects a more recent bottleneck in the breed's history. Based on studbook entry registration, in BAR a limited number of breeding animals has been used in recent generations, with one stallion being predominantly used. This stallion has sired 236 offspring due to its breeding value for height at withers, with the first being born in 2003 and the last in 2022. Those breeding choices might have caused the more recent inbreeding in BAR breed. Furthermore, this breed is undergoing

selection to enhance its market desirability, with a focus on novel traits related to gaits and rideability [29].

#### Signatures of selection

In this research, we used medium-density genotype data to detect signs of selection in various Italian horse breeds. Several genome regions selected between and within separate breeds were identified. To prevent discussion of potential false positive results, we focused on ROH islands present in at least 70% of the animals within a breed, and including at least five SNPs. Both private and shared signatures of selection will be discussed below within category of traits potentially under selection, which were defined based on the QTLs and genes located within the region of interest.

#### Selection signatures related to exterior traits

Throughout history, horses have been bred first based on their physical attributes. Conformation and morphological traits are closely linked to sports performances, such as show jumping and dressage. Other exterior traits, such as coat color, contribute to horses' aesthetic, making them desirable for recreational activities and as companions. Moreover, physical characteristics can impact on horse's longevity and health [30].

In this study, several ROH islands overlapped with QTLs related to exterior traits, with the majority located on ECA3 and shared between TPR and MUR. These two breeds are known for their muscular structure, size, strength, and stamina, as they were traditionally used to work in agriculture and transportation [31, 32]. Nowadays, they serve different purposes, with TPR horses primarily used for meat production [33] and heavy draft work, while MUR horses mainly employed for light draft and saddle. Therefore, we hypothesize that, although we found shared signatures of selection in TPR and MUR, they might be caused by different reasons. The selection program of TPR horses uses genetic evaluation of linear morphological traits such as muscle mass and diameters due to the main breeding goal of meat production, as well as expression and temperament for heavy draft work [31]. Therefore, we believe that the signatures of selection in body measurements QTL rich regions were in TPR due to the specific breeding goal towards meat and heavy draft purposes. The presence of signatures of selection within those QTLs was found in other draft breeds such as the Noriken, which corroborates our hypothesis [6]. On the other hand, the breeding association of MUR horse assesses several morphological traits by linear scoring method, with a breeding goal towards taller horses [24]. This latest aspect has grown in importance, especially in recent years, with an increased demand for MUR horses for military purposes. Specific prerequisites for these horses include a well-built structure and a height at withers above 170 cm. Thus, in the MUR breed, those signs of selection might be driven by the current interest in morphological requirements towards specific riding purposes. Within the signatures of selection on ECA3, some interesting genes corroborate the hypothesis of selection pressure for body size and height at withers in the two breeds. For example, within the ECA3 signature of selection in MUR and TPR, the *LCORL (Ligand Dependent Nuclear Receptor Corepressor Like)* gene is located, and it is known to play a significant role in determining skeletal size and stature in several animal species. In horses, *LCORL* has been strongly associated with height at the withers [34].

While the gene LCORL is not the sole determinant of height at withers, its impact on this trait is well-documented in the literature. Notably, when looking at the closest SNP to the LCORL gene, only two breeds, HAF and BAR, exhibited homozygosity for the G allele. Interestingly, these two breeds are both ponies and share a common breeding purpose, which includes an increase in height at withers. To further investigate the potential effect of selective breeding on the change in allele frequency in this region, we focused on BAR horses, where measured height at withers was available. Interestingly, the height of BAR horses in this study increased over time, as mentioned in previous studies [35], and confirmed from our results. In addition, there has been a corresponding change in the allele frequencies, supporting the hypothesis that the SNP BIEC2\_99523 might be associated with height at withers with a specifically positive effect of the minor allele (G) in pony breeds. This finding could be valuable for future breeding plans, as this SNP is present in the SNP chip used for routine genotyping of horses, serving as a good proxy for the LCORL gene, which is not covered by SNPs.

Additionally, in the same chromosome, also the ZC3H18 (Zinc Finger CCCH-Type Containing 18) gene is located, which has been found to be associated with body weight in several other species, comprising broilers [36]. The same gene has also been identified under potential selection in Clydesdale and Shire horse breeds, where the GG genotype is fixed and has been related to an increased body weight and body mass [37]. Furthermore, within a private ROH island in TPR in the same chromosome but different position (3: 107047112–107709841), two genes were found: the FAM184B (Family With Sequence Similarity 184 Member B) and the DCAF16 (DDB1 And CUL4 Associated Factor 16). The FAM184B has been linked to body size in Welsh pony [38], as well as conformation, locomotion, and specifically on hock circumference and body length in Spanish purebred horses [39]. Finally, the gene DCAF16 has been linked to average daily weight gain in cattle [40] and growth traits in donkeys [41]. Thus, combining all the signatures of selection on ECA3,

we can hypothesize that selective breeding towards meat production over the years in TPR has led to the fixation of specific alleles in genes involved in body size. Whereas, still looking at ECA3, it is possible to observe in MUR breed selective breeding for taller horses, particularly for military and riding purposes. This selection process has likely driven the fixation of specific alleles in genes associated with skeletal development, contributing to the observed signatures of selection in body size and height at withers QTLs rich region.

ECA9 showed a private ROH island for MAR horse, including four QTLs related to withers height. This breed is famous for being the first Italian sport horse breed, and it is known for its competitive attitude and performance in sports. MAR horse was one of the earliest breeds to implement a morphological scoring system in its breeding program in Italy, with withers height being the main trait for selection. A recent article has highlighted the development of a new scoring method to estimate breeding value from morphological traits, comprising height at withers [42]. This interest in withers height suggests that selection for morphology in MAR resulted in homozygosity in a different region to that observed for TPR and MUR. These findings are consistent with the polygenic nature of height [43] and indicate that different genomic regions may have undergone selection for the same trait, depending on the breed.

Coat color is a specific breeding objective for two of the evaluated breeds in this study. In the case of HAF, chestnut coat color is a distinctive feature, while in MUR breed, only black and gray coat colors are allowed. In the other breeds, inclusive criteria are based on coat color. Only shades from bay to black are allowed in BAR and MAR breeds, while TPR breed has a wider range of accepted coat colors, with a major preference for chestnut as stated by the breeding association (ANAREAI, ANAMF and ANACAITPR). Coat color in horses is gene-regulated with a key role of two genes: MC1R and ASIP [44]. Therefore, we further looked at the genotype frequency of the SNPs closest to the MC1R and ASIP genes, which showed interesting trends, especially in the breeds where we found a sign of selection. As a result, ROH islands in the region where the MC1R gene is located were found in two of the target breeds, HAF and TPR. As expected, horses with chestnut coat color were mostly homozygous for the A allele in the SNP closest to the *MC1R* gene (BIEC2\_816499), a pattern confirmed in both the HAF and TPR breeds. In TPR breed, there were some heterozygous horses since chestnut is the most common coat color, although not the only one allowed. Therefore, we hypothesize that the selection signature in HAF and TPR within the MC1R region, and the genotype frequency of the BIEC2\_816499 SNP, are likely due to proximity and potential linkage disequilibrium with causative mutation for chest coat color.

For the SNP closest to the ASIP gene (BIEC2\_618993), we observed fixation in MUR horse breed, where only black (prevalent) or gray (very rare) coat colors are allowed. Therefore, those results suggest that the identified polymorphisms in the proximity of the MC1R (BIEC2\_816499) and the ASIP (BIEC2\_618993) genes have potential as markers for the Extension and Agouti loci, respectively, due to their proximity and likely high linkage disequilibrium with the causative mutations. This result might allow breeders and researchers to efficiently predict coat color traits without the need for an extra cost of targeted genetic testing, making using these markers a practical and economical option. Nevertheless, in MUR (18.2%) and BAR (14.9%), the genotype frequencies did not follow the expected results based on breed of belonging. Therefore, when more accurate results are needed, we recommend targeted genotyping for the specific causative mutations.

Finally, a shared ROH island on ECA3 among TPR and HAF overlapped with known QTLs for white markings. This region has already been identified as potentially under selection in previous studies [6] for HAF, as well as for Akhal Teke, Arabian and Gidran horses. Interestingly, for TPR and HAF, the studbook inclusion criteria allow the presence of white markings on the face and legs. In contrast, this is not permitted for the MUR horse and is penalized for the BAR horse.

#### Selection signatures related to health traits

Health-related traits are relevant both for their economic value and for animal welfare. Local breeds are generally considered more resistant to disease and better adapted to their environments [45], but their limited population size poses a risk due to the restricted genetic pool. In this context, horses' health faces significant challenges from various risk factors, including climate change and the growing threat of antimicrobial resistance [46].

We identified signatures of selection associated with health traits on ECA 3, 10, and 15. In these regions, QTLs linked to chronic progressive lymphedema, guttural pouch tympany, osteochondrosis, navicular bone morphology, and hypersensitivity to insect bites have been identified. Interestingly, both BAR and HAF breeds exhibited signatures of selection in regions where QTLs for osteochondrosis and navicular bone morphology are located, on ECA3 and ECA 10. It is noteworthy that in the same shared region on ECA3, genes associated with hoof health, like the *WWP2* (*WW Domain Containing E3 Ubiquitin Protein Ligase 2*), have been found [37]. This shared ROH island might be attributed to selective pressures linked to the horse type and the traditional utilization of those breeds. Indeed, both breeds were used in mountain areas for draft purposes; thus, this might have shaped the hoof to be strong and resistant. On top of that, we cannot exclude the hypothesis that the management of these horses, which are often kept barefoot regardless of the terrain, might have contributed to this shared ROH island. Indeed, both breeds are commonly raised in semi-extensive conditions, often freely grazing in mountain pastures. Therefore, the environment might have influenced the robustness of these breeds, particularly in terms of hoof health. In line with this hypothesis, a previous study has found that the hooves of the HAF breed contain various minerals that contribute to their strength [47], suggesting that the use of horseshoes is unnecessary [48].

The ROH island identified on ECA10, common between BAR and HAF, contained QTL associated with chronic progressive lymphedema. This disease is present in several draft horse breeds [49], which highlights the genetic similarity between the two Italian breeds and other European draft breeds. Chronic progressive lymphedema is known to be a multifactorial condition with a genetic basis, and various studies have linked it to altered elastin metabolism. Furthermore, it has been genetically correlated with hair diameter and skinfold thickness [50], both traits of interest in BAR and HAF horses. Thus, we suppose that the target region could be under selection for hair diameter and skinfold thickness rather than to contrast progressive lymphedema, which has not been documented in those breeds.

Additionally, a private ROH island found in HAF breed on ECA3 and ECA11 overlapped with QTLs related to insect bite hypersensitivity (IBH). This condition is caused by sensitivity to the saliva of Culicoides spp [51]. The prevalence of IBH is influenced by factors such as habitat, climate, and breed, with a genetic predisposition playing a significant role [52]. Cold habitats with minimal warm days per year are associated with a lower disease prevalence. Considering that HAF horses inhabit high-altitude regions with challenging terrain and cold temperatures in the northern part of Italy, these environmental factors may have contributed to the selection of animals adapted to this climate and not affected by the disease [53]. In addition, as mentioned earlier, HAF horses likely underwent a selection for hair density, which plays a role in protecting the horses from insects in the pasture during summer and the harsh climate during winter.

This study found that all horse breeds except MAR had signatures of selection in regions, although different ones, where QTLs related to osteochondrosis are located, which is a skeletal issue in young horses and a component of developmental orthopedic disease [54]. Osteochondrosis is especially common in heavier, cold-blooded breeds, often linked to rapid weight gain and larger body

size [55]. A previous study found a positive correlation between the presence of the disease and body size [52], which may help explain why heavier breeds in this study have shown selection signatures in this region. These breeds may have been subjected to selective pressures to mitigate the effects of osteochondrosis, as they are more prone to weight-related skeletal issues than lighter, more athletic breeds like the MAR. Interestingly, the absence

athletic breeds like the MAR. Interestingly, the absence of such a selection signature in MAR breed could be due to its lighter body size and athletic purpose, where rapid weight gain is less of a concern. A valuable explanation could also lie in the breeding practices employed for the Maremmano breed. Since the 1990s, a concerted effort has been made to select against osteochondrosis through performance tests and radiographic analysis. Nowadays, positive individuals are not classified as top stallions (A class), but from the 1990s, heavy selection was actively carried out to reduce the prevalence of this condition. This long-term selection strategy likely contributed to the absence of a detectable selection signature in the MAR, as the focus on athleticism and skeletal soundness became integral to the breeding objective.

#### Sport performances

In recent decades, increased attention has been given to sport performances as primary traits for selection in various transboundary breeds. While the selection on gait or performance traits in local breeds is not as advanced, studbooks have started evaluating horses during performance tests. The transition prioritizing performance traits is well established for the HAF horse, and it is helped by the large number of horses subscribed to the studbook, with over 12,000 horses registered only in Italy. Evidence of selection signatures has been discovered for HAF breed on ECA7, where genes associated with bone remodeling such as ACP5 (type 5 acid phosphatase) [56] and heart muscle development like ECSIT (Evolutionarily conserved signaling intermediate in Toll) [57] are located. The heart size is known to be related to training with an increase in heart chambers size due to aerobic training [58]; since HAF horses are employed for sport performance comprising endurance races, this trait is important for the success in these competitions. It looks like the selection acted to make horses more prone to developing a good heart size and help them with racing performances.

# Conclusions

In this study, we used a population genetics approach to identify genomic regions under selection across five distinct Italian horse breeds. These breeds include both warm-blooded and cold-blooded, providing a comprehensive overview of genetic diversity within the Italian horse population. Our analysis focused specifically on inbreeding estimation from genotype data, in order to account for the variability present in those breeds. For some of them, tools like optimal contribution selection have been implemented in order to help breeders determine the best mating strategies, helping the breeders make informed decisions. We expect the next generations to be able to quantify the effect of these tools, resulting in a decrease in inbreeding in the populations. Along with the study of the inbreeding status homozygous hotspots study has been performed, which allowed us to pinpoint various quantitative trait loci and genes linked to various traits. Identifying these genomic regions is particularly useful as it highlights the peculiarities and essential traits that are characteristic of each breed. Understanding these genetic variations can inform future breeding selection strategies, helping a better conservation of the peculiarities of local breeds and enhancing at the same moment their performances.

#### **Materials and methods**

#### Animal inclusion criteria

Samples from Italian breeds collected for this study were part of the National Rural Development Program (PSRN) project, namely Equinbio and Equinbio.2 (PSRN 2014-2020-10.2, ID: J59H18000030005; Approval D.M. 6695, 21/02/2018). The PSRN is a tool through which the ministry of agricultural, food and forestry policies supports and enhances the potential of rural areas. Hair bulbs were collected from 1620 animals belonging to five Italian breeds and privately owned by individual breeders. These breeds were chosen since they are the most abundant in Italy and originate from geographically diverse regions across the country, spanning north to south: HAF from Trentino [59], TPR from Veneto [14], BAR from Emilia Romagna [25], MAR from Toscana [23], and MUR from Puglia [32]. On top of that, from a previous study, they were among the most diverse in terms of genetic diversity within the Italian equine gene pool [17].

#### Genotyping and quality control

Hair samples were sent to the AGROTIS S.R.L.—LABO-RATORIO GENETICA E SERVIZI (Cremona, Italy) for DNA extraction and genotyping. The data were collected over seven years, analyzed in different sample pools and with different Illumina's array: GGP Equine 70k° and Equine80k Select array. This latest bead chip was designed using data generated by the Equine Genome Mapping Workshop and Broad Institute's Equine Genome Sequencing Project, incorporating SNPs from Arabian, Andalusian, Akhal-Teke, Icelandic, Standardbred, Thoroughbred, and Quarter Horse breeds.

The initial data screening comprised the remapping of the files to the most recent version of the equine genetic map (EquCab3) using Unix commands and following **Table 2**Number of sampled horses per breed, their proportioncompared to the total population alive (FAO-DAD.is), and thehorse type

Breed	N. of sampled	N. animal	% Sampled	Horse type
	horses	alive		
BAR	473	3416	13.85	Cold-blooded
HAF	277	12,000	2.31	Cold-blooded
MAR	296	6030	4.91	Warm-blooded
MUR	85	4879	1.74	Warm-blooded
TPR	367	8100	4.53	Cold-blooded

the pipeline described in Beeson et al., 2019 [60]. Subsequently, the 55,972 SNPs in common between the two SNP chips were retained. SNP names were recoded for consistency across all files. PLINK v. 1.9 [61] software was used to identify the presence of any duplicated horses by estimating Identity by State (IBS) values. A threshold of 0.90 was employed to detect duplicates, which were then removed. Only autosomal chromosomes were included in the following analyses.

The quality control (QC) was performed using PLINK v1.9 software. Poorly genotyped data were removed based on criteria for missing genotypes per SNP (>0.10) and missing SNPs per sample (>0.10) [62]. No pruning was performed for minor allele frequency (MAF), deviation from Hardy-Weinberg equilibrium (HWE), or strong linkage disequilibrium (LD) following the recent indications provided for selection signatures studies [62]. The QC resulted in the removal of 1,147 variants due to missing genotype data and 36 horses due to missing SNPs per sample. A total of 54,825 variants and 1498 horses passed the filters and QC. In Table 2, details on the sampled horses are available.

Population structure was assessed using principal component analysis (PCA) performed in R (version 4.3.1). The PCA was used as a quality control measure to detect outliers or horses that did not cluster within the breed of affiliation and to visualize genetic relationships and clustering patterns between breeds.

#### Runs of homozygosity

The detectRUNS package [63] in R (version 4.3.1) was employed to analyze the runs of homozygosity, genomic regions where identical haplotypes are inherited from each parent [20], using a sliding window approach. The ROH regions were searched separately within the genomic information of each horse breed. The following parameters were used to detect ROH: Minimum 15 SNPs in a run, minimum length of a run equal to 500 kb, Maximum gap between consecutive SNPs in a window: 1 Mb, lower density limit: 1 SNP per 10 kb, and one missing and one heterozygous SNP allowed in a run. ROH segments were classified into six categories depending on their length: 0.5-1 Mb, 1-22 Mb, 2–4 Mb, 4–8 Mb, 8–16 Mb, and >16 Mb.

# Inbreeding

The genomic inbreeding coefficient (FROH) was estimated as the sum of individual ROH lengths divided by the total length of autosomal chromosomes covered by SNPs. The formula is:

$$F_{ROH} \frac{\sum_{k} \text{length (ROH_k)}}{L}$$

where:

- Σ is the sum across all ROH segments for an individual;
- length\_i is the length of the i-th ROH segment;
- L is the total length of the autosomal chromosomes covered by SNPs (2266 MB).

Descriptive statistics among population inbreeding levels were estimated. The expected age of inbreeding decreases from the first (0.5-1 MB) to the last (>16 MB) class of ROH length since a shorter ROH reflects a more ancient inbreeding.

# Signatures of selection and gene ontology

To minimize false positives arising from population bottlenecks or other historical events, ROH islands shared by more than 70% of horses within a breed were considered signatures of selection specific to that breed, as suggested in the literature [64]. Signatures of selection, also called ROH island, with fewer than 5 SNPs) have been excluded from the analysis. After this filtering, the remaining ROH islands were discussed. The EquCab3.0 genomic coordinates of these regions were used to retrieve candidate gene lists from the Ensembl genome browser (Release 112, May 2024). The horse QTL database (https://www.animalgenome.org/cgi-bin/QTLdb/E C/index, Release 54– September 2024) was then used to identify reported equine QTL regions overlapping with the candidate genes. Overlaps were categorized by traits:

- 1. Exterior traits (withers height, white markings, body weight, overall body size).
- 2. Health traits (insect bite hypersensitivity, guttural pouch tympany, osteochondrosis, osteochondrosis dissecans).
- 3. Gaits (alternate gaits, racing distance, racing performance).

Further gene descriptions used in this manuscript were based on GeneCards (http://www.genecards.org/).

#### Focus on coat color and height at withers

Based on ROH islands found in QTL-rich regions for coat colors, height at withers, and insect bite hypersensitivity (IBH) in horses, the genotype frequency of SNPs in the proximity of QTL/or known causative mutations was further assessed. For coat colors, we found a ROH island in both HAF and TPR, covering the MC1R gene (responsible for the Extension locus). Therefore, the genotype frequency of two SNPs closest to this gene (BIEC2\_816499 and BIEC2\_816577) were further evaluated in all breeds. Due to the combined effect of the Extension and the Agouti loci in coat color determination, we further assessed also the genotype frequency of two SNPs closest to the ASIP (the Agouti locus) gene on ECA22 (BIEC2\_618993 and BIEC2\_589347). We compared genotype frequencies among breeds to evaluate if those SNPs were possibly in linkage disequilibrium with Extension and the Agouti loci since coat color is a distinctive trait regulated in the disciplinary of each studbook. Specifically in the BAR horse, where both black and bay horses are allowed in the studbook and coat color data were available, genotype frequency was calculated within bay and black BAR horses [65]. A similar approach was used for the two SNPs near the LCORL gene (ligand-dependent nuclear receptor corepressorlike), associated with body size in horses: BIEC2\_104317 and BIEC2\_99523. These SNPs encompass a ROH island found in the MUR and TPR breeds. Thanks to the availability of data on birth year and height at withers in the BAR breed, a specific focus on this breed was performed. BAR horses were classified by year of birth cohorts (<2007, 2007–2015, and >2015) to assess the height at withers and genotype frequency variation across time.

#### Abbreviations

ACP5	Type 5 Acid Phosphatase
ASIP	Agouti Signaling Protein
BAR	Bardigiano
BL	Body length
BSIZE	Overall body size
BW	Body weight
CANNON	Cannon bone circumference
CHCIR	Chest circumference
CHRLYMP	Chronic progressive lymphedema
CONFS	Conformation score
DCAF16	DDB1 And CUL4 Associated Factor 16
ECA	Equine Chromosome
ECSIT	Evolutionarily Conserved Signaling Intermediate in Toll
FAM184B	Family With Sequence Similarity 184 Member B
GB	Genome Browser
GPT	Guttural pouch tympany
HAF	Haflinger
HOCKC	Hock circumference
HWE	Hardy-Weinberg Equilibrium
IBH	Insect Bite Hypersensitivity
IBS	Identity by State
KNEEC	Knee circumference
LCORL	Ligand Dependent Nuclear Receptor Corepressor Like
LD	Linkage Disequilibrium
MAF	Minor Allele Frequency
MAR	Maremmano

MC1R	Melanocortin 1 Receptor
MUR	Murghese
NAVBM	Navicular bone morphology
OSTEO	Osteochondrosis
OSTD	Osteochondrosis dissecans
PCA	Principal Component Analysis
PSRN	National Rural Development Program
QC	Quality Control
QTLs	Quantitative Trait Loci
ROH	Runs of Homozygosity
SNPs	Single Nucleotide Polymorphisms
SUSBITE	Insect bite hypersensitivity
SUSLAR	Recurrent laryngeal neuropathy
TPR	Tiro Pesante Rapido
WHIMAR	White markings
WITHIH	Withers height
WWP2	WW Domain Containing E3 Ubiquitin Protein Ligase 2
ZC3H18	Zinc Finger CCCH-Type Containing 18

# **Supplementary Information**

The online version contains supplementary material available at https://doi.or g/10.1186/s12917-025-04794-w.

Supplementary Material 1

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#### Author contributions

V.A. and M.A. design the work, V.A. formal data analysis, V.A. wrote original draft, M.A., A.Su, C.S, A.G., V.P., S.M., K.C., E.M., A.O., R.M., S.C. and A.Sa. writing—review and editing, A.Su, R.M., S.C. and A.Sa supervision. All authors read and approved the final manuscript.

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#### Data availability

Restrictions apply to the availability of these data, which were used under license for this study. Data are available upon reasonable request and with the permission from breeding associations from the corresponding author.

#### Declarations

#### Ethics approval and consent to participate

Animal Care and Use Committee approval was not obtained for this study because all the analyses were performed using pre-existing datasets provided by Italian Breeding associations within national Projects called Equinbio and Equinbio.2 where the animal study design was reviewed and approved by the MIPAAF—PSRN 2014–2020—10.2, ID: J59H18000030005; Approval D.M. 6695, 21/02/2018. The hair sampling procedure was non-invasive and caused no harm or discomfort to the animals. Informed consent was obtained from all horse owners prior to sample collection.

#### **Consent for publication**

Not applicable.

#### **Competing interests**

The authors declare no competing interests.

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