Genetic Analysis of White Facial and Leg Markings in the Swiss Franches-Montagnes Horse Breed

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Abstract

White markings and spotting patterns in animal species are thought to be a result of the domestication process. They often serve for the identification of individuals but sometimes are accompanied by complex pathological syndromes. In the Swiss Franches-Montagnes horse population, white markings increased vastly in size and occurrence during the past 30 years, although the breeding goal demands a horse with as little depigmented areas as possible. In order to improve selection and avoid more excessive depigmentation on the population level, we estimated population parameters and breeding values for white head and anterior and posterior leg markings. Heritabilities and genetic correlations for the traits were high ($h^2 > 0.5$). A strong positive correlation was found between the chestnut allele at the melanocortin-1-receptor gene locus and the extent of white markings. Segregation analysis revealed that our data fit best to a model including a polygenic effect and a biallelic locus with a dominant-recessive mode of inheritance. The recessive allele was found to be the white trait-increasing allele. Multilocus linkage disequilibrium analysis allowed the mapping of the putative major locus to a chromosomal region on ECA3q harboring the KIT gene.

Depigmentation phenotypes are widely known in mammalian species (Nocka et al. 1990; Spritz 1998; Reinsch et al. 1999), including horses. Phenotypes may vary from tiny depigmented body spots to white head and leg markings, further on to large white spotting and finally nearly complete depigmentation in white-born horses (Bowling and Ruvinsky 2000). Similar to other species, depigmentation phenotypes in the horse are sometimes associated with pleiotropic effects, resulting in severe conditions, for example, lethal dominant white (Pulos and Hutt 1969; Haase et al. 2007), the overo lethal white foal syndrome (Santschi et al. 2001), or melanoma (Henner, Poncet, Guérin, et al. 2002). Depigmentation phenotypes and color variation are thought to be a result of domestication processes, which are sometimes accompanied by behavioral changes—for example, tamability (Grandin 1998; Trut 1999; Dobney and Larson 2006; Stachurska et al. 2007). These complex associations may be partly explained due to modifications during neural crest–derived cell development and/or interactions in biochemical pathways involving tyrosinase. So far, in the horse, 4 different depigmentation phenotypes (roan–RN; sabino–SB, tobiano–TO, and dominant white–W) have been independently mapped to a chromosomal region on ECA3 harboring the KIT gene for the KIT receptor tyrosine kinase. For a more complete review on horse, coat color genetics consider Rieder (2006) and Sponenberg (2003).

White markings result from the lack of melanocytes in the hair follicles and the skin (Silvers 1979). In mammals, melanocytes in skin and hair follicles are clonally derived from primordial melanoblasts in the neural crest. A sharp middorsal separation exists for the primordial melanoblasts in the neural crest so that migration and clonal proliferation occur autonomously on each side of the embryo. The result of this process can be observed, for example, in embryo splitting and cloning experiments, respectively (Lewis 2005). Melanoblasts enter the limb buds and migrate toward the target area at the distal end. A completely pigmented head or leg depends on the complete migration and clonal proliferation of the melanoblasts in the mesoderm of the developing fetus, thus ensuring that limbs and the head acquire a full complement of melanocytes. According to Woolf (1998), white markings result from the absence of melanoblasts at the distal end of the particular body part bud as it begins to elongate and differentiate, and the amount of whiteness is a function of the distance between

the most distal melanoblasts and the tip of the developing body part bud. Stochastic events influence the migration of melanoblasts into and within a body part bud, causing variation in the quantitative expression of common white head and leg markings (Woolf 1995). Dam foal regression analyses suggest that about two-thirds of the phenotypic variance are attributable to genetic differences (Woolf 1990).

Although Woolf (1990) concluded that complex genetic systems and nongenetic factors determine the presence of common white facial and leg markings in his data set of Arabian horses from the United States of America, he found an overall heritability of total whiteness on the body of 0.77. This result indicates an important genetic component influencing these particular phenotypes. Similar findings are known for German sport horse populations (Nebe 1984).

Apart from high heritabilities, a significant influence of basic coat color phenotypes on the expression of white markings was reported by different authors (Nebe 1984; Woolf 1991). Thus, chestnut-colored horses showed significantly more extensive white markings compared with bay and black horses. These basic color phenotypes are the result of different alleles at the Extension (E/e) and Agouti (A/a) loci. Both chestnut and black segregate independently (Extension maps to ECA3p12 and Agouti maps to ECA22q15), and in a recessive manner in the horse, with chestnut being epistatic over black (Rieder et al. 2001). Woolf (1992) concluded that the difference in the quantitative expression of white markings in chestnut and bay horses is either due to pleiotropic action of alternate alleles at the Extension (E/e) and Agouti (A/a) loci or due to closely linked alleles that are part of the polygenic system influencing the variation of common white markings. At that time, Woolf could not know that the extension locus, encoded by the melanocortin-1-receptor gene (MC1R), and the KIT gene for a receptor tyrosine kinase are located on the same equine chromosome—3p12 and 3q21-22, respectively. Current linkage data from the horse indicate that MC1R and KIT are separated by only about 20–34 cM (Penedo et al. 2005; http://www.vgl.ucdavis.edu/equine/caballus/). Interaction between MC1R and KIT alleles is also reported in pigs, even though the 2 genes are not located on the same porcine chromosome—SSC6p15 and SSC8p12, respectively (Marklund et al. 1998; Kijas et al. 2001).

Furthermore, Woolf (1998) reported on the directional and anteroposterior asymmetry of common white leg markings, showing that both types of asymmetry have a genetic basis. However, much more genetic variation is present for anteroposterior asymmetry than for directional asymmetry.

Depending on the breeding goals for particular horse registries, market demands, and finally performance, white markings are traits under specific selection. In the Swiss Franches-Montagnes (FM) horse population, white markings have more than doubled in size and occurrence during the past 30 years, although the official breeding goal demands a horse with as little depigmented areas as possible. On the other hand, request on market and individual preferences led to a converse selection.

Goals of the present study included the genetic analysis of the traits and the development of selection tools in order to be able to manage the described contradictory situation adequately in the future and to avoid excessive white (depigmentation) on the population level. Thus, we first estimated population parameters and breeding values for white head and anterior and posterior leg markings. Systematic effects and correlations among the traits and parameters were studied. Second, segregation analysis was performed in order to find a mode of inheritance that would fit best to our data. Third, association analysis was done on a genotyped panel of phenotypically extreme horses (no or few white markings vs. extended amount of white markings) to unravel the chromosomal location of a putative major gene responsible for this type of depigmentation.

Materials and Methods

Animals and Phenotypes

The data comprise 23 019 recorded horses and 33 214 animals in the pedigree list, all of them registered to the studbook of the Swiss Franches-Montagnes Horse Breeding Association, Avenches, Switzerland.

Phenotypes were coded, scored, and registered from electronically available standardized horse identification forms of the studbook administration, as follows: we distinguished between the head, anterior and posterior limbs, and the body. First, the head was divided into the front, the bridge, the nose, and a rest (i.e., depigmented eyes). Each part of the head, except the nose, was attributed with score values starting from 0 (no white marking in that part) up to 3 (entirely white part). The nose (including nose and upper and lower lips) was attributed with scores up to a value of 6. For depigmented eyes, a score value of 2 was attributed per eye. Then, the 4 limbs were divided into a part up to the fetlock, up to half cannon, up to the knee or the hock, respectively, the knee and the hock themselves, and the most proximal part above the knee or the hock, respectively. All limbs were independently scored and each of the mentioned limb parts received a score value of 1, in case white markings were found within the defined area (thus a maximum score value of 5 per limb). All other white markings not located on the mentioned head and limb scopes were considered as body markings. A score value of 1 was given per such marking. Then, all parts of the head were summarized to a score value "total head" (maximum 16). The same procedure was obeyed with all 4 limbs and the body, resulting in a score value for "total anterior limbs" (maximum 10), "total posterior limbs" (maximum 10), and "total body" (score value according to the number of white markings found on the body). Finally, the sum of score values for total head, "total forelimbs," "total hindlimbs," and total body resulted in an overall score value for a particular horse. For the variance component analysis and the mixed inheritance model, we restricted the analysis to head and limb markings, due to only very few horses in our data expressing white markings on any other body parts.

However, horses with white markings on the body were included in the association analysis.

Three data sets according to coat color were considered for the analysis with the mixed inheritance model. For the first set, chestnut-colored horses with both chestnut parents were selected, grand- and greatgrandparents were included, if they had been registered as chestnuts. The resulting set contained 801 horses with recorded white markings. An identically selected set of bay-colored horses included 2 319 animals with recorded white markings. The third set of horses was selected without considering coat color. In this case, a horse and its ancestors were included if all his parents and grandparents were scored and recorded for white markings, great- and greatgreatgrandparents only, if they had been scored and recorded for white markings. This set contained 4 331 horses.

Finally, a randomly selected sample of 111 FM horses, consisting of 2 phenotypically extreme groups (43/68), according to their degree of white markings (score 0–2 vs. score >18) was genotyped to study the association between the trait (total white markings on head, fore- and hindlimbs, and body) and markers on chromosome ECA3. Given the available samples to our study, we allowed for the one phenotypic extreme "no white markings" to "few white markings," represented by score values from 0 to 2. The other phenotypic extreme ranged from score value 18 up to 35 (maximum found in the available sample set). Horses with score values in-between 2 and 18 were not considered for association analysis to guarantee a large enough phenotypic difference among groups.

Laboratory Analysis

DNA was extracted from blood samples of 111 FM horses using standard procedures. The horses were genotyped for alleles at the MC1R, agouti-signaling-protein (ASIP), and KITIntron3 loci according to Rieder et al. (2001) and Mau et al. (2004). Genotyping for alleles at the Agouti locus was included to study potential interaction between MC1R, ASIP, and white markings, even though ASIP does not map to ECA3. Moreover, microsatellite markers AHT036, COR028, SGCV018, TKY215, UCDEQ437, LEX057, AHT101, ASB023, and AHT097 were genotyped according to Glowatzki-Mullis et al. (2006). In addition, a newly detected microsatellite in the KIT gene completed this set of markers (Haase et al. 2007—GenBank accession number AM420315). Polymerase chain reaction (PCR) amplification was performed in a total volume of 15 µl with 10–50 ng template DNA, 2.1 µl GeneAmp 10× PCR buffer I, 4.5 µl deoxynucleoside triphosphates 1.25 mM, primer mix, and 0.4 µl AmpliTaq Gold DNA Polymerase. The cycling conditions included an initial activation step at 94 °C for 12 min, 30 cycles of 94 °C for 1 min (ramp 1 min), annealing at 58 °C for 1 min (ramp 1 min), extension at 72 °C for 1 min (ramp 1 min), and a final extension at 72 °C for 45 min. Amplification was carried out using a PE GeneAmp PCR 9600 or 9700 system (Applied Biosystems, Foster City, CA). PCR products were diluted with 80 µl distilled water. Each

diluted PCR (1.2 µl) was mixed with 0.4 µl GeneScan 500 LIZ Size Standard and 10.6 µl Hi-Di formamide. The denatured samples were run (POP-4; run temperature 45 °C) on a ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Data collection, extraction, and analysis were performed with 3100 Data Collection Software Version 1.0.1 and GeneScan Analysis Software Version 3.7 (Applied Biosystems).

Statistical Analyses

Quantitative Model

Multitrait variance component analysis was performed, including the whole set of recorded horses (23 019 animals) as described in the preceding paragraph, using a BLUP-animal-model with the ASREML software package (Gilmour et al. 2002). The basic coat colors (chestnut and bay) were taken as a fixed effect in the linear model. Breeding values were estimated for all horses in the pedigree file.

Mixed Inheritance Model

The mixed inheritance model in use for the segregation analysis is according to Janss et al. (1995). Two alleles are considered at the supposed major locus leading to 3 genotypes A1A1, A1A2, and A2A2 with frequency f+ for the trait-increasing allele (A2) and genetic effects described by Falconer and Mackay (1996) of −a, d, and a, respectively, where a is called the additive and d the dominance effect. The statistical procedure outlined by Janss et al. (1995) and implemented in the MAGGIC set of computer programs were used (Janss 1998). The method constructs chains of Monte Carlo realizations of the model parameters through Gibbs sampling. The statistical significance of the model parameters was evaluated by the highest posterior density region (HPDR) according to Box and Tiao (1973) and Scott (1992). The region was constructed such that 95% of the sampled parameter values were within the borders of this $(1−\alpha)$ region. The HPDR allows, for example, the following reasoning: if the region for a variance component or a frequency includes the boundary value of zero, then this parameter is not of importance for this particular trait. For more details, see Hagger et al. (2004).

Association and linkage disequilibrium analysis were performed using the DISEQ software package including DISMULT (Terwilliger 1995).

Results

Variance Component Analysis

In Table 1, the mean score, the fixed effect of chestnut over bay (i.e., the effect of the e/e genotype vs. E/e and E/E genotypes at the MC1R locus), phenotypic, and genetic variances are presented. Estimated heritabilities, phenotypic, and genetic correlations for the 3 traits from the quantitative analysis are in Table 2. High heritabilities were found for all traits, with the estimate for white markings on the head

Table 1. Population parameters for white markings in FM horses[a]—part 1

	Head	Forelimbs	Hindlimbs
Mean	7	2.3	5
ΔChestnut–Bay	2.1	0.8	1.3
σ^2 Phenotype	8.24	5.12	5.84
σ^2 Genotype	5.69	2.69	3.37

[a] Scale values for parameters result from the phenotyping scoring system described in the Materials and Methods.

considerably higher than for both corresponding limb traits. Estimates for the genetic correlations were also high, clearly highest between the 2 limb traits, but somewhat lower and of identical size between the head and the 2 limb traits.

The mean difference (scores) for the amount of whiteness, between chestnut and bay coat color bearing horses, was found to be significantly different for the 3 traits under consideration ($P < 0.05$). Furthermore, a distinct phenotypic anteroposterior asymmetry was found in our FM horse data (Table 1). Our findings are in perfect accordance with the various results described by Woolf, using data of a completely different horse population.

Based on these populations parameters breeding values estimated breeding value (EBV) for white markings were estimated and summarized to an index (index = EBV-head + EBV-forelimbs + EBV-hindlimbs). The mean index was set to 100, and the standard deviation was set to 20. All 3 traits were equally weighted. This transformation resulted in about two-thirds of the horses carrying an index between 80 and 120 and one-third of the horses being either below or above the index 80 or 120, respectively. The relative means of the breeding values per year were taken to display the genetic trend for white markings (Figure 1) for a time frame of about 30 years.

Segregation Analysis

Results from the analyses of the 3 data sets with the mixed inheritance model are summarized in Table 3. The estimates for the additive effect decrease in size from head to hindlimbs to forelimbs, respectively. Estimates for head and hindlimbs are, however, very similar for the 3 color sets. A larger variation can be observed between the forelimb estimates. Large, negative dominance effects were found from all color·trait combinations, therefore suggesting the

Table 2. Population parameters for white markings in FM horses[a]—part 2

	Head	Forelimbs	Hindlimbs
Head	0.69	0.65	0.67
Forelimbs	0.44	0.52	0.83
Hindlimbs	0.46	0.51	0.58

[a] Heritabilities for white head, forelimbs, and hindlimbs markings on the diagonal; genetic and phenotypic correlations among the traits above and below the diagonal, respectively.

trait-decreasing allele at the major locus to be the dominant one. Complete dominance, that is, a − |d| = 0, is indicated with less than 5% error probability for head markings of bay horses as well as for forelimb and hindlimb markings of chestnut horses. Nearly complete dominance (i.e., a − |d| very small) could also be observed for forelimb markings in the bay- and the mixed-colored data sets. The clearly smallest polygenic heritabilities were obtained for the forelimb markings from all color sets. The high total heritabilities for these 3 groups point to a nearly complete genetic control of forelimb markings by the supposed major locus. For head and hindlimb markings, the differentiation between major locus and polygenic influence is less distinct, a medium to high polygenic heritability always contributes to a high total heritability. The clearer the complete dominance inheritance at the major locus is expressed, the lower the polygenic inheritance.

Association Analysis

Multilocus linkage disequilibrium analysis using a total of 12 markers (2 single nucleotide polymorphisms and 10 microsatellites) in our panel of case ($n = 68$; extended white markings) and control ($n = 43$; no white or very few markings) FM horses revealed highest likelihood ratio test (LRT) chi-square values (LRT = 27.84) and logarithm of odds scores ($Z = 6.04$) for the putative major locus at a position closest to markers AHT101 and MSKITI7 on ECA3q. This chromosomal region also harbors the KIT gene (Figure 2). It is interesting to note that including the frequencies of MC1R alleles—chestnut (e) and nonchestnut (E)—to the analysis did influence the results. MC1R itself revealed significant association with the markings (LRT = 16.16; $Z = 3.5$). This result is in agreement with the one presented before, describing a significant mean difference between chestnut and bay coat color bearing horses and their relative amount of body whiteness. In our data, no additional effect was found when considering also the genotypes at the agouti locus. However, the frequency of the recessive black allele (a) is known to be low in the FM breed (~10% according to Henner, Poncet, Aebi, et al. 2002). Only few horses in the panel were found to be carriers (6 out of 43 in the control group with no or few white markings and 13 out of 68 in the case group with extended white markings), most horses were homozygous for the nonblack allele (A).

Discussion

We studied the genetics of white markings in horses using a data set of an autochthonous Swiss horse breed. White head and limb markings are part of the variation within the piebald phenotypes. Our data support the segregation of a recessive single gene accounting for 20–80% (Table 3) of the total heritability for the traits under study (head, forelimbs, and hindlimbs markings). Our results strengthen previous analyses from Woolf (1989, 1990, 1991, 1992, 1995, 1998—Arabian horses) and Nebe (1984—German Sport horses). These populations do not share a common
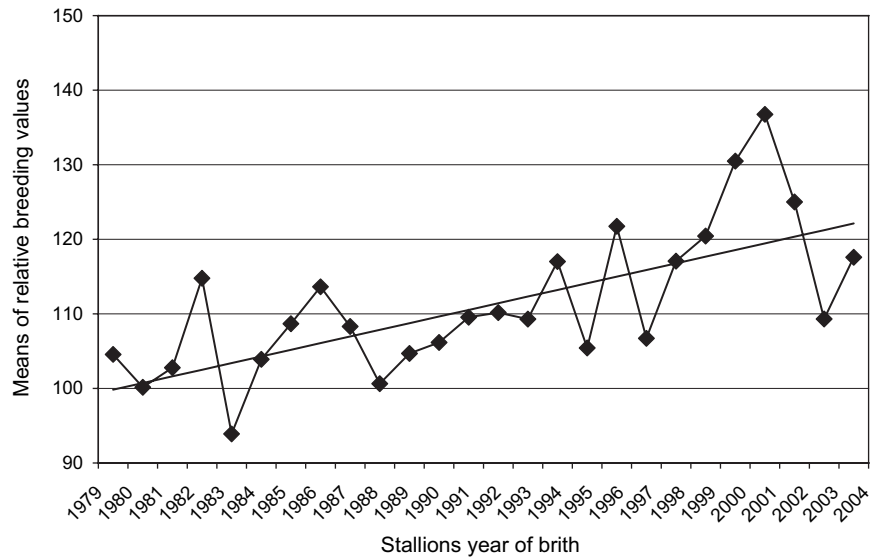
Figure 1. Overall genetic trend for white markings in the FM breed (index combining head, fore-, and hindlimb EBVs).

breed history, although some directed migration is known in the FM breed, especially during the time of breed consolidation in the second half of the 19th century. Therefore, the comparable results obtained in the mentioned horse populations point toward a rather old genetic background for white markings, dating from a time period before the formation of modern horse breeds.

To our knowledge, this is the first time that breeding values for a coat color phenotype were estimated in a modern horse population to allow more directed matings, in order to meet particular market demands (e.g., "painted" sells well; dressage horses are selected for as little white markings as possible), and in the mean time to prevent the FM breed in loosing part of its phenotypic characteristics (conservation of animal genetic resources). The latter aspect appears to be even more important, when considering the potential pathological consequences of excessive depigmentation in livestock species (e.g., oculocutaneous symptoms like photosensitivity, nystagmus, skin cancer, infection risk, neurological disorders, and deafness).

As also shown in the previous studies mentioned above, a significant difference in the expression of white markings was found between the chestnut and nonchestnut phenotypes. This result might be due to linkage disequilibrium between the (e)-allele at MC1R and the recessive allele at the putative major locus mapped to ECA3q close to the KIT gene. Alternatively to linkage disequilibrium, the observed epistasis between MC1R and the yet unknown major gene might be due to a functional interaction of MC1R and putative KIT signaling pathways. Such interactions have been shown in pigs carrying the (E^+) and (E^P) alleles at MC1R and the (I), (i), or (I^P) alleles at the KIT locus. Depending on the genotypes, an augmentation of pigment spots was observable (Marklund et al. 1998; Kijas et al. 2001). According to these authors, this is due to a more or less severe KIT dysfunction in the developing melanoblasts,

as a result of particular KIT genotypes (e.g., I/I animals vs. I/i and I^P/i, respectively). In addition, the same authors propose that a white color background in pigs lacking the dominant white (I)-allele at KIT might be due to a defect in melanocyte migration/survival in the absence of functional MC1R expression—which is exactly the case in chestnut (e/e) horses.

Table 3. Segregation analysis: marginal posterior means of mixed inheritance model parameters of 3 data sets according to coat color*

Parameter	Color	Head	Forelimb	Hindlimb
Additive effect—a	Chestnut	2.12	1.42	1.84
	Bay	2.10	1.16	1.73
	Mixed	2.22	1.29	1.86
Dominance effect—d	Chestnut	−1.71	−1.46	−1.90
	Bay	−1.93	−1.01	−1.04
	Mixed	−1.72	−1.19	−1.38
a − |d|	Chestnut	0.41	−0.05**	−0.06**
	Bay	0.15**	0.15	0.69
	Mixed	0.51	0.10	0.48
Polygenic heritability	Chestnut	0.50	0.09**	0.40
	Bay	0.25	0.09	0.25
	Mixed	0.29	0.11	0.38
Total heritability	Chestnut	0.62	0.47	0.50
	Bay	0.46	0.46	0.47
	Mixed	0.52	0.41	0.51
Fraction of the total heritability explained by the recessive single gene	Chestnut	0.19	0.81	0.20
	Bay	0.46	0.80	0.47
	Mixed	0.44	0.73	0.26

* See text, scale values for parameters result from the phenotyping scoring system described in the Materials and Methods.
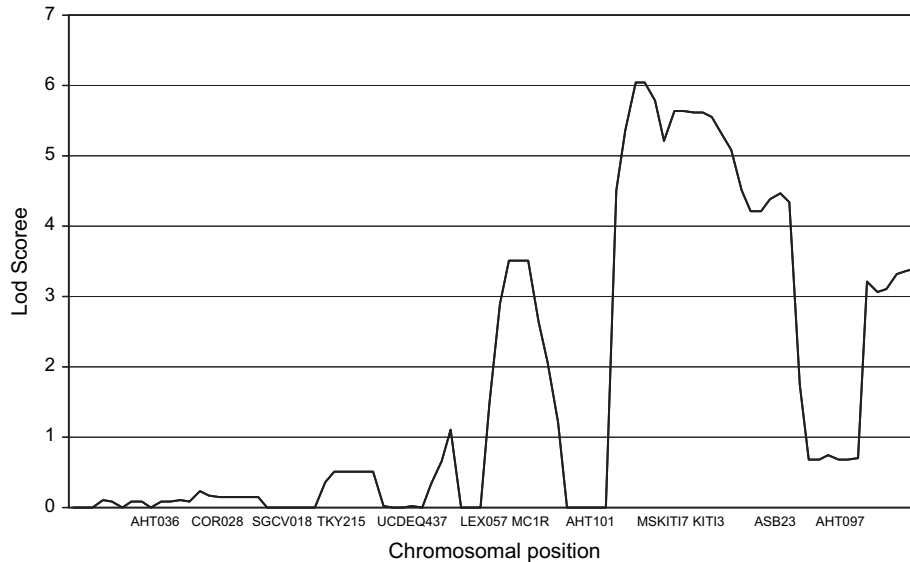** 0.0 in 95% HPDR included.

Figure 2. Linkage disequilibrium mapping results for white markings using a set of markers on ECA3, including also alleles at MC1R.

Our association analysis indicated that the putative major gene for white markings is located at or near the KIT locus. However, further studies are necessary to prove that the KIT gene indeed is the putative major gene for white markings. As mentioned before, we could not find an interaction between alleles at the agouti locus and the amount of white markings in our data. However, this might be biased by the low variation of ASIP detected in our sample set.

It is interesting to note that the genetic correlations for all 3 traits (head, forelimbs, and hindlimbs markings) are considerably higher than the phenotypic correlations (Table 2). This is an indication that genetic effects are more important in the expression of the markings than are environmental effects. These findings are also supported by the results of the segregation analysis, underlining the impact of a major locus, supplemented by a polygenic component, on the 3 traits, respectively.

According to Trut (1999) white markings, also known as piebald, are a common trait to all domestic animal species. In his remarkable 40 years selection experiment toward "human-friendly," "domesticated" foxes, Belyaev (1979) pointed out that piebald phenotypes were the first physical changes, his research group noted among the selected tamed foxes. Belyaev (1979) proposed a hierarchical model of genes within the genome to explain the process and the effect of domestication in animal species. Belyaev et al. (1981) named their piebald locus "star gene." It is not known yet whether KIT is the star gene. Belyaev's hypothesis was critically reviewed by Dobney and Larson (2006) who underlined the fact that, even though behavioral and morphological changes are evident between wild and domestic animals, these changes must be the result not only of individual gene products but also of countless additional pleiotropic interactions over the course of development.

However, the concept of major genes in animal breeding, which explains a considerable fraction of the total genetic variation of a given trait, supports the hypothesis of key single-gene products. These are responsible for the expression of particular phenotypes, conceding the fact that pleiotropy is crucial in mammals, carrying only a limited number of genes in their genome.

We mentioned that depigmentation phenotypes and color variation are thought to be a result of the domestication process, accompanied sometimes by behavioral changes. We studied a modern horse population where behavioral traits are not a firsthand selection criteria nowadays. Therefore, it was not possible to analyze piebald variation and behavioral changes in our horse sample. However, once the alleles at the major locus for white markings are detected, it would be interesting to investigate their occurrence and frequency in different modern horse breeds as well as in ancient DNA samples of true wild horses from archeological sites.

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