Synergy between *MC1R* and *ASIP* for coat color in horses (*Equus caballus*)¹

Songyang Shang,^{†,2} Yan Yu,^{†,2} Yuxin Zhao,^{†,2} Wanyi Dang,^{†,2} Junpeng Zhang,[†] Xia Qin,[†]
David M. Irwin,[‡] Qin Wang,^{||} Fei Liu,[§] Zhenshan Wang,[¶] Shuyi Zhang,[†] and Zhe Wang^{†,3}

[†]Institute of Equine Sciences, College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, China; [‡]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada M5S 1A8; ^{||}Chinese Academy of Inspection and Quarantine, Beijing 100176, China; [§]Shenyang Institute of Animal Husbandry and Veterinary Science, Shenyang 110034, China; and [¶]College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, China

ABSTRACT: Through domestication and human selection, horses have acquired various coat colors, including seven phenotypes: black, brown, dark bay, bay, chestnut, white, and gray. Here we determined the genotypes for melanocortin-1 receptor (*MC1R*) and agouti signaling protein (*ASIP*) in 709 horses from 15 breeds. We found that the E^EE^E genotype frequency at *MC1R* decreased from dark to light colors (black = 64.5%, brown = 67.5%, dark bay = 47.0%, bay = 16.5%, and chestnut = 0.0%), whereas the A^AA^A genotype frequency at *ASIP* increased as coat color lightened (black = 0.0%, brown = 22.9%, dark bay = 69.2%, and bay = 83.0%). When combined genotypes at *MC1R* and *ASIP* were examined, different advantage genotype combinations were found for each color: black $E^EE^E-A^aA^a$ (64.5%),

brown $E^EE^E-A^AA^a$ (47.0%), dark bay $E^EE^E-A^AA^A$, and $E^EE^e-A^AA^A$ (36.2% and 33.0%, totally 69.2%), bay $E^EE^e-A^AA^A$ (69.6%), and chestnut $E^eE^e-A^AA^A$ (62.6%). The χ^2 test showed that the phenotypes of horse coat colors were significantly related with the genotypes of *MC1R* and *ASIP* ($p < 0.001$). Furthermore, in contrast to a previous study where A^aA^a was only found in black, chestnut, and gray horses, we also found this allele in brown, dark bay, bay, and white horses. These results indicated that *MC1R* and *ASIP* may synergistically affect the levels of melanin in equine coat colors and that additional genes are likely involved in regulating coat colors, especially for white and gray colors. Our research provides new data for further studies on the synergetic actions of *MC1R* and *ASIP* in coat color of horses.

Key words: *ASIP*, coat color, horse, *MC1R*, polymorphism

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INTRODUCTION

Presently, there are more than 400 distinct horse breeds and various coat colors due to human selective action. Among them, black, bay, chestnut, and gray are common colors. Gray horses that are born with original coat color but then gradually lose hair pigmentation with age (Rosengren Pielberg et al., 2008). The bay coat color phenotype contains three subphenotypes, brown, dark bay, and bay, from dark to light. White is a relatively rare coat color, which is due

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²These authors contributed equally to this work.

³Corresponding author: zwang@syau.edu.cn

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to a complete coat of white hair and a largely or fully unpigmented skin (Haase et al., 2007). Two strongest candidate genes that influence the coat color in horses are melanocortin-1 receptor (*MC1R*) and agouti signaling protein (*ASIP*), both of which are in the same signaling pathway (Cone, 2006; Hoekstra et al., 2006; Campagna et al., 2017; Sponenberg and Bellone, 2017).

MC1R protein located on the surface of melanocytes and activated by melanocyte stimulating hormone (MSH) leading to the production of eumelanin, which is the wild-type allele E^E (Chen et al., 2017). The *MC1R* C901T mutation generates a recessive allele (E^e) that leads to the production of only pheomelanin within melanocytes (Neves et al., 2017). The eumelanin is a black pigment, whereas pheomelanin is a red/yellow pigment (Jackson, 1994). Melanocytes that are homozygous for the recessive mutation (E^eE^e) at *MC1R* cannot be activated by MSH, leading to the production of only pheomelanin (Suzuki et al., 1996; Neves et al., 2017).

ASIP encodes the agouti signaling protein (wild-type allele A^A), an antagonist to MSH that can block the function of *MC1R* by inhibiting eumelanin production in horse body melanocytes (Lu et al., 1994). An 11-bp deletion mutation at base 192 in exon 2 of *ASIP* (allele A^a) lead to loss of agouti signaling protein function, yielding a black phenotypic in the horse (Rieder et al., 2001). Combination of specific genotypes at *MC1R* and *ASIP* result in three basic phenotypes: black ($E^E E^- - A^A A^a$), bay ($E^E E^- - A^A A^-$), and chestnut ($E^e E^e - A^A A^-$ or $E^e E^e - A^a A^a$) (Neves et al., 2017). The superscript dash character ($-$) in the E^- and A^- alleles means any genotype at that gene.

Although a statistically significant association between *MC1R* and coat color has been found, this was based on a limited number of horses ($n = 120$) and only three colors were investigated (Rieder et al., 2001). Another statistical analysis of coat colors based on 153,778 horses generated a hypothesis that *MC1R* genotypes have a direct effect on eumelanin levels, but indicated that other genes are involved in this process (Sakamoto et al., 2017). While the genotype distribution of *MC1R* has been studied, the distribution of *ASIP* genotypes in horses with different coat colors remains unclear and how the combination of the two important genes correlate with the coat color has not been studied.

In this study, we determined the *MC1R* and *ASIP* genotypes in over 700 horses with seven different coat colors and examined the frequencies of

each genotype for both gene combinations in all phenotypes to characterize genotype distribution to coat colors in horses.

MATERIALS AND METHODS

Identifying the Phenotype of Equine Coat Colors

Horse coat colors were classified according to previous studies (Rieder, 2009; Neves et al., 2017; Sponenberg and Bellone, 2017; Figure 1a). Briefly, gray horses are born with an original coat color (such as black, bay, or chestnut) but gradually loose hair pigmentation at ages (6 to 8 yr) but maintained the dark skin. Some gray horses do not loose hair pigmentation but change to darker phenotype by decreasing white and increasing the black or brown hairs. The other six colors are from dark to light, black, brown, dark bay, bay, chestnut, and white. The black color is common in horse breeds such as Friesian and Percheron, which have black hairs on every part of their body, although some have white makings. Most horses with black color will fade to a brownish color when regularly exposed to the sunlight. Horses with brown coat color have black pigment in the mane, tail and legs, and have a nearly-black phenotype of their whole body, but reddish or tan pigments around their eyes, muzzle, and abdomen between the elbow and stifle. The dark bay horse coat color is a black mane, tail and legs, and very dark brown or reddish hair at the head, neck, back and hip, but with brown or reddish pigment at the belly. The bay horse coat color, the most common coat color, has a brown or reddish body, and a black mane, legs, and tail. Chestnut horses are reddish with no black color on their entire body including the mane. White horses are relatively rare and differ from gray horses as they are born with white hair and remain white for life.

Sample Collection and DNA Extraction

Hair roots from a total of 709 adult horses were collected from 15 breeds (566 Thoroughbreds, 65 Mongolians, 22 Warmbloods, 14 Arabians, 12 Akhal-tekcs, nine American standardbreds, five Draft horses, four Friesians, three Ponies, two Budyonnies, two Polos, two Orlov trotters, one Andalusian, one Clydesdale, and one Shetland) and represented all seven coat colors. Photos and legitimate registration information of all the horses from the China Horse Industry Association were used to confirm coat colors. A list of the coat color, breeds, and quantity of collected horses was shown

in Table 1. Genomic DNA was extracted from hair roots using the TIANamp Genomic DNA kit (Tiangen Biotech, China) according to manufacturer's manual with the exception that the lysis time was reduced from 1 h to 10 min. The concentration of the extracted DNA was estimated by measuring the OD260 using a NanoDrop 2000 (ThermoFisher Scientific). Each DNA sample was diluted to 20 to 50 ng/μL to prepare for PCR amplification.

PCR Amplification

To determine the identities of the genotypes at the *MC1R* (i.e., E^-E^-) and *ASIP* (i.e., A^-A^-) genes in each horse, primers for amplification of

polymorphic gene sequences were designed based on the horse genome EquCab 3.0 using the software Primer premier v5.0 (Lalitha, 2000). The primer sequences for *MC1R* were *MC1R*-forward 5'-TGACCACCAACCAGACGGA-3', *MC1R*-reverse 5'-CGAGACAGAGCAGGACAGC-3', and for *ASIP* were *ASIP*-forward 5'-GGCTCTGAGAAATGGAGGGTAG-3', *ASIP*-reverse 5'-ATATCCTTAACGCTTCCCCAAC-3'. PCR reactions (20 μL) containing 20 to 50 ng genomic DNA, 0.5 μM of each primer, 10 μL 2× EasyTaq PCR Supermix (Transgen Biotech, China) and the appropriate amount of ddH₂O. Condition for *MC1R* PCR was an initial incubation and enzyme activation at 95 °C for 5 min,

Figure 1. Horse coat colors and variation in the *MC1R* and *ASIP* genes. (a) Typical examples of horse coat colors investigated in this study. (b) Sanger sequence maps of variations in *MC1R*. Missense mutations in *MC1R* are indicated in the red boxes. (c) Sanger sequence maps of variations in *ASIP*. Position of the 11 bp deletion in *ASIP* was indicated by a red box (presence of sequence) and arrows (deleted sequence).

Table 1. Breeds and quantity of different coat color horses used in this study

Coat color	Breeds and quantity	Total
Black	17 Mongolians, 8 Thoroughbreds, 4 Friesians, 1 Arabian, 1 Warmblood	31
Brown	79 Thoroughbreds, 3 Warmbloods, 1 Akhal-teke	83
Dark bay	179 Thoroughbreds, 3 Warmbloods, 2 Akhal-tekcs, 1 American standardbred	185
Bay	146 Thoroughbreds, 5 American standardbreds, 4 Warmbloods, 4 Akhal-tekcs, 2 Arabians, 2 Budyonnies, 1 Pony	164
Chestnut	101 Thoroughbreds, 9 Warmbloods, 5 Draft horses, 3 Akhal-tekcs, 3 Arabians, 2 American standardbreds	123
White	48 Mongolian, 4 Thoroughbreds, 2 Akhal-tekcs, 1 Andalusian	55
Gray	49 Thoroughbreds, 8 Arabians, 2 Warmbloods, 2 Polos, 2 Ponies, 2 Orlov trotter, 1 Shetlands, 1 American standardbred, 1 Clydesdale	68

followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 59 °C for 30 s and extension of 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR condition for *ASIP* was same as for *MC1R*, except that the primer annealing temperature was 61 °C.

Sanger Sequencing, Genotyping, and Statistical Analysis

PCR products were firstly checked by 1% agarose gels and then directly sequenced by the Sanger method on a 3730xl DNA Analyzer (Applied Biosystems) and genotypes (E^-E^- and A^-A^-) at *MC1R* and *ASIP* were deduced from the sequences. The *MC1R* PCR fragment amplified here included the mutation position C901T that had previously been used to genotype *MC1R* in horses (Marklund et al., 1996). The presence of homozygous cytosines at mutation position C901T was genotyped as $E^E E^E$, with heterozygous thymine and cytosine being genotyped as $E^E E^e$, and homozygous thymine genotyped as $E^e E^e$. Similarly, the PCR amplified *ASIP* fragment contained the 11 bp deletion in exon 2 and was used to genotype *ASIP* in horses (Rieder et al., 2001). Fragments homozygous for the deletion were genotyped as $A^a A^a$, heterozygous for the deletion was genotype as $A^A A^a$, and homozygous for nondeletion was genotyped as $A^A A^A$. The frequencies of each genotype (E^-E^- or A^-A^-) and each genotype combination ($E^-E^- - A^-A^-$) were calculated for each coat color. To reveal the potential association between genotypes of *MC1R* and *ASIP* and phenotypes of the horse coat colors, a χ^2 test for independence was performed on R v3.52 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.R-project.org>; accessed February 26, 2019) using the equation of $\chi^2 = T [\sum (O_{ij}^2 / R_i C_j) - 1]$. The results of the test

were shown in Supplementary Figure S1 (Cox and Lewis, 1966; Krajewski and Matthews, 2010).

RESULTS

Typical examples of the investigated coat colors are shown in Figure 1a. PCR products containing the mutation positions in *MC1R* (501 bp) and *ASIP* (1,006 bp) were successful amplified and sequenced in 709 horses allowing identification of the genotypes (E^-E^- and A^-A^-) for both *MC1R* and *ASIP* (Figure 1b and c). A summary of the genotypes at *MC1R* and *ASIP*, and genotype frequencies for the seven horse coat colors are shown in Figure 2 and Table 2.

MC1R Genotypes and Horse Coat Colors

The frequency of the $E^E E^E$ genotype at *MC1R* generally decreased as coat color changed from dark (black) to light (white) colors, in contrast to the frequency of the $E^E E^e$ genotype that mainly increased from black to bay color (Figure 2a). Furthermore, the $E^E E^e$ genotype was the advantage genotype observed in bay color horses (83.5%), dark bay horses had the genotypes $E^E E^E$ and $E^E E^e$ at similar frequencies (47.0% and 53.0%, respectively), while most black (64.5%) and brown (67.5%) colored horses had the $E^E E^E$ genotype (Figure 2a). The changes in the frequencies of the three genotypes ($E^e E^e$, $E^E E^e$, and $E^E E^E$) were in keeping with coat color changes from light to dark (chestnut, bay, dark bay, brown, and black). A majority (63.6%) of the white horses possess the $E^e E^e$ genotype at *MC1R*, with none of them having the $E^E E^E$ genotype (Figure 2a). All chestnut colored horses had the $E^e E^e$ genotype (Figure 2a), as found in a previous study (Marklund et al., 1996). No advantage genotype ($E^E E^E$, $E^E E^e$ or $E^e E^e$) was found in the gray horses. The χ^2 test results show that the phenotypes

Figure 2. Genotype frequencies for *MC1R* (a) and *ASIP* (b) in seven tested coat colors.

of horse coat colors were significantly related with the genotypes of *MC1R* ($p < 0.001$, Table 3).

ASIP Genotypes and Horse Coat Colors

Unlike previous reports that the A^aA^a genotype at *ASIP* was only in black, chestnut, and gray colored horses, we found the A^aA^a genotype in horses of all seven coat colors, with even 16.4% of the white colored horse being homozygous for the 11 bp deletion (Figure 2b). From dark (brown) to light (bay) colors, the frequency of the A^aA^a genotype gradually decreased, while the A^AA^A genotype increased in these coat colors (Figure 2b). The advantage genotype found in brown horse was A^aA^a (65.1%), while in the dark bay, bay, and chestnut horse the advantage genotype changed to A^AA^A (ranging from 62.6% to 83.0%). The A^AA^A and A^aA^a genotypes had the same frequencies (41.8% and 41.8%, respectively) in white colored horses, with gray colored horses having a slightly greater frequency of the A^AA^A genotype than the A^aA^a genotype (50.0% and 38.2%), respectively. As previously reported (Rieder et al., 2001), all horses with black color coats were A^aA^a . The χ^2 test results show that the phenotypes of horse coat colors were significantly related with the genotypes of *ASIP* ($p < 0.001$, Table 3).

Genotype Combinations and Horse Coat Colors

When the combined genotypes at *MC1R* and *ASIP* were considered together, advantage genotype combinations for each coat color were identified (Table 2). Horses with black color coats only had two genotype combinations, with most having $E^FE^E-A^aA^a$ (64.5%) and remaining being $E^EE^E-A^aA^a$ (35.5%). For brown color horses, 83.2% of the horses were found to have one of three genotype combinations, $E^FE^E-A^aA^a$ (47.0%), $E^FE^E-A^AA^A$ (18.1%), or $E^FE^E-A^AA^A$ (18.1%) (Table 2). Similarly, three genotype combinations, $E^FE^E-A^AA^A$ (36.2%), $E^FE^E-A^aA^a$ (33.0%), and $E^FE^E-A^aA^a$ (18.9%), were found in 88.1% of the dark bay colored horses (Table 2). While for bay colored horses, a single-genotype combination, $E^FE^E-A^AA^A$, was found in a majority (69.6%) of these horses, and the addition of two other genotypes, $E^FE^E-A^AA^A$ (13.4%) and $E^FE^E-A^aA^a$ (13.4%), explained 96.4% of these bay colored horses (Table 2). Only three genotype combinations were found in chestnut colored horses, the majority of which were $E^FE^E-A^AA^A$ (62.6%) and the remaining being $E^FE^E-A^aA^a$ (35.0%) and $E^FE^E-A^aA^a$ (2.4%) (Table 2). For white colored horse, 85.4% had one of the four following genotype combinations, $E^FE^E-A^AA^A$ (34.5%), $E^FE^E-A^aA^a$ (21.8%), $E^FE^E-A^aA^a$ (20.0%), and $E^FE^E-A^aA^a$ (9.1%),

Table 2. Numbers of horses and frequencies of *MC1R*–*ASIP* genotype combinations in the seven coat colors

Coat color*	E^FE^E			E^FE^E			E^FE^E		
	A^aA^a	A^AA^A	A^AA^A	A^aA^a	A^AA^A	A^AA^A	A^aA^a	A^AA^A	A^AA^A
Black (31)	64.5% (20)	0	0	35.5% (11)	0	0	0	0	0
Brown (83)	2.4% (2)	47.0% (39)	18.1% (15)	9.6% (8)	18.1% (15)	4.8% (4)	0	0	0
Dark bay (185)	1.6% (3)	9.2% (17)	36.2% (67)	1.1% (2)	18.9% (35)	33.0% (61)	0	0	0
Bay (164)	1.2% (2)	1.8% (3)	13.4% (22)	0.6% (1)	13.4% (22)	69.6% (114)	0	0	0
Chestnut (123)	0	0	0	0	0	0	2.4% (3)	35.0% (43)	62.6% (77)
White (55)	0	0	0	9.1% (5)	21.8% (12)	7.3% (4)	7.3% (4)	20.0% (11)	34.5% (19)
Gray (68)	4.4% (3)	17.6% (12)	8.8% (6)	1.5% (1)	25.0% (17)	11.8% (8)	5.9% (4)	7.4% (5)	17.6% (12)

*The number of horses with each coat color and genotype combination is in parentheses.

Table 3. The genotype distribution of *MC1R* and *ASIP* gene

Gene	Genotype	Phenotype class							χ^2 value
		Black	Brown	Dark bay	Bay	Chestnut	White	Gray	
<i>MC1R</i>	E^E	51	139	272	191	0	20	68	$\chi^2 = 445.64$ $P < 0.01$
	E^e	11	27	98	137	246	50	68	
	E^EE^E	20	56	87	27	0	0	21	$\chi^2 = 672.82$ $P < 0.01$
	E^EE^e	11	27	98	137	0	20	26	
	E^eE^e	0	0	0	0	123	35	21	
<i>ASIP</i>	A^a	62	74	62	31	49	41	42	$\chi^2 = 286.74$ $P < 0.01$
	A^A	0	92	308	297	197	69	94	
	A^aA^a	31	10	5	3	3	9	8	$\chi^2 = 406.63$ $P < 0.01$
	A^AA^A	0	54	52	25	43	23	26	
	A^AA^A	0	19	128	136	77	23	34	

Table 2). In contrast to the other coat colors, all nine genotype combinations were found in the gray colored horses; however, most of them (72.0%) were $E^E E^e - A^A A^a$ (25.0%), $E^E E^E - A^A A^a$ (17.6%), $E^e E^e - A^A A^A$ (17.6%), or $E^E E^e - A^A A^A$ (11.8%, **Table 2**).

DISCUSSION

MC1R has a demonstrated role in hair and coat color variation in humans and in a number of other mammalian species (Healy et al., 2001; Römpler et al., 2006; Candille et al., 2007; Dreger and Schmutz, 2010; Våge et al., 2014). In previous research, the reddish chestnut horse coat color is due to the $E^e E^e$ genotype, while horses with the genotypes $E^E E^E$ and $E^E E^e$ present with darker coat colors (black and brown) (Marklund et al., 1996). Our results show that the advantage genotype at *MC1R* change with coat color in horses, through a spectrum from dark to light, i.e., black ($E^E E^E$), brown ($E^E E^E$), dark bay ($E^E E^E$ and $E^E E^e$), bay ($E^E E^e$), chestnut ($E^e E^e$), and white ($E^e E^e$). In black horses, no $E^e E^e$ genotypes were found, and similarly, in white no $E^E E^E$ genotypes at *MC1R* were found. These results suggest that *MC1R* affects horse coat color through the melanogenesis process, with the E^E allele enhancing melanogenesis and the E^e allele inhibiting it. Previous research on *MC1R* and coat color variation in the Arabian dromedary camel had indicated that the $E^E E^E$ genotype was associated with the dark phenotype (black, brown, and light brown), whereas the $E^E E^e$ and $E^e E^e$ genotypes were associated with light coat colors (e.g., white) (Almathen et al., 2018). While a minority of the tested horses does not conform to this expectation, this might be due to other genes participating in the eumelanin process or genes that regulate *MC1R* expression in these horses. In general, our results demonstrate that $E^E E^E$ genotype is associated with dark coat color phenotypes, including black, brown, dark bay, and bay, whereas $E^E E^e$ genotype is associated with the light phenotypes.

Most of the horses with dark bay, bay, and chestnut coat colors examined here possessed the $A^A A^A$ genotype at *ASIP*, with only the brown horse having $A^A A^a$ as an advantage genotype. Meanwhile, we found that the genotype of brown horses at last has one allele E^E or A^a . This observation might account for the deeper coat color in brown horses. The distribution of the advantage genotypes with coat color suggests that the A^a allele is associated with dark phenotypes (e.g., black and brown), whereas the A^A

allele is associated with lighter phenotypes (e.g., bay and chestnut). This pattern is consistent with *ASIP* acting through MSH antagonism to inhibit eumelanin production (Lu et al., 1994).

The 11 bp deletion mutation in exon 2 (the A^a allele) of *ASIP* leads to loss of function for the agouti signaling protein and thus should cause dark phenotypes in the horse (Rieder et al., 2001). Previous studies have reported that the $A^a A^a$ genotype only occurs in black, chestnut, and gray-colored horses (Rieder et al., 2001); however, in this study, we found that the $A^a A^a$ genotype is also found in brown, dark bay, bay, and even white-colored horses with frequencies between 1.8% and 16.4%. These horses with the $A^a A^a$ genotype did not show a darker color than other horses with $A^A A^A$ genotype of the same coat color. These results suggest that another gene(s) might take the place of *ASIP* to block melanin production promoted by *MC1R*. Based on the hypothesis that all black or gray (which were born black) horses were $A^a A^a$ at *ASIP* (Rieder et al., 2001), researchers had speculated on the coat colors of ancient horses by analyzing DNA from fossils. These analyses lead to the conclusion that black horse had appeared in the Copper Age (Ludwig et al., 2009), but our new findings might question this.

When the genotypes at both *MC1R* and *ASIP* were considered, the advantage genotype combinations for coat colors across black to chestnut were: black ($E^E E^E - A^a A^a$), brown ($E^E E^E - A^A A^a$), dark bay ($E^E E^E - A^A A^A$ and $E^E E^e - A^A A^A$), bay ($E^E E^e - A^A A^A$), and chestnut ($E^e E^e - A^A A^A$). The differences in the advantage genotype combinations correlate with color deepness. The variations of the advantage genotype combinations (from $E^E E^E - A^a A^a$ to $E^e E^e - A^A A^A$) with the darkness of the coat colors (from dark to light colors) indicate that *MC1R* and *ASIP* synergistically regulate the production of melanin for horse coat color. All nine genotype combinations were found in the gray horse, perhaps due to their various original coat colors.

Among nine possible genotype combinations for *MC1R* and *ASIP*, 97.6% to 100% black and chestnut horses were represented by only two of them, 83.2% to 96.4% of the brown, dark bay, and bay horses had three of them, and 72.0% to 85.4% of the white and gray horses had four of them (**Table 2**). These results indicate that coat color in horses is dominated by these two genes, but that other genes and loci also participated with the rare genotypes to generate coat colors (Metallinos et al.,

1998; Rosengren Pielberg et al., 2008; Holl et al., 2010; Hauswirth et al., 2012, 2013).

Through statistical analyses of *MC1R* and *ASIP* genotypes in seven coat color phenotypes in horses, we found that these two genes synergistically and advantaged influence the amount of melanin in black, brown, dark bay, bay, and chestnut horses, but also found that some other genes likely participate in generating the white and gray coat colors. Our results provide a molecular basis for further research on the mechanism of horse and other mammalian coat colors.

Conflict of interest statement. None declared.

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