A mutation in the SLC45A2 gene causing the cremello phenotype in a Turkish wild horse

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ABSTRACT

Coat color not only aids in the identification of horses but also plays a significant role in the accurate diagnosis and prevention of genetic disorders associated with specific coat colors. A wide variety of coat colors are observed phenotypically in horses, and these are determined by numerous different genes. This study aimed to determine whether a wild foal brought to Afyonkarahisar in 2017 as part of the "Wild Horse Control and Rehabilitation Project," which is affiliated with the 8th Regional Directorate of Nature Conservation and National Parks of the Ministry of Forestry and Water Affairs, exhibited the cream coat color, which is a rare phenotype among horses. Accordingly, the presence of a mutation in the 2nd exon of the SLC45A2 gene (solute carrier family 45 member 2, also known as MATP or AIM-1) was investigated. Seven Thoroughbred horses with basic dark colors (chestnut or bay) were used as a control group. The DNA sequencing analysis revealed a point mutation in the 2nd exon of the SLC45A2 gene, specifically NC_009164.3:g.31690653 G>A (rs1140980396). It was concluded that this mutation may lead to the expression of the cream gene (C^{Cr}) and the resulting cremello phenotype in wild horses, which are one of the native genetic resources of Türkiye. However, it is considered that more comprehensive and detailed studies are needed to accurately determine the phenotypic coat color variations observed in feral horses, their corresponding genotypes, and the allele frequencies within the population.

Introduction

Prior to domestication, coat color variation among horse breeds was limited. Although with domestication and selective breeding, certain coat colors have been preferentially propagated based on breeder preferences. This has increased homozygosity of recessive alleles and, along with gene mutations over time, contributed to the emergence of diverse coat color phenotypes (14, 23, 29). Coat color in horses is determined by the presence or absence of melanin pigments in the skin and hair. These pigments are synthesized in small vesicles known as melanosomes, which are found within pigment cells called melanocytes. There are two types of melanosomes: eumelanosomes and pheomelanosomes. Eumelanosomes produce eumelanin, which gives rise to dark brown to black colors, and pheomelanosomes produce pheomelanin, responsible for red to yellow colors. Coat colors in animals vary depending on the presence and distribution of these two pigments in the body (20, 31, 33).

Black, chestnut, and bay are considered the basic coat colors in horses, and their expression is determined by two genetic loci: Extension (E) and Agouti (A) loci. The extension locus has two alleles, E^+ and E^e, with E^+ being dominant. The E^+ allele increases the synthesis of eumelanin, while the E^e allele enhances pheomelanin. Therefore, a horse with the E^+-A^aA^a genotype will have a black coat color, while a horse with the E^eE^e-- genotype will have a chestnut coat color. Phenotypically, in black coat color, the whole body is black, and in chestnut coat color, the whole body is reddish-brown. The A^A allele at

the Agouti locus causes black pigmentation to be distributed in the mane, tail, and lower legs, while the A^a allele results in spreading blackness throughout the body. A^A is dominant and can only be expressed in the presence of the E^+ allele. When a horse with the E^+-A^A- genotype will have a bay coat color. Phenotypically, in this coat color, the body is reddish-brown; the mane, tail and lower legs are black. These basic coat colors can be altered by the alleles in the Champagne, Cremello, Dun, and Silver Dapple loci, which reduce pigmentation intensity and cause dilution, resulting in the emergence of new coat colors (6, 14, 23, 25, 28).

Cremello is a rare coat color in horses resulting from dilution. The C^+ allele in the cream locus has no effect on coat color, while the C^{Cr} allele causes a dilution of the color. The C^{Cr} allele exhibits incomplete dominance. In the heterozygous state (C^+C^{Cr}) affects pheomelanin, transforming red to yellow, but does not impact eumelanin. As a result, E^eE^e--C^+C^{Cr} and E^+-A^A-C^+C^{Cr} genotypes are formed, and palomino and buckskin coat colors are observed in the phenotype, respectively. In this context, palomino represents the diluted form of chestnut, while buckskin is the diluted variant of bay. While palomino's whole body is yellow, buckskin's body is yellow; the mane, tail, and lower legs are black. When it is present in the homozygous state ($C^{Cr}C^{Cr}$), its effect on pheomelanin increases even more, transforming red into cream (ivory) color, and it has a little effect on eumelanin. In that case, it causes the formation of E^eE^e--$C^{Cr}C^{Cr}$ and E^+-A^A-$C^{Cr}C^{Cr}$ genotypes, creating cremello and perlino coat colors, respectively. Cremello represents the diluted form of chestnut, and perlino the diluted form of bay. In cremello, the whole body is cream (ivory); in perlino, the body is cream, and the mane, tail, and lower legs are light brown. These horses have pink skin and blue eyes. Locus, alleles, actions, and comments of basic coat colors and cremello are given in Table 1, and the effects of the cream gene on basic coat colors are given in Table 2. The cremello phenotype has been attributed to a mutation in the SLC45A2 gene (Solute Carrier Family 45 Member 2), also known as MATP (Membrane-associated Transport Protein), which is located on chromosome 21 (1, 7, 17, 18, 23, 28). The MATP gene encodes a transporter protein involved in melanin transfer and pH balance. Proper pH allows copper (Cu) to activate tyrosinase, essential for melanin synthesis. MATP malfunction disrupts this process, leading to abnormal melanin levels (4, 5, 16, 26, 30, 36).

Table 1. Locus, alleles, actions, and comments of basic coat colors and cremello

Locus	Allele	Symbol	Action	Comment
Extension	Black Chestnut	**E^+** **E^e**	Dominant Recessive	Causes black Causes chestnut
Agouti	Bay Black	**A^A** **A^a**	Dominant Recessive	Causes bay Causes black
MATP (Membrane Associated Transport Protein) (Cream)	Wild Cremello	**C^+** **C^{Cr}**	Intermediate Intermediate	Allows intense color Heterozygotes have red diluted to yellow; black unaffected; homozygotes have red to cream, pink skin, and blue eyes

Table 2. The effects of the cream gene on basic coat colors

Coat colors	
Basic Dark Colors	**Diluted Colors**
Black (E^+-A^aA^a) Whole body is black.	(E^+-$A^aA^aC^+C^{Cr}$) There is no change in coat color. (E^+-$A^aA^aC^{Cr}C^{Cr}$) Slight dilution in coat color.
Chestnut (E^eE^e--) The whole body is reddish-brown.	**Palomino** (E^eE^e--C^+C^{Cr}) The whole body is yellow.
	Cremello (E^eE^e--$C^{Cr}C^{Cr}$) The whole body is cream (ivory); the skin is pink, and the eyes are blue.
Bay (E^+-A^A-) The body is reddish-brown; the mane, tail, and lower legs are black.	**Buckskin** (E^+-A^A-C^+C^{Cr}) The body is yellow; mane, tail and lower legs are black.
	Perlino (E^+-A^A-$C^{Cr}C^{Cr}$) The body is cream (ivory); the mane, tail, and lower legs are light brown; the skin is pink; and the eyes are blue.

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Today, wild horses (Yılkı) live in groups of 15-20 in different regions of Türkiye. The main regions are Karaman (Karadağ), Kayseri (Erciyes Mountain), Manisa (Spil and Yunt Mountains), Afyonkarahisar (Akdağ, Kumalar Mountain, and Emirdede Plateau), Samsun (Kızilirmak Basin), İzmir (Gediz Basin), Antalya (Eynif Plain and Bey Mountains), and Kaz Mountains (11). In 2017, the Karaman Branch Directorate, affiliated with the 8th Regional Directorate of Nature Conservation and National Parks of the Ministry of Forestry and Water Affairs, carried out the 'Wild Horses Control and Rehabilitation Project' to manage wild horse populations, provide care, and reduce their numbers to sustainable levels. Some captured horses were adopted by citizens free of charge. The aim of this study is to investigate the presence of a mutation in the SLC45A2 gene in a foal with a cream coat phenotype, which was brought to Afyonkarahisar as part of the "Wild Horses Control and Rehabilitation Project."

Materials and Methods

***Animal Material*:** Eight horses were used in this study. These horses consist of a cremello wild foal, which was brought to the enterprise of a breeder in Afyonkarahisar province, and among the horses distributed within the project named "Yılkı Horses Control and Rehabilitation Project," and seven thoroughbred horses with basic dark colors (chestnut or bay) were used as a control group. Photographs of the cream (ivory) hair, pink skin, and blue eyes of the cremello wild foal are presented in Figure 1.

***Blood Samples and DNA Isolation*:** The blood samples were collected from the horses' vena jugularis into an ethylene-diaminetetraacetic acid (EDTA) vacuum tube and stored at +4°C until the DNA isolation. The GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate DNA from blood, following the manufacturer's instructions. The DNA

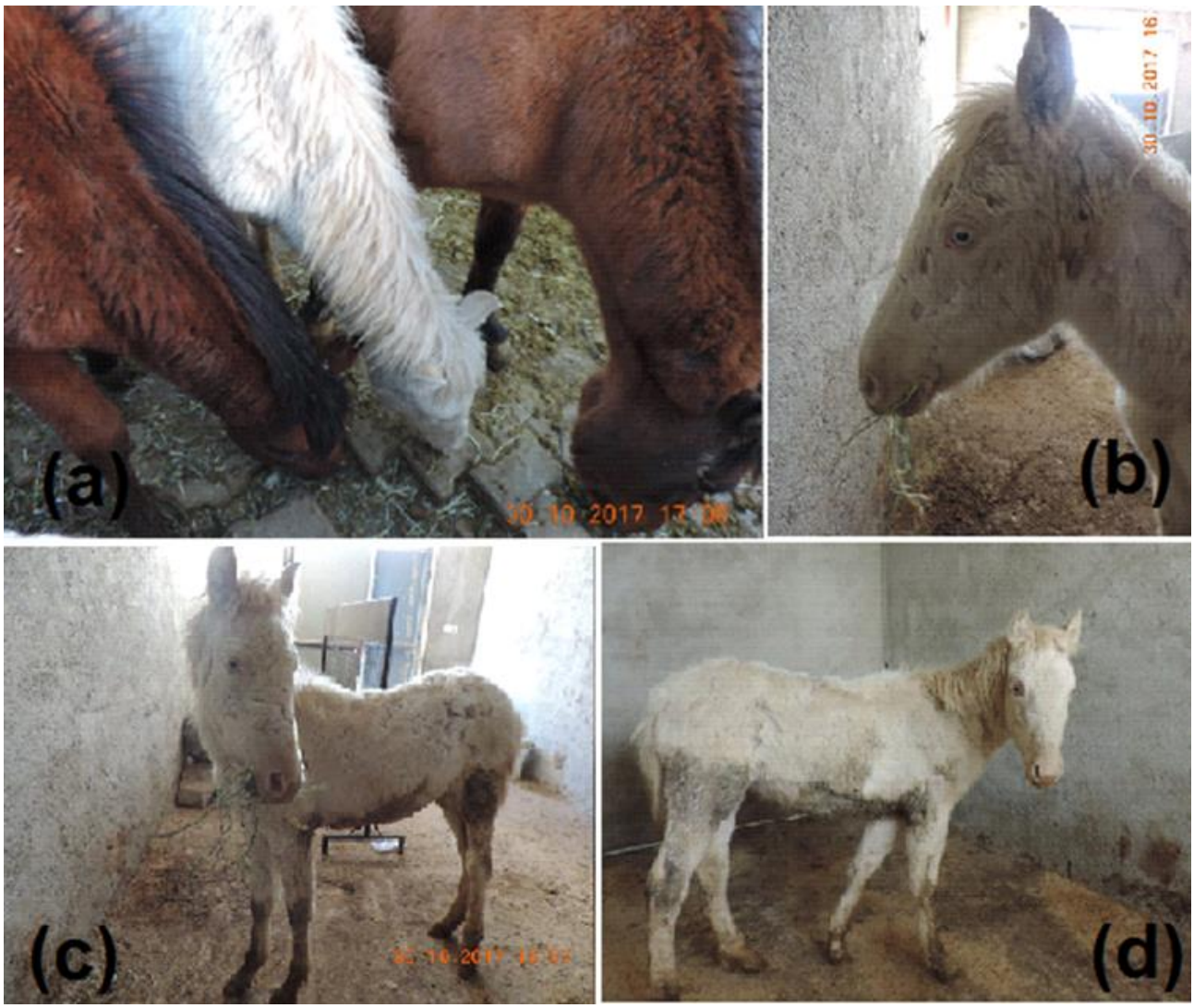

Figure 1. The cremello wild foal. (a) The cremello wild foal between bay foals. (b) Blue eyes at the cremello wild foal. (c-d) Cream (ivory) hair and pink skin at the cremello wild foal.

quantity was measured using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA), and its quality was evaluated with a Thermo MultiSkan GO device (Thermo Scientific, USA). The concentration was then adjusted to 20 ng/µl.

***PCR and Sequencing*:** Primers were designed using the Fast PCR Professional 6.1.2 package program with the NC_091704 reference sequence from NCBI (15). The formation of dimers and hairpins between primers was controlled by the same programme. The PCR reaction contained 40 ng DNA, 0.2 mM forward primer (5'-GAGAGAGCTTGATGACAGGAA-3'), 0.2 mM reverse primer (5'-AAATGCACTGGGAGACTGAGC-3'), 2 mM MgCl2, 1x PCR buffer, 0.2 mM of deoxynucleotide triphosphate mixture (dNTP), and 1 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Finally, the mixture was diluted to 20 µl with ultra-distilled water. The PCR machine was programmed to run as follows: an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes.

The PCR products were purified using ExoSAP-IT. The BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Life Technologies Corporation, Austin, TX, USA) was used for sequencing PCR, following the manufacturer's instructions. The resulting products were cleaned using the ethanol/sodium acetate/EDTA precipitation method. The samples were diluted with 15 µl of Hi-Di Formamide and loaded into the genetic analyzer (ABI 3500, Thermo).

The Sequencher (v.5.4.6) software package (Gene Codes Corporation, Ann Arbor, MI, USA) was utilised to automatically merge forward and reverse pairs, visualise Sanger sequencing chromatograms, and refine the consensus sequence to 426 bp in length. Then, the edited samples were aligned using the Bioedit (v.7.0.9) Sequence Alignment program (12).

Results

After PCR analysis, a band of 426 bp was recorded, and a point mutation was identified in the cremello foal by DNA sequencing analysis (Figure 2). Specifically, a guanine-to-adenine substitution was identified at the g.31690653th base (rs1140980396) in the 2nd exon of the SLC45A2 gene (NCBI Reference Sequence, NC_009164.3), located on chromosome 21, resulting in a D153N amino acid substitution in the cremello foal. No mutation was observed in any of the Thoroughbred horses of basic dark color.

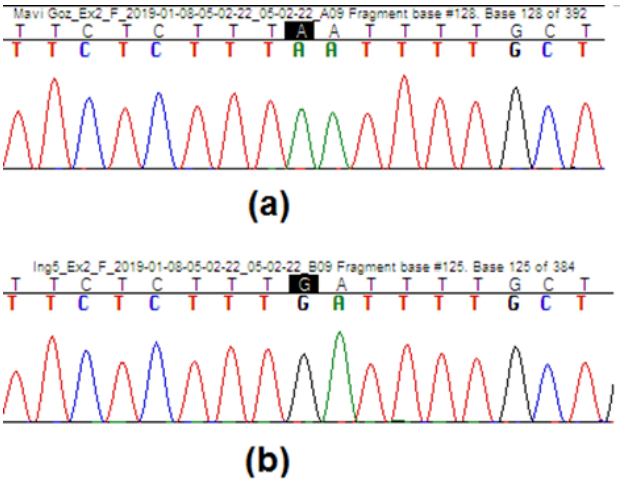

Figure 2. DNA sequencing analysis. (a) DNA sequence analysis of cremello horse. (b) DNA sequence analysis of thoroughbred horses of basic dark color.

Discussion and Conclusion

Coat color in animals is determined by the amount and distribution of two primary pigments in the skin and hair: black eumelanin and red pheomelanin. In horses, it has been reported that when the cream gene is present in a heterozygous state ($C^{Cr}C^{+}$), partial dilution of bay and chestnut base colors results in buckskin and palomino phenotypes. In contrast, a homozygous cream gene ($C^{Cr}C^{Cr}$) leads to full dilution, giving rise to the cremello and perlino phenotypes, which are characterized by pink skin and blue eyes (13, 14, 25, 28). Adalsteinsson (1) proposed that a single allele of this gene is responsible for the palomino and buckskin phenotypes in Icelandic horses through the dilution of pheomelanin. The presence of the cremello allele in different horse breeds (Akhal Teke, Andalusian, Appaloosa, Arabian, Barb, Connemara Pony, Don, Dutch Warmblood, Gypsy Cob, Gypsy Vanner, Hispano-Arabian, Icelandic Horse, Kabardin, Kırgız, Lewitzer, Lusitano, Marwari, Miniature Horse, Missouri Fox Trotter, Morgan Horse, Mustang, Norwegian Fjord Horse, Oldenburg, Paint Horse, Pura Raza Espanola, Quarter Horse, Rocky Mountain Horse, Shetland Pony, Standardbred, Spanish Purebred, Tennessee Walking Horse, Thoroughbred, Türkmen, Vyatka Horse, Welsh Pony, and Yomund) has been reported by various researchers (2, 3, 19, 24).

SLC45A2, a transporter protein involved in melanin synthesis (5), has been shown to play a crucial role in pigmentation across multiple species. Mutation in this gene is known to reduce melanin synthesis to varying degrees, leading to hypopigmentation of the eyes, skin, and hair. Such mutations have been associated with pigmentation disorders across multiple species, including oculocutaneous albinism type 4 (OCA4) in humans (22),

white coat color in mice (8), white phenotype in tigers (34) and Doberman Pinschers (32), as well as the gold phenotype in Medaka fish (9) and the silver phenotype in chickens (10). Previous studies in horses have also identified a mutation in the 2nd exon of the SLC45A2 gene, located on chromosome 21, that is completely linked to the cream locus (17, 18, 21, 27). Mariat et al. (18) reported that all cremello horses were homozygous A/A, all palomino and buckskin horses were heterozygous A/G, and all bay horses were homozygous G/G genotype in the 2nd exon of the MATP gene. In the present study, a wild foal exhibiting a phenotype with a whole body that is cream, pink skin, and light blue eyes was identified, consistent with the description of the cremello phenotype. As a result of DNA isolation, PCR, and sequencing performed on the blood sample of the cremello wild foal, a point mutation, g.31690653 G>A, was detected in the 2nd exon of the SLC45A2 gene, which has been reported to cause the cream coat color.

Yoshihara et al. (35) conducted a study aimed at obtaining genetic and phenotypic data on various traits of the Taishu horse, one of the native Japanese breeds currently at risk of extinction, in order to develop an effective breeding strategy based on these findings. It was determined that all horses included in the study exhibited basic dark coat colors, which are chestnut, black, and bay. Phenotypically, no instances of gray, frame overo, sabino, or cream coat colors were observed, and the absence of the alleles responsible for these phenotypes was confirmed, thereby validating the phenotypic predictions. Nakamura et al. (21) conducted a study on the Kiso horse, another native breed in Japan, and identified bay, chestnut, and buckskin coat colors phenotypically. Their genetic analyses revealed the presence of the C^{Cr} allele in the MATP gene, which is responsible for the buckskin phenotype. Both studies emphasized the importance of documenting and conserving these endangered breeds, recognized as native genetic resources, to preserve their unique characteristics through the identification and characterization of their genetic traits. The number of studies on indigenous horse breeds in Türkiye is quite limited. Moreover, no research has been identified that aims to determine the phenotypic and genetic characteristics of wild horses living in different regions and national parks. This study has demonstrated the presence of the cream coat color, rarely observed in horses, and its associated $C^{Cr}C^{Cr}$ genotype in wild horses. However, comprehensive studies are required to accurately determine the phenotypic coat color variations observed in these horses, their corresponding genotypes, and the allele frequencies within the population. Furthermore, as these horses represent a native genetic resource in terms of equine diversity in Türkiye, it is considered essential to document and characterize their genetic and phenotypic traits. Moreover, the identification and systematic documentation of the genetic and phenotypic characteristics of these horses, recognized as a local genetic resource in Türkiye, are of great importance for preserving the variation within the population. This will ensure the sustainability of their genetic diversity and provide a solid foundation for future scientific research.

In conclusion, coat color in horses is not only a key to identifying horses but also an important indicator for the accurate diagnosis and prevention of coat color-related genetic disorders. This study suggests that the identified point mutation in the 2nd exon of the SLC45A2 gene may be responsible for the presence of the cream allele (C^{Cr}) and the cremello in wild horses, which represent a valuable genetic resource within domestic animal populations. Further research into equine coat pigmentation is expected to contribute significantly to breeding strategies aimed at preserving genetic diversity and improving desirable phenotypes.

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Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (49533702/137).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ÖGH, KÇ and ME were responsible for the study design. ÖGH and KÇ collected the data. ME performed data analysis. ÖGH wrote the paper. All authors read and approved the final article.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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