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A De Novo *MITF* Deletion Explains a Novel Splashed White Phenotype in an American Paint Horse

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Abstract

Splashed white is a coat color pattern in horses characterized by extensive white patterning on the legs, belly, and face often accompanied by blue eyes and deafness. Three mutations in *microphthalmia-associated transcription factor (MITF*) and two mutations in *Paired Box 3 (PAX3*) have been identified that explain splashed white patterns (SW1–SW5). An American Paint Horse stallion with a splashed white phenotype and blue eyes, whose parents were not white patterned, was negative for the 5 known splashed white variants and other known white spotting alleles. This novel splashed white phenotype (SW6) was hypothesized to be caused by a de novo mutation in *MITF* or *PAX3*. Analysis of whole-genome sequencing using the EquCab3.0 reference genome for comparison identified an 8.7 kb deletion in *MITF* on ECA16 (NC_009159.3:g.21551060-21559770del). The deletion encompassed part of intron 7 through the 3′ UTR of exon 9 of MITF, including the helix-loop-helix DNA-binding domain (ENSECAT00000006375.3). This variant is predicted to truncate protein and impair binding to DNA. Sanger sequencing confirmed the stallion was heterozygous for the *MITF* deletion. No single nucleotide polymorphisms (SNPs) or structural variants were identified in *PAX3* or any of the other candidate genes that were unique to the stallion or predicted to affect protein function. Genotyping five of the stallion's splashed white offspring, including one all white foal, found that they were also heterozygous for the deletion. Given the role of MITF in producing white pattern phenotypes, and the predicted deleterious effect of this mutation, this 8.7 kb deletion is the likely causal variant for SW6.

Subject areas: Gene action, regulation and transmission
Key words: coat color, horse, pigmentation, white spotting

White spotting patterns in horses have been associated with a number of alleles affecting melanocyte development, migration, survival, or proliferation. These include variants in the *endothelin receptor type B* (*EDNRB*), *KIT proto-oncogene receptor tyrosine kinase* (*KIT*), *microphthalmia-associated transcription factor* (*MITF*), *paired box gene 3* (*PAX3*), and *transient receptor potential cation channel subfamily M member 1* (*TRPM1*) genes. Frame overo patterning is caused by a missense variant (p. Ile118Lys, O allele) in the *EDNRB*

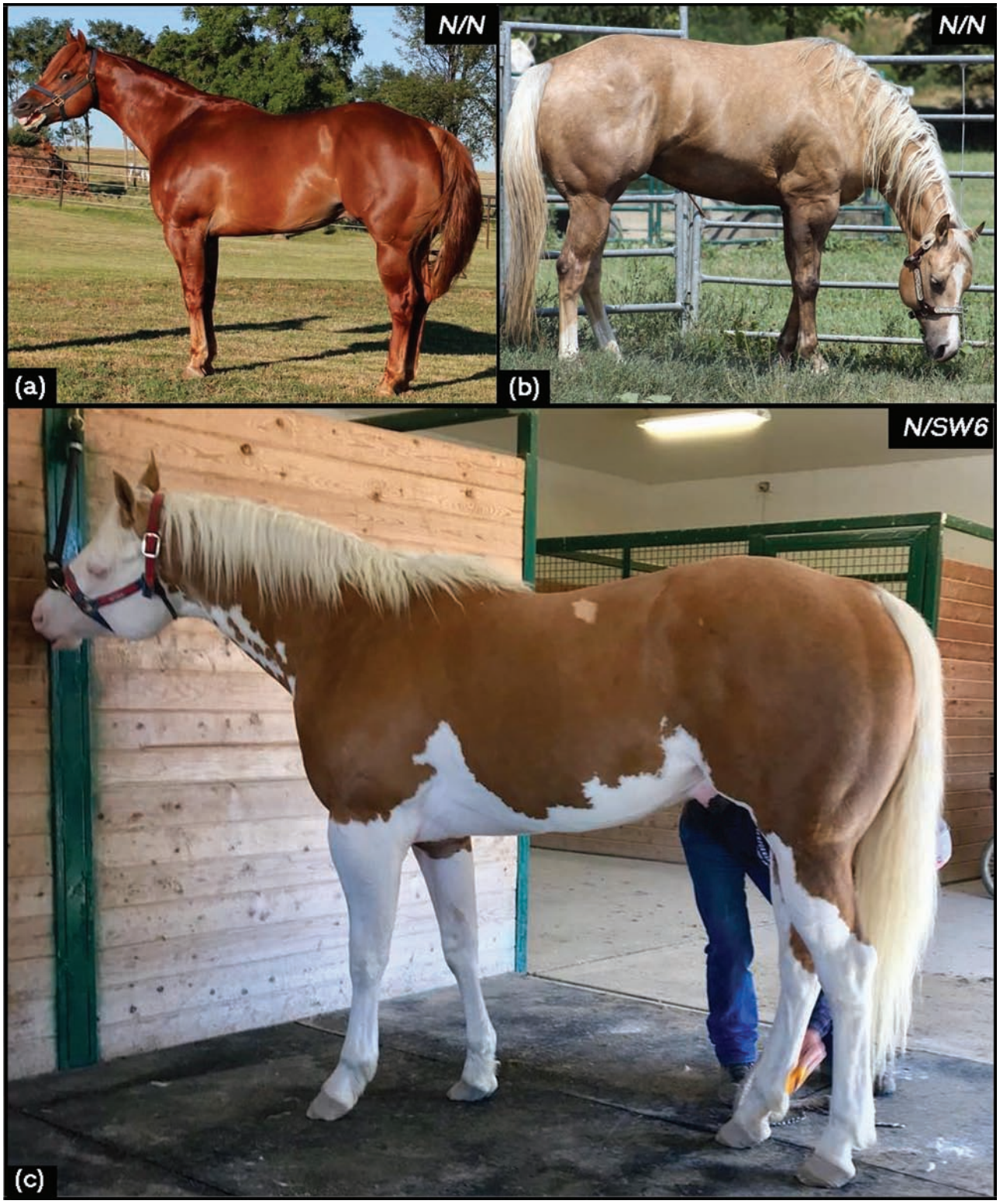

Figure 1. SW6 phenotype. A novel splashed white phenotype (**c**) resulted from a cross involving 2 parents without white spotting patterns (**a**). Sire of the identified splashed white stallion (in **c**) displaying a clear solid chestnut phenotype without any extensive face and leg markings. (**b**) Dam of the identified stallion (**c**) showing a palomino phenotype with minimal face and leg markings. (**c**) Paint stallion with a novel splashed white phenotype unexplained by any known splashed white or white patterning variants. Reported are the genotypes of each horse for the de novo SW6 mutation identified in this study.

gene in a number of breeds (Metallinos et al. 1998), whereas a variant of *TRPM1* (LP) causes leopard complex spotting in the Appaloosa, among other breeds (Bellone et al. 2013). Multiple variants (W1–W28, SB1, TO) in or involving the *KIT* gene are responsible for a variety of white spotting patterns, including dominant white, sabino-1, and tobiano (Brooks and Bailey 2005; Brooks et al. 2007; Hauswirth et al. 2013). Some of these are breed dependent and others like SB1, TO, and W20 occur in multiple breeds.

The splashed white phenotype is a complex trait with varying amounts of white, ranging from white patterning on the limbs and face, up to a nearly all white pattern. The classic splashed white pattern is distinctive with ventrally distributed white. It usually consists of high white markings on 3–4 of the limbs, extensive face markings referred to as bald or apron faces (a large amount of white covering the majority of the face that often extends from the lower lip to beyond the level of the eyes), occasional belly spots, and variable blue irides. Homozygous splashed white horses are more extensively marked than heterozygotes, and are sometimes completely white. Some splashed white horses are also deaf (Magdesian et al. 2009).

The splashed white phenotype has been reported to be associated with 5 alleles to date, 3 of those in the *MITF* (SW1, SW3, and SW5) and 2 in *PAX3* (SW2 and SW4) genes (Hauswirth et al. 2012, 2013; Henkel et al. 2019). SW1 is caused by a 10 bp insertion in melanocyte specific promoter and occurs in multiple breeds, whereas SW3 is due to a frameshift mutation in exon 5 and is limited to one family of American Paint Horses (Hauswirth et al. 2012). SW5 is the result of a 63 kb deletion involving exons 6–9 and also is limited to one family of American Paint Horses (Henkel et al. 2019). SW2 and SW4 represent different missense mutations in exon 2 of the PAX3 gene (Hauswirth et al. 2012, 2013). SW2 has been identified in the American Paint Horse, the American Quarter Horse, Norikers, and Lipizzaners (Druml et al. 2018), whereas SW4 was found in a single family of Appaloosas (Hauswirth et al. 2013). Macchiato, a phenotype with some similarities to splashed white, was found as a de novo missense mutation in exon 6 of the *MITF* gene in a Franches-Montagnes stallion (Blatter et al. 2013). Mutations in *EDN3* and *SOX10* have not yet been associated with pigment alterations in horses, but they have been associated with pigment disorders and deafness in Waardenburg syndromes in humans and are therefore potential candidate genes for unexplained white spotting patterns (Wang et al. 2014; Chandra Mohan 2018). Mutations in the *KITLG* gene have also not been reported in horses with white spotting phenotypes, but they are associated with pigment alterations in humans, cattle, and dogs, and therefore this gene was also evaluated in this study as a candidate for white patterning (Seitz et al. 1999; Amyere et al. 2011; Weich et al. 2020).

A dual registered American Paint and Quarter Horse stallion with a splashed white phenotype (Figures 1 and 2), was tested by the Veterinary Genetics Laboratory for the 5 known splashed white variants (SW1–SW5), as well as other known white spotting patterns (TO, W5/W10/W20/W22, O, LP, Appaloosa pattern-1, and SB1). This stallion did not have any of these white spotting pattern alleles. As neither the stallion's sire nor the dam had a white spotting pattern or extensive face and leg markings (Figure 1), we hypothesized that this splashed white phenotype was caused by a de novo, dominant mutation in *PAX3* or *MITF*.

Methods

DNA from the stallion was isolated from whole blood using a Gentra Puregene DNA isolation kit with the protocol as previously described (Mack et al. 2017). This sample and DNA extracted from hair follicles of the sire and dam were tested by the University of California (UC) Davis, Veterinary Genetics Laboratory for known white pattern alleles (SW1–SW5, TO, W5/W10/W20/W22, SB1, O, LP, and Appaloosa pattern-1). Routine parentage DNA testing was also performed by the Veterinary Genetics Laboratory to determine if the sire and dam qualify as parents of the stallion.

DNA from the stallion was also whole-genome sequenced on the NovaSeq platform with 2 × 150 bp paired end reads and an average insert size of approximately 300 base pairs. Sequencing data were processed utilizing the HTStream pipeline (https://github.com/ibest/HTStream) and were aligned to the reference assembly, EquCab3.0 using Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009). Variants were called utilizing the variant caller FreeBayes (Garrison E. FreeBayes source repository; https://github.com/ekg/freebayes), and annotated with SnpEff (Cingolani et al. 2012). The consensus classifier PredictSNP was utilized to predict the functional consequence of identified variants (Bendl et al. 2016). Coding variants in the candidate genes known to cause white spotting phenotypes in horses and other species, namely *MITF, KIT, KITLG, PAX3, EDNRB, EDN3, SOX10, and TRPM1*, were prioritized for further investigation. Additionally, sequencing data from these genes and one Mb of flanking sequence were visualized in Integrative Genomics Viewer (IGV) to look for structural variants (Robinson 2011). Genes, transcript ID, and genomic positions are summarized in Supplementary Table S2. Variants were filtered based on their presence in the splashed white sample under investigation, but absent in 16 horses from 4 breeds (Haflinger, Tennessee Walking Horse, Shetland Pony, and Friesian) that were available from other studies in the laboratory. Data were deposited at the European Nucleotide Archive (ENA, study accession numbers PRJEB36403 (horse under investigation in this study), PRJEB28306 (1 Shetland Pony), PRJEB36381 (1 Tennessee Walking Horse), PRJEB36380 (4 Friesians and 4 Haflingers), and PRJEB30871 (6 Haflingers).

To validate the identified deletion, primers were designed utilizing NCBI's primer blast (Ye et al. 2012; Supplementary Table 1). Polymerase chain reaction (PCR) was performed with a volume of 20 µL using 5.0 pmol of primers, 15 ng of DNA, 1× PCR buffer with 2.0 mM $MgCl_2$, 1 mM dNTP, and 0.1 µL FastStart Taq DNA polymerase (Roche Applied Science, Indianapolis, IN). The PCR products were visualized on a 2% ethidium bromide gel, and the amplicons were purified using an EdgeBio Quickstep 2 PCR purification kit, as per the manufacture's protocol (EdgeBio, Gaithersburg, MD). They were subsequently sequenced using BigDye Terminator v3.1 and ABI 3730 Genetic Analyzer (Applied Biosystems, at ThermoFisher Scientific, Grand Island, NY).

Additionally, 11 offspring were genotyped for the MITF deletion with PCR and gel electrophoresis as for the sire. These samples were also tested at the Veterinary Genetics Laboratory, UC Davis for the same known white spotting variants as the stallion and his parents.

Results

Average coverage for the sample under investigation was 52X (PRJEB36403). In screening the 8 prioritized candidate genes, 2 variants were identified: *EDNRB* (NC_009160.3:g.50488216T>C, ENSECAT00000026836.2, c.763A>G, p.Ile255Val) and *KIT* (NC_009146.3:g.79545351C>T, ENSECAT00000014037.3, c.2259G>A, p.Val753Val) (Table 1). Specifically, a SNP in *EDNRB*

Table 1. Coding SNPs identified in pigmentation candidate genes

Gene	Variant	Type	Variant ID	Transcript ID
EDNRB	c.763A>G, p.Ile255Val	missense	rs1135871119	ENSECAT00000026836.2
KIT	c.2259G>A, p.Val753Val	synonymous	rs1139103739	ENSECAT00000014037.3

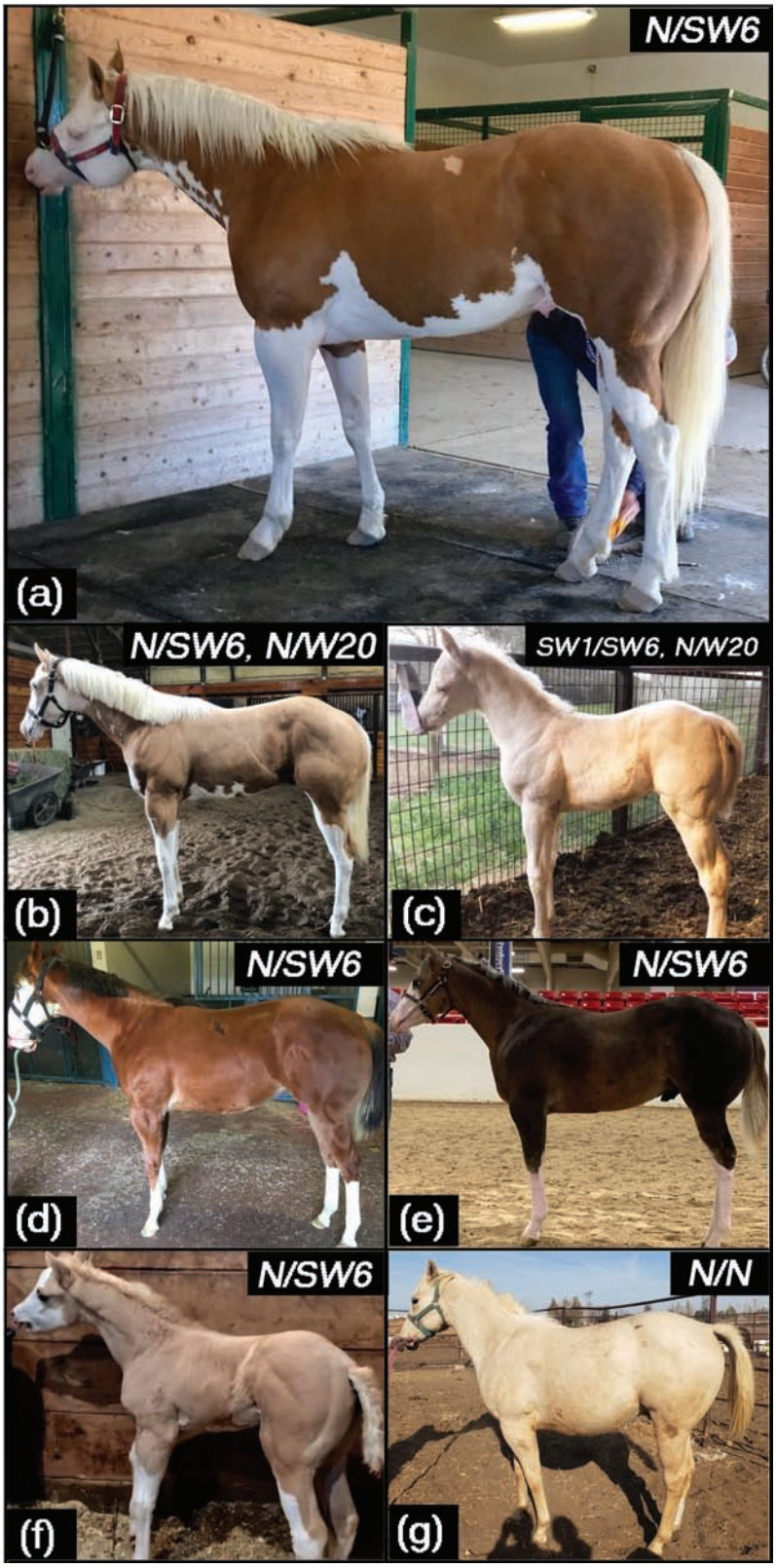

Figure 2. Phenotypes of the SW6 Paint horse half-sibling family. (**a**) Paint stallion identified with a splashed white phenotype unexplained by any known splashed white or other white patterning variants. (**b**) Palomino offspring with similar phenotype to the sire (**a**) despite also being heterozygous for W20. (**c**) All-white offspring of the stallion (in **a**), this foal is a compound heterozygote for SW6 and SW1 and also has one copy of W20. (**d**–**e**) Splashed white offspring of the stallion with less pronounced white patterning than the stallion. (**f**) Palomino splashed white offspring of the stallion with more extensive white patterning on the face and legs compared to **d** and **e**. (**g**) Solid palomino offspring of the SW6 Paint stallion.

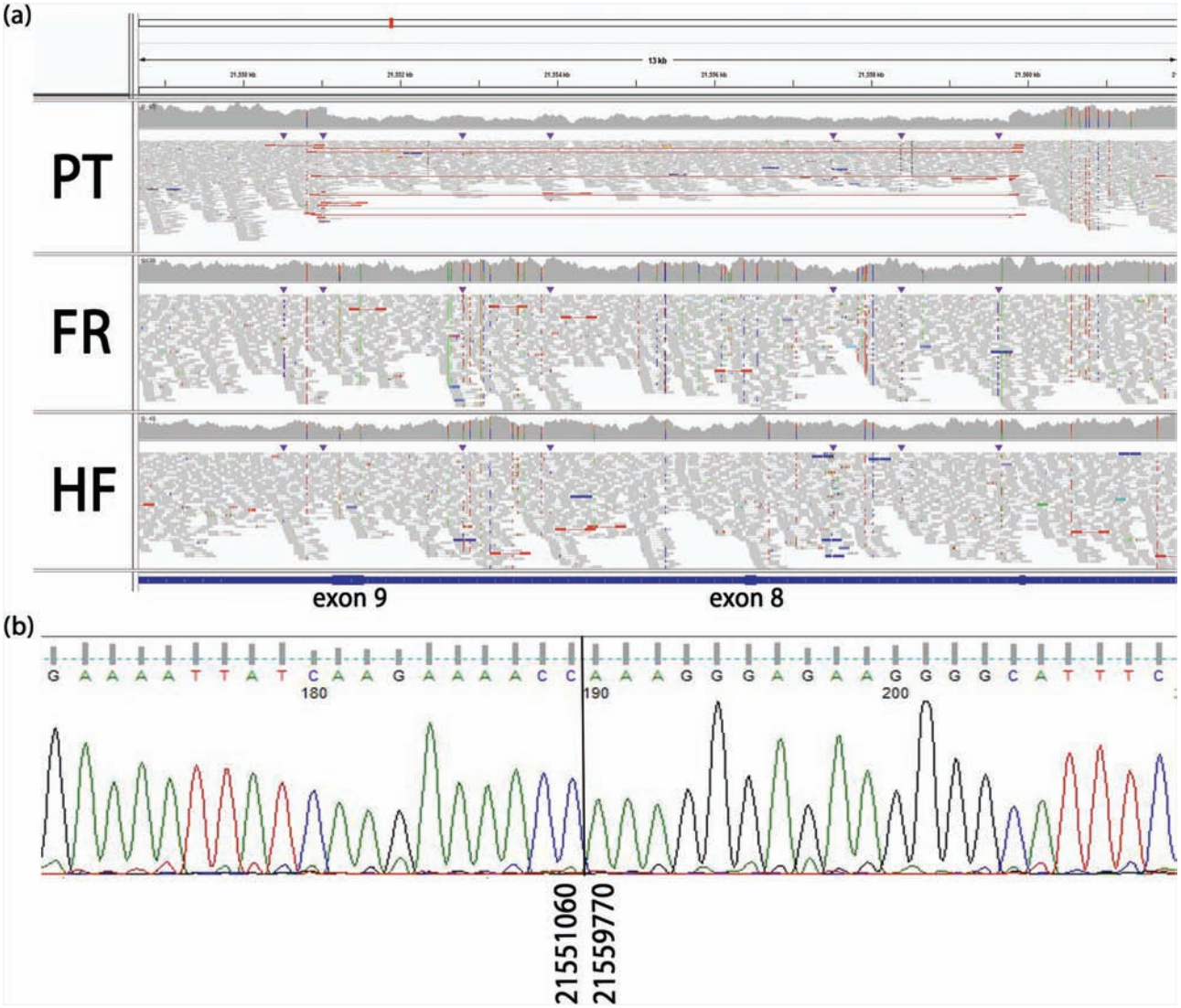

Figure 3. De novo deletion detected in MITF in the splashed white Paint Stallion. (**a**) Integrative Genomics Viewer (IGV) image of the 8.7 kb deletion present in the splashed white stallion which involved intron 7 and exons 8 and 9. Displayed is the splash white stallion (denoted as PT), and 2 nonsplashed white horses from 2 other breeds namely, Friesian (denoted as FR) and Haflinger (HF). Represented are the coverage tracks (top panel) and sequence reads/alignment (bottom panel) shown for each individual. The red lines of the alignment track of the paint horse stallion indicate the insert sizes were larger than expected for those aligned reads. Additionally, there is a significant drop in sequencing coverage for this stallion in this region compared with the other horses examined despite the higher than average coverage over all. Taken together both of these observations provided evidence of a deletion. (**b**) Validation and identification of boundaries by Sanger sequencing. Displayed is the electropherogram of the deletion in the Paint stallion with the breakpoints (labeled) by genomic position.

(c.763A>G, p.Ile255Val) was identified as homozygous alternate in the affected stallion and homozygous reference in all other samples screened. This variant was previously identified by others and submitted to dbSNP, however breeds and population frequency have not been reported (rs1135871119). This variant was predicted to be neutral to protein function by the consensus classifier PredictSNP with 83% accuracy (Supplementary Figure 1) and was therefore not investigated further. The *KIT* variant (c.2259G>A, p.Val753Val) was identified as heterozygous in the Paint stallion and was predicted to be synonymous and neutral to protein function, and therefore not pursued further (Table 1). This variant was also previously reported in dbSNP (rs1139103739).

Visualizing sequencing data in IGV identified a novel 8710 bp deletion in *MITF* (Figure 3a). Sanger sequencing confirmed this deletion and identified the boundaries (NC_009159.3:g.21551060-21559770del) in the stallion under investigation (Figure 3b). The stallion was heterozygous for the 8710 bp deletion, but neither his sire nor dam had the mutation. The parents of the stallion were confirmed by the standard parentage analysis routinely performed by the Veterinary Genetics Laboratory at UC Davis. Taken together, this finding supports the deletion to be a novel, de novo mutation in *MITF.* We have called this variant SW6, for SW6.

The deletion encompassed part of intron 7 through the 3′ untranslated region of exon 9 of *MITF*, including the helix-loop-helix DNA-binding domain (ENSECAT00000006375.3). Assuming this deletion would impact proper splicing of intron 7, it is predicted to cause a premature stop codon after proline 237 and thus impair protein function by removal of a portion of the DNA-binding

Table 2. Coat color genotypes and phenotypes for splashed white Paint Horse stallion and half-sibling family

Gene	Phenotype[a]	SW6[b]	Other white patterning genotypes[c]
Paint Stallion	Splashed white	N/SW6	None
Sire	Solid	N/N	None
Dam	Solid	N/N	None
Offspring_1	Splashed white	N/SW6	N/W20[d]
Offspring_2	Splashed white	N/SW6	None
Offspring_3	Splashed white	N/SW6	None
Offspring_4	Splashed white	N/SW6	None
Offspring_5	All white	N/SW6	N/SW1[e], N/W20
Offspring_6	Sabino like	N/N	None
Offspring_7	Solid	N/N	None
Offspring_8	Solid	N/N	None
Offspring_9	Frame overo	N/N	N/O[f]
Offspring_10	Solid	N/N	None
Offspring_11	Solid	N/N	None

[a]Refer Figures 1 and 2 for additional phenotype information.
[b]Splashed white 6 (SW6) genotype.
[c]Genotypes for commercially available white patterning variants offered by the UC Davis Veterinary Genetics Laboratory.
[d]Dominant white 20 (W20) allele.
[e]Splashed white 1 (SW1) allele.
[f]Frame Overo (O) allele.

domain. No unique structural variants were identified in any of the other candidate genes of the stallion.

Using a PCR assay, offspring of the stallion with similar splashed white phenotypes (N = 4) were screened and all were heterozygous for the SW6 deletion (Figure 2, Table 2). One additional completely white foal with blue irides was found to be a compound heterozygote at *MITF*, with both $MITF^{prom1}$ (SW1) and SW6, and was additionally heterozygous for W20 (Figure 2c). One of the heterozygous SW6 offspring was also heterozygous for W20 (Figure 2b), and the 3 remaining splashed white offspring had no other known white spotting mutation (Figure 2d–f). Of the 5 offspring with the SW6 allele, all had 1 or 2 blue eyes. Three of the dams of these splash white offspring were phenotypically solid Quarter horses, with brown eyes, no leg markings, and minimal face markings. DNA from these mares was not available for testing. One additional mare, registered as solid bred American Paint Horse, had 2 brown eyes, 2 hind stockings, and a blaze. This mare was heterozygous for W20, and NN for all other white spotting alleles, including SW6. The fifth mare was phenotypically a frame overo-splashed white mare, with white patterning that covered approximately 50% of her trunk and neck, as well as, extensive leg and face markings including a bald face and 4 white limbs. This mare also had 2 blue eyes, and was the dam of the all-white colt.

The newly identified variant (SW6) was absent in the stallion's offspring without this splashed white phenotype (N = 6). One filly was phenotypically frame overo like her dam and was confirmed by DNA testing to be heterozygous for the EDNRB variant causing the frame phenotype. One additional foal exhibited a sabino-like phenotype with white markings on all 4 limbs, a blaze, belly spot and 2 brown eyes, and was not as extensively patterned as the stallion or the 5 splashed white foals. This foal did not have the SW6 deletion or any other known white spotting alleles.

Discussion

Using whole-genome sequencing and a candidate gene approach we identified a newly recognized potentially causal mutation for a splashed white phenotype in horses. The mutation was determined to be a de novo mutation in the *MITF* gene of this individual, as neither his sire nor dam was found to harbor the mutation and neither had the splashed white phenotype (Figure 1). The stallion under investigation and 4 of his splashed white offspring, as well as one all white foal, were heterozygous for this mutation. It is unknown if homozygosity is viable, as homozygous horses have not yet been detected.

SW1 has been found in multiple breeds of horses and is speculated to impact *MITF* expression and thus melanocyte proliferation and development (Hauswirth et al. 2012). In a previous study, a SW1/SW3 compound heterozygote resulted in an all-white phenotype (Hauswirth et al. 2012). Consistent with compound heterozygosity resulting in a more pronounced phenotype, a compound heterozygous colt (SW1/SW6) who was also heterozygous for W20 in our study was noted to be all white with blue eyes. *MITF* encodes for a transcription factor important in the regulation of melanocyte development, with high expression in skin and melanocytes. MITF regulates several pigmentation genes, including *TYR*, *TRP-1*, *TRP-2*, *PMEL*, and *TRPM1*, which participate in the production and deposition of melanin (Vachtenheim and Borovansky 2010). In this way, mutations such as SW6 would also be expected to impact the differentiation, proliferation or survival of melanocytes, leading to a splashed white phenotype or an all-white phenotype in compound heterozygotes.

The *MITF* gene is highly conserved across species, and mutations in *MITF* cause similar phenotypes in humans, mice, cattle, and dogs with patchy white spotting, frequent blue eyes, and sometimes deafness (Philipp et al. 2011; Körberg et al. 2014). In humans, variants in *MITF* have been associated with Waardenburg (WS) and Tietz (TS) syndromes which are characterized by sensorineural deafness and hypopigmentation of the skin, hair, and irides (Zhang et al. 2012; Grill et al. 2013). Similar white spotting and deafness have been reported in horses with previously identified splashed white mutations in horses (SW1–SW5) (Hauswirth et al. 2013; Henkel et al. 2019). Macchiato, a white spotted phenotype in horses caused by a missense mutation in exon 6 of *MITF*, is thought to be analogous to Tietz syndrome with the affected horse exhibiting deafness and low progressive sperm motility (Hauswirth et al. 2012; Blatter et al. 2013). Because melanocytes are also found in the inner ear and contribute to hearing, mutations in *MITF* can impact the function of the inner ear (Henkel et al. 2019). The deletion presented here is predicted to result in a loss of function that dysregulates melanocytes, and it is likely that some horses with SW6 are also deaf. It is unknown whether any of the horses in this study with SW6 are in fact deaf, but this warrants further study.

The finding of another mutation associated with splashed white phenotypes in horses will contribute to breeding of American Paint horses who strive to produce white spotting phenotyping. White spotting is complex, and this variant could occur with other white spotting alleles, producing even more extensive white pattern phenotypes; this was noted in the compound heterozygous colt (SW6/SW1) with W20 that was all white.

In conclusion, this study revealed the second structural variant in the *MITF* gene (the first being SW5) associated with a splashed white depigmentation phenotype in American Paint Horses. Both SW5 and SW6 involve the basic loop helix DNA binding domain

of the protein. We propose that SW6 impacts proper splicing of the transcript causing a premature stop codon and impairing protein function by removal of a portion of the DNA-binding domain. Confirmation with investigation of complementary DNA (cDNA) and protein analysis would substantiate this proposed impact. However, given the role of MITF in melanocyte migration and development, and its role in producing splashed white phenotypes in horses (SW1, SW3, SW5), this 8.7 kb deletion is the likely causal variant for what is proposed here as splashed white 6 (SW6).

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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