Communication

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Identification of a Novel Haplotype Associated with the Roan Coat Color in the American Quarter Horse

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Simple Summary

The roan pattern is described as white hairs interspersed in varying intensity throughout the body, barrel, hips, chest, and upper legs on an otherwise solid colored coat. The head and legs often exhibit few to no white hairs. Previous studies mapped the locus for roan to the *KIT* region and observed linked variants in a small number of breeds. In a recent report we presented evidence for two independent haplotypes, *RN1* and *RN2*, in the *KIT* region that account for approximately 74% of roan horses. In the current report, using whole genome sequencing of unknown roan samples, we present a third allele, *RN3*, found in the American Quarter Horse. This allele accounts for an additional 75% of horses displaying the roan phenotype that are negative for *RN1* and *RN2*. In our sample set of horses, these three haplotypes account for 80%-90% of roan horses and more than 90%-95% of our Quarter Horse population studied in this report.

Abstract

The roan coat color is described as the dispersion of white hairs within an otherwise solid background color coat. This phenotype is primarily expressed on the body of the horse, with the head and legs exhibiting few to no white hairs. Previous studies mapped the locus for roan to the *KIT* region and observed linked variants in a small number of breeds. In a recent report we presented evidence for two independent haplotypes, *RN1* and *RN2*, in the *KIT* region that account for approximately 74% of roan horses. In the current report, using whole genome sequencing for unknown roan samples, we present a third novel allele, *RN3*, found in American Quarter Horse, that accounts for an additional 75% of horses negative for *RN1* and *RN2* displaying the roan phenotype. In our sample set of horses, these three haplotypes account for about 80%-90% of roan horses and more than 95% of our Quarter Horse population studied.

Keywords: horses; roan; KIT; American Quarter Horse

1. Introduction

Roan is inherited as a dominant epistatic trait, whereby white hairs are expressed throughout the base coat colour on the animal's body [1]. The roan coat color has been observed in other livestock species and is likely associated with the *KIT* or *KITLG* gene [2]. In horses, variants within the *KIT* locus were linked to roan for the Noriker horse [3] and Icelandic horse [4]. Recently, we published a study analyzing multiple breeds, wherein we validated the original variant found by Grilz-Segers (2020) in Noriker horses, while discovering a second variant [5] as well. That study showed the *RN1* haplotype to be present in multiple breeds, and the *RN2* haplotype to be present mostly in breeds of American heritage. In addition to roan, other polymorphisms in the *KIT* gene cause different white spotting phenotypes in horses, such as Tobiano, Sabino, and Dominant White variants [6]. In our previous paper we suggested using whole genome sequencing to discover other alleles for roan. This

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manuscript presents our first new roan allele discovered this way, and in doing so, demonstrates the usefulness of this methodology. As many breeders use coat color genotypes to inform breeding decisions, this new roan haplotype may aid in the selection of horses that will provide the expected outcome.

2. Materials and Methods

Genomic DNA for a set of horses (N=16), consisting of *RN1*, *RN2*, unknown roan, and non-roan horses, was whole genome sequenced using a commercial service to 40x average depth per genome (WGS) (Seqmatic, Fremont, CA, USA). Subsequently, the raw sequence data was aligned to the horse reference genome EquCab 3.0 [7] using BWA-MEM2 [8]. IGV version 2.16.2 [9] was used to screen the *KIT* region on chromosome 3 in the resulting bam files for the presence of new variants that can potentially distinguish *RN3* from *RN1*, *RN2*, and non-roan samples in the case-control dataset under the assumption that *Roan* is a dominant trait.

The selected candidate variants were tested for association using a Fisher's exact test in a set of 95 roan horses and 214 non-roan horses that were genotyped by our standard workflow [10]. In short, genomic DNA was extracted from hair samples using the Puregene Extraction Kit following the manufacturer's protocol (QIAGEN, Inc., Germantown, MD, USA), or retrieved from previously stored and extracted gDNA, where applicable, and subsequently sequenced using hybrid capture on the commercially available platform by Etalon, Inc., using 2 × 150 bp reads on a NextSeq1000 instrument (Illumina, San Diego, CA, USA). The resulting sequences had base call quality scores ≥ Q30 and read depth >40× for all regions under investigation after alignment to EquCab3.0. Candidate variants passing the statistical threshold were then analyzed for putative co-segregation and assigned into "roan haplotypes". For roan horses, coat color was confirmed through owner-provided pictures, owner outreach for coat color confirmation, or online pedigree search tools, e.g. allpedigrees.com. For non-roan horses, no outreach was performed unless the color predicted by the genotypes did not match written records.

3. Results and Discussion

Using whole genome sequencing and analysis on several *RN1*/*RN1*, *RN2*/*RN2*, *RN1*/*RN2*, *RN2*/*n*, unknown roan horses, as well as five non-roan horses (Table 1), we identified many variants that segregated between roan and non-roan horses. We included the *RN1* and *RN2* samples to ascertain if the same SNPs as described previously were called, thus confirming their association to roan. For *RN3*, we selected 12 variants that segregated separately from *RN1* and *RN2* and were not present in the non-roan horses. We then designed hybrid capture probes for five of these variants spaced throughout the region of interest and analyzed these on 80 roan horses without *RN1* or *RN2* alleles and 214 non-roan horses.

Table 1. Samples examined by whole genome sequencing.

Haplotype	# horses
RN1/*RN1*	5
RN2/*RN2*	1
RN1/*RN2*	2
RN2/*n*	1
Unknown roan	2
Control (non-roan)	5

Tests of association for the five candidate variants using Fisher's exact test revealed that three loci were not linked to the roan phenotype in this larger sample set and thus were discarded (all $p > 10^{-10}$ with odds <6). Additionally, the allele frequencies of the excluded variants were in excess of 25% in both roan and non-roan samples. The remaining two variants were present in 75% of the roan horses without an *RN1* or *RN2* allele. The SNP chr3:79428717 was present in 5% of non-roan horses

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whereas SNP chr3:79656505 was only present in *RN3* horses. These two assays were strongly associated with the phenotype (all $p < 10^{-15}$ with odds of >20, Fisher's exact test) and were assigned roan haplotype *RN3* (Table 2). This roan haplotype had $p < 10^{-15}$ with odds of >60 (Fisher's exact test).

Table 2. The newly identified *RN3 haplotype defining* variants.

Roan Alleles	*Allele 1 (Ref)	Allele 2 (RN)	AF † Allele 2 (Rn/Non-Rn)	Accession Number
RN3				
chr3:79428717	T	G	0.494/<0.05	rs1142018742
chr3:79656505	G	A	0.494/0.00	No rs ID

* all positions on EquCab3.0. † Allele frequencies of the respective roan alleles in the roan sample set and the non-roan sample set.

The newly discovered roan haplotype termed Roan 3 or *RN3* was present in 60 (75%) of the tested roan samples without a known roan allele. This *RN3* haplotype was only found in the American Quarter Horse and American Paint Horse only. Therefore, this haplotype can be considered a relatively young allele. This haplotype is strongly tagged by a G>A substitution at chr3:79656505, a variant not present on the horse reference genome, and this may constitute a private variant in this specific population. Within the American Quarter Horse, we examined the extended pedigrees of several horses carrying this variant (https://www.Allbreedpedigree.com and https://quarterhorseresource.com/), and assuming a dominant trait, we found the roan allele *RN3* tracing to the "Kitch roan mare" (born 1901, AQHA registry U0073276). Current major Quarter Horse sires likely transmitting this roan haplotype are Zippos Mr Good Bar (born 1984), VS Code Blue (2007) and VS Code Red (2007). All 60 horses tested with this allele were heterozygous for *RN3*.

Taking the newly discovered *RN3* allele, together with the previously validated two alleles *RN1* and *RN2*, it is estimated that these three alleles explain over 85%-90% of the roan phenotypes among the samples tested. Of the last group of 80 unknown roan samples tested for this publication, there remains 20 horses with an unknown roan allele present. These 20 horses were divided over 10 different breeds (Table 3). Thus, it is likely that there remain several additional roan variants yet to be discovered.

Table 3. Remaining unknown unexplained roan phenotypes in diverse horse breeds.

Breed	# Horses
American Quarter Horse	6
Gypsy Cob	3
Arabian	2
Shire	2
Tennessee Walker	2
Appaloosa	1
Belgian Draft	1
Clydesdale	1
Friesian Cross	1
Mangalarga Marchador	1

4. Conclusions

As proposed in our previous paper, we used whole genome sequencing to generate an average of 40x coverage for a sample of horses including some unknown roan variants. Analysis of the *KIT* region confirmed the previously detected roan variants and provided new variants defining a third roan allele *RN3*. Together with the roan *RN1* and *RN2* alleles discovered previously, these three alleles seem to account for the vast majority of roan phenotypes in American Quarter Horses and American Paint Horses, and all three alleles were likely proliferated by popular stallions. As such,

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we expect the majority of roan horses in the American Quarter Horses to be assigned one of these three haplotypes. Overall, the *RN3* haplotype is likely present in <1% of all horses.

For other breeds such as Arabian, Clydesdale, Tennessee Walker and Shire horses, further research into the genetic basis of these roan phenotypes needs to be performed, and is ongoing. So far, only *RN1* is detectable in these breeds, while other alleles might yet be discovered. This exploratory study using whole genome sequencing of a small number of roan samples showed the ease in discovering new alleles associated with the roan coat color. However, the limited number of non-roan horses examined by WGS increased the likelihood of variant drop out due to chance missingness of low frequency variation in the population. By sampling more broadly using WGS in the future, we hope to limit that dropout.

As with Dominant White, where several alleles were identified in only a single individual or a small family as a result of sporadic mutations, we expect that this will also be the case for roan. It is our plan to sequence a number of unknown roans in other breeds to discover their specific roan alles.

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Conflicts of Interest: All authors are affiliated with Etalon, Inc., which offers diagnostic testing for coat color and disease testing.

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