The Journal of Veterinary Medical Science

NOTE

Wildlife Science

# Variation in the *MC1R*, *ASIP*, and *MATP* genes responsible for coat color in Kiso horse as determined by SNaPshot™ genotyping

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*J. Vet. Med. Sci.*
81(1): 100–102, 2019
doi: 10.1292/jvms.18-0458

Received: 4 August 2018
Accepted: 7 November 2018
Published online in J-STAGE: 22 November 2018

**ABSTRACT.** Kiso horse is a breed of Japanese native horses. In this study, to clarify coat color gene variation in Kiso horses, we used SNaPshot™ genotyping to evaluate variation in *MC1R*, *ASIP*, and *MATP* genes at the *Extension* (*E*), *Agouti* (*A*), and *Cream dilution* (*C*) loci. The coat color of 149 horses was documented. The coat color of 140, 3, and 6 horses was bay, chestnut, and buckskin, respectively. Furthermore, the frequency of alleles *E*, *e*, *A*, *a*, *C*, and *Cr* was 0.80, 0.20, 0.86, 0.14, 0.98, and 0.02, respectively. Current status of coat color genes in Kiso horses was clarified, and this information will help plan further conservation of the horses.

**KEY WORDS:** coat color gene, Kiso horse, SNaPshot genotyping

Kiso horse is a breed of Japanese native horses that originated from the mountainous Kiso region of Central Japan. Similar to other native Japanese horses [5, 12], Kiso horses have lost their position as a working horse owing to the movement of population from countryside to cities, changes in the lifestyle of Japanese citizens, and modernization of agriculture. Their population had decreased to approximately 30 in the 1970s [3, 4]. Owing to the efforts of concerned people, the number has currently increased to approximately 150 [9, 10]. However, Kiso horses are in danger of extinction because of the aging of horses and their owners, who have spent a traditional lifestyle with horses, and also because of the lack of successors.

In an effort to increase the number, the horses are backcrossed to obtain a typical Kiso horse. Along with a small founder population, this human intervention has decreased the genetic diversity in Kiso horses. Currently, Kiso horses are medium-sized, with height at withers and chest circumference of approximately 130 and 176 cm, respectively [9]. In addition, they possess traditional characteristics: 66% of the horses have dorsal stripes and 72% of them have knock-knee—considered an ideal characteristic to walk on mountains [9]. Moreover, most of the surveyed horses in 2011 (i.e., 92.8%) had bayish coat color without white spots [7, 9].

Variation in coat color is not often observed in wild animals but is common in domestic animals [2]. In horses, various coat colors have been recorded, such as bay, black, chestnut, gray, and white. The coat color is an important factor influencing the value of animals, and it sometimes directly determines the use and demand of horses. In Japan, horses with rare coat colors are chosen to become Shinme (or Jinme, considered as sacred horses), which are dedicated to Japanese shrines for use in rites and festivals.

Currently, in Kiso horses, only three coat colors remain: bay, chestnut, and buckskin [9]. In horses, these coat colors are controlled by the genes *MC1R*, *ASIP*, and *MATP* (*SLC45A2*) at the *Extension* (*E*), *Agouti* (*A*), and *Cream dilution* (*C*) loci [1, 8, 13]; SNaPshot™ analysis enables genotyping of these genes [6]. As Kiso horses have limited coat color variation—bay, chestnut, and buckskin—SNaPshot genotyping could be a useful tool to screen such genetic variation. Therefore, we conducted SNaPshot genotyping of Kiso horses and determined the genetic variation in these genes within the study population. In addition, we have discussed possible future coat color variations in Kiso horses.

The present study was performed in accordance with the Ethical Guidelines of the Animal Care and Use Committee of the Gifu University. We surveyed 149 Kiso horses from 2009–2015. The phenotypic traits including the coat color of these horses were confirmed, and their blood sample was collected from the jugular vein. These samples were analyzed in the laboratory as soon as possible, and the DNA was extracted using the MFX-2000 MagExtractor System according to the manufacturer's protocol (Toyobo, Osaka, Japan).

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**Table 1.** Primers for PCR of the gene fragment and SNaPshot to detect variation of *MC1R*, *ASIP*, and *MATP* genes

| Primers | | Sequences | Detectable mutation |
|---|---|---|---|
| For PCR | *MC1R*-F | 5′-CCTACCTCGGGCTGACCACCAA-3′ | |
| | *MC1R*-R | 5′-GAGAGGACACTAACCACCCAGATG-3′ | |
| | *ASIP*-F; TestADEx2-F | 5′-CTTTTGTCTCTC'TTTGAAGCATTG-3′ | |
| | *ASIP*-R | 5′-GCTAGCTAGTACAGAAAGAT-3′ | |
| | *MATP*-F | 5′-TTGCTGACCGAAGGAAGAAG-3′ | |
| | *MATP*-R | 5′-GAAATGCACTGGGAGACTGA-3′ | |
| For SNaPshot to detect the genotypes | *E/e* (C/T) primer | 5′-GACTATCTGCTGCCTGGCCGTGT-3′ | C901T on *MCIR* |
| | *e /e* (C/T) primer | 5′-(GACT) 4TGCTCACCAGCAGGT-3′ | G903A on *MCIR* |
| | *A/a* (T/G) primer | 5′-(GACT) 5GAAGATCTCTTCTTCTTTTCTGCT-3′ | exon 2 of *ASIP* |
| | *C/Cr* (G/A) primer | 5′-(GACT) 7CACCATGATAGGTGTGGTTCTCTTT-3′ | G72A on exon 2 of *MATP* |

**Table 2.** Coat color variation in Kiso horses

| Colors | *N* | % |
|---|---|---|
| Bay | 140 | 94.0 |
| Chestnut | 3 | 2.0 |
| Buckskin | 6 | 4.0 |

**Table 3.** Allele frequencies in the loci *E*, *A*, and *C* in Kiso horses

| | *MC1R* | | *ASIP* | | *MATP* | |
|---|---|---|---|---|---|---|
| | *E* | *e* | *A* | *a* | *C* | *Cr* |
| Number of allele | 237 | 61 | 257 | 41 | 292 | 6 |
| Allele frequency | 0.80 | 0.20 | 0.86 | 0.14 | 0.98 | 0.02 |

Total number of horses were 149.

The genotype of the loci *E*, *A*, and *C* was analyzed using the SNaPshot Multiplex kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), according to the method of Kakoi *et al.* (2009) [5]. Briefly, the polymerase chain reaction (PCR) primers for *MC1R*, *ASIP*, and *MATP* were designed as shown in Table 1, and multiplex PCRs were carried out using a reaction mixture of total volume 15 $\mu l$ containing <20 *n*g DNA, 0.33 $\mu$M of each primer, 2.5 $\mu$M $MgCl_2$, 0.33 $\mu$M dNTPs, 10 × reaction buffer, and 0.5 U AmpliTaq Gold (Thermo Fisher Scientific), under the following thermal conditions: incubation at 95°C for 10 min; 30 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec; and a final incubation at 72°C for 10 min. The PCR products were purified using ExoSAP-IT® (Affymetrix-USB products, Cleveland, OH, U.S.A.). Genotyping was performed to detect mutations using the SNaPshot Multiplex kit (Applied Biosystems) with 0.02–0.1 $\mu$M primers, as shown in Table 1. The genotypes were determined using an automated DNA sequencer (Applied Biosystems 3130xl genetic analyzer; Thermo Fisher Scientific).

The coat color of the surveyed Kiso horses was bay (140 horses, 94% of the population), chestnut (3 horses, 2.0%), and buckskin (6 horses, 4.0%) (Table 2). All the surveyed horses were well genotyped, and the frequency of the alleles E, e, A, a, *C*, and *Cr* was 0.80, 0.26, 0.86, 0.16, 0.98, and 0.02, respectively (Table 3). The phenotype of the coat color was consistent with the genotype. All three studs were bay, of which two were *E*/*E*, *A*/*A*, *C*/*C*, and one was *E*/*e*, *A*/*A*, *C*/*C*.

Through SNaPshot analysis, we were able to genotype *MC1R*, *ASIP*, and *MATP* in Kiso horses, and we also evaluated the frequency of genes responsible for coat color. The data acquired are important to understand the status of coat color genes, plan mating to produce horses with expected colors, and conserve the genetic variation in Kiso horses.

Ninety-four percent of the Kiso horse population was bay, and the frequency of the alleles *E* and *A* was 0.80 and 0.86, respectively. This suggests that the loci *E* and *A* of Kiso horse are fixed as *E*/*E*, *A*/*A*. Moreover, all the three studs were bay, of which only one was *E*/*e*, *A*/*A*, whereas the other two were *E*/*E*, *A*/*A*. Consequently, uniformity in bay coat color was observed in Kiso horses, and this uniformity will be accelerated in the future. It partially reflects the effort of stakeholders, who have purified the population to obtain Kiso horses with desired traits, and our result suggested that the purification of Kiso horses may be successful.

The recessive alleles *e* and *a* have remained in the Kiso horse population. As there is a stud carrying the allele *e*, the number of horses carrying the allele *e* and with chestnut-colored coat *(e/e, A/-)* might increase in the population. Although the frequency of the recessive allele *a* is limited, there is still a possibility to revive black horses-(*E*/-, *a*/*a*) in the Kiso horse population. In addition, a rare *Cr* allele was detected in the population, and therefore, palomino (*e*/*e*, *C*/*Cr*) and double dilutes (*Cr*/*Cr*) might appear in the future. Consequently, variation in coat color in Kiso horses can be increased by carefully planned breeding.

In domestic animals, fixation of specific phenotype does not always directly indicate a reduction in the genetic diversity of a population. Variation in coat color of Kiso horses has been decreasing, but genetic diversity in the current Kiso horses, evaluated by microsatellites and mitochondrial DNA markers, has been suggested to be relatively conserved [10, 11], because the genetic background of a founder population (ancestors of 3–4 generations) might be diverse. Thus, it is not appropriate to evaluate genetic diversity of the horses based on the coat color variation.

In this study, we have clarified the current status of coat color genes in Kiso horses. It is the stakeholders who have to decide whether the priority is purification of the breed or regulation of their coat color variation in the future.

ACKNOWLEDGMENTS. This study was supported in part by JSPS KAKENHI (Grant Number 26290072) and by a grant from Kiso town for the conservation of Kiso horses.

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