



## NOTE

Wildlife Science

# Decreased genetic diversity in Kiso horses revealed through annual microsatellite genotyping

Mako NAKAMURA<sup>1)</sup>, Teruaki TOZAKI<sup>1,2)</sup>, Hironaga KAKOI<sup>2)</sup>,  
Kotono NAKAMURA<sup>1)</sup>, Reza RAJABI-TOUSTANI<sup>1)</sup>, Yasunori OHBA<sup>1,3)</sup>,  
Tatsuya MATSUBARA<sup>1)</sup> and Masaki TAKASU<sup>1,3)\*</sup>

<sup>1)</sup>Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

<sup>2)</sup>Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

<sup>3)</sup>Education and Research Center for Food Animal Health (GeFAH), Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

**ABSTRACT.** The Kiso horse is native to Japan and is on the verge of extinction. Here, we used microsatellites to characterize changes in their genetic diversity over time. We divided a population of Kiso horses that genotyped during 2007–2017 into three groups based on birth year: Group 1, 1980–1998 (70 horses); Group 2, 1999–2007 (61 horses); and Group 3, 2008–2017 (42 horses). We genotyped 31 microsatellites to calculate average number of alleles, observed heterozygosity, and expected heterozygosity. All indicators decreased across age groups. The results indicate that Kiso horses have been experiencing a drop in genetic diversity, and the population is expected to experience further decline unless appropriate measures are implemented.

**KEY WORDS:** genetic diversity, Kiso horse, microsatellite

*J. Vet. Med. Sci.*  
82(4): 503–505, 2020  
doi: 10.1292/jvms.19-0535

Received: 28 September 2019  
Accepted: 20 February 2020  
Advanced Epub:  
9 March 2020

The Kiso horse is native to the Kiso area, a mountainous region in central Japan. Medium-sized horses with an average height at withers of 133 cm, the Kiso breed is characterized by long body length and short legs [3, 8].

Historically, these horses had important agricultural functions and were indispensable to the region's residents. They were beasts of burden, their excrement was used as compost for improving farmland, and foals commanded decent cash income. However, post-World War II industrialization eliminated demand. A 1965 census confirmed 510 horses, but this number had decreased to 32 in 1975 [3, 4]. Concerned about population reduction, local residents established the Kiso Horse Conservation Association (KHCA) in 1969. Efforts of the KHCA eventually resulted in a population recovery to about 150 horses [3, 4]. However, this number remains critically low and cannot be viewed optimistically in terms of the breed's future survival [12, 13].

Currently, the population pyramid of Kiso horses is stationary or contractive, suggesting a decline in the near future. In addition, the horses exhibit a high coefficient of inbreeding [12]. Recovery from such circumstances requires sophisticated and scientifically sound strategies. Thus, in 2007, we began a conservation project to implement a variety of conservation activities [13, 14]. Despite a decade of such efforts, the Kiso-horse population size continues to be low, suggesting that current conservation strategies are ineffective in increasing the breed's population size or genetic diversity.

As part of the conservation project, we annually surveyed and genotyped microsatellites of newborn foals for individual identification and parentage analysis. Microsatellites are short, non-coding repetitive DNA sequences with high mutation rate and polymorphism. These characteristics make them ideal for assessing population genetic background [1, 2]. Typically, microsatellite-based genetic diversity assessments use average allele number and heterozygosity as indicators. Consequently, in this study, we aimed to determine variation in the genetic diversity of Kiso horses over time, using microsatellite-derived markers. Our work should benefit efforts to validate and improve the Kiso-horse conservation program.

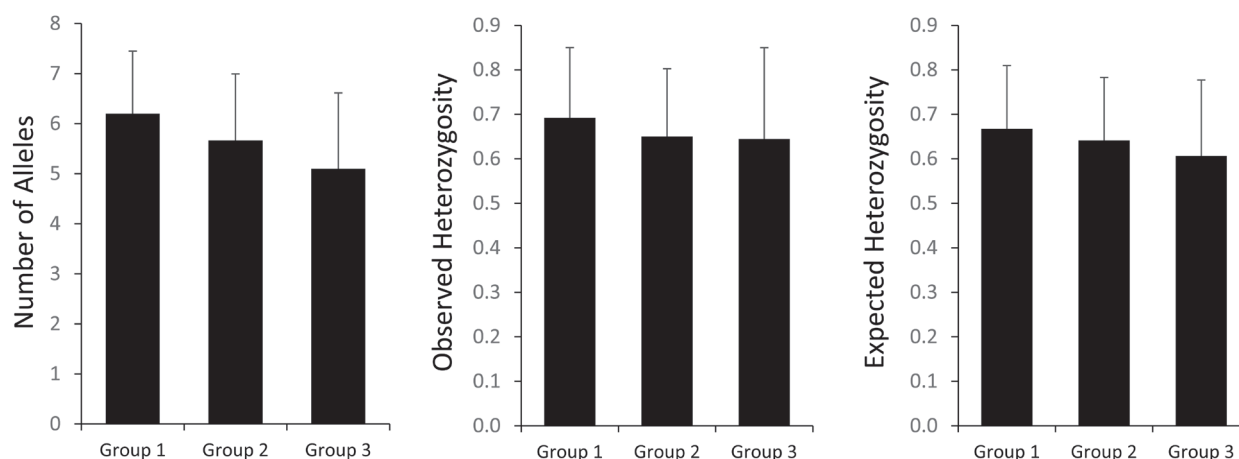
During 2007–2017, blood samples were collected from 163 Kiso horses (32 stallions, 17 geldings, and 114 mares registered by the KHCA), born between 1980 and 2017 (inclusive). Horses were divided into three groups based on birth year: Group 1, 1980–1998 (5 stallions, 6 geldings, 49 mares); Group 2, 1999–2007 (8 stallions, 11 geldings, 42 mares); and Group 3, 2008–2017 (19 stallions, 23 mares). The previously published data [13] and newly acquired 38 genotyped data from 38 foals were pooled

\*Correspondence to: Takasu, M.: takasu@gifu-u.ac.jp

©2020 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)



**Fig. 1.** The  $N_a$  (average allele count),  $H_e$  (expected heterozygosity), and  $H_o$  (observed heterozygosity) in Kiso horses divided into three groups based on birth year: Group 1, 1980–1998 (5 stallions, 6 geldings, 49 mares); Group 2, 1999–2007 (8 stallions, 11 geldings, 42 mares); and Group 3, 2008–2017 (19 stallions, 23 mares). For Groups 1–3, average  $N_a$  was  $6.2 \pm 1.2$ ,  $5.7 \pm 1.3$ , and  $5.1 \pm 1.5$ , respectively. Average  $H_o$  was  $0.692 \pm 0.158$ ,  $0.650 \pm 0.152$ , and  $0.644 \pm 0.141$ , respectively. Lastly, average  $H_e$  was  $0.668 \pm 0.142$ ,  $0.642 \pm 0.141$ , and  $0.606 \pm 0.171$ , respectively. Bar=standard deviation.

for re-analysis of change in Kiso-horse genetic diversity over time, using allele number and heterozygosity. Sampling procedures followed the Regulations for Animal Experiments at Gifu University and was approved by the institution's Committee for Animal Research and Welfare (#15137).

Blood samples collected from the jugular vein were used for genomic DNA extraction, following manufacturer protocol (DNeasy Blood & Tissue Kit, QIAGEN K.K. Japan, Tokyo, Japan). Thirty-one microsatellites were genotyped, including 17 routine markers (AHT4, AHT5, ASB2, ASB17, ASB23, CA425, HMS3, HMS6, HMS7, HTG4, HTG10, LEX3, LEX33, TKY19, TKY28, TKY321, VHL20) and 14 complement parentage-testing markers (TKY279, TKY287, TKY294, TKY297, TKY301, TKY312, TKY325, TKY333, TKY337, TKY341, TKY343, TKY344, TKY374, TKY394) [5, 15]. Allelic discrimination was based on the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee of the International Society for Animal Genetics (ISAG). Microsatellites were genotyped by published procedures [6, 9–11]. Indicators of genetic diversity were average number of alleles ( $N_a$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ). Genotype-based variables ( $N_a$ ,  $H_o$ ,  $H_e$ ) were calculated in GENEPOP version 4.2 [7], and their change over time was evaluated.

Every selected microsatellite was well-amplified and successfully genotyped for all 163 Kiso horses. All three indicators decreased over time (Fig. 1). For Groups 1–3, average  $N_a$  was  $6.2 \pm 1.2$  (range: 4–9),  $5.7 \pm 1.3$  (range: 3–8), and  $5.1 \pm 1.5$  (range: 2–8), respectively. Average  $H_o$  was  $0.692 \pm 0.158$  (range: 0.267–0.900),  $0.650 \pm 0.152$  (range: 0.279–0.836), and  $0.644 \pm 0.141$  (range: 0.167–0.905). Lastly, average  $H_e$  was  $0.668 \pm 0.142$  (range: 0.272–0.816),  $0.642 \pm 0.141$  (range: 0.258–0.823), and  $0.606 \pm 0.171$  (range: 0.156–0.796).

Here, we successfully characterized fluctuations in genetic diversity of Kiso horses using microsatellites: Decreases in  $N_a$ ,  $H_o$ , and  $H_e$  indicated that genetic diversity was declining over time.

A reduction in genetic diversity may be due to decreasing numbers of private horse owners who had kept one or two horses with constant foal production. As aging private horse owners gave up keeping horses, breeding became limited to Kisoumanosato, the Conservation Center for Kiso Horses, where limited number of animals are available for reproduction [4]. To make matters worse, space, staff, and cost limitations at Kisoumanosato result in fewer than 10 females mating and fewer than five foals born annually [12]. Furthermore, keeping stud stallions is difficult for the breeding center, which currently only houses three stud stallions (sires of recent foals), likely contributing to decreases in genetic diversity. Consequently, if this situation does not change, the breed will fall into the extinction vortex: as population size shrinks, the inbreeding coefficient increases and genetic diversity will further decrease.

We recommend that the KHCA and associated administrative agencies use our data to determine the direction of future conservation strategies. Among the possible improvements are management decisions that address limitations at Kisoumanosato. For example, some horses can be contracted to kept by KHCA members to lighten management load at the breeding center. Year-long pasturing could also be implemented because it requires comparatively less manpower in caring for the horses. However, the most important reforms may be on the sociological aspect: Kiso-horse economic value can be broadened through developing other applications for them.

As we only evaluated genetic diversity using microsatellite polymorphisms to determine average allele number and heterozygosity, future studies should consider other analyses for a more fine-tuned picture of Kiso horse genetics. However, we believe it is essential to perform continuous microsatellites genotyping so that we can provide timely evaluations of genetic diversity. In addition, our microsatellite-based genetic survey of this endangered breed should be a valuable reference for conservation of other endemic Japanese horses with a small population size [6, 9–11].

ACKNOWLEDGMENT. A part of this research was supported by a grant from the government of Kiso town.

## REFERENCES

1. Frankham, R., Ballou, J. D. and Briscoe, D. A. 2009. Genetic diversity. pp. 41–65. *In*: Introduction to Conservation Genetics, 2nd ed. (Frankham, R., Ballou, J. D. and Briscoe, D. A. eds.), Cambridge University Press, Cambridge.
2. Fernández, J., Villanueva, B., Pong-Wong, R. and Toro, M. Á. 2005. Efficiency of the use of pedigree and molecular marker information in conservation programs. *Genetics* **170**: 1313–1321. [[Medline](#)] [[CrossRef](#)]
3. Ito, M. 1996. Life with Kiso Horses. Kaida Village and Kiso Horse Conservation Association, Kaida (in Japanese).
4. Japan Equine Affairs Association 2008. The Kiso horse. pp. 133–161. *In*: Japanese Native Horses-Their Preservation and Utilization, Japan Equine Affairs Association, Tokyo (in Japanese).
5. Kakoi, H., Nagata, S. and Kurosawa, M. 2001. DNA typing with 17 microsatellites for parentage verification of racehorses in Japan. *Anim. Sci. J.* **72**: 453–460.
6. Kobayashi, I., Akita, M., Takasu, M., Tozaki, T., Kakoi, H., Nakamura, K., Senju, N., Matsuyama, R. and Horii, Y. 2019. Genetic characteristics of feral Misaki horses based on polymorphisms of microsatellites and mitochondrial DNA. *J. Vet. Med. Sci.* **81**: 707–711. [[Medline](#)] [[CrossRef](#)]
7. Raymond, M. and Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* **86**: 248–249. [[CrossRef](#)]
8. Scherf, B. D. 2000. Farm animal genetic resources. pp. 37–646. *In*: World Watch List of Domestic Animal Diversity, 3rd ed., Food and Agriculture Organization of the United Nations, Rome.
9. Senju, N., Tozaki, T., Kakoi, H., Almunia, J., Maeda, M., Matsuyama, R. and Takasu, M. 2017. Genetic characterization of the Miyako horse based on polymorphisms of microsatellites and mitochondrial DNA. *J. Vet. Med. Sci.* **79**: 218–223. [[Medline](#)] [[CrossRef](#)]
10. Senju, N., Tozaki, T., Kakoi, H., Shinjo, A., Matsuyama, R., Almunia, J. and Takasu, M. 2017. Genetic diversity of the Yonaguni horse based on polymorphisms in microsatellites and mitochondrial DNA. *J. Vet. Med. Sci.* **79**: 425–431. [[Medline](#)] [[CrossRef](#)]
11. Senokuchi, A., Ishikawa, S., Tozaki, T., Takasu, M., Kakoi, H., Misumi, K. and Hobo, S. 2018. Genetic analyses for conservation of the traditional Tokara horse using 31 microsatellite markers. *J. Equine Sci.* **29**: 97–104. [[Medline](#)] [[CrossRef](#)]
12. Takasu, M., Hiramatsu, N., Tozaki, T., Kakoi, H., Hasegawa, T., Maeda, M., Kusuda, S., Doi, O., Murase, T., Mukoyama H., Huricha 2011. Population statistics and biological traits of endangered kiso horse. *J. Equine Sci.* **22**: 67–72. [[Medline](#)] [[CrossRef](#)]
13. Takasu, M., Hiramatsu, N., Tozaki, T., Kakoi, H., Nakagawa, T., Hasegawa, T., Huricha., Maeda, M., Murase, T. and Mukoyama, H. 2012. Genetic characterization of the endangered Kiso horse using 31 microsatellite DNAs. *J. Vet. Med. Sci.* **74**: 161–166. [[Medline](#)] [[CrossRef](#)]
14. Takasu, M., Ishihara, N., Tozaki, T., Kakoi, H., Maeda, M. and Mukoyama, H. 2014. Genetic diversity of maternal lineage in the endangered Kiso horse based on polymorphism of the mitochondrial DNA D-loop region. *J. Vet. Med. Sci.* **76**: 1451–1456. [[Medline](#)] [[CrossRef](#)]
15. Tozaki, T., Kakoi, H., Mashima, S., Hirota, K., Hasegawa, T., Ishida, N., Miura, N., Choi-Miura, N. H. and Tomita, M. 2001. Population study and validation of paternity testing for Thoroughbred horses by 15 microsatellite loci. *J. Vet. Med. Sci.* **63**: 1191–1197. [[Medline](#)] [[CrossRef](#)]