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Genetic characterization of Japanese native horse breeds by genotyping variants that are associated with phenotypic traits

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Concerns have been raised about the loss of genetic diversity in Japanese native horses because of their declining populations. In this study, we investigated the genetic variation of four genes, myostatin (MSTN), ligand-dependent nuclear receptor corepressor like (LCORL), doublesex and mab-3 related transcription factor 3 (DMRT3), and 5-hydroxytryptamine receptor 1A (HTR1A), which are associated with horse phenotypic traits, in six Japanese horse breeds (Hokkaido, Kiso, Noma, Misaki, Tokara, and Yonaguni). MSTN, LCORL, DMRT3, and HTR1A showed polymorphisms in the Kiso; Hokkaido and Noma; Hokkaido; and Kiso, Tokara, and Yonaguni breeds, respectively. The Misaki did not show polymorphisms in any of the genes. This study may serve as a basis for developing future breeding strategies focusing on traits in Japanese native horses.

Key words: *breeding, conservation, insertion and deletion, single-nucleotide variant*

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The Hokkaido, Kiso, Misaki, Taishu, Noma, Tokara, Miyako, and Yonaguni horse breeds are registered as Japanese native horse breeds by the Japan Equine Affairs Association. The origins of all Japanese native horses have not been fully elucidated. However, Japanese native horses are suggested to have originated from Mongolian horses imported via the Korean Peninsula in the 3rd to 5th centuries [14, 23, 24]. They subsequently evolved in various places throughout Japan, becoming the indigenous breeds that exist today.

Although native horses were extensively bred in Japan and used to carry loads or farm before the early 20th century, motorization has led to their current population size being less than 2,000 individuals [17, 18, 20]. At present, the population of each Japanese native horse breed has decreased to 40–150 horses, except for the Hokkaido (over 1,000 horses). Consequently, concerns have been raised about the loss of genetic diversity in Japanese native horses [23].

Since native horses have been bred in limited local areas and specific breeding programs, current Japanese native horses have body sizes and characteristic phenotypes specific to each breed. For instance, the Hokkaido exhibits an ambling gait or pace [1]. The Hokkaido and Kiso also have withers heights of approximately 130 cm, whereas that of the Noma is smaller (110 cm) [23].

Many genetic variants associated with the phenotypes and traits of horses were identified in the Horse Genome Project [26]. For example, insertion of a short interspersed nuclear element (SINE) in the promoter region of the myostatin (*MSTN*) gene is associated with muscle content and myofiber type [25], and a single-nucleotide variant (SNV) in the cis-element of the ligand-dependent nuclear receptor corepressor-like (*LCORL*) gene affects withers height and body weight [12]. Similarly, an SNV in the coding region of the doublesex and mab-3-related transcription factor 3 (*DMRT3*) gene is associated with gait [2], and an SNV in the 5-hydroxytryptamine receptor 1A (*HTR1A*) gene is associated with tractability in specifically Thoroughbreds [8].

In this study, we aimed to analyze the variants of these four genes in six Japanese horse breeds, namely the Hokkaido, Kiso, Noma, Misaki, Tokara, and Yonaguni

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(Fig. 1). The results of this study may serve as a basis for developing an enhanced breeding strategy that maintains traditional phenotypic traits in Japanese horses.

We surveyed all living or breeding individuals of the Kiso, Noma, Misaki, Tokara, and Yonaguni. Considering that there are more than 1,000 Hokkaido horses, we randomly collected samples from regions where they are living. Genomic DNAs from 84 Hokkaido, 58 Kiso, 48 Noma, 72 Misaki, 123 Tokara, and 97 Yonaguni horses were used for genotyping in this study. Genomic DNA was extracted from whole blood using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions.

With the exception of the genomic DNA for the Noma horses, we used genomic DNAs collected in our previous studies [1, 9, 17, 19, 20]. The sampling procedures for the genomic DNAs of the Kiso, Yonaguni, Misaki, and Noma were approved by the Gifu University Animal Care Committee (2021-132, 2022-068, 17207). For the Hokkaido and Tokara horses, genomic samples were extracted from a portion of the blood collected for contagious disease control and other clinical purposes.

In this study, g.66608679T>C and g.66619237delinsSINE in *MSTN*, g.68603064A>G in *LCORL*, g.22391254C>A in *DMRT3,* and g.10175848G>A in *HTR1A* were genotyped (Table 1). Primers and probes for g.66608679T>C, g.68603064A>G, and g.22391254C>A were obtained from previous studies [2, 4, 5, 21], and those for g.66619237delinsSINE and g.10175848G>A were designed using the Primer Express Software (v3.0; Thermo Fisher Scientific, Waltham, MA, USA; Table 1). Genomic locations of the five variants in this study were based on EcuCab3.0 (GCA_002863925.1), and genotyping was performed using QuantStudio 5 (Thermo Fisher Scientific) with TaqMan GTXpress Master Mix (Thermo Fisher Scientific). Allele frequencies were calculated for each genotype.

At g.66608679T>C in *MSTN*, the Noma and Misaki did not display any polymorphisms. In contrast, the Hokkaido, Yonaguni, and Tokara showed low frequencies of the C-allele (0.03–0.12), whereas the Kiso exhibited a high frequency of the C-allele (0.42; Table 2). At g.66619237delinsSINE in *MSTN*, only the Kiso showed a polymorphism (Del, 0.69; In, 0.31; Table 2). The genotypes of the other breeds were all homogeneous within the breeds (Del, 1.00). At g.68603064A>G in *LCORL*, the frequencies of the C-allele were 0.02 and 0.05 in the Hokkaido and Noma, respectively (Table 2); the other breeds did not display any polymorphisms. At g.22391254C>A in *DMRT3*, only Hokkaido exhibited polymorphism; the frequency of the A-allele was 0.77 (Table 2). At g.10175848G>A in *HTR1A*, the frequencies of the A-allele were 0.01, 0.05, and 0.73 in the Kiso, Yonaguni, and Tokara, respectively (Table 2). The other breeds did not show any polymorphisms.

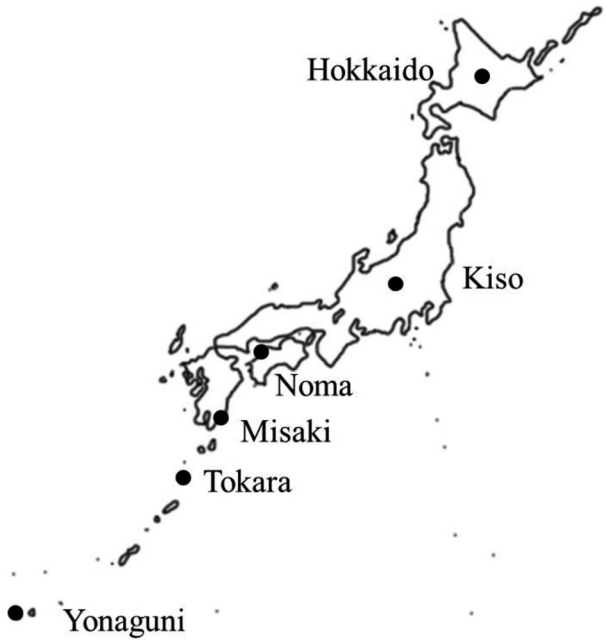

Fig. 1. Breeding locations of the six Japanese horse breeds used in this study. The six Japanese horse breeds studied are bred mainly in the following regions: the Hokkaido, Hokkaido prefecture; Kiso, Nagano prefecture; Noma, Ehime prefecture; Misaki, Miyazaki prefecture; Tokara, the Tokara islands in Kagoshima prefecture; and Yonaguni, Yonaguni island in Okinawa prefecture. Studies using genome-wide single nucleotide polymorphisms have demonstrated that these breeds have a strong tendency to genetically segregate from each other [23].

In Thoroughbreds, g.66619237delinsSINE and g.66608679T>C in *MSTN* are in complete linkage disequilibrium (Del-T, In-C) [16]. Homozygotes for the In-C haplotype have high proportions of fast-twitch fibers, heavy body weights, and short-distance race suitability [16], whereas homozygotes for the Del-T haplotype have high proportions of slow-twitch fibers, light body weights, and long-distance race suitability [6, 7]. In this study, g.66619237delinsSINE exhibited polymorphism in only the Kiso, whereas g.66608679T>C showed polymorphisms in the Hokkaido, Kiso, Yonaguni, and Tokara, indicating that the two variants were not in complete linkage disequilibrium in Japanese native horses. We hypothesized that the Japanese native horse population originally had a low frequency of the C-allele and lacked the In-allele. The C- and In-alleles might have been introduced when Kiso horses were crossbred with European horses. The Kiso experienced a bottleneck effect, and backcrossing with Kiso lineage horses was promoted. With the increased number of horses, the number of Kiso horses with the C- and In-alleles also possibly increased

Table 1. Probe and primer sequences of five variants in four genes

Gene and variant	Reference number, location, and description	Probe and primer sequences	References/ accession numbers
MSTN	rs397152648	MGB probe VIC: AATGCACCAAGTAATTT	[5]
g.66608679T>C	Chr18: 66608679 (EquCab3.0)	MGB probe FAM: ATGCACCAAATAATTT	
	SNV of intron 1	Forward: CCAGGACTATTTGATAGCAGAGTCA	
		Reverse: GACACAACAGTTTCAAAATATTGTTCTCCTT	
MSTN	No reference number	MGB probe VIC: ATAAAAAGCCACTTGGAATACAGTA	[4]
g.66619237delinsSINE	Chr18: 66610287 (EquCab3.0)	MGB probe FAM: CCCCGTGGCCGAGT	GCA_002863925.1
	Deletion/insertion of SINE	Forward: CAATCATAGATCCTGACGACACTTGT	
	(227 bp) in promotor	Reverse: ACAACTTGCCACACCAGTGAAT	
LCORL	rs68603064	MGB probe VIC: CATTCCAGCTTATTTCTGTA	[21]
g.68603064A>G	Chr3: 107374136 (EquCab3.0)	MGB probe FAM: CATTCCAGTTTATTTCTGTAC	
	60 kb upstream from LCORL	Forward: CCAAATTTGCCTGGCTAGAGA	
		Reverse: TGTTCCCTGTGATTCTGCCTTT	
DMRT3	rs1150690013	MGB probe VIC: CTGCCGAAGTTCG	[3]
g.22391254C>A	Chr23: 22391254 (EquCab3.0)	MGB probe FAM: CTCTGCCTAAGTTCG	
	Ser301STOP	Forward: CCTCTCCAGCCGCTCCT	
		Reverse: TCAAAGATGTGCCCGTTGGA	
HTR1A	rs1148692440	MGB probe VIC: CCGCTCCCTTCTTC	GCA_002863925.1
g.10175848G>A	Chr21: 10175848 (EquCab3.0)	MGB probe FAM: TCCGCTCTCTTCTTC	
	Gly237Arg	Forward: CCGCAAGACAGTCAAGAAGGT	
		Reverse: CGCCATTTGCGCTCTTCTT	

Minor groove binder (MGB) probes were labelled with VIC or FAM. SINE: short interspersed nuclear element.

compared with the other Japanese native breeds. Although we did not collect phenotypic measurements for individual horses, horses with the In-allele were considered to have relatively high muscle masses.

LCORL is associated with withers height and body weight in horses [13, 21, 22]. Its expression is affected by g.68603064A>G, which is located on the transcription factor binding site approximately 60 kb upstream of the gene [12]. The withers heights of horses with G-alleles, such as G/G and G/A, are higher than those of horses with the A/A genotype [22]. In Japanese native horses, low frequencies of the A/G genotype were found in Hokkaido and Noma horses. Therefore, differences in withers height within these breeds may be caused by *LCORL*. However, differences among Japanese breeds may also be caused by other genes associated with this trait, such as *HMGA2* and *ZFAT*, since the A-allele was the most common allele in all the breeds [10, 11].

Gait is an important trait of horses. In *DMRT3*, nonsense mutation at g.22391254C>A causes the full-length protein to lack 174 amino acid residues [2]. The homologous nonsense mutation (A/A genotype) is associated with the ability to pace, which is a two-beat gait that allows the horse to move both legs on the same side of the body in a synchronized manner. Among the Japanese native breeds assessed in this study, only the Hokkaido displayed polymorphisms. This result agrees with the findings of Promerová *et al.* [15]. Interestingly, the Yonaguni did not exhibit any polymorphisms in our study, yet a recent study by Chandra Paul *et al.* [3] demonstrated the presence of the A-allele in the Yonaguni. This discrepancy can be attributed to variation in the sampling times; their data derived from samples collected several decades ago, whereas our data were gathered in the last few years. We investigated almost all the individuals in the current Yonaguni population. Thus, the A-allele associated with pace had already disappeared from the current population. Traditional horseracing had once been held in the Ryukyu Kingdom area (currently Okinawa prefecture), and according to oral tradition, there were horses with the pace gait in that area. This result suggests that the A-allele at g.22391254C>A was also present in the Yonaguni, a breed of native horses in the Ryukyu Kingdom area.

HTR1A is a serotonin receptor, and Thoroughbreds with the A-allele at rs1148692440 show low tractability [8]. When the G-allele at g.10175848G>A changes to the A-allele, glycine at the 237th amino acid residue of *HTR1A* is replaced with arginine. Japanese native breeds have relatively mild temperaments, so they can be easily controlled. Interestingly, however, the A-allele frequencies were high in the Tokara in this study. Tokara horses have traditionally been used for sugar cane cultivation, and when the breed was discovered, it had a small population of approximately 10 individuals. Thus, many of the individuals possibly had the A-allele at that time. However, its population has now

Table 2. Genotype counts and allele frequencies of 5 variants in Japanese native horse breeds

MSTN g.66608679T>C	Total	Genotype count			Allele frequency	
		T/T	C/T	C/C	T	C
Hokkaido	84	69	14	1	0.90	0.10
Kiso	58	17	33	8	0.58	0.42
Noma	48	48	0	0	1	0
Misaki	72	72	0	0	1	0
Tokara	123	117	5	1	0.97	0.03
Yonaguni	97	73	24	0	0.88	0.12
MSTN g.66619237delinsSINE	Total	Genotype frequency			Allele frequency	
		Del/Del	Del/In	In/In	Deletion	Insertion
Hokkaido	84	84	0	0	1	0
Kiso	58	24	32	2	0.69	0.31
Noma	48	48	0	0	1	0
Misaki	72	72	0	0	1	0
Tokara	123	123	0	0	1	0
Yonaguni	97	97	0	0	1	0
LCORL g.68603064A>G	Total	Genotype frequency			Allele frequency	
		A/A	A/G	G/G	A	G
Hokkaido	84	81	3	0	0.98	0.02
Kiso	58	58	0	0	1	0
Noma	48	43	5	0	0.95	0.05
Misaki	72	72	0	0	1	0
Tokara	123	123	0	0	1	0
Yonaguni	97	97	0	0	1	0
DMRT3 g.22391254C>A	Total	Genotype frequency			Allele frequency	
		C/C	C/A	A/A	C	A
Hokkaido	84	7	24	53	0.23	0.77
Kiso	58	58	0	0	1	0
Noma	48	48	0	0	1	0
Misaki	72	72	0	0	1	0
Tokara	123	123	0	0	1	0
Yonaguni	97	97	0	0	1	0
HTR1A g.10175848G>A	Total	Genotype frequency			Allele frequency	
		G/G	G/A	A/A	G	A
Hokkaido	84	84	0	0	1	0
Kiso	58	57	1	0	0.99	0.01
Noma	48	48	0	0	1	0
Misaki	72	72	0	0	1	0
Tokara	123	10	47	66	0.27	0.73
Yonaguni	97	88	9	0	0.95	0.05

grown to over 100 individuals, and the bottleneck effect has likely increased the A-allele frequency.

In this study, we genotyped five variants in *MSTN*, *LCORL*, *DMRT3*, and *HTR1A*, which are associated with horse phenotypes, in six Japanese horse breeds. Our results suggest that trait-associated variants lost genetic diversity. All breeds, except for the Hokkaido, experienced a drastic population reduction as a result of motorization. Therefore, the changes in allele frequencies are possibly due to bottlenecks rather than modern breeding [23]. Loss-of-trait-associated variants, such as g.22391254C>A in *DMRT3*, possibly complicate the reproduction of specific phenotypes in each breed.

Considering that almost all individuals representing each breed, except for the Hokkaido, were genotyped in this study, we deemed that all alleles in each population can be detected. Therefore, identifying individuals with low-frequency alleles or desirable traits is beneficial for conserving native horses. Since this study was not tied to phenotypes, we believe future studies linked to actual

phenotypes would be useful for breeding plans. This study provides the basic information necessary to maintain the characteristic traits and phenotypes in each native horse breed.

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