Genetic Characterization of Japanese native horse

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INTRODUCTION

Large number of local horses have historically been raised in Japan for drafting, packing, and riding utilities in transportation, agriculture, and military purpose, but the population of these Japanese native horses has dramatically reduces in recent times, and currently only eight local populations of Japanese native horse breeds, namely Hokkaido, Kiso, Noma, Taishu, Misaki, Tokara, Miyako, and Yonaguni breeds, have remained for mainly conservation purpose in several locations of Japan (Nozawa 1992; Ichikawa 1984; Hayashida 1958). While the population sizes of these horses are markedly small ranging from tens to 200 animals, except for the Hokkaido population, which includes more than 1,000 animals (Takasu et al. 2011; Onogi et al. 2017; Senju et al. 2017a; Senju et al. 2017b; Senokuchi et al. 2018; Kobayashi et al. 2019), these native horse breeds may have unique genetic characteristics. Since such unique genetic characteristic can

Genotype distribution and allele frequencies of the genes associated with reproductive traits and hereditary disorders in Japanese native horses

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ABSTRACT

Eight breeds of horse, namely Hokkaido, Kiso, Noma, Taishu, Misaki, Tokara, Miyako, and Yonaguni horses, have currently been conserved as the Japanese native horse. Since to uncover their genetic properties involved in reproductive traits and hereditary disorders is important for their breeding and conservation programs, we investigated genotype distribution and allele frequencies of the genes associated with reproductive traits and hereditary disorders in the populations of the eight Japanese native horse breeds. We genotyped single nucleotide polymorphisms of $FKBP6$ (g.11040315G>A and g.11040379C>A), $CRISP3$ (c.622G>A and c.716A>G), and $PLCZ1$ (g.45586821C>T) genes associated with stallion fertility including semen qualities and impaired acrosome reaction, and found that both desirable and undesirable alleles of $FKBP6$ and $CRISP3$ genes are present in the populations, while only undesirable allele of $PLCZ1$ was observed in these populations. We also genotyped single nucleotide polymorphisms of $GYS1$ (c.926G>A), $RYR1$ (c.7360C>G), and $SCN4A$ (c.4248C>G) genes which are associated with polysaccharide storage myopathy, malignant hyperthermia, and hyperkalaemic periodic paralysis, respectively, and found that no mutant alleles responsible for these hereditary disorders are present in the populations of Japanese native horse breeds. These findings will be informative for future breeding and conservation programs for these horse breeds.

Key words: Japanese native horse, fertility genes, disease genes.

INTRODUCTION

Large number of local horses have historically been raised in Japan for drafting, packing, and riding utilities in transportation, agriculture, and military purpose, but the population of these Japanese native horses has dramatically reduces in recent times, and currently only eight local populations of Japanese native horse breeds, namely Hokkaido, Kiso, Noma, Taishu, Misaki, Tokara, Miyako, and Yonaguni breeds, have remained for mainly conservation purpose in several locations of Japan (Nozawa 1992; Ichikawa 1984; Hayashida 1958). While the population sizes of these horses are markedly small ranging from tens to 200 animals, except for the Hokkaido population, which includes more than 1,000 animals (Takasu et al. 2011; Onogi et al. 2017; Senju et al. 2017a; Senju et al. 2017b; Senokuchi et al. 2018; Kobayashi et al. 2019), these native horse breeds may have unique genetic characteristics. Since such unique genetic characteristic can

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be valuable for maintaining the genetic diversity of the domestic horse populations (Nozawa et al. 1998; Tozaki et al. 2003; Kakoi et al. 2007; Raudsepp et al. 2012; Usuga et al. 2018; Restrepo et al. 2018). We also genotyped missense mutations of c.926G>A in GYS1 gene responsible for PSSM (McCue et al. 2008a; 2008b), c.7360C>G in SCN4A gene responsible for MH (Aleman et al. 2009), and c.4248C>G of SCN4A gene responsible for HYPP (Rudolph et al. 1992).

The genotype data of these genes in the Japanese native horses will be informative for future breeding and conservation programs to prevent the incidences of reproductive defects and hereditary disorders caused by increased inbreeding situation of these local populations of Japanese native horses.

**MATERIALS AND METHODS**

**DNA samples of Japanese native horses**

A total of 221 genomic DNA samples of eight Japanese native horses, including Hokkaido (23), Kiso (30), Noma (32), Taishu (21), Misaki (29), Tokara (29), Miyako (32), and Yonaguni (25) breeds, were used for the present study. These DNA samples were extracted from the blood samples of Japanese native horses that are collected during 1971 to 1994 as a part of field research for Asian native livestock conducted by the Society for Researches on Native Livestock (Nozawa et al. 1998) and had been stored in deep freezer at -80°C. The extraction of DNA from these blood samples was performed according to the standard phenol/ chloroform method.

**Genotyping of the FKBP6 and CRISP3 genes by direct sequencing**

Genotypes of FKBP6 (g.11040315 G>A, g.11040379 C>A) and CRISP3 (c.622G>A, c.716A>G) genes were determined by direct sequencing of the PCR products. A 395-bp segment of the FKBP6 gene that contains both g.11040315 G>A and g.11040379 C>A SNPs and a 378-bp segment of the CRISP3 gene that contains both c.622G>A and c.716A>G SNPs were amplified by PCR using primer pairs indicated in Table 1. PCR were carried out in a 10 µl reaction mixture containing 2.0 µl of genomic DNA (10 ng/µl), 0.3µl of 0.2µM primers, 1.0 µl of 2.0mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer and 0.1 µl (0.5U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) for
35 cycles of denaturation at 94°C for 30 s and annealing temperature indicated in the Table 1 for 30-60 s, and extension at 72°C for 30-60 s using Thermal Cycler Dice Touch (Takara Bio, Japan). After PCR amplification, the PCR products were treated with ExoSAP-IT (Thermo Fisher Scientific, MA, USA) and subjected to Sanger sequencing to determine the genotypes of these SNPs.

Genotyping of the PLCZ1, CRISP3, GYS1 RYR1, and SCN4A genes by PCR-RFLP

Genotypes of PLCZ1 (g.45586821C>T), GYS1 (c.926G>A), RYR1 (c.7360C>G) and SCN4A (c.4248C>G) were determined by digestion of the PCR fragment by restriction enzymes. Segment of the PLCZ1, CRISP3, GYS1 RYR1, and SCN4A genes that contain g.45586821C>T, 926G>A, 7360C>G, and c.4248C>G SNPs, respectively, were amplified by PCR using primer pairs indicated in Table 1. PCR were carried out in a 10 µl reaction mixture containing 2.0 µl of genomic DNA (10 ng/µl), 0.3µl of 0.2µM primers, 1.0 µl of 2.0mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer and 0.1 µl (0.5U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) for 35 cycles of denaturation at 94°C for 30 s, annealing at temperatures indicated in the Table 1 for 30-60 s, and extension at 72°C for 30-60 ss using Thermal Cycler Dice Touch (Takara Bio, Japan). After PCR amplification, the PCR products of the PLCZ1, GYS1 RYR1, and SCN4A genes were digested with MwoI, Hpy CH4V, Bam HI, and Taq αI restriction enzymes (New England Biolabs, USA; Toyobo, Japan), and the digested products were electrophoresed in 2-3% agarose gel in TAE buffer, stained with GR Red (Bio-craft), and visualized with UV trans-illuminator. To confirm accuracies of the genotyping by PCR-RFLP, we directly sequenced the amplified fragments of PLCZ1, GYS1 RYR1, and SCN4A genes from at least one sample each of the eight Japanese native horse populations.

Table 1. Genotyping conditions for FKBP6, PLCZ1, CRISP3, GYS1, RYR1 and SCN4A genes.

| Gene   | Polymorphism     | Amino acide       | Primer sequence (5’ to 3’) | Length | Anneling temperature | Restriction enzyme | Genotyping | References       |
|--------|------------------|-------------------|----------------------------|--------|----------------------|-------------------|------------|------------------|
| FKBP6  | g.11040379 C>A   | His 166Asp        | F:ACAGGCCGATGACAGAAGC      | 395 bp | 57°C                 | Sequencing        | Schirmpf et al. 2015 |
|        | g.11040315 G>A   | Synonymous        | R:CTGGTCTCCCTCTCTGTC       |        |                      |                   |            |                  |
| CRISP3 | c.622G>A, c.716G>A | Glu208Lys, Gln239Arg | F:TCGAGAAGTGAAAGGCCCAT     | 378 bp | 56°C                 | Sequencing        | Hamann et al. 2007 |
|        |                  |                   | R:TTGGAAACTGGTTGCAACTAGC   |        |                      |                   |            |                  |
| PLCZ1  | g.45586821C>T    | Intronic          | F:GCTCTGTTGACCGCTCTCAG    | 295 bp | 57°C                 | Mwo 1             |            | Schirmpf et al. 2014 |
|        |                  |                   | R:ATGGCCTCACTTCTGAGAT     |        |                      |                   |            |                  |
| GYS1   | c.926G>A         | Arg309His         | F:TAAGACATGACGCCTCCCTCAG  | 230 bp | 56°C                 | Hpy CH4V          |            | McCue et al. 2008b |
|        |                  |                   | R:AGCTGTCCTCCCCCTCTAGAC   |        |                      |                   |            |                  |
| RYR1   | c.7360G>C        | Arg245Gly         | F:CGCTGTGCTGGAGCTC        | 455 bp | 59°C                 | Bam HI            |            | Akeman et al. 2009 |
|        |                  |                   | R:GAAGGATGGCCACATTG       |        |                      |                   |            |                  |
| SCN4A  | c.4248G>C        | Phe1416Leu        | F:CTTGTGACGAAGCGAAGCTG    | 408 bp | 60°C                 | Taq αI            |            | Rudolph et al. 1992 |
|        |                  |                   | R:CTCTAATGTCCTTTGCTCAT    |        |                      |                   |            |                  |
monoallelic in the Japanese native horse breeds. The genotype distributions of all these SNPs in the eight horse populations were not significantly different from those expected from Hardy Weinberg equilibrium ($p < 0.05$).

**FKBP6** has originally been identified as the gene essential for spermatogenesis and male meiosis in mouse and rat (Crackower et al. 2003; Noguchi et al. 2008) and later reported to associate with stallion fertility in horse. By GWAS, Raudsepp et al. (2012) reported that the A alleles of both g.11040315 G>A and g.11040379 C>A as well as AA haplotype of these two SNPs are significantly associated with Impaired Acrosomal Reaction (IAR) of sperm in Thoroughbred horses. Schrimpf et al. (2015) also reported association between g.11040379 C>A and estimated breeding values for the paternal component of the pregnancy rate per oestrus cycle (EBV-PAT) in Hanoverian horses. **CRISP3** encodes secretary protein of male genital tract that occupies major fraction of seminal plasma proteins of stallion (Novak et al. 2010). Hamann et al. (2007) reported that c. 622G>A of this gene is significantly associated with the fertility of stallions in Hanoverian horses, Restrepo et al. (2019) also reported significant association of several SNPs of this gene including c. 622G>A and c.716 A>G with semen qualities such as mortality, vitality, and morphology in Colombian Creole horses, and Gottschalk et al. (2016) reported an association of genomic region including **CRISP3** gene with semen quality identified by GWAS in German warmblood horses. **PLCZ1** is the gene for phospholipase C zeta 1 of sperm which plays an important role in fertilization by triggering Ca$^{2+}$ oscillation to activate oocyte (Saunders et al. 2002) and several SNPs of this gene were reported to be associated with estimated breeding value of the paternal component of the pregnancy rate per estrus cycle (EBV-PAT) in Hanoverian stallions, in particular, g.45586821C>T showed highest association with EBV-PAT (Schrimpf et al. 2014).

The genotyping results of **FKBP6** and **CRISP3** in the present study indicated that the SNPs of these genes which were originally identified in European and American breeds are also present in the Japanese native horses. This is the first report for the presence of these SNPs associated with stallion reproductive traits in native horse populations outside of European and American countries. The present finding of the presence of the SNPs of these genes in Japanese native horse populations suggests that these SNPs have spread into wide variety of horse populations, while we could not exclude a possibility of introgression of these SNPs from western horses, since western horses have historically been introduced to Japanese native horse populations to improve their physiques for military purposes before World War II (Ichikawa 1984). The average frequencies of the undesirable alleles of **FKBP6** (g.11040315 G>A), **FKBP6** (g.11040379 C>A), **CRISP3** (c.622G>A), and **CRISP3** (c.716 A>G) in the populations of the Japanese native horse are not so high, being 0.55, 0.49, 0.09, and 0.35, respectively, but some of these populations showed high frequencies of the undesirable alleles (Table 2, 3). In particular, the frequency of the A allele of **FKBP6** (g.11040379 C>A) is remarkably high (0.93) in the Misaki horse population (Table 2), and more than 20% of the horses in this population were homozygous for AA haplotype of **FKBP6** (g.11040315 G>A and g.11040379 C>A). Because of Misaki horse is feral horse population with minimum human intervention (Kobayashi et al. 2019), whether

| Table 2. Genotype distributions and allele frequencies of **FKBP6** gene. |
|---------------------------------------------------------------|
| **FKBP6** (g.11040315 G>A)                                   | **FKBP6** (g.11040379 C>A) |
| **Number of samples**                                      | **Genotype distribution** | **Allele frequencies** | **Genotype distribution** | **Allele frequencies** |
|                                                          | GG   | AG   | AA   | G     | A     | CC   | CA   | AA   | C     | A     |
| Hokkaido                                                | 23   | 1    | 11   | 11    | 0.28  | 0.72 | 8    | 10   | 5    | 0.57  | 0.43  |
| Kiso                                                    | 30   | 2    | 20   | 8     | 0.4   | 0.6  | 4    | 21   | 5    | 0.48  | 0.52  |
| Noma                                                    | 32   | 0    | 15   | 17    | 0.23  | 0.77 | 30   | 2    | 0    | 0.97  | 0.03  |
| Taishu                                                  | 21   | 3    | 11   | 7     | 0.4   | 0.6  | 6    | 12   | 3    | 0.57  | 0.43  |
| Misaki                                                  | 29   | 6    | 13   | 10    | 0.43  | 0.57 | 0    | 4    | 25   | 0.07  | 0.93  |
| Tokara                                                  | 29   | 18   | 6    | 5     | 0.72  | 0.28 | 6    | 13   | 10   | 0.43  | 0.57  |
| Miyako                                                  | 32   | 11   | 16   | 5     | 0.6   | 0.4  | 8    | 16   | 8    | 0.5   | 0.5   |
| Yonaguni                                                | 25   | 7    | 11   | 7     | 0.5   | 0.5  | 7    | 10   | 8    | 0.48  | 0.52  |
| **Total**                                               | 221  | 48   | 103  | 70    | 0.45  | 0.55 | 69   | 88   | 64   | 0.51  | 0.49  |
stallions of Misaki horse show higher incidence of IAR due to high allelic frequency of these alleles is currently unclear. Since higher stallion reproductive performance is important for the breeding of Japanese native horses to maintain the considerable number of horses in the population, the present findings of the distribution of the alleles of the genes associated with stallion reproductive traits will be informative for the breeding and conservation of these breeds.

Since we could not detect the C allele of PLCZ1 (g.45586821C>T) in the horses of all eight Japanese native horse breeds, it is likely that this polymorphism has not been present in the ancestral Japanese native horse populations. Therefore, this SNP was suggested to be relatively new SNP that are restricted in some particular western breeds of horse including Hanoverian horse. Since this is the first report of the allele frequency of PLCZ1 (g.45586821C>T) in horse breeds other than Hanoverian horse (Schrimpf et al. 2014), further investigation of this SNP in other horse populations is required to figure out the distribution and origin of this SNP in the domestic horses.

Next, we investigated the SNPs of the genes associated with hereditary disorders including GYS1, RYR1, and SCN4A genes by PCR-RFLP. The results of the genotyping indicated that all horses of the Japanese native horse populations examined were homozygous for the normal alleles of GYS1 (c.926G>A), RYR1 (c.7360C>G), and SCN4A (c.4248C>G) and no mutant alleles associated with the disorders were observed (Table 4). The SNP of GYS1 (c.926G>A) causes a missense mutation of Arg309His that is responsible for PSSM characterized by increased muscle glycogen concentration, abnormal polysaccharide storage accumulation in myofibres, and sign of painful cramping and progressive muscle atrophy (McCue et al. 2008b). Incidences of this disorder has been reported in genetically distinct breeds of horse and the mutation is predicted to be originated from the old population of domestic horses before the establishment of the diverse modern horse breeds.
(McCue et al. 2008a; Tryon et al. 2009; Baird et al. 2010; Druml et al. 2016). The SNP of RYR1 (c.7360C>G) causes a missense mutation of Arg2454Gly that is responsible for MH, a pharmacogenetic disorder triggered by halogenated aesthetics and other non-aesthetics factors including exercise and stress, and typical symptoms of this disorder are tachycardia, hyperthermia, muscle rigidity, rhabdomyolysis, respiratory, and metabolic acidosis (Aleman et al. 2009). Incidences of MH have been reported in Quarter horse, Thoroughbred, Appaloosa, Arabian, and pony breeds (Aleman et al. 2004). The SNP of SCN4A (c.4248C>G) causes a missense mutation of Phe1416Leu that is responsible for HYPP characterized by episodic attacks of muscle tremors, weakness and paralysis with increased serum potassium concentration (Rudolph et al. 1992). Incidences of this disorder has been reported in Quarter hose breeds (Rudolph et al. 1992; Tryon et al. 2009). The present genotyping data suggested that the mutant alleles of these genes, which were observed in diverse western horse breeds (Rudolph et al. 1992; Aleman et al. 2004, 2009; McCue et al. 2008a, b; Tryon et al. 2009; Baird et al. 2010; Schwarz et al. 2011; Druml et al. 2016) were not present in the populations of Japanese native horses. Therefore, it is likely that these mutant alleles have not been introgressed to Japanese native horse populations from these western breeds, while exotic horses have historically been introduced to Japanese native horse populations (Ichikawa 1984).

In conclusion, we investigated genotypes of the FKBP6, CRISP3, and PLCZ1 genes associated with stallion reproductive traits and GYS1, RYR1 and SCN4A genes associated with hereditary disorders in the populations of Japanese native horse breeds and found that the desirable alleles associated with higher stallion reproductive performance are present in these populations and the mutant alleles responsible for hereditary disorders are not present in these populations. These findings will be informative for breeding and conservation programs for these breeds.

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