Study on genetic diversity and characteristics of Japanese native horse populations

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Study on genetic diversity and characteristics of Japanese native horse populations

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by

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Dedication

to

My beloved wife

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List of Abbreviations

%	*Percentage*
µL	*Micro Litre*
^{0}C	*Degree Celcius*
AA	*Amino Acid*
bp	*Base Pair*
c	*Coding*
cm	*Centimetre*
del	*Deletion*
DNA	*Deoxyribonucleic Acid*
dNTP	*Deoxyribonucleotide-triphosphates*
DW	*Distilled Water*
EBV-PAT	*Estimated Breeding Values for the Paternal component of the Pregnancy rate per Oestrus cycle*
FAO	*Food and Agricultural Organization*
g.	*Genomic*
GWAS	*Genome Wide Association Study*
h	*Haplotype diversity*
HT	*Haplotype*
HWE	*Hardy-Weinberg Equilibrium*
KASP	*Kompetetive Allele Specific PCR*
kg	*Kilogram*
mM	*Mili Molar*
mtDNA	*Mitochondrial DNA*
N	*Number*
ng	*Nano Gram*
N-J	*Neighbour Joining*
P	*Protein*
p	*Probability*
rPm	*Rotation Per Minute*
SNPs	*Single Nucleotide Polymorphisms*
TAE	*Tris-Acetic Acid-EDTA*
TE	*Tris-EDTA*
TNESU	*Tris-Nacl-EDTA-SDS-Urea*
U	*Unit*
YBP	*Year Before Present*
π	*Nucleotide Diversity*

Abstract

A 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, namely Hokkaido, Kiso, Noma, Taishu, Misaki, Tokara, Miyako, and Yonaguni breeds, have remained for mainly conservation purpose in several locations of Japan. While the population sizes of these horses are markedly small except Hokkaido, these native horse breeds may have unique genetic characteristics. Since such unique genetic characteristics can be valuable for maintaining the genetic diversity of the domestic horse population, efforts must be taken to conserve the Japanese native horses. Currently, various genetic indexes are used to assess the genetic diversity of a population, including microsatellite markers and mitochondrial DNA (mtDNA). In addition, due to recent advances in molecular genetic analysis of domestic animals including genome wide association study (GWAS), various single nucleotide polymorphisms (SNPs) or mutations of the genes associated with particular traits of horse including physical performance, body conformation, coat color, reproductive performance as well as hereditary disorders, are also used to evaluate the genetic characteristics of the horse populations.

In this study haplotype of mtDNA, Y chromosome haplotypes and genotypes of genes associated with physical performance, body conformation, reproductive traits, hereditary disorders as well as coat colors were investigated to reveal the genetic diversity and characteristics of these Japanese native horse populations.

Haplotype of mtDNA D-loop region was analyzed to assess the relationships of the maternal lineage between the eight populations of Japanese native horses.

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The results obtained from these populations indicated the presence of 15 different haplotypes with haplotype and nucleotide diversities of 0 to 0.874 and 0 to 0.023, respectively, except the Noma and Tokara which showed no variation of mtDNA. Furthermore, Neighbour Joining (N-J) tree showed few common haplotypes in these populations. In addition, about 44% of Japanese native horse shared *X3c1* haplotypes which is regarded as ancient haplotype and few other ancient haplotypes were also observed in these populations. Next, to investigate origin and relationship of patriline of these populations, allelic states of 24 Y chromosome haplotype-indicative SNPs were analyzed. As of the result, total 8 Y chromosome haplotypes were detected in these native horses. All the horses from Kiso, Misaki and some of Miyako populations have haplotypes which was influenced by English Thoroughbred, and about 36%, of the Japanese native horse have haplotype which was influenced by Arabian horse breed. In addition, a small percentage of these populations have a haplotype that was thought to be influenced by Iberian and North African Barb. However, the remaining horses have unique haplotypes which predicted to separate from the root of the group regarded as modern horse groups or those separate from root of phylogenetic tree of domestic horse at earlier ages. These findings suggested that the Japanese native horse populations have retained ancestral genetic features in both maternal paternal lines.

Japanese native horses are small horses used mainly for riding, agricultural transport and packing loads. In this study, the SNPs of the genes associated with physical performance, body conformation and locomotion traits including *LCORL, ZFAT, HMGA2, LASP1, MSTN* and *DMRT3* genes, which are associated with increased wither height, increased muscle mass and ambling gait, were genotyped in Japanese native horses by PCR-RFLP and/or direct sequencing. As the results of the genotyping, both two alleles of *ZFAT* g.75550059 C>T, *HMGA2* g.81481064 C>T, *LASP1* g.23259732 A>G, and

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MSTN g.66493737C>T were observed in the all population of these horses except for *ZFAT* g.75550059 C>T in Misaki and *MSTN* g.66493737C>T and *LASP1* g.23259732 A>G in Noma which were mono allelic. Similarly, both two alleles of *LCORL* g.105547002 C>T in Hokkaido, Noma, Miyako and Kiso, and of *DMRT3* g.22999665C>A in Hokkaido, Miyako and Yonaguni were observed, whereas remaining were mono allelic for these genes. It also showed that, average minor allele frequencies were, *0.03,* 0.22, 0.21, 0.16, 0.07 and 0.03, for *LCORL* g.105547002 C>T, *ZFAT* g.75550059 C>T, *HMGA2* g. 81481064 C>T, *LASP1* g. 23259732 A>G, *MSTN* g.66493737C>T and *DMRT3* g.22999665C>A, respectively. The presences of the minor alleles of these genes at low frequencies suggest a possibility that these horse populations have not been under strong selection pressure for particular body composition and locomotion traits. However, relatively high frequency of the allele of *DMRT3* gene associated with gaitedness in Hokkaido population suggest a possibility that this horse population has been under strong selection pressure for locomotion traits including gaitedness. The present findings of the presence of these minor alleles in Japanese native horses will be informative for future selection, breeding and conservation.

Since the eight native horse populations have currently been conserved in Japan as small populations, to uncover their genetic properties involved in reproductive traits and hereditary disorders is important for their breeding and conservation programs. Therefore, genotype distribution and allele frequencies of the genes associated with stallion reproductive traits and hereditary disorders in the populations of the eight Japanese native horse breeds were investigated. The genotyping results of 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 showed that both desirable and

undesirable alleles of *FKBP6* and *CRISP3* genes were present in the populations, while only undesirable allele of *PLCZ1* was observed in these populations. Mutation 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, were also investigated and found that no mutant alleles responsible for these hereditary disorders were present in the populations of Japanese native horse. Since higher reproductive performance and healthy condition 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 reproductive traits and hereditary disorders will be informative for the conservation of these breeds.

Correct animal identification is important for selection and breeding, and coat color is one of the indexes for the identification. To uncovers the coat color genotype, mutation of *MC1R* c.901C>T and *ASIP* c.2174-2184del genes associated with basic coat colors of chestnut, bay and black, *MATP* c.457G>A with cream dilution, and *TBX3* g.18227267+1066G>T and g.18227267 1.6 del with dun coat color were genotyped, and found that both alleles *A, a* in *MC1R* and *E, e* in *ASIP* for basic coat color were present in all of these horse populations, while cream dilution allele C^{cr} was present in only three populations. In addition, two (*d1,d2*) of the three alleles, *D,d1* and *d2,* of *TBX3* gene were present in all Japanese native horse populations that were not associated with dun color but shows primitive markings, such as dorsal stripe. These findings suggested that these populations have retained ancestral features of the coat color gene.

The present findings of the genetic diversities of maternal and paternal lines indicated by mtDNA and Y chromosome haplotypes and distributions of the alleles of genes associated with physical performance, body conformation,

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reproductive traits, hereditary disorders, and coat color in the Japanese native horse populations will be informative for future breeding and conservation programs. In addition, the present findings that Japanese native horses have retained some ancestral genetic features in maternal and paternal lines and coat color gene will be important for genetic characterization of Japanese native horse populations.

CHAPTER 1

General Introduction

1.1: INTRODUCTION

Farming and animal domestication were fundamental steps in human development, contributing to the rise of larger settlements and more stratified societies and eventually, great civilizations. Horse is one of the domesticated animals that had played a very significant role in the human civilization (Diamond, 2002; Gupta, 2004; Ludwig *et al.*, 2009). The Tarpan (*Equss ferus*), a wild European horse already been extinct in last century (Bokonyi, 1974a ; Zeuner, 1963; http://www.ansi.okstate.edu/breeds/horses/), are regarded as the ancestors of present day horses and the Przewalski horse (*Equss Przewalskii)* the only remaining wild horse in the world, the closest living wild relative species of the present domestic horses (*Equus caballus*). Moreover, archaeological data and coat color variations indicated that horse was probably first domesticated in Eurasian steppe around 5,000 years ago (Ludwig *et al.*, 2009; Outram *et al.*, 2009; Warmuth *et al.,* 2012) and both domesticated stallions and mares spread out from this area, and then additional wild mares were added from local herds (Christa *et al.,* 2012). Since the domestication of horses, the history of utilization of horses can be traced from the rise and fall of empires, the conquest of entire continents, great battles, developments of transport systems, mail, agriculture, forestry progress and in times of war and peace (Bowling and Ruvinsky, 2000). During the middle of the 19th century, heavy breeds of horses were developed for agricultural and forestry works, coal mines, as power to other pieces of heavy machinery and for pulling carts. Horses have been also used by military forces for expeditions, riding, and transportation. The mechanization of transport and agriculture increased the

attention of many horse breeds for the development of breeds for sport and leisure activities. The role of the horses has mirrored the changes in the human society from war horse to draft horse to today's sport or companion animal (Waran, 2002). In recent times, one of the promising and emerging areas for the use of many breeds of horses is for competitive events or as sports animals. The development of leisure activities for horses reflects a regular decrease in the number of draft horses and a constant increase in the number of sport horses (Langlois *et al.,* 1983). Sport horse breeds are intended to be used in competitions for the major international equestrian disciplines of dressage, jumping, three day eventing, racing, trotting, endurance, and vaulting. In recent years, horses are also used in tourism, medical therapy, hobby, social rehabilitation, or social eventing, aesthetic and for cultural values. Horses became progressively used for transportation, agriculture and forestry, leisure, recreation, sports, meat and therapeutic riding (Hausberger *et al.*, 2008; Splan, 2004; Anderson *et al.,* 1999). Besides this, the equine industry plays a significant role in the socio-economic and environmental sector of a country. Data of FAOSTAT (2008) shows that there are 58.8 millions of horses in the world and a total of 786 breeds of horses were reported as of January 2006 which is 10.33 % of the total number of livestock breeds, whereas excluding 87 extinct horse breeds, there are 570 local breeds, 63 regional trans boundry breeds and 66 international trans boundry breeds (Khadka, 2010). Since the domestication, horses spread all over the world and locally adapted in various environment. But the number of native animals has recently been decreasing worldwide (Food and Agriculture Organization of the United Nations (FAO): Commission on Genetic Resources for Food and Agriculture, 2015). Due to mechanization and modernization of cities the number of native horses also decreasing rapidly. In Japan, the horse industry produced 7000 Thoroughbreds annually from 2010 to 2014 whereas; only about 200 animals belonging to eight

Japanese native breeds were produced annually during the same period (http://www.maff.go.jp/j/chikusan/kikaku/lin/pdf/27_zentai.pdf#page=70).

Mongolian horse represents ancient horse population of Euresian steppe; have not subject to artificial selection, reported to ancestor of Japanese native horses. According to the literature, Japanese horse populations are descended from Mongolian horses through the Korean peninsula and have spread all over Japan since there were no horses in Japan about 2,000 years ago and these populations localized to the particular areas in Japan and affected by the gene flow of each other (Tozaki, 2003). Since then, a 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 (Senokuchi *et al.,* 2018; Senju *et al.,* 2017a; Senju *et al.,* 2017b; Kobayashi *et a.l,* 2019; Takasu *et al.*, 2011; Onogi *et al.,* 2017), these native horse breeds may have unique genetic characteristics which can be valuable for maintaining the genetic diversity of the domestic horse populations (Nozawa *et al.*, 1998; Kakoi *et al.,* 2007; Tozaki *et al.,* 2003). So, origin, physical characteristics including wither height and coat color as well as purposes of use of the local population were reported various times are described below.

Hokkaido originated from horses of a Mongolian lineage (Nozawa *et al.*, 2001; Tozaki *et al.,* 2003) that were transported from the main island of Japan around the 15th century (Miyakami, 2006; Kondo, 2012) and were used as pack

horses until about the mid-1960s (Kondo, 2012). It has a patient disposition and comparatively small body size (male: 127–135 cm, female: 123–133 cm) (Nozawa, 1997; Kondo, 2012). Moreover, horses have been recognized as a good fat stock, given their ability to grow well on a roughage diet (Kondo, 1998; Clauss *et al.,* 2003; Miyakami, 2006). Furthermore, Hokkaido is a globally interesting horse breed in terms of naturally pace gait and exhibits a rich variation in coat color. Pedigree record has registered more than 10 coat colors including chestnut, bay, black, grey, palomino, buckskin, double dilutes, chestnut roan, bay roan and black roan. (Hachinohe, 1982), which is make Hokkaido horse valuable genetic resource for future coat color investigation.

Kiso horse is a breed of Japanese native horses that originated from the mountainous Kiso region of central Japan and historically, which had cultivated the poor highlands as well as used for transportation in rugged mountainous areas. These horses are medium-sized, with height at withers and chest circumference of approximately 130 and 176 cm, respectively. In addition, they possess traditional characteristics including dorsal stripes and knock-knee. (Takasu *et al.,* 2011). Moreover, most of the surveyed horses (92.8%) in 2011 had bayish coat color without white spots (Mukoyama, 2007; Takasu *et al.,* 2011). Furthermore, in these horses, various coat colors have been recorded, such as bay, black, chestnut, gray, and white. But, currently it has only three coat colors: bay, chestnut, and buckskin (Takasu *et al.,* 2011; Nakamura et *al.,* 2019).

Noma horse is a pony breed originating in Imabari, Ehime Prefecture, and is the smallest horse among Japanese native horses with the average withers height of 110cm to 120cm. The common coat color was gray in Edo period but nowadays it is mostly bay or chestnut. Moreover, it was bred actively due to its physical strength with a little vegetation and didn't require horse shoes for

carrying goods up to 70kg. Consequently, Noma was used for farming and conveyance *(*http://www.minnano jouba.com/mame_chishiki02_en.html*)*.

Taishu is another small Japanese native horse with 110 cm to 130 cm withers height that has been bred at Tsushima city, Nagasaki Prefecture. The original coat was black in color but nowadays bay or chestnut are more common. Like other Japanese native horses, the Taishu are quiet, withstand in the lean diet and strong legs with hard hooves that won't require horse shoes. Therefore, it has been used for farming, transporting woods, agricultural products and daily goods. *(*http://www.minnano-jouba.com/mame_chishiki02_en.html). Historical evidence shows the genetic introgression of the Anglo-Arabian into the Taishu during WWII, which caused doubt concerning the purity of the breed (Tezuka *et al.,* 2018)

Misaki population inhabits a limited meadow area in Toi Cape, Miyazaki Prefecture, which is located in southwestern Japan. This population is maintained under similar to those in the wild (Kaseda, 1981; Kaseda *et al.,* 1982) and phenotypic traits of the ancestral or 'pure' Japanese native breed are well maintained in this population (Kaseda, 1984). The bay and black-types are most commonly observed in coat color but now white markings and chestnut coat color also observed which had previously never been seen in Misaki horses due to gene flow from exotic breeds (Kaseda, 1984*)*. The wither height of Misaki horse ranges from 100cm to 120cm and weighting around 300kg, categorized in a mid-sized horse breed. It has a bold body with the large head, thin legs that were traditionally used for farming but not used as a riding horse. (http://www.minnano-jouba.com/mame_chishiki02_en.html*)*.

Tokara horse was confirmed in 1952 by Hayashida *et al.,* (1956) as a native breed on Takarajima, a small island at the southern end of the Tokara chain of islands in Japan. The Tokara horse appears to have originated from

approximately 10 unimproved horses introduced from Kikaijima , one of the Amami Islands near the Tokara Islands, in 1897 (Hyashida *et al.,* 1956). The Tokara is one of the smallest and pure breed horses in Japanese native horse breeds that stand from 100cm to 120cm with bay coat color. It is best known for their tolerance to heat and has been used for agriculture and conveyance. Nowadays it is simply grazed in this area. While other Japanese native horses have been used as a riding horse, there is no specific use for the Tokara at this moment. *(*http://www.minnanojouba.com/mame_chishiki02_en.html*).*

Miyako horse is a Japanese breed native to Miyako Island that is far southwestern region of Okinawa Prefecture. The horse is small-sized horses with 110–120-cm wither height and mostly bay or dun in color. (https://en.wikipedia.org/wiki/Miyako_horse). They have very hard hooves that can withstand the rough coral limestone trails on the island and can tolerate strenuous work even when provided a poor diet. Therefore, despite their small size, Miyako horses are valued by islanders as excellent workhorses, because of their docile and obedient nature. Moreover, Miyako horses were more commonly owned by the working class and became a popular means of transport among islanders. Since, Miyako horses could not meet the heavy demands of fieldwork for sugar industry and transport that expanded after World War II, Miyako horses were crossbred with western horses to improve their physiques, and the number of purebreds decreased rapidly (Senju *et al.,* 2017a).

Yonaguni horse is highly pure breed to Yonaguni Island in westernmost Japan, also called Okinawan breed. The horses are mostly bay colored and small, with a withers height of 110 to 120 cm. The Yonaguni horse was indispensable to life on the island, and each family on the island had at least one horse for transportation before World War II (Shinjo, 2010).

General Introduction

In spite of having small body size, diverse coat color, and suitable adaptability in harsh environmental condition as well as multiple utilities for different purposes; these breeds are facing considerable risks of extinction. Moreover, origin and ancestry of these populations are still in debate. But, these breeds can become important genetic resource for future genetic investigation. While the population sizes of these horses are markedly small, therefore, efforts must be taken to conserve the Japanese native horses. Since, conservation genetics is essential to understand the genetic diversity of an endangered species; various genetic indexes are used to assess the genetic diversity of a population, including microsatellite markers and mitochondrial DNA (Frankham *et al.* 2009). Genetic diversity is important in conservation as decreased genetic diversity associated with reduced fitness, diminished population growth and higher extinction risk by inbreeding, inbreeding depression, accumulating deleterious mutation and genetic drift. Various efforts have been taken to increase the genetic diversity so far including formation of conservation society in several breeds. But conservation genetics strategy, an interdisciplinary subfield of population genetics, that aims to understand the dynamics of genes in populations principally to avoid extinction, has not been taken. Specific genetic techniques are used to assess the genomes of a species regarding specific conservation issue as well as general population structure. Some of these techniques include the analysis of single nucleotide polymorphisms (SNPs) or mutations, mitochondrial DNA (mtDNA), and Y chromosome haplotypes have been applied on conservation genetics strategy. Since, native breeds may provide genetic resources of characteristics useful for adaptation to changing environments; efforts must be taken to conserve the Japanese native horses as well as other endangered horse.

Therefore, in this study mitochondrial D-loop region (mtDNA) and Y chromosome haplotypes for study of origin and ancestry of paternal, and

maternal lineage as well as allele frequencies and genotype distribution of genes regarding single nucleotide polymorphisms (SNPs) and mutation on physical performance, body conformation, locomotion trait, coat color, reproductive traits and hereditary disorders in Japanese native horse population were investigated.

The mtDNA and Y chromosome haplotypes result as well as genotype data of these genes in the Japanese native horses will be informative for future breeding and conservation programs.

1.2: OBJECTIVES

1. Analysis of mitochondrial DNA (mtDNA) and Y chromosome haplotypes in Japanese native horses.
2. A Study on genotyping of genes related to wither height, body conformation and locomotion traits in Japanese native horses.
3. A Study on genotyping of genes related to reproductive traits and hereditary disorders in Japanese native horses.
4. A Study on genotyping of genes related to coat color in Japanese native horses.

CHAPTER 2

Analysis of mitochondrial DNA (mtDNA) and Y chromosome haplotypes in Japanese native horses

2.1: INTRODUCTION

There are 8 breeds of horses native to Japan, all of them are in danger of extinction except Hokkaido, and there are calls for scientific evidence-based conservation of the breed. These horses are important not only as a unique genetic resource but also as a living asset that symbolizes the regional culture. Conservation of these horses holds much significance for building a more diverse society and for preserving regional identity. With the rapid progression of mechanization, the demand for horses disappeared and breeders of these horses undertook actions to conserve the breed. These efforts resulted in the recovery of the number of horses. However, there are still concerns about the various factors that can reduce diversity, including the harmful effects of inbreeding. It is necessary to conserve the diversity of theses horse. In conservation program, the maintenance of genetic diversity is a major objective; it is essential for a population to be able to face environmental changes in the future and to respond to long-term selection, either natural or artificial, for traits of economic or cultural interest (Frankham *et al.,* 2009)

In evolutionary biology, the diversity of mitochondrial DNA (mtDNA), in particular the D-loop region, is analyzed to assess the close relationship of the maternal lineage between breeds and within the species (Brown *et al.,* 1979; Harrison, 1989; Hutchison *et al.,* 1974) which is essential to optimize both conservation and utilization strategies. Furthermore, the rate of base substitution of mtDNA is 5- to 10- folds greater than that of nuclear DNA, which makes mtDNA an ideal target for analysis when determining inter and intra species

maternal relationship in evolutionary biology (Brown *et al.,* 1979). For suchpurposes, the mtDNA D-loop has been sequenced in various equids (Beja-Pereira *et al.,* 2004; McGahern *et al.,* 2006; Royo *et al.,* 2005; Prystupa *et al.,* 2012; Zhang *et al.*, 2012; Hristov *et al.,* 2016; Sziszkosz *et al.,* 2016; Cieslak *et al.,* 2010; Cieslak *et al., 2017*; Cozzi *et al., 2004*). The mtDNA analyses of ancient and modern domestic horses revealed that horse had multiple maternal origins (Vilá *et al.,* 2001; Lei *et al.,* 2009; Cieslak *et al.,* 2010). As a result, several researchers reported on origin ad ancestry of Japanese local populations that is still in debate. mtDNA as well as microsatellite markers analysis study (Kakoi *et al.,* 2007; Ishida *et al.,* 1995; Tozaki *et al.,* 2003) proposed origin of Japanese native horse from Mongolia but Hayashida (1958) proposed a two-wave migration hypothesis and reported Japanese native horses had been imported into Japan from southern China and Mongolia at different time periods.

In this study, diversity of the mtDNA D-loop region were analyzed and attempted to elucidate the maternal relationship of Japanese native horse population and compared genomic analysis data with previous study.

On other hand, mutations in the paternally transmitted portion of the Y chromosome can help to investigate paternal lineages. In contrast to the plenty of mutations in the mitochondrial genome and on the Y chromosome variation of pre-domestic horses (Lippold *et al.,* 2011), no diversity was detected in the Y chromosome of domestic horses awhile (Brandariz-Fontes *et al.,* 2013; Lindgren *et al.,* 2004). In the last few years, a few polymorphic sites were found in modern horses leading to a small number of haplotypes in contemporary domestic stallions (Ling *et al.,* 2010; Wallner *et al.,* 2013, 2017; Kreutzmann *et al.,* 2014, Felkel *et al.,* 2018, 2019; Han *et al.,* 2019).

Mitochondrial DNA (mtDNA) and Y chromosome haplotypes

Using high throughput sequencing technology Wallner *et al.,* (2013) identified the polymorphic Y-chromosomal markers useful for tracing paternal lines and concluded that the nucleotide variability of the modern horse Y chromosome is extremely low, resulting in six haplotypes (HTs), all clearly distinct from the Przewalski horse. Whereas, the most widespread haplotype 1 (HT1) is ancestral and the other five haplotypes apparently arose on the background of HT1 by mutation or gene conversion after domestication, and HT2 and HT3 are widely distributed at high frequencies among modern European horse breeds. As of the report of Kakoi *et al.,* (2018) three Y chromosome haplotypes have been observed in 159 male Japanese native horse populations by genotyping five Y-linked loci. Most of the Japanese native populations have the JHT-1 haplotype, which is widely distributed throughout Japan, and HT2, HT3 haplotypes observed in only few of these populations. Furthermore, they reported due to low Y chromosome haplotypes variation in Japanese native horse it was difficult to find origin of fixed patriline in each population and interpretation of the distribution of population by classifying them. But there is possibility of retaining more genetic variability in Japanese native horse. As Japanese local breeds, often from remote regions, are generally not intensively selected, they could also retain private variation already lost in strongly selected modern breeds. Autosomal, mtDNA , as well as Y chromosome haplotype studies on ancient horses, indicate more genetic variability in rural breeds (Lippold *et al.,* 2011; Warmuth *et al.,* 2012; Librado *et al.,* 2017). These results could not show much diversity, rather stated the fixation of each haplotype that influence independent breeding and genetic drift in each population and suggested that, updated technology may help in elucidating the origin of the fixed patriline in each population which could lead to an interpretation of the distribution of the populations of ancient Japanese native horses. Since, with the availability of a horse Y chromosome haplotype reference sequence and a suite of variants

screened from a wide range of horse breeds, it has become possible to construct relatively high resolution Y chromosome haplotypic genealogies of modern domestic horses (Wallner *et al.*, 2017; Felkel *et al.*, 2018, 2019; Han *et al.*, 2019). Using these resources and taking into account the retention of ancient Y chromosome variation in Asian male horses (Wallner *et al.*, 2017; Felkel *et al.*, 2018, 2019; Han et *al.*, 2019), in this study Y chromosome variation in Japanese native horse populations that may reveal a signature of ancient paternal variation, is now absent in many modern lineages were analyzed.

By genetic analyses of Japanese native horse population using the mtDNA and Y chromosome haplotypes, relationships of the maternal lineage between the eight populations of Japanese native horses, and fixed origin and relationship of patriline of these populations were revealed which will be informative for selection, breeding and future conservation of these populations.

2.2: *OBJECTIVES*

Analysis of mitochondrial DNA (mtDNA) and Y chromosome haplotypes in Japanese native horses.

2.3: *MATERIALS AND METHODS*

2.3.1: *MATERIALS FOR DNA ANALYSIS*

a) SAMPLING

A large number of genomic DNA samples of eight Japanese native horses were used for this study. These DNA samples were extracted from the blood samples of Japanese native horses (Fig 2.1) that are collected during 1971 to 1994 as a part of field research for Asian native livestock conducted by The Society for Research on Native Livestock (Nozawa *et al.*, 1998) and had been stored in freezer at -80°C. The extraction of DNA from these blood

samples was performed according to the standard phenol/chloroform method.

b) DNA EXTRACTION

Firstly, 0.2% Nacl was taken into 15 ml blood tube to wash blood cell. Then, blood filled tubes were centrifuged at 5000 rpm for 6-7 minutes at room temperature and removed supernatants, and kept the precipitates as well as repeated the 2-3steps until clear. 3000 µl TNESU-8 buffer and 80 µl Proteinase-K were taken into tube and incubate 37°C for overnight. 3000 µl mixture of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added in previous mixture and vortexed for 5-10 minutes, and cetrifuged the tube at 12000 rpm for 10 minutes at room temperature (16°C). Top layer was transferred into new tube and previous two steps were repeated, adding 3000 µl Chloroform: Isoamyl alcohol (24:1). Then, 2 volume of 100% ethanol was added in tube until 12-14 ml of tube and was converted gently. Collecting aggregates of DNA and transfer to 7 µl tube with 70% Ethanol. Finally, the tube was centrifuged at 12000 rpm for 5 minutes at 4°C temperature and ethanol was removed, dried for 10 minutes. Later, 5-200 µl of TE buffer were added in the tube and stored for future analysis.

c) PREPARATION OF DNA SAMPLE

To measure the nucleic acid concentration of DNA sample NanoDrop 2000 (Thermo Fisher Scientific, Waltham. Mass) was used and the DNA solution was adjusted to the required concentration with distilled water (DW).

2.3.2: *METHODS OF DNA ANALYSIS*

a) METHODS OF mtDNA ANALYSIS

To analysis the mtDNA from selected 183 samples, a 722 bp fragment of the D-loop region of mtDNA was amplified using a pair of primer F: CTAGCTCCACCATCAACACC and R: ATGGCCCTGAAGAAAGAACC.

PCR reaction were carried out in 10 µl reaction mixture containing 2.0 µl of genomic DNA (10 ng/ µl), 0.3µl of 0.2µM primers , 1.0 µl of 2.0 mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer, 0.1 µl (0.5 U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) for 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 60 sec, and extension at 72°C for 30 sec using Thermal Cycler Dice Touch (Takara Bio, Japan). Then PCR products were electrophoresed in 2-3% Agarose gel in TAE buffer at 135 volt 15-30 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator. PCR products of mtDNA D-loop were purified and prepared for sequencing according to Table 2.1. The obtained sequence data were aligned using MEGA7 and sequences were truncated to 15,494– 15,740 bps to accommodate published short sequences and finally generated a dataset in length of 247 bp, between 15494 and 15740 of reference mtDNA genome sequence X79547. Neighbor joining tree was made comparing with published data (Cieslak *et al.,* 2010). To estimate haplotype and nucleotide diversity DNA SP.5 Software was used.

b) METHODS OF Y CHROMOSOME HAPLOTYPES ANALYSIS

A set of male horse (81) representing eight Japanese native horse population were retained for Y chromosome haplotype analysis. The allelic states of 23 Y chromosome haplotype-indicative single nucleotide variants and one insertion/deletion variant (indel) were determined with LGC Kompetitive allele specific (KASP) assays (Fig 2.2) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories) according to Wallner *et al.,* 2017, Felkel *et al.*, 2018 and Felkel *et al.,* 2019). Detailed information for the haplotype-indicative markers is shown in Table 2.2. Based on the allelic states of these loci haplogroup clustering were inferred according to network identified by Felkel *et al.,* (2018), Han *et al.,* (2019), Wallner *et al,* 2017 and unpublished, by manually imputing the allelic states of 159 Markers. The

observed Japanese Y chromosome haplotypes were visualized using Median-Joining network generated with NETWORK 4.6.1.6 and by applying the Y chromosome haplotypes phylogeny generated by Felkel *et al.,* (2018) as a framework (Fig 2.4).

2.4.1: *RESULT AND DISCUSSION (mtDNA)*

In this study, mtDNA D-loop region of eight Japanese native horses were analyzed. As of the result, the sequences of the 247bp (15594- 15740) obtained from eight populations of Japanese native horses indicate the presence of 15 different haplotypes. Kiso have 10 haplotype with highest number as compared to Miyako, Misaki, Hokkaido, Taishu, Yonaguni, Noma and Tokara have 4, 3, 3, 2, 2,1 and 1 haplotypes, respectively. The haplotype and nucleotide diversity value were estimated 0 to 0.874 and 0 to 0.023, respectively, except the Noma and Tokara which revealed no diversity as it possessed single haplotype. Particularly, the haplotype and nucleotide diversity value were highest in Kiso horse (Table 2.3). Furthermore, Table 2.4 shows that, there are 30 segregating sites and six common (*JP-1, JP-2, JP-3, JP-4, JP-10* and *JP-14)* haplotypes among the populations, and the remaining haplotypes have unique for each haplotype, whereas some of Hokkaido, Kiso and Miyako possess new haplotype. I drawn a N-J tree to confirm the diversity of Japanese native horse comparing the results from the 87 haplotypes of ancient and modern diverse breeds of horses reported by Cieslak *et al.,* 2010 with Japanese native population and found about 44% of Japanese native horse cluster of *X3c1* haplotype which is regarded as ancient haplotype mostly found in modern horses. Furthermore, ancient lineage of haplotype *A,B1,X2,D2,D2d,K2* and X7a1 also observed in these populations (Table 2.5, Fig 2.3).

Mitochondrial DNA (mtDNA) and Y chromosome haplotypes

Ishida *et al.,* (1995) conducted a phylogenetic study on thoroughbreds, Japanese (Hokkaido) horses, Mongolian horses, and Przewalskii's horses using mtDNA D-loop region and suggested that the Asian horses were similar to each other and distinct from thoroughbreds.

Furthermore, Using 33 biochemical genetic loci and horses belonging to Japanese native horses, Asian as well as European horses Nozawa *et al.,* (1998) investigated the phylogenetic relationships between native and other horses, and concluded that native Japanese and Asian horses had descended from Mongolian horses. Evolution of Japanese native horses was inconsistent with the two-wave migration reported by Hyashida (1958).

Tozaki *et al.,* (2003) analyzed the genetic variation and phylogenetic relationship of Japanese native horses, Asian mainland horse as well as European horses using 20 microsatellite loci, and supported that Japanese horses originated from Mongolian horses through the Korean Peninsula as well as the genetic relationship of Japanese horses corresponded to their geographical distribution.

In addition, Kakoi *et al.,* (2007) reported on 318 horses from 11 populations including seven Japanese native horses and European light- and heavy-breed from 15437-15847 (411bp) of mtDNA D-loop region and stated 12 haplotypes with 33 variable sites, whereas Hokkaido, Kiso, Taishu and Misaki had three haplotypes, Noma and Yonaguni had two haplotypes, and Tokara had only one. There was no common haplotype across all populations. Furthermore, haplotypes and nucleotides diversity values were ranged from 0.14-0.62 and 0.001-0.010,respectively, except Tokara which revealed no diversity and analyzed haplotypes were distributed across six cluster including (A-F), and concluded that each Japanese native population was formed by the distribution across Japan of the founder populations derived from Mongolian horses and the

genetic construction of each population appears to have been derived from independent breeding in each local area and affected by drastic genetic drift in recent times that highlighting the evolutionary process for elucidation of ancestry.

Takasu *et al.,* 2014 reported on 136 Kiso horses based on 411 bp from 15,437 to 15,847 of mtDNA D-loop region and found that, number of haplotype, haplotype and nucleotide diversity were 7, 0.79 ± 0.01, and 0.017 ± 0.009, respectively. The results suggested the diversity of maternal lineage in the Kiso horse was reasonably maintained. Furthermore, they also suggested that various horses that came to Japan stayed at Kiso region and became ancestors of Kiso horse and also genetically supported the theory that the Kiso horse was historically improved by other Japanese native horse breeds. Finally, the distribution result suggested that diversity of maternal lineage would possibly be reducing in Kiso horses.

Senju *et al.,* 2017a reported on 78 Yonaguni horses by genotyping 32 microsatellites and 411 bp mtDNA D-loop from 15437-15847 and found that the average number of alleles, observed heterozygosity, and expected heterozygosity were 4.4, 0.591 and 0.601, respectively. In addition two mtDNA haplotypes were confirmed and suggested that genetic diversity of Yonaguni horses was not particularly low in comparison with that of other breeds that are at risk of extinction.

Senju *et al.,* 2017b reported on 35 Miyako horses by genotyping 32 microsatellites and 411 bp mtDNA D-loop from 15437-15847 and found that the average number of alleles, observed heterozygosity, and expected heterozygosity were 4.2, 0.701 and 0.649 respectively. In addition, one mtDNA haplotype was confirmed and suggested Miyako horses have experienced a recent genetic bottleneck.

Mitochondrial DNA (mtDNA) and Y chromosome haplotypes

Recently, Kobayashi *et al.,* 2019 reported on 77 Misaki horses by genotyping 32 microsatellites and 411 bp mitochondrial DNA D-loop from 15437-15847 and found that the average number of alleles, observed heterozygosity, and expected heterozygosity were 3.4, 0.509, and 0.497, respectively. Furthermore, three mtDNA haplotypes were confirmed and suggested that Misaki horses experienced a bottleneck, but it was neither severe nor recent.

A study on 207 ancient reminds and worldwide 1754 modern horse breeds by Cieslak *et al.,* (2010) was done based on the sample set ranged from Alaska and North East Siberia to Iberian Peninsula, and from late Pleistocene to modern times. They found 87 ancient haplotypes, 39 haplotypes were confirmed to survive in modern breeds including X2, D3, X2b, X3c1, I, F, B1 and A, and remaining 48 ancients haplotypes were extinct including B1a, *B1b*, B2, B3, C, C1, *D1*, *D2a*, D2b, *D2c*, D2f, *D3a*, *E1*, *G*, G2, Gx4a, H1b, I2a, J, K2b2, K3a1, *X1*, X2d, X2c, X3a, X3b, X3c1a, X3c2, X3d, X4, X5a, X6, X6a, X6b, X6c, X7, X7a2, X8, X8a, X9–12, X13, X14–15, X16 and X17. They concluded that, genetic variation in modern horses may be due to multiple origins, large number of female founder and large scale introgression of local lineages into domestic stock. Furthermore, suggested that, huge diversity of horse's mtDNA is not consequence of breeding but feature that already present in wild horse populations.

Therefore, according to this study number of haplotypes, nucleotides as well as haplotype diversity were more than previous study (Kakoi *et al.,* 2007; Takasu *et al.,* 2014; Senju *et al.*, 2017a, b; Kobayashi *et al.*, 2019), particularly in Kiso and Miyako indicating Japanese native horses are in alarming situation for conservation now. The population of these Japanese native horses has dramatically reduces in recent times and mostly reared for conservation purposes in some local areas. So, there might be possibility of

reducing genetic diversity due to inbreeding or genetic drift. Moreover, the sample of our study is relatively older than previously reported study.

Since, few Japanese native horse have common haplotype indicating that, they might be possesses common ancestor. According to the literature as well as previous reports Japanese horse populations are descended from Mongolian horses through the Korean peninsula and have spread all over Japan as there were no horses in Japan about 2,000 years ago and these populations localized to the particular areas in Japan, and affected by the gene flow of each other (Tozaki, 2003). Sharing of common haplotypes between Hokkaido and Kiso may be explained by Hokkaido were imported from the main island of Japan around the 15th century (Miyakami, 2006; Kondo, 2012), whereas Kiso lived in Main island of Kiso region, Nagano Prefecture, there might be possibility of gene flow in each other. While, sharing of common haplotypes of other horses are still unresolved. In addition, *X3c1* haplotypes sharing mostly in all of these populations may be explained by founder population of these native horses derived from same ancestor. Furthermore, the two Okinawan horse breeds, known to have originated during the reign of the Ryukyu Kingdom, have traditionally been known to have a close relationship which was later confirmed by Senju *et al.,* (2018) and stated that genetic relationship of the Okinawan horse breeds may be close, also suggested that, as origin of Tokara horses are geographically close to Okinawa. Therefore, it is possible that Tokara horses might be genetically close to the Okinawan horses.

Since, most of Japanese native horse clustered in *X3c1, A, B1, X2, D2, D2d, K2* and *X7a1* haplotype which is regarded as ancient haplotype mostly found in modern horses. Therefore, Japanese native horses might be possessed ancestral genetic features maternally.

2.4.2: *RESULTS AND DISCUSSION (Y CHROMOSOME HAPLOTYPES)*

In this study, allelic states of 24 Y chromosome haplotype-indicative markers were analyzed using KASP assays. As the result, total 8 Y chromosome haplotypes were detected among the 81 Japanese native horses (Fig 2.4). The number of horses exhibiting each Y chromosome haplotype and the percentage of the Y chromosome haplotypes in Japanese native horse populations are provided in Table 2.6. About 14% (n=11) of the Japanese native horses haplotypes were located at HAT, which lies at the root of the crown group (A-L-S-T) that includes modern horse breeds. All the horses from the Kiso, Misaki and some of Miyako populations have haplotypes Ta and Tb-d. About 36% (n= 29) of the Japanese native horse have haplotypes A and Ao-1. Where, about 4% among all horses and about 30% of Miyako horse have haplotype H, a haplotype recently reported by Felkel *et al.,* (2019). It is noteworthy that about 27% (n=22) of Japanese native horses were situated between the root nodes HAT and NRMIJYHAT (Fig 2.4), in particular about 22% (n=18) of horses had RMIJYHAT haplotypes, a root from M to R, whereas R has been dated to little earlier than N, a node has been dated to 1400 Year before Present (YBP) (unpublished). Furthermore, Yonaguni (n=3), Miyako (n=1) had MIJYHAT haplotype root between HAT to M group including I, J and Y. This findings indicate that, one-third of Japanese native horses Y chromosome lineages have roots as old as earlier of 1400 YBP

Wallner *et al.,* (2017) resolve the Y chromosome genealogy of modern horses by screening 1.46 Mb of the male-specific region of the Y chromosome (MSY) in 52 horses from 21 breeds based on highly accurate pedigree data and estimated the de novo mutation rate of the horse MSY, and showed that various modern horse Y chromosome lineages split much later than the domestication of the horses. Furthermore, they showed that apart from few private Northern European haplotypes (N and I, time of most

recent common a ncestor dated to about 1400 years), all modern horse breeds clustered together in a roughly 700-year-old crown haplogroup (A-L-S-T) that was transmitted to Europe by the import of Oriental stallions, that consisted of two major subclades: the Original Arabian lineage (A) and the Turkoman horse lineage (T), whereas English Thoroughbred MSY was derived from the Turkoman lineage, and showed that English Thoroughbred sires are largely responsible for the predominance of this haplotype in modern horses. Furthermore, Iberian origin horses contain haplogroups L and S also fall in A-L-S-T crown group also (Fig 2.5)

Recently Felkel *et al.*, (2018) analyzed additional 52 horses from Five European breeds and Seven Asian breeds in addition to previous sample reported by Wallner *et al.*, 2017 and found additional variants and 42 haplotypes in domestic horses. All European horses fall in previous defined A-L-S-T crown group. Moreover, Out of new variants, some were detected solely in Asian breeds, resulting in total eight private haplotype. Only seven of the 13 Asian samples fall into the crown group, out of which three introduced private haplotypes (C, Ta, Ao-m). Three Asian samples represent private haplotypes (Y, J and M dated to 1062±288.72 and 1232.56±332.58, respectively Year before present (YBP), all branching off after the Northern European group (dated 1385.1±333.23 YBP). The remaining three Asian horses group far outside the previous MSY phylogeny and support a new haplogroup (HG-O) (Oa,Ob,Oc) defined by new variants. HG-O introduces a deep, 5527 ± 872-year old split from the rest of the lineages. They concluded that, Asian horses from rural breeds significantly increase Y chromosome haplotype diversity and have private haplotype that deepen the Y chromosome phylogeny suggesting that these horses have been less subjected to demographic force and, hence, have retained autochthonous variation. Moreover, Asian horses form crown haplotypes phylogeny suggesting that Asian crown haplotypes comprised of

intensively bred breeds from the Asian continent, hence waves of migration and founder effects have led to a severe reduction of haplotype diversity in modern breeds (Fig 2.6).

Han *et al., (*2019) reported on male horses representing five Chinese Mongolian populations for 43 Y chromosome haplotype-indicative single nucleotide variants and one insertion/deletion variant by KASP assay and found 11 haplotypes. Furthermore, 38% of the Chinese Mongolian horse haplotypes were located at the roots of branches outside of previously reported crown group, whereas remaining fall in crown group indicating that most of the Chinese Mongolian horses carried modern haplotypes as a direct result of recent attempts at breed improvement and authentic Chinese Mongolian horses appear to have retained an ancient signature of paternal lineages that has not previously been described in extant horse populations.

Recently Felkel *et al.,* (2019), analyzed Y-chromosomal haplotype variants in 130 intensively selected and rural breeds, and nine Przewalski's horses. Among domestic horses they confirmed the presence of dominance of a crown haplogroup in central European and North American breeds, and they also distinguished 58 haplotypes based on 211 variants, forming three major haplogroups within crown group. In addition to previously identified two haplogroups, A and T observed in Arabian/Coldblooded and Thoroughbred horses, respectively, they characterized a third haplogroup H, observed in Iberian lines and a North African barb horse, previously defined as LS and C group. Furthermore, they resolved a historic controversy of English Thoroughbred founder stallions distinguishing the patrilines and suggested that, just after domestication of horses in 5,500 years ago, two instant radiations in the history of Central and Northern European Y-chromosomal lineages have occurred.

Since in this result, haplotypes were widely distributed regarding crown and out crown group, indicating that diverse male lineages have been incorporated into present day Japanese native horses. Despite the broad coverage of haplotype, a large portion of these population falls in root node HAT indicating Japanese native horses may harbour male lineage diversity that could not be identified into the haplotypes of modern European horse. The presences of Ta and Tb-d haplotype in kiso, Misaki and Miyako population can be explained by introduction of western exotic breeds in these populations for improvement program (Ishikawa 1984; Obata T; Kaseda 1984). Particularly, Misaki, horses were almost exclusively within the Ta clade is in agreement with recent finding that a stallion named Komatsu-go that descended from the foreign Trotter breed was once introduced into the Misaki population in 1913 (Kaseda, 1984). Furthermore, this result was confirmed by previous study from Kakoi *et al.,* (2018). The presences of haplotypes A and Ao-1 at a large number in Japanese native horse population indicating influence of Arabian horse, as it is regarded as Japanese native horses were originated from Mongolian horse, since A haplotypes arose in Chinese Mongolian horse; a horse populations that supposed to originated in Arabian Peninsula (Han *et al.,* 2019). Moreover, presences of A haplotype in Taishu may be explained by genetic introgression of the Anglo-Arabian into the Taishu during WWII (Tezuka *et al.*, 2019). Furthermore, the presences of haplotypes H in Miyako horse may be explained by influence of Iberian or North African Barb horse.

In addition, one-third of Japanese native horses have unique haplotypes which predicted to separate from the root of the group regarded as modern horse groups or those separate from root of phylogenetic tree of domestic horse at earlier ages. Furthermore, the presence of both unique haplotypes and modern influenced haplotypes in Tokara, Yonaguni and Miyako horses can be explained by the common paternal origin of these population which was explained by

Senju *et al.,* (2018) reported that, two Okinawan horse breeds are genetically close to each other, since both of these population originated during the reign of the Ryukyu Kingdom. Furthermore, origin of Tokara horses are geographically close to Okinawa therefore, it is possible that Tokara horses might be genetically close to the Okinawan horses. Therefore, this statement may be confirmed from the study of Hyashida (1958), who reported two wave of migration of Japanese horses, either from Mongolia or from Southern China.

2.5: *CONCLUSION*

It can be concluded that, the genetic diversity of Japanese native horses are reducing due to small population size with the advancement of time. Haplotypes of mtDNA D-loop region are ancestral and after transported of these populations in Japan they have been affected by gene flow of each other and few populations may be possess common ancestor. This information will be helpful for future breeding and conservation of Japanese native horse population. Some of these horses carried modern Y chromosome haplotypes as a direct result of recent attempts at breed improvement and some Japanese native horses appear to have retained an unique signature of paternal lineages that has not previously been described in these horse populations. This is the first report on Japanese native horses have unique ancestral Y chromosome haplotypes.

These findings suggested that the Japanese native horse populations have retained ancestral genetic features in both maternal paternal lines.

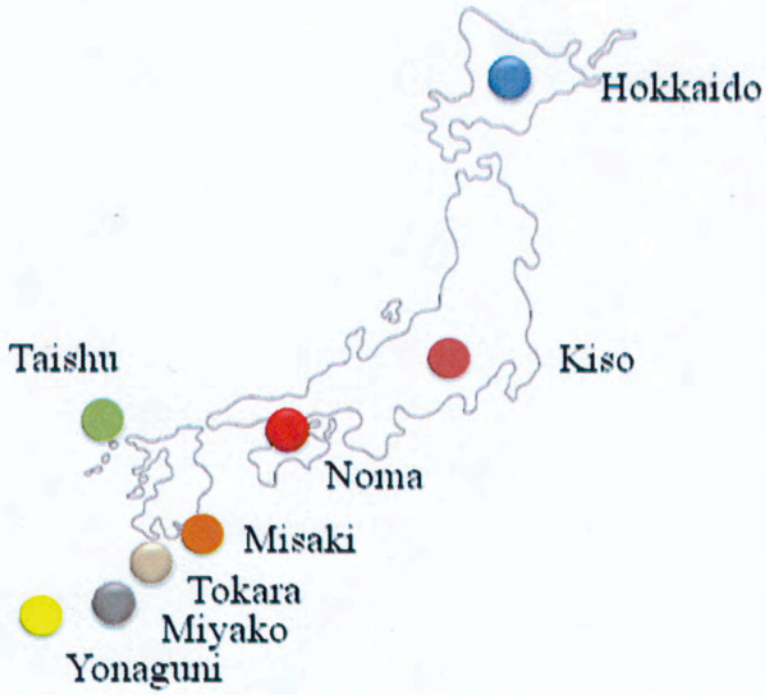


Fig. 2.1: Geographical location of 8 Japanese native horse populations

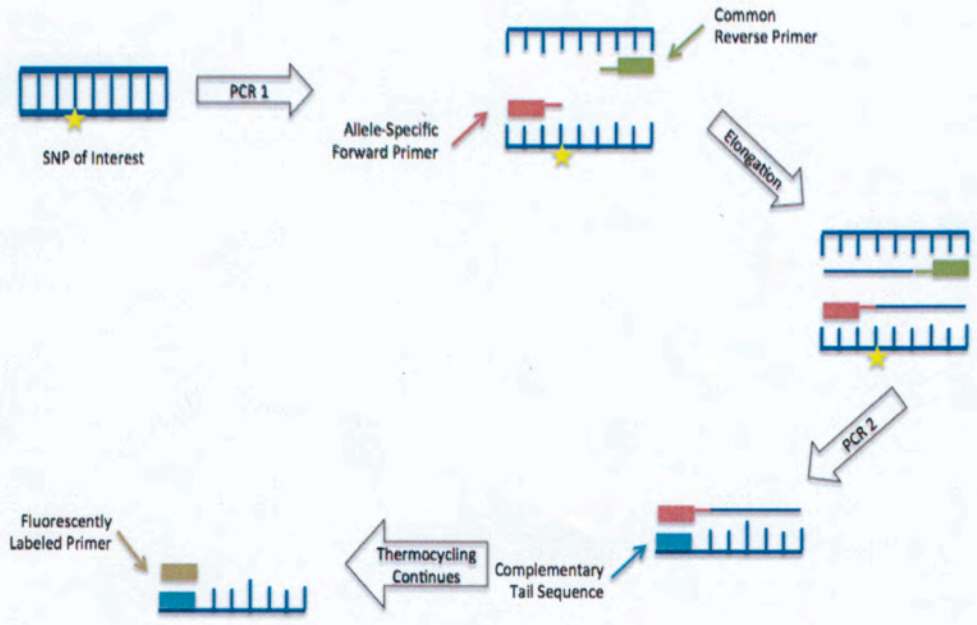


Fig. 2.2: Schematic drawing of kompetitive allele specific PCR (KASP)

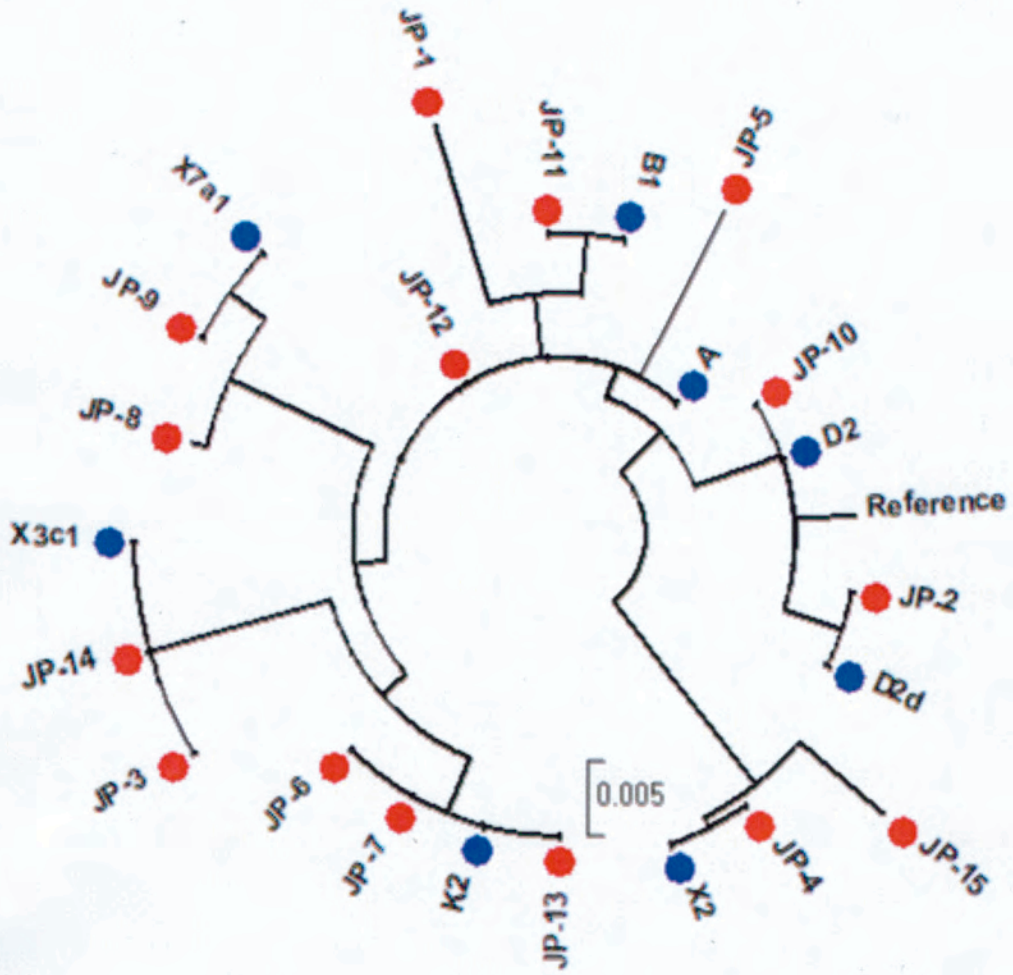


Fig. 2.3: Neighbour-Joining (N-J) tree based on 247 bp of mtDNA D-loop region in Eight Japanese native horse breeds including reference from Cieslak et al., 2010 (Bootstrap value 1000); Red circle indicated Japanese native horse haplotype and Blue circle indicated obtained haplotype from Cieslak et al., 2010.

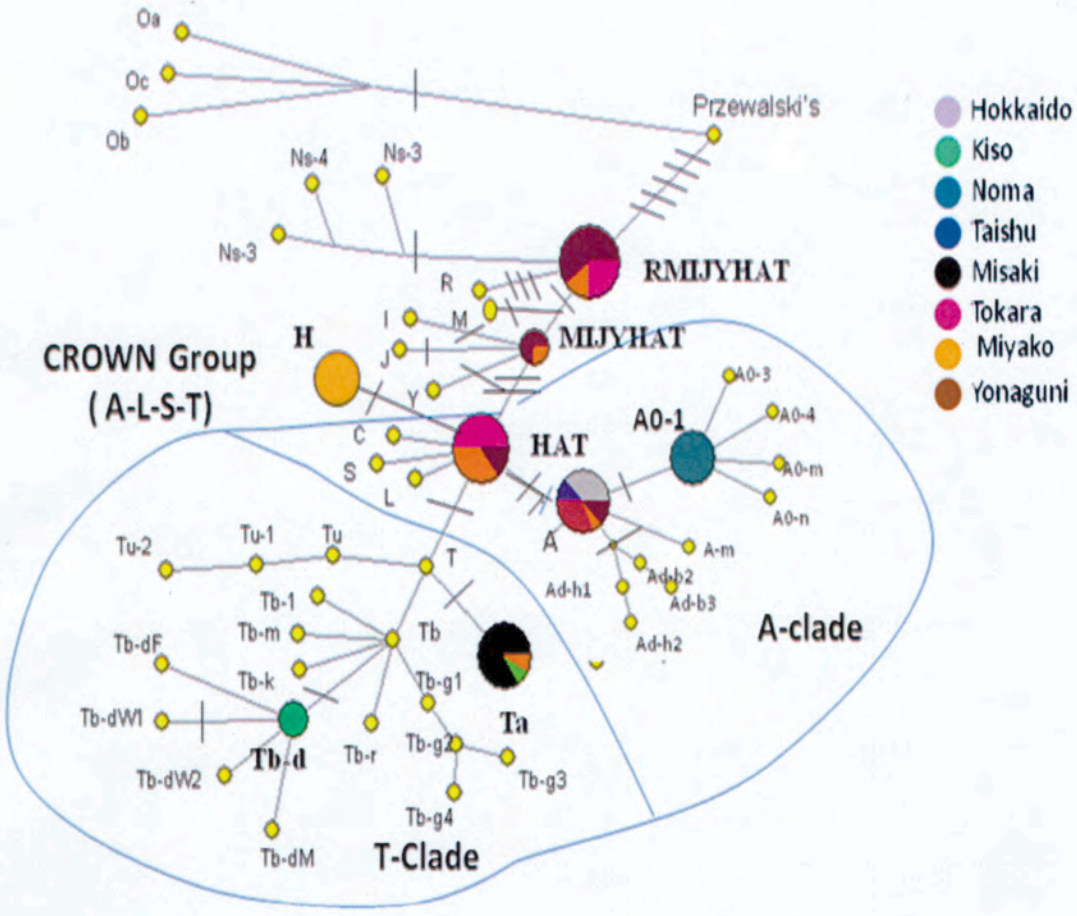


Fig. 2.4: Y chromosome haplotypes network for 129 male horse (81Japanese native horse, 48 domestic horse and Przewalski's from Felkel et al.,2018, 2019 and Wallner et al.,2017). The network is rooted with Przewalski's horse. Highlighted haplotypes found in this study and hash marks along branches correspond to number of mutation studied.

Mitochondrial DNA (mtDNA) and Y chromosome haplotypes

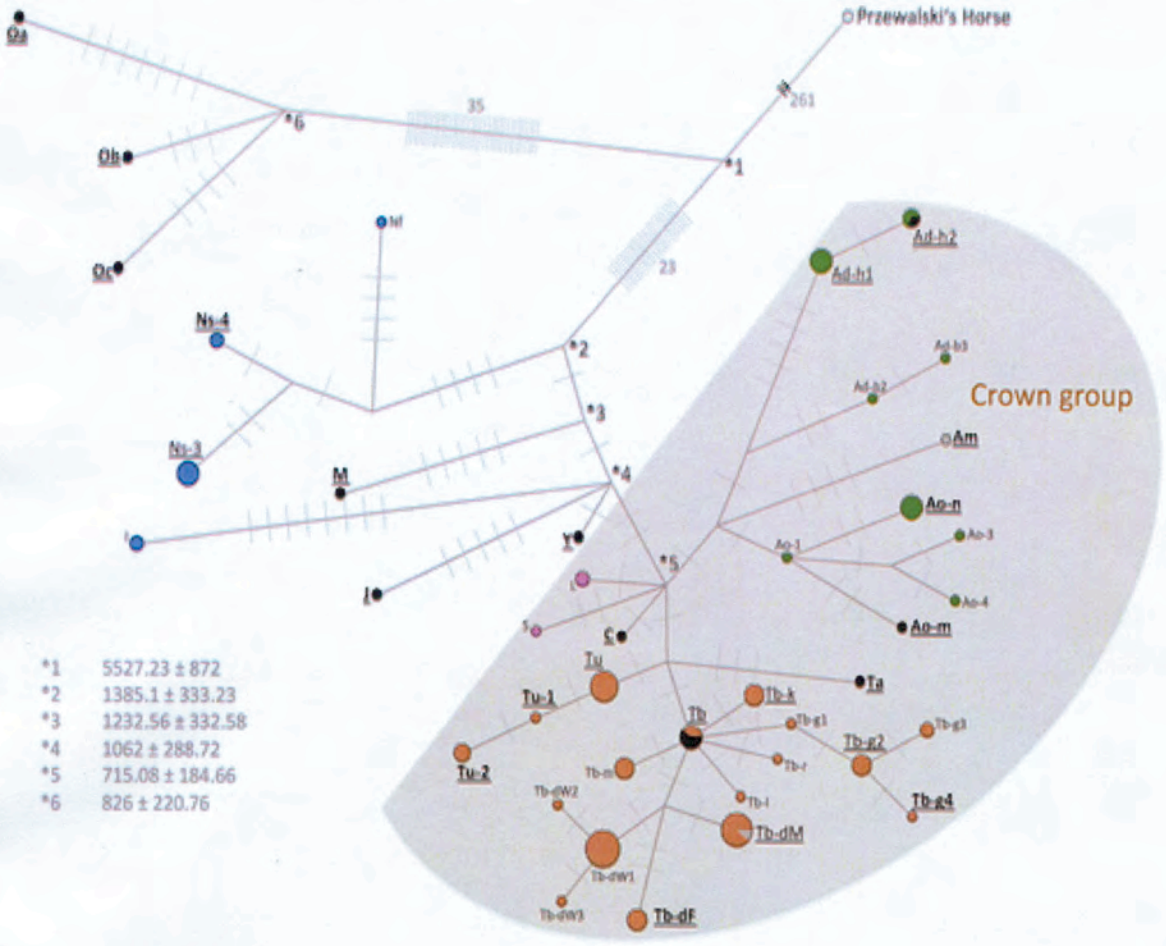


Fig. 2.5: Y chromosome haplotype network of male horses, 52 horses of 21 breeds from Wallner et al., 2017, 52 horses from five European and seven Asian breeds from Felkel et al., 2018 and a Przewalski's horse. Hashmarks along branches correspond to number of mutations; the number of mutations is shown for long branches. Asian samples are shown in black, American samples in grey and Northern European samples in blue. Haplotypes within the shaded area are part of the crown group (ALST) previously defined by Wallner et al., 2017. Haplogroup (HG) A is shown in green, HGs L and S in pink and HG T in orange. Haplotypes detected in the Felkel et al., 2018 appended dataset are underlined, and new haplotypes defined in this study are bold. Datings of nodes (marked by arterisks) and standard deviation were calculated by assuming a generation time of 7 years. The network is rooted with the Przewalski's Horse.

Table 2.1: PCR product preparation for purification and sequencing

Purification		**Sequencing (Genewitz)**		**Sequencing (Eurofins DNA)**	
PCR product	5.00 µl	PCR product	2.00 µl	PCR product	2.50 µl
EXOSAP-IT	0.50µl	Primer (F)	0.50µl	Primer (F)	0.96µl
DW	3.50 µl	DW	12.50µl	DW	17.54µl
Total	9.0 µl	Total	15.0 µl	Total	15.0 µl
Incubation Program for purification: 37 °C for 30 min 80 °C for 15 min 4°C for 5 min					

Mitochondrial DNA (mtDNA) and Y chromosome haplotypes

Table 2.2: Haplotype with indicative Markers (According to Wallner et al, 2017, Felkel et al, 2018 and 2019)

Markers	rA (T)	rAF (A)	rAO (C)	rAX (C)	rBF (G)	rBG (CTT)	rC (C)	rD (-)	rW (A)	rX (T)	sAA (C)	sAZ (T)	sEB (A)	sEE (G)	sHE (G)	sEN (G)	sES (G)	sQ (C)	sCO (C)	sPZ (A)	fTO (C)	qBG (C)	qDL (G)	qCA (A)
Haplotype	**T**	**Ad**	**I**	**HAT**	**YIJHAT**	**MYIJHAT**	**Tb-d**	**Tb-dW1**	**A**	**Ao-1**	**M**	**J**	**DW**	**DW**	**DW**	**DW**	**DW**	**Y**	**O**	**Ta**	**H**	**R***	**R***	**R***

* Unpublished and DW= Domestic Western

Table. 2. 3: Comparisons of number of haplotypes, haplotype and nucleotide diversity of mtDNA D-loop region within different Japanese native horse populations with previous reports.

Population	No. of Samples	No. of Haplotypes					Haplotype diversity (*h*)			Nucleotide diversity (π)		
		This study	Kakoi *et al.* 2007	Takasu *et al.*, 2014	Senju *et al.*,2017	Kobayashi *et al.*, 2019	This study	Kakoi *et al.*, 2007	Takasu *et al.*, 2014	This study	Kakoi *et al.*, 2007	Takasu *et al.*,2014
Hokkaido	30	3	3	-	-	-	0.544	0.14	-	0.020	0.004	-
Kiso	27	10	3	7	-	-	0.874	0.62	0.79 ± 0.01	0.023	0.009	0.017 ± 0.009
Noma	30	1	2	-	-	-	0.000	0.26	-	0.000	0.007	-
Taishu	35	2	3	-	-	-	0.330	0.43	-	0.010	0.010	-
Misaki	26	3	3	-	-	3	0.50	0.28	-	0.011	0.006	-
Tokara	30	1	1	-	-	-	0.000	0.00	-	0.000	0.00	-
Miyako	22	4	-	-	1	-	0.593	-	-	0.010	-	-
Yonaguni	22	2	2	-	2	-	0.415	0.53	-	0.001	0.001	-
Total	222	15	12				0.846			0.021		

Table 2. 4: Haplotypes of the mitochondrial DNA D-loop region and their distribution in Japanese native horses compared with reference sequence X79547 (15494-15740bps), Kakoi et al.,2007 and Takasu et al.,2014

15494	15495	15496	15521	15534	15538	15542	15585	15596	15597	15598	15600	15602	15603	15604	15615	15616	15617	15635	15649	15650	15657	15659	15666	15703	15709	15720	15723	15737	15740	Hokkaido	Kiso	Noma	Taishu	Misaki	Tokara	Miyako	Yonaguni	Haplotype	Kakoi *et al.*,2007 haplotype	Takasu *et al* .,2014 haplotype
T	***T***	***A***	***G***	***C***	***A***	***C***	***G***	***A***	***A***	***T***	***G***	***C***	***T***	***G***	***A***	***A***	***T***	***C***	***A***	***A***	***T***	***T***	***G***	***T***	***C***	***G***	***C***	***T***	***A***	**Reference X79547**										
-	C	-	A	-	-	-	-	G	-	-	-	T	-	-	-	-	C	-	-	-	-	-	-	-	-	A	-	C	-	7						1		JP-1	H39	-
-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	4	1							JP-2	H3	K6
-	C	-	-	-	-	T	-	-	G	-	-	T	-	-	-	-	-	T	-	G	-	-	A	C	-	A	-	-	-	19	2			5		12	16	JP-3	H10	-
C	C	G	-	T	-	-	A	-	-	-	-	T	C	-	-	-	-	-	G	-	-	-	-	-	-	A	-	-	-		2		7					JP-4	H28	-
-	C	-	-	-	G	-	A	-	-	-	A	T	-	-	-	-	-	-	-	G	-	-	-	-	T	A	-	-	-		3							JP-5	H14	K5
-	C	-	-	-	-	-	-	-	-	-	-	T	-	A	-	-	-		-	-	-	-	-	C	-	A	-	-	G		6							JP-6	H47	K2
-	C	-	-	-	-	-	A	-	-	-	-	T	-	A	-	-	-	-	-	-	-	-	-	C	-	A	-	-	G		1							JP-7	H46	K1
-	C	-	-	-	-	-	-	-	-	C	-	T	-	-	G	G	-	-	-	-	-	-	-	-	-	A	-	-	-		1							JP-8	-	-
-	C	-	-	-	-	-	-	-	-	C	-	T	-	-	G	G	-	-	-	-	-	-	-	C	-	A	-	-	-		3							JP-9	-	K3
-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-		7		28	3				JP-10	H1,2	K4
-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	C	-	-	-	-	C	-	-	-	A	-	-	-		1							JP-11	H18	K7
-	C	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	G	-	-	-	-	-	A	-	-	-			30						JP-12	H5	-
-	C	-	-	-	-	-	-	-	G	-	-	T	-	A	-	-	-	-	-	G	-	-	-	C	-	A	-	-	G					18				JP-13	H44	-
-	C	-	-	-	-	T	A	-	G	-	-	T	-	-	-	-	-	T	-	G	-	-	A	C	-	A	-	-	-						30	8	6	JP-14	H12	-
C	C	G	-	T	-	-	A	-	-	-	-	-	C	-	-	-	-	-	G	-	C	-	-	-	-	A	-	-	-							1		JP-15	-	-
Total= 222																														30	27	30	35	26	30	22	22			

Table. 2. 5: Haplotypes of the mitochondrial DNA D-loop region and their distribution in Japanese native horses compared with Cieslak et al., 2010 (Excluding hotspot region 15585,15597,15604,15650)

15494	15495	15496	15521	15534	15538	15542	15585	15596	15597	15598	15600	15602	15603	15604	15615	15616	15617	15635	15649	15650	15657	15659	15663	15703	15709	15720	15723	15737	15740	JP haplotype	Hokkaido	Kiso	Noma	Taishu	Misaki	Tokara	Miyako	Yonaguni	Cieslak et al.,2010 haplotypes	Haplotype (%)
T	*T*	*A*	*G*	*C*	*A*	*C*	*G*	*A*	*A*	*T*	*G*	*C*	*T*	*G*	*A*	*A*	*T*	*C*	*A*	*A*	*T*	*T*	*G*	*T*	*C*	*G*	*C*	*T*	*A*	Reference X79547										
-	C	-	A	-	-	-	-	G	-	-	-	T	-	-	-	-	C	-	-	-	-	-	-	-	-	A		C		JP-1	7						1		-	3.60
-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	JP-2	4	1							D2d	2.25
-	C	-	-	-	-	T	-	-	G	-	-	T	-	-	-	-	-	T	-	G	-	-	A	C	-	A	-	-	-	JP-3,14	19	2			5	30	20	22	X3c1	44.14
C	C	G	-	T	-	-	A	-	-	-	-	T	C	-	-	-	-	-	G	-	-	-	-	-	-	A	-	-	-	JP-4		2		7					X2	4.05
-	C	-	-	-	G	-	A	-	-	-	A	T	-	-	-	-	-	-	-	G	-	-	-	-	T	A	-	-	-	JP-5		3							-	1.35
-	C	-	-	-	-	-	-	-	-	-	-	T	-	A	-	-	-	-	-	-	-	-	-	C	-	A	-	-	G	JP-6,7,13		7			18				K2	11.26
-	C	-	-	-	-	-	-	-	-	C	-	T	-	-	G	G	-	-	-	-	-	-	-	-	-	A	-	-	-	JP-8		1							-	0.45
-	C	-	-	-	-	-	-	-	-	C	-	T	-	-	G	G	-	-	-	-	-	-	-	C	-	A	-	-	-	JP-9		3							X7a1	1.35
-	C	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	JP-10		7		28	3				D2	17.11
-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	C	-	-	-	-	C	-	-	-	A	-	-	-	JP-11		1							B1	0.45
-	C	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	G	-	-	-	-	-	A	-	-	-	JP-12			30						A	13.51
C	C	G	-	T	-	-	A	-	-	-	-		C	-	-	-	-	-	G	-	C	-	-	-	-	A	-	-	-	JP-15							1		-	0.45
Total=222																															30	27	30	35	26	30	22	22		

Table 2.6: Y chromosome haplotype distributions in Japanese native horses (%)

Population	N	Crown group						Out Crown group	
		T		A		H	HAT	NRMIJYHAT root	
		Tb-d	Ta	Ao-1	A			RMIJYHAT	MIJYHAT
Hokkaido	7	**0**	**0**	**0**	**7 (100)**	**0**	**0**	**0**	**0**
Kiso	4	3 (75)	1 (25)	0	0	0	0	0	0
Noma	12	0	0	12 (100)	0	0	0	0	0
Taishu	2	0	0	0	2 (100)	0	0	0	0
Misaki	11	0	11 (100)	0	0	0	0	0	0
Tokara	17	0	0	0	5 (29.41)	0	7 (41.18)	5 (29.41)	0
Miyako	10	0	1 (10.0)	0	1 (10.0)	3 (30.0)	2 (20.0)	2 (20.0)	1 (10.0)
Yonaguni	18	0	0	0	2 (11.11)	0	2 (11.11)	11 (61.10)	3 (16.66)
Total	81	3 (3.70)	13(16.04)	12 (14.81)	17 (21.00)	3 (3.70)	11 (13.58)	18 (22.22)	4 (4.94)
		16 (19.75)		29 (35.82)				22 (27.16)	

CHAPTER 3

A Study on genotyping of genes related to wither height, body conformation and locomotion traits in Japanese native horses

3.1: *INTRODUCTION*

The horses are historically most important domestic animal for human life, culture and civilization, since they are essential for agriculture, transportation and warfare. For this purpose horses have mainly served people by their physical performance. So, horse breeds have been intensively selected and actively bred for traits related to physical performance, such as body composition and locomotion traits and many different breeds with different physical traits, such as muscular power, stamina, pattern of locomotion and body composition, have been established by selection (Petersen *et al.,* 2013a). But the role of horses are less important today, except those used for racing and recreational riding, since they are being replaced by artificial machines. Therefore, numbers of horses are dramatically reduced. As a result, most of local horse populations are facing extinction and genetic diversity of worldwide horse populations expected to be reduced. Japanese native horses with small body size have been raised for drafting, packing and riding utilities in transportation, agriculture and military purpose. Particularly, Hokkaido, Miyako and Yonaguni population of Japanese native horses have historical evidence of naturally occurring pace capability which is regarded as important traits for riding horse as well as important for future genetic research, which adapted them for specific environment. Due to dramatic reduction of these horse populations except Hokkaido, remaining horses are raised for conservation purpose in several location of Japan. So, these populations are in risk

of extinction. Although several conservation society already have been formed to conserve these populations for rational breeding and conservation program, assessing genetic characteristics regarding physical performance, body conformation and locomotion traits is our prime concern. Therefore, in this study genes associated with wither height; body conformation as well as locomotion traits were genotyped.

As a result of recent advances in molecular genetic analysis of horse including genome-wide association studies (GWAS) various polymorphisms of the genes involved in Physical performance, body conformation and locomotion traits have been identified in genetically diverse horses breeds (Hill *et al.,* 2010; Tozaki *et al.,* 2010; Andersson *et al.,* 2012; Makvandi-Nejad *et al.,* 2012; Signer-Hasler *et al.,* 2012), and have revealed that allele frequencies of these genes significantly differ among breeds with different purposes, such as breeds for racing, riding and draughting (Makvandi-Nejad *et al.,* 2012; Petersen *et al.,* 2013b; Promerová *et al.,* 2014). Of those, the ligand-dependent nuclear receptor compressor-like (*LCORL*), Zinc finger and AT hook domain containing (*ZFAT*), High-mobility group AT-hook 2 *(HMGA2),* LIM and SH3 domain protein 1 (*LASP1),* Myostatin (*MSTN*) and doublesex and mab-3-related transcription factor 3 gene (*DMRT3*) genes associated with horse wither height, body conformation and locomotion traits are of special interest since increased characteristics of this traits results in increased performance of horses.

Therefore, we genotyped Single nucleotide polymorphism (SNP) of the *LCORL* g.105547002 C>T, *ZFAT* g.75550059 C>T, *LASP* g. 23259732 A>G, *HMGA2* g. 81481064 C>T genes which are associated with withers height of the horse (Fig 3.1) (Makvandi- Nejad *et al.*, 2012; Signer-Hasler *et al.*, 2012). The SNP of *MSTN* g.66493737C>T gene is associated with racing performance and body composition of racehorses (Hill *et al.*, 2010; Tozaki *et al.*, 2010), and

a nonsense mutation of the *DMRT3* g.22999655 C>A gene has major effects on the locomotion pattern of the horse (Fig 3.2) (Andersson *et al.,* 2012, Han *et al.,* 2015; Promerova *et al.,* 2014).

The genotype data of these genes in the Japanese native horses will be informative for future breeding and conservation programs to increase the physical performance of local populations of Japanese native horses.

3.2: *OBJECTIVES*

A Study on genotyping of genes related to wither height, body conformation and locomotion traits in Japanese native horses.

3.3: *MATERIALS AND METHODS*

3.3.1: *MATERIALS FOR DNA ANALYSIS*

SAMPLING

After extraction of DNA from blood shown in Chapter 2, 247 samples were randomly choosed from the eight Japanese native horse population and nucleic acid concentration of DNA samples were measured for analysis (shown in Chapter 2).

3.3.2: *METHODS OF DNA ANALYSIS*

a) POLYMERASE CHAIN REACTION (PCR)

For genotyping of functional genes that are associated with Physical performance, body conformation and locomotion in Japanese native horses, were amplified using PCR. PCR reaction were carried out for genes *LCORL* g.105547002 C>T, *ZFAT* g.75550059 C>T, *HMGA2* g. 81481064 C>T, *LASP1* g. 23259732 A>G, *MSTN* g.66493737C>T, and *DMRT3* g.22999665C>A, in 10 µl reaction mixture containing 2.0 µl of genomic DNA (10 ng/ µl), 0.3µl of

0.2µM primers , 1.0 µl of 2.0 mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer, 0.1 µl (0.5U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) and distilled water (DW). Primer pairs, target length and PCR conditions used for genotyping shown in Table 3.1 and Table 3.2 and for reaction PCR Thermal Cycler Dice Touch (Takara Bio, Japan) used. Then PCR products were electrophoresed in 2-3% Agarose gel in TAE buffer at 135 volt 15-30 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator.

b) POLYMERASE CHAIN REACTION AND FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

PCR products of *LCORL* g.105547002 C>T, *ZFAT* g.75550059 C>T, *LASP1* g. 23259732 A>G, *MSTN* g.66493737C>T, *DMRT3* g.22999665C>A, genes were incubated in incubator/thermal cycler for enzyme digestion using enzymes (New England Biolabs, Tokyo, Japan) shown in Table 3.3. After complete enzyme digestion, digested products were electrophoresed in 2-3% Agarose gel/ Nusieve gel in TAE buffer at 135 volt 20-40 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator and genotyped according to Table 3.3.

c) *DNA PURIFICATION AND SEQUENCING*

PCR products of *HMGA2* g. 81481064 C>T was purified and prepared for Sanger Sequencing (see Chapter 2, Table 2.1).

c) *GENOTYPING OF FUNCTIONAL GENES*

Obtained result from PCR/ PCR-RFLP and sequencing were analyzed. In addition, Hardy-Weinberg equilibrium (HWE) equation was calculated to observe the genotype distribution.

3.4: *RESULT AND DISCUSSION*

In this study, we investigated the SNPs of the genes associated with physical performance, body conformation and locomotion traits including *LCORL, ZFAT, HMGA2, LASP1, MSTN* and *DMRT3* genes in Japanese native horses by PCR-RFLP and/or direct sequencing. As the results of the genotyping, both two alleles of *ZFAT* g.75550059 C>T, *HMGA2* g. 81481064 C>T, *LASP1* g. 23259732 A>G, and *MSTN* g.66493737C>T were observed in the all population of Japanese native horse breeds except for Misaki in *ZFAT* g.75550059 C>T and Noma in *MSTN* g.66493737C>T and *LASP1* g. 23259732 A>G were mono allelic. Similarly, both two alleles of *LCORL* g.105547002 C>T in Hokkaido, Noma, Miyako and Kiso and of *DMRT3* g.22999665C>A in Hokkaido, Miyako and Yonaguni were observed, whereas in remaining of breeds were mono allelic for this two genes. The genotype distributions of these SNPs in the eight populations were not significantly different from hardy Weinberg Equilibrium, with few exceptions (Table 3.4, Table 3.5 and Table 3.6).

While average minor allele frequencies of *LCORL* g.105547002 C>T, *ZFAT* g.75550059 C>T, *HMGA2* g. 81481064 C>T, *LASP1* g. 23259732 A>G, *MSTN* g.66493737C>T and *DMRT3* g.22999665C>A in the population of Japanese native horse are small, being 0.03, 0.22, 0.21, 0.16, 0.07 and 0.03, respectively, some of these populations showed relatively medium frequencies of minor alleles (Table 3.4, Table 3.5 and Table 3.6). In particular, the frequency of minor allele of *LCORL* g.105547002 C>T (for Miyako), *ZFAT* g.75550059 C>T (for Hokkaido, Noma, Yonaguni, Taishu), *HMGA2* g. 81481064 C>T (all horses) and *LASP1* g. 23259732 A>G (for Hokkaido, Miyako, Taishu, Kiso),

MSTN g.66493737C>T (for Miyako, Kiso) and *DMRT3* g.22999665C>A (for Hokkaido) is medium (Table 3.4, Table 3.5 and Table 3.6).

LCORL is thought to encode a transcription factor associated with measures of skeletal frame size and adult height in humans and several animals (Gudbjartsson *et al.,* 2008; Weedon *et al.,* 2008 Hirschhorn *et al.,* 2009, Lettre *et al.,* 2011; Lindholm-Perry *et al.,* 2011; Soranzo *et al.,* 2009; Takasuga *et al.,* 2016) and later it was reported to be associated with height of horses (Makvandi-Nejad *et al.,* 2012; Boyko *et al.,* 2014), possibly linked to a transcription factor associated with genes involved in the development of bony skeleton (Metzger *et al.,* 2013). A single nucleotide polymorphism (SNPs) *LCORL* g.105547002 C>T located upstream of the gene, is significantly associated with withers height in various horse breeds. The C allele of this SNP is the minor allele associated with increased withers height (Makvandi-Nejad *et al.*, 2012; Signer-Hasler *et al.,* 2012; Tozaki *et al.*, 2016), which was later confirmed by Junior *et al.,* (2018) who reported that, in Brazilian pony breeds there is a close relationship between *LCORL* gene and height variation. Furthermore, the bioinformatic analysis performed with the Genomatrix® software (SNP Inspector) of the *LCORL* g.105547002 C>T suggests the interruption of a TFIID transcription factor binding site as a biological mechanism involved in stature reduction. This transcription factor participates in the expression of genes associated with skeletal development which was confirmed by Metzger *et al.,* (2013). Metzger *et al.,* (2013) who identified that the T/T genotype was highly associated with all pony breeds up to the limit value of 1.48 m for the withers height. Horses ranging from 1.30 m to 1.60 m at the withers showed T/T and C/T genotypes, while the taller and heavier horses showed predominantly the C/C genotype, associated with greater stature. Levels of transcripts in the *LCORL* gene were 40% lower in the C/T heterozygote

relation to the C/C homozygote, whereas this genotype presented 56% less transcription than the T/T genotype (smaller horses).

ZFAT encodes a protein that likely binds DNA and functions as a transcriptional regulator involved in apoptosis and cell survival, and it has an essential role in hematopoietic differentiation in blood islands and mice homozygous for a knockout of the gene die as embryos (Tsunoda *et al.,* 2010). In GWAS, a region from 74,795,013 to 76,254,733 bp including *ZFAT* was identified as a candidate region for withers height in horses (Makvandi-Nejad *et al*., 2012). *ZFAT* is associated with body height in various human populations (Allen *et al.,* 2010, Takeuchi et *al*., 2009) and later it was reported to, associated with withers height in several horse breeds (Makvandi-Nejad *et al*., 2012; Signer-Hasler *et al*., 2012; Metzger *et al.,* 2013).Of which, A SNP g.75550059 C>T in *ZFAT* is strongly associated with withers height in various horse breeds. The minor allele T of this SNP is associated with increased withers height (Makvandi-Nejad *et al*., 2012). Moreover, Signer-Hasler *et al*., (2012) reported A SNP g.74798143A>G on the same gene in Franches-Montagnes (FM) horse that is strongly associated with withers height and other body composition. A allele of this SNP is the minor allele associated with increased withers height.

The *HMGA2* locus has a well-known role in height determination in horses and many other species including humans and dogs (Weedon *et al*., 2008; Boyko *et al*., 2010). Furthermore, microdeletions in humans, which include *HMGA2* lead to proportionate short growth, whereas overexpression is associated with tumorigenesis (Buysse *et al.,* 2009; Zhang *et al.,* 2014). *HMGA2* deficient mice display the "pygmy" phenotype and *HMGA1 / HMGA2* double knock-out mice the "superpygmy" phenotype, which is characterized by even smaller body size, but also increased embryonic mortality and reduced

lifespan (Federico *et al.,* 2014; Zhou *et al.,* 1995). The HMGA2 protein is a DNA-binding protein with a function in transcription regulation (Reeves *et al.,* 2010). The protein contains 3 AT-hook DNA binding motifs. The AT-hook contains a central RGR motif, which directly contacts the minor groove of DNA (Huth *et al.,* 1997). The binding motif is usually PRGRP (Fonfria-Subiros *et al.,* 2012) but in the first AT-hook it is changed to GRGRP. The entire AT-hook contains additional positively charged amino acids, which help to bind the negatively charged DNA. Its optimal DNA binding sequence has been determined in a SELEX study (Cui T *et al.,* 2007). A SNP g.81481064C>T in *HMGA2* is strongly associated with withers height in various horse breeds. The minor allele C of this SNP is associated with decreased withers height (Makvandi-Nejad *et al.*, 2012). Later revealed that, A SNP c.83G>A (p.G28E) in *HMGA2* is strongly associated with withers height in Shetland pony and A allele of this SNP is associated with decreased withers height (Frischknecht *et al.,* 2015).

LASP1 may behave as an adaptive molecule in pathways involved in cell signaling or organization of cytoskeletal architecture. Through expression analysis, it was observed that *LASP1* transcripts are detectable in murine embryos of 7.5 to 17.5 days after fertilization and are present at varying levels in all of their tissues as adults. The *LASP1* encoded protein binds to actin and is responsible for regulating cell migration, proliferation and focal adhesion, along stress fibers and leading edges (lamellipodia, filopodia and pseudopodia). It is linked to both intramembranous ossification and endochondral ossification. LASP1 protein includes two acting binding sites and one SH3 domain, which is involved in binding to special focal adhesion proteins. These binding properties make *LASP1* a candidate structural protein that mediates the formation of protein complexes. The *LASP1* gene and its LIM and SH3 proteins have already

been related to several physiological pathways involved in vertebrate growth, and can affect animal stature. Among them, the expression of *LASP1* can affect the formation of cartilaginous tissue and the process of osteogenic differentiation. In addition, *LASP1* is required for cell migration (Schreiber *et al.,* 1998; Chew *et al.,* 2002; Joos *et al.,* (2008; Hermann-Kleiter *et al.,* 2009; Hu *et al.,* 2014; Lin *et al.,* 2004). A SNP g.23259732G>A in *LASP1* is strongly associated with withers height in various horse breeds. The minor allele A of this SNP is associated with increased withers height (Makvandi-Nejad *et al.*, 2012) later which was confirmed by Junior *et al.,* (2018) who reported that, in *LASP1* gene, the alleles of the mutation (G > A), presenting a difference in animals' heights. Allele A showed association with horses of higher stature while the G allele, even in heterozygosis, was related to smaller height in Brazilian pony indicating this SNP is capable of discriminating height of horses. Jun *et al.,* (2014) also evaluated the Marwari horses and identified *LASP1* as the candidate gene for stature.

Myostatin (*MSTN)* is a member of the transforming growth factor-β family with a key role in inhibition of muscle growth by negative regulation of both myoblast proliferation and differentiation. Variants of the *MSTN* gene encoding myostatin are associated with muscle hypertrophy phenotypes in a range of mammalian species, most notably cattle, dogs, mice, and humans. In particular, whippet racing dogs that are heterozygote for a *MSTN* polymorphism have significantly greater racing ability than both homozygote wild-type dogs and homozygotes for the mutation that have an increased musculature that is detrimental to performance (Mosher *et al.,* 2007; Schuelke *et al.,* 2004; Grobet *et al.,* 1997; McPherron *et al.,* 1997). It has suggested that an intronic variant in *MSTN* is predictive of the best race distance for the Thoroughbred (Hill *et al.* 2010); specifically, these studies suggest that horses homozygous for the "C"

allele (g.66493737C>T) are better suited for short distance racing, heterozygotes are more capable middle-distance racers, and homozygotes for the "T" allele have greater stamina for long-distance races. In addition to predicting optimal racing distance, *MSTN* has been implicated as important to racing success (McGivney *et al.,* 2012; Tozaki *et al.,* 2010) and also as having a role in body composition (Tozaki *et al.*, 2011). Later, revealed that, *MSTN* to be associated with proportions of muscular fiber types in Quarter Horse (Petersen *et al.,* 2013b). The C allele is the minor allele associated with higher proportions of Type 2B muscular fiber and lower proportions of Type 1 muscular fiber.

The doublesex and mab-3-related transcription factor 3 gene (*DMRT3*) encodes an important transcription factor involved in the coordination of the locomotor system controlling limb movement. Horses show considerable variation in the pattern of locomotion. The three naturally occurring gaits in all equids are, in order of increasing speed, walk, trot and canter/gallop. Some horses can use alternate gaits, typically at intermediate speed, and 'gaitedness' is a trait upon which many specialized breeds have been developed. Ambling gaits are four-beat gaits in which footfall pattern, foot placement and timing are often unique to specific breeds (Anderson *et al.,* 2012). So, the ability of horses to perform alternate gaits, ambling, in addition to common gaits, walk, trot and canter/gallop, is an important locomotion trait of the horse, since it affects smoothness of the locomotion. The gait trait has a strong genetic basis and horses of only limited breeds can perform the ambling gaits (Albertsdóttir *et al.*, 2011). Recently, a nonsense mutation of C to A in *DMRT3* (p.Ser301STOP) was reported to have major effects on the 'gaitedness' of the horse (Andersson *et al.,* 2012) and minor allele A more likely to responsible for performing ambling gaits.

Genotyping of genes related to wither height, body conformation and locomotion traits

The presence of the minor alleles for *MSTN* gene has been reported in Mongolian and Siberian horses at frequencies of 0.003 to 0.44, and 0.03 to 0.16, respectively by Bower *et al.,* (2012) and Padilh *et al.,* (2018) reported in Brazilian Sport Horses about the presence of the minor alleles for *MSTN* were 0.25.

Furthermore, Okuda *et al.,* (2017) reported, the minor alleles for *LCORL* gene in Myanmar horse at frequencies of 0.08 to 0.27, whereas for *MSTN* and *DMRT3* were 0.05 to 0.23 and 0 to 0.04, respectively. The presences of the minor alleles for *LCORL, ZFAT, HMGA2* and *LASP1* in Spanish horses that competes the highest standards in international dressage events at frequencies of 0.47, 0.09, 0.95 and 0.22, respectively (Sevane *et al.,* 2017).

The presence of the minor alleles for *DMRT3* gene has been reported in Iranian horses at frequencies of 0.3 to 0.10 and some of Japanese horses at frequencies of 0 to 0.72 (Promerová *et al.*, 2014) and 0 to 0.82 in Chinese horses (Han *et al.,* 2015). According to Amano *et al.*, (2018), Animals exhibiting pace and pace/trot had AA for *DMRT3:*Ser301Ter in high frequencies (100% and 81.8% respectively) confirming strong association between A allele this SNP and pace in Hokkaido horse, whereas 14.3% of the animals exhibiting trot also had AA for this SNP indicating additional genetic factors and/or environmental factors also involved in gait determination.

The findings obtained in the present study were comparable to those previously reported (He *et al.*, 2015; Signer-Hasler *et al.*, 2012; Sevane *et al.,* 2017; Bower *et al.,* 2012; Promerová *et al.*, 2014; Okuda *et al.,* 2017; Han *et al.,* 2015; Amano *et al.*, 2018). This is the first comprehensive survey for the presence of these SNPs associated with physical performance, body conformation and locomotion traits in Japanese native horse population.

The present findings of the presence of the SNPs of these genes in Japanese native horses suggest that, these SNPs are supposed to be relatively old polymorphisms or mutations that arose before establishment of the modern horse breeds, presumably originating from wild horse populations (*Equus ferus ferus*) that were extinct in the 19th century, and thus observed in various breeds of horse worldwide. Our findings of the presence of the minor alleles of these genes in Japanese native horse populations, as well as previous reports demonstrated wide distribution of these minor alleles in Asian native horse and European populations. Therefore, the present findings further support this supposition for the origins of these alleles, since these native horses are likely to have been genetically isolated and distinct from modern Western horse breeds, while we could not exclude the possibility of introgression of these SNPs from modern western horse breeds, since some of the Japanese native horses were historically crossed with exotic breeds (Ishikawa, 1984).

However, there were remarkable differences in the allelic frequencies of particular genes among the populations in the world. Since, Japanese native hoses were mainly used for riding, agricultural transport and packing loads. Therefore, present findings of the distribution of alleles of these genes associated with physical performance, body conformation and locomotion traits will be informative since selection and breeding of native horse by increased frequencies of desirable minor allele in the population will help in increased wither height, body conformation as well as locomotion trait. While, differences between native horses and the other populations might be due to the fact that these populations has been maintained with a unique breeding strategy and the intensive use of a few sires of a particular blood line as well as gene flows have actively occurred from foreign breeds into Japanese native horses. Particularly, presence of minor allele A for *DMRT3* Ser 301 STOP is high in Hokkaido can

be explained by, Hokkaido is a globally interesting horse breed and genetically valuable resource in terms of variations in two traits, namely occurring pace gait, which has been favored probably in the long history as a pack and riding horse (Andersson *et al.,* 2012) and exhibits a rich variation in coat color (Hachinohe, 1982). Hokkaido can become an important genetic resource for adaptation to future environment changes, considering the characteristics acquired during the adaptation to a cold climate and roughage diet. On the other hand, Okinawan horses including Miyako and Yonaguni had historical evidence of naturally occured pace ability for riding, there might be possibility of gene flow from the other horse within Japanese native horse breeds. Moreover, Presences of higher wither height related alleles exists in almost all populations indicating multiple origin of these population, although Hayashida (1958) reported that based on body size, these horses could be classified into two groups: Hokkaido, Kiso, and Misaki are considered medium sized horses, and the others are considered small and hypothesized that the founder populations of the Japanese native horses had been imported into Japan from southern China and Mongolia at different time periods. These findings also suggest that, with few exceptions these populations have not been under strong selection pressure for particular traits such a physical performance, body composition and locomotion pattern.

3. 5: *CONCLUSION*

The presences of the minor alleles of these genes at low frequencies suggest a possibility that these horse populations have not been under strong selection pressure for particular body composition and locomotion traits. However, relatively high frequency of the allele of *DMRT3* gene associated with gaitedness in Hokkaido population suggest a possibility that this horse population has been under strong selection pressure for locomotion traits including gaitedness. The present findings of the presence of these minor alleles in Japanese native horses will informative for future selection, breeding and conservation.

Fig 3.1: Wither height in horse

Fig 3.2: Ambling gait in horse

Table 3.1: Primer, target length, Amino acid substitution and references for genotyping LCORL, ZFAT, HMGA2, LASP1, MSTN and DMRT3 in Japanese native horses

Gene	Polymorphism	AA Subs.	Primer Pairs (5' to 3')	Target length	References
LCORL	g.105547002 C>T		F:GCCATCTATTTGCATGTTCTTG R:GGCAAGTTCATAGGCTGGTTC	347 bp	Metzger *et al.*, 2013.
ZFAT	g.75550059 C>T		F:GCAGAGACCCTTTGAGACC R:GCACCATTTATGTTCCTTCA	389 bp	Sevane *et al.*, 2016
HMGA2	g. 81481064 C>T		F:TGATTTCAGTGTTGCTTCTCT R:TTTATGTTGTTATCTGCCTGTG	246 bp	Sevane *et al.*,2016
LASP1	g. 23259732 A>G		F:ACACCCCAACACATACAACCC R: CAGGGGCATGTGCAGCTA	177 bp	This study
MSTN	g.66493737 C>T		F:ATTTGATAGCAGAGTCATAAAGGAAAAGTA R:CTGCGATCCTGCTTTACCCA	132 bp	Polasik *et al,* 2015.
DMRT3	g.22999665C>A	p.Ser 301STOP	F:CGACAAAGACACCGACCAGA R: CCGATCCCACGGACCATT	485 bp	Han *et al.*, 2015

Genotyping of genes related to wither height, body conformation and locomotion traits

Table 3.2: PCR conditions

Genes	PCR Conditions			
LCORL	94°C 30 sec 1 cycle	94°C 30 sec. 60°C 30 sec. 72°C 40sec 35 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle
ZFAT	95°C 10 min 1 cycle	95°C 30 sec. 53°C 60 sec. 72°C 30sec 40 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
HMGA2	95°C 10 min 1 cycle	95°C 45 sec. 58°C 60 sec. 72°C 30sec 40 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
LASP1	95°C 10 min 1 cycle	95°C 45 sec. 55°C 30 sec. 72°C 30sec 40 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
MSTN	95°C 5 min 1 cycle	95°C 45 sec. 55°C 45 sec. 72°C 45sec 35 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle
DMRT3	94°C 5min 1 cycle	94°C 20 sec. 65°C 20 sec. 72°C 20sec 35 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle

Genotyping of genes related to wither height, body conformation and locomotion traits

Table 3.3: Genotyping by sequencing and Restriction enzyme with cleavage site, incubation temperature and time of incubation

Gene	Restriction enzyme	Cleavage site	Incubation temperature (°C)	Incubation Time	Genotyping
LCORL	*Alu1*	5….AG/CT…3	37	1 hour	C=235,57,55; T=292,55
ZFAT	*HpyCH4V*	5…TG/CA…3	37	1 hour	T=267,122; C=369
LASP1	*Aci1*	5…C/CGC…3	37	1 hour	G= 48, 129 ; A= 177
HMGA2	Sequencing				T and C
MSTN	*Rsa1*	5…GT/AC…3	37	2-3 hour	T=132; C= 103, 29
DMRT3	*Dde1*	5..C/TNAG..3	37	1 hour	C=485; A=413, 72

Table 3.4: Genotype distribution and allele frequencies of LCORL and ZFAT gene

Population	No. of samples	***LCORL* g.105547002 C>T**						***ZFAT* g.75550059 C>T**					
		Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
		T/T	T/C	C/C	T	C		C/C	TC	T/T	C	T	
Hokkaido	31	26	05	00	0.92	0.08	0.24	05	20	06	0.48	0.52	2.64
Kiso	30	28	02	00	0.97	0.03	0.03	28	02	00	0.97	0.03	0.03
Noma	33	30	03	00	0.95	0.05	0.07	07	14	12	0.42	0.58	0.57
Taishu	32	32	00	00	1.00	0.00	NA	23	08	01	0.84	0.16	0.08
Misaki	30	30	00	00	1.00	0.00	NA	30	00	00	1.00	0.00	NA
Tokara	30	30	00	00	1.00	0.00	NA	28	02	00	0.97	0.03	0.03
Miyako	36	29	07	00	0.90	0.10	0.42	23	11	02	0.79	0.21	0.20
Yonaguni	25	25	00	00	1.00	0.00	NA	17	06	02	0.80	0.20	1.56
Total	**247**	**230**	**17**	**00**	**0.97**	**0.03**	**0.03**	**161**	**63**	**23**	**0.78**	**0.22**	**16.50***

***Significantly deviation from Hardy Weinberg Equilibrium (HWE) ($P < 0.05$).**

Table 3.5: Genotype distribution and allele frequencies of HMGA2 and LASP gene

Population	No. of samples	***HMGA2* g. 81481064 C>T**						***LASP* g. 23259732 A>G**					
		Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
		T/T	T/C	C/C	T	C		G/G	G/A	A/A	G	A	
Hokkaido	31	13	18	00	0.71	0.29	5.19*	23	08	00	0.87	0.13	0.68
Kiso	30	16	13	01	0.75	0.25	0.73	14	12	04	0.67	0.33	0.30
Noma	33	13	17	03	0.65	0.35	0.60	33	00	00	1.00	0.00	NA
Taishu	32	21	11	00	0.83	0.17	1.38	20	11	01	0.80	0.20	0.12
Misaki	30	25	04	01	0.90	0.10	2.01	25	05	00	0.92	0.08	0.25
Tokara	30	20	07	03	0.78	0.22	2.93	18	12	00	0.80	0.20	1.88
Miyako	36	25	11	00	0.85	0.15	1.17	15	21	00	0.71	0.29	6.10*
Yonaguni	25	20	04	01	0.88	0.12	1.47	24	01	00	0.98	0.02	0.01
Total	**247**	**153**	**85**	**09**	**0.79**	**0.21**	**0.45**	**172**	**70**	**05**	**0.84**	**0.16**	**0.48**

***Significant deviation from Hardy-Weinberg Equilibrium (P < 0.05).**

Table 3.6: Genotype distributions and allele frequencies of MSTN and DMRT3 gene

Population	No. of samples	*MSTN* g.66493737C>T						*DMRT3* g.22999665 C>A					
		Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
		T/T	T/C	C/C	T	C		C/C	C/A	A/A	C	A	
Hokkaido	31	29	02	00	0.97	0.03	0.03	20	10	01	0.81	0.19	0.34
Kiso	30	21	10	00	0.84	0.16	1.14	31	00	00	1.00	0.00	NA
Noma	33	33	00	00	1.00	0.00	NA	33	00	00	1.00	0.00	NA
Taishu	32	22	08	01	0.84	0.16	0.06	31	00	00	1.00	0.00	NA
Misaki	30	29	01	00	0.98	0.02	0.008	30	00	00	1.00	0.00	NA
Tokara	30	26	04	00	0.93	0.07	0.15	30	00	00	1.00	0.00	NA
Miyako	36	29	06	01	0.89	0.11	0.88	34	02	00	0.97	0.03	0.03
Yonaguni	25	23	02	00	0.96	0.04	0.04	24	01	00	0.98	0.02	0.01
Total	**247**	**212**	**33**	**02**	**0.93**	**0.07**	**0.32**	**233**	**13**	**01**	**0.97**	**0.03**	**2.79**

***Significantly deviation from Hardy Weinberg Equilibrium (HWE) ($P < 0.05$.**

CHAPTER 4

A Study on genotyping of genes related to reproductive traits and hereditary disorders in Japanese native horses

4.1: *INTRODUCTION*

Selection and breeding of horses based on genetics, desired performance and conformational characteristics are frequently done but knowledge on genetic effect influencing stallion fertility seems to be increasing importance. Moreover, less desirable or undesirable trait can be expanded in the equine genome since modern horse breeds have been established by selective breeding for a variety of desirable traits including athletic abilities for instance, racing speed or gaits. Since, 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 remained for mainly conservation purpose in several locations of Japan. Due to their small population sizes, these breeds are facing considerable risks for increased inbreeding situation. Since increased inbreeding situation can cause the inbreeding depression with increased incidences of lethal hereditary disorders and reduced reproductive performance, assessing genetic characteristics regarding the reproductive traits and hereditary disorders is particularly important for rational breeding and conservation program of these Japanese native horses with small population sizes. Therefore, we investigated allele frequencies and genotype distribution of the genes associated with reproductive traits and hereditary disorders in the present study.

As a result of recent advances in molecular genetic analysis of domestic animals including genome wide association study (GWAS), various single

nucleotide polymorphisms (SNPs) of the genes associated with particular reproductive traits have been identified in diverse horse breeds (Schrimpf *et al.,* 2014; Raudsepp *et al.,* 2012; Hamann *et al.,* 2007; Restrepo *et al.,* 2019; Usuga *et al.,* 2018). Out of these genes, FK506 binding protein 6 (*FKBP6*), cysteine-rich secretory protein-3 (*CRISP3*), and phospholipase C zeta 1 (*PLCZ1*) genes associated with stallion reproductive traits are of special interest, since reproductive defects of stallions could cause serious impacts on the breeding of domestic horses with small population size. On the other hand, many hereditary disorders have been reported in various horse breeds and their causative mutations of genes have been identified (McCue *et al*., 2008b; Aleman *et al*., 2009; Rudolph *et al.,* 1992). Of those, Polysaccharide Storage Myopathy (PSSM), Malignant Hyperthermia (MH), and Hyperkalaemic Periodic Paralysis (HYPP) caused by mutations of glycogen synthase 1 (*GYS1*), ryanodine receptor 1 (*RYR1*), and sodium voltage-gated channel alpha subunit 4 (*SCN4A*) genes, respectively are of special interest since incidences of these disorders are frequently observed in particular breeds (McCue *et al*., 2008a; Baird *et al.,* 2010; Tryon *et al*., 2009). Therefore, we genotyped SNPs of g.11040379 C>A and g.11040315 G>A in *FKBP6* gene that are associated with Impaired Acrosome Reaction (IAR) of sperm (Raudsepp *et al*., 2012; Schrimpf *et al.,* 2015), c. 622G>A and c.716A>G in *CRISP3* gene that are associated with stallion fertility and semen quality (Haman *et al*., 2007; Restrepo *et al.,* 2018 and Usuga *et al.,* 2018) and g.45586821 C>T in *PLCZ1*gene that is associated with stallion fertility (Schrimpf *et al*., 2014).We also genotyped missense mutations of c.926G>A in *GYS1*gene responsible for PSSM (McCue *et al.,* 2008a; 2008b), c.7360C>G in *RYR1*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.

4.2: *OBJECTIVES*

A Study on genotyping of genes related to reproductive traits and hereditary disorders in Japanese native horses.

4.3. *MATERIALS AND METHODS*

4.3.1: *MATERIALS FOR DNA ANALYSIS*

SAMPLING

After extraction of DNA from blood shown in Chapter 2, 221 samples were randomly choosed from the eight Japanese native horse population and nucleic acid concentration of DNA samples were measured for analysis (shown in Chapter 2).

4.3.2: *METHODS OF DNA ANALYSIS*

a) POLYMERASE CHAIN REACTION (PCR)

For genotyping of functional genes that are associated fertility and hereditary disorders in Japanese native horses were amplified using PCR. PCR reaction were carried out for genes *CRISP3* c.622G>A; c.716G>A , *FKBP6 g.*11040379 C > A; *g.*11040315 G>A, *PLCZ1* g.45586821C>T, *GYS1* c.*926* G>A, *RYR*1c.7360C>G, *SCN4A* c.4248C>G, in a 10 µl reaction mixture each containing 2.0 µl of genomic DNA (10 ng/ µl), 0.3µl of 0.2 µM primers , 1.0 µl of 2.0mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer, 0.1 µl (0.5U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) 0.5U and distilled water (DW). Primer pairs, target length and PCR conditions used for genotyping shown in Table 4.1 and Table 4.2, and for reaction PCR Thermal Cycler Dice Touch (Takara Bio, Japan) used. Then PCR products were

electrophoresed in 2-3% Agarose gel in TAE buffer at 135 volt 15-30 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator.

b) *POLYMERASE CHAIN REACTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)*

PCR products of *PLCZ1* g.45586821C>T, *GYS1* c.*926* G>A, *RYR*1c.7360C>G and c.*SCN4A* 4248C>G genes were incubated in incubator/thermal cycler for enzyme digestion using enzymes (New England Biolabs, Tokyo, Japan) shown in Table 4.3. After complete enzyme digestion, digested products were electrophoresed in 2-3% Agarose gel/ Nusieve gel in TAE buffer at 135 volt 20-40 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator and genotyped according to Table 4.3.

c) *DNA PURIFICATION AND SEQUENCING*

PCR products of *CRISP3* c.622G>A; c.716G>A, *FKBP6 g.*11040379 C > A and *g.*11040315 G>A were purified and prepared for Sanger sequencing according to Chapter 2, Table 2.1.

d) *GENOTYPING OF FUNCTIONAL GENES*

Obtained result from PCR/ PCR-RFLP and sequencing were analyzed, Whereas, Hardy-Weinberg equilibrium (HWE) equation was calculated to observe the genotype distribution.

4.4: *RESULTS AND DISCUSSION*

In this study, we investigated the SNPs of the genes associated with stallion reproductive traits including *FKBP6, CRISP3* and *PLCZ1* genes in the Japanese native horses by PCR-RFLP and/or direct sequencing of the PCR products. As the results of the genotyping, both two alleles of *FKBP6* (g.11040315 G>A),

FKBP6 (g.11040379 C>A), *CRISP3 (c. 622G>A*), and *CRISP3* (*c.716 A>G)* were observed in the populations of the eight Japanese native horse breeds, except for Noma, and Tokara in which *CRISP3* (622G>A) was mono-allelic, and Misaki in which *CRISP3* (c. 622G>A) and *CRISP3 (*c.716 A>G) were mono-allelic (Table 4.4 and Table 4.5). On the other hand, all horses in these populations were TT genotype of *PLCZ1* (g.45586821C>T*)* and no C allele was observed in these populations (Table 4.6), indicating that this gene is mono-allelic in the Japanese native horse breeds. The genotype distributions of these SNPs in the eight populations were not significantly different from Hardy Weinberg Equilibrium, with few exceptions (Table 4.4 and Table 4.5).

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 are relatively old SNPs that spread into wide variety of horse populations, while we could not exclude a possibility of introgression of these SNPs from western horses, since, exotic 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 *4.4* and Table *4.5).* 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 4.4), 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 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* (926G>A), *RYR1* (7360C>G), and *SCN4A* (4248C>G) and no mutant alleles associated with the disorders were observed (Table 4.6). 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; Baird *et al.,* 2010; Druml *et al.,* 2016; Tryon *et al.,* 2009). 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) cause 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 have 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 (Druml *et al.*, 2016; Schwarz *et al.,* 2011; Baird *et al.,* 2010; McCue *et al.,* 2008a, b; Aleman *et al.,* 2004, 2009; Rudolph *et al.,* 1992; Tryon *et al.*, 2009), 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).

4.5: *CONCLUSION*

Since higher reproductive performance and healthy condition 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 reproductive traits and hereditary disorders will be informative for the conservation of these breeds.

Table 4.1: Primer, target length, Amino acid substitution and references for genotyping FKBP6, PLCz1, CRISP3, GYS1, RYR1* and *SCN4A* genes in Japanese native horses

Gene	Polymorphism	AA Subs.	Primer Pairs (5' to 3')	Target Length	References
CRISP3	c.622G>A; c.716G>A	Glu208Lys Gln239Arg	F:TCGAGAAGTGAAAGGCCCAT R:TTGGAATCAGCTTTGCAACTAGC	378 bp	Hamann *et al.*, 2007
FKBP6	g.11040379 C > A g.11040315 G>A	His 166 Asp; Synonymous	F:ACACGGCAGTAGACAGAAGC R:CTGGGTCCCCTCTCTTAGTC	395 bp	R Schirmpf *et al.*, 2015
PLCz1	g.45586821C>T		F:GCTTCTGTTAGCCCCTTCTCA R:ATGGCCTCACTTTCTCTGCATT	295 bp	R Schirmpf *et al.*,2014
GYS1	*c.926* G>A	Arg309His	F:TGAAACATGGGACCTTCTCC R: AGCTGTCCCCTCCCTTAGAC	230 bp	McCue *et a.*, 2008b
RYR1	*c.*7360C>G	Arg2454Gly	F:CGCTGTCATGGAGCTCC R:GAAGGATGCCGACATCTTG	455 bp	Aleman *et al.*, 2009
SCN4A	c.4248C>G	Phe1416Leu	F:CTTTGTGACGAAGCAGGTGT R:CCTCATGTGCCTTTGTGCAT	408 bp	Rudolph *et al.*, 1992

Table 4.2: PCR conditions

Genes	PCR Conditions			
CRISP3: c.622G>A, c.716A>G	95°C 10 min 1 cycle	95°C 30 sec. 56°C 30 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
FKBP6: g.11040379C > A, g.11040315 G>A	95°C 5 min 1 cycle	94°C 30 sec. 57°C 60 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
PLCz1	94°C 2 min 1 cycle	94°C 30 sec. 57°C 30 sec. 72°C 30sec 35 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle
GYS1	94°C 30 sec 1 cycle	94°C 30 sec. 56°C 30 sec. 72°C 60sec 35 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle
RYR1	95°C 10 min 1 cycle	95°C 30 sec. 59°C 60 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
SCN4A	95°C 10 min 1 cycle	95°C 30 sec. 60°C 60 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle

Table 4.3: Genotyping by Restriction enzyme, their cleavage site, incubation temperature and time of incubation/sequencing

Gene	Restriction enzyme	Cleavage site	Incubation temperature (°C)	Incubation Time	Genotyping
PLCz1	*Mwo1*	5..GCNNNNN/ NNGC…3	60	1 hour	C: 295bp, T: 194, 101bp
GYS1	*HpyCH4V*	5…TG/CA…3	37	1 hour	G: 152 78bp, A: 98, 78, 54bp
RYR1	*BamHI*	5..G/GATCC… 3	37	1 hour	G: 455bp, C: 229, 226bp
SCN4A	*Taqα1*	5…T/CGA…3	65	1 hour	G: 21, 387 bp, C: 21, 192, 195 bp
CRISP3: 622G>A; c.716A>G	Sequencing				G and A
FKBP6: g.11040379 C > A ;g.11040315 G>A	Sequencing				C and A

Table 4.4: Genotype distributions and allele frequencies of FKBP6 gene

Population	*FKBP6* (g.11040315 G>A)							*FKBP6* (g.11040379 C>A)					
	Number of samples	Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
		GG	AG	AA	G	A		CC	CA	AA	C	A	
Hokkaido	23	1	11	11	0.28	0.72	0.74	8	10	5	0.57	0.43	0.31
Kiso	30	2	20	8	0.4	0.6	4.53*	4	21	5	0.48	0.52	4.83*
Noma	32	0	15	17	0.23	0.77	3	30	2	0	0.97	0.03	0.03
Taishu	21	3	11	7	0.4	0.6	0.16	6	12	3	0.57	0.43	0.58
Misaki	29	6	13	10	0.43	0.57	0.214	0	4	25	0.07	0.93	0.16
Tokara	29	18	6	5	0.72	0.28	6.74*	6	13	10	0.43	0.57	0.21
Miyako	32	11	16	5	0.6	0.4	0.009	8	16	8	0.5	0.5	0
Yonaguni	25	7	11	7	0.5	0.5	0.36	7	10	8	0.48	0.52	0.99
Total	**221**	**48**	**103**	**70**	**0.45**	**0.55**	**0.76**	**69**	**88**	**64**	**0.51**	**0.49**	**9.13***

***Significantly deviation from Hardy Weinberg Equilibrium (HWE) ($p < 0.05$).**

Table 4.5: Genotype distributions and allele frequencies of CRISP3 gene

Population	Number of samples	*CRISP3* (c.622G>A)						*CRISP3* (c.716 A>G)					
		Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
		GG	GA	AA	G	A		AA	AG	GG	A	G	
Hokkaido	23	13	9	1	0.76	0.24	0.13	12	9	2	0.72	0.28	0.028
Kiso	30	21	9	0	0.85	0.15	0.94	7	17	6	0.52	0.48	0.54
Noma	32	32	0	0	1	0	NA	15	16	1	0.72	0.28	1.8
Taishu	21	11	8	2	0.71	0.29	0.09	6	14	1	0.62	0.38	3.59
Misaki	29	29	0	0	1	0	NA	29	0	0	1	0	NA
Tokara	29	29	0	0	1	0	NA	6	18	5	0.52	0.48	1.71
Miyako	32	27	5	0	0.92	0.08	0.23	12	13	7	0.58	0.42	0.9
Yonaguni	25	23	2	0	0.96	0.04	0.04	8	11	6	0.54	0.46	0.33
Total	**221**	**185**	**33**	**3**	**0.91**	**0.09**	**1.14**	**95**	**98**	**28**	**0.65**	**0.35**	**0.12**

***Significantly deviation from Hardy Weinberg Equilibrium (HWE) ($p < 0.05$)**

Table 4.6: Genotype distributions of PLCZ1, GYS1, RYR1, and SCN4A genes

Population	No. of samples	Genotype distribution											
		***PLCZ1* (g.45586821C>T)**			***GYS1* (c.926 G>A)**			***RYR1* (c.7360C>G)**			***SCN4A* (c.4248C>G)**		
		CC	CT	TT	GG	GA	AA	CC	CG	GG	CC	CG	GG
Hokkaido	23	0	0	23	23	0	0	23	0	0	23	0	0
Kiso	30	0	0	30	30	0	0	30	0	0	30	0	0
Noma	32	0	0	32	32	0	0	32	0	0	32	0	0
Taishu	21	0	0	21	21	0	0	21	0	0	21	0	0
Misaki	29	0	0	29	29	0	0	29	0	0	29	0	0
Tokara	29	0	0	29	29	0	0	29	0	0	29	0	0
Miyako	32	0	0	32	32	0	0	32	0	0	32	0	0
Yonaguni	25	0	0	25	25	0	0	25	0	0	25	0	0
Total	**221**	**0**	**0**	**221**	**221**	**0**	**0**	**221**	**0**	**0**	**221**	**0**	**0**

CHAPTER 5

A Study on genotyping of genes related to coat color in Japanese native horses

5.1: INTRODUCTION

Equine coat color is an important phenotypic trait, predominantly within the context of correct animal identification (Cieslak *et al.,* 2013) because it can support the selection of undesired colors and allow planning for the future matings. Furthermore, the coat color is an important factor influencing the value of animals, and it sometimes directly determines the use and demand of horses. Since, the domestication of horses various coat color have been observed in diverse horses population as variation in coat color is not often observed in wild animals but is common in domestic animals (Cieslak *et al.,* 2011). Assessing coat color, it is easy to take future conservation strategy for numerically small population of horses which will be a valuable genetic resource for coat color investigation. 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. In Japanese native horses, various coat colors have been reported including basic, dilution, dun, gray, etc (Hachinohe, 1982; Takasu *et al.,* 2011; Nakamura *et al.,* 2019; Kaseda, 1984; http://www.minnano-jouba.com/mame_chishiki02_en.html). Since, the population sizes are small in Japanese native horses and these breeds are facing considerable risks for increased inbreeding situation, variation in coat color might be decreasing recently. Therefore, in this study allele frequencies and genotype distribution of the genes associated coat color were investigated.

As a result of recent genetic analysis studies these coat colors are reported to be controlled by several genes (Marklund *et al.,* 1996; Rieder *et al.,* 2001; Mariat *et al.*, 2003; Imsland *et al.,* 2016). Of those Melanocortin Receptor-1 (*MC1R*),

Agouti Signaling Protein *(ASIP),* Membrane associated transporter protein *(MATP)* and T-box 3 *(TBX3*) genes are associated with basic, cream dilution and dun coat color, respectively.

While, Melanocortin Receptor-1 (*MC1R*) Protein located on the surface of melanocytes and activated by melanocyte stimulating hormone (MSH) leading to the production of eumelanin, which is the wild type allele $MC1R^{E}$ (Chen *et al.,* 2017). The *MC1R c.*901C>T mutation generates a recessive allele ($MC1R^{e}$) that lead to the production of only pheomelanin within melanocytes (Neves *et al.,* 2017). The eumelanin is a black pigment while pheomelanin is a red/yellow pigment (Jackson, 1994). Melanocytes that are homozygous for the recessive mutation $MC1R^{ee}$ cannot be activated by MSH, leading to the production of only pheomelanin (Suzuki *et al.,* 1996; Neves *et al.,* 2017). (Fig 5.1). Agouti Signaling Protein *(ASIP)* encodes the agouti signaling protein (wild type allele $ASIP^{AA}$), an antagonist to MSH that can block the function of *MC1R* by inhibiting eumelanin production in horse body melanocytes (Lu *et al.*, 1994) (Fig 5.1). An *ASIP* c.2174-2184del (allele $ASIP^{a}$) leads to loss of agouti signaling protein function, yielding a black phenotypic in the horse (Rieder *et al.*, 2001). Combination of specific genotypes at *MC1R* and *ASIP* result in three basic phenotypes: black ($MC1R^{E\text{-}e\text{-}}$ $ASIP^{A\text{-}aa}$), bay ($MC1R^{E\text{-}e\text{-}}$ $ASIP^{A\text{-}a\text{-}}$) and chestnut ($MC1R^{ee\text{-}E\text{-}}$ $ASIP^{A\text{-}a\text{-}}$) (Neves *et al.,* 2017; Shang *et al.,* 2019) (Fig 5.2). Furthermore, the cream gene (C^{Cr}) is an incomplete dominant allele with a distinct dosage effect. The DNA sequence responsible for the cream colors is the cream allele, which is at a specific locus on the *MATP* gene (Locke *et al.,* 2001). A mutation in the *MATP* gene were first described by Mariat *et al.*, 2002, which was also confirmed by Brooks *et al.,* 2005 and Georgescu *et al.,* 2007. Its general effect is to lighten the coat, skin and eye colors. When one copy of the allele is present, it dilutes "red" pigment to yellow or gold, with a stronger effect on the mane and tail, but does not dilute black color to any

significant degree. When two copies of the allele are present, both red and black pigments are affected; red hairs still become cream, and black hairs become reddish. A single copy of the allele has minimal impact on eye color, but when two copies are present, a horse will be blue-eyed in addition to a light coat color. The cream gene is responsible for a number of horse coat colors. Horses that have the cream gene in addition to a basic coat color that is chestnut will become palomino (*MC1R* $^{ee\text{-}E\text{-}}$ *ASIP* $^{A\text{-}a\text{-}}$ *MATP* CC^{cr}) if they are heterozygous, having one copy of the cream gene, or cremello (*MC1R* $^{ee\text{-}E\text{-}}$ *ASIP* $^{A\text{-}a\text{-}}$ *MATP* $C^{cr}C^{cr}$) if they are homozygous. Similarly, horses with a bay coat and the cream gene will be buckskin (*MC1R* $^{E\text{-}e\text{-}}$ *ASIP* $^{A\text{-}a\text{-}}$ *MATP* CC^{cr}) or perlino (*MC1R* $^{E\text{-}e\text{-}}$ *ASIP* $^{A\text{-}a\text{-}}$ *MATP* C^{cr} C^{cr}). A black base coat with the cream gene becomes the not-always-recognized smoky black (*MC1R* $^{E\text{-}e\text{-}}$ *ASIP* $^{A\text{-}aa}$ *MATP* CC^{cr}) or a smoky cream (*MC1R* $^{E\text{-}e\text{-}}$ *ASIP* $^{A\text{-}aa}$ *MATP* $C^{cr}C^{cr}$) (Fig 5.2). The Dun coat color in the horse is typically characterized by a diluted base coat and presence of dun characteristics including a dorsal stripe, shoulder stripe, leg barring, eye shadows, etc (Fig 5.3C). According to Imsland *et al.,* (2016), the dun dilution effect is due to the presence of a 1.6 kb insert within the downstream region of the TBX3 gene (the dominant D allele), whereas absence of this fragment on both chromosomes results in non-dilute (d2, non-dun 2 allele) coats and absence of dun-related characteristics. There is also another variant located within the 1.6 kb insert (a G>T, SNP)), that disrupts the dun dilution effect (d1–non-dun 1 allele), but leaves evidence of the other dun phenotype characteristics like the dorsal stripe. Thus, horses carrying d1/d1 and d1/d2 genotypes often possess a dark dorsal stripe and other 'primitive' dun-related markings (Fig.5.3; A, B).

The genotype data of these genes in the Japanese native horses will be informative for future breeding and conservation programs by decreasing the inbreeding situation of these local populations of Japanese native horses due to proper animal identification by genotyping.

5.2: *OBJECTIVES*

A Study on genotyping of genes related to Coat Color in Japanese native horses.

5.3: *MATERIALS AND METHODS*

5.3.1: *MATERIALS OF DNA ANALYSIS*

SAMPLING

After extraction of DNA from blood shown in Chapter 2, 247 samples were randomly choosed from the Eight Japanese native horse population and nucleic acid concentration of DNA samples were measured for analysis (shown in Chapter 2).

5.3.2: *METHODS OF DNA ANALYSIS*

a) POLYMERASE CHAIN REACTION (PCR)

For genotyping of genes that are associated coat color in Japanese native horses were amplified using PCR. PCR reaction were carried out for genes *MC1R* c.901C>T, *MATP* c.457G>A, *ASIP* c.2174-2184del, *TBX3* g.18227267+1066G>T, g.18227267 1.6 kb del, in 10 µl reaction mixture containing 2.0 µl of genomic DNA (10 ng/ µl), 0.3µl of 0.2µM primers , 1.0 µl of 2.0 mM dNTP, 2.0 µl of 5X Go Taq Green PCR buffer, 0.1 µl (0.5 U) of Go Taq DNA Polymerase (Promega Corporation WI, USA) and distilled water (DW). Primer pairs, target length and PCR conditions used for genotyping shown in Table 5.1, 5.2 and for reaction PCR Thermal Cycler Dice Touch (Takara Bio, Japan) used. Then PCR products were electrophoresed in 2-3% Agarose gel in TAE buffer at 135 volt 15-30 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator. Genotyping result of *TBX3* g.18227267 1.6 del, and *ASIP* c.2174-2184del, were obtained from directly By PCR (See Table 5.3).

b) POLYMERASE CHAIN REACTION–FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

PCR products of *MC1R* c.901C>T and *MATP* c.457 G>A genes were incubated in incubator/thermal cycler for enzyme digestion using enzymes (New England Biolabs, Tokyo, Japan) shown in Table 3. After complete enzyme digestion, digested products were electrophoresed in 2-3% Agarose gel/ Nusieve gel in TAE buffer at 135 volt 20-40 minutes, stained with 6x GR Red (Bio-craft), and visualized under using UV trans-illuminator and genotyped according to Table 5.3.

c) DNA PURIFICATION AND SEQUENCING

PCR products of *TBX3* SNP.18227267+1066G>T were purified and prepared for Sanger sequencing according to Chapter 2, Table 2.1.

d) *GENOTYPING OF FUNCTIONAL GENES*

Obtained result from PCR/ PCR-RFLP and sequencing were analyzed, whereas, Hardy-Weinberg equilibrium (HWE) equation was calculated to observe the genotype distribution.

5.4: *RESULT AND DISCUSSION*

In this study mutation of the gene associated with horse coat color including *MC1R, ASIP*, *MATP* and *TBX3* genes in Japanese native horses were investigated by PCR-RFLP and /or direct sequencing of the PCR products. As the result of genotyping both two alleles of *MC1R* c.901C>T and *ASIP* c.2174-2184del were observed in the population of eight Japanese native horse breeds, whereas *MATP* c.457G>A was mono-allelic in all horses except Hokkaido, Kiso and Miyako. On the other hand, the genotyping results of *TBX3* in Japanese native horses indicated that out of three allele D, d1 and d2 , only two alleles d1 , d2 of g.18227267+1066 G>T and g.18227267: 1.6 kb deletion were observed

in all local populations with an exceptions. The genotype distributions of these SNPs in the eight populations were not significantly different from Hardy Weinberg Equilibrium (Table 5.4 and Table 5.6).

On the other hand, the average allele frequencies of the e, a, $C,^{cr}$ and D for *MC1R* c.901C>T, *ASIP* c.2174-2184del, *MATP* c.457G>A, T*BX3* were 0.49, 0.55, 0.03 and 0.00 respectively. But, some of this population showed high frequencies of the e and a allele. Particularly in Hokkaido high frequency of e and a alleles were observed. In addition, relatively high frequencies of C^{cr} for *MATP* gene were also observed in Hokkaido. (Table 5.4 and Table 5.6). Moreover, frequencies of C^{cr} for *MATP* gene were also observed in Kiso and Miyako but value was small.

In this study, frequency of coat color distribution by genotyping four genes in the eight populations of Japanese native horses (Table 5.5 and table 5.7) were also investigated. The average frequency of Chestnut, black, bay, buckskin, palomino, smoky black, dun, non dun1 and non dun2 coat colors is 0.25, 0.28, 0.42, 0.012, 0.037, 0.08, 0.00, 0.97 and 0.03, respectively.

Mutation in *MC1R* have been associated with coat color mutants assigned to *E* locus in mouse, human, cattle, sheep, pig, goat, Chicken and Fox (Robbins *et al.,* 1993; Velverde *et al.,* 1995; Klungland *et al.*, 1995; Yang *et al.,* 2013; Andersson *et al.*, 2003; Luca *et al.,* 2009; Ran *et al.*, 2016, Vage *et al.*, 1997) and later reported to associated with coat color in horses. According to Marklund *et al.,* (1996) sequence analysis of 144 horses from 12 western breeds revealed a single missense mutation Ser 83 Phe in the *MC1R* allele that is associated with the chestnut coat color. Mutation in *ASIP* have been associated with coat color mutants assigned to A locus in some domestic animals, such as dogs, cats, and pigs (Kerns *et al.*, 2004, Eizirik *et al.,* 2003, Drogemuller *et al.*, 2006) and later reported in horse. In 24 black coat colored horse out of 9

different western breeds , *ASIP* c.2174-2184del associated with horse recessive black coat color (*ASIP*^{aa}) and causes frame-shift mutation on coding sequence of gene that acts as loss of function mutation (Rieder *et al.,* 2001). Furthermore, Mutation in *MATP* have been associated with Oculocutaneous Albinism in Dog, coat color in Braunvieh cattle and Human, (Winkler *et al.,* 2014; Rothammer *et al.*, 2017; Jung *et al.*, 2012, and later reported to associated with coat color in horses. A SNP *MATP* c.457 G>A (N153D) in *MATP* in horse was associated with cream dilution (Mariat *et al.,* 2003).

A study on 11,688 Thoroughbred foals and their parents done by Stachurska *et al.,* (2008), and reported that the frequency of recessive a, e and g alleles were 0.1552, 0.4877 and 0.9773, respectively for *ASIP MC1R*, and *STX17* in the offspring. In Iberian Cantabrian coast horse breeds mainly black or bay colored coats, but occasionally chestnut coat color found by Rendo *et al.,* (2009), using SNaPshot genotyping technique, where chestnut allele frequency ranged between 0.156-0.322 in pony breeds and 0.604-0.716 in heavy breeds. Kakoi *et al.,* (2009) reported genetic variations at the five coat colour loci in Thoroughbred and Misaki native horses, and found allele frequencies at the polymorphic E and A loci for *MC1R* and *ASIP*: bay, 0.662; black, 0.070; chestnut, 0.268. in Thoroughbred population and bay, 0.792; black, 0.129; chestnut, 0.080 in Misaki native horses. Stachurska *et al.*, (2012) reported in Hulcul horse population that, recessive allele frequency for A, E, D, To and G loci were 0.521, 0.115, 0.878, 0.929 and 0.997, respectively on *ASIP, MC1R,* Dun, *KIT* and *STX17* gene. Reissman *et al.,* (2016) investigated the presence/absence of coat-color-associated alleles in 1093 domestic horses of 55 worldwide breeds and 20 specimens of Przewalski's horse and also genotyped for 12 coat color-associated alleles of five genes and found that the alleles for the basic colorations (bay, black, and chestnut) are widely distributed and occur in nearly all breeds, while alleles leading to dilutions or patterns are rare in

domestic breeds and were not found in Przewalski's horse. In 149 recent documented kiso horses 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 for *MC1R*, *ASIP*, and *MATP* genes when genotyping was done by SNaPshot™ (Nakamura *et al.,* 2019).

The dun pigmentation is suggested to be the wild-type allele for the domestic horse coat (Ludwig *et al.,* 2009) that was confirmed later by Imsland *et al.,* (2016), and reported that the dun dilution effect is due to the presence or absence of a 1.6 kb insert within the downstream region of the TBX3 gene (the dominant D allele), and G>T SNP variant located within the 1.6 kb insert in horse. Later, Stefaniuk-Szmukier *et al.*, (2017) shown the occurrence of dun genotype in primitive polish konik breed and the analysis *TBX3* gene from 93 of this horse sample revealed that this horse can be considered as dun. Genotyping of the *TBX3* gene variants (1.6 kb in/del polymorphism and the non dun-related SNP) in 74 randomly selected Hucul horses revealed the presence of all six possible genotypes with the d1/d1 at the highest frequency (0.27) and d2/d2 the lowest (0.05) whereas, the d1 allele frequency reached 0.50, whereas D and d2 equaled 0.30 and 0.20, respectively, and the *ASIP* genotype frequencies were AA – 0.19; Aa – 0.39 and aa – 0.42 (Mackowski *et al.* 2019). Ezoe *et al.,* (2019) genotyping *TBX3* gene variants (1.6 kb in/del polymorphism and the SNP) in four horses population from Kazakhstan, Laos, Nepal and Vietnam revealed the presence of five possible genotypes except D/D with the d1d1 at the highest frequency (0.34) and D/d1, D/d2 the lowest (0.05) in average, whereas, the D (dun) allele frequency was ranged from 0.03 to 0.05 with average 0.05 in four populations. Moreover, frequency for d1 was ranged from 0.25 to 0.67 with average 0.52 and frequency for d2 was ranged from 0.27 to 0.71 with average 0.43 in these populations.

However, genotypic distribution and allelic frequencies obtained in the present study were comparable to those previously reported (Nakamura *et al.,*

2019; Rendo *et al.*, 2009; Kakoi *et al.*, 2009; Reissman *et al.*, 2016; Stachurska *et al.*, 2008, 2012; Mackowski *et al.*, 2019; Stefaniuk-Szmukier *et al.*, 2017; Ezoe *et al.*, 2019). Coat color distribution frequencies were obtained in the present study was comparable to those previously reported (Hachinohe, 1982; Takasu *et al.*, 2011; Nakamura *et al.*, 2019; Kaseda, 1984; http://www.minnano-jouba.com/mame_chishiki02_en.html). According to Bowling (1996) horses with the same color description may not have the same genotype as we found some discripencies in our horse genotyping result. The genotype result of *MC1R, ASIP, MATP* and *TBX3* in the present study indicated that, the SNPs of these genes which were identified in diverse world horse population also present in the Japanese native horses except dun gene. Since we could not detect dun allele of *TBX3* in the horses of all eight Japanese native horses, it is likely that polymorphism has not been present in the ancestral Japanese native horse population, although this SNPs was suggested to be old that are restricted in particular breed but presence of the allele in Asian breeds indicates that, further investigation is required in Japanese native by horses increasing the sample size. Furthermore, these Japanese native populations may have selection pressure for d1 allele unlike Asian modern horses that was already excluded from modern horse breeds. There were remarkable differences in the allelic frequencies of particular genes among the populations. In particular, the allelic frequencies of C^{cr} of *MATP* were remarkably different in Hokkaido, Kiso and Miyako from those of the other populations. These differences between Hokkaido, Kiso and Miyako and the other populations might be due to the fact that these populations has been maintained with a unique breeding strategy and the intensive use of a few sires of a particular blood line as well as gene flows have actively occurred from foreign breeds into Japanese native horses. Furthermore, Hokkaido exhibits a rich variation in coat color and the pedigree record has registered more than 10 coat colors (chestnut,

bay, black, grey, palomino, buckskin, double dilutes, chestnut roan, bay roan and black roan) (Hachinohe, 1982), which will be a valuable genetic resource for coat color investigation in native horse breeds. It was reported that, Miyako and Kiso horses were affected by improvement programs that crossbred with western horse to improve their physiques that is suitable for modern military before World War II (Senju *et al.,* 2016; Hendricks *et al.,* 1995; Obata *et al.,* 1993). Presences of basic coat color alleles with high frequencies in these populations can be explained by the history of domestic horses, crosses among breeds/lineages were common, resulting in a widespread distribution of coat-color-associated alleles, starting with their initial introduction in the gene pool of domestic horses. On the one hand, the alleles for the basic colorations (bay, black, and chestnut) in the *MC1R* gene and in the *ASIP* gene are at least 6300 years old and were already found in pre-domestic times. Consequently, the color alleles for bay, black, and chestnut occur in nearly all breeds. Presences of dilution color alleles can be explained by the restrictions in breeding goals resulted in the absence of specific coat color-associated alleles in several breeds, but recessive alleles are hidden sometimes in very low frequencies and survive in the genetic underground for many generations without phenotypic expression (Reissman *et al.,* 2016). However, there were remarkable differences in the allelic, genotypic and coat color distribution, among the populations. So, more samples to be studied to reveal the exact result of coat color and also further investigation of more coat color related genes is necessary to uncover the genetics of unknown color in Japanese native horses.

5.5: *CONCLUSION*

In conclusion, the genotyping result of the *MC1R, ASIP, MATP* and *TBX3* genes associated with basic, cream dilution and dun coat color in Japanese native horse breeds revealed that basic color allele present in high proportion in these populations whereas cream dilution and dun allele present very little or absent. These findings suggested that these populations have retained ancestral features of the coat color gene. These findings will be informative for future breeding and conservation programs for these breeds.

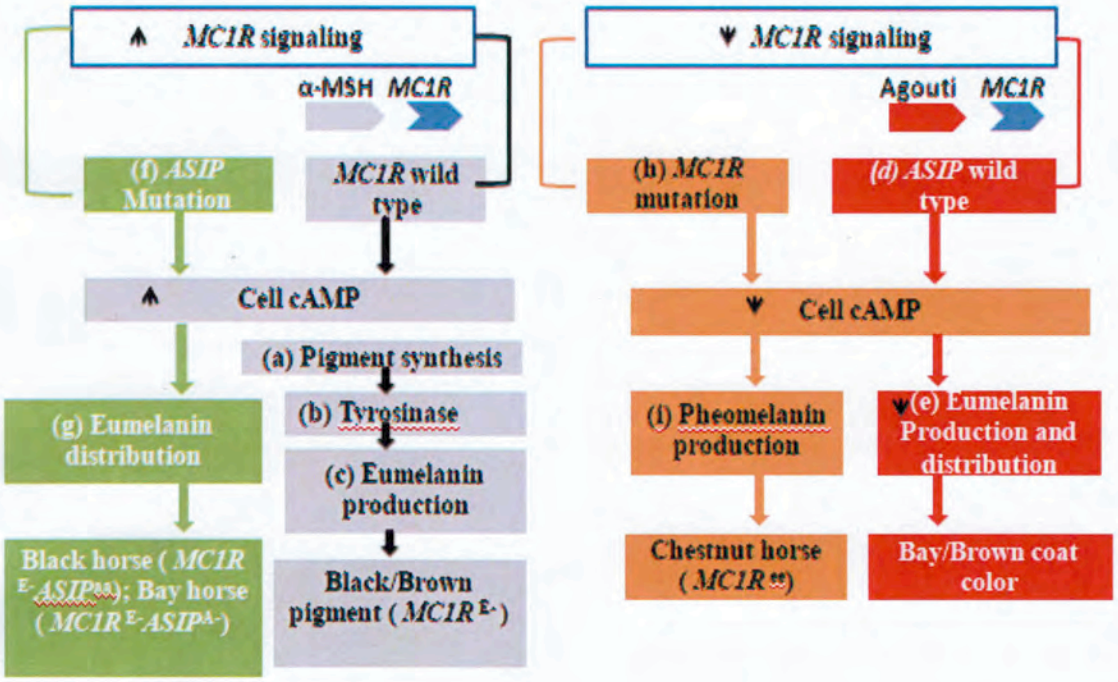


Fig 5.1: MC1R signaling in coat color formation in horse. Normal MC1R signaling (gray box and black arrow): MC1R signaling is activated by binding of the α-MSH to the MC1R receptor, resulting in increased celllular cyclic – AMP (cAMP) level leading to increased melanin synthesis (a) and stimulation of Tyrosine (b) leading to eumelain (black/brown pigment production (c). Normal ASIP signaling (Red boxes and arrow) (d) antagonizes the MC1R receptor resulting in a decrease in MC1R signaling and decrease eumelanin production (e) and leading to wild type bay/ brown coat color (in Wild type MC1R). Altered MC1R signaling in horse with ASIP deletion (green box and arrow): With ASIP deletion(f), antagonistic effects of ASIP is lost, resulting relative increase in MC1R signaling and increased cAMP signaling level compared with wild type. Horse homozygous for ASIP mutation ($ASIP^{aa}$) produce more eumelanin (g) resulting in black coat color. Horses with MC1R mutation (h) (brown box and arrow): MC1R signaling altered and cAMP level decreases that shifts eumelanin to pheomelanin (i) leading to chestnut coat color ($MC1R^{ee}$).

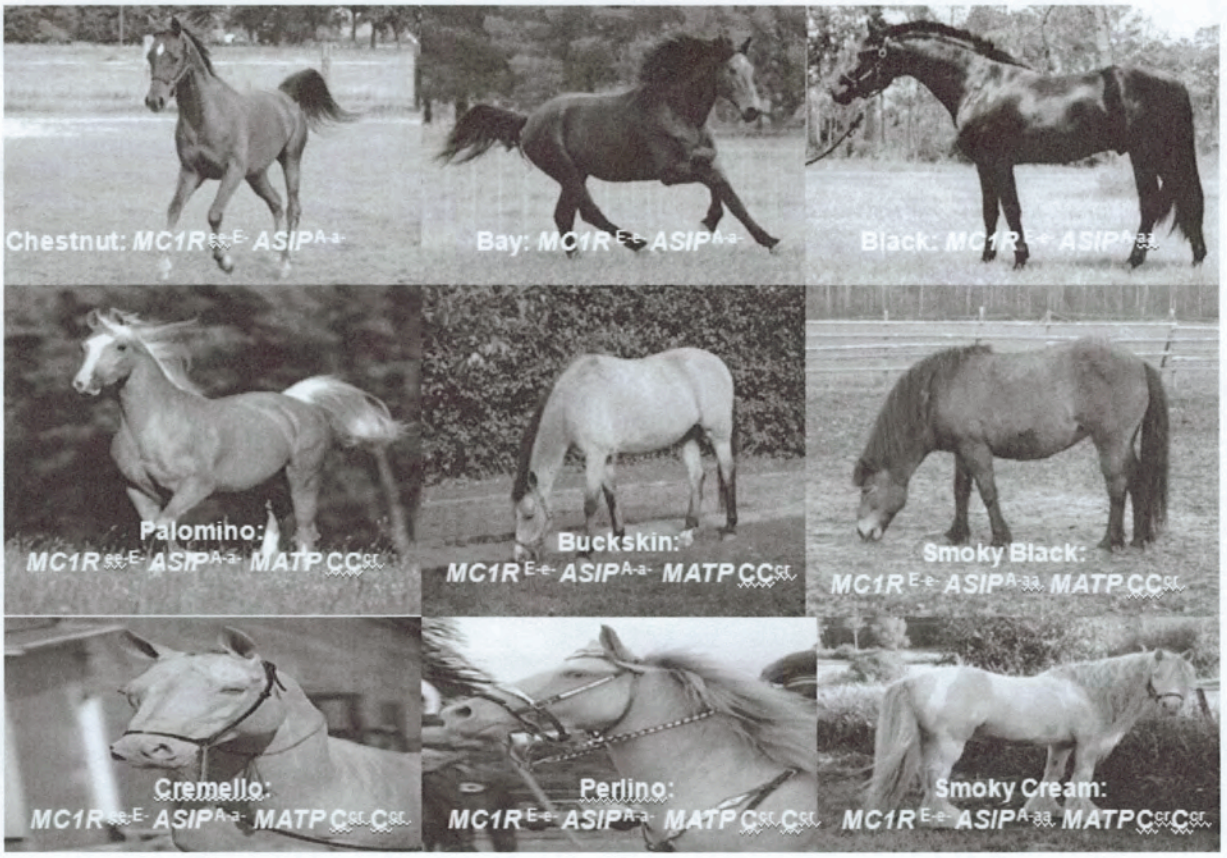
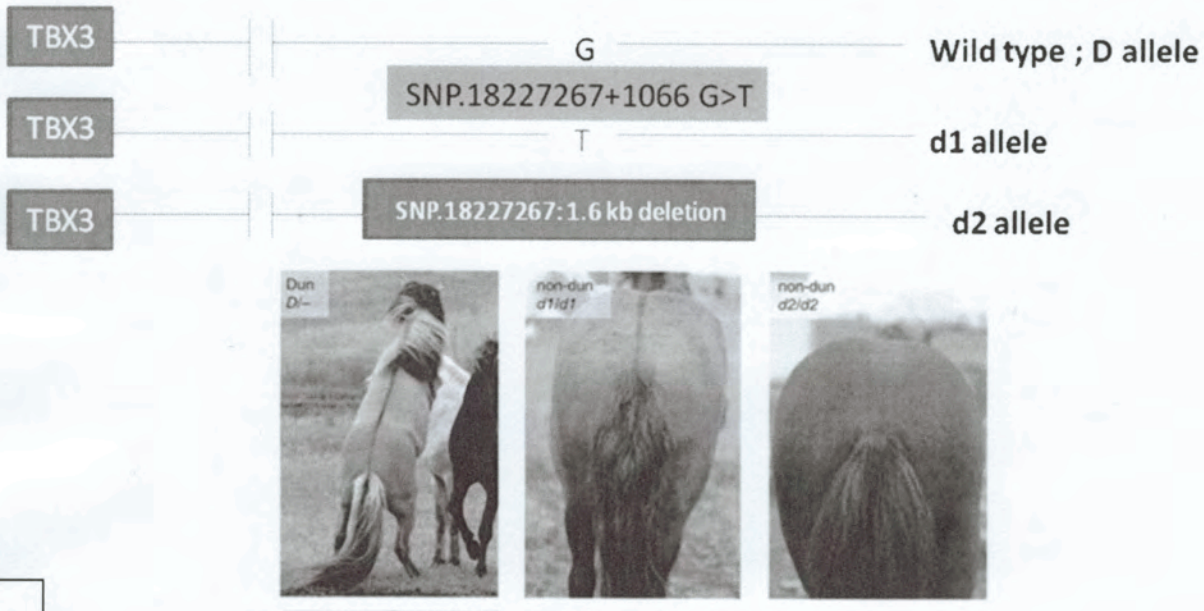


Fig.5.2: Horse coat color controlled by MC1R, ASIP, and MATP genes

A



B

Genotype	*D/D, D/d1, D/d2*	*d1/d1, d1/d2*	*d2/d2*
Coat color	**dun**	**Non-dun -1**	**Non-dun -2**
Primitive marking	**present**	**present**	**absent**

C

Fig 5.3: Determination of dun coat color By Imsland et al., 2016.A. Mutation on TBX3 region; B. Genotype and phenotype in horse C. Primitive Markings

Table 5.1: Primer, target length, Amino acid substitution and references for genotyping MC1R, ASIP, MATP* and *TBX3* in Japanese native horses

Gene	Polymorphism	AA Subs.	Primer Pairs (5' to 3')	Target Length	References
MC1R	C901phe	S83T	F:CCTACCTCGGGCTGACCACCAA R:GAGAGGACACTAACCACCCAGATG	460 bp	This study
MATP	G457A	N153D	F:TTGTTGACCGAAGGAAGAAG R:GAAATGCACTGGGAGACTGA	327 bp	This study
ASIP	c.2174-2184del		F:CTTTTGTCTCTCTTTGAAGCATTG R:GCTAGCTAGTACAGAAAGAT	329 bp	This study
TBX3	SNP.18227267 +1066 G>T		F:TAAGCCTCCAGACACCCAAG R:CAGCTCCCGTCTCCCTAGAT	240 bp	Stefaniuk-szmukier *et al.*, 2016
	SNP.18227267 : 1.6del		F:CAAGACGCAAGGCTCTTTCT R:CGTTTCTTTAAGGGCTCGTG	In 1837 or Del 215 bp	

Table 5.2: PCR conditions

Genes		PCR Conditions			
MC1R		95°C 10 min 1 cycle	95°C 30 sec. 60°C 60 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
ASIP		95°C 10 min 1 cycle	95°C 30 sec. 60°C 30 sec. 72°C 60sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
MATP		95°C 10 min 1 cycle	95°C 30 sec. 60°C 60 sec. 72°C 30sec 35 cycles	72°C 10 min 1 cycle	20°C ∞ 1 cycle
TBX3	*IN/DEL*	94°C 5 min 1 cycle	94°C 30 sec. 58°C 30 sec. 72°C 30sec 33 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle
	SNP	94°C 5 min 1 cycle	94°C 30 sec. 58°C 30 sec. 72°C 30sec 33 cycles	72°C 5 min 1 cycle	20°C ∞ 1 cycle

Genotyping of genes related to coat color

Table 5.3: Genotyping by PCR/sequencing / Restriction enzyme , their cleavage site, incubation temperature and time of incubation

Gene	Restriction enzyme	Cleavage site	Incubation temperature (°C)	Incubation Time	Genotyping
MC1R	*Taqα1*	5…T/CGA…3	65	1 hour	C=460 bp ;T=276, 184 bp
MATP	*Mse1*	5…T/TAA..3	37	1 hour	G=327 bp; A=215,112 bp
ASIP	PCR				Normal= 329 bp Mutation= 318bp
*TBX3*g.18227267+1066G>T	Sequencing				G=dun; T= d1
TBX3 1.6del	PCR				Normal= dun/d1, Deletion=d2

Table 5.4: Genotype distributions and allele frequencies of MC1R, ASIP and MATP gene

Population	*MC1R c.901 C>T*						*ASIP c.2174-2184del*						*MATP c.457 G>A*					
	Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE	Genotype distribution			Allele frequencies		Chi-square value for HWE
	EE	***Ee***	***ee***	***E***	***e***		***AA***	***Aa***	***aa***	***A***	***a***		***CC***	***CC^{cr}***	***C^{cr}C^{cr}***	***C***	***C^{cr}***	
Hokkaido	02	05	24	0.15	0.85	3.80	00	12	19	0.19	0.81	1.79	21	10	00	0.84	0.16	1.14
Kiso	09	18	04	0.58	0.42	1.14	10	14	07	0.55	0.45	0.24	29	02	00	0.97	0.03	0.03
Noma	03	17	13	0.35	0.65	0.60	02	19	12	0.35	0.65	2.36	33	00	00	1.00	0.00	NA
Taishu	11	14	06	0.58	0.42	0.16	12	15	04	0.63	0.37	0.04	31	00	00	1.00	0.00	NA
Misaki	15	13	02	0.72	0.28	0.13	03	18	09	0.40	0.60	1.80	30	00	00	1.00	0.00	NA
Tokara	18	09	03	0.75	0.25	1.20	00	08	22	0.13	0.87	0.71	30	00	00	1.00	0.00	NA
Miyako	02	16	18	0.28	0.72	0.42	22	11	03	0.76	0.24	0.84	35	01	00	0.89	0.11	0.07
Yonaguni	15	09	01	0.78	0.22	0.05	10	08	07	0.56	0.44	3.07	25	00	00	1.00	0.00	NA
Total	**75**	**101**	**71**	**0.51**	**0.49**	**8.18***	**59**	**105**	**83**	**0.45**	**0.55**	**4.96***	**234**	**13**	**00**	**0.97**	**0.03**	**0.18**

***Significantly deviation from Hardy Weinberg Equilibrium (HWE) (P < 0.05).**

Table 5.5: Coat color distribution (basic and cream dilution)

Populations	No. of samples	Chestnut: *(MC1R $^{e-E-}$ ASIP $^{A-a-}$)*	Black: *(MC1R $^{E-}$ ASIP $^{A-aa}$)*	Bay: *(MC1R $^{E-}$ ASIP $^{A-a-}$)*	Buckskin: *(MC1R $^{E-}$ ASIP $^{A-a-}$ MATP CC^{cr})*	Palomino: *(MC1R $^{e-E-}$ ASIP $^{A-a-}$ MATP CC^{cr})*	Smoky Black: *(MC1R $^{E-}$ ASIP $^{A-aa}$ MATP CC^{cr})*	Perlino: *(MC1R $^{E-}$ ASIP $^{A-a-}$ MATP C^{cr} C^{cr})*	Cremello: *(MC1R $^{e-E-}$ ASIP $^{A-a-}$ MATP C^{cr} C^{cr})*	Smoky Cream: *(MC1R $^{E-}$ ASIP $^{A-aa}$ MATP C^{cr}C^{cr})*
Hokkaido	31	0.48	0.10	0.07	0.00	0.29	0.07	0.00	0.00	0.00
Kiso	31	0.13	0.19	0.61	0.07	0.00	0.00	0.00	0.00	0.00
Noma	33	0.40	0.27	0.33	0.00	0.00	0.00	0.00	0.00	0.00
Taishu	31	0.19	0.10	0.71	0.00	0.00	0.00	0.00	0.00	0.00
Misaki	30	0.07	0.30	0.63	0.00	0.00	0.00	0.00	0.00	0.00
Tokara	30	0.10	0.50	0.40	0.00	0.00	0.00	0.00	0.00	0.00
Miyako	36	0.50	0.44	0.028	0.028	0.00	0.00	0.00	0.00	0.00
Yonaguni	25	0.04	0.28	0.68	0.00	0.00	0.00	0.00	0.00	0.00
Total	**247**	**0.25**	**0.28**	**0.42**	**0.012**	**0.037**	**0.008**	**0.00**	**0.00**	**0.00**

Genotyping of genes related to coat color

Table 5.6: Genotype distributions and allele frequencies of TBX3 gene

Populations	No. of samples	*TBX3* g.18227267+1066 G>T; g .18227267 1.6del								
		Genotype distributions						Allele frequencies		
		D/D	***D/ d 1***	***D/ d 2***	***d 1/ d 1***	***d 1/d 2***	***d 2/d 2***	***D***	***d 1***	***d 2***
Hokkaido	20	0	0	0	4	16	0	0.00	0.60	0.40
Kiso	28	0	0	0	17	11	0	0.00	0.80	0.20
Noma	29	0	0	0	5	18	6	0.00	0.48	0.52
Taishu	26	0	0	0	26	0	0	0.00	1.00	0.00
Misaki	22	0	0	0	4	18	0	0.00	0.60	0.40
Tokara	17	0	0	0	4	12	1	0.00	0.59	0.41
Miyako	34	0	0	0	2	32	0	0.00	0.53	0.47
Yonaguni	25	0	0	0	18	7	0	0.00	0.84	0.14
Total	**201**	**0**	**0**	**0**	**80**	**114**	**7**	**0.00**	**0.68**	**0.32**

Table 5.7: Coat color distribution in Japanese native horse(dun, non dun1 and non dun2)

Populations	No. of samples	Dun Color (D/D, D/d1, D/d2)	Non dun 1 (d1/d1, d1/d2)	Non dun 2 (d2/d2)
Hokkaido	20	0	1.0	0
Kiso	28	0	1.0	0
Noma	29	0	0.79	0.23
Taishu	26	0	1.0	0
Misaki	22	0	1.0	0
Tokara	17	0	0.94	0.06
Miyako	34	0	1.0	0
Yonaguni	25	0	1.0	0
Total	**201**	**0**	**0.97**	**0.03**

CHAPTER 6

General Conclusion

6.1: MITOCHONDRIAL DNA (mtDNA) AND Y CHROMOSOME HAPLOTYPES IN JAPANESE NATIVE HORSES

In this study, number of haplotypes, nucleotides as well as haplotype diversity of mtDNA were more than those of previous reported, particularly in Kiso and Miyako. Furthermore, few Japanese native horses have common haplotype. In addition, ancient X3c1 haplotypes shared in a large number of these populations were found in modern horse breeds. While the genetic diversity of Japanese native horses are reducing due to small population size with the time advance, haplotypes of these horses are relatively ancestral and after importation of these populations in Japan they have been affected by gene flow of each other, however few population may be possess common ancestor. This information will be helpful for future breeding and conservation of Japanese native horse population. On the other hand, Y chromosome haplotypes of Japanese native horses were widely distributed inside and outside of crown group which includes modern horse breeds. The presences of Ta and Tb-d haplotype in kiso, Misaki and Miyako population, A and Ao-1 haplotype at large number in other Japanese native horse population, and H haplotype in Miyako population indicating influence of English, Arabian and Iberian as well as North African Barb horse. Furthermore, relatively higher percentage of these populations falls in root node of crown group indicating Japanese native horses may harbour male lineage diversity that could not be identified into the haplotypes of modern European horse. In addition, one-third of Japanese native horses have unique haplotypes which predicted to separate from the root of the group regarded as modern horse groups or those separate from root of

phylogenetic tree of domestic horse earlier ages. Furthermore, most of these horses carried modern haplotypes as a direct result of recent attempts at breed improvement; Japanese native horses appear to have retained an ancient signature of paternal lineages that has not previously been described in these horse populations. The presence of both unique haplotype and modern influenced haplotypes in few populations may be due to common paternal origin of these populations. This is the first report on Japanese native horses that have unique ancestral Y chromosome haplotypes. Therefore, this study will be helpful for future breeding and conservation of these populations.

6.2: GENES RELATED TO WITHER HEIGHT, BODY CONFORMATION AND LOCOMOTION TRAITS IN JAPANESE NATIVE HORSES

As the results of the genotyping, both alleles of *ZFAT* g.75550059 C>T, *HMGA2* g. 81481064 C>T, *LASP1* g. 23259732 A>G, and *MSTN* g.66493737C>T were observed in the all population of Japanese native horse breeds with few exception. Similarly, both alleles of *LCORL* g.105547002 C>T in Hokkaido, Noma, Miyako and Kiso and of *DMRT3* g.22999665C>A in Hokkaido, Miyako and Yonaguni were observed, whereas remaining breeds were mono allelic for this two genes. The presences of the minor alleles of these genes at low frequencies suggest a possibility that these horse populations have not been under strong selection pressure for particular body composition and locomotion traits. However, relatively high frequency of the minor allele of *DMRT3* gene associated with gaitedness in Hokkaido population suggest a possibility that this horse population has been under strong selection pressure for locomotion traits including gaitedness. The present findings of the presence of these minor alleles in Japanese native horses will informative for future selection, breeding and conservation.

6.3: GENES RELATED TO REPRODUCTIVE TRAITS AND HEREDITARY DISORDERS IN JAPANESE NATIVE HORSES

As the genotyping result of single nucleotide polymorphisms of *FKBP6* (g.11040315G>A g.11040379C>A) and *CRISP3* (c.622G>A and c.716A>G) genes found that both desirable and undesirable alleles of *FKBP6* and *CRISP3* genes are present in the populations, while only undesirable allele *of PLCZ1* (g.45586821C>T) gene was observed in these populations. Furthermore, genotyping result of single nucleotide polymorphisms of *GYS1* (c.926G>A), *RYR1* (c.7360C>G), and *SCN4A* (c.4248C>G) genes found that no mutant alleles responsible for these hereditary disorders are present in the populations of Japanese native horse breeds. Since higher reproductive performance and healthy condition 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 reproductive traits and hereditary disorders will be informative for the conservation of these breeds.

6.4: GENES RELATED TO COAT COLOR IN JAPANESE NATIVE HORSES

As the genotyping result of mutation of *MC1R* c.901C>T, *ASIP* c.2174-2184del, *MATP* c.457G>A, *TBX3* g.18227267+1066G>T and g.18227267 1.6 del genes, associated with basic, cream dilution and dun coat colors, found that both alleles for basic (chestnut, bay and black) color of horse were present in all of this populations, while both cream dilution alleles were present only in three population indicating gene flow from exotic breeds except Hokkaido population. In addition, two (*d1,d2*) of the three alleles *D,d1* and *d2* of *TBX3* dun gene were present in all Japanese native horse populations that were not associated with dun color but shows primitive markings, such as dorsal stripe.

General Conclusion

These findings suggested that these populations have retained ancestral features of the coat color gene. Furthermore, coat color distribution were not diverse in Japanese native horses, except few populations indicating these population of horses, particularly Hokkaido population will be a valuable genetic resource for coat color investigation in future. These findings will be informative for future breeding and conservation programs for these horse breeds.

The present findings of the genetic diversities of maternal and paternal lines indicated by mtDNA and Y chromosomal haplotypes and distributions of the alleles of genes associated with physical performance, body conformation, reproductive traits, hereditary disorders, and coat color in the Japanese native horse populations will be informative for future breeding and conservation programs. In addition, the present findings that Japanese native horses have retained some ancient genetic features in maternal and paternal lines and coat color gene will be important for genetic characterization of Japanese native horse populations.

CHAPTER 7

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Dissertation Exposure

The following paper and proceeding from this dissertation have been published (or accepted for publication):

Paper

1. Ripon Chandra PAUL, Yu OKUDA, Trung Ba NGUYEN, Thu Nu Anh LE, Takayuki IBI, Yoshi KAWAMOTO, Ken NOZAWA, Tetsuo KUNIEDA.2019. Genotype distribution and allele frequencies of the genes associated with reproductive traits and hereditary disorders in Japanese native horses. The Journal of Animal Genetics, Vol. 47, N0. 2, (2019).

Conference Proceedings

1. Genetic characterization of Japanese native horses. Ripon Chandra PAUL, Yu OKUDA, Yoshi KAWAMOTO, Ken NOZAWA, Tetsuo KUNIEDA. 124th Japan Society of Animal Science Meeting. (Tokyo, Japan, 2018, March).
2. Genetic characterization of Japanese native horses. Ripon Chandra PAUL, Yu OKUDA, Yoshi KAWAMOTO, Ken NOZAWA, Tetsuo KUNIEDA. 18th Asian-Australasian Animal Production Congress. (Kuching, Malaysia, 2018, August).