



# Genetic characterization of the Miyako horse based on polymorphisms of microsatellites and mitochondrial DNA

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**ABSTRACT.** To help plan conservation of the endangered Miyako horse, a biological resource of the Miyako Islands in Japan, we characterized the genetics of the breed by genotyping 32 microsatellites and identifying mitochondrial DNA haplotypes. We also calculated genetic distances between individuals based on the proportion of shared alleles and visualized the genetic relationships with a phylogenetic tree. Two important results were obtained. One is that accurate pedigree registration of the horse by using microsatellites is possible, as the exclusion power of parentage testing is 0.999998. Another is that the current genetic diversity of the horses was clarified. The average number of alleles, observed heterozygosity and expected heterozygosity were 4.2, 0.701 and 0.649, respectively, for the 35 analyzed horses. The probability values for bottleneck models (infinite allele model: 0.00000; stepwise mutation model: 0.00026; and two-phase model: 0.00000) suggested that Miyako horses have experienced a recent genetic bottleneck. Only one mitochondrial haplotype was identified. Consequently, genetic diversity within the population is relatively well-maintained despite a very small population size (41 at the time of the study), and the first priority in conservation of the Miyako horse is to increase the population size.

**KEY WORDS:** conservation genetics, microsatellite DNA, mitochondrial DNA, Miyako horse

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The Miyako horse (Fig. 1) is a Japanese breed native to Miyako Island, the main island of the Miyako Islands in the far southwestern region of Okinawa Prefecture. The horses belonging to this breed are classified as small-sized horses, because of their 110–120-cm height [20, 22]. 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 [22, 23].

The ancestors of the Miyako horse are believed to have been introduced via the main island of Okinawa, where the capital of the Ryukyu Kingdom was located [22, 23]. During the Ryukyu Kingdom era (1429–1879), horses were bred on the Miyako Islands as gifts to China and for riding by the ruling class. Then, as modernization of Japan progressed during the Meiji period (1868–1912), Miyako horses were more commonly owned by the working class and became a popular means of transport among islanders [22, 23]. In time, as the Islands' sugar industry developed, the horses were used for working in sugarcane fields [22, 23]. However, as the sugar industry further expanded after World War II, the small Miyako horses could not meet the heavy demands of fieldwork and transport. Thus, Miyako horses were crossbred with western horses to improve their physiques, and the number of purebreds decreased rapidly.

To make matters worse, workhorses themselves were replaced by automobiles and farm machines. A 1976 census found that only 14 relatively pure Miyako horses remained, prompting concern pertaining to the risk of their extinction and leading to the foundation of the Miyako Horse Conservation Society (MHCS) in 1980 [22, 23]. The MHCS members and others who recognized the importance of these horses as a cultural asset collected Miyako horses from other islands, such as Aguni Island, and bred them. The number of Miyako horses increased to 41 in 2013 [22, 23]. However, the number of Miyako horses has not been sufficiently restored, and they are likely experiencing an extinction vortex. An extinction vortex is a chain of events that begins with a high frequency of inbreeding in a small population with low genetic variation, leading to inbreeding depression and thus further decline

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**Fig. 1.** Appearance of the Miyako horse. The Miyako horse is one of eight breeds of horse native to Japan. It is small, with a withers height of 110 to 120 cm.

in the population size [7].

When planning conservation of rare animal species, one must view the population in terms of conservation genetics [7]. In the case of Miyako horse, an accurate registration system that enables administration of the population based on genetic information is a primary need. Understanding the genetic diversity of the population will tell us the true scope of the conservation problem and help the MHCS to develop a breeding program to avoid future inbreeding depression. For these purposes, we can use microsatellites and mitochondrial DNA.

Microsatellites are short repetitive sequences with a high mutation rate [15]. Compared with classical polymorphism or mutational analyses, microsatellites are a superior tool to determine the genetic structure of a population and relationships between individual animals [6, 9]. Mitochondrial DNA is maternally inherited and also prone to mutation [10]. Consequently, its rate of base substitution (evolution rate) is 5- to 10-fold greater than that of nuclear DNA, which makes mitochondrial DNA an ideal target for analysis when determining inter- and intra-species maternal relationships in evolutionary biology [4].

The Miyako horse is a traditional livestock breed representing the biological and cultural diversity of Okinawa. However, conservation genetic data of the Miyako horse are limited. In this study, as an initial effort to gain necessary information for the conservation of the Miyako horse, the Miyako horse population was characterized in terms of conservation genetics by using microsatellite and mitochondrial DNA polymorphisms.

## MATERIALS AND METHODS

### *Animals*

This study was conducted in August 2013, using 35 Miyako horses (MIYAKO 01–35; 17 males and 18 females) of the total 41 horses (21 males and 20 females) registered with the MHCS. The average age of the animals was 5.6 years, ranging from 2 months to 15 years. Blood samples were collected with ethical considerations for the research and the welfare of the horses. Genomic DNA was extracted with the MFX-2000 MagExtractor System (Toyobo, Osaka, Japan) according to the manufacturer's protocols.

### *Microsatellite genotyping*

A panel of 18 markers (*AHT4*, *AHT5*, *ASB2*, *ASB17*, *ASB23*, *CA425*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG4*, *HTG10*, *LEX3*, *LEX33*, *TKY19*, *TKY28*, *TKY321* and *VHL20*) and a panel of 14 markers (*TKY279*, *TKY287*, *TKY294*, *TKY297*, *TKY301*, *TKY312*, *TKY325*, *TKY333*, *TKY337*, *TKY341*, *TKY343*, *TKY344*, *TKY374* and *TKY394*) were used in this study. These marker panels are used as routine and complement parentage systems in the Japanese Thoroughbred Registration, respectively. Allele discriminations are based on the consensus of the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee, International Society for Animal Genetics (ISAG).

Microsatellites were genotyped by the procedures described in Kakoi *et al.* [12] and Tozaki *et al.* [30] with minor modifications.

To confirm that each marker does not show linkage disequilibrium, the coefficient of disequilibrium,  $D'$ , was calculated using SNPalyze ver 8.0 (Dynacom, Chiba, Japan). No strong linkage disequilibrium was observed between any pair of markers.

### Mitochondrial DNA sequencing

The 411-bp reference sequence of the mtDNA D-loop region between positions 15,437 and 15,847 [33] from each horse was analyzed based on PCR amplification and direct sequencing using the forward primer 5'-CTAGCTCCACCATCAACACC-3', and the reverse primer 5'-ATGGCCCTGAAGAAAGAACC-3'. The amplicon was sequenced using a BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Science, Waltham, MA, U.S.A.). Haplotype was determined as previously described [13, 26], based on homology search using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information.

### Parentage verification

Microsatellite genotypes were used to determine the accuracy of the current pedigree registration for nine parent–offspring pairs. Genotype results were manually compared to test whether the registered parent–offspring relationships adhere to Mendelian laws of inheritance. If false parent–offspring pairs were identified, genotyping was repeated to rule out technical error. Two-marker exclusion was used to confirm false parent–offspring pairs. This criterion is based on the consensus of the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee, ISAG.

### Analyses

Number of alleles ( $N_a$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ) and fixation index [18, 32] were calculated using the statistical program GENEPOP version 4.2 [19]. Exclusion power (PE) was also calculated based on PE1 as described in Jamieson *et al.* [11], which is for one-parentage exclusion. *LEX3* on the X chromosome was excluded from the PE calculation and the remaining analyses.

The probability of a past genetic bottleneck was evaluated using BOTTLENECK version 1.2.02 under the assumptions of the infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM) [5, 27]. The effective population size ( $N_e$ ) was calculated based on  $H_e$  and  $H_o$  as follows:  $N_e = 1 / \{ 2 (H_o - H_e) / H_e \} + 1 / [ 2 \{ (H_o - H_e) / H_e + 1 \} + 1 ]$  [21].

A phylogenetic tree was constructed using Populations 1.2.30 [14] to visualize the relationships between individuals. Genetic distance was calculated based on the proportion of shared alleles ( $D_{SA}$ ) between each pair of individuals [2]. A neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 6.06 [28]. In NJ phylogenetic trees, individuals that are closely related, such as parent–offspring pairs and siblings, are placed close together. Therefore, NJ phylogenetic trees are useful for visually representing the genetic relationships between individuals.

## RESULTS

### Parentage verification

All microsatellites were well amplified and successfully genotyped for all 35 Miyako horses. When the nine parent–offspring pairs given in the pedigree report were tested, two were found to be false based on our criterion using microsatellite genotypes.

PEs for the 17-microsatellite routine testing panel and for the 14-microsatellite complement testing panel were 0.9998 and 0.9993, respectively. Total PE for the 31 microsatellites was 0.9999998, which fulfills the criteria of exclusion power for Thoroughbred Registration.

### Population genetics

The average  $N_a$  was 4.2 and ranged from 2 to 7. The average  $H_o$  was 0.701; maximum 0.914 for *TKY301*; and minimum 0.286 for *HMS2*. The average  $H_e$  was 0.649; maximum 0.820 for *TKY301*; and minimum 0.324 for *TKY294* (Table 1). The average  $F_{IS}$  was  $-0.075$ .

A Wilcoxon test implemented by BOTTLENECK showed significant deviation from mutation–drift equilibrium due to an excess of observed heterozygosity under IAM ( $P=0.00000$ ), SMM ( $P=0.00026$ ) and TPM ( $P=0.00000$ ), respectively. In other words, the Miyako horse has experienced a genetic bottleneck. Moreover, the  $N_e$  value was 6.7.

### Phylogenetic analysis

The  $D_{SA}$  was calculated, and the genetic distance between individuals was determined. Using  $D_{SA}$  data, a NJ phylogenetic tree of individuals was successfully constructed (Fig. 2), and the genetic distance between individuals was visualized.

### Mitochondrial DNA sequencing

The 35 Miyako horses had only one mitochondrial DNA haplotype (Accession number: AB329598). The haplotype is identical to that of two other native Japanese populations: the Tokara horse [13] and the Yonaguni horse (unpublished data).

## DISCUSSION

In this study, we genetically characterized the Miyako horse using microsatellite and mitochondrial DNA analyses. Here, 35

**Table 1.** The number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He) and Fixation index ( $F_{IS}$ ) in each locus

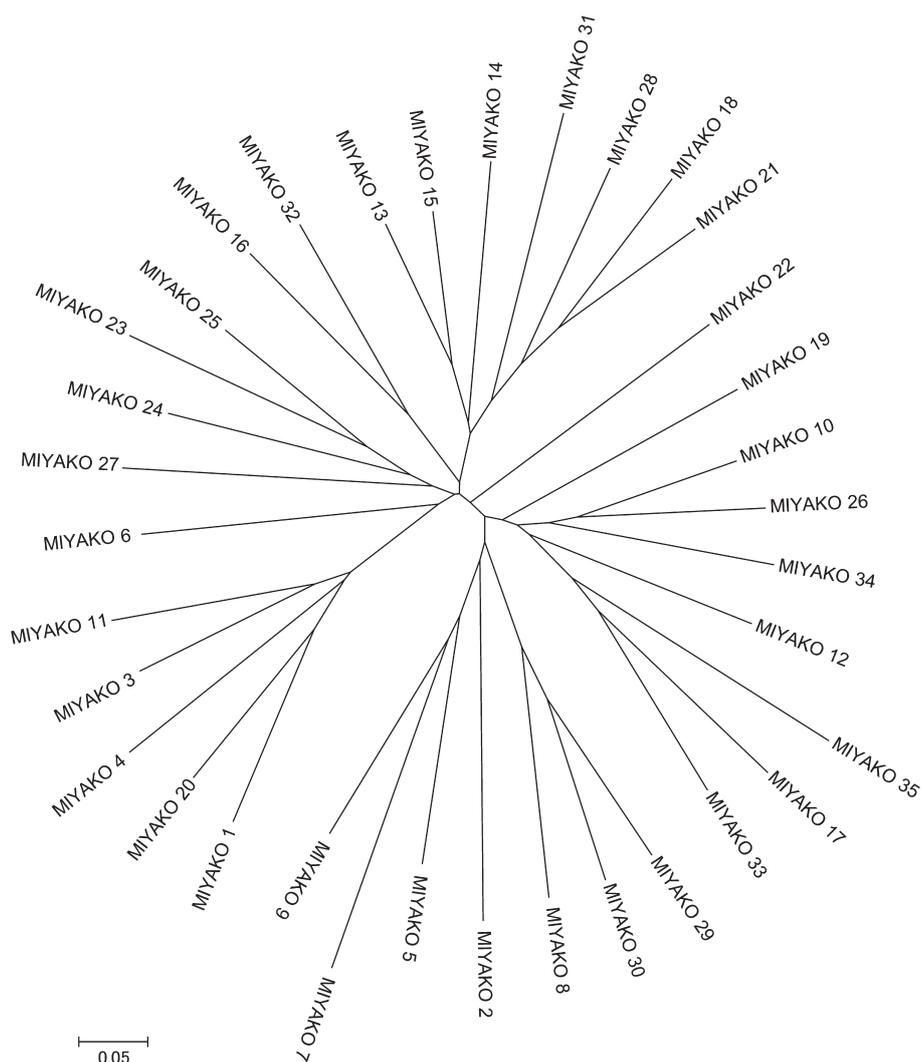
Locus	Na	Ho	He	$F_{IS}$
<i>AHT4</i>	5	0.800	0.677	-0.182
<i>AHT5</i>	4	0.629	0.712	0.117
<i>ASB2</i>	4	0.629	0.557	-0.129
<i>ASB17</i>	3	0.743	0.598	-0.243
<i>ASB23</i>	2	0.371	0.449	0.172
<i>CA425</i>	4	0.743	0.730	-0.017
<i>HMS2</i>	2	0.286	0.359	0.204
<i>HMS3</i>	3	0.657	0.594	-0.106
<i>HMS6</i>	5	0.829	0.687	-0.205
<i>HMS7</i>	5	0.829	0.753	-0.100
<i>HTG4</i>	4	0.800	0.693	-0.155
<i>HTG10</i>	5	0.886	0.764	-0.160
<i>LEX33</i>	4	0.800	0.730	-0.096
<i>TKY19</i>	5	0.743	0.717	-0.036
<i>TKY28</i>	7	0.743	0.711	-0.044
<i>TKY279</i>	6	0.657	0.610	-0.078
<i>TKY287</i>	4	0.714	0.656	-0.090
<i>TKY294</i>	2	0.343	0.324	-0.057
<i>TKY297</i>	5	0.714	0.717	0.004
<i>TKY301</i>	6	0.914	0.820	-0.115
<i>TKY312</i>	4	0.686	0.657	-0.044
<i>TKY321</i>	5	0.829	0.656	-0.264
<i>TKY325</i>	5	0.629	0.684	0.081
<i>TKY333</i>	5	0.800	0.748	-0.069
<i>TKY337</i>	3	0.600	0.534	-0.124
<i>TKY341</i>	3	0.600	0.569	-0.055
<i>TKY343</i>	4	0.771	0.604	-0.278
<i>TKY344</i>	5	0.829	0.798	-0.038
<i>TKY374</i>	4	0.800	0.698	-0.147
<i>TKY394</i>	3	0.600	0.602	0.003
<i>VHL20</i>	4	0.771	0.709	-0.088
Mean	4.2	0.701	0.649	-0.075

Miyako horses, 85% of the 41 horses registered in the MHCS and the Japan Equine Affairs Association (JEAA), were successfully genotyped. This genetic information can contribute to reconstruction of the breed pedigree registration system, because PE (using 31 microsatellites) was highly reliable. Genetic diversity of the Miyako horses was relatively high for such a small population. However, there was no diversity found in the mitochondrial DNA. Therefore, mitochondrial DNA is not a useful source of genetic markers for administration and conservation of this breed.

Our study suggests that parentage testing using microsatellites might be useful for pedigree management in Miyako horses. Pedigree reports have been prepared according to parent-offspring relationships based on breeding history. However, pedigree registration based only on breeding history is not sufficient, because identification of the sire could be incorrect in cases of accidental pregnancy. In fact, within the nine recorded parent-offspring pairs examined here, there were two inconsistencies between the pedigree reports and the genetic information. In this small population, such inconsistencies could have a significant impact on the future makeup of the population, and pedigree management is of utmost importance. Parentage testing using microsatellites should be performed for all newborn Miyako foals to make the pedigree record reliable.

The  $D_{SA}$  data and the NJ phylogenetic tree generated from it enable us to understand the genetic distances among individuals. The NJ phylogenetic tree is important, because it is a convenient way for the MHCS and horse owners, who are neither familiar with professional genetic analyses nor willing to use long streams of numeric  $D_{SA}$  data, to grasp genetic relationships among the individual Miyako horses for planning future conservation breeding.

Our results provide significant insight into the genetic diversity of the Miyako horse population. The average Na and Ho were 4.2 and 0.701, respectively. They were comparable to other rare horse breeds worldwide, including Frederiksborghesten (Denmark), Garrano (Portugal), Jaca Navarra (Spain), Kiso (Japan), Knabstrupper (Denmark), Lipicacanac (Croatia), Lipicai (Hungary), Lipizzaner (Austria), Lipizzano (Italy), Misaki (Japan), Noma (Japan), Pottoka (Spain), Skyros (Greek), Sorraiana (Portugal), Tokara (Japan), Tsushima (Japan) and Yonaguni (Japan) [1, 3, 13, 16, 17, 24, 25, 29, 31]. Moreover, the average  $F_{IS}$  was -0.075. These results suggest that genetic diversity within the Miyako horse population is relatively well-maintained despite the small population size, low variation in mitochondrial DNA and the history of a bottleneck effect. This may be because the Miyako horse population has been reconstructed only within the last three decades, using founder horses collected from other islands and



**Fig. 2.** A neighbor-joining phylogenetic tree of the genetic distances ( $D_{SA}$ ) among individual Miyako horses. The numbers in the tree are the identification numbers of individual horses.

that supposedly had diverse genetic backgrounds.

However, the Miyako horse is still at risk of extinction, because of its very small population size. Its  $N_e$  is 6.7, very less compared to  $N_e$  of 50 that is required for avoiding inbreeding depression [8]. Thus, the first task in conserving the Miyako horse is to increase its population. Fortunately, male horses are traditionally not castrated, and many young females are currently available for breeding. Therefore, with continuing microsatellite analysis for newborn foals in order to determine clear parentage; to monitor genetic diversity of the population; to select mating pairs based on  $D_{SA}$ , the population of this breed should increase without further deleterious effects of inbreeding, and genetic variation can be restored.

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