



Genetic diversity of the Yonaguni horse based on polymorphisms in microsatellites and mitochondrial DNA

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ABSTRACT. Thirty-two microsatellites and a mitochondrial DNA haplotypes of endangered Yonaguni horses were analyzed to establish a pedigree registration system and to understand their genetic diversity for planning effective conservation. Blood samples were collected from 78 of the 130 horses in existence, and DNA was extracted and genotyped. There were two major findings. One is that it is possible to use microsatellites for Yonaguni horse pedigree registration in the future because the power of exclusion of parentage testing is reliable at 0.999998. The second is the clarification of the current genetic diversity of Yonaguni horses. The average number of alleles, observed heterozygosity, expected heterozygosity and fixation index were 4.4, 0.591, 0.601 and 0.016, respectively, for the analyzed horses. The probability of a genetic bottleneck, under the assumptions of the stepwise mutation model, was 0.432, suggesting that the genetic structure of the horses was not influenced by a recent bottleneck. Genetic distance between individuals was visualized by a phylogenetic tree based on the proportion of shared alleles. Structure analysis based on Bayesian clustering revealed the possibility that Yonaguni horses comprise four or five subpopulations. Consequently, although only two haplotypes were identified in the mitochondrial analysis, genetic diversity of Yonaguni horses was not particularly low in comparison with that of other breeds that are at risk of extinction.

KEY WORDS: conservation genetics, microsatellite, mitochondrial DNA, Yonaguni horse

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The Yonaguni horse (Fig. 1) is a breed native to Yonaguni Island in westernmost Japan. The horses are mostly bay colored and small, with a withers height of 110 to 120 cm. Because a pedigree registration system for Yonaguni horses has not been fully established, the exact number is unknown, but estimates show approximately 130 Yonaguni horses in total, on and off the island [25, 26].

Yonaguni horses are bred on two of the three public ranches on Yonaguni Island [25], East (30 ha) and North (80 ha). All Yonaguni horses here have owners but are allowed to graze freely on the island throughout the year. There are currently two stallions on the North ranch and one on the East ranch. Mating is done entirely naturally.

Yonaguni horses are also kept on private guest ranches, where ranchers cultivate relationships between people and the horses. Activities, such as riding, petting and playing, in the sea with the horses (so-called “*umiuma*”) have met with great approval on and off the island, making the Yonaguni horses more widely known [26]. Unlike the two public ranches mentioned earlier, Yonaguni horses at this location are maintained in a modern equestrian style.

Little has been recorded about Yonaguni horses, and their origin remains unknown. However, as against other Japanese horses, the Yonaguni horse is considered a highly pure breed [26]. The reason for this is that Yonaguni Island was exempted from a 1939 order to castrate Japanese native studs called the Stud Horse Control Law [26].

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. With the increase in sugarcane production after World War II, the number of Yonaguni horses increased to over 600. However, with more widespread use of farm machinery in the 1960s, the demand for horses lessened, and the number of horses that were bred dramatically decreased. The number of Yonaguni horses declined to 42 by 1983, but due to efforts by the Yonaguni Horse Conservation Society (YHCS), established in 1975, the population of Yonaguni horses recovered

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

and is currently maintained at about 130 individuals [25, 26]. The breed has been designated endangered by the Food and Agriculture Organization of the United Nations [24]. Therefore, proactive conservation of the breed is necessary.

Microsatellites and mitochondrial DNA are widely used in the characterization of animal genetic resource diversity, which is essential to optimize both conservation and utilization strategies. Microsatellites are short repeating sequences of DNA with a high mutation rate [18]. Therefore, microsatellites are useful for evaluating population diversity and the relationships between individuals within a population [9, 11]. Mitochondrial DNA is maternally inherited and also prone to mutation [12], and mitochondrial DNA polymorphisms have therefore been extensively used in phylogenetic and genetic diversity analyses, as well as to identify the maternal lineages of breeds [4, 5, 17].

For this study, we obtained genetic information about Yonaguni horses based on microsatellites and mitochondrial DNA to establish a pedigree registration system, because such a system will enable appropriate breeding management. We also evaluated the genetic diversity of the breed, because understanding population genetics is necessary for conserving populations of rare animals [10] and little is known about the genetic diversity of Yonaguni horses.

MATERIALS AND METHODS

Samples

Seventy-eight Yonaguni horses (YO1–YO78)—13 stallions, 7 castrated males and 58 females—were used in this study. The horses sampled were from the North ranch (YO1–YO27) including two stallions; the East ranch (YO28–YO37), including one stallion; a municipal guest farm (YO38–YO46); two individual private farms on Yonaguni Island and on the island of Okinawa (YO53–YO74 and YO75–YO78, respectively); and the populations of four individual private owners (YO47–YO52).

Blood collection

Blood samples were collected in accordance with ethical guidelines by the Animal Care and Use Committee of Gifu University (#15137), ensuring the welfare of the horses. To avoid duplicate sampling because of the horses' free roaming on the North and East ranches, horses were herded into one location, and each released after sampling.

DNA extraction

Genomic DNA was extracted from the blood samples with the MFX-2000 MagExtractor System (Toyobo, Osaka, Japan) according to the manufacturer's protocols.

Microsatellite genotyping

In all, 32 microsatellites were used in this study. These microsatellites, which included 18 routine markers (*AHT4*, *AHT5*, *ASB2*,

ASB17, *ASB23*, *CA425*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG4*, *HTG10*, *LEX3*, *LEX33*, *TKY19*, *TKY28*, *TKY321* and *VHL20*) and 14 complement parentage testing markers (*TKY279*, *TKY287*, *TKY294*, *TKY297*, *TKY301*, *TKY312*, *TKY325*, *TKY333*, *TKY337*, *TKY341*, *TKY343*, *TKY344*, *TKY374* and *TKY394*), are routinely used in Japanese thoroughbred registration. All are used in the Horse Comparison Test of the International Society for Animal Genetics (ISAG), and allele discriminations are based on the consensus of the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee of the ISAG. Microsatellites were genotyped using procedures described by Kakoi *et al.* [14] and Tozaki *et al.* [33] with minor modifications. To confirm that markers do not show linkage disequilibrium, we calculated the linkage disequilibrium coefficient D' using SNPalyze ver 8.0 (Dynacom, Chiba, Japan). No strong linkage disequilibrium was found between any pair of markers, and we therefore used all markers for subsequent analyses.

Mitochondrial DNA sequencing

A 411-bp reference sequence of the mtDNA D-loop region between positions 15,437 and 15,847 [29] from each horse was analyzed based on PCR amplification and direct sequencing using the forward primer 5' CTAGCTCCACCATCAACACC-3' and 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.). The haplotypes were determined according to Kakoi *et al.* [15] and Takasu *et al.* [29], based on a homology search using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>).

Statistical analyses

Number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (F_{IS}) [21, 35] were calculated using the statistical program GENEPOP version 4.2 [23]. Exclusion power (PE) for one-parentage exclusion, as described by Jamieson *et al.* [13], was also calculated. *LEX3*, on the X chromosome, was excluded from the PE calculation and the above mentioned analyses of N_a , H_o , H_e and F_{IS} . The possibility of a bottleneck effect, an historical rapid decline in the herd population causing fixation of a gene that would adversely affect future conservation, was evaluated using BOTTLENECK version 1.2.02 software under the assumptions of the stepwise mutation model (SMM) [6, 30].

Genetic distance was calculated based on the proportion of shared alleles (D_{SA}) between individuals [2] using Populations 1.2.30 [16]. A neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 6.06 [31], and the relationships among individuals were visualized to enhance the practical utility of the D_{SA} data.

Population structure was analyzed using Structure ver 2.3.4 [22], performing 20 independent runs with 100,000 steps of burn-in and a 100,000-step Markov chain Monte Carlo for each potential number of clusters (K), for $K=1-10$. Finally, we calculated delta K using STRUCTURE HARVESTER v. 0.6.94 [7] and the method described by Evanno *et al.* [8] to identify the most likely K . This procedure was repeated five times to confirm reliability of the results.

RESULTS

Microsatellite genotyping

All microsatellites were well-amplified and successfully genotyped for all 78 Yonaguni horses. All parent-offspring pairs that had been recorded as pedigree information were confirmed with microsatellites. No exclusions were observed in any of the parent-offspring pairs analyzed. PEs for the 18-microsatellite panels as routine tests and that for the 14-microsatellite panels as complement tests were 0.9988 and 0.9987, respectively. Total PE for all 32 microsatellites was 0.999998, which fulfilled the criteria of PE in thoroughbred registration.

The average N_a of Yonaguni horses was 4.4 and ranged from 3 to 6. The average H_o was 0.591; maximum 0.744 for *TKY287*; and minimum 0.141 for *ASB23*. The average H_e was 0.601; maximum 0.750 for *TKY287*; and minimum 0.135 for *ASB23*. The mean F_{IS} was 0.016 (Table 1).

The probability value tested under the SMM assumptions in BOTTLENECK was 0.432, and the distribution of allele frequency across all loci formed the normal “L-shaped” distribution, indicating the existence of relatively large number of rare alleles [18]. Thus, it was concluded that Yonaguni horses have not experienced a recent bottleneck.

Phylogenetic and structure analyses

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. However, subpopulations were not easily identified in the NJ phylogenetic tree. The delta K_s obtained from STRUCTURE HARVESTER were four (four times) and five (one time). It was therefore estimated that Yonaguni horses could be divided into four or five subpopulations (Fig. 3).

Mitochondrial DNA sequencing

Yonaguni horses had only two mitochondrial DNA haplotypes (Accession numbers AB329624 and AB329598). One of these haplotypes (AB329598) is shared with other Japanese native populations: the Tokara horse [15] and the Miyako horse (unpublished data).

Table 1. The number of allele (Na), Observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (F_{IS}) at each locus

Locus	Na	Ho	He	F_{IS}
<i>AHT4</i>	4	0.449	0.504	0.109
<i>AHT5</i>	5	0.667	0.599	-0.114
<i>ASB2</i>	3	0.641	0.663	0.034
<i>ASB17</i>	5	0.603	0.647	0.069
<i>ASB23</i>	4	0.141	0.135	-0.046
<i>CA425</i>	6	0.615	0.701	0.123
<i>HMS2</i>	4	0.603	0.614	0.018
<i>HMS3</i>	4	0.628	0.636	0.012
<i>HMS6</i>	3	0.603	0.554	-0.087
<i>HMS7</i>	4	0.628	0.602	-0.044
<i>HTG4</i>	4	0.539	0.572	0.058
<i>HTG10</i>	6	0.321	0.368	0.130
<i>LEX33</i>	4	0.718	0.663	-0.083
<i>TKY19</i>	3	0.359	0.432	0.168
<i>TKY28</i>	4	0.692	0.653	-0.060
<i>TKY279</i>	5	0.654	0.635	-0.029
<i>TKY287</i>	4	0.744	0.750	0.008
<i>TKY294</i>	3	0.526	0.589	0.107
<i>TKY297</i>	5	0.564	0.579	0.026
<i>TKY301</i>	6	0.667	0.694	0.039
<i>TKY312</i>	4	0.628	0.587	-0.071
<i>TKY321</i>	6	0.615	0.620	0.007
<i>TKY325</i>	4	0.577	0.637	0.094
<i>TKY333</i>	4	0.718	0.743	0.034
<i>TKY337</i>	4	0.449	0.419	-0.071
<i>TKY341</i>	4	0.628	0.605	-0.039
<i>TKY343</i>	5	0.718	0.715	-0.004
<i>TKY344</i>	3	0.615	0.658	0.065
<i>TKY374</i>	6	0.654	0.666	0.018
<i>TKY394</i>	4	0.654	0.683	0.043
<i>VHL20</i>	5	0.718	0.710	-0.012
Mean	4.4	0.591	0.601	0.016

DISCUSSION

In this study, 78 blood samples were collected from Yonaguni horses, representing about 60% of the 130 total population estimated by the Japan Equine Affairs Association [24], and microsatellite genotyping information was successfully obtained from each. There are two important implications of this study. One is that the genotyping information obtained here will contribute to establish certification of Yonaguni horse pedigree in future, as currently, neither certificates of pedigree nor clear pedigree control exist for Yonaguni horses. This certification can be a useful tool for paternity assessment, individual identification and breeding management. The second is that current genetic diversity of the horses was clarified, and while it is not especially high, it can be maintained by conserving the present population size.

This study brings us a large step closer to being able to issue pedigree certificates for Yonaguni horses. Pedigrees for these horses have not been established to this day, because most of them mate freely under feral conditions. To be specific, the North ranch, which is the main habitat of Yonaguni horses, has two stallions in pasture, making it difficult to determine the paternity of foals born there. However, we believe the 32 microsatellites genotyped will resolve this uncertainty regarding paternity. Additionally, the NJ tree constructed from the D_{SA} data provides a better understanding of the genetic distances between individuals (Fig. 2). This is important practically, because this approach is user-friendly for the YHCS and horse owners, both of whom are neither familiar with genetic analyses nor willing to use long streams of numeric D_{SA} data to grasp genetic relationships between the individual Yonaguni horses when planning future conservation breeding. The NJ tree could be used by the YHCS and horse owners, along with other information, to select mating pairs with lower consanguinity.

The genetic integrity of Yonaguni horses can probably be conserved, if the number of individuals can be maintained. This is supported by the fact that even though the samples obtained for this study represented 60% of all Yonaguni horses, the average Na (4.4) and Ho (0.591) were not nearly as low as those of horse breeds said to be at risk of extinction in various other countries [1, 3, 14, 19, 20, 27, 28, 32, 34]. In addition, the genetic analysis of the Yonaguni horses did not suggest any recent bottleneck

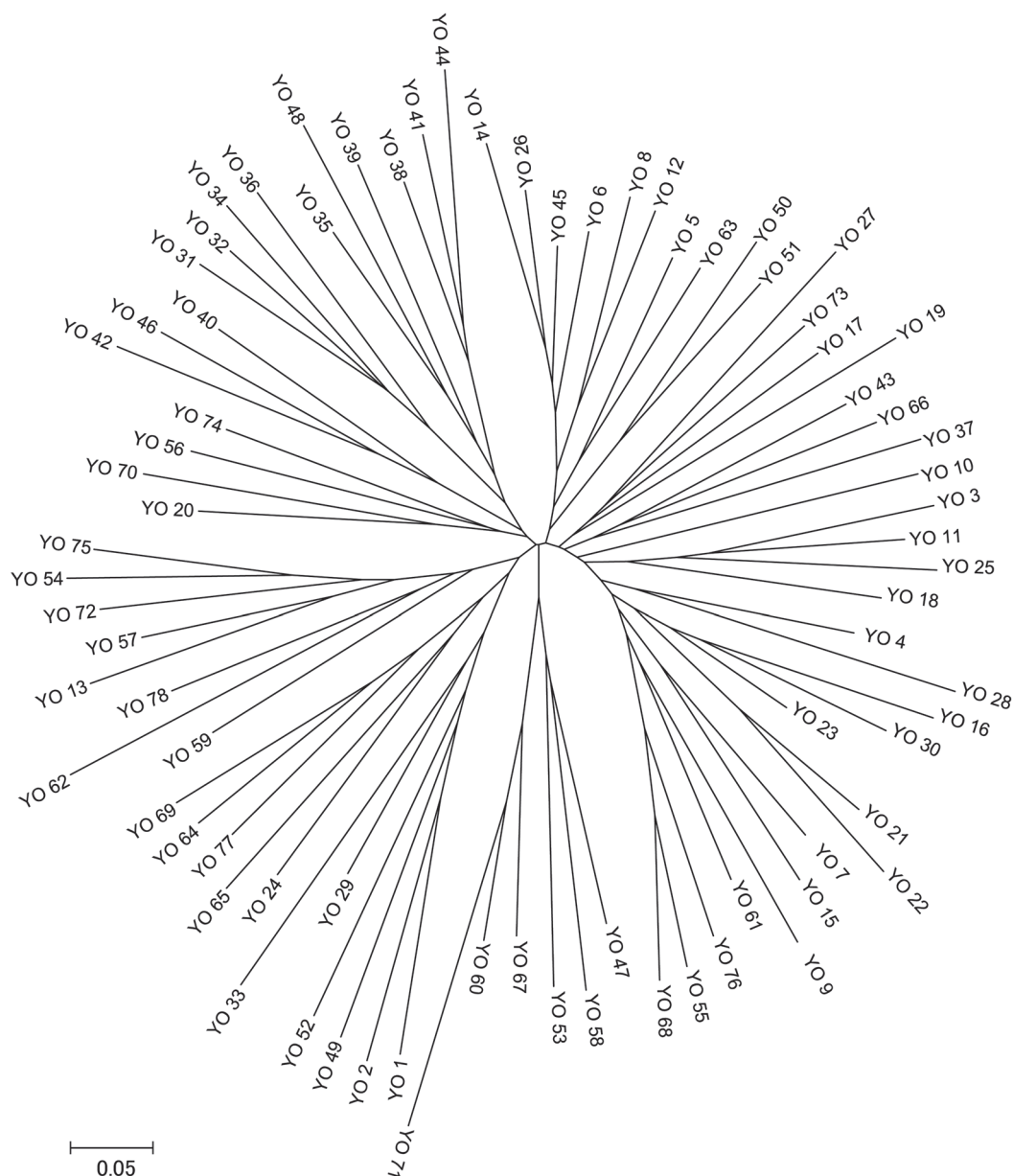


Fig. 2. A neighbor-joining phylogenetic tree of the genetic distances (D_{SA}) among individual Yonaguni horses. The numbers in the tree are the identification numbers of individual animals.

effect [10]. One reason for this is that Yonaguni horses mate naturally in the public ranches, requiring little, if any, intervention by ranchers.

We identified four or five subpopulations of Yonaguni horses using Structure (Fig. 3), but the clustering was unstable. However, the identified subpopulations of Yonaguni horses reflected the distribution of the ranches: two subpopulations on the north ranch (shown in structure A as green and blue and in structure B as green and purple), one on the east ranch (shown in structure A as yellow and in structure B as blue) and one on the private ranch (shown in structures A and B as red).

The 78 Yonaguni horses included here have only two mitochondrial DNA haplotypes, and this finding agrees with a previous study [15] in which the mitochondrial DNA of 19 horses was sequenced. The low number of haplotypes may be due to a reduction in maternal lineages associated with a decline in the number of individual horses, limited maternal origins for this breed or a combination of these factors.

It is very rare for endangered horse breeds as such Yonaguni horses to have experienced no bottleneck, and as mentioned before, we think this is owing to the distinct semi-wild rearing style adopted by the public ranches. The prospects for future populations of Yonaguni horses might be favorable so long as the pasturelands and the rearing style are maintained. However, in most cases, the time, effort and costs associated with rearing cause the rearing and maintenance of horses to become burdensome, though the rearing of Yonaguni horses may not be associated with many of these problems, since they are grazed yearlong and mate naturally.

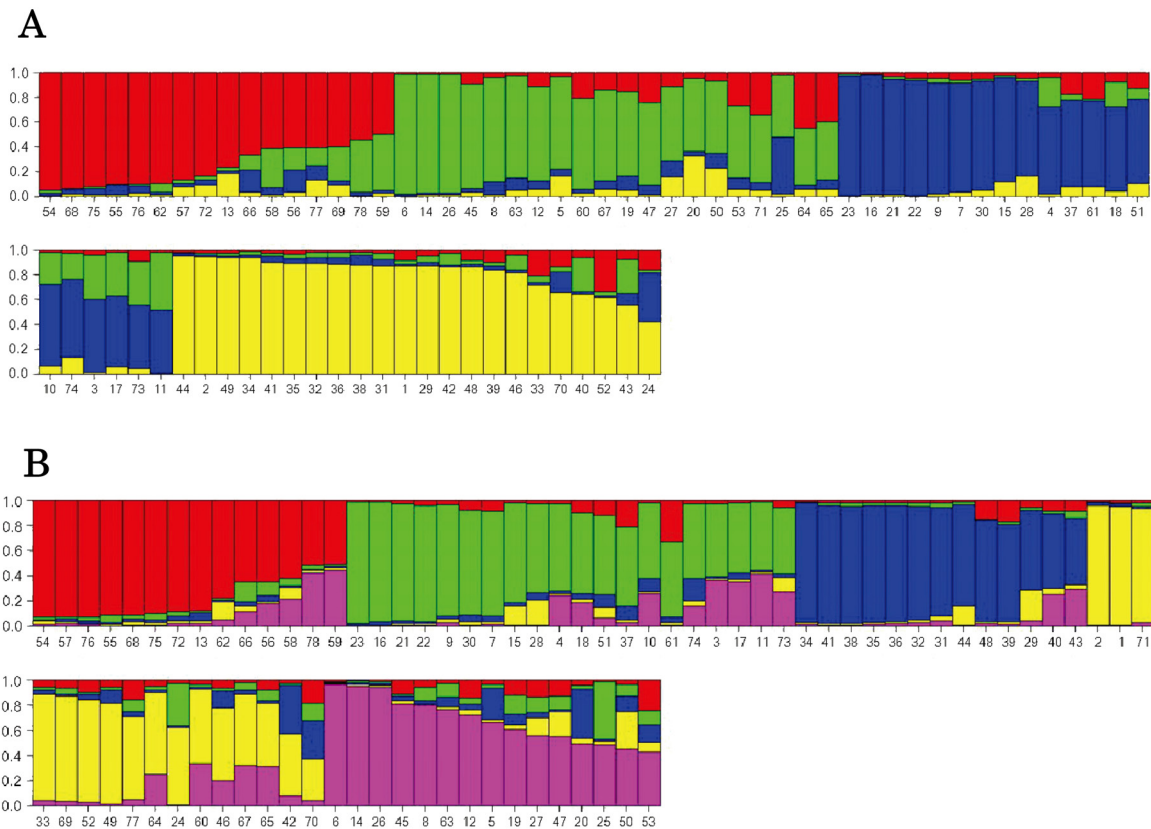


Fig. 3. Bayesian clustering suggesting the Yonaguni horse has 4 or 5 subpopulations. The software STRUCTURE HARVESTER indicated (A) $K=4$ in five analyses, and (B) $K=5$ in one. The numbers below each bar are the identification numbers of individual animals.

This rearing style entails little manpower or cost for owners and breeders and also ensures that the horse population is maintained at a size that the pastureland can hold. Thus, this semi-wild rearing style has played, and will continue to play, an important role in maintaining the Yonaguni horse breed. Consequently, based on our results, we suggest that pedigree control using our obtained data and active maintenance of current pasturelands may be important to effectively conserve Yonaguni horses.

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