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Microsatellite Variation in Japanese and Asian Horses and Their Phylogenetic Relationship Using a European Horse Outgroup

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Abstract

The genetic relationships of seven Japanese and four mainland-Asian horse populations, as well as two European horse populations, were estimated using data for 20 microsatellite loci. Mongolian horses showed the highest average heterozygosities (0.75–0.77) in all populations. Phylogenetic analysis showed the existence of three distinct clusters supported by high bootstrap values: the European cluster (Anglo-Arab and thoroughbreds), the Hokkaido-Kiso cluster, and the Mongolian cluster. The relationships of these clusters were consistent with their geographical distributions. Basing our assumptions on the phylogenetic tree and the genetic variation of horse populations, we suggest that Japanese horses originated from Mongolian horses migrating through the Korean Peninsula. The genetic relationship of Japanese horses corresponded to their geographical distribution. Microsatellite polymorphism data were shown to be useful for estimating the genetic relationships between Japanese horses and Asian horses.

In the domestic livestock species of Japan, the populations of native horses (*Equus caballus*) have been insulated from other horse populations and relatively free of artificial selection. Currently there are eight local populations of Japanese horses: Hokkaido, Kiso, Misaki, Noma, Taishu, Tokara, Miyako, and Yonaguni. The phylogenetic relationships among these Japanese horse populations is not well elucidated. Horses native to Japan have been kept in their local areas to avoid extinction, although there was previously some gene flow between the Japanese horse populations. The population sizes of these Japanese horses range from 35 to 100 horses, except for the native horses from Hokkaido, which include more than 2,000 horses (Nozawa 1992).

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

Hayashida (1958) classified Japanese horses into two groups according to body size and location. Medium-size and small-size horses are 130–140 cm and 110–120 cm in withers height, respectively. The medium-size horses—Hokkaido, Kiso, and Misaki horses—live mainly on the three main islands of Japan (Hokkaido, Honshu, and Kyushu). The small-size horses—Noma, Taishu, Tokara, Miyako, and Yonaguni horses—live on islets (Shikoku, Tsushima, Tokara, Miyako, and Yonaguni) south and west of the main islands. Based on this classification, Hayashida (1958) proposed a two-wave migration hypothesis for the evolution of Japanese horse populations.

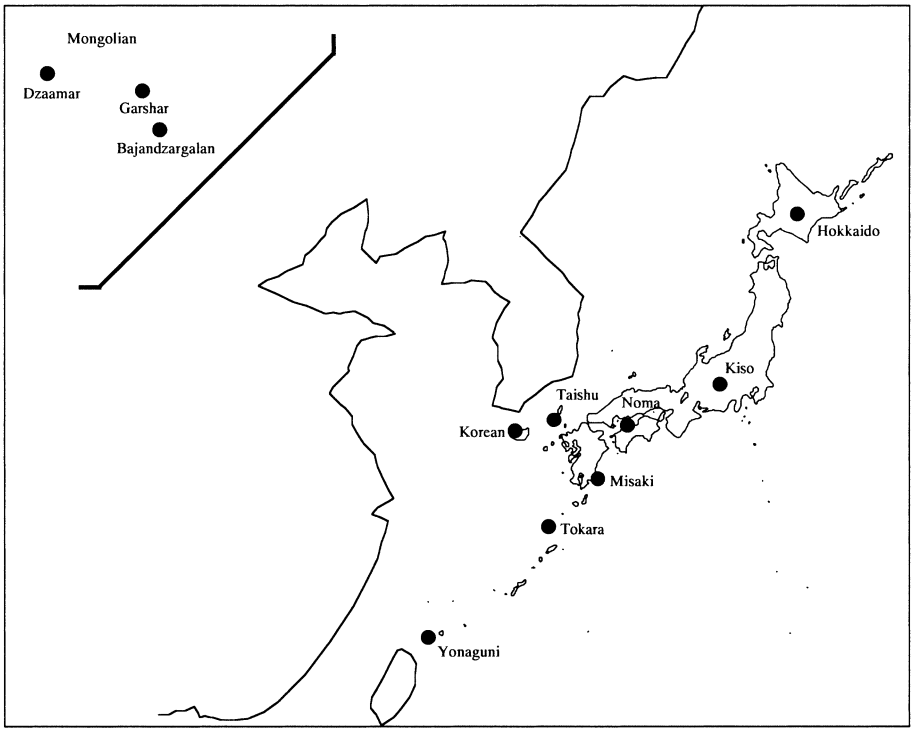

Figure 1. The location of seven Japanese horses, one Korean horse, and three Mongolian horses.

However, a recent study using the protein polymorphisms of 22 loci from 2,415 horses belonging to 34 horse populations in Japan and Asia (Nozawa et al. 1998) was inconsistent with this two-wave migration. The phylogenetic relationships among Japanese horses could not correspond to the geographical distribution in Japan (Nozawa et al. 1998). The difference between the phylogenetic relationship and the geographical distribution might be a consequence of a small population size, a bottleneck effect, and low polymorphism for protein loci. The 22 loci examined had monomorphism or two to three alleles in Japanese horses and were less informative for the determination of a phylogenetic relationship, although useful for Asian horses.

Recently many microsatellites were isolated from horse genome DNA (Tozaki et al. 2000a,b,c, 2001b). Microsatellites, in particular the (CA/TG)n repeats, which were constructed with tandemly repeated DNA sequences consisting of poly(dC-dA)/(dG-dT) repeats, have been found to be common in all eukaryotic genomes, occurring once for every 30–60 kb (Litt and Luty 1989; Stallings et al. 1991; Weber and May 1989). As microsatellites are also frequently polymorphic and evolutionarily conserved in the eukaryotic genome, they provide useful markers for comparative studies of genetic variation, parentage testing, and studies of gene flow (Bjørnstad and Røed 2001; Cunningham et al. 2001; Tozaki et al. 2001a) and have recently been the markers of choice for analyses of population structure in wild and domesticated species (Bowcock et al. 1994; Kim et al. 2001; Takahashi et al. 1998).

The genetic relationships of many horse populations in Europe have been investigated recently using microsatellites (Canon et al. 2000; Vila et al. 2001). However, the genetic relationships of horse populations in Japan have not been investigated using microsatellites.

In the present study, we analyzed 20 microsatellite loci, which were originally characterized in thoroughbred horses to infer the phylogenetic relationships among Japanese and Asian horses. In addition, we compared the levels of polymorphism between Japanese and Asian horses.

Materials and Methods

Animals

Sampling localities are shown in Figure 1. We collected blood samples from 60 Mongolian horses: Dzaamar ($n = 22$), Bajandzargalan ($n = 18$), and Garshar ($n = 20$). In addition, we collected blood samples from a total of 135 Japanese horses: Hokkaido ($n = 24$), Kiso ($n = 21$), Misaki ($n = 16$), Noma ($n = 16$), Taishu ($n = 10$), Tokara ($n = 24$), and Yonaguni ($n = 24$). We also collected blood samples from Korean horses ($n = 21$), thoroughbred horses ($n = 25$), and Anglo-Arab horses ($n = 18$). No horses were injured in any way during the collecting of samples. Genomic DNAs were prepared from whole blood samples. Genomic DNAs from thoroughbred horses were extracted using the MagExtractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Following digestion with proteinase K (2 mg/ml) in extraction buffer (10 mM Tris, 10

mM EDTA, 0.1 M NaCl, 2% SDS), genomic DNAs from Asian horses were extracted by phenol/chloroform, precipitated with ethanol, washed, and resuspended in a sterile TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Microsatellites

In this study we used the following 20 microsatellite loci: *TKY2* (Sakagami et al. 1995); *TKY3* (Tozaki et al. 1995); *HTG3*, *HTG4*, *HTG5*, *HTG6* (Ellegren et al. 1992); *HTG7*, *HTG10*, *HTG15* (Marklund et al. 1994); *HMS1*, *HMS2*, *HMS3*, *HMS5*, *HMS7*, *HMS8* (Guérin et al. 1994); *VHL20* (van Haeringen et al. 1994); and *AHT4*, *ASB1*, *ASB2*, and *ASB3* (Swinburne et al. 2000).

Genotyping

Forward primers of all the microsatellites were labeled with Cy-5 (Amersham Biosciences, Piscataway, NJ). Each polymerase chain reaction (PCR) amplification was performed in a total volume of 20 μl of the following mixture: 20–50 ng of equine genomic DNA, 5 pmol of a Cy-5-labeled forward primer, 5 pmol of reverse primer, 200 μM of dNTP, 2 μl of 10× reaction buffer; and 0.1 U of r*Taq* polymerase (Takara Bio, Shiga, Japan). PCR amplification entailed initial denaturation (94°C, 4 min); 30 cycles of 1 min each at 94°C, 55–60°C, and 72°C; and then 60 min at 72°C for final extension in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). The final extension step at 72°C for 60 min promoted a complete nontemplated 3′-nucleotide addition. The amplified fragments were analyzed through electrophoresis with an ALF express DNA sequencer (Amersham Biosciences) using an 8% polyacrylamide gel containing 7 M urea (Biomate, Tokyo, Japan). Finally, the raw data obtained were converted to dinucleotide repeat polymorphic band patterns using a Fragment Manager (Amersham Biosciences).

Estimation of Genetic Distances and the Construction of a Phylogenetic Tree

Alleles were designated according to PCR product size and allele frequencies were estimated by direct counting. Genetic differences among populations were estimated by calculating the D_A distance (Nei 1983) and $(\delta\mu)^2$ distance (Goldstein et al. 1995). A neighbor-joining (NJ) tree was constructed from the distances (Saitou and Nei 1987), and 1,000 bootstrap replicates were generated.

Results

Heterozygosities and the Number of Alleles

Polymerase chain reaction amplification of 20 (CA/TG)n microsatellites, which were mainly isolated from the thoroughbred horse genome, was successful with minor modifications of the original condition for the 13 horse populations studied. Allele frequencies and heterozygosities were calculated. (The allele frequencies are available from the senior author upon request.) Despite possible inbreeding, most loci displayed polymorphism between individuals. Allele frequency distributions and heterozygosities of 20 microsatellites showed unique features for each population. Although the other populations were polymorphic, with 2 to 10 alleles at all loci, the Misaki and Tokara populations were monomorphic at three loci (*HTG7*, *HMS3*, and *HMS8*) and six loci (*HTG5*, *HTG6*, *HTG7*, *HMS8*, *ASB1*, and *ASB2*), respectively; these loci also showed relatively less polymorphism than the other loci when the other horse populations were analyzed.

A total of 130 alleles were detected in this study and the average number of alleles was 4.6 (Table 1). The observed number of alleles and average heterozygosities for all populations are shown in Tables 1 and 2. The average heterozygosity was 0.62, ranging from 0.34 in the Tokara population of Japanese horses to 0.77 in the Bajandzargalan population of Mongolian horses. Four populations of Japanese horses (Misaki, Noma, Tokara, and Yonaguni) showed lower heterozygosity than the average value. Similar to the heterozygosity data, the observed number of alleles ranged from 42 in the Tokara population to 125 in the Dzaamar population, and the average allele number was 4.6. The average allele numbers of the aforementioned four populations were lower than the average value. In particular, low heterozygosities and a small number of alleles were observed in the Tokara and Misaki populations. Conversely, high heterozygosities and a large number of alleles were observed in three populations of Mongolian horses. Three populations of Mongolian horses had more alleles than the other populations at 17 loci. The average heterozygosity for Japanese horses was less than for any other population. Furthermore, the number of alleles per locus also tended to be fewer among Japanese horses.

Genetic Distances and Population Relationships

Estimates of the genetic distances among the 13 populations based on the allele frequency data on 20 microsatellites are presented in Table 3. The D_A values always range between 0 and 1 and are nonlinearly related to the number of gene substitutions. Mean genetic distances ranged from 0.057 (Bajandzargalan to Garshar populations), 0.065 (Bajandzargalan to Garshar populations), and 0.075 (Bajandzargalan to Garshar populations) to 0.491 (Tokara to thoroughbred populations) and 0.481 (Misaki to Noma populations), while the $(\delta\mu)^2$ distance ranged from 0.057 (Bajandzargalan to Garshar populations), 0.065 (Bajandzargalan to Garshar populations), and 0.075 (Bajandzargalan to Garshar populations) to 0.491 (Tokara to thoroughbred populations) and 0.481 (Misaki to Noma populations). Considering all distance measures, the three populations of Mongolian horses were closest.

D_A and $(\delta\mu)^2$ distance matrices were used to build phylogenetic trees with the NJ method. The NJ method for constructing evolutionary trees from distance data has been reported to be one of the most effective methods (Saitou and Nei 1987). Figures 2 and 3 show the phylogenetic tree

Table 1. The number of alleles and average alleles of 20 microsatellites

	Population	*TKY2*	*TKY3*	*HTG3*	*HTG4*	*HTG5*	*HTG6*	*HTG7*	*HTG10*	*HTG15*	*HMS1*	*HMS2*	*HMS3*	*HMS5*	*HMS7*	*HMS8*	*AHT4*	*ASB1*	*ASB2*	*ASB3*	*VHL20*	Average
Japanese	Hokkaido	6	3	5	5	6	5	4	5	4	4	8	5	2	5	5	7	4	7	5	7	5.1
	Kiso	3	4	5	3	4	2	4	6	4	4	6	5	3	5	4	7	5	9	6	6	4.8
	Misaki	3	2	3	3	3	2	1	4	2	2	3	1	4	5	1	4	4	7	2	4	3.0
	Noma	3	2	2	4	3	3	4	2	3	4	6	4	3	4	4	3	4	6	4	5	3.7
	Tokara	2	2	2	2	1	1	1	2	2	4	2	3	2	4	1	3	1	1	4	2	2.1
	Taishu	3	3	2	5	2	5	3	5	4	5	5	5	3	4	4	5	3	6	4	5	4.1
	Yonaguni	3	3	5	4	3	4	4	6	2	3	4	4	2	4	3	5	4	4	3	5	3.8
Korean	Cheju	6	3	6	5	7	5	5	10	5	5	6	7	3	5	4	8	5	10	5	8	5.9
Mongolian	Dzaamar	6	4	5	6	7	8	4	9	6	6	6	6	3	6	5	9	5	9	7	8	6.3
	Bajandzargalan	6	4	6	6	6	7	3	7	7	5	7	8	3	8	5	7	5	6	6	8	6.0
	Garshar	6	4	6	6	5	6	3	6	6	6	5	7	3	7	5	10	5	9	6	8	6.0
European	Anglo-Arab	3	5	5	4	6	4	2	6	3	4	5	5	3	6	3	4	3	6	5	8	4.5
	Thoroughbred	3	4	3	4	4	5	2	6	3	3	5	5	4	5	4	4	3	8	4	4	4.2
Average		4.1	3.3	4.2	4.4	4.4	4.4	3.1	5.7	3.9	4.2	5.2	5.0	2.9	5.2	3.7	5.9	3.9	6.8	4.7	6.0	4.6

Table 2. Heterozygosity for 20 microsatellites in each population

	Population	*TKY2*	*TKY3*	*HTG3*	*HTG4*	*HTG5*	*HTG6*	*HTG7*	*HTG10*	*HTG15*	*HMS1*	*HMS2*	*HMS3*	*HMS5*	*HMS7*	*HMS8*	*AHT4*	*ASB1*	*ASB2*	*ASB3*	*VHL20*	Average
Japanese	Hokkaido	0.68	0.58	0.49	0.51	0.60	0.54	0.72	0.66	0.27	0.63	0.73	0.67	0.41	0.78	0.74	0.84	0.67	0.86	0.34	0.80	0.63
	Kiso	0.66	0.73	0.51	0.48	0.57	0.47	0.73	0.82	0.34	0.46	0.85	0.73	0.51	0.70	0.75	0.71	0.73	0.84	0.78	0.81	0.66
	Misaki	0.62	0.52	0.34	0.19	0.55	0.48	0.00	0.77	0.53	0.46	0.53	0.00	0.68	0.76	0.00	0.30	0.58	0.80	0.18	0.30	0.43
	Noma	0.70	0.07	0.53	0.67	0.66	0.46	0.70	0.37	0.59	0.73	0.81	0.67	0.57	0.64	0.72	0.43	0.71	0.72	0.72	0.76	0.61
	Tokara	0.45	0.29	0.48	0.41	0.00	0.00	0.00	0.45	0.51	0.67	0.23	0.55	0.51	0.64	0.00	0.57	0.00	0.00	0.54	0.43	0.34
	Taishu	0.52	0.21	0.53	0.78	0.11	0.54	0.61	0.82	0.63	0.78	0.85	0.86	0.72	0.80	0.38	0.71	0.66	0.88	0.53	0.82	0.64
	Yonaguni	0.12	0.61	0.66	0.69	0.58	0.60	0.39	0.64	0.20	0.24	0.58	0.54	0.49	0.70	0.62	0.62	0.59	0.69	0.49	0.72	0.54
Korean	Cheju	0.72	0.14	0.82	0.42	0.64	0.66	0.81	0.84	0.56	0.57	0.85	0.82	0.62	0.71	0.74	0.87	0.61	0.88	0.77	0.81	0.69
Mongolian	Dzaamar	0.81	0.79	0.80	0.54	0.83	0.67	0.67	0.85	0.41	0.72	0.81	0.83	0.68	0.81	0.72	0.84	0.70	0.87	0.82	0.84	0.75
	Bajandzargalan	0.84	0.66	0.75	0.79	0.77	0.79	0.77	0.81	0.56	0.73	0.81	0.78	0.59	0.85	0.75	0.80	0.77	0.84	0.80	0.86	0.77
	Garshar	0.82	0.66	0.76	0.70	0.70	0.76	0.74	0.75	0.68	0.77	0.80	0.76	0.61	0.86	0.72	0.89	0.75	0.80	0.71	0.81	0.75
European	Anglo-Arab	0.41	0.56	0.71	0.56	0.48	0.65	0.55	0.85	0.60	0.69	0.45	0.72	0.70	0.81	0.68	0.72	0.66	0.88	0.71	0.80	0.66
	Thoroughbred	0.40	0.64	0.58	0.56	0.12	0.61	0.69	0.83	0.63	0.71	0.52	0.76	0.64	0.80	0.62	0.76	0.60	0.85	0.48	0.77	0.63
Average		0.60	0.50	0.61	0.56	0.51	0.56	0.57	0.73	0.52	0.63	0.68	0.67	0.60	0.76	0.57	0.70	0.62	0.76	0.61	0.73	0.62

Table 3. Genetic distances among all pairs of populations of Japanese, Asian, and European horse populations calculated using DA (above diagonal) and $(\delta\mu)^2$ (below diagonal)

	Hokkaido	Kiso	Misaki	Noma	Tokara	Taishu	Yonaguni	Korean	Dzaamar	Bajandzargalan	Garshar	Anglo-Arab	Thoroughbred
Hokkaodo		0.175	0.408	0.266	0.426	0.243	0.314	0.172	0.152	0.180	0.174	0.301	0.339
Kiso	0.491		0.459	0.351	0.448	0.281	0.320	0.213	0.180	0.193	0.197	0.296	0.306
Misaki	4.410	5.293		0.481	0.472	0.421	0.445	0.341	0.374	0.376	0.374	0.434	0.442
Noma	1.967	2.674	3.270		0.405	0.274	0.332	0.199	0.250	0.264	0.239	0.327	0.384
Tokara	5.151	5.559	6.630	3.414		0.458	0.385	0.375	0.382	0.376	0.371	0.435	0.491
Taishu	0.800	1.169	3.604	2.473	5.555		0.309	0.182	0.173	0.209	0.164	0.262	0.290
Yonaguni	1.409	1.794	2.802	1.748	4.644	1.225		0.274	0.278	0.274	0.246	0.405	0.376
Korean	0.776	1.394	2.079	1.692	5.012	0.888	0.852		0.110	0.128	0.110	0.171	0.197
Dzaamar	0.454	0.885	2.664	1.438	4.194	0.621	0.728	0.343		0.075	0.065	0.187	0.224
Bajandzargalan	0.623	1.104	2.637	1.502	4.165	0.695	0.698	0.347	0.162		0.057	0.197	0.219
Garshar	0.425	1.063	3.172	1.445	4.821	0.982	0.748	0.335	0.251	0.185		0.202	0.220
Anglo-Arab	2.273	2.329	3.006	3.148	5.670	1.759	1.438	1.271	1.561	1.554	1.850		0.082
Thoroughbred	2.803	2.830	2.598	3.309	5.653	2.318	1.605	1.429	1.912	1.710	2.115	0.195	

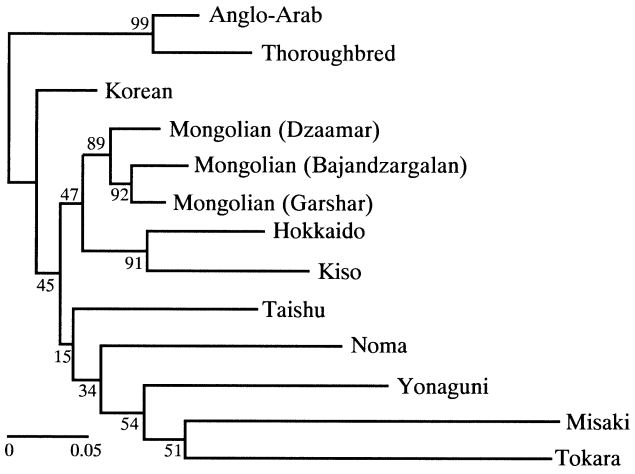

Figure 2. Neighbor-joining dendrogram (Saitou and Nei 1987) of Japanese, Mongolian, and Korean horse varieties based on D_A (Nei 1983). The numbers represent the percentage of bootstrap values from 1,000 replications of resampled loci. European horses are positioned as an outgroup. The linear scale relates the branch lengths to D_A units.

obtained from D_A and $(\delta\mu)^2$ distance values using the NJ method. However, these trees showed some differences from each other. In order to test the reliability of the tree topography, dendrograms from 1,000 replications of resample loci were constructed using both distances. High bootstrap values were obtained when using the D_A distance, which indicated the high confidence of the obtained trees. The D_A distance clustered together both European populations with a bootstrap value of 99%, and Mongolian populations with bootstrap values of 89% and 92%. Hokkaido and Kiso horses clustered with a bootstrap value of 91%.

Discussion

The task of resolving the genetic relationships among Japanese horses is a difficult one, because each population size of Japanese horses is small and Japanese horses had substantial gene flow between the populations (Nozawa 1992). Recently, many microsatellites were isolated from the horse genome (Tozaki et al. 2000a, 2000b, 2000c, 2001b), and the microsatellites showed multiple alleles as well as high heterozygosity among European horse breeds such as thoroughbreds. Microsatellites may be a useful approach for resolving the relationships of Japanese horse populations.

In this study we analyzed the allele frequencies and genotype distributions of Japanese horses to investigate a phylogenetic relationship among the populations. In addition, we analyzed the allele frequencies and genotype distributions among Mongolian and Korean horses to consider the ancestral populations for Japanese horse populations.

Twenty microsatellites, which were isolated from the thoroughbred genome, showed high heterozygosity and

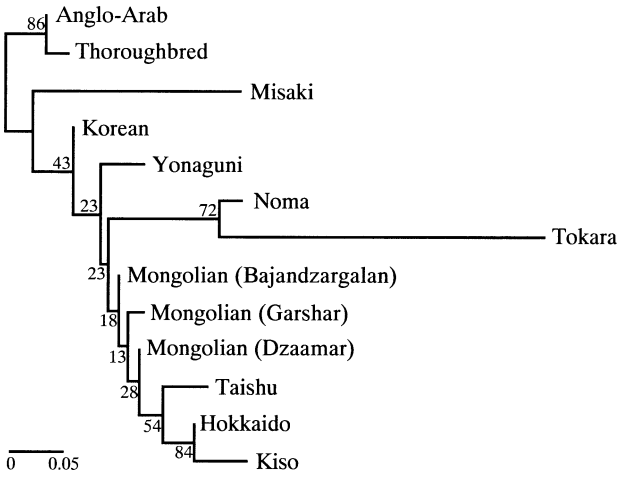

Figure 3. Neighbor-joining dendrogram (Saitou and Nei 1987) of Japanese, Mongolian, and Korean horse varieties based on $(\delta\mu)^2$ (Goldstein et al. 1983). The numbers represent the percentage of bootstrap values from 1,000 replications of resampled loci. European horses are positioned as an outgroup. The linear scale relates the branch lengths to $(\delta\mu)^2$ units.

were used for analyzing allele frequencies of Asian horse populations, Anglo-Arab breeds, and thoroughbreds. The average heterozygosity within populations analyzed in this study was 0.62 (range 0.34–0.77). Nozawa et al. (1998) investigated genetic variation among Asian horses using 22 blood protein polymorphisms; this study showed an average heterozygosity between 0.0584 and 0.1257. The microsatellites tested in our study were therefore effective tools in analyzing the genetic population structure of Asian horses.

The average heterozygosity within each population of Mongolian horses ranged from 0.75 to 0.77. Mongolian horses had the highest values in all populations, demonstrating that Mongolian horses have retained the largest amount of genetic variation of all the populations studied.

In contrast, the average heterozygosity within each population of Japanese horses ranged between 0.34 and 0.66. In addition, Mongolian horses possessed all the alleles found in Japanese horses. These results are attributed to the fact that Mongolian horses are descended from the ancestral populations of Japanese horses. This assumption is supported by the historical fact that native horses on the Asian continent were frequently transported to Japan through the Korean peninsula.

The Tokara population showed the lowest heterozygosity in this study. Furthermore, the Tokara population showed the lowest value of protein polymorphisms (Nozawa et al. 1998). According to the literature, the population size of the Tokara group has decreased to low numbers in the past, but has recently increased due to breeding. Thus the main reason for this low-level genetic variation is most likely the population bottleneck.

The phylogenetic reconstructions using D_A and $(\delta\mu)^2$ gave similar clustering results. However, the different clustering was not unexpected since the bootstrap values in both trees were low. The D_A tree showed much higher values than the $(\delta\mu)^2$ tree. This difference can be explained by the fact that $(\delta\mu)^2$ is based on the assumption that microsatellites follow a stepwise mutation model, while D_A is based on the infinite allele model. All the populations in Japan were derived from one original horse population, which was transported about 2,000 years ago through the Korean peninsula. Furthermore, these populations localized to the particular areas in Japan mentioned in this article. After localization, the populations were affected by the gene flow of each other. Thus allele frequencies of Japanese horses might not be the effect of a stepwise mutation of microsatellites, indicating that the D_A tree might be suitable for this study. In addition, Takezaki and Nei (1996) studied the efficiencies of several genetic distance measures, such as D_A (Nei 1983), D_C (Cavalli-Sforza and Edwards 1967), D_{SW} (Shriver et al. 1995), and $(\delta\mu)^2$ (Goldstein et al. 1995), when applied to microsatellites. Their computer simulation showed that the D_A and D_C distances are most efficient in obtaining the correct tree topology (Takezaki and Nei 1996).

In this study, our phylogenetic tree showed several clusters, including the European cluster (Anglo-Arab, thoroughbreds), the Hokkaido-Kiso cluster, and the Mongolian cluster, because of high bootstrap values. The relationship of the clusters to each other corresponds to their geographical distributions. In particular, the Hokkaido and Kiso populations clustered with a high bootstrap value. This relationship corresponds to the historical fact that the Hokkaido population was made up of horses transported from Honshu, Japan's mainland, for the purpose of agriculture. The Kiso population is geographically located in the area nearest to the Hokkaido population in Japan. Although Misaki and Tokara populations have long branch lengths and low bootstrap values, these populations were clustered into one group close to the Yonaguni population. The long branch lengths for the Tokara and Misaki populations in the tree might be caused by a bottleneck effect. The Misaki, Tokara, and Yonaguni populations were also close to the Noma population genetically. Furthermore, the genetic relationship of the Taishu population was close to the Mongolian, Korean, Hokkaido-Kiso groups, and the four populations. The genetic relationships constructed in this study corresponded to geographical distribution in Japan. Furthermore, the correspondence between the genetic relationship and the geographical distribution supports the hypothesis that Japanese horses are descended from Mongolian horses through the Korean peninsula and have spread all over Japan, because there were no horses in Japan about 2,000 years ago, according to the literature.

In conclusion, we analyzed the genetic variation and phylogenetic relationship of Asian and Japanese horses using microsatellites, and we support the view that Japanese horses originated from Mongolian horses through the Korean Peninsula. The genetic relationship of Japanese horses to each other corresponds to their geographical distribution. Our results also show that several horse populations in Japan have low genetic variation. Because almost all Japanese horses are in small populations, breeders should monitor

genetic variation to protect these horse populations. Genetic marker data, as described here, could be an effective tool.

In the future, an investigation of the pedigree of populations and the genetic variations in those populations will be necessary. As our data show, microsatellites are a useful tool for studying the genetic relationships among closely related horse breeds. Since the microsatellites in this study are highly polymorphic, they can also be applied for parentage testing. Thus we believe that using microsatellites is important in the evaluation of genetic variations.

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References

Bjørnstad G and Røed KH, 2001. Breed demarcation and potential for breed allocation of horses assessed by microsatellite markers. Anim Genet 32:59–65.

Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR, and Cavalli-Sforza LL, 1994. High resolution of human evolutionary trees with polymorphic microsatellites. Nature 368:455–457.

Canon J, Checa ML, Carleos C, Vega-Pla JL, Vallejo M, and Dunner S, 2000. The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data. Anim Genet 31:39–48.

Cavalli-Sforza LL and Edwards AW, 1967. Phylogenetic analysis. Models and estimation procedures. Am J Hum Genet 19(Suppl. 19):233.

Cunningham EP, Dooley JJ, Splan RK, and Bradley DG, 2001. Microsatellite diversity, pedigree relatedness and the contributions of founder lineages to thoroughbred horses. Anim Genet 32:360–364.

Ellegren H, Johansson M, Sandberg K, and Andersson L, 1992. Cloning of highly polymorphic microsatellites in the horse. Anim Genet 23:133–142.

Goldstein DB, Ruiz Linares A, Cavalli-Sforza LL, and Feldman MW, 1995. Genetic absolute dating based on microsatellites and the origin of modern humans. Proc Natl Acad Sci USA 92:6723–6727.

Guérin G, Bertaud M, and Amigues Y, 1994. Characterization of seven new horse microsatellites: HMS1, HMS2, HMS3, HMS5, HMS6, HMS7 and HMS8. Anim Genet 25:62.

Hayashida S, 1958. Ancestry of the native horses of Japan [in Japanese, with English summary]. Jpn J Zootech Sci 28:329–334.

Ishida N, Oyunsuren T, Mashima S, Mukoyama H, and Saitou N, 1995. Mitochondrial DNA sequences of various species of the genus *Equus* with special reference to the phylogenetic relationship between Przewalskii's wild horse and domestic horse. J Mol Evol 41:180–188.

Kim KS, Tanabe Y, Park CK, and Ha JH, 2001. Genetic variability in east Asian dogs using microsatellite loci analysis. J Hered 92:398–403.

Litt M and Luty JA, 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet 44:397–401.

Marklund S, Ellegren H, Eriksson S, Sandberg K, and Andersson L, 1994. Parentage testing and linkage analysis in the horse using a set of highly polymorphic microsatellites. Anim Genet 25:19–23.

Nei M, 1983. Genetic polymorphism and the role of mutation in evolution. In: Evolution of genes and proteins (Nei M and Koehn R, eds). Sunderland, MA: Sinauer Associates; 165–190.

Nozawa K, 1992. Origin and ancestry of native horses in eastern Asia and Japan [in Japanese, with English summary]. Jpn J Equine Sci 3:1–18.

Nozawa K, Shotake T, Ito S, and Kawamoto Y, 1998. Phylogenetic relationships among Japanese native and alien horses estimated by protein polymorphisms. J Equine Sci 9:53–69.

Saitou N and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Sakagami M, Tozaki T, Mashima S, Hirota K, and Mukoyama H, 1995. Equine parentage testing by microsatellite locus at chromosome 1q2.1. Anim Genet 26:123.

Shriver MD, Jin L, Boerwinkle E, Deka R, Ferrell RE, and Chakraborty R, 1995. A novel measure of genetic distance for highly polymorphic tandem repeat loci. Mol Biol Evol 12:914–920.

Stallings RL, Ford AF, Nelson D, Torney DC, Hildebrand CE, and Moyzis RK, 1991. Evolution and distribution of (GT)n repetitive sequences in mammalian genomes. Genomics 10:807–815.

Swinburne J, Gerstenberg C, Breen M, Aldridge V, Lockhart L, Marti E, Antczak D, Eggleston-Stott M, Bailey E, Mickelson J, et al., 2000. First comprehensive low-density horse linkage map based on two 3-generation, full-sibling, cross-bred horse reference families. Genomics 66:123–134.

Takahashi H, Nirasawa K, Nagamine Y, Tsudzuki M, and Yamamoto Y, 1998. Genetic relationships among Japanese native breeds of chicken based on microsatellite DNA polymorphisms. J Hered 89:543–546.

Takezaki N and Nei M, 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics 144:389–399.

Tozaki T, Inoue S, Mashima S, Ohta M, Miura N, and Tomita M, 2000a. Sequence analysis of trinucleotide repeat microsatellites from an enrichment library of the equine genome. Genome 43:354–365.

Tozaki T, Kakoi H, Mashima S, Hirota K, Hasegawa T, Ishida N, Miura N, and Tomita M, 2000b. The isolation and characterization of 18 equine microsatellite loci, TKY272-TKY289. Anim Genet 31:149–150.

Tozaki T, Kakoi H, Mashima S, Hirota K, Hasegawa T, Ishida N, Miura N, and Tomita M, 2000c. The isolation and characterization of 34 equine microsatellite loci, TKY290-TKY323. Anim Genet 31:234–236.

Tozaki T, Kakoi H, Mashima S, Hirota K, Hasegawa T, Ishida N, Miura N, Choi-Miura NH, and Tomita M, 2001a. Population study and validation of paternity testing for thoroughbred horses by 15 microsatellite loci. J Vet Med Sci 63:1191–1197.

Tozaki T, Mashima S, Hirota K, Miura N, Choi-Miura NH, and Tomita M, 2001b. Characterization of equine microsatellites and microsatellite-linked repetitive elements (eMLREs) by efficient cloning and genotyping methods. DNA Res 8:33–45.

Tozaki T, Sakagami M, Mashima S, Hirota K, and Mukoyama H, 1995. ECA-3: equine (CA) repeat polymorphism at chromosome 2p1.3-4. Anim Genet 26:283.

van Haeringen H, Bowling AT, Stott ML, Lenstra JA, and Zwaagstra KA, 1994. A highly polymorphic horse microsatellite locus: VHL20. Anim Genet 25:207.

Vila C, Leonard JA, Gotherstrom A, Marklund S, Sandberg K, Liden K, Wayne RK, and Ellegren H, 2001. Widespread origins of domestic horse lineages. Science 291:474–477.

Weber JL and May PE, 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44:388–396.

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