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Genetic analyses for conservation of the traditional Tokara horse using 31 microsatellite markers

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In order to promote conservation of the traditional Tokara horse in its remaining three breeding areas in Japan (Nakanoshima, Kaimondake, and Iriki), we genotyped 123 horses using 31 microsatellite markers and determined their genetic diversity. On average, the number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) among all horses were 3.0, 0.424, 0.481, and 0.108, respectively. Compared with other endangered horse breeds, we found that, even though the size of the Tokara horse population has recently increased, the N_A, H_O, and H_E of Tokara horses are still notably lower than those of other breeds. Neighbor-joining tree and STRUCTURE analysis showed that the current population of Tokara horses is divided into three subpopulations, corresponding to their respective feeding and breeding areas: Nakanoshima, Kaimondake, and Iriki. This subdivision was also reflected in the N_A of microsatellite DNAs, with four, three, and four different loci showing single alleles in Nakanoshima, Kaimondake, and Iriki horses, respectively. These alleles are considered to have become fixed as a consequence of breeding within the limited number of horses in each area. Since Tokara horses are currently strongly divided into subpopulations, it is vitally important to exchange several horses among their different breeding units in order to maintain the genetic diversity of the Tokara horse as a unique breed. The data obtained in this study contribute toward explaining the history of Tokara horses and also provide important information for future monitoring of population diversity and guiding conservation measures for this endangered breed.

Key words: *conservation, genetic diversity, Japan, microsatellite DNA, Tokara horse*

J. Equine Sci.
Vol. 29, No. 4
pp. 97–104, 2018

In Japan, there are eight horse breeds (Hokkaido, Kiso, Misaki, Noma, Taishu, Tokara, Miyako, and Yonaguni) [28] that are registered as native to Japan by the Japan Equine Affairs Association (JEAA). Among these breeds, the Tokara horse (Fig. 1) was confirmed in 1952 by Hayashida *et al*. [10] as a native breed on Takarajima (Fig. 2), a small island at the southern end of the Tokara chain of islands in Japan, and it has been registered as a natural treasure by Kagoshima Prefecture.

The Tokara horse appears to have originated from approximately 10 unimproved horses introduced from Kikaijima (Fig. 2), one of the Amami Islands near the Tokara Islands, in 1897 [10]. Thereafter, this small population interbred for almost 50 years, and by 1943, the number of Tokara horses had increased to approximately 100. Subsequently, however, the population size gradually decreased as a result of less use of these domestic animals in agricultures because of industrialization and motorization, and by 1952, the population had declined to 43. In an effort to conserve the Tokara horse, most of the horses on Takarajima were relocated to the mainland of Kagoshima, where they were transferred to the Nature Park at Kaimondake, the Kagoshima University

Received: September 5, 2018
Accepted: October 29, 2018
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Fig. 1. Appearance of the Tokara horse breed. Tokara horses are one of the eight horse breeds native to Japan.

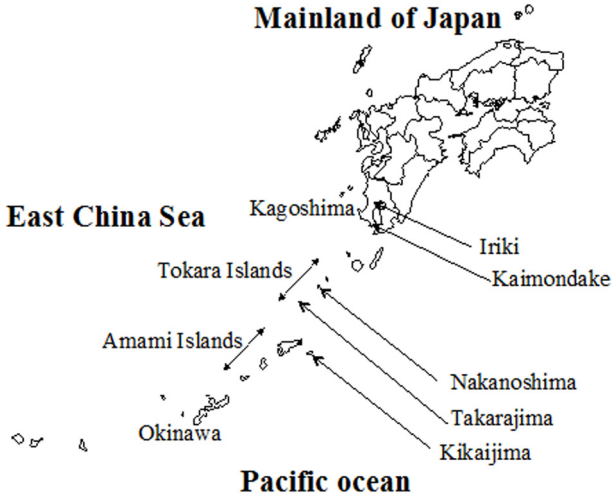

Fig. 2. Geographical locations of the feeding and breeding areas of Tokara horses.

Experimental Farm in Iriki, and Hirakawa Zoo in Kagoshima City (Fig. 2). In 1977, the sole male Tokara horse remaining on Takarajima was transported to Nakanoshima, one of the other Tokara Islands (Fig. 2). Thereafter, Tokara horses no longer inhabited Takarajima [19]. Subsequently, however, several horses were transferred to Nakanoshima from the mainland of Kagoshima for breeding purposes.

Today, Nakanoshima, Kaimondake, and Iriki have become major breeding areas of the Tokara horse. Although the overall population size had increased to 123 by 2016, breeding of the Tokara horse has been confined to each of the three subpopulations for over 50 years. Since there is currently no clear pedigree or genetic information for the Tokara horse population, it is essential to establish this information for future breeding plans.

Microsatellite DNAs, which are loci comprising a small number of tandem repeats, have a high mutation rate, giving rise to multiple alleles and high polymorphism [15]. Therefore, in conservation biology and population genetic studies, microsatellite DNAs are often used to characterize population structures and to determine genetic relationships within populations [7, 9]. For example, over 30 microsatellite DNAs have been developed for parentage testing in Thoroughbred horses [13, 29]. Such microsatellites have also been used in conservation-related studies for other native horses.

The breeding history of the Tokara horse indicates a potential risk of genetic homogeneity owing to the small size of the founder population and breeding within isolated areas for more than 50 years. Therefore, in the present study, we examined the genetic diversity of Tokara horses using 31 microsatellite markers in order to establish their genetic structure and diversity for the conservation of this breed.

Table 1. Number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}) for 123 Tokara horses

Locus	Nakanoshima				Kaimondake				Iriki				All			
	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}
AHT4	3	0.583	0.592	0.015	3	0.491	0.460	−0.070	3	0.333	0.339	0.016	4	0.455	0.483	0.057
AHT5	2	0.500	0.383	−0.314	2	0.421	0.478	0.120	1	0.000	0.000	-	2	0.293	0.353	0.172
ASB2	1	0.000	0.000	-	2	0.175	0.161	−0.087	1	0.000	0.000	-	2	0.081	0.078	−0.038
ASB17	3	0.708	0.543	−0.314	2	0.439	0.429	−0.022	3	0.429	0.396	−0.085	4	0.488	0.611	0.202
ASB23	2	0.542	0.439	−0.241	2	0.035	0.035	−0.009	2	0.190	0.174	−0.093	3	0.187	0.188	0.005
CA425	2	0.458	0.467	0.019	2	0.404	0.383	−0.055	2	0.238	0.212	−0.123	4	0.358	0.385	0.070
HMS2	2	0.500	0.507	0.014	3	0.614	0.567	−0.084	2	0.095	0.092	−0.038	3	0.415	0.456	0.091
HMS3	3	0.875	0.680	−0.295	3	0.579	0.627	0.078	3	0.643	0.529	−0.218	3	0.659	0.647	−0.017
HMS6	2	0.417	0.507	0.182	2	0.544	0.482	−0.130	2	0.357	0.471	0.245	2	0.455	0.502	0.093
HMS7	4	0.750	0.566	−0.333	5	0.702	0.687	−0.022	4	0.500	0.517	0.034	5	0.642	0.767	0.164
HTG4	3	0.583	0.503	−0.165	3	0.526	0.552	0.047	2	0.500	0.492	−0.017	3	0.528	0.543	0.028
HTG10	2	0.625	0.488	−0.287	2	0.088	0.085	−0.037	2	0.262	0.230	−0.139	2	0.252	0.494	0.490
LEX33	2	0.500	0.454	−0.104	2	0.439	0.454	0.035	3	0.548	0.544	−0.008	3	0.488	0.503	0.031
TKY19	3	0.583	0.478	−0.227	3	0.404	0.435	0.072	3	0.619	0.551	−0.126	3	0.512	0.633	0.191
TKY28	1	0.000	0.000	-	2	0.421	0.335	−0.258	2	0.286	0.248	−0.155	2	0.293	0.251	−0.168
TKY279	3	0.417	0.401	−0.041	2	0.491	0.407	−0.208	2	0.476	0.465	−0.025	3	0.472	0.600	0.214
TKY287	1	0.000	0.000	-	1	0.000	0.000	-	3	0.643	0.555	−0.160	3	0.220	0.279	0.215
TKY294	2	0.375	0.488	0.236	2	0.351	0.335	−0.047	1	0.000	0.000	-	2	0.236	0.496	0.525
TKY297	2	0.208	0.191	−0.095	2	0.263	0.231	−0.143	1	0.000	0.000	-	2	0.163	0.150	−0.084
TKY301	2	0.250	0.284	0.121	1	0.000	0.000	-	2	0.429	0.413	−0.038	2	0.195	0.227	0.142
TKY312	2	0.583	0.479	−0.224	2	0.474	0.504	0.060	2	0.548	0.506	−0.084	3	0.520	0.621	0.163
TKY321	4	0.708	0.696	−0.018	4	0.754	0.684	−0.103	3	0.381	0.371	−0.026	4	0.618	0.635	0.027
TKY325	3	0.875	0.664	−0.327	3	0.667	0.611	−0.093	2	0.643	0.471	−0.370	3	0.699	0.654	−0.069
TKY333	2	0.458	0.439	−0.046	2	0.439	0.415	−0.057	2	0.524	0.505	−0.038	2	0.472	0.461	−0.022
TKY337	2	0.583	0.479	−0.224	1	0.000	0.000	-	2	0.452	0.458	0.011	2	0.268	0.419	0.361
TKY341	3	0.708	0.653	−0.086	4	0.351	0.324	−0.084	2	0.524	0.477	−0.099	5	0.480	0.530	0.096
TKY343	4	0.708	0.609	−0.167	4	0.860	0.699	−0.232	3	0.595	0.488	−0.223	4	0.740	0.691	−0.072
TKY344	1	0.000	0.000	-	3	0.737	0.630	−0.171	2	0.357	0.354	−0.008	3	0.463	0.475	0.024
TKY374	3	0.708	0.606	−0.172	3	0.474	0.492	0.038	3	0.667	0.620	−0.075	4	0.585	0.630	0.071
TKY394	3	0.542	0.582	0.071	3	0.632	0.554	−0.141	2	0.095	0.092	−0.038	4	0.431	0.668	0.356
VHL20	2	0.542	0.488	−0.112	2	0.439	0.415	−0.057	2	0.500	0.506	0.012	2	0.480	0.491	0.024
Mean	2.4	0.493	0.441	−0.116	2.5	0.426	0.402	−0.059	2.2	0.382	0.357	−0.069	3.0	0.424	0.481	0.108

Materials and Methods

Animals

In 2016, 123 Tokara horses were registered by the JEAA. Blood samples were collected from each of the 123 horses (24 horses [Nos. 1–24] in Nakanoshima, 57 [Nos. 25–81] in Kaimondake, 42 [Nos. 82–123] in Iriki) using EDTA as an anticoagulant. Genomic DNA was extracted using a QIAamp® DNA Mini Kit (50) (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol.

Microsatellite DNA genotyping

The 31 microsatellite markers used in this study are listed in Table 1. These microsatellites have previously been used in Japan for parentage testing of racehorses. For amplification of these markers, we performed multiplex PCR according to Kakoi *et al*. [13] and Tozaki *et al*. [28]. Details regarding the primers and PCR conditions used are available upon request. The resulting PCR products were electrophoresed using a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.). Each marker was genotyped using the GeneMapper Software® (Applied Biosystems).

Individual diversity

The number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, and inbreeding coefficient (F_{IS}) were calculated according to Weir and Cockerham [30] using GENEPOP version 4.0.10 [21, 22]. Furthermore, we compared the means of N_A, H_O, and H_E with those of other endangered horses presented in the World Watch List for Domestic Animal Diversity (WWL-DAD).

Table 2. Diversity of microsatellites in native horses listed in the WWL-DAD*

Breed	Category	Population size	Mean number of alleles (N_A)	Observed heterozygosities (H_O)	Expected heterozygosities (H_E)	Country	Reference
Kiso	Critical	50♀, 5♂	6.3	0.67	0.66	Japan	[26]
Kiso	Critical	50♀, 5♂	3.8	0.68	0.69	Japan	[14]
Misaki	Critical	40♀, 25♂	3.4	0.52	0.51	Japan	[14]
Noma	Critical	30♀, 10♂	3.6	0.67	0.59	Japan	[14]
Yonaguni	Critical	60♀, 5♂	4.1	0.62	0.63	Japan	[14]
Lipizzano	Critical	54♀, 6♂	4.7	0.64	0.61	Italy	[1]
Lipican	Critical	48♀, 14♂	5.3	0.66	0.63	Slovakia	[1]
Tokara**	Critical-maintained	62♀, 61♂	3.0	0.42	0.48	Japan	This study
Tokara horse	Critical-maintained	60♀, 50♂	2.6	0.44	0.43	Japan	[14]
Tsushima	Critical-maintained	20♀, 5♂	4.6	0.66	0.65	Japan	[14]
Lipizzaner	Critical-maintained	100♀, 35♂	6.2	0.66	0.64	Austria	[1]
Sorraiana	Critical-maintained	60♀, 10♂	3.3	0.45	0.47	Portugal	[16]
Jaca Navarra	Endangered	240, 10♂	7.3	0.77	0.74	Spain	[25]
Knabstrupper	Endangered	170	7.3	0.71	0.77	Denmark	[27]
Lipicanac	Endangered	400, 200♀, 97♂	5.2	0.67	0.65	Croatia	[1]
Pottoka	Endangered	400♀, 170♂	8.1	0.75	0.76	Spain	[25]
Frederiksborgheste	Endangered-maintained	230	5.3	0.66	0.65	Denmark	[27]
Garrano	Endangered-maintained	1,000♀, 30♂	10.2	0.73	0.75	Portugal	[18]
Lipicai	Endangered-maintained	322♀, 24♂	5.8	0.71	0.68	Hungary	[1]

*World Watch List for Domestic Animal Diversity, 3rd ed. by FAO. **Results of this study.

Neighbor-joining tree and cluster analysis

Microsatellite Analyzer 4.05 [3] was used to analyze the genetic distance of individuals (DPS). NEIGHBOR implemented in PHYLIP version 3.695 [6] was used to convert the DPS data into a neighbor-joining (NJ) tree.

Structure version 2.3.4 [20] was used to estimate the population structure based on Bayesian analysis, with five repeated runs being performed for 2 to 10 populations (K). The length of burn-in was set to 10,000, and the number of Markov chain Monte Carlo (MCMC) iteration was set to 50,000. On the basis of the value of ΔK [5], determined using Structure Harvester [4], we estimated the optimal K value.

Results

Individual diversity

Table 1 shows the N_A, H_O, H_E, and F_{IS} values of all horses within the subpopulations from each of the three breeding areas (Nakanoshima, Kaimondake, and Iriki). Collectively, the horses showed multiple alleles at all the loci examined; however, those from the Nakanoshima, Kaimondake, and Iriki subpopulations showed single alleles at four, three, and four loci, respectively. With the exception of ASB 2 and TKY287, the fixed alleles differed among the different subpopulations.

For the Nakanoshima horses, the average N_A was 2.39, ranging from 1 to 4; H_O averaged 0.493, ranging from 0.000 in ASB2, TKY28, TKY287, and TKY344 to 0.875 in HMS2; H_E averaged 0.441, ranging from 0.000 in ASB2, TKY28, TKY287, and TKY344 to 0.696 in TKY321; and F_{IS} averaged −0.116, ranging from −0.333 in HMS7 to 0.236 in TKY294.

For the Kaimondake horses, the average N_A was 2.48, ranging from 1 to 5; H_O averaged 0.426, ranging from 0.000 in TKY287, TKY301, and TKY337 to 0.860 in TKY343; H_E averaged 0.402, ranging from 0.000 in TKY287, TKY301, and TKY337 to 0.699 in TKY343; and F_{IS} averaged −0.059, ranging from −0.258 in TKY28 to 0.120 in AHT5.

For the Iriki horses, the average N_A was 2.23, ranging from 1 to 4; H_O averaged 0.382, ranging from 0.000 in AHT5, ASB2, TKY294, and TKY297 to 0.667 in TKY374; H_E averaged 0.357, ranging from 0.000 in AHT5, ASB2, TKY294, and TKY297 to 0.620 in TKY374; and F_{IS} averaged −0.069, ranging from −0.370 in TKY325 to 0.245 in HMS6.

Tokara horses have been classified as a critical-maintained breed by the Food and Agriculture Organization (FAO) [23, 24]. When we compared the genetic diversity of Tokara horses with that of the other endangered breeds listed in the WWL-DAD (Table 2) [1, 14, 16, 18, 25–27], we found that, even though the population size has recently increased, the N_A, H_O, and H_E values were all lower than those of the other endangered breeds.

Genetic relationship among individuals

A NJ tree was generated in order to visualize the genetic relationships among individual horses. We found that Tokara horses appeared to be separated into three distinct genetic clusters, corresponding to each of the three breeding areas (Nakanoshima, Kaimondake, and Iriki) (Fig. 3). A single exception was horse No. 6 from the Nakanoshima subpopulation, which clustered with the Kaimondake group in the NJ tree. This, however, can be explained by the fact that this horse was recently transferred from the Kaimondake to Nakanoshima subpopulation.

Cluster analysis of the Tokara horse population

On the basis of the ΔK value of 107.3 obtained using Structure Harvester, we estimated the optimal K value to be 3, corresponding to the current breeding areas of the Tokara horses (Fig. 4). Again, the singular exception was horse No. 6, the genetic characteristics of which were more similar to those of Kaimondake horses than to those of the Nakanoshima subpopulation.

Discussion

This is the first study that has examined the genetic diversity and structure of Tokara horses based on an analysis of all 123 extant individuals registered in 2016, and we accordingly demonstrated that the breed is notably homogeneous when compared with the other Japanese native breeds (Table 2). This finding is consistent with historic records indicating a small founder population and a subsequent history of inbreeding.

In this study, we confirmed that Tokara horses are currently genetically divided into three subpopulations (Figs. 3 and 4) that correspond to their respective feeding and breeding areas: Nakanoshima, Kaimondake, and Iriki. These results reveal the fragmentation of the Tokara horse population and indicate that geographical isolation leads to reproductive isolation [2]. This undoubtedly reflects the breeding history of the Tokara horse population, which was translocated from its original location on Takarajima to three separate areas after 1953, within which they have bred in isolation for more than 50 years.

These findings were also supported by the N_A values obtained using microsatellite DNAs, which indicated that four, three, and four loci are represented by single alleles in the Nakanoshima, Kaimondake, and Iriki populations, respectively. With the exception of ASB 2 and TKY287, these loci differed among the three breeding areas (Table 1), indicating that the alleles have become fixed as a consequence of breeding amongst a limited number of horses. Accordingly, we can assume that each subpopulation has bred and evolved separately.

The loss of alleles occurs more rapidly than the loss of heterozygosity [17] and generally equates to a loss of genetic diversity [16], which in turn leads to a reduction in phenotypic diversity. However, although each subpopulation has several fixed loci, the population as a whole did not show single alleles at all of the loci examined (Table 1). Therefore, in order to maintain the genetic diversity of Tokara horses, it may be important to initiate a conservation program designed to prevent inbreeding within the same breeding regions.

Inbreeding and decreasing genetic diversity may carry the risk of extinction, particularly in small populations [8]. Inbreeding can lead to homozygosity at loci for deleterious recessive genes [11], which increases the risk of the expression of harmful phenotypes. Given that the Tokara horse population is probably undergoing a loss of genetic diversity as a consequence of breeding within isolated small populations, it will be important to maintain genetic diversity in order to prevent inbreeding depression in this breed.

In the past, the population of another Japanese native breed, the Noma horse, declined to less than 10 individuals, and as a conservation measure, these horses were bred via backcrossing [12]. Despite a similar situation in the Tokara horse, the N_A, H_O, and H_E of this breed are all lower than those of the Noma horse (Table 2). This tends to indicate that the founder Tokara horses were genetically close to each other, leading to the comparatively lower genetic diversity of the current population. Indeed, even though the current population has increased to 123 horses, the genetic diversity (N_A, H_O, and H_E) of Tokara horses is low compared with that of the other endangered horse breeds (Table 2). Therefore, it may be important to reconsider what constitutes an appropriate breeding management strategy for this breed, not only in terms of increasing the overall population size but also for maintaining and/or enhancing genetic diversity. In this regard, it is important to establish pedigree information for the Tokara horse based on the genetic information obtained in this study.

The NJ tree we generated enabled us to gain a better understanding of the genetic relationships among individual Tokara horses and can potentially contribute important information for breeding management. For example, analysis of the NJ tree data may enable us to identify horses that are genetically different from each other and indicate those horses that could be moved from one subpopulation to another in an effort to maintain/enhance genetic diversity.

The gender ratio was almost equal in all populations (Tables 1 and 2), while almost all the Tokara horses were allowed to graze outside. Therefore, it is considered that the current breeding management may be suitable for Tokara

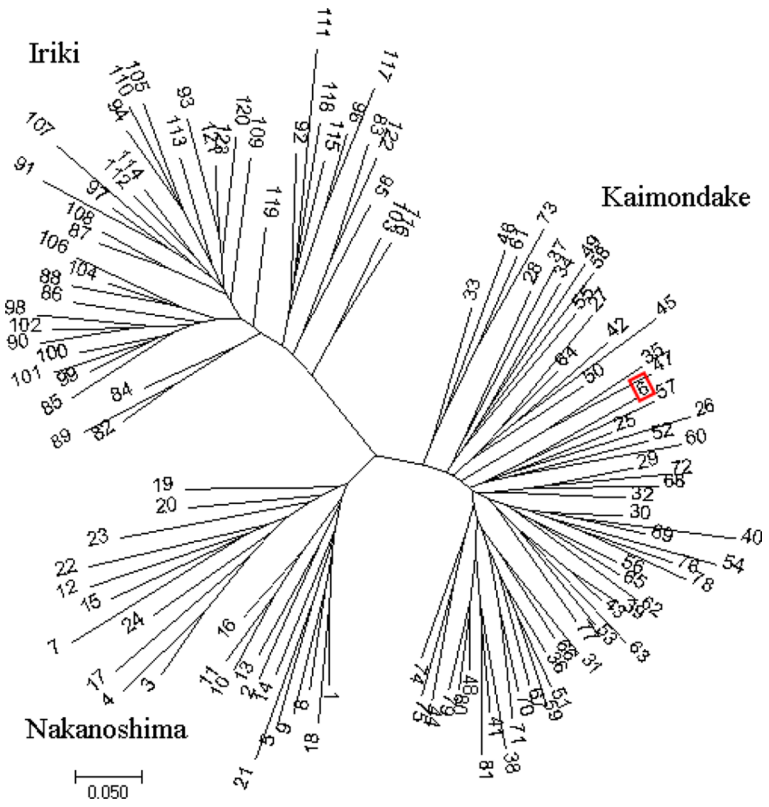

Fig. 3. A neighbor-joining tree based on genetic distances calculated from the ratio of shared alleles. Nos. 1–24 bred in Nakanoshima, Nos. 25–81 bred in Kaimondake, and Nos. 82–123 bred in Iriki. Horse No. 6 was moved from Kaimondake to Nakanoshima in the past, and it clustered with Kaimondake group in the neighbor-joining tree.

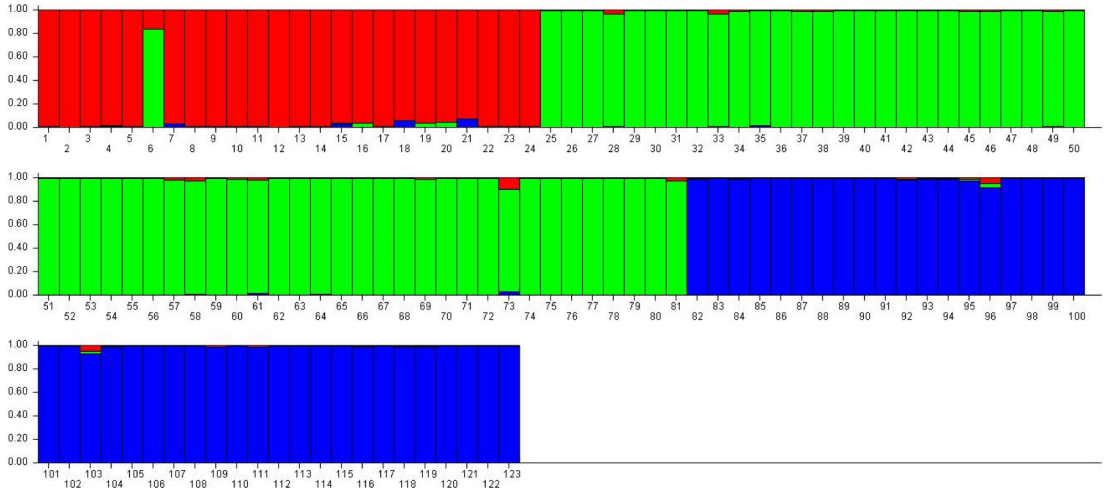

Fig. 4. Cluster analysis revealed that Tokara horses currently exist as three isolated subpopulations: subpopulation I (red), subpopulation II (green), and subpopulation III (blue). The optimal K value was estimated to be 3 (ΔK=107.3), as determined by Structure Harvester. Nos. 1–24 bred in Nakanoshima, Nos. 25–81 bred in Kaimondake, and Nos. 82–123 bred in Iriki. Horse No. 6 was moved from Kaimondake to Nakanoshima in the past, and its genetic characteristics are similar to those of other horses in the Kaimondake subpopulation.

horses in each area. However, as subgrouping was observed among breeding areas (Figs. 3 and 4), it may be necessary to review the breeding management by considering all the Tokara horses as one breed.

It is conceivable that breeding management for Tokara horses has been attempted at least once in the past, as one horse, No.6, was translocated from the Kaimondake to Nakanoshima group. Although there are no public records regarding the purpose of this translocation, and whether it was intentional or not, it represents a potentially effective example of the measures that can be used to maintain genetic diversity. Given that our genetic analyses succeeded in tracing this translocation event, they may contribute to future breeding plans for the Tokara horse.

In conclusion, the data obtained in the present study contribute to establishing the history of the Tokara horse population and will provide important information to support the future monitoring of population diversity and guide conservation measures for this endangered breed.

Acknowledgment

Part of this research was supported by the government of Kiso town, Nagano, Japan.

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