

Genetic Characterization of the Endangered Kiso Horse Using 31 Microsatellite DNAs

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ABSTRACT. In order to contribute to conservation of the endangered Kiso horse, we clarified their genetic information using 31 microsatellite DNAs, and genotyped 125 horses, 83% of the existing breed. First, we clarified the current status of the horses. The horses were confirmed to have experienced rapid loss of population causing a bottleneck, and their effective population size was much smaller than their census size. Moreover, the number of alleles (6.3), observed heterozygosity (0.674), and expected heterozygosity (0.662) were in the same range as other endangered horses all over the world. Therefore, although their inbreeding level was not so severe (F_{is} : -0.017), the Kiso horse is surely one of the endangered. Second, we obtained genetic information of individuals. This information allowed us to understand the genetic distance of individuals, and might help in development of a reproductive strategy concerning the genetic distance between the mating pairs. Moreover, there appeared to be 4 subpopulations of Kiso horse, and this result was in good agreement with their historical background. Third, we confirmed that the parentage test for identification using the 31 microsatellite DNAs was highly reliable (probability of exclusion: 0.999999993). This identification increases the reliability of stud certification, and is also helpful for effective management. Understanding the genetic diversity within the population and the relationships among individuals is important to ensuring effective management for maintenance of genetic variation, and this study may help in conservation of the endangered Kiso horse.

KEY WORDS: conservation, genetic diversity, Japan, Kiso horse, microsatellite DNA.

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Each community has unique features based on its historical and cultural background, and the Kiso area, a mountainous region of central Japan, has the Kiso horse (Fig. 1) as a symbol of the local culture. People in the Kiso area treasure the horses as old friends, and have created an original culture centered on horses. Historically, many horses have been kept in the Kiso area, and the area has been well-known for producing good horses. However, industrialization and motorization have replaced transportation and cultivation systems using horses. The Kiso horse has lost the value as a work animal, and their number decreased to 32 in 1975 [15]. At that time, those concerned about imminent extinction established the Kiso Horse Conservation Association to improve the infrastructure for conservation, such as designation of a natural monument in Nagano Prefecture. Fortunately, many people, not only in the Kiso region but also from outside the region, started to work for conservation of the horse. Moreover, a railway company agreed to conserve the horse, referring to the local culture, and established a conservation farm in 1969—however, the farm was closed in 1997, and the horses were dispersed. Today, the

number of horses has increased to 149 as a result of their efforts. However, the population of Kiso horses is still small, and the situation is not so optimistic.

In conservation programs, 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,



Fig. 1. Appearance of the Kiso horse. The Kiso horse is medium-sized horse with a long body, short legs, and a plump girth, and most are bays. The horse is one of the eight native horses in Japan, and the current population is 149.

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for traits of economic or cultural interest [12]. Of course, it is better to obtain as many individual data as possible to understand the current status of the population, and precise information about the population helps us plan a conservation strategy considering genetic diversity. In the case of Kiso horses, no one knows the genetic background of the population based on individual data, and therefore we must have a better understanding in order to conserve Kiso horses for generations to come.

The evaluation of genetic diversity of a population would be easier if faster evolving markers were used. Microsatellite DNAs, the few tandem repeat loci, have a high mutation rate, and show high polymorphism and a large number of alleles for a locus [20]. Therefore, microsatellite DNA may prove more informative than classical polymorphisms or sequence data for assessing the structure of the population and determining the relationship within a population [3, 14]. For this reason, population genetics and conservation biology often use microsatellite DNAs to characterize a population [11, 14], and so we clarified genetic information of Kiso horses using 31 microsatellite DNAs in order to contribute to their conservation in terms of genetic diversity in this study.

Table 1. Microsatellite DNAs analyzed in this study

Microsatellite	Chromosome	Sequence position on equCab2 of BroadInstitute	Accession number of NCBI
AHT4	chr24	23415722	Y07733
AHT5	chr08	Unknown	Y07732
ASB002	chr15	54612720	X93516
ASB017	chr02	30601026	X93531
ASB023	chr03	79279213	X93537
CA425	chr28	43085659	U67406
HMS3	chr09	16895938	X74632
HMS6	chr04	7229293	X74635
HMS7	chr01	162381813	X74636
HTG4	chr09	1497830	AF169165
HTG10	chr21	17139130	AF169294
LEX3	chrX	110524114	AF075607
LEX033	chr04	59500074	AF075635
TKY19	chr18	539493	AB048330
TKY28	chr06	66838196	AB048335
TKY279	chr16	6632374	AB033930
TKY287	chr17	4470525	AB033938
TKY294	chr27	19565304	AB034603
TKY297	chr01	62748186	AB034606
TKY301	chr23	21066889	AB034610
TKY312	chr06	17320169	AB034621
TKY321	chr20	61778074	AB034629
TKY325	chr29	27565193	AB044826
TKY333	chr28	2475777	AB044834
TKY337	chr04	29877413	AB044838
TKY341	chr16	81718708	AB044842
TKY343	chr11	12997597	AB044844
TKY344	chr05	92501364	AB044845
TKY374	chr01	98084756	AB044874
TKY394	chr24	33978924	AB048299
VHL20	chr30	18793939	X75970

MATERIALS AND METHODS

We collected blood samples from 125 horses using EDTA as an anticoagulant, from April 2008 to October 2009. The registered number of Kiso horses was 149, and we obtained samples from 83% of the whole breed. The horses were 12 males, 96 females, and 17 geldings, and the average age was 11.5 years, ranging from 1 to 29 years. Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.) according to the protocol of the manufacturer.

We employed the 31 microsatellites used for parentage testing of racing horses in Japan (Table 1). These markers were amplified by multiplex PCR according to Kakoi *et al.* [17] and Tozaki *et al.* [32] with a minor modification. Since the amplification in *TKY337* including a null allele was low when we used the original primer, this marker was analyzed alone by the following primers: forward primer, 5'-TAA-GACTCAAGAGGTCAATC-3', reverse primer, 5'-TACTCTCCAACCTCTTCCACT-3'. The information about primers used here and PCR conditions is available on request. We electrophoresed PCR products using the 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.), genotyped each marker using the GeneMapper Software® (Applied Biosystems), and confirmed the reliability of the present genotypes compared with the published data of the Horse Comparison Test from the International Society for Animal Genetics (ISAG). To confirm that each marker does not show linkage disequilibrium, we calculated D' using SNPalyze ver 8.0 (Dynacom, Chiba, Japan). No D' was >0.8 , and this result confirmed that there is no strong linkage disequilibrium between each marker.

In this study, we estimated the number of alleles (N_A), allelic frequency, observed heterozygosity (H_o), expected heterozygosity (H_e), and inbreeding coefficient (F_{IS}) according to Weir and Cockerham [34] of the respective markers using GENEPOP version 4.0.10 [25, 26], and compared the average N_A , H_o , and H_e with those of other endangered horses listed in the WWL-DAD (Table 2). We also determined the polymorphic information content (PIC) using Cervus 3.0 [19], and estimated the probability of exclusion (PE) according to Jamieson's formula [16].

To understand the genetic distance of individuals (D_{ps}), we calculated the proportion of shared allele among individuals using Microsatellite Analyzer 4.05 [6], and visualized the D_{ps} by a neighbor-joining (NJ) analysis using NEIGHBOR implemented in PHYLIP version 3.69 [10]. To estimate population structure, we performed Bayesian analysis using Structure ver 2.3.3 [24], and carried out the analysis of five replicate runs for the number of population (K) between 1 and 10. For each replication, 30,000 iterations were used. The best K value was estimated based on ΔK [9] implemented in Structure Harvester [8].

Since the two-phased model of mutation (TPM) provides a good description of the mutation process of simple sequence repetition, including microsatellite DNA [7], we examined the existence of a genetic bottleneck using BOT-

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

Breed	Category*	Population size*	Mean number of alleles	Observed heterozygosities	Expected heterozygosities	Country	Reference
Kiso**	Critical	50 ♀, 5 ♂	6.3	0.67	0.66	Japan	
Kiso***	Critical	50 ♀, 5 ♂	3.8	0.68	0.69	Japan	Kakoi <i>et al.</i> (2007)
Kiso****	Critical	50 ♀, 5 ♂	4.8	0.66	—	Japan	Tozaki <i>et al.</i> (2003)
Misaki	Critical	40 ♀, 25 ♂	3.4	0.52	0.51	Japan	Kakoi <i>et al.</i> (2007)
Misaki	Critical	40 ♀, 25 ♂	3	0.43	—	Japan	Tozaki <i>et al.</i> (2003)
Noma	Critical	30 ♀, 10 ♂	3.6	0.67	0.59	Japan	Kakoi <i>et al.</i> (2007)
Noma	Critical	30 ♀, 10 ♂	3.7	0.61	—	Japan	Tozaki <i>et al.</i> (2003)
Yonaguni	Critical	60 ♀, 5 ♂	4.1	0.62	0.63	Japan	Kakoi <i>et al.</i> (2007)
Yonaguni	Critical	60 ♀, 5 ♂	3.8	0.69	—	Japan	Tozaki <i>et al.</i> (2003)
Lipizzano	Critical	54 ♀, 6 ♂	4.7	0.64	0.61	Italy	Achmann <i>et al.</i> (2004)
Lipican	Critical	48 ♀, 14 ♂	5.3	0.66	0.63	Slovakia	Achmann <i>et al.</i> (2004)
Tokara pony	Critical-maintained	60 ♀, 50 ♂	2.6	0.44	0.43	Japan	Kakoi <i>et al.</i> (2007)
Tokara pony	Critical-maintained	60 ♀, 50 ♂	2.1	0.34	—	Japan	Tozaki <i>et al.</i> (2003)
Tsushima	Critical-maintained	20 ♀, 5 ♂	4.6	0.66	0.65	Japan	Kakoi <i>et al.</i> (2007)
Tsushima	Critical-maintained	20 ♀, 5 ♂	4.1	0.64	—	Japan	Tozaki <i>et al.</i> (2003)
Lipizzaner	Critical-maintained	100 ♀, 35 ♂	6.2	0.66	0.64	Austria	Achmann <i>et al.</i> (2004)
Skyros pony	Critical-maintained	53 ♀, 26 ♂	5.9	—	—	Greek	Bömcke <i>et al.</i> (2009)
Sorraiana	Critical-maintained	60 ♀, 10 ♂	3.3	0.45	0.47	Portugal	Luis <i>et al.</i> (2007)
Jaca Navarra	Endangered	240, 10 ♂	7.3	0.77	0.74	Spain	Solis <i>et al.</i> (2005)
Knabstrupper	Endangered	170	7.3	0.71	0.77	Denmark	Thirstrup <i>et al.</i> (2008)
Lipicanac	Endangered	400, 200 ♀, 97 ♂	5.2	0.67	0.65	Croatia	Achmann <i>et al.</i> (2004)
Pottoka	Endangered	400 ♀, 170 ♂	8.1	0.75	0.76	Spain	Solis <i>et al.</i> (2005)
Frederiksborgheste	Endangered-maintained	230	5.3	0.66	0.65	Denmark	Thirstrup <i>et al.</i> (2008)
Garrano	Endangered-maintained	1000 ♀, 30 ♂	10.2	0.73	0.75	Portugal	Morais <i>et al.</i> (2004)
Lipicai	Endangered-maintained	322 ♀, 24 ♂	5.8	0.71	0.68	Hungary	Achmann <i>et al.</i> (2004)

* World Watch List for Domestic Animal Diversity, 3rd ed. by FAO. ** Results of our study. *** Twelve horses were sampled to study the formation process of native Japanese horses. **** Twenty-one horses were sampled to study the phylogenetic relationship.

TLENECK version 1.2.02 [5] according to the TPM model. A population that has experienced a bottleneck shows a higher H_o than H_e under the Hardy-Weinberg equilibrium, and we performed the Wilcoxon signed-rank test to detect the deviation from the equilibrium. $P < 0.05$ was considered to be significant.

Effective population size (N_e) was calculated based on census data, the numbers of males/females (without geldings), in the current population. Furthermore, N_e based on genetic data, H_o and H_e , was also calculated as follows [29]:

$$N_e = 1 / \{2(H_o - H_e) / H_e\} + 1 / \{2\{(H_o - H_e) / H_e + 1\} + 1\}.$$

RESULTS

Table 3 shows N_A , H_o , H_e , F_{IS} , PIC , and PE for each microsatellite DNA. N_A was 6.3 on average, ranging from 4 to 9. H_o averaged 0.674, ranging from 0.288 in *TKY333* to 0.824 in *HMS6*, *TKY343*, and *TKY394*. H_e averaged 0.662, ranging from 0.299 in *TKY333* to 0.810 in *TKY19*. F_{IS} averaged -0.017 , ranging from -0.092 in *HMS6* to 0.244 in *TKY312*. PIC was 0.619 on average, ranging from 0.285 in *TKY333* to 0.778 in *TKY19*. PE was 0.441 on average, ranging from 0.161 in *TKY344* to 0.619 in *CA425*, and the combined PE of the 31 microsatellite DNAs was 0.999999993. Moreover, the confirmable records of 32 parentages were consistent with the results of parentage testing using the microsatellite DNAs.

We were able to obtain D_{ps} among individual horses, and visualized the D_{ps} using a NJ tree (Fig. 2). In the Bayesian

analysis, the structure shown in Fig. 3 demonstrated that the Kiso horse has 4 subpopulations ($K=4$, $\Delta K=70.394$).

The probability value in the Wilcoxon signed-rank test was 0.032, and deviated from the Hardy-Weinberg equilibrium according to the TPM. Therefore, this result confirmed that the Kiso horse genetically experienced a bottleneck in the past. Moreover, the N_e s based on the census data and genetic data were 45.8 and 28.1, respectively.

DISCUSSION

When we look at native horses all over the world, most of them are on the verge of extinction [13]. The Kiso horse experienced rapid loss of the population causing a bottleneck, which we confirmed here, and is categorized as one of such endangered breeds [27, 28]. In fact, the N_e s (45.8 and 28.1) were much smaller than their census size, and the difference between the N_e based on census data (45.8) and N_e based on genetic data (28.1) suggested that there was a selection bias for specific stallions. Moreover, comparison of the N_A , H_o , and H_e of the Kiso horse with other engendered breeds showed that the genetic diversity of the horse (N_A , H_o , and H_e on average were 6.3, 0.674, and 0.662, respectively) was in the middle level among the others listed in the WWL-DAD (ranging from 2.1–10.2 for N_A , 0.34–0.77 for H_o , and 0.43–0.77 for H_e) [1, 2, 18, 21, 22, 30, 31, 33]. Consequently, although the inbreeding level in the horse might not be so severe (F_{IS} : -0.017), these facts suggest that the Kiso horse is surely one of the endangered breeds.

The horse is still symbolic and very important for identi-

Table 3. The number of alleles, observed heterozygosity, expected heterozygosity, polymorphic information content, and probability of exclusion in each microsatellite locus

Markers	Number of alleles	Observed heterozygosity	Expected heterozygosity	F_{IS}^{**}	Polymorphic information content	Probability of exclusion
AHT4	6	0.712	0.658	-0.082	0.623	0.443
AHT5	6	0.664	0.629	-0.056	0.555	0.349
ASB2	8	0.712	0.699	-0.018	0.658	0.478
ASB17	6	0.392	0.429	0.086	0.405	0.251
ASB23	6	0.576	0.577	0.001	0.509	0.317
CA425	7	0.800	0.805	0.007	0.776	0.619
HMS3	6	0.800	0.770	-0.039	0.727	0.541
HMS6	6	0.824	0.755	-0.092	0.716	0.537
HMS7	6	0.696	0.647	-0.077	0.603	0.416
HTG4	4	0.536	0.510	-0.051	0.471	0.295
HTG10	7	0.776	0.745	-0.041	0.706	0.525
LEX3*	6	0.719	0.736	0.024	0.695	0.512
LEX33	6	0.752	0.699	-0.076	0.643	0.444
TKY19	7	0.776	0.810	0.042	0.778	0.616
TKY28	6	0.784	0.730	-0.074	0.694	0.516
TKY279	6	0.696	0.655	-0.062	0.592	0.396
TKY287	7	0.760	0.761	0.001	0.717	0.530
TKY294	4	0.456	0.440	-0.036	0.388	0.222
TKY297	7	0.768	0.788	0.026	0.759	0.598
TKY301	6	0.696	0.689	-0.011	0.647	0.460
TKY312	5	0.520	0.687	0.244	0.620	0.409
TKY321	5	0.632	0.615	-0.029	0.541	0.338
TKY325	9	0.768	0.713	-0.077	0.675	0.496
TKY333	5	0.288	0.299	0.038	0.285	0.164
TKY337	5	0.704	0.678	-0.039	0.622	0.422
TKY341	7	0.592	0.631	0.062	0.593	0.412
TKY343	9	0.824	0.796	-0.035	0.765	0.597
TKY344	5	0.344	0.317	-0.087	0.290	0.161
TKY374	7	0.776	0.743	-0.044	0.706	0.529
TKY394	9	0.824	0.758	-0.087	0.727	0.559
VHL20	6	0.720	0.756	0.048	0.714	0.531
Mean	6.3	0.674	0.662	-0.017	0.619	0.441

* LEX3 is an x-linked marker, and the value is estimated in the female population. ** F_{IS} : Coefficient of inbreeding estimated by Weir and Cockerham.

fyng the culture of each area in the world. Therefore, some efforts have made by breeder associations or governments to conserve native horses based on scientific ways [4, 13, 27]. Similarly, we started to work with the Kiso Horse Conservation Association to create a management program for conservation, and studied the genetic diversity of the Kiso horse in this study. As a result, we acquired two valuable pieces of information, the genetic information and reliable identification of individuals, for conservation of the horse.

We obtained genetic information on individuals accounting for 83% of the whole breed, and were able to calculate the D_{ps} . This information, simply visualized using a NJ tree, might help us to develop a reproductive strategy concerning genetic distance between the mating pairs. Moreover, the Bayesian analysis suggested that the population of Kiso horses contained 4 genetic components; most of the horses categorized as subpopulation I (Red) had belonged to the farm established by a railway company or were their offspring, horses categorized as subpopulation II (Green) were kept by a private equestrian club in Kiso Town, and horses categorized as subpopulations III (Blue) and IV (Yellow) were the lineage of stallions belonging to The Kiso Horse Conservation Association. This result was in good agreement

with the historical background of the horses. Historically, each horse owner or group of owners has individually tended to deal with the conservation issue, and so they might have had to mate horses within a small horse group, because of the limited number of horses. Our results might prove this tendency of mating genetically, and highlight one of the problems that accelerated the inbreeding level of the horse. Of course, although we admire the determination and efforts of owners for the conservation, the current situation of the horse seems to be a bit beyond their individual efforts now, and we would like to discuss this issue with all of horse owners in order to conserve the genetic diversity of the Kiso horse.

Reliable identification is important for effective management of the population [12]. PE based on the combined 31 microsatellites was 0.999999993, suggesting the reliability of a parentage test for identification of the Kiso horse as well as racing horses. This identification makes stud certification highly reliable and helps effective management for mating as well as obtaining genetic data for individuals. Hence, continuous typing of microsatellite DNAs for newborn foals may encourage appropriate management to conserve their genetic diversity, and we must therefore continue this analysis for conservation.

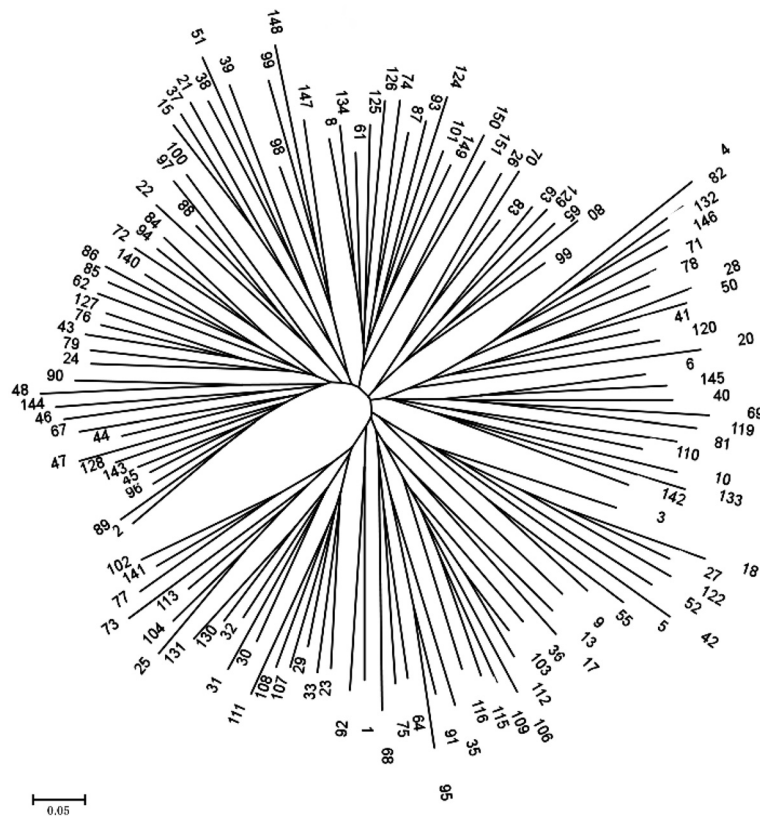


Fig. 2. The neighbor-joining tree based on the genetic distance estimated with the proportion of shared alleles.

In this study, we characterized genetics of the Kiso horse based on microsatellite DNAs in order to contribute to conservation. This horse is a symbol of the culture in the Kiso area, which involves a close relationship between men and horses, and extinction of the horse means loss of a piece of

our culture, leading to cultural uniformity and a crisis in regional identity. Therefore, awareness of the roles and values of genetic resources and concern for their rapid loss must be translated into effective action at the local, national, and global levels [27] in order to conserve biodiversity as

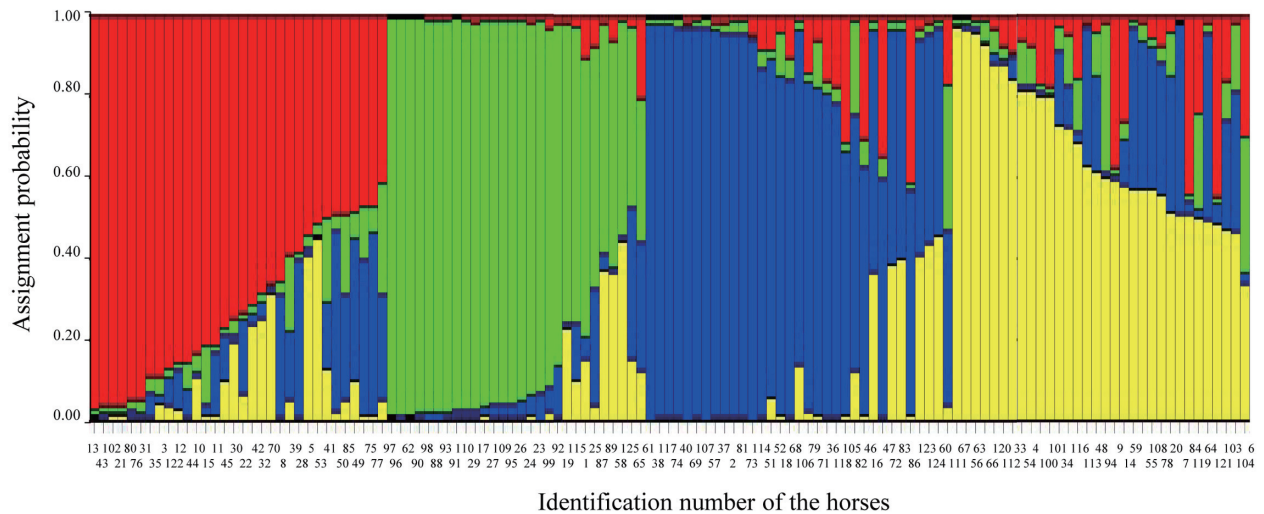


Fig. 3. The STRUCTURE suggested that the Kiso horse has 4 subpopulations: subpopulation I (Red), subpopulation II (Green), subpopulation III (Blue), and subpopulation IV (Yellow). $K=4$, $\Delta K=70.394$.

well as cultural diversity.

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