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Genetic variation and population structure of the sable *Martes zibellina* on eastern Hokkaido, Japan, revealed by microsatellite analysis

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Abstract. To assess the genetic variation and population structure of the sable *Martes zibellina* on eastern Hokkaido, Japan, we analyzed genotypes of 12 microsatellite loci on 48 individuals. Genotypes for all individuals examined were found to be different from each other. Mean observed and expected heterozygosites and allelic richness were calculated to be 0.52 (0.02–0.80), 0.58 (0.02–0.79) and 5.49, respectively. The genetic diversity of the eastern Hokkaido population was similar to those of farm-bred sables in Russia and other mustelids. STRUCTURE analysis showed that the sables of eastern Hokkaido were grouped into two genetic clusters. Eighty-nine percent (24/27) of individuals assigned to cluster 1 were distributed around Shiretoko Peninsula, whereas 81% (17/21) assigned to cluster 2 were distributed in the inner side of Hokkaido (Tokachi District). The two subpopulations could have been genetically differentiated due to geographic barriers such as higher mountains, lakes, rivers and solfatara areas, although the geographic isolation did not seem to be complete.

Key words: genetic diversity, geographic isolation, *Martes zibellina*, microsatellites, sable.

The sable *Martes zibellina* is a medium-sized carnivore of the family Mustelidae, and is distributed in eastern Russia, Sakhalin, North Korea, Mongolia, northern China, and Hokkaido Island of Japan (Wozencraft 2005). The Hokkaido population is classified as *M. z. brachyura*, and inhabits central, eastern and northern Hokkaido (Murakami 2009). They prefer areas with dense-tree cover or debris-rich microhabitats for resting and foraging (Miyoshi and Higashi 2005; Murakami 2009). Sables in Japanese cool-temperate mixed forests had home ranges of 0.50–1.78 km^2 (mean ± *SD*: 1.12 ± 0.495 km^2, $n = 6$) (Miyoshi and Higashi 2005). Xu et al. (1997) reported that an average home range size of males was 13.03 km^2 (9.13–17.33 km^2) and that of females was 7.18 km^2 (6.13–8.42 km^2) in Daxinganling Mountains, northern China.

Trapping sables in Hokkaido has been prohibited since 1920, because their population had decreased by over-trapping (Murakami and Ohtaishi 2000). The sables in Hokkaido were previously classified as Data Deficient (DD) category in the Red List of the Ministry of the Environment, Japan, because their distribution had been unknown. Murakami and Ohtaishi (2000) revealed that they are currently widespread throughout Hokkaido except the southwestern area. The sable is designated as Least Concern (LC) in the IUCN Red List (Abramov and Wozencraft 2008). Although distribution of the Japanese marten *M. melampus*, introduced from Honshu in 1940's (Inukai 1975), is currently expanding in southern, western and central Hokkaido, this species is not distributed in eastern Hokkaido (Murakami and Ohtaishi 2000).

There have been some genetic studies about the sable reported so far. Kurose et al. (1999) analyzed the phylogeny of mitochondrial DNA (mtDNA) cytochrome *b*, and reported that the level of genetic diversity was low and that there were no clear population structures. In addition, Inoue et al. (2010) examined the mtDNA control region, of which molecular evolutionary rate is the fastest among mtDNA genome, and reported that the population with a low genetic diversity was divided into three geographical subpopulations. The results between the two studies were different possibly because of use of

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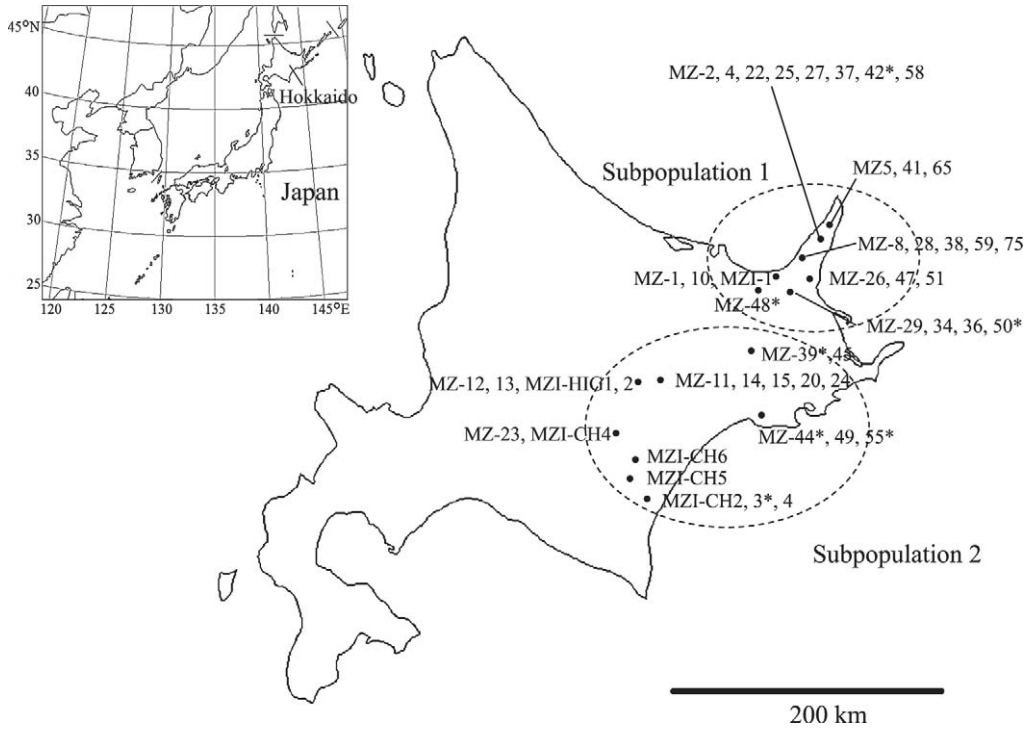

Fig. 1. Sampling locations of the sables in eastern Hokkaido. The map in the upper left shows the location of Hokkaido Island among the Japanese archipelago. Dots and numbers beside them indicate sampling locations and sample names, respectively, in Hokkaido. Numerals with asterisks show sables, which were not assigned to the major cluster in the sampling location.

different mtDNA gene markers and biased sample numbers. Although there was the above mentioned difference, both studies revealed that the nucleotide sequence differences between sables of Hokkaido and those of Russia were not so high.

Kashtanov et al. (2011a) studied the microsatellite diversity on the farm-bred sables, which were included in the industrial population of the Pushkinsky fur farm in Moscow region. The sable breeding began at the Pushkinsky fur farm in 1929 and the sable herd consisted of animals from natural populations, mainly from eastern Siberia and the Amur River basin. Later, sables captured in the Cis-Baikal region were supplemented there. The Pushkinsky population intensely experienced directed selection for dark fur (Kashtanov et al. 2011b). Kashtanov et al. (2011a) reported the number of alleles and heterozygosities at a total of six loci, and compared those of each locus with those of other mustelids such as the American marten *M. americanna* at four loci (Ma2, Ma3, Ma9 and Ma14) (Davis and Strobeck 1998) and the American mink *Neovison vison* at two loci (Mer041 and Mer175) (Fleming et al. 1999). These data showed that the number of alleles and values of expected heterozygosity for most loci in the sable are the same or very close to the related species.

So far, the study on variations of the nuclear DNA in any natural sable population have not been reported. Although Kurose et al. (1999) and Inoue et al. (2010) analyzed polymorphisms of mtDNA of sables in Hokkaido, the consequences did not coincide. In the present study, we analyzed microsatellite genotypes of the sable in eastern Hokkaido, and discuss the genetic diversity and population structure.

Materials and methods

Samples and DNA extraction

Tissue samples were obtained from 48 sables, most of which were roadkilled from 1991 to 2003. Figure 1 shows the sampling locations in Hokkaido. Total DNA was extracted from liver or muscle tissues using DNeasy Blood & Tissue Kit (Qiagen).

PCR amplification and microsatellite analysis

In the present study, we used 13 microsatellite loci (Mar02, Mar06, Mar08, Mar14, Mar15, Mar19, Mar21, Mar36, Mar43, Mar53, Mar56, Mar58 and Mar64), which were CA-repeat types or compound types devel-

oped in the European pine marten (Natali et al. 2010). Amplifications of polymerase chain reaction (PCR) were performed in 20 μl of the reaction mixture: 0.1 μl of r*Taq* DNA polymerase (5 units/μl, Takara), 2.0 μl of 10 × reaction buffer, 1.6 μl of dNTP, 0.2 μl of each primer (25 pmol/μl), 1.0 μl of DNA extacts and 14.9 μl of the distilled water. The 5' end of each forward primer was fluorescently labeled with FAM, NED, VIC or PET. After denaturing at 94°C for 5 min, 35 cycles were performed using a thermal cycler Takara TP-600 with the following program: denaturing 94°C for 40 sec; annealing at 50–55°C for 40 sec; extension at 72°C for 1 min; and then reaction completion at 72°C for 5 min. After each PCR product was electrophoresed using a DNA sequencer ABI3170, molecular sizes were measured with Gene Mapper 4.0, and alleles and genotypes were determined.

The PCR products were then sequenced to check whether microsatellite regions were included. A total volume of cycle PCR reaction mixture was 10 μl consisting of 0.4 μl of premix (BigDye Terminator Sequence Kit ver.1: ABI), 2.0 μl of diluent 5 × buffer, 1.6 μl of primer (1 pmol/μl), 1.0 μl of DNA product and 5.0 μl of distilled water. Cycle PCR amplifications were carried out in a thermal cycler Takara TP-600 using the following conditions: 30 cycle of denaturing at 94°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. Cycle PCR products were purified by isopropanol precipitation and then electrophoresed using a DNA sequencer ABI3170.

Data analysis

The population structure was estimated using STRUCTURE 2.3.3 software (Pritchard et al. 2000; Falush et al. 2003). We performed five runs at each value of *K* (1–5: number of subpopulations) with 100,000 burn-in and 100,000 Markov Chain Monte Carlo replicates after burn-in. In this analysis, we used admixture and correlated allele frequencies model. Values of log-likelihood ratio [LnP(D)] were calculated in order to estimate the number of clusters (*K*). When individuals had q-values (estimated membership in clusters) more than 0.6, they were assumed to be included in memberships of that particular cluster. Factorial correspondence analysis (FCA) was performed using GENETIX 4.05.2 (Belkhir et al. 1996–2004) to assess the patterns of the genetic relationships and analyze the population structure. Observed (*Ho*) and expected (*He*) heterozygosities were calculated using ALEQUIN 3.11 (Excoffier et al. 2005). Departures from Hardy-Weinberg equilibrium and linkage equilibrium were tested for each microsatellite locus using ALEQUIN 3.11. Allelic richness was calculated using FSTAT 2.9.3.2 (Goudet 2001). BOTTLENECK 1.2 (Piry et al. 1999) was used to evaluate recent declines in population size. In the present study, two bottleneck tests were performed. The first test was based on a detection of heterozygosity excess from the rapid loss of alleles in the population during bottleneck events (Cornuet and Luikart 1996). Heterozygosity excess was evaluated using the three microsatellite models (infinite alleles model (IAM), stepwise mutation model (SMM) and two phase model (TPM: 70% SMM and 30% IAM)) with Wilcoxon test. The second test was a qualitative graphical method for detecting the distortion of allele frequencies following a population bottleneck (Luikart et al. 1998). A population of stable effective size at mutation-drift equilibrium has a characteristic L-shaped distribution of allele frequencies, whereas a bottleneck population will show a mode-sifted distribution due to the loss of rare alleles.

Results

We detected PCR amplification in 12 of 13 candidate microsatellite loci, which were used in the present study, although we did not obtained no PCR products in one locus (Mar02); therefore, the 12 loci except Mar02 were used for the subsequent analysis. In addition, it was confirmed that each locus included microsatellite sequences.

Analysis of population structure

STRUCTURE analysis showed that the most optimal number of cluster was two ($K = 2$) (Fig. 2). The order of the individuals was arranged from north to south according to the sampling locations (Fig. 1). Based on the result, we divided the sable population in eastern Hokkaido into subpopulations 1 and 2. Figure 2 shows that 89% (24/27 individuals) from subpopulation 1 were assigned to cluster 1, whereas 81% (17/21 individuals) from subpopulation 2 were to cluster 2. It was found that the most individuals, which were not assigned to the major clusters to the subpopulations, were sampled near the boundary between the two subpopulations (MZ-39, MZ-44, MZ-48, MZ-50 and MZ-55) (Fig. 1).

In order to evaluate the genetic relationships among individuals, FCA analysis was performed based on allele frequency data (Fig. 3). The result showed that the members of subpopulation 1 consisted of one group, and were separated from those of subpopulation 2. Individuals

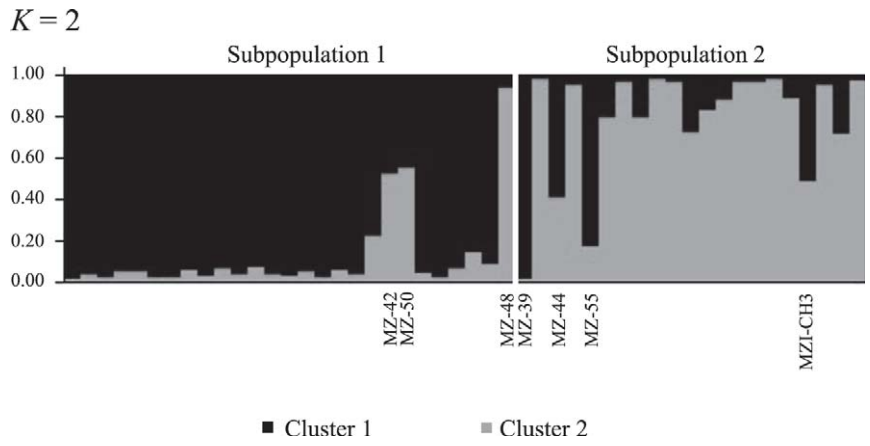

Fig. 2. Assignment test of the sables by STRUCTURE analysis, when the most optimal number of cluster was two ($K = 2$). The major cluster in subpopulation 1 was cluster 1 (dark color), and that in subpopulation 2 was cluster 2 (light color). Individuals from subpopulation 1, which were not assigned to cluster 1, were MZ-42, MZ-48 and MZ-50, whereas individuals from subpopulation 2, which were not assigned to cluster 2, were MZ-39, MZ-44, MZ-55 and MZI-CH3.

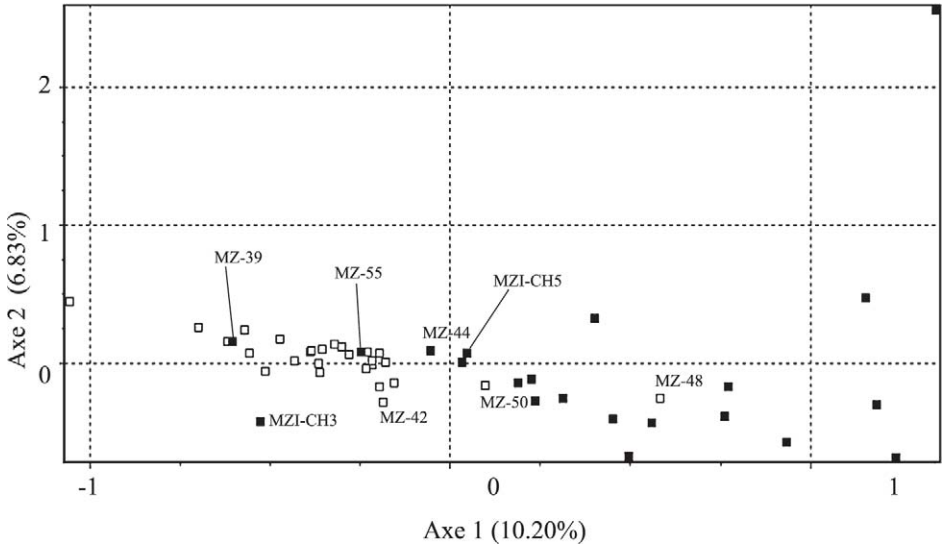

Fig. 3. Individuals' relationships in the two dimensions by FCA. Squares with the light color show individuals from subpopulation 1, and those with the dark color indicate individuals from subpopulation 2.

from subpopulation 1 (MZ-48 and MZ-50), which were sampled near the boundary between the two subpopulations, were located closely to those from subpopulation 2. In contrast, individuals from subpopulation 2 (MZ-39, MZ-44 and MZ-55), which were sampled near the boundary between the two subpopulations, were located closely to those from subpopulation 1.

Observed (*Ho*) and expected (*He*) hetelozigosities and allelic richness in all individuals and each subpopulation were calculated (Tables 1, 2 and 3, respectively). Subpopulation-specific alleles (private alleles) were found in both subpopulations (Tables 2 and 3): frequencies of private alleles were 17.8% (10/56 alleles) in subpopulation 1 and 30.8% (21/68 alleles) in subpopulation 2.

To detect recent effective population size reductions, two bottleneck tests for all individuals and each subpopulation were performed using BOTTLENECK. Although the first test showed significant heterozygosity excess under IAM in subpopulation 2 ($P = 0.026$), the test did not show significant under the other models. In addition, the second test indicated normal L-shaped distribution of allele frequencies. Microsatellites are generally considered to mutate according to intermediate TPM (Peery et al. 2012). This indicated that the sable population of eastern Hokkaido might not be experienced bottleneck events recently.

Table 1. Genetic diversity of 12 microsatellite loci in all sables. Allelic richness was calculated, based on minimum sample size 20

Locus	Number of samples	Number of alleles	Molecular sizes of alleles (bp)	Heterozygosity		Allelic richness
				Ho	*He*	
Mar06	50	11	216–240	0.65	0.73	8.51
Mar08	50	5	135–143	0.44	0.55*	4.26
Mar14	50	6	224–236	0.42	0.61*	5.40
Mar15	47	2	177–179	0.02	0.02	1.44
Mar19	50	5	198–208	0.56	0.52	4.78
Mar21	50	5	147–165	0.56	0.57*	4.46
Mar36	50	10	211–229	0.83	0.77*	7.39
Mar43	49	5	143–155	0.45	0.48	4.74
Mar53	50	8	225–249	0.65	0.79	7.67
Mar56	50	7	197–211	0.54	0.69*	5.66
Mar58	49	8	220–242	0.62	0.72	6.42
Mar64	50	6	176–186	0.48	0.48	5.10
Mean		6.50		0.52	0.58	5.49

*Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

Table 2. Genetic diversity of 12 microsatellite loci in subpopulation 1. Allelic richness was calculated, based on minimum sample size 20

Locus	Number of samples	Number of alleles	Molecular sizes of private alleles (bp)	Heterozygosity		Allelic richness
				Ho	*He*	
Mar06	27	8	216, 218	0.63	0.59	6.64
Mar08	27	3	–	0.37	0.40	3.00
Mar14	27	5	–	0.30	0.33*	4.61
Mar15	25	1	–	0.00	0.00	1.00
Mar19	27	5	–	0.48	0.48	4.68
Mar21	27	5	147, 165	0.59	0.62*	4.92
Mar36	27	5	211, 213	0.85	0.72*	4.74
Mar43	27	5	155	0.37	0.41	4.68
Mar53	27	5	–	0.63	0.69	4.94
Mar56	27	4	–	0.52	0.64	3.94
Mar58	27	7	240, 242	0.70	0.70	6.40
Mar64	27	3	178	0.33	0.39	2.94
Mean		4.67		0.48	0.50	4.37

*Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

Table 3. Genetic diversity of 12 microsatellite loci in subpopulation 2. Allelic richness was calculated, based on minimum sample size 20

Locus	Number of samples	Number of alleles	Molecular sizes of private alleles (bp)	Heterozygosity		Allelic richness
				Ho	*He*	
Mar06	21	9	232, 238, 240	0.67	0.83	8.90
Mar08	21	5	135, 143	0.52	0.61	4.95
Mar14	21	6	232	0.57	0.77	6.00
Mar15	20	2	179	0.05	0.05	2.00
Mar19	21	5	–	0.67	0.56	4.95
Mar21	21	3	–	0.52	0.50	3.00
Mar36	21	8	215, 219, 225, 227, 229	0.81	0.80	7.86
Mar43	20	4	–	0.55	0.57	4.00
Mar53	21	8	245, 247	0.67	0.86	7.95
Mar56	21	7	197, 201, 205	0.57	0.75*	6.91
Mar58	20	6	220	0.50	0.69	6.00
Mar64	21	5	182, 184, 186	0.67	0.54	4.91
Mean		5.67		0.56	0.63	5.62

*Significant departure from Hardy-Weinberg equilibrium ($P < 0.05$).

Table 4. Test of linkage disequilibrium among microsatellite loci

	Mar06	Mar08	Mar14	Mar15	Mar19	Mar21	Mar36	Mar43	Mar53	Mar56	Mar58	Mar64
Mar06												
Mar08	–											
Mar14	–	+										
Mar15	–	–	–									
Mar19	–	+	+	–								
Mar21	+	–	–	–	–							
Mar36	–	+	+	–	–	–						
Mar43	–	–	–	–	–	–	+					
Mar53	+	+	+	–	–	–	+	–				
Mar56	+	–	+	–	–	–	–	+	–			
Mar58	–	+	+	–	–	–	+	–	+	–		
Mar64	–	+	+	+	–	–	–	–	–	–	–	

+, statistically significant ($P < 0.05$).

Test of linkage disequilibrium and Hardy-Weinberg equilibrium

The results of disequilibrium test for all individuals showed statistically significant disequilibrium at 23/66 pairs of loci (Table 4). In addition, some deviations from linkage equilibrium in subpopulations were detected at six pairs of loci (Mar06/Mar53, Mar08/Mar36, Mar08/Mar64, Mar14/Mar43, Mar36/Mar43 and Mar53/Mar64) in subpopulation 1 and three pairs (Mar06/Mar36, Mar06/Mar56 and Mar08/Mar53) in subpopulation 2.

Departure from Hardy-Weinberg equilibrium ($P < 0.05$) was detected at five loci (Mar08, Mar14, Mar21, Mar36 and Mar56) for all individuals (Table 1). By contrast, the results showed departure from Hardy-Weinberg equilibrium at three loci (Mar14, Mar21 and Mar36) in subpopulation 1 (Table 2) and at a single locus (Mar56) in subpopulation 2 (Table 3). Because no pairs of loci were found to have departure from Hardy-Weinberg equilibrium in both subpopulations, data for all loci were applied to all analysis.

Discussion

Genetic division between subpopulations

Five departures from Hardy-Weinberg equilibrium for all individuals were found. Such departures from the equilibrium could have resulted from that some subpopulations, each of which had been genetically isolated, were treated as a single population (Wahlund effect: Wahlund 1928). On the other hand, three departures for subpopulation 1 and one departure for subpopulation 2 were detected. The observed departures from equilibrium in subpopulations might be due to the presence of smaller subpopulations to be recognized or the small sample size. In addition, the migration from or to outside of the population might cause departures from equilibrium.

STRUCTURE analysis showed that sables in eastern Hokkaido might be divided into two genetic clusters. Most individuals from subpopulation 1 were assigned to cluster 1, whereas most individuals from subpopulation 2 were assigned to cluster 2. There were alleles private to each subpopulation. These findings showed clear genetic differentiations between the two subpopulations. On the other hand, five individuals (MZ-39, MZ-44, MZ-48, MZ-50 and MZ-55), which were sampled near the boundary, were not assigned to the major cluster within that subpopulation. This means that there is some genetic interaction around the boundary. The result by FCA also supports this inference. Similarly, it is possible to distinguish subpopulation 1 from subpopulation 2 by another way. For example, subpopulation 1 is able to include MZ-39, MZ-44, MZ-45, MZ-49 and MZ-55. Subpopulation 1 members located within Shiretoko Peninsula is, however, better differentiated, as the result by STRUCTURE analysis (Fig. 2) shows that they well correspond with those of cluster 1. Higher mountains and lakes located between distributions of subpopulation 1 and subpopulation 2 might have served as their geographical barriers. In addition, long rivers such as Shari River and/or solfatara area caused by repeated volcanic events since 120,000 years ago (Gouchi 2007) also might prevent the migration between two subpopulations. Further study with additional samples from the boundary between subpopulations 1 and 2 could resolve the population structure and migration conditions.

Table 5. Genetic diversity in subpopulations of sables. Mitochondrial DNA haplotype sequences from Inoue et al. (2010) were used for calculation of haplotype and nucleotide diversities

Subpopulation	Number of samples	Number of haplotypes	Haplotype diversity (*SD*)	Nucleotyde diversity (*SD*)
Subpopulation 1	26	4	0.5446 (0.0774)	0.002032 (0.001536)
Subpopulation 2	18	8	0.8627 (0.0591)	0.003274 (0.002226)

Although the most optimal number of cluster was two ($K = 2$) in the present study, the result did not coincide with those of two previous studies about mtDNA phylogeny: Kurose et al. (1999) reported that the sables of Hokkaido have no clear geographic structures, whereas Inoue et al. (2010) reported that they were divided into three populations. In these studies, maternally inherited mtDNA, was used as a marker of phylogenetic analysis. Although there are little studies on sexual difference in home range size of the sable, a study of the American marten, which is a closely related species to the sable, reported that the home ranges of males were about three times larger than those of females (O'Doherty et al. 1997). This might be the reason why the previous mtDNA results were different from the result of the present study using biparentally inherited microsatellites. The difference in the sample numbers and the sampling locations between the previous two studies might be responsible for the different conclusions of the phylogenetic analyses.

Assessment of genetic diversity in the sables of eastern Hokkaido

The average *He* of the whole population was 0.58 (Table 1), whereas that in subpopulation 1 and subpopulation 2 was 0.50 and 0.63, respectively (Tables 2 and 3). No microsatellite data of continental sables were available in previous literatures, but *He* of farm-bred sables in Russia (0.57: Kashtanov et al. 2011a), which experienced intense selection, was very similar to that of the eastern Hokkaido sable in the present study. The average *He* of the Hokkaido sable was lower than that of the European pine marten in Tuscany, Italy (*He*: 0.400–0.856, mean *He*: 0.698; analyzed microsatellite loci except Mar02 were the same as those in the present study) (Natali et al. 2010).

The average *He* values of the eastern Hokkaido sables were similar to that reported from other mustelids such as the Canadian fisher *Martes pennanti* (*He*: 0.562–0.683, mean *He*: 0.623) (Kyle et al. 2001) and the North American wolverine *Gulo gulo* (*He*: 0.420–0.684, mean *He*: 0.63) (Kyle and Strobeck 2001). By contrast, the average *He* values of the eastern Hokkaido sable were higher than those of the Scandinavian wolverine (*He*: 0.269–0.376, mean *He*: 0.369) (Walker et al. 2001). Although the results are not directly comparable because population size, sampling location and analyzed loci were different, the present study shows that genetic diversity of the sable population in eastern Hokkaido was similar to those of other mustelids.

The comparison of heterozygosities, allelic richness and the number of identified alleles between subpopulations revealed the genetic diversity in subpopulation 1 was lower than that in subpopulation 2. In addition, FCA showed that genetic distances among individuals in subpopulation 1 were smaller than those in subpopulation 2 (Fig. 3). In the present study, using mtDNA control region sequence data (Inoue et al. 2010), the haplotype diversity and nucleotide diversity in subpopulations were newly calculated by ARLEQUIN (Table 5). Consequently, the genetic diversity of subpopulation 1 was lower than that of subpopulation 2, in agreement with the microsatellite variation revealed by the present study. Genetic drift or inbreeding might have resulted in the lower genetic diversity in subpopulation 1. Although bottleneck event was considered as one reason for the lower genetic diversity in subpopulation 1, BOTTLENECK analysis indicated that the sable population of eastern Hokkaido might not have experienced bottleneck events recently. Migration, considered by departure from Hardy-Weinberg equilibrium in the present study, might have erased genetic evidence of past bottleneck, as indicated by Cornuet and Luikart (1996). Otherwise, the smaller sampling area for subpopulation 1 might affect reduction in genetic diversity.

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