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Low genetic diversity in an isolated red fox (*Vulpes vulpes*) population on Mt. Hakodate, Japan, revealed by microsatellite analyses of fecal samples

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Abstract. Mt. Hakodate, Hokkaido, is a small mountain geographically isolated by the sea and urban area, and some red foxes (*Vulpes vulpes*) live there. In consideration of the specific geographical environment, we made a hypothesis that the fox population on Mt. Hakodate is small in size and isolated, and consequently its genetic variation is low, and then tested it. To investigate the genetic variation, we genotyped microsatellite loci using fecal samples collected noninvasively from 2009 to 2011. As the result, we successfully identified 35 foxes for the three years (12 in 2009, 11 in 2010, and 22 in 2011), and presumed eight of them were adults, indicating that the population size is extremely small. The genetic structures and degree of differentiations showed that the Mt. Hakodate population was genetically differentiated from those of the other populations. The urban area of Hakodate City could have played a role of a dispersal barrier for foxes. Additionally, the genetic diversity of the Mt. Hakodate population was lower than those of the other populations in Hokkaido. Our results suggested that the Mt. Hakodate population have low genetic diversity due to restricted gene flow and the small population size, supporting our hypothesis.

Key words: genetic structure, individual identification, noninvasive sample, population size.

The red fox, *Vulpes vulpes* (Carnivora: Canidae), is widely distributed across the northern hemisphere from the Arctic Circle to North Africa, Central America, the Asiatic steppes, and Australia (an introduced population), and has adapted to diverse environments including tundra, desert, forest, and urban areas (Macdonald and Reynolds 2008). The red fox also occurs on some small islands such as Assateague Island in the United States (Paradiso and Handley 1965) and Prince Edward Island in Canada (Sobey 2007). In Japan, one subspecies, *V. v. schrencki*, inhabits Hokkaido Island (Uraguchi 2015), and one of its populations is distributed on Mt. Hakodate, which is geographically isolated as “a land-tied island” (Kimura 2011).

Mt. Hakodate (41°45'32"N, 140°42'15"E), located near the base of the Kameda Peninsula, southern Hokkaido (Fig. 1A, 1B), is 334 m in altitude, roughly 9 km in circumference, and 326 ha in area. It is surrounded on three sides by the sea and on the landward side abuts the city of Hakodate (Fig. 1C). Mt. Hakodate was protected as a

military area from 1898 to 1945, and its natural environment remained relatively undisturbed for nearly half a century (Munakata 1980). The Hokkaido Government has designated Mt. Hakodate as a Wildlife Protection Area (353 ha) and Wildlife Special Protection Area (327 ha) (Hakodate City 2016). Though it borders a crowded urban area, it supports more than 600 plant species (Sugawara and Komatsu 1959) and provides habitat for both resident and migrating birds, of which approximately 150 species can be seen year-around (Munakata 1980). In mammal species other than the red fox, the chipmunk (*Tamias sibiricus*), introduced Japanese weasel (*Mustela itatsi*), two species of any mice (or shrews), and two bat species are distributed there (Kimura 2011). Vegetation and bird surveys have been conducted on Mt. Hakodate in the past because of the high diversity of species, but until now there have been no scientific studies on the resident terrestrial mammals including red foxes.

DNA analyses using noninvasively collected fecal samples can provide many kinds of biological informa-

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tion of target animals. For example, species and individual identifications have been often performed to obtain ecological data without harming animals. Kurose et al. (2005) analyzed the DNA from fecal samples to identify species and sex for four carnivore species, including the endangered Tsushima leopard cat (*Prionailurus bengalensis*), on Tsushima Island, Nagasaki Shimatani et al. (2008, 2010) likewise genetically identified five carnivore species from fecal samples collected in the Kushiro wetland, Hokkaido, and successfully identified individuals of the American mink (*Neovison vison*) there. Oishi et al. (2010) analyzed 59 fecal samples from red foxes in the Shiretoko National Park, Hokkaido, and ascertained that they derived from 22 individuals. This analysis can also provide much information important to conservation, such as population size, population structure, gene flow, and sex (Frankham 2010). Saito et al. (2016) identified 31 individuals from raccoon dog (*Nyctereutes procyonoides*) fecal samples on the grounds of the Imperial Palace, Tokyo, and examined the genetic structure of that population. Dallas et al. (2003) determined the sex from feces of the Eurasian otter (*Lutra lutra*), and attempted to estimate the sex ratio. Sex identification for red foxes has been previously conducted by using sex-specific markers designed based on genes on the Y chromosome, such as the *ZFY* gene (Ortega et al. 2004), *SRY* gene (Berry et al. 2007), and amelogenin gene (Moore et al. 2010).

Generally, isolated and small populations have low genetic variation due to genetic drift, restricted gene flow, and inbreeding. In fact, the phenomena have been seen in various animals such as the Galápagos penguin (*Spheniscus mendiculus*) (Nims et al. 2008), platypus (*Ornithorhynchus anatinus*) (Furlan et al. 2012), and Blakiston's fish owl (*Bubo blakistoni*) (Kohyama et al. 2015). In the present study, we made three hypotheses that the fox population on Mt. Hakodate is (1) small in size and (2) isolated, and consequently (3) its genetic variation is low. We then attempted to test the hypotheses. First, we determined microsatellite genotypes from fecal sample collected on Mt. Hakodate in order to identify individuals and to estimate population sizes. Then, we characterized the genetic structure of the population to determine whether the gene flow is restricted. Finally, we calculated indices of diversity to assess the genetic variation in the population. Furthermore, we designed a new sex-specific marker optimized for fecal DNA of foxes, and applied it to the field samples in this study to confirm the efficacy.

Materials and methods

Sampling and DNA extraction

Fecal samples were collected along nine hiking-trail courses on Mt. Hakodate (Irie-yama, Kannon, Yakushi-yama, Kyu-tozando, Shiomi-yama, Senjojiki, Nanamagari, Miyanomori, and Ezodate-yama), and from three towns (Kaminokuni, Matsumae, and Shikabe) in the southern part of the Oshima Peninsula (SOP) (Fig. 1). The continual fieldwork on Mt. Hakodate, designed so that all the hiking courses could be checked within the span of a month, was conducted one or more times per month in April–December 2009, April–November 2010, and April–December 2011. The sampling effort is not different substantially from year to year. One-off fieldwork was conducted also in Kaminokuni, Matsumae, and Shikabe in October or November 2011. Feces found in the field were placed into gamma-sterilized polypropylene conical tubes with sterilized forceps. Locations were determined with a handheld global positioning system (GPS) unit (GPS-CS3K, Sony) or a digital camera with a built-in GPS (Optio WG-1 GPS, Pentax). Fecal samples were transported to the laboratory at room temperature and then stored at -80°C for a week or more prior to DNA extraction, in order to inactivate tapeworm (*Echinococcus multilocularis*) eggs.

Total DNA was extracted from approximately 0.3 g of each fecal sample by using the QIAamp DNA Stool Mini Kit (Qiagen). As a positive control, DNA was extracted from the muscle tissue of a road-killed cub by using the DNeasy Blood & Tissue Kit (Qiagen).

Species-specific mitochondrial DNA amplification

To verify that the collected feces were from the red fox, a part of the mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR) with a red fox-specific primer pair fox-F1/R1 (Shimatani et al. 2008). To identify other mammal species presumed to occur on Mt. Hakodate, PCRs were performed with primer pairs specific for the domestic dog (*Canis familiaris*) (Shimatani et al. 2008) and domestic cat (*Felis catus*) (Kurose et al. 2005). The species-specific PCR amplifications were performed for all fecal DNA samples at least twice by following the protocols used in the previous studies.

Microsatellite genotyping

Genotyping was attempted for each fecal DNA sample identified as originating from the red fox. Initially the



Fig. 1. Sampling locations and points where fecal samples were obtained. (A) A map of East Asia and Japan. Dashed lines on Hokkaido Island indicate boundaries of red fox populations based on Oishi et al. (2011). (B) A map of the south part of the Oshima Peninsula (SOP) (enlargement of the boxed area in A). (C) A map of Mt. Hakodate (enlargement of the boxed area in B). The map in C was drawn Quantum GIS 1.7.1 Wrocław (QGIS Development Team 2011) using GIS Base Map Information obtained from the Geospatial Information Authority of Japan (<http://www.gsi.go.jp/kiban/>). Open and closed circles indicate red foxes and domestic cats, respectively. Gray heavy lines indicate hiking trails.

Table 1. Information on the microsatellite loci used for genotyping red foxes on Mt. Hakodate

Locus	Fluorescent label	Primer mix combination ^a	Allele size (bp)	Number of alleles	Genotyping success rate (%)	Reference ^b
DB1	6-FAM	A	130–152	4	79.0	1
DB3	VIC	C	125–131	3	81.5	1
DB4	PET	B	112–118	3	77.3	1
DB6	6-FAM	C	106–112	4	70.6	1
V142	NED	C	136–150	4	74.8	2
V374	NED	A	105	1	79.8	2
V402	VIC	B	81–85	3	84.0	2
V468	PET	A	87–91	3	81.5	2
V602	6-FAM	B	137–147	2	77.3	2
Mean				3.0	78.4	

^a Letters indicate combinations of primers included in the same mix.

^b References: 1, Lade et al. (1996); 2, Wandeler and Funk (2006).

following 10 microsatellite loci were selected for amplification: DB1, DB3, DB4, and DB6 (Lade et al. 1996), and V142, V374, V402, V468, V602, and V622 (Wandeler and Funk 2006). Locus V622 was later excluded because no PCR amplification was obtained for the locus. The 5' end of each forward primer was fluorescently labeled with 6-FAMTM, NEDTM, VIC[®], or PETTM (Applied Biosystems). PCR amplifications were performed with the QIAGEN Multiplex PCR Kit (Qiagen) and a TP600 DNA thermal cycler (Takara). Reactions were done in 5.0 µl volumes each containing 2.5 µl of 2× QIAGEN Multiplex PCR Master Mix, 0.9 µl of RNase-free water, 0.5 µl of primer mix (2.0 µM each primer) containing the forward and reverse primers for three loci, 0.1 µl of bovine serum albumin (BSA) solution (20 mg/ml, Roche), and 1.0 µl of template DNA. PCR thermal conditions were 95°C for 15 min; 35 cycles of 94°C for 30 sec, 57°C for 90 sec and 72°C for 1 min; and 60°C for 30 min. Primer mix combinations are given in Table 1. PCR products were subjected to electrophoresis in an ABI3730 DNA sequencer (Applied Biosystems), and molecular sizes were then measured with Peak Scanner 1.0 (Applied Biosystems) to determine alleles and genotypes.

For genotyping, each locus was amplified by PCR at least three times. Samples were considered homozygous for a locus when only one allele was detected for that locus in three amplifications. In cases when a PCR reaction detected two alleles for a locus in a particular sample, the locus was judged to be heterozygous only after the two alleles were detected in at least one additional independent PCR for the sample. Samples that yielded no PCR products for five or more loci were excluded from subsequent analyses.

To assess how many markers were sufficient to distinguish between different individuals using data from fecal samples, the probabilities of identity for unrelated individuals (P_{ID}) and siblings ($P_{ID-sibs}$) (Waits et al. 2001) were calculated with the program GIMLET 1.3.3 (Valière 2002). Mills et al. (2000) recommended a P_{ID} less than approximately 0.01 for estimating population size.

Individual identification and population size/density estimation

Determined genotypes were grouped by individuals with the program GIMLET 1.3.3. Population sizes of adults (one year of age and older) were inferred from three-year follow-up data based on individual identification. Individuals observed for a multi-year period were assumed to be adults, and were utilized to estimate population sizes. In contrast, individuals observed in a single-year were assumed to be young or adult. Based on this inferred population sizes, population densities were calculated from the area of the mountain (326 ha) assumed.

Sexing using the ZFX and ZFY genes

To determine the sex of red fox individuals from fecal samples more easily, we designed three new sex-specific primers based on partial sequences from each of the X and Y chromosomes, with which PCR fragments shorter than those in the previous studies (Ortega et al. 2004; Moore et al. 2010) were amplified: VVZFX-F1 (5'-TGA AGT TTT CAG ACC AGG GTT C-3'), VVZFY-F1 (5'-GGG TTT TTC TGT TAC CTC TTT TG-3') and VVZFYX-R1 (5'-CAT GAG TGA TCA AAC CAA GTT C-3'). These primers were designed based on the part of

the final intron sequence of the zinc-finger coding gene on the X (*ZFX*) and Y (*ZFY*) chromosomes (GenBank accession numbers AB622129 and AB622140, Tsubouchi et al. 2012). PCR reactions were performed in 20 μ l volumes each containing 0.2 μ l of *TaKaRa Taq*TM DNA polymerase (5 U/ μ l, Takara), 2.0 μ l of 10 \times PCR buffer, 1.6 μ l of dNTP mixture (2.5 mM each), 0.4 μ l of BSA solution (20 mg/ml, Roche), 0.2 μ l of each primer (2.5 μ M), 1.0 μ l of each DNA extract, and 14.4 μ l of distilled water. PCR thermal conditions were 94°C for 5 min; 35 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min; and 72°C for 10 min. PCR products were subjected to electrophoresis on a 3% agarose gel and visualized with ethidium bromide under an ultraviolet illuminator. These primers gave PCR products of 100 bp for *ZFX* and 131 bp for *ZFY* (Appendix 1), with one band and two bands indicating female and male, respectively. For confirmation, PCRs to determine sex were performed at least twice for each sample. The source of a sample was judged to be a male if at least two PCR amplifications showed both the *ZFX* and *ZFY* fragments, and to be a female if at least three PCR amplifications showed only the *ZFX* fragment, with no amplification of the *ZFY* fragment.

Data analyses

The genetic diversity and structure of the Mt. Hakodate population were compared with those of other populations on Hokkaido by using the microsatellite data of Oishi et al. (2011). The population structure was analyzed with STRUCTURE 2.3.4 (Pritchard et al. 2000). Ten runs were performed for each number of genetic clusters (*K*) from 1 to 10. Each run consisted of 1000 Markov Chain Monte Carlo (MCMC) iterations after a burn-in of 1000 iterations. To determine the most likely *K*, ΔK values were estimated by using STRUCTURE HARVESTER (Earl and vonHoldt 2012). The degree of genetic divergence between populations (pairwise F_{ST}) was estimated by using ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010). A population network based on F_{ST} values was constructed with the neighbor joining method in MEGA 6 (Tamura et al. 2013).

The effective population sizes (N_e) based on linkage disequilibrium (Waples and Do 2008) were calculated for the Mt. Hakodate and SOP populations by using NeEstimator 2.01 (Do et al. 2014). The mean numbers of alleles (*A*), observed and expected heterozygosity (H_o and H_e , respectively), and inbreeding coefficient (F_{IS}) were calculated and tested by using ARLEQUIN 3.5.1.2

(Excoffier and Lischer 2010). Allelic richness (A_R), normalized to the smallest number of samples, was calculated by using FSTAT 2.9.3.2 (Goudet 2002). Significance tests for F_{IS} values were performed by using Hardy-Weinberg exact test implemented in GENEPOP 4.2 (Raymond and Rousset 1995; Rousset 2008).

Results

Species identification

During the entire investigation, total 150 samples (49 in 2009, 48 in 2010, and 53 in 2011) of feces were collected on Mt. Hakodate, and species identification by PCR amplification of mtDNA fragments was successful for 122 (81.3%) of them (Table 2). The red fox was identified as the source of 98 (65.3%) of the samples, and the domestic cat as the source of 16 samples (10.7%). In addition, mtDNA fragments from both the red fox and the domestic cat were amplified from eight (5.3%) of the samples. From the remaining 28 samples (18.7%), no mtDNA fragments were amplified from the red fox, domestic cat, or domestic dog. The locations of samples identified as the red fox or domestic cat are shown in Fig. 1C. All six fecal samples obtained in places other than Mt. Hakodate were identified as the red fox, and were used for comparisons among populations.

Individual identification and population size/density estimation

The genotyping success rate per locus ranged from 70.6% to 84.0% (average, 78.4%), and 1–4 (average, 3.0) alleles were detected per locus (Table 1). The cumulative $P_{ID-sibs}$ and P_{ID} values for the nine loci examined were 0.015 and less than 0.001, respectively (Appendix 2), with the cumulative $P_{ID-sibs}$ value only slightly larger than the value of 0.01 recommended for estimating population size. The genotype data permitted individual identifica-

Table 2. Frequencies of identification of fecal samples to species, using species-specific primers

	2009	2010	2011	2009–2011
Red fox	31 (63.3%)	25 (52.1%)	42 (79.2%)	98 (65.3%)
Red fox + Domestic cat	3 (6.1%)	2 (4.2%)	3 (5.7%)	8 (5.3%)
Domestic cat	4 (8.2%)	6 (12.5%)	6 (11.3%)	16 (10.7%)
Domestic dog	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Not amplified	11 (22.4%)	15 (31.3%)	2 (3.8%)	28 (18.7%)
Total	49	48	53	150

Table 3. Number of fecal samples identified and sex of 35 red fox individuals identified in this study

Individual ID	N_{obs} for the entire period	N_{obs} per year			Sex
		2009	2010	2011	
MH01	7	2	5		M
MH02	10	8	1	1	F
MH03	8	3	1	4	F
MH04	1	1			U
MH05	6	3	3		F
MH06	1	1			M
MH07	1	1			F
MH08	2	2			F
MH09	2	1		1	M
MH10	1	1			F
MH11	1	1			F
MH12	6	1		5	M
MH13	3		3		M
MH14	1		1		M
MH15	1		1		M
MH16	2		1	1	M
MH17	1		1		M
MH18	1		1		F
MH19	4		1	3	M
MH20	3			3	M
MH21	3			3	M
MH22	2			2	F
MH23	1			1	M
MH24	1			1	U
MH25	1			1	F
MH26	1			1	F
MH27	4			4	M
MH28	1			1	U
MH29	1			1	F
MH30	1			1	U
MH31	1			1	U
MH32	3			3	F
MH33	1			1	M
MH34	1			1	M
MH35	1			1	M
Total N_{obs}	85	25	19	41	M40/F35/U10
Mean N_{obs}	2.4	2.1	1.7	1.9	
Total N_{ind}	35	12	11	22	M17/F13/U5

N_{obs} , number of fecal samples per individual; M, male; F, female; U, sex unknown; N_{ind} , total number of individuals. Shaded cells indicate time spans when an individual is confirmed as alive.

tion of 85 fecal samples for the three years (25 in 2009, 19 in 2010, and 41 in 2011), which were derived from 35 foxes (12 in 2009, 11 in 2010, and 22 in 2011) (Table 3). Of them, eight foxes (MH01, MH02, MH03, MH05, MH09, MH12, MH16, and MH19) were observed for a multi-year period (i.e., individuals expected to be adult),

and 11 foxes (MH04, MH06, MH07, MH08, MH10, MH11, MH13, MH14, MH15, MH17, and MH18) except foxes firstly observed for 2011 were observed only in a single-year (i.e., individuals expected to be juvenile). In both 2010 and 2011, the population size and density of adult foxes were estimated to be six (MH01, MH02, MH03, MH05, MH09, and MH12 in 2010; MH02, MH03, MH09, MH12, MH16, and MH19 in 2011) and 1.8 adult foxes/km², respectively.

Sex identification

Among the red fox 85 fecal samples identified individually, 40 samples came from males and 35 from females; sex could not be determined for the remaining 10 samples (Table 3). Sex was successfully determined for 30 of 35 individuals; these 30 individuals comprised 17 males and 13 females. Sex mismatch was not found among multiple fecal samples from the same individuals.

Genetic structure and diversity

Fig. 2 shows the genetic structure of the entire Hokkaido population including the population on Mt. Hakodate. The highest ΔK was calculated to be two (Appendix 3), indicating that the populations in southern Hokkaido, including the Mt. Hakodate and SOP populations, are separated from those in the rest of Hokkaido. The STRUCTURE analysis at $K \geq 4$ showed the population structure on Mt. Hakodate to be clearly different from that in Southern Hokkaido. Additionally, the red fox population on Mt. Hakodate was relatively homogeneous, indicating low genetic variation in this population. Genetic differentiation between the Mt. Hakodate population and other populations was also indicated by higher F_{ST} values (Fig. 3), which ranged from 0.13 to 0.16 between Mt. Hakodate and the other populations except the SOP population (Table 4), whereas F_{ST} values among other populations except the Mt. Hakodate population ranged from 0.00 to 0.12 (data not shown).

Table 5 shows barometers of the genetic diversity in the red fox populations on Hokkaido. The effective population size for the Mt. Hakodate population was 6.7 although that for the SOP population was infinite. The values for the mean number of alleles (A), allelic richness (A_{R}), and observed and expected heterozygosities (H_{O} and H_{E}) in the Mt. Hakodate population were lower than those in the SOP population and in the other populations on Hokkaido ($A = 5.000\text{--}8.375$, $A_{\text{R}} = 3.612\text{--}4.061$, $H_{\text{E}} = 0.625\text{--}0.671$, $H_{\text{O}} = 0.588\text{--}0.660$, not shown in the table). Significant heterozygote deficiency (i.e., deviations from

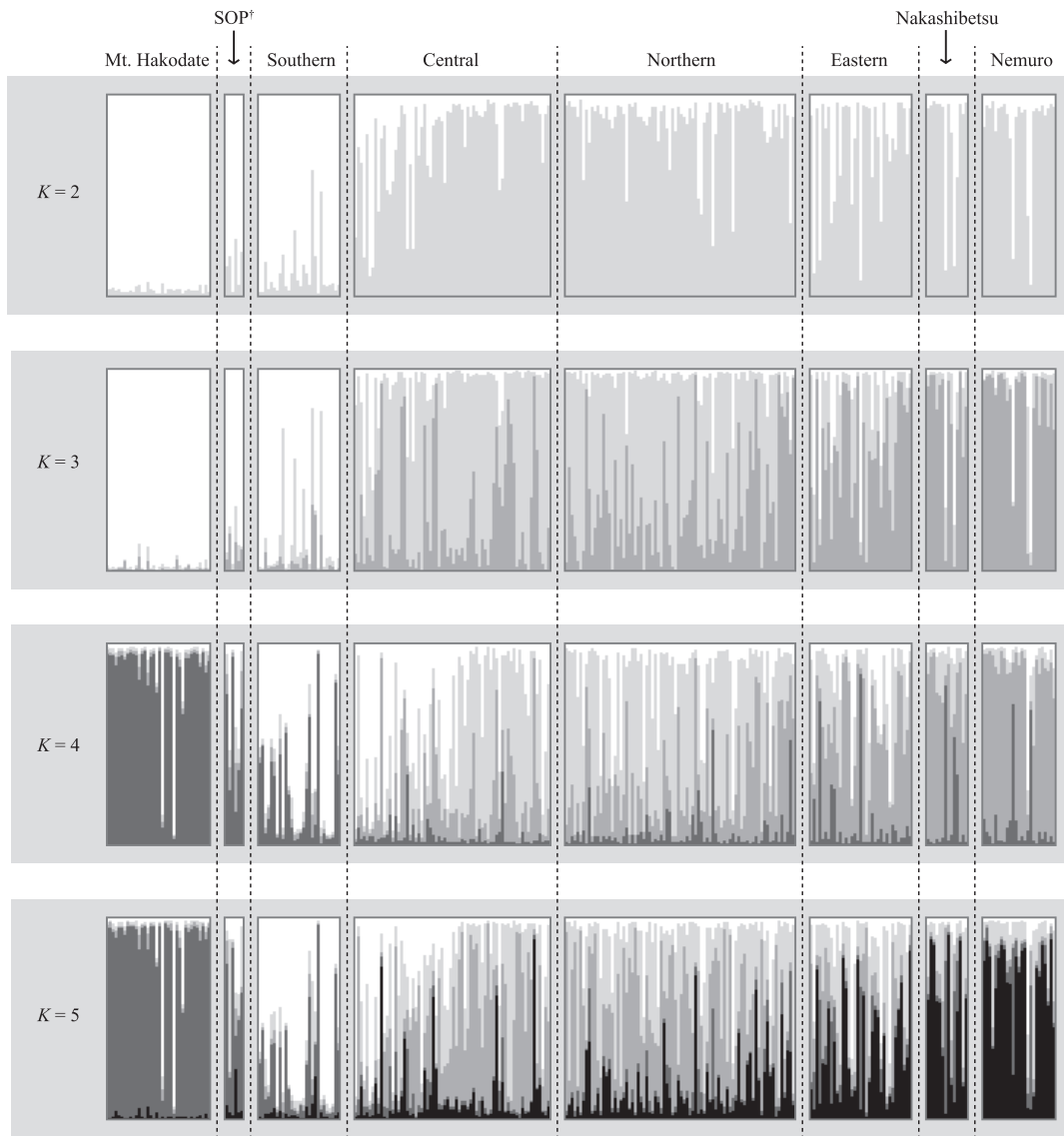


Fig. 2. Results of a STRUCTURE analysis ($K = 2-5$) of the Mt. Hakodate population and the other red fox populations in Hokkaido. The microsatellite data of Southern, Central, Eastern, Nakashibetsu, and Nemuro populations were cited from Oishi et al. (2011). †, the southern part of the Oshima Peninsula.

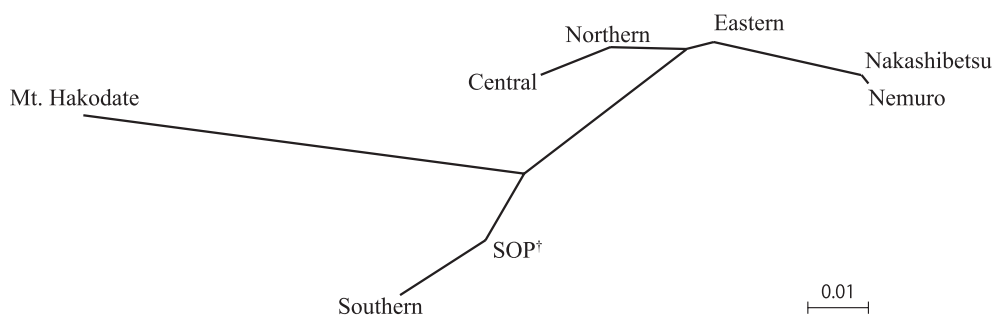


Fig. 3. Network of fox populations in Hokkaido, based on F_{ST} values from pairwise comparisons between populations. The microsatellite data of Southern, Central, Northern, Eastern, Nakashibetsu, and Nemuro populations were cited from Oishi et al. (2011). †, the southern part of the Oshima Peninsula.

Table 4. Pairwise F_{ST} values among red fox populations in Hokkaido, based on microsatellite data from Mt. Hakodate and the southern part of the Oshima Peninsula (SOP) (this study) and from other populations (Oishi et al. 2011)

	SOP	Southern	Central	Northern	Eastern	Nakashibetsu	Nemuro
Mt. Hakodate	0.08**	0.14***	0.16***	0.15***	0.13***	0.15***	0.16***
SOP	–	0.01 ^{ns}	0.09***	0.07***	0.06***	0.07***	0.09***

Significance of the F_{ST} values: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

Table 5. Microsatellite variation in the Mt. Hakodate and SOP populations

Populations	N	N_e (CI)	A	A_R	H_E	H_O	F_{IS}	P
Mt. Hakodate	36	6.7 (3.2–13.1)	3.250	2.592	0.476	0.454	0.047	0.004
SOP	6	∞ (14.6– ∞)	3.500	3.409	0.618	0.596	0.043	0.447

N , number of individuals; N_e , effective population size; CI, 95% confidence interval for N_e ; A , mean number of alleles; A_R , allelic richness; H_E , expected heterozygosity; H_O , observed heterozygosity; F_{IS} , inbreeding coefficient; P , P value for F_{IS} .

Hardy-Weinberg equilibrium) was detected in the Mt. Hakodate population ($F_{IS} = 0.047$, $P < 0.05$) although it was not detected in the SOP population ($F_{IS} = 0.043$, $P > 0.05$).

Discussion

Species composition and monitoring based on noninvasive genetic samples

Some previous studies have briefly surveyed other wild animal populations, including the red fox, using noninvasive genetic samples as in our study (Fernandes et al. 2008; Shimatani et al. 2008; Statham et al. 2012). Other, more extensive studies have used noninvasive samples to monitor species or individual distributions, population size, and genetic diversity across multiple years in various species, including the gray wolf (*C. lupus*) (Stenglein et al. 2010; Stansbury et al. 2014), San Joaquin kit fox (*V. macrotis mutica*) (Wilbert et al. 2015), Amur tiger (*Panthera tigris*) (Sugimoto et al. 2012), Far Eastern leopard (*P. pardus orientalis*) (Sugimoto et al. 2013), and sable (*Martes zibellina*) (Nagai et al. 2014), but there has been no similar previous study on the red fox.

Our study using fecal DNA revealed that both the red fox and domestic cat inhabit Mt. Hakodate. It is unclear whether the cats are feral, or are pets wandering from nearby residential areas. It is also unclear why DNA from both species was occasionally detected in single fecal samples. This might result from one of the species marking territory by defecating in the same place, or from foxes preying on cats, and in fact, Contesse et al. (2004) found cat remains in stomach contents of red foxes.

Success rate of species detection and genotyping

The amplification rate of mtDNA fragments (81.3%) in species identification in our study was within the range of approximately 60–96% in previous studies using fecal DNA from wild carnivorans (Kurose et al. 2005; Sugimoto et al. 2006; Shimatani et al. 2008). However, our success rates (70.6–84.0%; average, 78.4%) for genotyping microsatellite loci were high. Oishi et al. (2010) determined genotypes with success rates of 23.3–69.8% per locus in individual identification of red foxes in the Shiretoko National Park, Hokkaido. Nagai et al. (2014) genotyped sables in eastern Hokkaido with success rates of 47.8–76.1% per locus (average, 63.6%). Our high success rates were likely due to a high frequency of sampling and the method used to preserve fecal samples. We conducted sampling at least once a month and thus obtained relatively fresh feces. This was an important factor, because it is difficult to amplify microsatellite DNA from fecal samples that have remained in the open for more than three months (Piggott 2004). Furthermore, we preserved fecal samples by freezing, which according to some studies gives a higher genotyping success rate under specific conditions than samples preserved in ethanol (Piggott and Taylor 2003; Santini et al. 2007). Finally, for freezing samples we used a -80°C ultracold freezer rather than the standard -20°C freezer generally used for sample preservation.

Small population size

Our study showed that at least 11–22 (annual average, 15) foxes per year actually inhabited Mt. Hakodate, and indicated that the population size is small. However, the population size we estimated in this study could contain

the number of young foxes under one year old, which have high mortality (Yoneda and Maekawa 1982). In fact, the number of foxes observed only in a single-year (i.e., individuals expected to be juvenile) was larger than that of foxes observed for a multi-year period (i.e., individuals expected to be adult). Additionally, the adult population sizes (six adult foxes/year) inferred from the follow-up data were as small as the effective population size ($N_e = 6.7$), and correspond to up to three families if the sex ratio was equal. The numbers of adults of both sexes (three males and three females) assumed in 2010 might support that. This result indicated that the actual population size of foxes on Mt. Hakodate might be extremely small. On the other hand, the population densities estimated based on the putative numbers of adult foxes (1.8 adult foxes/km²) corresponds to rural level in comparison to typical densities (0.2–2.7 and 2–12 adult foxes/km² in rural and urban areas, respectively; Soulsbury et al. 2010). However, this estimation might be underestimated due to undetected individuals.

The number of individuals successfully genotyped in 2011 was largest for the three years. It is not necessarily appropriate to suggest that the number of individuals reflect the abundance because the rate of species identification is not equal throughout the years. Small proportion of unamplified samples in 2011 might be attributed to obtainment of better samples, short storage to DNA extraction, and improvement in laboratory technique.

Isolation by urban areas

Our microsatellite data compared with the data of Oishi et al. (2011) indicated that the genetic structure in the Mt. Hakodate population was greatly different from those of the other populations in Hokkaido, which means that the Mt. Hakodate population becomes geographically isolated from other populations by the urban development of Hakodate City. Although red foxes can disperse up to 302 km in straight-line distance (Allen and Sargeant 1993), the dense Hakodate urban area, which separates Mt. Hakodate from the rest of the Kameda Peninsula by only 8.5 km, likely inhibits gene flow between the two areas, and this has resulted in inbreeding on Mt. Hakodate. However, the Mt. Hakodate population has two individuals assigned to the Southern population. This indicates that foxes might fairly infrequently immigrate to Mt. Hakodate through the urban area or that major alleles in the Southern population might remain as a minor allele in the Mt. Hakodate population. The Mt. Hakodate population must have been a part of the south-

ern Oshima Peninsula population before development of the city, as our results show that the genetic structure of both populations are similar to each other.

Low genetic diversity

As our hypothesis, our microsatellite data indicated that the genetic diversity in the Mt. Hakodate population was low compared with those of the other populations in Hokkaido. The low genetic diversity we detected was corroborated by the P_{ID} values, which not only indicate the statistical confidence in individual identification but also are inversely correlated with the level of genetic diversity in natural populations (Paetkau and Strobeck 1994; Paetkau et al. 1998). The genetic diversity of the Mt. Hakodate population appears to be lower than that of the restricted population in the Shiretoko National Park, eastern Hokkaido, because the cumulative P_{ID-sib} in a study there by Oishi et al. (2010) was less than 0.01 at the seventh locus in only 22 samples, whereas in our study P_{ID-sib} was greater than 0.01 at the ninth locus in 36 samples, even though we used nearly the same microsatellite makers.

Our statistical results showed that deviations from Hardy-Weinberg equilibrium occurred in the Mt. Hakodate population. This indicates a small number of individuals, no random mating, and/or inbreeding in the population.

Formation process of the isolated population

As a conclusion of the present study, the Mt. Hakodate population geographically isolated has a low genetic diversity, which is due to gene flow restricted by the urban area of Hakodate City and the small population size leading to inbreeding and genetic drift. We then discussed the background in detail in the light of historical phenomena.

A possible scenario for how the Mt. Hakodate population became genetically isolated is as follows. Mt. Hakodate was an island previous to about 5000 years ago (Ganzawa 2002), when it became connected to the Hokkaido mainland. The red fox probably colonized Mt. Hakodate after that time, and could have moved freely between Mt. Hakodate and the rest of the Kameda Peninsula (Fig. 1B). At this early stage, some alleles were restricted to red foxes inhabiting the Kameda Peninsula. From the Meiji period (1868–1912), the city of Hakodate developed on the land bridge connecting Mt. Hakodate and rapidly increased in population (Nemoto 1990), resulting in a gradual decrease in gene flow between foxes on Mt. Hakodate and those on the rest of the Kameda

Peninsula. Isolation, genetic drift, and inbreeding could then have led to the present genetic structure unique to Mt. Hakodate.

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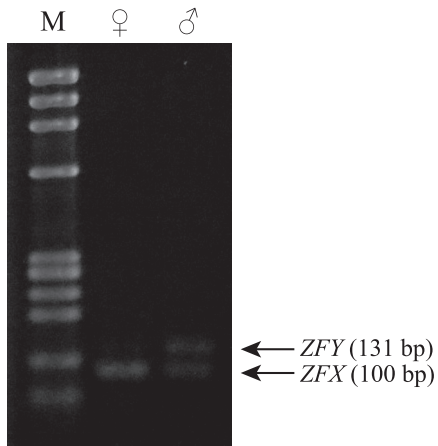
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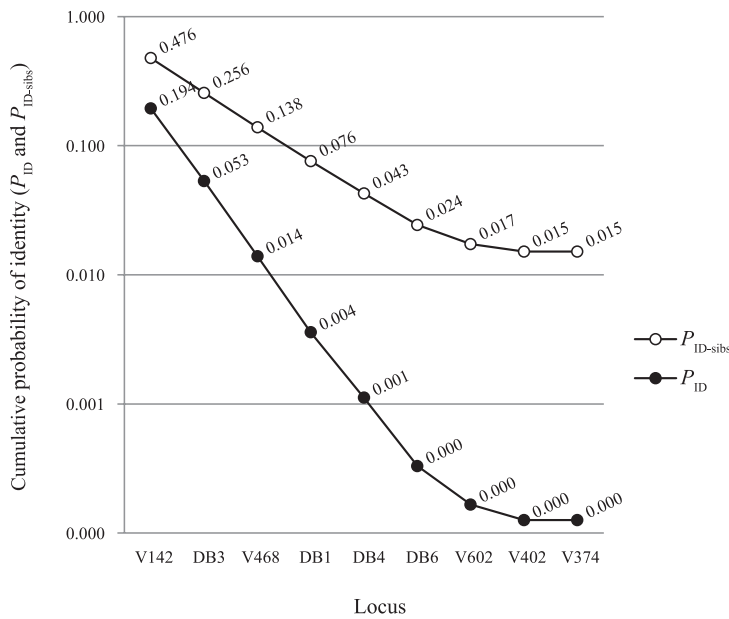
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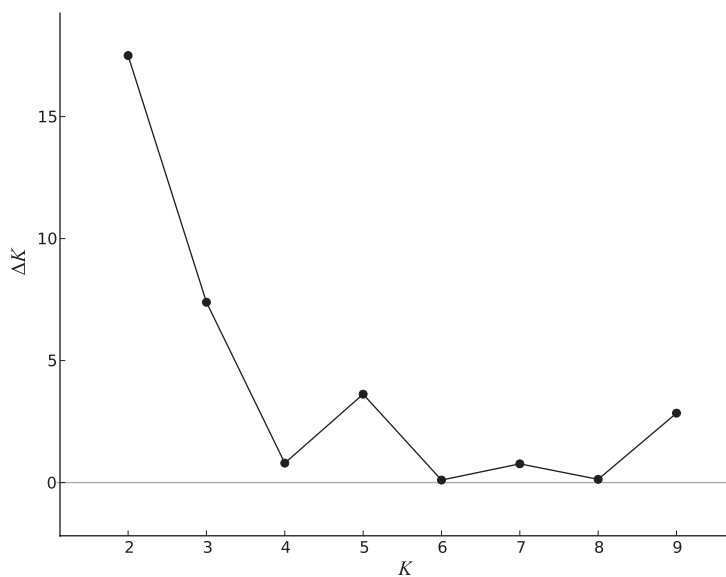
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Appendix 1. Photograph of a 3% agarose gel stained with ethidium bromide, showing bands of partial fragments of the fox *ZFX* and *ZFY* genes amplified by PCR from fecal DNA. M, size marker; ♂, male; ♀, female.



Appendix 2. Semilog plots of the cumulative probabilities of identity (P_{ID} and $P_{ID-sibs}$) calculated from fox microsatellite data in this study. The loci were arranged from left to right in order of increasing individual probability values.



Appendix 3. Changes in ΔK values calculated using STRUCTURE HARVESTER, based on a STRUCTURE analysis of the red fox populations in Hokkaido.