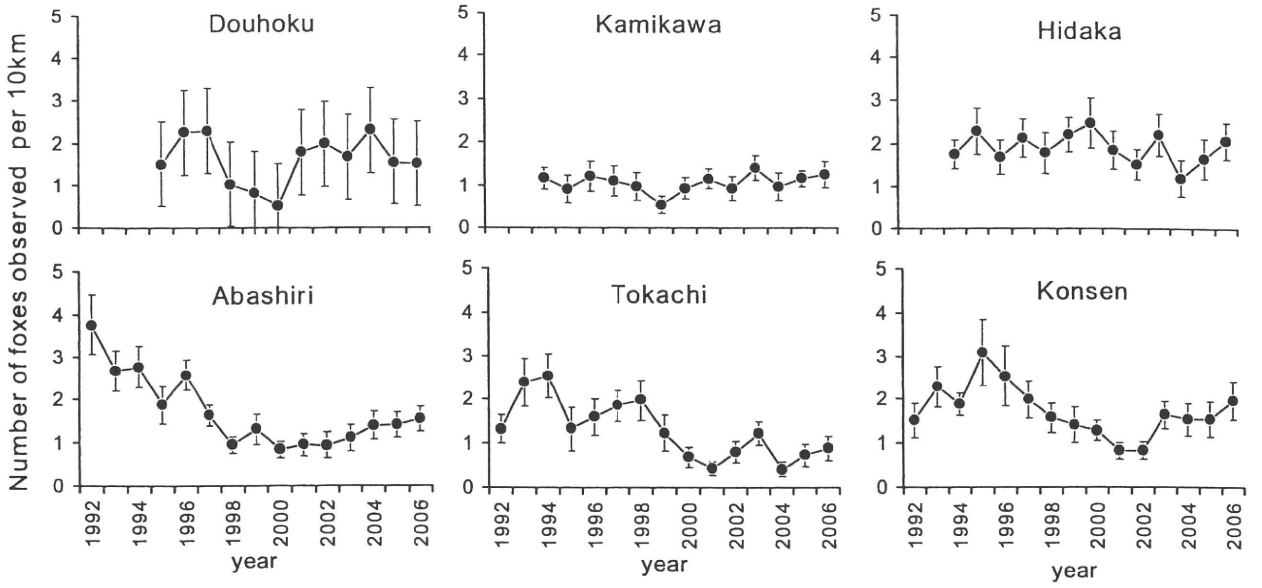

Fig. 2. Changes in relative abundance indices of red foxes *Vulpes vulpes* (number of foxes observed per 10 km by spotlight survey) in six study areas in Hokkaido, Japan, 1992–2006. Circles indicate mean values and bars indicate standard errors.

査地域内に含まれる全てのワナ設置箇所での野ネズミ類の合計捕獲数を，100トラップナイト当たりの捕獲数に換算し，これを各調査地域における野ネズミ類の密度指標値（頭/100TN）として用いた．各調査地域のワナ設置箇所数は年によって異なり，それぞれの箇所数は，道北22～58，上川45～85，日高43～100，網走73～124，十勝53～106及び根釧38～98であった．

捕獲がキツネの相対密度指標値の増減に及ぼす影響を調べるため，各調査地域において，各年のキツネ相対密度指標値の対前年比の値を目的変数，同じ年の捕獲数を説明変数とする単回帰分析を行った．同様に，野ネズミ類の資源量がキツネの相対密度指標値の増減に及ぼす影響を調べるため，各年のキツネの相対密度指標値を目的変数，前の年及び同じ年の野ネズミ類の密度指標値をそれぞれ説明変数とする単回帰分析を行った．有意水準はいずれも0.05とした．

結　　果

網走，十勝及び根釧におけるキツネの相対密度指標値の推移は類似しており，1990年代前半又は中ごろには約3頭/10 kmの最大値が記録されたが，その後減少し始め，2000年～2001年には1頭/10 kmを下回った（Fig. 2）．道北のキツネの相対密度指標値は，1996年～1997年の2頭/10 km前後から，2000年には約0.5頭/10 kmとなり，2001年には再び2頭/10 kmのレベルに戻るという変動を示したが，各年のデータのばらつきの大きさを考慮すると，明確な傾向は読み取れなかった（Fig. 2）．一方，上川と日高のキツネの相対密度指標値は，年変動はあるものの，上川は1頭/10 km，日高は2頭/10 km前後の水準で推移し，明確な増減の傾向は認められなかった（Fig. 2）．

キツネの捕獲数は，全ての調査地域で1990年代後半から2000年代前半にかけて減少した（Fig. 3）．単回帰分析の結果，全ての調査地域において，各年のキツネの相対密度指標値の対前年比とキツネ捕獲数の間に，有意な関係は認められなかった（$P>0.05$）．

野ネズミ類の密度指標値の動向には，いずれの調査地域においても2～4年間隔の周期性が認められた（Fig. 4）．単回帰分析の結果，日高では，同じ年のキツネの相対密度指標値と野ネズミ類の密度指標値との間に有意な関係が認められたが（回帰係数4.764，$P=0.028$，$r^2=0.366$），キツネの相対密度指標値と前年の野ネズミ類の密度指標値との間に有意な関係は認められなかった（$P>0.05$）．また，日高を除く全ての地域では，キツネの相対密度指標値と前年及び同年の野ネズミ類の密度指標値の間に有意な関係は認められなかった（$P>0.05$）．

考　　察

網走の知床半島や根釧の根室半島の局所個体群では，1990年代にキツネ個体数が急激に減少したことが明ら

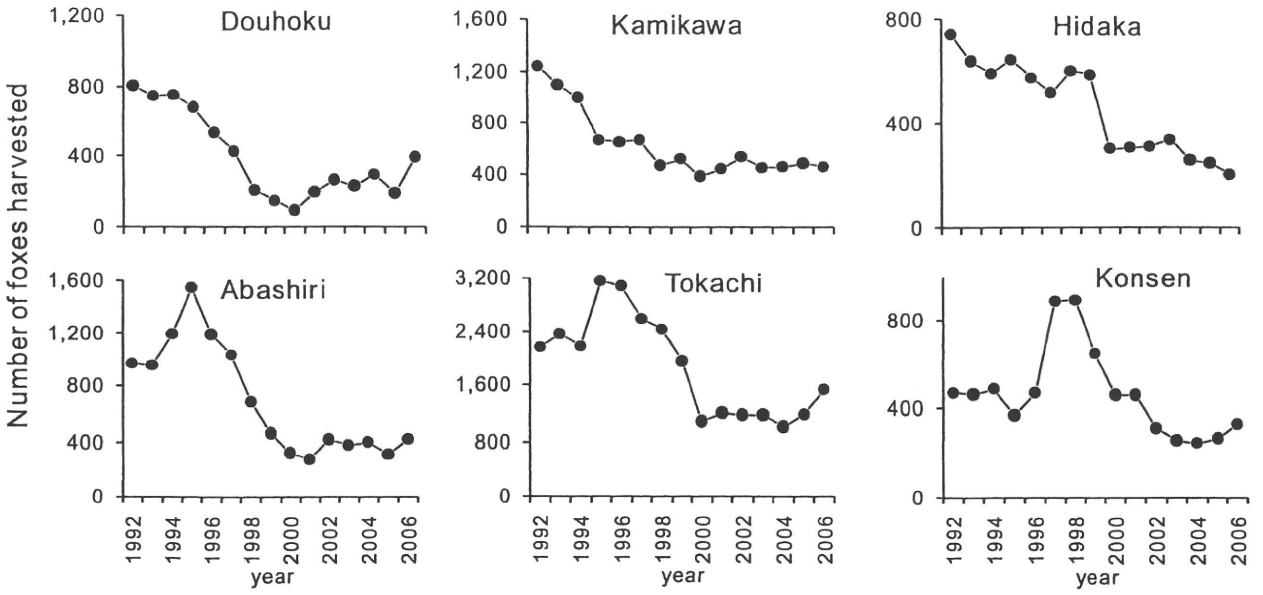

Fig. 3. Changes in annual number of red foxes (*Vulpes vulpes*) harvested in six study areas in Hokkaido, Japan. The annual number is the total number of foxes hunted in the previous winter plus the number culled in nuisance control from spring to autumn of the same year.

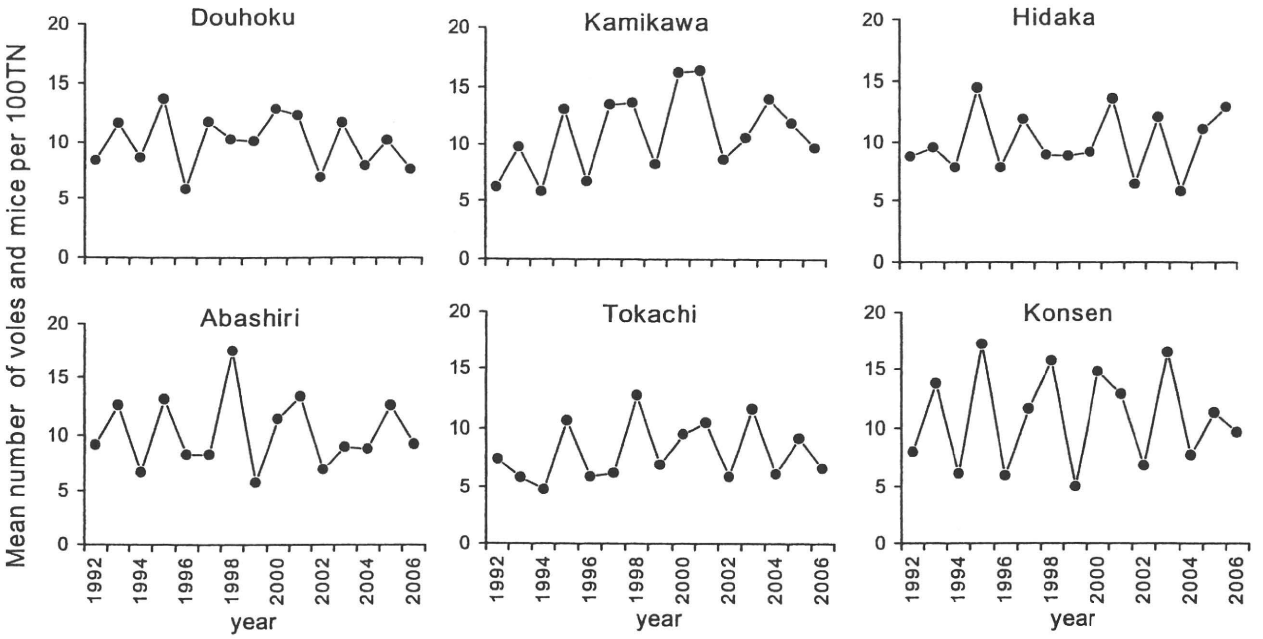

Fig. 4. Changes in abundance indices of voles and mice (number of voles and mice captured per 100 trap nights) in autumn in six study areas in Hokkaido, Japan, 1992–2006. These values were based on census data from the Hokkaido government (Program on Prediction on Voles Density). The number of trap stations ranged from 22 to 124 according to the year and the study area.

かにされている（塚田ほか 1999；高橋・浦口 2001）．本研究で確認された，網走，十勝及び根釧の 3 調査地域におけるキツネの相対密度指標値の 1990 年代の急激な減少は，それら局所個体群における先行研究の結果を支持するものであり，その時期に北海道東部の広い範囲でキツネ個体数が減少したと考えられる．キツネの個体数に影響を及ぼす要因としては，狩猟や駆除による捕獲，餌資源量の変化，生態的同位種との競争，薬剤汚染，疾病の流行などが考えられる．

北海道東部のキツネ個体群では，受ける捕獲圧の違いによって，平均寿命や成獣の生存率などが変化することが知られているが（Yoneda and Maekawa 1982），捕獲に

よる個体数への直接の影響については明らかにされていない．本研究においては，キツネの相対密度指標値の対前年比と捕獲数の間には有意な関係は認められず，捕獲がキツネの密度を下げたという証拠は得られなかった．

キツネなどの食肉目の個体数は，主要な餌動物の量が変化すると，0～1年程度遅れて同調する場合がある（Fuller and Sievert 2001）．しかし，本研究においては，日高では同じ年の野ネズミ類の密度がキツネ個体数に影響を及ぼしていた可能性があるもの，他の全ての調査地域でキツネの相対密度指標値と前の年及び同じ年の野ネズミ類密度指標値との間に有意な関係は認められず，今回観察された網走，十勝及び根釧の3調査地域におけるキツネ個体数の減少に，野ネズミ類の密度が直接的に関与していた可能性は低いと考えられた．

1990年代以降急速に北海道での分布域を拡大している外来種アライグマ（*Procyon lotor*）は，キツネと同様のニッチを占めるためその影響が懸念されている（池田2000）．しかし，1990年代後半にキツネ個体数が急減した3つの調査地域においては，2004年度までアライグマによる農業被害は確認されておらず（北海道野生鳥獣被害統計），少なくとも1990年代後半の時点において，アライグマの個体数はキツネの個体数に強い影響を及ぼすほどには達していなかったと考えられる．

また，平川（2001）は殺鼠剤フラトール（モノフルオール酢酸ナトリウム）の影響により，1950年代半ばから1970年代半ばにかけて北海道のキツネ密度が低下した可能性を指摘している．しかし，1974年にフラトールの使用は禁止され（三澤1985），その後，現在に至るまで二次毒性のある殺鼠剤は使用されていないため，1990年代後半のキツネの個体数減少が殺鼠剤によるものとは考えられない．

キツネに致死的な感染症の一つに疥癬があり（Mörner and Christensson 1984；Bornstein et al. 1995），疥癬の流行はキツネ個体群に深刻な影響を及ぼす場合がある（Lindström et al. 1994）．北海道でも，これまでに知床と根室の局所個体群において，疥癬が原因と考えられる個体数の減少が確認されている．知床半島では，キツネの疥癬が初確認された1994年を境に，ライトセンサスのキツネ観察数が40%程度まで減少した（塚田ほか1999）．また，1998年に疥癬が初確認された根室半島では，その直後からキツネのファミリー数が激減し，2000年には1997年以前の1/4まで減少した（高橋・浦口2001）．高橋・浦口（2001）は，1998年4月から1999年3月の間に北海道全域で捕獲されたキツネを検体として用い，全道的なキツネの疥癬流行状況調査を行った結果，道南の3支庁を除く11支庁で疥癬のキツネを確認した．1994年まで北海道ではキツネの疥癬は確認されていなかったことから，北海道では1990年代中ごろに一部地域で疥癬の流行が始まり，1990年代後半までには流行範囲が南部を除くほぼ全域に達していたと考えられる．その流行の時期は，本研究の3つの調査地域でキツネ個体数が減少した時期と概ね一致する．疥癬が流行するとキツネの個体数が減少するという現象は，スウェーデンの広域個体群でも報告されている（Lindström et al. 1994）．以上のように，北海道のキツネ個体数に影響を及ぼすと考えられる5つの要因について，本研究の3つの調査地域で認められたキツネの個体数減少に関与した可能性を検討した結果，疥癬流行の関与が最も強く疑われた．

一方で，同じく疥癬流行地域であった道北，上川及び日高では，顕著なキツネの相対密度指標値の減少は認められなかった．このことは，疥癬の流行状況やそれによる個体数への影響には地域差があったことを示唆する．また，ライトセンサスの調査努力量の不足によりそれらの地域の個体数変動を検出できなかった可能性もある（Ralls and Eberhardt 1997）．しかし，現時点ではいずれについても検証し得るデータがなく，今後明らかにしていく必要がある．

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ABSTRACT

Long-term population trends of red foxes (*Vulpes vulpes*) in Hokkaido, Japan

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We analyzed population trends of red foxes (*Vulpes vulpes*) in Hokkaido, Japan, from the number of foxes observed by spotlight surveys conducted in 1992–2006. The relative abundance indices of foxes in three of six study areas (Abashiri, Tokachi and Konsen) decreased to one-third in the 1990s. We were not able to detect the influence of hunting and food resources on these population declines. Since the timing of the decline in the fox population co-occurred approximately with the outbreak of sarcoptic mange in these areas, we strongly suspect that this epizootic participated in the reduction of fox populations. Though the mange had also spread among foxes in the three remaining study areas (Douhoku, Kamikawa and Hidaka), the relative abundance indices of foxes in these study areas did not decrease, but the cause is unclear.

Key words: *Vulpes vulpes*, red fox, population trend, spotlight survey, sarcoptic mange

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Non-invasive genetic identification of the red fox *Vulpes vulpes* in the Shiretoko National Park, eastern Hokkaido, Japan

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Abstract. In order to assess the genetic usefulness of feces that were obtained from field, we conducted genetic identification by microsatellite analysis on fecal samples of the red fox (*Vulpes vulpes*) collected in the Shiretoko National Park, Hokkaido. Consequently, 59 fecal samples resulted in 22 as the minimum number of individuals. The cumulative $P_{(ID)sibs}$ in the 22 samples was less than 0.01. Two pairs of fecal samples having the identical genotypes ($P_{(ID)sibs} < 0.01$) were considered to be dropped by the same foxes, and the distribution of these feces was almost overlapped with the home range of one fox, revealed by a previous study. Although few reports had revealed the absolute number of red foxes in field, the present study showed that the genetic analysis of their feces is useful for estimating a presumable number of individuals in the area. However, the lower genotyping success rates (23.3–69.8%) and genotyping reproducibility (53.5–88.4%) indicate the difficulty of genetic analysis by using these fecal samples. In order to improve the efficiency of the analysis, it may be effective to select and use a marker set which has smaller allele sizes, because the genotyping success rates and reproducibility increased when the average allele size decreased.

Key words: feces, genetic identification, non-invasive method, *Vulpes vulpes*.

The Shiretoko National Park in eastern Hokkaido, Japan (Fig. 1a) covers an area of 38,633 ha, and the primitive forests are still conserved in the park. The vertebrates such as the brown bear (*Ursus arctos*), steller's sea-eagle (*Haliaeetus pelagicus*) and Blakiston's fish owl (*Ketupa blakistoni*) and marine animals such as the chum salmon (*Oncorhynchus keta*), killer whale (*Orcinus orca*), sperm whale (*Physeter macrocephalus*), harbor seal (*Phoca vitulina*) consist of the specific food chain system. For conservation of the richness of the ecosystems and biodiversity, this area was registered as the World Nature Heritage in 2005 by the International Union for the Conservation of Nature and Natural Resources (IUCN).

The red fox (*Vulpes vulpes*) is also one of the mammals inhabiting this park. It plays an important role in the ecosystem as carnivore, and is an ecologically well-studied mammal in the Shiretoko National Park. Tsukada and Nonaka (1996) investigated the food habit of the red fox in the park, and revealed the utilization of provisions by human as secondary food supply. Tsukada (1997a) carried out the radio tracking of the red fox there, and reported that its home ranges were fluctuated with the seasonal resources and the human feeding. Tsukada et al. (1999) conducted the spotlight census in order to examine changes of the fox population size, and suggested the significant reduction of the population caused by sarcoptic mange. In addition, the red fox in Hokkaido carries the tapeworm *Echinococcus multilocularis* causing serious zoonosis, alveolar echinococcosis (Oku and Kamiya 2003). Nonaka et al. (1998) reported *E. multilocularis* infection in red foxes in Shiretoko by coproantigen detection, and recently the Hokkaido Institute of Public Health investigated the infection of *E. multilocularis* by egg examination with DNA identification about fox feces collected in the same area. The coproantigen or DNA positive rate based on the number of each positive feces is not the precise infection rate of the parasite among foxes, because of double-counting of feces from the same individual. However, the genetic identification technique in addition to the above method may lead to giving us the precise infection rate among foxes.

In the present study, to assess the usability of the fox feces that had been collected for examination of *E.*

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multilocularis, we conducted genetic identification of individuals by microsatellite analysis on the feces. In non-invasive DNA analysis, in general, feces are newly and systematically collected only for the purpose of genetic study (e.g. Banks et al. 2002; Lucchini et al. 2002; Flagstad et al. 2004; Bellemain et al. 2005; Piggott et al. 2006). For example, Shimatani et al. (2008, 2010) analyzed DNA of fecal samples younger than 24 hours, which were collected from field everyday in one sampling period. These studies above have successfully resulted in estimation of the population size, sex ratio, dispersal and relatedness. Because animal feces have been used for various purposes such as analyses of food habits, infection rates of parasite, estrous cyclicity, etc. (e.g. Tsukada 1997b; Nonaka et al.1998; Putoranto et al. 2007), if such feces already used and stored at laboratory for the above purposes are usable for genetic analysis, the non-invasive samples can bring more information to wildlife researches. By applying the fecal DNA analysis to the red fox feces, it will be also possible to non-invasively examine the ecological aspects of the red fox at the park. Moreover, the genetic identification technique would give us the precise infection rate of parasites among foxes. From these viewpoints, it is significant to conduct non-invasive genetic identification analysis on feces of the red fox.

Materials and methods

Sample collection and DNA extraction

The Hokkaido Institute of Public Health investigated fox feces in the Shiretoko National Park in 2007 (Fig. 1a). All fox feces were collected from both road shoulders along the Prefectural Route 93 within 8 km length (Fig. 1b) and examined the egg of *E. multilocularis*. In the present study, we used 59 fecal samples that had been collected and stored after the egg examination of *E. multilocularis* by the Hokkaido Institute of Public Health. The fecal samplings were conducted three times: late May, July–August, and early October of 2007. Because a fecal sampling was also conducted early October of 2006, the feces analyzed in the present study could be dropped in less than eight months. Based on morphological observation, fecal conditions were categorized arbitrarily as "Dried and whitened", "Dried", "Usual", "Fresher", and "Fresh and wet". In order to inactivate tape worm eggs and prevent echinococcosis infection, the feces were carefully put into polypropylene conical tubes in field, and then incubated at 70°C for three days at laboratory. Then, the fecal samples were preserved at 4°C until DNA extraction. Total DNA was extracted from 0.3 g in wet weight of each fecal sample by using the QIAamp DNA Stool Mini Kit (QIAGEN). As positive control, DNA extracted from the fox muscle tissues by using the DNA Tissue Kit (QIAGEN) was used. The total volume (200 μl) of the DNA solution was stored at 4°C.

PCR methods

In order to confirm whether the fecal samples are of the red fox, polymerase chain reaction (PCR) was performed with the primers specific to the red fox mitochondrial DNA (mtDNA) control region, using the method of Shimatani et al. (2008). Because mtDNA is more easily

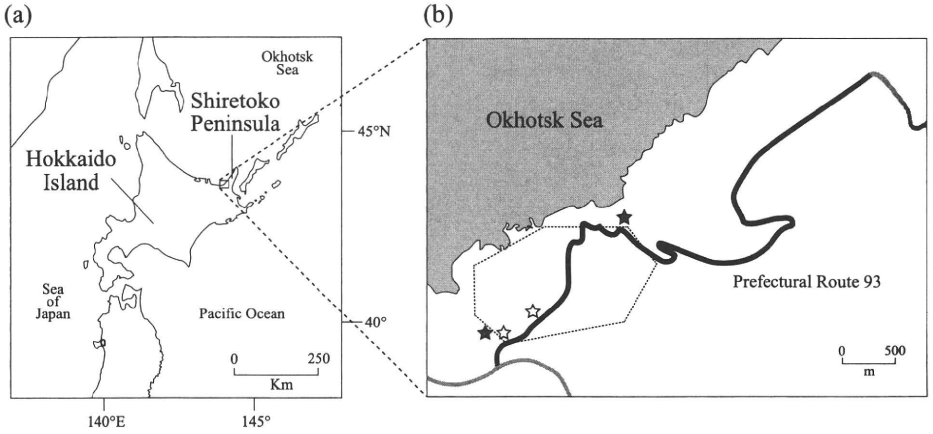

Fig. 1. (a) Location of the Shiretoko Peninsula on the Hokkaido Island. (b) The study area along Prefectural Route 93 in the Shiretoko Peninsula. The black line indicates the sampling area on the Route 93. The two open stars and two closed stars indicate the distribution of fecal samples which were consider to be dropped by the same foxes having the identical genotypes, respectively. Their genotypes are shown in Table 3. The areas closed by broken lines are the home ranges of one red fox revealed by Tsukada (1997a).

Table 1. Basic information of each microsatellite locus in the red fox

Locus	Allele size (bp)	Mean size (bp)	Number of alleles	Genotyping success rate (%)	Genotyping reproducibility (%)
DB1	133–152	138.1	6	51.2	55.8
DB3	125–129	126.7	3	51.2	53.5
DB4	111–135	116.5	5	51.2	55.8
DB6	106–112	108.4	3	37.2	67.4
V142	136–160	147.1	6	23.3	58.1
V374	100–113	106.3	4	46.5	72.1
V402	83–97	86.7	6	69.8	88.4
V468	84–94	89.1	4	65.1	74.4

PCR-amplified from fecal samples than nuclear DNA (Birky et al. 1989), for the micosatellite analysis, we used only the feces, in which mtDNA was successfully amplified. In order to avoid genotyping errors by a low quality of fecal DNA, PCR amplifications and microsatellite genotyping were carried out three times per locus per sample. Then, we chose allele bands that appeared two times or more for genotype. For each sample, 12 microsatellite loci were amplified using the following primers; DB1, DB3, DB4 and DB6 (Holmes et al. 1993); C213 (Ostrander et al. 1993); V142, V374, V402, V468, V502, V602 and V622 (Wandeler and Funk 2006). The PCR mixture of a total volume of 10.0 μl consisted of 1.0 μl of 10 × PCR buffer, 0.8 μl of dNTP mixture (2.5 mM), 0.3 μl of the primer above (5 pmol/μl), 0.1 μl of r*Taq* DNA polymerase (5 units/μl: TAKARA), 6.5 μl of distilled water and 1.0 μl of each sample extract.

The PCR amplification was started with denaturing 94°C for 3 min, then 30–40 cycles of amplification were performed with the following programs using a DNA thermal cycler (TAKARA TP600): denaturing 94°C for 1 min; annealing 52–60°C for 1 min; extension 72°C for 1 min, and reaction was completed at 72°C for 10 min. Before sizing microsatellite alleles, 2.5 μl of the Bromophenol blue loading solution (PROMEGA) were added to 2.5 μl of each PCR product, denatured at 95°C for 2 min, and chilled immediately on ice for 3 min. Then, we applied the products to an autosequencer HITACHI SQ-5500L, and determined molecular sizes of microsatellite alleles using the computer software FRAGLYS 3 (Hitachi). Because no clear results of four loci (C213, V502, V602 and V622) were obtained, these loci were excluded in the subsequent analysis.

Statistical analyses

Allele fragment size ranges, average allele sizes, the number of alleles, and genotyping success rates of each locus were counted. The genotyping reproducibility was calculated as rates of samples that had the finally decided genotypes same as the result in the first analysis of three genotypings. The fecal conditions were compared with success rates of microsatellite genotyping and mtDNA amplification rates.

The minimum number of individuals was estimated by comparing the genotypes. After excluding all the samples that were not identified as unique individuals, in order to evaluate whether the loci used were effective to identify individuals, the probability of a genotypic match ($P_{(ID)}$) was estimated by GIMLET version 1.3.2 (Valière 2002).

Results

In the present study, 43 of 59 samples were identified as fox feces by the mtDNA analysis. Basic information of each locus was shown in Table 1. All allele fragment sizes were less than 200 bp, and allele sizes of loci V402 and V468 were less than 100 bp. The number of alleles ranged from three to six through all the loci examined. The genotyping success rate was the lowest (23.3%) at V142, and the highest (69.8%) at V402. The genotyping reproducibility was the lowest (53.5%) at DB3, and the highest (88.4%) at V402. The genotyping success rate and reproducibility at each locus decreased when the average allele size increased (Figs. 2a, b).

There were no clear differences in genotyping success rates among condition levels of fecal samples (Table 2). In "Older feces" consisting of "Dried" and "Dried and whitened", the number of genotyped locus per sample was 4.21 and success rate of mtDNA analysis was 77.8%, whereas those values in Fresher feces consisting of "Fresh and wet", "Fresh" and "Usual" were 3.96 and 68.3%, respectively (Table 2).

The genotyping results of all fecal samples showed

Table 2. Conditions of feces in field and PCR success rates

Condition	Number of samples	Samples succeeded in mtDNA analysis	Genotyped microsatellite loci	Genotyped loci/ sample analysed	MtDNA amplification rate (%)
1. Fresh and wet	3	2	1	0.50	66.67
2. Fresher	2	1	6	6.00	50.00
3. Usual	36	25	104	4.16	69.44
4. Dried	7	6	27	4.50	85.71
5. Dried and whitened	11	8	32	4.00	72.73
Fresher (1 + 2 + 3)	41	28	111	3.96	68.29
Older (4 + 5)	18	14	59	4.21	77.78
Total	59	43	170	3.95	72.88

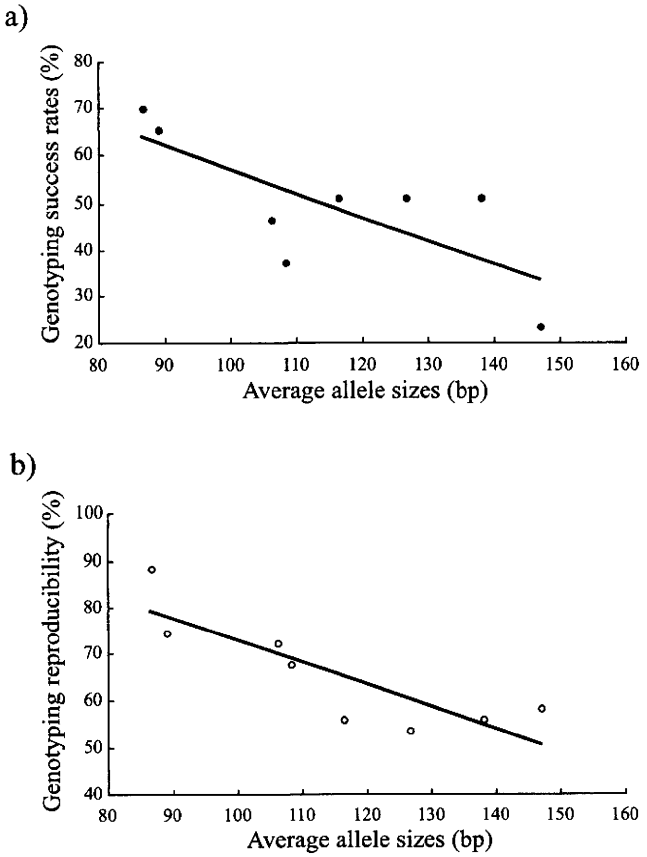

Fig. 2. (a) The relationships between genotyping success rates and average allele sizes, and (b) those between genotyping reproducibility and average allele sizes. The approximate lines were drown by Microsoft Excel X.

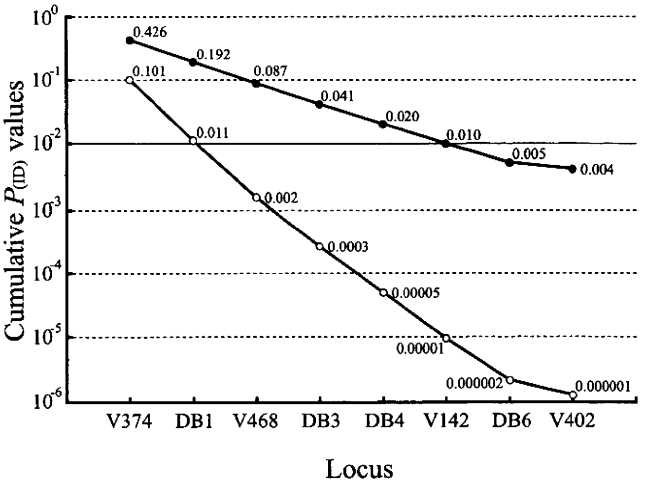

Fig. 3. Logarithmic plots of cumulative $P_{(ID)unbiased}$ (open circles) and $P_{(ID)sibs}$ values (closed circles).

that the minimum number of individuals was 22 (Table 3). There were two pairs of fecal samples, which were considered to have the identical genotypes ($P_{(ID)sibs} < 0.01$) (Table 3 and Fig. 1b). Cumulative $P_{(ID)sibs}$ in the 22 samples was less than 0.01 at the 7th locus (Fig. 3). In all possible combinations of seven in the eight loci, cumulative $P_{(ID)sibs}$ values were also estimated to be less than 0.01.

Discussion

The present study, using fecal samples collected for the purposes except genetic analysis, demonstrated that at least 22 individuals occurred in the sampling area of the Shretoko Peninsula in the sampling period (spring to autumn of 2007). Although there has been few report on the absolute number of individuals of the red fox in Japan, Nonaka et al. (1998) reported that 3–4 families have their distinct territories along the road in this study area. If one family consists of two adults (male and female) and an average of 3.5 cubs (Tsukada 1997b), a total of 16.5–22 individuals occur along the road. Actually, there might be additional individuals such as helpers (Macdonald 1979, Suzuki et al. 1983, Tsukada 1997c) and itinerants (Tsukada 1997c) in this area, and consequently the result of this analysis that a minimum of 22 individuals occurred in the sampling area is reasonable.

Table 3. Results of genetic identification of individuals

Sample code	Microsatellite loci								
	DB1	DB3	DB4	DB6	V142	V374	V402	V468	
S-07-29	AA	AA	CE	AA	DD	BC	BB	AC	
S-07-15	AA	AC	CE	AB	DD	BC	BB	AC	
S-07-23	AE	AA	CC	BB	BD	CC	BB	BC	☆
S-07-17	EF	BB	BB	AC	BD	BC	BB	BC	★
S-07-3	AB	AC	BB	BB	–	AD	BB	BD	
S-07-59	AF	AC	CC	AB	–	BC	BD	BC	
S-07-11	AF	AC	CE	AB	–	BB	BD	AB	
S-07-99	CC	AC	BC	AB	–	AB	BB	BB	
S-07-77	–	BB	BB	AC	BD	BC	BF	BC	
S-07-71	AA	–	–	AA	BE	BC	BB	BC	
S-07-60	AB	AC	BB	–	–	AA	BB	BB	
S-07-114	AE	CC	CC	AA	–	–	BB	BC	
S-07-85	AF	CC	CE	–	–	BB	BD	AB	
S-07-63	BD	AB	BB	–	–	AD	BB	BD	
S-07-16	–	BB	CC	AC	–	AA	BB	BC	
S-07-20	AA	AA	–	–	–	CC	BB	AC	
S-07-75	CC	–	CC	–	–	DD	BB	BC	
S-07-6	–	AA	BB	–	BF	–	BB	BD	
S-07-34	AA	BB	CC	–	–	–	–	CC	
S-07-36	–	BB	DD	–	–	–	BB	CC	
S-07-10	–	BB	–	–	–	BC	AB	BC	
S-07-89	–	–	–	BB	–	–	BB	DD	
S-07-25	–	–	–	–	–	–	BB	AC	
S-07-48	AA	–	–	–	–	–	–	–	
S-07-116	AA	–	–	AA	–	–	BB	–	
S-07-18	AE	AA	CC	BB	–	CC	BB	BC	☆
S-07-9	–	–	CC	–	–	–	BB	–	
S-07-32	EF	BB	BB	AC	BD	BC	BB	BC	★
S-07-5	–	–	–	–	CC	–	–	–	
S-07-113	–	–	–	–	–	BB	–	–	
S-07-38	–	–	–	–	–	–	BB	BB	
S-07-79	CC	–	–	–	–	–	–	–	
S-07-45	–	–	AA	–	–	–	–	–	
S-07-80	–	CC	–	–	–	–	–	–	
S-07-30	AA	–	–	–	–	–	CC	–	
S-07-4	–	–	–	–	–	–	EE	–	
S-07-42	–	–	–	–	AA	–	BB	CC	
S-07-69	–	–	–	–	–	–	–	DD	
S-07-22	–	–	–	–	–	–	–	–	
S-07-67	–	–	–	–	–	–	–	–	
S-07-68	–	–	–	–	–	–	–	–	
S-07-88	–	–	–	–	–	–	–	–	
S-07-112	–	–	–	–	–	–	–	–	

Each genotype was shown as a combination of alleles expressed as A–F. Samples were divided into two groups by the broken line. Because samples in the upper group have different genotypes at one or more loci from the others, they were considered as unique individuals. Samples in the lower group could not be identified as unique individuals. The two samples with the open stars and the different two with the closed stars are considered to have the same genotypes, respectively ($P_{(ID)sibs} < 0.01$).

The distribution of the feces that were considered to have been dropped from different individuals having the identical genotypes ($P_{(ID)sibs} < 0.01$) was almost overlapped with the home range of one fox revealed by Tsukada (1997a) (Fig. 1b). The $P_{(ID)}$ values of less than 0.01 is necessary for population size estimation (Mills et al. 2000), and such values in $P_{(ID)sibs}$ were earned without DB6 and V402 in the present study (Fig. 3). Moreover, in all possible combinations of seven out of the eight loci, $P_{(ID)sibs}$ values were estimated to be less than 0.01. Therefore, the combinations of the above six loci or all the combination of seven out of eight loci were considered effective to distinguish the fox individuals at the Shiretoko National Park. The present study showed two pairs of fox feces having $P_{(ID)sibs}$ values of less than 0.01 which are considered to be dropped by the same foxes (Table 3). Tsukada (1997a) reported that the average size of the fox's home range in this area was about 250 ha, constantly from May to October every year (Tsukada 1997a). Therefore, it is reasonable to consider that the distribution obtained by the fecal DNA analysis is not incongruent with the fox home range in Shiretoko. Increasing the number of samples would provide us more detailed structures of fox home ranges and population sizes in the Shiretoko National Park.

The present study did not always show that older feces resulted in lower success rates (Table 2). Although feces occurring in the field long time could have been exposed to sunlight and rain, and fecal DNA molecules could have been fragmented, the result of the present study indicates that older-looking feces sometimes included analyzable DNA as shown in Table 2.

On the other hand, the genotyping success rates of some loci in the present study were lower than those in other previous studies (e.g. Frantz et al. 2003; Piggott et al. 2006), indicating the difficulty of genetic analysis using fecal samples. The genotyping reproducibility values (53.5–88.4%, Table 1) show the unavoidable risks for genotyping errors. However, usage of better loci for identification would provide more reasonable investigations in lower cost and lower possibility of overestimate of population sizes by genotyping errors. Microsatellite loci having alleles in longer fragment sizes lead lower success rates and reproducibility of PCR amplification than the loci having shorter alleles (e.g. Hummel et al. 1999; Nielsen et al. 1999; Wandeler et al. 2003). Frantzen et al. (1998) also reported that short PCR fragments (100–200 bp) are significantly suitable to fecal identification analysis compared to longer fragments.

The present study, although some loci are in exceptions, also indicated that genotyping success rates and reproducibility likely correlated to average allele sizes (Table 1, Figs. 2a, b). Especially, because the higher genotyping reproducibility can bring us lower repeated times of genotyping, the improvement can decrease laboratory cost and time. It is reasonable to use loci that have smaller allele sizes to obtain more reliable results even if the fragment sizes were less than 100–200 bp.

Although the ideal methods were different from species to species, many transport and preservation methods were already reported for fecal non-invasive analysis. A simple drying transfer method by using silica beads was the best way in bear feces (Wasser et al. 1997). Preservation in DMSO/EDTA/Tris/salt solution was also effective in baboons (*Papio cynocephalus ursinus*) (Frantzen et al. 1998). The combination of GuSCN/Silica extraction method (Boom et al. 1990; Höss & Pääbo 1993) and the 70% ethanol-storage method brought high genotyping success rates in the Eurasian badger *Meles meles* (Frantz et al. 2003). Thus these methods may also improve the genotyping success rate. It could be more effective to develop the non-invasive method by improving the transportation and preservation methods and combination with the traditional field methods.

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Population Structures of the Red Fox (*Vulpes vulpes*) on the Hokkaido Island, Japan, Revealed by Microsatellite Analysis

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Abstract

In order to examine the population structures of the red fox (*Vulpes vulpes*) on the Hokkaido Island in Japan, we conducted analysis on 250 foxes from all over the island for 12 microsatellite loci. Assignment tests using the genotype data set showed that they were divided into 6 subpopulations. Of the 6, one was geographically isolated in the southern region and considered definitive subpopulation, whereas the other 5 were not. The slight differences among the latter 5 subpopulations were explained by the high adaptability and long dispersal of the red fox on the Hokkaido Island. Although there are few ecological data to explain the genetic differentiation of the southern population, we have proposed some hypotheses from the present ecological and geohistorical viewpoints. One convincing reason from the ecological viewpoint is the restriction of gene flow to southern Hokkaido from other areas due to geographical isolation resulting from the land shape. The other explanation is the geohistorical division of southern Hokkaido from other regions on the island during the last interglacial age, resulting in the isolation of the fox population.

Key words: *clustering, Hokkaido, microsatellite, population structure, red fox,* Vulpes vulpes

The fauna of the Japanese islands is geographically separated between the Hokkaido and Honshu Islands by the Tsugaru strait, which is a biogeographical demarcation called "Blakiston's line" (Blakiston and Pryer 1880, Figure 1). There are many endemic mammalian species of mammals in the main islands located south of Blakiston's line, and the endemic species reach about 40% of all Japanese mammalian species (Abe et al. 2005). On the other hand, the mammalian fauna on the Hokkaido Island, located north of Blakiston's line, exceptionally does not show such endemism of the other Japanese main islands (Abe et al. 2005), and the terrestrial fauna occurring in Hokkaido is rather similar to southern Siberian fauna (Fujimaki 1994). This is because that the Tsugaru strait (Blakiston's line) is considerable and separated Hokkaido from the other islands in the last interglacial age, whereas the Hokkaido Island was considered to be connected intermittently to the Eurasian continent via Sakhalin (Ohshima 1990).

Meanwhile, recent molecular genetic studies have revealed that the Hokkaido populations of mammals have particular and distinct features of the phylogeography and migration history. For example, the distribution of the 3 mitochondrial DNA (mtDNA) lineages of the brown bear (*Ursus arctos*) did not overlap, and each mtDNA lineage was considered to have immigrated separately to the Hokkaido Island (Matsuhashi et al. 1999). The genetic population structures of the sika deer (*Cervus nippon*) in Hokkaido reflected bottleneck effects, resulting from past heavy snows and hunting pressure (Nagata et al. 1998). The molecular phylogeography of other mammals on the Hokkaido Island, such as the least weasel (*Mustela nivalis*) (Kurose et al. 2005) and *Sorex* species (Ohdachi et al. 2001), also showed endemism according to the biogeographical history of the Hokkaido Island.

The red fox (*Vulpes vulpes*) is also widely distributed in Hokkaido, but its population structures have not yet been clarified. Inoue et al. (2007) examined the variations of the mtDNA cytochrome *b* gene and control region of the red fox on Hokkaido. Although they found some haplotype lineages in Hokkaido, their distributions were admixed throughout the entire island, and no clear genetic subdivisions were defined. This could have been caused by the high

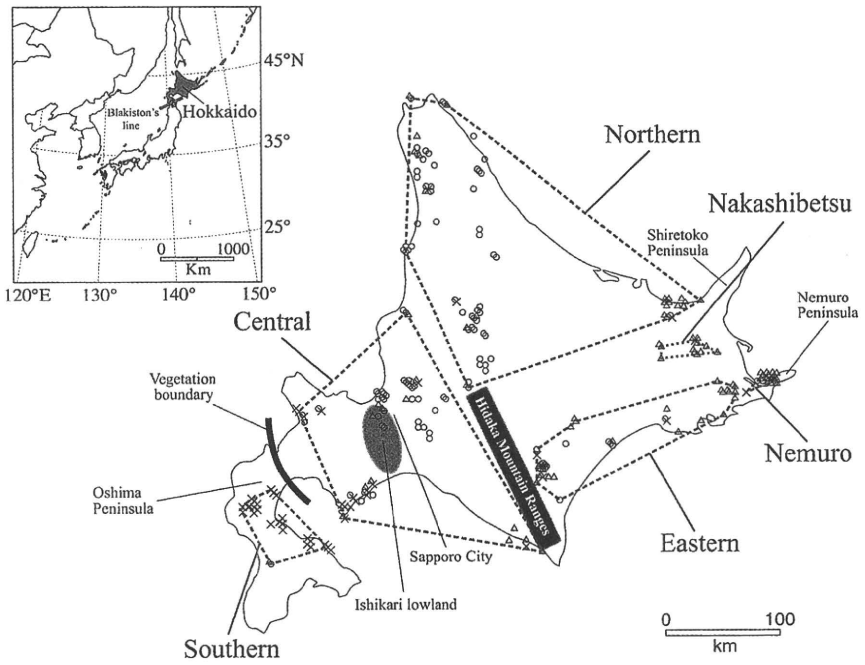

Figure 1. Locations of the red fox subpopulations on the Hokkaido Island, based on genetic clustering analyses of GENELAND and STRUCTURE at $K = 3$. Circles, triangles, and crosses show fox individuals, which were partitioned to segments of yellow, blue, and red, respectively, by the STRUCTURE results at $K = 3$ (see Figure 3). The individuals surrounding a polygon belong to the same subpopulation inferred by the GENELAND analysis. The vegetation boundary on the map was cited from Tatewaki (1958).

adaptability and dispersal ability of the red fox. This species is omnivorous (Yoneda 1981; Doncaster et al. 1990; Lucherini and Crèma 1994; Molsher et al. 2000), and its adaptability is as high as it is distributed widely in the northern hemisphere (Voigt and Macdonald 1984), including some urban areas (Teagle 1967). Behavioral studies revealed that the red fox can disperse over long distance (Storm et al. 1976; Hokkaido 1988).

However, elucidation of some unknown population structures on the island could also help to understand some ecological phenomena of the red fox in Hokkaido. For example, sarcoptic mange, which is a serious skin disease of red foxes caused by the mite (*Sarcoptes scabiei*) and spread by physical contact, has expanded from eastern to western Hokkaido but not to the Oshima Peninsula of southern Hokkaido (Takahashi and Uraguchi 2001). This may indicate a low level of migration (i.e., gene flow) between the southern and other populations of the red fox. Moreover, the Hokkaido Island has a complex geographic shape, such as narrow peninsulas and high mountain ranges, and climatic and vegetative variations between southern and other parts of the island (see Figure 1). These island conditions may also have been related to the formation of the fox population structures.

In addition, it is epidemiologically important to understand the fox population structures in Hokkaido to plan an effective program against zoonoses. This animal plays an important role as the host of a tapeworm (*Echinococcus multilocularis*) causing a serious zoonosis alveolar echinococcosis in Hokkaido (Oku and Kamiya 2003). In addition, although rabies has been exterminated in Japan, it remains possible that the red fox, which is the main rabies-infected animal in Europe, could spread the infection again in Hokkaido (Uraguchi 2008).

In order to further examine fox population structures in Hokkaido, we conducted biparentally inherited microsatellite analysis on 250 foxes collected throughout Hokkaido and then discuss gene flow among subpopulations and the process of population structuring of the red fox on the island.

Materials and Methods

Sample Collection

We examined muscle tissues of 250 foxes collected widely from Hokkaido for epidemiological survey on *E. multilocularis* infection conducted by the Hokkaido government. Most of the foxes were hunted in 2006, except for Nemuro and Nakashibetsu in 2003. Samples from Sapporo were obtained from road kills in 2001. The tissues were treated at 70 °C for 3 days for inactivation of parasites and then preserved in 99% ethanol at 4 °C.

DNA Extraction, PCR Amplification, and Data Analysis

Total DNA was extracted by a DNeasy Tissue Kit (QIAGEN) and stored in TE buffer at 4 °C. We amplified

Table 1 Basic information on the Hokkaido red fox subpopulations inferred by the GENELAND analysis

Subpopulation	Sample size (n)	Heterozygosities		Mean no. of alleles per locus (A)
		Observed (H_O)	Expected (H_E)	
Southern	28	0.59	0.61	4.78
Central	70	0.63	0.67	7.11
Northern	78	0.67	0.68	8.22
Eastern	41	0.67	0.67	6.33
Nakashibetsu	13	0.61	0.63	5.22
Nemuro	20	0.65	0.63	5.56

12 microsatellite loci for each sample, with the following primers of polymerase chain reaction (PCR): DB1, DB3, DB4, and DB6 (Holmes et al. 1993); C213 (Ostrander et al. 1993); V142, V374, V402, V468, V502, V602, and V622 (Wandeler and Funk 2006). The PCR amplification was carried out in 10 μl of the reaction mixture containing 1× PCR buffer, dNTP mixture (0.2 mM), 0.3 μl of each primer (0.15 pmol/μl), r*Taq* DNA polymerase (0.05 units/μl, TAKARA), and 1.0 μl of each DNA extract. The following PCR programs were performed using a DNA thermal cycler, TAKARA TP600: one cycle of denaturing at 94 °C for 3 min; 30–40 cycles of denaturing at 94 °C for 1 min, annealing at 52–60 °C for 1 min, extension at 72 °C for 1 min; and the reaction completion at 72 °C for 10 min.

Before sizing microsatellite alleles, 2.5 μl of the bromophenol blue loading solution (PROMEGA) were added to 2.5 μl of each PCR product, denatured at 95 °C for 2 min, and cooled down immediately on ice for 3 min. Then, we applied the products to an autosequencer HITACHI SQ-5500L and determined the molecular sizes of microsatellite alleles using computer software FRAGLYS 3 (HITACHI). Because no clear PCR results of 3 loci (C213, V374, and V502) were obtained, these loci were excluded from subsequent analysis.

Statistical Analysis

In order to infer the geographic boundaries and the number of subpopulations, we used GENELAND 3.1.4. program (Falush et al. 2003; Guillot, Mortier, and Estoup 2005). This was programmed on the basis of a Bayesian clustering model, assigning individuals to subpopulations from its genetic information (Guillot, Estoup, et al. 2005). GENELAND does not incorporate only genetic information but also spatial coordinates of sampling points at an earlier stage of simulation, making it possible to optimize the delineation of subpopulations (Guillot, Estoup, et al. 2005). We varied the number of subpopulations (K) from 1 to 12 in order to infer the possible number of K, and in this step, we set the Marcov Chain Monte Carlo (MCMC) parameters as 50 000 iterations and 50 thinning. Correlated and null allele model options were activated, and we used the default setting for other parameters and estimated K of our data set from the highest average posterior probability. The assignment of individuals to subpopulations was performed in a separate run, as suggested by Guillot, Estoup, et al. (2005). For these runs, K was set to the inferred number of subpopulations, the MCMC parameters were set to 100 000 iteration and 100 thinning, and other parameters were the same as the first step. Ten runs with fixed K were performed. For one of the 10 runs with the highest average posterior probability, the posterior probability of subpopulation membership was computed for each pixel of the spatial domain (400 × 400 pixels).

For each of the inferred subpopulations based on GENELAND results, deviations from Hardy–Weinberg and linkage equilibrium were examined by GENEPOP 3.4 (Raymond and Rousset 1995) (dememorization number = 1000; batch number = 100; number of iterations per batch = 10 000). The observed (H_O) and expected (H_E) heterozygosities were estimated by ARLEQUIN 3.1.1 (Excoffier et al. 2005). In addition, pairwise F_{ST} (Weir and Cockerham 1984) was estimated by ARLEQUIN, and we tested the significance of the observed F_{ST} values by 10 000 permutations of individuals among populations. We also estimated Nei's standard genetic distance (D_S; Nei 1978) among subpopulations by SPAGeDi 1.2 (Hardy and Vekemans 2002). Using the pairwise F_{ST} and D_S values, we constructed phylogenetic trees by the neighbor joining method (Saitou and Nei 1987) in MEGA 4 (Tamura et al. 2007).

After dividing populations into subpopulations by GENELAND, we also attempted to infer subpopulations and geographic boundaries by STRUCTURE 2.3.1 program (Pritchard et al. 2000; Falush et al. 2003). The STRUCTURE is also formed by the Bayesian clustering technique, similar to GENELAND, but spatial coordinates are not considered in its simulation process and used only as a method of visualizing the subpopulation membership. In STRUCTURE, it is sometimes difficult to estimate clear geographical boundaries among subpopulations if the subpopulations have

Table 2 F_{ST} (lower matrix) and D_S (upper matrix) among inferred red fox subpopulations

Subpopulation	Southern	Central	Northern	Eastern	Nakasibetsu	Nemuro
Southern		0.22	0.19	0.21	0.34	0.39
Central	0.10		0.04	0.05	0.18	0.17
Northern	0.09	0.02		0.03	0.11	0.16
Eastern	0.10	0.03	0.02		0.10	0.08
Nakashibetsu	0.14	0.07	0.04	0.04		0.08
Nemuro	0.17	0.08	0.07	0.04	0.03	

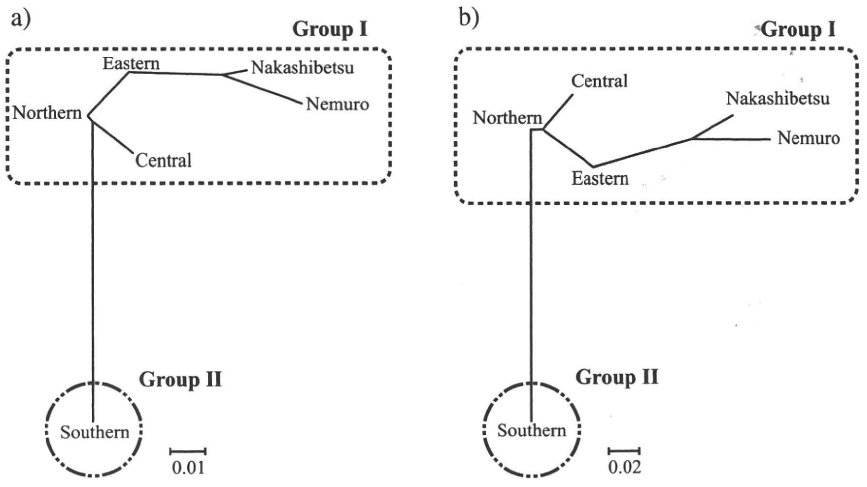

Figure 2. Neighbor joining relationships among the 6 red fox subpopulations, constructed by values of pairwise F_{ST} (**a**) and D_S (**b**).

shallow genetic structures. This clustering is, however, purely based on genetic information. In the present study, STRUCTURE was employed to examine the characteristics of the boundaries found by GENELAND. We then performed 5 runs at each value of the fixed parameter K (1–10: number of subpopulations). Each run consisted of 10 000 replicates of the MCMC after a burn-in of 10 000 replicates to examine the most likely number of subpopulations in the present data set by the highest average likelihood. The admixture model and correlated allele frequencies model were used because this combination is thought to provide the highest resolution in the case of a shallow population structure (Falush et al. 2003). We input the subpopulation information obtained from GENELAND analysis for later comparison. All other parameters were set to default values. From the highest average likelihood value, $K = 3$ was estimated as the most likely number of K, but if it would be difficult to detect clear population structure at $K = 3$, we performed additive runs (30 000 burn-in and 100 000 replicates) not only at $K = 3$ but from $K = 2$ to 6 to examine certain boundaries by the same method as Rosenberg et al. (2002).

In order to estimate the proportion of genetic variance explained by the subpopulations inferred by GENELAND and to assess the strength of each GENELAND boundary, we also conducted analysis of molecular variance (AMOVA) by ARLEQUIN. The AMOVA analysis examined the hierarchical proportion of total genetic variance in "among groups," "among populations within groups," "among individuals within populations," and "within individuals." We input the 6 subpopulations by GENELAND into "populations" and divided the subpopulations into 2 geographically connected "groups." Then, we considered the strength of boundaries by comparing the variance proportion values of "among groups."

In addition, we estimated resistance distances among subpopulations by CIRCUITSCAPE 3.5.1 based on circuit and random walk theory (McRae 2006). The resistance distances were then compared with F_{ST} values and geographic distances in order to consider the effects of the island shape on gene flow. We used a simple binary land/sea map created by DIVA GIS (LizardTech 2005) because of the limited information on the red fox habitat in Hokkaido. The resolution of the grid map of Hokkaido was 730 × 500. Each subpopulation was condensed into one node and located on the map, according to the average x, y coordinate of all samples belonging to each subpopulation. We chose the 8-neighbor connection option because it seemed to express the more natural dispersal of animals, and connections were calculated using the average resistance option. Geographic distances were also calculated from the average subpopulation locations described above using Excel X (Microsoft 2002). The resistance, geographic, and genetic distances were compared by the Mantel test and partial Mantel test on FSTAT 2.9.3.2 (Goudet 2001). P values were calculated after 10 000 randomizations.

Results

The GENELAND analysis indicated that the most likely value of K was 7 as the number of subpopulations in the total data set; however, there were only 6 subpopulations to which individuals were assigned. One of the inferred subpopulations was considered "ghost" subpopulation with no individuals assigned. This phenomenon has been previously reported particularly when subpopulations are slightly differentiated, and it is recommended that such subpopulations should be ignored (Guillot, Estoup, et al. 2005; Guillot, Mortier, and Estoup 2005). Therefore, we ignored the ghost subpopulation and named the other 6 subpopulations as follows: Southern ($n = 28$), Central ($n = 70$), Northern ($n = 78$), Eastern ($n = 41$), Nakashibetsu ($n = 13$), and Nemuro ($n = 20$) for sampling locations (Figure 1).

Although, in most subpopulations, no deviations from Hardy–Weingberg equilibrium were detected ($P > 0.05$), only the Central subpopulation ($P \leq 0.05$) had an H_O value

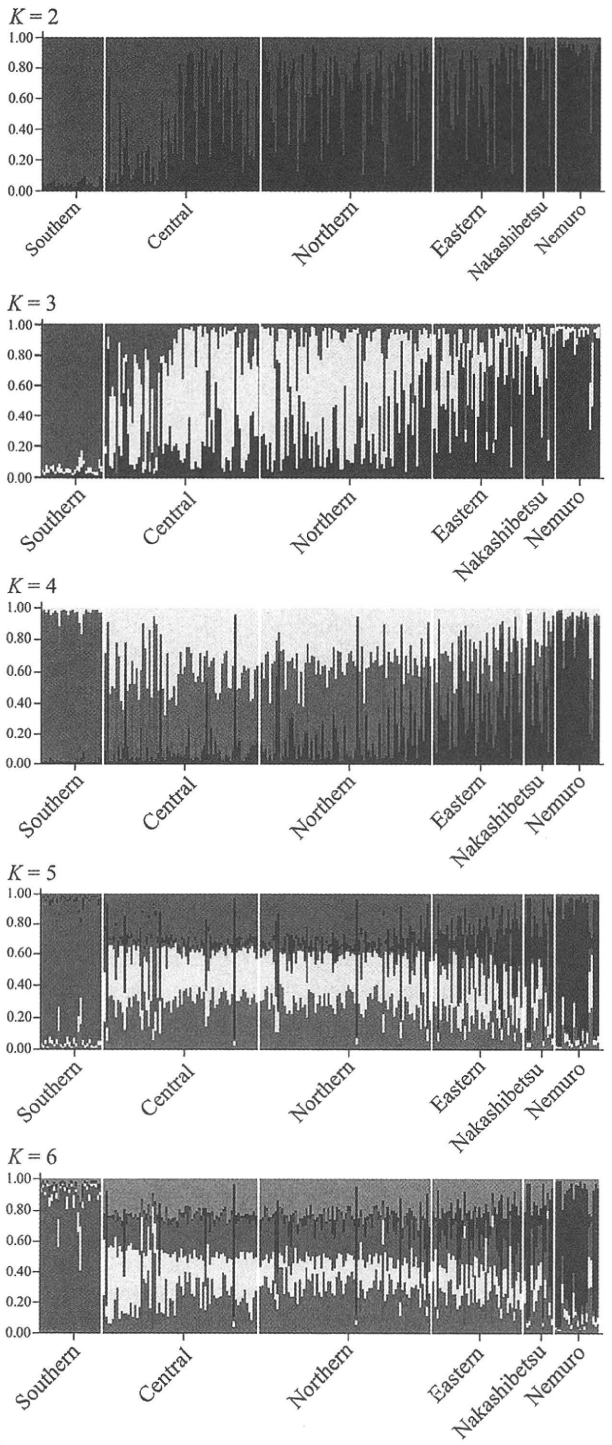

Figure 3. Assignments of individuals due to the STRUCTURE analysis on microsatellite allele data. One thin vertical line for each individual is partitioned into K colored segments that represent the individual's estimated assignment probabilities to each cluster. Six subpopulations were inferred as described below. (This figure appears in color in the online version of *Journal of Heredity*.)

lower than H_E (Table 1). This indicates that random mating has not occurred in the Central subpopulation and that there would be additional unknown subdivisions that could not be identified. Although the GENELAND analysis did not define the entire subdivision of the Central subpopulation, we treated the Central subpopulation in the manners same as the other subpopulations in subsequent analyses. No linkage disequilibrium in the subpopulations was found by the ARLEQUIN analysis.

Pairwise F_{ST} values between the Southern and other subpopulations (0.09–0.17) were higher than other F_{ST} values in all pairs of subpopulations (0.02–0.08 with $P < 0.01$) (Table 2). The D_S values between the Southern and other subpopulations (0.19–0.39) were higher than those of other combinations of subpopulations (0.03–0.18) (Table 2). The phylogenetic trees constructed using F_{ST} and D_S values (Figure 2) showed that the Southern subpopulation was genetically most differentiated from the other subpopulations.

The STRUCTURE analysis showed the existence of explicit boundaries between the Southern and other subpopulations at K = 3, 4, 5, and 6 as summarized in Figure 3. The results indicated that the most likely number of subpopulations was 3 with the highest average likelihood value. At K = 3, the geographical boundaries among the 3 clusters were unclear. With higher K numbers (4, 5, and 6) than 3, most individuals were not assigned to any subpopulations with more than 0.70 posterior probability of the population membership, indicating no clear population structures among them. At any number of K, however, the Southern subpopulation was always recognized as a distinctive group.

The result of the AMOVA analysis was similar to that of the STRUCTURE analysis. In all analyses, the proportions of variance in "individuals" reached more than 90% (Table 3), indicating that population structuring was weak throughout the Hokkaido Island. However, the highest percentage of variance among groups (6.9%, Table 3) was obtained when the whole population was classified into 2 groups: the Southern subpopulation versus the others. By contrast, the percentages of variance of "among groups" were less than 3.5% in the other combinations of subpopulations. This indicates that the boundary between Southern and Central subpopulation was clearer than the other boundaries.

Based on all results of genetic analyses, it was concluded that the GENELAND analysis divided the genotype data set into 6 subpopulations. Of the 6 subpopulations, the Southern subpopulation was genetically most differentiated.

The Mantel and partial Mantel test revealed that both the resistance (R) and geographic distances (S) significantly correlated to the genetic distances (G) ($P < 0.05$) (Figure 4). In both tests, the correlation between genetic and geographic distances were stronger than that between genetic and resistance distances (Mantel test: $R^2 = 0.30$ between G and R, $R^2 = 0.41$ between G and S) (partial Mantel test: $r_{RG|S} = 0.55$, $r_{SG|R} = 0.61$; Figure 4). Because the resistance distances were higher when either or both subpopulations were in peninsulas (Table 4), the land shape of the Oshima and Nemura Peninsulas could be related to the gene flow prevention.

Table 3 Differences of variance proportion depending on possible red fox subpopulation groupings based on AMOVA analysis

Grouping	Proportions of variance			
	Within individuals	Among individuals within populations	Among populations within groups	Among groups
Southern/others	90.4	−0.6	3.3	6.9
Northern/others	96.8	−0.6	7.4	−3.5
Eastern/others	97.2	−0.6	6.5	−3.1
Nakashibetsu/others	94.7	−0.6	5.2	0.8
Nemuro/others	92.7	−0.6	4.5	3.4
Southern, Central/others	95.5	−0.6	5.6	−0.4
Northern, Nakashibetsu/others	96.2	−0.6	6.8	−2.5
Eastern, Nemuro/others	95.2	−0.6	5.2	0.2
Nakashibetsu, Nemuro/others	93.0	−0.6	4.2	3.5
Southern, Central, Northern/others	94.8	−0.6	4.7	1.1
Southern, Central, Eastern/others	95.8	−0.6	6.3	−1.5

Discussion

Weak Structuring of the Red Fox Populations Due to Gene Flow on the Hokkaido Island

The present study revealed that the population structure of the red fox is not strongly differentiated throughout most parts of the Hokkaido Island, except for the Southern subpopulation. This is not inconsistent with the previous genetic study (Inoue et al. 2007) reporting no visible genetic structuring within Hokkaido, based on the distribution patterns of maternally inherited mtDNA haplotypes. Some red foxes on Hokkaido were reported to have dispersed more than 30 km (Hokkaido 1988; Uraguchi 2008). Such a shallow population structure could be explained by the high adaptability and far dispersal habit of this species. For example, although the Hidaka mountain range (Figure 1), which is the highest (more than 2000 m) in Hokkaido, could be a geographic barrier between the Central and Eastern subpopulations, the lower values of both F_{ST} (0.03) and D_S (0.05) between them in the present study indicate that geographic isolation by the mountain range is not so significant, leading to gene flow.

Genetic Differentiations of the Southern Subpopulation

The genetic differentiations between the Southern and other subpopulations were relatively stronger. Such population differentiation in southern Hokkaido has been reported in other species. For example, Matsuhashi et al. (1999) reported that the brown bear population in Hokkaido is clearly divided by the Ishikari lowland (Figure 1) based on mtDNA data and that the subpopulation in southern Hokkaido is genetically differentiated from other subpopulations. We here propose 2 hypotheses for the differentiation of the Southern population of the red fox from geohistorical and present ecological viewpoints.

From the geohistorical viewpoint, the geographical isolation between the Southern and other regions within Hokkaido in the past might have caused the genetic division. In the last interglacial age (130 000—60 000 years ago), the Ishikari lowland (Figure 1) was reported to have been under the sea, and the Oshima Peninsula was isolated from other areas of the Hokkaido Island (Japan Association for Quarternary Research 1987). Red foxes in Hokkaido might have been divided into 2 or more populations and been genetically differentiated during the last interglacial age.

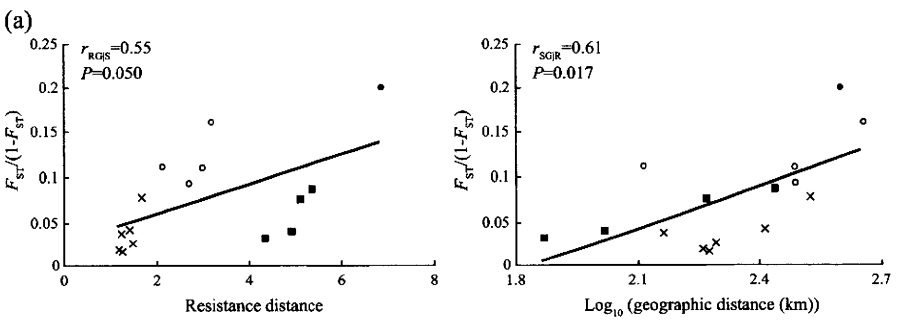

Figure 4. Plots of genetic, geographic, and resistance distances and the results of the partial Mantel test on all subpopulations. Partial correrations and *P* values are shown, where *G*, *S*, *R* indicate genetic, geographic, and resistance distances, respectively. Closed circle, plots between Southern and Nemuro subpopulations; open circles, those between Southern and other subpopulations; closed squares, those between Nemuro and other subpopulations; and crosses, those between inland subpopulations.

Table 4 Resistance (lower matrix) and geographic distances (km) (upper matrix) among red fox subpopulations

Subpopulation	Southern	Central	Northern	Eastern	Nakasibetsu	Nemuro
Southern		130	308	307	452	397
Central	2.12		183	196	336	274
Northern	2.70	1.21		189	260	186
Eastern	2.99	1.50	1.28		146	104
Nakashibetsu	3.18	1.69	1.43	1.26		74
Nemuro	6.86	5.37	5.11	4.92	4.34	

On the other hand, as an explanation for the subdivision from the present ecological viewpoint, we considered 2 possibilities. The first is that the genetic differentiation of the Southern population may have caused simply by the geographic shape of the distribution areas. The Oshima Peninsula, which covers a wide range of the Southern subpopulation (Figure 1), is the biggest peninsula in Hokkaido, and the narrow and complicated landform could have play the role of a dead end in gene flow. The resistance distance values were higher within a subpopulation in a peninsula (Table 4). The result suggests the idea of gene flow prevention due to the landform; however, resistance distances related to the Southern subpopulations were much lower than those related to the Nemuro subpopulation, although the genetic distances were opposite. This could be because of overestimation of the resistance distances of the Nemuro subpopulation due to the closed entrance to the Nemuro Peninsula. Because the brackish lake Onneto, which is located at the bottom of the peninsula, is part of the sea, we treated it as nonland data in the geographic input file for CIRCUITSCAPE; however, because it is dry at low tide and frozen in winter, the gene flow of foxes could occur more easily between the Nemuro and other inland subpopulations. Because it has not yet been considered that the genetic differentiation of the Southern subpopulation could be due to only resistance distance, we should discuss other possibilities to explain of the differentiation, as follows.

The other possibility from the present ecological viewpoint is that genetic differentiation might have resulted from natal-specific dispersal. Field studies of some vertebrates, such as the prairie deer mouse (*Peromyscus maniculatus*) (Wecker 1963), Toensend's ground squirrel (*Spermophilus townsendii*) (Olson and Van Horne 1998), and cuckoo (*Cuculus canorus*) (Vogl et al. 2002) indicated that individuals tend to disperse preferentially to habitats similar to their natal home ranges. Sacks et al. (2004) also reported that the population structure of the California coyote (*Canis latrans*) was a result of the intraspecific variability in habitat affinities. In Hokkaido, there are some differences in vegetation between the Oshima Peninsula including the Southern area and the other parts. The forests of Hokkaido, except for southern areas, are biogeographically regarded as the transition between temperate and subarctic zones and generally resemble those of the neighboring northern Asian mainland more than those of the Honshu Island in Japan (Tatewaki 1958; Kondo 1993). On the other hand, however, the vegetation in the Oshima Peninsula is relatively similar to that of the Honshu Island, covered by temperate and cool-temperate deciduous forests, and their border (Tatewaki 1958) almost corresponds with the boundary between Southern and Central subpopulations (Figure 1). Although there are no available reports on the differences in the red fox food habitat or behavior between the southern region and other parts of Hokkaido, there may be some behavioral differences related to these habitats. Natal habitat-biased dispersal could have formed a distinct boundary between the Southern and other subpopulations, as identified in the present study.

There is evidence supporting the present ecological hypothesis above. Recently, sarcoptic mange, which is a serious skin disease of red foxes caused by the mite *S. scabiei*, broke out on the Hokkaido Island. Fox mange was first found in the Shiretoko Peninsula in 1994 then appeared in the Nemuro Peninsula in 1998 (Figure 1). The 2 peninsulas are located on the eastern edges of Hokkaido. After finding mange in these areas, a survey was conducted throughout Hokkaido in 1999. Of the 458 foxes examined, 76 were infected with the mite. Although fox mange was found in 11 of the 14 administrative districts into which Hokkaido is divided, no infected foxes were found in the 3 districts of southern Hokkaido (Takahashi and Uraguchi 2001). This disease is spread by physical contact, such as grooming, mating, and fighting between foxes; therefore, the distribution of infected foxes suggests a low level of fox migration and subsequent gene flow between the Southern subpopulation and the others.

This is the first report examining the red fox population by microsatellite analysis using samples systematically collected on one island. It is interesting to know that the Southern subpopulation of Hokkaido has differentiated from the other subpopulations in spite of their high adaptability and wide dispersal. If the present fox ecological characteristics are responsible for the genetic differentiations, the results of the present study also could give a new insight into the issues of controlling alveolar echinococcosis and preventing of rabies reintroduction to Hokkaido. In order to clarify the reasons why the Southern subpopulation was genetically differentiated, further studies are needed not only from genetic analyses but also from ecological approaches.

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