Morphological and population genetical studies on the red fox (*Vulpes vulpes*) of Hokkaido Island

（キタキツネ集団の形態学的および集団遺伝学的研究）

PhD Dissertation

By

Yosuke Amaike

Department of Natural History Sciences

Graduate School of Science

Hokkaido University

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Abstract

In Chapter I, diversity of the red fox on Hokkaido Island was evaluated in morphological aspect. To investigate the morphological variation in an island population of the red fox (*Vulpes vulpes*), 25 cranial and 24 dental characters were measured for 225 specimens (137 males and 88 females) from Hokkaido Island, Japan. A Bayesian principal component analysis found only small differences among three groups identified genetically in previous studies. Concretely, analyses of variance and post-hoc tests detected clear differences in two functionally important measurements: postorbital constriction and upper fourth premolar length. In the postorbital constriction, relating to size of the temporal muscle, a difference with a gradient of increase from west to east on Hokkaido Island was found in both sexes. In the upper fourth premolar length, relating to body size, a difference between the Southern and Central groups was found in both sexes. Additionally, in differences in four measurements of skull width, skull height, mastoid width, and occipital condyle width, relating to braincase, were detected in only male. Subsequently, a correlation analysis showed that the postorbital constriction might be related to climatic conditions (e.g., minimum mean monthly temperature and snowfall). Endemic climate conditions and food habits appeared to contribute to the observed geographical variation in skull morphology.

In Chapter II, population genetic diversity of the red fox on Hokkaido Island was

evaluated using the genotypes of the major histocompatibility complex (MHC) gene as adaptive marker. To assess the genetic diversity of the population of the red foxes living on Hokkaido Island genotypes of the MHC class II *DRB* gene were examined for 232 individuals. As a result, 17 novel alleles of *DRB* exon 2 were identified from the foxes. In addition, it was found that the fox population of southern Hokkaido was genetically differentiated from those of central and eastern Hokkaido, possibly resulting from ecological and geographical isolation in southern Hokkaido. In addition, non-synonymous substitution rates exceeded synonymous substitution rates in antigen binding sites (ABS) of *DRB* exon 2, indicating that the red fox *DRB* alleles have evolved under positive selection. The Bayesian phylogenetic analysis with alleles of other canids showed that most *DRB* alleles in the red fox were grouped into “fox-like canids clade”, and that trans-species polymorphism was evident. This suggests that these alleles have evolved under balancing selection.

In Chapter III, genetic structure and population dynamics were investigated for the fox population isolated on Mt. Hakodate. Microsatellite genotypes of fecal samples, which were noninvasively collected from Mt. Hakodate for three years (2009–2011), were analyzed. Based on successfully obtained genotypes, 35 foxes were identified as 16 males, 13 females and 6 unknowns. The estimated population sizes were 20 in 2009,

25 in 2010, and 44 in 2011. Fox density in each year was found to be larger than that in rural areas previously reported, and those in the urban fox of Zurich, Switzerland, which was internationally famous for high density. The comparison of genetic distance and structure showed that the Mt. Hakodate population was differentiated from the other Hokkaido populations and strictly isolated by the sea and rural area as geographical barrier. Even small home ranges were overlapped between foxes. The results suggest that the fox population on this mountain has been maintained by the food diversity and abundance of anthropogenic food resource.

General Introduction

The red fox, *Vulpes vulpes* (Carnivora: Canidae), is one of the most widespread canids in the world, across the entire northern hemisphere from the Arctic Circle to North Africa, Central America, and Asia, and adapted to diverse environments such as tundra, desert, forest, step and urban area (Macdonald & Reynolds, 2008). They are classified to 45 subspecies in the world (Larivière & Pasitschniak-Arts, 1996; Aristov & Baryshnikov, 2001). Of them, two subspecies occur on the Japanese islands: *V. v. schrencki* on Hokkaido Island (lies in the northernmost) and *V. v. japonica* on Honshu, Shikoku and Kyushu Islands, and are geographically separated by Tsugaru Strait between Hokkaido and Honshu Islands, a biogeographical boundary known as Blakiston's Line (Blakiston & Pryer, 1880; Uraguchi, 2009). There are some reports on morphological and genetical variations between the two subspecies. Oishi *et al.* (2010a) investigated the skull morphology of the red fox throughout the Japanese islands, and reported that *V. v. schrencki* is smaller than *V. v. japonica*, which is an exception to Bergmann's rule. Inoue *et al.* (2007) examined mitochondrial DNA (mtDNA) in the red fox on the Japanese islands, and revealed that the haplotypes from the two subspecies were distinct from each other.

Variations were found not only between subspecies but also within subspecies. Inoue *et al.* (2007) also reported three maternally-inherited mtDNA groups in the *V. v.*

schrencki population on Hokkaido Island. This phenomenon is similar to the report on occurrence of three mtDNA of the brown bear (*Ursus arctos*) (Matsuhashi *et al.*, 1999). However, the distributions of mtDNA haplotypes in the red fox were not clearly separated on Hokkaido Island, in contrast to the brown bears reported in Matsuhashi *et al.* (1999). On the basis of microsatellite data, Oishi *et al.* (2011) reported that there are not large genetic differentiations among the red fox populations in most parts of Hokkaido Island, whereas the population of southern Hokkaido has been well differentiated from the others. This contrast might be attributed to the difference in genetic features between maternally inherited genes and biparentally inherited genes. Kutschera *et al.* (2013) analyzed the mtDNA phylogeography of the red fox in the entire Holarctic region including Hokkaido Island, and reported that red foxes colonized Hokkaido at least three times. These studies contributed to discussing phylogeography of the red fox on Hokkaido Island. However, it is still not enough for discussion on adaptive evolution because the data in the previous studies are based on only two neutral genetic markers: mtDNA and microsatellites. To further understand evolutionary background of the red fox on Hokkaido, it is necessary to study adaptive characteristics on morphology and functional genes.

To date, there is little comparable data on morphological variation in the red fox

population on Hokkaido. Oishi *et al.* (2010a) investigated interspecific variation of the red fox on the Japanese Islands, but did not analyze intrapopulation variation of the red fox within Hokkaido Island. In the brown bear, the geographical variation in accordance with Bergmann's rule was reported (Yoneda & Abe, 1976; Ohdachi *et al.*, 1992). So, it is interesting to investigate whether any geographical variation associated with adaptation occurs also within the Hokkaido population of the red fox. It is important to discuss the association among phylogenetic relationships, morphological features and environments.

To study genetic diversity of the functional genes, major histocompatibility complex (MHC) genes are one of appropriate genetic markers. In contrary to neutral markers such as mtDNA and microsatellites, the variability of MHC genes reflects evolutionarily relevant and adaptive processes within and between populations, and is very suitable to investigate various issues in evolutionary ecology and conservation (Sommer, 2005). Introduction of the MHC markers could produce a new discussion on phylogenetic history of the red fox on Hokkaido. In addition, except for one example of the Newfoundland red fox (*V. v. deletrix*) (Marshall *et al.*, 2016), no other molecular phylogenetic studies of the red fox based on MHC genes have been reported.

The relationship between genetic diversity and ecological features of red foxes

inhabiting a restricted area is focused in these days. One of the Hokkaido populations is distributed on Mt. Hakodate, southern Hokkaido, which is surrounded by the sea on three sides and the urban area on one side. The red fox population is supposed to have been maintained on such a small area. Genetic analyses of non-invasive samples such as feces can be applied to studying ecological characteristics of the isolated population.

On the basis of the above backgrounds, morphological and genetical variations of the red fox population on Hokkaido Island were investigated in the present study. In Chapter I of this dissertation, measurement of skull morphology of the red fox was done, and intrapopulation variations related to environment factors is discussed from the perspective of morphology. In Chapter II, polymorphisms of the MHC gene as a functional gene was examined, and whether the Hokkaido population has adaptively evolved thorough natural selection is discussed. In Chapter III, the molecular ecological feature of the red fox on Mt. Hakodate was studied using non-invasive fecal samples.

Chapter I

Geographical variation on skull morphology in the Hokkaido population of the red fox

Introduction

Imaizumi (1960) found that *V. v. schrencki* on Hokkaido has larger body size than *V. v. japonica* on Honshu, although only a few samples were examined. Oishi *et al.* (2010a) investigated the skull morphology of the red fox throughout the Japanese islands and reported that *V. v. schrencki* is smaller than *V. v. japonica*, which is an exception to Bergmann's rule. However for another canid species in Japan, the raccoon dog (*Nyctereutes procyonoides*), Haba *et al.* (2008) reported that skull measurements were larger for *N. p. albus* on Hokkaido than for *N. p. viverrinus* on Honshu, Shikoku and Kyushu, in accordance with Bergmann's rule. Inoue *et al.* (2007) detected three mitochondrial DNA (mtDNA) groups in the *V. v. schrencki* population on Hokkaido. In addition, Oishi *et al.* (2011) found from microsatellite data that the group in southern Hokkaido is genetically well differentiated from other groups on the island. These results parallel mtDNA studies of the brown bear (*Ursus arctos*); Matsuhashi *et al.* (1999) detected three lineages, distributed in central, eastern, and southwestern Hokkaido. Skull measurements from these populations increase from southwestern to northeastern Hokkaido, in agreement with Bergmann's rule (Yoneda & Abe, 1976; Ohdachi *et al.*, 1992). To date, however, there is a paucity of comparable data on morphological variation in the red fox population on Hokkaido. The goal of the present

study was to examine morphological variation in geographically distinct groups identified genetically on Hokkaido, to assess geographical variation and its possible association with either phylogeographic or environmental variables.

Material and Methods

SAMPLES

A total of 225 skull specimens (137 males and 88 females) of the red fox collected throughout Hokkaido (Fig. I-1) was measured. The skull specimens came mainly from animals shot by hunters, but partly from road kills, in 2005–2006. Fox ages were determined by examining the cementum layers in the canine teeth (Sasakawa *et al.*, 1980). The approximate age in months for individuals less than one-year old was estimated from collection dates. Skull specimens were classified into the three age stages defined by Sasakawa (1984): adults (over one-year old), subadults (7–11-months old), and juveniles (less than seven months old). According to Sasakawa (1984), fox growth is nearly complete at seven-months after birth; therefore, only adults and subadults were used in the analysis to minimize variation due to age differences. The sample collection included 10 specimens of uncertain age identified as either adults or

subadults based on cranial size and structure, dentition, and the collection date.

MEASUREMENTS

Referring to measurements made in previous studies (Saito, 1963; Sasakawa, 1984; Haba *et al.*, 2008; Oishi *et al.*, 2010a), 25 cranial and 24 dental characters (Fig. I-2) were measured to the nearest 0.01 mm, using a CD-S20C digital caliper (Mitutoyo). Skull measurements: rostrum length (RL), greatest length (GL), nasal length (NL), rostrum width (RW), interorbital constriction (IC), postorbital width (PW), postorbital constriction (PoC), zygomatic width (ZW), sphenion width (SphW), cranial width (W), skull height (SH), condylobasal length (CBL), length of upper tooth row (UT), palatal length (PL), distance between the first upper molars (MD), mastoid width (MtW), occipital condyle width (OCW), mandible length (ML), length of lower tooth row (LT), mandibular height (MH), mandibular thickness (MT), length between angular process and coronoid process (ACP), distance between the alveoli of the canine and p1 (c–p1), length of p1–p4 (p1–p4), and length of m1–m3 (m1–m3). Dental measurements were: length of upper canine (CL), width of upper canine (CW), height of upper canine (CH), length of upper premolar (P1L, P2L, P3L, and P4L), width of upper fourth premolar (P4W), length of upper molar (M1L and M2L), width of upper molar (M1W and M2W),

length of lower canine (cL), width of lower canine (cW), height of lower canine (cH), length of lower premolar (p1L, p2L, p3L, and p4L), width of lower fourth premolar (p4W), length of lower molar (m1L and m2L), and width of lower molar (m1W and m2W). Because some specimens had bullet holes or natural abrasion, some characters could not be measured and were treated as missing values.

GEOGRAPHICAL GROUPING BY GENETIC STRUCTURE

The individuals examined in the present study were identical to those examined genetically by Oishi *et al.* (2011). A cluster analysis of all individuals was conducted using the Bayesian clustering software GENELAND 4.0.3 (Guillot, Mortier & Estoup, 2005), based on microsatellite data (Oishi *et al.*, 2011) to determine genetic boundaries between groups. In the GENELAND analysis, the number of groups (*K*) was first varied from 1 to 10 to determine the optimal *K*. Markov chain Monte Carlo (MCMC) was performed by the following parameters: 1,000,000 iterations, sampling every 1,000 iterations, with a burn-in of 200,000, under the uncorrelated frequency and null allele models. Then, the optimal *K* was set, and 10 independent runs of 5,000,000 MCMC iterations (sampling every 5,000 iterations) were conducted with other parameters as in the prior step. The result with the highest average posterior probability showed the

individuals to be divided into three groups; Southern, Central, and Eastern (Table I-1 and Fig. I-1).

STATISTICAL ANALYSES

All statistical analyses were performed with R software 3.1.2 (R Development Core Team, 2014). To identify the trend and degree of morphological differentiation among groups, a principal component analysis (PCA) was conducted using PCAMETHODS version 1.52.0 (Stacklies *et al.*, 2007). Because most samples measured had missing values, a Bayesian PCA (BPCA) (Oba *et al.*, 2003), which can deal with missing values, was conducted. To statistically evaluate differences among groups for each morphological measurement, one-way multivariate analyses of variance (one-way MANOVAs) were performed using Pillai's trace. Subsequently, to confirm which variables contribute to the difference, univariate analyses of variance (ANOVAs) were conducted for each variable, and then conducted post-hoc multiple comparisons with the Tukey-Kramer method using MULTCOMP package version 1.3.1 (Hothorn, Bretz & Westfall, 2008). Correlations between each measurement and spatial and meteorological data were tested by using Pearson's correlation analyses, in which the minimum and maximum mean monthly temperature and snowfall were determined for

each sampling point from meteorological data spanning the past 30 years (1981–2010) (Japan Meteorological Agency website, http://www.jma.go.jp/). Additionally, to examine the relationships between the skull size and cold climate, the correlations between two measurements typically representing skull size (GL and ZW) and the minimum mean monthly temperature were tested. For each analysis, the significant level was set as 0.05.

Results

INTEGRATIVE COMPARISON FOR SKULLS AND TEETH

In the BPCA of skull measurements, the first (PC1) and second (PC2) principal component axes respectively explained 77.1% and 7.2% of the total variation in female, and 79.1% and 6.2% in male (Table I-2). In the BPCA of dental measurements, the PC1 and PC2 axes respectively explained 55.7% and 10.9% of the total variation in female, and 57.4% and 11.8% in male (Table I-2). The factor loadings of both sexes exhibited the same trends in skull and teeth. In the factor loadings for skulls, factors related to skull length such as GL, CBL, and ML contributed in a negative and major way to PC1, and factors related to skull width such as IC, PW, and ZW contributed in a negative and

major way to PC2 (Table I-2). In the factor loadings for teeth, factors related to canine size such as CH and cH contributed in a negative and major way to PC1, and in a positive and major way to PC2 (Table I-2). Fig. I-3 shows the distribution of BPCA scores, with the distributions for groups basically overlapping one another. The ANOVAs showed no significant difference in PC1 scores among groups for either skulls or teeth in both sexes, but in contrast, showed a significant difference in PC2 scores for skulls in both sexes and teeth in male (Table I-3).

STATISTICAL ANALYSES

The MANOVAs showed significant differences among groups in one or more variables for skulls in both male (Pillai's trace = 0.716, $P = 0.0065$) and female (Pillai's trace = 1.080, $P = 0.0058$) although they showed no significant difference for teeth in both male (Pillai's trace = 0.623, $P = 0.3429$) and female (Pillai's trace = 1.066, $P = 0.3821$). However, in the MANOVAs, 34 of 137 samples for male skulls, 22 of 137 samples for male teeth, 54 of 88 samples for female skulls, and 40 of 88 samples for female teeth were omitted due to missing values. Thus, because the results could possess low reliability, ANOVAs also on multivariate dataset without significant difference were performed. Basic statistics for skull and dental measurements for each

group and the ANOVA results are shown in Tables I-4 and I-5. Significant differences among groups were found for nine (IC, PoC, W, ZW, SH, MtW, OCW, MT and m1–m3) in male and two (IC and PoC) in female of 25 skull measurements (Table I-4), and two (P4L and m1L) in male and three (CL, P4L and M2L) in female of 24 dental measurements (Table I-5). In the post hoc test of multiple comparisons showed that IC, PoC, W, SH, MtW, OCW, P4L, and m1–m3 in male were significantly larger in the Eastern group than in the Southern group (Fig. I-4). In particular, PoC exhibited significant differences between the Eastern and Southern groups not only in male but also in female (Fig. I-4b). Additionally, the test showed that P4L was larger in the Central group than in the Southern group (Fig. I-4h). Table I-6 shows Pearson's correlation coefficient (*r*) between eight measurements exhibiting significant differences between the Southern and Eastern groups in the multiple comparison on one hand, and latitude, longitude, minimum maximum mean monthly temperatures, and snowfall on the other. The following significant correlations were found in male: m1–m3 and P4L with latitude; PoC, W, SH, MtW, OCW, and P4L with longitude; PoC and W with minimum mean monthly temperature; W with maximum mean monthly temperature; and PoC and MtW with snowfall. Similarly, the following significant correlations were found in female: P4L with latitude; IC, PoC, and MtW with longitude; IC, PoC, and

MtW with minimum mean monthly temperature; and IC and PoC with snowfall. In particular, the relationship between PoC and longitude was the most highly correlated in both sexes (male: $r = 0.38$, $P < 0.001$; female: $r = 0.39$, $P < 0.001$). In an additional correlation analysis, no significant correlation with minimum mean monthly temperature (MMT_{min}) was detected in GL (male: $r = 0.03$, $P = 0.7538$; female: $r = 0.08$, $P = 0.4512$) and ZW (male: $r = -0.05$, $P = 0.5779$; female: $r = -0.15$, $P = 0.1754$).

Discussion

VARIATION AMONG GROUPS

Geographical variation was detected in the skulls and teeth of the red fox population on Hokkaido Island. Morphological characters consolidated by the BPCA showed small differences among the three groups in skull width, mainly contributing to the second principal component, but no remarkable differences among groups in skull length, mainly contributing to the first principal component (Table I-3, Fig. I-3). In comparison with the principal component analyses performed by Oishi *et al.* (2010a), the morphological differences among groups on Hokkaido seemed smaller than that between the two Japanese subspecies, *V. v. schrencki* and *V. v. japonica*. The

morphological variation in the red fox on Hokkaido was similar to that reported by Huson & Page (1979) for another limited, small area: fox skulls in Wales are larger than those in Southeast England.

GEOGRAPHICAL DIFFERENCES IN SOME MEASUREMENTS

Some regional differences among groups were detected in some skull and dental measurements of both sexes or only male: interorbital constriction (IC), postorbital constriction (PoC), cranial width (W), skull height (SH), mastoid width (MtW), occipital condyle width (OCW), length of m1–m3 (m1–m3), and upper fourth premolar length (P4L) (Fig. I-4). In the present study, the clearest difference between the Southern and Eastern groups was shown in the postorbital constriction of both sexes (Fig. I-4b). Although there are no significant differences in the postorbital constriction between *V. v. japonica* and *V. v. schrencki* (Oishi *et al.*, 2010a), a regional difference within the latter subspecies was detected. Because the postorbital constriction positively correlated with longitude (Table I-6), the differences between groups might depend on degree of longitude. Postorbital constriction corresponds to size of temporal muscle in Carnivora and decreases with increasing muscle size (Radinsky, 1981). Accordingly, the temporal muscle of the foxes in eastern Hokkaido might be smaller than those in

western Hokkaido, suggesting a regional difference in masticatory ability. Some regional differences between the Southern and Eastern groups were also detected in the following skull and dental measurements of only male: interorbital constriction, cranial width, skull height, mastoid width, and occipital condyle width (Fig. I-4). Because the four measurements except interorbital constriction had positive correlations with longitude, the differences derived from degree of longitude. The interorbital constriction, cranial width, mastoid width, and occipital condyle width were affected to width of braincase in the left-right direction. The skull height is affected to height of braincase in the vertical direction. Thereby, the volume of braincase trends male-specifically to increase from west to east. However, because no significant differences were found in the zygomatic width (ZW) representing the greatest width of skull, the differences of skull could not significantly influence those of head size. Occipital condyle width is generally correlated with body weight in mammals (Martin, 1980). Therefore, the body weight of the red fox might have a tendency to increase from west to east. A significant difference between the Southern and Eastern groups was also found in the length of m1–m3 of only male. However, because no significant differences were detected in the lower second premolar length (m2L), the length of m1–m3 could reflect the lower first premolar length (m1L). While the male-specific characteristics might show the regional

difference of development in male foxes, there is also a possibility that significant differences were hard to detect at the same measurements in female due to the small sample size. Significant differences between the Southern and Central groups were found in the length of upper fourth premolar of both sexes (Fig. I-4h). The differences derived from degree of latitude in contrast to postorbital constriction because the length of upper fourth premolar had positive correlations with latitude not longitude (Table I-6). Upper fourth premolar is as functionally important a carnassial tooth as the lower first molar, and is significantly correlated with body weight, body length, and tail length (Yom Tov, Yom Tov & Baagoe, 2003). Thus, the body weight and length of the red fox might be larger in high latitude area. In the present study, similarly to the BPCA results, no regional differences in measurements relating to anteroposterior length of skull were detected in the ANOVAs and multiple comparisons.

A similar geographical cline in skull measurements was also reported in the brown bear on Hokkaido. Yoneda & Abe (1976) showed that some skull characters, including the mastoid width, are larger in the northeastern than in the southwestern region, although sample sizes were small. In addition, Ohdachi *et al.* (1992) reported that the mean cranial size of the brown bear increases from southern, through central, to northeastern Hokkaido in both sexes. By contrast, the skull length of the red fox was

found to be homogeneous throughout Hokkaido.

POSSIBLE CAUSES OF MORPHOLOGICAL VARIATION IN THE RED FOX ON HOKKAIDO

The results of this chapter suggested that a geographical cline in some morphological traits might be related to climatic conditions. However, the greatest length (GL) and zygomatic width (ZW), associated directly with body size, were not correlated with the minimum mean monthly temperature (MMT_{min}). Therefore, Bergmann's rule does not apply to variation in skull size in the red fox on Hokkaido. In contrast, the postorbital constriction, showing the most remarkable difference in the present study, was negatively correlated with minimum mean monthly temperature (MMT_{min}) and snowfall (SNOW) in both sexes. In other words, the red fox has the broader postorbital constriction with the lower temperature or the lighter snowfall. Because postorbital constriction is not directly related to body sizes, the relationships between postorbital constriction and minimum mean monthly temperature are not the case with Bergmann's rule. The differences of climatic and geographical conditions often affect the diets in animals. Because snow influences prey availability in the red fox (Halpin & Bissonette, 1988), the morphological variation on Hokkaido might be a

consequence of variation in diet among regions. In North America, the brown bear increases in size from the south to northwest, and is larger in the Pacific Northwest than in the interior (Rausch, 1963). Hilderbrand *et al.* (1999) and Mowat & Heard (2006) revealed by isotope analysis that the body size of the brown bear increases with the amount of salmon in the diet. The red fox on Hokkaido preys on the gray red-backed vole (*Myodes rufocanus*) throughout the year (Abe, 1975; Misawa, 1979). Vole populations on Hokkaido in turn show local variation in average density, with the highest average abundance in the Kushiro–Nemuro area (where the Eastern fox group is distributed) and generally lower abundance on the Oshima Peninsula (where the Southern fox group is distributed) (Saitoh, 1987; Saitoh, Stenseth & Bjørnstad, 1998). The foxes also prey on the sika deer (*Cervus nippon*); in the Shiretoko National Park at the northeastern tip of Hokkaido, the sika deer remains occurred in 16.7% fox feces, and the deer is a major item in the diet of foxes in spring (Tsukada & Nonaka, 1996). Populations of the sika deer are large and stable mainly in eastern Hokkaido, but relatively small and scattered in western and southern Hokkaido (Kaji, 1995). Thus, the skull and dental variation might have resulted from variation in food abundance. Cranium and mandible size, including mastoid width and upper fourth premolar length, are influenced by food abundance in the red fox (Englund, 2006). This is congruent with

the results in this chapter. More and detailed data on food utilization by the red fox throughout Hokkaido are needed to test the correlation between fox diet and morphological variation.

In general, the red fox is omnivorous, feeding on plants as well as animals. On Hokkaido, red foxes utilize mostly fruits, including *Actinidia arguta*, *Vitis coignetiae*, and *Prunus* spp., in autumn (Misawa, 1979; Tsukada & Nonaka, 1996). The morphological variation in southern Hokkaido might also reflect vegetation. A demarcation line separating climate and vegetation zones is the Kuromatsunai Lowland (see Fig. I-1) (Tatewaki, 1958), which corresponds to the boundary between the Southern and Central fox groups, and the genetic structure of the Hokkaido red fox population is highly related to regional differences in vegetation (Oishi *et al.*, 2011).

It was discussed that the geographical cline in sizes of only limited traits in the red fox of Hokkaido attributed adaptations to the cline of environment such as behavior patterns, climate conditions, food habits and vegetations. Asahara (2014) investigated morphological variations of the raccoon dog skulls within and between wild populations in Japan using two-dimensional geometric morphometric methods, and showed that the variation within subspecies reflect neutral evolution led by variability, evolvability and random drift, whereas the differences between subspecies is greatly affected by

adaptation to environment (e.g., climate). However, the present study showed the trends of geographical variation in skulls and teeth within the Hokkaido red fox population could have been caused by environmental adaptation. The wide variety of environments in Hokkaido could produce unique changes to the morphology of the red fox, in addition to other mammals such as the brown bear.

Chapter II

Genetic diversity of the MHC Class II *DRB* exon 2 in the Hokkaido population of the red fox

Introduction

In *V. v. schrencki* (that is, the Hokkaido population), some genetic and morphological differences have been reported so far. Amaike *et al.* (2015) reported that there are regional differences in cranial morphology with a gradient of increase from west to east on Hokkaido Island in the postorbital constriction, which relates to size of the temporal muscle. Inoue *et al.* (2007) reported occurrence of three lineages of mitochondrial DNA (mtDNA) within the Hokkaido population. Kutschera *et al.* (2013) analyzed the mtDNA phylogeography including the red foxes of the Hokkaido population, and reported that red foxes colonized Hokkaido at least three times. In addition, a microsatellite study revealed that the genetic structure of the subpopulation of southern Hokkaido is clearly different from those of central and eastern Hokkaido (Oishi *et al.*, 2011). On the other hand, there are no reports on characteristics of biparental genes possessing any function relating to adaptation.

The major histocompatibility complex (MHC) is a large cluster of closely related genes that play a critical role in immune response of vertebrates (Klein, 1986). The MHC genes are one of the most polymorphic genes in the vertebrate genome (Garrigan & Hedrick, 2003; Piertney & Oliver, 2006). They consist of two major groups, MHC

class I and II, encoding cell-surface glycoproteins that bind to intra- and extra-cellular peptides derived from pathogens, respectively, and present them to T cells, which trigger the appropriate immune response (Sommer, 2005; Jensen, 2007). The MHC class II proteins contain functionally important amino acids, antigen-binding sites (ABS), which directly bind to pathogen-derived peptides (Hughes & Nei, 1988). The second exon of MHC class II *DRB* gene has been spotlighted in many studies on the MHC variation, because the ABS codons display high levels of polymorphism in many species (Benoist *et al.*, 1983; Parham & Ohta, 1996; Hughes & Yeager, 1998). The diversity of MHC is closely associated with resistance to infectious disease (e.g. Grimholt *et al.*, 2003; Bonneaud *et al.*, 2006; Savage & Zamudio, 2011; Srithayakumar *et al.*, 2011). From a perspective of the immunological fitness, MHC genes, especially *DRB* gene, are enormously useful in conservation genetics (Hughes, 1991; Ujvari & Belov, 2011) such as genetic diversity assessments of endangered species (e.g. Marsden *et al.*, 2009; Kohyama *et al.*, 2015; Lau *et al.*, 2015). Recently, the variation of the MHC class II *DRB* alleles has also been used as a tool not only for evaluating diversity, but also for defining spatial patterns and geographical isolation in functional genes (e.g. Bowen *et al.*, 2006; Ekblom *et al.*, 2007; Alcaide *et al.*, 2008; Miller, Allendorf, & Daugherty, 2010). In terms of evolution, high genetic variation of MHC is considered to

be maintained by pathogen- or parasite-mediated balancing selection (Edwards & Hedrick, 1998; Spurgin & Richardson, 2010). Balancing selection leads not only to the maintenance of intraspecific diversity in alleles, but also to the extremely long-term persistence of allelic variation crossing over species, which is described as ‘trans-species polymorphism’ (Klein, 1987).

Characterization of the MHC class II *DRB* gene has been reported in some wild canid species: in the case of the red fox, Marshall *et al.* (2016) characterized *DRB* genes in the Newfoundland red fox (*V. v. deletrix*), and reported eight *DRB* alleles as the first genetic data from the red fox MHC. However, there are no studies on MHC from any other subspecies/populations of the red fox.

In the present study, geographic variation of MHC class II *DRB* in the Hokkaido population of the red fox is examined. Also discussed is the molecular evolution, such as trans-species polymorphism under (pathogen-driven) balancing selection, among local populations of the red fox on Hokkaido.

Material and Methods

SAMPLES AND DNA EXTRACTION

Muscle tissues were obtained from 232 red foxes collected widely from Hokkaido Island (Fig. II-1: locations 1–22) for epidemiological survey on parasite infection (in 2001, 2003 and 2006), conducted by the Hokkaido government (Oishi *et al.*, 2011). The tissue samples were treated at 70°C for 3 days for inactivation of parasites and then preserved in 99% ethanol at 4°C. Total DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN), and then stored at 4°C.

PCR AMPLIFICATION AND SEQUENCING

The partial sequence (237 base-pairs, bp) of MHC class II *DRB* exon 2 of the red fox was amplified by polymerase chain reaction (PCR) using the primer pair: Vu-DRBF1 (5′-GTC CCC ACA GCA CAT TTC TTG-3′: newly designed primer) and DM-2 (5′-TCG CCG CTG CAC CGT GAA GCT-3′: Hedrick, Lee & Parker, 2000). The former was newly designed as a genus-specific primer based on two *Canis* species: domestic dog (*Canis familiaris*, accession numbers U47338 and AJ630362) and coyote (*Canis latrans*, accession number EU400582). PCR amplification was performed in a 20 µl reaction volume containing 2.0 µl of 10× PCR Buffer (Mg^{2+} plus), 1.6 µl of dNTP mixture (2.5 mM each), 0.2 µl of each primer (25 pmol/µl), 0.2 µl of r*Taq* polymerase (5 U/µl, Takara), and 1.0 µl of DNA extract using a Thermal cycler Dice Touch®

(TP-350, Takara). The PCR was performed under the following condition: 5 min initial denaturation; 35 cycles of denaturation at 95°C for 40 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min; and additional extension at 72°C for 10 min. To confirm successful amplification, the PCR products were electrophoresed on a 3% agarose gel and visualized by ethidium-bromide staining. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN). Direct sequencing was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with an ABI 3730 DNA Analyzer (Applied Biosystems) following the manufacturer's instructions.

HAPLOTYPE PHASING

The MHC *DRB* alleles were reconstructed in DnaSP version 5.10.01 (Librado & Rozas, 2009) with the built-in program PHASE version 2.1.1 (Stephens, Smith & Donnelly, 2001), which avoid the cloning step, using a coalescent-based Bayesian method and running 1000 iterations after 200 burn-ins. Individuals possessing sequences with less than 90% probabilities of correct base at each site were excluded from the subsequence analyses. Obtained sequences were confirmed as those from the MHC class II *DRB* exon 2 by means of BLAST (Altschul *et al.*, 1990) of the NCBI

Genbank database. Nucleotide and the deduced amino acid sequences were checked and aligned with MEGA version 6.06 (Tamura *et al.*, 2013).

DATA ANALYSES

To identify genetically- and geographically-related populations, a spatial analysis of molecular variance (SAMOVA) was performed using SPADS version 1.0 (Dellicour & Mardulyn, 2014), based on 22 areas divided at the municipal level. The most likely number of groups (*K*) was determined using 10,000 iterations and 10 repetitions for each *K* from two to 10 groups. Additionally, populations were classified into five groups using microsatellite-based group definition of Oishi *et al.* (2011) for further analysis of genetic diversity. Note that the Nakashibetsu and Nemuro subpopulations were included in the Eastern group (Fig. II-1) because of small sample sizes. The allele frequency-based genetic differentiations (F_{ST}) among the groups were calculated by ARLEQUIN version 3.5.1.2 (Excoffier & Lischer, 2010). In addition, to represent spatial patterns of genetic diversity across the landscape, nucleotide diversity (π) and allelic richness (A_R) at each fox population were interpolated and mapped using GDivPAL function included in SPADS on R version 3.1.2 (R Development Core Team, 2014).

The values of π, 4Nμ for autosomal genes of diploid organisms (θ), and Tajima's D (D_T) based on allele frequency spectrum were calculated by DnaSP. Allele frequencies and A_R, standardized on the basis of the smallest number of samples, were calculated using FSTAT version 2.9.3.2 (Goudet, 2002). Observed and expected heterozygosities (H_O and H_E, respectively) were calculated and tested using ARLEQUIN.

The ABS positions were inferred from human ABS of the HLA-DR molecule (Bondinas, Moustakas & Papadopoulos, 2007). In order to reveal evidence for selection on ABS, values of nonsynonymous (d_N) and synonymous (d_S) substitution rates per site were calculated using MEGA with the Nei-Gojobori method (Zhang, Rosenberg & Nei, 1998) with Jukes-Cantor correction (Jukes & Cantor, 1969) to account for multiple hits. The ω (d_N/d_S) values were tested for significant differences from neutrality with a codon-based Z-test in MEGA.

A haplotype network was constructed due to PopART (Leigh & Bryant, 2015) using the median joining network methods (Bandelt, Forster & Röhl, 1999). A Bayesian phylogenetic analysis was performed to reconstruct the evolutionary relationships among alleles of the red fox, arctic fox, other canids (e.g. dog, grey wolf and coyote) and felids. KAKUSAN4 (Tanabe, 2007) under BIC4 (sample size = number of sites) selected HKY85_Gamma (first codon position), GTR_Gamma (second codon position),

and K80_Gamma (third codon position) as the optional substitution model. A phylogenetic tree was constructed by MrBayes version 3.2.6 (Ronquist & Huelsenbeck, 2003) with 5.0×10^7 generations. The convergence of the parameter values sampled from the chains was checked with Tracer version 1.6 (Rambaut *et al.*, 2014). The consensus tree was visualized using FigTree version 1.4.2 (Rambaut, 2014).

Results

GENOTYPING OF *VULPES VULPES* MHC CLASS II *DRB* ALLELES

PCR products were successfully sequenced from all 233 red fox samples, yielding 233 diploid sequences (237 base-pair, bp) with double peaks. More than two overlapping peaks, meaning existence of two or more loci, were not found. As the result of phasing, 17 haplotypes were found as new alleles in red foxes of Hokkaido. Although most of the phased sequences were supported with 100% probabilities of correct base at each site, one individual with a sequence supported with less than 90% probabilities was found and eliminated from the subsequent analyses. Hence, the final sample size of individuals was 232. No identical sequences to previously reported sequences were found in the DNA database, and the obtained 17 unique alleles were named as

*Vuvu-DRB*09–25* following the nomenclature proposed by Klein *et al.* (1990). The sequences will be deposited in Genbank. Among the sequences, 41 nucleotide and 20 amino acid sites were polymorphic (Figs. II-2 and II-3).

CLUSTER ANALYSIS

Although the SAMOVA showed the highest Φ_{CT} value (Φ_{CT} = 0.1294) for K = 3, there were one or more singleton populations in each group for K = 3–10. The optimal number of groups indicated by the SAMOVA was 2 (Φ_{CT} = 0.1287, no singleton population: Table II-1). It shows that all red foxes on Hokkaido (nos. 1–22 shown in Fig. II-1) were grouped into two major sections: one section (Southern group) including four subpopulations (nos. 1–4) of southern Hokkaido and the other section including subpopulations (nos. 5–22) of Central, Northern, Eastern, and Far Eastern groups. It was found that the Southern group was greatly differentiated from the other groups (Table II-2): Central (F_{ST} = 0.182, P < 0.001), Northern (F_{ST} = 0.227, P < 0.001), Eastern groups (F_{ST} = 0. 265, P < 0.001), and Far Eastern (F_{ST} = 0.207, P < 0.001). Additionally, the Eastern and Far Eastern groups were also slightly differentiated from Central and Northern groups (F_{ST} = 0.040–0.066, P < 0.001 each). In contrast, no statistically significant differentiations were found between the Central and Northern groups (F_{ST} =

0.005, $P < 0.153$) and between the Eastern and Far Eastern groups ($F_{ST} = 0.013$, $P < 0.171$). Figure II-4 shows the distribution patterns of π and A_R: both π and A_R in the Southern group were obviously lower than those in the other groups.

DIVERSITY OF MHC CLASS II *VUVU-DRB*

Table II-3 indicates a summary of statistics. The smallest and largest numbers for alleles (*A*) were $A = 5$ at Southern and $A = 13$ at Northern groups. Although A_R in the Southern group was 5.000, A_R in any of the other groups was about two-fold higher than the former. Allele frequencies of *Vuvu-DRB*s were shown in Fig. II-1 and Table II-4. The frequencies of *Vuvu-DRB*09, *10, *11*, and **13* were more than 75% in total. The most frequent allele was *Vuvu-DRB**10 (22.2%), which was also the most common (more than 15%) in each regional group. On the other hand, *Vuvu-DRB**09, which had the largest proportion (approximately 65%) in the Southern group, could play an important role in this area. In contrast, *Vuvu-DRB**12 made up less than 4% of the total alleles in the Southern group, whereas the frequency of this allele was more than 15% in each of the other groups. Tajima's *D* values were positive in most populations on Hokkaido, indicating that the *DRB* gene has a tendency toward balancing selection (Table II-2). In particular, significantly positive values were found in the Northern

population (D_T = 0.2124, $P < 0.05$) and the total population (D_T = 2.331, $P < 0.05$). By contrast, the negative value (D_T = −0.301, not significant) detected only in the Southern population indicates a tendency toward purifying selection.

SELECTION OF *DRB* FROM RED FOXES ON HOKKAIDO

Nonsynonymous substitution rates were almost equal to synonymous substitution rates (ω = 1.122, P = 0.871: Table II-5) for non-ABS codons, whereas significant excess of nonsynonymous substitution rates on the ABS codons and all positions (ABS + non-ABS) of *Vuvu-DRB* (ABS; ω = 2.720, P = 0.008: All sites; ω = 2.109, P = 0.013) (Table II-5) indicated that *Vuvu-DRB* has evolved under positive selection.

PHYLOGENETIC ANALYSES

Figure II-5 shows the haplotype network consisting of 17 alleles identified in the present study. In the network, the largest number of mutational steps (18 mutational steps) occurred between *Vuvu-DRB*16*/**19* and the other alleles.

The Bayesian phylogenetic tree among *DRB* alleles from the red fox, arctic fox, other Canidae, Felidae, and human (Fig. II-6) showed that all alleles from the Hokkaido

red foxes were included into the Canidae clade. A huge clade, named fox-like canids clade, consisted of alleles of only genus *Vulpes* together with alleles from the red fox of Newfoundland Island and the arctic fox. In the fox-like canids clade, the alleles from Hokkaido (*V. v. schrencki*) (*Vuvu-DRB*09–25*), Newfoundland (*V. v. deletrix*) (*Vuvu-DRB*01–08*), and arctic fox (*V. lagopus*) (*Vula-DRB*01–13*) were not divided into species/subspecies-specific clusters, and were discretely distributed. On the other hand, *Vuvu-DRB*16* and *Vuvu-DRB*19* were included into the wolf-like canids clade formed by *DLA-DRB*s and *Calu-DRB*s from four canid species (wolf, dog, coyote, and African hunting dog). The *V. v. schrencki DRB* alleles thus showed trans-species polymorphism not only among species, but also among genera in family Canidae.

Discussion

GEOGRAPHICAL VARIATION OF *VUVU-DRB* ALLELES ON HOKKAIDO

The results of the cluster analyses and the estimated genetic differentiations (F_{ST}) clearly showed that the genetic structure of the Southern group was largely different from those of the other groups in Hokkaido. The border between the Southern and the other groups corresponds with the Kuromatsunai lowland (see Fig. II-1). This border is

consistent with that of the previous report by Oishi *et al.* (2011), who investigated variation of microsatellites as neutral genetic markers. In addition, the nucleotide diversity (π) and allelic richness (A_R) in the Southern group were lower than those in the other groups. The Tajima's neutrality test suggested that the variation of the MHC alleles in the entire Hokkaido population is sustained by balancing selection. At the regional level, however, only the *DRB* alleles of Southern subpopulation might be under purifying selection (selective removal of deleterious alleles), whereas the others might be under balancing selection. In fact, the Southern group did not possess any locality-restricted alleles, although the other groups showed locality-restricted ones such as *Vuvu-DRB*18*, **20*, **23*, **24*, and **25*, indicating that these alleles might have been diverged and maintained by balancing selection against pathogens native in particular areas. On the other hand, *Vuvu-DRB*09*, which was at frequencies of 3–12% in the groups other than the Southern group, was the most frequent in the Southern group (65%). The regionally different allele selection could be attributed to geographical isolation adapting to specified environments, in addition to balancing selection. Actually, the vegetation on Oshima Peninsula, which is covered with the temperate deciduous forest zone, is endemic and similar to that of Honshu Island rather than the other parts of Hokkaido Island (Tatewaki, 1958). Oishi *et al.* (2011) reported that the number of

microsatellite alleles in the Southern subpopulation was the smallest among fox subpopulations of Hokkaido.

SELECTION IN RED FOXES ON HOKKAIDO

The present study also showed strong evidence of positive selection in the *DRB* exon 2 of red foxes on Hokkaido. The d_N/d_S ratios of more than 2.7 obtained here were highly similar to previously reported values in *Vulpes* species: 3.3 in *V. lagopus* (Ploshnitsa *et al.*, 2011) and 2.7 in *V. vulpes* of Newfoundland Island (Marshall *et al.*, 2016); and also in the other canine species: 2.61 in European and American wolves (*Canis lupus*), 2.58 in European wolves, 2.78 in American wolves, 1.98 in coyotes (*C. latrans*), and 2.75 in dogs (*C. l. familialis*) (Seddon & Ellegren, 2002). In contrast, although they are still more than one, relatively low levels of d_N/d_S ratios were also reported in other carnivorans: 1.40 in cheetahs (*Acinonyx jubatus*) (Castro-Prieto, Wachter & Sommer, 2010) and 1.364 in Japanese weasels (*Mustela itatsi*) and 1.414 in Siberian weasels (*M. sibirica*) (Nishita *et al.*, 2015). These variations of the d_N/d_S ratios could have resulted from species-specific selective pressure.

PHYLOGENETICAL RELATIONSHIPS OF *VUVU-DRB* ALLELES FROM RED

In general, most species of family Canidae were phylogenetically classified into three major clades: the fox-like canids clade, South American canids clade, and wolf-like canids clade, following by phylogenies based on allozymes (Wayne & O'Brien, 1987), mtDNA (Wayne *et al.*, 1997), and single nucleotide polymorphisms (SNPs) (Lindblad-Toh *et al.*, 2005). Tsuda *et al.* (1997) compared the mtDNA D-loop region of dogs/wolves with those of foxes and raccoon dogs (*Nyctereutes procyonoides*), and they estimated that the three genera, *Canis*, *Vulpes*, and *Nyctereutes*, diverged approximately 5–10 million years ago (Mya). Perini, Russo & Schrago (2010) examined three mitochondrial genes and the 22 nuclear genes from 27 canid species, and estimated that the divergences between fox-like canids and South American/wolf-like canids and between *V. lagopus* and *V. vulpes* ancestors occurred around 8.8 and 2.9 Mya, respectively. Zhao *et al.* (2016) inferred the divergence time in canids using the complete mitochondrial genome, estimating that the fox-like canids diverged from the South American-like/wolf-like canids around 8.6 Mya and that *V. lagopus* split from a lineage including *V. vulpes* around 3.4 Mya. In the Bayesian phylogenetic tree estimated in the present study, all *Vuvu-DRB* alleles obtained from the Hokkaido red fox did not form any species-specific monophyletic clades. This phylogenetic feature is consistent

with those of the Newfoundland red fox (Marshall *et al.*, 2016). Especially, *Vuvu-DRB*16* and *Vuvu-DRB*19* in the present study were grouped into the wolf-like canids clade with previously published allele of *Canis*. Thus, some of the *DRB* alleles derived from the ancestral species have been maintained not only among species but also among other genera of Canidae by a long-term balancing selection.

Chapter III

Population genetic diversity and home ranges of the red fox in Mt. Hakodate, revealed by microsatellite analysis using non-invasive fecal samples

Introduction

The red fox sometimes occurs on small islands such as Assateague Island in the United States (Paradiso *et al.*, 1965), and Prince Edward Island in Canada (Sobey, 2007). In Japan, one subspecies, *V. v. schrencki*, inhabits Hokkaido Island, and one of the populations is distributed on Mt. Hakodate, which is geographically isolated as “a land-tied island”.

Mt. Hakodate (41°45′32″N, 140°42′15″E) has an altitude of 334 m, a circumference of approximately 9 km, and an area of approximately 3.26 km^2, and is located at southwest of Hakodate City in southern Hokkaido. It is surrounded by the sea on three sides, and connected to Kameda Peninsula by the tombolo, which is currently occupied by the main urban area. Because the general citizens had been prohibited from entering Mt. Hakodate for fortification from about 1900 to 1945, the natural environment of Mt. Hakodate had been successfully maintained during about half a century. Therefore, Mt. Hakodate has approximately 600 plant species, although it is a tight area located next to the urban area. In addition, this mountain could be a favorable habitat for native wild birds and a resting-place for migrating birds, and approximately 150 species of birds can be seen year-round. Until now, Mt. Hakodate has been

designated as the Wildlife Special Protection Area (327 ha) by the Hokkaido Government (Sato, 1994).

In regard to the mammalian fauna on Mt. Hakodate, the red fox (*Vulpes vulpes*), chipmunk (*Tamias sibiricus*), introduced Japanese weasel (*Mustela itatsi*), two mouse species (the names are unknown) and two bat species (the names are unknown) are reported to live there (Kimura, 2011). In ecosystem on the mountain, the red fox is thought to stand at the top; however, it is not clear how the red fox population has been maintained in such a small mountain.

Recently, DNA analysis using non-invasively collected fecal samples allows us to know biological information on species, sex and individually of the target animals for conservation ecology and genetics. For example, Kurose, Masuda & Tatara (2005) performed DNA analyses using fecal samples from the Tsushima Island, Japan, and identified species and sex on four carnivores including the endangered Tsushima leopard cat. Shimatani *et al.* (2008, 2010) genetically identified five carnivora species from feces collected in the Kushiro wetland, Hokkaido, and succeeded to identify sex and individuality for American minks (*Neovison vision*). Oishi, Uraguchi & Masuda (2010b) analyzed 59 feces of the red fox in the Shiretoko National Park, Hokkaido, and ascertained that they derived from 22 individuals. Saito *et al.* (2016) successfully

identified 31 individuals using fecal samples of the raccoon dogs in the grounds of the Imperial Palace, Tokyo, and studied the genetic structure.

In the present study, to understand population genetic features of the red fox living on Mt. Hakodate, microsatellite genotypes were analyzed using fecal sample collected in the field. Then, the genetic diversity and population size were estimated, and the behavioral ranges of red foxes were calculated.

Material and Methods

SAMPLING AND DNA EXTRACTION

Fecal samples were non-invasively collected along nine hiking trails: Irie-yama, Kannon, Yakushi-yama, Kyu-tozando, Shiomi-yama, Senjojiki, Nanamagari, Miyanomori, and Ezodate-yama courses, on Mt. Hakodate, and three towns: Kaminokuni, Matsumae, and Shikabe, in southern Hokkaido (Fig. III-1). The field works on Mt. Hakodate were designed to cover all the hiking courses within a single month, and conducted one or more times per month in April–December of 2009, April–November of 2010, and April–December of 2011. The other field works in Kaminokuni, Matsumae, and Shikabe were conducted once in October or November of 2011. The feces were carefully collected into gamma sterilized polypropylene conical tubes using

sterilized tweezers in field. Location information was got using a handheld global positioning system (GPS) unit (GPS-CS3K, Sony, Tokyo, Japan) or a digital camera with a built-in GPS (Optio WG-1 GPS, Pentax, Tokyo, Japan) at the same time. The fecal samples were transported to laboratory at room temperature, and then stored at −80°C for more than one week until DNA extraction in order to inactivate parasites and the eggs.

Total DNA was extracted from approximately 0.3 g of the feces using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). As positive control, DNA extracted from the muscle tissue of a road-killed fox using the DNA Tissue Kit (QIAGEN, Hilden, Germany) was used.

SPECIES IDENTIFICATION

In order to make sure that the collected feces were dropped from the red fox, a fox-specific partial sequence of mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR), using the primer pair fox-F1/R1 (Shimatani *et al.*, 2008). To identify the other mammal species, which are presumed to occur on Mt. Hakodate, the other PCRs were performed with the domestic dog- and domestic cat-specific primer pairs reported by Shimatani *et al.* (2008) and Kurose *et al.* (2005), respectively. The species-specific PCR amplifications were performed for all

fecal DNA samples at least twice in accordance with the protocols of the previous studies.

GENOTYPING OF MICROSATELLITES

Genotyping was conducted for all samples identified as the red fox. For each fecal DNA sample, 10 microsatellite loci were amplified using the following primers; DB1, DB3, DB4 and DB6 (Lade *et al.*, 1996); V142, V374, V402, V468, V602 and V622 (Wandeler & Funk, 2006). The 5′ end of each forward primer was fluorescently labeled with FAM, NED, VIC or PET. The PCR amplification was performed using the QIAGEN Multiplex PCR Kit (QIAGEN, Germany) and a DNA thermal cycler (TP600, TAKARA, Japan). The PCR mixture of a total volume of 5.0 µl consisted of 2.5 µl of 2x QIAGEN Multiplex PCR Master Mix, 0.9 µl of RNase-free water, 0.5 µl of forward and reverse primer mix, 0.1 µl of bovine serum albumin (BSA) (Roche, Switzerland) and 1.0 µl of template DNA. The PCR protocol was one time of first denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 90 sec and extension at 72°C for 1 min; and one time of final extension at 60°C for 30 min. Primer mix combinations are shown in Table III-1. Preliminarily, V622 was excluded because no alleles were obtained. Each PCR product was electrophoresed using a DNA sequencer ABI3730, and then molecular sizes were measured with Peak Scanner

Software v1.0 (Life Technologies) to determine alleles and genotypes.

For individual identification, PCR was performed three or more times repeatedly for each sample, and attempted to amplify loci at least three times. Homozygotes for each locus were determined by at least three times of amplifications for genotyping. In case that a mixture of homozygous and heterozygous genotypes for a locus was detected, independent PCRs were additionally performed, and genotypes were determined to be heterozygous according to at least two appearances of both alleles. Samples, from which no PCR products were obtained at five or more loci, were excluded from the subsequent analyses.

The probability of identify for unrelated individuals and siblings (Waits, Luikart & Taberlet, 2001) was calculated using the program GIMLET (Valière, 2002) to assess how many markers are sufficient to distinguish between different fecal samples from individuals. The population size of the red fox living on Mt. Hakodate was estimated using the Two Innate Rates Model (TIRM) of the package CAPWIRE (Miller, Joyce & Waits, 2005; Pennell *et al.*, 2013) in R (R Development Core Team, 2014).

GENETIC STRUCTURE ANALYSES

To characterize the genetic structure and diversity of the Mt. Hakodate population, they were compared with those of the other population on Hokkaido using the

microsatellite data of Oishi *et al.* (2011). The population genetic structure was analyzed by STRUCTURE version 2.3.4 (Pritchard, Stephens & Donnelly, 2000). Ten runs were performed for each number of genetic clusters (K) from 1 to 10. Each run consisted of 1000 iterations of the Marcov Chain Monte Carlo (MCMC) after a burn-in of 1000 iterations. To determine the most likely K, ΔK values were estimated using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Allelic richness (A_R), standardized on the basis of the smallest number of samples, were calculated using FSTAT version 2.9.3.2 (Goudet, 2002). Degree of genetic divergences (F_{ST}), observed and expected heterozygosities (H_O and H_E, respectively), and inbreeding coefficient (F_{IS}) were calculated and tested using ARLEQUIN version 3.5.1.2 (Excoffier & Lischer, 2010). The basic genetic statistics was worked out by GENEPOP. The network tree based on F_{ST} was constructed by the neighbor joining method using MEGA version 6 (Tamura *et al.*, 2013).

SEXING USING *ZFX*/*ZFY* GENES

New sex-specific primers, which are able to amplify the partial final intron sequences of the zinc finger protein genes on X chromosome (*ZFX*) and Y chromosome (*ZFY*), were developed. Three primers VVZFX-F1 (5′-TGA AGT TTT CAG ACC AGG GTT C-3′), VV-ZFY-F1 (5′-GGG TTT TTC TGT TAC CTC TTT TG-3′) and

VVZFYX-R1 (5′-CAT GAG TGA TCA AAC CAA GTT C-3′) were newly designed, referring with sequences of GenBank accession numbers AB622129 and AB622140 (Tsubouchi *et al.*, 2012). The sexes of samples identified as red foxes were determined using the primers. The PCR was performed in a total volume of 20 µl containing 0.2 µl of r*Taq* DNA polymerase (5 units/µl, Takara), 2.0 µl of 10 × reaction buffer, 1.6 µl of dNTP, 0.4 µl of BSA and 0.2 µl of each primer (2.5 pmol/µl), 1.0 µl of each DNA extract and 14.4 µl of distilled water. The PCR cycling conditions were one time at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min; and final extension at 72°C for 10 min. The PCR products were run on a 3% agarose gel and visualized with ethidium bromide under an ultraviolet illuminator. These primers gave PCR products of 100 bp (partial X chromosome) and 131 bp (partial Y chromosome), respectively. One band and two bands indicate sexing for male and female, respectively.

ESTIMATION OF HOME RANGES

To investigate behavioral features of the red foxes on Mt. Hakodate, the home ranges were estimated with the minimum convex polygon (MCP) method using fecal sample position information of genetically identified individuals. The home range sizes of individuals were calculated using QGIS 1.7.1 (QGIS Development Team, 2011).

Results

SPECIES IDENTIFICATIONS

According to the PCR amplifications of mtDNA fragments, 98 (65.3%) of 150 fecal samples collected on Mt. Hakodate were successfully identified as the red fox. Of the 150 samples, 16 were judged as the domestic cat. Aside from these, the mtDNA fragments of both the red fox and the domestic cat were amplified from eight samples, suggesting existence of the prey-predator relationship between the two species or a mixture of feces from both species in the field. Consequently, the success rate of species identification by PCR amplification was 81.3% (122/150 fecal samples). In regard to the remaining 28 samples, neither PCR products for the red fox nor the domestic cat species were amplified. No samples were identified as the domestic dog. The locations of the samples identified as the red fox or the domestic cat was shown in Figure III-1C. All six fecal samples obtained at the sampling points other than Mt. Hakodate were identified as the red fox, and used for comparisons among populations in the subsequent analyses.

INDIVIDUAL IDENTIFICATIONS AND POPULATION SIZE ESTIMATION

Table III-1 shows the result: success rates of 70.6–84.0% (78.4% in average) and 1–4 (3 in average) alleles at each locus. Figure III-2 indicates the probability of identity ($P_{\text{ID-biased}}$ and $P_{\text{ID-sibs}}$) calculated from the genotyping data. The accumulate $P_{\text{ID-biased}}$ and $P_{\text{ID-sibs}}$ values for nine loci examined were less than 0.001 and 0.015, respectively. Mills *et al.* (2000) suggest that less than approximately 0.01 of P_{ID} is recommended for estimating population size. The $P_{\text{ID-sibs}}$ obtained in the present study is close to the recommended value. Using these genotype data, the individuals test was successful at 25 fecal samples in 2009, 19 fecal samples in 2010 and 41 fecal samples in 2011, and consequently 12, 11 and 22 foxes were found in 2009, 2010 and 2011, respectively (Table III-2). The CAPWIRE program estimated the fox population size on Mt. Hakodate in each year as follows: 20 [95% confidence interval (CI) = 15–33] in 2009, 25 [95% CI = 17–47] in 2010 and 44 [95% CI = 36–69] in 2011.

GENETIC STRUCTURE AND DIVERSITY

Figure III-3 shows the genetic structure of the entire Hokkaido population including the Mt. Hakodate population. The highest ΔK was calculated to be 2 (Fig. III-4), indicating the separation of the populations on southern Hokkaido, including Mt. Hakodate and Oshima, from those of the other regions in Hokkaido. This is congruent with Oishi *et al.* (2011). The STRUCTURE analysis at $K = 4$ or more showed that the

population structure on Mt. Hakodate was clearly different from that in the Southern part of Hokkaido. Additionally, the genetic structure of the red foxes on Mt. Hakodate was almost homogenized within the population, indicating that the genetic variation of the Mt. Hakodate population was low. The genetic differentiation of the Mt. Hakodate population was also indicated by F_{ST} values. The F_{ST} values between the Mt. Hakodate and the other populations excluding Oshima (south part of southern Hokkaido) were 0.13-0.16 whereas the F_{ST} values between the other populations were 0.00–0.12 (Table III-3). The network tree based on the F_{ST} values (Fig. III-5) also showed the genetic differentiation of the Mt. Hakodate population. Allelic richness (A_R), observed and expected heterozygosities (H_O and H_E) and inbreeding coefficient (F_{IS}) were shown in Table III-5. The means of allelic richness and observed and expected heterozygosities in the Mt. Hakodate population were the lowest among all populations of Hokkaido. These low genetic diversities could result from the long isolation of the Mt. Hakodate population within a small area.

SEX IDENTIFICATION

Table III-2 also shows the results of sex identification for each individual. Of all 85 fecal samples clarified for individuality, it became clear in the sex identification experiment that 40 and 35 samples were male and female, respectively, although it was

not able to determine about the ten remaining samples. In total 29 (82.9%) of 35 individuals were successfully sex-identified. The 29 individuals consisted of 16 males and 13 females. Complementally, no incongruent results of sexing were obtained from fecal samples identified as the same individuals.

ESTIMATION OF HOME RANGES

Figure III-6 indicates the estimated home ranges of identified individuals. The polygons showing home ranges were overlapped with each other on the mountain. Especially in the center of Mt. Hakodate (the south side of the mountaintop), home ranges of five individuals were overlapped. The home range sizes during the entire investigation estimated from 1.0 to 68.2 ha (14.4 ha in average) (Table III-2).

Discussion

SPECIES DETECTED BY NON-INVASIVE GENETIC SAMPLES

The present study using fecal DNA revealed that the red fox and domestic cat live on Mt. Hakodate. Traditionally, vegetation and bird surveys have been conducted on Mt. Hakodate because of the abundance of species. However, until now, there were no studies on biological information of terrestrial mammals living there. This is the first

report on population genetics on animals inhabiting Mt. Hakodate. In the present study, although inhabitation of the domestic cats was confirmed, it is unclear whether they are feral or house-kept cats, which appeared to come from the surrounding residential area. In addition, detection of fecal samples including DNAs from both of the two species suggested that the fox could occasionally eat the cats or vice versa, respectively, or that there was a chance of contact between feces from both species in the field.

HIGH SUCCESS RATE OF GENOTYPING

The present study obtained the higher success rate of species identification with fecal DNAs, compared with the previous studies on wild carnivorans, approximately 60–96% (Kurose *et al.*, 2005; Sugimoto *et al.*, 2006; Shimatani *et al.*, 2008). In addition, the genotyping was successful with a high determination rate (78.4% in average). Oishi *et al.* (2010b) determined genotypes with a probability of 23.3–69.8% in individual identification for the red foxes in the Shiretoko National Park, Hokkaido. Nagai, Murakami & Masuda (2014) genotyped the sables (*Martes zibellina*) in eastern Hokkaido, and reported that the probability of genotyping is 63.6 % on average (47.8–76.1%). The current genotyping rate higher than those in the previous studies could be attributed to a high frequency of the sampling on Mt. Hakodate, which could have increased the availability of DNA from fresh feces. Additionally, frozen preservation in

the present study yielded higher success rates. This is in agreement with Piggott & Taylor (2003) who showed that freezing of the relatively small feces was more effective for improving genotype success rate than preserving in ethanol.

HIGH POPULATION DENSITY LIKE URBAN FOXES

The present study estimated occurrence of at least 36 foxes on Mt. Hakodate during the three years, 2009–2011, including one road-killed cub. This data is invaluable for management of the small population. The number of the successfully genotyped individuals was the largest in 2011. It may be simply due to the large number of the collected samples in this year. The estimated population density in the present study was higher than other reports in many places of the world. Webbon, Baker & Harris (2004) surveyed the density of the red fox in rural Britain through fecal count, and reported that the mean fox density in landscapes ranged from 0.21–2.23 foxes/km^2. In the present study, especially the population density estimated in 2011 (13.5 foxes/km^2) was higher than 9.8–11.2 adult foxes/km^2 of the urban foxes in Zurich, Switzerland (Gloor, 2002). Thus, ecological features of the fox inhabiting Mt. Hakodate could be similar to those of urban foxes. The high population density of the Mt. Hakodate fox could result from the abundance of food resource on Mt. Hakodate or utilization of anthropogenic food resource in the circumjacent residential region. It is necessary to further investigate food

habits in the population.

SEX RATIO IN THE INDIVIDUAL-IDENTIFIED FOXES

According to the sex identification, although the sex ratio of the Mt. Hakodate fox was slightly biased toward male (proportion of male: 55.2%), the proportion of males in the present study is lower rather than that in the previous study (62.6–69.9%) (Uraguchi, Takahashi & Maekawa, 1991), which was done based on foxes killed by hunters in Hokkaido. In the three years of the investigation period, the estimated number of individuals was increased. Because the term was short, it is not clear whether such a shift is temporary or not.

SMALL HOME RANGE SIZE LIKE URBAN FOXES

The estimated home ranges of foxes were overlapped with each other, indicating that they share the environment and resources. The home ranges of fox have been reported by a number of previous studies until now. The smallest and largest mean home range sizes of fox were approximately 25 ha (White, Saunders & Harris, 1996) and 1600 ha (Jones & Theberge, 1982), respectively. To define the level of home range size, Adkins & Stott (1998) categorized the reported mean home range sizes into three classes: ‘small (< 100 ha)’, ‘medium (100–500 ha)’ and ‘large (> 500 ha)’. According to the definition,

the mean home range size of the Mt. Hakodate population (14.4 ha) is small, and at the same level as those of urban/suburban foxes, such as the foxes of Bristol, U.K. (45 ha) (Harris, 1980) and Boar's Hill, U.K. (71 ha) (Macdonald, 1981). The smaller home range on Mt. Hakodate could allow foxes to live with a higher density within a restricted area.

GENETIC VARIATION INFLUENCED BY GEOGRAPHICAL ISOLATION

The current result of the genetic structure analysis clearly revealed evidence that the Mt. Hakodate population was genetically differentiated from the Southern population of Hokkaido. This indicates that the Mt. Hakodate population has been geographically isolated by the sea and the main urban zone of Hakodate City for a long time. It is also likely that the genetic communication between Mt. Hakodate and the Kameda Peninsula of the Hokkaido Mainland was blocked by the urban area of minimum approximately 8.5 km in direct distance at least after development of the city. Because straight-line dispersal distances of foxes is up to 302 km (Allen & Sargeant, 1993), the genetic communication should be restricted by distance. The Mt. Hakodate population must have originated from the southern Hokkaido population before development of the city. Actually, the current results show that the genetic structure of the partial individuals from south area in southern Hokkaido is similar to that from Mt.

Hakodate. The results in the present study presented scenario how the Mt. Hakodate population has been genetically isolated. In an early stage, there were red foxes having some restricted alleles on the south edge of southern Hokkaido. Mt. Hakodate was originally a lonely island and connected to the Hokkaido mainland 5,000 years ago (Ganzawa, 2002). Probably, the red fox has colonized on Mt. Hakodate since that time, and could have come and gone between Mt. Hakodate and the mainland (see the map of Fig. III-1). From the Meiji period (1868–1912) in Japan, a modern city was developed on the land bridge connecting between Mt. Hakodate and the mainland with the population increase (Hakodate City, 1990). The frequency of genetic communication could have gradually decreased from that time. After them, inbreeding within the Mt. Hakodate population could have produced some genotypes unique to the south edge area. The lower genetic diversity of the Mt. Hakodate population indicates the progression of relative mating in this small population.

General Conclusion

This dissertation includes new findings on morphology, adaptive evolution and ecological genetics in the red fox of Hokkaido, compared with the previously reported population genetic features based on neutral genetic markers.

In Chapter I, geographical variation in the skulls and teeth of the red fox population on Hokkaido Island was discussed. Only the limited cranial characters showed the morphological differences with a gradient increase from west to east. Potential selective forces could have worked on adaptations to local habitat conditions such as climate conditions, food habits and vegetations.

In Chapter II, geographical variation of the MHC class II *DRB* allelic frequencies was found. The MHC diversity of the southern Hokkaido population was lower than those of the other populations in Hokkaido. This is consistent with previous findings based on neutral genetic markers (Oishi *et al.*, 2011). The MHC genes encode a set of cell surface proteins essential for the acquired immune system to recognize foreign molecules. Therefore, the bias could be attributed to regionally different pathogen-driven balancing selection.

In Chapter III, ecological characteristics of the red foxes isolated on Mt. Hakodate were revealed. The results showed that the population density on the Mt. Hakodate is very high. Mt. Hakodate is adjacent in the city area; therefore, Mt. Hakodate population

would be able to use specific urban resources such as food waste, like urban foxes. As the Mt. Hakodate population has been geographically isolated from adjacent rural area by the urban area and sea, the population of Mt. Hakodate is genetically differentiated from those of the other regions in Hokkaido.

The present study added new knowledges in the phylogeography of the red fox on Hokkaido Island. The red fox had been introduced from the Eurasian Continent to Hokkaido Island three times in the period from the Late Pleistocene to the Early Holocene (Kutschera *et al.*, 2013). However, the distribution patterns and its processes in Hokkaido after the colonization remained unclear. Based on the distribution pattern of the three mtDNA lineages shown by Inoue *et al.* (2007), red foxes could dispersed across Hokkaido regardless of the phylogenetic lineage and geographical conditions. However, increased temperature after the last glacial maximum (LGM) changed not only climatic pattern but also vegetation on Hokkaido. The environmental factors could have affected the distribution of the red fox (Oishi *et al.*, 2011), and then the Hokkaido population has been mainly separated into two group, Southern and the other subpopulations, in the short span of time. The Southern subpopulation was genetically and ecologically isolated from the other subpopulation, and adapted to the environment unique to southern Hokkaido, resulting in a reduction in genetic diversity. The state of

genetic differences was correlated with morphological differences. Although individual phenomena were shown in the present study, any direct relationships between genetic and morphological features are unclear. Finally, we found and analyzed a relatively rare case of the red fox population, which has been isolated from the rural area by the urban area and sea. In addition, the genetic diversity of the population was decreased by isolation. Although the Mt. Hakodate population has been isolated for only a hundred and several tens of years, it actually has adapted to the restricted area. The present data demonstrate that the red fox has the very high adaptability to the various environments.

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List of Tables

Table I-1. Numbers of red fox samples examined in the present study.

Groups	Males	Females	Total
Southern	18 (11/6/1)	10 (5/4/1)	28 (16/10/2)
Central	91 (44/43/4)	62 (29/29/4)	153 (73/72/8)
Eastern	28 (13/15/0)	16 (9/7/0)	44 (22/22/0)
Total	137 (68/64/5)	88 (43/40/5)	225 (111/104/10)

The numbers of adult, subadult and either are shown in parentheses.

Table I-2. Factor loadings of the Bayesian principal component analysis (BPCA) on skulls and teeth of the red fox on Hokkaido.

Skull					Tooth				
Measurements	Male		Female		Measurements	Male		Female	
	PC1	PC2	PC1	PC2		PC1	PC2	PC1	PC2
RL	-3.08	0.33	-2.80	0.47	CL	-0.32	0.08	-0.28	0.01
GL	-6.11	0.35	-5.51	0.23	CW	-0.19	0.02	-0.16	-0.03
NL	-2.67	0.09	-2.57	-0.13	CH	-0.95	0.54	-0.91	0.43
RW	-0.81	-0.29	-0.60	-0.14	P1L	-0.24	-0.07	-0.08	-0.06
IC	-1.04	-1.06	-0.80	-0.86	P2L	-0.46	-0.15	-0.38	-0.17
PW	-1.51	-2.15	-1.09	-2.12	P3L	-0.48	-0.16	-0.38	-0.17
PoC	-0.13	-0.72	0.13	-0.76	P4L	-0.54	-0.26	-0.36	-0.17
SphW	-0.33	-0.30	-0.47	-0.40	P4W	-0.35	-0.08	-0.28	-0.20
W	-0.64	-0.32	-0.40	-0.49	M1L	-0.36	-0.12	-0.30	-0.07
ZW	-2.29	-1.52	-1.87	-1.38	M1W	-0.47	-0.13	-0.41	-0.10
SH	-0.74	-0.40	-0.60	-0.54	M2L	-0.19	0.01	-0.10	-0.03
UT	-2.49	0.27	-2.43	0.19	M2W	-0.24	-0.02	-0.19	-0.10
CBL	-5.74	0.37	-5.20	0.27	cL	-0.29	0.11	-0.38	0.01
PL	-3.25	0.22	-2.92	0.43	cW	-0.21	0.01	-0.18	-0.02
MD	-0.62	-0.41	-0.52	-0.41	cH	-0.81	0.47	-0.80	0.33
MtW	-1.19	-0.11	-1.01	-0.46	p1L	-0.16	-0.08	-0.04	-0.05
OCW	-0.77	-0.02	-0.38	-0.18	p2L	-0.45	-0.17	-0.34	-0.18
ML	-4.48	0.17	-4.29	0.36	p3L	-0.38	-0.13	-0.31	-0.15
LT	-2.87	0.40	-2.51	0.08	p4L	-0.42	-0.18	-0.31	-0.13
MH	-0.71	-0.26	-0.69	-0.34	p4W	-0.23	-0.07	-0.11	-0.07
MT	-0.22	-0.09	-0.18	-0.08	m1L	-0.54	-0.21	-0.47	-0.16
ACP	-1.78	0.07	-1.48	-0.34	m1W	-0.25	-0.05	-0.18	-0.12
c–p1	-0.38	-0.24	-0.35	-0.26	m2L	-0.22	-0.04	-0.16	-0.04
p1–p4	-1.35	0.40	-1.50	0.23	m2W	-0.16	-0.06	-0.10	-0.09
m1–m3	-0.68	0.11	-0.41	0.11					
Contribution	0.79	0.06	0.77	0.07		0.57	0.12	0.56	0.11
Cummurative contribution rate	0.79	0.85	0.77	0.84		0.57	0.69	0.56	0.67

Table I-3. *F* statistics and *P* values in ANOVAs among groups at the first (PC1) and second (PC2) principal component scores of skull and dental measurements in the red fox on Hokkaido.

Region	Sex	PC1			PC2		
		F	*P*		*F*	*P*	
Skull							
	Male	0.54	0.5853	ns	7.15	0.0011	**
	Female	0.30	0.7439	ns	3.17	0.0472	*
Tooth							
	Male	0.62	0.5420	ns	3.89	0.0228	*
	Female	0.99	0.3776	ns	2.59	0.0809	ns

**: $P < 0.01$, *: $P < 0.05$, ns: not significant.

Table I-4. Means (mm) and standard deviations, and *F* -statistics (*F*) and *P* values (*P*) in ANOVAs among the Southern (S), Central (C), and Eastern (E) groups at skull measurements in the red fox on Hokkaido.

Measurements	Males						Females					
	N (S/C/E)	S	C	E	*F*	*P*	*N*	S	C	E	*F*	*P*
RL	18/91/28	61.71 ± 3.07	63.10 ± 3.01	62.53 ± 3.62	1.60	ns	10/61/16	59.59 ± 3.25	60.57 ± 2.96	59.42 ± 2.38	1.29	ns
GL	18/88/28	143.72 ± 6.30	145.38 ± 5.80	144.66 ± 6.87	0.61	ns	10/60/16	139.72 ± 5.83	139.81 ± 5.76	137.87 ± 3.94	0.81	ns
NL	18/91/28	56.16 ± 2.69	56.45 ± 3.18	56.27 ± 3.60	0.08	ns	10/62/16	53.87 ± 3.57	54.18 ± 3.23	52.51 ± 2.32	1.83	ns
RW	18/89/26	24.02 ± 1.04	23.65 ± 1.22	23.80 ± 1.28	0.77	ns	9/60/16	22.60 ± 0.92	22.62 ± 1.05	22.17 ± 0.84	1.26	ns
IC	16/91/28	26.47 ± 1.09	27.04 ± 1.83	27.82 ± 1.73	3.52	*	10/62/16	24.84 ± 1.08	25.44 ± 1.51	26.27 ± 1.66	3.12	*
PW	15/90/26	33.48 ± 1.69	33.77 ± 3.11	35.16 ± 2.95	2.52	ns	9/60/15	31.25 ± 2.67	31.82 ± 2.90	32.80 ± 2.36	1.04	ns
PoC	17/91/27	20.61 ± 1.35	21.23 ± 1.33	22.16 ± 1.22	8.24	***	10/61/16	20.46 ± 0.84	21.10 ± 1.30	21.72 ± 0.87	3.57	*
SphW	17/86/25	35.31 ± 1.22	35.69 ± 1.12	36.07 ± 1.07	2.38	ns	10/59/15	34.70 ± 1.39	34.95 ± 1.21	35.16 ± 0.62	0.48	ns
W	17/87/25	45.95 ± 1.21	46.35 ± 1.23	46.96 ± 1.22	3.83	*	10/59/15	45.53 ± 1.15	45.23 ± 1.17	45.50 ± 0.75	0.58	ns
ZW	17/87/26	75.48 ± 3.17	74.01 ± 3.10	75.45 ± 2.94	3.22	*	9/59/16	72.51 ± 1.99	70.68 ± 2.84	71.70 ± 1.60	2.57	ns
SH	18/85/25	39.77 ± 1.53	40.49 ± 1.17	40.76 ± 0.79	3.98	*	10/60/16	39.32 ± 1.34	39.20 ± 1.29	39.63 ± 1.17	0.70	ns
UT	18/91/28	62.61 ± 2.82	63.76 ± 2.54	63.69 ± 3.09	1.38	ns	10/62/16	60.97 ± 2.64	61.54 ± 2.75	61.55 ± 2.05	0.21	ns
CBL	18/87/26	136.16 ± 6.56	137.73 ± 5.44	137.55 ± 5.88	0.57	ns	10/61/16	132.43 ± 5.63	132.76 ± 5.46	131.73 ± 3.86	0.25	ns
PL	18/91/28	71.21 ± 3.71	71.93 ± 3.28	71.40 ± 3.68	0.50	ns	10/61/16	68.97 ± 3.10	69.63 ± 3.23	68.66 ± 2.16	0.74	ns
MD	18/89/27	17.76 ± 1.09	17.19 ± 1.33	17.36 ± 1.21	1.58	ns	10/60/16	17.29 ± 0.73	16.72 ± 1.26	16.94 ± 1.15	1.08	ns
MtW	18/85/25	44.10 ± 1.48	44.78 ± 1.73	45.47 ± 1.43	3.68	*	10/58/16	43.45 ± 1.68	43.96 ± 1.59	44.83 ± 1.56	2.72	ns
OCW	18/85/25	24.81 ± 1.27	25.53 ± 1.11	25.93 ± 1.17	5.04	**	10/62/16	24.51 ± 0.69	25.03 ± 0.85	25.11 ± 0.68	2.07	ns
ML	18/91/27	104.76 ± 4.72	105.79 ± 4.59	105.04 ± 4.54	0.55	ns	10/62/16	101.23 ± 4.59	101.36 ± 4.76	99.81 ± 2.50	0.79	ns
LT	15/90/27	70.36 ± 3.71	72.04 ± 2.83	72.10 ± 3.34	2.05	ns	9/61/16	68.50 ± 3.16	69.43 ± 2.95	69.35 ± 1.85	0.44	ns
MH	18/91/28	15.53 ± 1.22	15.33 ± 1.02	15.44 ± 0.90	0.35	ns	10/62/16	15.02 ± 0.86	14.81 ± 1.16	14.74 ± 0.70	0.22	ns
MT	18/90/28	6.87 ± 0.45	6.43 ± 0.38	6.58 ± 0.50	8.81	***	10/60/15	6.34 ± 0.26	6.28 ± 0.45	6.22 ± 0.36	0.27	ns
ACP	18/91/27	38.25 ± 2.22	37.83 ± 2.16	37.80 ± 2.09	0.32	ns	10/62/16	37.08 ± 1.44	36.15 ± 2.07	35.65 ± 1.39	1.73	ns
c–p1	17/91/26	4.27 ± 0.91	3.80 ± 0.83	4.01 ± 0.96	2.36	ns	10/60/16	3.85 ± 0.99	3.63 ± 0.89	3.79 ± 0.77	0.39	ns
p1–p4	17/91/28	33.57 ± 1.64	34.48 ± 1.56	34.37 ± 1.97	2.19	ns	10/62/16	32.64 ± 1.74	33.20 ± 2.24	33.43 ± 0.95	0.49	ns
m1–m3	15/89/27	24.84 ± 1.21	25.83 ± 1.02	26.00 ± 1.42	5.74	**	10/62/16	24.80 ± 1.08	25.01 ± 0.96	25.05 ± 0.71	0.25	ns

***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, ns: not significant.

Table I-5. Means (mm) and standard deviations of measurement values, and *F*-statistics (*F*) and *P* values (*P*) in ANOVA among the Southern (S), Central (C), and Eastern (E) groups at dental measurements in the red fox on Hokkaido.

Measurements	Males						Females					
	N (S/C/E)	S	C	E	*F*	*P*	*N* (S/C/E)	S	C	E	*F*	*P*
CL	18/89/27	6.79 ± 0.35	6.80 ± 0.43	6.66 ± 0.51	1.13	ns	10/60/16	6.12 ± 0.34	6.38 ± 0.43	6.15 ± 0.29	3.34	*
CW	18/89/28	4.39 ± 0.21	4.35 ± 0.26	4.31 ± 0.31	0.55	ns	9/58/16	4.04 ± 0.28	4.09 ± 0.26	3.93 ± 0.11	2.75	ns
CH	12/72/27	19.42 ± 0.92	19.63 ± 1.11	19.27 ± 1.36	0.98	ns	7/46/12	18.49 ± 1.16	18.50 ± 1.18	18.00 ± 1.08	0.90	ns
P1L	17/90/25	4.64 ± 0.25	4.73 ± 0.35	4.69 ± 0.45	0.46	ns	10/59/16	4.52 ± 0.31	4.67 ± 0.25	4.58 ± 0.23	1.84	ns
P2L	17/89/28	9.04 ± 0.51	8.96 ± 0.55	8.85 ± 0.75	0.60	ns	10/60/16	8.72 ± 0.39	8.71 ± 0.52	8.66 ± 0.48	0.06	ns
P3L	18/91/28	9.69 ± 0.49	9.70 ± 0.61	9.71 ± 0.69	0.01	ns	10/61/15	9.50 ± 0.50	9.37 ± 0.53	9.45 ± 0.36	0.37	ns
P4L	18/91/28	14.48 ± 0.59	15.17 ± 0.58	15.17 ± 0.99	8.08	***	10/60/16	14.03 ± 0.38	14.62 ± 0.63	14.52 ± 0.21	4.82	*
P4W	17/91/28	6.84 ± 0.42	6.87 ± 0.50	6.78 ± 0.56	0.39	ns	10/60/16	6.57 ± 0.37	6.53 ± 0.48	6.57 ± 0.40	0.08	ns
M1L	18/90/28	9.42 ± 0.56	9.59 ± 0.45	9.72 ± 0.60	1.96	ns	10/62/15	9.34 ± 0.36	9.36 ± 0.45	9.49 ± 0.48	0.52	ns
M1W	18/91/28	12.70 ± 0.51	13.00 ± 0.58	12.99 ± 0.76	1.87	ns	10/62/16	12.52 ± 0.44	12.63 ± 0.58	12.43 ± 0.48	0.91	ns
M2L	18/90/28	5.40 ± 0.33	5.40 ± 0.41	5.35 ± 0.35	0.20	ns	10/60/15	5.51 ± 0.39	5.25 ± 0.28	5.38 ± 0.33	3.67	*
M2W	17/91/27	8.61 ± 0.35	8.59 ± 0.52	8.55 ± 0.50	0.10	ns	10/60/16	8.37 ± 0.36	8.40 ± 0.52	8.39 ± 0.45	0.01	ns
cL	18/86/27	7.45 ± 0.40	7.21 ± 0.52	7.12 ± 0.46	2.55	ns	8/57/15	6.96 ± 0.40	6.82 ± 0.54	6.66 ± 0.32	1.03	ns
cW	18/88/27	4.75 ± 0.23	4.69 ± 0.30	4.57 ± 0.32	2.58	ns	8/57/15	4.26 ± 0.29	4.37 ± 0.28	4.24 ± 0.16	1.95	ns
cH	14/77/25	17.62 ± 0.66	17.45 ± 0.99	17.18 ± 1.39	0.93	ns	6/46/15	15.74 ± 0.84	16.43 ± 1.04	15.82 ± 0.74	3.13	ns
p1L	16/83/27	4.04 ± 0.22	4.16 ± 0.32	4.08 ± 0.36	1.39	ns	10/58/16	4.11 ± 0.22	4.11 ± 0.29	4.05 ± 0.23	0.33	ns
p2L	18/90/27	8.89 ± 0.47	8.87 ± 0.52	8.78 ± 0.80	0.27	ns	10/60/16	8.61 ± 0.29	8.52 ± 0.53	8.43 ± 0.33	0.45	ns
p3L	18/89/28	9.22 ± 0.38	9.26 ± 0.45	9.30 ± 0.68	0.14	ns	10/60/16	8.97 ± 0.44	8.96 ± 0.48	9.01 ± 0.28	0.07	ns
p4L	18/91/28	10.08 ± 0.42	10.25 ± 0.48	10.24 ± 0.75	0.77	ns	10/61/16	9.65 ± 0.37	9.82 ± 0.44	9.85 ± 0.36	0.80	ns
p4W	17/89/27	4.19 ± 0.26	4.30 ± 0.32	4.24 ± 0.41	1.06	ns	10/61/16	3.94 ± 0.22	4.08 ± 0.23	3.96 ± 0.20	2.77	ns
m1L	18/90/28	15.27 ± 0.56	15.73 ± 0.58	15.66 ± 0.98	3.37	*	10/62/16	14.87 ± 0.61	15.07 ± 0.67	15.11 ± 0.43	0.51	ns
m1W	18/90/27	6.00 ± 0.32	5.98 ± 0.32	6.11 ± 0.34	1.73	ns	10/62/16	5.83 ± 0.19	5.74 ± 0.30	5.80 ± 0.34	0.57	ns
m2L	18/91/27	6.93 ± 0.37	7.10 ± 0.38	7.10 ± 0.42	1.50	ns	10/61/16	7.05 ± 0.42	6.97 ± 0.35	6.98 ± 0.39	0.20	ns
m2W	18/88/26	5.45 ± 0.31	5.50 ± 0.30	5.39 ± 0.33	1.29	ns	10/62/16	5.37 ± 0.26	5.33 ± 0.28	5.39 ± 0.34	0.32	ns

***: $P < 0.001$, *: $P < 0.05$, ns: not significant.

Table I-6. Pearson's correlation coefficients *r* between measurements in skulls and teeth of the red fox on Hokkaido and latitude (LAT), longitude (LON), minimum mean monthly temperature (MMT_{min}), maximum mean monthly temperature (MMT_{max}), and snowfall (SNOW).

Measurements	LAT		LON		MMT_{min}		MMT_{max}		SNOW	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
IC	0.03 ns	-0.04 ns	0.15 ns	0.31 **	-0.10 ns	-0.27 *	0.16 ns	0.00 ns	-0.11 ns	-0.26 *
PoC	0.01 ns	0.06 ns	0.38 ***	0.39 ***	-0.28 ***	-0.25 *	-0.08 ns	-0.19 ns	-0.18 *	-0.24 *
W	0.08 ns	-0.04 ns	0.33 ***	0.15 ns	-0.23 **	-0.13 ns	-0.21 *	-0.09 ns	-0.06 ns	-0.12 ns
SH	0.07 ns	0.00 ns	0.22 *	0.13 ns	-0.15 ns	-0.15 ns	-0.13 ns	-0.06 ns	-0.03 ns	-0.09 ns
MtW	-0.05 ns	0.02 ns	0.23 **	0.31 **	-0.10 ns	-0.26 *	0.00 ns	-0.13 ns	-0.18 *	-0.19 ns
OCW	0.09 ns	0.09 ns	0.27 **	0.18 ns	-0.17 ns	-0.16 ns	-0.06 ns	-0.14 ns	-0.17 ns	-0.03 ns
m1–m3	0.18 *	-0.03 ns	0.16 ns	0.04 ns	-0.06 ns	-0.12 ns	-0.07 ns	0.04 ns	0.03 ns	-0.02 ns
P4L	0.29 ***	0.33 **	0.17 *	0.12 ns	-0.03 ns	-0.05 ns	-0.14 ns	-0.11 ns	0.13 ns	0.11 ns

***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, ns: not significant.

Table II-1. Assignment of fox populations of Hokkaido for each *K* in SAMOVA.

	Southern				Central							Northern							Eastern		Far Eastern	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	(4)	(14)	(10)	(24)	(12)	(28)	(16)	(10)	(50)	(44)	(6)	(16)	(32)	(12)	(16)	(4)	(10)	(30)	(52)	(16)	(14)	(44)
K = 2	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
K = 3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	2	2
K = 4	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	3	2	2	2	4	2	2
K = 5	1	2	1	1	3	3	3	3	3	3	4	3	3	3	3	3	3	3	3	5	3	3
K = 6	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	5	4	4	4	6	4	4
K = 7	1	2	1	1	3	3	3	3	3	3	3	3	3	4	5	6	3	3	3	7	3	3
K = 8	1	2	1	1	3	3	3	3	3	3	3	3	3	4	5	6	3	3	3	7	8	3
K = 9	1	1	1	1	2	3	4	4	4	4	2	4	5	4	6	6	4	5	7	8	9	9
K = 10	1	1	1	1	2	2	3	3	3	3	2	3	4	5	6	6	7	4	8	9	10	10

The numbers in header indicate locations (see Fig. II-1): 1, Otobe; 2, Mori; 3, Yakumo; 4, Imakane; 5, Iwanai; 6, Muroran; 7, Shiraoi; 8, Samani; 9, Sapporo; 10, Iwamizawa; 11, Furano; 12, Mashike; 13, Asahikawa; 14, Tomamae; 15, Teshio; 16, Wakkanai; 17, Esashi; 18, Shari; 19, Obihiro; 20, Kushiro; 21, Nakashibetsu; 22, Nemuro. Numbers of sequences obtained from diploid individuals (2N) are shown in parentheses. The same numbers for each *K* in table body indicate the local populations are genetically and geographically homogeneous. Underlines show singletons, of which groups consisted of only single genetic population.

Table II-2. Pairwise F_{ST} (lower matrix) and P values (upper matrix) among defined groups of the red fox on Hokkaido.

	Southern	Central	Northern	Eastern	Far Eastern
Southern		<0.001	<0.001	<0.001	<0.001
Central	**0.182**		0.153	<0.001	<0.001
Northern	**0.227**	0.005		<0.001	<0.001
Eastern	**0.265**	**0.062**	**0.040**		0.171
Far Eastern	**0.207**	**0.066**	**0.047**	0.013	

Significant F_{ST} values ($P < 0.05$) are indcated by bold.

Table II-3. Allele and sequence diversities of the MHC class II *DRB* in the red fox on Hokkaido.

	N	$2N$	A	A_R	H_E	H_O	π	θ (per site)	D_T	
Southern	26	52	5	5.000	0.546	0.500	0.020	0.022	-0.301	(Not significant)
Central	83	166	11	9.137	0.822	0.855	0.053	0.034	1.638	(Not significant)
Northern	60	120	13	10.745	0.841	0.850	0.060	0.035	2.124	($P < 0.05$)
Eastern	34	68	11	9.801	0.771	0.618	0.055	0.039	1.338	(Not significant)
Far Eastern	29	58	10	9.784	0.855	0.862	0.057	0.041	1.273	(Not significant)
All	232	464	17	10.768	0.850	0.780	0.054	0.030	2.331	($P < 0.05$)

N, number of individuals; $2N$, number of obtained sequences; A, number of alleles; A_R, allelic richness; H_E, expected heterozygosity; H_O, obserbed heterozygosity; π, nucleotide diversity; θ, $4N\mu$ for autosomal genes of diploid organisms, D_T, Tajima's D.

Table II-4. Frequencies of MHC class II *DRB* alleles in the red fox on Hokkaido.

	Southern	Central	Northern	Eastern	Far Eastern	All
*Vuvu-DRB*09*	**0.654**	**0.127**	0.033	0.044	**0.103**	**0.147**
*Vuvu-DRB*10*	**0.154**	**0.193**	**0.225**	**0.324**	**0.241**	**0.222**
*Vuvu-DRB*11*	0.077	**0.313**	**0.258**	0.074		**0.198**
*Vuvu-DRB*12*	0.038	**0.151**	**0.175**	**0.338**	**0.241**	**0.183**
*Vuvu-DRB*13*	0.077	0.054	0.008	0.015		0.032
*Vuvu-DRB*14*		0.006	0.025	0.015	**0.103**	0.024
*Vuvu-DRB*15*		0.030	0.067	0.044	0.034	0.039
*Vuvu-DRB*16*		0.024	0.058	0.015		0.026
*Vuvu-DRB*17*		0.048	0.083		0.017	0.041
*Vuvu-DRB*18*		0.006				0.002
*Vuvu-DRB*19*		0.048	0.025	**0.103**	0.086	0.050
*Vuvu-DRB*20*			0.017			0.004
*Vuvu-DRB*21*			0.008		0.017	0.004
*Vuvu-DRB*22*			0.017		0.086	0.015
*Vuvu-DRB*23*				0.015		0.002
*Vuvu-DRB*24*				0.015		0.002
*Vuvu-DRB*25*					0.069	0.009

Frequencies of more than 0.1 are indicated by bold.

Table II-5. Rates of non-synonymous (d_N) and synonymous (d_S) substitutions (± standard error, SE) for the antigen-binding sites (ABS) and non-ABS at the MHC class II *DRB* gene of the Hokkaido red fox.

Position	Number of codons	d_N (± SE)	d_S (± SE)	ω (d_N/d_S)	*P*
ABS	17	0.423 (± 0.101)	0.156 (± 0.078)	2.720	0.008
Non-ABS	62	0.021 (± 0.009)	0.018 (± 0.011)	1.122	0.871
All	79	0.088 (± 0.020)	0.042 (± 0.015)	2.109	0.013

Table III-1. Information of each microsatellite locus in the red fox on Mt. Hakodate.

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

*, Same characters indicate that their primers are included in same mix.

†, Lade *et al*. (1996)

††, Wandeler & Funk (2006)

Table III-2. Information of all 35 individuals identified in this study and the estimated home range sizes.

Individual ID	N_{obs}[a] for the entire period	N_{obs}[a] per year			Sex[b]	Home range sizes (ha)[c]
		2009	2010	2011		
MH01	7	2	5		M	9.7
MH02	10	8	1	1	F	68.2
MH03	8	3	1	4	F	27.8
MH04	1	1			U	–
MH05	6	3	3		F	9.3
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	8.2
MH13	3		3		U	3.5
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	9.0
MH20	3			3	M	3.4
MH21	3			3	M	1.0
MH22	2			2	F	–
MH23	1			1	M	–
MH24	1			1	U	–
MH25	1			1	F	–
MH26	1			1	F	–
MH27	4			4	M	1.1
MH28	1			1	U	–
MH29	1			1	F	–
MH30	1			1	U	–
MH31	1			1	U	–
MH32	3			3	F	17.5
MH33	1			1	M	–
MH34	1			1	M	–
MH35	1			1	M	–
Total N_{obs}[a]	85	25	19	41	M40/F35/U10	–
Mean	2.4	2.1	1.7	1.9	–	14.4
Number of pertinent individuals	35	12	11	22	M16/F13/U6	11

[a] N_{obs}, number of observations

[b] M, male; F, female; U, unknown.

[c] Individuals found at three or more sampling points were shown.

Table III-3. Pairwise F_{ST} (lower matrix) and P values (upper matrix) among red fox population on Mt. Hakodate and the other populations in Hokkaido using the published microsatellite data of Oishi *et al*. (2011).

	Mt.Hakodate	Oshima	Southern†	Central†	Northern†	Eastern†	Nakashibetsu†	Nemuro†
Mt.Hakodate	-	**	***	***	***	***	***	***
Oshima	0.08	-	ns	***	***	***	***	***
Southern†	0.14	0.01	-	***	***	***	***	***
Central†	0.16	0.09	0.08	-	***	***	***	***
Northern†	0.15	0.07	0.08	0.01	-	***	***	***
Eastern†	0.13	0.06	0.08	0.03	0.02	-	***	***
Nakashibetsu†	0.15	0.07	0.12	0.07	0.04	0.03	-	ns
Nemuro†	0.16	0.09	0.12	0.07	0.05	0.03	0.00	-

†, data of Oishi et al. (2011)

***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

Table III-4. Comparison of red fox microsatellite variation of the Mt. Hakodate population with those of the other populations in Hokkaido based on published data of Oishi *et al* . (2011).

Populations	N	A	A_R	H_E	H_O	F_{IS}
Mt.Hakodate	36	3.250	2.592	0.476	0.454	0.047
Oshima	6	3.500	3.409	0.618	0.596	0.043
Southern†	28	5.000	3.612	0.645	0.625	0.033
Central†	68	7.250	3.937	0.659	0.629	0.046
Northern†	80	8.375	4.061	0.671	0.651	0.030
Eastern†	35	6.375	3.995	0.658	0.660	-0.004
Nakashibetsu†	14	5.500	3.807	0.625	0.588	0.063
Nemuro†	25	6.125	3.737	0.630	0.629	0.001

†, data of Oishi *et al* . (2011)

N, number of individuals; A , number of alleles; A_R, allelic richness; H_E, expected heterozygosity, H_O, obserbed heterozygosity; F_{IS}, inbreeding coefficient

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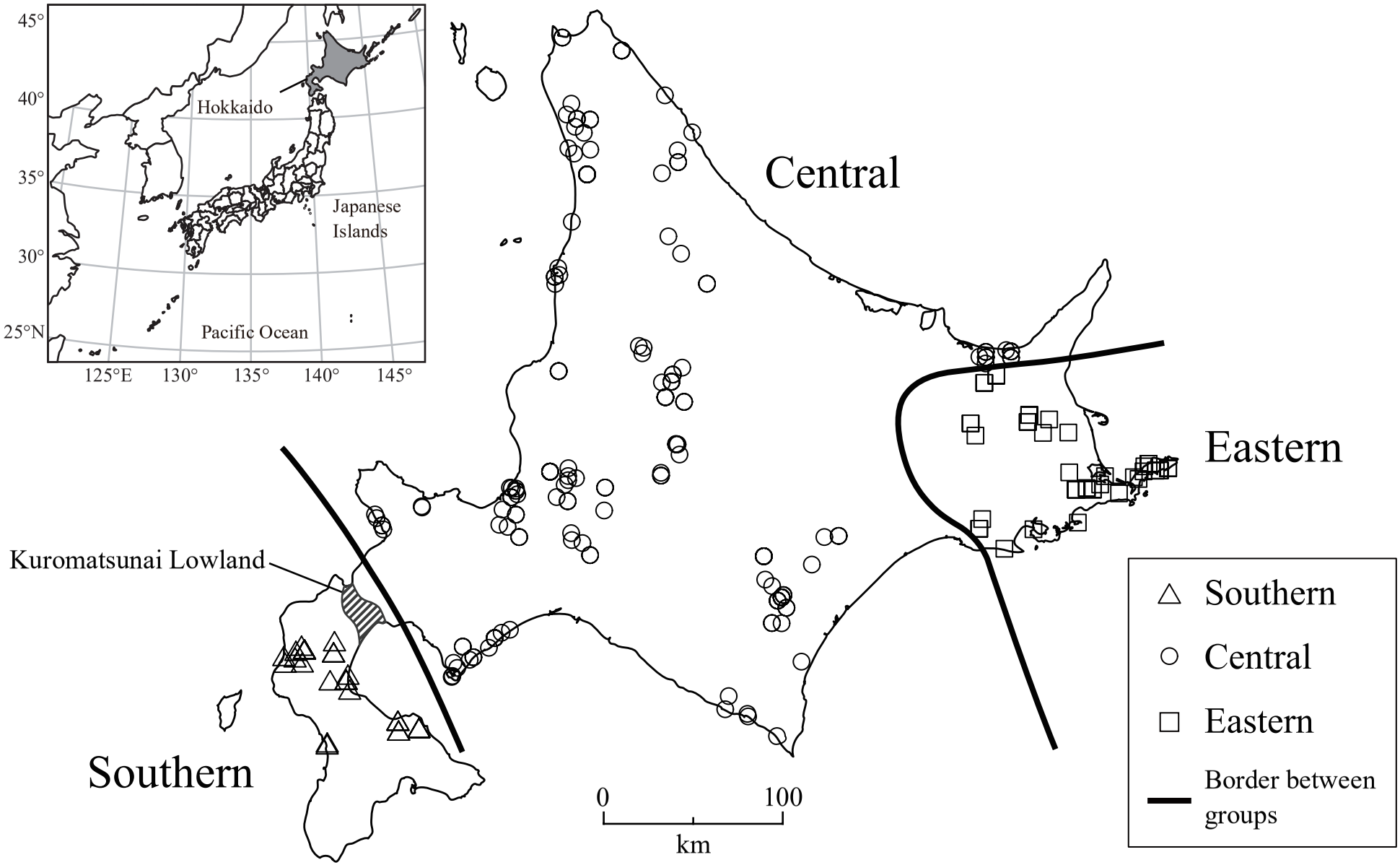

Figure I-1. Sample localities for the red fox (*Vulpes vulpes*) on Hokkaido Island, Japan, and geographical divisions based on a GENELAND analysis of the microsatellite data of Oishi *et al*. (2011). The Hokkaido population is geographically subdivided into three groups: Southern (triangles), Central (circles) and Eastern (squares). Each mark indicates an individual fox.

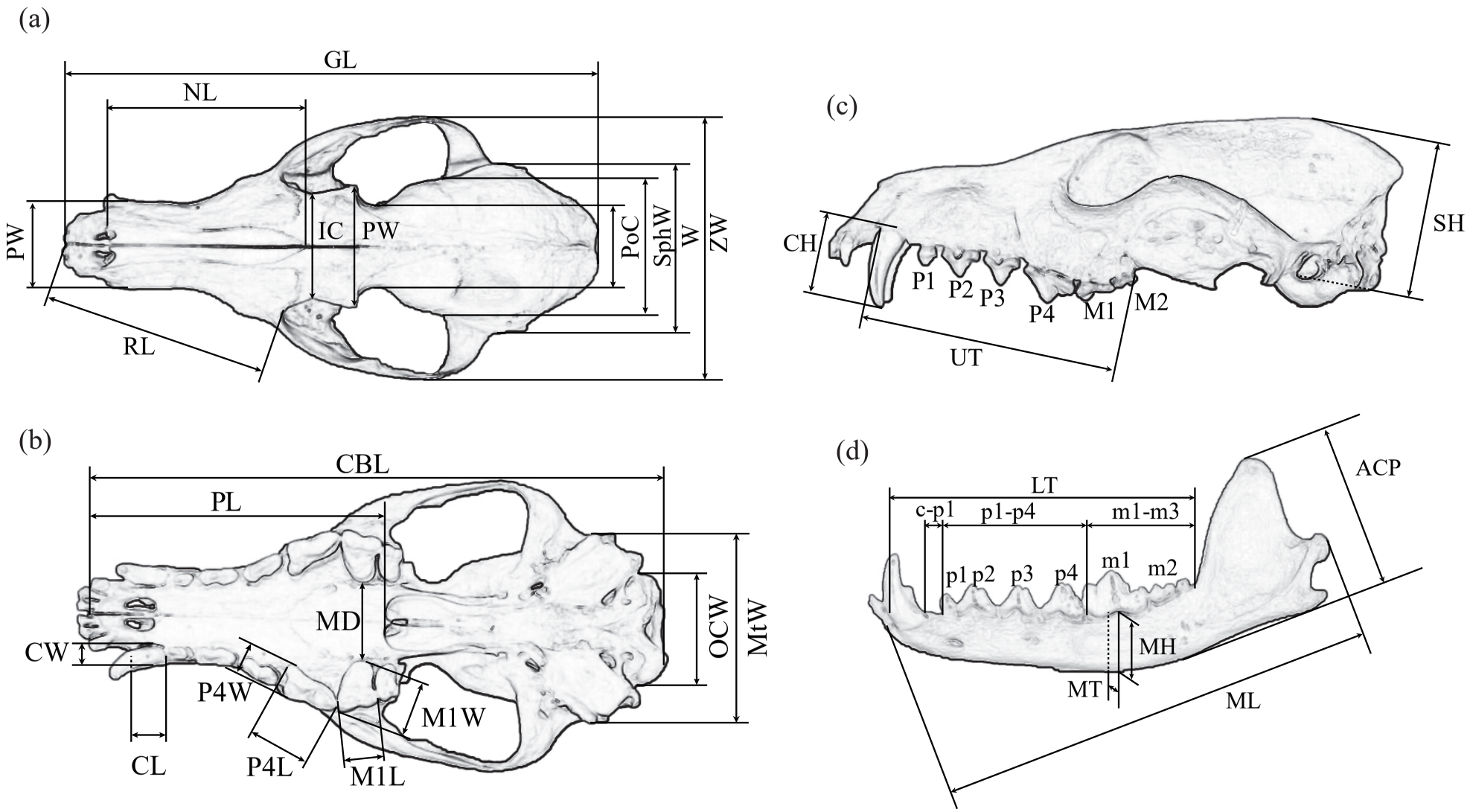

Figure I-2. Definitions of skull and dental measurements for the red fox on Hokkaido. (a) Dorsal view of skull, (b) ventral view of skull, (c) left view of skull, (d) left view of mandible. For definitions of abbreviations, refer to Materials and Methods.

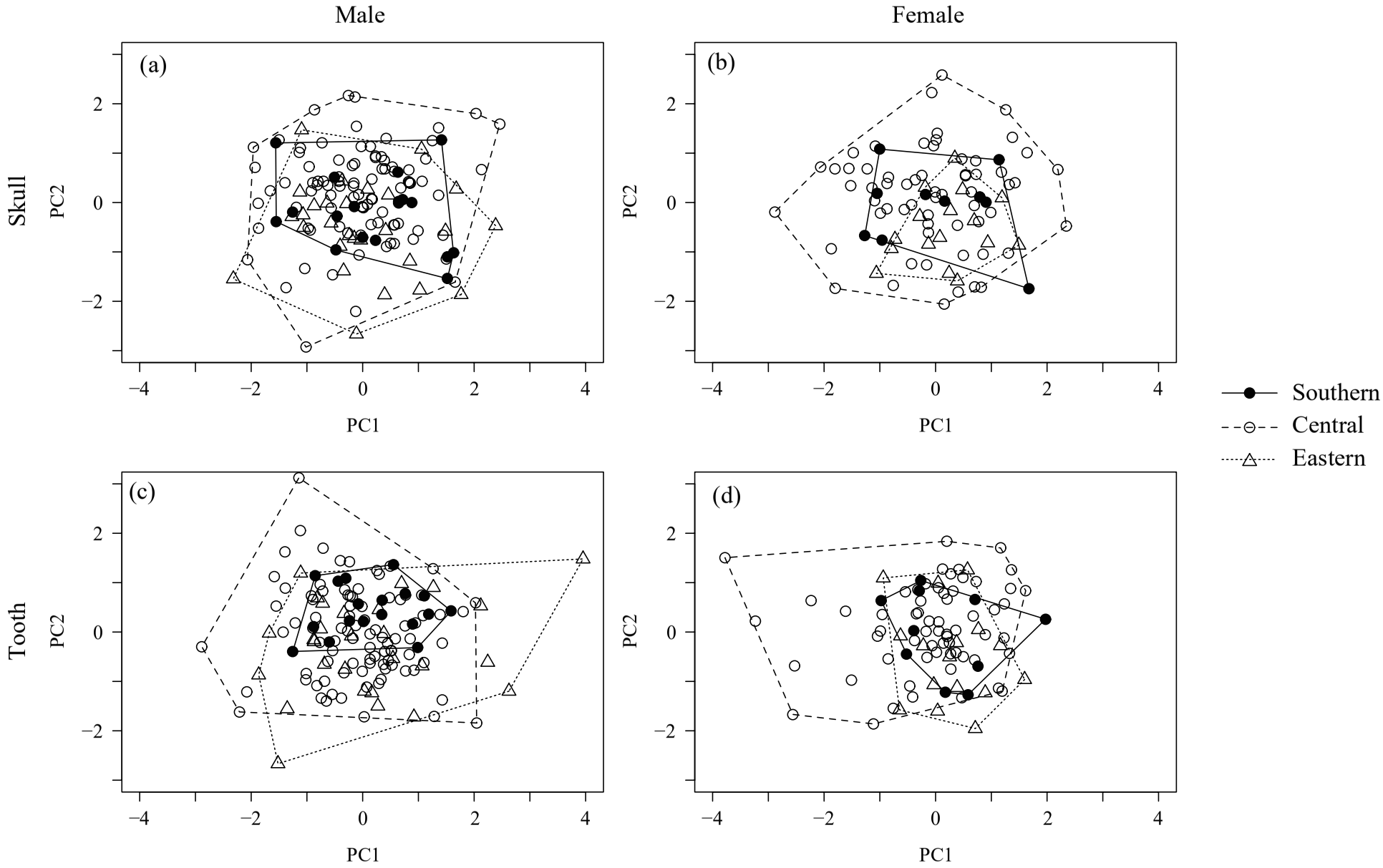

Figure I-3. Plots of the Bayesian principal component analysis (BPCA) scores for each group, based on measurements in (a) male skulls, (b) female skulls, (c) male teeth, and (d) female teeth. The horizontal and vertical axes indicate the first (PC1) and second (PC2) principal components, respectively. Closed circles, open circles, and triangles indicate individuals from the Southern, Central, and Eastern groups, respectively.

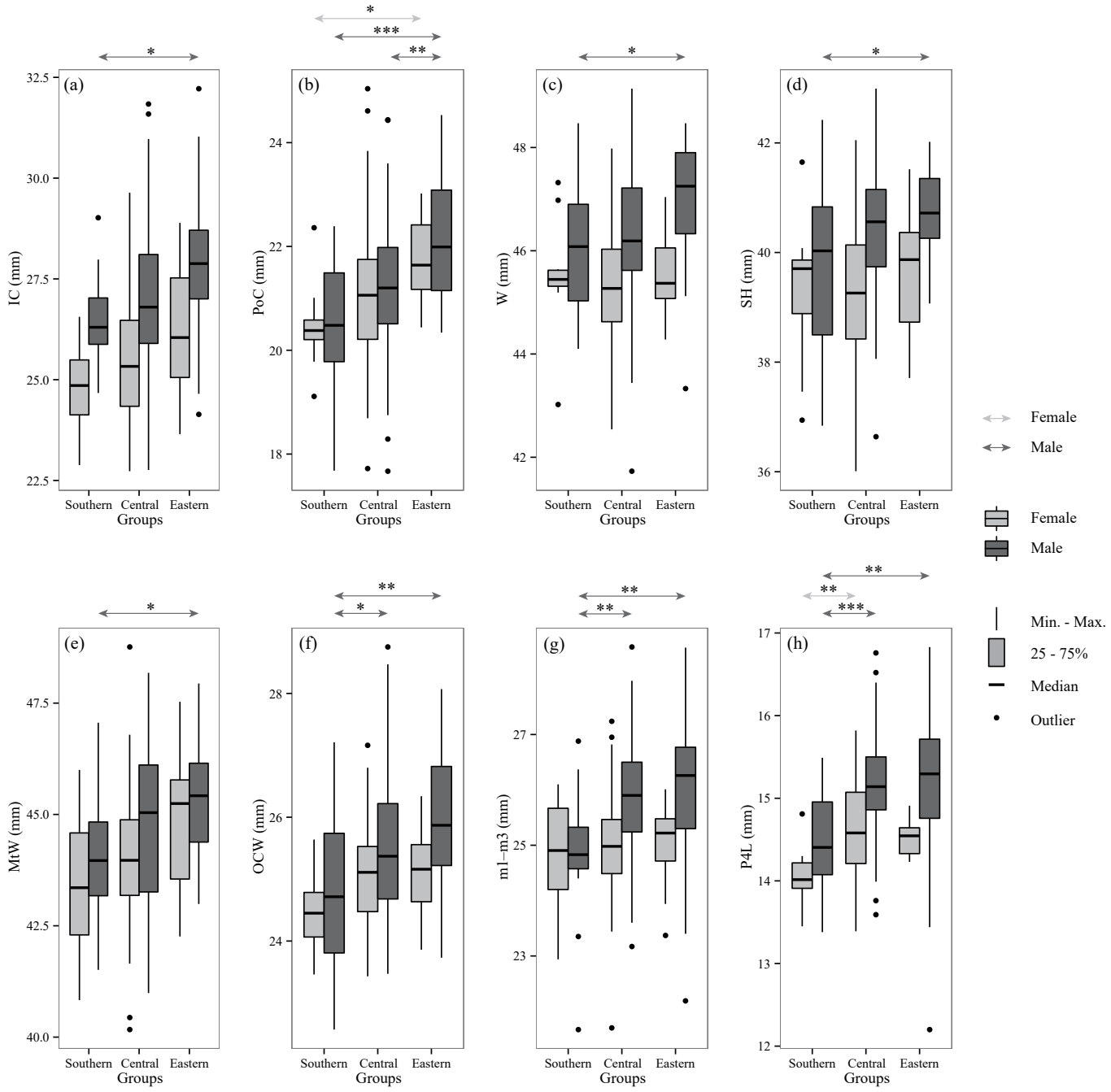

Figure I-4. Boxplots for (a) IC, (b) PoC, (c) W, (d) SH, (e) MtW, (f) OCW, (g) m1–m3, and (h) P4L for the red fox on Hokkaido. Double-headed arrows indicate significant differences between groups, detected by multiple comparisons. Significant levels are as the following: ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.

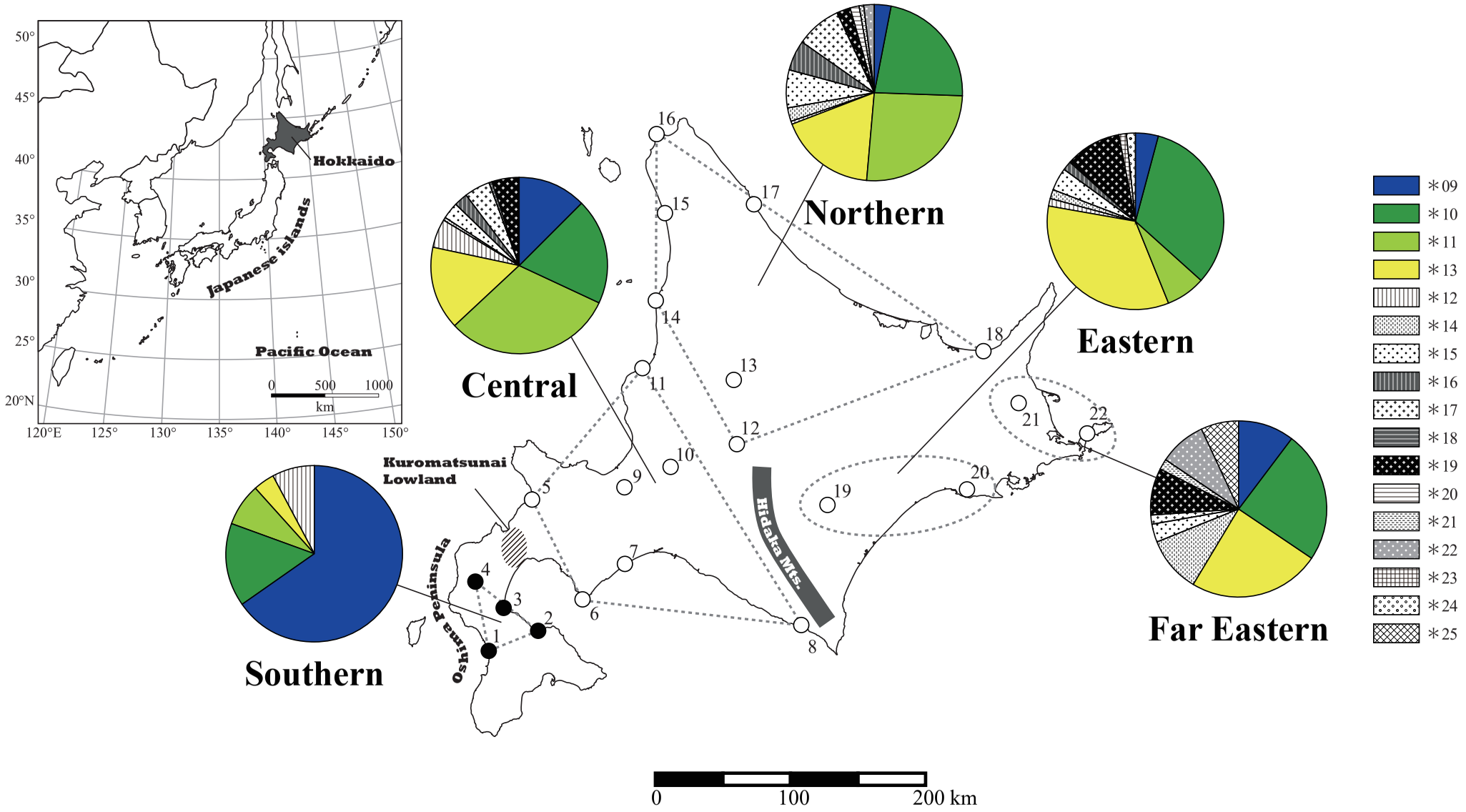

Figure II-1. Location of the Japanese islands (left) and Hokkaido Island (right). Sampling locations and geographical distribution of DRB allele frequencies of the red fox populations on Hokkaido Island. Open and solid circles indicate Clusters (Group) I and II, respectively, estimated by SAMOVA for $K = 2$ (see Table II-1, including the location names). The bar shows geographical distances.

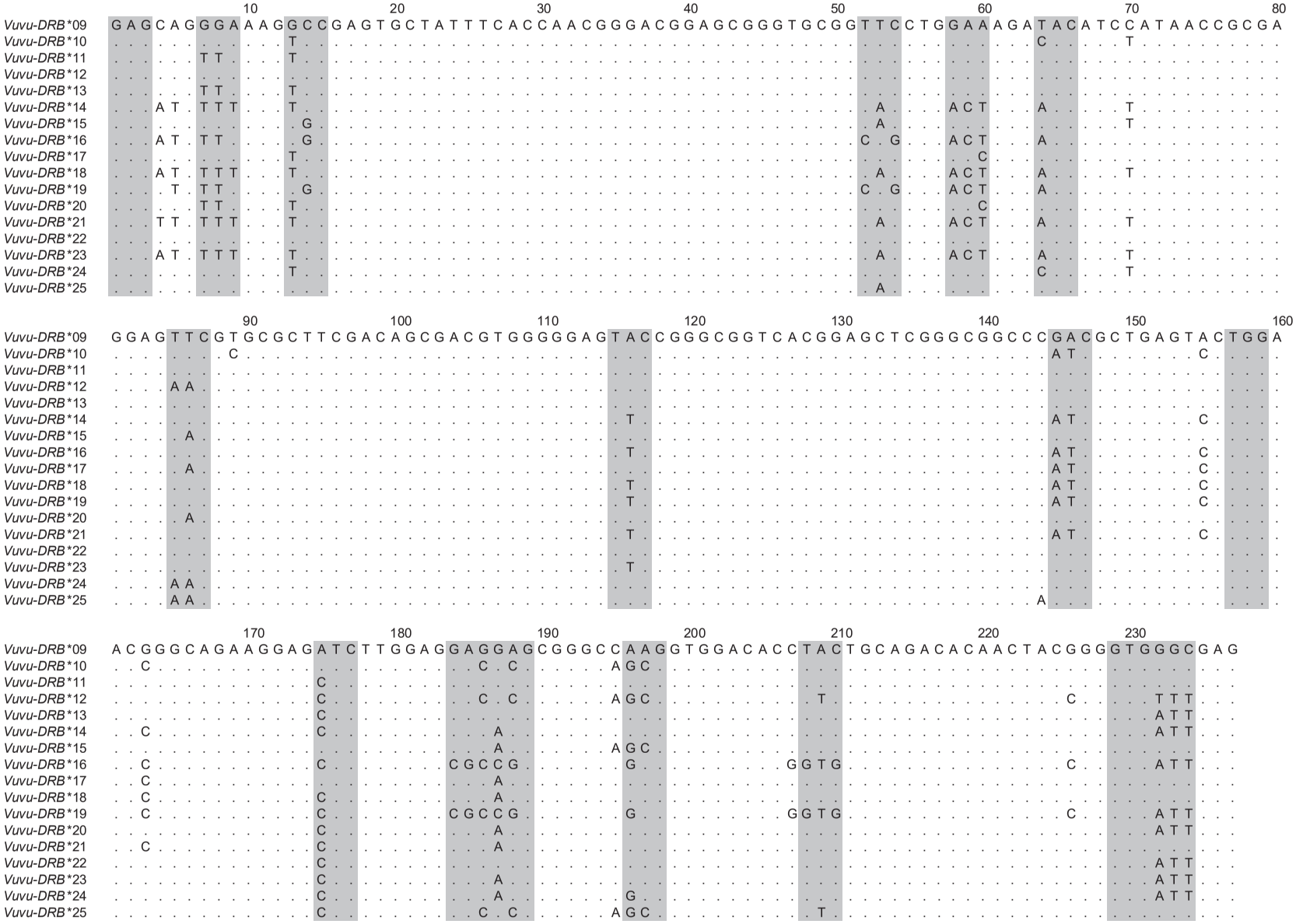

Figure II-2. Nucleotide sequences of MHC class II *DRB* exon 2 in the red fox on Hokkaido. Dots indicate identity with nucleotide of allele *Vuvu-DRB*09*. The codons for antigen binding sites (ABS) are shadowed in gray, according to human MHC (HLA) (Bondinas *et al*., 2007).

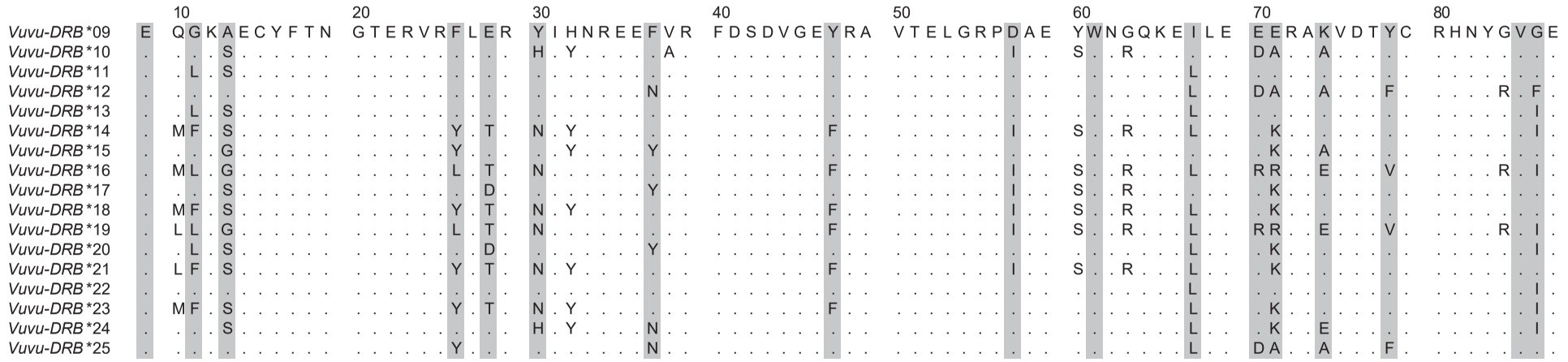

Figure II-3. Amino acid sequences deduced from MHC class II *DRB* exon 2 in the red fox on Hokkaido. Dots indicate identity with amino acid of allele *Vuvu-DRB*09*. The predicted antigen binding sites (ABS) are shadowed in gray, according to human MHC (HLA) (Bondinas *et al*., 2007).

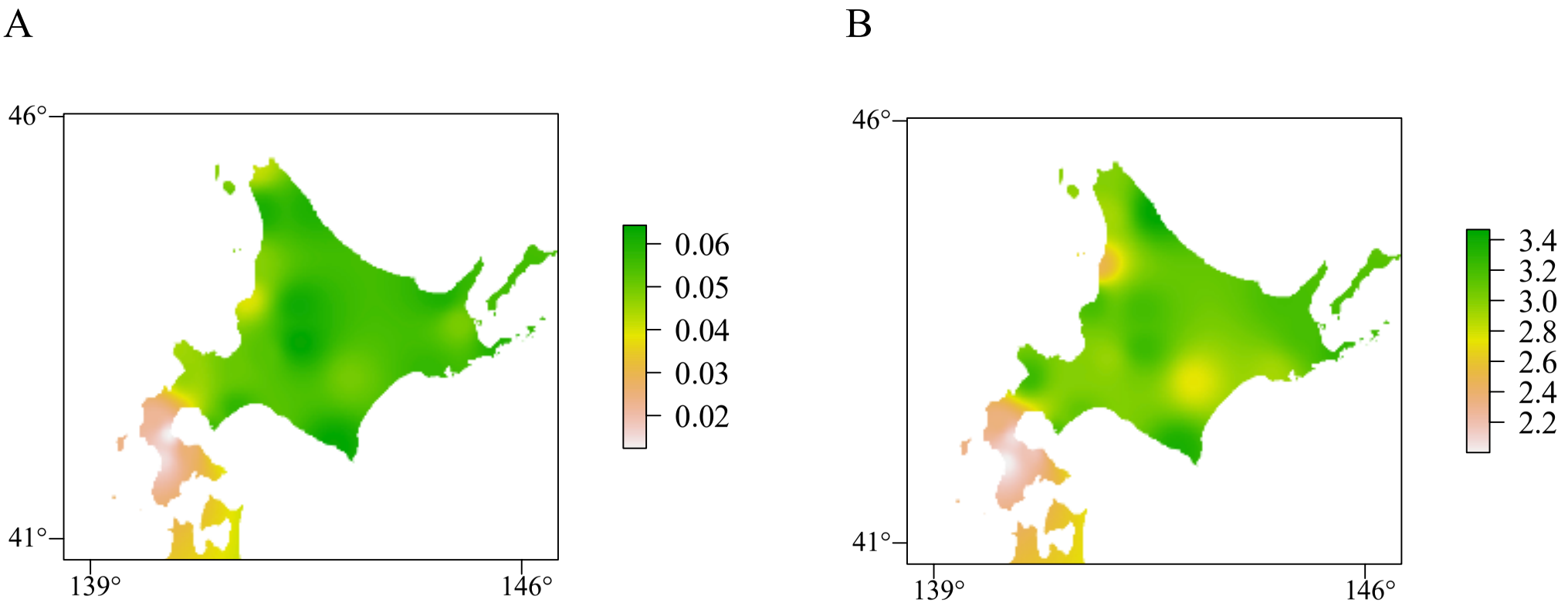

Figure II-4. Distribution patterns of nucleotide diversity (π) (A) and allelic richness (A_R) (B) in the red fox on Hokkaido.

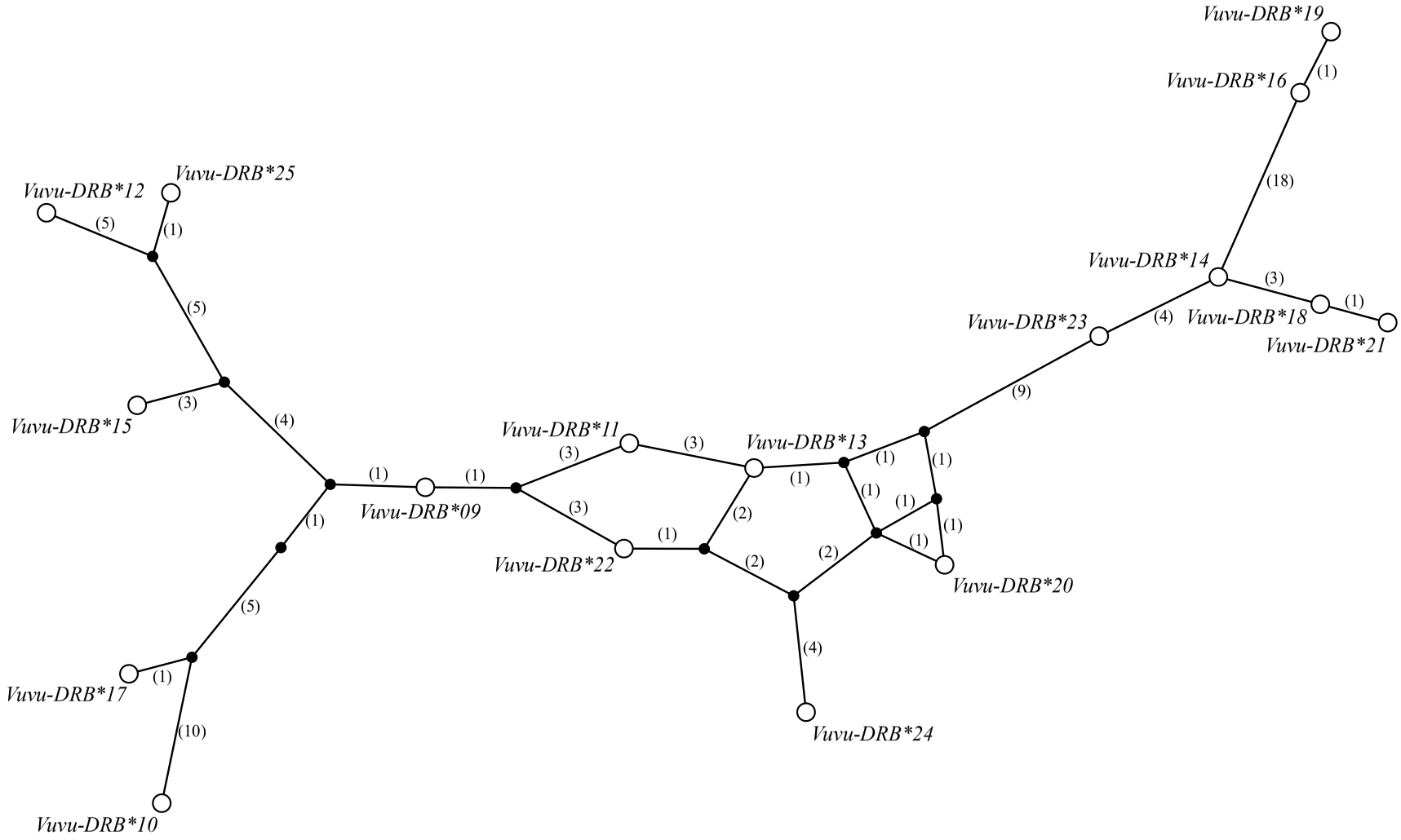

Figure II-5. Haplotype network of MHC class II *DRB* exon 2 sequences in the red fox on Hokkaido. Hypothesized alleles are indicated by small closed circles. The numbers of mutational steps are shown in parentheses.

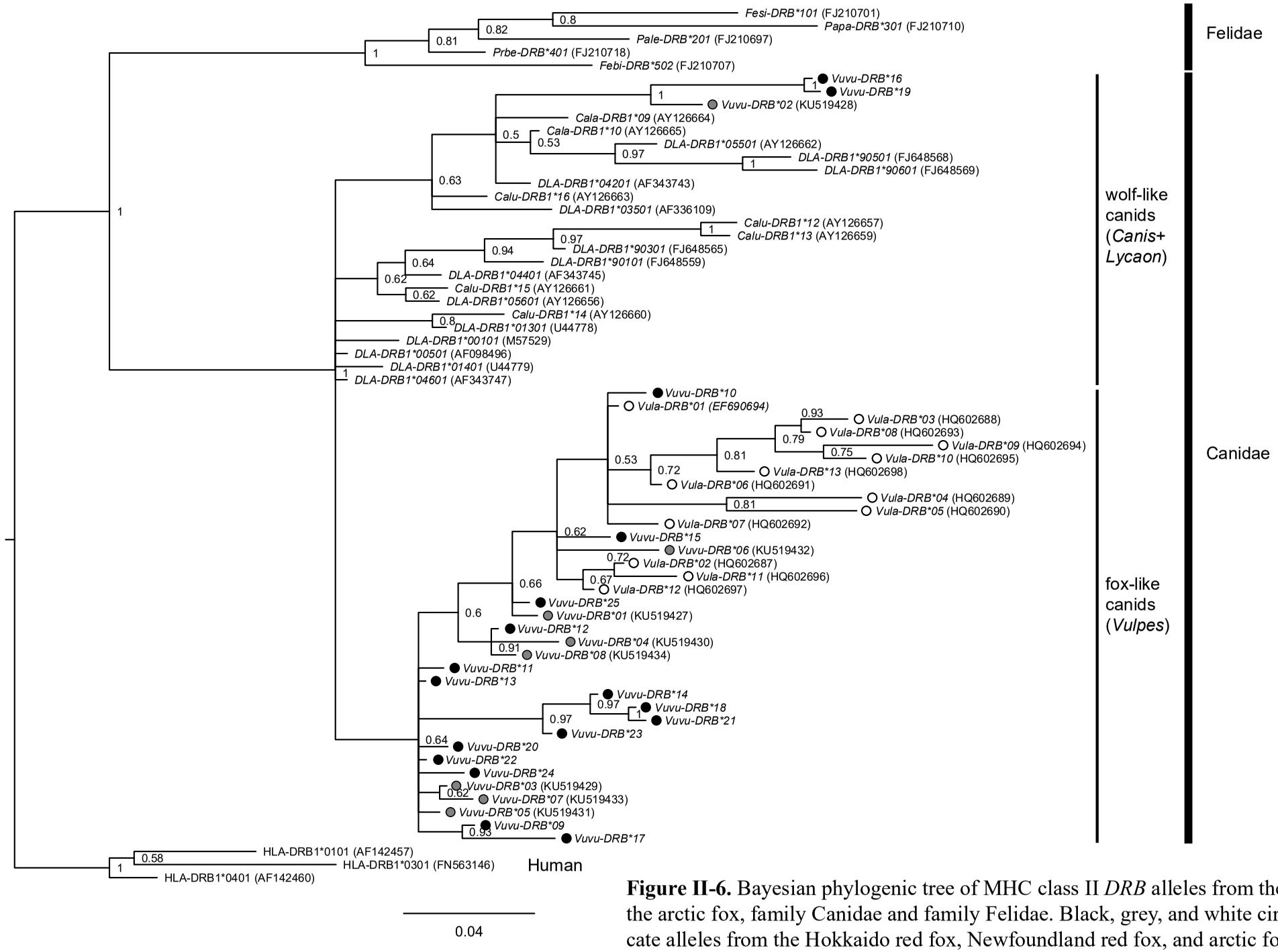

Figure II-6. Bayesian phylogenic tree of MHC class II *DRB* alleles from the red fox, the arctic fox, family Canidae and family Felidae. Black, grey, and white circles indicate alleles from the Hokkaido red fox, Newfoundland red fox, and arctic fox, respectively. Genbank accession numbers of previously published nucleotide sequences are indicated in parentheses. Numbers near nodes mean posterior probabilities.

Figure III-1. Feces sampling locations in this study. (A) All areas of Japan, (B) southern Hokkaido of Japan, and (C) Mt. Hakodate. White and black circles show sampling points of feces. Grey curves show climbing routes. Map of (C) was drawn by Quantum GIS 1.7.1 Wroclaw using the GIS Base Map Information obtained from Geospatial Information Authority of Japan (http://www.gsi.go.jp/kiban/).

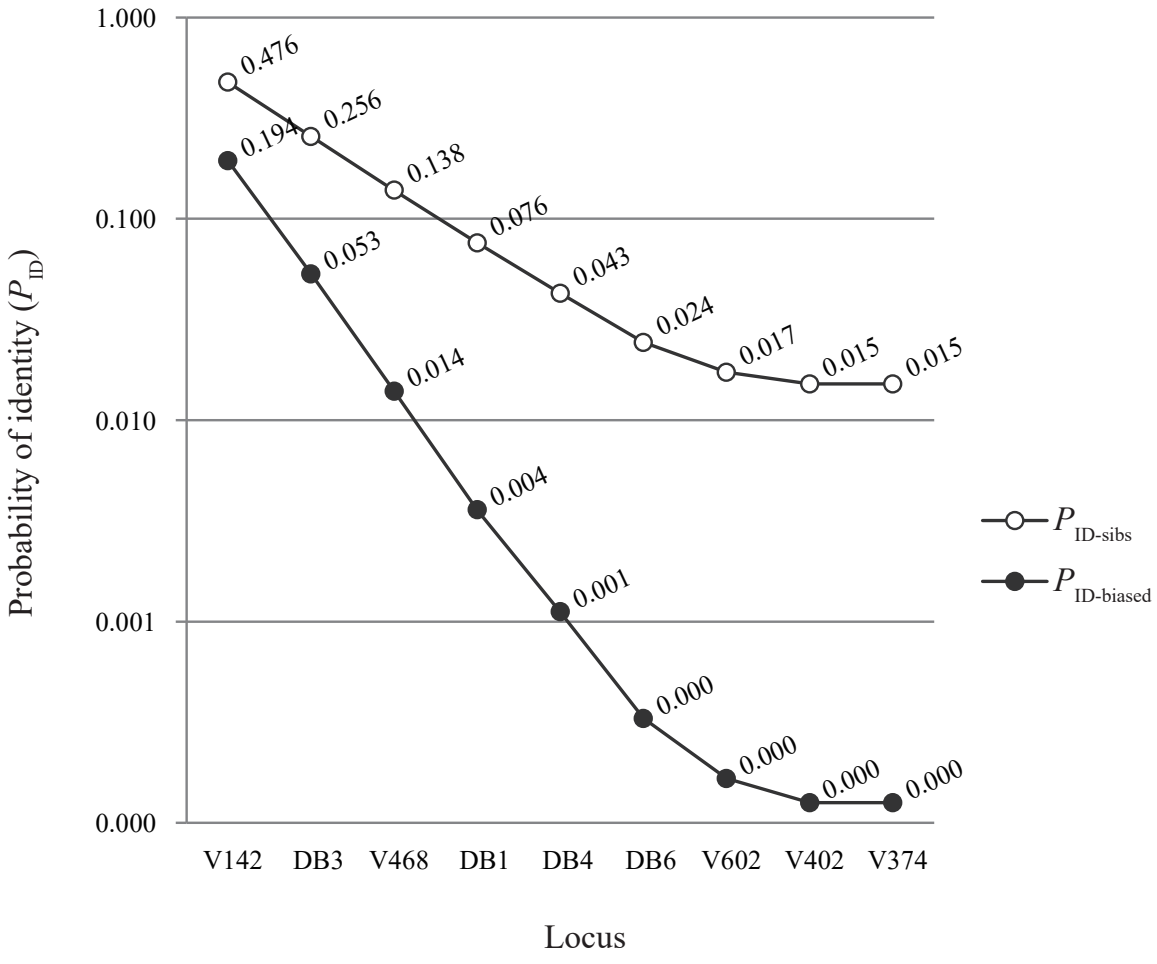

Figure III-2. Probability of identity (P_{ID} and $P_{ID\text{-}sibs}$) calculated from fox microsatellite data in this study.

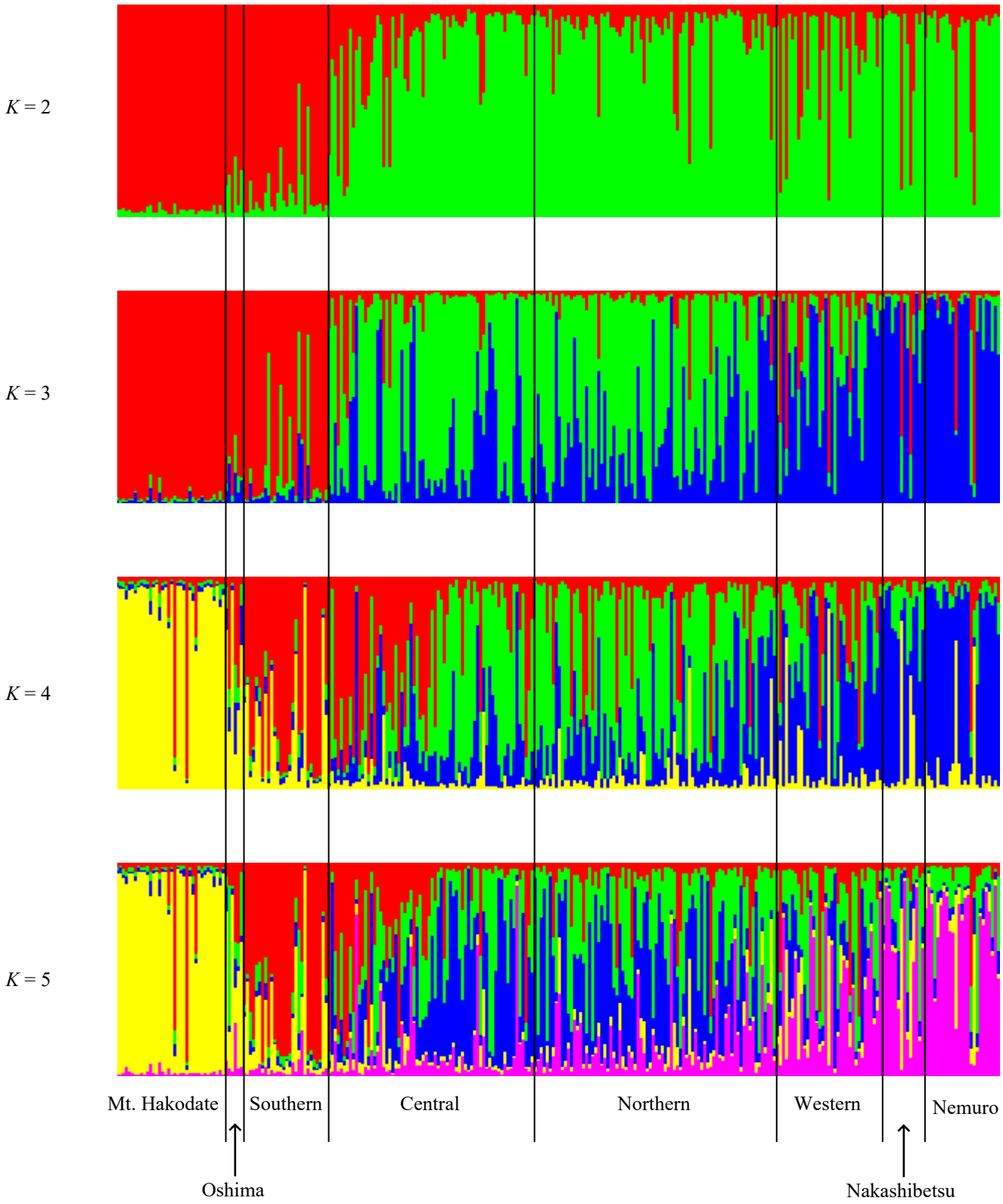

Figure III-3. Results of the STRUCTURE analysis (K = 2–5) of the Mt. Hakodate population and the other fox populations in Hokkaido.

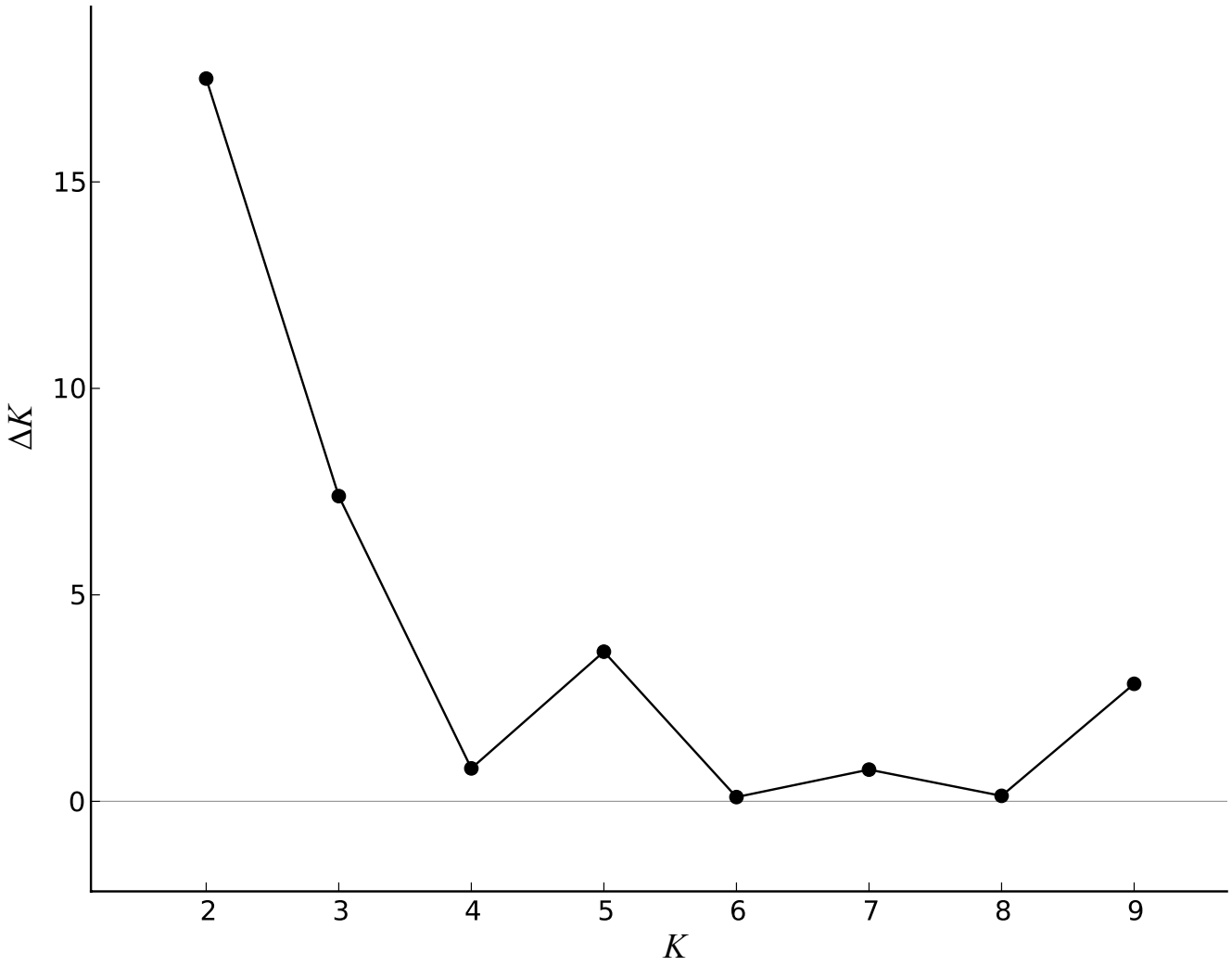

Figure III-4. Changes of the Δ*K* values calculated using STRUCTURE HARVESTER on the basis of the STRUCTURE results of the red fox populations in Hokkaido.

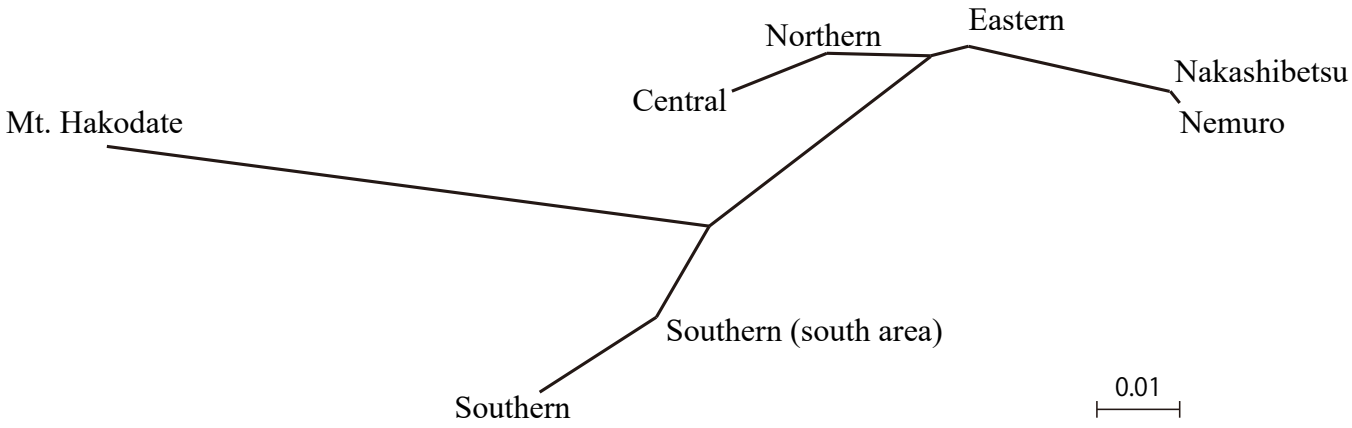

Figure III-5. Network of fox populations in Hokkaido, constructed by F_{ST}.

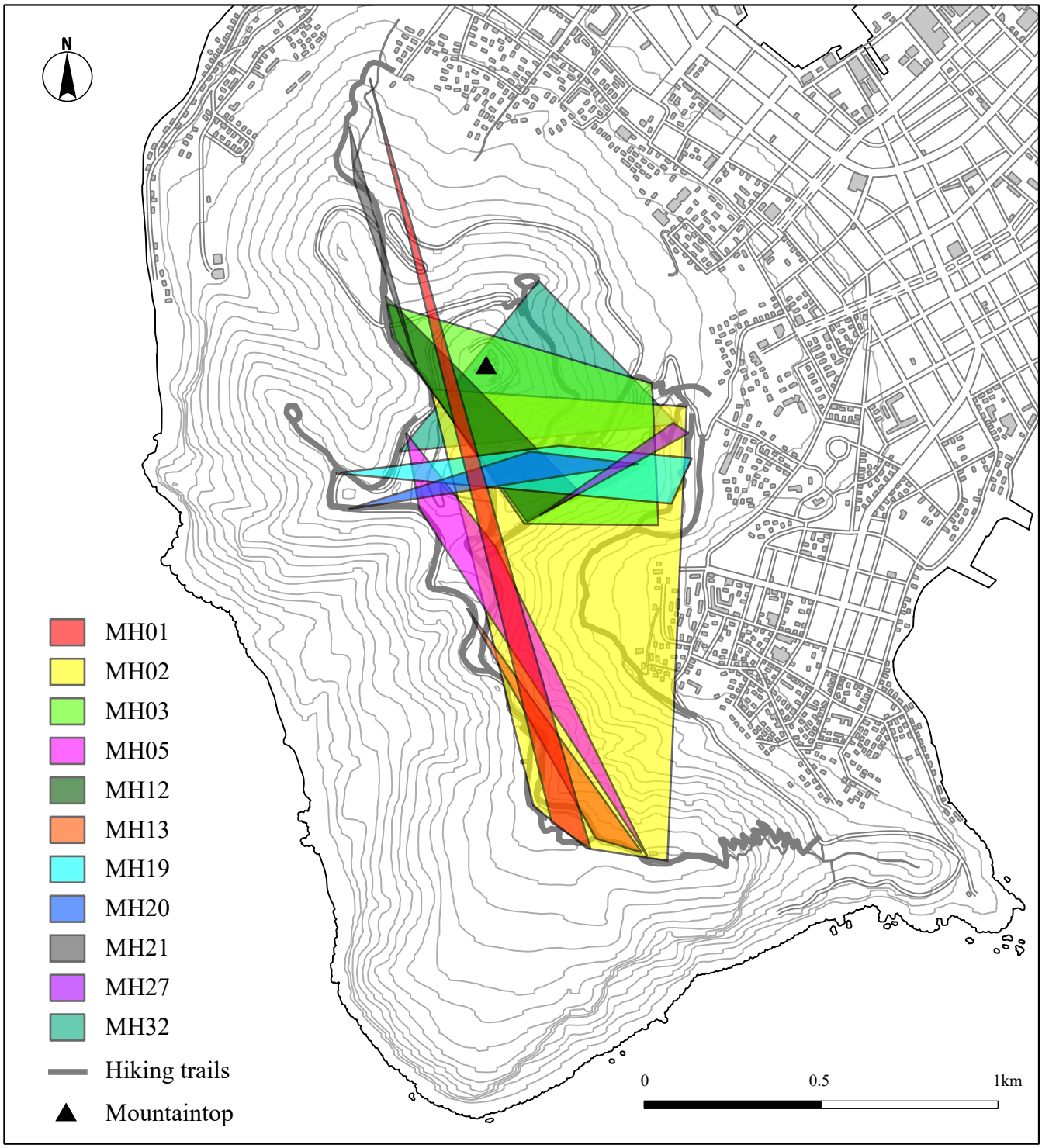

Figure III-6. Home range distributions of 11 fox individuals identified by fecal DNA analysis. They are shown by different colors for different individuals.