Studies on spatial genetic structure in an expanding sika deer (*Cervus nippon*) population of Hokkaido: the implication for management

個体数が増加し、分布が拡大しているエゾシカ個体群における遺伝的な空間構造に関する研究：
個体群管理と関連させて

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This thesis is dedicated to my parents,

OU Xiquan and YU Xiufang.

For their endless love, support and encouragement

Contents

Chapter 1

General introduction

Increase and expansion of deer population

In the late half of the 19th century, the numbers and range of deer declined due to overexploitation in Europe, North America and Japan. However, the population of deer recovered and increased rapidly across Europe, North America and Japan in response to changes in their environment and reductions of hunting and natural predators (Fuller & Gill 2001; Jedrzejewska et al. 1997; Leopold et al. 1947; McShea et al. 1997; Mysterud et al. 2000; Brown et al. 2000; Diefenbach et al. 1997; Porter & Underwood 1999; Kaji et al. 2010).

As deer become overabundant, deer damage to agriculture and forestry increased with negative economic values (Conover 1997). The deer damage is considered a major problem in the United States and in Austria, where their annual impacts are estimated at more than $750 million (Conover 1997) and more than $220 million (Reimoser 2003), respectively. And increased vehicle accident rate is now a serious problem in Europe, the United States, and Japan (Groot Bruinderink & Hazebroek 1996; Romin & Bissonette 1996).

Consequently, the serious concerns of deer management is how best to limit deer densities and the consequent impacts on ecosystem constituents and functions (Garrott et al. 1993) and how to make an effective management policy of deer population become a problem in different situation. In the USA, four feasible management options are using to control the density of deer, such as predator restoration, public hunting, sharpshooting and possibly immunocontraception (Warren 2011). Wildlife biologists have supplied much information to understanding the complex problem that may help us better manage instances of deer overabundance.

Population genetic structure

Knowledge of population genetic structure is important for estimation of the effective size of populations and for identification of management categories when making conservation plan (Wang & Caballero 1999; Nunney 2000). Moritz (1994) defined two distinct genetic criteria to assess priorities in conservation. One is evolutionary significant unit (ESU). Another one is management unit (MU),

representing sets of populations that are currently demographically independent (differing significantly in allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic relationship among alleles) (Moritz 1994). ESU is defined as a unit reflecting historically isolated sets of populations (identified by reciprocal monophyly of mitochondrial DNA and significant divergence of allele frequencies at nuclear loci). Recently, the definition of ESU (Allendorf et al. 2010) is: "A classification of populations that have substantial reproductive isolation which has led to adaptive differences so that the population represents a significant evolutionary component of the species". Mitochondrial haplotypes are especially powerful for identifying potential ESUs (Avise 1995; Moritz 1994) because of the special relevance of matrilines to population demography.

In management actions for conservation or exploitation (for instance hunting) of natural populations a critical need is that of defining appropriate management units (MUs). Management units may be considered as units of a population that are demographically independent (Moritz 1994), and therefore may be subject of specific monitoring and management actions. They differ from ESUs is that the focus of the management unit is on contemporary population structuring and short-term monitoring rather than historical factors (Fraser and Bernatchez 2001). In practice, MUs are in most cases devised on the basis of administrative boundaries, or of obvious barriers (rivers, mountain ridges, urban areas, etc.), but this is rarely an ecologically meaningful criterion. Hence, a genetic approach may resolve situations where geographical and administrative approaches are inadequate or ambiguous: if significant differences in allele frequencies at neutral marker loci are observed between two spatially separated groups of individuals, these groups may qualify as distinct MUs (Zannése et al. 2006). In synthesis, in conservation and management of wild animal biodiversity, molecular genetic markers have a fundamental role for identifying Evolutionary Significant Units, Management Units, and Metapopulations, and allow temporal monitoring of the genetic variability between and within these units. This information can be used to inform conservation and management plans by defining priorities (for instance when limited resources obligate to choose few among

many units for conservation actions), addressing the appropriate units for coordinated population demographic monitoring and active management actions (for instance for setting differentiated harvest quotas).

Population genetic structure has been used to understand how both landscape features and social structure affect dispersal pattern in ungulate. Some studies have attempted to use spatial genetic structure to identify management units of such recovered abundant populations (Coulon et al. 2006; Zannèse et al. 2006; Pérez-Espona et al. 2008; Frantz et al. 2012), suggesting that the landscape features serve as gene flow barriers. For red deer in the Scottish Highlands, the most important barriers to gene flow were identified as sea lochs, followed by mountain slopes, roads and forests (Pérez-Espona et al. 2008). Motorways have also been suggested as an effective barrier for another population of red deer in Belgium (Frantz et al. 2012). In contrast, Coulon et al. (2006) found no absolute barriers to roe deer populations in France; however, the combination of several linear landscape features was suggested to cause genetic differentiation. In additional, the studies of population genetic structure in other animals were also proved that the landscape features serve as gene flow barriers. Keyghobadi et al. (1999) discovered that forests were barriers to gene flow for alpine butterflies in Canada. Riley et al. (2006) found that population genetic structure in bobcats and coyotes corresponded to a major roadway. Castric et al. (2001) found that waterfalls were associated with more genetic isolation in brook charr in Maine. Funk et al. (2005) determined that mountain ridges and elevation differences were associated with genetic differentiation in Columbia spotted frogs in western Montana and Idaho. Sacks et al. (2004; 2008) found genetic structure in coyotes in California that was associated with habitat divisions. Epp et al. (2005) discovered that genetic diversity in desert bighorn sheep in California was limited by the anthropogenic barriers of highways, canals, and urban areas.

Molecular markers in population genetics

A recent profusion of population genetic studies reflects the utility of molecular methods in determining priorities for conservation (Avise 1995; Friesen 1997; Petit et

al. 1998). A wide array of molecular markers have been used to investigate population-level genetic studies, including the products of nuclear genes (e.g. allozymes, Kark et al.1999; Mahy et al. 1999), mitochondrial genes (e.g. cytochrome b, Friesen et al. 1996; Barber et al. 1999), restriction fragment length polymorphisms (RFLPs, Klein and Brown 1994) and random amplified polymorphic DNA (RAPDs, Nusser et al. 1996). However, as additional markers become available, the ability of researchers to detect population dynamics with greater precision and accuracy is increasing. Nuclear DNA (nDNA) and the mitochondrial DNA (mtDNA) are two broad classes in population genetics and molecular ecology (Jarne and Lagoda 1996; Baker and Marshall 1997; Luikart and England 1999).

Mitochondrial DNA is a small, haploid molecule inherited maternally, which is composed almost entirely of coding sequence, and free from recombination and have relatively high mutation rate. These features make mtDNA useful for phylogeographical studies (Weiss et al. 2000) and for analysis of spatial and temporal population structure (Laikre et al. 2002).

Microsatellites, which consist of short, tandemly repeated nucleotide sequences (generally 2-6 base pairs long), exhibit codominant inheritance and generally are selectively neutral (Colson and Goldstein 1999). The hypervariability and high mutation rate of microsatellites make them one of the most widely used types of molecular markers currently available in population genetics studies (Coulon et al. 2006; Pérez-Espona et al. 2008).

The study species

Life history of sika deer

The sika deer (*Cervus nippon*) in Hokkaido is a typical ungulate with number increasing and distribution expanding, which is a species with significantly concern for control and management. The number of sika deer (*Cervus nippon*) in Hokkaido decreased rapidly and closed to threaten level due to the overexploitation, habitat loss and snowfalls around 1900 (Inukai 1952). Three major subpopulations survived the bottleneck, these being located in the Akan, Hidaka, and Daisetsu mountain regions

(Nagata et al. 1998; Kaji et al. 2000). Due to a result of deer protection policies, an increase in grassland area, the extinction of wolves, and a decrease in the number of hunters, the population of sika deer recovered and the distribution expanded from eastern Hokkaido to south and northwestern parts by 1990s (Kaji et al. 2000). The damage of agriculture and forestry increased through the range of sika deer expanded and population is overabundant (Kaji, 1995; Hokkaido Government, 1986, 1994).

Migration pattern in eastern Hokkaido

The sika deer population in eastern Hokkaido originates from the Akan subpopulations (Nagata et al. 1998), which has recovered and expanded its distribution by the mid-1970s (Kaji et al. 2000). Female sika deer in eastern Hokkaido also exhibit the seasonal migration pattern with long distance (7.2-101.7 km) (Uno and Kaji 2006; Sakuragi et al. 2003; Igota et al. 2004). Igota et al. (2004) captured the female sika deers at the wintering area in the Shiranuka Hills in eastern Hokkaido which were radio-tracked during 1997-2001 to examine the factors affecting seasonal migration at the individual-landscape level. The summer home range of female sika deer were widely scattered over an area of 5734 km^2. Some of individuals, migrants, changed the low or high-altitude summer home range to the intermediate-altitude winter home range. Others of individuals, non-migrants, used the intermediate-altitude home range all year round in Shiranuka Hills. Female sika deer showed high site fidelities to their season home ranges in eastern Hokkaido.

Recent management policy

Adaptive management (AM) is applied in overabundant sika deer (*Cervus nippon*) populations management in Hokkaido, Japan, which is a management system for natural resources through continually improving management policies and practices, including both approaches of adaptive learning and feedback control (Walters 1986). The management goals of sika deer population in Hokkaido were: (1) to maintain the population at moderate density levels preventing population irruption, (2) to reduce damage to crops and forests, and (3) to sustain a moderate yield of

hunting without endangering the population (reviewed by Kaji et al. 2010). Kaji et al. (2010) purposed that hunting as resource management and culling for ecosystem management should be synergistically combined under adaptive management.

As the increasing population and distribution expanding of sika deer, Hokkaido Government responded promptly and established the Hokkaido Institute of Environmental Sciences (HIES) in 1991. Monitoring of sika deer populations was started using population indices such as spotlight counts, aerial surveys, catch per unit effort (CPUE; number of deer harvested per hunter-day), sighting per unit effort (SPUE; number of deer sighted per hunter day), and damage to agriculture and forestry. In 1998, the Conservation and Management Plan for Sika deer (CMPS) in eastern Hokkaido initiated adaptive management, including aggressive female harvesting. In 2000, the target areas were extended to include central Hokkaido, encompassing most parts of the deer distribution range in Hokkaido (Kaji et al. 2010). Based on distribution ranges of sika deer further expanding in western and northern Hokkaido, 12 monitoring units were set up in Hokkaido and assessed population status in each (HIES 1994). These ‘‘monitoring units’’ were not purely population based, but also relied on both geographical features and District office boundaries. Therefore, the study of population genetic structure of sika deer and the boundaries between subpopulations was needed to formulate an effective management policy in Hokkaido.

Research Objective

In this dissertation, I examined the spatial genetic structure in sika deer population in Hokkaido. The objectives were to:

1. Combine the genetic data and geographic data to test the temporal change of spatial genetic structure in an expanding sika deer population of Hokkaido, and what factor elicit such alterations to population structure.
2. Answer whether genetic differentiation occurs associated with migratory partitioning (migratory and philopatry).

Dissertation Structure

This dissertation is composed of four chapters, two of which are manuscripts to be submitted to peer-reviewed scientific journals (Chapter 2 & 3). In Chapter 1, I provide a general introduction to my research, and Chapter 4 contains the general discussion of my research and implications for management.

Chapter 2 focuses on the temporal change of spatial genetic structure of an expanding sika deer population of Hokkaido over a 15-year period, the effect of the dispersal of females from higher to lower density subpopulations, and the implications on control or manage sika deer population.

Chapter 3 focuses on the genetic differentiation associated with migratory habits in sika deer population of eastern Hokkaido.

Chapter 2

Temporal change in the spatial genetic structure of a sika deer population with an expanding distribution range over a 15-year period

Introduction

Human-based disturbance has caused a major decline in the size of many wildlife populations. Such disturbance includes habitat losses (through agriculture, logging, mining, tourisms, and development), over-exploitation, and pest control. Large reductions in population size have caused population fragmentation in many wildlife populations (Rueness et al. 2003; Manel et al. 2004; Kaji et al. 2010), which increases the risk of further reduction (i.e., through higher demographic stochasticity and lower genetic diversity). Recent genetic bottlenecks have been evidenced in various species as a result of this phenomenon (see Peery et al. 2012 for a review). Fragmented populations genetically differentiate from each other, leading to the formation of genetically distinct units, as is the case in restored populations [e.g., white-tailed deer (DeYoung et al. 2003); roe deer (Coulon et al. 2006); red deer (Pérez-Espona et al 2008; Frantz et al. 2012); black bear (Saitoh et al. 2001; Ishibashi and Saitoh 2004); brown bear (Manel et al. 2004); lynx (Rueness et al. 2003)]. Fortunately some populations have recovered mainly through conservation activities (Rueness et al. 2003; Manel et al. 2004; Coulon et al. 2006). As a result, the distribution ranges of such populations have expanded. This phenomenon raises the question of whether the spatial genetic structure altered during the population expansion. If so, what factors elicit such alterations in the structure? Through answering these questions, we could identify the management unit of a wildlife population. Studies on spatial genetic structure potentially provide valuable data to inform conservation and management policies (Crandall et al. 2000; Moritz 2002).

Deer are a typical example of wildlife that has experienced a large reduction and subsequent recovery in population size. Over-exploitation during the last half of the 19th century caused the number and distribution range to decline drastically worldwide (Staines 1977; DeYoung et al. 2003; Côté et al. 2004; Nielsen et al. 2008). However, effective protection measures enabled some deer populations to recover and even became overabundant, particularly in the North America and Europe (McShea et al. 1997; Brown et al. 2000; Mysterud et al. 2000; Fuller and Gill 2001; Ward et al.

2008; Warren 2011; Pérez-González et al. 2012). Some studies have attempted to use spatial genetic structure to identify management units of such recovered abundant populations (Coulon et al. 2006; Zannèse et al. 2006; Pérez-Espona et al 2008; Frantz et al. 2012), suggesting that the landscape features serve as gene flow barriers. For red deer in the Scottish Highlands, the most important barriers to gene flow were identified as sea lochs, followed by mountain slopes, roads and forests (Pérez-Espona et al 2008). Motorways have also been suggested as an effective barrier for another population of red deer in Belgium (Frantz et al. 2012). In contrast, Coulon et al. (2006) found no absolute barriers to roe deer populations in France; however, the combination of several landscape features was suggested to cause genetic differentiation.

While such studies provide valuable information about the spatial genetic structure of wildlife populations, the sampling in these studies was only conducted over short timeframes, and thus could not be used to answer the question about the stability of the population structure. Some subpopulations might merge into one, while others might remain distinct. In particular, the spatial genetic structure of a population that is rapidly recovering from a bottleneck is expected to change.

The sika deer (*Cervus nippon* Temminck, 1838) population in Hokkaido, Japan, is ideal for studying temporal changes in spatial genetic structure during population recovery, and for examining the effect of density variation on population structure. Prior to the Japanese colonization of Hokkaido in the late 19th century, the sika deer had been abundant throughout Hokkaido. Over-exploitation, habitat loss (due to agricultural development and timber extraction in lowland forests), and heavy snowfalls contributed to a rapid decline in deer numbers around 1900 (Inukai 1952). To conserve the sika deer population, the Hokkaido government established hunting regulations (i.e., the prohibition of hunting and/or bucks-only hunting; Kaji et al. 2010). Three major subpopulations survived the bottleneck, these being located in the Akan, Hidaka, and Daisetsu mountain regions (Fig. 1; Nagata et al. 1998; Kaji et al.

2000). As a result of deer protection policies, an increase in grassland area, the extinction of wolves, and a decrease in the number of hunters, the sika deer population recovered, with the distribution range extending (Kaji et al. 2010). By the mid-1970s, the sika deer population occupied most available habitats in the eastern half of Hokkaido, and then, spread to all potential habitats by the 1990s (Kaji et al. 2000). However the success of the protection measures caused the sika deer to become overabundant, which led to the damage of agricultural land, forests, and natural vegetation, in addition to increased number of traffic collisions. Consequently, hunting regulations were gradually relaxed to place some control on sika deer population numbers (Kaji et al. 2010). In 1998, the Conservation and Management Plan for Sika deer (CMPS) in eastern Hokkaido initiated adaptive management, including aggressive female harvesting. In 2000, the target areas were extended to include central Hokkaido, encompassing most parts of the deer distribution range in Hokkaido (Kaji et al. 2010). Nagata et al. (1998) carried out intensive sampling of sika deer between 1991 and 1996 in Hokkaido, and reported that three major mitochondrial DNA (mtDNA) haplotypes were distributed in the three core ranges (Akan, Hidaka, and Daisetsu) that remained after the historical bottleneck. Therefore, by integrating the samples most recently collected by our research group with those of Nagata et al. (1998), it might be possible to identify temporal changes in the spatial genetic structure during the expansion of this population.

In this study, we aimed to infer the subpopulation structure of a sika deer population inhabiting Hokkaido Island during two distinct periods (Period I : 1991–1996 ; Period II : 2008–2010), which spanned a 15-year timeframe. We analyzed the mtDNA (D-loop) and microsatellite DNA (nine loci) from samples collected by Nagata et al. (1998) combined with samples recently collected by our research group. Since mtDNA molecules tend to be inherited maternally in animals (Avise 2004), the spatial genetic structure revealed by mtDNA sequence variation is a combined product of maternal lineage structure that has accumulated for multiple generations and individual dispersal during a single generation. In comparison,

microsatellite DNA molecules are biparentally inherited; thus, microsatellite DNA-based structure reflects accumulated dispersal of both sexes for multiple generations. Therefore, if dispersal behavior differs between sexes, different spatial structures would be detected between mtDNA and microsatellite DNA-based analyses. Since deer tend to exhibit male-biased dispersal (Nelson 1993; Clutton-Brock et al. 2002), microsatellite DNA-based structures would show more homogenous patterns in comparison with mtDNA-based structures. Based on these analyses, we discuss the overall population management unit and relevance of the study results for conservation actions.

Materials and methods

Study area and sika deer

The study area, Hokkaido Island, is the northernmost island of Japan, which is mountainous and extensively forested (61% of the total area). The island covers an area of about 77,984 km^2, and is located at latitudes of 41°24' -- 45°31'N and longitudes of 139°46' -- 145°49'E. The island has four distinct seasons, with cool humid summers and cold snowy winters. From 2002 to 2011, the annual average temperatures of Sapporo ranged between 8.8 ℃ and 9.8 ℃, while the annual precipitations ranged between 843 mm and 1325 mm (Japan Meteorological Agency, http://www.data.jma.go.jp/obd/stats/etrn/index.php). It is warm in the southwestern part of the island and cool in the northeastern part (Stenseth et al. 1998). Less snow accumulates in the eastern part of the island compared to the western part (Kaji et al. 2000).

The sika deer is widely distributed throughout eastern and northeastern Asia, from the Ussuri region of Siberia to northern Vietnam, Taiwan, and Japan (Ohtaishi 1986; Whitehead 1993). It is likely that the wide distribution range of the sika deer, extending from subarctic to tropical zones, has resulted in the species exhibiting

considerable morphological variation among populations (Ohtaishi 1986; Terada et al. 2012). The sika deer of Hokkaido is classified as a subspecies, *C. n. yesoensis*, based on its large body size and antlers, in addition to the yellowish-red color of the summer pelage (Imaizumi 1949; Whitehead 1993).

Sample collection

We used 168 sika deer samples (muscle or liver tissue) that had been collected by Nagata et al. (1998) throughout Hokkaido from 1991 to 1996 (Period I). A further 648 samples were collected from 2008 to 2010 (Period II) by the authors of the current study in cooperation with the Hokkaido Government and the Hokkaido Hunters Association. Frozen muscle tissues (ca. 10 × 10 × 3 cm) that had been stored at -15 ℃ was supplied by hunters, in addition to information about the gender, age (adult: ≥1 year old; juvenile: <1 year old), and geographic location from which each sample was obtained. Since the number of samples differed between the two periods, 169 samples (110 males and 59 females) from Period II were selected from the 648 samples, based on sample size, sex ratio, and sample locations from Period I (112 males and 56 females; Fig. 1). Samples from the southern area were not used, because experimental reintroductions were conducted at this location during 1980 and 1981 (Kaji et al. 2000, 2010), with the possible unofficial introduction of deer from outside of Hokkaido (Terada et al. 2013).

In addition to the above datasets, 359 samples were selected from the 648 samples collected during the second period based on the following two steps: (1) juvenile and young individuals were removed, and (2) when several individuals were sampled at the same location, one individual was randomly selected for that location. These 359 samples were used for resampling analyses (see “Resampling analyses” section).

Molecular analysis

Total genomic DNA was extracted from muscle tissue using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocol. The

D-loop region (606 base-pairs) was amplified using primer L15926 (5′-CTAATACACCAGTCTTGTAAACC-3′) (Kocher et al. 1989) and primer H597 (5′-AGGCATTTTCAGTGCCTTGCTTTG-3′) (Nagata et al. 1998). PCR amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a 25-µl reaction mixture containing 1 µl of the DNA extract, 2.5 µl of 10× PCR Buffer, 2.5 µl dNTP, 0.1 µl AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1 µl of each primer (12.5 µM), and 16.9 µl UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). After incubation at 95 ℃ for 10 min, cycling was performed for 35 cycles of 1 min at 94 ℃, 1 min at 53 ℃, and 1min at 72 ℃, with a postcycling extension at 75 ℃ for 10 min. After removing excess primers and dNTP, the PCR products were labeled using the L15926 primer and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were then determined using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems). Sequences were analyzed using Sequence Scanner 1.0 (Applied Biosystems), and aligned using MEGA 4.0 (Tamura et al. 2007).

We used nine microsatellite loci: OarFCB193, BM203, BM888 (Talbot et al. 1996); Cervid14 (Dewoody et al. 1995); IDVGA55 (Mezzelani et al. 1995); INRA040 (Vaiman et al. 1994); TGLA127 (Slate et al. 1998); MM12 (Beja-Pereira et al. 2004); and BM4107 (Bishop et al. 1994). PCR amplification was performed in a 25-µl mixture containing 1 µl DNA Template, 12.5 µl AmpliTaq Gold 360 Master Mix (Applied Biosystems), 0.25–1 µl Reverse primer (12.5 µM), 0.25–1 µl Forward primer (12.5 µM) end-labeled with a fluorescent dye (NED, PET, VIC or 6-FAM), and 9.5–11 µl UltraPure DNase/RNase-Free Distilled Water. After incubation at 95 ℃ for 10 min, cycling was performed for 30–35 cycles of amplification (30 sec at 94 ℃, 30 sec at melting temperature [T_m], 30 sec at 72 ℃), with a postcycling extension at 72 ℃ for 7 min. The PCR products were resolved in an ABI PRISM 3100-Avant Genetic Analyzer, and allele sizes were determined for each locus using Gene Mapper 4.0 (Applied Biosystems). To maximize the quality of the genotype data, the presence

of null alleles and genotyping errors (such as large allele dropout and stuttering) was examined using Micro-Checker 2.2.3 (van Oosterhout et al. 2004).

Mitochondrial DNA data analysis

Standard population genetic analyses

The number of haplotypes, nucleotide diversity (π, Nei 1987), and haplotype diversity (*h*, Nei 1987) were estimated using DnaSP 5.10.01 (Librado and Rozas 2009).

Inferring subpopulations

To infer the spatial genetic structure, we used the software GENELAND 4.0.3 (The Geneland development group 2012). GENELAND assigns an individual to a subpopulation based on the genetic information and spatial coordinates, implementing a Bayesian MCMC approach (Falush et al. 2003; Guillot et al. 2005). To infer subpopulation structure and to estimate other parameters, the MCMC was run 10 times, allowing the number of subpopulations (*K*) to vary between 1 and 10, and using the correlated allele frequency model with the following parameters: 100,000 MCMC iterations, maximum rate of Poisson process being set to 100, maximum number of nuclei being set to 300, and the uncertainty of spatial coordinates being set to 5 km, according to a grid on a map of Hokkaido used by hunters (Kaji et al. 2000). We calculated the average logarithm posterior probability for each of the 10 runs. To verify the consistency of the inferred subpopulation structures, we compared the subpopulation patterns of the 10 runs. When more than 90% of runs showed the same subpopulation pattern, we assumed that the consistency of the population was supported, and selected the run that had the highest average logarithm posterior probability for subsequent analyses. The posterior probability of population membership for pixels was computed with a burn-in of 10,000 iterations, and the number of pixels was set to 200 along both the *x*- and *y*- axes. Finally, the posterior probability of population membership was computed for each pixel of the spatial domain, and the population membership of individuals to the modal population was

inferred.

The frequency and number of haplotypes, nucleotide diversity (π), and haplotype diversity (h) was estimated for each subpopulation in the two periods using DnaSP in the same way as it was used for the whole samples. Genetic differentiation (F_{ST}; Weir and Cockerham 1984) between inferred subpopulations for each period was assessed based on haplotypes frequency differences using ARLEQUIN 3.5 (Excoffier et al. 2010). Differences in the frequency of haplotypes among subpopulations for each period were examined using Fisher's exact test. Significance was assumed when $P < 0.05$.

Microsatellite data analysis

Standard population genetic analyses of the whole population

Standard population genetic analyses were performed with the whole samples collected during each period. The mean number of alleles (MNA) per locus, expected heterozygosity (H_E), and observed heterozygosity (H_O) were calculated using the Excel Microsatellite Toolkit (Park 2001). The Hardy–Weinberg equilibrium for each locus was tested for each locus using heterozygote deficiency, and was tested globally using the Markov chain method implemented in GENEPOP 4.0 (Rousset 2008; parameter values for the test at the locus level: dememorization number = 10,000, number of batches = 200, number of iterations per batch = 2,000; parameter values for the global test: dememorization number = 1,000, number of batches = 100, number of iterations per batch = 1,000). Linkage disequilibrium was also examined using GENEPOP 4.0 (parameter values: dememorization number = 10,000, number of batches = 800, number of iterations per batch = 8,000).

Inferring subpopulations

The subpopulation structures were inferred by GENELAND 4.0.3 based on microsatellite DNA data using the uncorrelated frequency model in the same way as it was inferred for mtDNA, except for the parameter set and the number of runs.

Parameters were set as follows: 500,000 MCMC iterations, maximum rate of the Poisson process was fixed to 100, maximum number of nuclei was fixed to 300, and the uncertainty of spatial coordinates was set at 5 km. The MCMC was run 30 times, allowing K to vary between 1 and 10, to verify the consistency of the inferred subpopulation structures. The posterior probability of population membership was computed for each pixel of the spatial domain (200 × 200 pixels), and the population membership of individuals to the modal population was inferred. When more than 90 % of runs showed the same subpopulation pattern, we assumed that the consistency of the subpopulation was supported, and selected the run that had the highest average logarithm posterior probability for further analyses.

Pairwise F_{ST} values were estimated among subpopulations for each periods using ARLEQUIN 3.5.

Resampling analyses

To test the null hypothesis that the datasets for Period I and Period II were sampled from the same mother population and to validate the robustness of the original results on spatial genetic structure, we randomly resampled 169 samples from the 359 samples collected for Period II. One-hundred datasets were obtained through this resampling procedure. Spatial genetic analyses using GENELAND 4.0.3 were performed for each of the 100 resampled datasets using mtDNA and microsatellite DNA features, and the results for the original two datasets from Period I and Period II were compared with those produced by the 100 datasets. In addition mtDNA-based and microsatellite DNA-based structures were also inferred by using the 359 samples.

Temporal comparison of subpopulations

To examine genetic differentiation between the two periods, F_{ST} was calculated for each subpopulation between the two periods using ARLEQUIN 3.5. Differences in the frequency of haplotypes were also examined for each subpopulation between the two periods using Fisher's exact test.

Sighting per unit effort (SPUE)

To test our hypothesis that the spatial genetic structure of a population is altered due to gene flow from high to low density subpopulations, a density index of deer was analyzed in relation to the observed subpopulation structures. The Hokkaido Government requested that hunters report the date and location of hunting, the number and sex of harvested deer, and the total number of live deer that were observed. Sightings per unit effort (SPUE, i.e., the number of deer sighted per hunter-day) were converted to a density index based on the reported number of observed deer (Uno et al. 2006). We organized the SPUE for each of 3,599 blocks (approx. 5 km × 4.6 km) placed as a grid on a map of Hokkaido from 2000 to 2010 according to Kaji et al. (2000). This information was used to determine the spatial variation of deer density. Of note, CMPS targeted management activity on a deer subpopulation in eastern Hokkaido in 1998, after which the target areas were extended to include the eastern and central parts of Hokkaido in 2000, covering most parts of the deer distribution range, except for the south-western peninsula (Kaji et al. 2010). Since SPUE could only be obtained from the areas targeted by the CMPS, we focused on the SPUE from of 2,448 blocks between 2000 and 2010. SPUE was not reported for certain blocks that were not entered by hunters. Since hunters did not anticipate successful hunting in these blocks, the SPUE of these blocks was treated as zero. The averaged SPUE was obtained for the subpopulations, and used as a density index. The proportion of blocks where SPUE was higher than zero was also used to determine the proportion of deer presence.

Results

Mitochondrial DNA analyses

Six hundred and two base pairs of the mtDNA D-loop region were sequenced for all samples (n = 168 for Period I and n = 169 for Period II). Five haplotypes based on four variable nucleotide sites (base substitution between A and G at nt 178, 251, and 499; base substitution between C and T at nt 457) were found, and were identical to

those reported by Nagata et al. (1998): type-a, type-b, type-c, type-d, and type-f. These haplotype sequences were deposited in the databases of GenBank, EMBL, and DDBJ by Nagata et al. (1998) under the following accession numbers: D50128 (a-type), D50129 (b-type), AB004297 (c-type), AB00428 (d-type), and AB004300 (f-type). Haplotype frequencies were similar between Period I (1991–1996) and Period II (2008–2010; Table 1; Fisher's exact test = 0.251). The main haplotypes were type-a, type-b, and type-d, which constituted 92.3 % of all samples in Period I and 93.5 % of all samples in Period II. The geographic distributions of the haplotypes are presented in Fig. 2. In both periods, type-a was the most widespread haplotype, while type-b was primarily distributed in the northern and central regions. The type-d haplotype was primarily distributed in the southern area. Haplotype and nucleotide diversity were low and similar for both periods (Table 1).

Subpopulation structure

Four subpopulations (*K*) were estimated in nine out of 358 10 runs for Period I (Fig. 2a). In Period II, three clusters were supported by all 10 runs (Fig. 2b). According to the sampling locations, we named the four subpopulations from Period I and the three subpopulations from Period II as follows: 1990mtN1 (*n* = 80), 1990mtN2 (*n* = 40; northern Hokkaido), 1990mtE (*n* = 27; eastern Hokkaido), and 1990mtC (*n* = 21; central Hokkaido); 2000mtN (*n* = 85), 2000mtE (*n* = 47), and 2000mtC (*n* = 37), respectively. The location of the boundary between eastern and northern Hokkaido remained stable for both periods, and it ran through Abashiri city, Kitami city, Ashoro town, and Urahoro town (Fig. 2). The boundary that divided the northern subpopulation into two subpopulations (1990mtN1 and 1990mtN2) was only observed during Period I. The boundary that separated the central subpopulation from all other subpopulations shifted slightly northwards between the two periods.

The type-a haplotype dominated all other haplotypes in the eastern subpopulation during both periods (1990mtE and 2000mtE; Table 1). The type-b haplotype was dominant in the northern subpopulations during both periods (1990mtN1, 1990mtN2,

and 2000mtN), although type-d was also common in 1990mtN2. Type-c was the most common haplotype in the central subpopulation during Period I, followed by type-a and type-d (1990mtC), while type-c was replaced as the most common haplotype by type-d during Period II (2000mtC). Haplotype frequencies differed significantly among subpopulations during both Period I (Fisher's exact test, $P < 0.0001$) and Period II ($P < 0.0001$).

Genetic differentiation among subpopulations was large and highly significant (Table 2). The pairwise F_{ST} estimated for Period I (0.206–0.461) was generally higher compared to that estimated for Period II (0.175–0.377).

Eighty-five of the 100 resampled datasets showed consistent patterns for subpopulation structure, with two, 34, and 49 datasets supporting $K = 2$, $K = 3$, and $K = 4$, respectively. The subpopulation structure of the 34 datasets supporting three subpopulation resembled the one of the original dataset for Period II. The basic subpopulation structure of the 49 datasets supporting four subpopulations was similar to the one of the original dataset for Period II. However, the subpopulation corresponding to 2000mtC was divided into two subpopulations resembling the structure based on the 359 sample-analyses (Fig. 2c; see the next paragraph). The subpopulation structure of the two datasets supporting two subpopulations lacked the subpopulation corresponding to 2000mtE. When focusing on the northern area (Period I: 1990mtN1, 1990mtN2, and 1990mtE; Period II: 2000mtN and 2000mtE), all of the resampled datasets showed the different subpopulation structure from that of Period I, indicating that 1990mtN1 and 1990mtN2 were merged together in Period II. Therefore, the null hypothesis that the datasets for Period I and Period II were sampled from the same mother population was rejected.

All of the 10 runs using the 359 samples showed that the number of subpopulations was four, and the subpopulation structure was consistent across the 10 runs (Fig. 2c). The subpopulation structures from the 169 samples and the 359

samples for Period II resembled each other, except for the central subpopulation being separated into two in the analysis using the 359 samples. Based on the 359 samples, the structure of the northern subpopulations evidently differed from those collected during Period I. The area of 1990mtN2 was coupled with that of 1990mtN1 in the 359 sample-based structure. Both boundaries that were observed in the analysis using the 169 samples were confirmed in the analysis using the 359 samples.

Differences between the two periods

Genetic differentiation between the two periods was examined using the pairwise F_{ST} and Fisher's exact test for each subpopulation. For the eastern and central subpopulations, no significant genetic differentiation was detected between the two periods (Table 3). For the northern subpopulations, we combined the two subpopulations from Period I (1990mtN1 and1990mtN2) into one subpopulation, and examined the genetic differentiation with the equivalent subpopulation in Period II (2000mtN). These subpopulations also showed no significant genetic differentiation (Table 3). We then divided the 2000mtN into two units (2000mtN1′ and 2000mtN2′) based on the boundary separating 1990mtN1 and 1990mtN2, and again examined the genetic differentiation. Genetic differentiation was found between 1990mtN2 and 2000mtN2′, whereas 1990mtN1 and 2000mtN1′ did not show any significant genetic differentiation.

Microsatellite DNA analyses

The mean number of alleles and heterozygosity are presented in Table 4. No significant deviation from the Hardy–Weinberg equilibrium expectation was detected for any of the loci when the significance values for multiple comparisons were adjusted using the Bonferroni correction. The analysis of each pair of loci showed no significant linkage disequilibrium after the Bonferroni correction for multiple tests. The number of alleles was the same between Period I and Period II for all loci, except BM4107 (Table 4). Observed frequencies of alleles were not significantly different between the periods for any loci (Fisher's exact test, $P > 0.090$).

Subpopulation structure

The inferred number of subpopulations was two, and the subpopulation structure was consistent across the 30 runs for Period I. The subpopulation structure with the highest average logarithm posterior probability is presented in Fig. 3(a). The subpopulations were named as 1990msN (n = 151) and 1990msC (n = 17) according to the sampling locations. The modal number of populations was one in all 30 runs for Period II (Fig. 3b).

Genetic differentiation among subpopulations was estimated using F_{ST}. The pairwise F_{ST} was significantly large among the subpopulations (1990msN–1990msC) in Period I (Table 5). The samples from Period II were grouped into two units according to the boundaries of the two subpopulations in Period I, with the two units being named as 2000msN′ and 2000msC′. Although pairwise F_{ST} for Period II (2000msN'–2000msC′) was lower compared to that for Period I, significant genetic differentiation was obtained (Table 5).

Eighty-nine of the 100 resampled datasets showed consistent patterns for subpopulation structure, with 34, 41, and 14 datasets supporting K = 1, K = 2, and K = 3, respectively. The population structure of the 34 datasets supporting one subpopulation was the same as the one of the original dataset for Period II, of course. In the 41 datasets supporting two subpopulations three patterns of the subpopulations structure were shown; (1) the subpopulations structure of 16 datasets resembled the one of the original dataset for Period I, (2) in 24 datasets, one subpopulation occupied most of Hokkaido together with another small subpopulation being located on eastern part, and (3) in the remaining one datasets the population was almost evenly divided into eastern and western subpopulations. The 14 datasets supporting three subpopulations showed a combined structure of the above patterns (1) and (2). Because the considerable number of resampled datasets showed the subpopulation structure resembling the one of the original dataset for Period I or Period II, the

hypothesis that the datasets for Period I and Period II were sampled from the same mother population was not rejected.

Two subpopulations were consistently detected across the 30 runs in the analysis using the 359 samples (Fig. 3c). The inferred subpopulation structure resembled that from the 168-sample analysis for Period I, although the boundary separating the northern subpopulation from the central one shifted slightly northwards in the 359-sample analysis for Period II.

Differences between the two periods

Genetic differentiation between the two periods was examined using the pairwise F_{ST} for each subpopulation (Table 5). No significant differentiation was observed in the comparison between 1990msN (n = 151)–2000msN′ (n = 143) and between 1990msC (n = 17)–2000msC′ (n = 26).

Sighting per unit effort (SPUE)

The density index represented by the averaged SPUE was compared among subpopulations and years. Subpopulations were defined using the mtDNA-based structure for Period I, because the subpopulation structure for Period I might represent the basis of changes in the spatial genetic structure between the two periods. SPUE was not analyzed using on the microsatellite DNA-based subpopulations, because the microsatellite DNA-based structure using the 169 samples for Period II is inconclusive (see Discussion).

The number of blocks for the four subpopulations detected by the mtDNA-based analysis (1990mtC, 1990mtE, 1990mtN1, and 1990mtN2) was 413, 418, 882, and 735, respectively. The highest average density (±SD) in 2000 was in block 1990mtE (4.06 ±3.20), followed by 1990mtC (3.48 ±3.55), 1990mtN1 (2.69 ±2.89), and 1990mtN2 (1.76 ±2.49), with no substantial overlap in the 95 % CIs among the subpopulations

(Fig. 4). The density of all subpopulations gradually increased between 2000 and 2010, with the highest values being obtained in 2010; 1990mtE: 4.77 ±3.24; 1990mtC: 5.68 ±4.91; 1990mtN1: 4.39 ±4.42; and1990mtN2: 3.36 ±4.08. The subpopulations of 1990mtN1 and 1990mtE showed similar densities during 2005–2010, while the densities of 1990mtC and 1990mtN2 substantially differed to all other densities obtained during this period. The subpopulation with the highest density shifted from 1990mtE to 1990mtC in 2004, while 1990mtN2 remained the lowest subpopulation throughout 2000–2010. The highest rate of increase for density was recorded in 1990mtN2 (1.91), followed by 1990mtC (1.63), 1990mtN1 (1.63), and 1990mtE (1.17).

In contrast to the subpopulation densities, there was a clear variation in the temporal change in the proportion of presence (i.e., the proportion of blocks where SPUE was higher than zero) among subpopulations (Figs. 4 and 5). In 2000, the proportion of presence was highest in 1990mtE (0.87), followed by 1990mtN1 (0.73), 1990mtC (0.73), and 1990mtN2 (0.52). The proportion of presence was stable in 1990mtE, with the value for 2010 (0.89) remaining similar to that obtained in 2000. The proportion of presence for 1990mtC and 1990mtN1 was comparative in most years (Fig. 4), showing a gradual increase until 2004, after which the proportion became saturated at around 0.8. There was a noticeable change in the proportion of presence for 1990mtN2, which showed a major increase from 0.52 in 2000 to 0.73 in 2004, and continued to increase at a lower rate until 2009.

Throughout the study period, the proportion of presence for 1990mtN2 remained substantially lower compared to all other subpopulations. While the proportion of presence for 1990mtE was higher compared to all other subpopulations until 2003, it exhibited only a minor difference to 1990mtC and/or 1990mtN1 in all subsequent years.

Discussion

The results of this study partly support our prediction that the spatial genetic structure of a rapidly recovering population changes. The mtDNA-based structures changed between the two study periods, whereas the microsatellite DNA-based structures did not change. Here, we discuss the processes of structural change when considering the effects of population density and inter-sexual differences in dispersal pattern. The management implications are also presented in relation to the observed differences in the mtDNA- and microsatellite DNA-based subpopulation structures.

Temporal changes in subpopulation structure

Between Period I (1991–1996) and Period II (2008–2010), the number of subpopulations decreased from four to three based on the mtDNA analyses. One major change was the fusion of subpopulations 1990mtN1 and 1990mtN2 into subpopulation 2000mtN. In contrast, the changes in subpopulation structure observed by microsatellite DNA analysis were unclear.

Genetic structuring is primarily determined by the dispersal capability and habitat requirements of a given population (Slatkin 1987). Since sika deer were distributed throughout Hokkaido Island in the past, and the sika deer population has recently spread again to most areas of Hokkaido, most areas of Hokkaido are likely to meet the habitat requirements of sika deer. Furthermore, major changes in agricultural pasture and forest area, which are considered to have contributed to the recovery of deer distribution and abundance (Kaji et al. 2010), were not reported during the two study periods (http://www.e-stat.go.jp/; Department of Fishery and Forestry, Hokkaido 1990-2010), while pasture areas increased and many natural forests were converted into artificial forests during the 1960s and 1970s. Therefore, habitat features might not represent a major factor explaining the change in the spatial genetic structure during the study periods. Alternatively, dispersal behavior and its associated factors (such as population density and geographic structure) might explain the documented changes in the spatial genetic structure of this population. 1990mtN1 and

1990mtN2 were genetically distinct in Period I, but had merged into one subpopulation in Period II (Fig. 2). Since no significant genetic differentiation was observed between 1990mtN1+1990mtN2 and 2000mtN, mtDNA features that were different between 1990mtN1 and 1990mtN2 for Period I might have been homogenized by the dispersal of deer by Period II. However, the change in the structure between 1990mtN1 and 1990mtN2 was different. Genetic differentiation was found between 1990mtN2 and 2000mtN2′, whereas 1990mtN1 and 2000mtN1′ did not show significant genetic differentiation (Table 3). This result indicates that 1990mtN2 might have received more immigrants compared to 1990mtN1. For instance, there might have been more frequent dispersal from 1990mtN1 to 1990mtN2 compared to dispersal in the reverse direction. Spatial variations in changes in SPUE, as a density index, were consistent with this interpretation. The density of 1990mtN1 was substantially higher compared to 1990mtN2 across the periods. In addition, many vacant habitat blocks in the region of 1990mtN2 became occupied between the two study periods, while the other subpopulations occupied most blocks by 2000 (Figs. 4 and 5).

The microsatellite DNA-based subpopulations for Period I appeared to have become considerably mixed by Period II (Figs. 3a and 3b). However, pairwise F_{ST} values do not support this change in subpopulations, because genetic differentiation was significant between the hypothetical subpopulations (2000msN'–2000msC') during Period II as well as between the subpopulations (1990msN–1990msC) during Period I. Furthermore, no significant differentiations was observed in the comparison between 1990msN–2000msN′ and between 1990msC–2000msC′ (Table 5). In addition, the hypothesis that the datasets for Period I and Period II were sampled from the same mother population was not rejected by resampling analyses. Similar subpopulation structures were observed both in the 168-sample analysis for Period I and the 359-sample analysis for Period II (Fig. 3). Therefore, the microsatellite DNA-based subpopulation structure may not have changed across the two study periods; hence, the result indicating that no spatial genetic structure was detected

during Period II might have been caused by a sampling bias.

Sex specific density dependent movement

Spatial genetic structures were more heterogeneous in the mtDNA-based structure compared to the microsatellite DNA-based structure (Figs. 2 and 3). These results indicate that gene flow among subpopulations is asymmetric between mtDNA and microsatellite DNA. This phenomenon might be explained by assuming the presence of male-biased dispersal and female philopatry. Male-biased dispersal occurs in most mammalian species (Greenwood 1980), including deer (Nelson 1993; Clutton-Brock et al. 2002). Male movement contributes towards homogenizing the microsatellite DNA-based structure; however, its contribution is limited for the mtDNA-based structure, because the inheritance system differs between mtDNA and microsatellite DNA; specifically, mtDNA is maternally inherited, while microsatellite DNA is bi-parentally inherited. The result demonstrating that the subpopulation structure of 1990mtE (2000mtE) was maintained within a larger microsatellite DNA-based structure indicates that female movement was limited in these areas, whereas males moved between the mtDNA-based subpopulations. Hence, the phenomenon might be explained by male-biased dispersal and female philopatry.

We have suggested that the fusion of the 1990mtN1 and 1990mtN2 into 2000mtN is explained by the dispersal from 1990mtN1 to 1990mtN2 along a density gradient. The mtDNA features of 1990mtN2 for Period I were significantly altered by Period II. Females are expected to contribute to this alteration. Male immigrants might also alter mtDNA features; however, their contribution is expected to be minor because only females pass mtDNA features onto the next generation.

Although we explained the change in the mtDNA-based subpopulation structure based on the dispersal of females along the density gradient, a density gradient does not always generate mtDNA gene flow. Although SPUE and the proportion of presence were higher in 1990mtE compared to 1990mtN1 in the early 2000s, mtDNA

gene flow from 1990mtE to 1990mtN1was not observed. These results indicate that density effects have a limited influence on dispersal. Other factors might also contribute to the structuring of deer subpopulations.

Factors influencing the formation subpopulations

Two boundaries remained stable throughout the two study periods with respect to the mtDNA-based structure: (1) the west-to-east boundary, separating the northern subpopulations from the central subpopulations traversed the same locations, and (2) the north-to-south boundary in eastern Hokkaido, separating the eastern subpopulation from other subpopulations (Fig. 2). The first boundary was also observed in the microsatellite DNA-based structures from the 168-sample analysis of Period I and the 359-sample analysis of Period II. The first boundary might limit the movement of both sexes, while the second boundary might have had a larger effect on female dispersal, because this boundary was only valid for the mtDNA-based structure.

Igota et al. (2004) captured 57 female sika deer at Shiranuka, which is located 25–30 km east of the boundary separating the eastern subpopulation from the other subpopulations (Fig. 1). The authors subsequently released the deer after attaching radio-telemetry units, and tracked their movements patterns during 1997–2001. Long-distance seasonal migration was recorded for 39 females (35.1 ±3.6 km; mean ±SD), whereas the remaining females were non-migrants or the tracking units failed. The migratory direction was north or east in the majority of cases, with none of the females crossing the east boundary. This tracking study supports the presence of this boundary for the female component of the population, although there was a lack of information about males.

The sampling resolution of the current study might not be sufficiently high enough to obtain a detailed picture about boundaries. Further studies that use a greater number of samples collected by spatially uniform and intensive sampling effort are required to identify the exact landscape barriers to gene flow.

Management implications

CMPS has been focusing on reducing the size of the subpopulation in eastern Hokkaido, because increased deer abundance in recent years has caused severe agricultural damage. When the target areas for population control were extended to central Hokkaido, the management units (MUs) were defined as western and eastern subpopulations, which roughly correspond to 1990mtN2+C and 1990mtN1+E, respectively. However, these management units should be revised based on the subpopulation structure identified in this study.

The current study raises the question of which subdivisions of a population should be used when mtDNA and nuclear DNA analyses present different results. Since mtDNA is maternally inherited, the mtDNA-based subdivisions indicate maternally structured groups. In comparison, nuclear DNA-based subdivisions indicate the effect of gene flow by both males and females; thus, reflecting groups that have the potential for future local adaptation. Therefore, from an evolutionary perspective, a nuclear DNA-based analysis of the subpopulation structure should be adopted. However, from a demographic perspective, an mtDNA-based structure should be adopted. For example, the CMPS has been encouraging hunters to target female sika deer in areas of high population density, because the removal of adult females with high reproductive value represents the most efficient strategy of controlling abundant populations. In other words, the survival rate of adult females strongly influences the population growth rate of wildlife populations (Escos et al. 1994; Walsh et al. 1995; Uno 2006). This interpretation raises the question of whether female-biased hunting effort in 2000mtE would impact 2000mtN. Our study results indicate that this would not be the case, because the dispersal of females between 2000mtE and 2000mtN was limited in both study periods at least. Therefore, the 2000mtN population size would continue to increase, despite the successful control of the 2000mtE subpopulation. Through understanding the genetic structuring and gene flow among the subpopulations, we are able to demonstrate that these two

subpopulations should be managed separately, which differs to the interpretation based on the microsatellite DNA-based management unit.

Genetic monitoring presents a promising tool for conservation and management (Schwartz et al. 2007). Our study shows the power of spatial genetic analyses, and the importance of periodical monitoring, for the management of wildlife. In conclusion, the combination of spatial genetic analyses with behavioral, geographic, and demographic datasets would increase the power of these different, yet related, wildlife population analysis techniques.

Table 1 Diversity of mtDNA for each inferred sika deer subpopulation during Period I (1991–1996 and Period II (2008–2010).

	N	*h*	*π*	*H*	type-a	type-b	type-c	type-d	type-f
Period I									
1990mtE	27	0.359	0.001	2	21	6	0	0	0
					77.8%	22.2%	0.0%	0.0%	0.0%
1990mtC	21	0.710	0.002	4	6	1	9	5	0
					28.6%	4.8%	42.9%	23.8%	0.0%
1990mtN1	80	0.359	0.001	3	17	62	0	1	0
					21.3%	77.5%	0.0%	1.2%	0.0%
1990mtN2	40	0.703	0.002	5	6	17	1	13	3
					15.0%	42.5%	2.5%	32.5%	7.5%
Total	168	0.637	0.002	5	50	86	10	19	3
					29.8%	51.2%	5.9%	11.3%	1.8%
Period II									
2000mtE	47	0.457	0.001	3	32	14	0	1	0
					67.5%	30.3%	0%	2.2%	0%
2000mtC	37	0.670	0.002	3	11	0	10	16	0
					29.7%	0%	27.0%	43.2%	0%
2000mtN	85	0.517	0.002	4	21	55	0	8	1
					24.7%	64.7%	0%	9.4%	1.2%
Total	169	0.669	0.002	5	64	69	10	25	1
					37.9%	40.8%	5.9%	14.8%	0.6%

N: the number of samples; *H*: the number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; type-a, type-b, type-c, type-d, type-f: the frequency of haplotype a, b, c, d, and f

Table 2 Genetic differentiation among subpopulations estimated by pairwise F_{ST} (*P* value) and Fisher's exact test based on mtDNA features in Period I (1991–1996) and Period II (2008–2010)

Subpopulation combination	F_{ST} (*P*-value)	Fisher's exact test
Period I		
1990mtC–1990mtE	0.214 (<0.0001)	<0.0001
1990mtN1–1990mtE	0.461 (<0.0001)	<0.0001
1990mtN1–1990mtC	0.457 (<0.0001)	<0.0001
1990mtN2–1990mtE	0.409 (<0.0001)	<0.0001
1990mtN2–1990mtC	0.289 (<0.0001)	<0.0001
1990mtN1–1990mtN2	0.206 (<0.0001)	<0.0001
Period II		
2000mtC–2000mtE	0.209 (<0.0001)	<0.0001
2000mtN–2000mtE	0.175 (<0.0001)	<0.0001
2000mtN–2000mtC	0.377 (<0.0001)	<0.0001

P value are the probability of F_{ST} values being at least as extreme as the observed values, assuming that the null hypothesis ($F_{ST} = 0$) is true

Table 3 Temporal comparisons of genetic differentiation among the sika deer subpopulations using F_{ST} (P-value) and Fisher's exact test between Period I (1991–1996) and Period II (2008–2010) for subpopulations based on mtDNA features

Subpopulation combination	F_{ST} (P-value)	Fisher's exact test
1990mtE–2000mtE	-0.015 (0.550)	P = 0.740
1990mtN1+1990mtN2–2000mtN	-0.008 (0.676)	P = 0.802
1990mtC–2000mtC	0.048 (0.198)	P = 0.219
1990mtN1–2000mtN1'	0.017 (0.162)	P = 0.091
1990mtN2–2000mtN2'	0.039 (0.081)	P = 0.035

Table 4 Genetic variation per locus based on microsatellite DNA features of the whole sika deer population of Hokkaido for Period I (1991–1996) and Period II (2008–2010)

	Period I				Period II				Fisher's
Locus name	*A*	H_O	H_E	*P*-value	*A*	H_O	H_E	*P*-value	Exact test
OarFCB193	2	0.327	0.313	0.800	2	0.260	0.277	0.292	$P = 0.367$
BM203	2	0.387	0.373	0.751	2	0.284	0.322	0.095	$P = 0.166$
BM888	3	0.512	0.517	0.214	3	0.503	0.518	0.423	$P = 0.559$
INRA040	3	0.589	0.655	0.027	3	0.592	0.655	0.023	$P = 0.995$
Cervid14	2	0.476	0.501	0.312	2	0.462	0.501	0.190	$P = 0.818$
BL42	2	0.399	0.455	0.074	2	0.432	0.442	0.443	$P = 0.625$
IDVGA55	6	0.768	0.781	0.273	6	0.751	0.807	0.076	$P = 0.090$
MM12	2	0.464	0.486	0.341	2	0.503	0.479	0.795	$P = 0.695$
TGLA127	2	0.208	0.187	1.000	2	0.189	0.209	0.184	$P = 0.625$
BM4107	3	0.417	0.486	0.022	5	0.527	0.522	0.519	$P = 0.603$

Fisher's exact tests were performed to test the differences in allele frequencies between the periods. *A*: the number of allele per locus; H_O: observed heterozygosity; and H_E: expected heterozygosity.

Table 5 Spatial and temporal comparisons of genetic differentiation for the sika deer subpopulations based on the microsatellite DNA features

Subpopulation combination	F_{ST}	*P*-value
1990msN–1990msC	0.048	<0.0001
2000msN'–2000msC'	0.017	<0.0001
1990msN–2000msN'	0.000	0.812
1990msC–2000msC'	0.004	0.342

The individuals in Period II were divided into 2000msN' and 2000msC' by the boundary of two subpopulations in Period I. Genetic differentiation was estimated by pairwise F_{ST}. *P* values are the probability of F_{ST} values being at least as extreme as the observed values, assuming that the null hypothesis ($F_{ST} = 0$) is true.

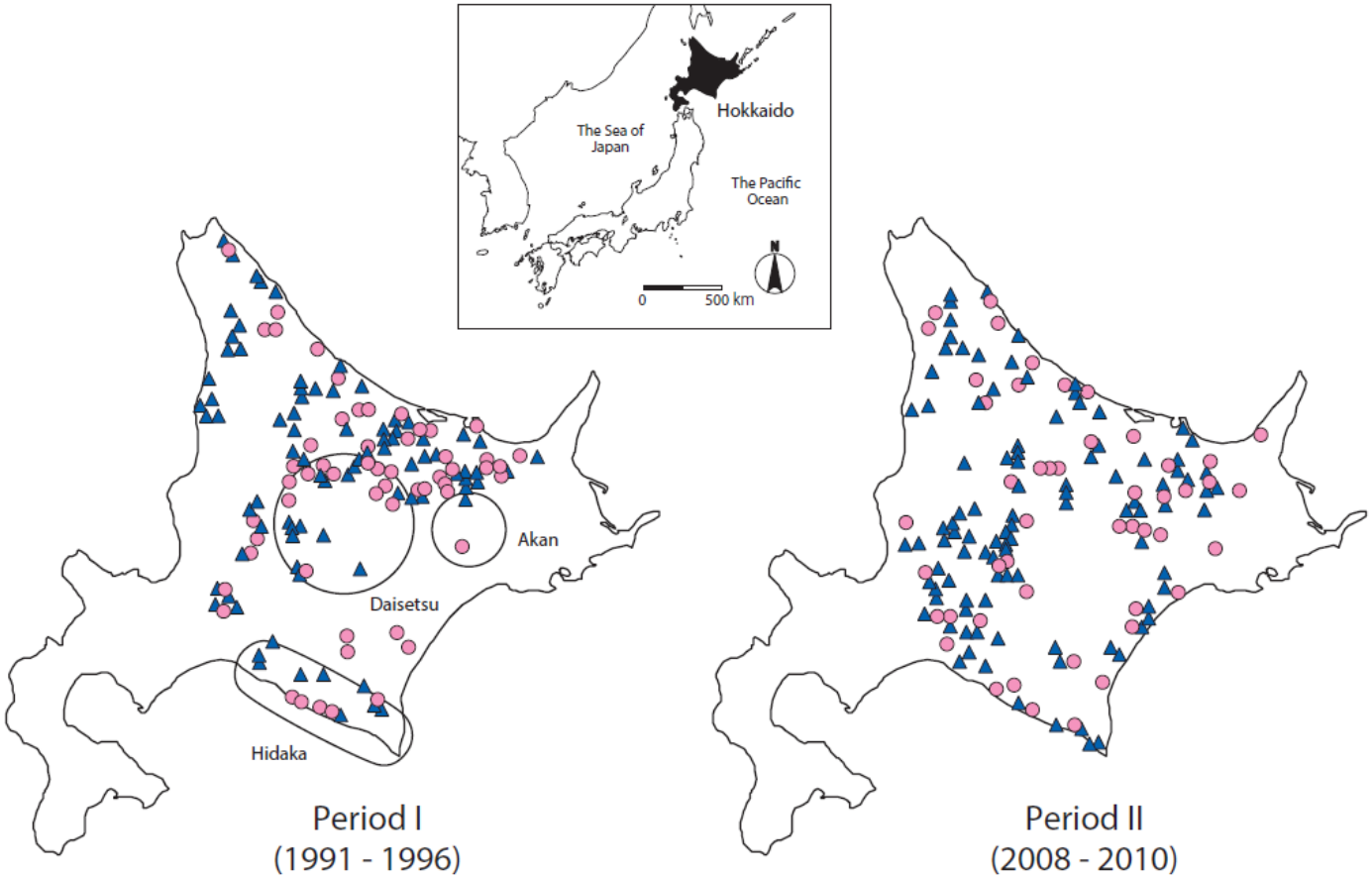

Fig. 1 Study area and sampling locations of sika deer in Hokkaido, Japan, during (a) Period I: 1991–1996, and (b) Period II: 2008–2010. *Circles* indicate female samples; *triangles* indicate male samples.

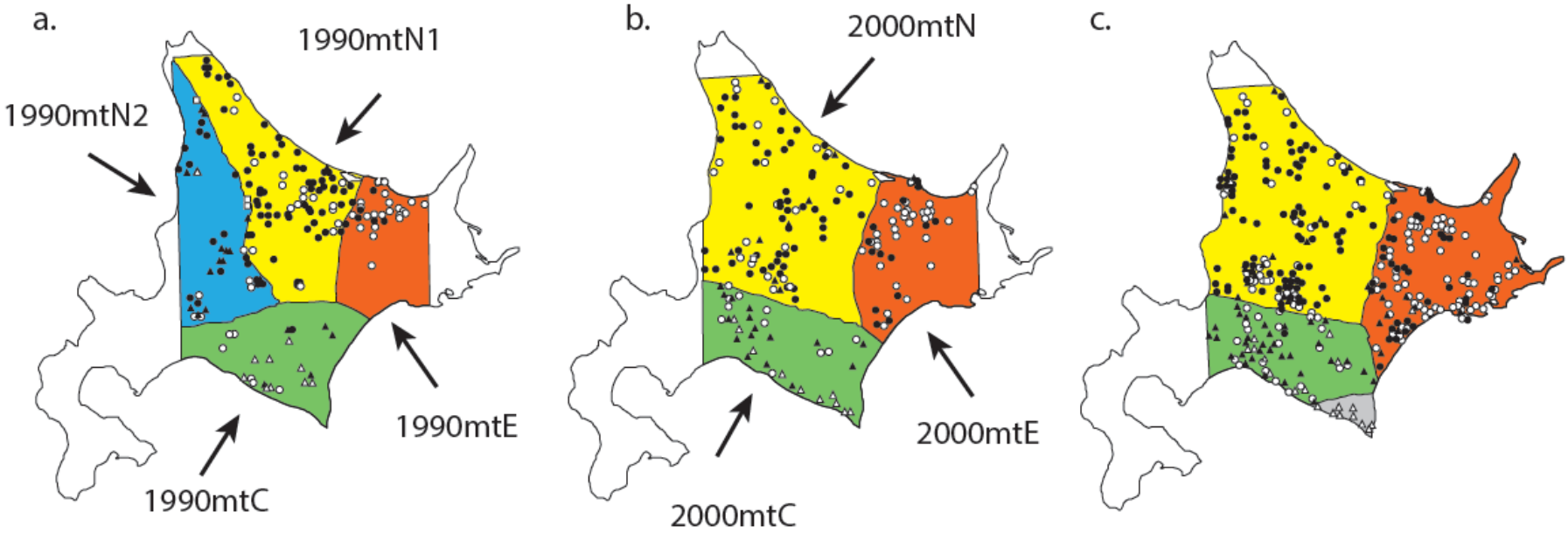

Fig. 2 Map of the subpopulations based on mtDNA haplotypes (D-loop). Memberships of individual sika deer were deduced by GENELAND 4.0.3 for (a) Period I (1991–1996, *n* = 168), (b) Period II (2008–2010, *n* = 169), and (c) Period II (2008–2010, *n* = 359). Samples with haplotype-a are represented by *open circles*, type-b by *solid circles*, type-c by *open triangles*, type-d by *solid triangles*, and type-f by *open squares*. Four subpopulations were named 1990mtN1, 1990mtN2 (northern Hokkaido), 1990mtE (eastern Hokkaido), and 1990mtC (central Hokkaido) during Period I, and three subpopulations were named 2000mtN, 2000mtE, and 2000mtC during Period II.

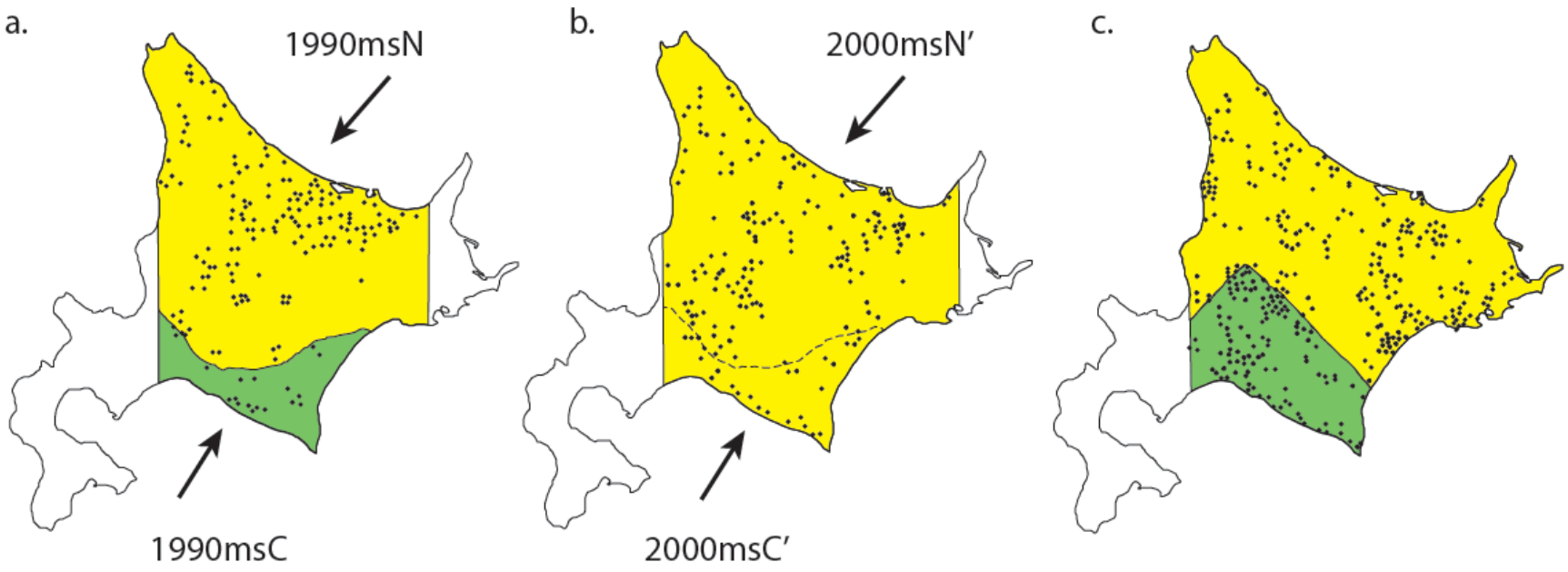

Fig. 3 Map of the subpopulations based on microsatellite DNA features. Memberships of individual sika deer were deduced by GENELAND 4.0.3 for (a) Period I (1991–1996, n = 168), (b) Period II (2008–2010, n = 169), and (c) Period II (2008–2010, n = 359). Subpopulations were named as 1990msN (northern Hokkaido) and 1990msC (central Hokkaido) during Period I. Dots indicate sampling locations. Although the subpopulation structure was unclear for Period II, samples from Period II were grouped into two units according to the boundary of the two subpopulations in Period I (a broken line), with the two units being named as 2000msN′ and 2000msC′.

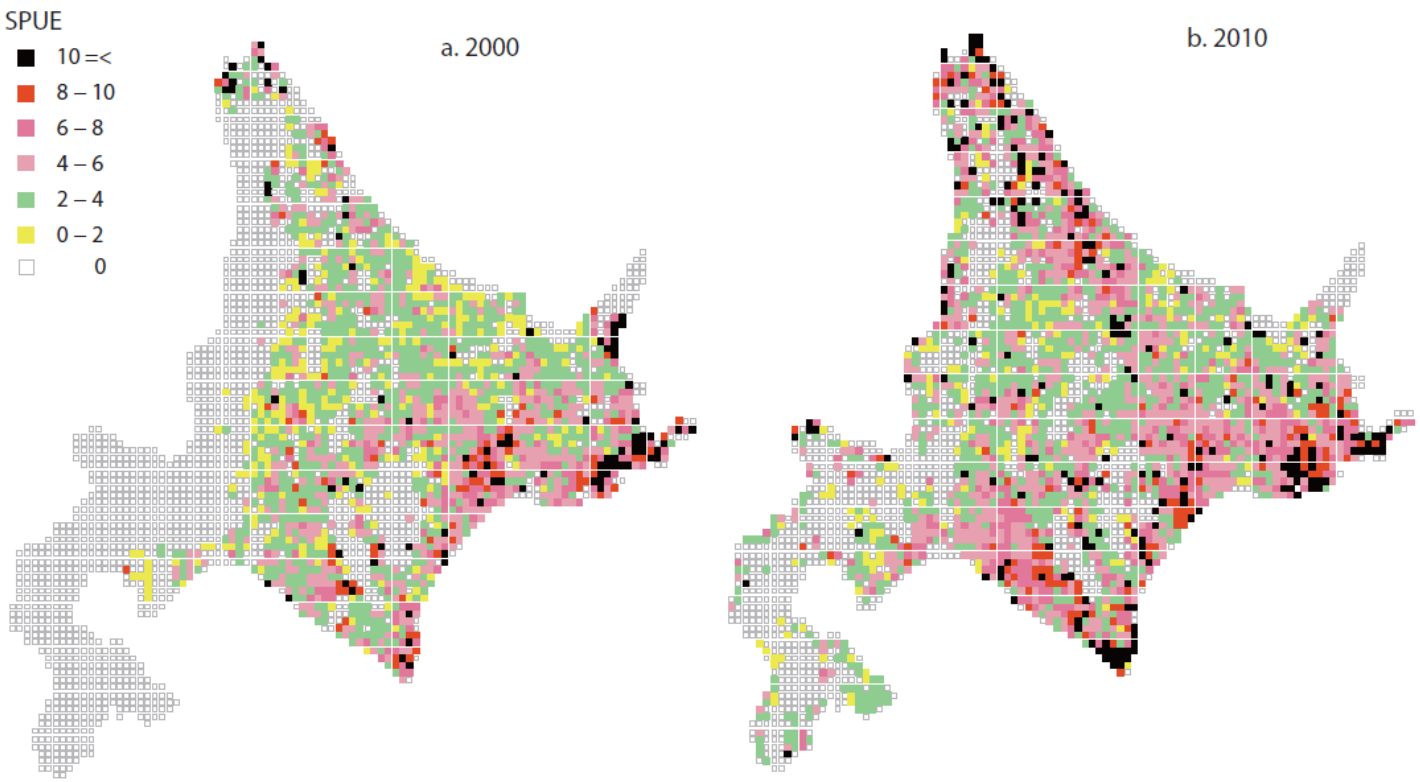

Fig. 4 (a) Temporal changes in sika deer sightings per unit effort (SPUE, i.e., the number of deer sighted per hunter-day) reported by hunters. SPUE was averaged for four subpopulations based on the mtDNA-based structure. Gray zones denote the 95 % confidence interval. (b) Temporal changes in the proportion of sika deer presence. The proportions represent the number of blocks where SPUE was higher than zero, and were organized for the four mtDNA-based subpopulations. Gray zones denote 95 % confidence intervals.

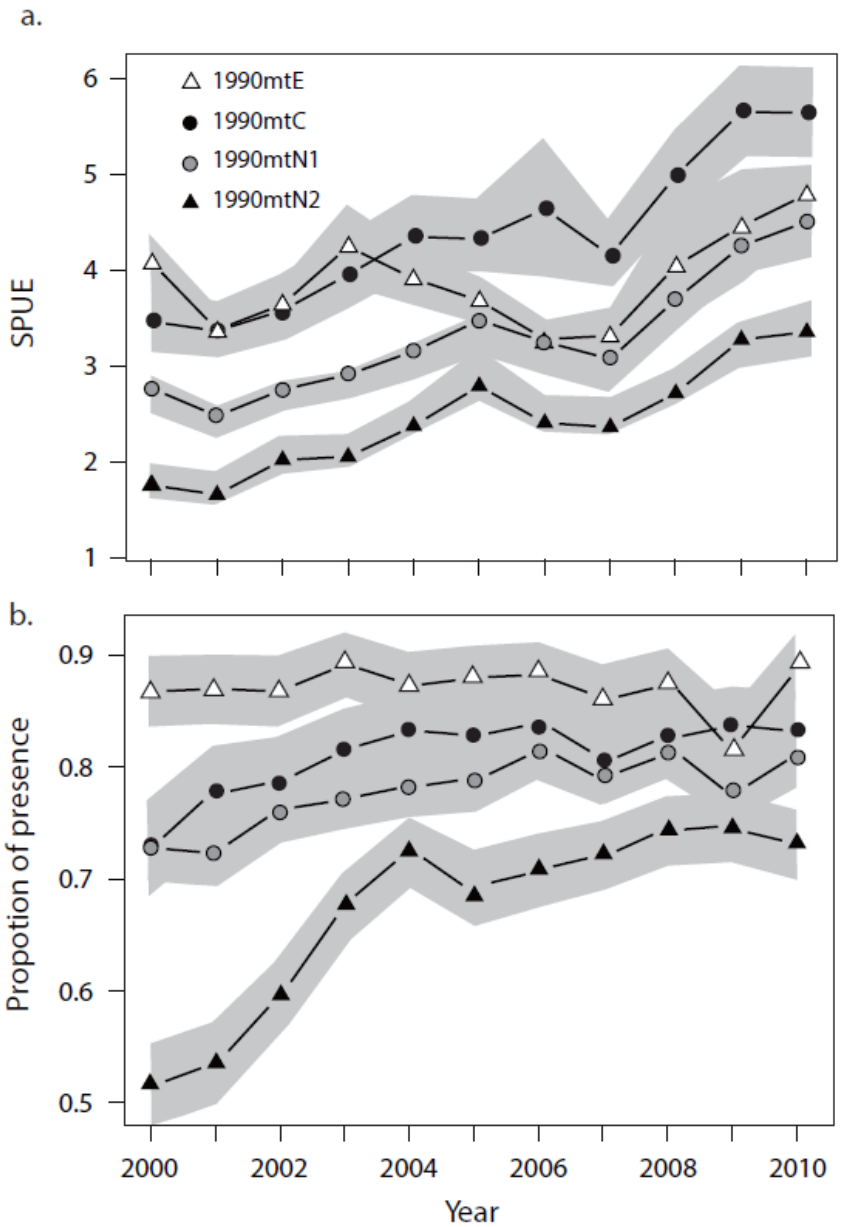

Fig. 5 Sightings of sika deer per unit effort (SPUE, i.e., the number of deer sighted per hunter-day) reported by hunters in Hokkaido for (a) 2000 and (b) 2010. The sightings were averaged for 3,599 blocks (about 5 km ×4.6 km), and placed as a grid on a map of Hokkaido. SPUE was not reported for blocks not entered by hunters. Since hunters did not anticipate successful hunting in these blocks, the SPUEs of these blocks were treated as zero, and presented as open blocks. SPUEs were classified into seven ranks at intervals of two from zero to more than 10, and were represented by a gray gradation (this is presented in color in the online version).

Chapter 3

Genetic differentiation associated with migratory habits in a deer population

Introduction

Deer is one of the most influential ecosystem engineers in terrestrial ecosystems, which modify resources available to other species in a community, as well as being a pest that damages agricultural and forestry products. They can strongly modify vegetation structure (McNaughton et al. 1988; Russell et al. 2001; Rooney and Waller 2003; Danell et al. 2006). Reduction in vegetative cover due to their grazing and browsing results in the alteration of co-occurring animal assemblages including small rodents (Parsons et al. 2013), birds (McShea and Rappole 1997; Berger et al. 2001; Martin 2007; Ogada et al. 2008), and invertebrates (Miyashita et al. 2004; Takada et al. 2008; Sakai et al. 2012). The management and control of deer populations are, therefore, critical for ecosystem management, and various programmes have been conducted to control or manage an overabundant population of deer (e.g., Kaji et al. 2010).

A management unit is a basic unit of management and conservation of wildlife and defined as geographical areas with restricted interchange of the individuals of interest with adjacent areas (Taylor and Dizon 1999). Molecular approaches are commonly used for delimiting pertinent management units and other criteria of wildlife populations (Zannèse et al. 2006; Morin et al. 2010). Some studies that identify the management units of wildlife populations using analyses of the spatial genetic structure of populations have suggested that the landscape features serve as gene flow barriers (Coulon et al. 2006; Zannèse et al. 2006; Pérez-Espona et al. 2008; Frantz et al. 2012). In contrast, Coulon et al. (2006) found no absolute barriers to roe deer populations, although the combination of several landscape features was suggested to cause genetic differentiation. While such studies provide valuable information about the management of wildlife populations, the sampling in these studies was only conducted over short timeframes, and thus could not be used to answer the question about the stability of the population structure. Some subpopulations might merge into one, while others might remain distinct. Ou et al. (2014) compared subpopulation structures of the sika deer (*Cervus nippon* Temminck,

1838) population of Hokkaido, Japan, during two periods (1991–1996 and 2008–2010), using mitochondrial DNA (mtDNA; D-loop) and microsatellites (9 loci). The number of gene-based subpopulations decreased across the 15-year period; specifically from four to three subpopulations based on mtDNA. The fusion of the two northern subpopulations caused the change to the mtDNA-based structure, which might be explained by the dispersal of females from higher to lower density subpopulations. In comparison, a stable subpopulation structure was found in the north and central population separated by a west-to-east boundary using both mtDNA and mitochondrial DNA based analyses. Why and how the northern mtDNA-based subpopulations were coupled? Why some other subpopulations were stable? These questions about how a population is structured is an essential challenge in landscape genetics of wildlife.

Long-distance migratory habits between wintering and feeding habitats may play a key role in forming the spatial genetic structure of a population, together with the dispersal from their natal sites. In several long-distance migrant birds, genetic differentiation has been shown to closed match the partitioning of migratory routes (Ruegg and Smith 2002; Ruokonen et al. 2004; Jones et al. 2005; Riou et al. 2012), whereas in others it is failed to show genetic differentiation associated with migratory partitioning (Avise et al. 1992; Dallimer et al. 2003). In the cases where the site fidelity is high and gene flow between different migratory groups is limited, seasonal migration may contribute to maintaining the spatial genetic structure of a population. However, if gene flow between different migratory groups is common, migration may contribute to the fusion of subpopulations.

Sika deer in Hokkaido show two types of migratory behaviors (migratory and philopatry; Igota et al. 2004). Long-distance migration was observed between wintering and summer feeding habitats by radio-tracking; migration distances ranged between 7.2 and 101.7 km. Although high site fidelities to their seasonal home ranges are shown (Igota et al. 2004; Sakuragi et al. 2004), genetic differentiation associated

with migratory partitioning is unknown. Therefore, genetic analyses of migrants and philopatric individuals may contribute to understanding the processes of subpopulation structure formation.

In this study, we analyzed mitochondrial DNA (mtDNA) and microsatellites DNA (msDNA) features of the sika deer that were radio-tracked by Igota et al. (2004), in order to answer whether genetic differentiation occurs associated with migratory partitioning (migratory and philopatry). Subpopulation structure was observed based on a summer home range map using the software GENELAND 4.0.3, which assigned an individual to a subpopulation based on the genetic information and spatial coordinates, implementing a Bayesian MCMC approach (Falush et al. 2003; Guillot et al. 2005), and significant genetic differentiation was detected between philopatric individuals and migrants toward north. Based on these analyses, we discuss the role of seasonal migration in forming the spatial genetic structure of the sika deer population in Hokkaido. This is the first report on genetic differentiation associated with migratory habits in deer, while genetic differentiations are observed among different migratory types in marine mammals and birds.

Materials and methods

Study area and sika deer

The study area, Hokkaido Island, is the northernmost island of Japan, which is mountainous and extensively forested (61% of the total area). The island covers an area of about 77,984 km^2, and is located at latitudes of 41°24′ to 45°31′N and longitudes of 139°46′ to 145°49′E. The island has four distinct seasons, with cool humid summers and cold snowy winters. From 2002 to 2011, the annual average temperatures of Sapporo ranged between 8.8 ℃ and 9.8 ℃, while the annual precipitations ranged between 843 mm and 1325 mm (Japan Meteorological Agency, http://www.data.jma.go.jp/obd/stats/etrn/index.php). It is warm in the southwestern part of the island and cool in the northeastern part (Stenseth et al. 1998). Less snow

accumulates in the eastern part of the island compared to the western part (Kaji et al. 2000).

Seasonal migration of sika deer was observed in eastern Hokkaido by Igota et al. (2004). Sixty female and 3 male sika deer were captured along the upper stream of the Shoro River in the Shiranuka Hills (43°13′N, 143°53′E), which is a representative wintering area of sika deer in eastern Hokkaido (Hokkaido Institute of Environmental Sciences 1995). Each of deer was equipped with radio tags and tracked (see Igota et al. 2004 for details). The mean annual precipitation was 1,399mm and the mean annual temperature at Shiranuka was 5.2 ℃ with monthly means of -8.2℃ in February and 18.6℃ in August (National Land Agency of Japan 1992). Snow cover persists from December to late March or April. Mean snow depth in February in the wintering area in the study years varied between 34 and 80 cm.

Sample collection

Sixty female and three male sika deer samples (blood) were collected by Igota et al. (2004) in 1997-2003 in eastern Hokkaido. Each sample has the information about gender and age (adult: ≥1 year old; juvenile: <1 year old). We focused comparable 39 adult female deer, which were captured and radio-tracked in 1997-1998 (Table 1).

Molecular analysis

Total genomic DNA was extracted from muscle tissue using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The D-loop region (606 base-pairs) was amplified using primer L15926 (5′-CTAATACACCAGTCTTGTAAACC-3′) (Kocher et al. 1989) and primer H597 (5′-AGGCATTTTCAGTGCCTTGCTTTG-3′) (Nagata et al. 1998). PCR amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) in a 25-μl reaction mixture containing 1 μl of the DNA extract, 2.5 μl of 10× PCR Buffer, 2.5 μl dNTP, 0.1 μl AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1 μl of each primer (12.5 μM), and 16.9 μl

UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). After incubation at 95 ℃ for 10 min, cycling was performed for 35 cycles of 1 min at 94 ℃, 1 min at 53 ℃, and 1 min at 72 ℃, with a postcycling extension at 75 ℃ for 10 min. After removing excess primers and dNTP, the PCR products were labeled using the L15926 primer and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences were then determined using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems). Sequences were analyzed using Sequence Scanner 1.0 (Applied Biosystems), and aligned using MEGA 4.0 (Tamura et al. 2007).

We used 16 microsatellite loci: OarFCB193, BM203, BM888, BL42 (Talbot *et al*. 1996), Cervid14 (Dewoody et al. 1995), INRA040 (Vaiman *et al*. 1994), TGLA127, TGLA337, CSSM43, RM188, IDVGA55 (Slate *et al*. 1998), MM12 (Beja-pereira *et al*. 2004), BM4107, BM6506 (Bishop *et al*. 1994), ETH225, CSSM19 (Kuhn *et al.* 1996). PCR amplification was performed in a 25-μl mixture containing 1 μl of DNA Template, 12.5 μl of AmpliTaq Gold 360 Master Mix (Applied Biosystems), 0.25–1 μl of Reverse primer (12.5 μM), 0.25–1 μl of Forward primer (12.5 μM) end-labeled with a fluorescent dye (NED, PET, VIC or 6-FAM), and 9.5–11 μl of UltraPure DNase/RNase-Free Distilled Water. After incubation at 95 ℃ for 10 min, cycling was performed for 30–35 cycles of amplification (30 sec at 94 ℃, 30 sec at melting temperature (T_m), 30 sec at 72 ℃), with a postcycling extension at 72 ℃ for 7 min. The PCR products were resolved in an ABI PRISM 3100-Avant Genetic Analyzer, and allele sizes were determined for each locus using Gene Mapper 4.0 (Applied Biosystems). To maximize the quality of the genotype data, the presence of null alleles and genotyping errors (such as large allele dropout and stuttering) were examined by using Micro-Checker 2.2.3 (Van Oosterhout et al. 2004).

Mitochondrial DNA analyses

Standard population genetic analyses

The number of haplotypes, nucleotide diversity (π, Nei and Tajima 1987), and haplotype diversity (*h*, Nei and Tajima 1987) were estimated using DnaSP 5.10.01

(Librado and Rozas 2009).

Subpopulation clustering

To infer the spatial genetic structure, we used the software GENELAND 4.0.3 (The Geneland development group 2012). GENELAND assigns an individual to a subpopulation based on the genetic information and spatial coordinates, implementing a Bayesian MCMC approach (Falush et al. 2003; Guillot et al. 2005). To infer subpopulation structure and to estimate other parameters, the MCMC was run 10 times, allowing the number of subpopulations (K) to vary between 1 and 10, with the following parameters: 100,000 MCMC iterations, maximum rate of Poisson process being set to 100, maximum number of nuclei being set to 300, and the uncertainty of spatial coordinates being set to 5 km, according to a grid on a map of Hokkaido used by hunters (Kaji et al. 2000). We calculated the average logarithm posterior probability for each of the 10 runs. In order to verify the consistency of the inferred subpopulation structures, we compared subpopulation patterns between the 10 runs. The posterior probability of population membership for pixels was computed with a burn-in of 10,000 iterations, and the number of pixels was set to 200 along both the x and y axes. Finally, the posterior probability of population membership was computed for each pixel of the spatial domain, and the population membership of individuals to the modal population was inferred.

The frequency and number of haplotypes, nucleotide diversity (π), and haplotype diversity (h) of two clusters were estimated using DnaSP in the same way as the whole 40 samples. Pairwise F_{ST} was calculated using ARLEQUIN 3.5 (Excoffier et al. 2010) to examine genetic differentiation between the two clusters. Differences in the frequency of haplotypes were also examined between the two clusters using Fisher's exact test.

Microsatellite DNA analyses

Standard population genetic analyses of the whole population

Standard population genetic analyses were performed with 16 microsatellite loci of 39 sika deer samples. The mean number of alleles (*A*) per locus, expected heterozygosity (H_E), and observed heterozygosity (H_O) were calculated using the ARLEQUIN 3.5 (Excoffier et al. 2010). The Hardy–Weinberg equilibrium for each locus was tested using heterozygote deficiency, and globally using the Markov chain method implemented in GENEPOP 4.2 (Rousset 2008; parameter values for the test at the locus level: dememorization number = 10,000, number of batches = 200, number of iterations per batch = 2,000; parameter values for the global test: dememorization number = 1,000, number of batches = 100, number of iterations per batch = 1,000). Linkage disequilibrium was also examined using ARLEQUIN 3.5 (Excoffier et al. 2010).

Subpopulation clustering

The subpopulation structures were inferred by GENELAND 4.0.3 based on microsatellite DNA data using the uncorrelated frequency model and the correlated frequency model in the same way as it was inferred for mtDNA, except for the parameter set and the number of runs. Parameters were set as follows: 500,000 MCMC iterations, maximum rate of the Poisson process was fixed to 100, maximum number of nuclei was fixed to 300, and the uncertainty of spatial coordinates was set at 5 km. The MCMC was run 30 times, allowing *K* to vary between 1 and 10, to verify the consistency of the inferred subpopulation structures. The posterior probability of population membership was computed for each pixel of the spatial domain (200 × 200 pixels), and the population membership of individuals to the modal population was inferred.

Pairwise F_{ST} was calculated using ARLEQUIN 3.5 to examine genetic differentiation between the two clusters.

Correlations between relatedness and geographic distance

Genetic distance

We used Lynch and Ritland's (1999) measure of relatedness (r) by the software GenAIEx 6.5 (Peakalla and Smouse 2006) to estimate a genetic distance between all pairs of individuals in eastern Hokkaido. We use 16 microsatellite loci data to calculate the index r between all pairs of individuals. The value of r can range from –1 to 1, with negative values indicating unrelated dyads and positive values indicating some degree of relatedness.

Geographic distance

For each pair of individuals, we calculated geographic distance in kilometers based on great circle distances using the haversine (Sinnott 1984). The haversine formulate is

$$D = 2R \arctan\left(\frac{\sqrt{hav(\theta)}}{\sqrt{1-hav(\theta)}}\right),$$

Where $hav\ (\theta) = \sin^2\left(\frac{\delta 1 - \delta 2}{2}\right) + \cos \delta 1 \cos \delta 2 \sin^2\left(\frac{\alpha 1 - \alpha 2}{2}\right)$.

D is the distance between two points specified by (latitude, longitude) coordinates (α_1, δ_1) and (α_2, δ_2), with a central angle of θ between the two points. R is the radius of the Earth, which we assume to be 6,371 km.

We examined the correlation between genetic relatedness and geographic distance using GenAIEx 6.5 (Peakalla and Smouse 2012). We compared the matrix of geographic distance to the corresponding matrix of genetic relatedness using the Mantel test (Mantel 1967). The r values were the standardized Mantel statistic calculated as the usual Pearson correlation coefficient between two matrices. The P values were estimated using the randomization method with 9,999 permutations and the significance level is 0.05.

Results

Mitochondrial DNA analyses

Six hundred and two base pairs of mtDNA D-loop region were sequenced for 39 samples. Four haplotypes based on four variable nucleotide sites (base substitution between A and G at nt 178, 251, and 499; base substitution between C and T at nt 457)

were found for 39 samples, and were identical to those reported by Nagata et al. (1998): type-a, type-b, type-c, and type-d. Those haplotype sequences were deposited in GenBank, EMBL, and DDBJ by Nagata et al. (1998) under the following accession numbers: D50128 (a-type), D50129 (b-type), AB004297 (c-type), and AB00428 (d-type). The main haplotypes were type-a and type-b which constituted 89.7% of 39 samples. Haplotype and nucleotide diversity were low for all the 39 samples (Table 2).

Clustering analyses

The geographic distributions of the haplotypes (haplotype a, b, c, d) are presented in Fig. 1. Type-a was most widely spread, whereas most deer with haplotype b and d were distributed in the west half of the study area.

The analyzed individuals were grouped into two clusters. The number of clusters was consistent through 10 runs of the analyses. The maps of the highest average logarithm posterior probability are shown in Fig. 2; the subpopulation structure consisted of the two clusters that were named by cluster mt1 and mt2. Thirty individuals were assigned into cluster mt1 and 9 were assigned into cluster mt2. Haplotype-a and type-b was the main haplotype in cluster mit1 and mt2, respectively (Table 1). Deer assigned into cluster mt1 were distributed in Shiranuka Hills (10), northern part (15) and eastern part (5), while the cluster mt2 is composed of deer distributed in northern part (7) and eastern part (2) in study area (Fig. 1). Based on the definition of sika deer migration behavior by Igota et al. (2004), cluster mt1 consisted of migrants and non-migrants, while only migrants were found in cluster mt2. Any clear boundary was not found between cluster mt1 and mt2.

Haplotype and nucleotide diversity were lower in cluster mt1 than mt2 (Table 2). Genetic differentiation between two clusters was large (F_{ST} = 0.47) and highly significant based on both F_{ST} value (P < 0.0001) and haplotype frequencies (Fisher's exact test, P = 0.0003).

Microsatellite DNA analyses

Thirty-nine samples were successfully genotyped in 16 loci. The mean number of alleles (*A*) per locus, expected heterozygosity (H_E), and observed heterozygosity (H_O) are presented in Table 3. The mean number of alleles per locus is 2-7. The Hardy–Weinberg equilibrium for each locus was tested using heterozygote deficiency, and globally using the Markov chain method implemented in GENEPOP 4.2. No significant deviation from the Hardy–Weinberg equilibrium expectation was detected for 15 loci, when significance values for multiple comparisons were adjusted using the Bonferroni correction, while one loci (RM188) showed the significant deviation. The analysis of each pair of loci showed no significant linkage disequilibrium after Bonferroni correction for multiple tests.

Clustering analyses

The analyzed individuals were grouped into two clusters using the correlated frequency model, which is consistent by 30 runs, whereas no spatial structure was detected using the uncorrelated frequency model. Since the results from analyses using the uncorrelated frequency model is generally conservative in comparison with those using the correlated frequency model (The Geneland development group 2012), and the studied population may satisfy the assumption of the correlated frequency model that allele frequencies tend to be similar in different populations, we used the results of analyses using the correlated frequency model thereafter. The maps of the highest average logarithm posterior probability are shown in Fig. 3. The two clusters were named by cluster ms1 and cluster ms2. Fifteen individuals were assigned into cluster ms1, while 24 were assigned into cluster ms2. The cluster ms1 was located in Shiranuka Hills (10), northern part (1) and eastern part (4); and cluster ms2 is composed of the samples distributed in northern part (21) and eastern part (3). Based on the definition of sika deer migration behavior by Igota et al. (2004), cluster ms1 consisted of migrants and non-migrants, while only migrants were found in cluster ms2. Any clear boundary was not found between cluster ms1 and ms2.

Genetic differentiation between the two clusters was significant (F_{ST} = 0.019, P = 0.018).

Correlations between relatedness and geographic distance

Pairwise relatedness (Lynch and Ritland 1999) was obtained between all pairs of individuals (n = 741) based on microsatellite DNA features. The values varied from -0.198 to 0.286, and the average value was -0.013. Relatedness between pairs of cluster ms1 ranged from -0.166 to 0.286 (average: -0.003). Relatedness between pairs of cluster 2 ranged from -0.182 to 0.243 and average value is -0.002. Relatedness between cluster1 and cluster 2 individuals ranged from -0.198 to 0.211 (average: -0.024). The geographic distances of pairs were 0.084 - 120.426 km. Average pairwise distances were 33.910 km within cluster ms1, 26.723 km within cluster ms2, and 38.102 km between Cluster 1 and Cluster 2.

Mantel test with all pairs of 39 individuals showed no significant correlation between relatedness and geographic distance (n = 741, r = -0.025, P = 0.110). Correlation between relatedness and geographic distance was also examined for the pairs within cluster ms1 and ms2 and between the two clusters (Fig. 4). The results presented the relatedness do not correlate significantly with geographic distance (within cluster ms1, n = 105, r = 0.063, P = 0.523; within cluster 2, n = 276, r = -0.033, P = 0.581; between two cluster, n = 360, r = -0.007, P = 0.890).

Discussion

Genetic differentiation associated with migratory habits

The two clusters detected based on mtDNA analyses showed highly significant genetic differentiation (Fig. 2). However, these clusters did not perfectly match migratory habits (migrants and non-migrants) according to the definition by Igota et al. (2004). Cluster mt1 consisted of migrants and non-migrants, while only migrants were found in cluster mt2. Similarly, individuals belonging to the two clusters

detected based on microsatellite DNA analyses (Fig. 3) did not perfectly match migratory habits (migrants and non-migrants). Cluster ms1 consisted of migrants and non-migrants, while only migrants were found in cluster ms2. Individuals belonging to both of cluster mt2 and ms2 were seven, which were distributed in a compact area about 40 km north from the capture site. In comparison, individuals belonging to both of cluster mt1 and ms1 were 13, and 10 of them were non-migrants staying round the capture site. The seven and 10 individuals were distinctive about both mtDNA and microsatellite DNA features, and they showed the same migratory habit; the seven were migrants that moved toward the north from their wintering site in summer, while the 10 were non-migrants that did not leave their wintering site in summer. These results indicate that there were genetically distinctive group associated with migratory habits in a sika deer population in Hokkaido.

Although they were distinctive about microsatellite DNA features, pairwise relatedness was not higher within the clusters in comparison with the relatedness between the clusters and did not show the correlationship with geographic distance.

These results on the genetic differentiation and the relatedness indicate that the group associated with migratory habits may be conducted on the basis of matrilineage, but they were not close relatives. The matrilineage of deer continue to use the same area over generations with minimal dispersal (Nelson and Mech 1999). However, by mating with genetically distant males the relatedness of the group may be diluted.

Subpopulation structure and migration

The sika deer was abundant throughout Hokkaido prior to the colonization of the island by the Japanese in the late 19th century. Over-exploitation, habitat loss (due to agricultural development and timber extraction in lowland forests), and heavy snowfall contributed to a rapid decline in deer numbers around 1900 (Inukai 1952). To conserve the sika deer population, the Hokkaido government established hunting regulations (i.e., the prohibition of hunting and/or bucks-only hunting; Kaji et al.

2010). Nagata et al. (1998) reported that three major subpopulations having three different mtDNA haplotypes survived the bottleneck, which were located in the Akan, Hidaka, and Daisetsu mountain regions (Fig. 5; Nagata et al. 1998; Kaji et al. 2000). Following the implementation of deer protection policies, an increase in grassland area, the extinction of wolves, and a decrease in the number of hunters, the sika deer population recovered, and their distribution range expanded (Kaji et al. 2010).

Recently four subpopulations were detected for the entire sika deer population of Hokkaido based on mtDNA-based analyses using a large number of samples (n = 359) collected during 2008–2010 (Fig. 5; Ou et al. 2014). Subpopulation mtE, mtC1+mtC2, and mtN may match the surviving populations in Akan, Hidaka, and Daisetsu. In other words deer may dispersed from the three core areas (Akan, Hidaka, and Daisetsu) to various areas in Hokkaido. Akan, Hidaka, and Daisetsu may hold some high-quality wintering areas for refugia, where the core populations could survive the bottleneck. During the low density years deer may have completed their life cycle around the wintering areas, and thus may have not performed seasonal migration. According to the increase of their density they may have begun looking for better habitats for feeding in summer, and such behavioral change may have contributed to expanding their distribution range.

Shiranuka, in which we captured the radio-tracked deer, is one of major wintering areas of deer in eastern Hokkaido. The long-distance seasonal migration from Shiranuka to the east edge of Hokkaido that was observed by Igota et al. (2004) suggests that deer wintering in Shiranuka can be a source of the range expansion to eastward. Such seasonal migration may have been conducted on the basis of matrilineage, and then some deer may have settled themselves in a portion of their summer range even in winter, although female deer usually show high fidelity to seasonal ranges and migration routes (Igota et al. 2004; Sakuragi et al. 2004). The core population of Akan may hold several high-quality wintering areas beside Shiranuka. From these wintering areas deer may have dispersed to northward. Similar

processes are expected for the range expansion of the other two subpopulations (mtN and mtC1+mtC2).

Seasonal migration is "persistent movement across different habitat in response to seasonal changes in resource availability and quality" (Feldhamer et al. 1999). In sika deer of Hokkaido, spring migration from winter to summer range occurs in April-May in accord with snow-melt, while autumn migration from summer to winter range occurs during October-February (Uno and Kaji 2000; Igota et al. 2009). Since the mating season of sika deer (October-January; Suzuki et al. 1996) roughly match the autumn migration season in Hokkaido, migratory females may mate with males from various summer ranges on their way to wintering areas. Therefore, microsatellite DNA feature may be more prone to being homogenized than mtDNA feature. The low relatedness within the clusters can be explained by these processes. Furthermore the following expectation can be drawn: microsatellite DNA-based structure may be simpler than mtDNA-based structure, because mtDNA features may be maintained in each of migratory groups, while microsatellite DNA features may be exchanged among migratory groups. Indeed the subpopulation structure is simple in microsatellite DNA-based analyses in comparison with that from mtDNA-based analyses (Ou et al. 2014).

The present study focuses on migration of females, because matrilineage is important to understand the space use of deer (Nelson and Mech 1999), and females are essential components for management of deer populations (Uno 2006). The present study confirmed the importance of matrilineage in migration and the formation of spatial structure of a population. However, in order to develop a deeper understanding of the formation process of the spatial genetic structure, we need to investigate males migration behavior together with research into their genetic features.

Table 1 Basic information about individuals that were radio-tracked. All were females, whose age was one year or older at the capture. Summer ranges were estimated in August of the capture year, while winter home ranges were estimated in March of the next year of the capture.

		Age at	Location of winter range		Location of summer range	
ID No.	Capture date	capture	latitude	longitude	latitude	longitude
1	4/10/1997	3+	43.192	143.959	43.191	143.957
2	4/10/1997	3+	43.187	143.960	43.239	143.909
3	4/10/1997	3+	43.203	143.947	43.203	143.949
4	4/11/1997	1	43.178	143.982	43.424	144.411
5	4/11/1997	1	43.200	143.949	43.196	143.950
6	4/11/1997	3+	43.200	143.944	43.330	144.331
7	4/12/1997	3+	43.176	143.978	43.328	144.040
8	4/12/1997	3+	43.192	143.980	43.454	144.014
9	4/12/1997	3+	43.190	143.957	43.617	143.729
10	4/12/1997	3+	43.176	143.980	43.314	143.932
11	4/13/1997	3+	43.200	143.949	43.197	143.948
12	4/14/1997	3+	43.188	143.979	43.344	144.665
13	4/14/1997	3+	43.186	143.968	43.339	144.084
14	4/15/1997	3+	43.200	143.948	43.197	143.946
15	4/15/1997	3+	43.214	143.938	43.210	143.937
16	4/15/1997	3+	43.190	143.957	43.190	143.957
17	4/16/1997	3+	43.192	143.956	43.274	144.994
18	4/16/1997	3+	43.182	143.960	43.410	144.039
19	4/17/1997	3+	43.192	143.958	43.187	143.960
21	3/17/1998	2	43.203	143.948	43.527	143.993
22	3/18/1998	3+	43.210	143.937	43.506	143.901
23	3/18/1998	2	43.219	143.932	43.568	144.203
24	3/18/1998	3+	43.201	143.947	43.350	143.970
25	3/18/1998	3+	43.200	143.948	43.203	143.945
27	3/18/1998	3+	43.194	143.958	43.323	144.024
28	3/19/1998	3+	43.198	143.951	43.338	143.987
30	3/20/1998	3+	43.201	143.949	43.502	143.970
31	3/18/1998	3+	43.218	143.932	43.330	144.079
32	3/19/1998	2	43.208	143.939	43.486	143.993
33	3/19/1998	1	43.239	143.946	43.375	145.184
34	3/19/1998	3+	43.205	143.943	43.205	143.944
38	3/21/1998	1	43.209	143.939	43.584	144.144
39	3/22/1998	1	43.214	143.931	43.302	143.739
41	3/19/1998	3+	43.207	143.943	43.507	143.901
42	3/21/1998	3+	43.218	143.933	43.619	144.823
46	3/20/1998	3+	43.204	143.948	43.290	143.933
50	3/22/1998	3+	43.220	143.931	43.498	143.969
51	3/23/1998	3+	43.215	143.942	43.489	143.990
52	3/23/1998	3+	43.175	143.979	43.315	144.361

Table 2 Genetic diversity and haplotype frequency of mtDNA for each inferred cluster of radio-tracked sika deer.

					Haplotype frequency			
	N	*h*	*π*	*H*	type-a	type-b	Type-c	Type-d
Cluster mt1	30	0.349	0.001	3	24	4	0	2
					80.0%	13.3%	0.0%	6.7%
Cluster mt2	9	0.583	0.002	4	1	6	1	1
					11.1%	66.7%	11.1%	11.1
Total	39	0.530	0.002	4	25	10	1	3
					64.1%	25.6%	2.6%	7.7%

N: the number of samples; *h*: haplotype diversity; π: nucleotide diversity; *H*: the number of haplotypes; type-a, type-b, type-c, type-d, type-f: the frequency of haplotype a, b, c, d, and f

Table 3 Genetic variation of each locus of microsatellite DNA in radio-tracked sika deer (n = 39).

Locus name	A	H_O	H_E	P-value
OarFCB193	2	0.359	0.330	1.000
BM203	2	0.410	0.360	0.650
BM888	3	0.462	0.451	1.000
INRA040	3	0.590	0.669	0.637
Cervid14	2	0.436	0.505	0.520
BL42	2	0.410	0.410	1.000
IDVGA55	6	0.872	0.813	0.503
MM12	2	0.333	0.441	0.153
TGLA127	2	0.205	0.186	1.000
BM4107	3	0.564	0.564	0.465
ETH225	7	0.821	0.782	0.025
CSSM19	5	0.667	0.632	0.128
CSSM43	3	0.333	0.346	0.804
RM188	7	0.615	0.740	0.004
BM6506	3	0.692	0.575	0.058
TGLA337	4	0.718	0.734	0.525

A: the number of allele per locus; H_O: observed heterozygosity; and H_E: expected heterozygosity and P-value for the exact test for Hardy-Weinberg equilibrium.

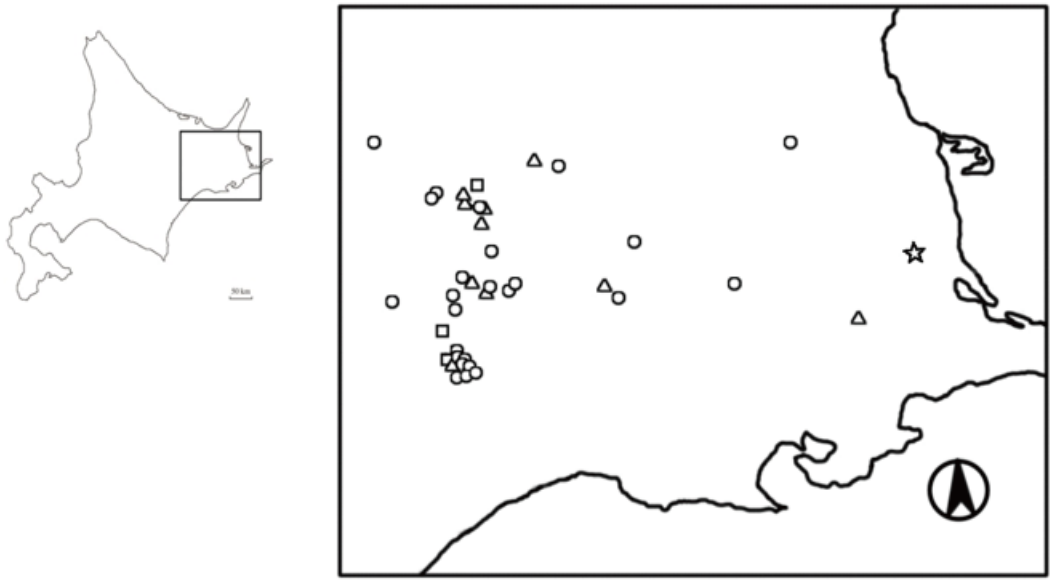

Fig. 1. Study area and the capture location (an arrow). The locations of summer home ranges are illustrated by symbols. Haplotype a, b, c, d are shown by circles, triangles, pentagrams and squares, respectively.

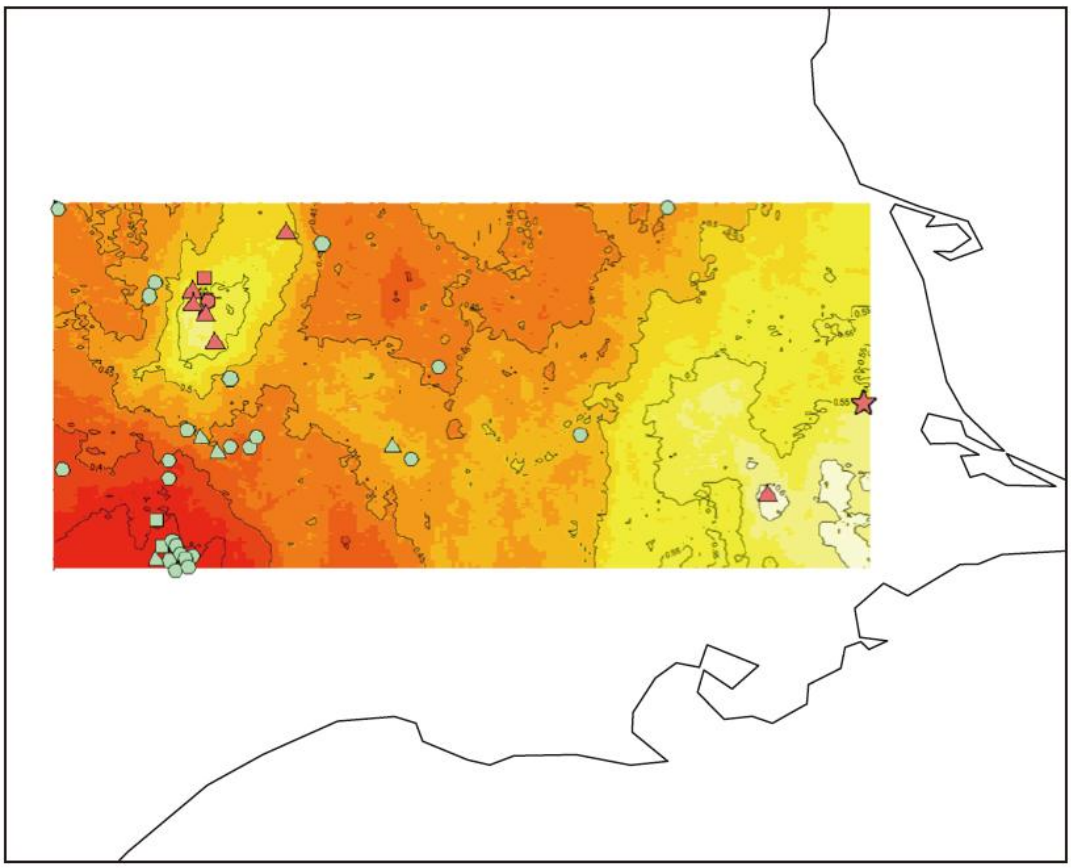

Fig. 2. Topographic map based on posterior probability of clustering by GENELAND 4.0.3. using mtDNA data. Areas with higher probability to include individuals belonging to the cluster mt2 are illustrated by lighter color. Haplotype a, b, c, and d are shown by circles, triangles, star, and squares, respectively. Green symbols denote individuals belonging to cluster mt1, red symbols for cluster mt2.

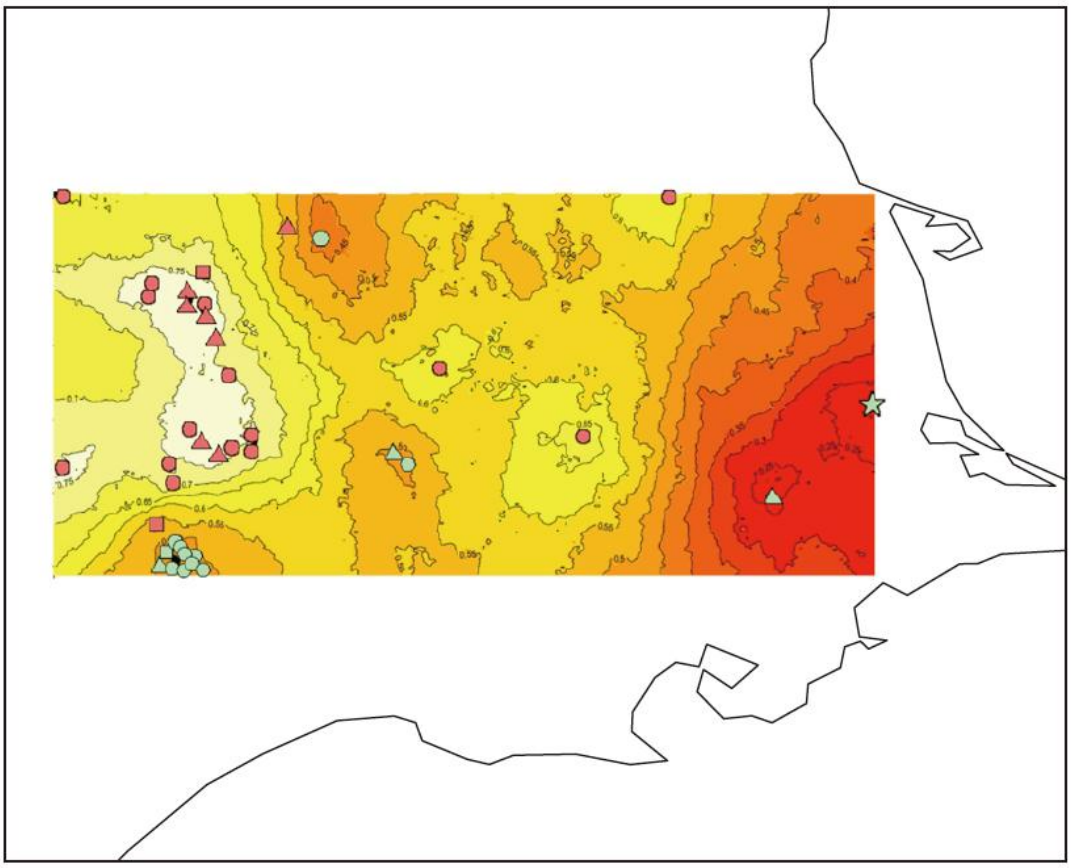

Fig. 3. Topographic map based on posterior probability of clustering by GENELAND 4.0.3. using microsatellite DNA data. Areas with higher probability to include individuals belonging to the cluster ms2 are illustrated by lighter color. Haplotype a, b, c, and d are shown by circles, triangles, star, and squares, respectively. Green symbols denote individuals belonging to cluster ms1, red symbols for cluster ms2.

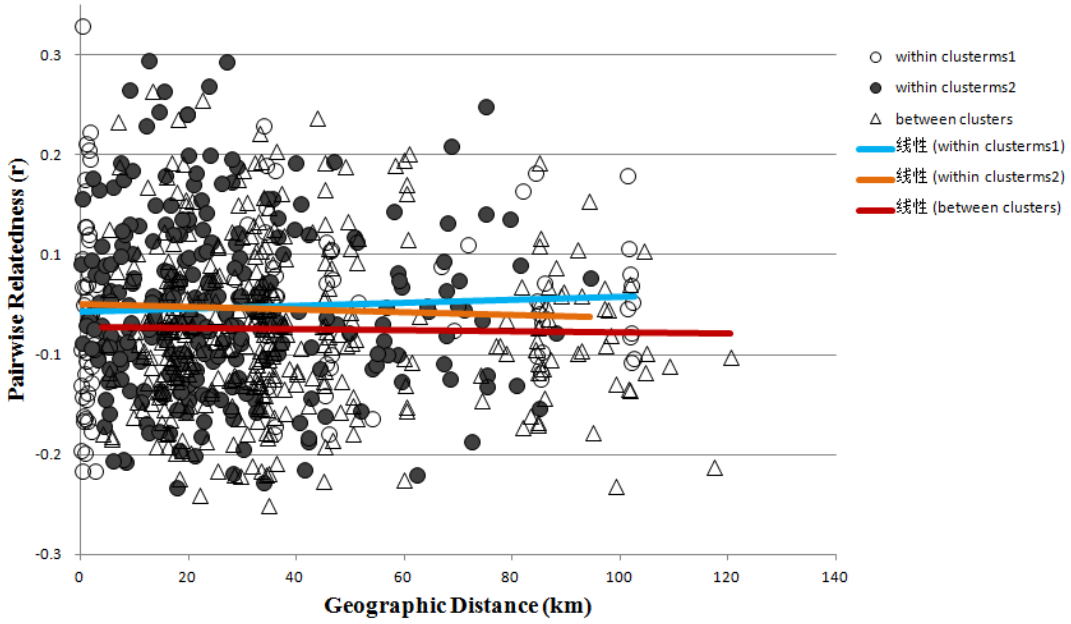

Fig. 4. Linear relationship between relatedness and geographic distance for each pair of individuals. Open circles: pairs within cluster ms1; solid circles: pairs within cluster ms2; triangles: pairs between the clusters.

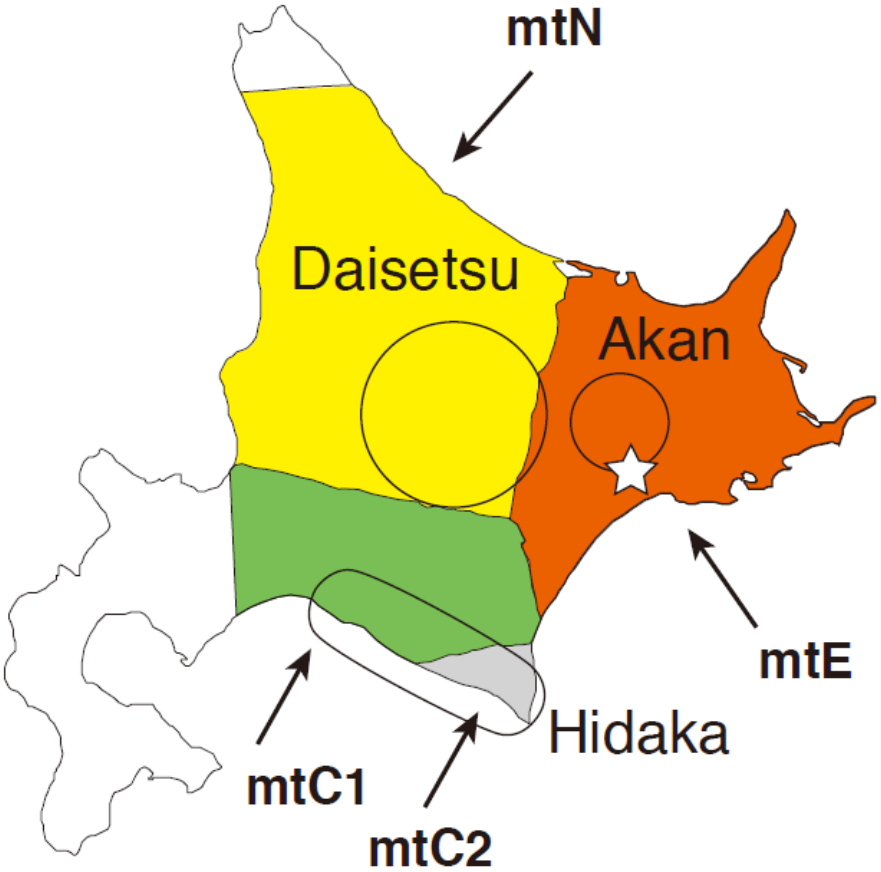

Fig. 5. Subpopulation structure of a sika deer population based on mtDNA analyses in Hokkaido (Ou et al. 2104). Open circles show three core subpopulations which survived the bottleneck from the late19th to the early 20th century. A star shows the location of the capture site (Shiranuka).

Chapter 4

General Discussion

Spatial genetic structure

Overabundance and expanding ungulate populations cause critical social problems in Europe, America and Japan. Ecology, evolution and geographic knowledge are necessary for formulating management policy. In this thesis, I used the geographic data, genetic data, demographic data and behavior data to infer the spatial genetic structure of sika deer population in Hokkaido. Temporal changes of spatial genetic structure were estimated by a landscape genetic approach combining the geographic data (coordinate information) and genetic data (mtDNA and microsatellite) (Chapter 2). A factor affecting the spatial genetic structure based on mtDNA data was explained by demographic data (Chapter 2). Behavior (migration) was a key factor to form the spatial genetic structure (Chapter 3). Therefore, studying spatial genetic structure on the basis of different scales and disciplines of deer ecology can provide useful information to work out the management.

In Chapter 2, I investigated the temporal change of spatial genetic structure by an expanding distribution over a 15-year period. The number of gene-based subpopulations decreased over a 15-year period. The two northern subpopulations in Period I were coupled into one subpopulation in Period II. Then I estimated the spatial variation of deer density (SPUE). The fusion of the two northern subpopulations caused the change to the mtDNA-based structure, which might be explained by the dispersal of females from higher to lower density subpopulations. In Chapter 3, I examined genetic differentiation associated with migratory partitioning. The results indicated that there were genetically distinctive groups associated with migratory habits in a sika deer population in Hokkaido.

This study is the first to examine the temporal change of spatial genetic structure on ungulate. The results of this study shows that the significant temporal change was the fusion of subpopulations 1990mtN1 and 1990mtN2 into subpopulation 2000mtN in northern mtDNA-based structure. This result is an indication that the spatial genetic structure may change during the ungulate population expanding. In addition, the mtDNA-based structure in eastern and central Hokkaido did not change between two periods. These results indicated that the spatial genetic structure may be stable during

the ungulate population expanding.

Dispersal and migration

Dispersal and migration are the key movements for forming the spatial genetic structure. 'Dispersal' is defined as a process by which individuals were influenced to leave their natal range and establish a new distinct home ranges (Halls 1984). Individuals usually continue along a path of dispersal until an area is found that fulfills their habitat needs. 'Migration' is defined as a process when an individual moves from one home range to a different home range and then returns to the original. Generally, movement occurs between two ranges, summer and winter. Migration from winter to summer range is referred to as "spring migration" while migration from summer to winter is known as "fall migration".

In this study, the temporal changes of spatial genetic structure have been explained by the pattern of dispersal and migration. Dispersal of female sika deer may explain the fusion of spatial genetic structure. During the sika deer population expanding, the female sika deer disperse from high density to low density for reproduction and survival. The dispersal can offer better opportunities for access to higher quality habitat, increased reproduction, avoidance of competition or predators, increased gene flow, and a higher probability of offspring having desirable genotypic traits (Bekoff 1977). Benefits for individuals that choose to disperse may include increased availability of resources (Conmins et al. 1980), decreased probability of inbreeding (Holzenbein and Marchinton 1992), and higher likelihood of surviving to reproduce (Horn 1983). However, dispersing deer may face increased energy expenditures and risk of mortality as they transition to areas. Deer that disperse tend to have lower survival rates than philopatric individuals (Nicholson et al. 1997). Migration of female sika deer may explain the stability of spatial genetic structure. Female sika deer show high site fidelity of migration between summer and winter ranges in eastern Hokkaido (Igota et al. 2004). The mating season of sika deer (October-January; Suzuki et al. 1996) roughly match the autumn migration season in Hokkaido, migratory females may mate with males from various summer ranges on

their way to wintering areas. Knowledge of dispersal and migration patterns is important in understanding the suitability and effects of efforts to manage habitat and populations (Nelson and Mech 1992).

Implications for Management

The information acquired from my study objectives could assist the Conservation and Management Plan for Sika deer (CMPS) in development of future management for deer population in Hokkaido. Specifically, information regarding sika deer population genetic structure in Hokkaido could be used as a basis for making the management unit. Now the plan makes recommendations for reducing the size of the subpopulation in eastern Hokkaido, because increased deer abundance in recent years has caused severe agricultural damage (Kaji *et al.* 2010). And the management unit is defined as western and eastern subpopulations. My study shows three subpopulations, northern, eastern and central subpopulations of mtDNA-based structure. The management units should be revised based on the subpopulation structure identified in this study.

Final considerations

The potential for the use of landscape genetics in conservation is obvious (Manel *et al.* 2003). The ability of identify geographical barriers to gene flow is great importance for the preparation of species and landscape action plans. In this study, a stable west-to-east boundary separated the north and central subpopulation in mtDNA-based structure. And a north-to south boundary in eastern Hokkaido maintained stability in the eastern subpopulation versus all other subpopulations. The landscape features around two boundaries are various landscapes, such as city area, forest, farmland, mountains, rivers, railway, and highway. Therefore, further research should be force on examining whether these boundaries are geographical barrier to gene flow between subpopulations and which landscape feature influence the dispersal of sika deer.

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