MOLECULAR ECOLOGICAL STUDIES OF SIKA DEER INHABITING CENTRAL JAPAN

【日本中部地域に生息するニホンジカの分子生態学的研究】

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ABSTRACT

At present, origin and geographical distribution of some mammalian species have been increasing with their number including distribution range in central Japan at an alarming rate. The present doctoral dissertation is focusing especially for sika deer (*Cervus nippon*). Rapid expansion of this species has resulted serious ecological imbalance subsequently loss of biodiversity. To elucidate the ecology of increasing wild sika deer is an important issue from the viewpoint of biodiversity, agriculture, forestry and conservation purposes. Genetic analysis with the advantage of widely used nuclear DNA microsatellite has been emerged as most popular, versatile marker type for ecological applications, available for inferring population structure and dynamics. This study will clarify the genetic characteristics of sika deer in Toyama Prefecture, entering routes of multiple groups from adjacent Prefectures. Since the number of deer populations in Toyama Prefecture is increasing every year, continuous monitoring is necessary in near future. This dissertation aims to disentangle the contribution of background and understanding current population structure using 11 polymorphic microsatellite loci. The present study identified that hybridizing with a resident and introduced species to enhance genetic diversity, including gene flow and dispersal pattern around Toyama Prefecture from neighboring Prefectures.

This thesis demonstrates mainly five chapters, such are:

I) The first part briefly illustrates general introduction of the study.

II) In the second part, previously maternally-inherited mitochondrial DNA analysis detected individuals possessing exogenous haplotypes of sika deer mainly from central part of Toyama Prefecture, which was thought to be derived from artificially introduced individuals. This study assessed the effects of hybridization on the exogenous and indigenous

haplotypes in the major occurrence area; this has created a fear of alteration of indigenous ecosystem. Using NewHybrids software the results reliably distinguished that hybrids between two species are fertile and produce viable offspring in backcrosses with both parental species. Furthermore, this study elucidated the contribution of genetically divergent ancestral sources and subsequent loss of pure parental species due to continuous mating across generations in the central part of Toyama Prefecture.

III) In the third part, population genetic diversity, genetic structure, admixture and migration paths were identified. Deviations from the Hardy-Weinberg Equilibrium (HWE) were observed, resulting from the mixture of samples from different sources. Pairwise F_{ST}, Fisher's exact test and molecular variance (AMOVA) among Toyama and neighboring Prefectures populations showed low but significant differentiation ($F_{ST} = 0.028$, $P < 0.0001$). Additionally, factorial correspondence analysis (FCA) and principal coordinate's analysis (PCoA) identified genetic similarity between regional neighboring Prefectures. Detected genetic continuities seem more related to the effect of past historical events. Historical migration results identified dispersal into Toyama Prefecture from all three directions, thus suggesting sika deer entered from multiple routes. Although, migration into Toyama Prefecture was high especially from one to the east whereas from Toyama Prefecture to south. Furthermore, present study results highlight that the corridors may still be functional as there is evidence of contemporary migration. This was also confirmed Bayesian STRUCTURE results, suggested populations of Toyama Prefecture derive from multiple ancestral sources, and consequently five distinct clusters were present in neighboring Prefectures populations. Each of the Prefectures has strong biasness of one particular cluster. However; Toyama Prefecture showed multiple lines of genetic structure and maximum admixture across the central region.

IV) Finally, the main goal was to assess whether any isolation-by-distance pattern in data sets using mantel tests. The result suggested significant but weak positive relationship between geographic and genetic distance, (Rxy = 0.14, R^2 = 0.02, $P < 0.002$). Additionally, spatial genetic structure results indicated presence of non-random spatial structuring and genetic association among individuals at larger distances. Individuals below this threshold, share a higher proportion of genes, than spatially distant individuals. Furthermore, both male and female sika tended to be dispersed further away from the natal area, although interestingly slight higher indication was observed for female bias dispersal. This result was also supported with negative Assignment index analysis (AIc) provided the evidence of female biased dispersal among Toyama and neighboring Prefectures.

V) Lastly, from these findings, it could be concluded that gene flow among neighboring population was high and started occurring many years before from the present study period. Additionally, present study observed central part of Toyama Prefecture mixed with multiple ancestral sources and mostly prevailed with polluted gene pool. Therefore, concern has been expressed that conservation strategy should be taken to maintain genetic integrity in Toyama Prefecture.

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

Biodiversity is the foundation for numerous ecosystem services for all living organisms that sustained provision of a wide set of ecosystem services, affected by global environmental change (Chapin et al., 2000; Cardinale et al., 2012). Recent evidences suggest that the disturbance of biodiversity caused by direct and indirect consequence of human activities and climate changes such as overhunting, introduction of alien species, and destruction of habitat (Frankham et al., 2002; Vié et al., 2009). These disturbances contribute to biodiversity loss and ecosystem degradation. For instance, most analyses of the current loss of biodiversity emphasize the patterns of species decline and extinctions (Short and Smith, 1994; Anderson, 1995; Lomolino and Channell, 1995; Pimm et al., 1995; MacPhee, 1999; Channell and Lomolino, 2001; Ceballos and Ehrlich, 2002; Contreras-Balderas et al., 2003; Ceballos et al., 2010, 2017; Woinarski et al., 2015; Estrada et al., 2017). On the other hand, some species increased drastically with their numbers and distribution ranges, also promoting the modification of ecosystems (Flowerdew and Ellwood, 2001; Fuller and Gill, 2001; Ohashi et al., 2013a, b; Tamate, 2013; Fabbri et al., 2014; Yamazaki et al., 2016). Nevertheless, either increase or decrease of species has a negative impact on biodiversity (Pennekamp et al., 2018). Knowledge of the gene pollution sources and impacts on ecosystems is important not only for a better understanding on the ecosystem responses to pollutants but also to formulate prevention measures.

During recent decades, genetic population studies provide empirical data for monitoring and predicting long-term changes in demography and population structure (Tamate et al., 2000). Such studies highlighted the genotypes of current population's structure to identify the

genetic variation within populations. Thus, involving the examination and modeling of changes in the frequencies of genes and alleles in populations over space and time (Hartl and Clark, 1997; Hedrick, 2000; Habel et al., 2015). Identification of population expansion, admixture and dispersal patterns of wild animals that contribute to mapping the origin species for effective conservation and management purposes (Frankham et al., 2002; Antonio and Marco, 2015; Habel et al., 2015; Kalb and Bowman, 2017).

Recently invasive alien species pose a significant threat to biodiversity; might share traits that allowed them to capitalize on the various elements (Dukes and Monney, 1999). Invasive or introduced species exacerbated by the potential of hybridization and may dramatically influence the establishment, spread, species declines and native habitat degradation, infecting them without any diseases resistance, or alter ecosystem functions (Vitousek et al., 1997; Wilcove et al., 1998; Akashi and Nakashizuka, 1999; D'Antonio and Meyerson, 2002; Gurevitch and Padilla, 2004; Diaz et al., 2006; McDevitt et al., 2009; Takatsuki, 2009; McGeoch et al., 2010; Darling, 2011; Veale et al., 2015; Pūraitė and Algimantas, 2016; Krojerová-Prokešováet al., 2017).

Nowadays in the Japanese Archipelago, habitat of sika deer ranges widely and became abundant from Hokkaido to Kyushu Islands particularly in the northern and central part of Honshu Islands (Japan Ministry of the Environment, 2004; Nagata, 2009). Decreased hunting is the most likely cause of population increase in sika deer in Japan at present days (Takatsuki, 2009). Recent expansion of sika deer affects vegetation in agricultural, forested, and alpine habitats (Nagata et al., 1999; Nagata, 2009; Takatsuki, 2009). According to a report from the Forestry Agency of Japan, approximately 8,800 ha of forest areas were affected by wildlife in 2014, about 80% being attributable to over-browsing by deer (Noguchi, 2017). Additionally,

artificial internal- and external- introduction of sika deer exacerbates this situation. For example, sika deer derived from Japan have been introduced to a wide range of habitats in America and Europe approximately 100 years ago, and this had a tremendous impact on local ecosystems, including hybridization with and/or displacement of native deer (McDevitt et al., 2009; Baranc˘ekova´ et al., 2012; Tamate, 2013; Kalb and Bowman, 2017).

Previously based on mitochondrial DNA sequence, two genetically distinct lineages (Northern and Southern Japan groups) were detected throughout the Japanese archipelago (Nagata et al., 1999). Furthermore, haplotypes of Northern Japan group were found in individuals of the Northern Kinki District, Honshu Island, Japan. However, some studies have reported the occurrence of an alternative haplotype in sika deer. For instance, in the Southern Kanto region, which is within the range of occurrence of the Northern Japan group of sika deer, some individuals possessing a haplotype belonging to the Southern Japan group were found. It has been suggested that such individuals or their ancestors have been artificially introduced to the region (Yuasa et al., 2007).

Northern and Southern lineages genetically distinct as well as morphology and food habit are also different. Sika deer show striking variation from north to south; for example, Northern sika deer has large and heavy in body size while southern sika is small (Yokoyama et al., 2000). Geographical location causes sika deer ecologically different in food habit due to available vegetation; Northern sika deer eat graminoids, particularly dwarf bamboos while Southern sika deer browse leaves and fruits (Nagata, 2009).

Toyama Prefecture is located approximately at the center of the Japanese Archipelago, with plains covering an area of 30 km^2, and mountains of several thousand meters' elevation on three sides, with the fourth side bordered by the sea. Recently, some wild animals were tremendously increasing in Toyama Prefecture; for example, wild boar and sika deer populations. Genetic investigations have already been carried out for wild boar and elucidation of population structure and migration path (Yamazaki et al., 2015, 2016) using both mitochondrial DNA (mtDNA) and microsatellite markers analysis. During the Meiji era in Japan (1868-1912), the number of sika deer decreased by over hunting to produce furs in Toyama Prefecture. It has been reported that the appearance and disappearance in multi snowy area and alpine belt on Japan sea side, was not a habitat in past. However, the number of individuals increased in recent years and continues to expand each year (Nambu, 1999; Nambu and Yoshimura, 2002; Toyama Prefecture, 2017). Since, in Toyama Prefecture and surrounding areas, genetic analysis of sika deer has hardly been performed, therefore the genetic information is insufficient. From the mitochondrial DNA (mtDNA) analysis, Yamazaki (2018) explored the current existences of haplotypes. Sika deer in Toyama Prefectures mainly from the Northern Japan lineage group, whereas some individuals showed haplotypes corresponded to the Southern Japan lineage group, especially at high frequencies in the central Toyama. However, expansion of invasive haplotypes and the current population structure using microsatellite marker has yet to be investigated.

Identification of population units within species is a crucial task for management authority to create guideline for management practices. Microsatellite markers proved efficient and cost-effective to address many questions in molecular ecological studies and powerful to assessing population genetic structure. Microsatellite markers or Simple Sequence Repeat (SSR) was widely used for DNA profiling in molecular ecology for

conservation genetics, fingerprinting and phylogenetic studies due to their co-dominant, hyper-variable and multi-allelic nature at each locus (polymorphism), can easily amplified with the polymerase chain reaction (PCR) (Dimitry et al., 2006; Miah et al., 2013; Senan et al., 2014). It represents a unique type of tandemly repeated genomic sequences, which are abundantly distributed across genomes and mostly used to understanding genetic diversity, information of migration; distinguish the relatedness of individuals (Selkoe and Toonen, 2006). Several recent reviews detail the myriad of genetic analysis and encourage ecologists to use genetic approaches for ecological questions they can address (Quelleret al., 1993; Bossart and Prowell, 1998; Davies et al., 1999; Luikart and England, 1999; Shoemaker et al., 1999; Sunnucks, 2000; Manel et al., 2002; 2005; Beaumont and Rannala, 2004; Pearse and Crandall, 2004). Microsatellite markers were widely used for deer populations to investigate the genetic similarity or dissimilarity and gene flow between sika deer populations based on the analysis of allele frequencies at different loci (Tamate et al., 2000; Goodman et al., 2001; Diaz et al., 2006; McDevitt et al., 2009; Pūraitė and Algimantas, 2016; Krojerová-Prokešová et al., 2017; Konishi et al., 2017). However, to date, sika deer expansion studies have been limited to a few areas in Yamagata Prefecture (Sato et al., 2013). Thus, the present study was conducted to elucidate genetic population structure of recently expanded sika deer population in Toyama Prefecture using polymorphic microsatellite DNA markers to determine the degree of genetic admixture from different ancestors.

This doctoral dissertation will be explained as follows:

In the second chapter, previously maternally-inherited mitochondrial DNA analysis detected individuals possessing exogenous haplotypes of sika deer mainly from central part of Toyama Prefecture, which was thought to be derived from artificially introduced individuals. In the present study microsatellite analysis was used to find out the effects of hybridization on the exogenous and indigenous haplotypes; this has created a fear of alteration of indigenous ecosystem.

In the third chapter, using genetic analysis of sika deer in Toyama and surrounding Prefectures (Nagano, Niigata, Ishikawa, Gifu, Aichi and Fukui) clarified population genetic structure, admixture and migration path of sika deer population inhabiting in Toyama Prefecture. This chapter explored the regional uniqueness, group structure, and entry route in Toyama Prefecture.

Lastly, in the fourth chapter, showed whether any isolation-by-distance pattern present or not in data sets and spatial genetic autocorrelation according the genetic and geographical distance. Furthermore, sex biasness in dispersal pattern was also examined.

Analysis of population structure, understanding the processes and patterns of gene flow, local adaptation, identifying migrant individuals and detection of hybridization requires a detailed knowledge to establish a framework and effective management strategies and/or conservation policies for the indigenous ecosystem as well as wild animal species.

CHAPTER 2

HYBRIDIZATION BETWEEN NATIVE AND INTRODUCED INDIVIDUALS

2.1. Introduction

Invasive species was considered the 2nd most cause of global biodiversity loss can have far-reaching mostly harmful effects on ecosystem such as disturbance of indigenous habitats, alteration of biological interaction, damage of agricultural crops, and subsequent loss of biodiversity (Akashi and Nakashizuka, 1999; McNeely et al., 2001; Wittenberg and Cock, 2001; Baskin, 2002; Côté et al., 2004; Takatsuki, 2009). Invasive animals have been often introduced outside of their natural range and distribution area with closely related species, even within conspecific distribution area for commercial activities, biological control, and environmental education, or through unintentional introduction, thereby occasionally resulting in intraspecific and interspecific genetic hybridizations (Rhymer and Simberloff, 1996; Park, 2004). Hybridization significantly leads to a disturbance in genetic diversity and/or outbreeding depression, i.e. genetic pollution (Frankham et al., 2002). The introduced species or hybrids animals raised particular interest to understand how they affect the structure and composition of invaded ecosystems and conservation status of indigenous habitats. First of all, they are able to modify native plant and animal community which was essential to balance the ecosystem functioning (Côté et al., 2004; Mysterud, 2006). Another main problem of hybridization is introgression, if introgression does occur and continuing between species it can be highly destructive to the integrity of locally adapted species and may eventually be extinct of the original species via genetic mixing (Rhymer and Simberloff, 1996; Marco et al., 2016).

Sika deer derived from Japan had been introduced throughout North America, Europe, and Oceania, leading to the replacement and/or hybridization with native deer in some

regions (Diaz et al., 2006; McDevitt et al., 2009; Kalb and Bowman, 2017; Krojerová-Prokešováet al., 2017). After introduction of sika deer, have expanded distributional range and populations due to their ability to hybridize with local red deer (*Cervus elaphus*) was primary concerns about hybridization including damage of habitats and forestry (Swanson and Putman, 2009).

Recently, Yamazaki (2018) examined the mtDNA haplotypes of sika deer collected from Toyama Prefecture, Central Japan, and detected haplotypes belonging to both lineages, despite the region is within the occurrence range of the northern lineage haplotypes. Especially, in the central part of Toyama Prefecture, sika deer individuals possessing the southern lineage haplotype, having high homology with those detected in the Yakushima Island, off the southern coast of Kyushu, Japan, appeared frequently together with the northern lineage. The southern lineage haplotype is thought to be derived from the individuals, which had been captive-bred in the central part of Toyama Prefecture, and might have subsequently escaped (Nambu, 1999; Yamazaki, 2018). The identification and characterization of hybrids generally based on morphological approach although only morphology to detect hybrids has proven problematic due to un availability of known hybrids samples and hybrids offspring has been usually taken on the basis of morphological intermediacy from the parental species (Esquer-Garrigos et al., 2015).

The present study explored the genetic composition of the sika deer using microsatellite markers and determined the degree of genetic disturbance in the central part of Toyama Prefecture, to conserve the native gene pools.

2.2. Material and methods

2.2.1. Samples

In the present study, DNA samples extracted by Yamazaki (2018), samples of 83 sika deer collected from Toyama City, the central part of Toyama Prefecture between 2013 and 2016 were subjected to microsatellite genotyping (Fig. 1). Samples were provided by hunters who had legal permits. Tissue samples were stored at room temperature (25°C) in 99% ethanol before DNA extraction. DNA was extracted using a Gentra Puregene Tissue Kit (QIAGEN) according to the protocol provided by the manufacturer. These samples were separated into two groups: group I (n = 44) included individuals possessing the native (northern lineage) haplotypes (Cn01, Cn03, Cn04, Cn07, and Cn10) and group II (n = 39) included individuals possessing the introduced (southern lineage) haplotype (Cn11), according to the mtDNA analysis of Yamazaki (2018).

2.2.2. Microsatellite genotyping

The samples were genotyped using 13 nuclear microsatellite markers, such as loci BL42, BM3628, BM6506, BM6438, and BMC1009 (Bishop et al., 1994); BM203, BM888, and OarFCB193 (Talbot et al., 1996); Cervid14 (DeWoody et al., 1995); CSSM019 (Moore et al., 1992); ETH225 (Kühn et al., 1996); IDVGA29 (Mezzelani et al., 1995); and RM188 (Barendse et al., 1994) (Table 1). Polymerase chain reaction (PCR) was carried out according to a multiplex standard protocol using the Type-it Microsatellite PCR Kit (QIAGEN). Multiplex mixes set A (BL42, BM203, BM888, BMC1009, BM6438, and CSSM019) and set B (BM3628, BM6506, Cervid14, ETH225, IDVGA29, OarFCB193, and RM188) loci were amplified, respectively. The microsatellite forward primers were labeled with the fluorescent

dyes 6-FAM, VIC, NED, or PET. The products of PCR were analyzed using the 3130 Genetic Analyzer (Applied Biosystems). The allelic size was scored using the GeneScan-500 LIZ Size Standard (Applied Biosystems) and analyzed using the GeneMapper version 3.7 (Applied Biosystems).

2.2.3. Genetic data analysis

2.2.3.1. Genetic Diversity

Deviation from the Hardy-Weinberg Equilibrium (HWE) was tested using GENEPOP version 4.2 (Raymond and Rousset, 1995) with the following chain parameters: 10000 dememorizations, 100 batches, and 10000 iterations. At first, all the samples were tested together and then individually for each group. GENETIX version4.02 (Belkhir et al., 1996-2004) was used to calculate the observed heterozygosity (H_O) and expected heterozygosity (H_E). The MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004) was used to test for technical artefacts such as PCR stuttering, null alleles and large allele dropout. The number of alleles and the unique alleles of per locus were counted using the FSTAT version 2.9.3.2 (Goudet, 1995). Pairwise F_{ST} and exact tests were conducted using the ARLIQUEN version 3.5.1.2 (Excoffier and Lischer, 2010) and GENEPOP version 4.2 (with the same setting used for HWE test) respectively, to quantify the genetic differences between two groups based on gene frequency.

2.2.3.2. Hybridization analysis

To detect hybridization between native and introduced gene pools, NewHybrids version 1.1 beta3 (Anderson, 2003) was used to calculate the posterior probability of individual belonging to six genotypic classes: two Parental classes (A and B), first generation hybrid (F_1), second generation hybrid (F_2), and backcrosses hybrids. The posterior distributions were run total 550,000 Markov chain Monte Carlo (MCMC) iterations (the first 50, 000 steps were discarded as burn-in) and uniform priors were chosen for allele frequencies and mixing proportions to reduce the effect of very low frequency alleles. The result was also checked with total 250,000 and 350,000 Markov chain Monte Carlo (MCMC) iterations with discarding 50, 000 burn in respectively in all case. However long or short run did not influence the result.

2.2.3.3. Population structure

Bayesian clustering algorithm available in STRUCTURE version 2.3.4 (Pritchard et al., 2000) was used to infer individual genetic ancestry. I used the settings of the admixture model with correlated allele frequencies; all other parameters used the default settings. To estimate the number of clusters (K), 10 independent runs with K = 1-10 were conducted with 100,000 iterations, followed by a burn-in period of 200,000 iterations of Markov Chain Monte Carlo (MCMC) reps after burn in. The most likely K value was determined according to the ad hoc statistic ΔK based method, developed by Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and von Holdt, 2012).

2.3. Results

Out of the 13 microsatellite markers tested in the present study, 12 markers-amplified loci were found to be polymorphic; however, the locus IDVGA29 was monomorphic. Highest number of alleles (N_A = 11) was detected in both group I and II at the locus CSSM019, whereas lowest number of alleles (N_A = 2) was detected in group I at the locus BM203. MICRO-CHECKER did not indicate any null alleles or genotyping errors such as large allele dropout or stuttering. Since locus CSSM019 showed deviation from the HWE in both total population and separate groups and locus IDVGA29 was found monomorphic, both were excluded for further analysis. After exclusion total 63 alleles were found in the present study. Seven out of 33 test samples indicated significant deviations from the HWE (Table 2). Three deviated loci were detected in the total samples (combined group I and II) and two deviated loci were detected in each of group I and II. Most loci deviated from HWE showed lower H_E when compared with the H_O. Considerable differences in allele distribution between two groups were observed, such as the loci BM203, BM888, BM3628, BM6438, BM6506, and Cervid14. The present study found that seven alleles of group II were absent in group I, whereas three alleles of group I were absent in group II. The H_O for whole loci was 0.662 for the total sample, 0.638 for group I, and 0.688 for group II (Table 2). Pairwise F_{ST} value (F_{ST} = 0.019; $P < 0.001$) and exact test result ($P < 0.001$) indicated significant genetic difference between the two groups.

Posterior probabilities of assigned individuals to the genotype classes (Parent A and B, F_1-hybrid, and F_2- or backcross hybrids) were obtained with NewHybrids program (Fig. 2). The posterior probabilities of Parent A tended to be higher in group I individuals (0.06 - 0.97, average: 0.73) than in group II individuals (0.00 - 0.93, average: 0.47). The posterior

probabilities of Parent B were marginally high in group II individuals (0.00 - 0.66, average: 0.08) than in group I (0.00 - 0.25, average: 0.02). Furthermore, the posterior probabilities of hybrid categories (F_1, F_2, and backcross hybrids) tended to be higher in group II individuals (0.07 - 0.95, average: 0.45) than in group I individuals (0.03 - 0.80, average: 0.26). Only 11% (5/44) of sika deer samples in group I showed extremely high posterior probabilities (≥ 0.95) to the Parent A category; however, none of the individuals showed high posterior probabilities to the Parent B category. The posterior probabilities in almost all individuals contained those belonging to multiple categories including F_1, F_2 or backcross hybrids.

Bayesian structure analysis revealed the height ΔK value as K = 5 (Fig. 3). Individuals were assigned to five different clusters and the percentage of membership coefficient (Q) was admixed in the central region of Toyama Prefecture. This study did not found any dominant genetic cluster present in current sika deer population between the two studied groups (Fig. 4).

2.4. Discussion

From the assessment of genetic composition of sika deer, deviations from HWE were observed in both the total and separate groups possessing native and introduced haplotypes, suggesting that the sampled sika deer in central part of Toyama Prefecture cannot be considered a single random mating population. Random mating can be prevented by certain population dynamics, such as shrinkage population size, division of individual population by geographical isolation, expansion in population, and/or mixing of populations from multiple origins (Futuyma, 1998). Furthermore, most loci significantly deviated from the HWE (Table

2) and the observed heterozygosity (H_O) was higher than expected (H_E), implying mixing of different gene pools (Frankham et al., 2002).

In the ancestral estimation using NewHybrids, existence of two Parental sources and multiple elements were identified. Among 83 sampled individuals 40% individuals showed mixed ancestry (Fig. 2). The results showed, individuals that were assigned a high probability of a single assumed ancestor Parent A was less, probably suggesting that such individuals corresponded to pure native ones. Other individuals showed an admixture of multiple genetic classes, including those of opposing Parent B and various degrees of hybrids. These results indicate the existence of multiple ancestors and their genetic changes in the current sika deer population in the central part of Toyama Prefecture. Total 11% (5/44) pure Parent A individuals with introgressed genomes are common, suggesting the possibility that hybridization occurred several generations before sampling (Goodman et al., 1999). Similar kind of study showed in 15.5% hybrid individuals in the studied region between native red deer (*C. elaphus*) and invasive sika deer (*C. nippon*) in Eastern Europe (Biedrzycka et al., 2012). In Kintyre Peninsula of Scotland, native red deer and invasive sika deer showed overall 6.9% mixed ancestry only one site where 43% individuals were hybrids (Senn and Pemberton, 2009).

Subsequently, STRUCTURE also confirms the existence of many distinct gene pools in central part of Toyama Prefecture, suggesting that there were five genetically divergent ancestral sources. Current study result showed there was no pure or dominant genetic cluster present in the studied population in central Toyama. Probably one particular cluster dominancy was lost due to introgression of gene pool between indigenous and exogenous sika deer populations and also the possibility of continuous mating across generations.

Although Toyama Prefecture should be within the occurrence range of northern lineage haplotypes of sika deer, Yamazaki (2018) reported that individuals possessing southern lineage haplotypes in this region appeared frequently together with the northern lineage. Based on the phylogeographical analysis, southern lineage mtDNA haplotype was closely related with haplotype, which was detected from individual in Yakushima Island (Yamazaki, 2018). Some of the introduced sika deer in central part of Toyama Prefecture might have been originated from Kyushu (see Nambu, 1999). Additionally, some individuals possessing the southern lineage mtDNA haplotype in this study less than two years old, suggesting mating among native, introduced, and their descendant individuals might have continued since introduction of sika deer in the central part of Toyama Prefecture.

Some instances of interspecific, including introduced species, hybridization in deer have been reported around the world (Goodman et al., 1999; Diaz et al., 2006; McDevitt et al., 2009; Matsumoto et al., 2015; Pūraitė and Algimantas, 2016; Kalb and Bowman, 2017; Krojerová-Prokešová et al., 2017). However, examples of intraspecific hybridization of deer, i.e. mating between native and domestic introduced individuals, are limited. As intraspecific hybridization is one of the important issues in conservation (Allendorf et al., 2001), continuous proliferation due to both interspecific and intraspecific hybridizations will further lead not only to the expansion of genetic pollution in sika deer, but also to the ecological imbalance in native ecosystem. Therefore, to control the increasing number of sika deer in Toyama Prefecture as well as regions where sika deer is increasing, need to take immediate measures such as increasing hunting pressure, enforcement of strict legislation, and safety practices to prevent further spread (Kaji, 2010; Iijima and Nagaike, 2015).

CHAPTER 3

POPULATION STRUCTURE, ADMIXTURE, AND MIGRATION PATTERNS

3.1. Introduction

During recent decades, researcher's interest is growing for population genetic structure and viability of species due to many abundant or extinction of species under the impact of human population growth and actions (Whiteley et al., 2006; Gaston and Fuller, 2007; Basto et al., 2016). Delimitation of species units and understanding the structure is primary importance for management and conservation biology (Coulon et al., 2006; Hunter and Gibbs, 2006). Genetic diversity seen within and among species is influenced by a complex interplay of ecological and evolutionary processes (Tinnert et al., 2016). Demographic and evolutionary population structures are interlinked through dispersal and gene flow events (Futuyma, 1998; Bohonak, 1999; Eric et al., 2005). Dispersal is the universal tendency to spread from territorial populations that are often limited by natural (e.g., mountains, rivers) or artificial (e.g., fences, motorways, hunting) barriers (Frantz et al., 2012; Niedzialkowska et al., 2012; Li et al., 2013; Sawaya et al., 2014; Sun et al., 2016). Gene flow defines the movement and integration of genes from one population into another through dispersal and mating. Studies on gene flow examine current population structure by estimating essential genetic differences among populations (Goodman et al., 2001; Pérez-Esponaet al., 2008; Senn and Pemberton, 2009; Genovart et al., 2013). Analysis of population structure and identifying migrant individuals establishes a framework that can be used to protect the indigenous ecosystem. In conservation biology genetic approaches have become the most pragmatic way to elucidate interference of current population structures via gene flow (Richard and Linda, 2007).

Recent rapid flux of sika deer is now recognized as one of the serious ecological issues worldwide such as alter forest structure, agricultural crops (Côté et al., 2004; Takatsuki, 2009). Researchers previously found sika deer derived from Japan have been introduced to a

wide range of habitats in America and Europe approximately in the 19th Century, and this has had a tremendous impact on local ecosystems such as have bred extensively with native deer and displacement has occurred on population structure (Diaz et al., 2006; McDevitt et al., 2009; Tamate, 2013; Swanson and Putman, 2009; Kalb and Bowman, 2017; Krojerová-Prokešová et al., 2017).

Recently, the number of sika deer is increasing in Toyama Prefecture and damaging ecosystems, agriculture crops and forestry. There are also concerns about the impact on alpine plants (Toyama Prefecture, 2017). From microsatellite DNA analysis deduced in chapter 2, showed hybridization occurred between native and introduced individuals over multiple generations. Therefore, the influence of such hybridization on current population structure of Toyama Prefecture should be clarified. Effective conservation strategies for indigenous ecosystems, such as identifying major dispersal routes, should be outlined to manage uncontrolled sika deer populations. In this study; genetic population structure of a recently expanded sika deer population was elucidated in Toyama Prefecture and its surrounding areas using polymorphic microsatellite DNA markers. In this chapter, 1) genetic diversity, 2) population structure, and 3) migration path using both contemporary and historical migration were shown.

3.2. Materials and methods

3.2.1.Samples

Tissue samples (muscle or ear tip) from 247 sika deer were collected from throughout Toyama and adjacent Prefectures between 2013 and 2017. Samples used by Yamazaki (2018) and (chapter 2) were also used (Fig. 1). For population genetic analysis, the samples from each intra-Prefectural regions (east, central, and west) of Toyama Prefecture were treated separately (Fig. 1, Table 3). Sika deer samples originated from 165 males, 76 females, and 6 individuals of unknown sex. Most of the data from the central region of Toyama Prefecture were obtained from (chapter 2).

3.2.2. Data analysis

3.2.2.1. Genetic diversity

MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004) was used to check the occurrence of genotyping errors due to PCR stuttering, non-amplified alleles (null alleles), and large allele dropout. Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008) with the following chain parameters: 10,000 dememorizations, 100 batches, and 10,000 iterations. First all samples from Toyama Prefecture together tested and then tested each of the three regions in Toyama and the surrounding Prefectures separately. Allelic richness (*AR*) or private allelic richness (*PAR*) was estimated using the rarefaction statistical approach, implemented using HP-RARE (Kalinowski, 2005). GENETIX version 4.02 (Belkhir et al., 1996-2004) was used to calculate the observed heterozygosity (H_O) and expected heterozygosity (H_E).

3.2.2.2. Population differentiation analysis

The pairwise F_{ST} and Fisher's exact tests were conducted using the ARLIQUEN version 3.5.1.2 (Excoffier and Lischer, 2010) and GENEPOP version 4.2 respectively, to quantify the genetic differences between the populations based on gene frequency. GENEPOP was used to quantify the genetic difference between the populations using the pairwise F_{ST} and Fisher's exact test based on gene frequency. Bonferroni (Rice, 1989) and Benjamini-Yekutieli (Narum, 2006) methods were used to adjust the significances levels of pairwise F_{ST} and Fisher's exact test, respectively, when multiple comparisons were performed. Nei's genetic similarities and distances calculated based on pairwise population matrix of different populations using GenAlEex version 6.5 (Peakall and Smouse, 2012). In addition, factorial correspondence analysis (FCA) implemented in GENETIX version 4.02 (Belkhir et al., 1996-2004) was used to visualize the genetic relationship among individuals. PCoA (Principal Coordinate Analysis) was carried out using GenAlEx version 6.5 (Peakall and Smouse, 2012). PCoA was calculated among all of the individuals and also Nei's pairwise genetic distance from geographic distance based on Global Position System coordinates.

The Bayesian clustering algorithm available in STRUCTURE version 2.3.4 (Pritchard et al., 2000) was used to infer individual genetic ancestry. I used the settings of the admixture model with correlated allele frequencies; all other parameters used the default settings. To estimate the number of clusters (K), 10 independent runs with K = 1 - 10 were conducted with 100,000 iterations, followed by a burn-in period of 200,000 iterations of Markov Chain Monte Carlo (MCMC) reps after burn in. The most likely K value was determined according to the ad hoc statistic ΔK based method, developed by Evanno et al. (2005) using STRUCTURE HARVESTER (Earl and von Holdt, 2012).

The within and among population differentiation was investigated by analyses of molecular variance (AMOVA) using Arlequin 3.5.1.2 (Excoffier and Lischer, 2010). In order to see whether differences between the population structures, a spatial analysis of molecular variance was performed using SAMOVA 2.0 (Dupanloup et al., 2002) to identify groups of populations that are phylogeographically homogeneous and maximally differentiated from each other, taking into account the geographic distances. This analysis permits identification of the maximally differentiated groups that correspond to predefine. SAMOVA was run successively with a different K (the putative number of populations as different groups), ranging from 2 to 6 using the average latitude and longitude of all sample localities within each population (Qingzhang et al., 2014; Stephen et al., 2015). Analyses were run twice for each value of K to check consistency between runs. For each run, 100 simulated annealing processes were performed. This simulation aims at maximizing the fixation index among groups (F_{CT}), which is the proportion of total variance due to differences between groups of populations. For each simulation, the population indexes among groups (F_{CT}), among individuals within populations (F_{IS}), and among population within groups (F_{SC}), within individuals (F_{IT}) variation between groups were calculated. To test the genetic relationships between the population different groups were considered as follows: (K = 2 groups): (TOY-E, TOY-C, TOY-W) and (NIG, NGN, GIF, ISK, AIC, FUK); (K = 3 groups): (TOY-E, TOY-C, TOY-W, GIF, AIC); (NIG, NGN) and (FUK, ISK); (K = 4 groups): (TOY-E, TOY-C, TOY-W); (NIG, NGN); (GIF, AIC) and (FUK, ISK); (K = 5 groups): (TOY-E, TOY-C, TOY-W, NIG, NGN); (GIF); (AIC); (FUK) and (ISK); (K = 6 groups): (TOY-E); (TOY-C); (TOY-W); (NIG, NGN); (GIF, AIC) and (FUK, ISK).

3.2.2.3. Gene flow

To evaluate the magnitude and direction of gene flow four Bayesian approaches were used. To estimate recent migration, GENECLASS2, STRUCTURE and BAYESASS were used and for historic gene flow MIGRATE-n 3.6.11were used.

First, to estimate contemporary migration patterns between the populations GENECLASS2 (Piry et al., 2004) was used to detect the first-generation migrants (F_0). The likelihood-based Bayesian methods of Rannala and Mountain (1997) were used and the simulated likelihood distribution was assessed using the Monte Carlo re-sampling method of Paetkau et al. (2004), with 1000 simulated individuals and type I errors 0.05 and 0.01.

Second, STRUCTURE (Pritchard et al., 2000) was used to verify the robustness of GENECLASS2 results using population information with the same parameters settings as described above to detect recent migrant ancestry (MIGRPRIOR = 0.03 and 0.05) and migrant descendants within only two generations (GENSBACK = 2). Priori choices did not affect the results, so I only report the results for MIGRPRIOR = 0.05.

Third, BAYESASS version 1.3 (Wilson and Rannala, 2003) was used to trace each individual's recent migration ancestry within the last two to three generations. BAYESASS runs were performed a total of 8×10^6 MCMC iterations, by discarding the first 2×10^6 steps as burn-in and sampling at every 2,000 iteration intervals of the remaining 6×10^6 MCMC chain. Individual assignments and immigrant ancestries were calculated at a migration rate prior of 0.05, however the result also checked with the prior rate (0.01) which did not affect the results and all other settings were kept at default.

Finally, to determine historic gene flow between populations, the Bayesian coalescent approach implemented in MIGRATE-n 3.6.11 were used (Beerli and Felsenstein, 2001; Beerli, 2006, 2009). MIGRATE-n was used to estimate the mutation-scaled population size theta ($\theta = 4N_e\mu$, where N_e is the long-term effective population size and μ is the mutation rate per locus per generation), and the mutation-scaled immigration rate ($M = m/\mu$, where m is the proportion of immigrants and μ is the mutation rate per locus per generation). These results were estimated using a Bayesian inference with a full migration matrix model. The Brownian-motion model was chosen with constant mutation rates and uniform priors were set for both θ and M (min = 0, max = 20, delta = 50). Calculation was performed with a burn-in of 10,000 iterations for each chain and one long chain with 100 increments and 20,000 recorded steps in each chain. A static heating scheme at four temperatures (1.0, 3.0, 6.0 and 10) was used with a swapping interval of 1. The parameter was chosen based on the performance of multiple trial runs with different values. For migration analysis, the samples were divided according to the geographical locations of Toyama such as eastern side (NIG & NGN), southern side (GIF) and western side (ISK) of Toyama Prefecture. The result was considered as the median values of θ and M with posterior distribution values (0.025 - 0.975) as 95% confidence interval estimates. As θ and M estimates from MIGRATE-n are compounded by the mutation rate, the effective number of immigrants (N_em) per generation in each Prefecture was calculated by multiplying M by θ to avoid making an assumption about mutation rates (Hull et al., 2010; Genovart et al., 2013; Miryeganeh et al., 2014; Baltazar-Soares and Eizaguirre, 2016).

In addition, Maximum likelihood approach was also evaluated with the default setting parameter. The present study evaluated the goodness of fit of data under several different migration scenarios. The models tested included:

1. A full migration model in which unrestricted migration was assumed among all populations.
2. An n-dimensional island model assuming equal reciprocal migration among all populations and equal population sizes.
3. A migration model assuming the stepping stone (type I), which hypothesizes that bi-directional migration occurs between geographical neighboring populations.
4. A migration model assuming the stepping stone (type II), following the hypothesis that symmetric migration occurs between the geographical neighboring populations but not among other populations.
5. A migration model assuming the Source-Sink (type I), which hypothesizes that non-restricted migration occurs among TOY populations (Source) but unidirectional migration occurs from the populations (Sink)
6. A migration model assuming the Source-Sink (type II), which hypothesizes that non-restricted migration occurs among NIG and NGN populations (Source) but unidirectional migration occurs from the populations (Sink)

Full model was run twice, and default search strategy was used to confirm the convergence of parameter estimates. Alternative models were each run once, with the initial θ values being the estimates from the final run of the full model. Likelihood estimates of all models were compared to the full model using a likelihood-ratio test to evaluate goodness of fit of the model in order to determine which migration model was statistically best supported by the data. The Akaike Information Criterion (AIC) was used to compare the migration models with different combinations of parameters (θ, M). However, considering the programming options of MIGRATE-n, successful application of Bayesian inference is often simpler than maximum likelihood (Beerli, 2006).

3.3. Results

3.3.1. Genetic diversity and population structure

Of the 13 microsatellite DNA markers, 11 were polymorphic among the populations, although allele frequency at each locus was different in each population. Since IDVGA29 and ETH225 loci were monomorphic, both were excluded from further analysis. MICRO-CHECKER confirmed that none of the remaining loci (except AIC and FUK) showed any evidence of scoring errors due to large allele dropout or stutter packs in any of the populations. Sample sizes of Aichi and Fukui Prefectures were small, and MICRO-CHECKER did not produce any results for these populations. A total of 27 of 110 tests indicated deviations from HWE (Table 4). For the Toyama Prefecture samples, significant heterozygote excess was observed at OarFCB19, BM888, Cervid14, BM203, and BM3628 loci, and heterozygote deficits were observed at CSSM019, RM188, and BMC1009 loci (Table 4). None of the marker pair tests showed any LD after Bonferroni correction.

The number of alleles per locus ranged from 4 (loci BM203 and BL42) to 11 (locus CSSM019) (Table 4). The lowest *PAR* value was 0.080 in TOY-C and AIC, and the highest *PAR* value was 0.150 in GIF. The H_O for all loci ranged from 0.742 to 0.909 for NIG and FUK and the H_E for all loci ranged from 0.568 to 0.740 for ISK and TOY-W (Table 5).

Results of pairwise F_{ST} and Fisher's exact test showed significant differences between TOY-E and TOY-C and between TOY-E and TOY-W, after correction (Table 6). There were significant differences each of the Toyama Prefecture regions simultaneously showed significant differences from NIG, NGN, and GIF (Table 6). Nei's genetic similarities were very high ranges (86 - 99%) among populations of sika deer in Toyama and neighboring

Prefecture. Inversely, values of genetic distances were very low between different populations (Table 7). The two-dimensional plot derived from FCA showed that the total variation for axis 1 was 34.33%, while that for axis 2 was 18.29% (Fig. 5). Individuals in geographically neighboring regions tended to be moderately genetically close. This study observed GIF showed distinct genetic features that can be regarded as a separate group. Genetic features of other populations extensively overlapped. Of the three Toyama populations, TOY-E showed mostly close genetic features to those of NIG and NGN. PCoA detected axes 1 and 2 (49.71%) and (22.03%) of the genetic variation respectively. The result showed that TOY-W and TOY-C region of Toyama Prefecture including ISK was located at same quadrant whereas TOY-E was showed similarity with NIG and NGN. GIF, AIC and FUK were clustered together on the upper quadrant respectively (Fig. 6).

Bayesian clustering analysis showed a peak in the mean posterior probability (LnP (D)) with the ΔK value as K = 5 including a second highest value as K = 2 (Fig. 2). The results considering K = 5 indicated that each of the Prefectures had strong biases towards one particular cluster, for example, NIG, NGN and AIC were assigned mostly to cluster 5 (red color), GIF and ISK were assigned mainly to cluster 3 (black), FUK was assigned to cluster 4 (green) (Figs. 7, 8). On the other hand, Toyama Prefecture population did not show any dominant genetic cluster. Populations TOY-C and TOY-W showed mixed ancestry of five clusters, whereas TOY-E showed the similarity with NIG and NGN. However, when the second highest value as K = 2 was considered, sika deer from TOY-E, NIG, NGN and AIC were mostly assigned to cluster 1, while TOY-W, ISK, and FUK were assigned to cluster 2. TOY-C was assigned mostly to cluster 2, but GIF were slightly biased towards cluster 1 (Figs. 7, 8). Frequency of cluster 2 individuals tended to be higher in the peripheral regions of Toyama Prefecture.

Analysis of molecular variance (AMOVA) showed low but significant differentiations among the groups (F_{ST} = 0.028, P < 0.0001), among population within groups F_{SC} = 0.007, P < 0.0007 and among individuals within populations (F_{CT} = 0.021, P < 0.0001) (Table 8). In the present study, revealed that almost 97.12% of the total variance could be attributed to variation within the population's level, while among groups level variation represented only 2.18%. Spatial analysis of molecular variance (SAMOVA) according to the average latitude and longitude of all sample localities within each population showed F_{CT} value was low among the different groups. The F_{CT} value was ranged (0.024 to 0.026) for K = 2 – 6, including the significant P values. SAMOVA, K = 4 showed slightly high F_{CT} (0.026) value among them, the groups level showed that (39.08) of the variance occurs, while (26.3) of the variance were among the population within groups level (Table 9).

3.3.2. Detection of recent migrants and admixed individuals

GENECLASS2 identified a total of 29 (P < 0.05) individuals as first-generation (F_0) migrants between Toyama and neighboring areas, with a self-assignment probability of 64.4%. Of the 29 individuals, 24 migrated to Toyama from NIG (2), NGN (14), GIF (4), ISK (3), and AIC (1) and 5 individuals migrated from Toyama (Table 10). However, GENECLASS2 also identified 1 individual migrated from NIG to NGN, 3 individuals migrated from NGN to NIG, AIC and GIF respectively which was not shown in (Table 10). Using sampling location as population information (popinfo) to test for migrants was identified based on the percentage of membership coefficient (Q). The results suggested that resident individuals of Toyama Prefectures showed a high membership coefficient (Q > 0.90). Four individuals were potential migrants whose resident ancestry ranged Q (0.2 - 0.6). However, some individuals with Q (0.7 - 0.8) were not classified as either migrants or

residents, therefore those individuals were considered as admixed. Both GENECLASS2 and STRUCTURE identified same individuals as first-generation migrants, except for a few, probably as GENECLASS2 was not designed to detect admixed individuals (Richard and Linda, 2007). Nevertheless, when the threshold value (0.01) was used, the total number of first-generation migrants between Toyama Prefecture and the surrounding areas decreased. Contemporary migration results from the program BAYESASS suggested bidirectional migration may have occurred recently among the studied population (Table 11), however the amount was not same in all direction. Gene flow is profound from the eastern region NIG & NGN to TOY and TOY to GIF & AIC, ISK & FUK respectively (Fig. 9).

3.3.3. Historical migration

It was examined the historic gene flow between Toyama Prefecture and surrounding areas using MIGRATE-n. The estimates of mutation-scaled effective population size θ values and mutation-scaled historical migration rate (M) are shown in (Table 12). The present study observed bidirectional dispersal occurred between the populations using the Bayesian approach. However, observed gene flow was asymmetric between the populations; for example, M was high in the direction from NIG & NGN to TOY, whereas TOY to GIF & AIC (Fig. 10). Additionally, the effective number of migrants (N_em) into Toyama Prefecture was high as 2.72 individuals from NIG & NGN (Table 12). Furthermore, Model selection based on AIC (Akaike's information criterion) confirmed the model 5, Source-Sink (type I) as the best model according to the maximum likelihood approach (Table 13). Estimated of the historical migration for Source-Sink model percentiles of 95 % confidence intervals with (25.0% - 97.5%) were reported. For model 5, the historical source sink model, theta values (θ) ranged 1.7 to 2.1 and the migration rate (M) ranged from 2.2 to 9.7 (Fig. 11).

3.4. Discussion

In this study, genetic structures and migration patterns of Japanese sika deer populations in Toyama Prefecture and the surrounding areas were analyzed using 11 polymorphic microsatellite DNA markers. Deviations from HWE, detected mainly in populations in the central region of Toyama Prefecture, suggested non-random mating and/or sub-structuring in sika deer populations. Random mating can be prevented by certain population dynamics, such as a decrease in population size, geographical isolation, population expansion, and/or mixing of populations from multiple origins (Futuyma, 1998). Furthermore, most loci significantly deviated from HWE and H_O was higher than H_E, implying a mixing of different gene pools (Frankham et al., 2002). Additionally, present study did not detect LD or lower pairwise F_{ST} values among the populations, which might suggest that the admixtures occurred between populations in the past. The present study observed *PAR* value was low in Toyama Prefecture's individuals, which could be possible due to genetic drift and inbreeding. Loss of private allelic richness probably in sampled populations might share alleles with their parent's population rendering them no longer unique to each population (Allendorf and Luikart, 2007). Based on FCA, PCoA and STRUCTURE results, sika deer populations of Toyama and adjacent Prefectures were not genetically distinct and or freely mixed with respective neighboring regions, indicating genetic connectedness or admixture among them (Thévenon et al., 2004).

STRUCTURE analysis suggested that optimal K value was 5, indicating that all the sampled individuals exhibited admixture from different gene pools, probably resulting from topographical features of Toyama and surrounding Prefectures which is surrounded by high mountains that inhibit free dispersal, or the presence of multiple divergent ancestral sources.

These ancestral sources might be the result of natural dispersal and overlapping of two or more groups that were once geographically separated from each other, and hybridization between genetically different lineages has occurred (Eva and Yamazaki, 2018). Natural dispersal and overlapping among populations can be elucidated from genetic analysis (Frankham et al., 2002; Niedzialkowska et al., 2012; Genovart et al., 2013). In this study, genetic similarities tended to be observed among the geographically neighboring populations of sika deer. When assumed K = 2, It was observed that individuals of the eastern region of Toyama Prefecture were highly biased towards cluster 2, whereas NIG & NGN were mostly in cluster 2. Conversely, individuals from the central and western regions of Toyama Prefecture were biased towards cluster 1. Similar regional similarity was observed across varying numbers of ancestral sources (K = 5).

Results of molecular variance (AMOVA) among all populations showed significant low genetic differentiation. Higher values of genetic similarity and lower values of genetic distance between different populations indicate a higher rate of gene flow (Minhas et al., 2018). SAMOVA analysis provided very small but significant spatial population structure for the populations of sika deer. Populations were divided into four different groups that considered according to geographical location of Toyama Prefecture for example, eastern side (NIG, NGN), western side (FUK, ISK) and southern side (GIF, AIC) of Toyama Prefecture. This result was consistent with the STRUCTURE result indicating sika deer sample from the studied sample were not same ancestry but shown very close relationship especially from the geographically neighboring Prefecture to each region.

GENECLASS2 and STRUCTURE identified first generation migrant as well few individuals with admixed ancestry. Identification of admixed suggesting gene flow is

occurred at least in the recent past (Yumnam et al., 2014; Thapa et al., 2018). Many migrants especially individuals from NIG & NGN Prefectures were males, possibly indicating a male bias in dispersal, as female philopatry is generally the case for most cervid species (Clutton-Brock et al., 1982; Goodman et al., 2001; Coulon et al., 2004). Furthermore, BAYESASS 1.3 focuses on contemporary migration rates (past two generations) suggested migration among groups might have occurred at the present time. Compared with the results from contemporary and historical gene flow both are similar, mostly sika deer entered into Toyama Prefecture from the direction of east and migrated from Toyama to south and west. The result was roughly consistent according to mtDNA analysis of Yamazaki (2018), revealed current sika deer individuals in Toyama Prefecture migrated from the east and south-west. Migration not only of sika deer but also wild boar occurred recently in Toyama Prefecture from each geographically neighboring region (Yamazaki et al., 2016). On the other hand, AIC confirmed Source-Sink (type I) as the best model. Using the source-sink model of population demography, MIGRATE-n revealed considerable differences in population size between the source (Toyama populations) versus the sink (other populations), possibility that theta was biased by the difference in sample size. Accuracy of coalescent F_{ST}-based study estimation can be affected by a difference in sample size among study populations (Beerli, 1998; Beerli and Felsenstein, 2001). Therefore, the present study acknowledges that sample sizes of the neighboring Prefecture are relatively small.

A previous study of sika deer suggested that dissuasion of sika deer dispersal using artificial constructions resulted in genetic divergence among local populations (Goodman et al., 2001; Yuasa et al., 2007). However, in Toyama Prefecture, there are no conspicuous artificial constructions for sika deer, especially in mountainous areas. Therefore, the genetic population structure of sika deer around Toyama Prefecture has more likely been affected by

natural dispersal patterns, rather than artificial isolation. Previously, it was thought that sika deer migrated to Toyama Prefecture via multiple routes (Nambu and Yoshimura, 2002). In a recent study, Yamazaki (2018) also indicated similar dispersal patterns of sika deer according the mtDNA analysis. There were no absolute barriers that facilitated genetic differentiation for the sika deer population among Toyama and neighboring Prefectures.

Although the habitat range of sika deer has recently been expanding almost all over the Japanese Archipelago, the previous habitat was divided between the Kinki and Koshin regions in central Honshu Island, Japan (e.g. Japan Ministry of the Environment, 2004). Considering their geographical relationship and genetic composition, as well as the dispersal patterns inferred in this study, it is conceivable that the genetically divergent ancestral sources might have derived from gene pools in these regions.

Consequently, sika deer around Toyama Prefecture are thought to derive from multiple origins, including at least two and or more genetically and geographically distinct natural sources, as well as an introduced gene pool. Therefore, it is a serious concern that continuing dispersal pattern may spread hybrid individuals from Toyama Prefecture. The present study proposes further migration pattern should be monitored and focused more intensively with increasing sample size. Sample size might be a factor of inconsistency of migration analysis; however, several points support the validity of the present study. First, levels of polymorphism were high, all loci were polymorphic (almost all sampled individuals). For such highly polymorphic loci, lower sample sizes should not affect estimates of heterozygosity, allele frequencies, and subsequent statistics (Hale et al., 2012). Moreover, estimated high population structures ($5 \geq K \geq 2$) of subpopulations also support the result. Estimating the migration rate and dispersal patterns of sika deer will contribute to

establishing effective measures for management and conservation of the indigenous ecosystem. The hidden genetic structure results may provide helpful information for predicting the genetic dynamics of sika deer at the forefront of their range expansion.

CHAPTER 4

SPATIAL GENETIC STRUCTURE, SEX-BIAS DISPERSAL AND INDIVIDUAL'S RELATEDNESS

4.1. Introduction

In evolutionary biology the spatial structure resulting from the genomic variation among natural population is primarily influenced by the population density, breeding system, and environmental heterogeneity (Pometti et al., 2018). Population's evolutionary patterns are often explained in genetic relatedness among individuals, become an important measure in many areas of biology (Hedrick and Lacy, 2015). Knowledge of the degree of inbreeding and relatedness between individuals due to recent common ancestry is pivotal concepts in many research areas like quantitative genetics, conservation genetics, forensics, evolution and ecology (Wright, 1921, 1922; Ritland, 1996; Lynch and Ritland, 1999; Weir et al., 2006). Relatedness between interacting individuals is required to predict evolutionary consequences of social interaction and optimize conservation strategies in conservation genetics (Hamilton, 1964; Oliehoek et al., 2006).

Two individuals are genetically related because of their shared genealogical history or common ancestors in the recent past. The number of common ancestors and their distances (number of generations) to a pair of individuals determine the extent of relatedness between individuals. Related individuals have more similar genotypes at each locus because their alleles have a higher probability of identity by descent compared to unrelated individuals. As a result, they also tend to have a higher similarity in the phenotype of a quantitative trait (Wang, 2017). Spatial distribution of genetic variation can provide perspectives into current and past population dynamics (Wang et al., 2012). Long term existence of spatial genetic structure decreases effective population size, affecting population genetic diversity and even progeny fitness (Kalisz et al., 2001; Fenster et al., 2003). Both theoretical and empirical studies have shown that gene flow plays a critical role in determining the extent of

relatedness among adjacent individuals and levels of local random genetic drift. Thus gene flows become the predominant determinant of spatial genetic structure in the absence of selection. Spatial genetic structure in turn is influenced by a range of factors such as mating systems, genetic discontinuities arising from historical events and the extent of dispersal (Vekemans and Hardy, 2004; Hardy et al., 2006; Gonzales et al., 2010; Segelbacher et al., 2010; Wang et al., 2012).

In the present study of sika deer, low levels of genetic differentiation were identified between geographically close regions of Toyama and neighboring Prefectures (Niigata, Nagano, Gifu, Ishikawa, Aichi and Fukui) described in chapter 3. On the other hand, Toyama Prefecture populations showed loss of allelic richness probably due to genetic drift and/or inbreeding. Thus these species could generate population structure at fine geographic scales despite the signals of gene flow at large geographic scales. Fine-scale population structure as a result of philopatric behavior can provide evolutionary benefits under certain conditions, such as maintaining local environmental adaptation, decrease of high dispersal costs, increase probability of mate encounter with low population densities and enhance cooperation among members of a social group (Bilde et al., 2005; Bonte et al., 2012; Stelkens et al., 2012; Margarita et al., 2015). However, fine-scale population structures increase the risk of extreme co-ancestry within populations and can lead to inbreeding depression, a reduction in individual fitness due to increasing levels of genetic homozygosity and expression of partially deleterious alleles (Charlesworth and Charlesworth, 1999; Margarita et al., 2015). The negative effects of inbreeding depression on fitness have been demonstrated in many species of threatened populations with low effective population sizes (Ralls et al., 1988; Richards, 2000; Frankham, 2010; Margarita et al., 2015).

Dispersal is the common phenomena often resulted with sex-biasness. Such as: female-biased dispersal was observed in monogamous birds whereas male-biased dispersal was predominant in polygynous mammals since females benefit from familiarity with resources in their territory and can afford better parental care (Greenwood, 1980; Dobson, 1982; Williams and Rabenold, 2005; Xiang-Yi and Kokko, 2018). Dispersal from inhabited areas is a normal practice of animals, strategically evolved under inbreeding avoidance, mortality risk, adaptivity and benefits with environment, resource competition, mating competition (Greenwood, 1980; Moore and Ali, 1984; Pusey, 1987; Perrin and Mazalov, 1999). The balance among the strategic factors ultimately determine sex dependent evolutionary stable pattern. The present study was aimed to investigate the population spatial structure, autocorrelation of genetic with geographic distance including whether the dispersal is sex-biased or not, and individual's relatedness. These findings will further help to understand that (1) the individuals are more genetically related to each other than would be expected at random, (2) females are more genetically related than males or not.

4.2. Materials and methods

4.2.1. Samples

Tissue samples (muscle or ear tip) from 247 sika deer were collected throughout Toyama and adjacent prefectures between 2013 and 2017. Samples used by Yamazaki (2018) and (chapter-2 and 3) were also used. For population spatial genetic structure and relatedness analysis, the samples from Toyama Prefecture and overall studied region were treated separately.

4.2.2. Spatial genetic structure

To characterize spatial genetic structure, isolation by distance (IBD) and spatial autocorrelation patterns were examined. First to determine whether a significant correlation existed between pairwise co-dominant genotypic and geographical distances by applying mantel tests for across Toyama and neighboring Prefectures population considering overall (i.e., both sexes combined) and each sex separately using GenAlEx 6.5 (Peakall and Smouse, 2012). For Toyama Prefecture population's mantel test was also applied. Global Position System technology was used to collect latitude and longitude coordinates used for regression analyses in conjunction with genetic distances.

Secondly, spatial autocorrelation analysis was conducted in GenAlEx 6.5 (Peakall and Smouse, 2012) to examine the spatial extent of genetic structure and to determine if dispersal patterns were sex-biased. A significant positive value of (r) indicates that individuals are more genetically similar than expected by chance, while a negative significant (r) indicates that individuals are less closely related than is expected by chance. When the value of (r) is

not significantly different from zero, this indicates random spatial distribution, where individuals are just to be situated next to closely related individuals as they are unrelated individuals. Thereby, a positive autocorrelation coefficient only for the first lowest distance classes would reflect high local genetic similarity. The present study compared the overall patterns of spatial genetic structure and patterns of spatial genetic structure between sexes using the "multiple population analysis". This analysis combines datasets from multiple populations (in this case, males and females of Toyama, Niigata, Nagano, Gifu, Ishikawa, Aichi and Fukui). For each sex of Toyama Prefecture populations "single population analysis" was applied. Autocorrelation coefficients, (r) between pairwise genetic and geographic distance matrices were calculated for variable distance classes ranging from 1 to 85 km. Spatial genetic structure is tested against the null hypothesis of no autocorrelation (r = 0) by generating 95% confidence intervals (CI) for each distance class via permutation (999 simulations) and bootstrapping (999).

Furthermore, using GenAlEx 6.5 (Peakall and Smouse, 2012) assignment tests were calculated separately for male and female individuals of across the population. In case of Toyama Prefecture population assignment tests were also calculated together (all regions) and each of region separately. Comparison of the average AIc (assignment index) between sexes was made with the nonparametric statistical analysis Mann-Whitney U test, and the significance level was ($P < 0.05$). Significant difference in average AIc between males and females indicates the presence of sex bias dispersal. Negative AIc indicates individuals that are more likely to be dispersed, and positive AIc indicates more philopatry individuals.

4.2.3. Individuals relatedness

Pairwise genetic relatedness, (*R*) among individuals of all studied populations (N = 247) and both of male and female individuals was evaluated separately. Furthermore, Toyama Prefecture populations together and both sex were calculated. In addition, relatedness of each herd were also evaluated, a total 47 tissue samples were collected from 20 herds. 16 herds were captured from Toyama and 2 were from Nagano and Ishikawa respectively. This analysis was conducted using Spagedi version 1.3b (Hardy and Vekemans, 2002). To estimate coefficient of relatedness (*R*) by Lynch and Ritland (1999) were used and standard errors of the average pairwise (*R*) were estimated by jack-knifing over loci.

4.3. Result

4.3.1. Spatial genetic structure and autocorrelation

The results of Mantel tests between genetic and geographic distance matrices are shown in Table 14. For all sampled population and combined data set (males and females) showed a significant positive relationship between genetic and geographic distance. Furthermore, separately male and female also exhibit significant positive relationship. On the other hand, females of Toyama Prefecture populations showed non-significant negative relationship between genetic and geographic distance (Table 14).

Fig. 12 shows correlograms of spatial genetic autocorrelation analysis for two different distance class sizes of combined sika deer population. In the correlogram with smaller distance classes (Fig. 12A), *r* (the combined estimate of *rc* across populations) between genetic and geographic distance was positive up to 4 km and significant at 1 and 3 km (*r* =

0.029, 0.014, $P < 0.028$) with a first x-intercept of 5 km. Although, closer examination of the contributing to each distance class revealed that further some points showed significantly positive autocorrelation (14, 17, 29, 30, and 39 km) ($0.041 \geq r \geq 0.018$, $P < 0.029$), empirically confirming the presence of non-random spatial structuring and genetic association among individuals at distances < 39 km (Fig. 12A). This pattern was clearer when distance class sizes were increased (Fig. 12B), with larger distance classes of 5 km, r was positive up to 15 km and significant at only first distance class at 5 km ($r = 0.010$, $P < 0.003$) with an x-intercept of 20 km and again became significant at distance class of 40 km ($r = 0.24$, $P < 0.006$) but the general trend was toward more negative r values with increasing geographic distance, a pattern that is indicative isolation by distance (Diniz-Filho and Telles, 2002). The combined populations of entire data set, there is an indication neighborhood size of sika deer approximately at least 40 km. Furthermore, the spatial autocorrelation correlograms for male and female sika deer was also shown separately. Male shows positive and significant up to 2 km ($r = 0.016, 0.021$, $P < 0.025$) including a significant positive autocorrelation at distance class 17 and 29 km ($r = 0.030, 0.054$, $P < 0.025$) with a first x-intercept at 4 km (Fig. 13A). Female showed positive autocorrelation at 1km ($r = 0.067$, $P < 0.004$) with a first x-intercept at 2 km including positive autocorrelation at distance class 4, 22, 24 and 61 km ($0.141 \geq r \geq 0.057$, $P < 0.015$) (Fig. 14A). These results indicated that neighborhood size of sika deer approximately 29 km for male and 61 km for female. On the other hand in the larger distance class male showed autocorrelation at first distance class ($r = 0.11$, $P < 0.007$) (Fig. 13B), whereas female did not show any significant positive autocorrelation at any distance class (Fig. 14B). The overall extent of significant positive autocorrelation indicating genetic structure was finer in large geographic scales for both sexes, though with possible indication of female-bias in dispersal distance.

In addition, Fig.15 showed the spatial autocorrelation analysis of sika deer only for Toyama Prefecture individuals. In the correlogram *r* (the combined estimate of *rc* across Toyama populations) between genetic and geographic distance was positive and significant at 1, 7, 14, 15, 17, 29, 30, 39 and 70 km ($0.075 \geq r \geq 0.012$, $P < 0.05$) (Fig. 15A). For larger distance class spatial positive and significant autocorrelation was observed at 5, 30 and 40 km ($r = 0.011, 0.024$, $P < 0.011$) with an x-intercept of 10 km (Fig. 15B). Male of Toyama Prefecture showed significant positive autocorrelation at 2, 17, 23 and 29 km ($0.052 \geq r \geq 0.024$, $P < 0.05$) with an x-intercept of 4 km (Fig. 16A) and female sika deer of Toyama Prefecture showed significant positive autocorrelation at 1, 22, 24 km ($0.212 \geq r \geq 0.085$, $P < 0.016$) (Fig. 17A). For larger distance class males were positive and significant at 5 km ($r = 0.010$, $P < 0.035$) with an x-intercept of 15 km (Fig. 16B) for females no significant autocorrelation was observed (Fig. 17B).

4.3.2. Sex-biased dispersal

Assignment index analysis provided the evidence for sex-biased dispersal among male and female individuals of whole sample and subsequently collected from Toyama Prefecture. Assignment index (AIc) for male of whole studied sample was (0.147) and for female (-0.318) were significant $P < 0.019$ (Fig. 18). These results indicated that female bias dispersal present among sika deer population of Toyama and neighboring Prefectures. Assignment index (AIc) for Toyama Prefecture's male (0.122) and female (-0.332) were not significant, $P = 0.106$ (Fig. 19). Positive AIc values observed in males indicated their higher probability of originating from the sampled population while negative AIc of females indicate their higher probability of being immigrants. Furthermore the result was slightly different while each region of Toyama Prefecture was considered separately. Eastern side AIc for male

was (-0.208) and female was (0.956) (Fig. 20); western side AIc for male was (-0.224) and female was (0.336) (Fig. 21) indicating male biasness in dispersal although they were not significant, $P > 0.5$. On the other hand, central side of Toyama Prefecture AIc for male (0.240) and for female (-0.677) became significant ($P = 0.012$) indicating female biasness in dispersal (Fig. 22).

4.3.3. Relatedness among individuals

Relative kinship estimation based on the microsatellite data of across all studied populations (*R*) was shown in Fig. 23. The pairwise relatedness of overall populations (total 30381 pairs) is (-0.004 ± 0.00) average ± SE ; for males (-0.006 ± 0.00) and females (-0.013 ± 0.00). These results were not significantly different from the average pairwise relatedness of Toyama Prefecture for combined populations (-0.006 ± 0.00); males (-0.008 ± 0.00) and females (-0.023 ± 0.00) ($P < 3.90$, two-tailed, t-test) (Fig. 24). The overall pairwise kinship estimation suggested that individuals are weakly or moderately related. Furthermore, the average pairwise (*R)* was estimated between and within each herd (Table 15). Four pairs of individuals within each herd had (*R*) values larger than 0.50 suggesting they may be related at least full sib level and four pairs of individuals larger than 0.25 probably related at least the half-sib level (Table 15). Theoretical *R* value for first order relatives 0.50 and second orders relatives is 0.25 (Wilson et al., 2005).

4.4. Discussion

Spatial genetic structure results indicated the presence of non-random spatial structuring and genetic association among individuals at larger distances. Individuals below this threshold, share a higher proportion of genes, than spatially distant individuals (Wultsch et al., 2016; Thapa et al., 2018). Genetic structure in the studied sika deer population was manifested at a large scale corresponding to the spatial proximity of closely related individuals. Furthermore, the overall result of kin relatedness indicated that individuals were weakly or moderately related. This was essentially due to the majority of individuals belonging from different families (Arriagada et al., 2018). On the other hand, high pairwise relatedness occurred mostly within each pair of herd than those between herds, thus indicating within pair of herds individuals were more genetically close than distant individuals (Jennifer et al., 2011; Clinton et al., 2013). The result of dispersal between sex and spatial genetic structure indicated that the slight lack of female philopatry. Negative AIc value of female, indicating dispersal is mainly female-biased. Additionally, when Prefectural regions were considered separately, eastern and western parts of Toyama Prefecture showed evidence of male bias dispersal. These results were consistently observed and described in the chapter 3, where most of the first generation individuals were migrated from the geographically neighboring Prefectures.

Although, both male and female sika tended to be dispersed further away from the natal area. The wide spread pattern of male-biased dispersal is common in deer species (Comer et al., 2005; Nussey et al., 2005; Frantz et al., 2008; Cullingham et al., 2011; Grear et al., 2010; Miller et al., 2010; Pérez-Espona et al., 2010). Several studies for deer analysis revealed different patterns of spatial genetic structure generally observed in females rather than males. In case of red deer, strong male-biased dispersal and female philopatry was

observed (Nussey et al., 2005; Frantz et al., 2008, Pérez-Espona et al., 2010). Non-random spatial association among female white-tailed deer have also been observed, whereas males were found unrelated at all distances, indicating strong female philopatry and the formation of matrilines (Comer et al., 2005; Grear et al., 2010; Miller et al., 2010; Cullingham et al., 2011). These results of various deer species were contrast from the sika deer results of the present study. The current study results showed both male and females individuals were positively related in larger distance. However, some studies also reported lack of female philopatry for roe deer species. For example, no difference was observed between the sexes of roe deer, lacking sex-biased dispersal and weak polygynous mating system (Bonnot et al., 2010). Additionally, in south-western Spain, red deer populations showed strong female-biased dispersal along with high proportion of young males. Female based dispersal was hypothesized due to inbreeding depression, choice of high quality males, whereas young males might disperse as a result of inbreeding avoidance, mating competition (Pérez-González and Carranza, 2009; Clutton-Brock, 1989). Very few reports till yet showed female based sex-biased dispersal patterns in sika deer species. Although seasonal migrations were reported for sika deer in Japan, depending on snow falls, bamboo grass, coniferous cover, calving season etc (Miura, 1974; Maruyama, 1981; Ito and Takatsuki, 1987; Takatsuki et al., 2000). Study reports showed snow falling might cause Hokkaido females to migrate 35.1 km on an average (Igota et al., 2004); while Kyushu females migrated 2 to 7.5 km might be due to resource competition (Yabe and Koizumi, 2003).

From the current study results, negative AIc values indicated occurrence of significant female bias dispersal in the central region of Toyama Prefecture as well as entire data set of all Prefectural regions. Female biased dispersal incidents can be explained due to possible inbreeding depression, choice of actively mating high quality males, whereas male biased

dispersal observed in eastern and western Toyama, might be resulted from high mating competition, inbreeding avoidance, strong female philopatry and the formation of matrilines (Packer, 1979; Waser et al., 1986; Pusey, 1987; Clutton-Brock, 1989; Wolff, 1994; Perrin and Mazalov, 1999). These results were evidently described in the chapter 2 of the present research work, indicating mating does occur between two genetically dissimilar lineages resulting hybridization (Eva and Yamazaki, 2018). While in the chapter 3 obtained results established the phenomena of genetic drift and inbreeding events in the Toyama Prefecture populations due to loss of private allelic richness (Eva and Yamazaki, 2019). To further accurately characterize in details of spatial genetic structure and dispersal pattern among male and female sika deer obtained from the present study results, it will be better idea to equalize both male and female sample sizes in future studies.

CHAPTER 5

GENERAL DISCUSSION

5.1. General Discussion

This doctoral dissertation explored the formation process of genetic diversity of native sika deer genome in Toyama Prefecture based on of genetic structure, hybridization with introduced species and migration events from the neighboring Prefectures. Geographical distribution is important in the formation of biological diversity. A single species can inhabit in a wide range and exhibit great diversity brought by natural selection. For example, deriving from same species sika deer was identified in two different lineages with genetic, morphological like body structures, food habit variations etc. It is clear from the present study, even only one or a few species are capable of infecting host species. In chapter 2, introgression of an introduced gene pool and subsequent continuous mating between native and introduced sika deer was identified, mainly in the central region of Toyama Prefecture. Introduced individuals could possibly share a common allele with the native individuals around Toyama Prefecture. The population genetic structure also suggested the existence of multiple ancestral sources probably reflecting the origin of hybridization. According to mtDNA analysis of Yamazaki (2018), a passage breeding individual derived from Yakushima Island, is thought to be the origin of introduced individuals in Toyama Prefecture, shared many common alleles that observed in the current study. Another possibility is that continuous mating between native and introduced individuals led to the formation of a common allele composition. Admixture sometimes causes the genetic traits of the resultant population to change because more advantageous alleles are likely to be selected during a merger of populations. Furthermore, recently bi-directional contemporary migration was identified among the neighboring Prefectures described in chapter 3. Considering the historical gene flow, individuals entered into Toyama from all three directions although mostly from (east) suggesting admixture of different gene pools in Toyama Prefecture. Such

kind of sympatric distribution of the two groups of sika deer has been observed in eastern Shikoku Island (Yamada et al., 2006). Tamate (2009) explained secondary contact between genetically differentiated populations can lead to the formation of a new admixture population, which was observed in the present study. Similar pattern of genetic variability was also explained for the other mammalian species. For instance, Japanese monkeys, Asian black bear and Japanese hare exhibited the existence of different geographical origins of population (Kawamoto et al., 2007; Tamate, 2013; Nunome et al., 2014). Moreover, multiple mixing was also observed in wild boar, domestic pig, and Inobuta that leads to establishment of new population of *Sus scrofa* thus creating risks not only with alterations in the genetic structure, but also fitness enhancement of wild boar population (Takahashi, 2018). There was an indication of non-random spatial structuring and genetic association among individuals at larger distances (neighborhood size of sika deer approximately at least 40 km) described in chapter 4. Therefore, genetic similarities were highly observed among the geographically neighboring populations potentially exhibit freely dispersal. Free ranging domestic animals have some serious consequences, such as crossbreeding with related wild animals, resulting in disturbance of genetic diversity, pathogen transmission, hybridization, behavioral modification (Webley et al., 2007; Ohdachi et al., 2009; Scandura et al., 2011; Tamate, 2013; Murakami et al., 2014; Berteaux et al., 2015; Biosa et al., 2015; Twardek et al., 2017). In such situations, the present study result will help in supporting conservation efforts and establish effective management strategies for wild animal species.

5.2. Conservation and Management

Genetic assimilation via hybridization between native and introduced individuals of sika deer captured from Toyama Prefecture concluded existence of multiple ancestors and their genetic changes raised concerns and harmful consequences beyond the loss of genetic integrity in the central part of Toyama Prefecture. Three ways to specify hybridization can be a conservation threat are hybrid populations might have greater probability of extinction overall, aesthetically or intellectually undesirable (e.g., ecologically inauthentic relative to a non-hybrid native species), and/or deleterious impacts on other native species or ecosystem (Fitzpatrick et al., 2015).

In the present study focusing on hybridization, hybrids might spread ranges, expansion of invasion could result possible loss of existing origins (native deer and natural resources). Careful monitoring of introgression and its fitness consequences is needed in central region of Toyama Prefecture to ensure native sika will not go genetically extinct in future. From conservation perspective, it is crucial to discover introgression process over time and space affecting overall fitness in threatened species. Conservation goals should be based on the empirically measured consequences of hybridization, rather than on the levels of hybridization (Stronen and Paquet, 2013; Jackiw et al., 2015; Bohling, 2016; Nussberger et al., 2018).

Long term conservation can be established by considering some measures should be addressed prior to deciding on threat level, such are as follows:

- Do the source populations from the expanding taxon overwhelm the introgressed individuals?

- Or are introgressed domestic genes accumulating in the introduced population, leading to its genetic extinction?

- Are introgressed individuals suffering from out breeding depression?

Consequently, levels of gene flow are particularly important for conservation issue. Movement of individuals and genes in spaces affects many important ecological and evolutionary properties of populations (Hanski and Glipin, 1997). Estimation the movement rate of genes over population helps to determine the possibility of local adaption on complex landscapes. Furthermore, dispersal affects the persistence of local populations, species extinctions rates, the evolution of species ranges, synchrony of population size changes, and many other important ecological properties (Whitlock and McCauley, 1999). Mills and Allendorf (1996) suggested that a minimum of one and a maximum of 10 migrants per generation are appropriate for management purposes. Therefore, the population should be monitored intensively and the major dispersal route can be covered by fences, to prevent excessive gene flow.

A successful wildlife management plan should be ecologically sound, economically practical and realistically attainable. Deer management plan is crucial although it allows

recording current property and looking back past conditions, following improvements, making changes in the management strategies based on previous records. Regulated hunting of deer has proven to be an effective management tool, and alternative techniques, such as trapping, strict legislation, safety practices and birth control drugs can be used to manage over abundant deer; however, it is expensive and impractical over large areas (Ellingwood and Caturano, 1988; Kaji, 2010; Iijima and Nagaike, 2015). Continuously, a selective culling should be a good recommendation to avoid range expansion of sika deer (both native and hybrids) of these expanded species. For example, as young sika males disperse first therefore pioneering stags could be the best choice for culling (Baiwy et al., 2013). Specified Wildlife Conservation and Management Plans for sika deer include avoiding the extinction of local populations, reducing the conflicts between deer and humans, such as damage to agriculture and forestry, fencing around the distributed deer zone, reducing deer population sizes, and conserving ecosystems (Uno et al., 2007).

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Tables and Figures

Table 1. Characterization of 13 microsatellite loci used in the present study for sika deer analysis

Name	Annealing	Primer	Sequence	Reference
	Temperature (℃)			
BL42	56	Forward	5'-CAAGGTCAAGTCCAAATGCC-3'	Bishop et al. (1994)
		Reverse	5'-GCATTTTTGTGTTAATTTCATGC-3'	
BM3628	56	Forward	5'-CTGAGATGGACTCAGGGAGG-3'	
		Reverse	5'-GTTGGATTGGAAAGGTTAGGC-3'	
BM6438	56	Forward	5'-TTGAGCACAGACGACACAGACTGG-3'	
		Reverse	5'-ACTGAATGCCTCCTTTGTGC-3'	
BMC1009	56	Forward	5'-GCACCAGCAGAGAGGACATT-3'	
		Reverse	5'-ACCGGCTATTGTCCATCTTG-3'	
BM6506	58/60	Forward	5'-GCACGTGGTAAAGAGATGGC-3'	
		Reverse	5'-AGCAACTTGAGCATGGCAC-3'	
BM203	56	Forward	5'-GGGTGTGACATTTTGTTCCC-3'	Talbot et al. (1996)
		Reverse	5'-CTGCTCGCCACTAGTCCTTC-3'	
BM888	48	Forward	5'-AGGCCATATAGGAGGCAAGCTT-3'	
		Reverse	5'-CTCGGTGAGCTCAAAACGAG-3'	
OarFCB193	56	Forward	5'-TTCATCTCAGACTGGGATTCAGAAAGGC-3'	
		Reverse	5'-GCTTGGAAATAACCCTCCTGCATCCC-3'	
Cervid14	65	Forward	5'-TCTTCTTGCGTCTCCTGCATTGAC-3'	DeWoody et al. (1995)
		Reverse	5'-AATGGCACCCACTCCAGTATTCTTC-3'	
CSSM019	56	Forward	5'-TTGTCAGCAACTTCTTGTATCTTT-3'	Moore et al. (1992)
		Reverse	5'-TGTTTTAAGCCACCCAATTATTTG-3'	

Table 1. continued

Name	Annealing Temperature (℃)	Primer	Sequence	Reference
ETH225	56	Forward	5'-GATCACCTTGCCACTATTTCCT-3'	Kühn et al. (1996)
		Reverse	5'-ACATGACAGCCAGCTGCTACT-3'	
IDVGA29	50/52	Forward	5'-CCCACAAGGTTATCTATCTCCAG-3'	Mezzelani et al. (1995)
		Reverse	5'-CCAAGAAGGTCCAAAGCATCCAC-3'	
RM188	58/60	Forward	5'-GGGTTCACAAAGAGCTGGAC-3'	Barendse et al. (1994)
		Reverse	5'-GCACTATTGGGCTGGTGATT-3'	

Table 2. Summary of the sika deer microsatellite data from the central part of Toyama Prefecture

Locus	Observed values	Total sample (n = 83)	Group I (n = 44)	Group II (n = 39)
BL42	N_A	3	3	3
	N_P	0	0	0
	H_E	0.543	0.518	0.568
	H_O	0.530	0.455	0.615
	P	0.379	0.688	0.313
BM3628	N_A	7	6	7
	N_P	0	0	1
	H_E	0.750	0.736	0.750
	H_O	0.868	0.841	0.897
	P	**0.010**	**0.008**	0.150
BM6506	N_A	7	6	7
	N_P	0	0	1
	H_E	0.622	0.622	0.614
	H_O	0.542	0.523	0.564
	P	0.158	**0.009**	0.497
BM6438	N_A	7	7	5
	N_P	0	2	0
	H_E	0.756	0.784	0.670
	H_O	0.711	0.727	0.692
	P	0.087	0.558	0.067
BMC1009	N_A	5	5	5
	N_P	0	0	0
	H_E	0.570	0.566	0.570
	H_O	0.615	0.636	0.590
	P	0.498	0.757	0.659
BM203	N_A	4	2	4
	N_P	0	0	2
	H_E	0.501	0.375	0.589
	H_O	0.434	0.409	0.462
	P	0.072	0.705	0.105
BM888	N_A	9	7	9
	N_P	0	0	2
	H_E	0.729	0.710	0.738
	H_O	0.747	0.750	0.744
	P	0.687	0.894	0.326

Table 2. continued

Locus	Observed values	Total sample (n = 83)	Group I (n = 44)	Group II (n = 39)
OarFCB193	N_A	7	7	7
	N_P	0	0	0
	H_E	0.827	0.833	0.805
	H_O	0.988	1.000	0.974
	P	**0.032**	0.444	**0.009**
Cervid14	N_A	6	5	5
	N_P	0	1	1
	H_E	0.679	0.613	0.716
	H_O	0.735	0.614	0.872
	P	**0.007**	0.059	**0.027**
ETH225	N_A	4	4	4
	N_P	0	0	0
	H_E	0.497	0.457	0.521
	H_O	0.494	0.500	0.487
	P	0.545	0.561	0.732
RM188	N_A	4	4	4
	N_P	0	0	0
	H_E	0.690	0.669	0.705
	H_O	0.615	0.568	0.667
	P	0.253	0.32	0.664
Whole loci	H_E	0.651	0.626	0.659
	H_O	0.662	0.638	0.688

N_A: Number of alleles; n : Number of the individuals genotyped; N_P: Number of personal allele; H_E , H_O: expected and observed heterozygosites, respectively; P : P value of test for deviation from Hardy-Weinberg equilibrium (P <0.05). Tests that are significant are in bold.

Table 3. Prefectures and regions of sika deer samples used in the present study. The number of individuals with introduced haplotypes is indicated in parentheses

Prefecture	Region	Abbreviation	Male	Female	Unknown	Total
Toyama						
	Eastern	TOY-E	23	5	1	29
			(6)	(0)	(0)	(6)
	Central	TOY-C	82	29	2	113
			(22)	(28)	(7)	(57)
	Western	TOY-W	15	10	1	26
			(3)	(7)	(2)	(12)
Niigata		NIG	6	6	-	12
			(0)	(0)		(0)
Nagano		NGN	20	11	-	31
			(0)	(0)		(0)
Gifu		GIF	10	10	1	21
			(0)	(0)	(0)	(0)
Ishikawa		ISK	9	1	-	10
			(0)	(0)		(0)
Aichi		AIC	-	2	1	3
			-	(0)		(0)
Fukui		FUK	-	2	-	2
			-	(0)	-	(0)

Table 4. Allele frequencies, expected heterozygosity (H_E), observed heterozygosity (H_O), and the probability of departure from Hardy-Weinberg equilibrium (P) for all Japanese sika deer samples

	Location	TOY- All	TOY- E	TOY- C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
	Number (N)	168	29	113	26	12	31	21	10	3	2
Locus											
OarFCB19	Allele										
	115	0.170	0.207	0.164	0.154	0.375	0.194	0.095	0.200	0.333	0.500
	125	0.104	0.017	0.120	0.135	0.042	0.113	0.071	0.050	0.167	0.000
	129	0.268	0.310	0.243	0.327	0.292	0.290	0.381	0.300	0.000	0.500
	131	0.128	0.155	0.124	0.115	0.167	0.210	0.071	0.150	0.000	0.000
	133	0.033	0.035	0.035	0.019	0.042	0.065	0.071	0.100	0.000	0.000
	135	0.205	0.155	0.217	0.212	0.042	0.081	0.214	0.150	0.333	0.000
	137	0.092	0.121	0.097	0.039	0.042	0.048	0.095	0.050	0.167	0.000
	H_E	0.821	0.797	0.827	0.791	0.740	0.809	0.776	0.810	0.722	0.500
	H_O	0.994	1.000	0.991	1.000	1.000	1.000	0.952	1.000	1.000	1.000
	P	**0.000**	0.659	**0.000**	**0.031**	0.339	0.267	0.340	0.920	1.000	1.000
CSSM019	Allele										
	145	0.107	0.138	0.097	0.115	0.000	0.194	0.119	0.150	0.333	0.000
	147	0.051	0.069	0.049	0.039	0.000	0.081	0.071	0.200	0.000	0.000
	149	0.086	0.086	0.089	0.077	0.000	0.129	0.143	0.050	0.167	0.250
	151	0.199	0.172	0.208	0.192	0.167	0.226	0.310	0.150	0.000	0.500
	153	0.095	0.069	0.106	0.077	0.000	0.000	0.119	0.150	0.000	0.000
	155	0.149	0.121	0.159	0.135	0.125	0.113	0.143	0.050	0.000	0.000
	157	0.069	0.052	0.066	0.096	0.083	0.016	0.024	0.000	0.167	0.000
	159	0.021	0.052	0.013	0.019	0.167	0.065	0.048	0.000	0.000	0.000
	161	0.048	0.035	0.040	0.096	0.292	0.129	0.000	0.000	0.333	0.000
	163	0.128	0.207	0.111	0.115	0.167	0.048	0.024	0.250	0.000	0.250
	165	0.048	0.000	0.062	0.039	0.000	0.000	0.000	0.000	0.000	0.000
	H_E	0.882	0.870	0.878	0.885	0.809	0.852	0.827	0.825	0.722	0.625
	H_O	0.899	0.966	0.858	1.000	0.917	0.774	0.762	0.800	1.000	1.000
	P	0.532	0.885	**0.000**	0.287	0.461	**0.034**	**0.005**	0.545	0.465	1.000
RM188	Allele										
	145	0.042	0.086	0.022	0.077	0.083	0.177	0.024	0.000	0.000	0.000
	147	0.015	0.035	0.000	0.058	0.042	0.048	0.000	0.150	0.000	0.000
	149	0.330	0.172	0.376	0.308	0.125	0.113	0.310	0.350	0.500	0.500
	151	0.131	0.086	0.128	0.192	0.083	0.065	0.000	0.000	0.000	0.000
	153	0.310	0.517	0.283	0.192	0.458	0.500	0.214	0.250	0.500	0.500
	155	0.033	0.017	0.040	0.019	0.000	0.000	0.191	0.150	0.000	0.000
	157	0.086	0.035	0.093	0.115	0.208	0.065	0.167	0.000	0.000	0.000
	163	0.054	0.052	0.058	0.039	0.000	0.032	0.095	0.100	0.000	0.000
	H_E	0.765	0.683	0.748	0.807	0.715	0.694	0.785	0.760	0.500	0.500
	H_O	0.714	0.690	0.717	0.731	0.500	0.710	0.714	0.600	1.000	1.000
	P	**0.000**	0.206	**0.008**	0.315	**0.012**	**0.012**	0.206	**0.002**	0.400	1.000
BM6506	Allele										
	202	0.122	0.052	0.150	0.077	0.042	0.081	0.071	0.050	0.000	0.500
	204	0.455	0.414	0.469	0.442	0.417	0.323	0.333	0.500	0.333	0.000
	206	0.024	0.017	0.031	0.000	0.042	0.016	0.048	0.000	0.000	0.000
	208	0.083	0.103	0.053	0.192	0.083	0.048	0.191	0.000	0.167	0.000
	210	0.164	0.259	0.155	0.096	0.083	0.210	0.095	0.150	0.333	0.250
	212	0.140	0.138	0.133	0.173	0.292	0.226	0.262	0.300	0.000	0.250
	216	0.012	0.017	0.009	0.019	0.042	0.097	0.000	0.000	0.167	0.000
	H_E	0.724	0.729	0.712	0.722	0.722	0.783	0.768	0.635	0.722	0.625
	H_O	0.726	0.655	0.726	0.808	1.000	0.839	0.810	0.700	1.000	1.000
	P	0.054	0.056	0.431	0.325	0.252	0.110	**0.043**	0.407	0.465	1.000

Table 4. continued

	Location	TOY- All	TOY- E	TOY- C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
	Number (N)	168	29	113	26	12	31	21	10	3	2
BM888	Allele										
	202	0.113	0.207	0.093	0.096	0.125	0.113	0.286	0.050	0.000	0.250
	204	0.060	0.017	0.062	0.096	0.083	0.113	0.071	0.100	0.167	0.000
	206	0.176	0.190	0.168	0.192	0.375	0.226	0.095	0.050	0.000	0.000
	208	0.333	0.224	0.363	0.327	0.167	0.210	0.429	0.450	0.500	0.250
	210	0.149	0.241	0.133	0.115	0.125	0.194	0.048	0.250	0.000	0.250
	212	0.074	0.069	0.080	0.058	0.042	0.048	0.071	0.100	0.167	0.250
	214	0.015	0.000	0.013	0.039	0.000	0.032	0.000	0.000	0.167	0.000
	218	0.080	0.052	0.089	0.077	0.083	0.065	0.000	0.000	0.000	0.000
	H_E	0.807	0.805	0.796	0.814	0.785	0.835	0.713	0.710	0.667	0.750
	H_O	0.881	0.828	0.894	0.885	0.917	0.871	0.952	0.700	1.000	1.000
	P	0.129	0.820	0.155	**0.011**	0.857	0.164	0.058	0.503	1.000	1.000
Cervid14	Allele										
	226	0.417	0.448	0.412	0.404	0.375	0.629	0.429	0.250	0.500	0.750
	228	0.104	0.190	0.084	0.096	0.375	0.129	0.095	0.050	0.000	0.000
	234	0.170	0.103	0.195	0.135	0.000	0.048	0.119	0.100	0.167	0.250
	236	0.080	0.069	0.097	0.019	0.208	0.081	0.214	0.200	0.167	0.000
	238	0.003	0.000	0.000	0.019	0.000	0.000	0.048	0.000	0.000	0.000
	240	0.036	0.017	0.031	0.077	0.042	0.065	0.024	0.000	0.167	0.000
	242	0.054	0.017	0.049	0.115	0.000	0.016	0.024	0.250	0.000	0.000
	244	0.137	0.155	0.133	0.135	0.000	0.032	0.048	0.150	0.000	0.000
	H_E	0.757	0.723	0.755	0.771	0.674	0.573	0.742	0.800	0.667	0.375
	H_O	0.804	0.759	0.788	0.923	0.500	0.581	0.714	1.000	1.000	0.500
	P	**0.000**	0.785	**0.006**	0.101	**0.012**	0.577	0.218	0.789	1.000	-
BM203	Allele										
	223	0.592	0.690	0.580	0.539	0.667	0.694	0.595	0.500	0.500	0.500
	225	0.048	0.035	0.044	0.077	0.000	0.016	0.167	0.050	0.000	0.000
	229	0.336	0.241	0.350	0.385	0.208	0.194	0.143	0.400	0.167	0.250
	233	0.024	0.035	0.027	0.000	0.125	0.097	0.095	0.050	0.333	0.250
	H_E	0.533	0.464	0.539	0.556	0.497	0.472	0.588	0.585	0.611	0.625
	H_O	0.571	0.483	0.531	0.846	0.583	0.484	0.619	0.900	1.000	1.000
	P	0.114	0.549	0.117	**0.005**	1.000	0.642	0.187	0.105	1.000	1.000
BM3628	Allele										
	204	0.006	0.017	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	206	0.006	0.000	0.009	0.000	0.083	0.016	0.000	0.000	0.000	0.000
	208	0.015	0.017	0.013	0.019	0.000	0.000	0.024	0.000	0.000	2.000
	210	0.250	0.172	0.266	0.269	0.042	0.129	0.119	0.100	0.500	0.500
	212	0.089	0.172	0.075	0.058	0.375	0.290	0.310	0.350	0.167	0.000
	214	0.054	0.086	0.044	0.058	0.167	0.129	0.119	0.000	0.000	0.000
	216	0.113	0.086	0.120	0.115	0.083	0.081	0.048	0.100	0.167	0.000
	218	0.336	0.397	0.323	0.327	0.083	0.290	0.167	0.250	0.167	0.250
	220	0.131	0.052	0.146	0.154	0.167	0.065	0.214	0.200	0.000	0.250
	H_E	0.783	0.765	0.782	0.777	0.781	0.787	0.799	0.755	0.667	0.625
	H_O	0.899	0.862	0.903	0.923	0.750	0.903	0.714	0.900	1.000	0.500
	P	**0.000**	0.092	**0.000**	0.289	0.344	0.056	**0.026**	**0.010**	1.000	0.333

Table 4. continued

	Location	TOY- All	TOY- E	TOY- C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
	Number (N)	168	29	113	26	12	31	21	10	3	2
BL42	Allele										
	250	0.048	0.052	0.040	0.077	0.000	0.210	0.071	0.000	0.000	0.000
	252	0.000	0.000	0.000	0.000	0.000	0.048	0.000	0.000	0.000	0.000
	256	0.467	0.466	0.478	0.423	0.708	0.565	0.571	0.650	0.667	0.750
	258	0.485	0.483	0.482	0.500	0.292	0.177	0.357	0.350	0.333	0.250
	H_E	0.544	0.548	0.537	0.565	0.413	0.604	0.541	0.455	0.444	0.375
	H_O	0.577	0.724	0.575	0.423	0.417	0.516	0.524	0.300	0.000	0.500
	P	0.140	0.183	0.317	0.144	1.000	0.166	0.077	0.480	0.201	-
BMC1009	Allele										
	280	0.015	0.017	0.013	0.019	0.000	0.016	0.000	0.000	0.000	0.000
	282	0.021	0.000	0.031	0.000	0.000	0.000	0.095	0.000	0.000	0.000
	286	0.256	0.207	0.266	0.269	0.250	0.274	0.381	0.200	0.500	0.500
	288	0.301	0.276	0.310	0.289	0.250	0.177	0.095	0.400	0.000	0.000
	290	0.107	0.138	0.106	0.077	0.083	0.113	0.119	0.150	0.000	0.000
	292	0.000	0.000	0.000	0.000	0.000	0.048	0.024	0.000	0.000	0.000
	296	0.009	0.017	0.009	0.000	0.042	0.000	0.024	0.050	0.000	0.000
	302	0.292	0.345	0.266	0.346	0.375	0.371	0.262	0.200	0.500	0.500
	H_E	0.747	0.743	0.751	0.718	0.726	0.740	0.753	0.735	0.500	0.500
	H_O	0.708	0.828	0.708	0.577	0.750	0.774	0.810	0.700	1.000	1.000
	P	**0.000**	0.298	**0.000**	**0.001**	0.514	**0.021**	**0.012**	0.278	0.399	1.000
BM6438	Allele										
	268	0.310	0.241	0.336	0.269	0.000	0.065	0.048	0.250	0.000	0.000
	270	0.176	0.138	0.137	0.385	0.208	0.242	0.191	0.300	0.000	0.000
	272	0.012	0.017	0.013	0.000	0.000	0.000	0.000	0.000	0.000	0.250
	274	0.018	0.086	0.004	0.000	0.042	0.016	0.000	0.000	0.000	0.000
	276	0.036	0.017	0.044	0.019	0.042	0.000	0.071	0.100	0.000	0.250
	278	0.048	0.017	0.058	0.039	0.000	0.032	0.095	0.100	0.000	0.250
	280	0.250	0.328	0.261	0.115	0.333	0.226	0.476	0.150	0.500	0.250
	282	0.152	0.155	0.146	0.173	0.375	0.419	0.119	0.100	0.500	0.000
	H_E	0.784	0.783	0.773	0.735	0.701	0.709	0.706	0.795	0.500	0.750
	H_O	0.726	0.690	0.761	0.615	0.833	0.839	0.619	0.700	1.000	1.000
	P	0.066	0.258	0.436	0.308	0.440	**0.007**	0.078	0.777	0.401	1.000

Table 5. Summary of genetic diversity for Japanese sika deer samples of Toyama and neighboring Prefectures

	TOY-E	TOY-C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
Allelic Richness (*AR*)	2.750	2.760	2.810	2.690	2.740	2.790	2.800	2.650	2.730
Private allelic richness *(PAR)*	0.090	0.080	0.100	0.120	0.130	0.150	0.110	0.080	0.130
Expected heterozygosities (H_E)	0.719	0.736	0.740	0.688	0.714	0.715	0.568	0.727	0.611
Observed heterozygosities (H_O)	0.771	0.768	0.794	0.742	0.754	0.755	0.864	0.745	0.909

Table 6. *P*- values for Fisher's exact test of population differentiation (upper diagonal) and pairwise F_{ST} values (lower diagonal) based on microsatellite allele frequencies

	TOY- E	TOY- C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
TOY- E	-	**0.0002**	0.0066	**0.0008**	**0.0004**	0.0193	**<0.0001**	0.1824	0.1344
TOY- C	**0.010**	-	0.0267	**<0.0001**	**<0.0001**	0.0054	**<0.0001**	0.1103	0.5135
TOY-W	0.013	0.001	-	**<0.0001**	**<0.0001**	0.0554	**<0.0001**	0.1422	0.1369
NIG	0.022	**0.047**	**0.041**	-	0.0603	**<0.0001**	**<0.0001**	0.1409	0.0570
NGN	**0.015**	**0.040**	**0.033**	0.008	-	**<0.0001**	**<0.0001**	0.5100	0.1361
GIF	**0.028**	**0.027**	**0.028**	**0.031**	**0.033**	-	0.0176	0.0611	0.4166
ISK	0.017	0.009	0.007	0.035	**0.039**	0.018	-	0.1556	0.5948
AIC	0.014	0.011	0.019	0.018	0.005	0.030	0.005	-	0.9525
FUK	0.006	-0.002	0.007	0.040	0.002	0.015	-0.016	-0.045	-

Test that are significant after Bonferrnoni correction are in bold (critical value of specific $P = 0.0013889$), and after Benjamin-Yekutieli correction are underlined ($P = 0.011977$)

Table 7. Pairwise population matrix of Nei's genetic (A) genetic similarities and (B) distance exhibited by 11 markers in sika deer population of Toyama and neighboring Prefectures

(A)

	TOY-E	TOY-C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
TOY-E	1.000								
TOY-C	0.971	1.000							
TOY-W	0.963	0.999	1.000						
NIG	0.940	0.865	0.880	1.000					
NGN	0.960	0.885	0.903	0.977	1.000				
GIF	0.919	0.921	0.913	0.912	0.906	1.000			
ISK	0.954	0.976	0.979	0.903	0.890	0.947	1.000		
AIC	0.963	0.968	0.938	0.957	0.990	0.982	0.906	1.000	
FUK	0.995	1.016	0.980	0.904	1.011	1.053	0.956	1.132	1.000

(B)

	TOY-E	TOY-C	TOY-W	NIG	NGN	GIF	ISK	AIC	FUK
TOY-E	0.000								
TOY-C	0.029	0.000							
TOY-W	0.038	0.001	0.000						
NIG	0.062	0.145	0.128	0.000					
NGN	0.041	0.123	0.102	0.023	0.000				
GIF	0.084	0.082	0.091	0.092	0.099	0.000			
ISK	0.047	0.024	0.021	0.103	0.117	0.054	0.000		
AIC	0.038	0.033	0.064	0.044	0.010	0.019	0.099	0.000	
FUK	0.005	0.000	0.020	0.100	0.000	0.000	0.045	0.000	0.000

Table 8. Analysis of molecular variance (AMOVA) of populations of sika deer population of Toyama and neighboring Prefectures

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage variation	Fixation indices	*P*-value
Among groups	3	38.77	0.09	2.18	$F_{CT} = 0.021$	$P < 0.0001$
Among population within groups	5	26.61	0.03	0.69	$F_{SC} = 0.007$	$P < 0.0007$
Among individuals within populations	485	1969.59	4.06	97.12	$F_{ST} = 0.028$	$P < 0.0001$
Total	493	2034.98	4.18	100.0	-	-

Table 9. Spatial analysis of molecular variance (SAMOVA) analysis of sika deer population of Toyama and neighboring Prefecture showing hight F_{CT} result of K=4

SAMOVA result for K=4						
Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage variation	Fixation indices	*P*-value
Among groups	3	39.08	0.11	2.57	F_{CT}= 0.025	$P < 0.0001$
Among population within groups	5	26.30	0.03	0.70	F_{SC} = 0.007	$P < 0.0001$
Among individuals within populations	238	926.10	-0.17	-3.98	F_{IS} = -0.041	$P > 0.0500$
Within individuals	247	1043.50	4.22	100.71	F_{IT} = -0.007	$P > 0.0500$
Total	493	2034.98	4.19	100	-	-

Table10. Contemporary migration patterns of Japanese sika deer between Toyama and neighboring prefectures. GENECLASS2 and STRUCTURE showing first generation migrants (F0)

ID	Sampled population	Origin of population	(*P* -value)	Sex	Resident ancestry (Q)	Migrated ancestry (Q)
CN14-17	TOY-E	NIG	0.031	Male	-	-
CN14-25	TOY-E	NGN	0.020	Male	0.750	0.250
CN14-53	TOY-E	NGN	0.008	Unknown	0.773	0.227
CN16-43	TOY-E	NGN	0.014	Male	-	-
CN17-36	TOY-E	NGN	0.012	Male	-	
CN15-11	TOY-E	GIF	0.030	Male	0.772	0.228
CN17-32	TOY-E	AIC	0.047	Male	-	-
CN17-33	TOY-E	ISK	0.003	Male	0.212	0.788
CN13-02	TOY-C	GIF	0.028	Male	0.794	0.206
CN14-26	TOY-C	NGN	0.019	Male	-	
CN15-18	TOY-C	NGN	0.003	Male	0.587	0.413
CN14-42	TOY-C	NGN	0.017	Male	-	-
CN16-14	TOY-C	NGN	0.041	Male	-	-
CN16-27	TOY-C	NGN	0.015	Male	-	-
CN17-22	TOY-C	NGN	0.015	Female	0.583	0.417
CN14-52	TOY-C	NGN	0.020	Male	-	
CN16-25	TOY-C	ISK	0.019	Male	-	-
CN15-22	TOY-C	GIF	0.044	Male	-	-
CN17-08	TOY-C	-	-	Female	0.751	0.249
CN17-20	TOY-C	ISK	0.027	Female	-	
CN17-30	TOY-W	NIG	0.017	Male	0.398	0.602
CN17-19	TOY-W	NGN	0.035	Male	0.723	0.277
CN14-39	TOY-W	NGN	0.014	Male	-	-
CN16-44	TOY-W	NGN	0.036	Male	-	-
CN17-37	TOY-W	-	-	Male	0.748	0.252
CN17-17	TOY-W	GIF	0.043	Male	-	-
CN16-NGN01	NGN	TOY	0.020	Female	-	-
CN15-GIF05	GIF	TOY	0.014	Female	-	-
CN17-NGN02	NGN	-	-	Male	0.782	0.218
CN13-AIC02	AIC	TOY	0.029	Female	-	-
CN13-FUK01	FUK	TOY	0.000	Female	-	-
CN17-ISK01	ISK	TOY	0.002	Male	0.755	0.245

Table 11. Contemporary migration patterns of Japanese sika deer between Toyama and neighboring Prefectures using BYESASS software.The populations into which individuals are migrating are listed in the rows, while the sources of the migrants are listed in the columns. Values along the diagonal are proportions of individuals derived from the source populations

	TOY	NIG & NGN	GIF & AIC	ISK & FUK
TOY	**0.87 (0.82- 0.91)**	0.093 (0.062-0.132)	0.014 (0.0002 - 0.053)	0.022 (0.007 - 0.042)
NIG & NGN	0.012 (0.0002 - 0.042)	**0.88 (0.82 - 0.94)**	0.015 (0.0001 - 0.049)	0.088 (0.039 - 0.144)
GIF & AIC	0.150 (0.0531- 0.255)	0.069 (0.006 - 0.167)	**0.75 (0.69 - 0.86)**	0.026 (0.001 - 0.079)
ISK & FUK	0.218 (0.095 - 0.308)	0.038 (0.0004 - 0.129)	0.025 (0.0001 - 0.108)	**0.72 (0.67 - 0.81)**

Table 12. Bayesian estimates of historic gene flow among populations of Japanese sika deer in Toyama and neighboring prefectures

A

	Bayesian Analysis: Posterior distribution table		
Population size (θ)	0.025	0.975	median
$\theta 1$ (TOY)	0.09	1.51	1.29
$\theta 2$ (NIG & NGN)	0.61	1.09	0.86
$\theta 3$ (GIF & AIC)	0.15	1.27	0.47
$\theta 4$ (ISK & FUK)	0.00	0.29	0.14

B

Migration rates (M)	0.025	0.975	median
$M\_2 \rightarrow 1$ (NIG & NGN > TOY)	8.12	9.16	8.44
$M\_3 \rightarrow 1$ (GIF & AIC > TOY)	0.00	0.33	0.28
$M\_4 \rightarrow 1$ (ISK & FUK > TOY)	0.00	1.37	0.59
$M\_1 \rightarrow 2$ (TOY > NIG & NGN)	0.86	2.17	3.78
$M\_3 \rightarrow 2$ (GIF & AIC > NIG & NGN)	1.78	4.54	3.54
$M\_4 \rightarrow 2$ (ISK & FUK > NIG & NGN)	2.01	5.09	3.53
$M\_1 \rightarrow 3$ (TOY > GIF & AIC)	3.66	8.51	6.00
$M\_2 \rightarrow 3$ (NIG & NGN > GIF & AIC)	6.71	7.75	4.49
$M\_4 \rightarrow 3$ (ISK & FUK > GIF & AIC)	1.52	7.62	4.74
$M\_1 \rightarrow 4$ (TOY > ISK & FUK)	0.00	1.15	0.48
$M\_2 \rightarrow 4$ (NIG & NGN > ISK & FUK)	6.69	9.86	7.30
$M\_3 \rightarrow 4$ (GIF & AIC > ISK & FUK)	0.60	3.27	1.70

C

Effective number of migrant (Nem = $\theta * M/4$)			
Population size (θ)	Migration rates (M)	4Nem	Nem/4
$\theta 1$ (TOY)	NIG & NGN > TOY	10.89	2.72
$\theta 1$ (TOY)	GIF & AIC > TOY	0.36	0.09
$\theta 1$ (TOY)	ISK & FUK > TOY	0.76	0.19
$\theta 2$ (NIG & NGN)	TOY > NIG & NGN	3.24	0.81
$\theta 3$ (GIF & AIC)	TOY > GIF & AIC	2.82	0.71
$\theta 4$ (ISK & FUK)	TOY > ISK & FUK	0.69	0.17

Bayesian estimates of mutation-scaled effective population sizes and migration rates among the sika deer poulation,95% confidence Interval (CI) are reported. A. Mutation scaled effective population sizes (θ) values of each population, B. Mutation-scaled immigration rate, M and C. Effective number of migrants ($N_e m$).

Table 13. Results of migration models selection apporch

	Hypothesis	*L* n *L*	AIC
1	Full model	-6499.2	13030.4
2	N-island model	-19976.7	39963.3
3	Stepping stone model: type I	-13811.8	27647.6
4	Stepping stone model: type I	-12193.1	24394.3
5	Source-Sink model: type I	-2482.2	4978.4
6	Source-Sink model: type II	-3083.6	6181.1

Maximun likelihood estimates of historic gene flow among populations of Japanese sika deer in Toyama and neighboring prefectures. Log-likelihood (*Ln L*) and Akaike's information criterion (AIC) values measured to the fit of the data, considering different parameterizations into account. The lower value of AIC indicate a better fit.

Table 14. Outcomes of Mantel tests for correlation Rxy between the pairwise genetic and geographic distance matrices and *P* for tests of significance by random permutation.

Sample		Rxy	*P*
All Prefectures population		0.140	0.002
	Male	0.170	0.010
	Female	0.129	0.010
Toyama Prefecture population		0.024	0.310
	Male	0.048	0.090
	Female	-0.020	0.480

Table 15. Average pairwise relatedness between and within each herd (Gray color) was shown in diagonal. Pairwise relatedness within each herd, gretar than 0.50 shown as undelined and bold, greater than 0.25 shown as underlined only. Herd sizes are included in parenthesis

	herd 1	herd 2	herd 3	herd 4	herd 5	herd 6	herd 7	herd 8	herd 9	herd 10	herd 11	herd 12	herd 13	herd 14	herd 15	herd 16	herd 17	herd 18	herd 19	herd 20
herd 1 (2)	-0.185																			
herd 2 (2)	0.014	-0.176																		
herd 3 (2)	-0.092	0.259	**0.647**																	
herd 4 (2)	0.157	0.183	0.091	**0.519**																
herd 5 (4)	0.121	0.041	-0.073	0.224	0.051															
herd 6 (3)	-0.042	0.032	-0.146	-0.031	0.041	0.058														
herd 7 (3)	-0.225	-0.119	0.018	-0.167	-0.032	-0.111	0.145													
herd 8 (2)	-0.127	-0.085	0.014	-0.206	0.010	0.093	0.255	0.239												
herd 9 (2)	-0.004	-0.100	-0.249	-0.106	0.101	0.034	-0.010	0.064	0.328											
herd 10 (2)	0.080	0.014	-0.072	0.150	0.108	0.079	-0.121	-0.054	-0.071	0.083										
herd 11 (2)	0.002	-0.171	-0.244	-0.073	-0.130	-0.059	-0.250	-0.124	-0.112	0.105	**0.933**									
herd 12 (2)	-0.028	-0.042	-0.009	-0.031	0.055	0.031	0.030	0.002	0.073	0.043	-0.047	-0.267								
herd 13 (4)	-0.054	0.029	0.061	0.137	0.033	0.025	-0.068	-0.100	-0.103	0.130	0.066	0.173	0.286							
herd 14 (3)	-0.070	0.032	-0.058	-0.076	-0.004	0.011	-0.038	-0.048	-0.056	-0.032	-0.084	0.039	0.061	0.118						
herd 15 (2)	-0.093	-0.040	0.023	0.147	-0.054	0.030	-0.093	-0.087	-0.241	-0.085	-0.023	-0.075	0.067	-0.181	0.246					
herd 16 (2)	-0.156	0.027	-0.107	-0.083	-0.082	-0.031	-0.091	-0.094	-0.207	-0.042	-0.100	-0.092	-0.013	0.032	0.164	**0.511**				
herd 17 (2)	-0.021	-0.179	-0.047	-0.100	0.025	0.016	0.119	0.257	0.129	-0.100	0.001	0.061	-0.066	-0.089	-0.138	-0.189	0.340			
herd 18 (2)	-0.120	-0.004	-0.057	-0.140	-0.086	-0.075	-0.005	0.108	0.093	0.020	-0.024	-0.063	-0.159	-0.122	-0.216	-0.026	0.130	-0.147		
herd 19 (2)	-0.219	-0.100	-0.078	-0.039	-0.079	-0.046	0.018	0.006	-0.180	-0.017	0.003	0.018	0.044	-0.051	0.024	0.147	-0.122	-0.014	0.277	
herd 20 (2)	-0.098	0.004	-0.074	0.061	-0.055	-0.428	-0.053	-0.055	0.004	-0.091	-0.060	-0.004	-0.094	-0.184	0.131	-0.075	0.018	0.042	-0.033	-0.013

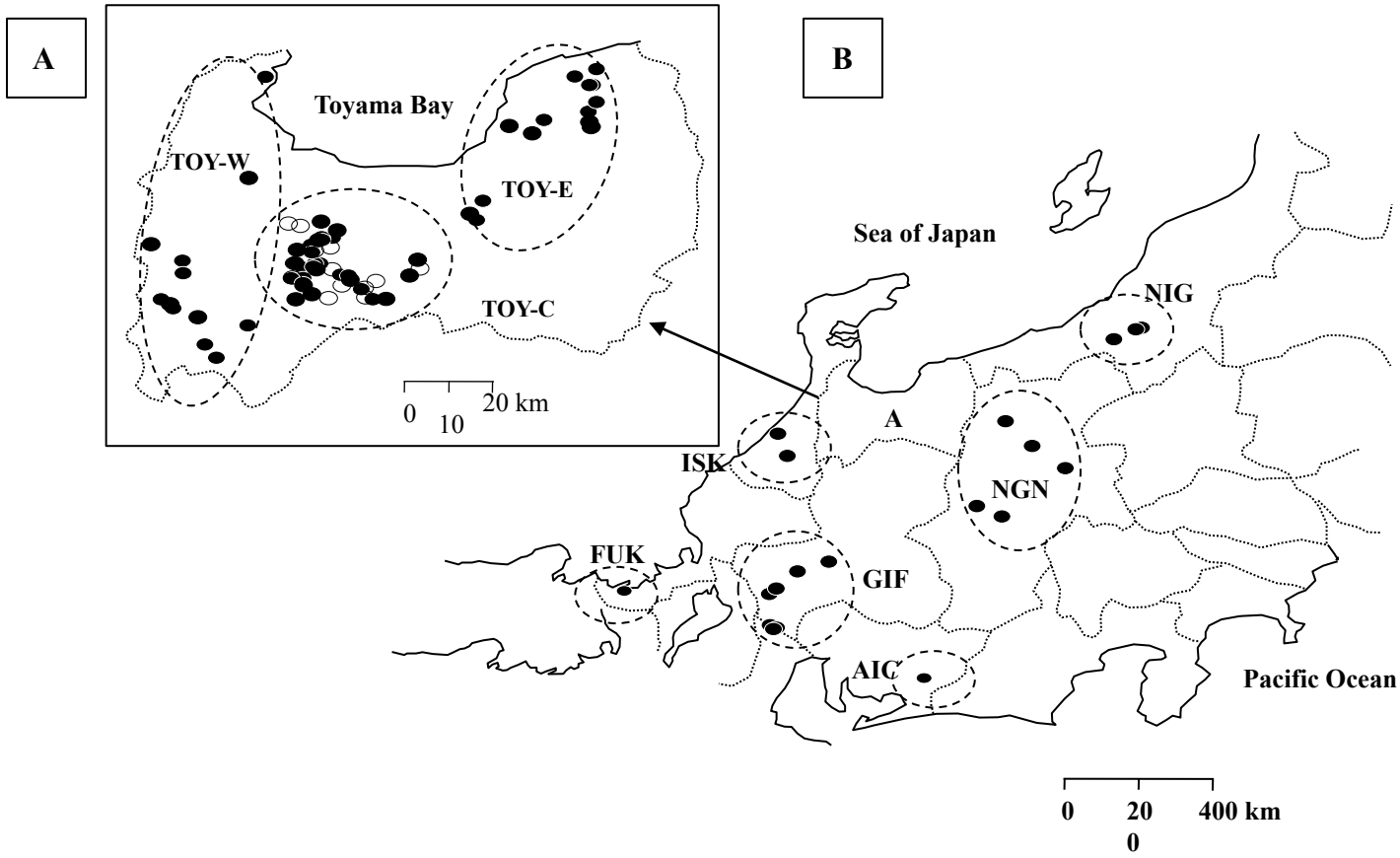


Figure 1. Approximate sampling locations and regional grouping of Japanese sika deer around (A) Toyama and (B) neighboring prefectures. Solid and open circles indicate the approximate collection sites of the sika deer individuals.

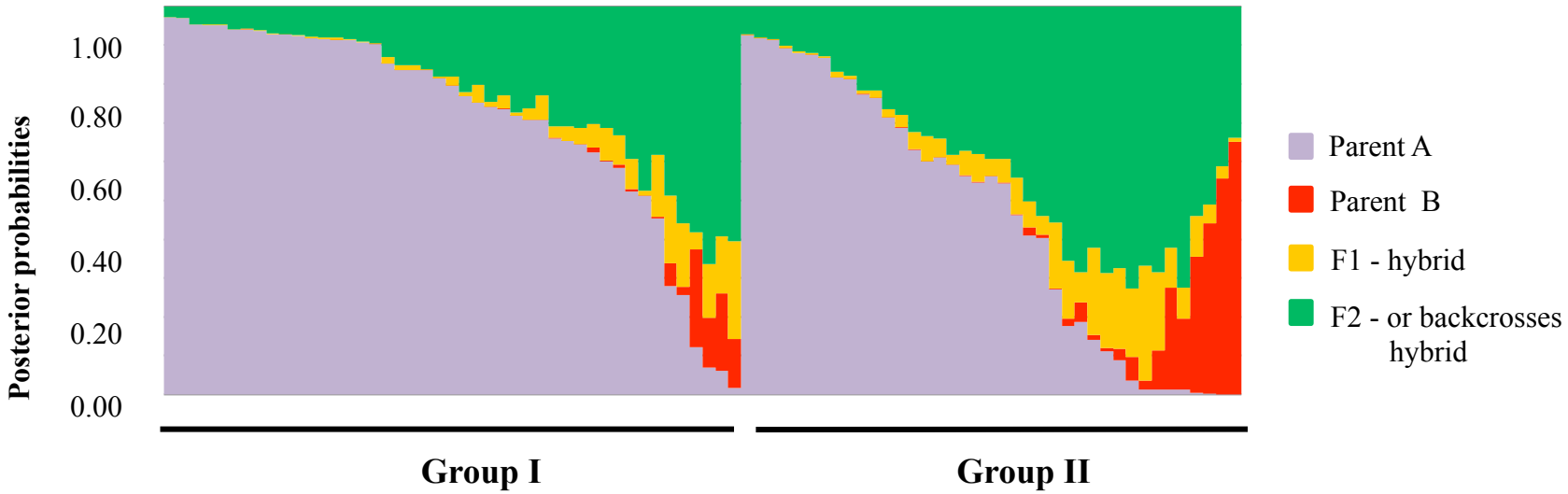


Figure 2. Bar graph showing NewHybrids result of sika deer individuals in the central part of Toyama Prefecture. Each bar represents the composition of assumed source classes.

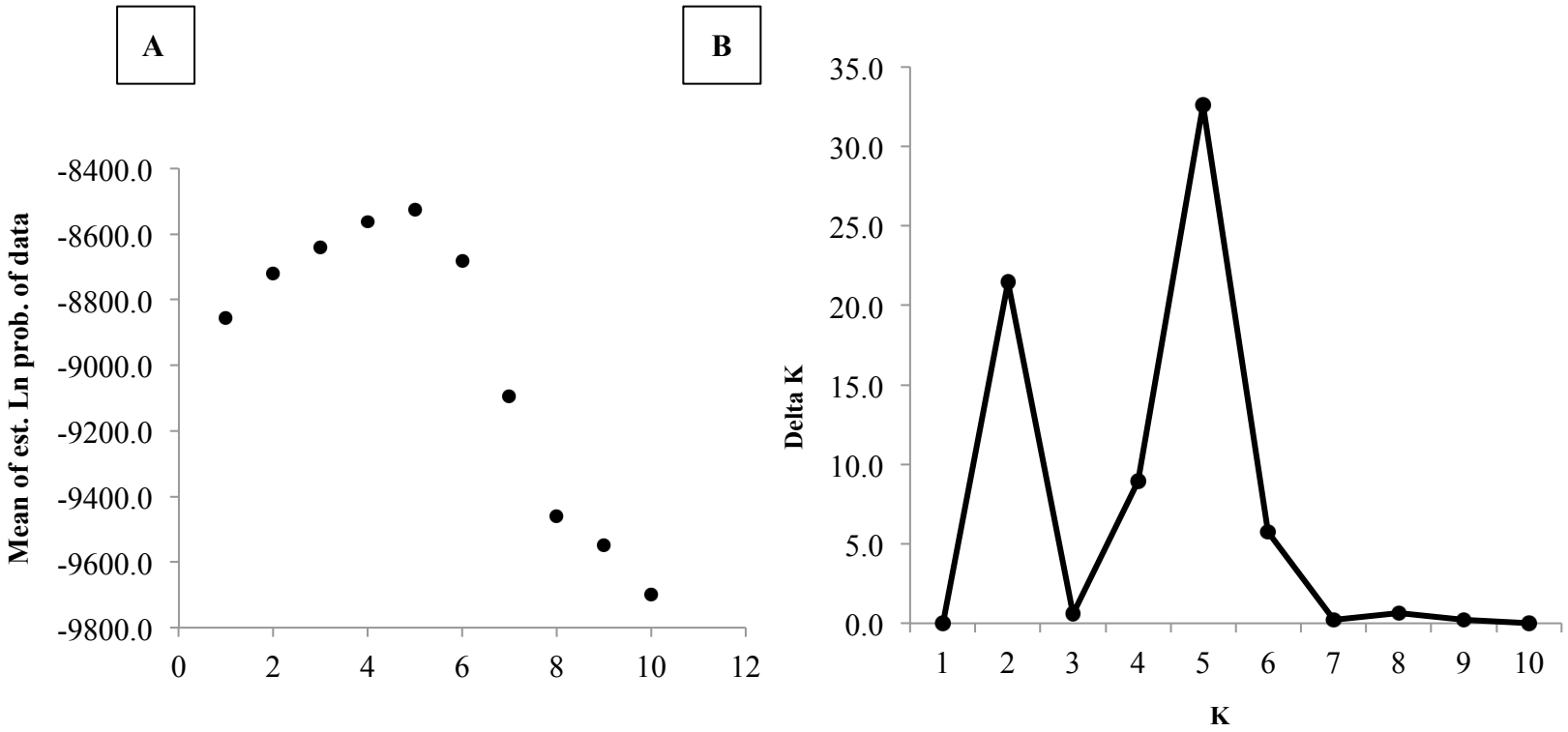


Figure 3. (A) Mean posterior probabilities and (B) delta K, height K = 5 including a 2nd height K = 2 was observed in the studied population.

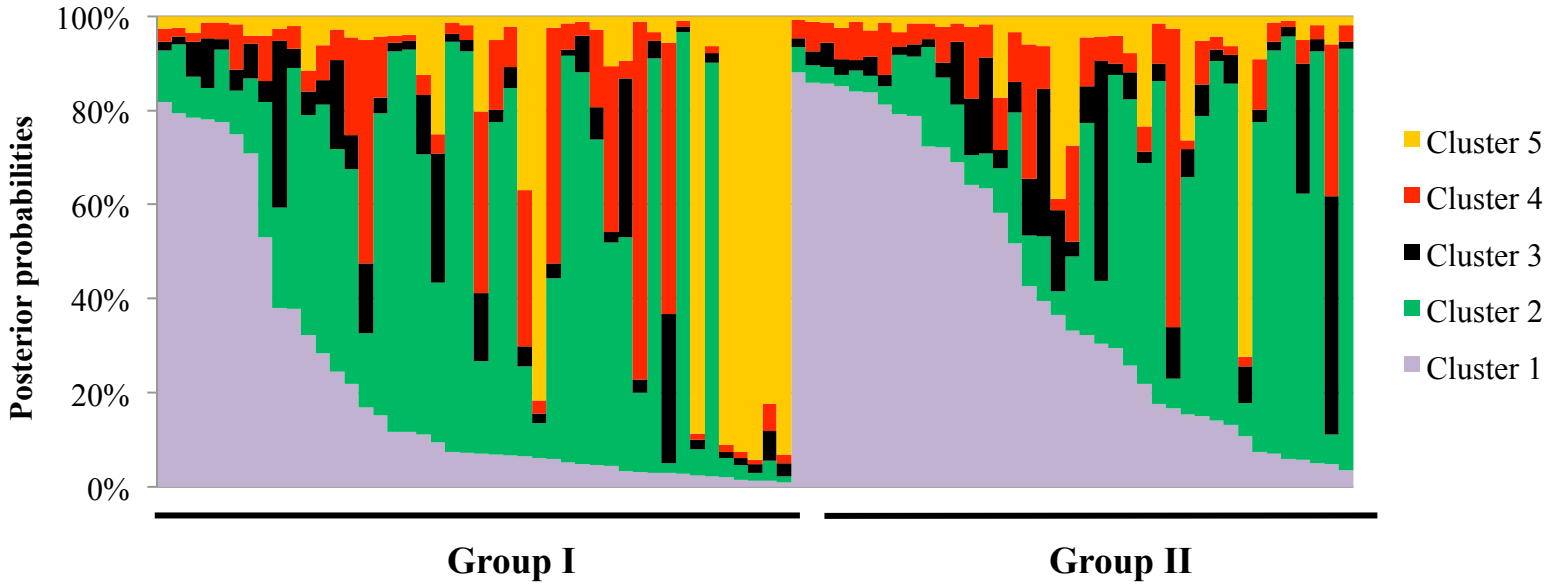


Figure 4. Bar graph showing STRUCTURE result of the sika deer individuals in the central part of Toyama Prefecture indicating admixture of multiple clusters in both groups.

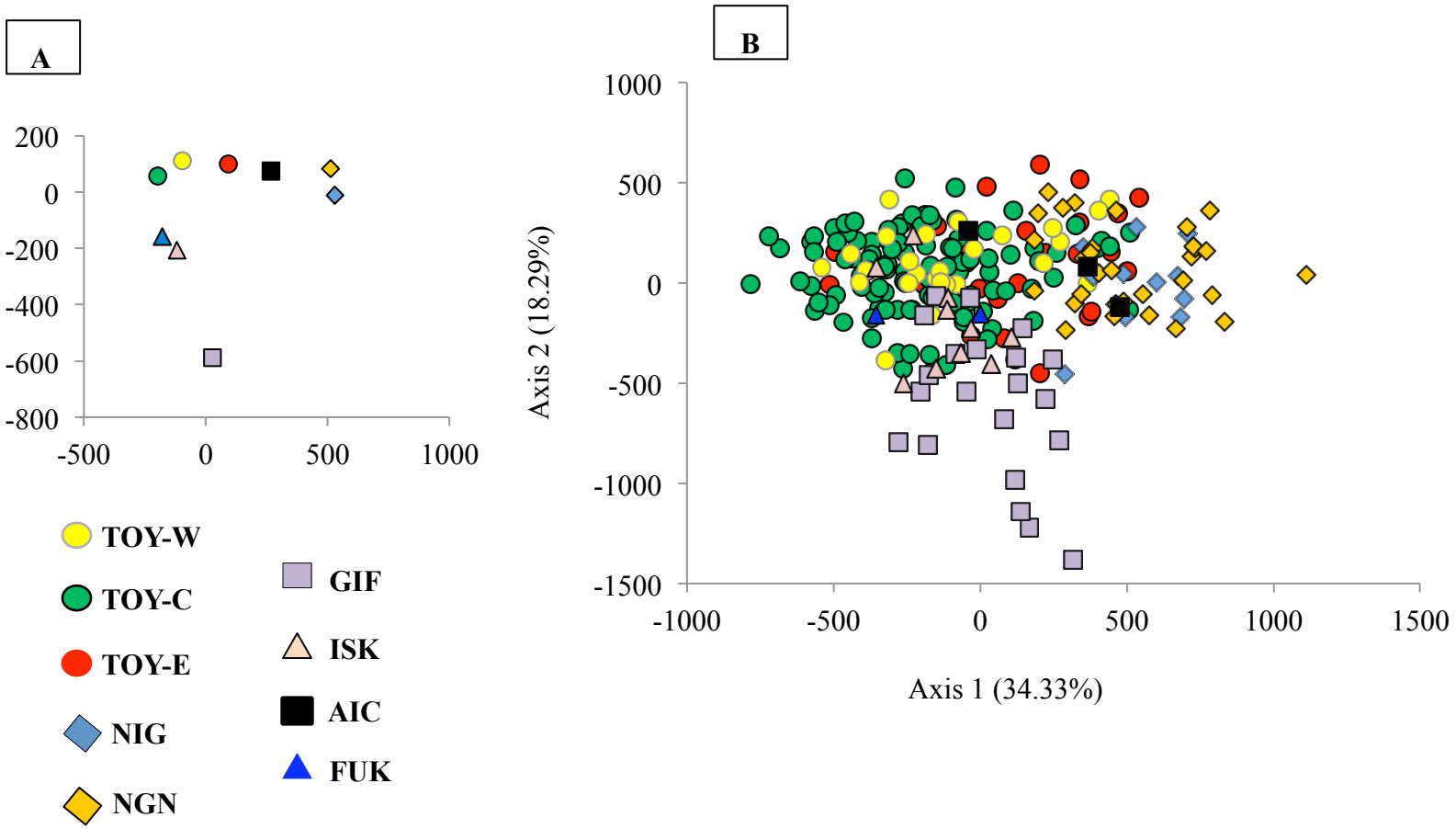


Figure 5. Two-dimensional plots of the microsatellite genotypes of Japanese sika deer at regional (A) and individual (B) levels derived from a factorial correspondence analysis.

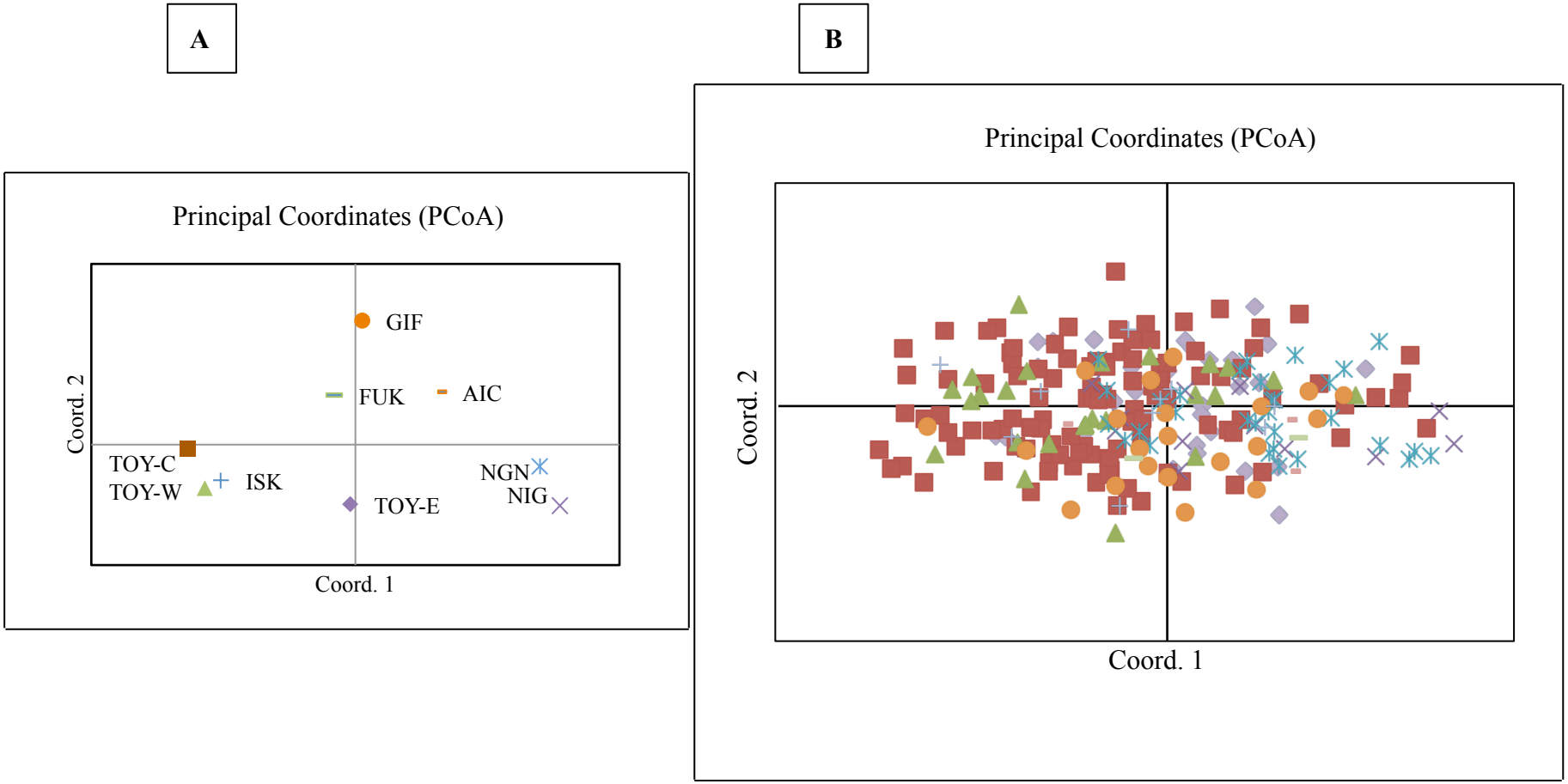


Figure 6. Principal coordinates analysis (PCoA) based on Nei's genetic distances (A) from geographic distance using Global Position System coordinates (B) among the sika deer populations.

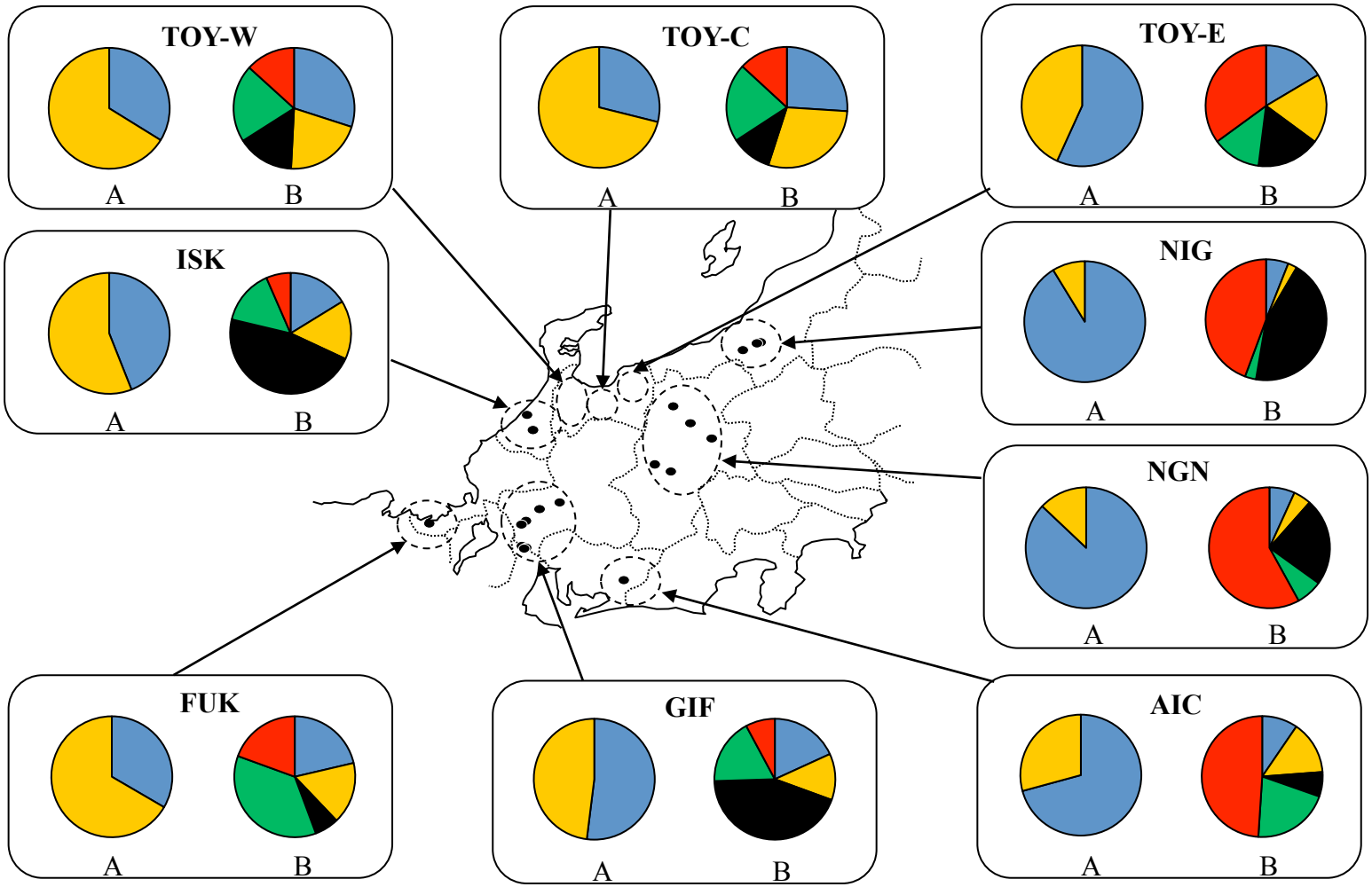


Figure 7. Composition of two as (A) (left) and five (B) (right) clusters in each regional group of Japanese sika deer.

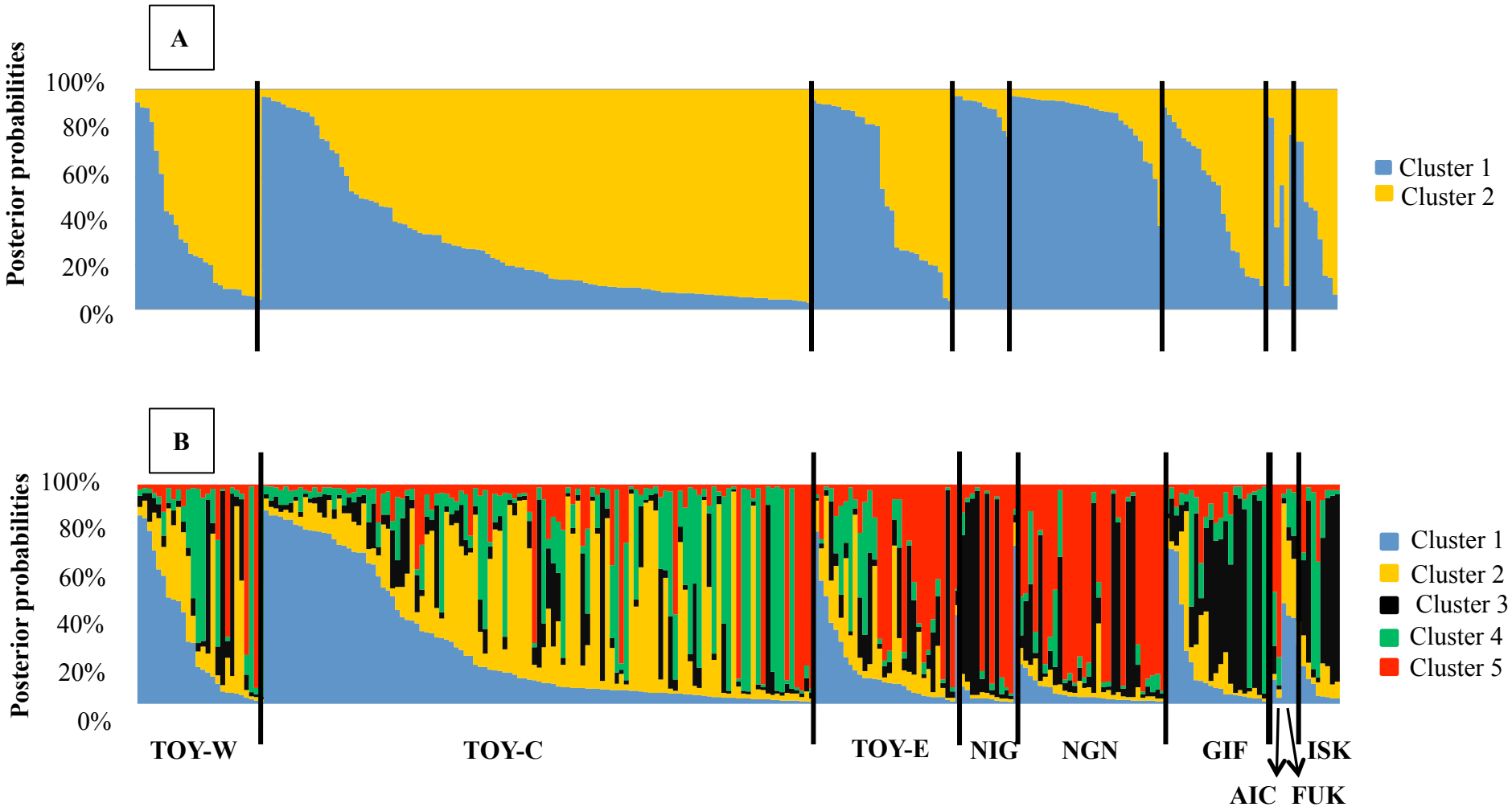


Figure 8. Bar charts showing composition of two (A) and five (B) clusters in each regional group of Japanese sika deer.

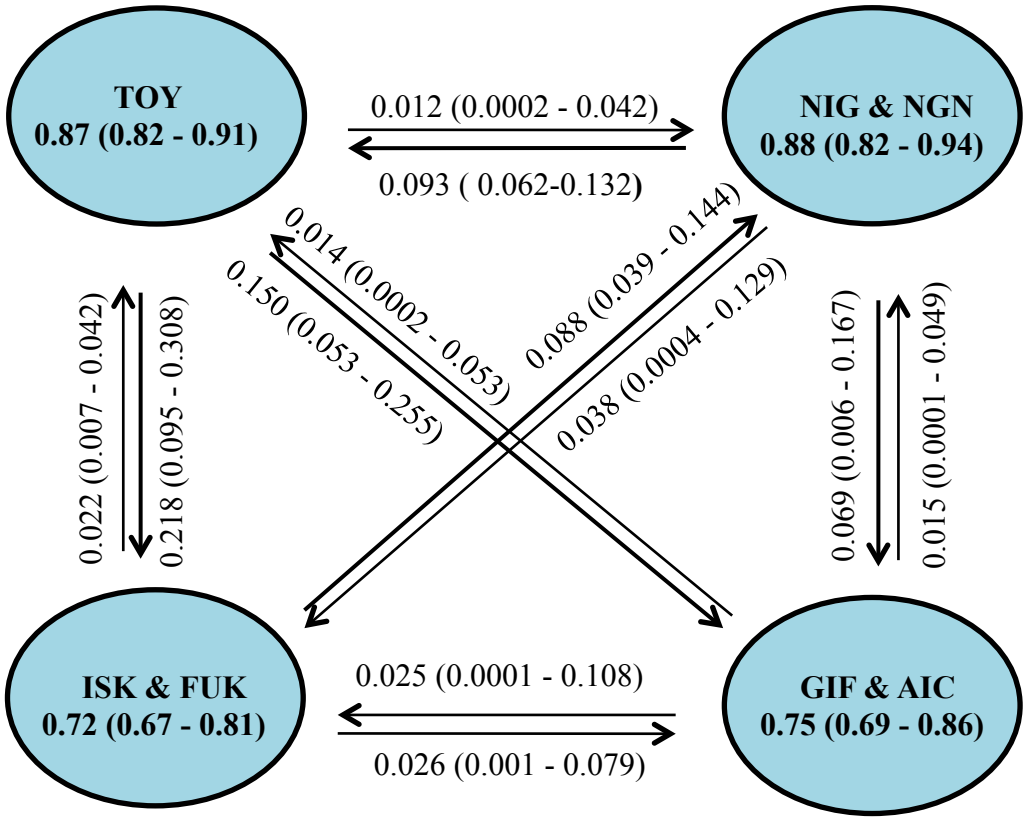


Figure 9. Contemporary gene flow patterns among populations of sika deer. The values above and bellow arrows represent migration rates in the direction of the arrow, thickness of arrows indicating relative amount of directional gene flow. Numbers inside circles represent proportions of non-migrants (self population) and numbers in brackets are 95% confidence intervals.

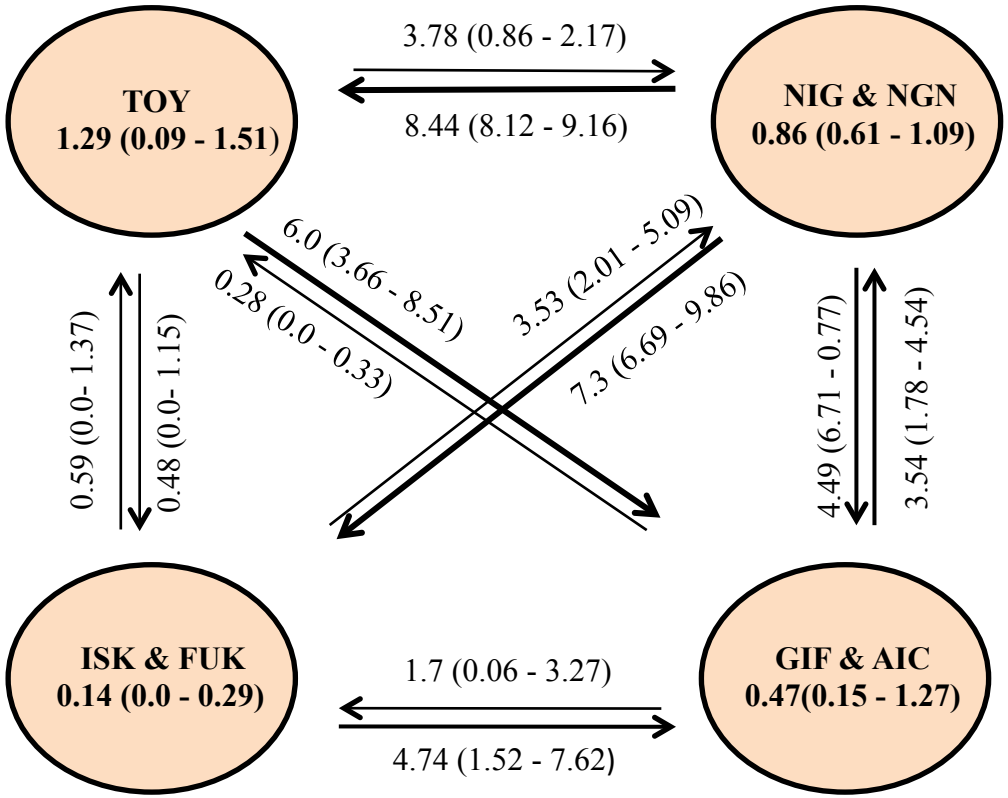


Figure 10. Comparison of historical gene flow among Japanese sika deer around Toyama and neighboring Prefectures. Numbers inside circles represent effective population sizes, and number of above and below arrows represent migration rates in the direction of the arrow. Numbers in brackets are 95% confidence intervals. Thickness of arrows scaled according to their values.

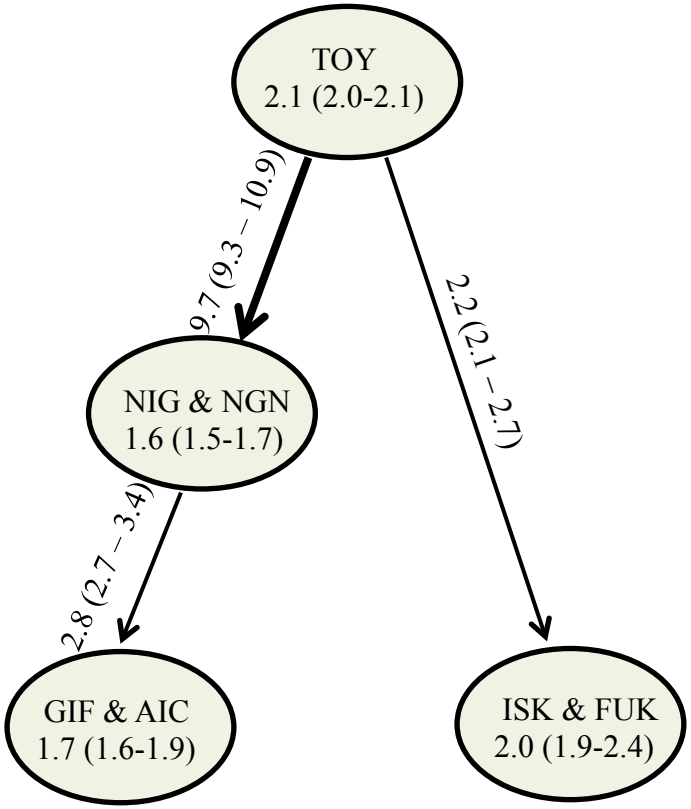


Figure 11. Historical gene flow patterns among populations of sika deer of source sink model. Arrows direction of gene flow between the population, relative thickness of arrows relative amount of directional gene flow.

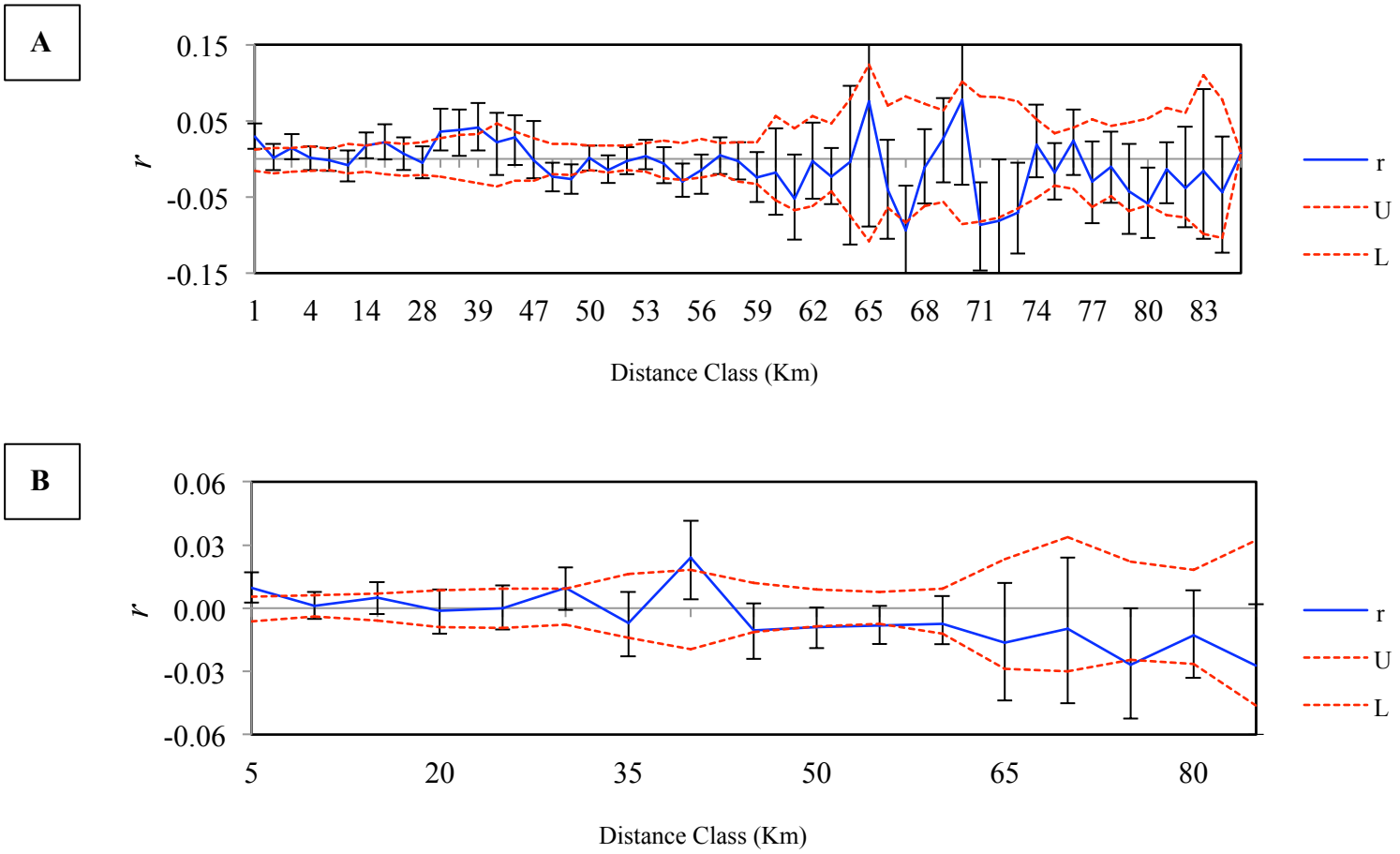


Figure 12. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for all pairs of individual of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

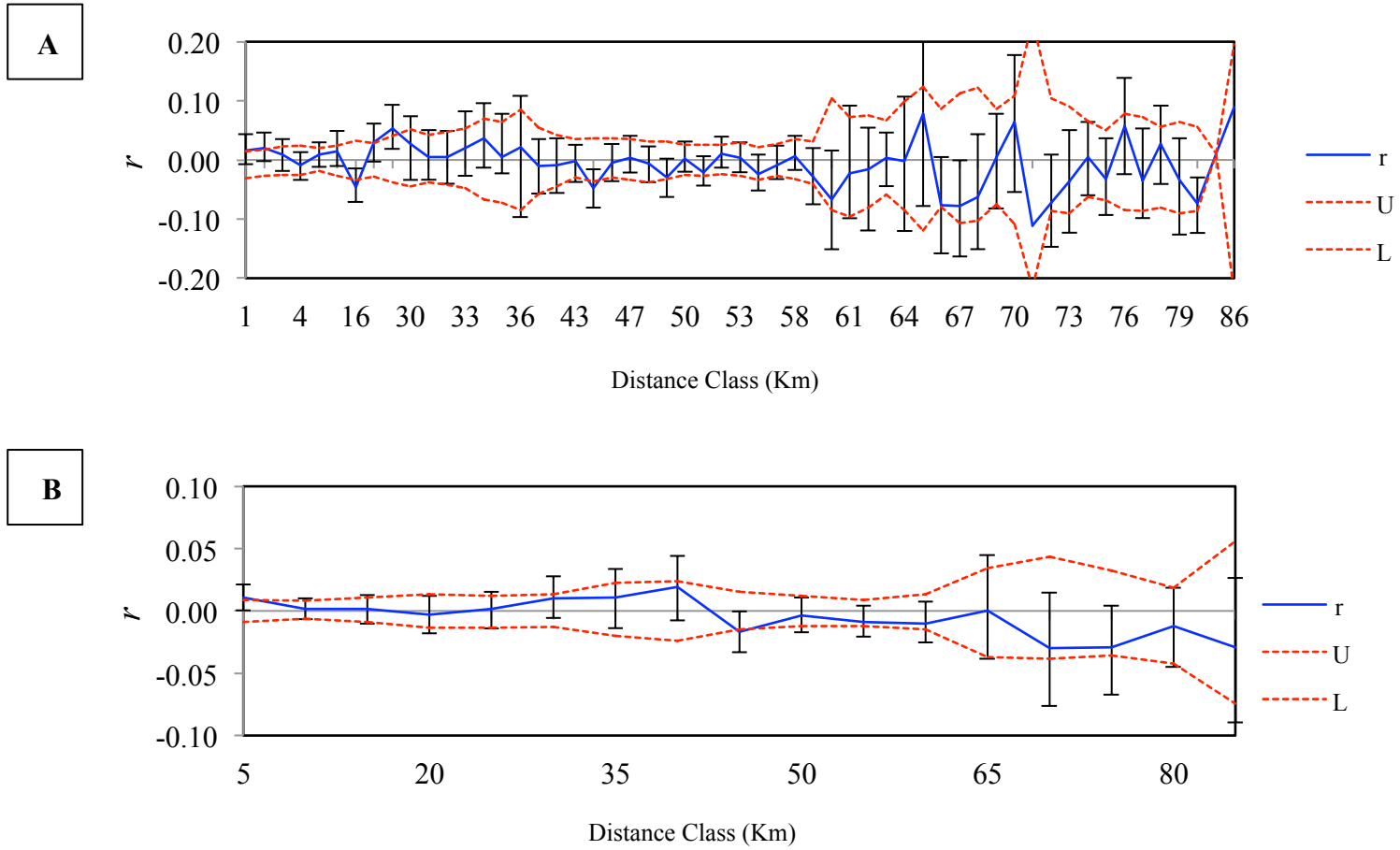


Figure 13. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for all pairs of males of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

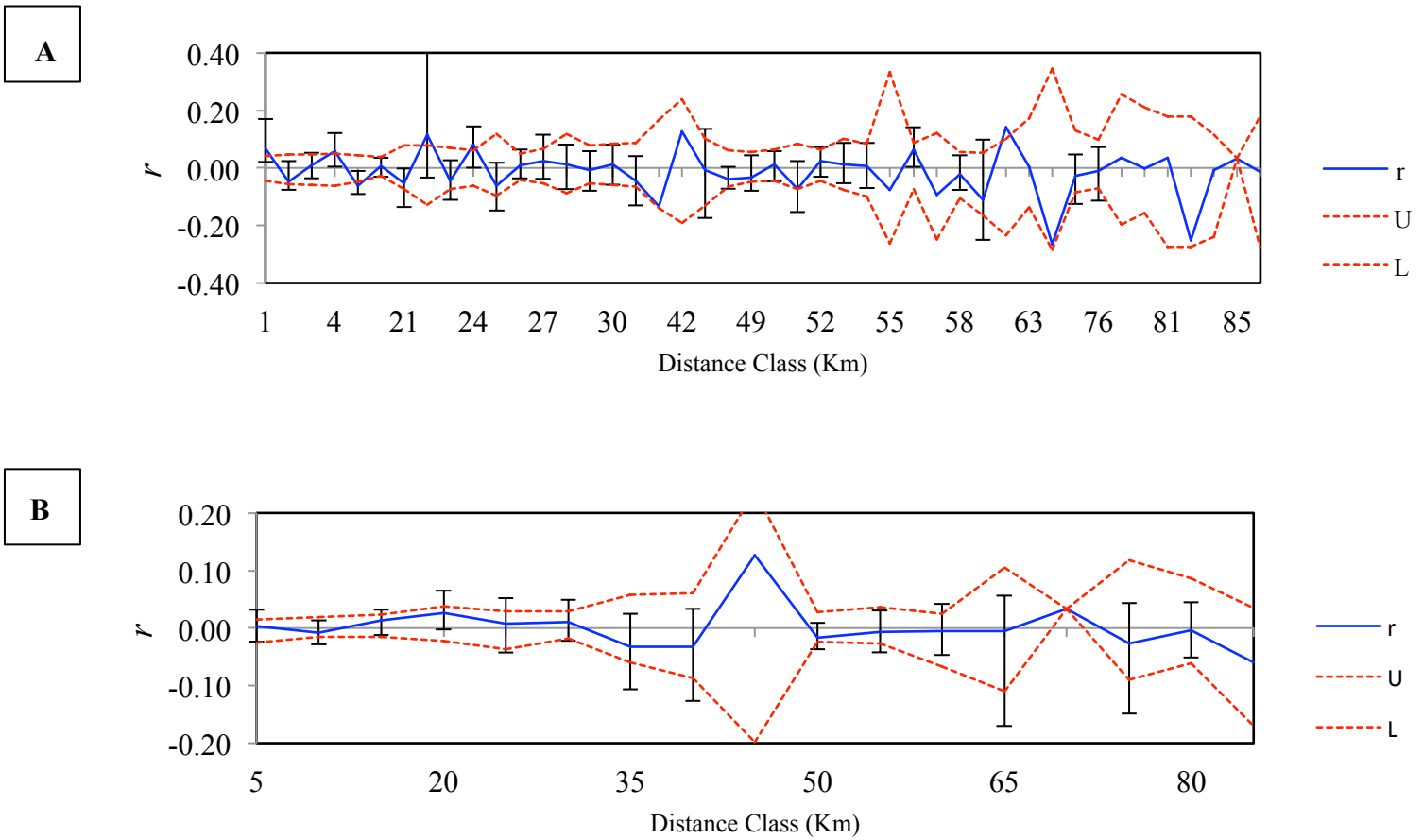


Figure 14. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for all pairs of females of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

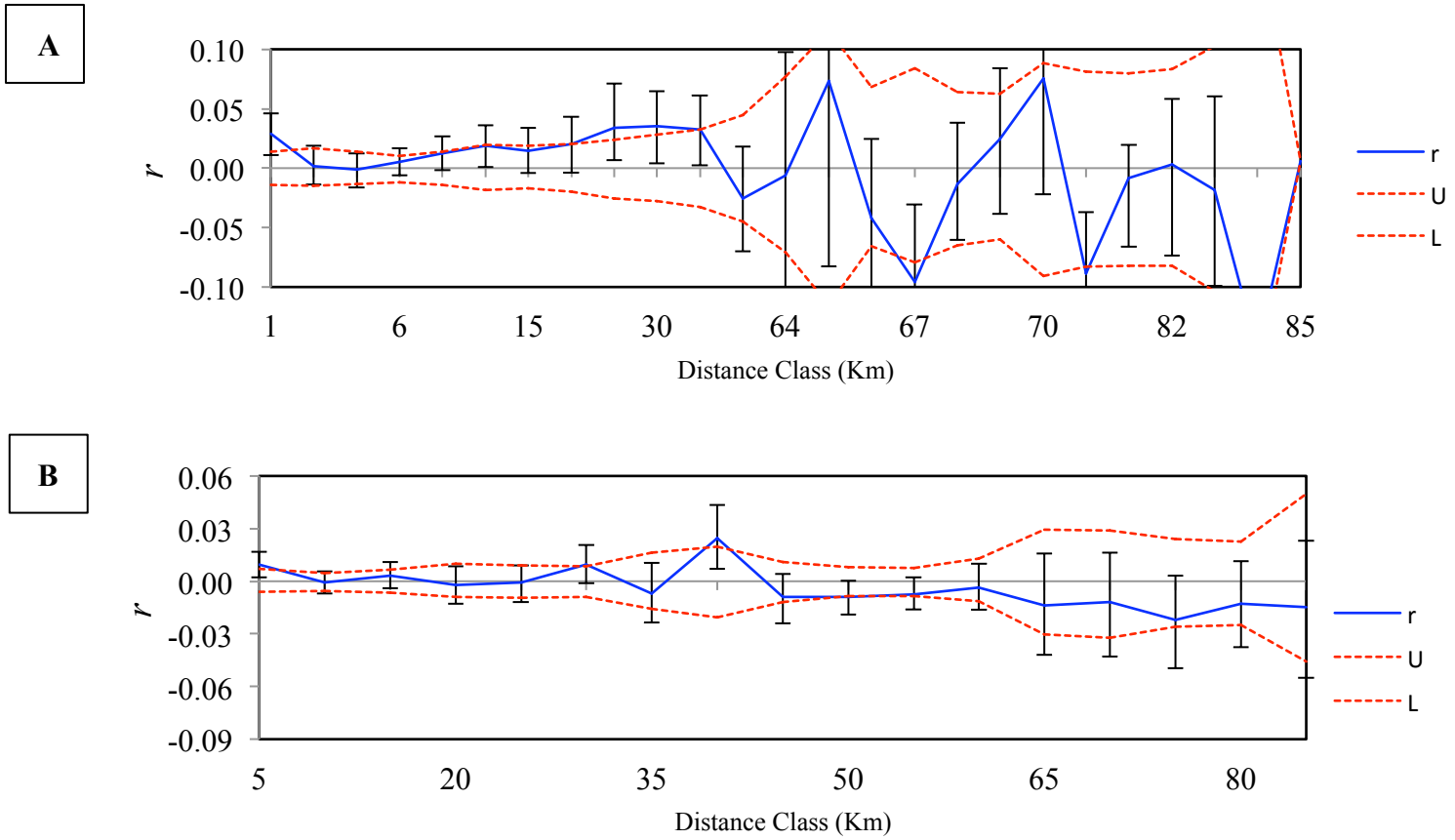


Figure 15. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for Toyama Prefecture's individual of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

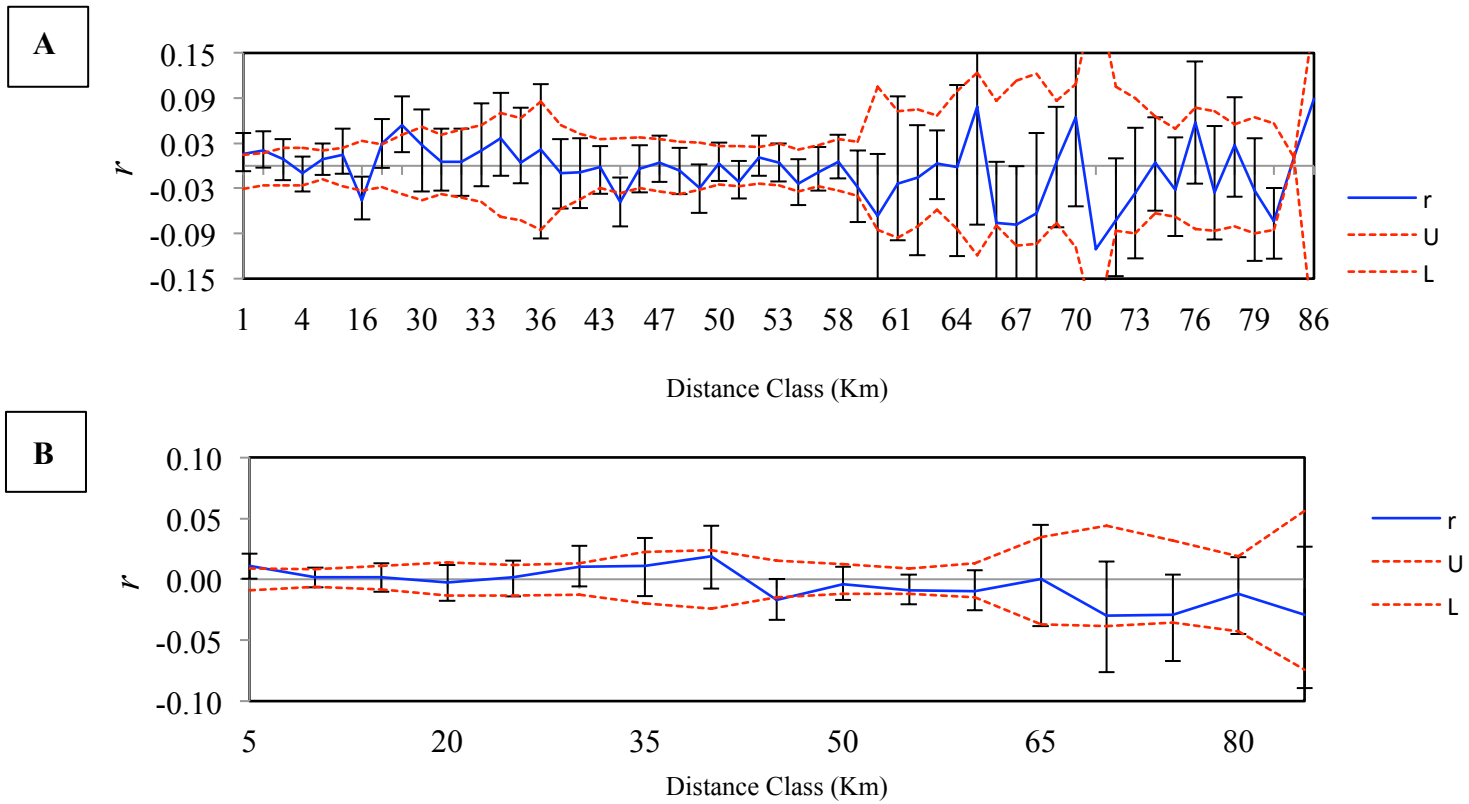


Figure 16. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for males of Toyama Prefecture's individual of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

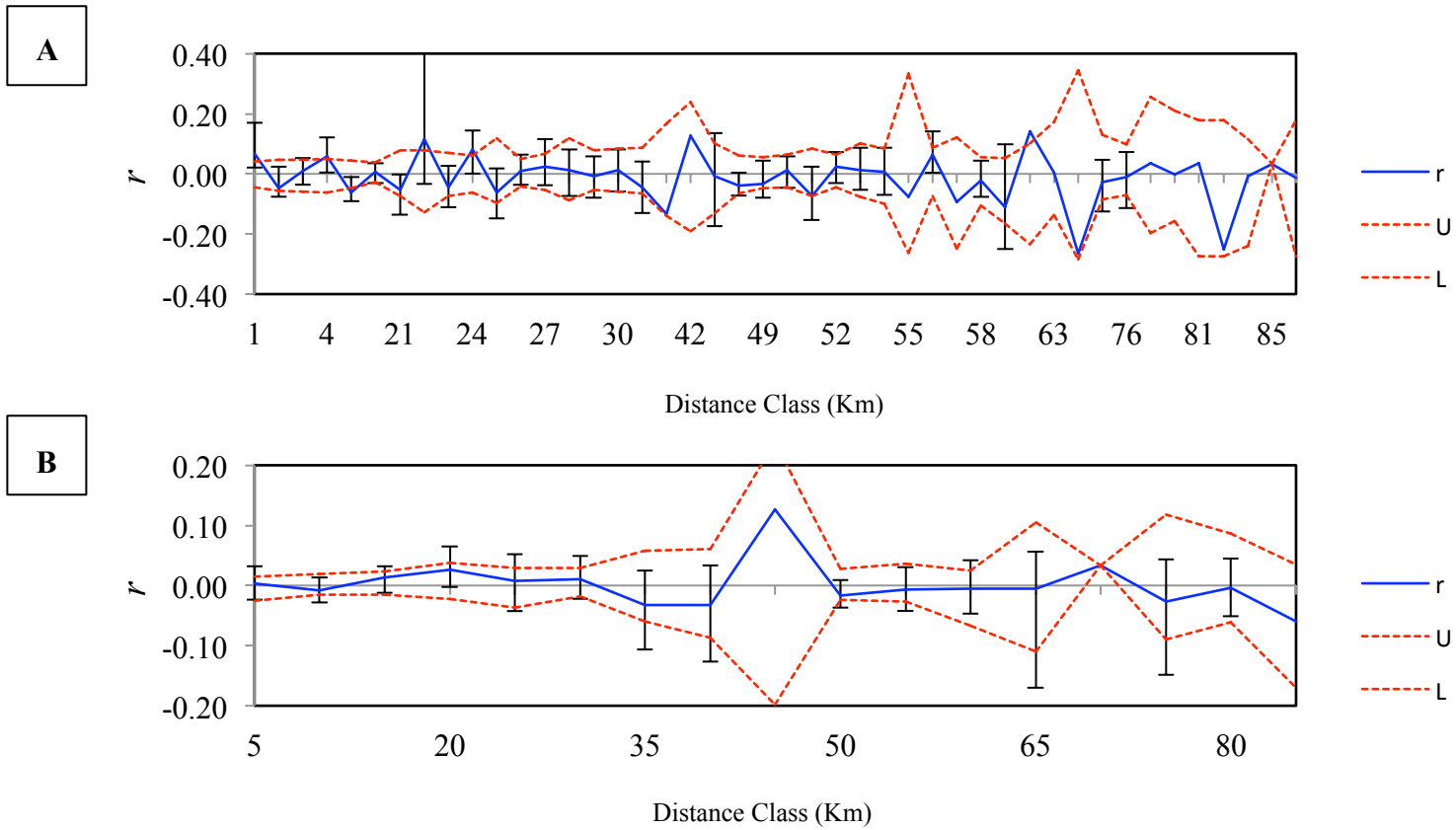


Figure 17. Spatial autocorrelation correlograms of the coefficient *r* as a function of distance for females of Toyama Prefecture's individual of sika deer in solid line considering distance classes of 1 km (A) and 5 km (B). Dashed red lines represent upper (U) and lower (L) bounds of the null distribution based on 999 random permutations. Error bars represent 95% confidence intervals about *r* based on 999 bootstraps.

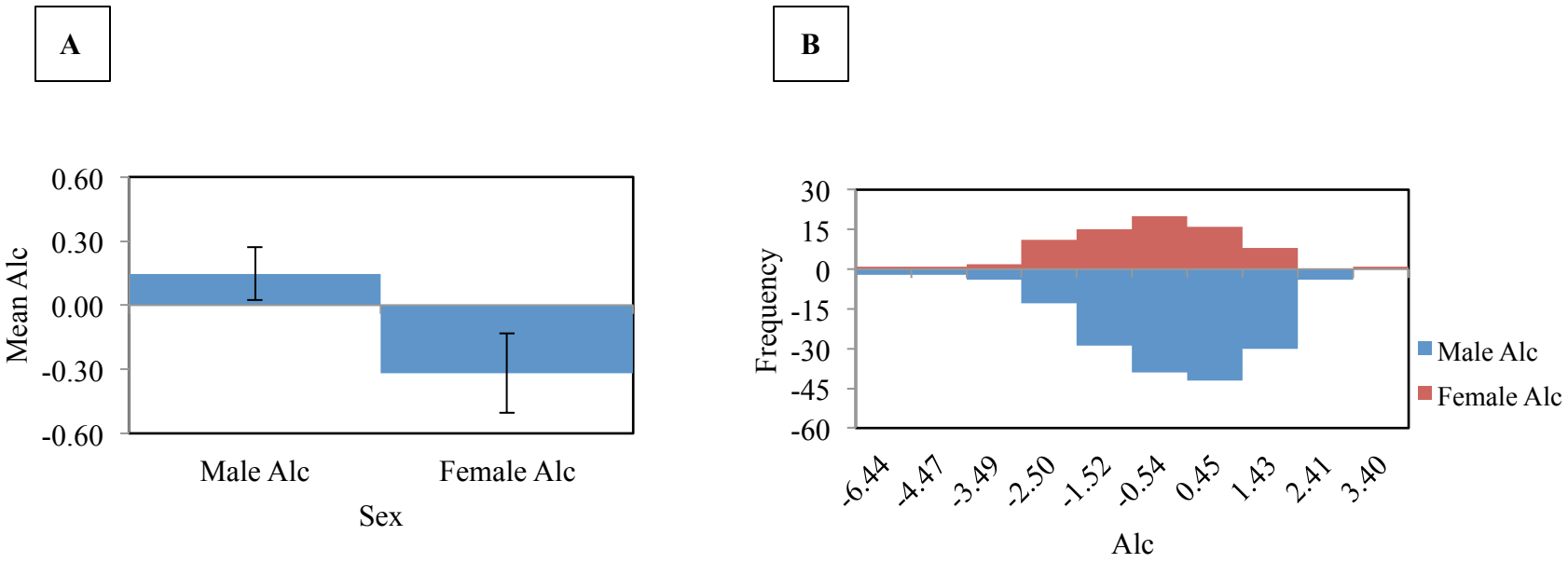


Figure 18. Frequency distributions of the corrected assignment index (AIc) for across the studied populations. AIc values differed among sexes, (A) male vs female mean assignment bias and (B), females having on average negative values.

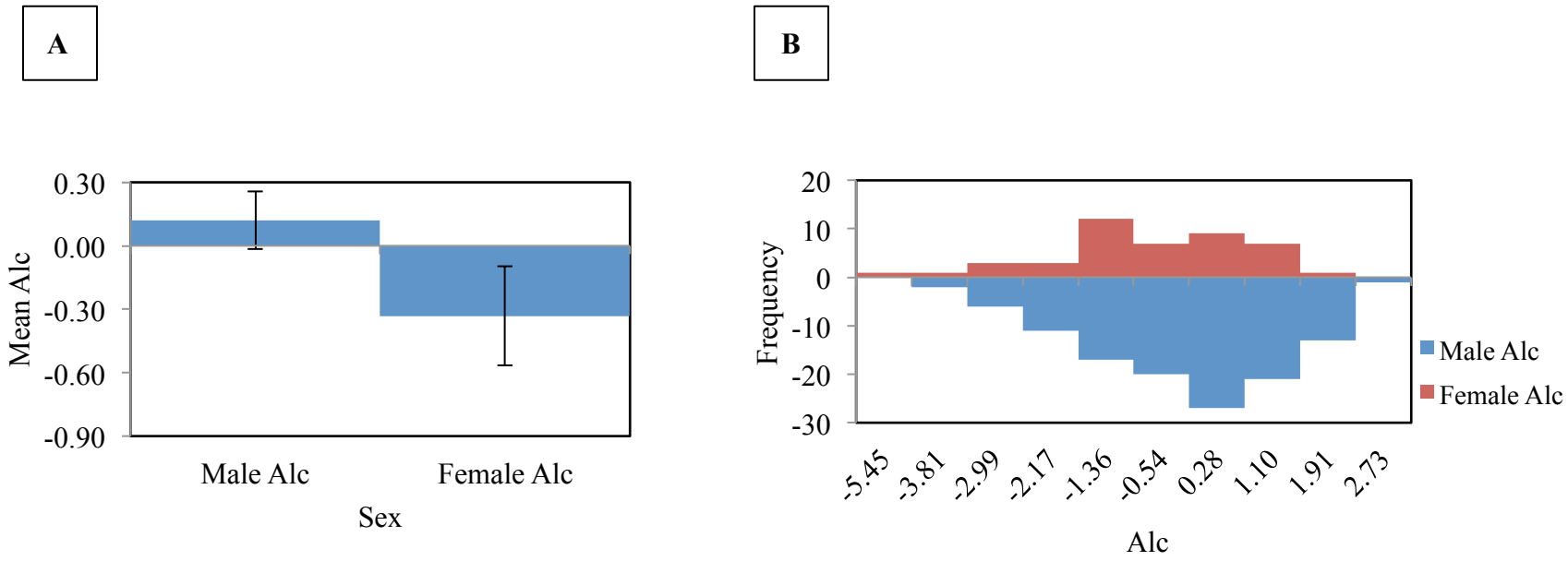


Figure 19. Frequency distributions of the corrected assignment index (AIc) of Toyama Prefecture populations. AIc values differed among sexes, (A) male vs female mean assignment bias and (B), females having on average negative values.

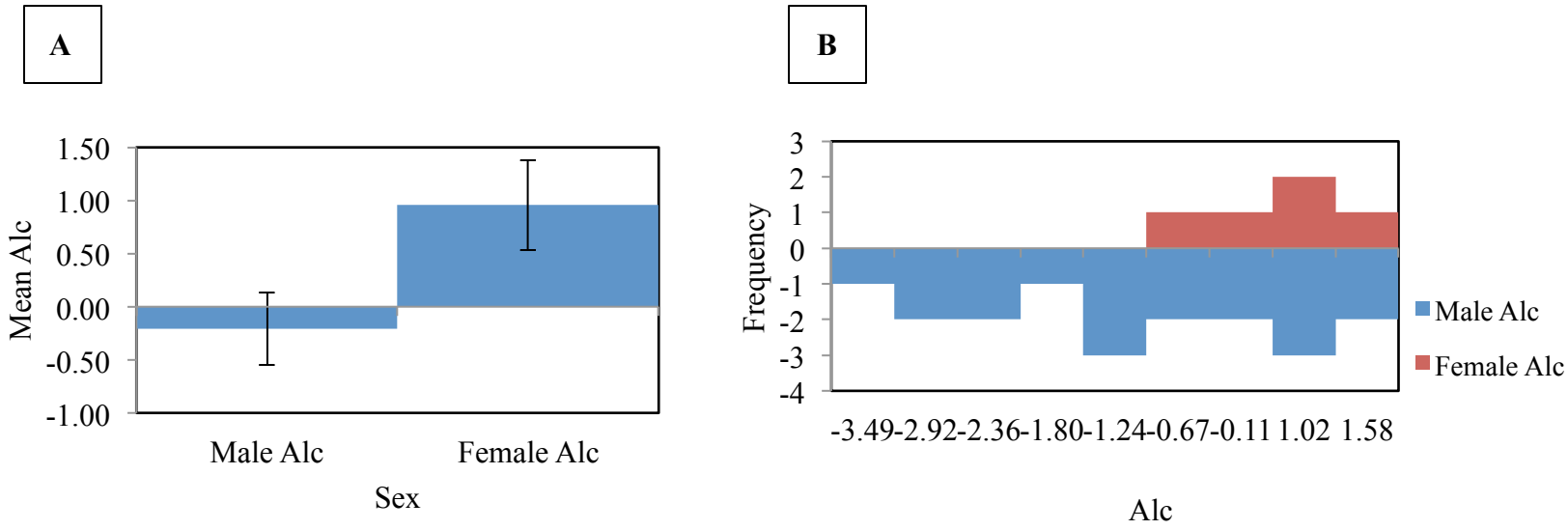


Figure 20. Frequency distributions of the corrected assignment index (AIc) for eastern part of Toyama Prefecture populations. AIc values differed among sexes, (A) male vs female mean assignment bias and (B), males having on average negative values.

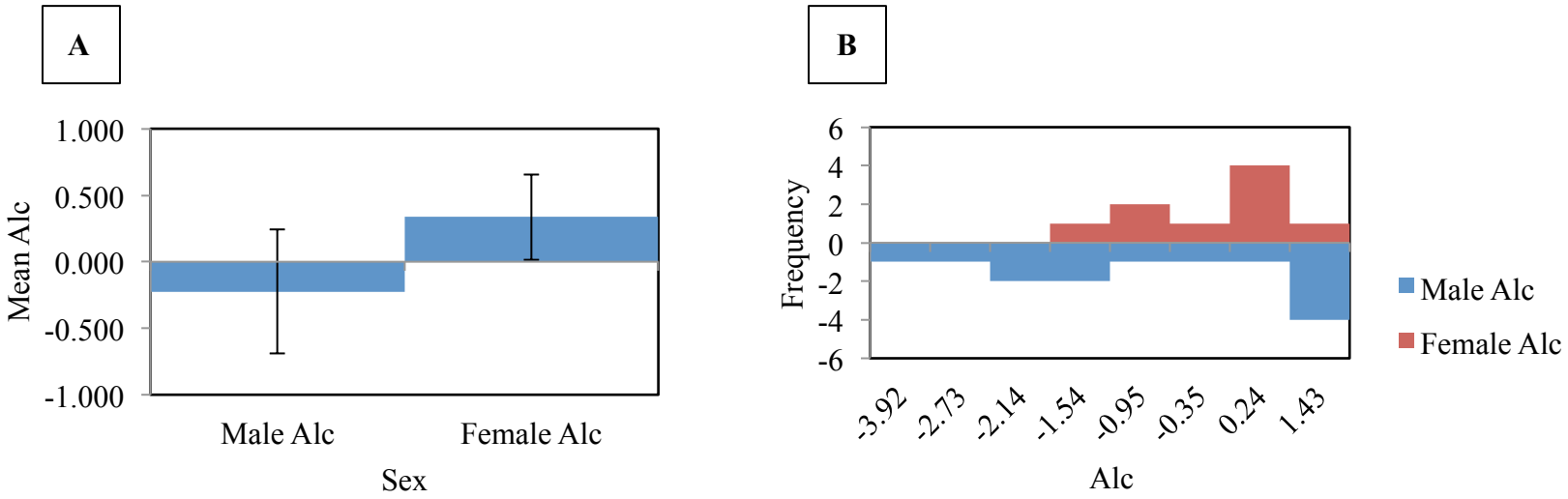


Figure 21. Frequency distributions of the corrected assignment index (AIc) for western part of Toyama Prefecture populations. AIc values differed among sexes, (A) male vs female mean assignment bias and (B), males having on average negative values.

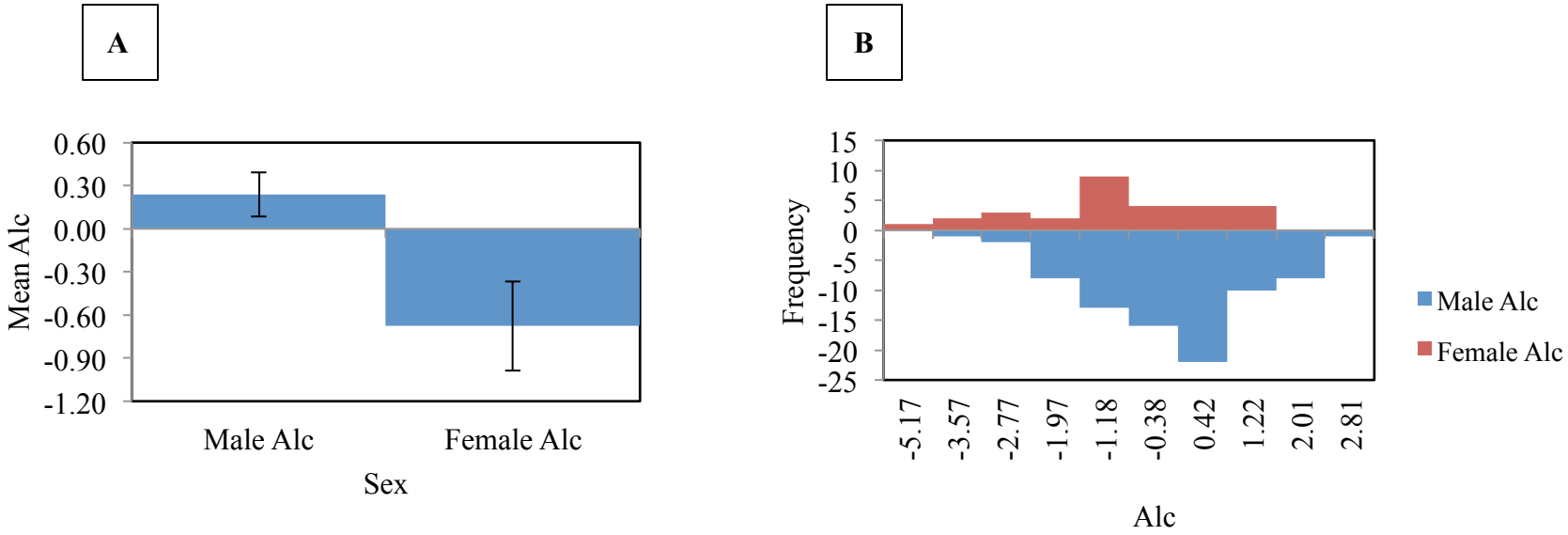
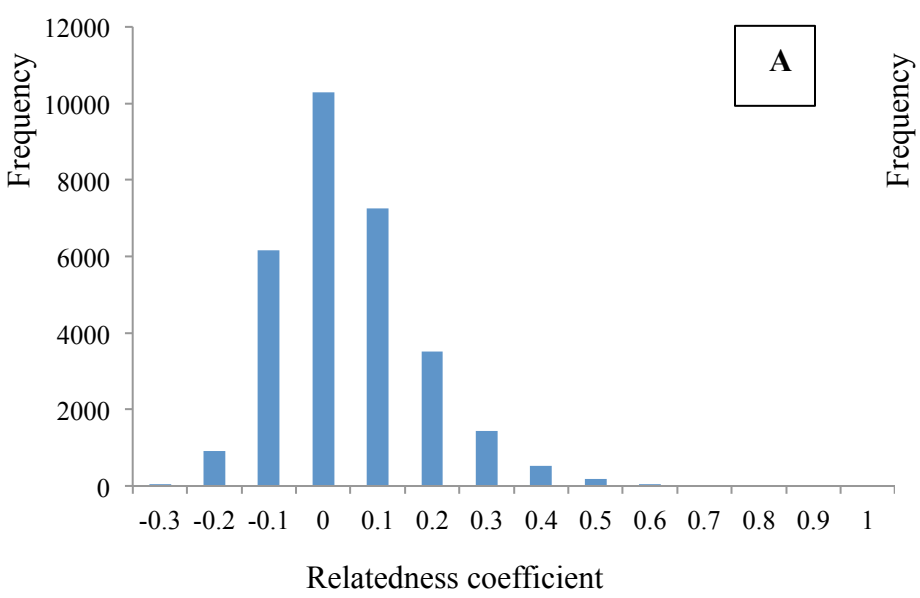
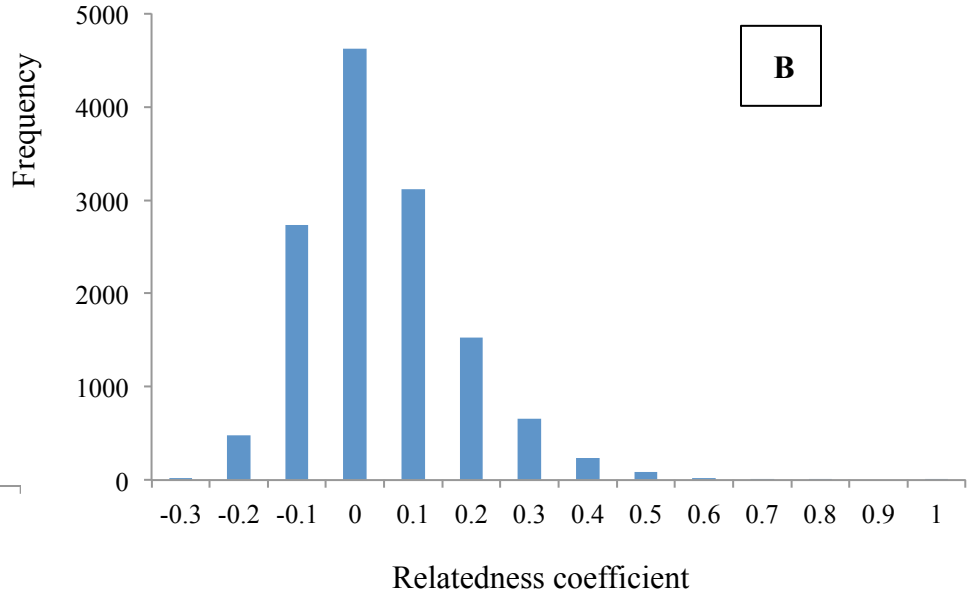


Figure 22. Frequency distributions of the corrected assignment index (AIc) for central part of Toyama Prefecture populations. AIc values differed among sexes, (A) male vs female mean assignment bias and (B), females having on average negative values.





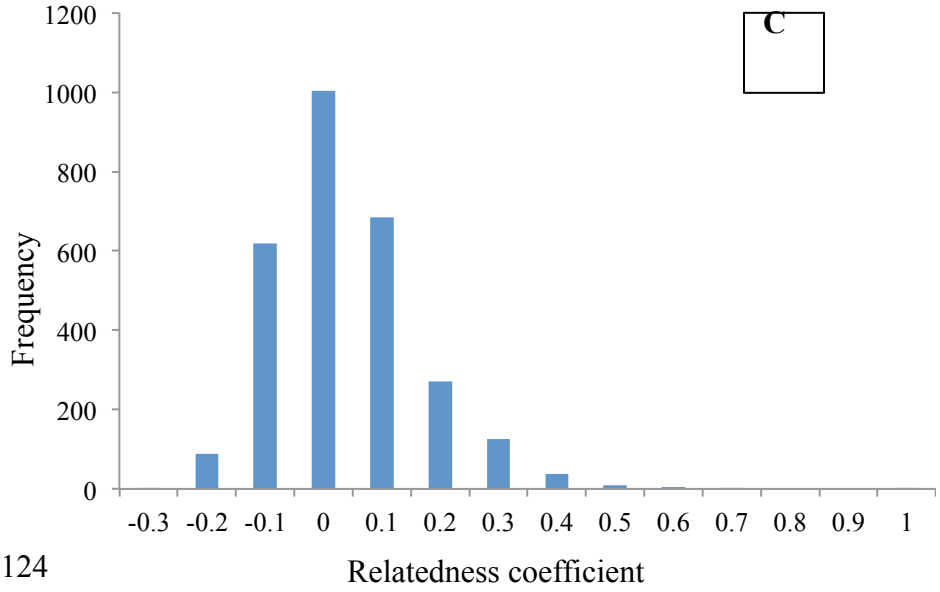
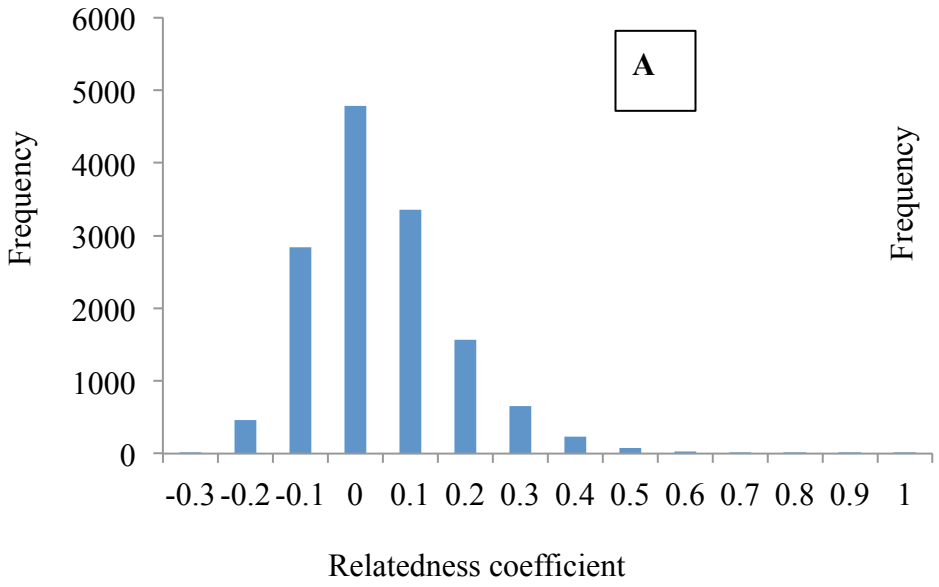
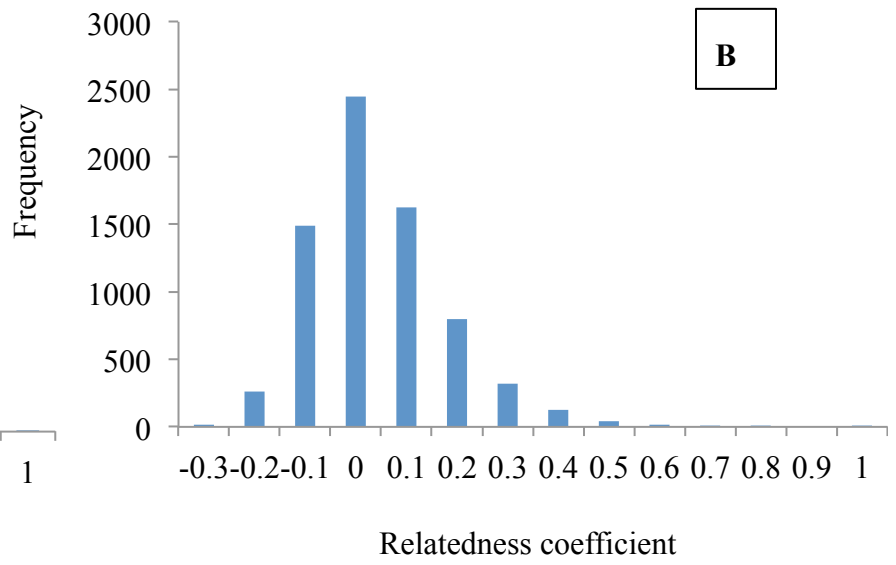


Figure 23. Distribution of pairwise kinship coefficients among sika deer populations, (A) All pair of sampled individuals (B) all pairs of males and (C) all pairs of females respectively.





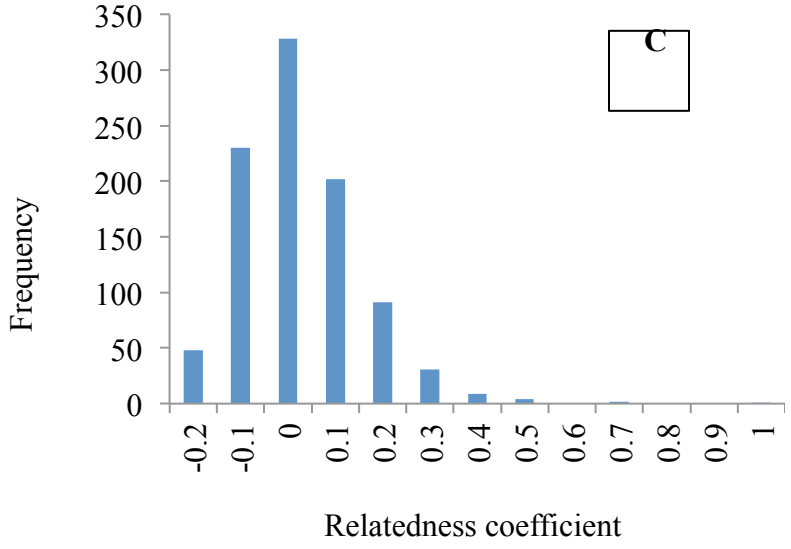


Figure 24. Distribution of pairwise kinship coefficients among sika deer populations only in Toyama Prefecture, (A) All pair of Toyama's individuals (B) all pairs of Toyama's males and (C) all pairs of Toyama's females respectively.