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# Genetic Variation and Population Structure of the Japanese Sika Deer (*Cervus nippon*) in the Tohoku District Based on Mitochondrial D-loop Sequences

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The sika deer (*Cervus nippon*) once inhabited the entire Tohoku District, the northeastern part of the main island of Japan. Currently, they are isolated as three discontinuous populations on Mt. Goyo, the Oshika Peninsula, and Kinkazan Island. To assess the genetic diversity and relationships among the sika deer populations in the Tohoku District, we analyzed the mitochondrial DNA D-loop sequences from 177 individuals. We detected a total of five haplotypes. Three haplotypes were present in the population from Mt. Goyo at a haplotype diversity of  $0.235 \pm 0.061$ , two haplotypes in the population from the Oshika Peninsula at  $0.171 \pm 0.064$ , and only one haplotype was detected in the population from the Kinkazan Island. A significant genetic differentiation was observed among all population pairs. Collectively, our data supports the observed population bottlenecks in the past. Four of the five haplotypes were specific to one of the three populations, whereas only one haplotype was shared between the Mt. Goyo and the Oshika Peninsula populations. This common haplotype may indicate a common ancestral population in the Tohoku District. Conversely, the D-loop haplotypes were completely different among the Kinkazan Island and Oshika Peninsula populations. The lack of a shared haplotype indicates that female gene flow between the two populations is very limited and that the 0.6 km strait acts as a strong barrier.

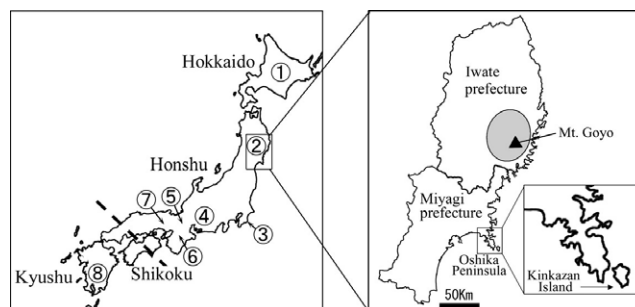
**Key words:** *Cervus nippon*, phylogeny, sika deer, mtDNA, Tohoku

## INTRODUCTION

Sika deer (*Cervus nippon*) are found throughout the Japanese Archipelago from the Hokkaido to the Kyushu Islands, although their habitat has been fragmented by human activities over the last 100 years (Tamate, 2009). In the middle of the 18th century, sika deer were abundant in the Tohoku District, located in the northeastern Honshu Island, and caused terrible damage to agricultural products (Walker, 2001). Their population shrunk dramatically between the late 18th and the early 20th centuries, probably due to overhunting and extraordinarily heavy snowfall (Tokida et al., 1980; Takatsuki, 1992; Walker, 2001). As a result, only three habitats are left in this district: (1) Mt. Goyo and neighboring areas in the Iwate Prefecture, (2) the Oshika Peninsula, and (3) Kinkazan Island in the Miyagi Prefecture (Fig. 1).

Mt. Goyo area is the largest sika deer habitat in the Tohoku District and the number of deer has been increasing for the last three decades. The Oshika Peninsula is located at the southern end of the mountainous region on the Pacific

coast of the Tohoku District more than 90 km from Mt. Goyo area. The population density of sika deer in this peninsula was so scarce that they were not well known before the 1990s (Miyagi Prefecture, 2010). During this period, the veg-



**Fig. 1.** Sika deer habitats in the Tohoku District and the locations of the previously reported haplotypes used in the present study. The encircled numbers from 1 to 8 indicate localities of haplotypes at the prefecture level (1. Hokkaido: Hka and Hkb; 2. Iwate: Gyo1; 3. Chiba: Ama1, Boso1b, and Kmo1; 4. Gifu: Kna1 and Kna2; 5. Shiga: Tga1; 6. Nara: 7Nra1; 7. Hyogo: 7Hyg1, 7hyg2, and Wda1; 8. Miyazaki: Mya1). The dashed line on the left map represents the boundary between the southern and northern mtDNA haplogroups in the Japanese Archipelago (Nabata et al., 1999; Yamada et al., 2006).

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etation in the region was not affected by the deer (Takatsuki and Hirabuki, 1998). However, in the last 20 years the deer population has rapidly increased, and deer herbivory has caused agricultural damage as well as modification of the vegetation (Miyagi Prefecture, 2010). Kinkazan Island is approximately 0.6 km off the coast of the Oshika Peninsula and is 9.6 km<sup>2</sup> in area. The deer on this island have been protected for religious reasons. Hundreds of deer live perennially on Kinkazan Island, and their population size is probably at the upper limit of the carrying capacity for this island (Takatsuki et al., 1994). Swimming deer have occasionally been seen in the strait between Kinkazan Island and the Oshika Peninsula. Therefore, migrations from Kinkazan Island could be responsible for the recent rapid increase of the deer population in the Oshika Peninsula.

The D-loop region of mitochondrial DNA (mtDNA) is a powerful genetic marker and is frequently used for analyzing the population structure of sika deer in Japan (Nagata et al., 1999; Yamada et al., 2006; Yuasa et al., 2007; Yoshio et al., 2008). The maternal lineage of the Japanese sika deer is clearly divided into southern and northern groups (Nagata et al., 1999). Due to the large genetic divergence between the southern and northern mtDNA groups, the colonization of sika deer in the Japanese Archipelago may have occurred from the Asian Continent through the Korean Peninsula and the Sakhalin Peninsula. The present boundary between these two populations is located in the western part of the Honshu and Shikoku Islands (Fig. 1) (Nagata et al., 1999; Yamada et al., 2006). Although the genetic structures of Japanese sika deer populations have been analyzed using D-loop region in several areas, the genetic structure of the populations in the Tohoku District is unknown. The objective of the present study was to survey maternally inherited mtDNA and assess the genetic diversity and relationships among the three sika deer populations in the Tohoku District.

## MATERIALS AND METHODS

### Animal collection and DNA extraction

We collected soft tissue samples (ear auricle, kidney, or skeletal muscle) from 177 sika deer from Mt. Goyo ( $n = 78$ ), the Oshika Peninsula ( $n = 54$ ), and Kinkazan Island ( $n = 45$ ) (Fig. 1). Samples were obtained from individuals culled under deer population control programs of the local governments between 2002 and 2010 on Mt. Goyo and between 2007 and 2010 in the Oshika Peninsula. On Kinkazan Island, ear snip samples were collected from deer that were briefly captured or found dead between 2009 and 2010.

Tissue samples were chopped into small pieces, fixed by soaking in 80% ethanol, and stored at room temperature. Fixed tissues were pretreated twice with 30× volume of 10 mmol/L EDTA solution (pH 8.0). DNA was extracted from the pretreated tissues (20–40 mg) using the QuickGene DNA tissue kit (FUJIFILM, Tokyo, Japan) according to the manufacture's instructions.

### Amplification and sequencing of deer MtDNA D-loop region

A portion of the mtDNA D-loop region was amplified by PCR using the primer pair LD5 (5'-AAGCCATAGCCCCACTATCAA-3') and HD2 (5'-CCTGAAAAAGAACCAGATG-3') (Nagata et al., 1998). PCR reactions were performed using TaKaRa Ex-Taq DNA polymerase (Takara Biotechnology, Shiga, Japan) according to the manufacturer's instructions under the following conditions: denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min. The 600

bp PCR product was purified using a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The purified PCR product was directly sequenced with a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using either LD5 or HD2 or both primers, and then analyzed on an ABI 3100 DNA sequencer (Applied Biosystems).

### Data analysis

The partial D-loop sequences (approximately 600 bp) of sika deer were aligned with previously reported Japanese sika deer D-loop haplotypes (Nagata et al., 1999; Yamada et al., 2006; Yoshio et al., 2008) including Hka (AB012365) and Hkb (AB012364) from Hokkaido; Gyo1 (AB012371) from Iwate; Kmo1 (AB012366), Ama1 (AB012372), Boso1a (AB247654), and Boso1b (AB247654) from Chiba; Kna1 (AB012368) and Kna2 (AB012369) from Gifu; Tga1 (AB012370) from Shiga; 7Nra1 (AB248233) from Nara; Wda1 (AB012373), 7Hyg1 (AB248234), and 7Hyg2 (AB248235) from Hyogo; and Mya1 (AB012375) from Miyazaki Prefecture (Fig. 1). In these haplotypes, only Mya1 belongs to the southern Japan group.

The molecular phylogeny was evaluated using the maximum parsimony (MP), neighbor-joining (NJ), and Bayesian methods with MEGA 4.0 (Tamura et al., 2007) and MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Mya1 was used for outgroup to root the trees. The tree reliability of MP and NJ methods was estimated by bootstrap percentages after 10,000 replicates. The posterior probabilities for the Bayesian consensus tree were calculated using four independent Markov chains run for 500,000 Markov chain Monte Carlo generations, with tree sampling every 100 generations and a burn-in of 1250 trees.

Haplotype diversities for the three sika deer populations in the Tohoku District were estimated using Arlequin v3.1 (Excoffier et al., 2005), based on computations at the haplotype and nucleotide level. We also calculated the  $F_{ST}$  values between pairs of the three populations to gauge genetic differentiation.

## RESULTS

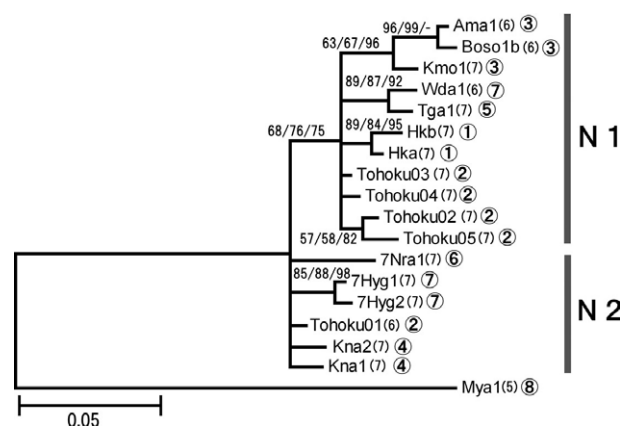
Five haplotypes (Tohoku 01, 02, 03, 04, and 05) in the partial D-loop region were found among 177 sika deer from the Tohoku District. These nucleotide sequences were deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers AB646745–AB646749. A comparison of the five Tohoku haplotypes with previously reported sequences revealed that Tohoku 01 was identical to Gyo1 identified in the Iwate Prefecture (Nagata et al., 1999). However, DNA sequences of the other four Tohoku haplotypes were not identical to any previously reported sequences. The polymorphic sites and frequencies of the Tohoku haplotypes are listed in Table 1. Three haplotypes were found on Mt. Goyo (Tohoku 01, 02, and 03), two in the Oshika Peninsula (Tohoku 03 and 04), and only one haplotype in Kinkazan Island (Tohoku 05) (Table 1). Although Tohoku 03 was shared among Mt. Goyo and the Oshika Peninsula populations, the other four haplotypes were unique to their population.

The D-loop region in Japanese sika deer has a block of tandem repeats comprising approximately 40 bp, and the number of repeated units differs between the two major mtDNA populations, with five or less repeats in the southern group and six or more in the northern group (Nagata et al., 1999). Since the haplotype Tohoku 01 had six repeats and the other four haplotypes had seven, all the Tohoku haplotypes found in this study were classified as northern groups.

Figure 2 shows the phylogenetic tree of the sika deer D-loop sequences constructed using the Bayesian method.

**Table 1.** Comparison of nucleotide sequence of sika deer D-loop haplotypes (polymorphic sites are shown) and numbers of individuals sharing each haplotype in Tohoku District.

Haplotype	Nucleotide position of polymorphic sites											Population		
	1	2	2	2	3	3	3	3	4	4	4	Goyo	Oshika	Kinkazan
	5	5	0	5	7	» <sup>b</sup>	1	2	3	5	1			
	0	3	0	5	3		1	3	3	5	4	(n = 78)	(n = 54)	(n = 45)
Tohoku01	– <sup>a</sup>	C	C	T	39-bp deletion		T	C	T	A	C	68	0	0
Tohoku02	A	C	C	C	(d8 <sup>c</sup> )		C	T	T	G	T	5	0	0
Tohoku03	–	C	C	T	(d8)		C	T	T	A	T	5	49	0
Tohoku04	–	C	C	T	(d8)		C	T	C	A	T	0	5	0
Tohoku05	A	T	T	T	(d8)		C	T	T	G	T	0	0	45

<sup>a</sup>The (–) sign indicates a single base gap.<sup>b</sup>The 39-bp sequence gap probably because of a deletion of a tandem repeat unit was detected on Tohoku01 haplotype.<sup>c</sup>The d8 is a 39-bp tandem repeat unit reported by Nagata et al. (1999).**Fig. 2.** Molecular phylogeny of sika deer D-loop haplotypes using the Bayesian method. The scale shows 0.05 expected changes per site. Mya1 (Southern Japan haplotype) was used as outgroup. The values indicated on the nodes are bootstrap percentages for the maximum parsimony and neighbor-joining methods and percentages of Bayesian posterior probabilities, respectively. Values less than 50% are not shown. Digits from 5 to 7 in parentheses indicate the copy numbers of repeat units in the haplotypes. The encircled numbers from 1 to 8 indicate the localities in Fig. 1.

The phylogenetic trees constructed using the other two methods also had a similar topology. In the phylogenetic tree, the northern Japanese sika deer D-loop sequences were classified roughly into two subgroups, N1 and N2. Tohoku 02, 03, 04, and 05 were clustered within N1 subgroup. However, Tohoku 01 was situated away from the other Tohoku haplotypes and was classified as N2 subgroup.

The estimated haplotype diversities were  $0.235 \pm 0.061$  ( $\pm$ SD) for Mt. Goyo and  $0.171 \pm 0.064$  for the Oshika Peninsula. This index was 0 for the Kinkazan Island because there was only one haplotype. The  $F_{ST}$  values were 0.843 between Mt. Goyo and the Oshika Peninsula, 0.850 between Mt. Goyo and Kinkazan Island, and 0.977 between the Oshika Peninsula and Kinkazan Island.

## DISCUSSION

### Molecular phylogeny of the sika deer D-loop sequences in the Tohoku District

As shown in Fig. 2, the D-loop haplotypes in the north-

ern group of sika deer were classified into N1 and N2 subgroups. Four out of the five Tohoku haplotypes were clustered together with the N1 subgroup, which also included Hokkaido haplotypes. In particular, the Tohoku 03 haplotype was most closely related to Hka, which is the dominant haplotype in Hokkaido (Nabata et al., 2004), and they differed by only two transitions. These results support the hypothesis that the northern lineage of Japanese sika deer in the Honshu Island migrated from the Asian continent by way of Hokkaido (Nagata et al., 1999).

The N1 subgroup comprised 11 haplotypes detected in six prefectures located across broad areas of central and eastern Honshu and Hokkaido, while the N2 sub-

group comprised Tohoku 01 and five haplotypes detected in three prefectures in central Honshu. However, there was no clear boundary between the geographical distributions of N1 and N2 subgroups (Figs. 1, 2). This sporadic distribution of the N1 and N2 subgroup in Honshu suggests that these two haplogroups derived from a common ancestral stock of the northern group rather than from different ancestral populations. It is possible that random genetic drift have caused the loss of either one of the two subgroups in local populations.

Of the five Tohoku haplotypes, only Tohoku 01 harbors six repeats and the others have seven repeats (Fig. 2, Table 1). The coexistence of haplotypes with different number of repeats was also observed in the Chiba and Hyogo Prefectures (Yamada et al., 2006; Yoshio et al., 2008). In the case of the Chiba Prefecture, all the detected haplotypes were clustered together in spite of the difference in the number of repeat units (Fig. 2). In contrast, Wada1 (six repeats) and 7Hyg1 (seven repeats) haplotypes found in Hyogo Prefecture were clearly divided into different subgroups, similar to those found on Mt. Goyo (Fig. 2, Table 1). The pattern of phylogenetic clustering of the haplotypes suggests that changes in repeat numbers between six and seven have occurred multiple times in the northern Japanese sika deer lineages.

### Genetic diversity and relationships among sika deer populations in the Tohoku District

In the Tohoku District, the D-loop haplotype diversities ranged from 0 (Kinkazan Island) to 0.235 (Mt. Goyo). These values were lower than 0.38 in Chiba prefecture (Yoshio et al., 2008), and those of subpopulations in southern Kantoh District in Honshu (Yuasa et al., 2007); 0.532 (Mt. Tanzawa), 0.531 (foot of Mt. Fuji), 0.533 (Mt. Minobu), and 0.353 (Okutama). The haplotype diversities in the Tohoku District were comparable with the lowest level among the previously reported (Yuasa et al., 2007): Mt. Yatsugatake (0.211), Izu Peninsula (0.143), and Hakusyu (0.063). In addition, four of the five Tohoku haplotypes were unique to one of the three populations. The low haplotype diversities within the populations and high  $F_{ST}$  values between populations indicate that the sika deer in the Tohoku District underwent severe bottlenecks and that the three populations were highly fragmented. Our results agree with the historical records of sika deer in the Tohoku District (Takatsuki, 1992; Walker, 2001). Among the three populations in the Tohoku District, haplo-

type diversity on Mt. Goyo ( $0.235 \pm 0.061$ ) was higher compared with the other two and was approximately 37% higher than in the Oshika Peninsula ( $0.171 \pm 0.064$ ). This can be explained by the difference in habitat size because the forested area on Mt. Goyo is much larger compared with the Oshika Peninsula. Tohoku 03 was shared between the Mt. Goyo and Oshika Peninsula populations and its presence suggests that the current sika deer populations on Mt. Goyo and in Oshika Peninsula shared a common ancestral population in the Tohoku District. On Kinkazan Island, only one haplotype was detected in the 45 individuals assayed, which constitutes approximately 10% of the total population. This D-loop haplotype has become fixed in the Kinkazan sika deer, most likely due to a founder effect, which is an inevitable event on a small island. The population size of sika deer on Kinkazan Island has fluctuated greatly depending on snow level (Takatsuki et al., 1994), these bottlenecks may also have contributed to the loss of genetic variation. On the other hand, Goodman et al. (2001) analyzed nine autosomal microsatellite loci and reported that both average heterozygosity (0.54) and allelic diversity (3.22) of the sika deer population on Kinkazan Island were comparable to those observed in other populations with larger habitat size. Because all the nine loci showed heterozygote excess, Goodman et al. (2001) concluded that there was a natural selection to maintain levels of genetic diversity on Kinkazan Island. These contrasting patterns of gene diversity between nuclear and mtDNA marker indicate that combination of genetic markers with different hereditary mode are very important to understand the population structure of sika deer.

The lack of shared haplotypes between the Kinkazan Island and the Oshika Peninsula populations indicates that female gene flow between the two populations is very limited and the 0.6 km strait has acted as a strong barrier. Although male-mediated gene flow could not be denied by present mtDNA analysis, immigration from Kinkazan Island had little effect on the recent population expansion of sika deer in Oshika Peninsula.

In recent years, sika deer populations have been increasing and their range has been expanding in the Tohoku District, most likely due to agricultural and forestry decline. Consequently, sika deer have reemerged in regions where they had not been found for many decades (Miyagi Prefecture, 2010). In this study, we identified four Tohoku haplotypes that were population-specific. These can be used for the identification of local migration of sika deer in the Tohoku District.

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