Microsatellite DNA variations of the sika deer, *Cervus nippon*, in Hokkaido and Chiba

Junko NAGATA¹, Ryuichi MASUDA², Koichi KAJI³, Keiji OCHIAI⁴, Masahiko ASADA⁴ and Michihiro C. YOSHIDA²

¹ Wildlife Management Laboratory, Forestry and Forest Products Research Institute, Ibaraki 305-8687, Japan

Fax. +81-298-73-1543, e-mail. junbe@ffpri.affrc.go.jp

² Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

³ Hokkaido Institute of Environmental Sciences, Sapporo 060-0819, Japan

⁴ Natural History Museum and Institute, Chiba, Chiba 260-0852, Japan

Abstract. To study genetic diversity of populations of the sika deer, *Cervus nippon*, in Hokkaido, polymorphism of three microsatellite loci (OarFCB193, BOVIRBP and INRA040) were examined and compared with that of the population of Chiba Prefecture in the Japanese main island (Honshu). The microsatellite genotype frequency significantly well agreed with the Hardy-Weinberg expectation at each locus of each population except for INRA040 of the Chiba population. Average observed heterozygosity ($H_o = 0.21 \pm 0.11$) of the Hokkaido population was relatively smaller than that of the Chiba population ($H_o = 0.23 \pm 0.09$). Moreover, observed heterozygosities of OarFCB193 and BOVIRBP of the Hokkaido population were lower than that of the Chiba population, and the number of alleles observed at each locus was smaller in the Hokkaido population than in the Chiba population. These results indicate a lower genetic diversity in the Hokkaido population, resulting from their historical bottleneck(s) previously reported. The present study provides information of useful microsatellite markers and gives an insight for better understanding population genetics of the Japanese sika deer.

Key words: bottleneck, *Cervus nippon*, heterozygosity, microsatellite DNA, sika deer.

The Japanese sika deer, *Cervus nippon*, is a large herbivore which is distributed through the Japanese islands. Overpopulation of the sika deer occurring on small islands such as Kinkazan of Miyagi Prefecture, Goto of Nagasaki Prefecture, and Nakanoshima of Hokkaido has caused not only severe damage to island forests, but also suffered from their own malnutrition (Ohtaishi 1986, Kaji *et al.* 1988, Whitehead 1993, Takatsuki 1994, Kaji 1995).

In Hokkaido, which is the northernmost island of Japan, the sika deer population suffered from a crash due to heavy snow falls especially during the winter of the year 1879 in the Meiji era of the Japanese history (Inukai 1952) and then most of local populations were extinct (Hokkaido Government 1986). After then, because Hokkaido Government controlled harvest of the sika deer

for conservation, the population remarkably recovered from a very small number during the last 40 years (Kaji *et al.* 1988, Hokkaido Government 1986, 1994, Kaji 1995). To date, the sika deer has been populated most densely in the central and eastern areas of Hokkaido. Between 1980 and 1993, the annual harvests reported by regular hunters increased from approximately 3,500 to 26,000 animals in Hokkaido (Kaji 1995). Recently, increasing of damage to agricultural crops, plantations, natural forests and traffic accidents brought by the sika deer has become the severest social problems in Hokkaido. Therefore, it is urgently needed to control and manage the sika deer population (Hokkaido Government 1994).

On the other hand the Hokkaido population of the sika deer is likely to have reduced a level of genetic variation through the bottleneck(s) despite a large population size, because genetic drift by bottleneck is reported to generally drive neutral genetic variability to a very low level (Nei *et al.* 1975, Chakraborty and Nei 1977, Viard *et al.* 1996). Such a decrease of genetic variability can lead to inbreeding depression, loss of evolutionary flexibility and greater susceptibility to some disease resulting in possible extinction of the population (O'Brien and Evermann 1988). The harvest without considering genetic diversity could bring depression of viable potential in the population. However, because scientifically definitive data on the sika deer genetic diversity in Hokkaido was lacking, it was very necessary and urgent to investigate genetic variation in the population for control and conservation.

Microsatellite DNA is known as a highly polymorphic and neutral Mendelian marker of nuclear genome (Queller *et al.* 1993). This kind of DNA region includes tandem repeats of 1-5 base units, and alleles are defined as the number of polymorphic repeats. Such hypervariable numbers of repeats are used extensively for identifying individuals and paternity as well as for population genetic studies (Queller *et al.* 1993, Abernethy 1994, Pépin *et al.* 1995, Viard *et al.* 1996).

The objective of the present study is to clarify genetic variation of such microsatellites in the sika deer population of Hokkaido and then to understand population diversity for conservation and management. We here present alleles and their frequencies obtained at their microsatellite loci and discuss genetic diversity and characteristics of the Hokkaido population and Chiba population in the middle of Honshu, the mainland of Japan.

MATERIALS AND METHODS

1. Animal collections and DNA extraction

A total of 110 sika deer samples (blood, muscle or liver tissues) were obtained in Hokkaido from 1991 to 1996 in cooperation with scientists, town offices and hunters. Especially, Nature Conservation Department of Hokkaido Government kindly supported systematic sampling collection. Sampling was done as widely as possible in various areas of Hokkaido, and as to avoid an inclination of areas. No animals with known familial relationships were

used for analysis. Thirteen liver samples of the Chiba population were used as a control population.

From whole blood or tissues, total DNA was extracted using the phenol/proteinase K/sodium dodecyl sulfate (SDS) method of Sambrook *et al.* (1989) with a slight modification (Masuda and Yoshida 1994). Using a small glass homogenizer, a small piece of tissue (approximately $2 \times 2 \times 2$ mm) was homogenized in 500 μ l of STE buffer (100 M NaCl/10 mM Tris, pH7.5/1 mM EDTA) containing a final concentration of 0.5% SDS and 5 μ g/ml of proteinase K. The homogenate was incubated at 37°C overnight. In case of blood, 100 μ l was treated in 400 μ l of STE buffer. DNA was purified by extracting at least twice with the equal volume of phenol/chloroform (1 : 1) and once with chloroform/isoamyl alcohol (24 : 1). These procedures were done in 1.5 ml microcentrifuge tubes. Extract without any animal tissue was used as a negative control in the following polymerase chain reaction (PCR) amplification.

2. Microsatellite analysis

Microsatellite loci established from the bovine or sheep were used for analysis: BOVIRBP, OarFCB193 (Abernethy 1994), and INRA040 (Vaiman *et al.* 1994) (Table 1). PCR amplification of microsatellites was performed using a PCR reagent kit (GibcoBRL) according to the manufacturer's instruction. One microliter of the DNA extract was subjected to PCR amplification in a total volume of 25 μ l of a reaction mixture including 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, each dNTP at 0.2 mM, 1.25 U of Taq DNA polymerase and each Texas Red-labeled primer at 0.5 μ M. After the first step of denaturing at 94°C for 5 min, 26–30 cycles of amplification were realized (94°C for 15 sec, 55°C for 15 sec, 72°C for 20 sec) followed by reaction completion at 72°C for 10 min. PCR products (0.2–1.0 μ l) were then loaded with 2 μ l of the loading buffer on an 6–8% Long Ranger gel (FMC BioProducts) and run for 10 hours using DNA Sequencer SQ-5500 (Hitachi).

Molecular sizes of alleles at each locus were identified from difference of electrophoretic mobility of PCR product bands using the computer program FRAGLYS ver. 2 (Hitachi). To assess genetic variability within each population, expected heterozygosity (*he*) was calculated for each locus using the

Table 1. PCR primer sequences for microsatellite DNA analysis in the present study.

Microsatellite locus	Primer sequence (5'–3')	References
BOVIRBP	TGTATGATCACCTTCTATGCTTC GCTTTAGGTAATCATCAGATAGC	Abernethy (1994)
OarFCB193	TTCATCTCAGACTGGGATTGAGAAAGGC GCTTGGAATAACCCTCCTGCATCCC	Abernethy (1994)
INRA040	TCAGTCTGGAGGAGAGAAAAC CTCTGCCCTGGGGATGATTG	Vaiman <i>et al.</i> (1994)

formula $h_e = 1 - \sum X_i^2$, where X_i is the i th allele frequency of the locus in the population. An average of expected heterozygosity (H_e) was calculated using the formula $H_e = \sum h_{e_j} / r$, where h_{e_j} is heterozygosity of the j th locus and r is the number of analyzed loci. Microsatellites genotype frequencies were tested against the Hardy-Weinberg's expectation for each locus in the population using the computer program Arlequin ver. 1.0 (Schneider *et al.* 1997). Genetic differentiation between populations was estimated with Weir and Cockerham's (1984) F_{st} value. The significance of F_{st} value was tested using the permutation procedure in Arlequin ver. 1.0.

RESULTS

At two microsatellite loci (OarFCB193 and BOVIRBP), clear bands of 100–150 base-pairs (bp) were identified as alleles. The INRA040 locus provided prominent bands (188–240 bp) with a few weak bands (Table 2). Animals from the Hokkaido ($n=93$ for OarFCB193, $n=108$ for BOVIRBP, and $n=100$ for INRA040) and the Chiba ($n=13$ for all loci) populations were analyzed to estimate allele frequencies for each microsatellite locus. At OarFCB193 locus, two alleles in Hokkaido and four alleles in Chiba were found. At INRA040 locus, two alleles in Hokkaido and five alleles in Chiba were found (Table 2),

Table 2. Microsatellite variation in the Hokkaido and Chiba populations.

Locus			Hokkaido	Chiba
OarFCB193	No. individuals		93	13
	Allele* & frequency	130	0.87	0.08
		128	0	0.08
		123	0	0.08
		109	0.13	0.78
	h_o		0.24	0.39
	h_e		0.23	0.39
BOVIRBP	No. individuals		108	13
	Allele* & frequency	144	1	0.96
		140	0	0.04
	h_o		0	0.08
	h_e		0	0.07
INRA040	No. individuals		100	13
	Allele* & frequency	240	0	0.04
		238	0	0.08
		202	0.32	0.81
		190	0	0.04
		188	0.68	0.04
	h_o		0.39	0.23
	h_e		0.44	0.38
Average over 3 loci $\pm$ SE	No. individuals		100.33 $\pm$ 4.33	13.00 $\pm$ 0.00
	A		1.67 $\pm$ 0.33	3.67 $\pm$ 0.88
	H_o		0.21 $\pm$ 0.11	0.23 $\pm$ 0.09
	H_e		0.22 $\pm$ 0.13	0.28 $\pm$ 0.11

*Molecular sizes (bases) refer to allele name

h_o and H_o : observed heterozygosity.

h_e and H_e : expected heterozygosity.

while the BOVIRBP locus showed monomorphism in the Hokkaido population and two alleles in the Chiba population (Table 2). Between Hokkaido and Chiba populations there were some common alleles: two alleles at OarFCB193, one allele at BOVIRBP and two alleles at INRA040.

Observed heterozygosities (h_o) of OarFCB193, BOVIRBP and INRA040 were 0.24, 0 and 0.39, respectively, in the Hokkaido population, and 0.39, 0.08 and 0.23, respectively, in the Chiba population. The average observed heterozygosity (H_o) was 0.21 ± 0.11 for the Hokkaido population and 0.23 ± 0.09 for the Chiba population (Table 2). The microsatellite genotype frequency significantly agreed with the Hardy-Weinberg expectation in each locus in the population except at INRA040 in the Chiba population ($p=0.06$) (Table 3). The difference between Hokkaido and Chiba was statistically significant (Fst value = 0.072, $p=0.03$ in permutation tests).

DISCUSSION

Microsatellite analysis has more advantage than allozyme analysis and multilocus DNA fingerprinting, because of higher polymorphism and easier genotyping from a small amount of DNA. Nozawa *et al.* (1985) analyzed 28 allozyme loci from 20 individuals in the Hokkaido sika deer population and reported only two polymorphic loci with low variability: the average heterozygosity was 0.0158. By contrast, our results of microsatellite analysis showed much higher values ($H_o = 0.21 \pm 0.11$) than their allozyme data (Table 2).

Abernethy (1994) indicated that the sika deer population introduced to Scotland showed monomorphism at the BOVIRBP locus and two alleles for OarFCB193. In the present study, the BOVIRBP locus of the Hokkaido population was also monomorphic and showed a low value (0.08) of heterozygosity in the Chiba population. These data suggest the BOVIRBP locus is not so hypervariable in the sika deer. Pépin *et al.* (1995) reported that the number of alleles for INRA040 were nine in the goat ($n=60$) and 44 in the cattle ($n>36$). Our results revealed that the sika deer in Japanese islands have at least four alleles for OarFCB193, five alleles for INRA040 and two alleles for BOVIRBP. In the present study, two other loci (INRA003 and INRA023) could not be PCR-amplified with primers reported by Pépin *et al.* (1995).

The genotype frequency significantly well agreed with Hardy-Weinberg expectations at all loci in both populations except for the INRA040 locus in the Chiba population (Table 3). The observed heterozygosities of OarFCB193 and BOVIRBP were same level as the expected heterozygosity, while the observed heterozygosity of INRA040 was lower than the expected heterozygosity (Table 2). Some heterozygotes such as null/normal genotypes, however, might have been counted as homozygotes of normal alleles, because it is difficult to distinguish homozygotes of normal alleles from null/normal genotypes with a single band of PCR product. Our results show that null allele may exist at INRA040 locus. Jarne and Lagoda (1996) suggested that null alleles may disturb population studies leading to underestimate of heterozygosity. The

INRA040 locus in the present study is likely in that case. From this reason, we compared heterozygosities of OarFCB193 and BOVIRBP between Hokkaido and Chiba. At both loci, heterozygosities of the Hokkaido population were less than those of the Chiba population (Table 2). Besides that, allele numbers at each locus in the Hokkaido population were much smaller than those of the Chiba population (Table 2). These results suggest a higher degree of homogeneity in the Hokkaido population. This supports a low genetic variety of mitochondrial DNA in the Hokkaido population, shown by our previous analysis (Nagata *et al.* 1998). Some bottlenecks of the sika deer recorded in Hokkaido history (Inukai 1952) could have induced such a low genetic diversity in the population.

The present results provide invaluable information for understanding genetic variety and history of the Hokkaido sika deer population, leading to the range of application of molecular genetics to conservation biology of the sika deer in Japan.

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