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Authors: Tamate, Hidetoshi B., Shibata, Kiyotaka, Tsuchiya, Takeshi, and Ohtaishi, Noriyuki

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Assessment of Genetic Variations within Populations of Sika Deer in Japan by Analysis of Randomly Amplified Polymorphic DNA (RAPD)

HIDETOSHI B. TAMATE[1], KIYOTAKA SHIBATA[1], TAKESHI TSUCHIYA[2] and NORIYUKI OHTAISHI[3]

[1]*Departments of Biotechnology and* [2]*Basic Sciences, Senshu University of Ishinomaki, Ishinomaki 986-80, and* [3]*Department of Environmental Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060, Japan*

ABSTRACT—Random amplified polymorphic DNA (RAPD) was exploited as a genetic marker to assess the level of genetic variation in populations of Sika deer, *Cervus nippon*, in Japan. DNA samples were collected from three local populations in Japan, namely, Kinkazan, Goyozan and Ashoro populations. Four arbitrary primers, when used individually, amplified an average of five RAPD fragments in the polymerase chain reaction (PCR). The number of polymorphic bands was scored to calculate band-sharing coefficients within populations. Average band-sharing coefficients revealed a higher degree of homogeneity in the Kinkazan population. Samples collected from larger populations, namely, Ashoro and Goyozan, revealed greater polymorphism than samples from the Kinkazan deer. Our data suggest that RAPD is useful as a marker for detecting genetic variations in populations of Sika deer with reduced levels of genetic diversity.

INTRODUCTION

The habitats of Sika deer on the Japanese islands have become fragmented over the past decades [15]. As a consequence, local populations of deer are now isolated and confined to small areas. Demographic studies have shown that sizes of some populations are very small, being the order of one hundred or less. Since gene flow between populations is non-existent, loss of genetic variability, which sometimes leads to local extinction, has been anticipated in distinct subspecies of these deer. Although genetic analysis of wild populations is prerequisite for conservation of this species, little is known about the current genetic status of Sika deer in Japan.

One of the major problems in genetic studies of Sika deer is the lack of appropriate markers. The most commonly used genetic markers in population studies are isoenzymes, restriction fragment length polymorphism (RFLPs) and mitochondrial DNA haplotypes. However, only a few intrapopulational variations can be detected in Sika deer with those markers [4, 8, 16]. Tamate and Tsuchiya have analyzed RFLPs of deer mitochondrial DNA and found very few variations among samples from Kinkazan, Goyozan and Ashoro populations [16]. Nagata and Masuda reported that Shiretoko (Hokkaido) and Goyozan deer shared identical sequences for both mitochondrial cytochrome b gene and 12S rRNA gene [8]. Those markers are, therefore, not applicable to our analysis. For this reason, we have chosen RAPD as an alternative marker in an attempt to assess the level of genetic variability within populations.

Genetic analysis using RAPD has several advantages over analyses with other genetic markers: (i) analysis of RAPD requires only a small amount of DNA, usually 10 ng or so; (ii) large numbers of samples can be analyzed simultaneously; (iii) large numbers of anonymous markers distributed over the entire complement of chromosomes can be surveyed; and (iv) divergence of even a fraction of a percent among genomes can be detected [17–19]. While several problems, both technical and theoretical, still remain in RAPD analysis, this technique has been successfully exploited in studies of genetic variations [5], pedigrees [12], and structures of populations [2].

In the present study, we analyzed RAPD in an attempt to determine the extent of genetic variations in three deer populations of various sizes. The aim of this study was to determine the usefulness of RAPD in the genetic analysis of deer populations and, also, to test the hypothesis that small populations have a reduced level of genetic variation as compared with larger populations.

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MATERIALS AND METHODS

The sites of collection are shown in Figure 1. We collected samples from 105 individual deer from the following sites in Japan: Goyozan (Mt. Goyo), Iwate Prefecture (n=18); Kinkazan, Miyagi Pref. (n=69); and Ashoro, Hokkaido (n=18). Blood was taken from live-captured animals and was mixed immediately with anticoagulant as described elsewhere [16]. During collection of samples, we took special care not to injure the animals in any way, as described previously [6]. Livers and muscles were also collected from hunter-killed deer and these were sent to our laboratory without freezing.

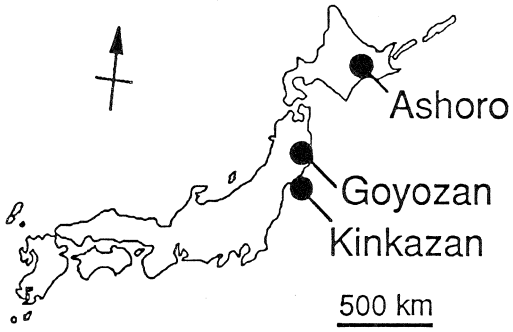

FIG. 1. Map showing locations of the populations of Sika deer that were sampled. Closed circles on the map indicate the locations of collection sites.

To remove impurities that might interfere with the polymerase chain reaction (PCR), we prepared template DNA by a two-step extraction method, as follows. Blood samples were first centrifuged to remove serum and then cells were homogenized in extraction buffer [11] that contained 0.5% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.5) and 100 mM EDTA. Liver and muscles were homogenized in TNM buffer, which contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1.5 mM $MgCl_2$. A stock solution of EDTA (pH 8.0) was then added to each homogenate to a final concentration of 1 mM. After homogenization, proteinase K was added to all samples at 50 μg/ml. The homogenates were incubated at 56°C for 1 hr and extracted twice with an equal volume of a mixture of phenol and chloroform (1:1, v/v), and then with chloroform. DNA was precipitated in ethanol, resuspended in 1 ml of the extraction buffer and incubated with 30 μg of DNase-free RNase A for 1 hr at 37°C. Each sample was extracted again with the mixture of phenol and chloroform, precipitated in ethanol, and resuspended in sterilized water. We determined the amount of resuspended DNA by agarose gel electrophoresis and with a spectrophotometer, and we adjusted the final concentration of DNA in all samples to 1 ng/μl.

Arbitrarily primed PCR [17] was carried out in 50-μl aliquots of a reaction mixture that contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% (w/v) gelatin, 3 mM $MgCl_2$, 0.1% Triton X-100, 0.5 units of Taq polymerase (Perkin-Elmer Cetus, USA), 200 pmol of primer and 10 ng of the template DNA. Sets of arbitrary primers were purchased from Bex, Tokyo, Japan. The nucleotide sequences of the primers are listed in Table 1. Amplification was performed in a thermal cycler (Perkin-Elmer Cetus). The reaction mixture was first heated to 95°C for 2 min, then it was subjected to 40 cycles of 95°C for 30 s, 42°C for 2 min and 72°C for 2 min. A final incubation at 72°C for 5 min was included for complete extension of the amplified products.

Aliquots of each amplified sample were subjected to electrophoresis on a 0.8% agarose gel (type II medium EEO; Sigma, St.Louis, MO, USA) in TAE buffer as described previously [11]. The gels were stained with ethidium bromide and photographed. Sizes of amplified fragments were estimated by comparison of their mobilities with those of standard DNA markers: a *Hin*d III digest of bacteriophage λDNA and a 100 base-pair ladder (Pharmacia, Sweden).

One of the major difficulties in RAPD analysis is the reproducibility of banding patterns [19]. A presence of faint bands is also troublesome. Therefore, we took several precautions to eliminate such ambiguities. The quantity and quality of template DNAs used in PCR were carefully controlled. All pipetting procedures were performed with filtered pipette tips, in a class II safety cabinet (Hitachi, Japan). We repeated PCR at least twice with the same template DNA, and scored only reproducible RAPDs of length between 0.3 kb and 2 kb because the PCR was found to be most reliable in this range.

To assess genetic variability within populations, we calculated the band-sharing coefficient (BS) between individuals using the formula $BS=2n_{xy}/(n_x+n_y)$, where n_x and n_y are numbers of DNA fragments amplified in individuals X and Y, respectively, and n_{xy} is the number of DNA fragments shared by them [5]. An average band-sharing coefficient (ABS) was calculated using the formula $ABS=\Sigma BS/S$, where S is the number of pairwise comparisons between individuals in a population. For a sample population that consists of n individuals, $S=n(n-1)/2$. For example, S=153 for the Goyozan population in which 18 deer were sampled. The band-sharing data obtained for each primer were ranked and subjected to nonparametric analysis to test the effect of population in determining the band-sharing levels.

RESULTS AND DISCUSSION

To obtain distinct banding patterns, we tested 70 RAPD primers in an initial screening with PCR by using a template DNA from a single animal. Of those, 34 primers gave clear RAPD patterns that corresponded to products of PCR that ranged in length from 0.2 to 2.5 kb, while others produced smears or no bands at all. The second screening of the primers was done by PCR in which four DNA samples from each population were used as templates; we tested 26 primers from the initial screening and found that twenty-one of them gave polymorphic DNA bands, while others resulted in monomorphic patterns (Table 1). Since monomorphic DNA bands were not informative in comparing genetic variabilities between populations, we used primers that yielded polymorphic DNAs within populations for further analysis. Four 12mers (09A, 52A, 06B and 60B) were chosen arbitrarily for this purpose. Figure 2 shows an example of RAPD of the agarose gel electrophoresis. An average of five bands of RAPD was generated with the chosen primers in each reaction. With all four primers, samples from Kinkazan deer showed very few variations in RAPD.

Values of ABS obtained for each populations with the four different primers are listed in Table 2. Statistical analy-

TABLE 1. Nucleotide sequences of random primers

primer	sequence	RAPD*
00A	ATCAgCgCACCA	M
02B	AgCCTTACggCA	P
05A	CCgCAgTTAgAT	nt
06A	ACTggCCgAggg	P
06B	CTCAAgCgTACA	P
08A	TTCggACgAATA	P
08B	gCCTTCgTTACg	P
09A	AgAATTggACgA	P
42B	gCTATggCAACg	nt
44B	CCTTggAACTCg	M
48B	AgAggTgTAAAT	P
49B	TTgCATAATCgT	P
50A	ATTggTgCAgAA	M
52A	AgAgACATAgTT	P
54A	AAggCgTgTTTA	P
55A	TACgCCggAATA	P
56A	AACATCTCCggg	P
57A	ATCATTggCgAA	P
58A	gTCATgCCTggA	M
60B	AgCCCTTATTTA	P
61B	CCAggCggTggT	nt
62B	ACTCCAAATgTg	P
64B	gAgACTATgAAA	P
67B	gCCCTTTTggAC	P
90A	ACTgAgCAACAA	nt
92A	TTCgAggATCgA	P
94A	TCTATggACCCT	nt
95A	TACgTggTAACA	P
97A	gTgTggAAgCCA	nt
99A	gCggTCAgCACA	M
101R	CgCgCCggTT	nt
104R	gCTggTggTT	nt
105R	gAgggTggT	P
108R	TTCgAgCCAg	P

*RAPD patterns were polymorphic (p), monomorphic (M), or not tested (nt) in the second screening.

sis revealed significant differences in the ABS levels among the populations ($P<0.05$) for each of the primers. The mean ABS was 0.97 for Kinkazan deer. By contrast, the mean values of ABS for Ashoro and Goyozan deer were 0.89 and 0.84, respectively. The data suggests higher degree of homogeneity among individuals in the Kinkazan population.

In the present study, we chose the Kinkazan deer as a model of a small, isolated population. It should be noted that isolation of the Kinkazan deer have occured very recently as a result of habitat fragmentation which was caused by human activities during the 19th and 20th centuries. In the winter of 1988, the deer on Kinkazan Island experienced a crash and the number of the deer dropped by about 50% [13]. Although recovery resulted in about 550 deer by 1994 (Takatsuki *et al.* personal communication), the genetic diversity of this population can be assumed to have been severely reduced. This assumption was confirmed by our RAPD data: the Kinkazan population had a lower level of genetic variation than the other two local populations.

The populations of Goyozan and Ashoro deer are much larger than that of Kinkazan deer. The number of deer in Goyozan has been estimated to be several thousand or more [14]. The deer in Ashoro belong to a very large population that is spread over the eastern part of Hokkaido Island. Although there are no definitive data for Hokkaido deer, the size of this population is assumed to be at least 230,000. The RAPD data revealed that Ashoro and Goyozan deer are genotypically more diverse than the deer in the Kinkazan population. Our finding is consistent with the results of previous studies [1, 9, 10] that showed a correlation between population size and genetic variability: larger populations have greater heterozygosity.

It should be noted that genetic data obtained from wild populations may vary according to sampling patterns, sizes of sampling units and intensity of sampling [10]. We collected samples with different sampling patterns for each population. In Kinkazan Island, about 15% of the entire population was sampled within a 4-day field study. Ashoro deer were sampled at a single hunting post during a month in the hunting season. Goyozan deer, by contrast, were sampled at various sites in the Goyozan area over the course of two years. If deer in Ashoro and Goyozan constitute a metapopulation, as proposed elsewhere [7, 9], different sampling patterns would be expected to result in biased genetic data. We cannot exclude such a possibility because the sizes of samples were small in the present study. Studies of intrapopulational structures in deer populations are in progress to clarify this problem.

We did not find population- or breed-specific RAPD bands, as was reported in previous studies [5, 12]. The absence of such population-specific RAPD suggests that the three populations are still genotypically close even though they are geographically separate at the present time.

Our study demonstrates that RAPD can provide good estimates of genetic variations in population studies. However, the theoretical basis for the analysis of RAPD data has not been established. Clark and Lanigan [3] have proposed a method for estimating the degree of sequence divergence between populations using RAPD data. To apply their method, several criteria must be fulfilled: allelism of RAPD bands must be determined and single substitutions of nucleotides should result in loss of bands. These criteria are, however, difficult to apply in many cases including the present case. Further statistical studies that provide a theoretical background for RAPD analysis are necessary.

Decreased genetic variability can lead to inbreeding depression, a loss of evolutionary flexibility and greater susceptibility to diseases. Assessment of genetic variation is, therefore, an important step in conservation of endangered species. However, it is often difficult to find appropriate genetic markers for small populations with re-

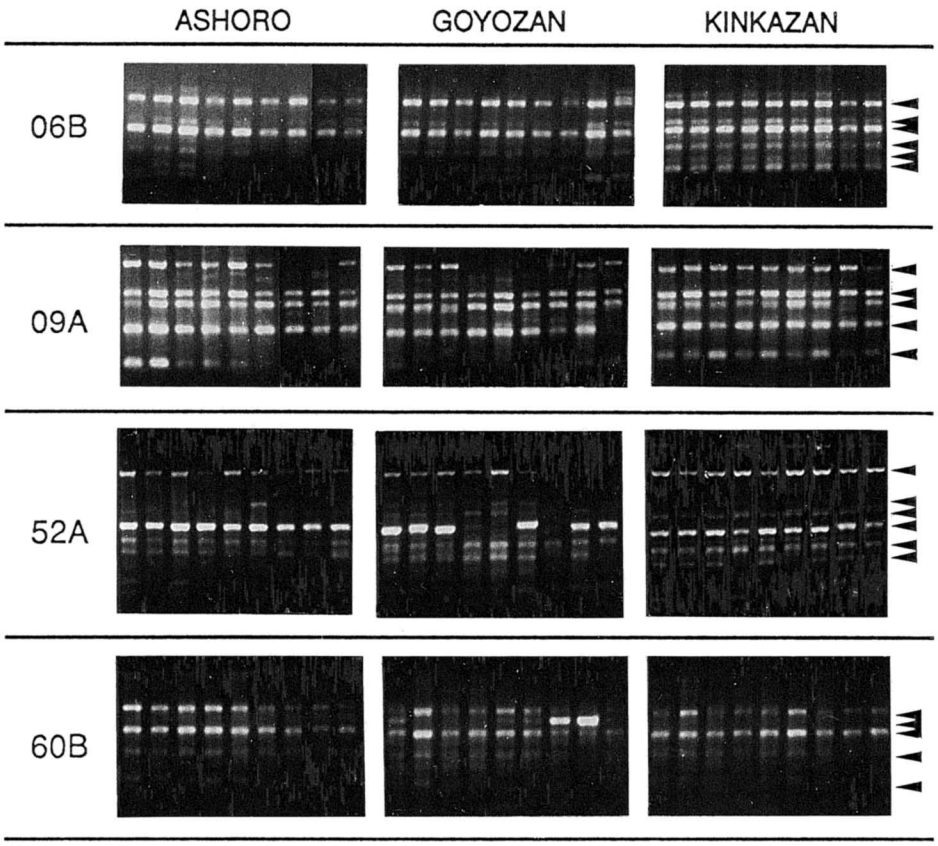

FIG. 2. DNA polymorphism among the Ashoro, Goyozan and Kinkazan populations of Sika deer, as revealed by RAPD. Photographs are arranged in numerical order according to primer numbers (06B, 09A, 52A and 60B). For each population, RAPD patterns for nine samples are shown as examples. Arrowheads indicate the bands scored in this study.

TABLE 2. Average band-sharing coefficients within three populations of Sika deer

Primer	Locality		
	Ashoro	Goyozan	Kinkazan
09A	0.86	0.84	0.98
52A	0.87	0.78	0.91
06B	0.86	0.88	1.00
60B	0.96	0.84	1.00
mean	0.89	0.84	0.97

duced levels of genetic variability. The present study demonstrates that RAPD provides detailed information about polymorphism in small populations. Use of RAPD provides, therefore, a useful alternative to the use of other genetic markers in monitoring the genetic status of wildlife.

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