Application of DNA Fingerprinting in the Hokkaido Brown Bear (*Ursus arctos yesoensis*)

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ABSTRACT. DNA fingerprinting employing a minisatellite Myo probe was used for individual identification and paternity determination in Hokkaido brown bears (*Ursus arctos yesoensis*). We used two restriction enzymes, *Hinf*I and *Hae*III to make DNA fingerprints. Band patterns obtained from randomly selected bears were compared with each other, and the probability x that fragment in an individual was also present in the other was 0.69 for *Hinf*I and 0.83 for *Hae*III. The value for *Hinf*I (0.69) was similar to that obtained from other species, such as dog and domestic animals, and the mean probability of all fragments was calculated to be 2.5×10^{-2} . The results suggest that DNA fingerprinting applying the combination of *Hinf*I and Myo is available for individual identification. On the other hand, the ability to determine paternity seemed to be insufficient owing to the lack of paternal fragments, although the band patterns reflected the correct relationships between child and father.—**KEY WORDS:** brown bear, individual identification, minisatellite, paternity determination.

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The habitat of bears, including the Hokkaido brown bear (*Ursus arctos yesoensis*) is becoming smaller world wide, and the number of bears is decreasing as well [4, 17, 23]. Recently, for the purpose of conserving this animal, basic studies have been performed on North American bears [15]. In these studies, it was suggested that morphological diversity depends on differences of habitat [1, 19, 21, 22]. However, there are only a few reports about genetic diversity [24]. It is important for maintenance of a viable population to estimate the genetic relationships among different populations and the degree of inbreeding in small populations.

In humans, the use of highly polymorphic minisatellites was reported by Jeffreys *et al.* [11]. The DNA fingerprint provided by hypervariable minisatellites is unique to an individual and has been applied for individual identification and paternity determination [6–8, 12]. Moreover, the DNA fingerprint is useful for paternity determination in dogs, cats and birds, and for the estimation of the degree of inbreeding in chickens [2, 14, 16].

We aimed to apply DNA fingerprinting for maintenance of viable bear populations using the hyperpolymorphic minisatellite “Myo” probe, which has been previously applied to a Japanese population [8], a Japanese macaque group [9] and grey red-backed voles [10]. And as the first step, this study investigated whether DNA fingerprinting can be applied for individual identification and paternity determination of a captive Hokkaido brown bear population, in which relationships among the bears are partially known.

MATERIALS AND METHODS

Animals: Forty-seven bears, including 4 families kept in Noboribetsu Bear Park, were used in this study. Some of the bears were captured in Hokkaido while the others

were born in the park.

Preparation of DNA: To extract DNA, blood was collected from bears using a Venoject (Terumo) with heparin sodium as an anticoagulant, and white blood cells (WBCs) were separated by centrifugation at 3,000 rpm for 10 min. WBCs were resuspended in TNE buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA) containing 0.5% w/v sodium dodecyl sulfate (SDS) and 50 µg/ml of proteinase K, and incubated overnight at 37°C. After three treatments with phenol and one with chloroform, DNA was extracted, and precipitated by addition of three volumes of ethanol.

Digestion and electrophoresis: Ten micrograms of each DNA sample was digested with *Hinf*I or *Hae*III restriction enzyme. After digestion, the DNA was subjected to electrophoresis in 15 cm long 0.8% agarose gel at 30 V for 21 to 24 hr, denatured and then transferred to a nitrocellulose membrane filter [25].

DNA probe: The minisatellite Myo probe for hybridization was labelled with ³²P using the Multiprime DNA labelling system (Amersham). The consensus sequence of the Myo was 5'-GACCGAGGTCTAAAGCTGGAGG-TGGGCAGGAAG-3' [6].

Hybridization and washing: The filters were prehybridized for over 4 hr in 5×SSC, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% SDS and 10 µg/ml yeast tRNA at 55°C, and hybridized overnight after addition of the probe (2.0×10^6 cpm/ml). Washing was first performed in 1 × SSC at room temperature, and then in 0.25 × SSC at 42°C. Autoradiography was carried out in two exposure periods (overnight and 3 days) at –80°C.

RESULTS

Individual identification: The DNA fingerprints obtained by *Hinf*I and *Hae*III restriction enzymes are

Fig. 1. DNA fingerprints of bears. DNAs from 15 randomly sampled bears (lanes 1–15) were digested with *HinfI* (a) and *HaeIII* (b), electrophoresed and then hybridized with the minisatellite probe Myo. (●) indicate the fragments used for the analysis. *HindIII*-digested lambda DNA was used as a size marker.

Table 1. DNA fingerprint variability in bears

DNA fragment size (kb)	No. of fragments per individual $\pm$ SD		Probability x		Mean allelic frequency, q	
	<i>HinfI</i>	<i>HaeIII</i>	<i>HinfI</i>	<i>HaeIII</i>	<i>HinfI</i>	<i>HaeIII</i>
9.4–23.1	1.4 $\pm$ 0.9	1.2 $\pm$ 1.2	0.39	0.64	0.22	0.40
4.4–9.4	2.2 $\pm$ 0.9	2.7 $\pm$ 2.4	0.64	0.66	0.40	0.41
2.0–4.4	6.3 $\pm$ 1.2	6.8 $\pm$ 1.1	0.77	0.87	0.52	0.64
2.0–23.1	9.9 $\pm$ 1.9	10.8 $\pm$ 4.2	0.69	0.83	0.44	0.59

The DNA fingerprints from 45 bears were compared and the number of DNA fragments for each restriction enzyme are shown. The band patterns were analyzed from autoradiographs exposed for long and short periods.

The x represents the probability that fragment in an individual is present in the other. Probability x is related to the mean frequency of q by $x = 2q - q^2$.

shown in Fig. 1a and 1b, respectively. About ten polymorphic DNA fragments were observed in the region longer than 2.0 kilobases in each column. We calculated the variability of these fragments from Fig. 1 (a, b) according to the methods described by Jeffreys *et al.* [12] and the results are shown in Table 1. The mean probability x that a fragment in an individual was present in the other was calculated using 45 randomly selected pairs from 41 adult bears for *HinfI* and 33 pairs for *HaeIII*. In comparison, co-migrating fragments in each individual that had roughly similar autoradiographic intensities were thought to be derived from the identical allele of the same locus. When a fragment that had strong autoradiographic intensity in an individual co-migrated with a fragment in the other with weak intensity, the latter was ignored even if there was a difference in the amount of DNA applied. According to previous reports, if co-migrating fragments in an individual and the other are derived from an identical dimorphic locus, it is considered that the fragments are one of two alleles of the locus [8, 13]. Thus, these alleles (+) are dominant compared to the other alleles (–). When the frequency for the allele (+) is q and that for allele (–) is $(1-q)$, the probability x that an allele (+) in an individual is also present in the other is related to the allele frequency q by $x = 2q - q^2$, which is the sum of q^2 (+/+) and $2q(1-q)$ (+/–) [8, 13].

As can be seen in Table 1, the probability that all fragments obtained in an individual agreed with those of

Fig. 2. DNA fingerprints of a bear family using *HinfI* and Myo. O is the offspring of M (mother) and F1 (father), and F2 is the father of the other family. The arrowheads indicate paternal fragments.

Table 2. Application to paternity determination

	No. of paternal fragments		Probability ^{a)}	
	Hinfl	HaeIII	Hinfl	HaeIII
family 1	2	1	0.48	0.83
family 2	2	0	0.48	—
family 3	3	5	0.33	0.39
family 4	4	3	0.23	0.57

a) Probability that a bear has all paternal fragments.
 $P=x^n$ n: No. of paternal fragments.

the other at fragment sizes over 2.0 kilobases was $0.69^{9.9} = 2.5 \times 10^{-2}$ for *Hinfl* and $0.83^{10.8} = 1.3 \times 10^{-1}$ for *HaeIII*.

Paternity determination: One set of DNA fingerprints from four families in which their relationships were clear is shown in Fig. 2. Comparing band patterns of a mother and her child, some fragments were specific to the child. It is assumed that those fragments are paternal fragments and that a male bear which has all the paternal fragments is the father. The probability of a male bear that has all paternal fragments in each case is presented in Table 2.

DISCUSSION

In the first report about DNA fingerprinting, using minisatellite probes 33.6 and 33.15 for humans by Jeffreys *et al.*, the probability x was 0.08–0.27 at the range of 4.0–20.0 kilobases [12]. In animals, many works using various minisatellite probes have been reported and the results were described as follows. The values for dogs and cats were 0.56 and 0.47, respectively [14]. For domestic animals, the values were 0.27–0.68 using four minisatellite probes [5]. These results show that genetic variation was small in these animals compared with that in humans. It was shown that the probability x for a Japanese population is 0.192 in the range of 4.4–23.1 kilobases using the minisatellite Myo [7]. It is considered that the Myo probe is very useful for individual identification and paternity determination. Some researchers reported application of the Myo probe to animals, although there was no mention about the probability x [9, 10]. Since the value (0.69) obtained in this study is similar to that described for other animals using other minisatellite probes, DNA fingerprinting using the Myo probe also appears an informative way for individual identification of bears.

In paternity determination, the band patterns reflect exact relationships among families, but it seems that the data are insufficient (Table 2). When there are many paternal bands from two or three children as in family 4, it is easy to determine the father, but the lack of a paternal band makes determination impossible. If we use other minisatellite probes and/or together with Myo, we may be able to obtain sufficient data for paternity identification.

From the results of this study, we can consider various applications for DNA fingerprinting of bears. We are conducting research into the habitat and behavior of the

Hokkaido brown bear by attaching a telemeter and radiotracking device. The profiles obtained make individual identification of bears easy even if markers are lost because of unforeseen accidents. In bear parks, in which many bears are kept, there is continuous contact among bears, therefore, identification of each individual is very difficult. Until now, the caretaker's careful observation has been the best method of identifying bears. However, DNA fingerprinting can also be used effectively in such conditions.

From a different point of view, DNA fingerprinting can be applied to assess the variety of genes. Such an assessment was reported for inbreeding lines of chickens [16]. From measurement of the variety of genes among the groups, it is possible to investigate the relationships between the groups and the degree of inbreeding of an isolated population. The data in this study would be valuable when compared with data from wild populations to estimate the degree of inbreeding on this population.

DNA fingerprinting, when applied to ecology and eco-management, especially in birds [2, 3, 18] and in some mammals [20], has revealed many interesting behaviors. These kind of genetic markers could also be a useful tool for managing Hokkaido brown bear.

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