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Polymerase chain reaction-restriction fragment length polymorphism
(PCR-RFLP) method for mtDNA typing in Hokkaido brown bear
(*Ursus arctos yesoensis*)

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Abstract

To develop an easy method of typing of mitochondrial DNA (mtDNA) in Hokkaido brown bears (*Ursus arctos yesoensis*), the PCR-RFLP technique was improved using four restriction enzymes : *Mbo* 1, *Cfr* 13 I, *TspE* 1, and *Fok* 1. This approach identified seven groups of mtDNA haplotypes, HB1/2/5-7, HB3, HB4, HB8/9, HB10/11, HB12 and HB13 from 102 brown bears of northern, central and eastern Hokkaido.

Key words : Hokkaido brown bear ; mitochondrial DNA (mtDNA) ; PCR-RFLP method

Based on sequences of the 5' variable region in the mtDNA control region (Fig. 1), 17 haplotypes were identified from the brown bear (*Ursus arctos yesoensis*) population on Hokkaido Island²⁾. Generally, DNA sequencing is a costly and time-consuming method of identifying haplotypes. Polymerase Chain

Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is superior in identifying large numbers of samples at less cost and time when sequencing data are available. In this study, a PCR-RFLP method was established for easy typing of Hokkaido brown bear mtDNA using sequence data reported previ-

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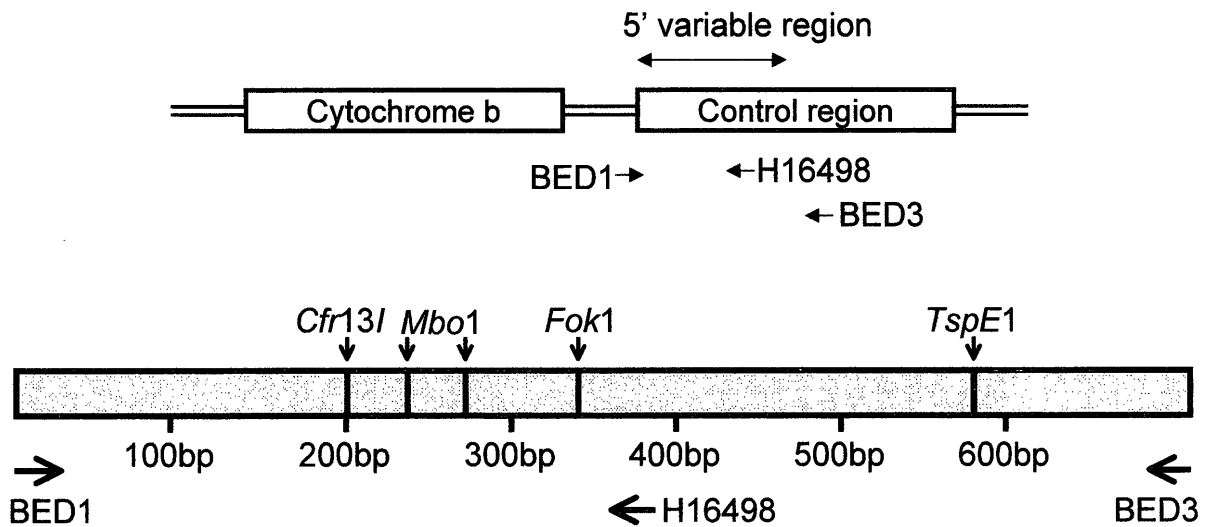


Fig. 1. Schematic diagram of mtDNA control region of the brown bear. Arrows indicate the primers for PCR and/or sequencing. Diagnostic restriction sites are shown in lower diagram.

Table 1. Presence or absence of diagnostic restriction sites in mtDNA types identified from Hokkaido brown bears

MtDNA type ^{a)}	MboI		Cfr13 I	FokI	TspE1	Nos. of samples ^{b)}
	235bp	272bp	201bp	341bp	579bp	
HB1/2/5-7	-	+	-	-	-	23 (9)
HB 3	-	+	-	-	+	6 (1)
HB 4	-	+	-	+	-	5 (4)
HB 8 / 9	-	+	+	-	-	4 (2)
HB10/11 (HB14/15) ^{c)}	-	-	-	-	-	7 (2)
HB12	+	-	+	-	-	4 (1)
HB13 (HB16/17) ^{d)}	+	-	-	-	-	48 (2)

Presence, + ; absence, -.

a) Haplotypes refer to those of Matsushashi et al. (1999)¹⁾.

b) Numbers of samples sequenced in this study. The numbers in parentheses indicate the numbers of samples quoted from Matsushashi et al. (1999)¹⁾.

c) HB10/11 and HB14/15 shared the same restriction sites between them.

d) HB13 and HB16/17 shared the same restriction sites between them.

ously²⁾.

We sampled livers or muscle tissues from 102 brown bears killed in north-central and eastern Hokkaido. These bears had been hunted legally or were killed for pest control

from 1995 to 2001.

Tissues were preserved in 70% ethanol until analysis. Total DNA was extracted using the conventional proteinase K/phenol/chloroform method³⁾. Briefly, approximately 2 x 2 x

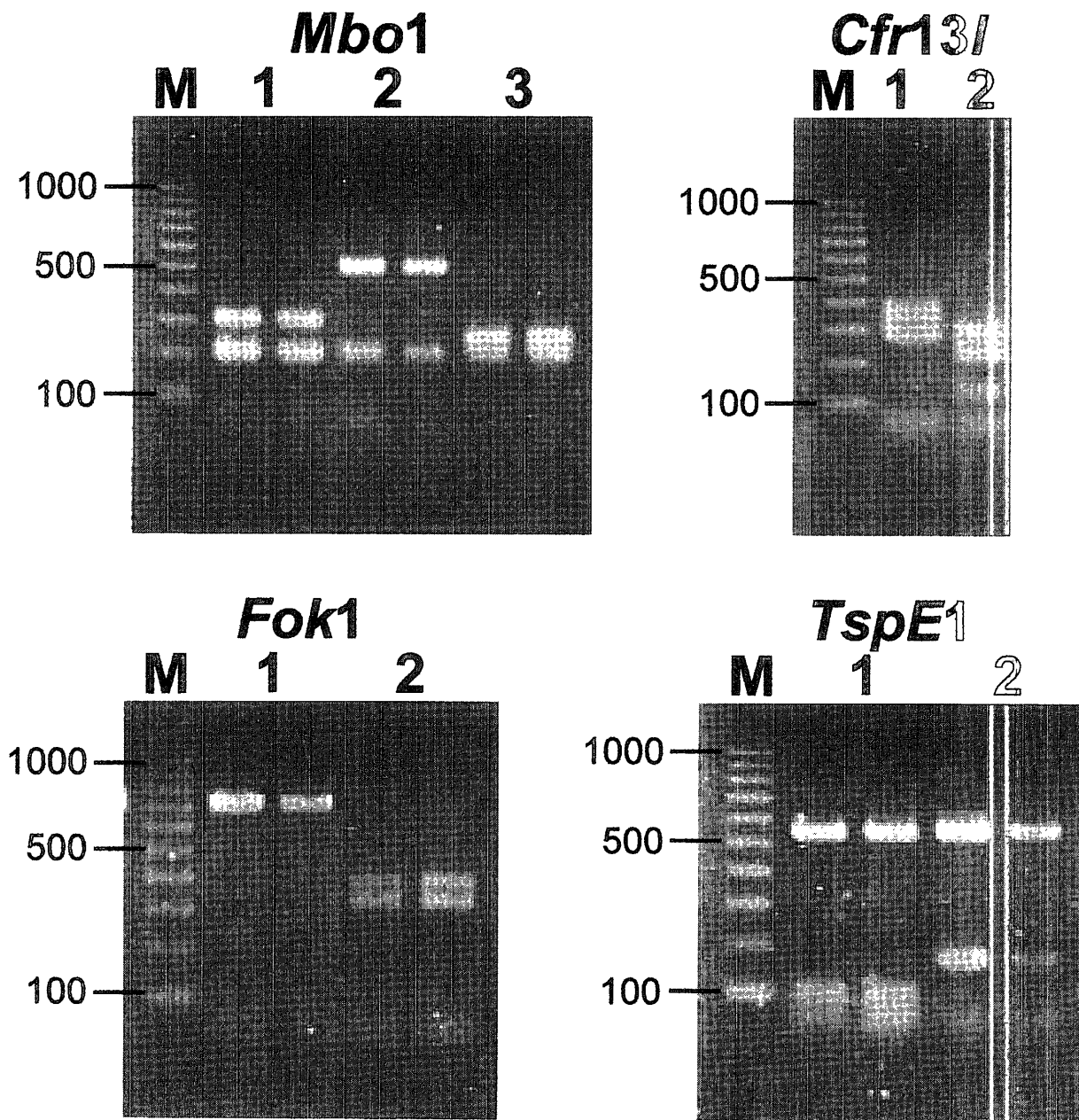


Fig. 2 . Gel image for the PCR-RFLP method.

*Mbo*1 : 1 , HB1-9 ; 2 , HB12 or 13 ; 3 , HB10 or 11 ;

*Cfr*13 I : 1 , HB1-7, 10, 11, or 13 ; 2 , HB 8 / 9 , or 12 ;

*Fok*1 : 1 , HB 1 - 3 or 5 -13 ; 2 , HB 4 ;

*TspE*1 : 1 , HB 3 ; 2 , HB 1 , 2 , or 4 -13 ;

The molecular weight marker (100-1000bp) is in the far left lane.

2 mm of tissue was washed with STE buffer (containing 10 mM of pH 8.0 Tris-HCl, 1 mM of pH 8.0 EDTA, and 150 mM NaCl), and homogenized in 0.5 ml of STE buffer using a

small glass homogenizer. After addition of 25 μ l of 10% SDS and 5 μ l of 50 mg/ml proteinase K, the mixture was incubated at 37°C overnight, and then purified by extraction at

least twice with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with an equal volume of chloroform/isoamyl alcohol (24 : 1). Purified DNA solutions were stored at 4 °C until they were used as the templates for PCR.

The 5' end of the mtDNA control region was amplified using PCR with two primers²⁾ (Fig. 1): BED1, 5' - AGCAACAGCTCCAC-TACCAG- 3' ; BED3, 5' - CGATTTAGTGGC-GTTTATGTAC- 3'. PCR was performed in 20 µl of a mixture containing 0.8 µl of 10 ng/µl DNA extract, 0.1U of rTaq polymerase (Sigma, St. Louis, MO USA), 0.4 µl of 10 mM dNTP, and 0.4 µl of 25 pmol for each of the above two primers. The reaction was conducted using a thermal cycler (Model PE 2000, Perkin Elmer Inc., Wellesley, MA, U.S.A.) as follows: one cycle of 94°C for 5 min and then 35 cycles of 94°C for 30 sec ; 59°C for 30 sec ; 72°C for 1 min. The reaction was completed at 72°C for 7 min.

Restriction enzymes were selected with reference to sequences of the brown bear mtDNA control region²⁾. Restriction enzymes *Mbo*1, *Cfr*13 I, *TspE*1, and *Fok*1 were used to classify the 13 haplotypes into several groups. These haplotypes had been identified in a previous study and had been reported to distribute in the north-central and eastern area of Hokkaido. A reaction mixture of 15 µl, containing 2.5 µl of PCR product and 0.5U of the enzyme, was incubated overnight at 37°C (*Mbo*1, *Cfr*13 I, and *Fok*1) and at 65°C (*TspE*1). The mixture was then run on 2 - 4 % agarose gel, stained with ethidium bromide and detected using an UV illuminator.

Diagnostic restriction sites in each haplotype are shown in Table 1 and Fig. 1. The gel images are shown in Fig. 2. *Mbo*1-digested PCR products amplified with BED1/BED3 gave three patterns of fragment lengths that were classified as haplotypes HB1-9, HB10/11 and HB12/13. *Cfr*13 I gave two patterns of

RFLP. The first pattern was identified in HB 8 / 9 / 12, while another was found in HB1-7 / 10 / 11 / 13. *TspE*1 yielded two patterns of digestions identified in HB3 or others. *Fok*1 digested only one haplotype, HB4. Thus, with the four restriction enzymes the 13 haplotypes were classified into 7 groups as follows: HB1 / 2 / 5 -7, HB3, HB4, HB8/9, HB10/11, HB12 and HB13. Additionally, DNA of samples for which haplotypes are known was digested with the above restriction enzymes (n=17), and all samples were typed accurately.

To confirm the accuracy of the PCR-RFLP method, sequencing was performed for all 102 samples with two primers (Fig. 1): BED1²⁾; H16498, 5' - CCTGAACTAGGAACCAGATG-3'¹⁾. These primers amplified a fragment that included mutations involving restriction sites.

The sequence data and accurate typing of known samples indicated that this PCR-RFLP method has high reliability. However, not all the haplotypes reported in the previous study²⁾ were always separated in this study. Since no other restriction enzymes were found to be able to work on the sequences of the 13 haplotypes, some types of RFLP included more than one haplotype. In addition, as shown in Table 1, restriction patterns for HB10/11 and HB14/15 were the same. HB13 and HB16/17 also have the same patterns for restriction enzymes *Mbo*1, *Cfr*13 I, *TspE*1, and *Fok*1.

To resolve the inadequate typing in HB1 to 13, information regarding longer sequences is required for use of other appropriate restriction enzymes. In order to distinguish HB10/11 from HB14/15 and to distinguish HB13 from HB16/17, geographic distribution must be included in the consideration of mtDNA typing. There are three clusters distributed allopatrically in north-central, eastern, and southwestern Hokkaido²⁾. Haplotypes HB1 to HB9 are included in Cluster A spreading in

north-central Hokkaido, and these haplotypes are distinguished from others by digestion with *Mbo*I. HB10 to 13 (Cluster B) are distributed in the eastern side of Cluster A, and HB14 to 17 (Cluster C) are located in the southwestern side of Cluster A; thus, mtDNA typing by RFLP is not so confused.

In conclusion, our PCR-RFLP method enabled mtDNA typing of brown bears in central and eastern Hokkaido. The method lessens both the cost and time involved in identifying haplotypes. The application of additional restriction enzymes with references to longer sequence data could further classification of mtDNA typing of Hokkaido brown bears.

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