Short communication

The first detection of *Babesia* species DNA from Japanese black bears (*Ursus thibetanus japonicus*) in Japan

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ABSTRACT

In this study, we tried to detect protozoan blood parasites from the liver or blood of 156 Japanese black bears (*Ursus thibetanus japonicus*) in Iwate Prefecture of Japan by polymerase chain reaction. Two amplicons (approximately 540 bp and 480 bp) were detected by amplification for V4 hyper-variable regions of the 18S rRNA gene. Approximately 540-bp products were obtained in 119 samples (76.3%) and were considered to be DNA of *Hepatozoon ursi*. Approximately 480-bp products were obtained in 22 samples (14.1%) and were considered to be DNA of *Babesia* species.

The nucleotide sequences (1635 bp) of the 18S rRNA gene of *Babesia* sp. were very similar (99.3%) to those (AY190123, AY190124) of *Babesia* sp. detected previously from *Ixodes ovatus*. Phylogenetic analysis showed that *Babesia* sp. detected in this study closely related to *Babesia* sp. derived from raccoons in Japan and the U.S.A. This is the first report of *Babesia* species detected from Japanese black bears.

Keywords: *Babesia* sp.; *Hepatozoon ursi*; *Ursus thibetanus japonicas*; Japan; 18S rRNA; PCR

Recently, protozoan blood parasites including the genera *Babesia* and *Hepatozoon* have been reported from wild animals in the world [1-8]. The Japanese black bear (*Ursus thibetanus japonicus*), which is a subspecies of the Asian black bear, *Ursus thibetanus* inhabiting eastern Asia and the Russian Far East, is widely distributed throughout the Honshu and Shikoku regions of Japan. Although parasitic helminthes such as filarial and *Trichinella* nematodes have been found in Japanese black bears [9-12], detection of blood protozoa has been limited in a single species of *Hepatozoon ursi* [13, 14]. We report here the first detection of *Babesia* species as well as *H. ursi* from Japanese black bears in Iwate Prefecture, Japan.

Blood or liver samples were obtained from 156 Japanese black bears hunted in 21 localities of Iwate Prefecture (38.8 to 40.4°N, 140.7 to 141.9°E), Japan and kept at -80°C until DNA extraction. Total DNA was extracted from each sample by using the PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and

stored at -25°C. First, we amplified the V4 hyper-variable region of the 18S rRNA gene of protozoa according to the method of Gubbels et al. [15]. Briefly, the PCR mixture contained 9 µl of total DNA, 2.5 µl of 5×Go *Taq* buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$), 0.1 µl (25 mM) of dNTP, 0.13 µl（50 pmol/µl）of each primer (RLB-F, RLB-R), 0.063 µl of Go *Taq* DNA polymerase (Promega, Madison, U.S.A.) and 0.577 µl of Milli-Q® water and was prepared in a total volume of 12.5 µl. PCR conditions were initially 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec, and a final extension step at 72°C for 5 min. All amplifications were performed using a TaKaRa PCR Thermal Cycler Dice™ mini (TaKaRa BIO INC, Shiga, Japan). Amplification products with different sizes were purified by using High Pure PCR Clean Up Micro Kits (Roche Diagnostics GmbH, Mannheim, Germany). PCR products were directly sequenced by using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, U.S.A.), RLB-F and RLB-R primers, an ABI PRISM® 3100 - Avant Genetic Analyzer (Applied Biosystems, California, U.S.A.) and Sequencing Analysis ver. 3.7 (Applied Biosystems, California, U. S. A.). The nucleotide sequences obtained were assembled by using ATGC ver. 6 (Genetics, Tokyo, Japan) and analyzed by using BLAST program version 2.0 of National Center for Biotechnology Information for detection of homologous sequences.

For samples in which sequences of the V4 region were closely related to those of *Babesia* spp., the full sequence of the 18S rRNA gene was also determined by the above-described PCR amplification and using three sets of primers, RIB19 (5'-CGGGATCCAACCTGGTTGATCCTGC-3') [16] and RLB-R (5'-TCTTCGATCCCCTAACTTTC-3') [15], SeqF3

(5'-CAGAGTATCAATTGGAGGGC-3') and SeqR2 (5'-TCGATGGACGCATCAGTG-3') [17], and SBs-F (5'-TCCTTCAGCACCTTGAGAGA-3') and SBs-R (5'-ACTTCCCTAGGCAAAACCGA-3') designed in the present study.

The determined sequences were aligned with the sequences registered in GenBank by using Clustal X ver. 1.83 and were analyzed phylogenetically by the Neighbor-Joining method using PAUP* 4.0 (Sinauer Associates, Massachusetts, U.S.A.). The stability of the phylogenetic tree obtained was estimated by 1000 replications of bootstrap analysis.

Two amplicons with different sizes (approximately 540 bp and 480 bp) were detected by amplification for the V4 hyper-variable region (Fig. 1). Approximately 540-bp products were obtained in 119 (76.3%) of the 156 samples. The sequences of 27 amplicons were completely consistent, extremely similar (99.6%, 545/547 bp) to those of *Hepatozoon ursi* (EU041717, EU041718), and belonged to the same clade as *Hepatozoon ursi* in the phylogenetic tree (Fig. 2). Therefore, the 540-bp amplicons were considered to be DNA sequences of *Hepatozoon ursi*.

On the other hand, approximately 480-bp products were detected from 22 samples (14.1%), of which 18 samples (11.5%) also produced the 540-bp amplicons (*H. ursi*) in addition to the 480-bp amplicons (Fig. 1, lane 3). The sequences of the 480-bp amplicons obtained from 9 samples were completely consistent and highly homologous to those of *Babesia* spp. The sequences (1635 bp) of the 18S rRNA gene in the 9 samples were also completely consistent and similar (98.0 – 99.3%) to those for *Babesia* sp. (AY190123, AY190124) from *Ixodes ovatus*, *Babesia* sp. (AB251608) from

raccoons in Japan, *Babesia* sp. (DQ028958) from raccoons in the U. S. A. and *Babesia* sp. (DQ329138) from panthers in the U.S.A. The phylogenetic tree (Fig. 3) on sequences of the 18S rRNA gene indicated that these *Babesia* protozoa detected from Japanese black bears, *Ixodes ovatus*, raccoons and panthers belonged to the same clade, showing close relationships each other.

The sequences determined in this study were registered in DNA Data Bank of Japan (DDBJ) as AB586027 for *Babesia* sp. Iwate248 and AB586028 for *Hepatozoon ursi.*

Hepatozoon ursi was described as a new species in 2008 and was detected in 100% of Japanese black bears in Gifu Prefecture, in the central region of Japan [14]. The present study conducted in Iwate Prefecture, in the northeastern region of Japan, also showed high detection rates (76.3%). Therefore, *H. ursi* may be widely prevalent in Japanese black bears throughout Japan. Further studies on genotypes of *H. ursi* will be needed in geographical populations of Japanese black bears, because the present genotype derived from Iwate differed from that from Gifu.

Babesia species has not been detected in American black bears (*Ursus americanus*) in the U.S.A. [18] and in European brown bears (*Ursus arctos*) in Sweden [19]. Until recently, there have been no reports on detection of *Babesia* species from bears of the family Ursidae [20]. However, *Babesia* sp. has recently been detected from a Japanese brown bear (*Ursus arctos yesoensis*) in Hokkaido, Japan [21]. Sequence similarlity of the 18S rRNA gene was 95.8% (1499/1565 bp) between the two *Babesia* species (AB586027 and AB480557) obtained from Japanese black bears and a Japanese brown bear, and the similarity was low compared with 98.3% (1695/1725 bp) between *Babesia divergens* (AY098643) and *B. odocoilei* (U16369), 96.7% (1595/1649 bp) between *B.*

bigemina (FJ426361) and *B. motasi* (AY260180), and 95.4% (1618/1696 bp) between *B. ovata* (AY603401) and *B. major* (EU622907). Additionally, the phylogenetic tree of the 18S rRNA gene indicated that *Babesia* sp. Iwate248 (AB586027) detected in this study belonged to a cluster distinct from that of *Babesia* sp. UR1 (AB480557) detected in a Japanese brown bear, which was supported by high boot strap value (98.4). Therefore, *Babesia* sp. Iwate248 is probably distinct species from *Babesia* sp. UR1 detected from a Japanese brown bear.

Acknowledgements

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Legends to figures

Fig. 1. PCR amplicons of the V4 hyper-veriable region. Lanes 2 to 13: samples obtained from bears, Lane14: DNA-negative (distilled water), Lane15: *Babesia* DNA-positive (*Babesia rodhaini*), Lanes 1 and 16: 100-bp DNA ladder markers.

Fig. 2. Phylogenetic tree based on sequences of the 18S rRNA gene of *Hepatozoon* spp. An underline indicates *Hepatozoon* sp. detected in this study. A scale bar indicates 10% divergence. Numbers on branches are bootstrap values. *Toxoplasma gondii* (L37415) was used as an outgroup.

Fig. 3. Phylogenetic tree based on sequences of the 18S rRNA gene of *Babesia* spp. An underline indicates *Babesia* sp. detected in this study. A scale bar indicates 1% genetic divergence. Numbers on branches show bootstrap values. *Toxoplasma gondii* (L37415) was used as an outgroup.

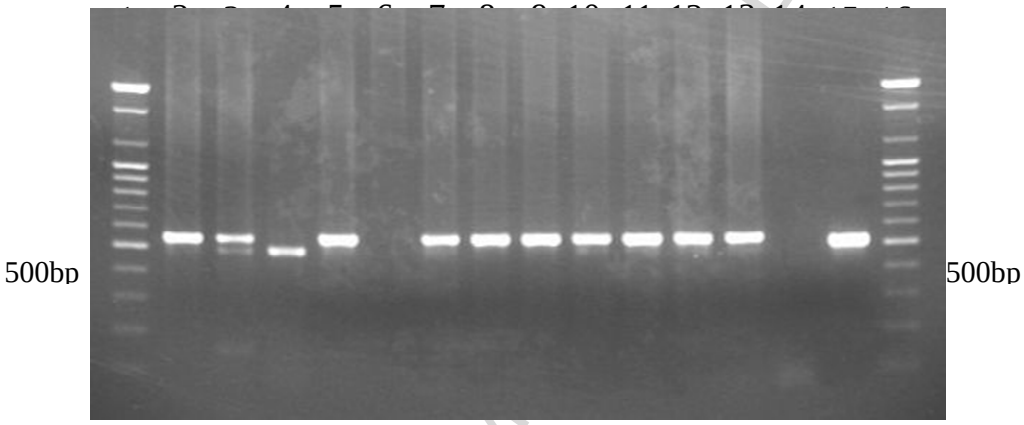


Fig. 1

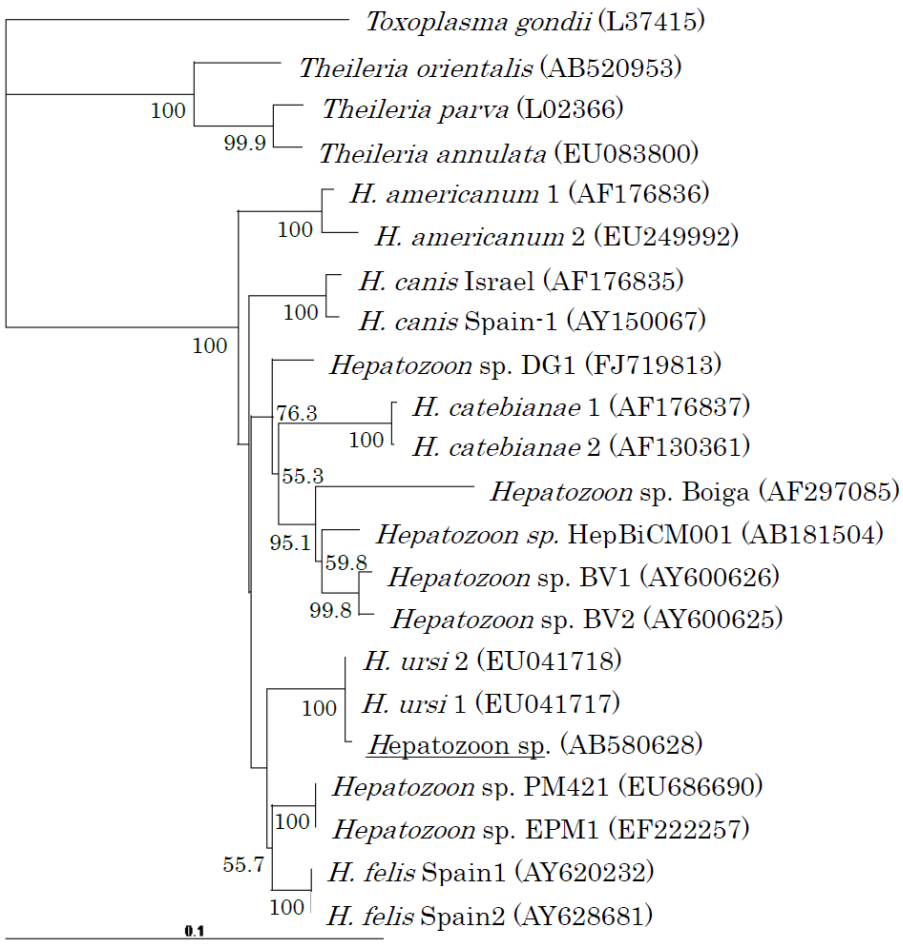


Fig. 2

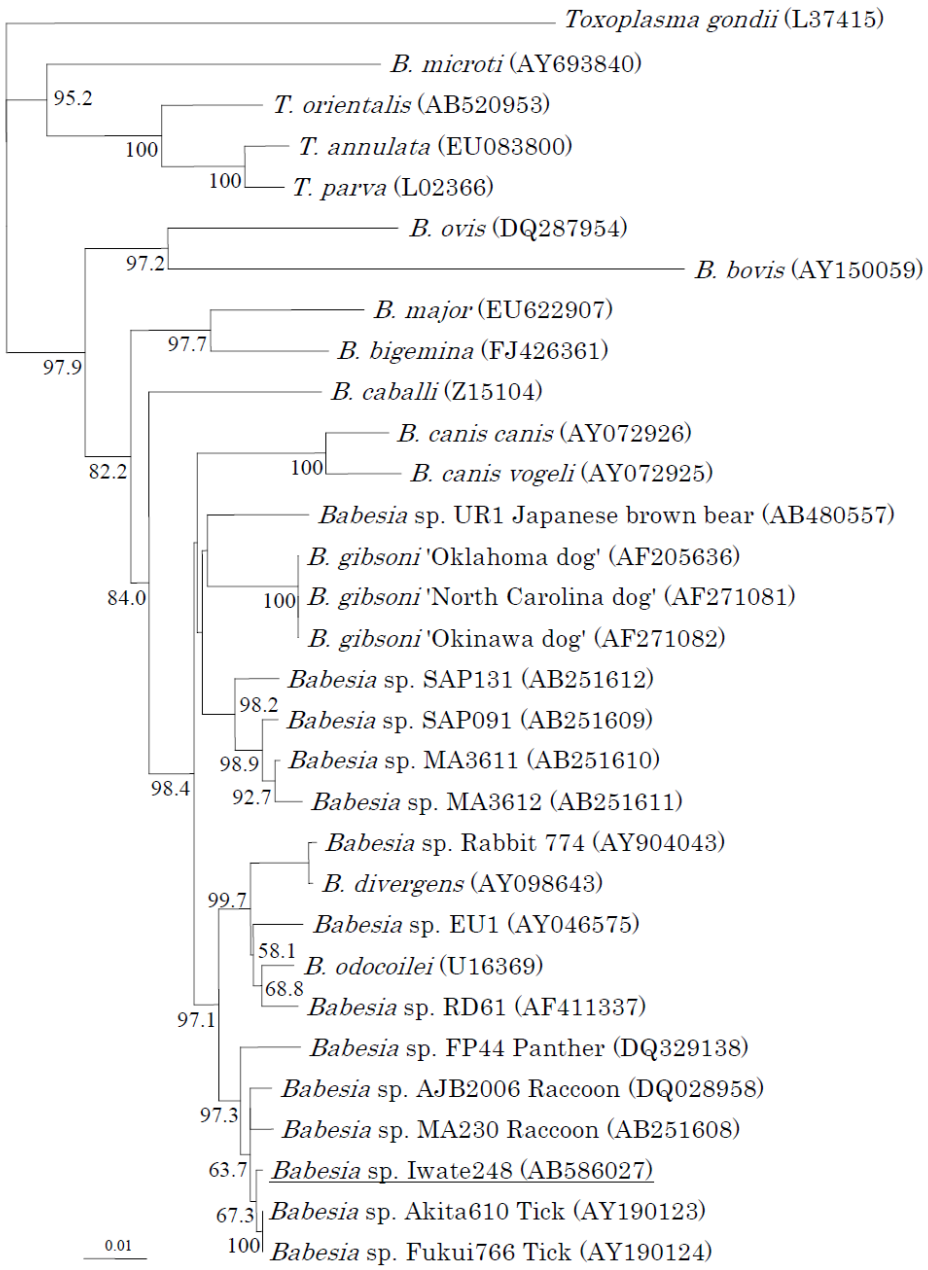
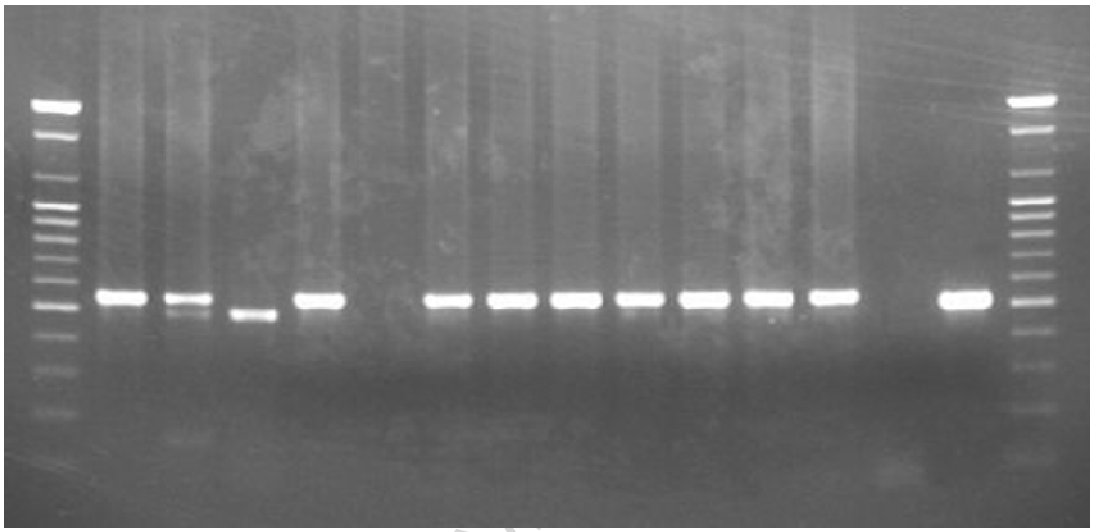


Fig. 3

Graphical Abstract



Highlights

>*Babesia* species and *Hepatozoon ursi* were detected from Japanese black bears (*Ursus thibetanus japonicus*) in Iwate Prefecture of Japan by polymerase chain reaction. >The detection rates were 14.1% for *Babesia* sp. and 76.3% for *H. ursi*. >*Babesia* sp. detected in this study phylogenically related to *Babesia* sp. derived from raccoons in Japan and the U.S.A. >This is the first report of *Babesia* species detected from Japanese black bears.