Molecular detection of apicomplexan protozoa in Hokkaido brown bears (*Ursus arctos yesoensis*) and Japanese black bears (*Ursus thibetanus japonicus*)

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Abstract

Many tick-borne pathogens (TBPs) are present in wildlife. The objective of this study is to reveal the role of wild bears in maintaining TBPs. A total of 49 brown bears (*Ursus arctos yesoensis*) from Hokkaido, and 18 Japanese black bears (*Ursus thibetanus japonicus*) from Tochigi and 66 Japanese black bears from Nagano were examined by two molecular methods, reverse line blot (RLB) hybridization and nested PCR. A total of 5 TBPs (*Hepatozoon ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and *Babesia microti*) were detected from bear blood DNA samples. *B. microti* was detected from blood DNA samples of Japanese black bear for the first time, with the prevalence of 6.0% (5/84). Out of detected pathogens, *H. ursi*, *Babesia* sp. UR2 like pathogens and *Cytauxzoon* sp. UR1 were considered as three of the most prevalent TBPs in bears. The prevalence of *H. ursi* were significantly higher in Japanese black bear (0% vs 96.4%) while that of *Babesia* sp. UR2-like group was higher in Hokkaido brown bears (89.8% vs 40.5%). The prevalence of *Babesia* sp. UR1 were significantly higher in Japanese black bears from Tochigi (44.4%), comparing to those from Nagano (18.2%). The prevalence of the detected TBPs were significantly higher in adult bears, comparing to those in younger bears. The present study suggests that Japanese bear species contribute in the transmission of several TBPs in Japan. The expanding

distribution of bears might cause the accidental transmission of TBPs to humans and domestic animals.

Keywords: *Bears, RLB, Apicomplexan protozoa, Babesia microti, Cytauxzoon.*

Declarations

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1. Introduction

Ticks are important arthropod vectors that transmit a variety of pathogens, such as viruses, bacteria, and protozoans (Estrada-Pena and Jongejan 1999). The incidence of tick-borne diseases (TBDs) is increasing worldwide by several factors (de la Fuente and Estrada-Pena 2012), including the developed diagnostic tool, the global climate change, and the habitat expansion of ticks and wild mammals (Medlock et al. 2013; Medlock and Leach 2015). In Japan, the recurrence of neglected human TBDs, such as tick-borne encephalitis (TBE) and severe fever with thrombocytopenia syndrome (SFTS), raised public concerns and highlighted the importance of studies on TBDs (National Institute of Infectious Diseases 2017; National Institute of Infectious Diseases 2016). Many tick-borne pathogens (TBPs) are maintained in lifecycles that include ticks and wild vertebrate animals in nature and could be accidentally transmitted to humans and domestic animals (Baneth 2014). Several TBPs were detected from bear species around the world, one of the most common wild large mammals in Japan, and some of these are zoonotically or economically important pathogens (Leydet and Liang 2013; Moustafa et al. 2015; Yabsley et al. 2009). Bears might act as reproductive hosts for many tick species due to the wide home range bears use (Hazumi and Maruyama 1987; Izumiyama and

Shiraishi 2004) and act as one of the most important hosts of many tick species (Katsuhiro 2010; Tsunoda T 2001; Zolnik et al. 2015). Nowadays, the range of bear population is expanding in Japan. This decreases the distance between humans and bears (Takahata et al. 2013; Sato 2017) and might increase the risk of transmission of TBPs from bears to humans and domestic animals. However, it remains unknown how wild bears have contributed to the life cycle of TBPs and whether TBPs in wild bears are infectious to humans and other animals. Hence, it is important to study current situation and prevalence of TBPs in bear species in Japan.

In Japan, bears have a unique habitat pattern. There are two different bear species, the Japanese black bear (*Ursus thibetanus japonicus*) and the Hokkaido brown bear (*Ursus arctos yesoensis*). While Japanese black bears inhabit wide region of Honshu Island and Shikoku Island, Hokkaido brown bears inhabit only Hokkaido Island, north part of Japan (Hazumi and Maruyama 1987). Although there could be differences of prevalence of TBPs between the two different bear species in different habitats, there is no report to compare the prevalence of TBPs in these two different Japanese bears.

Apicomplexan protozoa infect various animals, including domestic and wild animals, and cause serious diseases (Morrison 2009). Out of these microbes, piroplasms, including Babesidae and Theileridae, and Hepatozoidae have been detected from bear species in Japan (Ikawa et al. 2011; Jinnai et al. 2010; Kubo et al. 2010). Generally, piroplasms are intracellular parasites that can be

transmitted to the animal hosts during the infestation of infected ticks on those animals. Some *Babesia* spp. were reported to infect humans and cause human babesiosis: e.g. *Babesia microti* and *B. microti*-like organisms, *Babesia duncani* and *B. duncani*-type organisms, *Babesia divergens* and *B. divergens*–like organisms, *Babesia venatorum*, and *Babesia* sp. KO1 (Vannier et al. 2008). To the best of our knowledge, four sequences of *Babesia* spp. (*Babesia* sp. UR1 [AB48055], *Babesia* sp. UR2 [AB704303], *Babesia* sp. Iwate248 [AB586027] and *Babesia* sp. EBB1021 [AB566229]) and one sequence of *Cytauxzoon* sp. (*Cytauxzoon* sp. UR1 [AB480558]) have been detected from bear species in Japan. Among them, *Babesia* sp. UR2, *Babesia* sp. Iwate248 and *Babesia* sp. EBB1021 were considered as one group of *Babesia* sp. (*Babesia* sp. UR2-like group) because those sequences of the amplified region of 18S rRNA gene were identical to each other. There was no report of *Theileria* spp. detected from bears. However, several *Theileria* spp. were detected from sika deer (*Cervus nippon*) that share habitats with bears in Japan (Elbaz et al. 2017; Watanabe et al. 2016). In Hokkaido, *Theileria* sp. Thrivae is the common pathogen that is frequently seen in Hokkaido sika deer (*Cervus nippon yesoensis*) (Lee et al., 2019).

Hepatozoon spp. are classified as Hepatozoidae, and usually infected through ingestion of hematophagous arthropods, such as mites, ticks, or insects that contain sporulated oocysts (Smith 1996). Schizogony occurs in various organs of the intermediate hosts, and merozoites invade leukocytes in mammals. Then,

merozoites become gametocytes (Smith 1996). *Hepatozoon ursi* was detected from Japanese black bears in Gifu prefecture with the prevalence of 100% (44/44) in 2008 (Kubo et al. 2010). Also, in the previous research conducted in Iwate prefecture in 2011, *H. ursi* was detected from 119 Japanese black bears out of 156 (76.3%) (Ikawa et al. 2011). On the other hand, several *Hepatozoon* spp. other than *H. ursi* were detected from rodents in Hokkaido (Moustafa et al. 2017) while there was no report of *H. ursi* from Hokkaido brown bears, except for the histological report that a small number of schizonts of *Hepatozoon* spp. were observed in the lung of one Hokkaido brown bear (4.0%, 1/25) (Kubo et al. 2010).

The purpose of this study was to reveal the role of wild bears in transmission of TBPs. Here, TBPs infecting Hokkaido brown bears and Japanese black bears were detected by using the reverse line blot (RLB) hybridization and nested PCR. Several apicomplexan protozoa, which have been detected from bears, were targeted. Other piroplasms, which were considered as zoonotically and/or economically important protozoa, were also included.

2. Materials and Methods

2.1. Bear blood samples and DNA extraction

Whole blood samples were obtained from a total of 49 Hokkaido brown bears from Shiretoko peninsula, eastern part of Hokkaido Island, during 2010 to 2018. In addition, a total of 18 and 66 Japanese black bears were captured from a wide area around Karuizawa town of Nagano and Ashio area of Tochigi,

157 respectively, during 2017 to 2018 (Fig. 1). Hokkaido brown bears and Japanese
158 black bears were caught for study or killed for nuisance control. Blood samples
159 were collected from the jugular or cephalic veins of anesthetized bears or from
160 hearts of killed bears as soon after death as possible in Na-EDTA tubes and kept
161 at －20 ℃ until DNA extraction. Total DNA was extracted from 300 µl of each
162 blood sample with the Wizard® Genomic DNA Purification Kit (Promega,
163 Madison, WI, USA) as the manufacturer's protocol and stored at －20 ℃ until
164 analysis.
165 2.2. Touchdown PCR and reverse line blot (RLB) hybridization
166 The extracted DNA samples were examined by touchdown PCR and RLB. The
167 PCR System 9700 (Applied Biosystems, Foster City, CA, USA), KOD-Plus-
168 Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Osaka, Japan) and the
169 primer pair RLB-F2 and RLB-R2 (Matjila et al. 2004) were used to amplify a
170 fragment of the 18S rRNA gene of apicomplexan protozoa. The PCR mixtures
171 consisted of 1.5 µl of 10×KOD-Plus-Neo buffer, 1.5 µl of dNTPs (2 mM), 0.9
172 µl of 25 mM $MgSO_4$, 0.45 µl of each primer (10 pmol/µl), 0.3 µl of KOD-Plus-
173 Neo DNA Polymerase, 1.0 µl of DNA and 8.9 µl of RNase-free water, with
174 total volume of 15 µl. The PCR conditions consisted of 94 ℃ for 5 min
175 followed by a touchdown step of 10 cycles of 10 sec at 94 ℃, 30 sec at 67 ℃,
176 30 sec at 68 ℃ with the annealing temperature decreasing every cycle by 1 ℃.
177 This was followed by 40 cycles of 10 sec at 94 ℃, 30 sec at 57 ℃, 30 sec at

68 ℃ and a final extension step of 7 min at 68 ℃. PCR products were examined by electrophoresis using a 1% agarose gel stained with ethidium bromide and visualized by UV illuminator.

RLB was carried out with the PCR products as described before (Elbaz et al. 2017; Kong and Gilbert 2006; Moustafa et al. 2016). Briefly, a 15 × 15 cm Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated by 20 ml 16% 1-ethy-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Subsequently, the membrane was washed gently with Milli-Q water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, Massachusetts). Oligonucleotide probes with C6 amino linker were obtained from Sigma Aldrich Co., LLC, Japan. A volume of 5 µl of each 100 pmol/µl probes were diluted in 0.5 M $NaHCO_3$ to a final volume of 170 µl. The slots of the miniblotter were filled with the 150 µl of each diluted oligonucleotide and the membrane was incubated at room temperature for 5 min. The membrane was inactivated in 250 ml 0.1M NaOH with gentle shaking for 8 min at room temperature, then washed with prewarmed 250 ml of 2×SSPE/0.1% SDS for 5 min at 60 ℃. Then the membrane was directly used or was stored in a sealed plastic bag with 15 µl of 20 mM EDTA, pH 8.

A volume of 10 µl of each PCR product was diluted in 2× SSPE/0.1% SDS to a final volume of 170 µl and heat-denatured at 99.9 ℃ for 10 min and immediately cooled on ice. A volume of 150 µl of the diluted PCR products

were applied into the slots of miniblotter, which contained the prepared membrane, and hybridized at 60 ℃ for 1 hr. The membrane was washed twice with 250 ml of 2× SSPE/0.5% SDS for 10 min at 52 ℃ and then incubated with diluted peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Walyham, MA, USA) for 1 hr at 42 ℃. Subsequently, the membrane was washed with 2× SSPE/0.5% SDS twice at 42 ℃ for 10 min and 2× SSPE twice at room temperature for 5 min to wash away the excess of NeutrAvidin. Finally, the membrane was incubated with 15 ml Immobilon TM Western Chemiluminescent HRP Substrate (Millipore, Japan) for 5 min at room temperature. The result was checked by Chemiluminescent imager (Billerica, MA, USA). For reuse, the membrane was washed twice in prewarmed 1% SDS at 90 ℃ for 30 min and washed with 250 ml of 20 mM EDTA at room temperature for 15 min. Finally, the membrane was stored in a plastic bag with 15 ml of 20 mM EDTA at 4 ℃ until next use.

To carry out RLB, we used a total of 32 oligonucleotide probes. Out of these, 6 probes were newly designed, and 17 probes were previously published (Table 1). Remaining 9 probes were previously designed and tested with the positive control, but the data was not published yet. New 6 probes were designed according to (Kong and Gilbert 2006). The sequences of 18S rRNA gene for target pathogens and the previously published ones of apicomplexan parasites were downloaded from the GenBank and aligned using MEGA (Molecular Evolutionary Genetics Analysis) software version 7 (Kumar et al. 2016). The

sites with polymorphic nucleotides were visually identified and used to design the species-specific probe sequences for each target pathogen and evaluated by OligoEvaluator software (Sigma Aldrich) to ensure optimum physical characteristics. The specificity of designed probes was checked by performing BLASTn (the Nucleotide Basic Local Alignment Search Tool) search and carrying out RLB against the positive controls.

2.3. Nested PCR

Nested PCR was carried out to amplify a segment of the 18S rRNA gene of each detected protozoan. The primer pair Piro0F/Piro6R (Kawabuchi et al. 2005) was used for the first-round PCR. The PCR mixture was prepared as mentioned above and the conditions consisted of 94 ºC for 5 min followed by 25 cycles of 40 sec at 94 ℃, 60 sec at 55 ℃, 60 sec at 72 ℃ and a final extension step of 5 min at 72 ℃.

To amplify the 18S rRNA gene of *Hepatozoon* spp., *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1, a total of three second-round PCRs were performed separately using 1.0 µl of the primary PCR products for each PCR and the primer sets, Hep F/Hep R (Inokuma et al. 2002), Bab-1F/Bab-2R and Cfnest F/Cfnest R (Nagamori et al. 2016), respectively (Table 2). Bab-1F/Bab-2R was newly designed for this study based on the sequence of *Babesia* sp. UR2-like group, *Babesia* sp. UR2 (AB704303), *Babesia* sp. Iwate248 (AB586027) and *Babesia* sp. EBB1021 (AB566229), by using Primer-BLAST

(Ye et al. 2012). The PCR profile of second-round PCR were same as that of the first-round PCR.

PCR products were examined by electrophoresis through 1% agarose gel stained with ethidium bromide and visualized by UV illuminator.

2.4. Sequence analysis of 18S rRNA gene

To confirm the results of RLB and nested PCR, some of the strongest positive samples for each protozoan were selected and sequenced. In addition to the three primer sets for *H. ursi*, *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1, new two primer sets were used to obtain the partial sequence of 18S rRNA gene of *Babesia* sp. UR1 and *B. microti*. Primer sets which were used are shown in Table 2. For *Babesia* sp. UR1, the same protocol was used as the previously mentioned nested PCR.

To obtain the sequence of *B. microti*, Bmt-F1 and RLB-R2 (Matjila et al. 2004) were used as a forward and reverse primers, respectively. The touch down PCR mixture and reaction was performed as mentioned above.

The PCR products were purified by Fast Gene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) and sequenced by the Big-Dye Terminator version 3.1 Cycle Sequencing Kit and ABI PRISM™ 310 genetic analyzer (Life Technologies Co., NY, USA). The obtained sequences were identified by using BLASTn search. We submitted the obtained sequences to DDBJ, and the accession numbers were LC431841 – LC431855.

2.5. Sequence analysis of β-tubulin gene

For further molecular characterization of *Babesia* sp. UR2-like group, a partial segment of the β-tubulin gene was sequenced. To avoid sequencing the untargeted TBPs other than *Babesia* sp. UR2-like group, samples that were positive for *Babesia* sp. UR2-like group, but negative for other *Babesia* or *Theileria* spp. were selected. In this study, a total of three positive samples, including one from a brown bear and two from black bears, were selected and sequenced. To amplify the β-tubulin gene, touchdown nested PCR was conducted and F34/R323 primers were used for the first-round PCR and F79/R206 primers for the second-round PCR (Caccio et al. 2000).

The first-round PCR mixture and reaction was performed as mentioned above with the annealing temperature of 62 ℃. For the nested PCR, 2.0 μl of primary PCR products were used. The obtained sequences were submitted to DDBJ and the accession numbers were LC432489 – LC432491.

2.6. Statistical analyses

Statistical analyses were performed to investigate the relevance of infection rate of each protozoon with methods to use, animal species, study area, sex, age class, and capture season. The results of *H. ursi*, *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1 by nested PCR and the results of *Babesia* sp. UR1 and *B. microti* by RLB hybridization were used for statistical analyses. Bears were divided into two groups, “Cub and Subadult (y≦3)” and “Adult (y>3)”, using

the estimated age class. The age class of female bears was determined by the body size and the existence of their young cubs. The age class of males was determined by body size only. In some cases, the age class was also estimated by analysis of cementum annuli present in the teeth (Fancy 1980). Cubs were not distinguished from subadults because of the small sample size of cubs. In addition, the capture season was separated into three groups, “Spring (from April to June)”, “Summer (from July to August)” and “Autumn (from September to November)” by the capture date of bears. Statistical analyses were performed using chi-square test or Fisher’s exact test when appropriate. P value less than 0.05 were considered significant. Data was analyzed using R software version 3.5.1 (The R Foundation for Statistical Computing, www.R-project.org).

2.7. Phylogenetic analyses

The obtained sequences were aligned with the sequences of closely related species registered in GenBank by using the program Clustal W Alignment and were analyzed phylogenetically by using MEGA software version 7 (Kumar et al. 2016). The phylogenetic tree was constructed by the Maximum Likelihood model based on Kimura 2-parameter model with invariant sites (Kimura 1980). For the phylogenetic analysis, the best-fit substitution model was selected by MEGA program. The stability of phylogenetic tree was assessed by 1,000 bootstrap replications.

3. Results

3.1. Touchdown PCR and RLB hybridization

A total of 49 brown bears from Hokkaido, 18 Japanese black bears from Tochigi and 66 Japanese black bears from Nagano were examined by touchdown PCR and RLB hybridization to detect apicomplexan parasites (Fig. 2). For the detection of *Babesia* sp. UR2-like group, two probes, "*Babesia* sp. UR2-1" and "*Babesia* sp. UR2-2" were designed. The probe "*Babesia* sp. UR2-1" that was designed first, might have had a low sensitivity because of the presence of a very weak secondary structure. The probe "*Babesia* sp. UR2-2" was re-designed to solve this problem. The RLB results showed that 0% (0/49), 83.7% (41/49), 73.5% (36/49), 14.3% (7/49), and 0% (0/49) of brown bear blood DNA samples were positive for *H. ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and *B. microti*, respectively (Table 3 and Table 4). The prevalence in Japanese black bears from Tochigi were 100% (18/18), 44.4% (8/18), 100% (18/18), 44.4% (8/18) and 16.7% (3/18), and in Japanese black bears from Nagano were 80.3% (53/66), 34.8% (23/66), 95.5% (63/66), 18.2% (12/66) and 3.0% (2/66) for *H. ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and *B. microti*, respectively (Table 3 and Table 4). By the results of RLB, there was no difference of the number of positive samples between the two probes, "*Babesia* sp. UR2-1" and "*Babesia* sp. UR2-2".

3.2. Nested PCR

Nested PCR was carried out to confirm the prevalence of the most prevalent three protozoa in bear blood DNA samples. The prevalence of *H. ursi*, *Babesia* sp. UR2-like group, and *Cytauxzoon* sp. UR1 were 0% (0/49), 89.8% (44/49) and 91.8% (45/49) in Hokkaido brown bears, 94.4% (17/18), 44.4% (8/18) and 88.9% (16/18) in Japanese black bears from Tochigi, and 97.0% (64/66), 39.4% (26/66) and 97.0% (64/66) in Japanese black bears from Nagano, respectively (Table 3).

3.3. Statistical analyses

The prevalence of *H. ursi* in Japanese black bears by nested PCR were significantly higher than those by RLB hybridization ($P<0.05$) (Fig. 3). The results for *H. ursi*, *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1 by nested PCR were used for the statistical analyses.

While the infection rate of *H. ursi* in Japanese black bears (96.4%) was significantly higher than that in Hokkaido brown bears (0%) ($P<0.001$), the infection rate of *Babesia* sp. UR2-like group in Japanese black bears (40.5%) was significantly lower than that in Hokkaido brown bears (89.8%) ($P<0.001$) (Fig. 4). There was no significant difference in the prevalence of *Cytauxzoon* sp. UR1 between Japanese black bears and Hokkaido brown bears. In addition, the prevalence of each TBPs in Japanese black bears collected from different study areas were compared. The infection rate of *Babesia* sp. UR1 in Japanese black bears from Tochigi (44.4%) was significantly higher than that from Nagano (18.2%) ($P<0.05$) (Fig. 5). Positive correlations of the prevalence of TBPs with

age class were observed. The infection rate of *Cytauxzoon* sp. UR1 in “Adult” Hokkaido brown bears (97.4%) was significantly higher than that in “Cub and Subadult” brown bears (70.0%) ($P<0.05$) (Fig. 6). In addition, the prevalence of *H. ursi* and *Babesia* sp. UR2-like group were significantly higher in “Adult” Japanese black bears (100% and 49.1%, respectively), comparing to “Cub and Subadult” black bears (89.7% and 24.2%, respectively) ($P<0.05$ and $P<0.05$, respectively) (Fig. 7). No significant correlation of prevalence of TBPs with sex or capture season was observed.

3.4. Phylogenetic analyses

To confirm the results of RLB and nested PCR, representative positive samples for each protozoan were selected and sequenced. The partial sequences of 18S rRNA gene for the detected protozoa by RLB or nested PCR were obtained. The obtained sequences from positive samples for four previously detected protozoa (*H. ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1 and *Babesia* sp. UR1) showed 99-100% identity with the sequences of 18S rRNA gene of each protozoa. In addition, a 152-168 bp segment of the 18S rRNA gene was obtained by sequencing the nested PCR products of *B. microti* positive samples, and the obtained sequences were identical to the previously published 18S rRNA gene of *B. microti*. Although the strain of the detected *B. microti* was not revealed by sequencing due to the short length of the obtained sequences, the RLB results showed that the detected strain is *B. microti*-Otsu/Hobetsu strain.

In addition, partial sequences of the β-tubulin gene from three positive *Babesia* sp. UR2-like group samples were obtained (Fig. 8). The obtained sequences were identical to each other and exhibited 89 - 90% similarity with β-tubulin gene sequences of *B. divergens* from cattle (*Bos taurus*) in Ireland [AB860328]. However, the query coverage between the obtained sequences and similar sequences were very low (61%). The phylogenetic analysis showed that the obtained β-tubulin gene sequences were clustered with *Babesia* sp. FP130 (Accession number: DQ329139) from a Florida panther (*Puma concolor coryi*), and the similarity was 89% but the query coverage was 52%.

4. Discussion

In this study, the current infection status of some apicomplexan pathogens were clarified from blood DNA samples of Hokkaido brown bears and Japanese black bears by two molecular methods, RLB hybridization and nested PCR. The results of RLB showed that the genetic materials from *H. ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1, and *B. microti* were present in bear blood DNA samples. Out of the five protozoa, *H. ursi*, *Babesia* sp. UR2-like pathogens, and *Cytauxzoon* sp. UR1 were the most prevalent protozoa in bear species in Japan.

The partial sequencing of the 18S rRNA gene by using RLB hybridization primers has failed for most of the detected TBPs due to the high prevalence of *Cytauxzoon* sp. UR1 and high genetic homology among the targeted protozoa. To detect and sequence these TBPs separately, nested PCR was conducted by

using a total of 12 primers, including five newly designed primers. The prevalence of the most prevalent three protozoa (*H.ursi*, *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1) were determined by nested PCR in addition to RLB. The obtained higher prevalence of *H. ursi* by nested PCR was as expected, because the sequences of RLB-F2/RLB-R2, which were used as primer sets for RLB, were not identical to the sequence of *H. ursi* (Table 5). These sequence differences of RLB primers might have affected the efficiency of amplification of the 18S rRNA gene of *H. ursi*.

In this study, *B. microti* was detected from blood DNA samples of Japanese black bear for the first time. *B. microti* is frequently seen in small mammals like rodents and this protozoan is the causative agent of human babesiosis (Homer et al. 2000). While there was no report of *B. microti* from Japanese black bear, this pathogen was reported from American black bears in New Jersey (Shaw 2015; Zolnik et al. 2015) and Oklahoma (Skinner et al. 2017), USA. In this study, it is suggested that Japanese black bears have a role in the life cycle of *B. microti*, which is one of the most important zoonotic pathogens. The expansion of bear populations may increase the chance of contact between bears and humans, which might contribute in transmitting this zoonotic pathogen to humans. In Japan, the first index case of human babesiosis via a transfusion of blood was found in 1999 (Matsui et al. 2000). Furthermore, several strains of *B. microti* were detected, for example: Kobe strain, Hobetsu strain, Otsu strain, and U.S. strain (Matsui et al. 2000; Saito-Ito et al. 2004; Wei et al. 2001; Zamoto et al.

2004a). Although this study could not identify the most closely related strain of the detected *B. microti* by sequencing, the detected strain was similar to *B. microti* Otsu strain (AB119446) or *B. microti* Hobetsu strain (AB050732) because the samples were positive for both "*Babesia microti* all" probe and "*Babesia microti* Otsu and Hobetsu type" probe by RLB hybridization.

By nested PCR, the infection rate of *H. ursi* in Japanese black bears were approximately 100% and were significantly higher than that in Hokkaido brown bears. The high prevalence of this protozoa in this study and previous studies conducted in Gifu and Iwate prefectures (Ikawa et al. 2011; Kubo et al. 2008) suggests that *H. ursi* is very common in Japanese black bears in Honshu Island of Japan. *Hepatozoon* species are transmitted by ingestion of arthropods that contain sporulated oocysts (Smith 1996). In addition to transmission by ingestion of vector hosts, it was suggested that vertical transmission and the predator-prey cycles could occur in the life cycle of *Hepatozoon* spp. (Johnson et al. 2008; Johnson et al. 2009; Murata et al. 1993). The chronic infection of *Hepatozoon* spp. has also been reported (Baneth 2011). The high prevalence of *H. ursi* in Japanese black bears may suggest that the above-mentioned infections occur in the transmission cycles of *H. ursi* in Japan.

H. ursi was not detected from Hokkaido brown bears in this study (0%, 0/51). This result suggested that *H. ursi* is rarely present in eastern part of Hokkaido. The habitat of the Hokkaido brown bears from which *Hepatozoon* spp. were

441 histologically detected was the south part of Hokkaido (4%, 1/25) (Kubo et al.
442 2010), which is geographically far from this study area. A further study is
443 required to confirm the presence or absence of *H. ursi* in Hokkaido brown bears
444 from south part of Hokkaido by using the molecular techniques.
445 The infection rate of *Babesia* sp. UR2-like group in brown bears was
446 significantly higher than that in black bears. This difference could be caused by
447 several factors, including the differences in susceptibility to this protozoan
448 group and the difference of tick abundance. In a previous study conducted in
449 Iwate prefecture, the infection rate of *Babesia* sp. Iwate248 (one of the *Babesia*
450 sp. UR2-like group) was 14.1% (22/156) in Japanese black bears (Ikawa et al.
451 2011). This infection rate is lower than those obtained from all study area in this
452 study. The difference of prevalence could be explained by the difference of
453 detection methods with different sensitivity. It is also possible that the
454 expanding spread of vector ticks and TBPs nowadays might have increased the
455 prevalence of this protozoan.
456 The infection rate of *Cytauxzoon* sp. UR1 was very high in bears from all study
457 areas. This result suggested that *Cytauxzoon* sp. UR1 is one of the most
458 common protozoa in bear species in Japan. Although there is no report of
459 infection routes other than tick biting (e.g. transovarial transmission), it was
460 previously suggested that infection of *Cytauxzoon* spp. could become chronic
461 and the host could carry the pathogen for whole life after infection (Zieman et

462 al. 2017). This chronic infection could be the cause of the high prevalence of
463 *Cytauxzoon* sp. UR1 in Japanese bears.
464 There were significant differences of prevalence of *Babesia* sp. UR1 between
465 different study areas. The prevalence of these protozoa were higher in Japanese
466 black bears from Tochigi, comparing to those from Nagano. In addition, the
467 infection rate of *B. microti* in Japanese black bears from Tochigi was higher
468 than that in bears from Nagano (P=0.06). In fact, the infection rate of *B. microti*
469 in Tochigi is so high if compared to previous reports of *B. microti* in wild
470 medium and large sized mammals, such as raccoon in Japan and American
471 black bears in USA (Kawabuchi et al. 2005; Shaw 2015; Skinner et al. 2017).
472 The high deer density could be one of the factors that increase the prevalence of
473 TBPs in Tochigi. According to latest governmental reports from Tochigi and
474 Nagano prefectures, it was suggested that the estimated deer density in the study
475 area of Tochigi (12.24 deer/km^2), which was estimated by compartment method
476 is higher than the mean deer density of the study area of Nagano (Mean:
477 5.50/km^2) . The high density of deer, which act as vector tick amplifiers, might
478 increase tick abundance and subsequently could increase the risk of infection
479 with TBPs, including even TBPs of which deer do not act as hosts (Bolzoni et
480 al. 2012; Kilpatrick et al. 2014; Mysterud et al. 2016).
481 The prevalence of *Cytauxzoon* sp. UR1 in brown bears had a positive
482 correlation with the age class. In addition, the prevalence of *H. ursi* and *Babesia*
483 sp. UR2-like group in black bears were also higher in “Adult” bears. In a

previous study, the prevalence of *Babesia* spp. were higher in adult American black bears (Shaw 2015). According to these findings, adult bears might have more opportunities to be infested by ticks than younger bears. In previous studies on tick burden of cattle, adult cattle bore significantly higher tick burden than juvenile cattle and calves (Lorusso et al. 2013; Rehman et al. 2017), and it was suggested that adult cattle could have more chances to carry ticks (Swai et al. 2005). Some factors such as the smaller body surface area of younger animals and the frequent chances to be groomed by their mothers could explain this phenomenon (Mooring et al. 2000). Also, the chronic infection of *Hepatozoon* spp. and *Cytauxzoon* spp. was suspected to occur (Baneth 2011; Zieman et al. 2017). This could explain the high prevalence of *H. ursi* and *Cytauxzoon* sp. UR1 in “Adult” bears because the probability to encounter pathogens increases as individuals live longer (Behnke et al. 1999).

The pathogenicity of the detected protozoa in bears has not been completely clarified. It is supposed that the infections of piroplasmid and *Hepatozoon* spp. in wild healthy animals are normally subclinical (East et al. 2008; McCully et al. 1975; Penzhorn 2006; Shock et al. 2011). However, the concomitant infection of pathogens or the exposure to highly stressful conditions potentially weakens the immune system of animals, and in such immune-compromised animals, the clinical symptoms may occur (Baneth et al. 1998; Kubo et al. 2006; Yabsley et al. 2005).

This study obtained a partial sequence of β-tubulin gene for the first time from *Babesia* sp. UR2-like group. The obtained three sequences were identical to each other and had similarity with the other *Babesia* spp., which suggests that the obtained sequences were a segment of the β-tubulin gene sequence of *Babesia* sp. UR2-like group. It is considered that the information of multiple genes is important and useful to detect and distinguish piroplasm species, which exhibit similar genotypes.

5. Conclusion

This study revealed that wild Japanese bear species were infected with a total of five tick-borne protozoa, including *H. ursi*, *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1, and *B. microti*. *H. ursi*, *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1 were the most prevalent three protozoa. This result should provide a basis for investigating *Cytauxzoon* sp. in domestic cats in Japan. In addition, *B. microti*, which is one of the zoonotically important pathogens, was detected from Japanese black bears for the first time. Furthermore, the prevalence of the detected protozoa in bears were modulated by animal species, habitats and age class of bears. This study suggests that Japanese bear species contribute in the transmission of several piroplasms.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Tables & Figures

Table 1

Probe list used in this study

Name	Sequence (5'→3')	Reference
Babesia, Theileria and *Hepatozoon* catch all	TAATGGTTAATAGGARCRGTWG	(Moustafa et al. 2016)
Theileria spp. all	ATTAGAGTGCTCAAAGCAGGC	(Matjila et al. 2008)
Babesia spp. all 1	ATTAGAGTGTTTCAAGCAGAC	(Matjila et al. 2008)
Babesia spp. all 2	ACTAGAGTGTTTCAAACAGGC	(Matjila et al. 2008)
Hepatozoon catch all	GCTTTGTAATTGGAATGATAGA	This study
Hepatozoon ursi	TTTAGCAATAGCGTCCTTTGA	This study
Cytauxzoon sp. UR1	TTTAAACCCTTTCCGGAGTAT	This study
Babesia sp. UR2-1	GATTTCTGCGTTATCGATTT	This study
Babesia sp. UR2-2	TTTCTGCGTTATCGATTTC	This study
Babesia sp. UR1	AATTTCTGCGTTCCCTCTT	This study
B. gibsoni	TACTTGCCTTGTCTGGTTT	(Matjila et al. 2008)
B. bigemina	CTCGTAGTTGTATTTCAGCCT	(Elbaz et al. 2017)
Babesia sp. Bab-sd-1	TTGCGTGCTGTTTGCCGT	(Moustafa *et al*., unpublished data)
Babesia sp. Bab-sd-2	GTTGGCTTTTCTTATTACTTTGA	(Moustafa *et al*., unpublished data)
B. bovis	GAGCATGGAATAACCTTGTAT	(Elbaz et al. 2017)
B. divergens and *B. capreoli*	GGTGTTAATATTGACTRATGTCGAG	(Moustafa et al. 2016)
B. venatorum	GAGTTATTGACTCTTGTCTTTAA	(Gigandet et al. 2011)
B. divergens-like	TTAATCATAACWGATGTTTTG	(Elbaz et al. 2017)
B. duncani	AGTTGAACTTCTGCCGCTT	(Moustafa et al. 2016)
B. rodhaini	TGTGGATTAGTGCGCAAG	(Elbaz et al. 2017)
B. microti all	GRCTTGGCATCWTCTGGA	(Matjila et al. 2008)
B. microti US	GGGTACTATTTTCCAGGAT	(Elbaz et al. 2017)
B. microti Otsu and Hobetsu	GGGTACTGTTTCCAGGGT	(Elbaz et al. 2017)
B. microti like Raccoon	TGTTTTTCATATTTTTCCAGT	(Moustafa *et al*., unpublished data)
B. microti JM1	GGGTCTTTTTCCAGGATTTACT	(Moustafa *et al*., unpublished data)
B. microti Kobe	GTTCTATTTTCCGGGATTTACT	(Moustafa *et al*., unpublished data)
B. ovata	GTATTTCAGCCCGTCGTAT	(Moustafa *et al*., unpublished data)
Theileria orientalis / *buffeli* / *sergenti* 1	TTTGAGTTTGTTATTGTGG	(Elbaz et al. 2017)
Theileria orientalis / *buffeli* / *sergenti* 2	TTTCTGAGTTTGTTTTTGCG	(Moustafa *et al*., unpublished data)
Theileria sp. Thrivae	ACGAGTGTCTGTATTGCG	(Elbaz et al. 2017)
T. capreoli	ATACGAGTTTTTGCATTGTG	(Moustafa *et al*., unpublished data)
T. luwenshuni	TGATGAGTTGATGTATTGTGG	(Moustafa *et al*., unpublished data)

Table 2
Primer list which we used in this study

Name	Sequence (5'→3')	Reaction and/or use	Reference
RLB-F2	GACACAGGGAGGTAGTGACAAG	Touchdown PCR for RLB forward primer	(Gubbels et al. 1999; Matjila et al. 2004)
RLB-R2	CTAAGAATTTCACCTCTGACAGT	Touchdown PCR for RLB reverse primer and Touchdown PCR for *Babesia microti* reverse primer	
Piro0F	GCCAGTAGTCATATGCTTGTGTTA	Nested PCR outer forward primer	(Kawabuchi et al. 2005; Zamoto et al. 2004b)
Piro6R	CTCCTTCCTYTAAGTGATAAGGTTCAC	Nested PCR outer reverse primer	
Hep F	ATACATGAGCAAAATCTCAAC	Nested PCR *Hepatozoon* spp. specific forward primer	(Inokuma et al. 2002)
Hep R	CTTATTATTCCATGCTGCAG	Nested PCR *Hepatozoon* spp. specific reverse primer	
Bab1-F	TCTGCGTTATCGATTTCGTC	Nested PCR *Babesia* sp. UR2-like group specific forward primer	This study
Bab2-R	AGAAGCAGACCGTAACGGAG	Nested PCR *Babesia* sp. UR2-like group specific reverse primer	
Cfnest F	TCGCATTGCTTTATGCTGGCGATG	Nested PCR *Cytauxzoon* spp. specific forward primer	(Nagamori et al. 2016)
Cfnest R	GCCCTCCAATTGATACTCCGGAAA	Nested PCR *Cytauxzoon* spp. specific reverse primer	
BabUR1 F2	TTTGCGGGTTCGCTTTTGG	Nested PCR *Babesia* sp. UR1 specific forward primer	This study
BabUR1 R1	CCCTCTAAGAAGCAAGCCGA	Nested PCR *Babesia* sp. UR1 specific reverse primer	
Bmt F1	GGATTTGGTGCCTTCGGGTA	Touchdown PCR *Babesia microti* specific forward primer	This study
F34	TGTGGTAACCAGATYGGWGCCAA	Nested PCR for β-tubulin gene outer forward primer	(Caccio et al. 2000)
R323	TCNGTRTARTGNCCYTTRGCCCA	Nested PCR for β-tubulin gene outer reverse primer	
F79	GARCAYGGNATNGAYCCNGTAA	Nested PCR for β-tubulin gene inner forward primer	
R206	ACDGARTCCATGGTDCCNGGYT	Nested PCR for β-tubulin gene inner reverse primer	

Table 3
Prevalence of three of the most prevalent protozoa in bears by RLB and nested PCR

Area	RLB			Nested PCR		
	Hepatozoon ursi	*Babesia* sp. UR2-like group	*Cytauxzoon* sp. UR1	*Hepatozoon ursi*	*Babesia* sp. UR2-like group	*Cytauxzoon* sp. UR1
Hokkaido (N=49)	0% (0/49)	83.7% (41/49)	73.5% (36/49)	0% (0/49)	89.8% (44/49)	91.8% (45/49)
Tochigi (N=18)	100% (18/18)	44.4% (8/18)	100% (18/18)	94.4% (17/18)	44.4% (8/18)	88.9% (16/18)
Nagano (N=66)	80.3% (53/66)	34.8% (23/66)	95.5% (63/66)	97.0% (64/66)	39.4% (26/66)	97.0% (64/66)

Table 4
Prevalence of other protozoa in bears by RLB

Area	*Babesia* sp. UR1	*Babesia microti*
Hokkaido (N=49)	14.3% (7/49)	0% (0/49)
Tochigi (N=19)	44.4% (8/18)	16.7% (3/18)
Nagano (N=66)	18.2% (12/66)	3.0% (2/66)

Table 5

Sequence of RLB-F2/RLB-R2 primer which we used for RLB and attachment site of *H. ursi* (EU041717, EU041718, HQ829437).

Target	Sequence of primer for touchdown PCR and attachment site of *H. ursi* (5'→3')																						
RLB-F2	G	A	C	A	C	A	G	G	G	A	G	G	T	A	G	T	G	A	C	A	A	G	
H. ursi	-	C	A	T	A	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
RLB-R2	C	T	A	A	G	A	A	T	T	T	C	A	C	C	T	C	T	G	A	C	A	G	T
H. ursi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-

Fig. 1. Maps of study areas.

(A) Map of Japan. Gray area indicates each prefecture which include the study areas.

(B) Map of Hokkaido prefecture. Gray area is the study area (Shiretoko peninsula).

(C) Map of Nagano prefecture. Gray area is the study area (around Karuizawa town).

(D) Map of Tochigi prefecture. Gray area is the study area (Ashio area).

Fig. 2. A representative RLB result. RLB.1,43: *Babesia* sp. UR2 positive control (Black bear from Nagano); 2,42: Negative control; 3-21: Brown bear blood; 22-41: Black bear blood derived 18S rRNA partial sequences. Oligonucleotide probes are listed on the left of the figure.

Fig. 3. Relevance between prevalence of three of the most prevalent protozoa in bears by RLB hybridization and nested PCR. The prevalence of *H. ursi* in only Japanese black bears (N=84) were used for the statistical analysis because no brown bear individuals were positive for *H. ursi*. The prevalence of *Babesia* sp. UR2-like group and *Cytauxzoon* sp. UR1 were calculated using both brown bear and black bear samples (N=133). The prevalence of *H. ursi* by nested PCR were significantly higher than those by RLB ($P<0.05$).

*: Significant differences assessed by Fisher's exact test ($P<0.05$).

Fig. 4. Comparison of prevalence of the detected blood protozoa in Hokkaido brown bears and Japanese black bears. The infection rate of *H. ursi* in brown bears was significantly lower than that in black bears ($P<0.001$) while the prevalence of *Babesia* sp. UR2-like group in brown bears were significantly higher ($P<0.001$).

***: Significant differences assessed by Fisher's exact test ($P<0.001$).

Fig. 5. Prevalence of blood protozoa in Japanese black bears from Tochigi compared with black bears from Nagano. The prevalence of *Babesia* sp. UR1 in Japanese black bears from Tochigi were significantly higher ($P<0.05$).

*: Significant differences assessed by Fisher's exact test ($P<0.05$).

Fig. 6. Comparison of prevalence of blood protozoa in Hokkaido brown bears with age groups. The group of “Cub and Subadult” included individuals whose estimated age were three or less than three. The group of “Adult” included individuals whose estimated age were more than three. The infection rate of *Cytauxzoon* sp. UR1 was significantly higher in “Adult” brown bears ($P<0.05$).
*: Significant differences assessed by Fisher’s exact test ($P<0.05$).

Fig. 7. Comparison of prevalence of blood protozoa in Japanese black bears with age groups. The group of “Cub and Subadult” included individuals whose estimated age were three or less than three. The group of “Adult” included individuals whose estimated age were more than three. The infection rate of *H. ursi* and *Babesia* sp. UR2-like group were significantly higher in “Adult” black bears ($P<0.05$ and $P<0.05$, respectively).
*: Significant differences assessed by Fisher’s exact test ($P<0.05$).

Fig. 8. The phylogenetic tree based on the β-tubulin gene of *Babesia* species and *Theileria* species, constructed by the Maximum Likelihood method based on the Kimura 2-parameter model using MEGA ver.7 program. The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 32.30% sites). Numbers at the nodes are bootstrap values supported from 1,000 replications. The scale bar represents 0.05 nucleotide substitutions per nucleotide site. Bold letters indicate the sequences of the β-tubulin gene of *Babesia* sp. UR2-like group obtained in this study. *Babesia bovis* (L00978) was

used as an out-group. The obtained sequences were clustered with *Babesia* sp. detected from Florida panther in USA (DQ329139) with 89% similarity and 52% query cover.