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

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26

27 **Abstract**

28 Many tick-borne pathogens (TBPs) are present in wildlife. The objective of this
29 study is to reveal the role of wild bears in maintaining TBPs. A total of 49
30 brown bears (*Ursus arctos yesoensis*) from Hokkaido, and 18 Japanese black
31 bears (*Ursus thibetanus japonicus*) from Tochigi and 66 Japanese black bears
32 from Nagano were examined by two molecular methods, reverse line blot
33 (RLB) hybridization and nested PCR. A total of 5 TBPs (*Hepatozoon ursi*,
34 *Babesia* sp. UR2-like group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and
35 *Babesia microti*) were detected from bear blood DNA samples. *B. microti* was
36 detected from blood DNA samples of Japanese black bear for the first time,
37 with the prevalence of 6.0% (5/84). Out of detected pathogens, *H. ursi*, *Babesia*
38 sp. UR2 like pathogens and *Cytauxzoon* sp. UR1 were considered as three of the
39 most prevalent TBPs in bears. The prevalence of *H. ursi* were significantly
40 higher in Japanese black bear (0% vs 96.4%) while that of *Babesia* sp. UR2-like
41 group was higher in Hokkaido brown bears (89.8% vs 40.5%). The prevalence
42 of *Babesia* sp. UR1 were significantly higher in Japanese black bears from
43 Tochigi (44.4%), comparing to those from Nagano (18.2%). The prevalence of
44 the detected TBPs were significantly higher in adult bears, comparing to those
45 in younger bears. The present study suggests that Japanese bear species
46 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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Consent to participate: (Not applicable)

Consent for publication (Not applicable)

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Code availability (Not applicable)

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,

respectively, during 2017 to 2018 (Fig. 1). Hokkaido brown bears and Japanese black bears were caught for study or killed for nuisance control. Blood samples were collected from the jugular or cephalic veins of anesthetized bears or from hearts of killed bears as soon after death as possible in Na-EDTA tubes and kept at -20°C until DNA extraction. Total DNA was extracted from 300 μl of each blood sample with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) as the manufacturer's protocol and stored at -20°C until analysis.

2.2. Touchdown PCR and reverse line blot (RLB) hybridization

The extracted DNA samples were examined by touchdown PCR and RLB. The PCR System 9700 (Applied Biosystems, Foster City, CA, USA), KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Osaka, Japan) and the primer pair RLB-F2 and RLB-R2 (Matjila et al. 2004) were used to amplify a fragment of the 18S rRNA gene of apicomplexan protozoa. The PCR mixtures consisted of 1.5 μl of 10 $\times$ KOD-Plus-Neo buffer, 1.5 μl of dNTPs (2 mM), 0.9 μl of 25 mM MgSO_4 , 0.45 μl of each primer (10 pmol/ μl), 0.3 μl of KOD-Plus-Neo DNA Polymerase, 1.0 μl of DNA and 8.9 μl of RNase-free water, with total volume of 15 μl . The PCR conditions consisted of 94°C for 5 min followed by a touchdown step of 10 cycles of 10 sec at 94°C , 30 sec at 67°C , 30 sec at 68°C with the annealing temperature decreasing every cycle by 1°C . This was followed by 40 cycles of 10 sec at 94°C , 30 sec at 57°C , 30 sec at

178 68 °C and a final extension step of 7 min at 68 °C. PCR products were
179 examined by electrophoresis using a 1% agarose gel stained with ethidium
180 bromide and visualized by UV illuminator.

181 RLB was carried out with the PCR products as described before (Elbaz et al.
182 2017; Kong and Gilbert 2006; Moustafa et al. 2016). Briefly, a 15 × 15 cm
183 Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated
184 by 20 ml 16% 1-ethy-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC)
185 (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature.

186 Subsequently, the membrane was washed gently with Milli-Q water for 2 min
187 and placed in Miniblotter MN45 (Immunetics, Boston, Massachusetts).

188 Oligonucleotide probes with C6 amino linker were obtained from Sigma
189 Aldrich Co., LLC, Japan. A volume of 5 µl of each 100 pmol/µl probes were
190 diluted in 0.5 M NaHCO₃ to a final volume of 170 µl. The slots of the
191 miniblotter were filled with the 150 µl of each diluted oligonucleotide and the
192 membrane was incubated at room temperature for 5 min. The membrane was
193 inactivated in 250 ml 0.1M NaOH with gentle shaking for 8 min at room
194 temperature, then washed with prewarmed 250 ml of 2×SSPE/0.1% SDS for 5
195 min at 60 °C. Then the membrane was directly used or was stored in a sealed
196 plastic bag with 15 µl of 20 mM EDTA, pH 8.

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

200 were applied into the slots of miniblottedter, which contained the prepared
201 membrane, and hybridized at 60 °C for 1 hr. The membrane was washed twice
202 with 250 ml of 2× SSPE/0.5% SDS for 10 min at 52 °C and then incubated with
203 diluted peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Wallyham,
204 MA, USA) for 1 hr at 42 °C. Subsequently, the membrane was washed with 2×
205 SSPE/0.5% SDS twice at 42 °C for 10 min and 2× SSPE twice at room
206 temperature for 5 min to wash away the excess of NeutrAvidin. Finally, the
207 membrane was incubated with 15 ml Immobilon TM Western
208 Chemiluminescent HRP Substrate (Millipore, Japan) for 5 min at room
209 temperature. The result was checked by Chemiluminescent imager (Billerica,
210 MA, USA). For reuse, the membrane was washed twice in prewarmed 1% SDS
211 at 90 °C for 30 min and washed with 250 ml of 20 mM EDTA at room
212 temperature for 15 min. Finally, the membrane was stored in a plastic bag with
213 15 ml of 20 mM EDTA at 4 °C until next use.

214 To carry out RLB, we used a total of 32 oligonucleotide probes. Out of these, 6
215 probes were newly designed, and 17 probes were previously published (Table
216 1). Remaining 9 probes were previously designed and tested with the positive
217 control, but the data was not published yet. New 6 probes were designed
218 according to (Kong and Gilbert 2006). The sequences of 18S rRNA gene for
219 target pathogens and the previously published ones of apicomplexan parasites
220 were downloaded from the GenBank and aligned using MEGA (Molecular
221 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 °C for 5 min followed by 25 cycles of 40 sec at 94 °C, 60 sec at 55 °C, 60 sec at 72 °C and a final extension step of 5 min at 72 °C.

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 CfneF/CfneR (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.

266

267 2.5. Sequence analysis of β -tubulin gene

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

277 The first-round PCR mixture and reaction was performed as mentioned above
278 with the annealing temperature of 62 °C. For the nested PCR, 2.0 μ l of primary
279 PCR products were used. The obtained sequences were submitted to DDBJ and
280 the accession numbers were LC432489 – LC432491.

281 2.6. Statistical analyses

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

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

405 In this study, *B. microti* was detected from blood DNA samples of Japanese
406 black bear for the first time. *B. microti* is frequently seen in small mammals like
407 rodents and this protozoan is the causative agent of human babesiosis (Homer et
408 al. 2000). While there was no report of *B. microti* from Japanese black bear, this
409 pathogen was reported from American black bears in New Jersey (Shaw 2015;
410 Zolnik et al. 2015) and Oklahoma (Skinner et al. 2017), USA. In this study, it is
411 suggested that Japanese black bears have a role in the life cycle of *B. microti*,
412 which is one of the most important zoonotic pathogens. The expansion of bear
413 populations may increase the chance of contact between bears and humans,
414 which might contribute in transmitting this zoonotic pathogen to humans. In
415 Japan, the first index case of human babesiosis via a transfusion of blood was
416 found in 1999 (Matsui et al. 2000). Furthermore, several strains of *B. microti*
417 were detected, for example: Kobe strain, Hobetsu strain, Otsu strain, and U.S.
418 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

al. 2017). This chronic infection could be the cause of the high prevalence of *Cytauxzoon* sp. UR1 in Japanese bears.

There were significant differences of prevalence of *Babesia* sp. UR1 between different study areas. The prevalence of these protozoa were higher in Japanese black bears from Tochigi, comparing to those from Nagano. In addition, the infection rate of *B. microti* in Japanese black bears from Tochigi was higher than that in bears from Nagano ($P=0.06$). In fact, the infection rate of *B. microti* in Tochigi is so high if compared to previous reports of *B. microti* in wild medium and large sized mammals, such as raccoon in Japan and American black bears in USA (Kawabuchi et al. 2005; Shaw 2015; Skinner et al. 2017).

The high deer density could be one of the factors that increase the prevalence of TBPs in Tochigi. According to latest governmental reports from Tochigi and Nagano prefectures, it was suggested that the estimated deer density in the study area of Tochigi (12.24 deer/km²), which was estimated by compartment method is higher than the mean deer density of the study area of Nagano (Mean: 5.50/km²). The high density of deer, which act as vector tick amplifiers, might increase tick abundance and subsequently could increase the risk of infection with TBPs, including even TBPs of which deer do not act as hosts (Bolzoni et al. 2012; Kilpatrick et al. 2014; Mysterud et al. 2016).

The prevalence of *Cytauxzoon* sp. UR1 in brown bears had a positive correlation with the age class. In addition, the prevalence of *H. ursi* and *Babesia* sp. UR2-like group in black bears were also higher in “Adult” bears. In a

484 previous study, the prevalence of *Babesia* spp. were higher in adult American
485 black bears (Shaw 2015). According to these findings, adult bears might have
486 more opportunities to be infested by ticks than younger bears. In previous
487 studies on tick burden of cattle, adult cattle bore significantly higher tick burden
488 than juvenile cattle and calves (Lorusso et al. 2013; Rehman et al. 2017), and it
489 was suggested that adult cattle could have more chances to carry ticks (Swai et
490 al. 2005). Some factors such as the smaller body surface area of younger
491 animals and the frequent chances to be groomed by their mothers could explain
492 this phenomenon (Mooring et al. 2000). Also, the chronic infection of
493 *Hepatozoon* spp. and *Cytauxzoon* spp. was suspected to occur (Baneth 2011;
494 Zieman et al. 2017). This could explain the high prevalence of *H. ursi* and
495 *Cytauxzoon* sp. UR1 in “Adult” bears because the probability to encounter
496 pathogens increases as individuals live longer (Behnke et al. 1999).
497 The pathogenicity of the detected protozoa in bears has not been completely
498 clarified. It is supposed that the infections of piroplasmid and *Hepatozoon* spp.
499 in wild healthy animals are normally subclinical (East et al. 2008; McCully et
500 al. 1975; Penzhorn 2006; Shock et al. 2011). However, the concomitant
501 infection of pathogens or the exposure to highly stressful conditions potentially
502 weakens the immune system of animals, and in such immune-compromised
503 animals, the clinical symptoms may occur (Baneth et al. 1998; Kubo et al. 2006;
504 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.

6. References

- Baneth G (2011) Perspectives on canine and feline hepatozoonosis. Vet Parasitol 181(1):3-11 doi:10.1016/j.vetpar.2011.04.015
- Baneth G (2014) Tick-borne infections of animals and humans: a common ground. Int J Parasitol 44(9):591-6 doi:10.1016/j.ijpara.2014.03.011
- Baneth G, Aroch I, Tal N, Harrus S (1998) *Hepatozoon* species infection in domestic cats: a retrospective study. Vet Parasitol 79(2):123-33
- Behnke JM, Lewis JW, Zain SN, Gilbert FS (1999) Helminth infections in

548 *Apodemus sylvaticus* in southern England: interactive effects of host age,
 549 sex and year on the prevalence and abundance of infections. J Helminthol
 550 73(1):31-44
 551 Bolzoni L, Rosa R, Cagnacci F, Rizzoli A (2012) Effect of deer density on tick
 552 infestation of rodents and the hazard of tick-borne encephalitis. II:
 553 population and infection models. Int J Parasitol 42(4):373-81
 554 doi:10.1016/j.ijpara.2012.02.006
 555 Caccio S, Camma C, Onuma M, Severini C (2000) The beta-tubulin gene of
 556 *Babesia* and *Theileria* parasites is an informative marker for species
 557 discrimination. Int J Parasitol 30(11):1181-5
 558 de la Fuente J, Estrada-Pena A (2012) Ticks and tick-borne pathogens on the
 559 rise. Ticks Tick Borne Dis 3(3):115-6 doi:10.1016/j.ttbdis.2012.03.001
 560 East ML, et al. (2008) A *Hepatozoon* species genetically distinct from *H. canis*
 561 infecting spotted hyenas in the Serengeti ecosystem, Tanzania. J Wildl
 562 Dis 44(1):45-52 doi:10.7589/0090-3558-44.1.45
 563 Elbaz E, et al. (2017) Molecular identification and characterization of piroplasm
 564 species in Hokkaido sika deer (*Cervus nippon yessoensis*), Japan. Ticks
 565 Tick Borne Dis 8(5):802-807 doi:10.1016/j.ttbdis.2017.06.007
 566 Estrada-Pena A, Jongejan F (1999) Ticks feeding on humans: a review of
 567 records on human-biting Ixodoidea with special reference to pathogen
 568 transmission. Exp Appl Acarol 23(9):685-715
 569 Fancy SG (1980) Preparation of mammalian teeth for age determination by

570 cementum layers: a review. Wildlife Society Bulletin (1973-2006)
 571 8(3):242-248
 572 Gigandet L, Stauffer E, Douet V, Rais O, Moret J, Gern L (2011) Prevalence of
 573 three zoonotic *Babesia* species in *Ixodes ricinus* (Linne, 1758) nymphs in
 574 a suburban forest in Switzerland. Vector Borne Zoonotic Dis 11(4):363-6
 575 doi:10.1089/vbz.2010.0195
 576 Gubbels JM, et al. (1999) Simultaneous detection of bovine *Theileria* and
 577 *Babesia* species by reverse line blot hybridization. J Clin Microbiol
 578 37(6):1782-9
 579 Hazumi T, Maruyama N (1987) Movements and Habitat Use of Japanese black
 580 bears in Nikko. bears: Their biology and management 7:275-279
 581 doi:10.2307/3872634
 582 Homer MJ, Aguilar-Delfin I, Telford SR, 3rd, Krause PJ, Persing DH (2000)
 583 Babesiosis. Clin Microbiol Rev 13(3):451-69
 584 Ikawa K, Aoki M, Ichikawa M, Itagaki T (2011) The first detection of *Babesia*
 585 species DNA from Japanese black bears (*Ursus thibetanus japonicus*) in
 586 Japan. Parasitol Int 60(2):220-2 doi:10.1016/j.parint.2011.02.005
 587 Inokuma H, Okuda M, Ohno K, Shimoda K, Onishi T (2002) Analysis of the
 588 18S rRNA gene sequence of a *Hepatozoon* detected in two Japanese
 589 dogs. Vet Parasitol 106(3):265-71
 590 Izumiyama S, Shiraishi T (2004) Seasonal changes in elevation and habitat use
 591 of the Asiatic black bear (*Ursus thibetanus*) in the Northern Japan Alps.

592 Mammal Study 29(1):1-8 doi:10.3106/mammalstudy.29.1

593 Jinnai M, et al. (2010) Molecular evidence of the multiple genotype infection of
 594 a wild Hokkaido brown bear (*Ursus arctos yesoensis*) by *Babesia* sp.
 595 UR1. Vet Parasitol 173(1-2):128-33 doi:10.1016/j.vetpar.2010.06.018

596 Johnson EM, Allen KE, Breshears MA, Panciera RJ, Little SE, Ewing SA
 597 (2008) Experimental transmission of *Hepatozoon americanum* to rodents.
 598 Vet Parasitol 151(2-4):164-9 doi:10.1016/j.vetpar.2007.10.017

599 Johnson EM, Allen KE, Panciera RJ, Ewing SA, Little SE (2009) Experimental
 600 transmission of *Hepatozoon americanum* to New Zealand White rabbits
 601 (*Oryctolagus cuniculus*) and infectivity of cystozoites for a dog. Vet
 602 Parasitol 164(2-4):162-6 doi:10.1016/j.vetpar.2009.05.028

603 Katsuhiro Y, Koji, Y., Shinsuke, K., Chinatsu, K., Yui, N., Ami N. (2010) Ixodid
 604 ticks collected from Japanese black bears in the Northern Kanto district,
 605 central Japan (Arachnida, Acarina). Bulletin of Ibaraki Nature Museum
 606 13:81-84

607 Kawabuchi T, Tsuji M, Sado A, Matoba Y, Asakawa M, Ishihara C (2005)
 608 *Babesia microti*-like parasites detected in feral raccoons (*Procyon lotor*)
 609 captured in Hokkaido, Japan. J Vet Med Sci 67(8):825-7

610 Kilpatrick HJ, LaBonte AM, Stafford KC (2014) The relationship between deer
 611 density, tick abundance, and human cases of Lyme disease in a residential
 612 community. J Med Entomol 51(4):777-84

613 Kimura M (1980) A simple method for estimating evolutionary rates of base

614 substitutions through comparative studies of nucleotide sequences. J Mol
 615 Evol 16:111-120
 616 Kong F, Gilbert GL (2006) Multiplex PCR-based reverse line blot hybridization
 617 assay (mPCR/RLB)--a practical epidemiological and diagnostic tool. Nat
 618 Protoc 1(6):2668-80 doi:10.1038/nprot.2006.404
 619 Kubo M, et al. (2010) *Hepatozoon* sp. infection in Hokkaido brown bears
 620 (*Ursus arctos yesoensis*). Japanese Journal of Zoo and Wildlife Medicine
 621 15(2):111-113 doi:10.5686/jjzwm.15.111
 622 Kubo M, Miyoshi N, Yasuda N (2006) Hepatozoonosis in two species of
 623 Japanese wild cat. J Vet Med Sci 68(8):833-7
 624 Kubo M, et al. (2008) *Hepatozoon ursi* n. sp. (Apicomplexa: Hepatozoidae) in
 625 Japanese black bear (*Ursus thibetanus japonicus*). Parasitol Int
 626 57(3):287-94 doi:10.1016/j.parint.2008.01.002
 627 Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary
 628 Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol
 629 33(7):1870-4 doi:10.1093/molbev/msw054
 630 Leydet BF, Jr., Liang FT (2013) Detection of human bacterial pathogens in ticks
 631 collected from Louisiana black bears (*Ursus americanus luteolus*). Ticks
 632 Tick Borne Dis 4(3):191-6 doi:10.1016/j.ttbdis.2012.12.002
 633 Lorusso V, et al. (2013) Ixodid ticks of traditionally managed cattle in central
 634 Nigeria: where *Rhipicephalus (Boophilus) microplus* does not dare (yet?).
 635 Parasit Vectors 6:171 doi:10.1186/1756-3305-6-171

636 Matjila PT, Leisewitz AL, Oosthuizen MC, Jongejan F, Penzhorn BL (2008)
 637 Detection of a *Theileria* species in dogs in South Africa. Vet Parasitol
 638 157(1-2):34-40 doi:10.1016/j.vetpar.2008.06.025
 639 Matjila PT, Penzhorn BL, Bekker CP, Nijhof AM, Jongejan F (2004)
 640 Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in
 641 South Africa. Vet Parasitol 122(2):119-25
 642 doi:10.1016/j.vetpar.2004.03.019
 643 Matsui T, et al. (2000) First documentation of transfusion-associated babesiosis
 644 in Japan. Rinsho Ketsueki 41(8):628-34
 645 McCully RM, Basson PA, Bigalke RD, De Vos V, Young E (1975) Observations
 646 on naturally acquired hepatozoonosis of wild carnivores and dogs in the
 647 Republic of South Africa. Onderstepoort J Vet Res 42(4):117-33
 648 Medlock JM, et al. (2013) Driving forces for changes in geographical
 649 distribution of *Ixodes ricinus* ticks in Europe. Parasites & Vectors 6(1):1
 650 doi:10.1186/1756-3305-6-1
 651 Medlock JM, Leach SA (2015) Effect of climate change on vector-borne disease
 652 risk in the UK. Lancet Infect Dis 15(6):721-30 doi:10.1016/s1473-
 653 3099(15)70091-5
 654 Mooring MS, Benjamin JE, Harte CR, Herzog NB (2000) Testing the
 655 interspecific body size principle in ungulates: the smaller they come, the
 656 harder they groom. Anim Behav 60(1):35-45
 657 doi:10.1006/anbe.2000.1461

658 Morrison DA (2009) Evolution of the Apicomplexa: where are we now? Trends
659 in Parasitology 25(8):375-382
660 doi:<https://doi.org/10.1016/j.pt.2009.05.010>

661 Moustafa MAM, et al. (2015) Molecular characterization and specific detection
662 of *Anaplasma* species (AP-sd) in sika deer and its first detection in wild
663 brown bears and rodents in Hokkaido, Japan. Infect Genet Evol 36:268-
664 274 doi:10.1016/j.meegid.2015.09.027

665 Moustafa MAM, et al. (2017) First molecular detection and characterization of
666 *Hepatozoon* and *Sarcocystis* spp. in field mice and voles from Japan.
667 Parasitol Res 116(8):2321-2325 doi:10.1007/s00436-017-5505-z

668 Moustafa MAM, et al. (2016) Dynamics, co-infections and characteristics of
669 zoonotic tick-borne pathogens in Hokkaido small mammals, Japan. Ticks
670 and tick-borne diseases 7(5):922-928 doi:10.1016/j.ttbdis.2016.04.014

671 Murata T, Inoue M, Tateyama S, Taura Y, Nakama S (1993) Vertical
672 transmission of *Hepatozoon canis* in dogs. J Vet Med Sci 55(5):867-8

673 Mysterud A, Easterday WR, Stigum VM, Aas AB, Meisingset EL, Viljugrein H
674 (2016) Contrasting emergence of Lyme disease across ecosystems. Nat
675 Commun 7:11882 doi:10.1038/ncomms11882

676 Nagamori Y, Slovak JE, Reichard MV (2016) Prevalence of *Cytauxzoon felis*
677 infection in healthy free-roaming cats in north-central Oklahoma and
678 central Iowa. JFMS Open Rep 2(1):2055116916655174
679 doi:10.1177/2055116916655174

680 National Institute of Infectious Diseases (2017) Infectious diseases weekly
681 report Japan. 2017, week28.,
682 National Institute of Infectious Diseases (2016) Infectious diseases weekly
683 report Japan. 2016, week32. .
684 Penzhorn BL (2006) Babesiosis of wild carnivores and ungulates. Vet Parasitol
685 138(1-2):11-21 doi:10.1016/j.vetpar.2006.01.036
686 Rehman A, Nijhof AM, Sauter-Louis C, Schauer B, Staubach C, Conraths FJ
687 (2017) Distribution of ticks infesting ruminants and risk factors
688 associated with high tick prevalence in livestock farms in the semi-arid
689 and arid agro-ecological zones of Pakistan. Parasit Vectors 10(1):190
690 doi:10.1186/s13071-017-2138-0
691 Saito-Ito A, Yano Y, Dantrakool A, Hashimoto T, Takada N (2004) Survey of
692 rodents and ticks in human babesiosis emergence area in Japan: first
693 detection of *Babesia microti*-like parasites in *Ixodes ovatus*. J Clin
694 Microbiol 42(5):2268-70
695 Sato Y (2017) The future of urban brown bear management in Sapporo,
696 Hokkaido, Japan: a Review. Mammal Study 42: 17-30, 14.
697 Shaw M (2015) *Babesia* spp. in *Ursus americanus* (black bear) in New Jersey.
698 Northeastern naturalist v. 22(no. 3):pp. 451-458pp. 08-2015 v.22 no.3
699 doi:10.1656/045.022.0303
700 Shock BC, et al. (2011) Distribution and prevalence of *Cytauxzoon felis* in
701 bobcats (*Lynx rufus*), the natural reservoir, and other wild felids in

702 thirteen states. Vet Parasitol 175(3-4):325-30
 703 doi:10.1016/j.vetpar.2010.10.009
 704 Skinner D, Mitcham JR, Starkey LA, Noden BH, Fairbanks WS, Little SE
 705 (2017) Prevalence of *Babesia* spp., *Ehrlichia* spp., and tick infestations in
 706 Oklahoma black bears (*Ursus americanus*). J Wildl Dis 53(4):781-787
 707 doi:10.7589/2017-02-029
 708 Smith TG (1996) The genus *Hepatozoon* (Apicomplexa: Adeleina). J Parasitol
 709 82(4):565-85
 710 Swai ES, Mbise AN, Kessy V, Kaaya E, Sanka P, Loomu PM (2005) Farm
 711 constraints, cattle disease perception and tick management practices in
 712 pastoral Maasai community-Ngorongoro, Tanzania, Livestock Research
 713 for Rural Development vol 17 (2)
 714 Takahata C, Nishino S, Kido K, Izumiyama S (2013) An evaluation of habitat
 715 selection of Asiatic black bears in a season of prevalent conflicts. Ursus
 716 24(1):16-26 Tsunoda T CS, Yamazaki K (2001) Ticks of the Asiatic black
 717 bear, *Ursus thibetanus*, in the Okutama Mts., Central Japan. Bulletin of
 718 Ibaraki Nature Museum 4:101-102
 719 Vannier E, Gewurz BE, Krause PJ (2008) Human babesiosis. Infect Dis Clin
 720 North Am 22(3):469-88, viii-ix doi:10.1016/j.idc.2008.03.010
 721 Watanabe Y, Fukumoto S, Harasawa R (2016) Prevalence of tick-borne
 722 hemolytic microbes in free-living sika deer (*Cervus nippon*) captured in a
 723 deer-overcrowded area. Japanese Journal of Zoo and Wildlife Medicine

724 21(1):17-27 doi:10.5686/jjzwm.21.17

725 Wei Q, et al. (2001) Human babesiosis in Japan: isolation of *Babesia microti*-
726 like parasites from an asymptomatic transfusion donor and from a rodent
727 from an area where babesiosis is endemic. J Clin Microbiol 39(6):2178-
728 83 doi:10.1128/jcm.39.6.2178-2183.2001

729 Yabsley MJ, Nims TN, Savage MY, Durden LA (2009) Ticks and tick-borne
730 pathogens and putative symbionts of black bears (*Ursus americanus*
731 *floridanus*) from Georgia and Florida. J Parasitol 95(5):1125-8
732 doi:10.1645/ge-2111.1

733 Yabsley MJ, Quick TC, Little SE (2005) Theileriosis in a white-tailed deer
734 (*Odocoileus virginianus*) fawn. J Wildl Dis 41(4):806-9
735 doi:10.7589/0090-3558-41.4.806

736 Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012)
737 Primer-BLAST: a tool to design target-specific primers for polymerase
738 chain reaction. BMC Bioinformatics 13:134 doi:10.1186/1471-2105-13-
739 134

740 Zamoto A, Tsuji M, Kawabuchi T, Wei Q, Asakawa M, Ishihara C (2004a) U.S.-
741 type *Babesia microti* isolated from small wild mammals in Eastern
742 Hokkaido, Japan. J Vet Med Sci 66(8):919-26

743 Zamoto A, et al. (2004b) Epizootiologic survey for *Babesia microti* among
744 small wild mammals in northeastern Eurasia and a geographic diversity
745 in the beta-tubulin gene sequences. J Vet Med Sci 66(7):785-92

746 Zieman EA, Jimenez FA, Nielsen CK (2017) Concurrent examination of
747 bobcats and ticks reveals high prevalence of *Cytauxzoon felis* in Southern
748 Illinois. J Parasitol 103(4):343-348 doi:10.1645/16-133
749 Zolnik CP, Makkay AM, Falco RC, Daniels TJ (2015) American black bears as
750 hosts of blacklegged ticks (Acari: Ixodidae) in the Northeastern United
751 States. J Med Entomol 52(5):1103-10 doi:10.1093/jme/tjv092

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759 **Tables & Figures**

Table 1
Probe list used in this study

Name	Sequence (5'→3')	Reference
<i>Babesia</i> , <i>Theileria</i> and <i>Hepatozoon</i> catch all	TAATGGTTAATAGGARCRGTWG	(Moustafa et al. 2016)
<i>Theileria</i> spp. all	ATTAGAGTGCTCAAAGCAGGC	(Matjila et al. 2008)
<i>Babesia</i> spp. all 1	ATTAGAGTGTTC AAGCAGAC	(Matjila et al. 2008)
<i>Babesia</i> spp. all 2	ACTAGAGTGTTC A AAGCAGGC	(Matjila et al. 2008)
<i>Hepatozoon</i> catch all	GCTTTGTAATTGGAATGATAGA	This study
<i>Hepatozoon ursi</i>	TTTAGCAATAGCGTCCTTTGA	This study
<i>Cytauxzoon</i> sp. UR1	TTTAAACCCTTTCCGGAGTAT	This study
<i>Babesia</i> sp. UR2-1	GATTCTGCGTTATCGATTT	This study
<i>Babesia</i> sp. UR2-2	TTTCTGCGTTATCGATTC	This study
<i>Babesia</i> sp. UR1	AATTCTGCGTTCCCTCTT	This study
<i>B. gibsoni</i>	TACTTGCCTTGTCTGGTTT	(Matjila et al. 2008)
<i>B. bigemina</i>	CTCGTAGTTGTATTCAGCCT	(Elbaz et al. 2017)
<i>Babesia</i> sp. Bab-sd-1	TTGCGTGCTGTTTGCCGT	(Moustafa et al., unpublished data)
<i>Babesia</i> sp. Bab-sd-2	GTGGCTTTTCTTATTACTTTGA	(Moustafa et al., unpublished data)
<i>B. bovis</i>	GAGCATGGAATAACCTTGTAT	(Elbaz et al. 2017)
<i>B. divergens</i> and <i>B. capreoli</i>	GGTGTTAATATTGACTRATGTCGAG	(Moustafa et al. 2016)
<i>B. venatorum</i>	GAGTTATTGACTCTGTCTTTAA	(Gigandet et al. 2011)
<i>B. divergens</i> -like	TTAATCATAACWGATGTTTTG	(Elbaz et al. 2017)
<i>B. duncani</i>	AGTTGAACCTCTGCCGCTT	(Moustafa et al. 2016)
<i>B. rodhaini</i>	TGTGGATTAGTGCGCAAG	(Elbaz et al. 2017)
<i>B. microti</i> all	GRCTTGGCATCWTCTGGA	(Matjila et al. 2008)
<i>B. microti</i> US	GGGTACTATTTTCCAGGAT	(Elbaz et al. 2017)
<i>B. microti</i> Otsu and Hobetsu	GGGTACTGTTTCCAGGGT	(Elbaz et al. 2017)
<i>B. microti</i> like Raccoon	TGTTTTTCATATTTTCCAGT	(Moustafa et al., unpublished data)
<i>B. microti</i> JM1	GGGTCTTTTCCAGGATTTACT	(Moustafa et al., unpublished data)
<i>B. microti</i> Kobe	GTTCTATTTCCGGGATTTACT	(Moustafa et al., unpublished data)
<i>B. ovata</i>	GTATTTCCAGCCCGTCGTAT	(Moustafa et al., unpublished data)
<i>Theileria orientalis</i> / <i>buffeli</i> / <i>sergenti</i> 1	TTTGAGTTTGTTATTGTGG	(Elbaz et al. 2017)
<i>Theileria orientalis</i> / <i>buffeli</i> / <i>sergenti</i> 2	TTTCTGAGTTGTTTTTGCG	(Moustafa et al., unpublished data)
<i>Theileria</i> sp. Thrivae	ACGAGTGCTGTATTGCG	(Elbaz et al. 2017)
<i>T. capreoli</i>	ATACGAGTTTTTGCAATTGTG	(Moustafa et al., unpublished data)
<i>T. luwenshuni</i>	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	
RLB-R2	CTAAGAATTTACCTCTGACAGT	Touchdown PCR for RLB reverse primer and Touchdown PCR for <i>Babesia microti</i> reverse primer	(Gubbels et al. 1999; Matjila et al. 2004)
Piro0F	GCCAGTAGTCATATGCTTGTGTTA	Nested PCR outer forward primer	
Piro6R	CTCCTTCCTYTAAGTGATAAGGTTTAC	Nested PCR outer reverse primer	(Kawabuchi et al. 2005; Zamoto et al. 2004b)
Hep F	ATACATGAGCAAAATCTCAAC	Nested PCR <i>Hepatozoon</i> spp. specific forward primer	
Hep R	CTTATTATTCCATGCTGCAG	Nested PCR <i>Hepatozoon</i> spp. specific reverse primer	(Inokuma et al. 2002)
Bab1-F	TCTGCGTTATCGATTTGTC	Nested PCR <i>Babesia</i> sp. UR2-like group specific forward primer	
Bab2-R	AGAAGCAGACCGTAACGGAG	Nested PCR <i>Babesia</i> sp. UR2-like group specific reverse primer	This study
Cfnest F	TCGCATTGCTTTATGCTGGCGATG	Nested PCR <i>Cytauxzoon</i> spp. specific forward primer	
Cfnest R	GCCCTCCAATTGATACTCCGGA	Nested PCR <i>Cytauxzoon</i> spp. specific reverse primer	(Nagamori et al. 2016)
BabUR1 F2	TTTGCGGGTTCGCTTTTGG	Nested PCR <i>Babesia</i> sp. UR1 specific forward primer	
BabUR1 R1	CCCTCTAAGAAGCAAGCCGA	Nested PCR <i>Babesia</i> sp. UR1 specific reverse primer	This study
Bmt F1	GGATTTGGTGCCTTCGGGTA	Touchdown PCR <i>Babesia microti</i> specific forward primer	This study
F34	TGTGGTAACCAGATYGGWGCCAA	Nested PCR for β -tubulin gene outer forward primer	
R323	TCNGTRTARTGNCCYTTRGCCCCA	Nested PCR for β -tubulin gene outer reverse primer	
F79	GARCAYGGNATNGAYCCNGTAA	Nested PCR for β -tubulin gene inner forward primer	(Caccio et al. 2000)
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		
	<i>Hepatozoon ursi</i>	<i>Babesia</i> sp. UR2-like group	<i>Cytauxzoon</i> sp. UR1	<i>Hepatozoon ursi</i>	<i>Babesia</i> sp. UR2-like group	<i>Cytauxzoon</i> 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	<i>Babesia</i> sp. UR1	<i>Babesia microti</i>
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 <i>H. ursi</i> (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	
<i>H. ursi</i>	-	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
<i>H. ursi</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	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.