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1 **Molecular detection of apicomplexan protozoa in Hokkaido brown bears**
2 **(*Ursus arctos yesoensis*) and Japanese black bears (*Ursus thibetanus***
3 ***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

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

49

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

51 **Declarations**

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56 **Conflicts of interest/Competing interests:** The authors declare that they have
57 no competing interests.

58 **Ethics approval:** All procedures involved in sample collection from live
59 animals in Hokkaido were conducted in accordance with the Guidelines for
60 Animal Care and Use of Hokkaido University, and were approved by the
61 Animal Care and Use Committee of the Graduate School of Veterinary
62 Medicine, Hokkaido University (Permit Number: 1106, 1151, 15009, 17005 and
63 18-0083).

64 **Consent to participate:** (Not applicable)

65 **Consent for publication** (Not applicable)

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68 LC432491.

69 **Code availability** (Not applicable)

70

71

72 **1. Introduction**

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

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

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

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

113 transmitted to the animal hosts during the infestation of infected ticks on those
114 animals. Some *Babesia* spp. were reported to infect humans and cause human
115 babesiosis: e.g. *Babesia microti* and *B. microti*-like organisms, *Babesia duncani*
116 and *B. duncani*-type organisms, *Babesia divergens* and *B. divergens*-like
117 organisms, *Babesia venatorum*, and *Babesia* sp. KO1 (Vannier et al. 2008). To
118 the best of our knowledge, four sequences of *Babesia* spp. (*Babesia* sp. UR1
119 [AB48055], *Babesia* sp. UR2 [AB704303], *Babesia* sp. Iwate248 [AB586027]
120 and *Babesia* sp. EBB1021 [AB566229]) and one sequence of *Cytauxzoon* sp.
121 (*Cytauxzoon* sp. UR1 [AB480558]) have been detected from bear species in
122 Japan. Among them, *Babesia* sp. UR2, *Babesia* sp. Iwate248 and *Babesia* sp.
123 EBB1021 were considered as one group of *Babesia* sp. (*Babesia* sp. UR2-like
124 group) because those sequences of the amplified region of 18S rRNA gene were
125 identical to each other. There was no report of *Theileria* spp. detected from
126 bears. However, several *Theileria* spp. were detected from sika deer (*Cervus*
127 *nippon*) that share habitats with bears in Japan (Elbaz et al. 2017; Watanabe et
128 al. 2016). In Hokkaido, *Theileria* sp. Thrivae is the common pathogen that is
129 frequently seen in Hokkaido sika deer (*Cervus nippon yesoensis*) (Lee et al.,
130 2019).

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

135 merozoites become gametocytes (Smith 1996). *Hepatozoon ursi* was detected
136 from Japanese black bears in Gifu prefecture with the prevalence of 100%
137 (44/44) in 2008 (Kubo et al. 2010). Also, in the previous research conducted in
138 Iwate prefecture in 2011, *H. ursi* was detected from 119 Japanese black bears
139 out of 156 (76.3%) (Ikawa et al. 2011). On the other hand, several *Hepatozoon*
140 spp. other than *H. ursi* were detected from rodents in Hokkaido (Moustafa et al.
141 2017) while there was no report of *H. ursi* from Hokkaido brown bears, except
142 for the histological report that a small number of schizonts of *Hepatozoon* spp.
143 were observed in the lung of one Hokkaido brown bear (4.0%, 1/25) (Kubo et
144 al. 2010).

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

151 **2. Materials and Methods**

152 2.1. Bear blood samples and DNA extraction

153 Whole blood samples were obtained from a total of 49 Hokkaido brown bears
154 from Shiretoko peninsula, eastern part of Hokkaido Island, during 2010 to 2018.
155 In addition, a total of 18 and 66 Japanese black bears were captured from a wide
156 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 $-20\text{ }^{\circ}\text{C}$ until DNA extraction. Total DNA was extracted from $300\text{ }\mu\text{l}$ 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 $-20\text{ }^{\circ}\text{C}$ 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 $1.5\text{ }\mu\text{l}$ of $10\times$ KOD-Plus-Neo buffer, $1.5\text{ }\mu\text{l}$ of dNTPs (2 mM), 0.9
172 μl of 25 mM MgSO_4 , $0.45\text{ }\mu\text{l}$ of each primer (10 pmol/ μl), $0.3\text{ }\mu\text{l}$ of KOD-Plus-
173 Neo DNA Polymerase, $1.0\text{ }\mu\text{l}$ of DNA and $8.9\text{ }\mu\text{l}$ of RNase-free water, with
174 total volume of $15\text{ }\mu\text{l}$. The PCR conditions consisted of $94\text{ }^{\circ}\text{C}$ for 5 min
175 followed by a touchdown step of 10 cycles of 10 sec at $94\text{ }^{\circ}\text{C}$, 30 sec at $67\text{ }^{\circ}\text{C}$,
176 30 sec at $68\text{ }^{\circ}\text{C}$ with the annealing temperature decreasing every cycle by $1\text{ }^{\circ}\text{C}$.
177 This was followed by 40 cycles of 10 sec at $94\text{ }^{\circ}\text{C}$, 30 sec at $57\text{ }^{\circ}\text{C}$, 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 (Immunitics, 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, Walyham,
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

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

228

229 2.3. Nested PCR

230 Nested PCR was carried out to amplify a segment of the 18S rRNA gene of
231 each detected protozoan. The primer pair Piro0F/Piro6R (Kawabuchi et al.
232 2005) was used for the first-round PCR. The PCR mixture was prepared as
233 mentioned above and the conditions consisted of 94 °C for 5 min followed by
234 25 cycles of 40 sec at 94 °C, 60 sec at 55 °C, 60 sec at 72 °C and a final
235 extension step of 5 min at 72 °C.

236 To amplify the 18S rRNA gene of *Hepatozoon* spp., *Babesia* sp. UR2-like
237 group and *Cytauxzoon* sp. UR1, a total of three second-round PCRs were
238 performed separately using 1.0 µl of the primary PCR products for each PCR
239 and the primer sets, Hep F/Hep R (Inokuma et al. 2002), Bab-1F/Bab-2R and
240 Cfnest F/Cfnest R (Nagamori et al. 2016), respectively (Table 2). Bab-1F/Bab-
241 2R was newly designed for this study based on the sequence of *Babesia* sp.
242 UR2-like group, *Babesia* sp. UR2 (AB704303), *Babesia* sp. Iwate248
243 (AB586027) and *Babesia* sp. EBB1021 (AB566229), by using Primer-BLAST

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

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

248

249 2.4. Sequence analysis of 18S rRNA gene

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

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

260 The PCR products were purified by Fast Gene Gel/PCR Extraction Kit (Nippon
261 Genetics Co., Ltd., Tokyo, Japan) and sequenced by the Big-Dye Terminator
262 version 3.1 Cycle Sequencing Kit and ABI PRISM™ 310 genetic analyzer (Life
263 Technologies Co., NY, USA). The obtained sequences were identified by using
264 BLASTn search. We submitted the obtained sequences to DDBJ, and the
265 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

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

300

301 2.7. Phylogenetic analyses

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

310 3. Results

311 3.1. Touchdown PCR and RLB hybridization

312 A total of 49 brown bears from Hokkaido, 18 Japanese black bears from
313 Tochigi and 66 Japanese black bears from Nagano were examined by
314 touchdown PCR and RLB hybridization to detect apicomplexan parasites (Fig.
315 2). For the detection of *Babesia* sp. UR2-like group, two probes, “*Babesia* sp.
316 UR2-1” and “*Babesia* sp. UR2-2” were designed. The probe “*Babesia* sp. UR2-
317 1” that was designed first, might have had a low sensitivity because of the
318 presence of a very weak secondary structure. The probe “*Babesia* sp. UR2-2”
319 was re-designed to solve this problem. The RLB results showed that 0% (0/49),
320 83.7% (41/49), 73.5% (36/49), 14.3% (7/49), and 0% (0/49) of brown bear
321 blood DNA samples were positive for *H. ursi*, *Babesia* sp. UR2-like group,
322 *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and *B. microti*, respectively (Table 3 and
323 Table 4). The prevalence in Japanese black bears from Tochigi were 100%
324 (18/18), 44.4% (8/18), 100% (18/18), 44.4% (8/18) and 16.7% (3/18), and in
325 Japanese black bears from Nagano were 80.3% (53/66), 34.8% (23/66), 95.5%
326 (63/66), 18.2% (12/66) and 3.0% (2/66) for *H. ursi*, *Babesia* sp. UR2-like
327 group, *Cytauxzoon* sp. UR1, *Babesia* sp. UR1 and *B. microti*, respectively
328 (Table 3 and Table 4). By the results of RLB, there was no difference of the
329 number of positive samples between the two probes, “*Babesia* sp. UR2-1” and
330 “*Babesia* sp. UR2-2”.

331 3.2. Nested PCR

332 Nested PCR was carried out to confirm the prevalence of the most prevalent
333 three protozoa in bear blood DNA samples. The prevalence of *H. ursi*, *Babesia*
334 sp. UR2-like group, and *Cytauxzoon* sp. UR1 were 0% (0/49), 89.8% (44/49)
335 and 91.8% (45/49) in Hokkaido brown bears, 94.4% (17/18), 44.4% (8/18) and
336 88.9% (16/18) in Japanese black bears from Tochigi, and 97.0% (64/66), 39.4%
337 (26/66) and 97.0% (64/66) in Japanese black bears from Nagano, respectively
338 (Table 3).

339 3.3. Statistical analyses

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

344 While the infection rate of *H. ursi* in Japanese black bears (96.4%) was
345 significantly higher than that in Hokkaido brown bears (0%) ($P < 0.001$), the
346 infection rate of *Babesia* sp. UR2-like group in Japanese black bears (40.5%)
347 was significantly lower than that in Hokkaido brown bears (89.8%) ($P < 0.001$)
348 (Fig. 4). There was no significant difference in the prevalence of *Cytauxzoon* sp.
349 UR1 between Japanese black bears and Hokkaido brown bears. In addition, the
350 prevalence of each TBPs in Japanese black bears collected from different study
351 areas were compared. The infection rate of *Babesia* sp. UR1 in Japanese black
352 bears from Tochigi (44.4%) was significantly higher than that from Nagano
353 (18.2%) ($P < 0.05$) (Fig. 5). Positive correlations of the prevalence of TBPs with

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

362 3.4. Phylogenetic analyses

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

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

384 **4. Discussion**

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

393 The partial sequencing of the 18S rRNA gene by using RLB hybridization
394 primers has failed for most of the detected TBPs due to the high prevalence of
395 *Cytauxzoon* sp. UR1 and high genetic homology among the targeted protozoa.
396 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.

419 2004a). Although this study could not identify the most closely related strain of
420 the detected *B. microti* by sequencing, the detected strain was similar to *B.*
421 *microti* Otsu strain (AB119446) or *B. microti* Hobetsu strain (AB050732)
422 because the samples were positive for both “*Babesia microti* all” probe and
423 “*Babesia microti* Otsu and Hobetsu type” probe by RLB hybridization.

424

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

438 *H. ursi* was not detected from Hokkaido brown bears in this study (0%, 0/51).

439 This result suggested that *H. ursi* is rarely present in eastern part of Hokkaido.

440 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/km²), which was estimated by compartment method
476 is higher than the mean deer density of the study area of Nagano (Mean:
477 5.50/km²) . 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

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).

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

512

513 **5. Conclusion**

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

524

525 **Conflicts of interest**

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

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759 **Tables & Figures**

760 **Table 1**
761 **Probe list used in this study**
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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	ATTAGAGTGTTTCAAGCAGAC	(Matjila et al. 2008)
<i>Babesia</i> spp. all 2	ACTAGAGTGTTTCAAACAGGC	(Matjila et al. 2008)
<i>Hepatozoon</i> catch all	GCTTTGTAATTGGAATGATAGA	This study
<i>Hepatozoon ursi</i>	TTTAGCAATAGCGTCCTTTGA	This study
<i>Cytauxzoon</i> sp. UR1	TTTAAACCCCTTCCGGAGTAT	This study
<i>Babesia</i> sp. UR2-1	GATTCTGCGTTATCGATTT	This study
<i>Babesia</i> sp. UR2-2	TTTCTGCGTTATCGATTTT	This study
<i>Babesia</i> sp. UR1	AATTCTGCGTCCCTCTT	This study
<i>B. gibsoni</i>	TACTTGCCCTGTCTGGTTT	(Matjila et al. 2008)
<i>B. bigemina</i>	CTCGTAGTTGTATTCAGCCT	(Elbaz et al. 2017)
<i>Babesia</i> sp. Bab-sd-1	TTGCGTGCTGTTTGCCGT	(Moustafa <i>et al.</i> , unpublished data)
<i>Babesia</i> sp. Bab-sd-2	GTGGCTTTTCTTATTACTTTGA	(Moustafa <i>et al.</i> , unpublished data)
<i>B. bovis</i>	GAGCATGGAATAACCTTGAT	(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>	AGTTGAACTTCTGCCGCTT	(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 <i>et al.</i> , unpublished data)
<i>B. microti</i> JM1	GGGTCTTTTCCAGGATTTACT	(Moustafa <i>et al.</i> , unpublished data)
<i>B. microti</i> Kobe	GTCTATTTTCCGGGATTTACT	(Moustafa <i>et al.</i> , unpublished data)
<i>B. ovata</i>	GTATTTCCAGCCGTCGTAT	(Moustafa <i>et al.</i> , unpublished data)
<i>Theileria orientalis / buffeli / sergenti</i> 1	TTTGAGTTTGTATTGTGG	(Elbaz et al. 2017)
<i>Theileria orientalis / buffeli / sergenti</i> 2	TTTCTGAGTTTGTTTTTCG	(Moustafa <i>et al.</i> , unpublished data)
<i>Theileria</i> sp. Thrivae	ACGAGTGTCTGTATTGCG	(Elbaz et al. 2017)
<i>T. capreoli</i>	ATACGAGTTTTTGCATTGTG	(Moustafa <i>et al.</i> , unpublished data)
<i>T. luwenshuni</i>	TGATGAGTTGATGTATTGTGG	(Moustafa <i>et al.</i> , unpublished data)

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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	CTCCTTCTTYTAAGTGATAAGGTTTAC	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	TCTGCGTTATCGATTTTCGTC	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	GCCCTCCAATTGATACTCCGGAAA	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	TCNGTRTARTGNCCYTTRGCCCA	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.