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1 Utilizing the attached hard ticks as pointers to the risk of infection by *Babesia* and *Theileria*
2 species in sika deer (*Cervus nippon yesoensis*), Japan
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24 **Abstract**

25 Ticks are hematophagous ectoparasites that have a significant impact on their animal hosts.
26 Moreover, along with mosquitoes, they are the main arthropod vectors of disease agents in
27 domestic animals, wildlife and humans. To investigate the occurrence and prevalence of
28 piroplasmids in the ticks, DNA was extracted from 519 hard ticks collected from 116 hunted
29 Hokkaido sika deer (*Cervus nippon yesoensis*). The success of the DNA extraction was
30 confirmed by touchdown PCR targeting the mitochondrial 16S rDNA gene of ticks. Touchdown
31 PCR and reverse line blot (RLB) hybridization targeting the 18S rRNA gene were used to detect
32 14 piroplasm species. All hard ticks parasitizing Hokkaido sika deer were identified as belonging
33 to the genera *Ixodes* and *Haemaphysalis*. A total of 163 samples (31.4%) were positive for
34 *Babesia* and *Theileria* spp. among different tick species according to RLB hybridization. The
35 DNA from 27.0% (140/519), 10.6% (55/519), 1.7% (9/519), 0.6% (3/519), 0.4% (2/519), and
36 0.4% (2/519) of ticks hybridized to the *Theileria* sp. Thrivae, *Theileria capreoli*, *Babesia*
37 *divergens*-like, *Babesia* sp. (Bab-SD), *Babesia microti* U.S., and *B. microti* Hobetsu
38 oligonucleotide probes, respectively. The partial sequencing and phylogenetic analyses of the
39 18S rRNA gene confirmed the RLB hybridization results. Further investigations are needed to
40 reveal the epidemiology and respective vectors of these pathogens.

41 **Keywords**

42 *Babesia*, Hard ticks, Reverse line blot, Sika deer, *Theileria*

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47 **Introduction**

48 Ticks are hematophagous ectoparasites that have significant impacts on their animal
49 hosts, such as by causing anemia and direct skin tissue damage. Moreover, along with
50 mosquitoes, they are the main arthropod vectors of disease agents in domestic animals and
51 humans (Dantas-Torres et al. 2012; Ferrolho et al. 2016; Masatani et al. 2017). Two major
52 families of ticks, Ixodidae (hard ticks) and Argasidae (soft ticks), which include more than 900
53 tick species, have been recorded globally (Chen et al. 2014). In Japan, 47 tick species have been
54 reported, of which 21 parasitize humans (Takano et al. 2014).

55 The incidence of tick-borne diseases (TBDs) and the emergence of novel TBD agents
56 have been increasing worldwide during the past two decades (Kernif et al. 2016; Piesman and
57 Eisen 2008). Hence, it is important to investigate the prevalence of tick-borne pathogens to
58 develop effective control strategies against TBDs. Tick-borne apicomplexan parasites, such as
59 *Babesia* and *Theileria* spp., are of great importance worldwide, as they cause diseases in
60 domestic and wild animals. In addition, some *Babesia* spp., such as *Babesia microti*, *Babesia*
61 *divergens*, and *B. divergens*-like, are considered zoonotic pathogens (Andersson et al. 2017).

62 In Japan, many studies have identified several *Babesia* and *Theileria* spp. in domestic
63 animals (Fujinaga 1981; Kim et al. 2004; Minami and Ishihara 1980; Ota et al. 2009; Sivakumar
64 et al. 2012), wild animals (Elbaz et al. 2017; Ikawa et al. 2011; Inokuma et al. 2004; Watanabe et
65 al. 2016) and in humans (Wei et al. 2001). These studies have provided information about the
66 potential vector hosts for *Babesia* and *Theileria* spp. in Japan. For example, *Haemaphysalis*
67 *longicornis* and *Ixodes ovatus* act as vectors for *Babesia ovata* (Sivakumar et al. 2014), and
68 *Ixodes persulcatus*, *Haemaphysalis megaspinosa*, *Haemaphysalis douglasi*, and *Ixodes ovatus*

69 act as vectors for *Theileria orientalis* (Yokoyama et al. 2012); zoonotic *Babesia* spp. such as *B.*
70 *microti* U.S. and Hobetsu strains have been detected in *I. ovatus* and *I. persulcatus*, respectively
71 (Zamoto-Niikura et al. 2012), and *B. divergens*-like in *I. persulcatus* (Zamoto-Niikura et al.
72 2018). Other studies have identified piroplasm species from ticks feeding on animals (Inokuma
73 et al. 2003; Iwakami et al. 2014; Masatani et al. 2017; Tatenno et al. 2015).

74 Moreover, the same tick may be co-infected with different piroplasm species (Abdallah et
75 al. 2017) or various other tick-borne pathogens that may have important implications for
76 transmitting pathogens to the animal host (Moutailler et al. 2016). Disease severity may be
77 enhanced if humans or animals are bitten by co-infected ticks, as reported for concurrent
78 babesiosis and Lyme diseases (Golightly et al. 1989; Grunwaldt et al. 1983). This could also
79 have serious consequences for the diagnosis and treatment of TBDs (Chen et al. 2014).

80 Sika deer (*Cervus nippon*) is a native cervid species in Japan (Ohdachi et al. 2009) that
81 has recently expanded in range and number due to environmental and climate changes
82 (Takatsuki 2009). Moreover, sika deer are one of the most important reproductive hosts for
83 several tick species at all developmental stages, such as *H. longicornis*, *Haemaphysalis*
84 *flava*, *Haemaphysalis yeni*, *H. megaspinosa*, *I. ovatus*, and *Amblyomma testudinarium*, which
85 have been collected from western and central Japan (Inokuma et al. 2002; Tsukada et al. 2014).
86 Although several piroplasm species have been detected in sika deer from Japan (Elbaz et al.
87 2017; Inokuma et al. 2004; Watanabe et al. 2016; Zamoto-Niikura et al. 2014), few molecular
88 epidemiological studies have been conducted to detect piroplasm species in host-feeding ticks
89 collected from sika deer. Two *Theileria* spp. have been reported in feeding *Haemaphysalis* spp.
90 collected from sika deer in southern Japan (Masatani et al. 2017). One of these *Theileria* spp. is

91 closely related to *Theileria sp.* Thrivae and the other to *T. capreoli*, both of which were detected
92 previously in sika deer (Elbaz et al. 2017; Watanabe et al. 2016).

93 In our previous study, four piroplasm species were identified in Hokkaido sika deer blood
94 samples: *B. divergens*-like, a novel *Babesia* species (here referred to as : *Babesia sp.* Bab-SD),
95 *Theileria sp.* Thrivae, and *T. capreoli* (Elbaz et al. 2017). In addition, *Theileria sp.* (sika 1) was
96 detected in sika deer collected from Hokkaido, Japan (Shibata et al. 2018) and was found to be
97 different from *T. orientalis*. *Theileria sp.* Thrivae is a non-pathogenic *Theileria sp.* that has been
98 reported from sika deer in Japan (Watanabe et al. 2016). A closely related *Theileria sp.* has been
99 detected in China and named “*Theileria cervi*” (Liu et al. 2016). However, *T. cervi* that was
100 reported from white-tailed deer and elk (*Cervus canadensis*) in USA and Canada (Chae et al.
101 1999) is genetically separated from *Theileria sp.* Thrivae and *T. cervi* of Japan and China,
102 respectively.

103 Many of tick-borne pathogens (TBPs) are zoonotic (Moustafa et al., 2016); for examples,
104 human granulocytic anaplasmosis, human babesiosis and human monocytic ehrlichiosis are
105 caused by zoonotic tick-borne pathogens and both wildlife and domestic animals act as reservoir
106 or amplifying hosts (Dantas-Torres et al. 2012). Thus, detecting pathogens in ticks collected
107 from animals is crucial to predict their spread and emergence and to protect humans and animals.
108 Hence, the present study investigated the occurrence and prevalence of piroplasmids in ticks
109 collected from sika deer in Hokkaido.

110 **Material and methods**

111 **Tick collection and identification**

112 A total of 519 ticks were collected from 116 Hokkaido sika deer (*Cervus nippon yesoensis*)
113 from May to November 2012, which were hunted for nuisance control procedures in two

114 different regions on the Shiretoko Peninsula in Hokkaido Prefecture (Shari and Shibetsu). All
115 collected ticks were either engorged with blood or questing on the surface of the sika deer (non-
116 engorged). The collected ticks were identified under a microscope according to their
117 morphological characteristics (Yamaguti et al. 1971). Subsequently, the ticks were separated into
118 groups according to species and stage and stored in 70% ethanol at -20°C until DNA extraction.

119 **DNA extraction**

120 Total DNA was extracted from ticks individually using the Wizard [®]Genomic DNA
121 purification Kit (Promega, Madison, WI, USA) per the manufacturer's instructions with
122 modifications (Taylor et al. 2013). The whole ticks were crushed in a 50/50 mixture of the
123 included cell and nuclear lysis solutions supplemented with 17.5 μl proteinase K, followed by
124 incubation at 60°C for 90 min. DNA concentration and quality were assessed by
125 spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Yokohama, Japan). The DNA
126 was stored at -20°C until analysis.

127 **Touchdown polymerase chain reaction (PCR) to validate tick DNA extraction**

128 The success of DNA extraction was confirmed by touchdown PCR targeting the mitochondrial
129 16S rDNA gene (*mt-rrs*) of ticks. Touchdown PCR was conducted using the PCR System 9700
130 (Applied Biosystems, Foster City, CA, USA), KOD-Plus-Neo high fidelity DNA polymerase kit
131 (Toyobo Co. Ltd., Tokyo, Japan), and Mt-rrs1 (5'- CTG CTC AAT GAT TTT TTA AAT TGC
132 TGT GG-3') and Mt-rrs2 (: 5'-CCG GTC TGA ACT CAG ATC AAG TA-3') primers (Ushijima
133 et al. 2003) to amplify a 401–416 bp fragment. The 25 μl PCR mixture consisted of 2.5 μl $10\times$
134 KOD-Plus-Neo buffer, 2.5 μl dNTPs (2 mM), 1.5 μl 25 mM MgSO_4 , 0.75 μl each primer (10
135 pmol/ μl), 0.5 μl KOD-Plus-Neo DNA polymerase, 15.5 μl molecular biology-grade water, and 1
136 μl DNA. The PCR conditions were 94°C for 2 min, a touchdown step of 10 cycles at 94°C for 10

137 s, 65°C for 30 s, and 68°C for 30 s with the annealing temperature decreasing by 1°C every cycle.
138 This was followed by 40 cycles of 98°C for 10 s, 55°C for 30 s, 68°C for 30 s, and a final extension
139 of 68°C for 7 min. The PCR products (5 µl) were mixed with 1 µl loading buffer (Nippon Gene
140 Co. Ltd., Tokyo, Japan) and examined by 1% agarose gel electrophoresis with ethidium bromide
141 staining, followed by visualization using a UV illuminator.

142 **Touchdown PCR and reverse line blot (RLB) hybridization**

143 Touchdown PCR targeting the 18S rRNA gene of *Babesia* and *Theileria* spp. was performed
144 using the PCR System 9700, KOD-Plus-Neo high fidelity DNA polymerase kit, and the RLB-F2
145 (5'-GAC ACAGGG AGG TAG TGA CAA G-3') and RLB-R2 (biotin-5'-CTA AGA ATT TCA
146 CCT CTG ACA GT-3') primer pair (Gubbels et al. 1999; Matjila et al. 2004). The PCR mixture
147 was used as described above to amplify a 460–540 bp segment. One microliter of molecular grade
148 water and genomic DNA samples of *B. divergens*-like and *Theileria* sp. Thruvae were used as
149 negative and positive controls for *Babesia* and *Theileria* spp., respectively. The PCR conditions
150 were 94°C for 2 min, a touchdown step of 10 cycles at 94°C for 10 s, 67°C for 30 s, and 68°C for
151 30 s with a 1°C decrease in annealing temperature every cycle. This was followed by 40 cycles at
152 98°C for 10 s, 57°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 7 min. The PCR
153 products were examined by 1% agarose gel electrophoresis with ethidium bromide staining
154 followed by visualization using a UV illuminator.

155 The PCR products were subjected to RLB hybridization as described previously (Kong and
156 Gilbert 2006; Moustafa et al. 2016) with modifications. Briefly, a 15 × 15 cm Biotodyne C
157 membrane (Pall Life Science, Ann Arbor, MI, USA) was activated with 20 ml 16% 1-ethyl-3-(3-
158 dimethyl-amino-propyl) carbodiimide (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room

159 temperature. The membrane was washed gently with MilliQ water for 2 min and placed in the
160 Miniblotter MN45 (Immunetics, Boston, MA, USA). This study included 19 oligonucleotide
161 probes: 17 previously published probes (Elbaz et al. 2017) and 2 probes designed in this study.
162 The designed probes (*T. capreoli* 5'-ATACGAGTTTTTGCATTGTG-3' and *Babesia* sp. (Bab-
163 SD) 5'-GTTGGCTTTTCTTATTACTTTGA-3') linked to a C6 amino linker were obtained from
164 Sigma Aldrich (Tokyo, Japan) and reconstituted as 100 pmol/ μ l solutions by using Tris-EDTA
165 (TE) buffer (PH 8.0) (Nippon Gene, Japan). Ten microliters of each 100 pmol/ μ l probe solution
166 were diluted in 0.5 M NaHCO₃ to a final volume of 170 μ l. The openings in the miniblotter were
167 filled with 150 μ l each diluted oligonucleotide, and the membrane was incubated for 5 min at room
168 temperature. The membrane was inactivated in 250 ml 0.1 M NaOH with gentle shaking for
169 exactly 8 min at room temperature, then washed with prewarmed 250 ml 2 \times SSPE/0.1% SDS at
170 60°C. Finally, the membrane was sealed in a plastic bag with 15 μ l 20 mM EDTA for future use.
171 Ten microliters of each PCR product were diluted in 2 \times SSPE/0.1% SDS to a final volume of 170
172 μ l, denatured by heating at 100°C for 10 min, and immediately cooled on ice. The denatured
173 mixtures were allowed to hybridize to the membrane at 60°C for 1 h. Afterwards, the membrane
174 was washed twice in 250 ml 2 \times SSPE/0.5% SDS at 52°C for 10 min and then incubated with
175 diluted peroxidase-labeled NeutrAvidin (Thermo Fisher Scientific, Waltham, MA, USA) at 42°C
176 for 45–60 min. The NeutrAvidin was removed by washing twice with 250 ml 2 \times SSPE/0.5% SDS
177 at 42°C for 10 min and with 2 \times SSPE twice at room temperature for 5 min. The membrane was
178 exposed to 15 ml Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Tokyo,
179 Japan) for 5 min and photographed using the Ez-Capture MG/ST (ATTO Corp. Tokyo, Japan). To
180 remove the PCR products, the membrane was washed twice in pre-warmed 1% SDS at 90°C for

181 30 min and washed with 250 ml 20 mM EDTA. The membrane was sealed in a plastic bag with
182 15 ml 20 mM EDTA and stored at 4°C for future reuse.

183 **Cloning and sequencing**

184 The RLB results were confirmed by cloning and sequencing 12 randomly selected positive
185 PCR products. A 460–540 bp fragment was amplified from the 18S rRNA gene of piroplasm
186 species using the KOD-Plus-Neo high fidelity DNA polymerase kit with the abovementioned
187 RLB-F2 and RLB-R2 primers and PCR conditions. The PCR products were examined by
188 electrophoresis, and the band corresponding to 460–540 bp was excised for purification using the
189 NucleoSpin® Gel and PCR clean up kit (Macherey-Nagel, Düren, Germany). The purified PCR
190 products were attached to dA nucleotides by mixing 0.9 µl 10× KOD-Plus-Neo buffer, 0.9 µl 2
191 mM dNTPs, 0.54 µl 25 mM MgSO₄, 6.66 µl each purified PCR product, and 1 µl 10× A-
192 attachment mix (Toyobo) at 60°C for 10 min. One microliter of each dA-attached product was
193 cloned into the pMD20 T-vector (Takara Bio, Otsu, Japan). Five colonies per sample were selected
194 and screened by EmeraldAmp MAX PCR (Takara Bio) as described in the manufacturer's manual.
195 The purified PCR products were sequenced using the Big-Dye Terminator version 3.1 Cycle
196 Sequencing Kit and purified using the BigDye® XTerminator™ Purification Kit (Applied
197 Biosystems). Subsequently, the purified products were sequenced using the ABI PRISM™ 310
198 genetic analyzer (Applied Biosystems). BLASTn searches were performed to compare the
199 sequences to reference sequences recorded in GenBank for identification.

200 **Phylogenetic analysis**

201 The 18S rRNA partial sequences were examined by BLASTn search against the *Babesia* and
202 *Theileria* spp sequences in GenBank and submitted to the DNA Data Bank of Japan (DDBJ). The
203 obtained sequences were aligned in MEGA software version 7 with representative 18S rRNA gene
204 sequences of the previously identified *Babesia* and *Theileria* spp. in the GenBank to know its
205 taxonomic position. The “Find Best DNA/Protein Models” option in MEGA 7 was used to select
206 the most suitable substitution model for the phylogenetic analysis. The phylogenetic trees were
207 constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and
208 Nei 1993) using the gamma distribution in the same software and a full 18S rRNA gene sequence
209 for *Plasmodium falciparum* (XR_002966679) was used as an outgroup. Node support was assessed
210 using 1,000 bootstrap replicates (Kumar et al. 2016).

211 **Statistical analysis**

212 Linear mixed models (LMM) was performed to investigate differences in the prevalence of
213 *Theileria* sp. *Thrivae* and *T. capreoli*. We fitted LMM with predictive variables (tick species, stage,
214 and status) in presence of sampling location as a random effect variable. We used “lmer” function
215 in R package lme4 to perform the LMM. The correlations among the detected piroplasm species
216 were investigated by the chi-square test. The statistical analyses were performed using SPSS
217 version 21.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered significant.

218 **Results**

219 **Identifying the tick species and detecting tick DNA**

220 The collected ticks were identified as belonging to the genera *Ixodes* and *Haemaphysalis*.
221 The *Ixodes* ticks included 330 (63.6%), 40 (7.7%), 11 (2.1%) and 110 (21.2%) *I. persulcatus*

222 nymph, larvae, adult and *I. ovatus* adults, respectively. The remaining collected ticks including
223 15 (2.9%), 11 (2.1) and 2 (0.4%) were identified as *H. japonica* adult, nymph and larvae,
224 respectively. Of these ticks, 70 (18.4%) and 66 (60%) of the collected *I. persulcatus* and *I.*
225 *ovatus* were engorged with blood; while 311 (81.6%), 44 (40%) and 28 (100%) of the collected
226 *I. persulcatus*, *I. ovatus* and *H. japonica* were non-engorged, respectively.

227 **Detection and identification of *Babesia* and *Theileria* spp. in tick samples by**
228 **touchdown PCR/RLB hybridization**

229 Touchdown PCR and RLB hybridization, used to detect a partial fragment of the 18S rRNA
230 gene of *Babesia* and *Theileria* spp., showed that 163 of the 519 (31.7%) DNA samples examined
231 were positive for *Babesia* and *Theileria* spp. including *Theileria* sp. Thirivae, *T. capreoli*, *B.*
232 *divergens*-like, *Babesia* sp. (Bab-SD), *B. microti* U.S., and *B. microti* Hobetsu (Table 1).

233 Furthermore, the prevalence of *Theileria* sp. Thirivae was the highest among the detected
234 piroplasms and was detected mostly in adult *I. ovatus*.. In addition, the highest prevalence
235 *Theileria* sp. Thirivae was found in the nymph stage of *H. japonica* (45.5%; 5/11) and adult of *I.*
236 *persulcatus* (36.4%; 4/11). (Table 1).

237 In addition, *T. carpreoli* was found in all tick species and stages except *H. japonica* larvae, and
238 the highest prevalence were observed in the adult stages of *I. ovatus*: 22.7% (25/110) and *H.*
239 *japonica*: 26.7% (4/15). A total of 7.5% (3/40) and 9.1% (1/11) of the DNA samples extracted
240 from engorged *I. persulcatus* larvae and adult *I. persulcatus* were positive for *T. capreoli*,
241 respectively.

242 The highest prevalence of *B. divergens*-like were 6.7% (1/15) in *H. japonica*. The
243 *Babesia* sp. (Bab-SD) was detected only in *Ixodes* spp., with prevalence of 0.3% (1/330) and

244 1.8% (2/110) in *I. persulcatus* nymphs and *I. ovatus* adults, respectively. In addition, *B. microti*
245 U.S. and Hobetsu were identified in *Ixodes* spp., with prevalence of 0.5% (2/381) in *I.*
246 *persulcatus* and 1.8% (2/110) in *I. ovatus*, respectively.

247 Of the 163 ticks positive for piroplasm species, the co-infection rate was 26.4% (43/163) and
248 included 39 double, 3 triple, and 1 quadruple infection cases. The prevalence of double co-
249 infection were 79.1% (34/43), 9.3% (4/43), and 2.3% (1/43) between *Theileria* sp. Thrivae and
250 *T. capreoli*, *Theileria* sp. Thrivae and *B. divergens*-like, and *T. capreoli* and *B. divergens*-like,
251 respectively (Table 2). Of the three ticks with triple co-infection, two were infected with
252 *Theileria* sp. Thrivae, *T. capreoli*, and *B. divergens*-like, and the third tick was infected with
253 *Theileria* sp. Thrivae, *Babesia* sp. (Bab-SD), and *B. microti* (Hobetsu type). In addition, only one
254 tick was infected with four pathogens: *Theileria* sp. Thrivae, *T. capreoli*, the *Babesia* sp. (Bab-
255 SD), and *B. divergens*-like. The highest co-infection rate, 53.5% (23/43), was detected in adult
256 ticks, whereas a 41.9% (18/43) co-infection rate was found in nymph ticks.

257 **DNA sequencing and molecular characterization**

258 The sequencing results yielded 12 partial 18S rRNA gene sequences (Table 3), which were
259 classified as six piroplasm species: two *Theileria* spp. and four *Babesia* spp. (Figs. 1 and 2). The
260 sequences from *I. persulcatus* (LC336717–LC336718), *I. ovatus* (LC336719), and *H. japonica*
261 (LC336720) were identical to those from the uncultured *Theileria* spp. (LC215385–LC271213)
262 and *Theileria* sp. Thrivae (AB981972), which were previously detected in sika deer in Japan and
263 are 99% similar to *Theileria* sp. sequences (LC169094 and LC169091) found in *Haemaphysalis*
264 sp. collected from sika deer and vegetation, respectively. Our sequences obtained from *I.*
265 *persulcatus* (LC336721 and LC336724), a *H. japonica* (LC336722), and *I. ovatus* (LC336723)

266 were identical to each other and were identical to the uncultured *Theileria* spp. sequence
267 (LC271206) from sika deer in Japan and 99% similar to sequences of *T. capreoli* (KJ188219)
268 from red deer in China and of *Theileria* sp. (LC169093) from a feeding *Haemaphysalis* sp.
269 detected on sika deer in Japan.

270 The obtained sequence (LC336725) was identical to that of the *Babesia* sp. (Bab-SD)
271 (LC215388), whereas the sequence LC336727 was identical to a *B. divergens*-like sequence
272 (LC215387) detected in sika deer from Japan. The sequences (LC336726 and LC336728) were
273 clustered with *B. microti*. The first sequence was identical to that of *B. microti* Hobetsu
274 (AB050732) from a field rodent (*Apodemus speciosus*), while the other sequence was identical
275 with that of the *B. microti* U.S. strain (LC127372) from *I. persulcatus* in Japan.

276 **Prevalence of *Theileria* spp. according to tick variables**

277 In this study, LMM test was used to analyze the results of *Theileria* spp., but not for *Babesia*
278 spp. because of the low number of positive *Babesia* spp. samples. The prevalence of *Theileria*
279 sp. *Thrivae* and *T. capreoli* were significantly higher in the engorged ticks than in the non-
280 engorged ones (LMM: $P < 0.001$ and $P < 0.05$), respectively (Table 4 and Fig. 3). Additionally, a
281 significant positive correlation between *Theileria* sp. *Thrivae* and *T. capreoli* was detected by
282 chi-square test ($P < 0.01$) (Table 5).

283 **Discussion**

284 Ticks are obligate blood feeders and important vectors of a large number of pathogens
285 infecting animals and humans (Azmi et al. 2016). Despite that many studies have reported
286 piroplasm species from sika deer in Japan (Elbaz et al. 2017; Inokuma et al. 2004; Shibata et al.

287 2018; Watanabe et al. 2016; Zamoto-Niikura et al. 2014), only few published reports are available
288 on piroplasm species from feeding ticks detected on sika deer in Hokkaido (Masatani et al. 2017).

289 Here, a total of six piroplasms, *Theileria* sp. Thirivae, *T. capreoli*, *B. divergens*-like,
290 *Babesia* sp. (Bab-SD), *B. microti* U.S., and *B. microti* Hobetsu, were identified. Hokkaido sika
291 deer are potential hosts for *Theileria* sp. Thirivae, *T. capreoli*, *B. divergens*-like, and the *Babesia*
292 sp. (Bab-SD) (Elbaz et al. 2017). However, *B. microti* strains have not been reported in
293 Hokkaido sika deer but were detected previously in rodents; therefore, the ticks infected with *B.*
294 *microti* strains in this study may have been infected during previous life stages when those ticks
295 fed on other animal hosts.

296 Our results revealed *Theileria* sp. Thirivae and *T. capreoli* are the most prevalent species in
297 the ticks examined, and both organisms were detected in all examined stages of *Ixodes* nymphs
298 and adults of *H. japonica*. This observation is in agreement with the high prevalence of *Theileria*
299 sp. Thirivae in sika deer, indicating the persistence of infection in sika deer (Elbaz et al. 2017).
300 Recently, a high prevalence of *Theileria* spp. in sika deer (97.8%; 89/91) was detected in Hokkaido,
301 Japan (Shibata et al. 2018). Here, most of the ticks positive for *Theileria* spp. were engorged with
302 sika deer blood, which may have caused this high prevalence. Although the prevalence of *T.*
303 *capreoli* in Hokkaido sika deer is unknown, the results of this study suggest that sika deer could
304 be the main animal host for this piroplasm species in the Hokkaido ecosystem. Moreover, the
305 finding that the sequences from ticks collected in Kyushu and Hokkaido were almost identical
306 suggests that *Theileria* sp. Thirivae and *T. capreoli* are distributed throughout Japan (Masatani et
307 al. 2017). However, this study cannot confirm that these tick species are the vector hosts to the
308 detected pathogens, because the blood meal from infected host could be the source of the positive

309 results. It is required to examine salivary glands of tick species to confirm that these ectoparasites
310 act as epidemiological vectors.

311 The prevalence of *Theileria* sp. *Thrivae* and *T. capreoli* in *H. japonica* in this study are
312 higher than the previously reported rates of 6.3% (2/32) and 12.5% (4/32) (Masatani et al. 2017),
313 respectively. This is possibly due to the higher sensitivity of the RLB technique compared with
314 conventional PCR assays. In addition, most of ticks that were examined in this study were
315 engorged with sika deer blood, which can lead to an increase in the prevalence of *Theileria* species.
316 Interestingly, both *Theileria* spp. were detected in the larval stage of *I. persulcatus*; however,
317 *Theileria* spp. are not known to be transmitted via a transovarian route (Bhattacharyulu et al. 1975).
318 Positivity for *Theileria* spp. was likely because all larvae were engorged with infected sika deer
319 blood.

320 The detected *Babesia* spp., *B. divergens*-like, *B. microti* U.S., and *B. microti* Hobetsu are
321 zoonotic pathogens. The highest rate of *B. divergens*-like recovery was from an *I. persulcatus*
322 nymph in this study and was in accordance with a previously published report on questing *I.*
323 *persulcatus* (1.3%; 11/845) from Japan (Zamoto-Niikura et al. 2018).

324 In this study, the *Babesia* sp. (Bab-SD) was detected in *Ixodes* spp. only. Surprisingly, the
325 *Babesia* sp. (Bab-SD) was found in a non-engorged *I. persulcatus* nymph, suggesting that the
326 larvae of this tick were infected before molting into nymphs, either from an infected reservoir host
327 or by transovarian transmission. However, not all larvae examined in this study were infected by
328 the *Babesia* sp. (Bab-SD); thus, further study on this *Babesia* sp. is needed to understand its life
329 cycle.

330 Sika deer are not hosts for *B. microti*; however, this study found that *Ixodes* spp. feeding
331 on sika deer were positive for *B. microti* strains. Furthermore, the *B. microti* U.S. and Hobetsu
332 types were detected in *I. persulcatus* and *I. ovatus*, respectively. This result is consistent with
333 previous studies reporting that *I. persulcatus* and *I. ovatus* are vectors for *B. microti* U.S. and *B.*
334 *microti* Hobetsu in Japan and Russia, respectively (Katargina et al. 2011; Rar et al. 2011; Rar et
335 al. 2014; Zamoto-Niikura et al. 2012). The prevalence of *B. microti* U.S. type in *I. persulcatus* in
336 this study was in accordance with the rates of those previously published reports from Japan and
337 Russia (Katargina et al. 2011; Rar et al. 2011; Rar et al. 2014; Zamoto-Niikura et al. 2012).

338 The partial 18S rRNA gene sequences were identified as belong to two *Theileria* spp. and
339 four *Babesia* spp. The two *Theileria* spp. clustered with *Theileria* sp. Thirivae and *T. capreoli*,
340 which were detected in sika deer in Japan. The *Babesia* spp. sequences were associated with four
341 branches. The first branch clustered with *B. divergens*-like sequences, the second branch with the
342 *Babesia* sp. (Bab-SD) sequences detected in sika deer in Japan (Elbaz et al. 2017), and the
343 remaining two with *B. microti* sequences, one with a previously published *B. microti* U.S. sequence
344 from *I. persulcatus* in Japan (Zamoto-Niikura et al. 2016) and the other with *B. microti* Hobetsu
345 type detected in a field rodent (*Apodemus speciosus*) (Wei et al. 2001).

346 The prevalences of both *Theileria* spp. detected in this study were significantly higher in
347 the engorged ticks than in non-engorged ones, which could be due to the high infection rates of
348 both *Theileria* spp. in sika deer blood, indicating the persistence of infection in sika deer.

349 Co-infection of ticks with more than one pathogen is not uncommon, and one tick can be
350 infected with up to five pathogens (Eshoo et al. 2014; Moutailler et al. 2016; Reis et al. 2011).
351 Multiple pathogens in individual ticks increases the risk of co-transmission to, and co-infections

352 in, humans and animals (Moutailler et al. 2016). In this study, a significant co-infection correlation
353 was shown between *Theileria* sp. Thrivae and *T. capreoli*, which may have occurred because both
354 *Theileria* spp. have similar life cycles. This was supported by the phylogenic analysis showing that
355 the two *Theileria* spp. belong to the same *Theileria* spp. cluster (Inokuma et al. 2008; Li et al.
356 2014). The association between *Theileria* and *Babesia* spp. reported in double, triple, or quadruple
357 co-infection is likely due to shared reservoir hosts or co-feeding during different life stages.
358 Additionally, the highest number of co-infection cases was observed in adult ticks, which may be
359 because of the greater opportunity of adult ticks to receive more pathogens from host feeding via
360 transstadial transmission.

361 **Conclusion**

362 Ticks and TBDs constitute a growing burden for human and animal health. This study
363 revealed that ticks collected from sika deer are exposed to a high diversity of piroplasm species
364 and demonstrated the ecology of these pathogens endemic to Hokkaido. The sika deer is an
365 amplifying host for *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like, and *Babesia* sp. (Bab-
366 SD). In addition, zoonotic TBPs, such as *B. divergens*-like and *B. microti* U.S. and Hobetsu, were
367 detected in the ticks of this study. Because this is the first report of piroplasm species detected in
368 feeding ticks collected from sika deer in Hokkaido, further investigations are needed to reveal the
369 epidemiology and respective vectors of these pathogens.

370 **Competing interests**

371 The authors declare that they have no competing interests.

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376 **English check**

377 The English in this document has been checked by at least two professional editors, both native
378 speakers of English. For a certificate, please see:

379 <http://www.textcheck.com/certificate/0sWd1W>

380 **Table 1** The prevalence of piroplasm species in ticks collected from Hokkaido sika deer by RLB

Tick samples				Detected piroplasm spp.						
Species	Stage	No.tested		<i>Theileria</i> sp. <i>Thrivae</i>	<i>T. capreoli</i>	<i>B. divergens</i> -like	<i>Babesia</i> sp. (Bab-SD)	<i>B. microti</i> U.S.	<i>B. microti</i> Hobetsu	Co-infection
		Engorged	Non-engorged							
<i>I. persulcatus</i>	Larvae	15	25	8 (20%)	3 (7.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
	Nymph	53	277	74 (22.4%)	21 (6.4%)	7 (2.1%)	1 (0.3%)	1 (0.3%)	0 (0.0%)	18
	Adult	2	9	4 (36.4%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	1 (10%)	0 (0.0%)	1
	Sub total	70	311	86 (22.6%)	25 (6.6%)	7 (1.8%)	1 (0.3%)	2 (0.5%)	0 (0.0%)	21
<i>I. ovatus</i>	Adult	66	44	45 (40.9%)	25 (22.7%)	1 (0.9%)	2 (1.8%)	0 (0.0%)	2 (1.8%)	20
	Sub total	66	44	45 (40.9%)	25 (22.7%)	1 (0.9%)	2 (1.8%)	0 (0.0%)	2 (1.8%)	20
<i>Haemaphysalis</i> <i>japonica</i>	Larvae	0	2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
	Nymph	0	11	5 (45.5%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
	Adult	0	15	4 (26.7%)	4 (26.7%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
	Sub total	0	28	9 (32.1%)	5 (17.9%)	1 (3.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
Total			519	140 (27%)	55 (10.6%)	9 (1.7%)	3 (0.6%)	2 (0.4%)	2 (0.4%)	43

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391 **Table 2.** A summary of co-infections between piroplasm species in ticks collected from Hokkaido sika deer by RLB

Co- infection	Piroplasms	<i>I. persulcatus</i>	<i>I. ovatus</i>	<i>H. japonica</i>	392
Double infection	<i>Theileria</i> sp. Thirivae and <i>T. capreoli</i>	15	18	1	34
	<i>Theileria</i> sp. Thirivae and <i>B. divergens</i> like	3	1	0	393
Triple infection	<i>T. capreoli</i> and <i>B. divergens</i> like	0	0	1	1
	<i>Theileria</i> sp. Thirivae, <i>T. capreoli</i> and <i>B. divergens</i> like	2	0	0	394
	<i>Theileria</i> sp. Thirivae, <i>Babesia</i> sp. (Bab-SD) and <i>B. microti</i> Hobetsu	0	1	0	1
Quadruple infection	<i>Theileria</i> sp. Thirivae, <i>T. capreoli</i> , <i>Babesia</i> sp. (Bab-SD) and <i>B. divergens</i> like	1	0	0	395

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408 **Table 3** Samples of sequencing

Number	Sample I.D.	Accession number	RLB results
1	130308 I.P.N	LC336717	
2	130304 I.P.A	LC336718	<i>Theileria</i> sp. Thrivae
3	120619D3 I.O.A	LC336719	
4	120618D21 H.N	LC336720	
5	120925D2 I.P.L	LC336721	
6	120610D14 H.A	LC336722	
7	120219D13 I.O.A	LC336723	<i>Theileria capreoli</i>
8	120219D3 I.P.N	LC336724	
9	12012D3 I.O.A	LC336725	<i>Babesia</i> sp. (Bab-SD)
10	120618D13 I.O.A	LC336726	<i>Babesia microti</i> Hobetsu
11	120928N2 I.P.N	LC336727	<i>Babesia divergens</i> -like
12	130305 I.P.A	LC336728	<i>Babesia microti</i> U.S.

409 I.P.N: *I. persulcatus* nymph, I.P.A: *I. persulcatus* adult, I.P.L: *I. persulcatus* larvae, I.O.A: *I. ovatus* adult,

410 H.N: *H. japonica* nymph and H.A: *H. japonica* adult

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415 **Table 4.** The final models for the prevalence of *Theileria* sp. Thrivae and *T. capreoli* in ticks collected from Hokkaido sika deer.

		Prevalence of <i>Theileria</i> sp. Thrivae					Prevalence of <i>Theileria capreoli</i>				
Random	Predictors	Estimate	Std. Err	df	t value	Pr(> t)	Estimate	Std. Err	df	t value	Pr(> t)
	(Intercept)	0.44	0.12	40.98	3.68	0.00 ***	0.29	0.07	141.83	3.92	0.00 ***
	Species_ <i>I. ovatus</i>	0.04	0.11	512.22	0.38	0.71	-0.04	0.07	434.92	-0.47	0.64
Location	Species_ <i>I. persulcatus</i>	-0.11	0.10	509.42	-1.11	0.27	-0.08	0.07	503.20	-1.14	0.26
	Stages_Larvae	-0.07	0.12	512.99	-0.57	0.57	-0.10	0.08	512.99	-1.21	0.23
	Stages_Nymph	0.06	0.10	512.67	0.58	0.56	-0.09	0.07	499.98	-1.20	0.23
	Status_Non-engorged	-0.18	0.05	512.25	-3.82	0.00 ***	-0.08	0.03	512.60	-2.36	0.02 *

421 **Significance codes:** 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

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433 **Table 5** Number of Ticks infected with *Theileria* sp. Thrivae and/or *T. capreoli*.

		<i>T. capreoli</i>		Total	434
		Positive	Negative		
<i>Theileria</i> sp. Thrivae	Positive	37*	103	140	435
	Negative	18	361	379	
	Total	55	464	519	436
					437

438 * Correlation between *Theileria* sp. Thrivae and *T. capreoli* was significant positive (P<0.01) using chi-square (χ^2) test in SPSS

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446 **Fig. 1** Phylogenetic tree based on nucleotide 18S rRNA gene sequences of *Babesia* spp. from
447 GenBank. Sequences from the present study are labeled in bold. The DNA sequences from this
448 study was deposited to DDBJ as accession number from LC336725 to LC336728. Phylogenetic
449 trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model
450 (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by
451 applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using
452 the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with
453 superior log likelihood value. A discrete Gamma distribution was used to model evolutionary
454 rate differences among sites (5 categories (+G, parameter = 0.2599)). The analysis involved 22
455 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was
456 a total of 174 positions in the final dataset. Evolutionary analyses were conducted in MEGA7
457 (Kumar et al. 2016).

458 **Fig. 2** Phylogenetic tree based on nucleotide 18S rRNA gene sequences of *Theileria* spp. from
459 GenBank. Sequences from the present study are labeled in bold. The DNA sequences from this
460 study was deposited to DDBJ as accession number from LC336717 to LC336724. Phylogenetic
461 trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model
462 (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by
463 applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using
464 the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with
465 superior log likelihood value. A discrete Gamma distribution was used to model evolutionary
466 rate differences among sites (5 categories (+G, parameter = 0.3910)). The analysis involved 31
467 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was

468 a total of 177 positions in the final dataset. Evolutionary analyses were conducted in MEGA7
469 (Kumar et al., 2016).

470 **Fig. 3** Descriptive analysis of the prevalence of *Theileria* sp. *Thrivae* and *T. capreoli* in (a)
471 engorged and non-engorged ticks and (b) the developmental stages of ticks

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473 **Declarations**

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477 **Conflicts of interest/Competing interests** (The authors declare that they have no competing
478 interests.)

479 **Ethics approval** (Not applicable)

480 **Consent to participate** (Not applicable)

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

482 **Availability of data and material** (The DNA sequence data were registered at the DNA
483 DataBank of Japan (DDBJ) under the accession numbers LC336717 - LC336728)

484 **Code availability** (Not applicable)

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682

Fig. 1

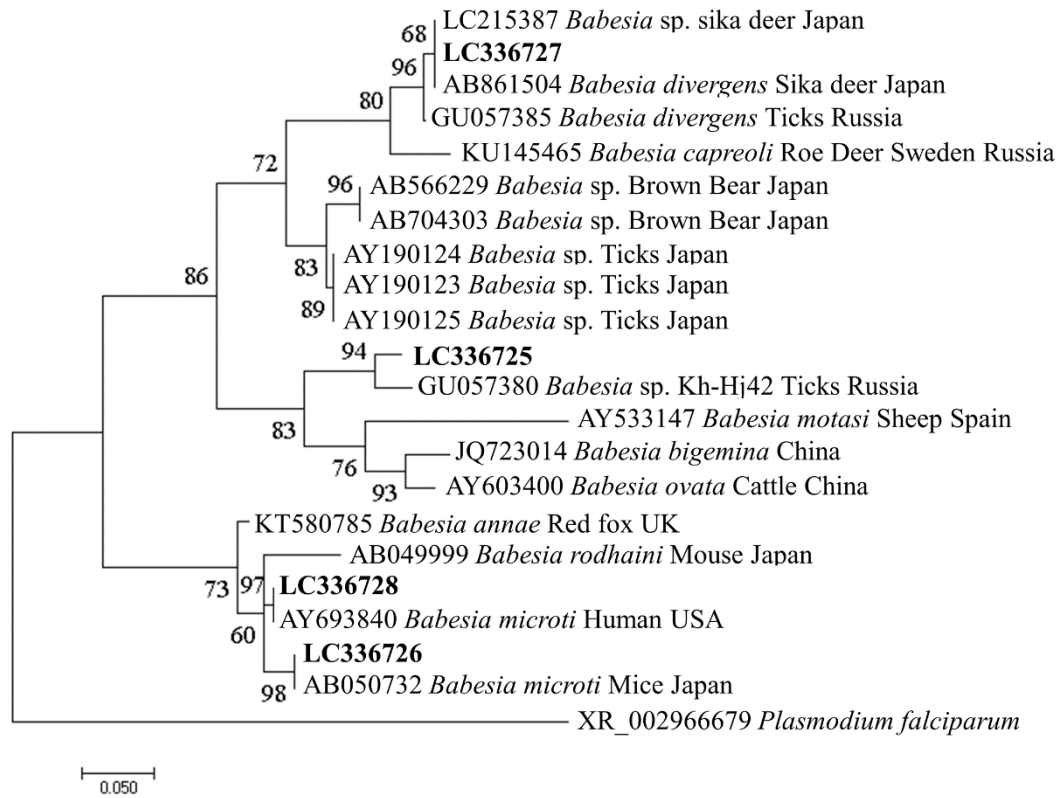


Fig. 2

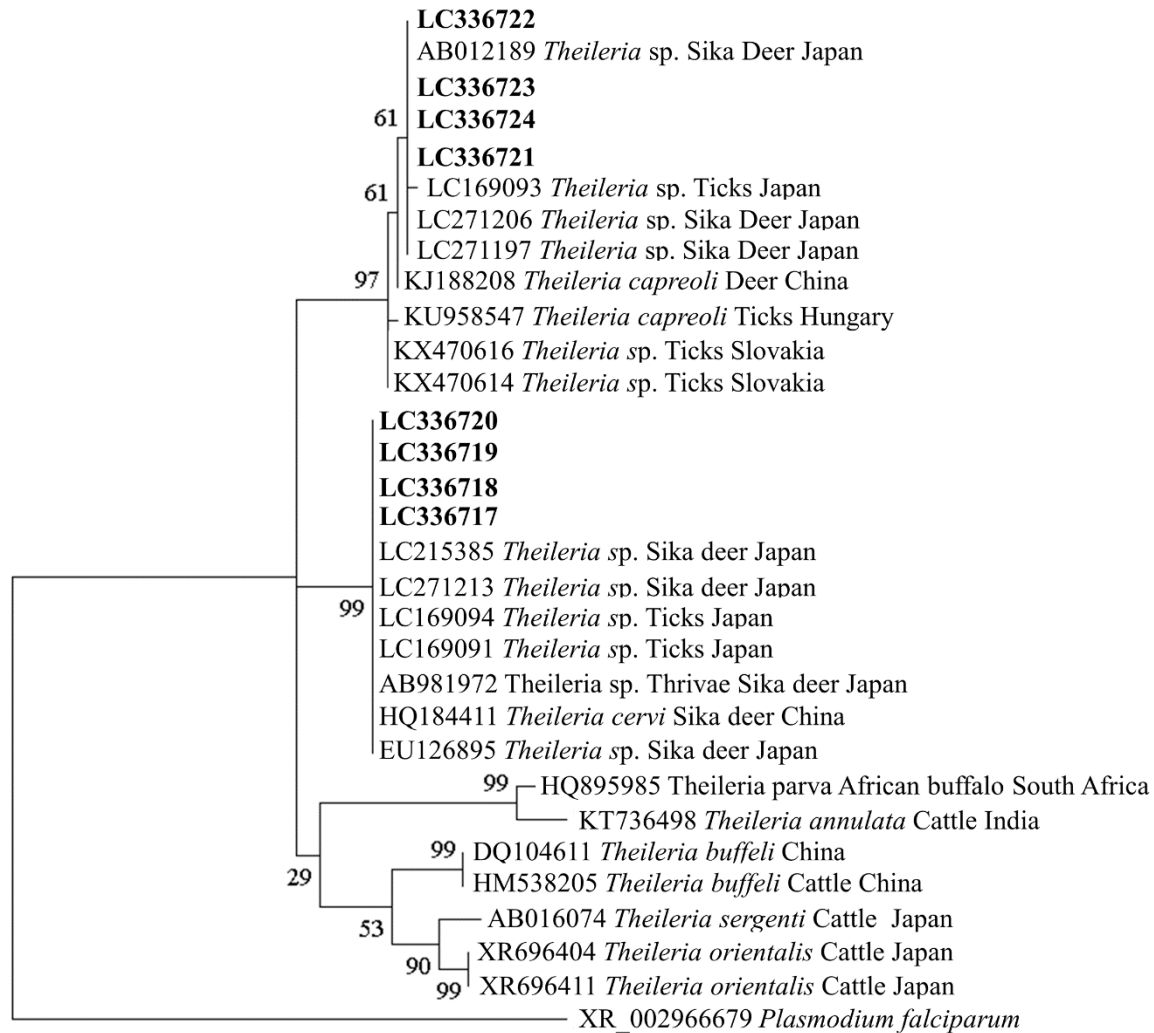


Fig. 3

