Title	Molecular identification and characterization of piroplasm species in Hokkaido sika deer (<i>Cervus nippon yesoensis</i>), Japan
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Abstract 45

Babesia and *Theileria* species are tick-borne protozoan parasites that have a 46
veterinary and zoonotic importance. In order to investigate the prevalence and genetic 47
diversity of these parasites, a total of 269 sika deer blood DNA samples collected 48
from Hokkaido, Japan, were examined for *Babesia* and *Theileria* species by touch- 49
down PCR targeting the 18S rRNA gene. Reverse line blot (RLB) hybridization was 50
then used to detect 12 piroplasm species. The results revealed that 95.5 % (257/269), 51
94.1 % (253/269), 14.1 % (38/269), 87.7 % (236/269) and 11.5 % (31/269) of the 52
examined PCR products hybridized with the probes which were designed to detect all 53
Babesia and *Theileria* spp., all *Theileria* spp., all *Babesia* spp., *Theileria* sp. Thrivae 54
and *Babesia divergens*-like, respectively. The 18S rRNA gene partial sequences were 55
divided into *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like and an undescribed 56
Babesia species. This study showed the first detection of the undescribed *Babesia* sp. 57
from Japan. Therefore, more studies are required to understand the ecology of the 58
newly detected tick-borne pathogens in Hokkaido. 59

Keywords 60

Babesia; *Theileria*; Sika deer; Reverse line blot. 61

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Introduction	67
<i>Babesia</i> and <i>Theileria</i> species are tick-borne protozoan parasites that have a	68
veterinary and zoonotic importance (Mans et al., 2015). <i>Babesia</i> and <i>Theileria</i> spp.	69
are obligatory intracellular parasites and genetically close to each other (Homer et al.,	70
2000), however, both have different life cycles. In the mammalian host, <i>Babesia</i> spp.	71
sensu stricto infect erythrocytes (Schreeg et al., 2016), while <i>Theileria</i> spp. infect both	72
leukocytes and erythrocytes (Li et al., 2014).	73
In cattle, East Coast fever and tropical theileriosis are caused by <i>T. parva</i> and <i>T.</i>	74
<i>annulata</i> , respectively, and exhibit fever, enlargement of lymph nodes and weight loss	75
(Mans et al., 2015; Olds et al., 2016). In addition, <i>B. bovis</i> and <i>B. bigemina</i> are highly	76
pathogenic in cattle and cause bovine babesiosis, a diseases which shows fever,	77
hemolytic anemia and hemoglobinuria (Decaro et al., 2013). <i>Babesia ovis</i> , <i>B. motasi</i>	78
and <i>B. crassa</i> cause sever disease in sheep which is characterized by anemia, fever,	79
icterus and hemoglobinuria (Hashemi-Fesharki, 1997).	80
To the best of our knowledge, there is no published description of clinical	81
manifestations in sika deer (<i>Cervus nippon yesoensis</i>) with piroplasmosis. However,	82
clinical piroplasmosis may occur in the case of exposure to high stressful conditions	83
such as malnutrition (Yabsley et al., 2005). The clinical symptoms of theileriosis in a	84
white-tailed deer (<i>Odocoileus virginianus</i>) fawn were previously reported from the	85
USA and included icterus and anemia (Yabsley et al., 2005).	86
One of the zoonotic <i>Babesia</i> spp. is <i>B. divergens</i> , which has been reported from	87
several deer species including reindeer, roe deer and red deer in Europe (Michel et al.,	88
2014). Recently, another zoonotic <i>B. divergens</i> -like has been detected in sika deer in	89
Japan (Zamoto-Niikura et al., 2014). Whereas, many <i>Theileria</i> spp. have been	90

detected in cervids, for example *T. cervi*, which is a non-pathogenic *Theileria* sp. that 91
has been reported from white-tailed deer and elk (*Cervus canadensis*) in USA and 92
Canada (Chae et al., 1999). However, another *T. cervi*, which is genetically separated 93
from *T. cervi* in the USA and Canada, has been found in sika deer from China (Liu et 94
al., 2016), but has been reported as *Theileria* sp. Thrivae in Japan (Watanabe et al., 95
2016). Nevertheless, all *Theileria* spp. have not been recognized as zoonotic 96
pathogens (Zanet et al., 2014). 97

The sika deer (*Cervus nippon*) is widely distributed in Asia and acts as a reproductive 98
host for several tick species such as *Heamaphysalis longicornis*, *H. flava*, *H. Yeni*, *H.* 99
megaspinosa, *H. Formosensis*, *H. kitaokai* and *Ixodes ovatus* were detected on sika 100
deer in Japan (Inokuma et al., 2002). In Japan, sika deer populations have an 101
important role in maintaining several tick-borne pathogens, such as *Anaplasma* sp. 102
Ap-sd (Moustafa et al., 2015), *Theileria* sp. Thrivae (Watanabe et al., 2016), *B.* 103
divergens-like (Zamoto-Niikura et al., 2014) and *Borrelia lonestari*-like (Lee et al., 104
2014) . In Hokkaido, sika deer population density has been expanding (Ijima et al., 105
2015) and they live in close proximity to human settlements and livestock. 106
Consequently, sika deer may facilitate the transmission of several tick-borne 107
pathogens to both humans and livestock. 108

In spite of the enormous research on the role of sika deer in the transmission of tick- 109
borne pathogens, a continuous survey is needed to detect the new emerging 110
piroplasmids in sika deer in Japan. Previously, conventional polymerase chain 111
reactions (PCR) have been used to detect *Babesia* and *Theileria* spp. in Japan 112
(Watanabe et al., 2016). Here, the reverse line blot hybridization (RLB) was applied 113
in this study for the specific detection of 12 different piroplasm species. This 114
technique is a useful tool in identifying many tick-borne pathogens in a large number 115

of samples (Kong and Gilbert, 2006) with high specificity and less cost than	116
sequencing a large number of samples. The objective of this study was to estimate the	117
infection rates and molecular characterization of several <i>Babesia</i> and <i>Theileria</i> spp.	118
among sika deer in Hokkaido.	119
Materials and methods	120
<i>Blood samples and DNA extraction</i>	121
Whole blood samples were obtained from the jugular vein of 269 sika deer (<i>C. nippon</i>	122
<i>yesoensis</i>) that were culled for nuisance control during 2010 to 2013 from Hokkaido	123
prefecture, Japan. These blood samples were collected in EDTA-Na containing tubes	124
and stored at -20 °C until used. DNA was extracted from the blood samples by using	125
the Wizard ®Genomic DNA purification Kit (Promega, Madison, WI, USA) as per	126
manufacturer's instructions and kept at -20 °C until analysis.	127
<i>Touch-down Polymerase Chain Reaction (PCR)</i>	128
A touch-down PCR targeting the 18S rRNA gene of <i>Babesia</i> and <i>Theileria</i> species	129
was performed by using PCR System 9700 (Applied Biosystems, Foster City, CA),	130
QIAGEN Multiplex PCR kits (Qiagen, Hilden, Germany) and the primer pair RLB-F2	131
and RLB-R2 (Gubbels et al., 1999; Matjila et al., 2004). To amplify a 460-540 bp	132
fragment, PCR mixtures composed of 9 µl of molecular grade water, 1.25 µl of each	133
primer (10 pmol), 12.5 µl of 2 x QIAGEN Multiplex PCR Master Mix and 1 µl DNA.	134
The PCR consisted of an activation step at 94 °C for 15 min, a touch down step of 12	135
cycles at 94 °C for 30 s, 69 °C for 90 s and 72 °C for 90 s with annealing temperature	136
decreasing every cycle by 1°C. This followed by 40 cycles at 94°C for 30 s, 57°C for	137
90 s and at 72°C for 90 s and a final extension step at 72°C for 10 min. PCR products	138
were stained with 10× Midori Green Direct (Nippon Genetics, Dören, Germany) and	139

examined by electrophoresis through 1% agarose gel and visualized by UV	140
illuminator.	141
<i>Oligonucleotide probes and reverse line blot (RLB) hybridization</i>	142
A total of 8 newly designed and 9 previously published oligonucleotide probes (Table	143
1) were received from Sigma Aldrich Co., LLC, Japan. The probe designing process	144
was performed according to (Kong and Gilbert, 2006). Briefly, the 18S rRNA gene	145
sequences for the target pathogens (Table 1) were downloaded from the GenBank and	146
aligned by using MEGA software version 6 (Tamura et al., 2013). The probe	147
sequences were selected for each target and evaluated by OligoEvaluator software	148
(Sigma Aldrich, MO, USA) to ensure optimum physical characteristics. Finally, the	149
selected probe sequences were searched in the Nucleotide Basic Local Alignment	150
Search Tool (BLASTn) (Altschul et al., 1990) and tested against the positive control	151
samples to ensure its specificity.	152
RLB was carried out with the PCR products as described before (Kong and Gilbert,	153
2006; Moustafa et al., 2016) with modifications. Briefly, a 15 x 15 mm Biodyne C	154
membrane (Pall Life Science, Ann Arbor, MI, USA) was activated for 10 min at room	155
temperature by 20 ml 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide	156
(EDAC) (Sigma Aldrich, MO, USA). The membrane was washed gently with milliQ	157
water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, Massa-	158
chusetts). A total of 17 oligonucleotide probes (Table 1) linked with C6 amino linker	159
were received from Sigma Aldrich Co., LLC, Japan, and reconstituted into 100 pmol	160
solutions. Ten microliters of each 100 pmol/μl probes were diluted in 0.5 M NaHCO ₃	161
to a final volume of 170 μl. The openings of the miniblotter were filled with a 150 μl	162
of each diluted oligonucleotide and the membrane was incubated at room temperature	163
for 5 min. The membrane was inactivated in 250 ml 0.1 M NaOH with gentle shaking	164

for exactly 8 min at room temperature, then washed at 60°C with prewarmed 250 ml
of 2× SSPE/0.1% SDS. Finally, the membrane was kept with 15μl of 20 mM EDTA
in a sealed plastic bag for the future use.

A total of 10 μl of each PCR product was diluted in 2× SSPE/0.1% SDS to a final
volume of 170 μl and denatured by heating at 99.9°C for 10 min and immediately
cooled on ice. The denatured mixtures were introduced to the membrane, and
hybridized at 60°C for 1 hr. Afterwards, this membrane was washed in 250 ml of 2×
SSPE/0.5% SDS twice at 52°C for 10 min and then incubated with diluted peroxidase
labeled NeutrAvidine (Thermo Fisher Scientific, Walyham, MA, USA) for 45-60 min
at 42°C. Subsequently, NeutrAvidine was washed away by 250 ml 2× SSPE/0.5%
SDS twice at 42°C for 10 min and 2× SSPE twice at room temperature for 5 min. To
visualize the results, the membrane was exposed for 5 min to 15 ml Immobilon™
Western Chemiluminescent HRP Substrate (Millipore, Japan), and photographed by
Ez-Capture MG/ST (ATTO Corp., Japan). To remove the PCR products, the
membrane was washed twice in pre-warmed 1% SDS at 90°C for 30 min and washed
with 250 ml of 20 mM EDTA. For the future reuse, the membrane was kept with 15
ml of 20 mM EDTA in a sealed plastic bag at 4°C.

Cloning and sequencing

To confirm RLB results, randomly selected twenty-one RLB-positive PCR products
were subjected to direct sequencing. The selected PCR products included eight, one
and twelve samples that hybridized with the probes which were designed to detect
Theileria sp. Thrivae, *Babesia divergens*-like and all *Babesia* and *Theileria* spp.,
respectively. To amplify a fragment of 460-540 bp from 18S rRNA gene of piroplasm
spp., KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Japan) and
the above-mentioned primers were used. PCR consisted of 2.5 μl of 10× KOD-Plus-

Neo buffer, 2.5 µl of dDNTPs (2mM), 1.5 µl of 25 mM MgSO₄, 0.75 µl of each 190
primer (10 pmol), 0.5 µl of KOD-Plus-Neo DNA polymerase and 1 µl of DNA. The 191
PCR conditions were 2 min at 94°C and 40 cycles of 10 s at 98°C, 30 s at 57°C and 30 192
s at 68°C, followed by a final extension 7 min at 68°C. The PCR products were 193
examined by electrophoresis and the positive bands at 460-540 bp were excised for 194
purification by NucleoSpin® Gel and PCR clean up (Macherey-Nagel, GM & CO KG, 195
Germany). The purified PCR products were sequenced by using the Big- Dye 196
Terminator version 3.1 Cycle Sequencing Kit and purified by BigDye® 197
XTerminator™ Purification Kit (Life Technologies Co., NY, USA). Subsequently, 198
the purified products were sequenced by ABI PRISM™ 310 genetic analyzer (Life 199
Technologies Co., NY, USA). BLASTn (Altschul et al., 1990) searches were 200
performed to compare the obtained sequences to reference sequences recorded in 201
GenBank for identification. 202

A total of 3 sequencing results with mixed signals were resolved by TA cloning. The 203
purified PCR products were A-attached by mixing 0.9 µl of 10× KOD-Plus-Neo 204
buffer, 0.9 µl of 2 mM dDNTPs, 0.54 µl of 25 mM MgSO₄, 6.66 µl of each purified 205
PCR product and 1µl of 10× A-attachment mix (Toyobo Co. Ltd.) at 60°C for 10 min. 206
One microliter of each A-attached product was cloned with pMD20 T-vector (Takara 207
Bio, Otsu, Japan). Five colonies per sample were selected and screened by 208
EmeraldAmp MAX PCR (Takara Bio, Otsu, Japan) as described in the manufacturer's 209
manual and sequenced as mentioned above. When a sequence was detected from one 210
clone, more 5 clones from the same sample were sequenced. The sequences were 211
considered genuine when they were recovered from more than 3 clones. 212

Phylogenetic analysis 213

A total of twenty-two 18S rRNA partial sequences were submitted to DDBJ. The
accession numbers were: (LC271196 - LC271213) from the direct sequencing of
eighteen PCR products and (LC215385 - LC215388) from cloning and sequencing of
three other PCR products. The obtained sequences were identified by using BLASTn
search against the sequences of *Babesia* and *Theileria* spp. in the GenBank. The
divergence distances between sequences were calculated by the Maximum Composite
Likelihood model in MEGA software version 6 (Tamura et al., 2013). The
evolutionary distance matrix was visualized as a heat map (Fig. 3 & 4) that was
developed by using the R Stats package in the R studio software (<http://www.R-project.org/>).

Results

A total of 269 sika deer blood DNA samples were molecularly screened for the
presence of *Babesia* and *Theileria* species by touch-down PCR and RLB
hybridization using 17 oligonucleotide probes (Table 1). The specificity of the newly
designed 8 oligonucleotide probes was ensured through examining the genomic DNA
samples of *B. microti*-US type, *B. microti*-Hobetsu type, *B. rodhaini*, *B. divergens*-
like, *B. bovis*, *B. bigemina*, *Theileria* sp. Thrivae and *T. orientalis* positive controls
(Fig. 1).

The results showed that 95.5% (257/269), 94.1% (253/269), 14.1% (38/269), 87.7%
(236/269) and 11.5% (31/269) of the sika deer blood DNA samples hybridized with
the *Babesia/Theileria* spp. catch-all, *Theileria* spp. catch-all, *Babesia* spp. catch-all,
Theileria sp. Thrivae and *B. divergens*-like oligonucleotide probes (Table 2),
respectively. In addition, 10.0% (27/269) of the PCR products hybridized with both
Theileria sp. Thrivae and *B. divergens*-like oligonucleotide probes, while 6.3%
(17/269) were positive for *Babesia / Theileria* spp. catch-all and *Theileria* spp. catch-

all probes, but negative for all species-specific probes (Fig. 2). Interestingly, 2.6% (7/269) hybridized with *Babesia* / *Theileria* spp. catch-all, *Theileria* spp. catch-all, *Theileria* sp. Thrivae and *Babesia* spp. 1 catch-all probes, but were negative for all *Babesia* spp. species-specific probes (Fig. 2).

The obtained 18S rRNA partial gene sequences were mainly divided into two *Theileria* and two *Babesia* spp. (Fig. 3 & 4). The similarity between the accession numbers (LC271208 - LC271213 and LC215385) was from 99 to 100%. Whereas sequence accession numbers (LC271196 - LC271207 and LC215386) were 99 - 100% similar to each other. The obtained sequence (LC215385) was identical to *T. cervi* (KT863532) from sika deer in China and 99% similar to *Theileria* sp. (AB012199 & AB012194) from sika deer in Japan, whereas sequence (LC215386) was identical to *Theileria* sp. (AB012189) from sika deer in Hokkaido, Japan and 99% similar to *T. capreoli* (KJ188219) from red deer in China. In addition, the obtained sequence (LC215387) was identical to *B. divergens*-like (KC465973 & KC465975) from sika deer in Japan, whereas sequence (LC215388) was 98% similar to *Babesia* sp. Kh-Hj42 (GU057380) from *Haemaphysalis japonica* in Russia.

Discussion

Sika deer act as reproductive hosts for several tick species, which may transmit several tick-borne pathogens to wildlife, livestock and human beings (Watanabe et al., 2016). During the last two decades, numbers of sika deer (*C. nippon*) populations in Hokkaido have been increasing (Ijima et al., 2015). In Japan, previous studies stated that sika deer act as reservoir hosts for *Theileria* sp. Thrivae and *B. divergens*-like (Ikawa et al., 2011; Inokuma et al., 2004; Zamoto-Niikura et al., 2014). In this study, we examined sika deer from Hokkaido for infection with 12 different piroplasm species by touch-down PCR and RLB hybridization. This study identified four

piroplasm species; *B. divergens*-like, undescribed *Babesia* sp., *Theileria* sp. Thrivae 264
 and *T. capreoli*. Unfortunately, no species-specific oligonucleotide probes were 265
 designed for the detected *Babesia* sp. and *T. capreoli*; both organisms were detected 266
 in this study by cloning and sequencing. The respective infection rates of the *B.* 267
divergens-like (11.5%) and *Theileria* sp. Thrivae (87.7%) detected in this study were 268
 similar to and higher than previously published reports from Japan (Ikawa et al., 2011; 269
 Watanabe et al., 2016; Zamoto-Niikura et al., 2014). This is possibly due to the higher 270
 sensitivity of RLB technique than conventional PCR assays. The hybridization results 271
 were confirmed by sequencing, which showed that all obtained sequences have the 272
 same hybridization sites as their species-specific probes. However, a total of 7 and 17 273
 PCR products, which were hybridized with the respective catch-all probes for *Babesia* 274
 and *Theileria* species, were negative for all other *Babesia* and *Theileria* species- 275
 specific probes. 276

The obtained partial 18S rRNA gene sequences were divided into two *Babesia* and 277
 two *Theileria* species. One *Babesia* sp. clustered with the previously published *B.* 278
divergens-like (Fig. 3) from sika deer in Japan (Zamoto-Niikura et al., 2014). The 279
 second *Babesia* sp. sequence was completely separated from the other *Babesia* spp. 280
 and clustered with a *Babesia* sp. which was detected in *Haemaphysalis japonica* from 281
 Russia (Fig. 3). Hence, additional research on this *Babesia* sp. is required to fully 282
 understand its molecular taxonomy and to identify its vector in Japan. 283

The phylogenetic position of the detected *Theileria* spp. in the sika deer of this study 284
 was among the benign theileriosis group, which includes *T. orientalis* and *T. buffeli* 285
 (Inokuma et al., 2008; Li et al., 2014). One *Theileria* spp. cluster was very similar to 286
Theileria sp. Thrivae (Fig. 4), which was reported from sika deer in Japan (Watanabe 287
 et al., 2016) and *T. cervi* from sika deer in China (Liu et al., 2016). However, this 288

cluster was completely divergent from *T. cervi* (U97054) that was detected in white-tailed deer and elk (*Cervus canadensis*) from the USA and Canada (Fig. 4), respectively (Chae et al., 1999). The second *Theileria* spp. cluster was closely related to *T. capreoli* (Fig. 4). Previously, *T. capreoli* 18S rRNA gene sequence (AB012189) was deposited into the GenBank from Japan, however, this study was not published. In addition, *Theileria* sp. Sola, which is 97.8% similar to *T. capreoli*, and was previously reported from Japan (Watanabe et al., 2016), was not detected in this study.

Conclusions

This study identified the role of sika deer in maintaining 4 different tick-borne protozoan parasites in Hokkaido, Japan with *Theileria* sp. Thrivae having the highest infection rates and *T. capreoli* needing more examinations to understand its ecology. Furthermore, a potentially novel *Babesia* sp. was detected in the sika deer samples of this study. However, this study lacks information about the prevalence of the undescribed *Babesia* sp. and *T. capreoli* in sika deer in Hokkaido. Future investigations will also be needed to identify the vector hosts for these organisms to fully understand the ecology of piroplasm spp. in Japan.

Competing interests

The authors declare that they have no competing interests.

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Probe	Sequence (5'-3')	Reference
<i>Babesia</i> / <i>Theileria</i> 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 A A CAGGC	Matjila et al. (2008)
<i>B. microti</i> all	GRCTTGGCATCWTCTGGA	Matjila et al. (2008)
<i>B. microti</i> Otsu and Hobetsu	GGGTACTGTTTCCAGGGT	This study
<i>B. microti</i> US	GGGTACTATTTTCCAGGAT	This study
<i>B. gibsoni</i>	TACTTGCCCTTGTCTGGTTT	Matjila et al. (2008)
<i>B. divergens</i> -like	TTAATCATAACWGATGTTTTG	This study
<i>B. rodhaini</i>	TGTGGATTAGTGCGCAAG	This study
<i>B. bigemina</i>	CTCGTAGTTGTATTT CAGCCT	This study
<i>B. bovis</i>	GAGCATGGAATAACCTTGTAT	This study
<i>B. venatorum</i>	GAGTTATTGACTCTTGTCTTTAA	Gigandet et al. (2011)
<i>B. divergens</i> & <i>B. capreoli</i>	GGTGTTAATATTGACTRATGTCGAG	Moustafa et al. (2016)
<i>B. duncani</i>	AGTTGAACTTCTGCCGCTT	Moustafa et al. (2016)
<i>Theileria</i> sp. Thrivae	ACGAGTGTCTGTATTGCG	This study
<i>Theileria orientalis</i> / <i>buffeli</i> / <i>sergenti</i>	TTTGAGTTTGTTATTGTGG	This study

TABLE 1 List of *Babesia* and *Theileria* spp. and their oligonucleotide probes used

for its detection

Total	No. of positive deer samples (%)
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		<i>Babesia</i> / <i>Theileria</i> all	<i>Theileria</i> spp. all	<i>Babesia</i> spp. all 1	<i>Theileria</i> sp. Thrivae	<i>B. divergens</i> -like
Fawn	108	102 (94.4)	98 (90.7)	26 (24.1)	86 (79.6)	25 (23.1)
Adult	161	155 (96.3)	155 (96.3)	12 (7.5)	150 (93.2)	6 (3.7)
Total	269	257 (95.5)	253 (94.1)	38 (14.1)	236 (87.7)	31 (11.5)

TABLE 2 Summary of *Babesia* and *Theileria* spp. detection in sika deer blood samples.

FIG 1 Validation of the newly designed oligonucleotide probes in this study. RLB
 result of *Babesia* and *Theileria* spp. genomic DNA derived 18S rRNA gene partial
 sequence (1-8). Oligonucleotide probes are listed on the left of the figure.

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FIG 3 Phylogenetic relationship between 18S rRNA gene sequences of *Babesia* spp. from GenBank. Sequences from the current study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number LC215387 and LC215388. The evolutionary distance matrix is visualized as a heat map. In the heat map, numbers in the table are presented as colored shades scaled from light to dark purple according to the degree of homology between sequences. The darker color the higher homology (less evolutionarily distant). White colors represent the same sequence

FIG 4 Phylogenetic relationship between 18S rRNA gene sequences of *Theileria* spp. from GenBank. Sequences from the current study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number LC215385 and LC215386. The evolutionary distance matrix is visualized as a heat map. In the heat map, numbers in the table are presented as colored shades scaled from light to dark blue according to the degree of homology between sequences. The darker color the higher homology (less evolutionarily distant). White colors represent the same sequence