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Title: Molecular identification and characterization of piroplasm species in Hokkaido sika deer (*Cervus nippon yesoensis*), Japan

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Abstract

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

Keywords

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

Introduction

Babesia and *Theileria* species are tick-borne protozoan parasites that have a veterinary and zoonotic importance (Mans et al., 2015). *Babesia* and *Theileria* spp. are obligatory intracellular parasites and genetically close to each other (Homer et al., 2000), however, both have different life cycles. In the mammalian host, *Babesia* spp. sensu stricto infect erythrocytes (Schreeg et al., 2016), while *Theileria* spp. infect both leukocytes and erythrocytes (Li et al., 2014).

In cattle, East Coast fever and tropical theileriosis are caused by *T. parva* and *T. annulata*, respectively, and exhibit fever, enlargement of lymph nodes and weight loss (Mans et al., 2015; Olds et al., 2016). In addition, *B. bovis* and *B. bigemina* are highly pathogenic in cattle and cause bovine babesiosis, a diseases which shows fever, hemolytic anemia and hemoglobinuria (Decaro et al., 2013). *Babesia ovis, B. motasi* and *B. crassa* cause sever disease in sheep which is characterized by anemia, fever, icterus and hemoglobinuria (Hashemi-Fesharki, 1997).

To the best of our knowledge, there is no published description of clinical manifestations in sika deer (*Cervus nippon yesoensis*) with piroplasmosis. However, clinical piroplasmosis may occur in the case of exposure to high stressful conditions such as malnutrition (Yabsley et al., 2005). The clinical symptoms of theileriosis in a white-tailed deer (*Odocoileus virginianus*) fawn were previously reported from the USA and included icterus and anemia (Yabsley et al., 2005).

One of the zoonotic *Babesia* spp. is *B. divergens*, which has been reported from several deer species including reindeer, roe deer and red deer in Europe (Michel et al., 2014). Recently, another zoonotic *B. divergens*-like has been detected in sika deer in Japan (Zamoto-Niikura et al., 2014). Whereas, many *Theileria* spp. have been

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

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

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

of samples (Kong and Gilbert, 2006) with high specificity and less cost than sequencing a large number of samples. The objective of this study was to estimate the infection rates and molecular characterization of several *Babesia* and *Theileria* spp. among sika deer in Hokkaido.

Materials and methods

Blood samples and DNA extraction

Whole blood samples were obtained from the jugular vein of 269 sika deer (*C. nippon yesoensis*) that were culled for nuisance control during 2010 to 2013 from Hokkaido prefecture, Japan. These blood samples were collected in EDTA-Na containing tubes and stored at -20 ºC until used. DNA was extracted from the blood samples by using the Wizard ®Genomic DNA purification Kit (Promega, Madison, WI, USA) as per manufacturer's instructions and kept at -20 ºC until analysis.

Touch-down Polymerase Chain Reaction (PCR)

A touch-down PCR targeting the 18S rRNA gene of *Babesia* and *Theileria* species was performed by using PCR System 9700 (Applied Biosystems, Foster City, CA), QIAGEN Multiplex PCR kits (Qiagen, Hilden, Germany) and the primer pair RLB-F2 and RLB-R2 (Gubbels et al., 1999; Matjila et al., 2004). To amplify a 460-540 bp fragment, PCR mixtures composed of 9 µl of molecular grade water, 1.25 µl of each primer (10 pmol), 12.5 µl of 2 x QIAGEN Multiplex PCR Master Mix and 1 µl DNA. The PCR consisted of an activation step at 94 ºC for 15 min, a touch down step of 12 cycles at 94 ºC for 30 s, 69 ºC for 90 s and 72 ºC for 90 s with annealing temperature decreasing every cycle by 1ºC. This followed by 40 cycles at 94ºC for 30 s, 57ºC for 90 s and at 72ºC for 90 s and a final extension step at 72ºC for 10 min. PCR products were stained with 10× Midori Green Direct (Nippon Genetics, Düren, Germany) and

examined by electrophoresis through 1% agarose gel and visualized by UV illuminator.

Oligonucleotide probes and reverse line blot (RLB) hybridization

A total of 8 newly designed and 9 previously published oligonucleotide probes (Table 1) were received from Sigma Aldrich Co., LLC, Japan. The probe designing process was performed according to (Kong and Gilbert, 2006). Briefly, the 18S rRNA gene sequences for the target pathogens (Table 1) were downloaded from the GenBank and aligned by using MEGA software version 6 (Tamura et al., 2013). The probe sequences were selected for each target and evaluated by OligoEvaluator software (Sigma Aldrich, MO, USA) to ensure optimum physical characteristics. Finally, the selected probe sequences were searched in the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990) and tested against the positive control samples to ensure its specificity.

RLB was carried out with the PCR products as described before (Kong and Gilbert, 2006; Moustafa et al., 2016) with modifications. Briefly, a 15 x 15 mm Biodyne C membrane (Pall Life Scince, Ann Arbor, MI, USA) was activated for 10 min at room temperature by 20 ml 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma Aldrich, MO, USA). The membrane was washed gently with milliQ water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, Massa-chusetts). A total of 17 oligonucleotide probes (Table 1) linked with C6 amino linker were received from Sigma Aldrich Co., LLC, Japan, and reconstituted into 100 pmol solutions. Ten microliters of each 100 pmol/µl probes were diluted in 0.5 M NaHCO3 to a final volume of 170 µl. The openings of the miniblotter were filled with a 150 µl of each diluted oligonucleotide and the membrane was incubated at room temperature for 5 min. The membrane was inactivated in 250 ml 0.1 M NAOH with gentle shaking

for exactly 8 min at room temperature, then washed at 60ºC 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 99.9ºC for 10 min and immediately cooled on ice. The denatured mixtures were introduced to the membrane, and hybridized at 60ºC for 1 hr. Afterwards, this membrane was washed in 250 ml of 2× SSPE/0.5% SDS twice at 52ºC for 10 min and then incubated with diluted peroxidase labeled NeutrAvidine (Thermo Fisher Scientific, Walyham, MA, USA) for 45-60 min at 42ºC. Subsequently, NeutrAvidine was washed away by 250 ml 2× SSPE/0.5% SDS twice at 42ºC 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 90ºC 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 4ºC.

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

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

Phylogenetic analysis

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

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

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

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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Agriculture and Veterinary Medicine, Japan) for providing us with the genomic DNA samples of the positive controls.

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Probe	Sequence (5'-3')	Reference
Babesia /*Theileria* all	TAATGGTTAATAGGARCRGTWG	Moustafa et al. (2016)
Theileria spp. all	ATTAGAGTGCTCAAAGCAGGC	Matjila et al. (2008)
Babesia spp. all 1	ATTAGAGTGTTTCAAGCAGAC	Matjila et al. (2008)
Babesia spp. all 2	ACTAGAGTGTTTCAAACAGGC	Matjila et al. (2008)
B. microti all	GRCTTGGCATCWTCTGGA	Matjila et al. (2008
B. microti Otsu and Hobetsu	GGGTACTGTTTCCAGGGT	This study
B. microti US	GGGTACTATTTTCCAGGAT	This study
B. gibsoni	TACTTGCCTTGTCTGGTTT	Matjila et al. (2008)
B. divergens-like	TTAATCATAACWGATGTTTTG	This study
B. rodhaini	TGTGGATTAGTGCGCAAG	This study
B. bigemina	CTCGTAGTTGTATTTCAGCCT	This study
B. bovis	GAGCATGGAATAACCTTGTAT	This study
B. venatorum	GAGTTATTGACTCTTGTCTTTAA	Gigandet et al. (2011)
B. divergens & B. capreoli	GGTGTTAATATTGACTRATGTCGAG	Moustafa et al. (2016)
B. duncani	AGTTGAACTTCTGCCGCTT	Moustafa et al. (2016)
Theileria sp. Thrivae	ACGAGTGTCTGTATTGCG	This study
Theileria orientalis /buffeli / sergenti	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 (%)

		Babesia /*Theileria* all	*Theileria* spp. all	*Babesia* spp. all 1	*Theileria* sp. Thrivae	*B. divergens*-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 *Theileia* spp. genomic DNA derived 18S rRNA gene partial sequence (1-8). Oligonucleotide probes are listed on the left of the figure.

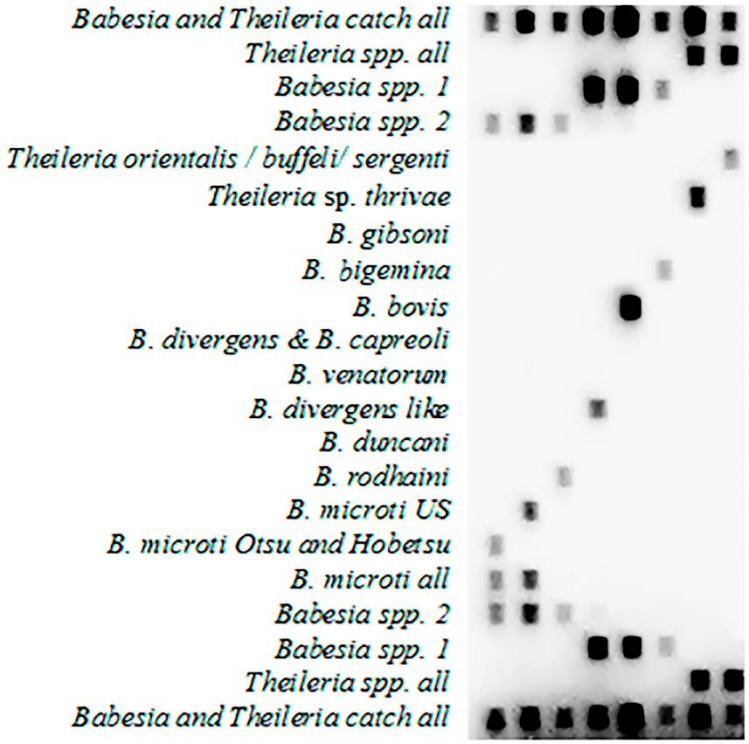


FIG 2 A representative RLB result. 1- 41: Sika deer blood derived 18S rRNA gene partial sequence. Oligonucleotide probes are listed on the left of the figure.

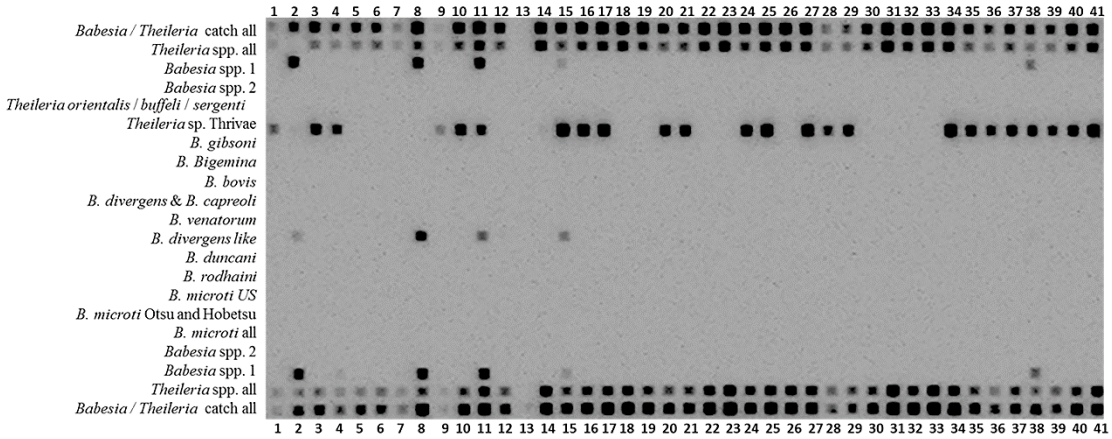


FIG 3 Phylogenetic relationship between 18S rRNA gene sequences of *Babesia* spp. from GenBank. Sequences from the current study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number LC215387 and LC215388. The evolutionary distance matrix is visualized as a 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

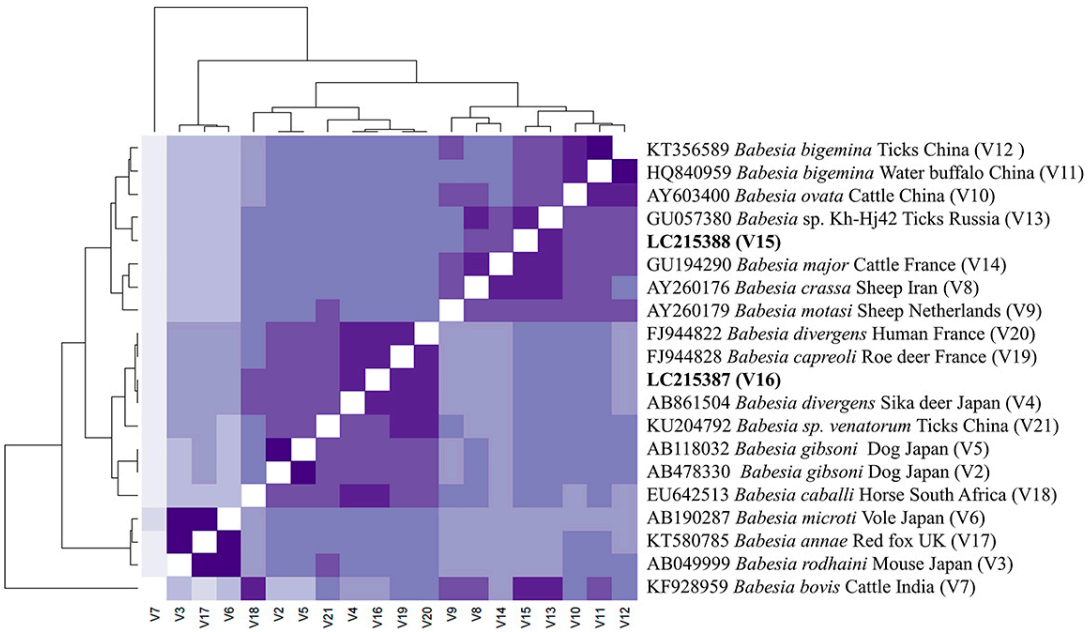


FIG 4 Phylogenetic relationship between 18S rRNA gene sequences of *Theileria* spp. from GenBank. Sequences from the current study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number LC215385 and LC215386. The evolutionary distance matrix is visualized as 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

