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Molecular evidence of zoonotic *Babesia* species, other than *B. microti*, in ixodid ticks collected from small mammals in the Republic of Korea

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Abstract

The occurrence of tick-borne infectious diseases, including zoonotic babesiosis, has become a serious concern in recent years. In this study, we detected *Babesia* spp. using polymerase chain reaction (PCR) amplification of the 18S rRNA of the parasites isolated from ixodid ticks collected from small mammals in the Republic of Korea (ROK). Sequence analysis of the PCR amplicon revealed the presence of *B. duncani*, *B. venatorum*, *B. capreoli/divergens*, and, the most prevalent, *B. microti* in the ticks. The molecular phylogenetic analysis showed that the four species-specific18S rRNA sequences clustered in four distinct clades. This is the first study to provide molecular evidence for the presence of zoonotic *Babesia* spp. other than *B. microti* in ticks in the ROK.

KEYWORDS
babesiosis, *Ixodidae*, ribosomal RNA, the Republic of Korea, tick

1 | INTRODUCTION

The incidence of tick-borne diseases is increasing worldwide, and this is attributed to the growth and geographical expansion of the tick populations. Considering the effect of tick-borne infections on human health, investigations of the geographical and seasonal distribution of ticks and the epidemiology of the associated pathogens are of importance (Gratz, 2006).

Human babesiosis is a zoonotic tick-borne disease caused by protozoan parasites belonging to the genus *Babesia*, which infect and destroy erythrocytes (Gray et al., 2010). This disease can also be transmitted through blood transfusion and organ transplantation, and even congenitally (Herwaldt et al., 2011; Vannier & Krause, 2012). The most common symptoms of human babesiosis are hemolysis, hemoglobinuria, fever, and hypoxia, which could be severe, moderate, or mild depending on the causative species and immunological status of the patient (Kirtz et al., 2012; Michel et al., 2014). Although more than 100 *Babesia* spp. have been shown to cause infections in animals (Gray et al., 2010; Vannier & Krause, 2012; Yabsley & Shock, 2013), only a few have been shown to be pathogenic to humans; among them, *B. microti* is the most prevalent followed by *B. divergens*, *B. duncani*, and *B. venatorum* (Fang et al., 2015; Leiby, 2011). Ticks of the genus *Ixodes* are the primary vectors of human babesiosis agents. *Ixodes scapularis* is the primary vector of *B. microti* in the United States (Hunfeld et al., 2008), whereas *I. spinipalpis*, *I. angustus*, *I. muris*, and *I. ricinus* are vectors in other parts of the world. *Ixodes ricinus* is the primary vector of *B. divergens* and *B. venatorum*, causative agents of human babesiosis mainly in Europe, while *I. persulcatus* is the most frequently encountered tick that transmits human *Babesia* parasites in Asia (Zamoto et al., 2004).

In the ROK, cases of human babesiosis have been sporadically reported, but most of them were imported (Kwon et al., 2018), while only two were endemic (Kim et al., 2007; Hong et al., 2019); however,

TABLE 1 Primers and PCR conditions for the detection of *Babesia* spp

Target	Primer sequence		Thermal cycles	Size
18S rRNA (Zintl et al., 2011)	1st PCR	BTH1F: 5′-CCTGAGAAACGGCTACCACATCT-3′ BTH1R: 5′-TTGCGACCATACTCCCCCCA-3′	94°C, 10 min; 45 cycles (95°C, 30 s; 68°C, 1 min; 72°C, 1 min), 72°C, 10 min	561 bp
	2nd PCR	GF2F: 5′-GTCTTGTAATTGGAATGATG-3′ GR2R: 5′-CCAAAGACTTTGATTTCTCTC-3′	94°C, 10 min; 40 cycles (95°C, 30 s; 60°C, 1 min; 72°C, 1 min), 72°C, 10 min	
β-tubulin (Zamoto et al., 2004)	1st PCR	Tubu93: 5′-GAYAGYCCCTTRCAACTAGAAAGAGC-3′ Tubu897R: 5′-CGRTCGAACGAACATTTGTTGHGTCARTTC-3′	95°C, 10 min; 35 cycles (95°C, 30 s; 58°C, 1 min; 72°C, 1 min 30 s), 72°C; 10 min	551 bp
	2nd PCR	Tubu192F: 5′-ACHATGGATTCTGTTAGATCYGGC-3′ Tubu782R: 5′-GGGAADGGDATRAGATTCACAGC-3′	94°C, 10 min; 45 cycles (94°C, 30 s; 61°C, 30 s; 72°C, 1 min), 72°C, 10 min	

the tick species responsible for the local transmission of the parasites were not identified. Among seven hard tick species known to bite humans in the ROK, *I. nipponensis* is the most frequently observed (Shin, 2014). Ixodids are common ectoparasites of ruminants and rodents (Kim et al., 2006; Kim et al., 2014; Shin et al., 2013).

Currently, the incidence of babesiosis is increasing globally. Although endemic babesiosis is not common in the ROK, surveillance of reservoirs and vectors of zoonotic *Babesia* spp. is necessary. In this study, we performed molecular detection of *Babesia* spp. in ticks collected from small mammals in various regions across the ROK.

2 | MATERIALS AND METHODS

2.1 | Collection and identification of ticks

Small mammals were surveyed from March to November, 2017 in the ROK. Animals were captured in several habitats, including waterways, farm roads, and mountain trails located around villages using Sherman folding live traps (BioQuip Products; Rancho Dominguez, CA) baited with cheese-spread biscuits. After their morphological classification, the small mammals were euthanized with compressed carbon dioxide, and ectoparasites were collected by suspending the animals over glass bowls filled with tap water for 24 h. Ticks that fell into the tap water were harvested and stored in 70% EtOH until identification. Individual ticks were placed on a chill table and identified by stereoscopic microscopy, according to the morphological classification keys (Yamaguti et al., 1971).

2.2 | Tick DNA extraction

Ticks were homogenized in PBS with zirconium oxide beads using a Precellys Evolution homogenizer (Bertin Technologies, Bretonneux, France). The homogenates were centrifuged, and the supernatant was collected. Tick genomic DNA was extracted using a MagMAX™ DNA Multi-Sample Ultra 2.0 Kit (Applied Biosystems, Foster City, CA, USA) and KingFisher® Flex magnetic bead absorption system (Model A5400630; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The extracted DNA was assessed for quantity and quality using a Nanodrop® 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C until used.

2.3 | PCR and nucleotide sequencing

Babesia spp. in ticks were identified based on the sequence of the 18S rRNA hypervariable region. Gene fragments were amplified by nested PCR using tick genomic DNA as a template; primers and thermocycling conditions are presented in Table 1. To confirm the identity of *B. microti*, nested PCR was also performed for *B. microti*-specific β-tubulin-encoding gene. The amplified products were subjected to electrophoresis in an automated QIAxcel® system (QIAgen, Hilden, Germany), purified, and sequenced using the Sanger method in an ABI PRISM® 3730xl Analyzer (Applied Biosystems, Foster City, CA, USA); each chromatogram was manually checked for quality using BioEdit® program ver. 7.2.5 (www.mbio.ncsu.edu/bioedit). The sequences were submitted to NCBI GenBank under accession numbers MT433886, MT433918, MT433920, MT433921, MT433922, and MT433923.

2.4 | Sequence analysis

The obtained *Babesia* sequences were subjected to BLASTN search to identify homologous genes available in GenBank. Multiple sequence alignment was performed using CLC Main Workbench Ver. 6.9 (CLC Bio, Aarhus, Denmark). Maximum likelihood phylogeny was evaluated using the neighbor-joining algorithm based on the Kimura 80 nucleotide substitution mode with 1000 bootstrap replicates. A phylogenetic tree of the 18S rRNA sequences was constructed using MEGA-X software.

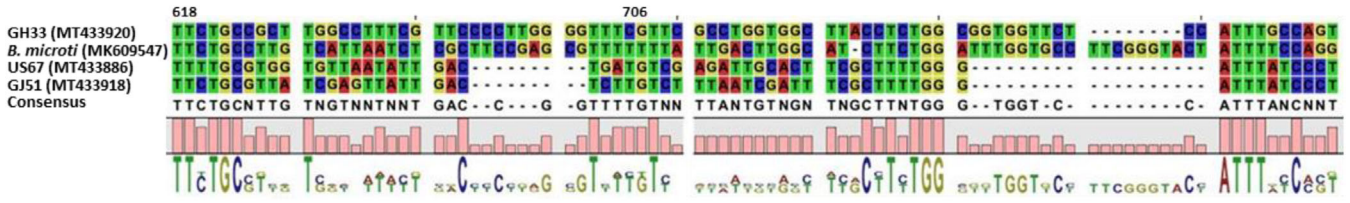

FIGURE 1 Multiple alignment of four 18S rRNA hypervariable region sequences of *Babesia* spp. identified in this study

3 | RESULTS

3.1 | Classification of small mammals and ticks

In our survey, *Apodemus agrarius* (black-striped field mouse) was the most prevalent small mammal, followed by *Crocidura lasiura* (Ussuri white-toothed shrew). Among the ectoparasites collected from them, ixodid ticks were morphologically classified. Ticks in the nymph stage were identified as *I. nipponensis* and *I. angustus*, whereas unidentifiable larval stage ticks were classified only to the genus *Ixodes*. Overall, ticks belonging to the genus *Ixodes* were the most prevalent in small mammals.

3.2 | PCR and sequencing analysis

To detect *Babesia* spp. in ticks, PCR amplification and sequencing of the 18S rRNA hypervariable region were performed. The results of multiple sequence alignment revealed four distinct 18S rRNA sequences of *Babesia* spp. (Figure 1). Among the four 18S rRNA sequences, the most prevalent one was 100% identical to that of *B. microti* isolated worldwide; the most prevalent sequence was identified in ticks from all sites examined in the study. Besides *B. microti*, other known zoonotic *Babesia* spp., *B. duncani*, *B. capreoli/divergens*, and *B. venatorum* were also identified. The 18S rRNA sequences amplified from *I. nipponensis* and *I. angustus* nymphs and *Ixodes* sp. larvae parasitizing *A. agrarius* from Goheung, Donghae, and Jeju showed 100% identity to those of *B. duncani* WA1, WA2, CA5, and CA6 isolates (Figure 2). The sequence of *Babesia* spp. of an *Ixodes* sp. larvae collected from *A. agrarius* in Uiseong was 99.82% identical to that of *B. capreoli* and *B. divergens*. As there was no sequence variation in the 18S rRNA hypervariable region between *B. capreoli* and *B. divergens*, we used the term "*B. capreoli/divergens*" in this study. Furthermore, the sequence of *Babesia* spp. from an *Ixodes* sp. larva from *C. lasiura* at Geoje was 99.08% identical to *B. venatorum*. None of the six tested samples were positive for *B. microti*-specific *β*-tubulin gene, as determined by PCR, indicating that there was no *B. microti* in the samples.

3.3 | Molecular phylogeny

A phylogenetic tree constructed based on multiple sequence alignment of the 18S rRNA sequences showed that four sequences of *B. duncani* (GH33, GH44, DH32, and JJ89) clustered in a big clade together with the sequences of *B. duncani* from North America (Figure 3). The 18S rRNA sequences of *B. venatorum* (GJ51) and *B. capreoli/divergens* (US67) clustered in two distinct clades. Overall, the phylogenetic analysis based on 18S rRNA sequencing revealed that *Babesia* spp. identified in this study belonged to four independent clades.

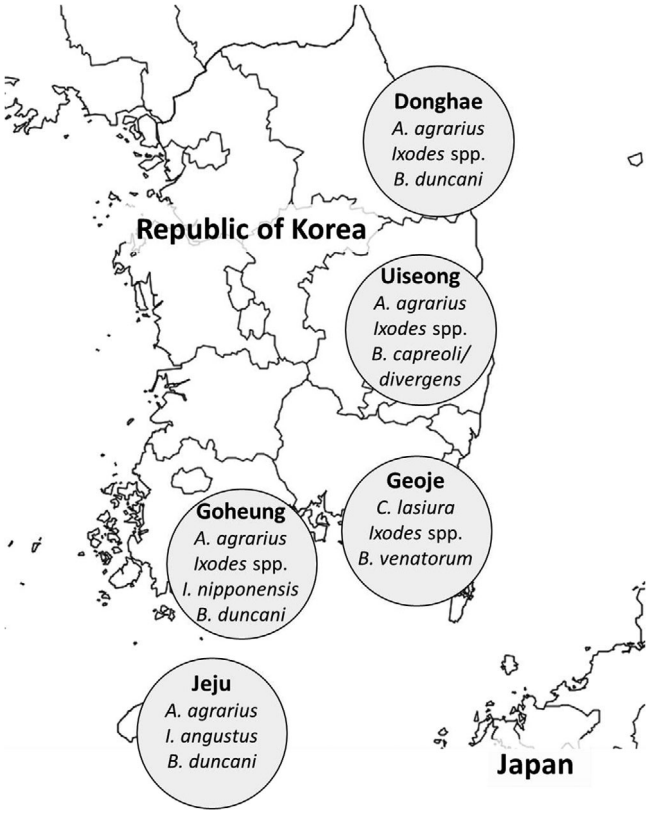

FIGURE 2 Geographical locations where zoonotic *Babesia* spp. were detected in the ROK. Circles show species of small mammals, ticks, and *Babesia* identified in each location

4 | DISCUSSION

Although seroprevalence and molecular diagnostic studies of babesiosis have been conducted in domestic and wild animals in the ROK, the identification of *Babesia* spp. directly from ticks inhabiting geographically isolated regions across the country has been rarely performed (Hong et al., 2019; Kang et al., 2013). To the best of our knowledge, in

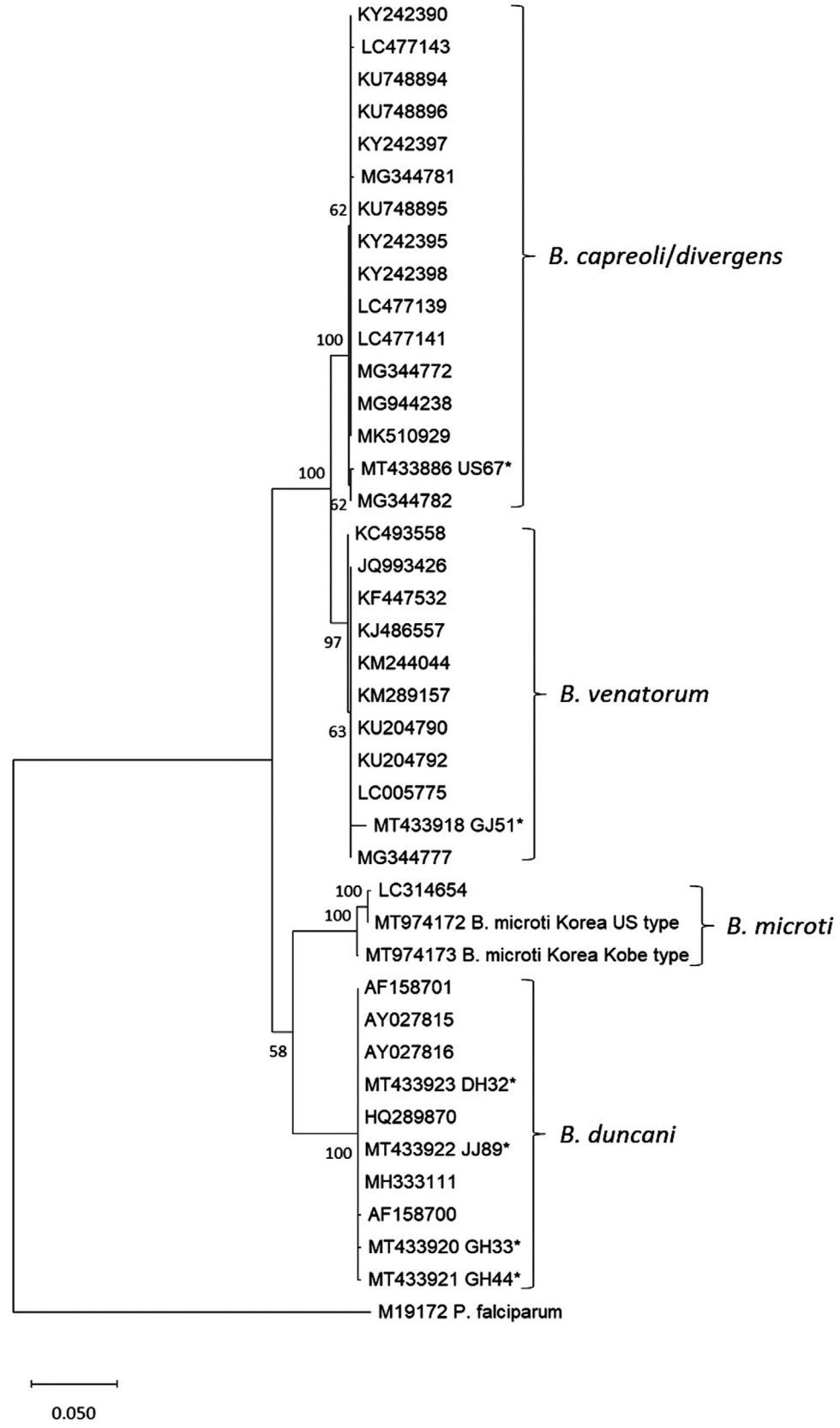

FIGURE 3 Phylogeny of *Babesia* spp. based on the 18S rRNA sequences. The phylogenetic tree was constructed using the neighbor-joining method. Scale bar shows an evolutionary distance of 0.05 nucleotide substitutions per position in the 18S rRNA sequence and numbers show bootstrap values (1000 replicates); *sequences identified in this study

this study, we identified *B. duncani*, *B. capreoli/divergens*, and *B. venatorum* in ticks parasitizing small mammals in the ROK for the first time.

Although *B. microti* is documented as the most widely distributed zoonotic *Babesia* sp., cases of animal infections have been reported in only small wild animals, but not domestic animals in the ROK (Hong et al., 2014; Hong et al., 2017; Hwang et al., 2017). Among the six species of small mammals captured in the country, only *A. agrarius* was positive for *B. microti*, as determined by PCR and indirect immunofluorescence assay (Hong et al., 2014). *Apodemus agrarius*, the most common wild rodent in rural areas in the country (Kim et al., 2013; Lee et al., 2009), is considered a reservoir of Hantaan virus, which causes hemorrhagic fever with renal syndrome (Lee et al., 1978; Lee et al., 1981), and *Leptospira interrogans*, which is an agent of leptospirosis (Cho et al., 1998). Furthermore, *O. tsutsugamushi* has been detected in chigger mites on *A. agrarius* in the ROK nationwide (Choi e al., 2018; Lee et al., 2009), indicating that this rodent is the dominant host of vectors transmitting zoonotic pathogens to humans. Although there is no information on the tick species associated with babesiosis in the two Korean patients reported previously (Hong et al., 2019; Kim et al., 2007), it is most likely that they originated from ticks on *A. agrarius*.

Since the first isolation of *B. duncani* type WA1 from a patient in the USA, human cases of *B. duncani* infection have been reported across the United States and Canada, with the highest incidence along the Pacific Coast (Scott, 2017; Scott and Scott, 2018; Swei et al., 2019). In this study, hypervariable regions of the 18S rRNA gene from four *Ixodes* ticks were identical to those of the WA1, WA2, CA5, and CA6 isolates of *B. duncani*. Although one 18S rRNA sequence identified in a Chinese tick (accession no. KX008042) was identical to that of the WA1 and CA5 isolates, there is no related publication. Therefore, to the best of our knowledge, the four sequences reported here represent the first evidence of *B. duncani* in ticks outside of North America.

Babesia divergens was associated with the first human case of babesiosis in 1957 in Europe (Skrabalo and Deanivič, 1957). Since then, the disease has been recorded worldwide. In this study, the 18S rRNA gene region identified in an *Ixodes* tick was similar to that in *B. capreoli* and *B. divergens*. The two *Babesia* spp. are closely related as evidenced by 99.83% identity in the 18S rRNA sequences; therefore, their identification based only on molecular analysis is challenging and should be performed considering even biological characteristics, including the spectrum of infected hosts (Malandrin et al., 2010). Recently, an 18S rRNA sequence of *Babesia* sp. was identified in a Korean water deer (*Hydropotes inermis argyropus*), and it was 92.2% identical to the sequence of *B. capreoli* and was distinct from the sequence of *B. divergens* (Shin et al., 2020).

Human infection with *B. venatorum* has been mainly reported in Europe (Häselbarth et al., 2007; Herwaldt et al., 2003). However, many cases of human babesiosis caused by *B. venatorum* have been recently described in China (Jiang et al., 2015; Sun et al., 2014), suggesting that the area of *B. venatorum* infectious to humans has expanded from Europe to Asia.

Although we detected various zoonotic *Babesia* spp. infected ticks, there is little information about autochthonous clinical babesiosis in the ROK. At least seven tick species have been documented in 38 previous reports on tick bite cases in the country, most of them related to *I. nipponensis* (Shin, 2014). In spite of the predominance of *Ixodes* spp. collected from small mammals (Kim et al., 2006; Kim et al., 2014; Shin et al., 2013), *H. longicornis* is the most commonly collected tick species in almost all areas in the ROK (Kim et al., 2014; Noh et al., 2019). Since the first case of severe fever with thrombocytopenia in the country (Kim et al., 2013), requests for the identification of ticks that have bitten humans have increased considerably in the Korea Disease Control and Prevention Agency, and over 80% of them were classified as *H. longicornis* (Yang et al., 2016). In a recent human case of babesiosis in the ROK, the tick was not specified, but *B. microti* and *B. motasi* genes were detected in *H. longicornis* and *H. flava* collected in the area around the patient's residence (Hong et al., 2019). In this study, *B. microti* 18S rRNA was found in one *H. longicornis* larva collected from *A. agrarius* from Jeju Island (data not shown). Therefore, detection of zoonotic *Babesia* spp. should be performed in both *Ixodes* and *Haemaphysalis* spp. in the country.

In summary, this is the first report for the molecular identification of *B. duncani*, *B. venatorum*, and *B. capreoli/divergens* in ticks of the ROK. It is possible that they are autochthonous species, as zoonotic babesiosis may have been overlooked in the country. Therefore, to prevent and prepare for the emergence of these zoonotic *Babesia* spp. in the ROK, extensive nationwide surveillance of ticks and their animal hosts should be performed.

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ETHICAL STATEMENT

The animal-handling protocol used in this study was based on the Institutional Animal Care and Use guidelines and was approved by the Ethical Committee of the Korea Centers for Disease Control and Prevention (KCDC-046-13-2A).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this study.

DATA AVAILABILITY STATEMENT

Data generated specifically for this study are included within the article. Materials obtained or generated for this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.581.

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