Detection of a *Babesia* sp. genotype closely related to marsupial-associated *Babesia* spp. in male *Haemaphysalis shimoga* from Sarawak, Malaysian Borneo

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**ABSTRACT.** In this study, *Babesia* screening was conducted in 55 rodents and 160 tick samples collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo. PCR targeting the 18S ribosomal DNA revealed the presence of *Babesia* spp. DNA detected in two questing male *Haemaphysalis shimoga* ticks collected from the oil palm plantation. Sequence analysis revealed that both sequences were identical and had 98.6% identity to a *Babesia macropus* sequence obtained from Eastern grey kangaroos (*Macropus giganteus*) in Australia. Phylogenetic tree revealed clustering with marsupial-associated *Babesia* spp. in the *Babesia* sensu stricto clade. Whether or not *H. shimoga* is the competent vector and the importance of the *Babesia* sp. detected in this study warrants more investigation.

**KEYWORDS:** Babesia, *Haemaphysalis shimoga*, ixodid tick, rodent, Sarawak

*Babesia* spp. are tick-borne protozoan parasites of the phylum Apicomplexa that infect erythrocytes of mammals and birds [33]. Since the discovery of *Babesia* species by Victor Babes in 1888 [1], over 100 *Babesia* spp. have been reported worldwide from a vast range of vertebrate hosts [15]. Furthermore, *Babesia* spp. are continuously being detected in new host species, including bears, badgers, kangaroos, and many more [3, 13, 27]. Recently updated phylogeny-based classification for piroplasmids designated *Babesia* spp. into *Babesia* sensu stricto (Clade X/(VI)), and *Babesia microti*-like (Clade I), Western clade (Clade III/(II), and Peircei group (Clade V) collectively as *Babesia* spp. in the *Babesia* sensu lato [15, 33]. Among the detected *Babesia* spp., *Babesia microti* is the main etiological agent related to marsupial-associated *Babesia* spp. [11]. In this study, *Babesia* screening was conducted in 55 rodents and 160 tick samples collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo. PCR targeting the 18S ribosomal DNA revealed the presence of *Babesia* spp. DNA detected in two questing male *Haemaphysalis shimoga* ticks collected from the oil palm plantation. Sequence analysis revealed that both sequences were identical and had 98.6% identity to a *Babesia macropus* sequence obtained from Eastern grey kangaroos (*Macropus giganteus*) in Australia. Phylogenetic tree revealed clustering with marsupial-associated *Babesia* spp. in the *Babesia* sensu stricto clade. Whether or not *H. shimoga* is the competent vector and the importance of the *Babesia* sp. detected in this study warrants more investigation. In the Southeast Asian region, there are several reports of *Babesia* spp. in humans, animals, and ticks. Two human cases inflicted by *B. microti* have been reported at the China-Myanmar border [40]. In addition, a previous study in Thailand reported a *Babesia* sp. detected in *Haemaphysalis lagrangei*, which was phylogenetically related to *Babesia* sp. KO1 from a human patient in Korea [39]. Dantrakool et al. [6] reported a *Babesia* sp. from *Bandicota indica* rats in Thailand, which morphologically resembled *B. microti*, but was closely related to *B. canis* in phylogenetic analysis. *Babesia bovis* and *B. bigemina*, the causative agents for bovine babesiosis, have been reported in Malaysia, Indonesia, Thailand, and Vietnam [4, 11, 23, 26, 31, 34]. Both *Babesia gibsoni* and *Babesia vogeli* have been detected in dogs from Malaysia and Thailand [8, 19, 30], while *B. vogeli* has been reported from the dogs in Cambodia [12, 14]. Additionally, *B. gibsoni* and *B. vogeli* were detected in *Rhipicephalus sanguineus* parasitizing dogs in Peninsular Malaysia [30]. Although several studies of *Babesia* spp. were conducted in Southeast Asian countries, knowledge of *Babesia* spp. in the region,
especially Sarawak Borneo, is still limited.

Previous studies undertaken in Sarawak state involved molecular and serological surveys in dogs [19] and cattle [31]. There has been no research into Babesia spp. in ticks and rodents from this state of Malaysia. Thus, we conducted a molecular survey on Babesia spp. in different tick and rodent species collected from two primary forests and an oil palm plantation in Sarawak, Malaysian Borneo, to identify the presence of Babesia spp.

A total of 160 questing and engorged ticks and 55 rodents were included for screening (Table 1), which were previously collected from two protected primary lowland (0–300 m) forests (Gunung Gading National Park: 1.69° N, 109.85° E, Kubah National Park: 1.61° N, 110.20° E) and an oil palm plantation (3.36° N, 113.69° E) in Sarawak. Morphological and molecular identifications were performed to confirm the genera and species for both tick and rodent samples as described previously [22] before the screening of Babesia. In brief, ticks and rodents were morphologically identified at the species or genus level using published identification keys [5, 17, 28, 29, 36] before molecular identification based on mitochondrial 16S ribosomal DNA (rDNA) for ticks [38] and cytochrome oxidase subunit I for rodents [32]. Of note, our previous study targeted only Ixodes ticks (n=32) for the detection of Borrelia spirochete [22], whereas, in the present study, the remaining 128 ticks were included. DNA was extracted from ticks and rodent spleens with the methods described by Lau et al. [22] using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) after crushing the ticks with Micro Smash MS-100R (TOMY, Tokyo, Japan). All samples used in this study were processed individually.

DNA from the ticks and rodent spleens were screened for Babesia spp. using polymerase chain reaction (PCR) targeting the 18S rDNA. Nested PCRs using BTH18S primer sets were carried out to screen all the samples (Table 1). The nested PCR used BTH18S 1st F and BTH18S 1st R primers in the first PCR and BTH18S 2nd F and BTH18S 2nd R primers in the second PCR, which yielded an approximately 1.4–1.6 kbp fragment [25]. All PCRs were conducted using KOD One PCR Master Mix (Toyobo, Osaka, Japan) with a reaction mixture of 20 µL. Then, the amplification products were observed with gel electrophoresis and purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), followed by Sanger sequencing in both directions for the 18S rDNA primer set. The Sanger sequencing was performed using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI Prism 3130x genetic analyzer (Applied Biosystems) following the manufacturer’s instructions.

Forward and reverse sequences were assembled and trimmed using the ATGC software version 9.0.0 (GENETYX, Tokyo, Japan) to obtain the consensus. Consensus sequences were then compared with those in public databases using BLASTn. MAFFT version 7.471 [16] was employed for sequence alignment with those reference sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications. The sequences obtained from public databases. The aligned sequences were then imported to MEGA X [21], where the Maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model with 1,000 bootstrap replications.

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Sampling and sample exportation were approved by the Forest Department Sarawak, Malaysia (Permit No. (91) JHS/NCCD/600-7/2/107 and Park Permit No. WL47/2018; Permit No. (11) JHS/NCCD/600-7/2/107 (Jld2) and Park Permit No.WL5/2019; Export Permit No.18650). All sampling methods were approved by the Animal Care and Use Committee of Hokkaido University, Japan (Approval No. 18-0081).

Overall, we investigated Babesia spp. in six different tick species, Ixodes granulatus, H. hystricis, H. shimoga, D. compactus, D. steini, and D. atrosignatus, and rodents collected in Sarawak, Malaysian Borneo. Babesia sequences were obtained from two tick samples (Sample IDs: HS55 and HS57) by nested PCRs. The Babesia-positive samples were male Haemaphysalis shimoga (2.9%, 2/69) collected from the oil palm plantation via flagging. Sequence analysis for BTH18S sequences revealed that both H. Shimoga samples had identical sequences. Furthermore, the sequences showed 98.6% (1,433/1,451 bp) identity with Babesia macropus detected from eastern grey kangaroos (Macropus giganteus) in Australia (JQ437265). Consistently, the phylogenetic tree based on the 18S rDNA sequences inferred that both our samples clustered in the Babesia sensu stricto clade (Clade X/(VI)) and were closely related to the marsupial-associated Babesia spp. (Fig. 1). None of the rodent spleen samples were positive for Babesia sp. in the screening with both 18S rDNA primer sets.

We detected the presence of Babesia sp. genotype in H. Shimoga, which is closely related to R. macropus, described from eastern

Table 1. Tick and rodent samples used in this study

| Rodent species           | Number of positive/tested |
|-------------------------|---------------------------|
| Leopodamy sabanus       | 0/3                       |
| Maxomys rajah           | 0/2                       |
| Maxomys whiteheadi      | 0/2                       |
| Rattus spp.             | 0/45                      |
| Sundamys muelleri       | 0/3                       |
| Total                   | 0/55                      |

N/A: not available.

Table 1. Tick and rodent samples used in this study

| Tick species          | Number of positive/tested |
|-----------------------|---------------------------|
| Ixodes granulatus     |                           |
| Female 0/22           | N/A                       |
| Nymph 0/5             | N/A                       |
| Larva 0/5             | N/A                       |
| Total 0/32            | N/A                       |
| Haemaphysalis hystricis|                           |
| Female N/A            | 0/1                       |
| Larva 0/4             | 0/33                      |
| Total 0/4             | 0/34                      |
| Haemaphysalis shimoga |                           |
| Male N/A              | 2/21                      |
| Female N/A            | 0/27                      |
| Nymph 0/18            | 0/3                       |
| Total 0/18            | 2/51                      |
| Dermacentor compactus |                           |
| Male N/A              | 0/2                       |
| Female N/A            | 0/2                       |
| Total N/A             | 0/4                       |
| Dermacentor steini    |                           |
| Female N/A            | 0/4                       |
| Larva 0/1             | 0/5                       |
| Total 0/4             | 0/9                       |
| Dermacentor atrosignatus|                         |
| Male N/A              | 0/2                       |
| Female N/A            | 0/2                       |
| Total N/A             | 0/4                       |
| Total                | 0/58                      |

2/102
grey kangaroos in Australia [7], and clustered with other marsupial-associated Babesia spp. However, there have been no records of marsupials on Borneo Island, even historically [28, 29]. The two positive samples in this study were male H. shimoga ticks collected from an oil palm plantation. Haemaphysalis shimoga was first described as H. cornigera subspecies from Southern India [36, 37], and its distribution included Cambodia, China, Myanmar, Thailand, and Vietnam [18, 24, 35]. To the best of our knowledge, H. shimoga has not been documented in Malaysia, or at least the species may not be identified in the available published literature from Malaysia. However, there have been records of the H. cornigera, both male and female individuals reported from Peninsula and Borneo Island [17], collected from sambar deer (Cervus unicolor), wild boar, and humans. Ixodes and Haemaphysalis ticks were speculated to likely be the tick vector of B. macropus [7, 9]. Another marsupial-associated Babesia sp., B. mackerrasorum, has been detected in H. bancrofti [2]. Nevertheless, the role of H. shimoga as the competent vector for the Babesia sp. detected in this study still requires further investigation.

The potential hosts and pathogenicity of the Babesia sp. detected in this study are unknown. However, the closely related B. macropus is of veterinary importance, with clinical symptoms such as severe anemia, emaciation, lethargy, and neurologic signs reported in macropods, with fatality also reported [7, 9]. The adult H. shimoga ticks are known to feed on sambar deer, but other hosts such as cattle, sheep, goats, and humans have also been recorded [10, 35]. Future investigation into the vector capacity and potential hosts of the Babesia sp. detected in this study will help understand its transmission cycle.

Babesia spp. were not detected from other tick species and rodent spleen samples, which could be due to the small sample size in this study. Future surveillance should encompass a more extensive sampling size to confirm the presence of other Babesia spp. in ticks and rodents. Nevertheless, this study reported a Babesia sp. genotype closely related to marsupial-associated Babesia spp. belonging to the Babesia sensu stricto clade for the first time from H. shimoga collected from the oil palm plantation. The importance of the Babesia sp. detected in this study warrants more investigation.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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31. Rahman WA, Lye YP, Chandrawathani P. 2010. The seroprevalence of bovine babesiosis in Malaysia. *Trop Biomed* 27: 301–307. [Medline] [CrossRef]
32. Robins JH, Hingston M, Matisoo-Smith E, Ross HA. 2007. Identifying *Rattus* species using mitochondrial DNA. *Mol Ecol Notes* 7: 717–729. [CrossRef]
33. Schnittger L, Rodriguez AE, Florin-Christensen M, Morrison DA. 2012. *Babesia*: a world emerging. *Infect Genet Evol* 12: 1788–1809. [Medline] [CrossRef]
34. Sivakumar T, Lan DT, Long PT, Yoshinari T, Tattiyapong M, Guswanto A, Okubo K, Igarashi I, Inoue N, Xuan X, Yokoyama N. 2013. PCR detection and genetic diversity of bovine hemoprotozoan parasites in Vietnam. *J Vet Med Sci* 75: 1455–1462. [Medline] [CrossRef]
35. Tanskul P, Stark HE, Inlao I. 1983. A checklist of ticks of Thailand (Acari: Metastigmata: Ixodoidea). *J Med Entomol* 20: 330–341. [Medline] [CrossRef]
36. Tanskul P, Inlao I. 1989. Keys to the adult ticks of *Haemaphysalis* Koch, 1844, in Thailand with notes on changes in taxonomy (Acari: Ixodidae). *J Med Entomol* 26: 573–601. [Medline] [CrossRef]
37. Trapido H, Hoogstraal H. 1964. *Haemaphysalis cornigera shimoga* subsp. n. from Southern India (Ixodoidea, Ixodidae). *J Med Entomol* 26: 573–601. [Medline] [CrossRef]
38. Ushijima Y, Oliver JH Jr, Keirans JE, Tsurumi M, Kawabata H, Watanabe H, Fukunaga M. 2003. Mitochondrial sequence variation in *Carlos capensis* (Neumann), a parasite of seabirds, collected on Torishima Island in Japan. *J Parasitol* 89: 196–198. [Medline] [CrossRef]
39. Wattanamethanont J, Kaewthamasorn M, Tiawsirisup S. 2018. Natural infection of questing ixodid ticks with protozoa and bacteria in Chonburi Province, Thailand. *Ticks Tick Borne Dis* 9: 749–758. [Medline] [CrossRef]
40. Zhou X, Li SG, Wang JZ, Huang JL, Zhou HJ, Chen JH, Zhou XN. 2014. Emergence of human babesiosis along the border of China with Myanmar: detection by PCR and confirmation by sequencing. *Emerg Microbes Infect* 3: e55. [Medline] [CrossRef]