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RESEARCH ARTICLE

First detection of *Babesia* sp. in Bornean sun bear (*Helarctos malayanus euryspilus* Horsfield) in Sabah, Malaysia

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ABSTRACT

The Bornean sun bear, a subspecies of the endangered Malayan sun bear, resides only on Borneo Island and little is known about diseases or parasites that may impact their health. In 2019, blood and ticks were collected from 46 captive bears held at the Bornean Sun Bear Conservation Centre in Sabah, Malaysia during annual health examinations in response to previous blood smear analysis that revealed presumptive haemoparasites in more than half the resident bears. Polymerase chain reaction detected a unique *Babesia* sp. in one of the bears. Disease surveillance of mosquitoes trapped along the outer perimeter of the bears' outdoor enclosure did not reveal any malaria parasites. This research marks the first documented case in Bornean sun bears of both a *Babesia* sp. and the Ixodes tick *Haemaphysalis* nr *koningsbergeri*. More research on incriminating the vector and the effects of *Babesia* infection on the health of Bornean sun bears is needed. Due to the zoonotic nature of babesiosis, mitigative actions should be taken to protect any humans that work with or come into close contact with these captive bears or their enclosures.

Keywords: *Babesia*; Bornean Sun Bear; *Haemaphysalis nr koningsbergeri*; Sabah.

INTRODUCTION

The Bornean sun bear (*Helarctos malayanus euryspilus* Horsfield), a subspecies of the Malayan sun bear resides only on Borneo Island (Meijaard, 2004). There is sparse information on diseases or parasites that may impact their health. Despite being protected under legislation in Malaysia, sun bears are still hunted for meat, gall bladders and bile (Gomez *et al*., 2020), while the cubs are also often captured for the illegal pet trade (Kunde, 2017).

The Bornean Sun Bear Conservation Centre (BSBCC) at Sandakan, Sabah was founded in 2008 to provide care and rehabilitation to former pets, orphaned cubs, and those rescued or confiscated by the Sabah Wildlife Department. BSBCC operates on a 2.5-hectare area in the Kabili-Sepilok Forest Reserve, providing individual indoor cages and an outdoor natural enclosure to its residents. In 2019, BSBCC housed 46 Bornean sun bears (19 males and 27 females) with mean age 9.4 years old (1-26) for males and 8.5 (1-18) years old for females. (Supplementary Table 1). All except seven are provided free access between their cages and the centre's fenced forest enclosure (BSBCC, 2019).

This study was initiated when haemoparasites were detected in the blood smears of the sun bears during routine health examinations in 2019. Ring shaped organisms suspected to be either *Plasmodium* sp. or *Babesia* sp. were observed in the red blood cells from Giemsa-stained blood smears (Figure 1).

The objective of this study was to investigate and identify haemoparasites in the sun bears kept at BSBCC, to help promote bears' health and to assess any zoonotic threat to the staff.

MATERIALS AND METHODS

Collection of blood samples and ticks

Annual health examinations, including taking blood samples, for all the resident bears were conducted by the Centre's veterinarian from April to December 2019. All bears were fasted for at least eight hours prior to being anaesthetized with a combination of 3 mg/kg Zoletil (Zolazepam/Tiletamine) and 1mg/kg Xylazine, administered via remote drug delivery system. Blood was collected via cephalic venipuncture in EDTA blood tubes. One set of EDTA samples was sent to Gribbles lab at Sandakan for hematological screening while another set was stored in -20°C freezer. In addition, a blood drop was also placed on an FTA card as a backup for DNA assay and stored at room temperature. The frozen blood samples and FTA cards were later transported by road in a cooler box with ice packs to the Universiti Malaysia Sabah laboratory (UMS) for haemoparasite investigation.

During the health examination procedure, ticks were also collected using forceps, kept in alcohol 70%, and later identified using published keys (Anastos, 1950; Tanskul & Inlao, 1989).

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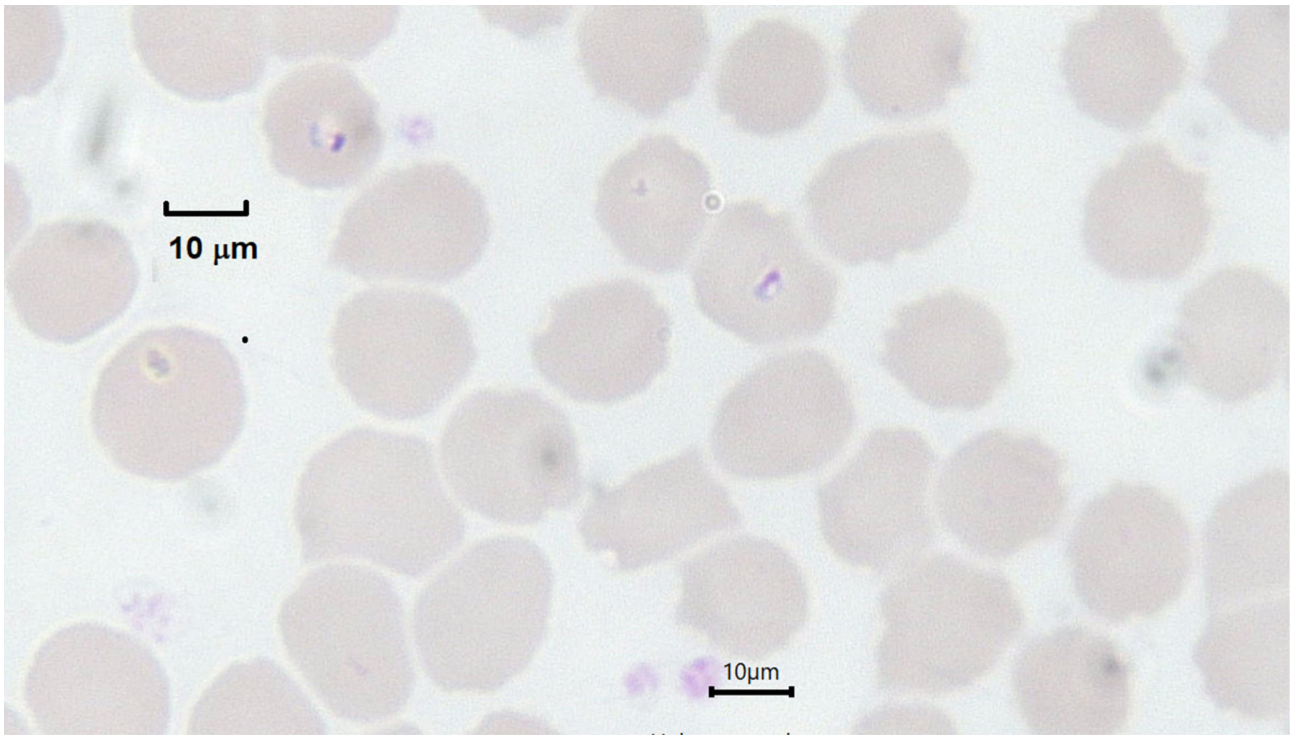

Figure 1. Blood smear of sun bear showing ring shaped haemoparasites.

Mosquito surveillance

In December 2019, mosquitoes were also trapped overnight from 1700-0700 for three days using two MosquitoMagnetic Traps (Woodstream Corporation) placed along the outer perimeter of the forested enclosure of the BSBCC. The mosquitoes were examined under microscopy and only *Anopheles* individuals were separated out, speciated using published keys (Sallum *et al*., 2005), stored individually in microfuge tubes and brought to UMS laboratory for PCR analysis.

DNA extraction

DNA was extracted from bear blood samples and *Anopheles* samples using the protocol described by (Phillips & Simon, 1995) and stored at -20°C until use. Blood samples were subjected to a control PCR for amplification of mammalian cytB gene using Cytb1 and Cytb2 primers (Meyer *et al*., 1995). *Anopheles* were subjected to a nested PCR targeting the *Anopheles* COII gene using the primers COIIF (5'-TCT AAT ATG GCA GAT TAG TGC A-3') and X2R (5'-TGA TTT AAG AGA TCA TTA CT TGC-3') for the first PCR reaction, and the primers X2F (5'- GGC AGA TTA GTG CAA TGA ATT -3') and COIIR (5'- ACT TGC TTT CAG TCA TCT AAT G -3') for the second PCR reaction (Hawkes *et al*., 2017).

The primers and protocol used for PCR reactions have been described for *Plasmodium* (Singh *et al*., 1999) and *Babesia* (Carret *et al*., 1999; Cacciò *et al*., 2002; Jefferies *et al*., 2003; Beck *et al*., 2009). The blood sample positive for *Babesia* was further subjected to PCR using CryptoF and CryptoR primers for the first PCR and PiroA1 and Bab2 for the second PCR. The resultant PCR product was purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA), cloned, and sent to Apical Scientific Sdn. Bhd. (Malaysia) for sequencing.

Phylogenetic analysis

The phylogenetic analysis was conducted using 98 sequences of 18S rRNA consisting of 87 *Babesia*, 2 *Cytauxzoon felis* and 9 *Theileria* sequences. The distances between our *Babesia* sequence and other sequences were estimated using pairwise p-distance with 1,000 bootstraps replications. The phylogenetic analysis was done using MEGA version X (Kumar *et al*., 2016). The phylogenetic tree was inferred using the maximum likelihood (ML) method and branch support was assessed with bootstrap values calculated from 1,000 replications. The heuristic search method was applied and the tree topology with the highest log likelihood value was selected.

RESULTS

All sun bears examined by the resident veterinarian were deemed clinically healthy. Furthermore there were no clinically significant hematology or serum biochemistry findings. Although the microscopic examination of the blood slides of 24/46 bears indicated the presence of ring shaped haemoparasites, none of the positive cases had shown any clinical symptoms. A total of two females, one male, and two nymph ticks were collected from different bears. These were identified as *Haemaphysalis nr koningsbergeri* Warburton & Nuttall.

Description of *Haemaphysalis nr koningsbergeri*

Female 1.7 mm long by 1.2 mm wide. Body oval, widest at mid-length. Scutum brown, oval, and slightly longer than wide, punctations medium, cervical grooves short, deep and parallel. Abdomen brown and uniformly covered with punctations; marginal groove distinct and short. Ventral body surface lighter, with numerous, medium punctations and a few, short, white hairs posteriorly; genital opening opposite coxae II and III; anal opening opposite spiracles; Capitulum basis nearly 2 times as wide as long; posterior margin concave; porose areas oval, large, widely separated, with a depression between them; hypostome long, widest at mid-length; dentition 4: 4, with approximately 13 teeth per file (Figure 2).

Detection and identification of haemoparasites

A total of 65 *Anopheles* from nine species were collected (Table 1). Of these *An. barbirostris*, *An. donaldi*, *An. roperi* and *An. whartoni* were the major species. *Plasmodium* DNA was not detected in any of the *Anopheles* or bear blood samples.

However, *Babesia* DNA was detected in one blood sample only (Figure 3). It has high sequence similarities (p-distance: 0.01201 ± 0.00365) with a group of *Babesia* isolates Kh-Hj441 and Kh-Hj540 from *Haemaphysalis japonica* ticks found in Russia and *Babesia* sp. UR1 isolated from a Hokkaido brown bear in Japan. Both groups of *Babesia* shared a common node with *B. gibsoni* but with a low bootstrap value (<70%) supporting the branch (Figure 4).

DISCUSSION

Although we had conducted PCR assays for *Plasmodium* in the sun bear blood samples, using the standard primers, we failed to detect *Plasmodium* spp. in any of the samples. This is not surprising as malaria parasites have never been reported in bears.

On the other hand, although the microscopic examination of the blood slides of 24/46 bears indicated the presence of ring shaped haemoparasites, none of the bears had shown any clinical symptoms. Furthermore, *Babesia* was detected in one sample only,

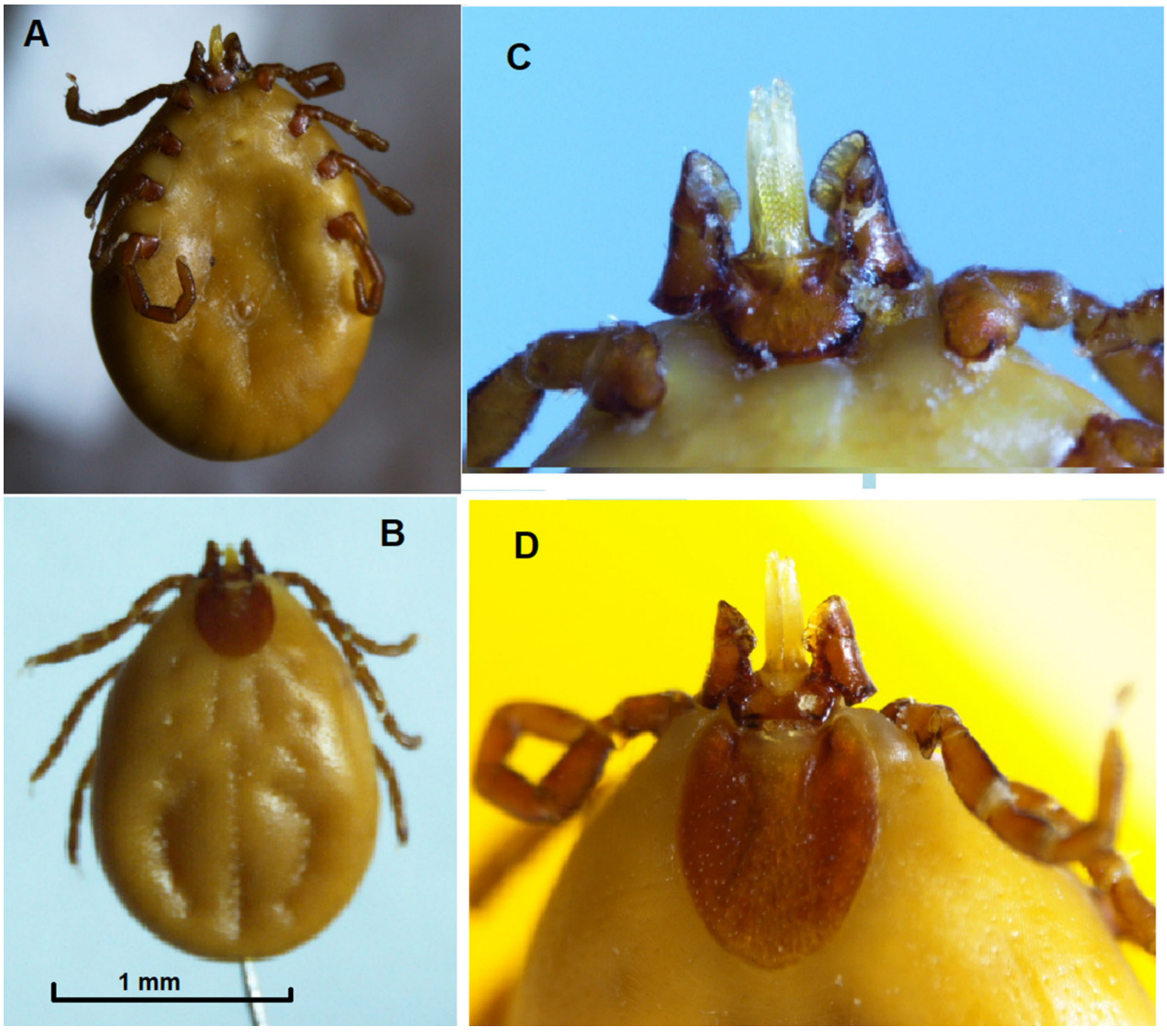

Figure 2. *Haemaphysalis nr koningsbergeri* Warburton & Nuttall female. A. Ventral view, B. Dorsal view, C. Capitulum ventral view, D. Capitulum dorsal view.

Table 1. *Anopheles* species caught at different sampling sites in the Bornean Sun Bear Conservation Centre. Abbreviation: BH2 bear house, J3, A3, C3 G3 and F3 are sites along the perimeter of the enclosed forest area for the bears. All 65 Anopheles tested negative for *Plasmodium* using PCR assay

Anopheles sp.	Sites						Total
	BH2	J3	A3	C3	G3	F3	
	16/12/2019		17/12/2019		18/12/2019		
An. balabacensis	0	0	0	2	0	0	2
An. barbirostris	0	0	0	7	0	0	7
An. donaldi	0	2	3	10	0	1	16
An. latens	1	1	0	1	0	1	4
An. letifer	0	0	1	2	0	0	3
An. montanus	0	0	0	2	0	0	2
An. roperi	0	0	0	6	2	0	8
An. tessellatus	0	0	0	1	0	1	2
An. whartoni	0	2	6	8	0	0	16
unidentified	0	0	2	2	0	1	5
Total individuals	**1**	**5**	**12**	**41**	**2**	**4**	**65**
Total species	**1**	**3**	**3**	**9**	**1**	**3**	**9**

Figure 3. Electrolysis gel showing blood sample of sun bear BSBCC46 positive for *Babesia* parasite using 3 pairs of PCR primers targeting the 18S rRNA. Lanes L: 100 bp DNA ladder (Promega); 1: PCR primers PiroA1 and Bab2; 2: PCR primers Bab1 and Bab2; 3: PCR primers PiroA1 and PiroB; 4: Negative control (No DNA template).

which could be due low parasitaemia, DNA degradation because of inappropriate transport protocol to UMS, and the non-ideal primers used.

The sole *Babesia* sp. detected revealed high sequence similarity (bootstrap value of 90%) with a group of *Babesia* found in a Hokkaido brown bear in Japan (Jinnai *et al*., 2010) and *H. japonica* in Russia (Rar *et al*., 2014). This *Babesia* group have been isolated from European badger, raccoon, dog and *Haemaphysalis* ticks. *Babesia* infection has also been recorded in the American black bears (Zolnik *et al*., 2015), giant panda (Yue *et al*., 2020) and Japanese black bears (Ikawa *et al*., 2011). It may be noted that most of the bears which had haemoparasites in the blood smears also harboured ticks. The association of *Babesia* and the tick should be further investigated to establish if *Haemaphysalis* sp. is the vector for *Babesia* in sun bear. These ticks could have been spread from small mammals in the enclosure as these in Sabah are heavily infested by tick species (Wells *et al*., 2013).

Reports of human babesiosis are on the rise. Infections by *B. microti* in the United States, *B. divergens* in Western Europe, *B. venatorum* in China (Ord & Lobo, 2015), *B. duncani* in Australia (Mayne, 2011), and *Babesia microti*-like organism in Taiwan (Shih *et al*., 1997) and *B. bovis* (Hoare, 1980) have been reported. It is an increasing global problem because of the expansion of tick habitats likely due to global warming and deforestation, coupled with the increased overlap of wildlife and human habitats. More research into *Babesia* spp. epidemiology, vector biology, ecology and effective intervention measure is needed.

This is the first record of *Babesia* and *Haemaphysalis nr koningsbergeri* (Ixodidae) in Bornean sun bear in Sabah. The BSBCC is the only Bornean Sun bear facility of its kind in the world and additional surveillance may not only prevent an outbreak of babesiosis among the resident bears, but also mitigate zoonotic transmission to the staff.

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Conflict of interest statement

The authors declare that they have no conflict of interest

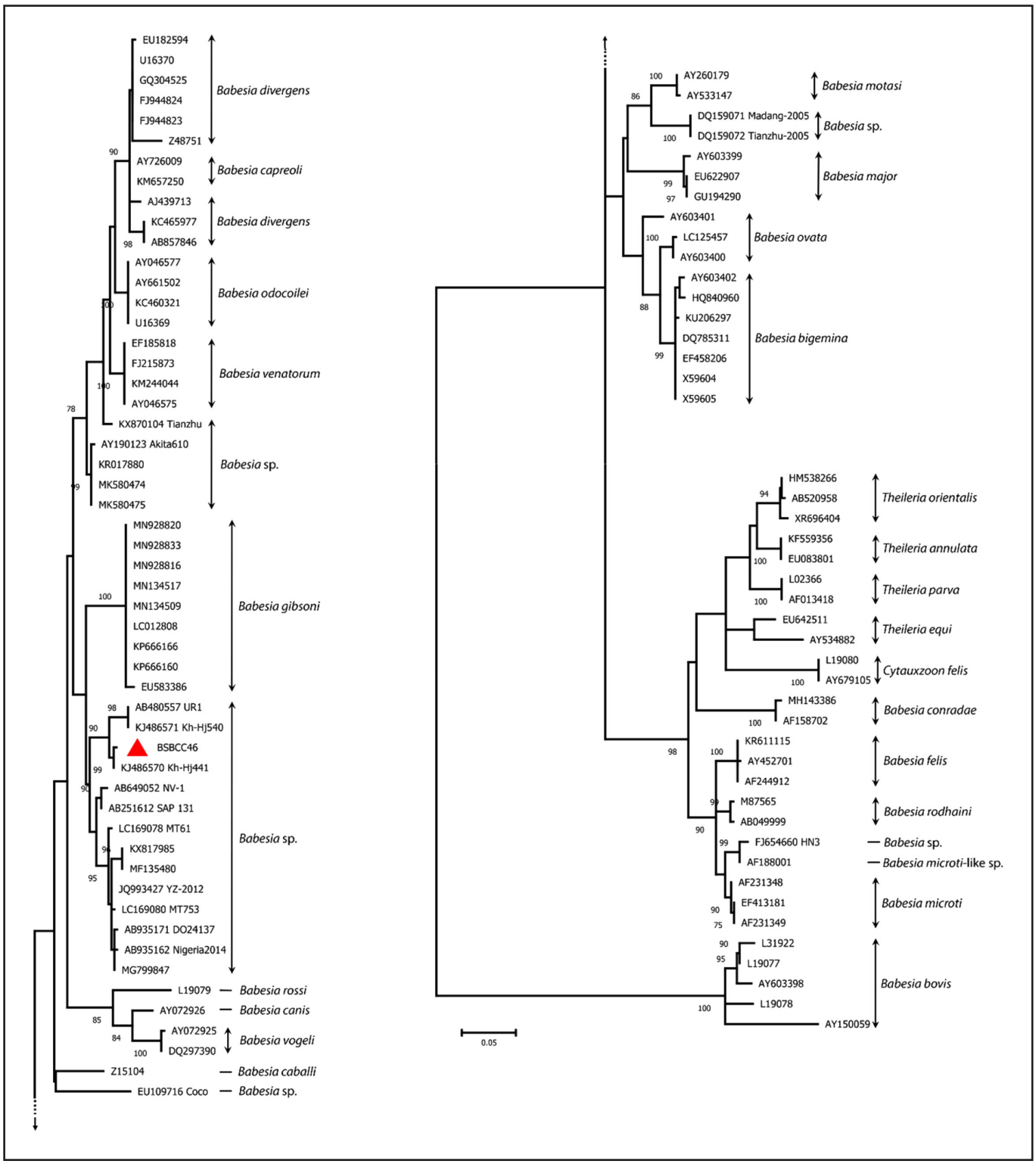

Figure 4. Phylogenetic tree constructed using 18S rRNA piroplasms sequences based on Kimura 2-parameter with invariable sites and gamma model. The *Babesia* sample in this study is marked with a red triangle. The bootstraps values (≥70%) are shown at the node of the branches. The bar below the tree represents nucleotide substitution per site. Other *Babesia* sequences were obtained from Genebank.

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