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Diversity of questing ticks and prevalence of tick-associated pathogens in Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri, Thailand

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ABSTRACT

Ixodid ticks are important vectors for tick-borne diseases distributed worldwide, including Thailand. Recreation areas within wildlife habitats are considered high-risk zones for tick exposure and tick-borne disease in humans. The study aimed to determine seasonal variations in tick diversity and pathogen prevalence in Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri, Thailand. From November 2021 to March 2023, a total of 1331 immature ticks were collected by dragging. The proportion of collected larvae was highest in February 2022, while the number of collected nymphs peaked in December 2021. Seven tick species were molecularly identified: *Haemaphysalis lagrangei*, *H. wellingtoni*, *H. shimoga*, *H. obesa*, *Dermacentor auratus*, *Rhipicephalus microplus*, and *Amblyomma integrum*. Of 80 tick pools, *Anaplasma*, piroplasms (*Babesia* and *Theileria*), *Bartonella*, and *Rickettsia* were detected in 10% (8/80), 3.75% (3/80), 1.25% (1/80), and 3.75% (3/80) of tick pools, respectively. Phylogenetic analysis grouped the newly generated sequences in the clades of *Anaplasma bovis*, *Babesia gibsoni*, *Theileria cervi*, *Bartonella henselae*, and *Rickettsia montanensis*. A seasonal pattern of pathogen appearance was detected during November to February, the cool season in Thailand. Based on our results indicating the highest peak of immature ticks and prevalence of pathogens, visitors should take precautions to avoid tick exposure during this season.

1. Introduction

Ixodid ticks (Acari: Ixodidae) are ectoparasites that infest domestic animals, wildlife, and occasionally humans. Ticks can transmit various types of pathogens to animals and humans *via* biting or blood-sucking and cause tick-borne diseases (TBDs) (de la Fuente et al., 2008; Brites-Neto et al., 2015). Most TBDs are zoonotic diseases considered an important public health concern. In Thailand, more than 53 species of ixodid ticks have been identified (Cornet et al., 2009). Tick-borne protozoan and rickettsial pathogens, as well as spotted fever group rickettsiae (SFGR), have been reported in several regions (Ahantarig et al., 2008; Malaisri et al., 2015; Sumrandee et al., 2016; Nooroong et al., 2018; Hirunkanokpun et al., 2022). Moreover, wild animals are considered significant reservoirs for these causative agents.

The family *Anaplasmataceae* includes intracellular gram-negative bacteria transmitted by ixodid ticks. Various species of *Anaplasma*, including *A. marginale*, *A. centrale*, *A. ovis*, and *A. bovis*, are obligatory bacteria that parasitize ruminants, whereas *A. platys* primarily infects dogs (Rymaszewska and Grenda, 2008). *Anaplasma phagocytophilum* is a pathogenic bacterium capable of infecting a broad spectrum of hosts, including humans as well as domestic and wild animals (Woldehiwet, 2010). *Ehrlichia* spp. are bacterial parasites that predominantly infect animal leukocytes (Yu and Walker, 2016). Further, wild animals can harbor pathogenic or zoonotic *Ehrlichia* spp. pathogens without showing clinical signs or symptoms, serving as potential sources of zoonotic transmission (Andre, 2018). Additionally, findings of *Anaplasma* spp. and *Ehrlichia* spp. in humans in Thailand were based on serological data, without supporting clinical evidence or pathogen isolation from humans (Heppner et al., 1997; Takhampunya et al., 2019).

In addition, protozoan parasites such as *Babesia* spp. and *Theileria*

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spp. are primarily transmitted by ticks and can infect the erythrocytes and/or leukocytes of numerous domestic and wild animals (Yabsley and Shock, 2013). While *Babesia* spp. have a broad host range, to date no species of *Theileria* has been identified as a causative agent of zoonotic infections (Yabsley and Shock, 2013; Karshima et al., 2021; Kumar et al., 2021). In wildlife, *Babesia* spp. infections can manifest with subclinical to clinical symptoms, including fever, hemolytic anemia, and mortality. Conversely, *Theileria* spp. infections in wildlife typically remain asymptomatic and act as a reservoir for infection (Bishop et al., 2004; Schnittger et al., 2012).

SFGR, transmitted by ticks, is distributed worldwide; many are emerging human pathogens (Piotrowski and Rymaszewska, 2020). Most tick-borne infections documented in humans in Thailand are caused by SFGR, with few cases confirmed through serological or molecular methods (Sirisanthana et al., 1994; Jiang et al., 2005; Gaywee et al., 2007; Ahantarig et al., 2008). Additionally, seropositivity for *Rickettsia conorii*, *R. helvetica*, and *R. felis* was determined in undifferentiated febrile illness patients in several regions of Thailand (Parola et al., 2003; Fournier et al., 2004; Ellis et al., 2006). In addition, there is molecular evidence of *Rickettsia* spp. detection in ticks in the country (Malaisri et al., 2015; Sumrandee et al., 2016; Nooroong et al., 2018; Hirunkanokpun et al., 2022; Usananan et al., 2022; Chaorattanakawee et al., 2024). Despite the lack of reports of SFGR infections in humans caused by tick bites in Thailand, the increasing number of people entering tick-endemic areas increases the potential for exposure to ticks carrying SFGR.

Bartonella spp. are intra-erythrocytic bacteria, transmitted among hosts by arthropod vectors (Chomel et al., 1996; Ellis et al., 1999). Based on molecular evidence, ticks were considered potential vectors for *Bartonella* spp. transmission (Chang et al., 2001; Kim et al., 2005; Rar et al., 2005; Tsai et al., 2011; Regier et al., 2017). Additionally, *Bartonella henselae* was isolated in blood culture or detected by PCR from human patients with a history of tick bites, emphasizing the point that ticks may serve as vectors for *Bartonella* spp. (Breitschwerdt et al., 2008; Maggi et al., 2013). Moreover, several studies in Thailand reported *Bartonella* spp. detection and isolation from animals and their ectoparasites (McKee et al., 2017; Pangjai et al., 2018; Saengsawang et al., 2021; Poofery et al., 2022) as well as human cases (Kosoy et al., 2008; Paitoonpong et al., 2008). To better understand bacterial circulation and transmission, there is a need for surveying for *Bartonella* spp. in ticks, especially in tick-endemic areas.

The Khao Kheow-Khao Chomphu Wildlife Sanctuary, located in the Chon Buri Province, Thailand, has been protected since 1974. It stands as an International Union for Conservation of Nature (IUCN) Category IV wildlife sanctuary, dedicated to safeguarding specific species and habitats through proactive management. Encompassing a diverse landscape of both dry and wet broadleaf forests, it spans an impressive area of 144.70 km^2. The sanctuary is home to a variety of wildlife, including 106 species of birds, 36 species of reptiles, 25 species of mammals, and 18 species of amphibians (Department of National Parks, Wildlife and Plant Conservation, unpublished data). Nonetheless, the sanctuary authorities have established designated areas where visitors can engage in recreational activities such as camping, bird and butterfly watching, hiking, and waterfall exploration, enhancing the sanctuary's appeal as a touristic destination. Therefore, it is deemed a high-risk zone for tick bites and tick-borne diseases in humans. To evaluate the risk of emerging tick-borne diseases and gain insights into seasonal variations in tick diversity and pathogen prevalence, we conducted longitudinal surveillance of bacterial, rickettsial, and protozoan pathogens in questing ticks within this wildlife sanctuary.

2. Materials and methods

2.1. Tick collection and morphological identification

Ixodid ticks were sampled monthly from November 2021 to March 2023 in Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri, Thailand (Fig. 1A and B). Two water-drinking areas for wildlife animals were chosen as sampling sites (Fig. 1C and D). Questing ticks were collected by dragging a white cloth over vegetation and visually searching for ticks along animal trails, with a minimum of 30 min spent at each site. All collected ticks were stored in microcentrifuge tubes with an RNA stabilization solution (RNA*later*™ Soln., Invitrogen, USA) and transported to the Veterinary Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University, for processing. All collected ticks were counted and identified according to genus, sex, and life stage under

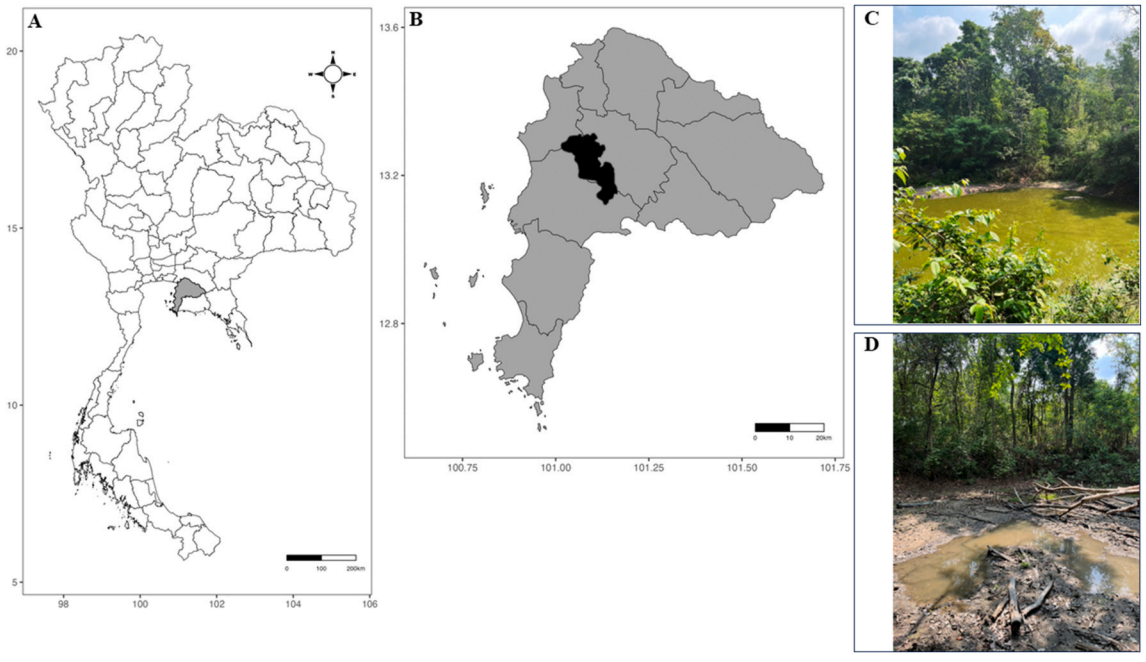

Fig. 1. Location of sampling sites. Chon Buri Province, Thailand shown in grey area (**A**), Khao Kheow-Khao Chomphu Wildlife Sanctuary shown in black area (**B**), sampling Site 1 (13°14′11″N, 101°02′40″E) (**C**), and sampling Site 2 (13°14′11″N, 101°02′37″E) (**D**).

a stereomicroscope using morphological identification keys (Hoogstraal et al., 1966, 1973; Tanskul et al., 1983; Hoogstraal and Wassef, 1985; Tanskull and Inlao, 1989). Immature ticks with similar morphologies and life stages were pooled (1–50 specimens/pool sample) according to the same sampling site and date. Tick species were further identified by PCR amplification. All samples were stored at −20 °C until DNA extraction.

2.2. DNA extraction from pooled tick samples and PCR amplification

Pooled tick samples were manually homogenized, and DNA was extracted using IndiSpin Pathogen Kit (Indical Bioscience, Leipzig, Germany), according to the manufacturer's instructions. Representative DNA samples of pooled ticks from each genus and life stage were selected and tested for tick species identification. All DNA samples were tested for the family *Anaplasmataceae*, piroplasms, *Bartonella* spp., and *Rickettsia* spp. using a PCR assay. The PCR mixture comprised a 25 µl reaction volume containing a DNA template, 10× PCR buffer (KOD One, TOYOBO Co., Ltd., Osaka, Japan), 10 µM of forward and reverse primers, and sterile distilled water. The PCR conditions included an initial denaturation at 98 °C for 1 min, followed by 35 cycles of denaturation and annealing. Denaturation occurred at 98 °C for 10 s in each cycle. The annealing temperature varied depending on the target: 55 °C for 30 s (tick identification), 53 °C for 30 s (family *Anaplasmataceae*), 50 °C for 30 s (piroplasms), 48 °C for 30 s (*Bartonella* spp.), and 45 °C for 30 s (*Rickettsia* spp.). The extension step was conducted at 68 °C for 5 s. The list of primers used and their sources is shown in Supplementary Table S1 (Regnery et al., 1991; Black and Piesman, 1994; Gubbels et al., 1999; Parola et al., 2003; Diniz et al., 2007). DNA from *Anaplasma marginale*, *Babesia canis*, *Bartonella henselae*, and *Rickettsia felis* (positive controls) and distilled water (negative control) were used as controls for PCR amplification. Pathogen PCR-positive products were purified using a GenepHlow Gel/PCR cleanup kit (Geneaid Biotech Ltd., Taipei City, Taiwan) and sent for nucleotide sequencing (U2Bio Co., Ltd, South Korea).

2.3. Nucleotide sequence analysis

All sequences from each pathogen PCR-positive sample were analyzed for the closest similarity with reference nucleotide sequences in the GenBank database using the NCBI nucleotide BLAST tool. All sequences were validated, aligned, and compared for genetic similarity using MegAlign (DNASTAR, Inc., USA). The number of nucleotide sequence types (ntSTs) and ntST diversity of tick species and pathogen-positive sequences were analyzed using DnaSP version 6.12.03 (Rozas et al., 2017). We analyzed the best-fit models for constructing phylogenetic trees using MEGA X (Kumar et al., 2018). Phylogenetic trees were generated using MEGA X with the maximum likelihood (ML) algorithm with the general time reversible (GTR) model plus gamma distribution (GTR+G+I) for tick species identification, Kimura 2-parameter model plus gamma distribution (K2+G) for *Anaplasma* spp. sequences, Tamura-Nei parameter model plus gamma distribution (TN93+G) for *Babesia* spp. and *Theileria* spp. sequences, and the Tamura 3-parameter model plus gamma distribution (T92+G) for *Bartonella* spp. and *Rickettsia* spp. Bootstrap support was inferred from 1000 replications.

2.4. Data analysis

Pathogen infection rates in pooled tick samples were evaluated using the bias-corrected maximum likelihood estimation (MLE) method with a 95% confidence interval, per 100 ticks in the PooledInfRate statistical software version 4.0 (Biggerstaff, 2009).

3. Results

3.1. Diversity of questing ticks

A total of 1331 immature stage ticks were collected from the wildlife sanctuary area in Chon Buri province, Thailand, from November 2021 to March 2023. However, no adult ticks were found in this study. The ticks were identified and sorted according to morphology, life stage, sampling

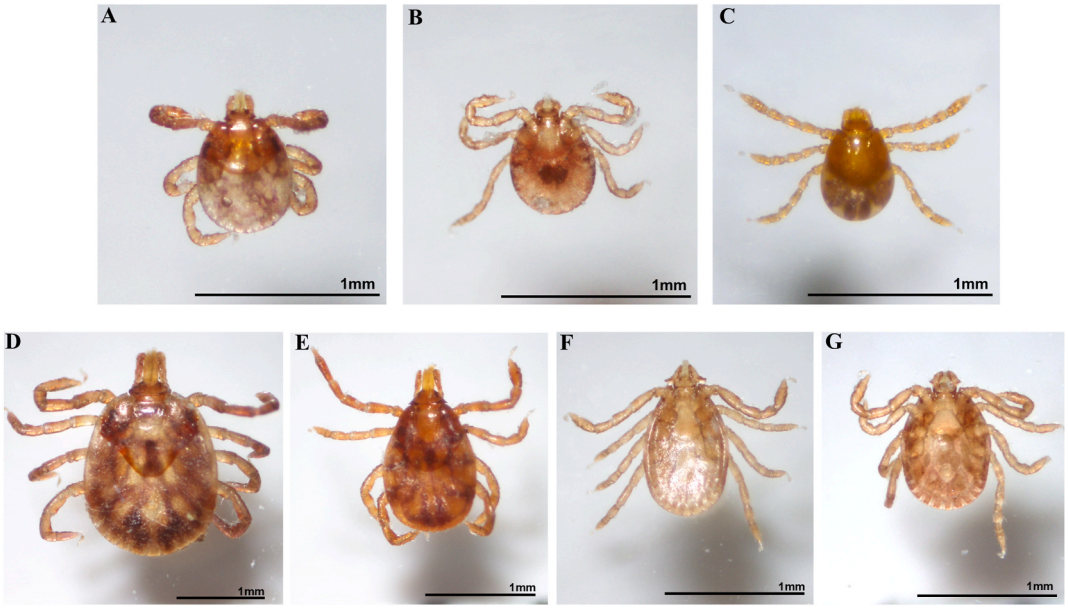

Fig. 2. Seven morphological forms in dorsal view of immature ticks collected in this study. **A** *Dermacentor* sp. larva related to *D. auratus* (GenBank: MT371592) with 99.75% similarity. **B** *Haemaphysalis* sp. larva related to *H. lagrangei* (GenBank: MG788690) with 100% similarity. **C** *Rhipicephalus* sp. larva related to *R. microplus* (GenBank: OQ725522) with 100% similarity. **D** *Amblyomma* sp. nymph related to *A. integrum* (GenBank: OP363195) with 100% similarity. **E** *Dermacentor* sp. nymph related to *D. auratus* (GenBank: MT371592) with 99.75% similarity. **F** *Haemaphysalis* sp. nymph related to *H. shimoga* (GenBank: KC170730) with 99.78% similarity. **G** *Haemaphysalis* sp. nymph related to *H. wellingtoni* (GenBank: MG283136) with 100% similarity.

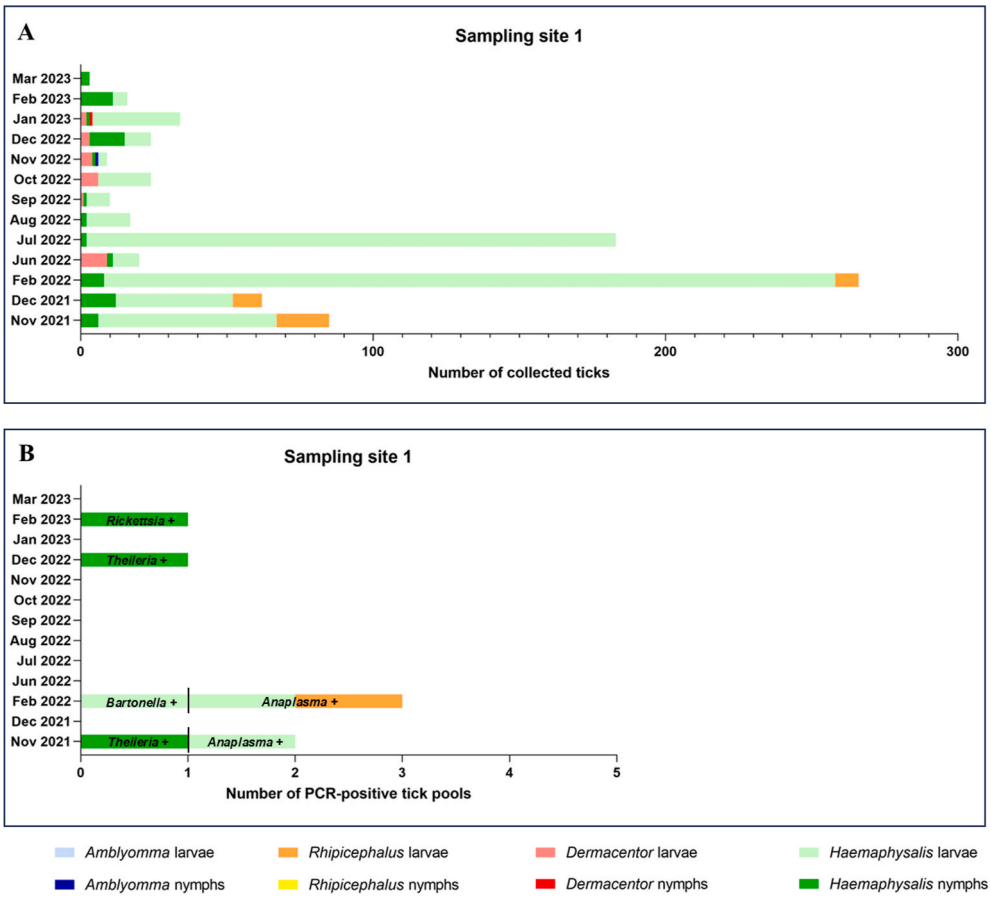

Fig. 3. The number of ticks collected (**A**) and PCR-positive tick pools (**B**) obtained at sampling Site 1, Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri Province, Thailand.

site, and collecting date. In both sampling sites, the number of collected larvae was highest in February 2022 (24.61%; 304/1235), while that of nymphs peaked in December 2021 (26.04%; 25/96). Three morphological forms were observed in the larvae, including *Dermacentor* sp.-like (Fig. 2A), *Haemaphysalis* sp.-like (Fig. 2B), and *Rhipicephalus* sp.-like (Fig. 2C). Four morphological forms were identified among nymphs, including *Amblyomma* sp.-like (Fig. 2D), *Dermacentor* sp.-like (Fig. 2E), and *Haemaphysalis* sp.-like (Fig. 2F and G). In sampling Site 1, a total of 753 ticks (690 larvae and 63 nymphs) were collected (Fig. 3A). The highest number of ticks (35.32%; 266/753) was collected in February 2022, followed by July 2022 (24.30%; 183/753). Based on the life stage, the number of collected larvae was highest in February 2022, while that of nymphs peaked in December 2021 and 2022. In this sampling site, *Haemaphysalis* larvae were the most frequently encountered species (83.53%; 629/753), followed by a small number of other tick species, including *Haemaphysalis* nymphs (8.10%; 61/753), *Rhipicephalus* larvae (4.78%; 36/753), *Dermacentor* larvae (3.32%; 25/753), *Dermacentor* nymph (0.13%; 1/753), and *Amblyomma* nymph (0.13%; 1/753) (Fig. 3A).

A total of 578 immature ticks (545 larvae and 33 nymphs) were collected from sampling Site 2 (Fig. 4A). The highest percentage of ticks (26.12%; 151/578) was collected in October 2022. The number of collected larvae at different life stages was highest in October 2022, while that of nymphs peaked in December 2021. *Haemaphysalis* larvae were mostly found (64.01%; 370/578) on this sampling site, followed by *Dermacentor* larvae (21.79%; 126/578), *Amblyomma* larvae (7.61%; 44/578), *Haemaphysalis* nymphs (3.11%; 18/578), *Amblyomma* nymphs (1.90%; 11/578), *Rhipicephalus* larvae (0.86%; 5/578), and *Dermacentor* nymphs (0.69%; 4/578) (Fig. 4A).

Additionally, ticks were sorted into 80 pooled samples, including 53 larval pools (14 *Dermacentor* sp.-like, 34 *Haemaphysalis* sp.-like, and 5 *Rhipicephalus* sp.-like) and 27 nymph pools (3 *Amblyomma* sp.-like, 3 *Dermacentor* sp.-like, and 21 *Haemaphysalis* sp.-like).

A total of 45 16S rRNA sequences of the ticks from representative samples (22 larval and 23 nymph pools) were validated, aligned, and grouped into nucleotide sequence types (ntSTs). In total, 17 ntSTs were revealed using DnaSP version 6.12.03, including ntST1 (10 sequences), ntST2 (7 sequences), ntST3 (4 sequences), ntST4 (1 sequence), ntST5 (1 sequence), ntST6 (1 sequence), ntST7 (1 sequence), ntST8 (1 sequence), ntST9 (2 sequences), ntST10 (3 sequences), ntST11 (2 sequences), ntST12 (1 sequence), ntST13 (1 sequence), ntST14 (1 sequence), ntST15 (5 sequences), ntST16 (3 sequences), and ntST17 (2 sequences). One sequence from each ntST was submitted into the GenBank database under the accession numbers PP831985-PP832001. The NCBI BLAST result showed that sequences from ntST1-9 showed 98.23–100% similarity with *H. lagrangei* from Thailand (GenBank: MG788690); ntST10 was identitical with *H. wellingtoni* from Thailand (GenBank: MG874023); ntST11 was identitical with *H. obesa* (GenBank:

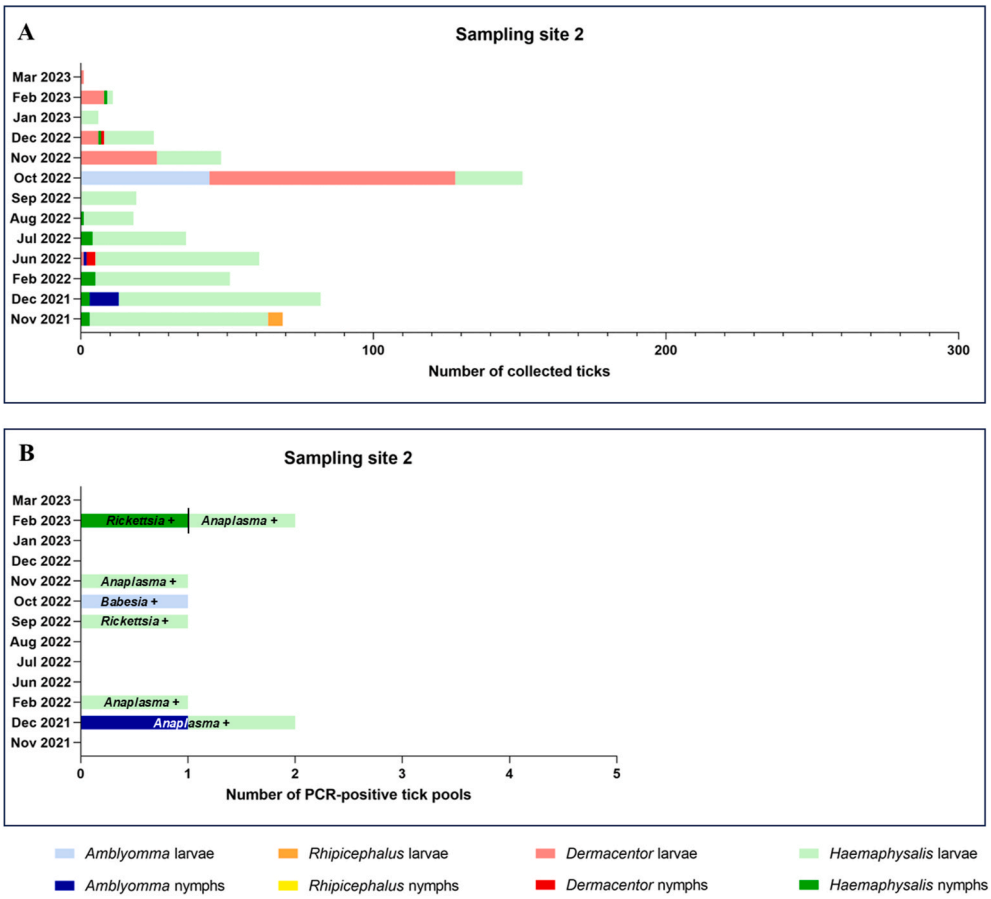

Fig. 4. The number of ticks collected (**A**) and PCR-positive tick pools (**B**) obtained at sampling Site 2, Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri Province, Thailand.

KC170732); ntST12-14 had 100%, 99.74%, and 99.23% similarity with *H. shimoga* from Thailand (GenBank: KC170730), respectively; ntST15 showed 99.74% similarity with *D. auratus* from Singapore (GenBank: MT371592); ntST16 was identical with *A. integrum* from India (GenBank: OP363195); and ntST17 was identical with *R. microplus* from Colombia (GenBank: MN650726). Next, 17 representative tick sequences from each ntST were used to construct a phylogenetic tree for comparison with reference tick sequences from the GenBank database (Fig. 5).

3.2. Pathogen detection in tick pools

Overall, PCR results showed that 10% (8/80), 3.75% (3/80), 1.25% (1/80), and 3.75% (3/80) of tick pools carried DNA of *Anaplasma* spp., piroplasms (*Babesia* spp. and *Theileria* spp.), *Bartonella* spp., and *Rickettsia* spp., respectively (Table 1). MLE of pathogen infection rate of tick pools indicated 0.64% for *Anaplasma* spp., 0.23% for piroplasms, 0.22% for *Rickettsia* spp., and 0.08% for *Bartonella* spp. No co-detection of pathogens was found.

Of the 15 pathogen PCR-positive samples, *Haemaphysalis* spp. were the most common ticks ($n = 12$) harboring pathogen DNA (Fig. 3B and 4B). At sampling Site 1, seven *Haemaphysalis* spp. pools were infected with pathogens. Among them, *H. lagrangei* pools tested positive for *Anaplasma* spp. ($n = 2$), *Bartonella* spp. ($n = 1$), and *Rickettsia* spp. ($n = 1$). Additionally, one *H. shimoga* pool was positive for *Anaplasma* spp. DNA. *Theileria* spp. DNA was detected in two *H. obesa* pools (Table 1 and Fig. 3B). At sampling Site 2, *Anaplasma* spp. DNA was found in three *H. lagrangei* pools. *Amblyomma integrum* ($n = 1$) and *Rhipicephalus microplus* ($n = 1$) pools also tested positive for *Anaplasma* spp. Moreover, *Rickettsia* spp. DNA was detected in two *H. lagrangei* pools, and *Babesia* spp. DNA was found in an *A. integrum* pool (Table 1 and Fig. 4B). The occurrence of pathogens at different collection times is shown in Fig. 3B and 4B. In questing ticks, *Anaplasma* spp. were found from November 2021 to February 2022 and from November 2022 to February 2023, while *Rickettsia* spp. were found in September 2022 and February 2023. *Bartonella* spp. and *Babesia* spp. were detected in February and October 2022, respectively. *Theileria* spp. was detected in November 2021 and December 2022.

3.2.1. Anaplasma spp. detection

Eight pooled tick samples (10.25%) were PCR-positive for the 16S rRNA gene of the family *Anaplasmataceae*: one *A. integrum* nymph pool; five *H. lagrangei* larval pools; one *H. shimoga* larval pool; and one *R. microplus* larval pool sample (Table 1). Eight sequences generated from *Anaplasma* spp. PCR-positive samples were grouped into six ntSTs. One representative sequence from each ntST was submitted to the

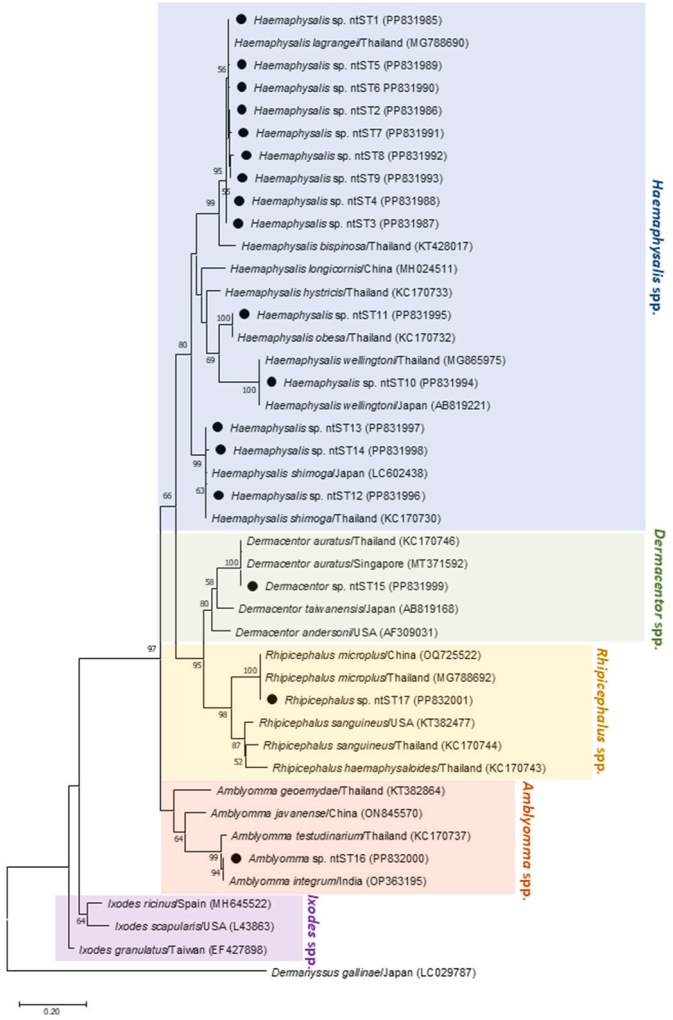

Fig. 5. Maximum likelihood (ML) tree of tick 16S rDNA gene sequences (primer cut; 405 bp) computed with the GTR+G+I model. The phylogenetic relationships of the newly generated sequences (black dots) and reference sequences from the GenBank database. *Dermanyssus gallinae* (GenBank: LC029787) was used as the outgroup.

GenBank database: ntST-A1 (3 sequences; GenBank: PP820820); ntST-A2 (1 sequence; GenBank: PP820821); ntST-A3 (1 sequence; GenBank: PP820822); ntST-A4 (1 sequence; GenBank: PP820823); ntST-A5 (1 sequence; GenBank: PP820824); and ntST-A6 (1 sequence; GenBank: PP820825).

BLAST results revealed that ntST-A1-A5 were closely related to several *Anaplasma* species such as *A. capra* (GenBank: LC432114), *A. marginale* (GenBank: OR520946), and *A. ovis* (GenBank: OQ380621), and ntST-A6 was closely related to *A. bovis* detected from a horse in South Korea (GenBank: MK028574), cattle in China (GenBank: MH255937), goat in China (GenBank: KP062958), and questing tick in China (GenBank: KP314248) with 100% similarity. The phylogenetic tree showed that the ntST-A6 sequence was clustered in the same clade as *A. bovis*, while ntST-A1-A5 sequences were grouped in the clade of other ruminant-associated *Anaplasma* spp. (Fig. 6).

3.2.2. Piroplasm detection

Three pool tick samples (3.84%) were PCR-positive for the 18S rRNA gene of piroplasms, including one *A. integrum* larval pool and two *H. obesa* nymph pool samples (Table 1). Three sequences were separated into three ntSTs and submitted to the GenBank database: ntST-P1 (1 sequence; GenBank: PP814794); ntST-P2 (1 sequence; GenBank:

Table 1
Pathogen detection rate and infection rate (MLE) from tick pools collected from Khao Kheow-Khao Chomphu Wildlife Sanctuary, Chon Buri Province, Thailand.

Pool samples	Positive samples for 16S rRNA gene of the *Anaplasmataceae* (345 bp)		Positive samples for 18S rRNA gene of piroplasms (460–520 bp)		Positive samples for 16S-23S intergenic spacer (ITS) region of *Bartonella* spp. (620 bp)		Positive samples for citrate synthase (*glt A*) gene of *Rickettsia* spp. (381 bp)	
	Detection rate	MLE (95% CI) (%)	Detection rate	MLE (95% CI) (%)	Detection rate	MLE (95% CI) (%)	Detection rate	MLE (95% CI) (%)
Nymphs (n = 27)	3.70% (1/27; *Ai*)	1.06 (0.06–5.15)	7.40% (2/27; *Ho*)	2.21(0.40–7.26)	(0/25)		7.40% (2/27; *Hl*)	2.13 (0.39–6.88)
Larvae (n = 53)	13.20% (7/53; *Hl, Hs, Rm*)	0.60 (0.27–1.19)	1.88% (1/53; *Ai*)	0.08 (0–0.40)	1.88% (1/53; *Hl*)	0.08 (0–0.40)	1.88% (1/53; *Hl*)	0.08 (0–0.39)
Total (n = 80)	10.00% (8/80)	0.64 (0.30–1.21)	3.75% (3/80)	0.23 (0.06–0.61)	1.25% (1/80)	0.08 (0–0.37)	3.75% (3/80)	0.22 (0.06–0.60)

Abbreviations: NA, not applicable; *Ai*, *A. integrum*; *Hl*, *H. lagrangei*; *Ho*, *H. obesa*; *Hs*, *H. shimoga*; *Rm*, *R. microplus*.

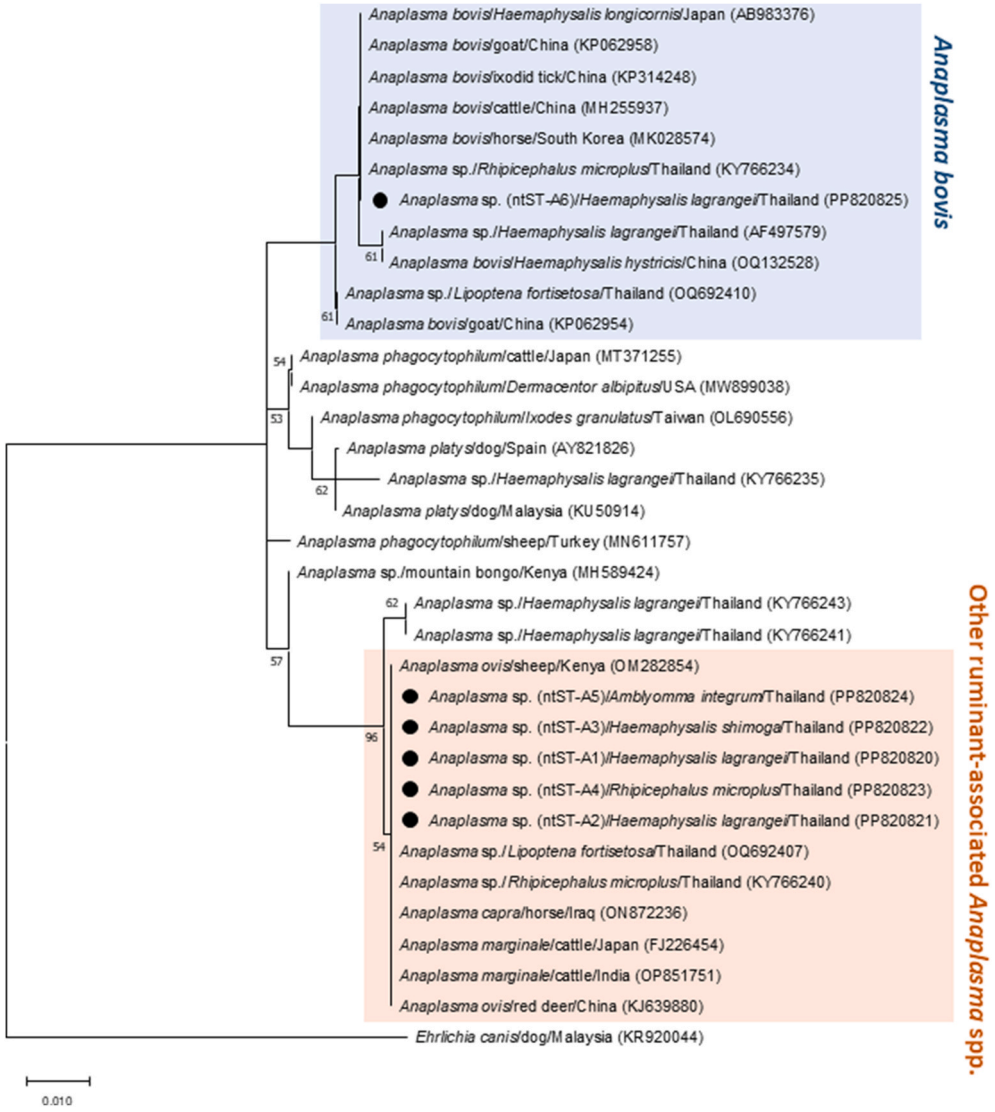

Fig. 6. Maximum likelihood (ML) tree of *Anaplasmataceae* 16S rRNA gene sequences (primer cut; 305 bp) computed with the K2+G model. The phylogenetic relationships of the newly generated sequences (*black dots*) and reference sequences from the GenBank database. *Ehrlichia canis* (GenBank: KR920044) was used as the outgroup.

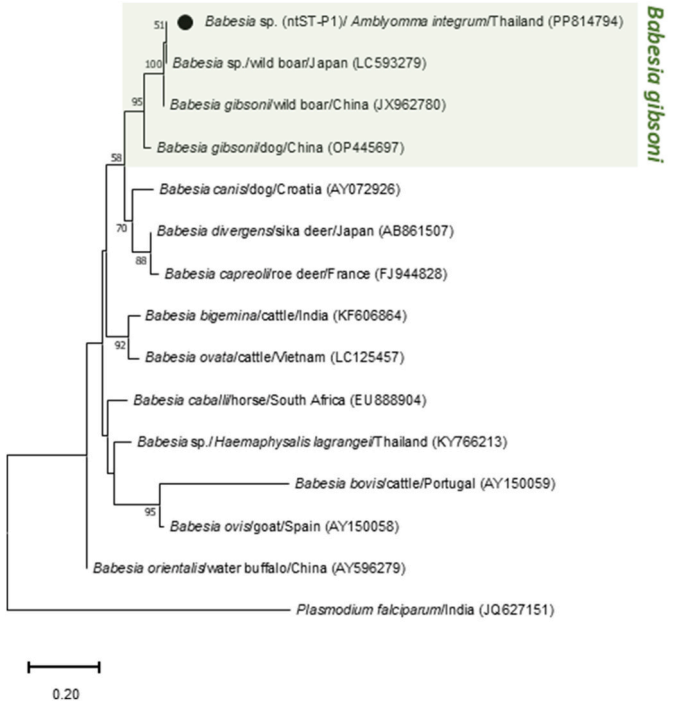

Fig. 7. Maximum likelihood (ML) tree of *Babesia* spp. 18S rRNA gene sequences (primer cut; 426 bp) computed with the TN93+G model. The phylogenetic relationships of the newly generated sequences (*black dot*) and reference sequences from the GenBank database. *Plasmodium falciparum* (GenBank: JQ627151) was used as the outgroup.

PP814795); ntST-P3 (1 sequence; GenBank: PP814796). BLAST results showed that ntST-P1 was closely related to a *Babesia* sp. detected in a wild boar in Japan (GenBank: LC593279) with 99.76% similarity, ntST-P2 and ntST-P3 were closely related to *Theileria* sp. in *H. lagrangei* ticks in Thailand (GenBank: JQ751277) with 100% and 99.53% similarity, respectively. Phylogenetic analyses showed that the new *Babesia* sp. sequence clustered together with *Babesia gibsoni* (Fig. 7), while two new *Theileria* sp. sequences were grouped in the clade of *Theileria cervi* (Fig. 8).

3.2.3. *Bartonella spp. detection*

One *H. lagrangei* larval pool (1.28%) was PCR-positive for the 16S-23S intergenic spacer (ITS) region of *Bartonella* spp. (Table 1). The newly generated sequence (ntST-BAR1) was then submitted to the GenBank database (GenBank: PP825052). BLAST results showed that the new sequence was closely related to that for *Bartonella henselae* detected in domestic cats in Brazil (GenBank: MT095053) with 99.83% similarity. In the phylogenetic tree, this sequence clustered in the clade of *B. henselae* (Fig. 9).

3.2.4. *Rickettsia spp. detection*

Three pooled *H. lagrangei* tick samples (3.84%), including one larval and two nymph pools, were PCR-positive for the *gltA* gene of *Rickettsia* spp. (Table 1). The new sequences were grouped into a ntST (ntST-R1), and a representative sequence was submitted to the GenBank database (GenBank: PP826458). BLAST analysis revealed that the new sequence was identical with *Rickettsia* sp. detected in *H. hystricis* ticks in Japan (GenBank: LC456206) and *R. montanensis* from questing ticks in Thailand (GenBank: OR524032). The phylogenetic tree showed that the new sequence grouped with *R. montanensis* and another *Haemaphysalis*-associated *Rickettsia* (Fig. 10).

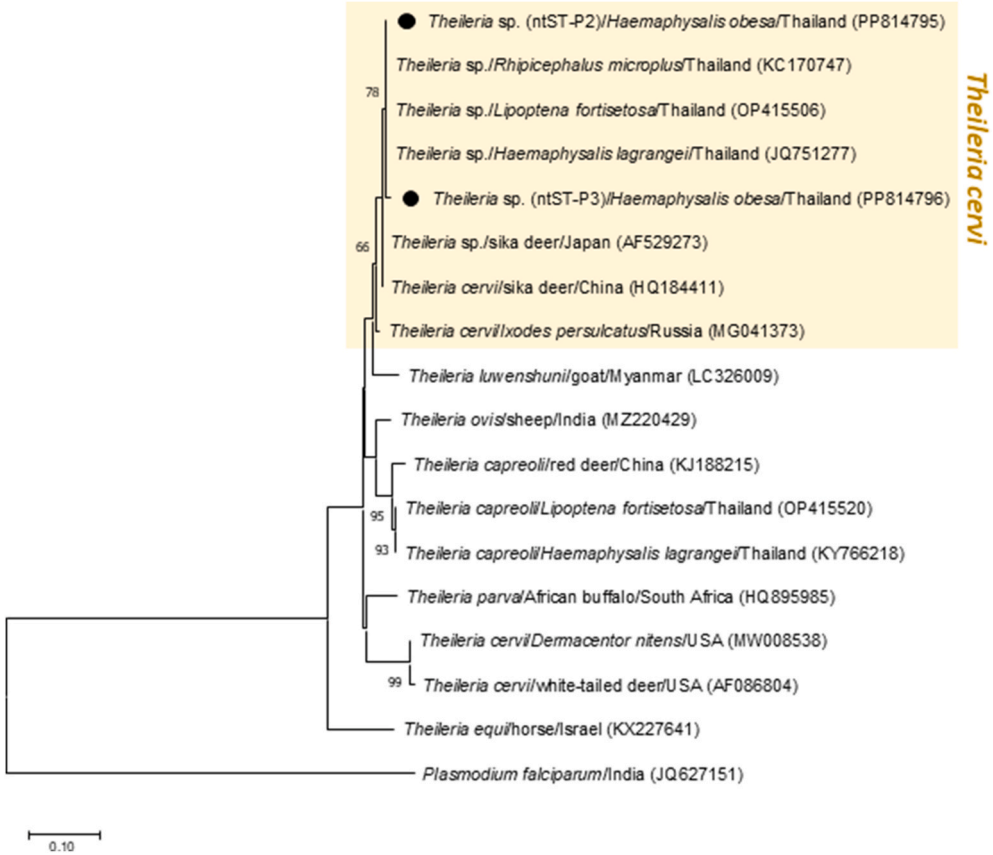

Fig. 8. Maximum likelihood (ML) tree of *Theileria* spp. 18S rRNA gene sequences (primer cut; 426 bp) computed with the TN93+G model. The phylogenetic relationships of the newly generated sequences (*black dots*) and reference sequences from the GenBank database. *Plasmodium falciparum* (GenBank: JQ627151) was used as the outgroup.

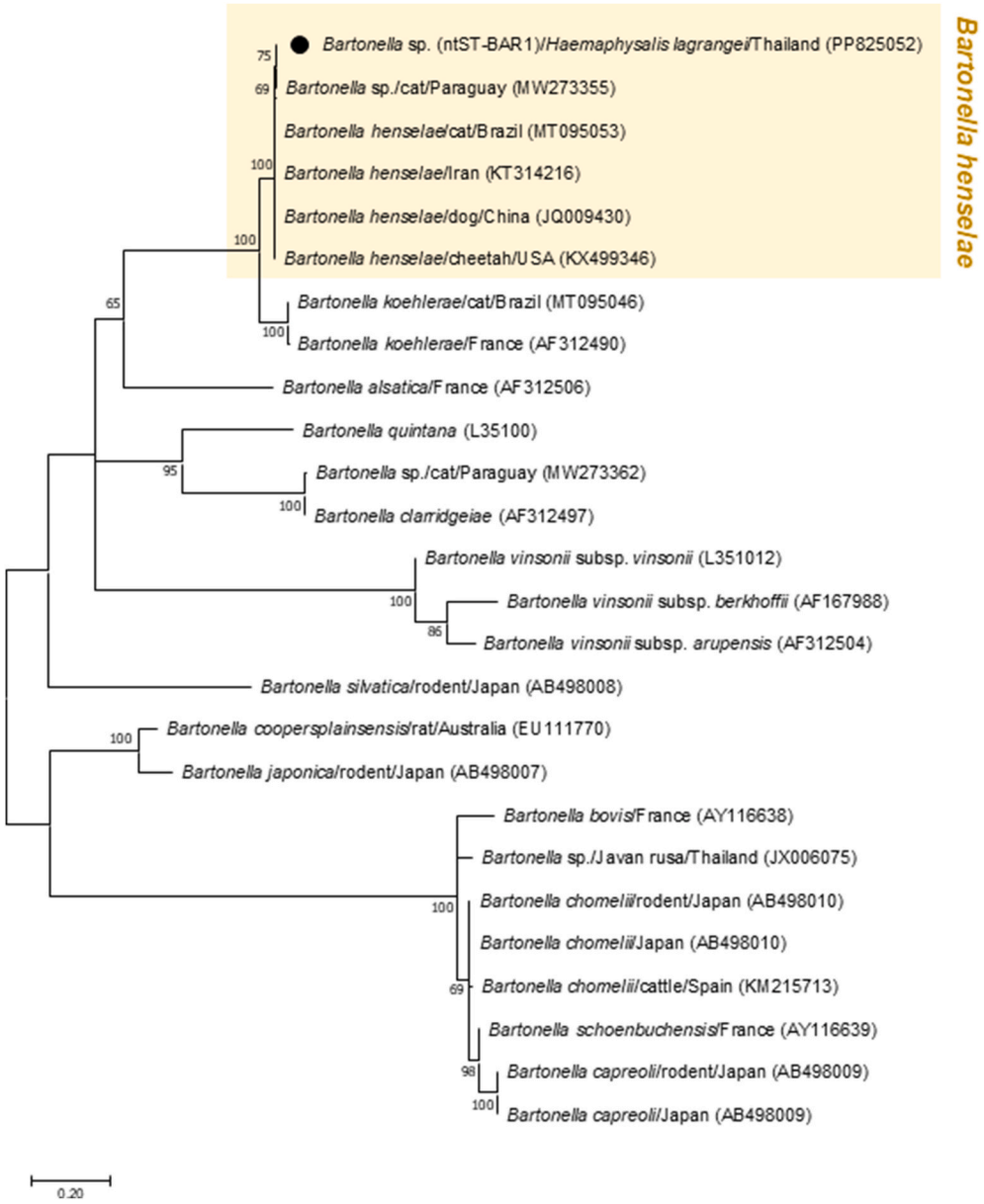

Fig. 9. Maximum likelihood (ML) tree of *Bartonella* spp. internal transcribed spacer region 16S-23S rRNA sequences computed with the T92+G model. The phylogenetic relationships of the newly generated sequence (*black dot*) and reference sequences from the GenBank database.

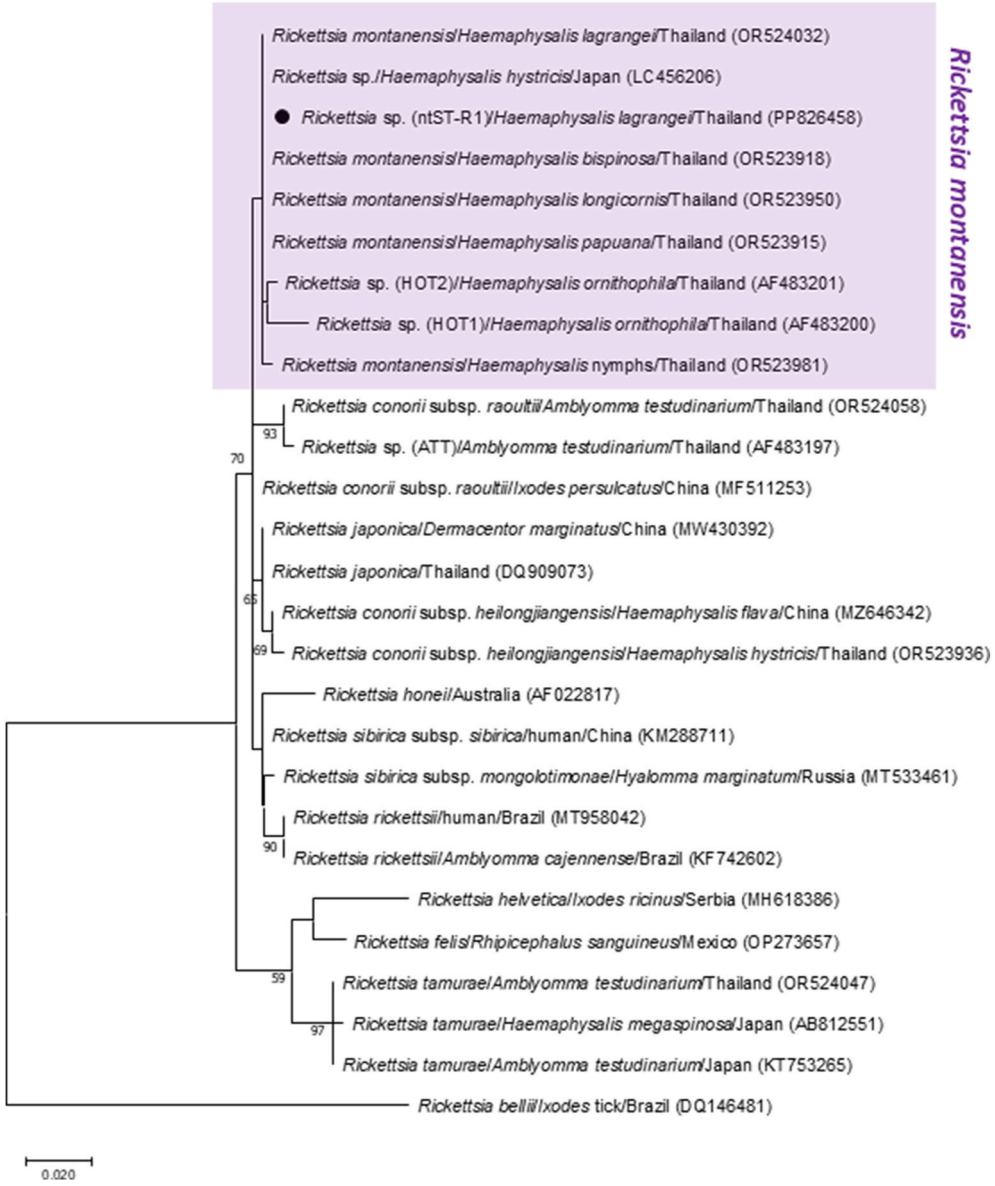

Fig. 10. Maximum likelihood (ML) tree of *Rickettsia* spp. *gltA* gene sequences (primer cut; 333 bp) computed with the T92+G model. The phylogenetic relationships of the newly generated sequence (*black dot*) and reference sequences from the GenBank database. *Rickettsia bellii* (GenBank: DQ146481) was used as the outgroup.

4. Discussion

In the present study, we collected 1331 questing ticks from a conserved wildlife habitat in Chon Buri, Thailand, and screened for the presence of pathogens. Among the seven tick species identified (*H. lagrangei*, *H. shimoga*, *H. obesa*, *H. wellingtoni*, *D. auratus*, *R. microplus*, and *A. integrum*) only *A. integrum* has not been previously recorded in Thailand (Tanskul et al., 1983). Most ticks were collected by dragging a white cloth over the vegetation, with the majority being in the larval stage. *Haemaphyslis* spp. appeared to be predominant (68.75%) in this area. The most frequent was *H. lagrangei*, primarily parasitizing wild ungulates (Hoogstraal et al., 1973; Tanskul et al., 1983), followed by *H. wellingtoni*, commonly infesting avian species. These findings are similar to previous reports from other wildlife habitats in Thailand (Malaisri et al., 2015; Wattanamethanont et al., 2018; Takhampunya et al., 2021). Further, *H. shimoga* and *H. obesa* have previously been found in mammals and humans in Thailand (Hoogstraal et al., 1966; Tanskul et al., 1983). *Dermacentor auratus* infests wild boars and small rodents, and occasionally humans (Hoogstraal and Wassef, 1985). Similar to Wattanamethanont et al. (2018), the lower occurrence of *R. microplus* (6.41%) compared to other genera, could be due to its single-host tick life cycle and the dragging collection technique. Additionally, *R. microplus* is commonly found in both wild and domestic ruminants in Thailand (Tanskul et al., 1983). Although Malaisri et al. (2015) reported collecting of two females of *A. integrum* in Thailand identified morphologically based on keys (Tanskul et al., 1983; Walker et al., 2000), neither key offered sufficient information for morphological identification of *A. integrum*. Therefore, we performed molecular identification to confirm its presence in Thailand. *Amblyomma integrum* is commonly found in India and Sri Lanka, where it parasitizes wild deer and cattle (Geevarghese et al., 1997; Ghosh et al., 2007).

Haemaphyslis lagrangei, the predominant tick identified in this study, is likely the most prevalent carrier (60.0%) of pathogens, including DNA from *Anaplasma* spp., *Bartonella* spp., and *Rickettsia* spp.

Bartonella spp. and *Rickettsia* spp. were only detected in *H. lagrangei*. However, *Anaplasma* spp. were found in multiple tick species, including *A. integrum*, *H. shimoga*, and *R. microplus*, in addition to *H. lagrangei*, similar to the previous reports in Thailand (Malaisri et al., 2015;

Wattanamethanont et al., 2018; Takhampunya et al., 2021). When specifically comparing immature tick pools, the prevalence of *Anaplasma* spp. detected in this study (10.00%) was lower than in a previous report (47.48%) (Wattanamethanont et al., 2018), but higher than in more recent studies (0.98%, see Takhampunya et al., 2021; 2.88% (7/243), see Chaorattanakawee et al., 2024). Variations in prevalence may highlight diverse factors, including geographical location, sampling methodologies, pool sizes, tick pool species, and host populations. In the present study, we identified two genotype groups of *Anaplasma* spp. First, a genotype closely related to *A. bovis* was detected in a larval pool of *H. lagrangei*. This was previously reported in *H. lagrangei* ticks collected from Malayan sun bear (*Helarctos malayanus*) (Parola et al., 2003), sambar deer (*Cervus unicolor*) (Sumrandee et al., 2016), and in wildlife habitats in Thailand (Wattanamethanont et al., 2018; Chaorattanakawee et al., 2024). These findings highlight the important role of *H. lagrangei* in *A. bovis* circulation in the country. In addition to *H. lagrangei*, molecular evidence detected *A. bovis* detection in questing *H. megaspinosa* in Japan and *H. longicornis* in Korea (Kim et al., 2003; Yoshimoto et al., 2010). Our sequence was identical with a sequence for *A. bovis* strain previously found in domestic animals and cattle across various countries. Despite previous detection in domestic goats (Aung et al., 2022), there have been no reports of *A. bovis* in wildlife in Thailand. However, previous research suggested a potential association between *A. bovis* infection in cattle and wild deer, indicating that wild animals might act as the natural reservoir for anaplasmosis (Jilintai et al., 2009). Secondly, *Anaplasma* DNA fragments from seven tick pools clustered with the clades of other ruminant-related *Anaplasma* spp., which included *A. capra*, *A. marginale*, and *A. ovis*. In the present study, the close homology of this partial 16S rRNA gene could not be distinguished among these three species of *Anaplasma*. However, only *A. marginale* and *A. ovis* have been detected in domestic ruminants as well as their infesting ticks (Aung et al., 2022) and ticks collected on wild ruminants in Thailand (unpublished data; GenBank: OQ352821). We implied that the seven new sequences could be either *A. marginale* or *A. ovis*. The finding of ruminant-associated *Anaplasma* spp. in several tick species suggests a high potential risk of bacterial transmission among wildlife in this habitat.

In the present study, we detected *Babesia* sp. in *A. integrum* and *Theileria* sp. in *H. obesa*. *Babesia* sp. was detected in only one questing tick pool (1.25%; 1/80), similar to a previous study (0.24%; 1/419) (Wattanamethanont et al., 2018). The new *Babesia* sp. sequence was closely related to those found in wild boars in Japan and China (Morikawa et al., 2021), all belonging to the clade of *B. gibsoni*. *Babesia gibsoni* is a small *Babesia* and a causative agent of babesiosis in dogs with a global distribution (Liu et al., 2022). However, no confirmed reports exist of *B. gibsoni* infection in either pigs or wild boars. As *B. gibsoni* has been considered to specifically infect canids, the findings of *B. gibsoni*-like and *A. testudinarium* on wild boars suggest the possibility of a new species capable of parasitizing hosts of the family Suidae (Masatani et al., 2017; Morikawa et al., 2021). Moreover, in Thailand, *B. gibsoni* has been reported in Asiatic wild dogs (*Cuon alpinus*) (Bhusri et al., 2022) and clouded leopards (*Neofelis nebulosa*) (unpublished; GenBank: MZ145286). The presence of *H. lagrangei*, *H. shimoga*, and *Amblyomma* ticks in Asiatic wild dogs (unpublished), makes it difficult to rule out the possibility of *B. gibsoni* transmission in the country among wildlife or from wildlife to domestic animals. Additionally, *B. gibsoni* was detected in *I. ricinus* tick in Germany, highlighting its potential role as a vector in that region (Schorn et al., 2011).

Theileria sp. DNA was detected from two *H. obesa* pools. The prevalence of *Theileria* spp. detection in this study (2.5%; 2/80) was lower than in previous reports in Thailand (12.08%; 18/419) and South Korea (3.80%; 3/79) (Wattanamethanont et al., 2018; Alkathiri et al., 2023). The two new *Theileria* sp. sequences were grouped in the clade of *T. cervi*, which mainly parasitizes cervid hosts, such as white-tailed deer (*Odocoileus virginianus*), pampas deer (*Ozotoceros bezoarticus*), and sika deer (*Cervus nippon*) (He et al., 2012; Silveira et al., 2013; Cauvin et al., 2019). In Thailand, *T. cervi* has been detected in *H. lagrangei*, *H. obesa*, and *R. microplus* collected from sambar deer, questing *H. lagrangei* ticks in a wildlife habitat, and keds (*Lipoptena fortisetosa*) from captive Eld's deer, highlighting the circulation of *T. cervi* in deer populations and their ectoparasites in the country (Sumrandee et al., 2015; Wattanamethanont et al., 2018; Tiawsirisup et al., 2023). While *T. cervi* infection typically manifests as mild symptoms in white-tailed deer, death due to *T. cervi* infection has been documented in reindeer (*Rangifer tarandus tarandus*) (Garner et al., 2012). Nonetheless, the pathogenic impact of *T. cervi* on the deer population in Thailand remains unclear. Future research on the association between *Theileria*-infected hosts and their infested ectoparasites would help better understand the current situation of the parasite infection in wildlife in the country.

To the best of our knowledge, this is the first report on *Bartonella* spp. detection in questing ticks in Thailand. The bacterial DNA was detected in a *H. lagrangei* larval pool but with a very low prevalence (1.25%; 1/80). Despite the lack of previous reports in Thailand, *Bartonella* spp. DNA has been found in questing *Ixodes* (*I.*) *pacificus* (19.2%) in the USA and *I. ricinus* (0.2–9.8%) in France (Chang et al., 2001; Halos et al., 2005; Cornet et al., 2009; Reis et al., 2011). Our sequence was grouped within the clade of *B. henselae*, which causes cat scratch disease in humans and companion animals, having domestic cats as reservoir hosts and cat fleas (*Ctenocephalides felis felis*) as competent vectors (Kordick et al., 1995; Chomel et al., 1996, 2006). In recent years, the detection of *B. henselae* DNA in several tick species has raised concerns about them being potential vectors for *Bartonella* spp. transmission (Chang et al., 2001; Kim et al., 2005; Rar et al., 2005; Tsai et al., 2011; Regier et al., 2017). Additionally, several reports showed *Bartonella* spp. detection in domestic animals and their ectoparasites in Thailand, but few studies related to wildlife species (McKee et al., 2017; Pangjai et al., 2018; Poofery et al., 2022). In fact, a novel *Bartonella* (Pangjai-1) has been detected and isolated from captive Rusa deer (*Rusa timorensis*) blood samples (Pangjai et al., 2018). *Bartonella henselae* has also been detected in Thai bats as well as in other known zoonotic species, such as *Bartonella ancashensis*, *Bartonella bacilliformis*, and *Bartonella australis* (Poofery et al., 2022). Notably, because *H. lagrangei* has a broad host range and an occasional presence in humans (Hoogstraal et al., 1973), it may play a role in *Bartonella* spp. transmission, at least mechanically.

Rickettsia sp. DNA was detected in both *H. lagrangei* nymphs and larvae. When comparing to questing immature tick pools collected in the country, the prevalence of *Rickettsia* sp. detected in this study (3.75%; 3/80) was lower than in previous studies (7.73% (13/168), see Takhampunya et al., 2021; 46.21% (61/132), see Chaorattanakawee et al., 2024). Phylogenetic analysis of the partial *gltA* gene of *Rickettsia* spp. indicated that our sequence belongs to the same clade as those found in *Haemaphysalis* spp. ticks in Thailand (Hirunkanokpun et al., 2003) and Japan (unpublished). These sequences were previously identified as a new *Rickettsia* strain, aligning with other SFGR but forming a distinct branch. Interestingly, the recently discovered *R. montanensis* in questing *H. lagrangei* ticks (GenBank: OR524032) in Thailand (Chaorattanakawee et al., 2024), was identical with our sequence and clustered within the same clade as previously published *Rickettsia* spp. sequences from *Haemaphysalis* spp. ticks in Thailand and Japan. Based on genetic and phylogenetic results, it is possible that our sequence represents *R. montanensis*. However, further genetic characterization of multiple genes is needed for confirmation. *Rickettsia montanensis* (formerly *R. montana*), a non-pathogenic SFGR to mammals, is primarily transmitted to humans in the USA by *Dermacentor variabilis* tick bites (McQuiston et al., 2012; Lippi et al., 2021; Snellgrove et al., 2021). Although various species of *Haemaphysalis* ticks collected in wildlife habitats harbored *R. montanensis* DNA, there are no reports of human transmission in Thailand (Chaorattanakawee et al., 2024). However, other SFGR closely related to *R. raoultii*, *R. belli*, *R. tamurae*, *R. monacensis*, as well as *R. montanensis* have been reported in a wide range of tick species (Hirunkanokpun et al., 2003; Doornbos et al., 2013; Malaisri et al., 2015; Nooroong et al., 2018; Takhampunya et al., 2021

Chaorattanakawee et al., 2024). Further molecular analyses of SFGR in tick vectors or bacterial seroprevalence are needed to update pathogen circulation and disease prevention in Thailand.

Our findings investigated questing tick population throughout the entire year, providing information on the seasonal patterns of tick density, developmental stages, and the circulation of tick-associated pathogens. The questing tick population and density in this area were highest in February for larvae and October for nymphs. Our finding differed from a previous study in Khao Yai National Park, the third largest national park in Thailand, reporting that larval density peaked from October to December with the highest number of nymphs found from December to February (Chaorattanakawee et al., 2024). In general, seasonal tick activity varies across regions and tick species, as reported in Brazil, the USA, and northern China (Cilek and Olson, 2000; Zheng et al., 2012; Meng et al., 2016; Dantas-Torres et al., 2021). These findings indicate that tick abundance and developmental patterns over the seasons are influenced by ecological factors, such as weather, environment, and host distribution. A seasonal occurrence pattern of *Anaplasma* was noted during two periods: from November 2021 to February 2022 and from November 2022 to February 2023. Consistent with a previous study, *Anaplasma* spp. were prominently detected in questing ticks collected in December (66.67%; 6/9) (Chaorattanakawee et al., 2024). Moreover, *Theileria* spp., *Bartonella* spp., and *Rickettsia* spp. were also identified in ticks collected in November, December, and February. As the cool season typically spans from November to February in Thailand, there may be an increased risk of tick-associated pathogen infection and transmission during this season.

5. Conclusions

Our comprehensive study of questing ticks in the wildlife sanctuary of Chon Buri, Thailand, provided valuable insights into the tick species present and their associated pathogens. We identified seven tick species, with *A. integrum* being first molecularly characterised in Thailand. Our findings indicate that *H. lagrangei* is the predominant tick species, playing an important role in the circulation of several pathogens within wildlife habitats. Notably, this is the first report on *Bartonella* spp. detection in questing ticks in Thailand. The observed seasonal patterns in tick density and pathogen prevalence indicate a heightened risk of tick-borne pathogen transmission from November to February. Visitors should take precautions to avoid tick exposure during this time period. Overall, this study underscores the importance of continued surveillance and molecular analysis to better understand the distribution and potential health impacts of tick-borne pathogens in wildlife habitats.

CRediT authorship contribution statement

Wittawat Wechtaisong: Data curation, Formal analysis, Validation, Writing – original draft. **Chalida Sri-in:** Data curation, Validation, Investigation. **Kritsada Thongmeesee:** Data curation, Validation, Investigation. **Elizabeth Riana:** Resources, Investigation. **Thuong Thi Huyen Bui:** Resources, Investigation. **Lyric C. Bartholomay:** Writing – review & editing. **Sonthaya Tiawsirisup:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Ethical approval

The area entry permission and sampling procedures were approved by the National Parks, Wildlife, and Plant Conservation Department and Chulalongkorn University Animal Care and Use Committee (No. 2231058). In addition, this study was approved by the Faculty of Veterinary Science Biosafety Committee of Chulalongkorn University (IBC 2231018).

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2024.100220.

Data availability

The data supporting the conclusions of this article are included within the article. The newly generated sequences were deposited in the GenBank database under the accession numbers as follows: 16S rRNA sequences of ticks (PP831985-PP832001); 16S rRNA gene of *Anaplasma* sp. (PP820820-PP820825), 18S rRNA of *Babesia* sp. (PP814794), 18S rRNA of *Theileria* sp. (PP814795, PP814796), 16S-23S ITS region of *Bartonella* sp. (PP825052), and *gltA* gene of *Rickettsia* sp. (PP826458).

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