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RESEARCH ARTICLE

Coxiella-like bacteria in *Haemaphysalis wellingtoni* ticks associated with Great Hornbill, *Buceros bicornis*

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ABSTRACT

Birds are known to be the most mobile hosts and are therefore considered to be hosts with potential to contribute to the long-distance spread and transmission of tick-borne pathogens. In the present study, ticks were collected from a hornbill nest at Chaiyaphum Province, Thailand. They were screened for the presence of *Coxiella* bacteria using conventional PCR. The evolutionary relationships of positive *Coxiella*-like bacteria (CLB) were analysed based on the gene sequences of *16S* rRNA, *groEL* and *rpoB*. Among all 22 tested ticks, CLB infections were found in 2 *Haemaphysalis wellingtoni* individuals. In a phylogenetic analysis, the *Coxiella 16S* rRNA gene detected in this study formed a separate clade from sequences found in ticks of the same genus. In contrast, the phylogenetic relationships based on *groEL* and *rpoB* revealed that these two genes from *H. wellingtoni* ticks grouped with CLB from the same tick genus (*Haemaphysalis*). This study is the first to report the presence of CLB in *H. wellingtoni* ticks associated with the Great Hornbill, *Buceros bicornis* in Thailand. Three genes of CLB studied herein were grouped separately with *Coxiella burnetii* (pathogenic strain). The effects of CLB in the ticks and *Buceros bicornis* require further investigation.

Keywords: Tick; *Coxiella*-like bacteria; *Haemaphysalis wellingtoni*; *Buceros bicornis.*

INTRODUCTION

Ticks are blood-sucking parasites second only to mosquitoes as vectors in human, domestic and wild animals. They are known to harbour several pathogens, such as viruses, bacteria and protozoa (Jongejan & Uilenberg, 2004). In addition to transmit a number of pathogenic bacteria (e.g., the genera *Anaplasma, Borrelia, Coxiella, Ehrlichia, Francisella* and *Rickettsia*), ticks have been documented to harbour endosymbionts that plays important roles in mutualistic symbiosis with their tick hosts. The endosymbionts can supplement ticks with nutrients, promote the reproductive fitness of ticks and influence pathogen transmission (Guizzo *et al*., 2017).

Coxiella-like bacteria (CLB) (mostly are endosymbionts in ticks) have been identified in hard ticks from the genera *Amblyomma*, *Haemaphysalis* and *Rhipicephalus* (Guizzo *et al*., 2017). They are involved in manipulating the reproductive fitness and maturation of *Haemaphysalis longicornis* ticks (Zhang *et al*., 2017). Moreover, they can interfere with the replication and transmission of a wide range of pathogens. For example, CLB impacts pathogen susceptibility in ticks such as this symbiont can defend their tick hosts against the pathogenic parasite of human babesiosis, *Babesia microti* of *Rhipicephalus haemaphysaloides* ticks (Li *et al*., 2018). In contrast, CLB was determined to be the cause of death of a female eclectus parrot (*Eclectus roratus*) (Vapniarsky *et al*., 2012).

Birds are known to be the most movable hosts and therefore are considered to be hosts with potential to contribute to the long-distance dispersion and transmission of tick-borne pathogens (Morshed *et al*., 2005; Waldenstrom *et al*., 2007; Capligina *et al*., 2014). A number of studies have shown that many bird species are parasitized by ticks, which carry pathogenic diseases, including Lyme borreliosis and tick-borne encephalitis (TBE) (Comstedt *et al*., 2006; Waldenstrom *et al*., 2007). The hard tick *Haemaphysalis ornithophila*, the principal vector for the pathogenic bacteria *Anaplasma* sp. in Taiwan, can be found in Swinhoe's pheasant *Lophura swinhoii* (Kuo *et al*., 2017). So far, there have been few reports of the microbial communities present in ticks collected on birds.

In Thailand, the infection of CLB in ticks from hornbills has never been studied. The objective of this study was to investigate the CLB infection in ticks associated with Great Hornbill, *Buceros bicornis*. The evolutionary relationships of the CLB were also analysed.

MATERIALS AND METHODS

Tick collection and identification

In June 2015, ticks were collected from a hornbill nest at Chaiyaphum Province (16°12'18.9"N 101°52'22.9"E), Thailand. They were preserved in 70% ethanol before morphological identification to

the genus/species level, stage of development (e.g., larva, nymph and adult) and sex under a microscope using a standard taxonomic key for *Haemaphysalis* ticks (Tanskul & Inlao, 1989). Identification of the ticks using molecular methods was also performed using previously published primers (16S+1/16S-1) for the amplification of the mitochondrial *16S* rDNA gene (mt *16S* rDNA) (Black & Piesman, 1994) to support the taxonomic identification of tick species. For ethical approval, all applicable national and international guidelines for the care and use of animals were followed. No vertebrate used in our study. The study was carried out according to the license number U1-05257-2559 from NRCT, Thailand, before the release of ministerial regulation about invertebrate used at the end of 2015.

DNA extraction

Ticks were cleaned three times each with 70% ethanol, 10% sodium hypochlorite and sterile distilled water to remove environmental debris. DNA extraction was performed from the whole (individual) tick using a Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) following the manufacturer's protocol. DNA products were then maintained at -20°C until their use as templates in PCR amplification.

Molecular analysis

First, tick samples were amplified using conventional PCR-based methods for the presence of *Coxiella* (Table 1). Then, samples (genomic DNA) of positive PCR products (in this study, *Coxiella* species were positive for *16S* rRNA) were used to amplify the *groEL* (60-kDa chaperone heat shock protein B) and *rpoB* (β subunit of bacterial RNA polymerase) genes, using nested PCR procedures (Table 1). The PCR conditions to detect *Coxiella* bacteria are as follows: *16S* rRNA:-initial denaturation at 95°C for 3 min; 35 cycles of denaturation 95°C for 1 min, annealing 58°C for 1 min, extension 72°C for 2 min; and final extension at 72°C for 10 min*, groEL:*-initial denaturation at 93°C for 3 min; 30 cycles of denaturation 93°C for 30 s, annealing 56°C for 30 s, extension 72°C for 1-2 min; and final extension at 72°C for 5 min (both first and second rounds) and *rpoB*:-initial denaturation at 93°C for 3 min; 35 cycles of denaturation 93°C for 30 s, annealing 56°C for 30 s, extension 72°C for 1-2 min; and final extension at 72°C for 5 min (both first and second rounds).

DNA purification, sequencing and phylogenetic analysis

Positive samples from PCR products were purified with a NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sequenced. The assembled sequences were subjected to BLASTn analysis and submitted to GenBank. Sequence alignment of the *16S* rRNA, *groEL* and *rpoB* genes in the present study was conducted using CLUSTALW. The reference sequences for the construction of phylogenetic trees were selected based on BLAST, previous publications and complete genome of the same region. Then, phylogenetic trees were constructed from the aligned DNA sequences according to the maximum likelihood (ML) method by bootstrap analysis of 1000 replicates using MEGAX. For the *16S* rRNA gene, the best fit approximation was the Kimura 2-parameter model with a gamma distribution (K2+G). For the *groEL* gene, the best fit approximation was the Tamura 3-parameter model with an invariant site (T92+I). The best fit model for the *rpoB* gene was the Tamura 3-parameter model with invariant site (T92+I) (Felsenstein, 1985; Kumar *et al*., 2018).

RESULTS

Tick identification

A total of 19 adult hard ticks (13 males, 6 females: engorged and unengorged) and 3 nymphs (partially engorged) were collected from a hornbill nest in Chaiyaphum Province, Thailand. All ticks were morphologically and molecularly identified as *H. wellingtoni*. For molecular identification, the partial mitochondrial *16S* rRNA gene from *H. wellingtoni* ticks in this work was 100% identical to the three reference sequences of ticks, including *H. wellingtoni* ticks from Trang (accession number: MG874021), Satun (accession number: MG874023) and Rayong (accession number: MG874024), collected from birds in Thailand. The DNA sequences of *H. wellingtoni* ticks in this study were submitted to GenBank with accession numbers MZ434930 (PK1528) and MZ434946 (PK1525).

***Coxiella 16S* rRNA**

Overall, 9.1% (2/22) of the examined ticks (1 male tick and 1 nymphal tick) were positive for *Coxiella*. The *16S* rRNA gene sequences of *Coxiella* obtained in this study (PK1525 and PK1528, with accession numbers MW116774 and MW116775, respectively) were found to be 100% identical to each other. Maximum likelihood phylogenetic analysis using the *16S* rRNA gene sequence showed that the two *16S* rRNA sequences of *Coxiella* sp. in *H. wellingtoni* ticks from this study grouped with other previously published *Coxiella 16S* rRNA sequences from ticks of the genus *Rhipicephalus* (GenBank: KP994843-France and KP994849-Ivory Coast) (Figure 1). Interestingly, the *Coxiella 16S* rRNA sequences detected in our study separated from the CLB in same ticks species previously reported, including genus *Haemaphysalis* in Northern Thailand, Nan (GenBank: MK671724), Northeastern Thailand, Chaiyaphum (GenBank: MG871183), Eastern Thailand, Rayong (GenBank: MG871190) and Southern Thailand, including Trang (GenBank: MG871191) and Satun (GenBank: MG871192). Moreover, *16S* rRNA sequences of *Coxiella* from this study formed a distinct clade apart from *C. burnetii* strains (GenBank: CP013667, CP001019, CP014565 and CP040059) (Figure 1).

***Coxiella groEL* gene**

The *groEL* gene sequences of *Coxiella* obtained in this study (GenBank: MW142221 and MW142222) were found to be 100% identical to each other. Phylogenetic analysis of the *groEL* gene indicated that two *groEL* sequences of CLB from *H. wellingtoni* ticks grouped together in the same clade with CLB of the same tick species isolated from southern Thailand, including Trang (GenBank: MG874471), Satun (GenBank: MG874470) and Eastern Thailand, Rayong (GenBank: MG874469). Moreover, the *groEL* gene sequences of *Coxiella* detected in our study formed a sister clade with the *Haemaphysalis obesa* tick in northeastern Thailand, Chaiyaphum (GenBank: MG874468). The *groEL* gene sequences obtained in this study formed a separate clade from the CLB sequences of different tick genera, including CLB isolated from the genus *Rhipicephalus* (GenBank: KP985520 and KP985524) and CLB from the genus *Ixodes* (GenBank: KP985500-France and KP985502-France). Additionally, *groEL* gene sequences of *Coxiella* from this study formed a distinct clade apart from the *C. burnetii* strain (GenBank: CP001019, CP014565, CP013667 and CP040059) (Figure 2).

***Coxiella rpoB* gene**

For the *Coxiella rpoB* gene, we were able to obtain only the *rpoB* gene sequence of *Coxiella* bacteria (GenBank: MW460003) from 1 nymph of a *H. wellingtoni* tick. The result of DNA sequencing of CLB in 1 adult male tick was not used because the band was too faint to obtain good quality DNA for sequencing. The ML phylogenetic analysis indicated that the *rpoB* gene sequence of CLB (GenBank: MW460003) obtained in this study grouped in the same clade as CLB from *Haemaphysalis* ticks from Southern Thailand, including Trang (GenBank: MG893017), Satun (GenBank: MG893016), Eastern Thailand, Rayong (GenBank: MG921604) and Northeastern Thailand, Chaiyaphum (GenBank: MG893014). In contrast, *rpoB* gene sequence of *Coxiella* bacteria was clustered in different clade with *Coxiella* endosymbionts of the different tick species, including CLB isolated from the genus *Rhipicephalus* (GenBank: KP985344,

Table 1. Primers for PCR amplification used in this study

Organism	Target gene	Primer name	Sequence (5'-3')	References
Tick (Acari)	mt *16S* rDNA	16S+1 16S-1	CCGGTCTGAACTCAGATCAAGT CTGCTCAATGATTTTTTAAATTGCTGTGG	Black and Piesman (1994)
Coxiella	*16S* rRNA	COX-F COX-R	GGGGAAGAAAGTCTCAAGGGTAA TGCATCGAATTAAACCACATGCT	Almeida *et al*. (2012)
	groEL	CoxGrF1 CoxGrR2 CoxGrF2 CoxGrR1	TTTGAAAAYATGGGCGCKCAAATGG CGRTCRCCAAARCCAGGTGC GAAGTGGCTTCGCRTACWTCAGACG CCAAARCCAGGTGCTTTYAC	Duron *et al*. (2015)
	rpoB	CoxrpoBF2 CoxrpoBR1 CoxrpoBF3 CoxrpoBR3	GGGCGNCAYGGWAAYAAAGGSGT CACCRAAHCGTTGACCRCCAAATTG TCGAAGAYATGCCYTATTTAGAAG AGCTTTMCCACCSARGGGTTGCTG	Duron *et al*. (2015)

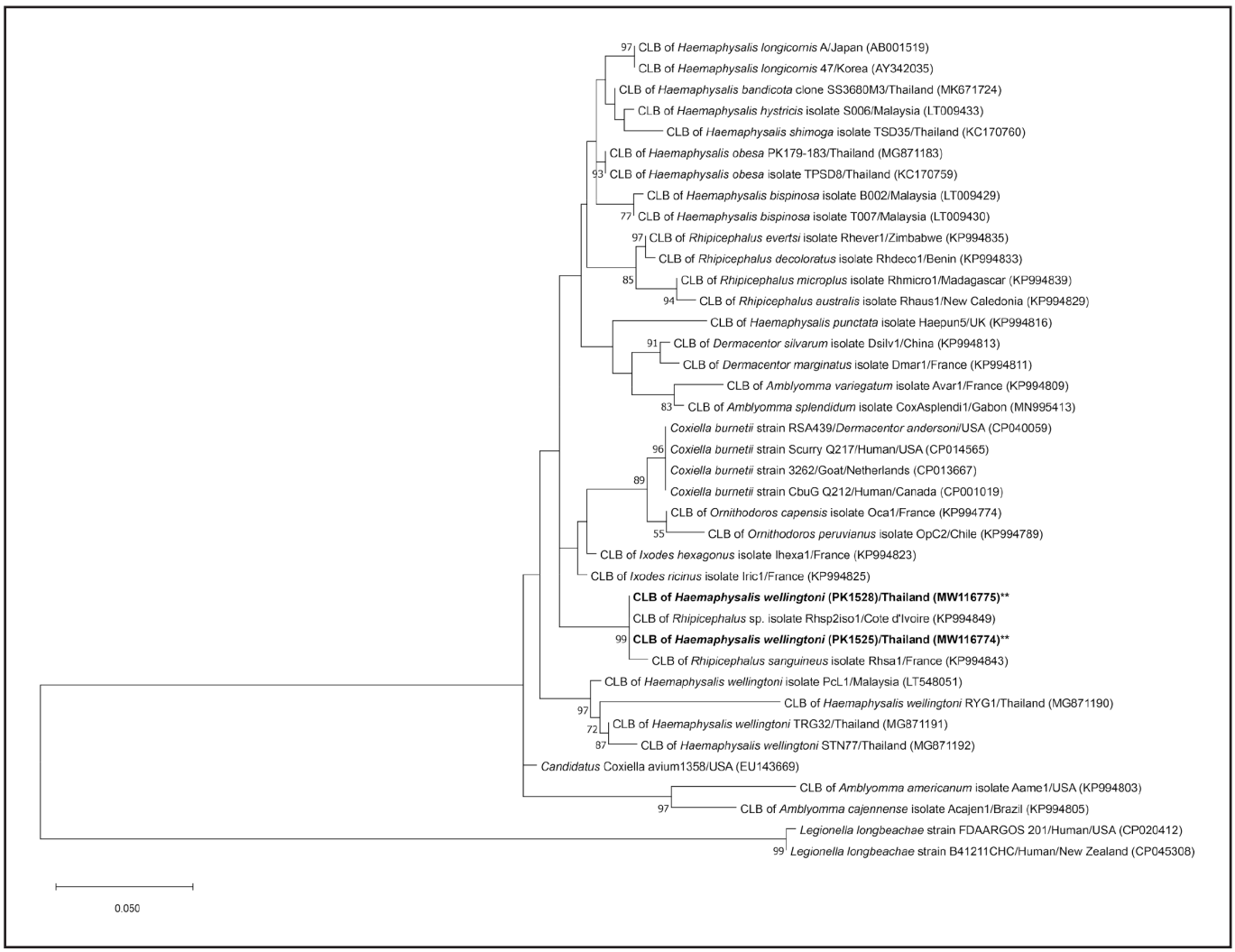

Figure 1. *Coxiella* bacterial phylogenetic tree constructed with 1000 bootstrap replicates (bootstrap values <50% are not shown) based on *16S* rRNA gene sequences by maximum likelihood (ML). Phylogenetic trees were constructed from the aligned DNA sequences according to the maximum likelihood (ML) method by bootstrap analysis of 1000 replicates using MEGAX. Sequences from this study are indicated with bold and asterisk. *Legionella longbeachae* was used as an outgroup. Scale bars represent the genetic variation for the length of the scale.

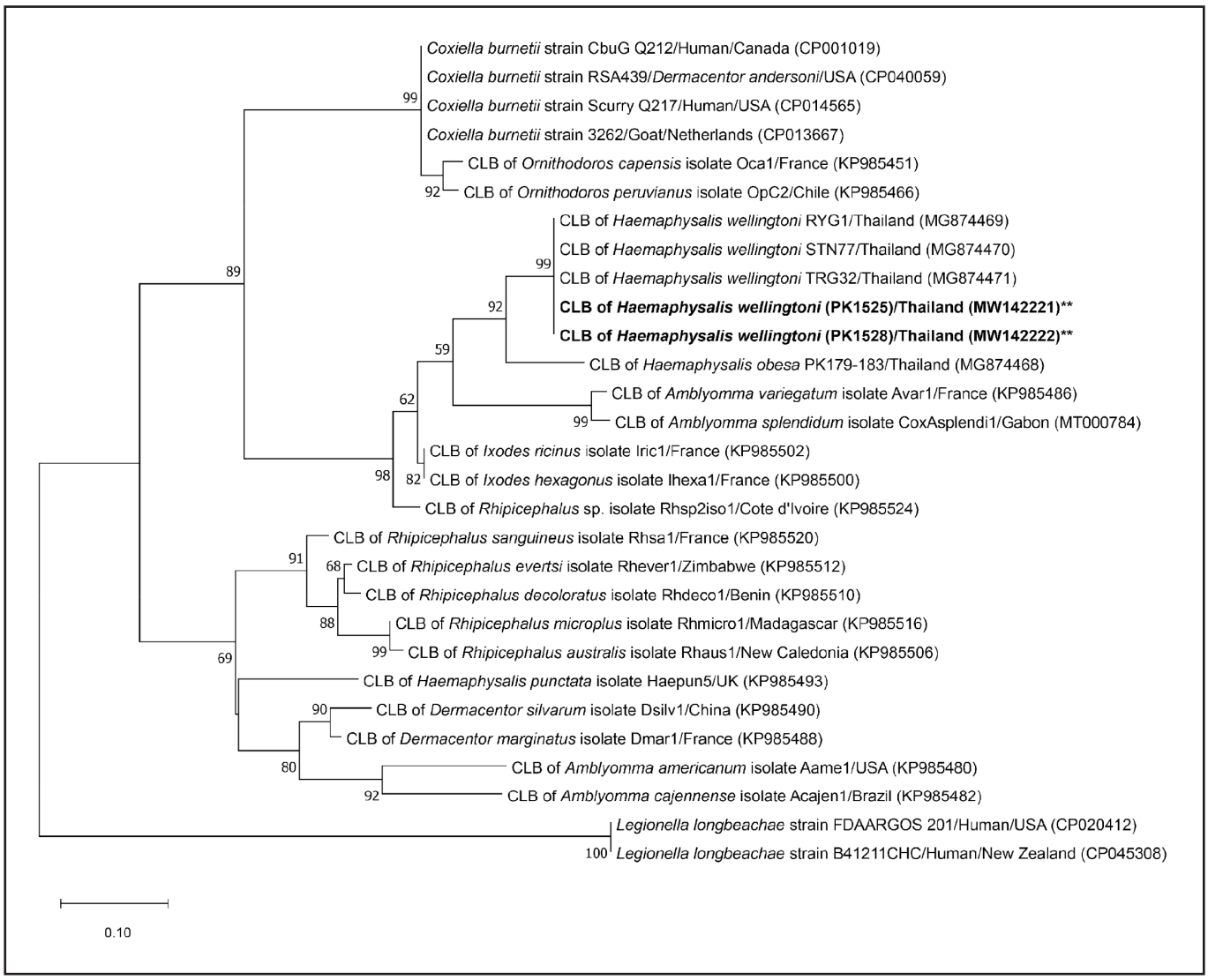

Figure 2. *Coxiella* bacterial phylogenetic tree constructed with 1000 bootstrap replicates (bootstrap values <50% are not shown) based on *groEL* gene sequences by maximum likelihood (ML). Phylogenetic trees were constructed from the aligned DNA sequences according to the maximum likelihood (ML) method by bootstrap analysis of 1000 replicates using MEGAX. Sequences from this study are indicated with bold and asterisk. *Legionella longbeachae* was used as an outgroup. Scale bars represent the genetic variation for the length of the scale.

KP985338) and CLB from the genus *Ixodes* (GenBank: KP985318-France and KP985320-France). The *rpoB* gene sequence of CLB from this study formed a distinct clade from those of the *C. burnetii* strain (GenBank: CP001019, CP014565, CP013667 and CP040059) (Figure 3).

DISCUSSION

Trinachartvanit *et al*. (2018) reported the presence of CLB, with a higher prevalence of infestation detected in *Haemaphysalis* ticks collected from birds. This is the first study describing the detection of CLB in *H. wellingtoni* ticks from Great Hornbill**,** *Buceros bicornis*. However, the limitation of this study is the low number of tick specimens, which may lead to low positive results for CLB. Therefore, a larger population size of ticks collected from hornbills should be investigated further.

The two *16S* rRNA genes of *Coxiella* sp. in *H. wellingtoni* ticks detected herein clustered with other *Coxiella 16S* rRNA sequences from ticks of the genus *Rhipicephalus,* not with *Haemaphysalis*. The result is in contrast to the *groEL* and *rpoB* phylogenetic analyses of CLB from the same individual tick. This study does not agree with Trinachartvanit *et al*. (2018) in terms of the phylogenetic relationships based on the *16S* rRNA and *rpoB* genes of CLB in which the *16S* rRNA gene sequences were located within a CLB group from the same species of ticks and the *rpoB* gene sequences of CLB from *H. wellingtoni* ticks were located within a CLB group from a different genus of ticks. More tick samples will need to be investigated to determine whether the *Coxiella 16S* rRNA, *rpoB* and *groEL* genes of CLB depend on tick species, vertebrate host, geographic distribution or the nature of the *Coxiella* bacteria itself. It has been reported that bacterial endosymbionts, which are nonpathogenic microorganisms, may have positive or negative effects on tick-borne pathogen transmission, depending on the nature of the tick/microbe interactions (Gall *et al*., 2016). Whether CLB are pathogenic or nonpathogenic to tick or Great Hornbill is not yet known. Because the CLB positive from this work were in engorged or partially engorged ticks. The CLB from this work may originate from either the hornbill or the tick itself. Different species of birds may be susceptible to CLB, but the disease appears to be sporadic (Shivaprasad *et al*., 2008). The roles of CLB in infected ticks or birds require further investigation.

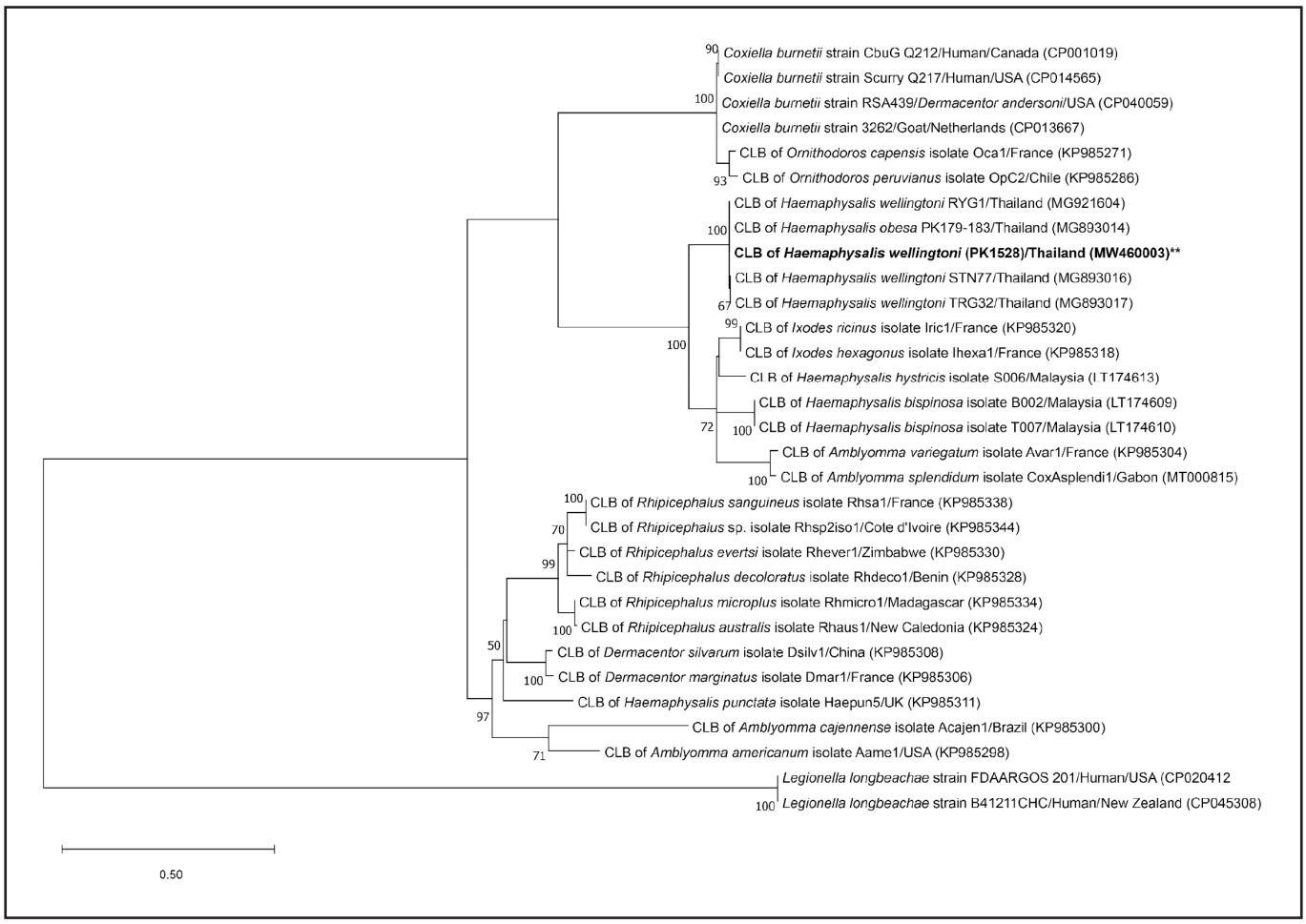

Figure 3. *Coxiella* bacterial phylogenetic tree constructed with 1000 bootstrap replicates (bootstrap values <50% are not shown) based on *rpoB* gene sequences by the maximum likelihood (ML) method. Phylogenetic trees were constructed from the aligned DNA sequences according to the maximum likelihood (ML) method by bootstrap analysis of 1000 replicates using MEGAX. Sequences from this study are indicated with bold and asterisk. *Legionella longbeachae* was used as an outgroup. Scale bars represent the genetic variation for the length of the scale.

CONCLUSION

This is the first study describing the detection of CLB in *H. wellingtoni* ticks associated with *Buceros bicornis* hornbill in Thailand. The interesting points are as follows: *16S* rRNA gene sequences of CLB from *H. wellingtoni* ticks in this study were placed in different clade from CLB of the same genus of ticks and *rpoB* gene sequence of CLB from *H. wellingtoni* tick in this study was grouped together in the same clade with CLB detected in the same species of ticks.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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