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Original article

Reptile-associated *Borrelia* spp. In *Amblyomma* ticks, Thailand

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ABSTRACT

A total of 127 *Amblyomma* ticks (*A. helvolum*, *A. varanense* and *A. geoemydae*) were collected from reptiles: water monitors (*Varanus salvator*), Bengal monitors (*Varanus bengalensis*), Burmese pythons (*Python bivittatus*), yellow-spotted keelbacks (*Xenochrophis flavipunctatus*), keeled rat snakes (*Ptyas carinata*) and elongated tortoises (*Indotestudo elongata*) from nine provinces in Thailand. The presence of *Borrelia* spp. of the *16S rRNA*, *flaB*, *glpQ*, *groEL* and *gyrB* genes was examined by conventional, semi-nested and nested PCR. Phylogenetic analyses using maximum likelihood method of housekeeping genes showed that most sequences of *Borrelia* spp. in these *Amblyomma* ticks belonged to the clade of reptile-associated (REP) borreliae. Interestingly, one *Borrelia* sp. in an *A. geoemydae* tick collected from an elongated tortoise clustered in the same clade as a *Borrelia* sp. detected from an *A. geoemydae*-infested turtle in Japan (it may belong to the same species given the identical sequences of their *16S rRNA*, *flaB* and *glpQ* genes) and formed the same group with tick-borne relapsing fever (RF) borreliae of *B. miyamotoi* and *B. theileri*. Our findings are the first report on the presence of *Borrelia* spp. in *A. helvolum* and *A. geoemydae* ticks from reptiles in Thailand adding to the geographic distribution of *Borrelia* spp. in Asia.

1. Introduction

Ticks are important vectors that transmit several pathogens, including viruses, bacteria and protozoa, which threaten animal and human health (Jongejan and Uilenberg, 2004). *Borrelia* spp. are Gram-negative bacteria with a spiral-shape belonging to the Spirochaetaceae family (Parola and Raoult, 2001). They are known as pathogens of arthropod-borne diseases of public health problems. Phylogenetic trees of *Borrelia* bacteria have been divided into three groups of borreliae, i.e., Lyme borreliosis/Lyme disease (LB), relapsing fever (RF) and reptile-associated (REP) (Takano et al., 2010). Lyme borreliosis etiological agents include *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. They are transmitted by hard ticks of several species of *Ixodes*, such as *I. ricinus, I. persulcatus* and *I. scapularis* (Steere et al., 2016).

Etiological agents of RF borreliae contain more than 20 species, e.g., *B. hermsii* in North America and *B. duttonii* in Afica. The main vectors of RF borreliae are soft ticks of the genus *Ornithodoros* (Wang, 2015). However, some RF borreliae are transmitted by hard ticks, such as *B. lonestari* (*Amblyomma*), *B. miyamotoi* (*Ixodes*) and *B. theileri* (*Rhipicephalus*) (Wang, 2015).

The third group of *Borrelia* spp. has been referred to reptile-associated (REP) borreliae (Takano et al., 2010, 2011), although a *Borrelia* sp. of this group has been found to be associated with echidna (Panetta et al., 2017; Loh et al., 2017; Gofton et al., 2018). *Borrelia turcica* sp. nov. was first discovered in *Hyalomma aegyptium* ticks collected from tortoises in Turkey (Guner et al., 2003, 2004). Phylogenetically, this group is separated from LB and RF borreliae. In addition, REP borreliae have also been detected in blood and tissue of reptiles and in the hard ticks of the genera *Amblyomma*, *Hyalomma* and *Bothriocroton* that infest several reptiles (Takano et al., 2010, 2011; Panetta et al., 2017).

The presence of *B. burgdorferi* has been detected in the blood from a dog in Chiang Mai Province (Thailand) by immunological and nested PCR assays (Sthitmatee et al., 2016). Additionally, REP *Borrelia* was recently reported in Thailand. A novel species of the reptile-associated group was discovered in an *A. varanense* tick from a reticulated python in Lopburi Province (Trinachartvanit et al., 2016). The aims of this study were to investigate the infection of *Borrelia* spp. in *Amblyomma* ticks from reptiles using conventional, semi-nested and nested PCR. Evolutionary relationship of *Borrelia* spp. was constructed using phylogenetic analyses of *16S rRNA* (*16S rDNA*), *flaB* (flagellin B), *glpQ* (glycerophosphoryl diester phosphodiesterase), *groEL* (a molecular

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chaperone) and *gyrB* (DNA gyrase subunit B) genes.

2. Materials and methods

2.1. Tick collection and identification

During 2014 to 2018, ticks were collected from six species of reptiles from nine provinces in Thailand namely, Pattani, Narathiwat, Satun, Songkhla, Uthai Thani, Chaiyaphum, Sisaket, Kalasin and Mahasarakham. The reptiles infested with ticks were captured by local people. All ticks were removed from reptiles using forceps under suitable conditions. Then, reptiles were released at the capture site. The ticks were placed in 1.5-ml microcentrifuge tubes with 70% ethanol, transported to the Faculty of Science, Mahidol University, Bangkok and stored at −20 °C. Standard morphological keys (Kohls, 1957; Voltzit and Keirans, 2002; Kadosaka et al., 2006) were used to identify tick species. Positive ticks for *Borrelia* spp. were further confirmed the species by molecular identification technique employing a set of 16S + 1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) and 16S-1 (TTAC GCTGTTATCCCTAGAG) primers (Black and Piesman, 1994) to detect the mitochondrial *16S rRNA* gene (mt *16S rRNA*). Then, the PCR products of mt *16S rRNA* of *Borrelia*-positive ticks were purified, sequenced and compared with existing sequences in GenBank.

2.2. DNA extraction

Ticks were washed three times each with 70% ethanol, 10% sodium hypochlorite and sterile distilled water. Then, they were placed in 1.5-ml microcentrifuge tubes with a 3 mm stainless steel bead and crushed using TissueLyser II (Qiagen, Hilden, Germany) for 30 s at 30 Hz. DNA was extracted from all tick samples using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.3. Molecular detection of Borrelia spp.

The presence of *Borrelia* spp. was performed by using conventional, semi-nested and nested PCR. A segment of *16S rRNA*, *flaB*, *glpQ*, *groEL* and *gyrB* genes was used to identify *Borrelia* bacteria in tick samples with specific primers. The primers used in this study are described in Table 1.

For the first screening, a segment of *16S rRNA* was amplified using conventional PCR with a primer set of 16SF1 and 16SR. Each PCR included 4 µl of DNA template, 9.3 µl of distilled water, 2 µl of 10x buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of dNTPs (10 mM each), 1 µl of 10 µM forward and reverse primers and 0.2 µl of *Taq* DNA polymerase. PCR conditions for the *16S rRNA* gene included an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 15s, annealing at 64 °C for 20 s, extension at 72 °C for 1.2 min and a final extension at 72 °C for 5 min. Ticks that were positive for *Borrelia 16S rRNA* were further amplified for the segment of the *flaB*, *glpQ*, *groEL* and *gyrB* genes. Nested PCR was performed for the *flaB* gene as previously described (Sato et al., 1997). Furthermore, semi-nested PCR of the *glpQ*, *gyrB* and *groEL* genes was performed by the protocol described previously in Loh et al. (2017). DNA amplifications were run on a SimpliAmp thermal cycler (Thermo Fisher Scientific, Massachusetts, USA). The PCR products obtained from the 5 genes were separated by electrophoresis on a 1% agarose gel. The gel was stained with ethidium bromide and visualized under UV light.

2.4. DNA sequencing and phylogenetic analysis

The PCR products from ticks positive for *Borrelia* spp. were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sequenced. All DNA sequences were edited and assembled using MEGA7 (Kumar et al., 2016). Then, DNA sequences were compared with available sequences in GenBank using BLASTn analysis (Altschul et al., 1990). DNA sequences were aligned by CLUSTALW multiple alignment and phylogenetic analysis was performed using MEGA7. The maximum likelihood method was used to represent the evolutionary relationship based on the best fit model for each gene, including the Hasegawa-Kishino-Yano (HKY) for *16S rRNA*, *groEL* and *gyrB* genes, Tamura 3-parameter (T92) for *flaB* gene and general time reversible (GTR) for *glpQ* gene. The bootstrap values were determined using 1000 replicates.

3. Results

3.1. Tick collection and identification

A total of 127 *Amblyomma* ticks were collected from 23 different reptiles including six water monitors, four Bengal monitors, four Burmese pythons, two yellow-spotted keelbacks, two keeled rat snakes and five elongated tortoises from different parts of Thailand (Table 2). Three species of ticks were identified namely, *A. helvolum*, *A. varanense* and *A. geoemydae*. Twenty-eight *A. helvolum* (26 females and two males) were collected from water monitors, Bengal monitors, yellow-spotted keelbacks and keeled rat snakes. Sixty-two *A. varanense* (23 females and 39 males) were collected from water monitors, Bengal monitors and Burmese pythons. Thirty-one *A. geoemydae* (six females and 25 males) were collected from elongated tortoises. Six *Amblyomma* sp. (nymphs)

Table 1
PCR primers used for *Borrelia* detection.

Target gene	Primer name	Nucleotide sequence (5' to 3')	PCR assay	Size (bp)	Reference
16S rRNA	16SF1	ATAACGAAGAGTTTGATCCTGGC	Conventional	1,350	Masuzawa et al. (1999)
	16SR	CAGCCGCACTTTCCAGTACG			
flaB	BflaPAD	GATCA(G/A)GC(T/A)CAA(C/T)ATAACCA(A/T)ATGCA	Nested	384	Sato et al. (1997)
	BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC			
	BflaPBU	GCTGAAGAGCTTGGAATGCAACC			
	BflaPCR	TGATCAGTTATCATTCTAATAGCA			
glpQ	*glpQ* fragment 1 (primary)-F	CATTAATTATAGCTCACAGAG	Semi-nested	599	Toledo et al. (2010); Loh et al. (2017)
	glpQ fragment 1 (primary)-R	AACAAGCATTATCAATTTTCC			
	glpQ fragment 1 (secondary)-F	AGTGGATATTTACCAGAACA		573	
	glpQ fragment 1 (secondary)-R	AACAAGCATTATCAATTTTCC			
groEL	*groEL* fragment 2 (primary)-F	ATTGCTATACTTACTGGAGG	Semi-nested	768	Toledo et al. (2010); Loh et al. (2017)
	groEL fragment 2 (primary)-R	TTACATCATTCCCATTCCAG			
	groEL fragment 2 (secondary)-F	GGTGTGCTTATTAGTGAGGA		750	
	groEL fragment 2 (secondary)-R	TTACATCATTCCCATTCCAG			
gyrB	*gyrB* fragment 2 (primary)-F	CAARGAGRCTTAGRGAACTTGC	Semi-nested	949	Schwan et al. (2005); Loh et al. (2017)
	gyrB fragment 2 (primary)-R	CCATCAACATCAGCATCAGC			
	gyrB fragment 2 (secondary)-F	CAARGAGRCTTAGRGAACTTGC		880	
	gyrB fragment 2 (secondary)-R	CCAACACCAGCWCCAAGAGA			

Table 2
Location, host, tick species, borrelial positive results and list of accession numbers of nucleotide sequences of each gene.

Location (province)	Host (number of ticks infected/ total)	Host no.	Tick				Gene with accession number				
			species	no. tested (no. F, M, N)	no. of *Borrelia 16S rRNA* positive (%)	code	*16S rRNA*	*flaB*	*glpQ*	*groEL*	*gyrB*
Pattani	water monitor (2/5)	1	*Amblyomma helvolum*	3 (3 F, 0 M)	0	–	–	–	–	–	–
		2	*Amblyomma helvolum*	2 (2 F, 0 M)	0	–	–	–	–	–	–
			Amblyomma varanense	1 (1 F, 0 M)	1 (100%)	**PTN177**	MK453297	MK462204	MK501781	MK462214	–
		3	*Amblyomma helvolum*	1 (1 F, 0 M)	0	–	–	–	–	–	–
		4	*Amblyomma helvolum*	3 (3 F, 0 M)	0	–	–	–	–	–	–
		5	*Amblyomma varanense*	9 (2 F, 7 M)	2 (22%)	**PTN926**	MK453298	MK462205	MK501782	MK462215	–
						PTN933	+*	+	+*	+	–
			Amblyomma sp.	1 (1 N)	0	–	–	–	–	–	–
Narathiwat	water monitor (1/1)	1	*Amblyomma helvolum*	2 (2 F, 0 M)	1 (50%)	**NWT72**	MK453299	MK462206	+*	MK462216	–
			Amblyomma varanense	3 (0 F, 3 M)	0	–	–	–	–	–	–
Satun	Bengal monitor (2/2)	1	*Amblyomma varanense*	6 (1 F, 5 M)	5 (83%)	STN1	+	+	+	+	–
						STN2	MK453300	MK462207	–	MK462217	MK462224
						STN3	MK453301	MK462208	–	MK462218	MK462225
						STN4	+	+	–	+	–
						STN6	+	+	–	+	–
		2	*Amblyomma varanense*	13 (7 F, 6 M)	2 (15%)	STN93	+	+	+	+	+
						STN96	+	+	–	+	–
			Amblyomma sp.	1 (1 N)	0	–	–	–	–	–	–
	elongated tortoise (1/1)	1	*Amblyomma geoemydae*	5 (0 F, 5 M)	1 (20%)	**STN357**	MK453305	MK462212	+*	MK462222	–
			Amblyomma sp.	1 (1 N)	0	–	–	–	–	–	–
Songkhla	elongated tortoise (0/2)	1	*Amblyomma geoemydae*	1 (1 F, 0 M)	0	–	–	–	–	–	–
		2	*Amblyomma geoemydae*	4 (4 F, 0 M)	0	–	–	–	–	–	–
Chaiyaphum	Burmese python (4/4)	1	*Amblyomma varanense*	3 (2 F, 1 M)	2 (67%)	**PK34**	MK453303	MK462210	+*	MK462220	MK462227
						PK36	+	+	–	+	+
		2	*Amblyomma varanense*	3 (0 F, 3 M)	1 (33%)	**PK37**	MK453304	MK462211	–	MK462221	MK462228
		3	*Amblyomma varanense*	3 (1 F, 2 M)	2 (67%)	PK41	+	+	–	+	+
						PK42	+	+	–	+	+
		4	*Amblyomma varanense*	4 (4 F, 0 M)	1 (25%)	PK43	+	+	–	+	+
Sisaket	Bengal monitor (1/2)	1	*Amblyomma helvolum*	2 (0 F, 2 M)	0	–	–	–	–	–	–
			Amblyomma varanense	7 (0 F, 7 M)	0	–	–	–	–	–	–
			Amblyomma sp.	1 (1 N)	0	–	–	–	–	–	–
		2	*Amblyomma helvolum*	4 (4 F, 0 M)	0	–	–	–	–	–	–
			Amblyomma varanense	10 (5 F, 5 M)	1 (10%)	**SSK18**	MK453302	MK462209	MK501784	MK462219	MK462226
	yellow-spotted keelback (0/2)	1	*Amblyomma helvolum*	4 (4 F, 0 M)	0	–	–	–	–	–	–
		2	*Amblyomma helvolum*	1 (1 F, 0 M)	0	–	–	–	–	–	–
Kalasin	keeled rat snake (0/1)	1	*Amblyomma helvolum*	5 (5 F, 0 M)	0	–	–	–	–	–	–
			Amblyomma sp.	2 (2 N)	0	–	–	–	–	–	–
Mahasarakham	keeled rat snake (0/1)	1	*Amblyomma helvolum*	1 (1 F, 0 M)	0	–	–	–	–	–	–
Uthai Thani	elongated tortoise (1/2)	1	*Amblyomma geoemydae*	7 (1 F, 6 M)	0	–	–	–	–	–	–
		2	*Amblyomma geoemydae*	14 (0 F, 14 M)	1 (7%)	**HKK19**	MK453306	MK462213	MK501785	MK462223	MK462229
Total	(12/23)			127 (55 F, 66 M, 6 N)	20 (15.7%)						

F = female, M = male, N = nymph.

* The faint bands that could not be sequenced. The bold codes are representatives from each location.

were collected from water monitor, Bengal monitors, keeled rat snake and elongated tortoise (Table 2).

Borrelia-positive ticks were amplified for mt *16S rRNA* to confirm species. The results of sequence analysis showed that one *A. helvolum* obtained from a water monitor had 99% identity with a partial sequence of mt *16S rRNA* of *A. helvolum* (KC170738) and was submitted to GenBank under accession number MK480198. The sequences of *A. varanense* obtained from water monitors, Bengal monitors and Burmese pythons showed 97–99% identity with a partial sequence of mt *16S rRNA* of *A. varanense* (KC170736). These sequences were submitted to GenBank under accession numbers MK480197, MK480199 and MK480200. In addition, one *A. geoemydae* obtained from an elongated tortoise was submitted to GenBank under accession number MK480201 and showed 99% identity with a partial sequence of mt *16S rRNA* of *A. geoemydae* (KT382864).

3.2. Detection of Borrelia spp.

The presence of *Borrelia* spp. was first screened by conventional PCR based on the *16S rRNA* gene and showed positive results in 20 out of 127 ticks (15.7%) from 50% (3/6) water monitors in Pattani and Narathiwat provinces, 75% (3/4) Bengal monitors in Satun and Sisaket provinces, 100% (4/4) Burmese pythons in Chaiyaphum province and 40% (2/5) elongated tortoises in Satun and Uthai Thani provinces. These included 4% (1/28) *A. helvolum* (collected from a water monitor), 27% (17/62) *A. varanense* (collected from water monitors, Bengal monitors and Burmese pythons) and 6% (2/31) *A. geoemydae* (collected from elongated tortoises) (Table 2). *Borrelia*-positive ticks were further amplified for the segments of the *flaB*, *glpQ*, *groEL* and *gyrB* genes. The presence of *Borrelia* spp. for the *flaB* and *groEL* genes showed positive results in 20 ticks (15.7%). Positive PCR results for the *glpQ* gene were detected in 10 ticks (7.9%), including one *A. helvolum*, seven *A. varanense* and two *A. geoemydae*. Eleven ticks (8.7%) were positive for the *gyrB* gene, including 10 *A. varanense* and one *A. geoemydae* (Table 2).

3.3. Sequence analysis of five genes and phylogenetic analysis

Representative positive sequences of *Borrelia* bacteria for each gene (PTN177, PTN926, NWT72, STN2, STN3, STN357, PK34, PK37, SKK18 and HKK19) were selected based on tick species, host and location. The DNA sequences were submitted to GenBank database under accession numbers: MK453297-MK453306 for *16 SrRNA*, MK462204-MK462213 for *flaB*, MK501781-MK501782, MK501784-MK501785 for *glpQ*, MK462214-MK462223 for *groEL* and MK462224-MK462229 for *gyrB*. Tick codes and accession numbers are shown in Table 2.

3.3.1. 16S rRNA

Ten representative positive sequences from a total of 20 positive ticks were submitted to GenBank database. Multiple sequence alignment of the *16S rRNA* gene revealed that the percent identity of *Borrelia* sequences obtained from this study ranged from 96.3 to 100% (Supplementary Table S1A). BLASTn analysis of the *16S rRNA* gene showed that *Borrelia* sequences from this study (except HKK19) were 98–99% identity with the REP borreliae group, including *Borrelia* sp. TA2 (AB529427) obtained from *A. exornatum* collected from a Savannah monitor, *Borrelia* sp. tAG85 (AB529377) obtained from *A. geoemydae*-infested turtle in Japan, *B. turcica* IST7 (CP028884) obtained from *H. aegyptium*-infested tortoise in Turkey, *Borrelia* sp. in *A. varanense* (KU497718) obtained from a snake in Thailand and *Borrelia* sp. BF16 (AB473538) obtained from *A. trimaculatum*-infested snake. Furthermore, one *Borrelia* sequence from HKK19 showed 100% identity with a partial sequence of *Borrelia* sp. in *A. geoemydae* (AB529435), which was isolated from a turtle in Japan and had a 98% identity with some species of the RF borreliae group, including *B. hermsii* strain MTW (CP005680) and *B. miyamotoi* strain CT13-2396 (CP017126) (Table 3).

Phylogenetic tree based on the partial sequences of the borrelial *16S rRNA* gene revealed that most *Borrelia* spp. in this study (nine out of 10 sequences) clustered within the clade of the REP borreliae, whereas *Borrelia* from HKK19 clustered with the RF borreliae. *Borrelia* from PTN177, PTN926, NWT72, STN2, STN3 and SSK18 were grouped together and closely related to *Borrelia* sp. TA2 (AB529427). In contrast, *Borrelia* from PK34 and PK37 formed a monophyletic group with *Borrelia* sp. in *A. varanense* (KU497718) and *Borrelia* sp. in BF16 (AB473538). While, *Borrelia* from STN357 clustered in the same clade as *B. turcica* IST7 (CP028884) and *Borrelia* sp. tAG85 (AB529377). On the contrary, *Borrelia* from HKK19 was closely related to *Borrelia* sp. in *A. geoemydae* (AB529435) and these sequences formed sister clade to *B. miyamotoi* strain CT13-2396 (CP017126) and *B. theileri* strain KAT (KF569941) of the RF borreliae group (Fig. 1).

3.3.2. flaB

Multiple sequence alignment of the *flaB* gene revealed that 10 *Borrelia* sequences obtained from this study shared 75.7–100% identity among themselves. The percent identity among nine sequences and *Borrelia* from HKK19 ranged from 75.7 to 83% (Supplementary Table S1B). BLASTn analysis of the *flaB* gene revealed that the nine sequences (except HKK19) showed 85–99% with *Borrelia* from the REP group. While, *Borrelia* sequence obtained from HKK19 showed 100% identity with a partial sequence of *Borrelia* sp. in *A. geoemydae* (AB529429) and 92% identity with *B. parkeri* strain HR1 (CP007022) and *B. miyamotoi* strain CT13-2396 (CP017126) from the RF borreliae group (Table 3).

For phylogenetic result based on the partial sequences of the borrelial *flaB* gene, most of the sequences clustered within the clade of REP borreliae, while the *Borrelia* from HKK19 clustered with the RF group (Fig. 2).

3.3.3. glpQ

Three of borrelial *glpQ*-PCR products (NWT72, PK34 and STN357) were not successfully sequenced and the presence of *Borrelia* spp. of the *glpQ* gene was not detected in STN2, STN3 and PK37. Multiple sequence alignment of the *glpQ* gene (four of them) revealed that the percent identity within *Borrelia* sequences obtained from PTN177, PTN926 and SSK18 ranged from 99.2 to 99.6% and showed 86–88% identity with the REP borreliae group. The percent identity among three sequences and *Borrelia* from HKK19 ranged from 67.1 to 67.7% (Supplementary Table S1C). A sequence of *Borrelia* obtained from HKK19 showed 100% identity with a partial sequence of *Borrelia* sp. in *A. geoemydae* (AB529433) and showed 89% identity with the RF borreliae group of *B. miyamotoi* strain CT13-2396 (CP017126) (Table 3).

Phylogenetic tree based on the partial sequences of the borrelial *glpQ* gene (Supplementary Fig. S1) was consistent with the phylogenetic tree based on the partial sequences of *16S rRNA* gene. Phylogenetic tree analyses confirmed that most of *Borrelia* spp. characterized in this study (except HKK19) clustered together with the clade of REP borreliae, whereas *Borrelia* from HKK19 clustered in the clade of the RF borreliae.

3.3.4. groEL

For the *groEL* genes, 10 representative positive sequences from a total of 20 positive ticks were also submitted to GenBank database. Multiple sequence alignment of the *groEL* gene revealed that nine *Borrelia* sequences obtained from this study (except HKK19) shared 82.4–100% identity among themselves. The percent identity among these sequences and *Borrelia* sequence from HKK19 ranged from 83.4 to 85.4% (Supplementary Table S1D). BLASTn analysis of the *groEL* gene revealed that nine sequences (except HKK19) showed 89–91% identity with *Borrelia* from the REP group. While, one *Borrelia* sequence obtained from HKK19 showed 92–93% identity with *Borrelia* from the RF group (Table 3).

Phylogenetic tree based on the partial sequences of the borrelial *groEL* gene also confirmed that most *Borrelia* spp. from this study clustered within the clade of REP borreliae. Interestingly, for this gene, *Borrelia* from PK34 and PK37 collected from Burmese pythons and

Table 3
Percent identity of each borrelial gene in this work with BLAST search.

Tick code	BLAST result with percent identity and accession number				
	16S rRNA	*flaB*	*glpQ*	*groEL*	*gyrB*
PTN177	*Borrelia* sp. TA2 (98%) (AB529427)	*Borrelia* sp. TA2 (87%) (AB529428)	*Borrelia* sp. tAG85 (88%) (AB529403)	*B. turcica* (90%) (CP028884)	–
PTN926	*Borrelia* sp. TA2 (99%) (AB529427)	*Borrelia* sp. TA2 (88%) (AB529428)	*Borrelia* sp. tAG85 (88%) (AB529403)	*B. turcica* (90%) (CP028884)	–
NWT72	*Borrelia* sp. TA2 (98%) (AB529427)	*Borrelia* sp. TA2 (87%) (AB529428)	–	*B. turcica* (90%) (CP028884)	–
STN2	*Borrelia* sp. TA2 (99%) (AB529427)	*Borrelia* sp. TA2 (87%) (AB529428)	–	*B. turcica* (91%) (CP028884)	*B. turcica* (89%) (CP028884)
STN3	*Borrelia* sp. TA2 (99%) (AB529427)	*Borrelia* sp. TA2 (87%) (AB529428)	–	*B. turcica* (91%) (CP028884)	*B. turcica* (89%) (CP028884)
SSK18	*Borrelia* sp. TA2 (99%) (AB529427)	*Borrelia* sp. TA2 (88%) (AB529428)	*Borrelia* sp. tAG85 (88%) (AB529403)	*B. turcica* (91%) (CP028884)	*B. turcica* (89%) (CP028884)
PK34	*Borrelia* sp. in *A. varanense* (99%) (KU497718)	*Borrelia* sp. in *A. varanense* (99%) (KT758064)	–	*B. turcica* (89%) (CP028884)	*Borrelia* sp. BF16 (99%) (AB473534)
PK37	*Borrelia* sp. in *A. varanense* (99%) (KU497718)	*Borrelia* sp. in *A. varanense* (99%) (KT758064)	–	*B. turcica* (89%) (CP028884)	*Borrelia* sp. BF16 (99%) (AB473534)
STN357	*Borrelia* sp. tAG85 (99%) (AB529377)	*Borrelia* sp. tAG85 (98%) (AB529323)	–	*B. turcica* (89%) (CP028884)	–
HKK19	*Borrelia* sp. in *A. geoemydae* (100%) (AB529435)	*Borrelia* sp. in *A. geoemydae* (100%) (AB529429)	*Borrelia* sp. in *A. geoemydae* (100%) (AB529433)	*B. parkeri* HR1 (93%) (CP007022)	*B. parkeri* HR1 (90%) (CP007022)

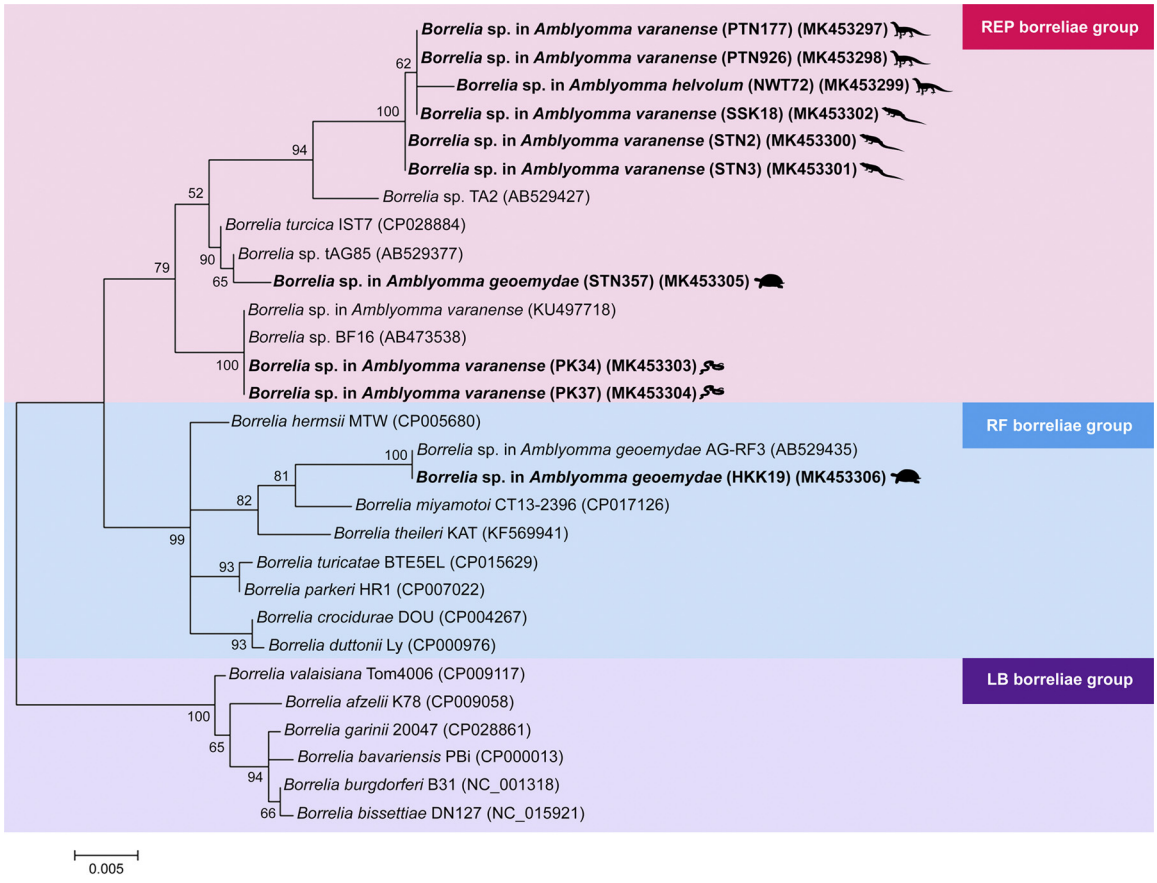

Fig. 1. Phylogenetic analysis of *Borrelia* spp. based on the partial sequences of the *16S rRNA* gene using the maximum likelihood method calculated under the HKY + G + I substitution model. The phylogenetic tree was constructed by MEGA7. Bootstrap values were determined with 1000 replicates and supported more than 50% shown above the branches. Bold type indicates the sequences of this study.
(= water monitor, = Bengal monitor, = Burmese python and = elongated tortoise)

Borrelia from STN357 collected from an elongated tortoise were grouped together in the same clade (Fig. 3). HKK19 *Borrelia* also clustered within the same clade of RF borreliae.

3.3.5. *gyrB*

The presence of *Borrelia* spp. of this gene was not detected in PTN177, PTN926, NWT72 and STN357. Multiple sequence alignment of the *gyrB* gene (six of them) revealed that the percent identity within five

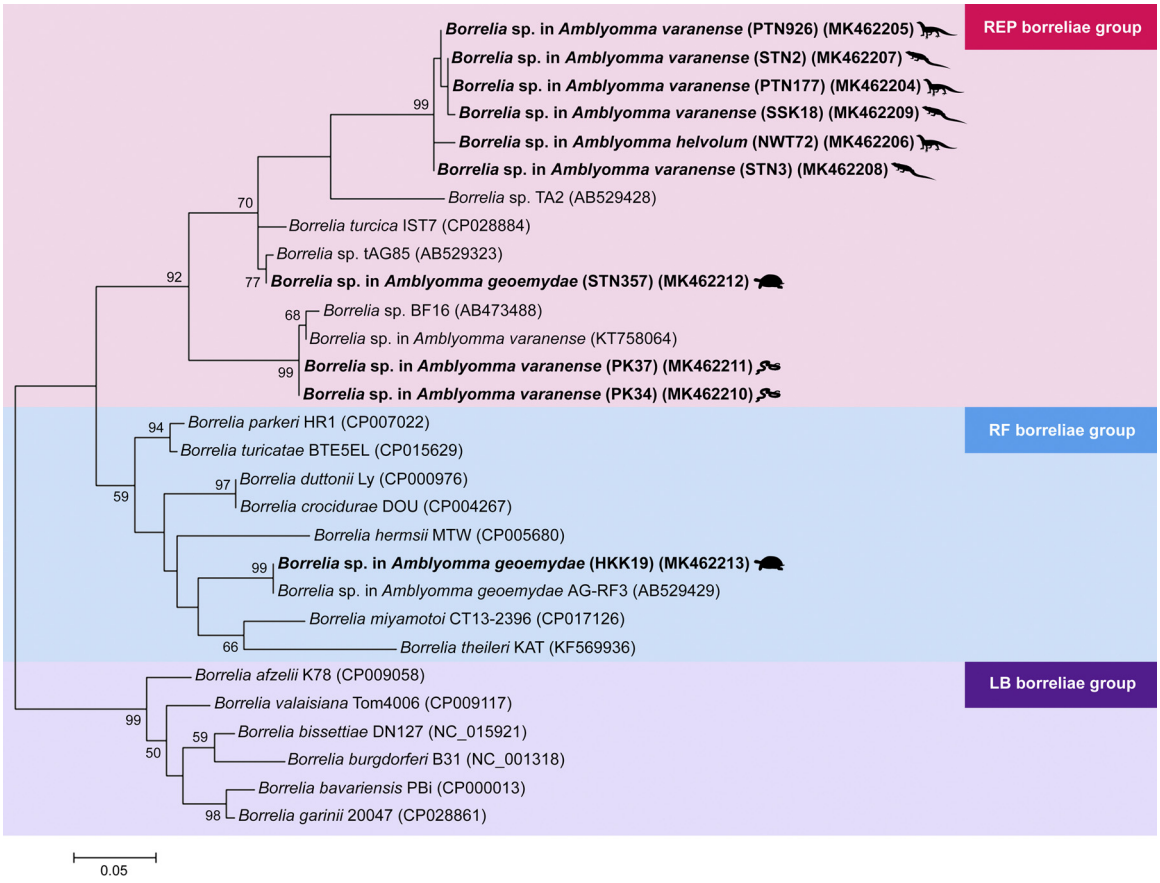

Fig. 2. Phylogenetic analysis of *Borrelia* spp. based on the partial sequences of the *flaB* gene using the maximum likelihood method calculated under the T92 + G substitution model. The phylogenetic tree was constructed by MEGA7. Bootstrap values were determined with 1000 replicates and supported more than 50% shown above the branches. Bold type indicates the sequences of this study.
(= water monitor, = Bengal monitor, = Burmese python and = elongated tortoise)

Borrelia sequences (except HKK19) ranged from 74.5 to 100% and showed 84–99% identity with the REP borreliae group. The percent identity among five sequences and *Borrelia* from HKK19 ranged from 72.7 to 73.9% (Supplementary Table S1E). A sequence of *Borrelia* obtained from HKK19 showed 89–90% identity with the RF borreliae group, including *B. parkeri* strain HR1 (CP007022) and *B. miyamotoi* strain CT13-2396 (CP017126) (Table 3).

Phylogenetic tree based on the partial sequences of the borrelial *gyrB* gene (Supplementary Fig. S2) revealed the same trend of group as the other phylogenetic trees (genes) from this study.

4. Discussion

In Thailand, *Amblyomma*-infesting reptiles have been reported in several studies (Sumrandee et al., 2014a, b, 2015; Trinachartvanit et al., 2016). *Amblyomma* ticks are potential vectors and carry several tick-borne pathogens and endosymbionts, including *Rickettsia*, *Anaplasma*, *Hepatozoon*, *Ehrlichia*, *Francisella* and *Borrelia* spp. (Ogrzewalska et al., 2019; Kho et al., 2015; Sumrandee et al., 2014b; Trinachartvanit et al., 2016). In this study, *Borrelia* spp. were detected in three species of *Amblyomma* ticks (*A. helvolum*, *A. varanense* and *A. geoemydae*) collected from four reptile species (water monitors, Bengal monitors, Burmese pythons and elongated tortoises) in six provinces of Thailand (Pattani, Narathiwat, Satun, Uthai Thani, Chaiyaphum and Sisaket).

Amblyomma helvolum and *A. geoemydae* have been found parasitizing humans (Audy et al., 1960; Kadosaka et al., 2006) and REP borreliae have been detected in reptiles (tortoises) and hard ticks (*Hyalomma*, *Amblyomma* and *Bothriocroton*) (Guner et al., 2003, 2004; Takano et al., 2010, 2011; Panetta et al., 2017). Trinachartvanit et al. (2016) found *Borrelia* sp. infected *A. varanense* ticks from a reticulated python in Lopburi Province, Thailand and it was closely related to the REP borreliae group. Our studies showed that 15.7% (20/127) of *Amblyomma* ticks were positive for *Borrelia* bacteria. Thus, REP *Borrelia* was detected in three species of *Amblyomma* ticks (*A. helvolum*, *A. varanense* and *A. geoemydae*) while RF-related *Borrelia* was detected in only one *A. geoemydae*. REP *Borrelia* detected in this study revealed a higher DNA sequence identity within the same reptile host group more than those *Borrelia* found in the different host groups. Our phylogenetic analysis of the *16S rRNA* showed the majority of samples clustered within the REP clade. Similar results were consistently observed in the phylogenetic trees of the *flaB*, *glpQ*, *gyrB* and *groEL* genes. Interestingly, only the *groEL* gene from *A. varanense* collected from Burmese pythons (PK34 and PK37) were identical to *Borrelia* sequence detected in *A. geoemydae* collected from an elongated tortoise (STN357). This seems to imply that *groEL* gene DNA sequence variations may be less dependent on tick species/and or vertebrate hosts than the other genes evaluated in our study. Though, more tick samples are needed for further investigation.

In Asia, the presence of the RF borreliae has been commonly found in Central, Western and East Asia (Wang and Schwartz, 2010). Recently, *Borrelia* was found in *Haemaphysalis hystricis* from Malaysia and it was closely related to the RF group, including *B. miyamotoi* and *B. theileri* (Khoo et al., 2017). Interestingly, we found one *Borrelia* obtained from *A. geoemydae* (HKK19) collected from an elongated tortoise whose partial sequence of *16S rRNA*, *flaB* and *glpQ* was identical to *Borrelia* sp. in *A. geoemydae* infested a turtle in Japan (Takano et al., 2012). These *Borrelia* were closely related to the RF borreliae in all phylogenetic trees in this study. Thus, our findings showed for the first time of the

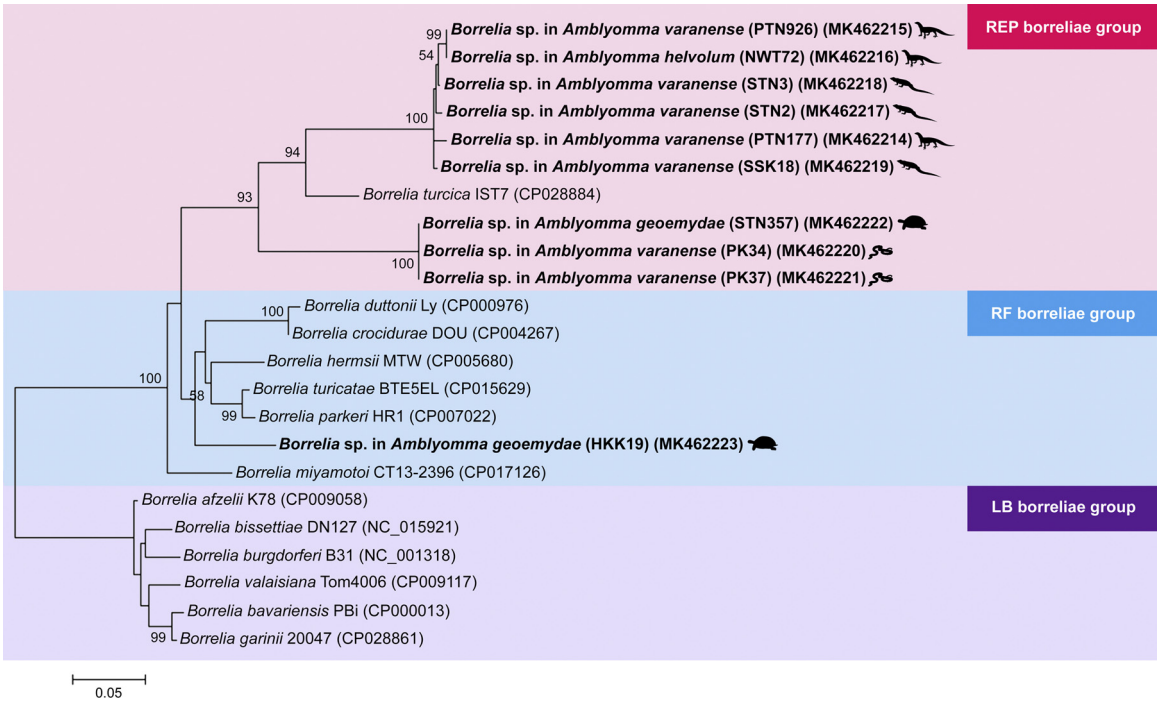

Fig. 3. Phylogenetic analysis of *Borrelia* spp. based on the partial sequences of the *groEL* gene using the maximum likelihood method calculated under the HKY + G substitution model. The phylogenetic tree was constructed by MEGA7. Bootstrap values were determined with 1000 replicates and supported more than 50% shown above the branches. Bold type indicates the sequences of this study.
(= water monitor, = Bengal monitor, = Burmese python and = elongated tortoise)

presence of RF *Borrelia* in an *A. geoemydae* tick in Thailand. These results seem to suggest that RF borreliae have been distributed throughout Southeast Asia. However, the pathogenicity of the RF *Borrelia* detected in an *A. geoemydae* from an elongated tortoise in our study is still unknown. Furthermore, *A. geoemydae* feeding on humans and exported reptiles have been reported (Kadosaka et al., 2006; Simmons and Burridge, 2000), which cause a serious concern on the distribution of pathogens by ticks. Additionally, the infection and the impact of REP borreliae in animals and humans in Thailand is unknown thus far.

Ethical statements

The protocol was performed according to the National Research Council of Thailand under license number U1-05257-2559.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.101315.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Audy, J.R., Nadchatram, M., LIM, B.-L., 1960. Malaysian parasites XLIX. Host distribution of Malayan ticks (Ixodoidea). Stud. Inst. Med. Res. Malaya 29, 225–246.
Black, W.C., Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. U.S.A. 91, 10034–10038.
Gofton, A.W., Margos, G., Fingerle, V., Hepner, S., Loh, S.-M., Ryan, U., Irwin, P., Oskam, C.L., 2018. Genome-wide analysis of *Borrelia turcica* and '*Candidatus* Borrelia tachyglossi' shows relapsing fever-like genomes with unique genomic links to Lyme disease *Borrelia*. Infect. Genet. Evol. 66, 72–81.
Guner, E.S., Hashimoto, N., Kadosaka, T., Imai, Y., Masuzawa, T., 2003. A novel, fast-growing *Borrelia* sp. Isolated from the hard tick *Hyalomma aegyptium* in Turkey. Microbiology 149, 2539–2544.
Guner, E.S., Watanabe, M., Hashimoto, N., Kadosaka, T., Kawamura, Y., Ezaki, T., Kawabata, H., Imai, Y., Kaneda, K., Masuzawa, T., 2004. *Borrelia turcica* sp. nov., isolated from the hard tick *Hyalomma aegyptium* in Turkey. Int. J. Syst. Evol. Microbiol. 54, 1649–1652.
Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129 (Suppl), S3–S14.
Kadosaka, T., Fujita, H., Goto, I., Kawabata, H., 2006. Human tick-bite by *Amblyomma geoemydae* in Ishigakijima island of Okinawa prefecture, Japan. Med. Entomol. Zool. 58 (Suppl), 71.
Kho, K.L., Koh, F.X., Tay, S.T., 2015. Molecular evidence of potential novel spotted fever group rickettsiae, *Anaplasma* and *Ehrlichia* species in *Amblyomma* ticks parasitizing wild snakes. Parasit. Vectors 8, 112.
Khoo, J.J., Lim, F.S., Tan, K.K., Chen, F.S., Phoon, W.H., Khor, C.S., Pike, B.L., Chang, L.Y., AbuBakar, S., 2017. Detection in Malaysia of a *Borrelia* sp. From *Haemaphysalis hystricis* (Ixodida: ixodidae). J. Med. Entomol. 54, 1444–1448.
Kohls, G.M., 1957. Malaysian parasites-XVIII. Ticks (Ixodoidea) of Borneo and Malaya. Stud. Inst. Med. Res. Malaya 28, 65–94.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.
Loh, S.-M., Gillett, A., Ryan, U., Irwin, P., Oskam, C., 2017. Molecular characterization of "*Candidatus* Borrelia tachyglossi" (family *Spirochaetaceae*) in echidna ticks, *Bothriocroton concolor*. Int. J. Syst. Evol. Microbiol. 67, 1075–1080.
Masuzawa, T., Fukui, T., Miyake, M., Oh, H.B., Cho, M.K., Chang, W.H., Imai, Y., Yanagihara, Y., 1999. Determination of members of a *Borrelia afzelii*-related group isolated from *Ixodes nipponensis* in Korea as *Borrelia valaisiana*. Int. J. Syst. Bacteriol. 49 (Pt 4), 1409–1415.
Ogrzewalska, M., Machado, C., Rozental, T., Forneas, D., Cunha, L.E., de Lemos, E.R.S., 2019. Microorganisms in the ticks *Amblyomma dissimile* Koch 1844 and *Amblyomma rotundatum* Koch 1844 collected from snakes in Brazil. Med. Vet. Entomol. 33, 154–161.
Panetta, J.L., Šíma, R., Calvani, N.E.D., Hajdušek, O., Chandra, S., Panuccio, J., Šlapeta, J., 2017. Reptile-associated *Borrelia* species in the goanna tick (*Bothriocroton undatum*) from Sydney, Australia. Parasit. Vectors 10, 616.
Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32, 897–928.

Sato, Y., Konishi, T., Hashimoto, Y., Takahashi, H., Nakaya, K., Fukunaga, M., Nakao, M., 1997. Rapid diagnosis of lyme disease: flagellin gene-based nested polymerase chain reaction for identification of causative *Borrelia* species. Int. J. Infect. Dis. 2, 64–73.

Schwan, T.G., Raffel, S.J., Schrumpf, M.E., Policastro, P.F., Rawlings, J.A., Lane, R.S., Breitschwerdt, E.B., Porcella, S.F., 2005. Phylogenetic analysis of the spirochetes *Borrelia parkeri* and *Borrelia turicatae* and the potential for tick-borne relapsing fever in Florida. J. Clin. Microbiol. 43, 3851–3859.

Simmons, L.A., Burridge, M.J., 2000. Introduction of the exotic ticks *Amblyomma humerale* koch and *Amblyomma geoemydae* (Cantor) (Acari: ixodidae) into the United States on imported reptiles. Int. J. Acarology 26, 239–242.

Steere, A.C., Strle, F., Wormser, G.P., Hu, L.T., Branda, J.A., Hovius, J.W., Li, X., Mead, P.S., 2016. Lyme borreliosis. Nat. Rev. Dis. Primers 2, 16090.

Sthitmatee, N., Jinawan, W., Jaisan, N., Tangjitjaroen, W., Chailangkarn, S., Sodarat, C., Ekgatat, M., Padungtod, P., 2016. Genetic and immunological evidences of *Borrelia burgdorferi* in dog in Thailand. Southeast Asian J. Trop. Med. Public Health 47, 71–77.

Sumrandee, C., Hirunkanokpun, S., Doornbos, K., Kitthawee, S., Baimai, V., Grubhoffer, L., Trinachartvanit, W., Ahantarig, A., 2014a. Molecular detection of *Rickettsia* species in *Amblyomma* ticks collected from snakes in Thailand. Ticks Tick. Dis. 5, 632–640.

Sumrandee, C., Hirunkanokpun, S., Grubhoffer, L., Baimai, V., Trinachartvanit, W., Ahantarig, A., 2014b. Phylogenetic relationships of *Francisella*-like endosymbionts detected in two species of *Amblyomma* from snakes in Thailand. Ticks Tick. Dis. 5, 29–32.

Sumrandee, C., Baimai, V., Trinachartvanit, W., Ahantarig, A., 2015. *Hepatozoon* and *Theileria* species detected in ticks collected from mammals and snakes in Thailand. Ticks Tick. Dis. 6, 309–315.

Takano, A., Goka, K., Une, Y., Shimada, Y., Fujita, H., Shiino, T., Watanabe, H., Kawabata, H., 2010. Isolation and characterization of a novel *Borrelia* group of tick-borne borreliae from imported reptiles and their associated ticks. Environ. Microbiol. 12, 134–136.

Takano, A., Fujita, H., Kadosaka, T., Konnai, S., Tajima, T., Watanabe, H., Ohnishi, M., Kawabata, H., 2011. Characterization of reptile-associated *Borrelia* sp. In the vector tick, *Amblyomma geoemydae*, and its association with Lyme disease and relapsing fever *Borrelia* spp. Environ. Microbiol. Rep. 3, 632–637.

Takano, A., Sugimori, C., Fujita, H., Kadosaka, T., Taylor, K.R., Tsubota, T., Konnai, S., Tajima, T., Sato, K., Watanabe, H., Ohnishi, M., Kawabata, H., 2012. A novel relapsing fever *Borrelia* sp. Infects the salivary glands of the molted hard tick, *Amblyomma geoemydae*. Ticks Tick. Dis. 3, 259–261.

Toledo, A., Anda, P., Escudero, R., Larsson, C., Bergstrom, S., Benach, J.L., 2010. Phylogenetic analysis of a virulent *Borrelia* species isolated from patients with relapsing fever. J. Clin. Microbiol. 48, 2484–2489.

Trinachartvanit, W., Hirunkanokpun, S., Sudsangiem, R., Lijuan, W., Boonkusol, D., Baimai, V., Ahantarig, A., 2016. *Borrelia* sp. Phylogenetically different from Lyme disease- and relapsing fever-related *Borrelia* spp. In *Amblyomma varanense* from *Python reticulatus*. Parasit. Vectors 9, 359.

Voltzit, O.V., Keirans, J.E., 2002. A review of Asian *Amblyomma* species (Acari, Ixodida, Ixodidae). Acarina 10, 95–136.

Wang, G., 2015. Chapter 104 - *Borrelia burgdorferi* and other *Borrelia* species. In: Tang, Y.W., Sussman, M., Liu, D., Poxton, I., Schwartzman, J. (Eds.), Molecular Medical Microbiology. Academic Press, Boston, pp. 1867–1909.

Wang, G., Schwartz, I., 2010. Genus *Borrelia*. In: Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L., Ludwig, W., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology: Volume Four: the Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes. Springer, New York, pp. 484–531.