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Low Genetic Diversity of Vector-Borne Haemoparasites in Dogs and Their Ticks Revealed Local and Long-Range Transmission in Peninsular Malaysia

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ABSTRACT

Molecular methods coupled with phylogenetic analysis are sensitive tools for detecting and classifying parasites. This study used nuclear and mitochondrial gene markers to investigate the host-vector interaction of the vector-borne haemoparasites. The population genetic structures of important vector-borne haemoparasites in dogs, namely, *Anaplasma platys*, *Ehrlichia canis*, *Babesia vogeli*, and *Babesia gibsoni*, were determined from the nuclear gene of 16S or 18S rRNA gene, *gltA* and *groESL* and mitochondrial gene of *COX1* across dogs and vector ticks. A total of 220 blood samples and 140 ticks were collected from shelter dogs in Peninsular Malaysia. Out of the positive samples for the vector-borne haemoparasites, 28 positive blood isolates and six tick isolates were selected and characterised. There was a low diversity in tick sequences, while varying degree of variability was observed in dogs' sequences. Overlapped haplotypes were observed in sequences of dogs and ticks, revealing the possibility of the same infection origin. No regional separation was detected, but similar haplotypes from different regions were observed. These findings contribute to the epidemiology of vector-borne haemoparasites in dogs in Malaysia.

Keywords: Dogs, genetic structures, haemoparasites, ticks

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INTRODUCTION

Ticks can transmit a wide range of diseases, including viral, bacterial, and protozoan, to animal hosts (Otranto & Wall, 2008). *Rhipicephalus sanguineus* sensu lato (*R. sanguineus*), the brown dog tick, now known as *Rhipicephalus linnaei* (Šlapeta et al., 2021) is an important vector of canine vector-borne haemoparasites (VBHs) in Southeast Asia (SEA), and the primary tick infesting dogs in Malaysia (Dantas-Torres, 2010; Low et al., 2017; Nguyen et al., 2019; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). The higher prevalence of VBHs in dogs and low prevalence in ticks were evident in previous studies (Galay et al., 2018; Low et al., 2018; Prakash, Low, Tan et al., 2018; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). Nevertheless, stray dogs were more susceptible to VBHs infection compared to pet dogs due to the high exposure to arthropod vectors (Cao et al., 2015). The infection rate in dogs strongly correlates to exposure to ticks and VBHs (Jennett et al., 2013).

The development of molecular methods combined with phylogenetic analysis provides a highly sensitive diagnostic tool for detecting and classifying these VBHs into their closely related species according to genetic makeup. Molecular methods and phylogenetic trees have been used in phylogenetic studies of parasites in co-relation to their geographical distribution, climate change effects, and host-specificity (Clare, 2011; Martin et al., 2006; Martins et al., 2009; Morgan et al., 2012). Polymerase chain reaction (PCR)-based detection methods are commonly used for pathogen detection and species identification. The gene markers, namely 16S rRNA and 18S rRNA, are markers that are commonly used in canine VBHs detection using molecular techniques (Koh et al., 2016; Low et al., 2018; Mokhtar et al., 2013; Nazari et al., 2013; Prakash, Low, Vinnie-Siow, et al., 2018). However, these common nuclear gene markers were reported to be highly conserved (Harrus & Waner, 2011; Kamani, Baneth, et al., 2013; Vargas-Hernandez et al., 2012; Zhang et al., 2008). Higher resolution marker such as the mitochondrial DNA (mtDNA) gene (e.g., cytochrome c oxidase I (*COX1*)) is more variable than the nuclear gene. Thus, the present work attempts to use the *COX1* gene, *groESL* gene, and citrate synthase (*gltA*) gene for the haplotype and nucleotide diversities analyses of selected VBHs.

Several studies in Malaysia have started focusing on phylogenetic analysis of VBHs in dogs and their arthropod vector, tick (Low et al., 2018; Prakash, Low, Vinnie-Siow, et al., 2018; Sipin et al., 2020). Nonetheless, further studies are required on the correlation of the presence of VBHs in dogs with the arthropod vector to investigate the origin of the infection. Therefore, this study aims to characterise VBHs in dogs and their ticks using molecular techniques, which focus on investigating the genetic overlap of VBHs in dogs and ticks and the origin of infection using phylogenetic and haplotype network analyses.

MATERIALS AND METHODS

Ethical Approval

The Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (AUP no: UPM/IACUC/AUP-R028/2018) approved the application for animal ethical clearance. Additionally, consent was obtained from the animal shelters before sampling activities were conducted. The owner of the shelters was briefed regarding the purpose of the study, and the handling of the animals during the sample collection was in accordance with animal ethics laws.

Parasites Isolation

Details of sample collection, tick identification, and DNA isolation were performed as described by Sipin et al. (2020). Briefly, a total of 220 dog blood samples and 140 tick samples were collected from 10 animal shelters in North (Pulau Pinang, Kedah, Perak), South (Johor), East (Pahang), and Central (Selangor) regions of Peninsular Malaysia for the detection of VBHs. Shelter dogs were restrained and subjected to blood and tick collection, where blood was sampled from the cephalic vein into ethylenediamine tetraacetic acid (EDTA) blood tubes, and ticks were removed using forceps and placed in small plastic tubes. The morphological inspection of ticks was performed under a stereomicroscope before DNA extraction.

A total of 28 positive blood isolates and six tick isolates screened from 220 shelter dogs were subjected to further analyses (Table 1). Out of the 28 positive blood isolates used in this study, 11 samples were from each North and Central region, five were from the East region, and only one was from the South region. In addition, four tick isolates used were from the North region and one isolate from each South and Central region.

Table 1
VBHs isolates subjected to molecular characterisation

Region	Sample	ID no.	VBH detected
North (N)	Blood	Nb15	*Anaplasma platys*
		Nb2	*Anaplasma platys*
		Nc13	*Anaplasma platys*
		Nd4	*Anaplasma platys*
		Na12	*Ehrlichia canis*
		Nb9	*Ehrlichia canis*
		Nc6	*Ehrlichia canis*
		Nc7	*Ehrlichia canis*
		Nd15	*Ehrlichia canis*
		Nb1	*Babesia gibsoni*
		Nd7	*Babesia vogeli*
	Tick	Nb17 (11)	*Ehrlichia canis*
		Nb17 (32)	*Ehrlichia canis*
		Nc15	*Ehrlichia canis*
		Nb4	*Babesia vogeli*

Table 1 *(continue)*

Region	Sample	ID no.	VBH detected
South(S)	Blood	Sa2	*Babesia gibsoni*
East(E)	Blood	Ea7	*Anaplasma platys*
		Ea8	*Anaplasma platys*
		Ea11	*Anaplasma platys*
		Ea6	*Ehrlichia canis*
		Ea14	*Ehrlichia canis*
	Tick	Ea8	*Anaplasma platys*
Central(W)	Blood	Wa19	*Anaplasma platys*
		Wd25	*Anaplasma platys*
		Wa19	*Ehrlichia canis*
		Wb20	*Ehrlichia canis*
		Wb23	*Ehrlichia canis*
		Wd1	*Ehrlichia canis*
		Wa1	*Babesia vogeli*
		Wb18	*Babesia vogeli*
		Wd2	*Babesia vogeli*
		Wb25	*Babesia gibsoni*
		Wd25	*Babesia gibsoni*
	Tick	Wd22	*Anaplasma platys*

Molecular Technique

DNA extraction of ticks and blood was conducted using commercially available extraction kits, DNEasy Blood and Tissue Kit (Qiagen, Germany). All DNA was eluted in 100 µl of elution buffer and stored at -20°C before PCR screening.

The PCR amplification for 16S rRNA and *groESL* gene for *A. platys*, 18S rRNA and *COX1* gene for *B. gibsoni*, 18S rRNA and *COX1* gene for *B. vogeli*, and 16S rRNA and *gltA* gene for *E. canis* from both blood and tick samples were performed with the cycling conditions indicated in Table 2.

PCR products were sequenced using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI PRISM 3730xl Genetic Analyser (Applied Biosystems, USA). The sequences obtained were subjected to the Basic Local Alignment Search Tool (BLAST) identity search to compare with known sequences of *B. gibsoni*, *B. vogeli*, *A. platys*, and *E. canis* available in the NCBI GenBank.

Phylogenetic Analysis

The sequences obtained via sequencing were aligned using DAMBE5 (Xia, 2018). MEGA X was applied for phylogenetic relationship determination using a maximum likelihood (ML) algorithm (Kumar et al., 2018). At least 1,000 replicates were used to estimate each species' bootstrap values. Nucleotide sequences of *A. platys* (16S rRNA and

Table 2
Primers and annealing temperatures for PCR reaction used in this study

Haemoparasites	Primer sequence (5'-3')	Gene target (bp)	Annealing temperature (°C)	Reference
Babesia gibsoni	Gib599F (5'-CTCGGCTACTTGCCTTGTC-3') Gib1270R (5'-GCCGAAACTGAAATAACGGC-3')	18S rRNA (690)	62	Otranto et al. (2009)
	Bg-cox1-F (5'-CTTCAGCCAATAGCTTTCTGTTTG-3') BG-cox1-R (5'-CCTGAGGCAAGTAAACCAAATAT-3')	*COX1* (150)	62	Qurollo et al. (2017)
Babesia vogeli	C172F (5'-GTTTATTAGTTTGAAACCCGC-3') C626R (5'-GAACTCGAAAAAGCCAAACGA-3')	18S rRNA (450)	57.5	Otranto et al. (2009)
	CoxBV-F (5'-TGAGTGGCGCAAATTTTGTA-3') CoxBV-R (5'-TGTCTGTCAAGAAAAACCATTAGC-3')	*COX1* (166)	62	Primers were designed based on accession no. KX426022.1 in the National Center for Biotechnology Information (NCBI) GenBank
Anaplasma platys	PlatysF (5'-AAGTCGAACGGATTTTTGTC-3') PlatysR (5'-CTTTAACTTACCGAACC-3')	16S rRNA (500)	60	Beall et al. (2008)
	GroESL-F (5'-AAGGCGAAAGAAGCAGTCTTA-3') GroESL-R (5'-CATAGTCTGAAGTGGAGGAC-3')	*groESL* (724)	60	Inokuma et al. (2002)
Ehrlichia canis	ECA (5'-AACACATGCAAGTCGAACGGA-3') HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3')	16S rRNA (400)	60	Wen et al. (1997)
	Ecanis(gltA)-F (5'-GCTGATCATGAGCAAAATGC-3') Ecanis(gltA)-R (5'-TTGACCAAAACCCATTAGCC-3')	*gltA* (400)	62	Primers were designed based on accession no. LC428206.1 in NCBI GenBank

groESL), *B. vogeli* (18S rRNA and *COX1*), *B. gibsoni* (18SrRNA and *COX1*), and *E. canis* (16S rRNA and *gltA*) in blood and tick samples were used for analyses in the phylogenetic tree. Furthermore, selected sequences in GenBank were used as an outgroup reference sequence for comparison to sequences obtained from the molecular work.

The present study nucleotide sequences were submitted to the NCBI GenBank database under the following accession numbers: *B. gibsoni* 18S rRNA [Blood (MN068981-84)], *B. vogeli* 18S rRNA [Blood (MN075251-54), Tick (MN194598)], *A. platys* 16S rRNA [Blood (MN075275-83), Tick (MN159064-65)], *A. platys groESL* [Blood (OP104926-35), Tick (OP184817)], *E. canis* 18S rRNA [Blood (MN075258-68), Tick (MN159066-67)], and *E. canis gltA* [Blood (OP104926-35), Tick (OP104936-37)].

Pairwise differences (pi) indices and population genetic estimators were calculated in ARLEQUIN version 3.5.2.1 using individual and combined dogs or tick samples sequences data, haplotype diversity h, nucleotide diversity π (Excoffier & Lischer, 2010). Meanwhile, minimum spanning trees were computed using HapStar version 0.7 (Teacher & Griffiths, 2011). Population genetic differences were estimated using ARLEQUIN by analysis of molecular variance (AMOVA) using predefined groups based on the host, comprising dogs and ticks.

RESULTS

Sequence Alignment

The phylogenetic analysis of VBHs obtained in the study was inferred from sequences, as presented in Table 3. The size of each studied gene and the similarity between blood and tick sequences are shown in Table 4. The size of the studied sequence ranges from 116 to 681 bp. Most of the nucleotides in the blood sequence were monomorphic (95.7-100%), with a few variable sites for dogs ranging from 1 to 10 bp. All tick sequences for this study's studied gene of interest were monomorphic.

Table 3
Number of sequences inferred for phylogenetic analysis for VBH species in the study

VBH	Gene	No. of sequences
Anaplasma platys	16S rRNA	11
	groESL	9
Ehrlichia canis	16S rRNA	13
	gltA	12
Babesia vogeli	18S rRNA	5
	COX1	5
Babesia gibsoni	18S rRNA	4
	COX1	3

Phylogenetic Analysis

A phylogenetic tree based on the 16S rRNA gene of *A. platys* and *E. canis* revealed no significant clades and failed to reflect any correlation with the host and pattern of parasite transmission. Most of the sequences appeared similar and sequences from other regions of the world (Figure 1), with a few different sequences (highlighted in blue). A similar result was observed for the *groESL*

Table 4
Sequences alignment of VBH from the study against outgroup sequence in GeneBank

VBH	Gene	Samples	Monomorphic	Polymorphic
Anaplasma platys	16S rRNA	Blood	257 bp (97.70%)	6 bp (2.30%)
		Tick	263 bp (100%)	-
	groESL	Blood	681 bp (100%)	-
		Tick	Only one tick sequence is available	-
Ehrlichia canis	16S rRNA	Blood	374 bp (95.65%)	17 bp (4.35%)
		Tick	391 bp (100%)	-
	gltA	Blood	230 bp (99.57%)	1 bp (0.43%)
		Tick	231 bp (100%)	-
Babesia vogeli	18S rRNA	Blood	421 bp (99.76%)	1 bp (0.24%)
		Tick	Only one tick sequence is available	
	COX1	Blood	131 bp (100%)	-
		Tick	131 bp (100%)	-
Babesia gibsoni	18S rRNA	Blood	616 bp (98.40%)	10 bp (1.60%)
		Tick	None tick positive	
	COX1	Blood	114 bp (98.28%)	1 bp (1.72%)
		Tick	None tick positive	

gene of *A. platys* (Figure 2) and the *gltA* gene of *E. canis* (Figure 3). In addition, a similar result was observed in the phylogenetic tree based on the 18S rRNA gene (Figure 4) and *COX1* gene (Figure 5) of *B. gibsoni* and *B. vogeli*, revealing no significant clades and low diversity.

For the 16S rRNA gene of *A. platys* [Figure 6(a)] and *E. canis* [Figure 6(b)] haplotype network, four haplotypes were observed, with one common haplotype that was shared between both hosts. Only two haplotypes were observed for the *E. canis gltA* gene haplotype network, where one common haplotype was shared between both hosts [Figure 6(c)]. Additionally, four haplotypes were detected for the 18S rRNA gene of *B. vogeli*, comprising three diverse haplotypes in dogs and one separate haplotype for ticks [Figure 6(d)].

Haplotype and Nucleotide Diversity

The haplotype and nucleotide diversity for *A. platys* 16S rRNA, *E. canis* 16S rRNA, and *gltA* genes revealed no variation in the ticks and a low variation in dogs (Table 5). The haplotype and nucleotide diversity for *B. vogeli* 18S rRNA and *B. gibsoni* 18S rRNA and *COX1* genes depicted a low level of variation in dogs. Comparisons of haplotype and nucleotide diversity were generated for ticks as none of the tick samples were positive for *B. gibsoni*, while only one tick sample was positive for *B. vogeli* (Table 5).

DISCUSSION

The population genetic structure of *A. platys*, *E. canis*, *B. vogeli*, and *B. gibsoni* among dogs and the associated vector-

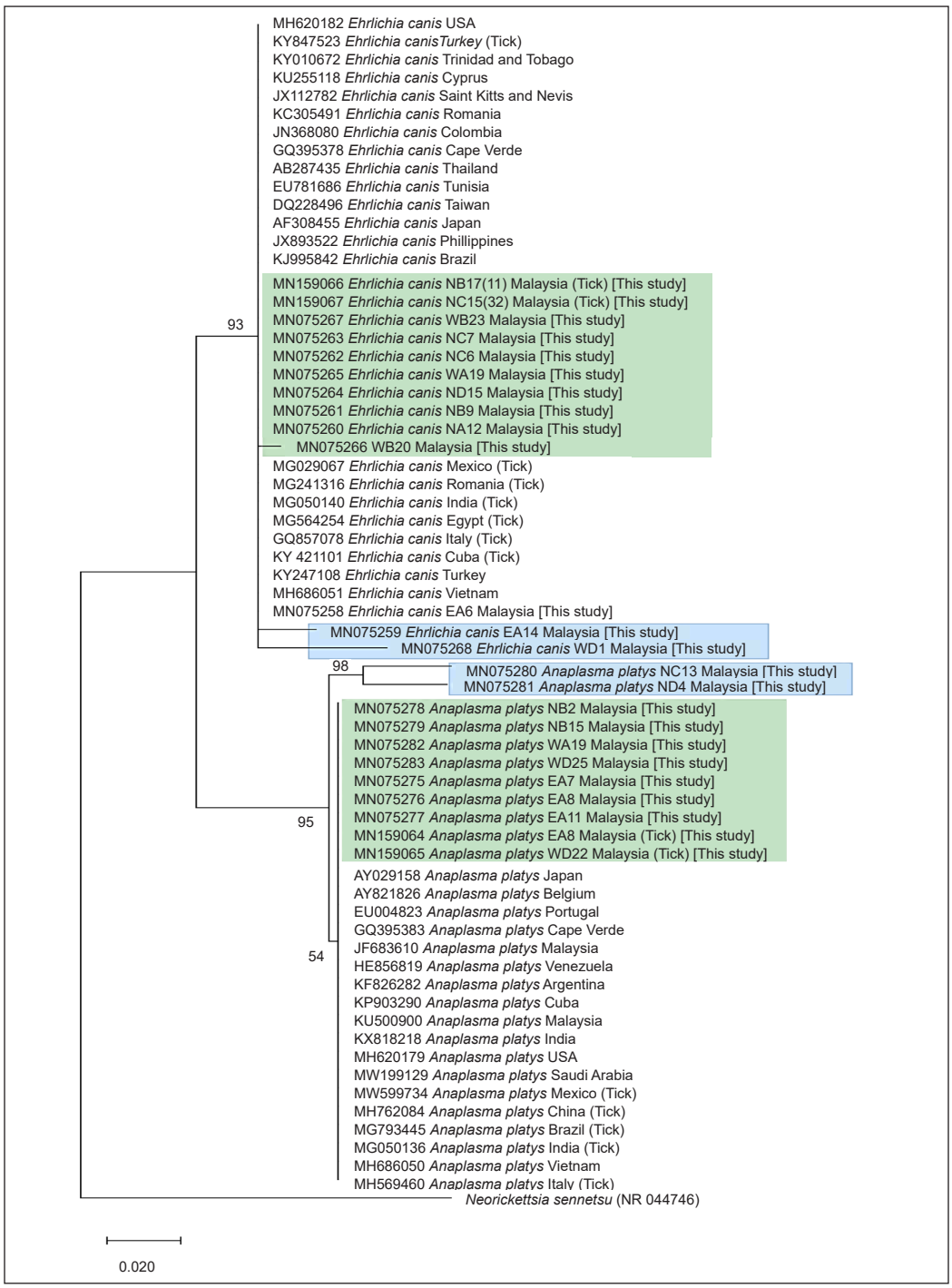

Figure 1. Phylogenetic analysis of *Anaplasma platys* and *Ehrlichia canis* based on the 16S rRNA gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Neorickettsia sennetsu* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

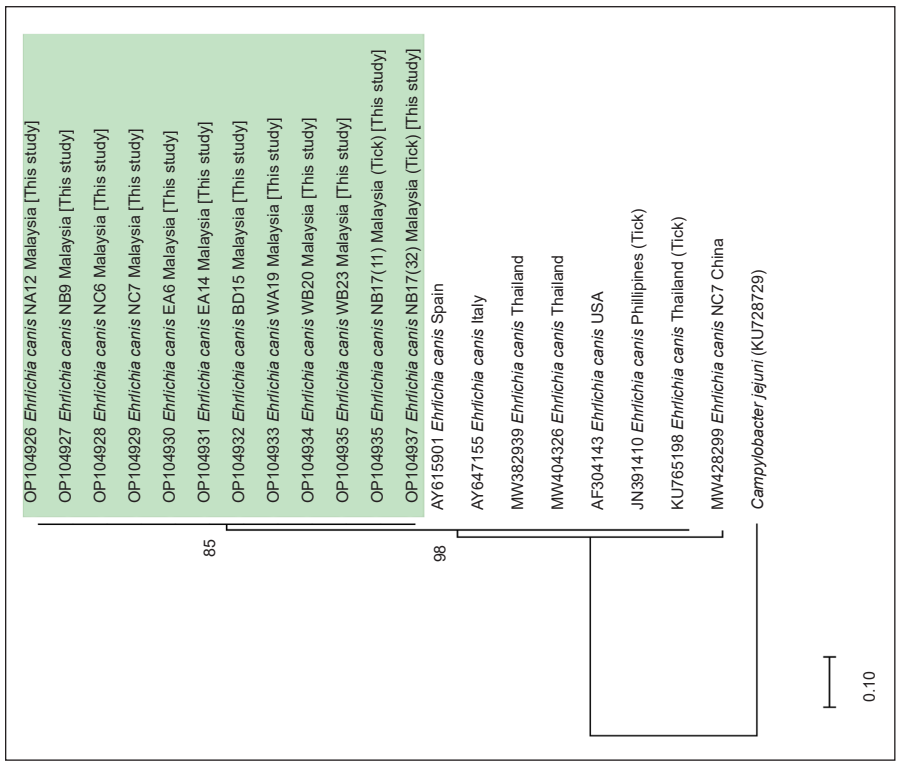

Figure 3. Phylogenetic analysis of *Ehrlichia canis* based on the *glnA* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Campylobacter jejuni* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

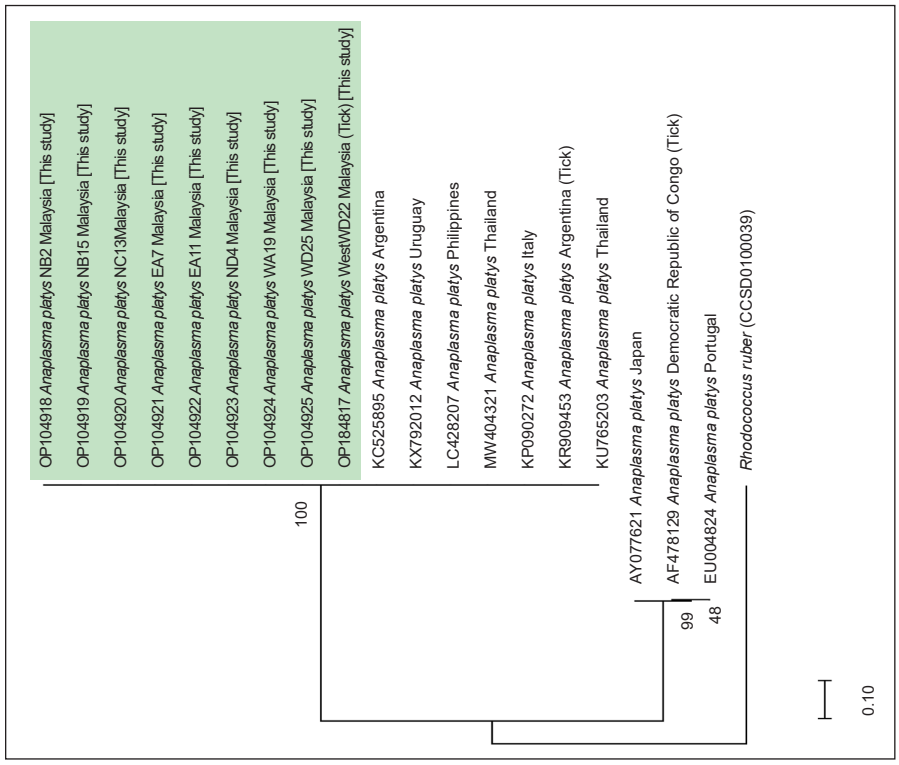

Figure 2. Phylogenetic analysis of *Anaplasma platys* based on the *groESL* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Rhodococcus ruber* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

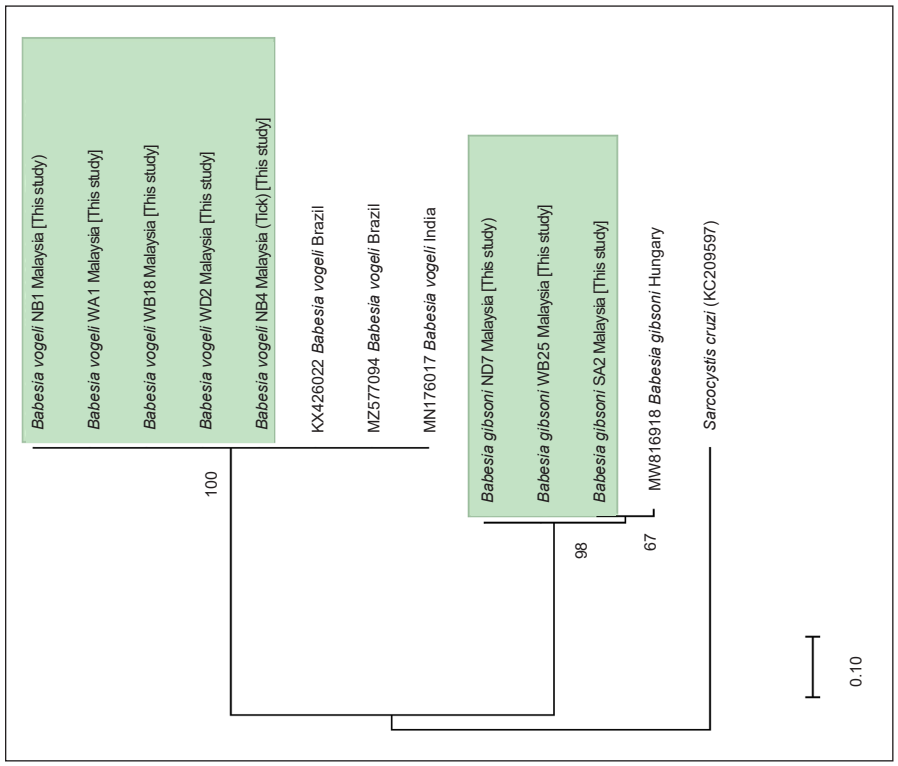

Figure 5. Phylogenetic analysis of *Babesia vogeli* and *Babesia gibsoni* based on the *COX1* gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Sarcocystis cruzi* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

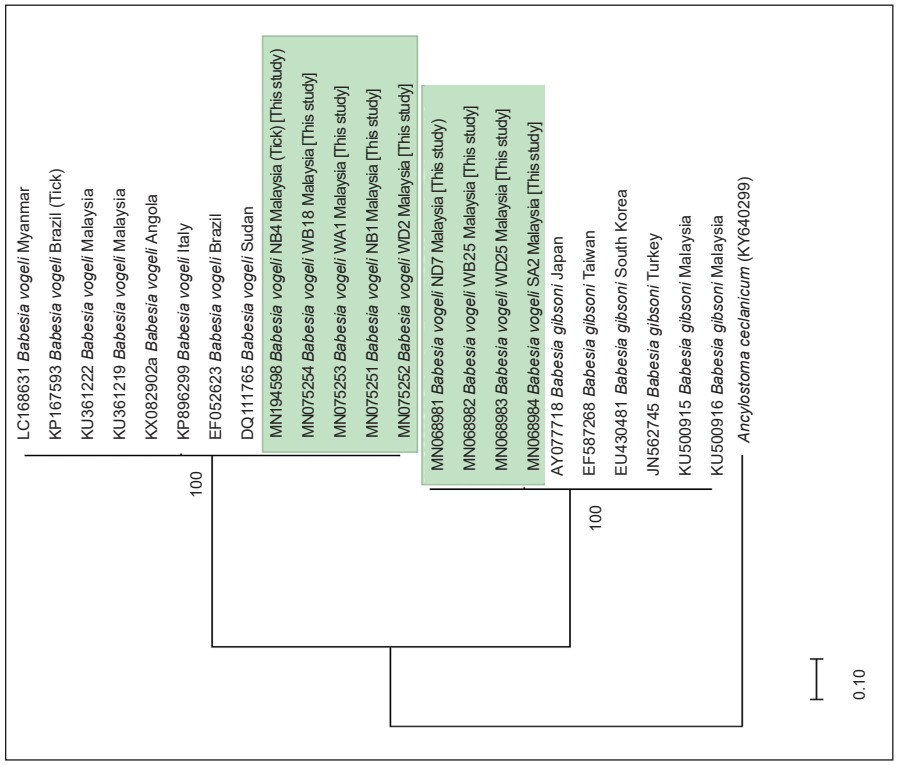

Figure 4. Phylogenetic analysis of *Babesia vogeli* and *Babesia gibsoni* based on the 18S rRNA gene obtained in this study and other countries using the Maximum Likelihood method (Kimura-two-parameter model). *Ancylostoma ceylanicum* is provided as an outgroup species. MEGA X software with 1,000 bootstrap replications was used to create the phylogenetic tree

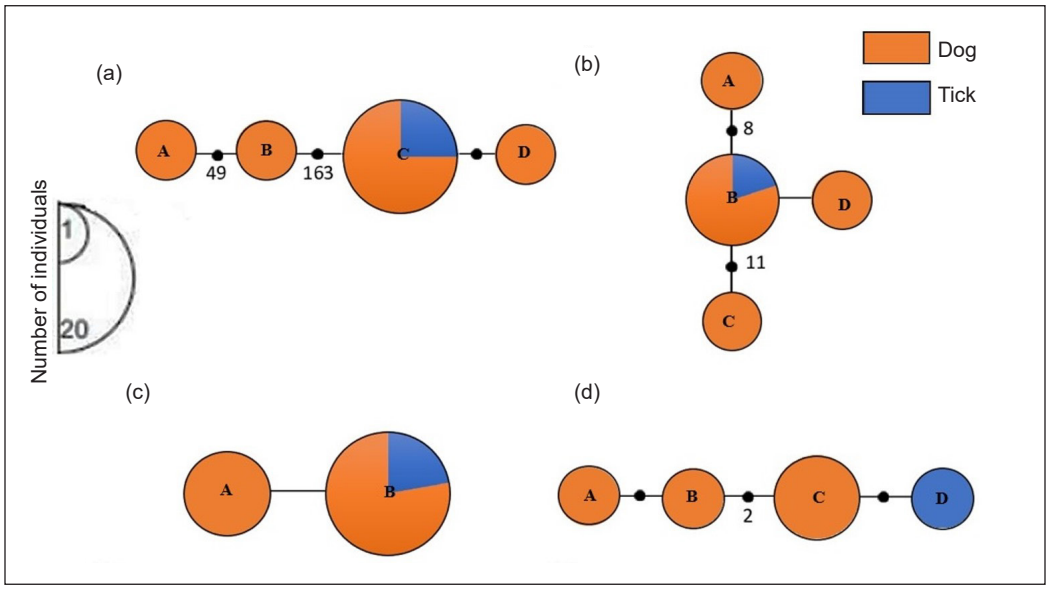

Figure 6. Haplotype network of *Anaplasma platys* 16S rRNA (a), *Ehrlichia canis* 16S rRNA (b), *Ehrlichia canis gltA* (c), and *Babesia vogeli* 18S rRNA (d) sequences. The size of the circles in the network is proportional to the frequency of the haplotype in the data set, and the coloured area identifies the sample collected

Table 5
Haplotype (h) and nucleotide diversities (π) based on the gene of interest of Anaplasma platys*,* Ehrlichia canis*,* Babesia vogeli*, and* Babesia gibsoni

Populations	Individuals	Haplotypes	Polymorphic sites	Haplotype diversity	Nucleotide diversity
Anaplasma platys 16S rRNA					
Blood	9	3	6	0.417	0.006
Tick	2	1	0	0.000	0.000
Ehrlichia canis 16S rRNA					
Blood	11	4	17	0.491	0.010
Tick	2	1	0	0.000	0.000
Ehrlichia canis gltA					
Blood	10	2	1	0.467	0.002
Tick	2	1	0	0.000	0.000
Babesia vogeli 18S rRNA					
Blood	4	2	1	0.667	0.002
Tick	-	-	-	-	-
Babesia gibsoni 18S rRNA					
Blood	4	2	10	0.500	0.008
Tick	-	-	-	-	-
Babesia gibsoni COX1					
Blood	3	2	2	0.667	0.013
Tick	-	-	-	-	-

borne haemoparasites in Malaysia were analysed in this study. This study revealed more variable genetic diversity in dogs as a host compared to the tick vector. Overlap was observed in ticks' and dogs' haplotypes except for the 18S rRNA gene of *B. vogeli*. However, the present study was unable to trace the origin of infection, most likely due to a low positive number of tick samples, overlapping of haplotypes between dogs and ticks, and the lack of clade separation according to regions in the phylogenetic tree analysis.

The low diversity in tick sequences and the more variations in dog sequences could be due to the movement of dogs and ticks as the carriers of VBHs. In terms of the tick vector, the haplotype in ticks has a low diversity due to the restricted movement to allow the genetic mixing or admixture event to occur. On the other hand, dogs can move in a broader range of geographical areas; thus, dogs are more likely to spread genetic variation, resulting in better haplotype diversity. It might explain why more haplotypes were observed in dogs than in ticks, as shown in the haplotype networks. The movement of stray dogs before being brought to the shelter might contribute to spreading ticks and haemoparasites. The role of dogs as long-distance hosts and carriers of the infection, as well as the capability of the tick vectors to remain attached to their host during travelling, might lead to the introduction of the same infection in other regions of the country. These events may contribute to the presence of the same haplotype in different regions observed in this study. Moreover, some of the sampled shelters accepting dogs from other regions of the country could also contribute to the haplotype sharing between sequences obtained from different regions. Another theory that might play a role in the dispersal of pathogens from one region to another is the presence of a variety of hosts available for tick infestation. Although primarily infesting dogs, *R. sanguineus* sensu lato, now known as *R. linnaei* (Šlapeta et al., 2021), could also infest a wide range of hosts such as mammals, birds, and humans (Dantas-Torres, 2010). The mobility of these hosts throughout the country might be considered one of the factors influencing the genetic dispersal and transmission of the infection among dogs and ticks in different regions, which can also explain the different haplotypes observed in the same shelter.

The haplotype networks obtained for the 16S rRNA gene of *A. platys* (Figure 6a), 16S rRNA gene of *E. canis* (Figure 6b), and the *gltA* gene of *E. canis* (Figure 6c) revealed that the tick isolates were sharing the same haplotype with some of the blood isolates. The most likely explanation was that the infection in both dogs and ticks originated from the same source, which could be either the tick vector collected, different ticks feeding on the same infected dogs, or the movement mentioned earlier. On the other hand, the tick sampled for the 18S rRNA gene of *B. vogeli* (Figure 6d) showed a separate haplotype from the rest of the blood samples. It might indicate that the infection in ticks does not share the same origin with all the blood samples

collected. A haplotype network was not presented for the *groESL* gene of *A. platys* since the sequence alignment result was monomorphic. Likewise, the haplotype network was not presented for the *COX1* gene of *B. vogeli* since only one tick sample was found positive; thus, the haplotype and nucleotide diversity for tick samples could not be generated.

The 16S rRNA gene of *E. canis* reflected a slightly higher haplotype (0.491) and nucleotide (0.00954) diversities compared to the *gltA* gene of *E. canis*. This result was proportional to the haplotype network for both genes as the 16S rRNA gene of *E. canis* depicted the presence of four haplotypes, whereas only two haplotypes were observed for the *gltA* gene of *E. canis*. The *gltA* or citrate synthase gene encrypts an important enzyme in the tricarboxylic acid cycle, which is present in almost all living cells and acts as an important regulator for intracellular adenosine triphosphate (ATP) production (Wiegarg & Remington, 1986). These results indicated that the *gltA* gene was more conserved compared to the 16S rRNA gene in contrast to a previous study (Inokuma et al., 2001). The *gltA* gene was different among *Ehrlichia* spp. sequences compared to the 16S rRNA gene. Although the differences were small, the result suggests that the *gltA* could be a tool for advanced molecular work and suitable for comparing closely related species but not within species (Inokuma et al., 2001).

Although *E. canis* is widely distributed globally and has been identified for a long time, little is known about its genetic diversity and strains from geographically distant regions (Hsieh et al., 2010). Recent studies have focused on 16S rRNA genes for molecular work, while there is a dearth of information on other genes (Alhassan et al., 2021; Çelik et al., 2021; Low et al., 2018; Malik et al., 2018; Selim et al., 2021; Sipin et al., 2020). The 16S rRNA gene sequences of *E. canis* have high similarity despite originating from South America, North America, Asia, Europe, Africa, and the Middle East. The similar characteristic of the gene recommends significantly conserved and limited information on the gene diversity. Some other genes that were found to be conserved among isolates worldwide include the OMP-1 family, Dsb, TRP19, and TRP140 (Aguiar et al., 2008; Aguirre et al., 2004; C.-C. Huang et al., 2010; Kamani, Lee, et al., 2013; Yu et al., 2007; Zhang et al., 2008). Two main antibody epitopes: TRP36 and gp36, in the tandem repeat region, were considered to vary among *E. canis* isolates. The studies of the *trp36* gene in *E. canis* strains from the United States, Brazil (only a single strain), Cameroon, Nigeria, and Taiwan *E. canis* identified variations in the number of tandem repeats while a divergent *E. canis* genotype has been identified in Israel (Zhang et al., 2008). A phylogenetic tree of *E. canis* strains based on the gp36 amino acid sequences revealed that the Taiwanese isolates fell into a separate clade, indicating the presence of a novel strain that is yet to be characterised (Hsieh et al., 2010).

Anaplasma platys isolates in the current study presented four haplotypes using the 16S rRNA gene, and only one haplotype was observed in the *groESL* gene (haplotype

network not presented). These results were similar to previous studies reporting low variation in both genes in the sequences of *A. platys* strains (De La Fuente et al., 2006; H. Huang et al., 2005; Martin et al., 2005; Unver et al., 2003). The 16S rRNA and *groESL* sequences may be useful for phylogenetic studies of *Anaplasma* species (Inokuma et al., 2002); however, they are disadvantageous for within-species variation and strain differentiation (De La Fuente et al., 2006). The low genetic variation of *A. platys* compared to other *Anaplasma* species might be due to the limited movement of infected dogs compared to infected cattle in *Anaplasma marginale* and/or the narrow host range of *Anaplasma ovis* in comparison to *Anaplasma phagocytophilum* in humans (De La Fuente et al., 2005).

For the genes targeted for the detection of *B. gibsoni*, the *COX1* dataset displayed higher haplotype (0.667) and nucleotide diversities (0.01258) compared to the 18S rRNA gene of *B. gibsoni*. It is expected since the *COX1* gene was considered to vary significantly compared to the 18S rRNA gene (Bilgic et al., 2010; Haanshuus et al., 2013; Isozumi et al., 2015). Small canine piroplasms, originally known as *B. gibsoni*, were found to be divided into three genetically distinct species with similar morphology and clinical signs. *Babesia conradae*, described in dogs in the western United States, was previously thought of as *B. gibsoni* (Conrad et al., 1991; Kjemtrup et al., 2006; Ionita et al., 2012). However, only *B. gibsoni* and *B. vogeli* infections were observed in the present work.

In this study, the 18S rRNA gene for *B. vogeli* revealed the presence of three haplotypes in dogs and one in ticks, while *COX1* displayed 100% similarity in all sequences in dogs and ticks (haplotype network not presented). In 18S rRNA, the haplotype in a tick did not overlap with any of the sequences in dogs. It is most likely that the tick was newly introduced into the study area, given that the haplotype in a tick was new and yet to be established in the studied dogs. The present work was in contrast with previous work in Brazil, where only one haplotype was observed from the 18S rRNA gene of *B. vogeli* observed in dogs (Moraes et al., 2015). The researchers concluded that the genotypes observed were the most common in the South American continent (Moraes et al., 2015). In the present study, dogs and ticks shared no common haplotype for the 18S rRNA gene of *B. vogeli*, which corroborates the phylogenetic analysis by reflecting low variation in sequences and non-host-specific population structure.

Analysis of molecular variance (AMOVA) depicted a higher level of variation within populations than among populations (Table 6). It indicates that the infection in ticks used in this study has little to no variation from the infections in dogs, consistent with the haplotype networks. There were no significant differences in genetic variation within the population, although the percentage of variations was high. This result could indicate a low to no genetic differentiation of parasite isolates included in this study but a high gene flow of

Table 6
Analysis of molecular variance (AMOVA) results for the gene of interest of Anaplasma platys*,* Ehrlichia canis*, and* Babesia vogeli *for all samples as a group*

Gene/ Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	FST
Anaplasma platys 16SrRNA					
Among population	1	14.182	- 5.750 Va	- 21.100	- 0.211
Within population	9	297.000	33.000 Vb	121.100	
Total	10	311.182	27.250		
Ehrlichia canis 16S rRNA					
Among population	1	0.336	- 0.460 Va	- 32.110	- 0.094
Within population	11	20.818	1.893 Vb	132.110	
Total	12	21.154	1.433		
Ehrlichia canis gltA					
Among population	1	0.150	- 0.018 Va	- 9.380	- 0.094
Within population	10	2.100	0.210 Vb	109.380	
Total	11	2.250	0.192		
Babesia vogeli 18S rRNA					
Among population	1	1.300	0.083 Va	6.670	0.067
Within population	3	3.500	1.167 Vb	93.330	
Total	4	4.800	1.250		

Note. d.f. = Degrees of freedom; FST = Fixation indices; Va = Among groups variance; Vb = Within groups variance

the vector-borne haemoparasites in the study area (Yin et al., 2013). This high gene flow could stem from the movements of the dog host in the study area, as mentioned earlier. However, the current result could also be due to the small number of parasite isolates used in this study since the genetic diversity could be affected by several factors, including the sample size (Araya-Anchetta et al., 2015). The haplotype networks showed that most parasite isolates were in the same haplotype, with only one parasite isolated in several other haplotypes. In addition, the pairwise differences (pi) demonstrated no significant results statistically (Table 7), indicating little genetic differentiation between the host, which agrees with the phylogenetic analysis.

All the phylogenetic trees generated from this study exhibited no specific pattern or separation between isolates of VBHs in dogs and ticks, as well as region-wise separation for all observed haemoparasites. Two main factors exist for the insufficient separation obtained in the present study. First, the insufficient sample size represents the population where 28 blood isolates and only four tick isolates were used for the characterisation and phylogenetic analyses. The low number of tick isolates was insufficient to represent the population, thus affecting the phylogenetic data analysis results. The low prevalence of VBHs in ticks was also observed in previous studies despite high detection in dogs

Table 7
Pairwise differences measures of haplotype frequencies (pi) based on the gene of interest of Anaplasma platys*,* Ehrlichia canis*,* Babesia vogeli*, and* Babesia gibsoni *sequences below diagonal and above diagonal,* p *values of significant (*=p < *0.05)*

Gene	Sample	Blood	Tick
Anaplasma platys 16SrRNA	Blood	-	0.991
	Tick	- 0.211	-
Ehrlichia canis 16S rRNA	Blood	-	0.991
	Tick	- 0.094	-
Ehrlichia canis gltA	Blood	-	0.991
	Tick	- 0.094	-
Babesia vogeli 18S rRNA	Blood	-	0.991
	Tick	0.067	-

(Chao et al., 2016; Foongladda et al., 2011; Galay et al., 2018; Inokuma et al., 2000; Livanova et al., 2018; Nguyen et al., 2019; Ybañez et al., 2012). Tick DNA isolation is difficult compared to blood due to the thick chitinous exoskeleton and the small amount of microbial nucleic acids usually present in ticks' DNA (Halos et al., 2004). Furthermore, inhibitors can interfere with PCR (Halos et al., 2004; Hill & Gutierrez, 2003; Hubbard et al., 1995). The other possible reason for the low prevalence of tick-borne haemopathogens (TBHs) in ticks might be due to the detachment of ticks infected with TBHs from the dog host before sample collection since most of the tick vectors rest in the environment (Dantas-Torres et al., 2008). The ticks sampled in this study were all attached to dogs during collection.

Second, the most likely reason is the conserved region of the genetic markers used in the present study. The use of *groESL* and *gltA* gene markers for *A. platys* and *E. canis* was due to the absence of previous works on the mtDNA marker for both pathogens. Low variation was observed despite using the *COX1* gene for *Babesia* spp. *COX1* is a mitochondrial gene that has been reported to be of more diversity than the nuclear gene. Mitochondrial genes have benefits in previous studies and have been used to identify variation within populations and cryptic species. Several mtDNA regions have served as relevant markers for genetic, epidemiological, and ecological studies (Hu & Gasser, 2006; Jex et al., 2010). mtDNA is acknowledged as a suitable marker in discovering the possibility of cryptic species when sequence data from small sample sizes were utilised (Blouin, 2002). However, *COX1* is still considered a conserved gene compared to other mtDNA. Other mtDNA genes have a more variable region and were suggested by researchers as useful in exploring variation in parasite populations due to the within-species variation, for example, NADH dehydrogenase subunit 4 (NAD4) and subunit 6 (NAD6) (Lv et al., 2012; Jabbar et al., 2013).

Most of the molecular studies have reported various isolates of the canine VBHs targeting the 18S rRNA or the 16S rRNA gene markers. Both gene markers were suitable and widely used for species identification of the VBHs worldwide (Abd-Rani et al., 2011; Adao et al., 2017; Andersson et al., 2017; Galay et al., 2018; Nazari et al., 2013; Pinyoowong et al., 2008; Yabsley et al., 2008). Nevertheless, the 18S rRNA and 16S rRNA genes are conserved and considered unreliable in distinguishing between strains or subspecies (Aktas et al., 2009; Criado-Fornelio et al., 2003; Tian et al., 2013). Most studies using these target genes reported amplifying fragments with a higher similarity percentage to other strains of that pathogen deposited in GeneBank.

CONCLUSION

A more variable gene marker region should be employed for detailed phylogenetic analysis (e.g., NAD4, NAD6, and mitochondrial DNA). The present study was unable to infer the actual transmission pattern in VBHs among dog and tick hosts, which may be due to the conserved region of the gene of interest and a low number of positive samples, especially from ticks. Future studies are recommended to identify and employ a more variable gene region, as well as a larger sample size, to allow a better resolution to elucidate the transmission patterns of VBHs among dogs and the infecting ticks.

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