# MOLECULAR CHARACTERISATION OF *Rhipicephalus sanguineus* (ACARI: IXODIDAE), AND OCCURRENCE OF PATHOGENS IN TICKS AND HOST DOGS

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KUALA LUMPUR

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**MOLECULAR CHARACTERISATION OF *Rhipicephalus sanguineus* (ACARI: IXODIDAE), AND OCCURRENCE OF PATHOGENS IN TICKS AND HOST DOGS**


## ABSTRACT

The brown dog tick*, Rhipicephalus sanguineus* is one of the ubiquitous ticks in the world and it is a well-recognized vector of many tick-borne diseases affecting dogs and occasionally humans. The taxonomic status of *R. sanguineus* has been long debated, there is a consensus that the populations of this tick species should be referred to as *R. sanguineus* sensu lato (s.l.) until its taxonomic status is resolved. The existence of two divergent lineage, the southern lineage and northern lineage in *R. sanguineus* isolates indicates the genetic variability of *R. sanguineus* s.l. The aims of the study were to molecularly characterize *R. sanguineus* in Malaysia by determining its lineage and detect various pathogens in *R. sanguineus* and host dogs. Phylogenetic analysis indicated that all *R. sanguineus* from Peninsular Malaysia fall into the northern lineage. Low genetic diversity was observed in this isolate as inferred from the cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S) genes. For pathogen detection, tick and dog blood samples were molecularly screened for the presence of pathogenic bacteria *Rickettsia*, *Ehrlichia*, *Anaplasma*, and protozoa *Babesia* and *Hepatozoon*. In the present study, *Rickettsia asembonensis*, *Ehrlichia canis*, *Anaplasma platys*, *Babesia gibsoni*, *Babesia vogeli*, and *Hepatozoon canis* were detected at varying frequencies. The prevalence of *R. asembonensis*, *E. canis*, *A. platys*, *B. gibsoni*, *B. vogeli* and *H. canis* in *R. sanguineus* ticks were as follows, 1.43%, 0.71%, 2.86%, 1.43%, 1.43% and 0.71%, respectively. For the dog blood samples, the prevalence was 12. 92% for *E. canis*, 3.33% for *A. platys*, 2.08% for *B. canis*, and 3.33% for *H. canis*. None of the blood samples were tested positive for *Rickettsia asembonensis*. Results from the present study indicated that a

variety of tick-borne diseases are present in Malaysian dogs and *R. sanguineus* may act as the potential vector in transmitting these diseases.

**Keywords**: *Rhipicephalus sanguineus*, dog, tick-borne pathogens, 16S rRNA gene, COI gene.

**PENCIRIAN MOLEKUL *Rhipicephalus sanguineus* (ACARI: IXODIDAE), DAN KEWUJUDAN PATOGEN DALAM SENGKENIT DAN PERUMAH ANJING**


**ABSTRAK**

Sengkenit anjing perang, *Rhipicephalus sanguineus* merupakan salah satu sengkenit yang terdapat di seluruh dunia dan dikenali sebagai vektor kepada pelbagai jenis penyakit yang menjangkiti anjing dan kadang-kala manusia. Status taksonomi *R. sanguineus* telah lama dibahaskan, menjurus kepada konsensus yang meyatakan bahawa species sengkenit ini harus dirujuk sebagai *R. sanguineus* sensu lato (s.l.) sehingga status taksonominya diselesaikan. Kewujudan dua keturunan yang berbeza: keturunan selatan dan keturunan utara di isolat *R. sanguineus* menunjuk kepelbagian genetik dalam *R. sanguineus* s.l. Matlamat kajian ini adalah untuk mencirikan molekul *R. sanguineus* di Malaysia dengan menentukan keturunannya, serta mengesan pelbagai jenis patogen yang boleh ditemui dalam *R. sanguineus* dan perumah anjing. Analisis filogenetik menunjukan bahawa semua *R. sanguineus* dari Semenanjung Malaysia adalah dari kelompok keturunan utara. Kepelbagaian genetik yang rendah telah diperhatikan dalam isolat ini, seperti yang ditunjukkan daripada analisis *cytochrome oxidase subunit I* (COI) dan *16S ribosomal RNA* (16S). Untuk pengesanan pathogen, sampel darah anjing dan sengkenit telah disiasat secara molekul untuk bakteria *Rickettsia*, *Ehrlichia*, *Anaplasma*, dan protozoa *Babesia* dan *Hepatozoon*. Dalam kajian ini, *Rickettisia asembonensis*, *Ehrlichia canis, Anaplasma platys*, *Babesia gibsoni, Babesia vogeli* dan *Hepatozoon canis* telah dikesan pada frekuensi yang berbeza. Kelaziman *R. asembonensis*, *E. canis*, *A. platys*, *B. gibsoni, B. vogeli* dan *H. canis* dalam *R. sanguineus* adalah seperti berikut 1.70%, 0.71%, 2.86%, 1.43%, 1.43% dan 0.71%. Untuk sampel darah anjing, kelaziman adalah 12.92% bagi *E. canis,* 3.33% untuk *A. platys,* 2.08% untuk *B. vogeli* dan 3.33% untuk *H. canis.* Tiada sampel darah diuji positif untuk *Rickettsia asembonensis*. Keputusan dari kajian ini menunjukkan bahawa pelbagai jenis penyakit bawaan sengkenit terdapat dalam anjing

Malaysia dan *R. sanguineus* mempunyai potensi untuk bertindak sebagai vektor dalam penyebaran penyakit tersebut.

**Kata Kunci**: *Rhipicehalus sanguineus*, anjing, patogen bawaan sengkenit, gen 16S rRNA, gen COI.

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| | | |
|---|---|---|
| & | : | And |
| < | : | Less than |
| > | : | More than |
| % | : | Percentage |
| BSF | : | Brazilian spotted fever |
| COI | : | Cytochrome c oxidase I |
| DNA | : | Deoxyribonucleic acid |
| EDTA | : | Ethylenediaminetetraacetic acid |
| et al. | : | et alia (“and others”) |
| gltA | : | Citrate synthase |
| ITS2 | : | Internal transcribed spacer 2 |
| NCBI | : | National Center for Biotechnology Information |
| n.d | : | Not determined |
| NJ | : | Neighbour-joining |
| OTU | : | Operational Taxonomic Unit |
| PAUP | : | Phylogenetic Analysis Using Parsimony |
| PBS | : | Phosphate-buffered saline |
| PCR | : | Polymerase Chain Reaction |
| RMSF | : | Rocky Mountain Spotted Fever |
| s.l | : | Sensu lato |
| spp. | : | Several species |
| s.s | : | Sensu stricto |

# CHAPTER 1: INTRODUCTION

## 1.1 Scope of study

*Rhipicephalus sanguineus* (Ixodidae) was first described in 1806 by Latrielle as *Ixodes sanguineus*. However, it was poorly described, and the description was acceptable for that time because the tick taxonomy was still new. The tick was later reclassified into the genus *Rhipicephalus* by Koch (Dantas-Torres et al., 2013). It is also known as the brown dog tick and it is well distributed around the globe (Dantas-Torres, 2008). Dogs are the most common host for *R. sanguineus*, however, this brown dog tick also parasitizes other hosts such as cattle, goats, horses, cats and human. The infestation of this tick causes considerable public health problems (Liu et al., 2013).

Rickettsial diseases in dogs such as anaplasmosis and ehrlichiosis are of substantial concern worldwide. Canine anaplasmosis is caused by *Anaplasma phagocytophilum* or *A. platys* which infects dogs worldwide (Sainz et al., 2015). Particularly, *A. phagocytophilum* is of zoonotic importance because it is well-known for causing human granulocytic anaplasmosis transmitted by several tick species including *Haemaphysalis*, *Ixodes* and *Rhipicephalus* (Brites-Neto et al., 2015). Canine ehrlichiosis, on the other hand, is primarily caused by *Ehrlichia canis* and vectored by the brown dog tick *R. sanguineus* (Skotarczak, 2003). *Ehrlichia chafeensis* and *Ehrlichia ewingii* have also been reported in dogs (Beall et al., 2012) and both are emerging zoonoses that cause human monocytotropic ehrlichiosis and human ehrlichiosis ewingii, respectively (Ismail et al., 2010). Infections of *Rickettsia* spp. in dogs and *R. sanguineus* have been little studied.

Canine babesiosis is an emerging infectious tick-borne disease with a worldwide distribution. *Babesia* infection in dogs was described for the first time in 1895 (Roncalli, 2001). Subsequently, with the advent of molecular techniques, several species of *Babesia*

including *Babesia canis*, *Babesia gibsoni*, *Babesia rossi* and *Babesia vogeli* have been found to infect dogs (Solano-Gallego et al., 2016). Although human infection with *B. canis* has been reported (Homer et al., 2000), in the context of zoonotic transmission, the potential of canine *Babesia* spp. to infect human remains uncertain. As far as the vectorial capacity is concerned, *R. sanguineus* is primarily known as a vector of *B. canis* (Dantas-Torres 2008),

Canine hepatozoonosis is a common infectious protozoan disease in dogs caused by *Hepatozoon canis* and *Hepatozoon americanum. Hepatozoon canis* is particularly prevalent in Asia, Africa and southern Europe whereas *H. americanum* is commonly reported in the Central America and northern South America (Baneth et al., 2000; Chhabra et al., 2013). While several studies have incriminated *R. sanguineus* as the main vector of *H. canis* (Baneth et al., 2007; Aktas & Özübek, 2017), a study from Brazil suggested that *R. sanguineus* may not be the vector or has little importance in the transmission of *H. canis* (Demoner et al., 2013).

## 1.2 Problem statement

Depending on factors such as temperature, humidity and host availability, the populations of *R. sanguineus* completely vary from region to region (Dantas-Torres, 2010), which is indeed a challenge for its taxonomic classification. The taxonomic status of *R. sanguineus* has long been debated among taxonomists around the world. *Rhipicephalus sanguineus* sensu stricto (s.s.) has been superficially described, and the unavailability of its type specimen making the situation worse. Currently, at least four operational taxonomic units (OTUs) have been reported in *Rhipicephalus* ticks from dog, some of which may represent species that have been described and placed in synonym with *R. sanguineus* s.s. or new species (Dantas-Torres et al., 2013). Nevertheless, there is

no consensus about the range of morphological variability within the species (Nava et al., 2015), further complicating the taxonomic assessment of *R. sanguineus*.

The DNA barcoding cytochrome c oxidase subunit I (COI) gene has been relatively little studied in *R. sanguineus* (Dantas-Torres et al., 2013; Almeida et al., 2017; Hornok et al., 2017), despite its value in discovering novel lineages in other species of *Rhipicephalus* (Burger et al., 2014; Low et al., 2015). One of the possible reasons for limited use can be the difficulty in amplifying the fragment of the COI gene, a common observation in the family Ixodidae (Lv et al., 2014b; Low et al., 2015; Ernieenor et al., 2017). Nevertheless, based on the data from these three mitochondrial genes, *R. sanguineus* represents three main divergent lineages: the southern lineage (temperate species), the northern lineage (tropical species), and a recently discovered the southeast European lineage. The lineage of Malaysian population, however, remains unknown.

There is a paucity of information on the role of *R. sanguineus* in Malaysia in transmitting tick-borne bacteria and protozoa in dogs, despite the infectious agents such as *Anaplasma* spp. and *Ehrlichia* spp. (Mokhtar et al., 2013; Nazari et al., 2013; Koh et al., 2016; Konto et al., 2017), *Babesia* spp. (Rajamanickam et al., 1985; Zulkifli et al., 2011; Mokhtar et al., 2013; Mohammed et al., 2017 and *H. canis* (Rajamanickam et al., 1985, Mohammed et al., 2017) have been reported in dogs at varying frequencies. Nevertheless, co-infections of these pathogens in ticks and dogs have not been fully understood.

## 1.3 Significance of study

Identification of genetically distinct species is of paramount importance in disease control strategies because different populations or lineages of vectors may present different susceptibility levels toward pathogen infections. There are at least four operational taxonomic units (OTUs) reported in *Rhipicephalus* ticks from dogs worldwide. The present study identified the lineage of *R. sanguineus*, for the first time in Malaysia.

Identification of tick-borne pathogens, especially co-infection in dogs is important for a prompt treatment because some of the clinical signs of infection might be overlapped and the treatments could be different, especially in the case of infection with both bacteria and protozoa. The detection of these pathogens will aid in the design of appropriate management strategies for the control of tick-borne diseases in Malaysia. Further, identification of the potential vectors in the disease transmission could be advantageous in developing effective tick control programs.

## 1.4 Aims and objectives

The main purposes of this study were to investigate the genetic lineage of *R. sanguineus* and detect different tick-borne pathogens in dogs and *R. sanguineus* in Peninsular Malaysia. Herein, the present study was carried out to address the following specific objectives:

1. To develop a pair of primers to solve the issue in the recovery of the COI gene fragment of *R. sanguineus*, at least in the setting of Malaysian samples.
2. To identify the genetic lineage of *R. sanguineus* in Malaysia based on the 16S and COI genetic markers.
3. To detect different tick-borne pathogens such as the bacteria *Ehrlichia* spp., *Anaplasma* spp. and *Rickettsia* spp., and the protozoa *Babesia* spp. and *Hepatozoon* spp. in both *R. sanguineus* and dogs.
4. To determine the co-infection patterns of tick-borne pathogens in both *R. sanguineus* and dogs.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Introduction to *R. sanguineus*

*R. sanguineus*, also known as the brown dog tick, is one of the ubiquitous ticks in the world and vectors to several pathogens affecting dogs and occasionally humans (Dantas-Torres, 2010). *Rhipicephalus* ticks fall under hard tick group, Ixodidae. The nomenclature of *R. sanguineus* is described as below:

Kingdom: Animalia

Phylum: Athropoda

Class: Arachnida

Order: Ixodida

Family: Ixodidae

Genus: *Rhipicephalus*

Species: *Rhipicephalus sanguineus*

*R. sanguineus* ticks are distributed worldwide and commonly found in the tropical and subtropical regions. It is classified as endophilic tick because it can adapt well in indoor living. Nevertheless, *R. sanguineus* is also able to survive in outdoor environments by the means of refuge (Gray et al., 2013). It is known to be monotrophic because all its developmental stages (larva, nymph and adult), feed on the same host species (Dantas-Torres, 2010).

However, *R. sanguineus* has been reported to feed occasionally on different hosts such as humans which are not its natural trophic chain. A previous study has examined

the weather impact associated with its attack on human, and the results showed an increase of tick attachment to human skin after exposure to different temperature, indicating that the *R. sanguineus* ticks can deploy different stratagem for its survival. (Parola et al., 2008; Dantas-Torres, 2010).

## 2.2 Taxonomic status of *R. sanguineus*

The taxonomic status of *R. sanguineus* is an ongoing process of debate, mainly because there is bona fide morphological description (Dantas-torres et al., 2017). Scientific works have been conducted on *R. sanguineus* for more than a century, there is no solid description nor affirmation about the range of morphological variability within the species. Several species in *R. sanguineus* group can be discriminated by studying the genital aperture (Gray et al., 2013). Currently, at least four operational taxonomic units (OTUs) have been reported in *Rhipicephalus* ticks from dog, some of which may represent species that have been described and placed in synonym with *R. sanguineus* sensu stricto (s.s.) or new species (Dantas-Torres et al., 2013). Therefore, there is a consensus that the population of this tick species should be referred as *R. sanguineus* sensu lato (s.l) until its taxonomic status is resolved (Nava et al., 2015).

## 2.3 Morphology of *R. sanguineus*

*R. sanguineus* has four pairs of legs which are slender and has no rings. The coxa I on *R. sanguineus* has large, distinct internal and external spur. Coxa II to IV anterior spur are small and are not visible dorsally. The basis capituli of *Rhipicephalus* has a hexagonal shape (Figure 2.3). The palp is wider than long, and the palp article are all small (Figure 2.1; Figure 2.4). Spiracular plates are comma shaped, large and posterior to the legs (Figure 2.3). The anal groove is posterior to the anus (Figure 2.3). The eye of *R. sanguineus* is slightly convex (Figure 2.2). Columns of teeth on hypostome are 3+3. The genital aperture is located between in the middle between coxa I and coxa II (Figure 2.1; Figure 2.3) (Anastos, 1950; Barker & Walker, 2014).

The caudal process of the male tick is protruded when fed (Figure 2.1). The punctuations on conscutum are conspicuous. The lateral grooves and festoon are both present in adult male and female R. sanguineus ticks (Figure 2.2; Figure 2.4). Meanwhile the adanal shield, accessory adanal shield are only found in adult male *R. sanguineus* (Figure 2.1). Size of a male adult *R. sanguineus* may range from 3.0 mm to 3.8 mm. (Barker & Walker, 2014; Anastos, 1950). A female *R. sanguineus* has a pale scutum instead of conscutum as male (Figure 2.4). Porose areas are circular and are broadly separated (Figure 2.4). An unfed female *R. sanguineus* can range from 3.0 mm to 4.5 mm while engorged or fully fed female may measure up to 12 mm in length (Anastos, 1950; Barker & Walker, 2014).

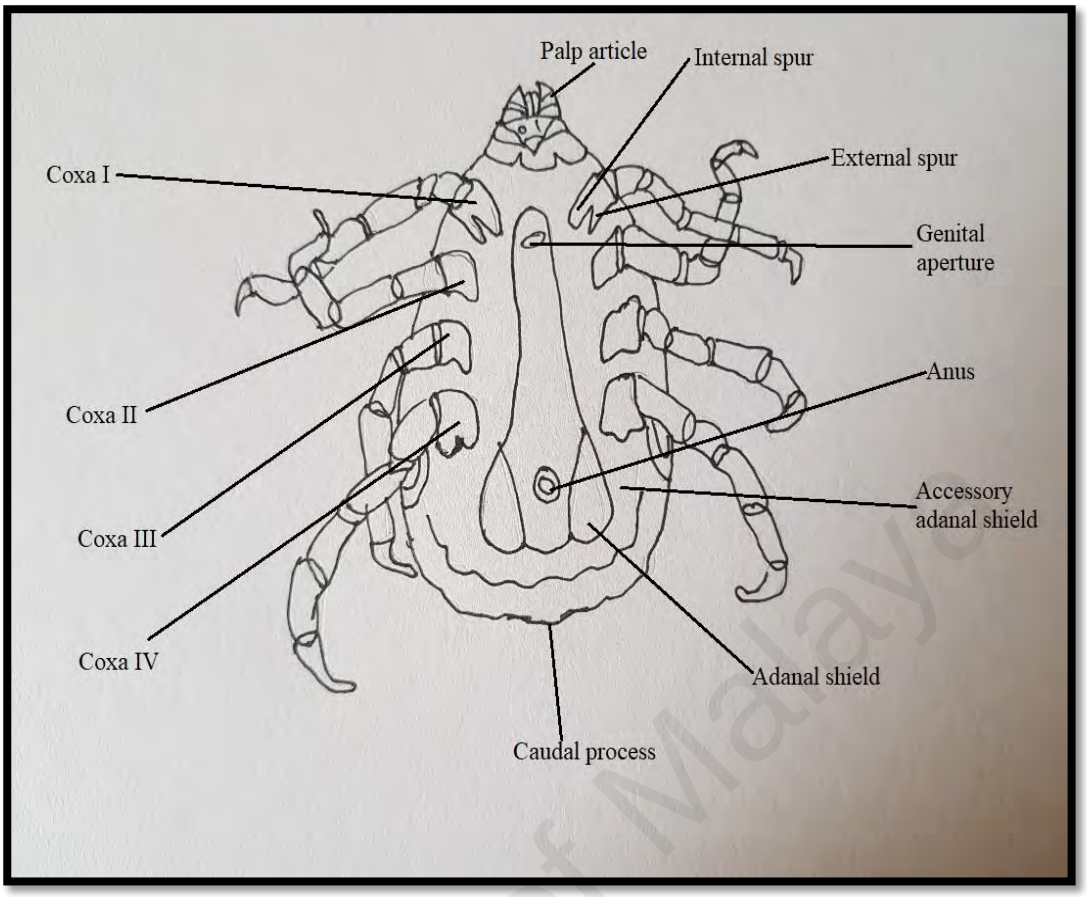


**Figure 2.1:** Ventral view of adult male *R. sanguineus*

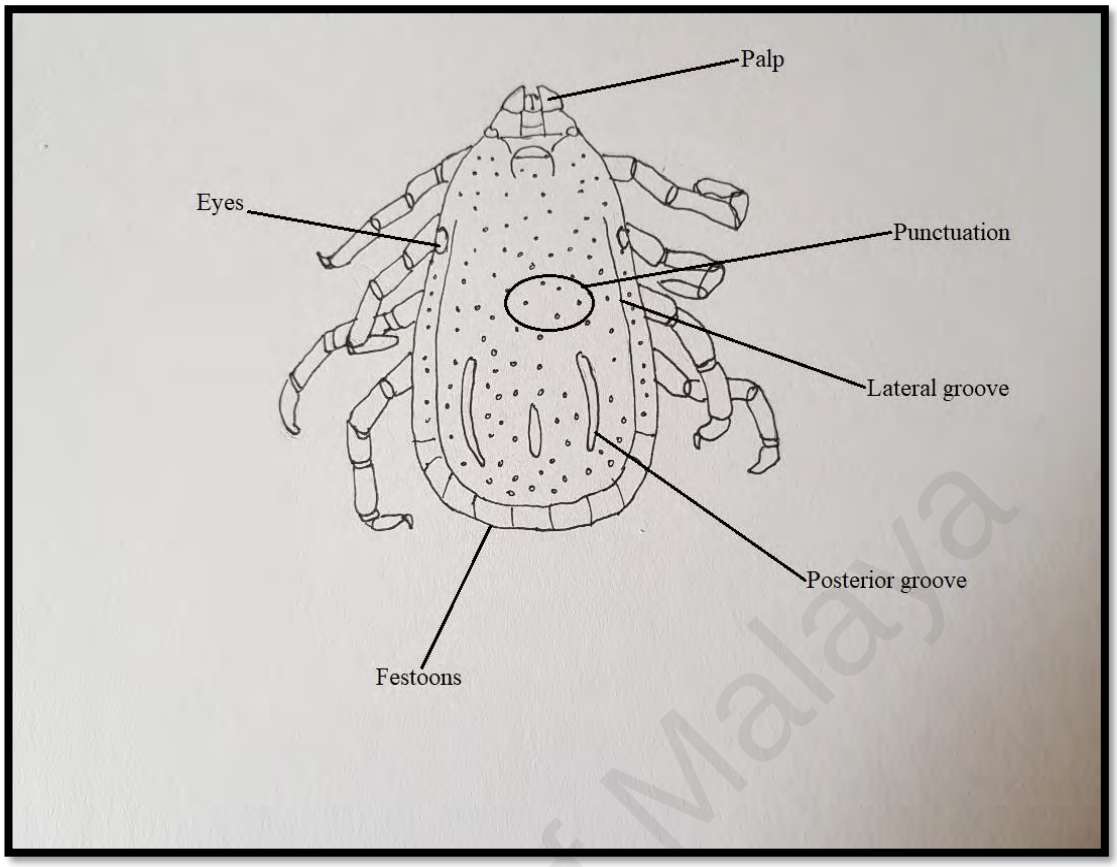


**Figure 2.2:** Dorsal view of adult male *R. sanguineus*

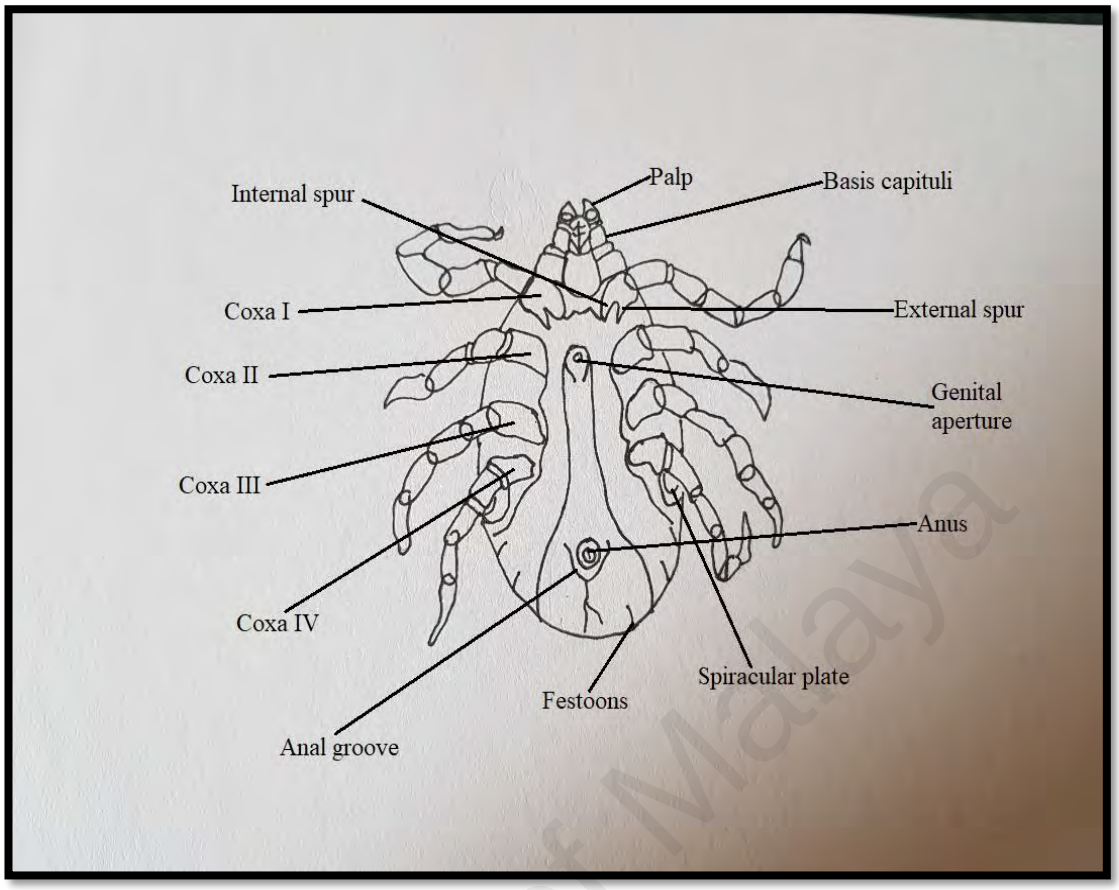


**Figure 2.3:** Ventral view of adult female *R. sanguineus*.

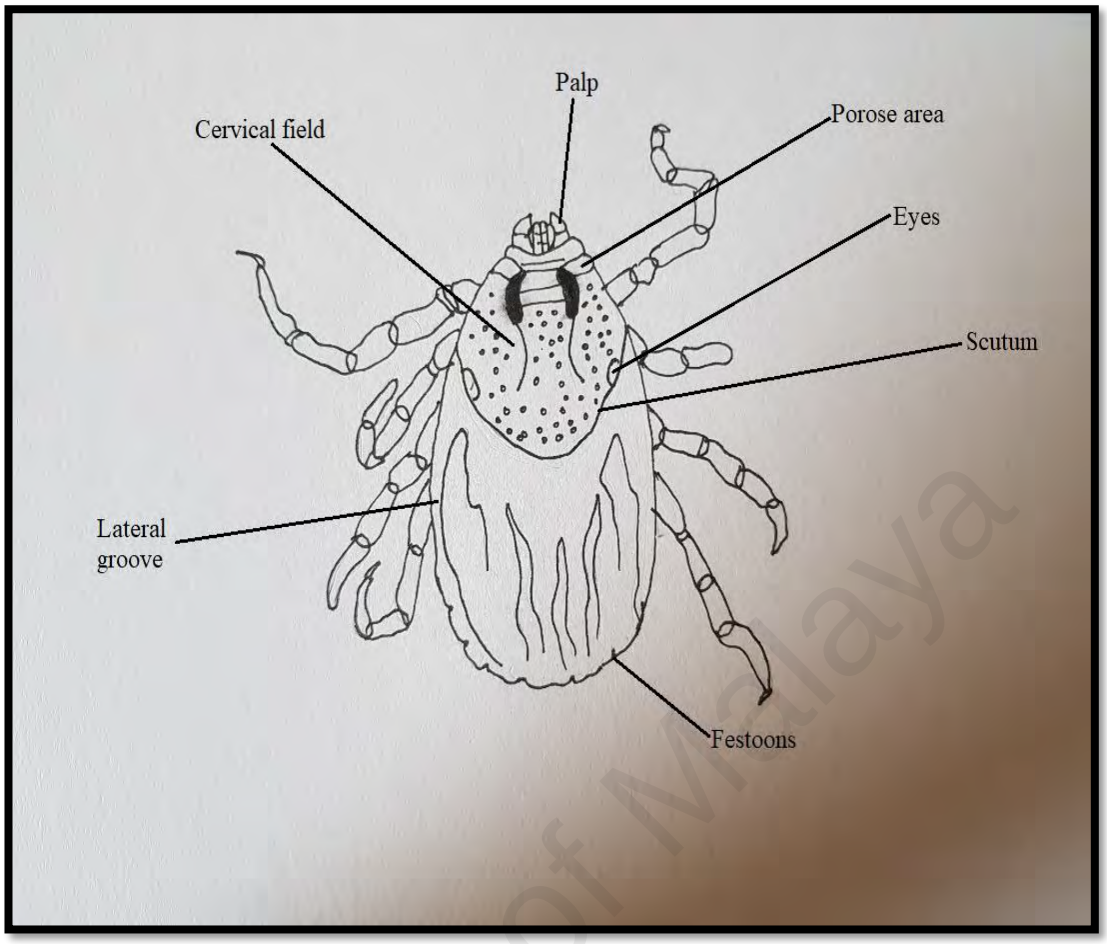


**Figure 2.4:** Dorsal view of adult female *R. sanguineus*

## 2.4 Developmental and life stages of *R. sanguineus*

The life cycle of *R. sanguineus* or brown dog tick, is identical to other tick species in the family Ixodidae. It is a three-host ticks which drop off from different host after the blood meal before each of their developmental stage. The life cycle of *R. sanguineus* takes part after the feeding of the female tick. Approximately seven days after continuously feeding on the host, the female *R. sanguineus* will become fully engorged and dropped off from the host. Egg-laying begins about three days, and the female tick will deposit as about 4000 to 5000 eggs in crevices or cracks inside or outside the house. The larvae will hatch from the eggs within three to five weeks. Once on the host, the six-legged larva feeds for five to fifteen days and detaches from host. The molting process will occur about one week to two weeks after blood meal and develops into nymphs. The nymphs will quest for the host and blood feed for three to thirteen days, and after detaching from the host, it takes about two weeks to mol and develops into adult stage. Both larval and nymphal stages can survive up to six to nine months without a blood meal, while the adult *R. sanguineus* can survive for eighteen months without feeding (Tick Encounter Resource Center. n.d). Depending on different region, the number of generations completed in a year varies up to three to four generations (Silveira et al., 2009)

## 2.5 On-host ecology of *R. sanguineus*

*R. sanguineus* ticks can infest a wide range of hosts, whether it is a domestic or a wild host from cats, rodents, birds and humans. However, the main host of *R. sanguineus* is dogs. The most frequent attachment sites of adult ticks are the ears, interdigital areas and armpit of the dogs (Lorusso et al., 2010). Parasitism on host is mainly affected by two factors, whether the environment is highly infested or presence of heavily infested dogs. Human parasitism by *R. sanguineus* varies according to the

tick population, for example in Europe, human parasitism is common especially during summer while in Brazil, it is less common. However, the like hood of host other than dogs attacked by *R. sanguineus* depends on the tick population in area. Other factors that affect the infestation of *R. sanguineus* is the use of treatments such as ectoparasiticides or tick repellents in the dog population. Prevalence of *R. sanguineus* infestation on dogs are at high level in endemic area of Southern Italy and North-eastern Thailand (Nithikathkul et al., 2005; Lorusso et al., 2010; Otranto et al., 2012). Tick resistance on dogs are measured by comparing various biological parameters such as weight of engorged female ticks, ticks yield, and egg production efficiency which contributes to indirect or direct evidence of tick resistance in dogs. The breed of the dog may also play a factor in the susceptibility from *R. sanguineus* infestation (Louly et al., 2009; Louly et al., 2010)

### 2.6 Off-host ecology of *R. sanguineus*

The three-host tick, *R. sanguineus* is commonly found in the environment instead on their host under the influence of its biotic and abiotic factors (Randolph, 2004). The nymphal and adult stages of *R. sanguineus* have a nocturnal drop off rhythm meanwhile the *R. sanguineus* larvae have a diurnal drop off periodicity (Dantas-Torres, 2008). *Rhipicephalus sanguineus* exhibits endophilic behavior, which it is mostly found indoor while most of the ixodid ticks are exophilic, found outdoor. *R. sanguineus* has been seen climbing walls and curtains of infested house, and several reports found *R. sanguineus* ticks crawling on sofa, furniture and walls (Dantas-Torres et al., 2006; Demma et al., 2005). In warmer zone, *R. sanguineus* may be active all year-round while in temperate zones *R. sanguineus* is active from late spring to early fall in different regions of the world. In France, the, higher number of adult *R. sanguineus* was observed in the summer while in the spring, higher number of immature *R. sanguineus* was observed (Dantas-Torres, 2010). In Italy, all stages of *R. sanguineus* are found during the spring and

summer meanwhile in Mexico, a higher prevalence of *R. sanguineus* was observed during summer, spring and fall compared to winter season (Cruz-Vasquez & Garcia-vasquez, 1999). In India, a high peak was observed from March to September (Das & Bhatia, 1994). In Greece, adults *R. sanguineus* were active on both spring and summer (Papadopoulos et al., 1996). In tropical areas, *R. sanguineus* may be less observed since they are often found inhabiting indoor areas (Dantas-Torres, 2008).

## 2.7 Medical and veterinary importance of *R. sanguineus*

*R. sanguineus* is a potent disease vector and can transmit various diseases to animals and humans. This tick acts as a main vector and alternative vector for various tick-borne pathogens around the world (de la Fuente et al., 2006; Karagenc et al., 2006; Rodriguez-Vivas et al., 2005; Santos et al., 2009). *R. sanguineus* plays role as a vector in the transmission of Rocky Mountain spotted fever in humans (Traeger et al., 2015). *Rhipicephalus sanguineus* is also known to be the main vector of canine hepatozoonosis which is transmitted by ingestion of infected ticks, different from other diseases caused by tick bite and commonly infects dogs in Africa, southern Europe, the Middle East and Asia. Other tick-borne diseases include Babesiosis (*Babesia gibsoni, Babesia canis canis,* and *Babesia canis vogeli*) and Canine Anaplasmosis (*Anaplasma platys*) (Shaw et al., 2001; Dantas-Torres, 2008). *R. sanguineus* is also suspected as a vector of *Leishmania chagasi*, a pathogen responsible for visceral leishmania (Coutinho et al., 2005). *Rhipicephalus turanicus*, is also known to serve as a vector for *E. canis* and *H. canis* (Harrus et al., 2011; Jarquín-Díaz et al., 2016). Hence, specific distinction of species within the *R. sanguineus* group is important to understand the ecology of pathogens, transmission, epidemiology and control of the diseases.

## 2.8 Ehrlichiosis

*Ehrlichia* is an obligate bacterium classified in the family Anaplasmataceae. *Ehrlichia* is a Gram-negative coccobacillus and it is well known for the transmission of a tick-borne disease known as Ehrlichiosis. The genus *Ehrlichi*a includes *Ehrlichia canis, Ehrlichia ewingii*, and *Ehrlichia chaffeensis* which are pathogenic to humans and canines. *E. canis* acts as the primary causative agent of Canine Ehrlichiosis. *E. canis* has been documented in humans as well, where it is not transferred through infected dogs but from its primary vector, *R. sanguinues* (Bowman et al., 2009). The transmission of *E. canis* is transstadial, the ticks may acquire *E. canis* by feeding either in larva or nymph stage, on acutely infected dogs and transmit the infection as nymph or adults (Groves et al., 1975). Male *R. sanguineus* is also able to acquire and transmit *E. canis* without the presence of female *R. sanguineus* (Bremer et al., 2005). Other than *R. sanguineus*, *Dermacentor variabilis* has shown to be able to transmit *E. canis* through transstadial transmission (Johnson et al., 1998). *E. chaffeensis*, the etiological agent of human monocytotropic ehrlichiosis (HME) is an emerging zoonotic pathogen and the primary vector of HME is the lone star tick, *Ambylomma americanum* (Ismail et al., 2010). *A. americanum* also transmits *E. ewingii* and has been confirmed as the vector of the pathogen (Wolf et al., 2000). However, both *E. ewingii* and *E. chaffeensis* have also been detected in *R. sanguineus* (Ndip et al., 2007).

## 2.9 Anaplasmosis

*Anaplasma platys* is a gram negative, obligate, intracellular bacteria which is related to the appearance of inclusion bodies in blood platelets. The probable vector for this bacterium is *R. sanguineus* (Ferreira et al., 2007). Tick vectors for the transmission of *A. platys* have not been fully characterized, despite *Dermacentor auratus* and *Hyalomma truncatum* were suggested as a possible vector for *A. platys* transmission.

Furthermore, there are no experimental transmission with these tick species have been demonstrated (de la Fuente et al., 2006). However, a study in Mongolia has detected the presence of *A. platys* in *Ixodes persulcatus* and *Dermacentor nutalli* (Javkhlan et al., 2014). *Anaplasma phagocytophilum*, another zoonotic tick-borne disease is well-known for causing human granulocytic anaplasmosis transmitted by several tick species including *Haemaphysalis*, *Ixodes*, and *Rhipicephalus* (Holden et al., 2003; Brites-Neto et al., 2015). The main vectors of *A. phagocytophilum* are *Ixodes scapularis* and *Ixodes pacifus* in the United States, meanwhile in Europe the main vector is *Ixodes ricinus*. *Anaplasma phagocytophilum* is transmitted transstadially by tick vectors (Ogden et al., 2003). These bacteria also cause canine anaplasmosis, an acute disease that occurs in dogs, 1 to 2 weeks after infection through tick bite (Ward, 2012).

## 2.10 Babesiosis

*Babesia* is a malaria-like parasite that infects the red blood cells. There are two types of *Babesia* spp. that cause Canine Babesiosis, namely *Babesia canis* and *Babesia gibsoni.* Large *Babesia* parasites on dogs such as *B. canis* are transmitted by *Dermacentor* ticks in Europe, *R. sanguineus* in tropical and subtropical regions (Uilenberg et al., 1989). There are three subspecies of *Babesia canis*: *B. canis, B. vogeli, Babesia rossi*. *B. rossi* is found in the South Africa transmitted by *H. leachi* which causes severe and often fatal hemolytic disease in dogs. *B. canis* is transmitted by *Dermacentor reticulatus* and causes hemolytic anemia with different degrees of severity. *B. vogeli* is found in North Africa, Middle East, Europe, Australia and Asia and it is transmitted by *R. sanguineus* (Baneth et al., 2004). *B. gibsoni* has been recognized as one of the important tick-borne pathogens that affects dogs worldwide. The specific vector for *B. gibsoni* has not been well established, therefore *R. sanguineus* is suspected to be the vector (Boozer & Macintire, 2005). While in other countries, *Haemaphysalis* ticks have been known to transmit *B. gibsoni,* such as *Haemaphysalis*

*longicornis* in Japan and *Haemaphysalis hystricis* in Taiwan. (Iwakami et al., 2014; Jongejan et al., 2018). *B. gibsoni* can also be transmitted without an ixodid vector through blood exchange during dogs biting and fighting (Irwin, 2009).

## 2.11 Hepatozoonosis

*Hepatozoon canis* is a protozoan parasite that infects leukocytes and parenchymal tissues in dogs around the world by the ingestion of ticks which contains mature oocyst. *R. sanguineus* is the main vector of *H. canis*, however, this protozoan also has been detected in other tick species that feed on dog blood such as *H. longicornis* and *Haemaphysalis flava* in Japan and *Ambylomma ovale* in Brazil (Table 2.1). Due to the close association with *R. sanguineus* and the cosmopolitan distribution of *R. sanguineus*, *H. canis* is probably known as the most widespread canine vector-borne diseases (Little et al., 2009; Otranto et al., 2011). *Rhipicephalus turanicus,* largely distributed in the Mediterranean sub-region, Africa and Asia, is also capable as a vector in the transmission of *H. canis* (Giannelli et al., 2016).

## 2.12 Rickettsiosis

Several *Rickettsia* spp. have been detected in *R. sanguineus* such as *Rickettsia rickettsii, Rickettsia conorii* and *Rickettsia masillae* (Eremeeva et al., 2006; Wikswo et al., 2007; Dantas-Torres, 2008). *R. rickettsi* causes Brazilian spotted fever (BSF) and Rocky Mountain spotted fever (RMSF) in canines. Rocky Mountain spotted fever is more prevalent in younger dogs that live outdoor. RMSF main vector is *Ambylomma sculptum,* meanwhile the main vector for BSF is *Ambylomma cajennense* (Guedes et al., 2005; Ueno et al., 2016). *Rickettsia typhi* is a typhus group rickettsia, the causative agent for Murine typhus, a human rickettsiosis. This pathogen is usually transmitted by *Xenopsylla cheopis* also known as the oriental rat flea through transovarial transmission. However, it also has been known to be transmitted by *R. sanguineus* (Farhang-Azad et

al., 1985; Dzul-Rosado et al., 2017). *Rickettsia asembonensis* (formerly *Candidatus Rickettsia asemboensis)* is distinct but similar to the closest relative, *R. felis* and is categorized in the group of *R. felis*-like organisms (RFLO). *Rickettsia asembonensis* is transmitted by *Ctenocephalides felis* also known as the cat flea. *R. masilliae*, is also transmitted by *R. sanguineus* from Spain and Switzerland and *R. turanicus* in Switzerland. Different genotypes of *R. massiliae* were also detected in *Rhipicephalus* spp. complex, such as Mtu5 strain in *R. senegalensis*, Mtu1 strain in *R. sulcatus*, and *R. lunulatus* in Central Africa (Beeler et al., 2011).

## 2.13 Summary of common pathogens in dogs and their vectors

**Table 2.1**: Summary of common pathogens in dogs and their vectors.

| Tick-borne disease | Pathogen | Main Vector | Potential vector |
|---|---|---|---|
| Anaplasmosis | *A. platys* | *R. sanguineus* (Ferreira et al., 2007) | *I. persulcatus*, *D. nutalli* (Javkhlan et al., 2014) |
| | *A. phagocytophilum* | *I. ricinus* (Svitálková et al., 2015) | *H. longicornis* (Kim et al., 2003)<br>*D. variabilis* (Holden et al., 2003)<br>*I. pacificus* (Holden et al., 2003) |
| Ehrlichiosis | *E. canis* | *R. sanguineus* (Bowman et al., 2009; Bremer et al., 2005) | *D. variabilis* (Johnson et al., 1998) |
| | *E. ewingii* | *A. americanum* (Wolf et al.,2000) | *R. sanguineus* (Ndip et al., 2007) |
| | *E. chafeensis* | *A. americanum* (Ismail et al., 2010) | *R.sanguineus* (Ndip et al., 2007) |
| Babesiosis | *B. gibsoni* | *H. longicornis* (Iwakami et al., 2014) | *R. sanguineus* (Boozer & Macintire, 2005)<br>*H. hystricis* (Jongejan et al., 2018) |
| | *B. vogeli* | *R. sanguineus* (Baneth et al., 2004) | |
| | *B. canis* | *D. reticulatus* (Uilenberg et al., 1989) | |
| | *B. rossi* | *H. leachi* (Uilenberg et al., 1989) | *H. elliptica* (Penzhorn, 2011) |
| Hepatozoonosis | *H. canis* | *R. sanguineus* (Little et al., 2009) | *H. longicornis* (Murata et al., 1991)<br>*H. flava* (Murata et al., 1991)<br>*A. ovale* (Forlano et al., 2005)<br>R. turanicus (Giannelli et al., 2016) |
| Rickettsiosis | *R. rickettsi* | *A. sculptum* (Ueno et al., 2016)<br>*A. cajennense* (Guedes et al., 2005) | *R. sanguineus* (Ortega-Morales et al., 2019) |
| | *R. typhi* | *X. cheopis* (Farhang-Azad et al., 1985) | *R. sanguineus* (Dzul-Rosado et al., 2017) |
| | *R. asembonensis* | *C. felis* (Silva et al., 2017) | |

## 2.14 Molecular markers for phylogenetics of ticks

The application of molecular markers in studying the phylogenetic of ticks yielded new insights into their taxonomic relationship and population structure. Different molecular markers have been used in ticks, all have their limitation, however choosing the right molecular marker appropriate to the objective can minimize the problems. Mitochondrial genes have been vastly used in molecular study. Mitochondrial ribosomal genes are divided into two categories, ribosomal genes and protein-coding genes. There are two mitochondrial genes not separated by internal transcribes spacers, 12S rDNA and 16S rDNA. The 28S rDNA gene and 18S rDNA gene are more conserved than 16S rDNA gene. 18S and 28S rDNA are likely to provide a powerful tool for resolving deeper level phylogenies. At low taxonomic level, the second transcribed spacer of the nuclear ribosomal gene cluster (ITS2) and the mitochondrial protein-coding gene cytochrome oxidase I (COI) together act as a powerful tool for phylogenetic study, however COI appears to evolve slightly faster (Cruickshank, 2002).

An assessment of four DNA fragments, COI, 16S rDNA, ITS2, 12S rDNA for the identification of Ixodid ticks demonstrated that 16S rDNA and COI were more dependable compared to 18S and ITS2 in terms of sequence recovery rates. As for the sequence length, the length of 12S, 16S and CO1 fragment is shorter compared to the length of ITS2 fragment, which leads to a lower quality of ITS2 sequence compared to the other three DNA fragments. Meanwhile 16S sequencing quality is the highest among the four loci. In terms of sequence alignment, CO1 fragment was found to be least complicated due to its protein-coding sequence and the absence of gaps within the alignment, leading to the idea of the use both CO1 and 16S rDNA as a suitable DNA marker for tick species identification (Lv et al., 2014b).

# CHAPTER 3: METHODOLOGY

## 3.1 Sample collection

A total of 206 tick specimens were collected from eight states representing the northern, southern, east coast and west coast regions of the Peninsular Malaysia (Table 3.1). Kedah was represented with 6 ticks from 2 dogs; Perlis with 5 ticks from 2 dogs; Perak with 12 ticks from 3 dogs, Selangor with 140 ticks from 41 dogs; Pahang with 11 ticks from 3 dogs, Terengganu with 11 ticks from 4 dogs, Johor with 11 ticks from 6 dogs and Malacca with 10 ticks from 3 dogs.

Sample collections were conducted in the morning until evening. Ticks were removed from dogs by using forceps and kept in a small sealable plastic bag. The tick samples were brought back to the laboratory in Institute of Biological Sciences, Faculty of Science, University of Malaya for species identification.

A total of 189 dog blood samples were collected from a veterinary hospital and 51 dog blood samples from an animal shelter in the state of Selangor, Peninsular Malaysia (Table 3.2) (Figure 3.1; Figure 3.2). Blood samples were collected via venipuncture by veterinarian and stored in EDTA tubes in 2-4ºc prior to DNA extraction.

**Table 3.1:** Collection sites for *R. sanguineus* in Peninsular Malaysia.

| State | District | Collection date | No. of ticks collected | No. of dogs |
|---|---|---|---|---|
| **Northern** | | | | |
| Kedah | Kulim | 22/04/17 | 6 | 2 |
| Perlis | Pauh | 26/05/17 | 3 | 1 |
| | Kangar | 27/05/17 | 2 | 1 |
| **West coast** | | | | |
| Perak | Ipoh | 19/04/17 | 12 | 3 |
| Selangor | Semenyih | 17/01/2016<br>06/12/2016 | 140 | 41 |
| **East coast** | | | | |
| Pahang | Kuantan | 17/04/2017 | 11 | 3 |
| Terengganu | Chukai | 28/05/2017 | 7 | 2 |
| | Kerteh | 27/05/2017 | 4 | 2 |
| **Southern** | | | | |
| Johor | Pekan Nanas | 17/06/2017 | 7 | 5 |
| | Tangkak | 19/06/2017 | 4 | 1 |
| Malacca | Bukit Katil | 30/04/2017 | 10 | 3 |
| | Total | | 206 | 64 |

**Table 3.2:** Dog blood collection sites in Selangor, Malaysia.

| Location | Dog blood sample (n) |
|---|---|
| Gasing Veterinary Hospital, Petaling Jaya | 189 |
| Animal shelter, Semenyih | 51 |
| Total | 240 |


**Figure 3.1:** Blood sample collection at Gasing Veterinary Hospital, Petaling Jaya, Selangor

**Figure 3.2:** Blood sample collection at animal shelter, Semenyih, Selangor

### 3.2 Microscopic observation

*R. sanguineus* ticks were identified and separated from other tick genera by using a set of morphological features: (1) Distinct anal groove. (2) Hexagonal basis capituli. (3) Mouth part as long as basis capituli. (4) Coxa I have both distinct internal and external spur. (5) Feston present. (6) Eyes present (7) Scutum elongate-oval, longer than wide and usually produced posteriorly. (8) Punctations more numerous anteriorly, with a very short, inconspicuous hair arising from each. (9) Genital opening opposite coxa II. (10) Hypostome short with 3:3 dentition. (Anastos, 1950). Under microscopic observation, the ventral view of the male *R. sanguineus* can be identified with the adanal plate on each side below coxa IV (Figure 3.3). Dorsal view of male *R. sanguineus* does not have scutum as female *R. sanguineus.* (Figure 3.4). In ventral view of female *R. sanguineus,* there is absence of adanal plate (Figure 3.5). In the dorsal view of female *R. sanguineus*, scutum is present covering less than half of the tick body (Figure 3.6).

All 206 ticks collected were identified as *R. sanguineus*. Life stage and gender of the *R. sanguineus* were recorded (Table 3.3). Specimens were then stored at -20 ºC prior to DNA extraction.

**Table 3.3:** Life stage and gender of collected *R. sanguineus* ticks.

| State | Adult Male | Adult Female | Nymph |
|---|---|---|---|
| Kedah | 1 | 4 | 1 |
| Perlis | 3 | 2 | 0 |
| Perak | 4 | 7 | 1 |
| Selangor | 45 | 77 | 18 |
| Pahang | 4 | 6 | 1 |
| Terengganu | 2 | 7 | 2 |
| Johor | 4 | 5 | 2 |
| Malaca | 3 | 7 | 0 |
| Total | 66 | 115 | 25 |

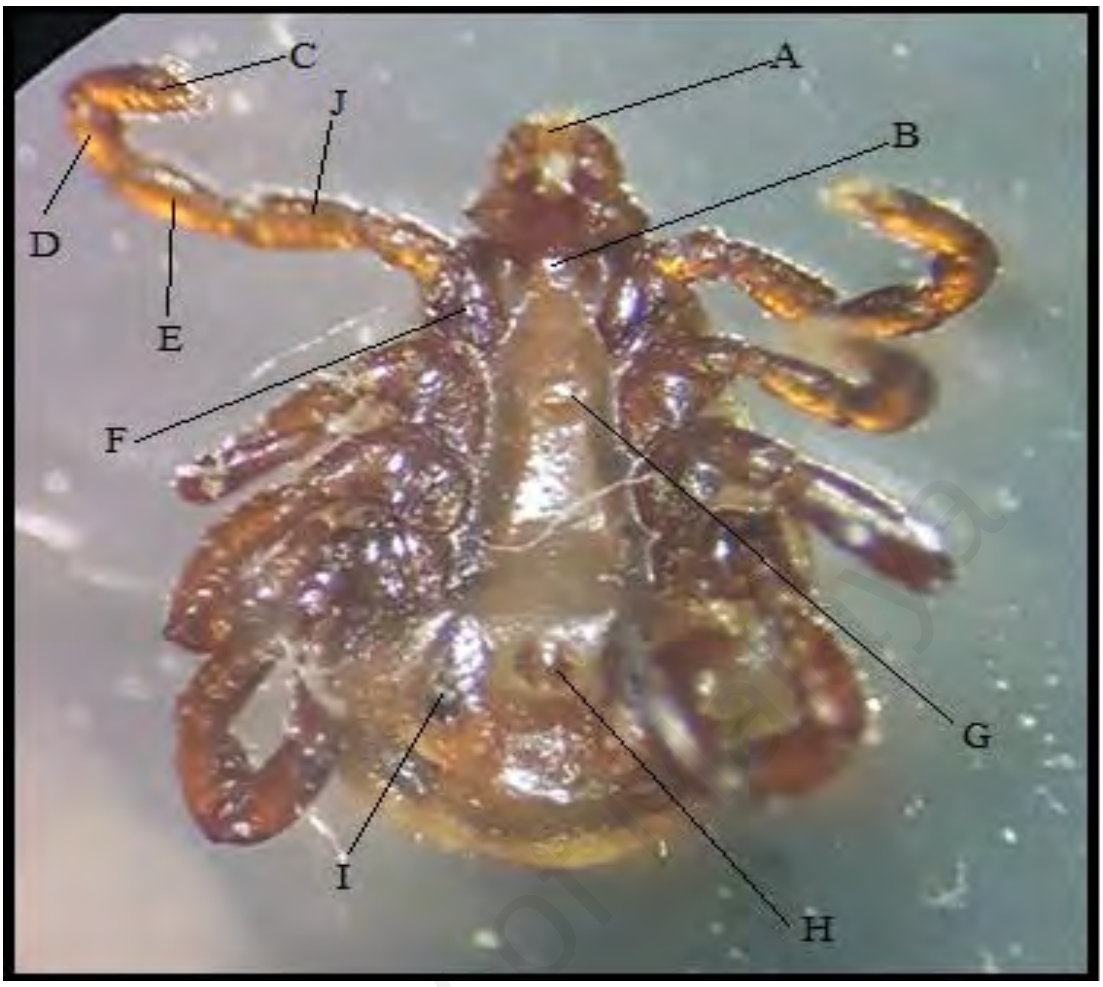


**Figure 3.3**: Ventral view of male *R. sanguineus*

A-J, Ventral view male *R. sanguineus*: A. Hypostome; B. Basis capituli; C. Tarsus; D. Metatarsus; E. Tibia; F. Distinct external and internal spur at coxa I; G. Genital aperture; H. Anus; I. Adanal plate; J. Femur.


**Figure 3.4**: Dorsal view of male *R. sanguineus*

A-G, Dorsal view male *R. sanguineus*: A. Basis capituli; B. Eyes; C. Lateral groove; D. Festoon; E. Punctation; F. Trochanater; G. Palpus.


**Figure 3.5:** Ventral view of female *R. sanguineus*

A-I, Ventral view female *R. sanguineus* s.l: A. Hypostome; B. Basis capituli; C. Distinct external and internal spur at coxa I; D. Genital aperture; E. Anus, F. Coxa IV; G. Coxa III; H. Coxa II; I. Coxa I.


**Figure 3.6:** Dorsal view of a female *R. sanguineus*

A-F, Dorsal view female *R. sanguineus*: A. Palpus e; B. Basis capituli; C. Eyes; D. Scutum; E. Punctation; F. Lateral groove.

### 3.3 DNA extraction of *R. sanguineus* ticks

Of 206 collected *R. sanguineus*, 176 specimens were used for DNA extraction. DNA of *R. sanguineus* tick was extracted from each specimen using the G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Inc., Seognam, South Korea). Tick specimen was taken out from freezer and placed in a petri dish. The specimen was then washed with distilled water and transferred to a sterilized 1.5 ml tube. 200 µl of Buffer CL, 20 µl of Proteinase K and 5 µl of RNase A solution were added into the 1.5 ml tube and mixed vigorously using a vortex mixer. The lysate was then incubated at 56 ºC in a water bath for 25 minutes. After the lysis was completed, 200 µl of Buffer BL was added into the sample tube and mixed thoroughly. The mixture was then incubated at 70 ºC for 5 minutes. The sample tube was then centrifuged at 13000 rpm for 5 minutes to remove un-lysed tissue particles. 350 µl of the supernatant was transferred into a new 1.5ml tube. 200 µl of absolute ethanol was added into the lysate. The mixture was mixed well by gently inverting 5 to 6 times. After mixing, the mixture in the 1.5ml tube was briefly centrifuged to remove the drops from inside of the lid. The mixture was then transferred into the spin column in a 2 ml collection tube without wetting the rim. The mixture was centrifuged at 13000 rpm for 1 minute and the filtrate was discarded. 700 µl of Buffer WA was added into the spin column and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded. 700 µl of Buffer WB was added into the spin column and centrifuged for 1 minute at 13000 rpm. The flow-through was discarded. 2 ml collection tube with the spin column was centrifuged again for 1 minute to dry the membrane. The flow through and the 2ml collection tube was discarded. The spin column was placed into a new 1.5ml tube. 50 µl was added directly onto the membrane of the spin column. The mixture was incubated for 1 minute at room temperature and then centrifuged for 1 minute at 13000 for elution. The spin column was discarded and the DNA in the tube was stored at -20 ºC.

### 3.4 DNA extraction of dog blood samples

A total of 240 dog bloods were extracted using the QIAamp DNA Mini and Blood Mini Extraction Kit. All blood DNA extraction steps were performed according to the manufacturer's instructions. 20 µl of QIAGEN proteinase K was pipetted into the bottom of a 1.5 ml microcentrifuge tube. 200 µl of blood sample was added to the microcentrifuge tube. If the sample volume was less than 200 µl, appropriate volume of PBS was added. 200 µl of Buffer AL was added to the sample. The sample was mixed by pulse-vortexing for 15 seconds. Sample was incubated in a water bath at 56 ºC for 10 minutes. The sample in the 1.5ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. 200 µl of ethanol (96-100%) was added to the sample. The sample was mixed again by pulse-vortexing for 15 seconds. The sample was briefly centrifuged after mixing to remove drops from the inside of the lid. The mixture was then transferred to the QIAamp Mini spin column 2ml collection tube without wetting the rim. The mixture was then centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIAamp Mini spin column was placed in a clean 2ml collection tube and the tube that contains the filtrate was discarded. 500 µl of Buffer AW1 was pipetted into the QIAamp Mini spin column and centrifuged at 6000 x g (8000rpm) for 1 minute. The QIAamp Mini spin column was placed in a clean 2ml collection tube and the tube that contains the filtrate was discarded. 500 µl of Buffer AW2 was pipetted into the QIAamp Mini spin column and centrifuged at 20000 x g (14000 rpm) for 3 minutes. The QIAamp Mini spin column was placed in a clean 2ml collection tube and centrifuged for 1 minute, while the old collection tube with filtrate was discarded. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. 200 µl of Buffer AE was pipetted into the QIAamp Mini spin column and incubated at room temperature for 1 minute. The mixture was then centrifuged at 6000 x g (8000 rpm) for 1 minute. The spin column was discarded and the DNA in the tube was stored at -20 ºC.

### 3.5 Phylogenetic analyses of *R. sanguineus* ticks in Peninsular Malaysia

For phylogenetic study, a total of 40 *R. sanguineus* comprised of five individuals from each of the eight study sites in Peninsular Malaysia were amplified using two different mitochondrial encoded 16S rRNA and COI genes.

### 3.6 16S ribosomal RNA (16S rRNA)

Extracted DNA of *R. sanguineus* ticks were amplified using 16S-F (5'-TTA AAT TGC TGT RGT ATT-3') and 16S-R1 (5'CCG GTC TGA ACT CAS AWC-3') primers. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA. PCR was run for 5 cycles (94ºC for 30s, 49ºC for 30s and 68ºC for 30s), 5 cycles (94ºC for 30s, 47ºC for 30s and 68ºC for 30s), 5 cycles (94ºC for 30s, 45ºC for 30s and 68ºC for 30s), 25 cycles (94ºC for 30s, 43ºC for 30s and 68ºC for 30s) followed by a final extension for 5min at 68ºC. PCR was conducted with an Applied Biosystems Veriti 96-Well Thermal Cycler. All amplification products were analyzed on 2% agarose gel after staining with SYBR® Safe DNA staining dye.

### 3.7 Cytochrome c oxidase subunit I (COI)

Extracted DNA of *R. sanguineus* ticks were amplified using the newly designed primers, TICKRSCOI_F (5'-GAT TTT GGT TAC TTC CTC CTT CTC T-3') and TICKRSCOI_R (5'GCC TAA TAA TCC AAT TGC TGC T-3'). Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA. PCR was run for initial denaturation at 94ºC for 3 min, 35 cycles (94ºC for 30s, 59ºC for 30s and 72ºC for 30s), followed by a final extension for 10min at 72ºC. PCR was conducted with an Applied Biosystems Veriti 96-Well Thermal Cycler. All amplification products were analyzed on 2% agarose gel after staining with SYBR® Safe DNA staining dye.

### 3.8 Molecular detection of tick-borne pathogens in both *R. sanguineus* tick and dog blood samples

A total of 240 dog blood samples and 140 *R. sanguineus* ticks were used for the molecular detection of tick-borne pathogens.

### 3.9 Molecular detection of *Ehrlichia* spp. and *Anaplasma* spp. in both *R. sanguineus* tick and dog blood samples

A nested PCR was used for the molecular screening of *Ehrlichia* spp. and *Anaplasma* spp. in both tick and dog blood DNA samples. The first amplification was performed to amplify the genus *Ehrlichia* and *Anaplasma*, using forward primer: ECB 5'-AGA ACG AAC GCT GGC AAG CC -3' and reverse primer: ECC 5'- CGT ATT ACC GCG GCT GCT GGC A -3'. Amplification was performed in a volume of 25 µl containing 12.5 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA PCR was run for 2 cycles (94ºC for 1 min, 45ºC for 2 min and 72ºC for 30s), 38 cycles (94ºC for 1min, 45ºC for 2 min and 72ºC for 31s) followed by a final extension for 5 min at 72ºC.

For second amplification, species specific primer was used for *Ehrlichia canis*, forward primer: ECA 5'-CAA TTA TTA TAA CCT TTT GGT TAT AAA T -3' and reverse primer: HE3 5'-ATA GGG AAG ATA ATG ACC TAT A -3'. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of PCR product from the first amplification. PCR was run for 3 cycles (94ºC for 1 min, 55ºC for 2 min and 70ºC for 1min 30s), 37 cycles (88ºC for 1min, 55ºC for 2 min and 70ºC for 1min 30s) followed by a final extension for 5 min at 72ºC. *Anaplasma platys* was amplified using forward primer: PLATYS 5'-TTT GTC GTAGCTTGC TAT G -3' and reverse primer: GA1UR 5'- GAG TTT GCC GGG ACT TCT TCT-3'. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl PCR product from the first

amplification. PCR was run for initial denaturation, 94ºC for 1 min, 39 cycles (94ºC for 1 min, 57.6ºC for 1 min and 72ºC for 1min) followed by final extension for 5min at 72ºC (Anderson et al., 1992; Dawson et al., 1994; Yabsley et al., 2008).

### 3.10 Molecular detection of *Rickettsia* spp. in both *R. sanguineus* tick and dog blood samples

For molecular screening of *Rickettsia* spp., *gltA* gene was amplified using forward primer: CS-239 5'-ATA CAT GAG CAA AAT CTC AAC-3' and reverse primer: CS-1069 5'-CTT ATT ATT CCA TGC TGC AG-3'. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA. PCR was run for initial denaturation, 95ºC for 5 min, 40 cycles (95º for 20s, 52ºC for 20s and 72ºC for 40s) followed by a final extension for 5 min at 72ºC (Labruna et al., 2004).

### 3.11 Molecular detection of *Babesia* spp. in both *R. sanguineus* tick and dog blood samples

For molecular screening of *Babesia* spp., 18S rRNA gene was amplified using forward primer: BAB 143-167 5'-CCG TGC TAA TTG TAG GGC TAA TAC A -3' and reverse primer BAB 694-667 5'-GCT TGA AAC ACT CTA RTT TTC TCA AAG -3'. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA. PCR was run for initial denaturation, 95ºC for 5min, 35 cycles (95º for 30s, 58ºC for 30s and 72ºC for 30s) followed by a final extension for 7 min at 72ºC (Almeida et al., 2012; Araujo et al., 2015).

### 3.12 Molecular detection of *H. canis* in both *R. sanguineus* tick and dog blood samples

For molecular screening of *H. canis*, 18S rRNA gene was amplified using forward primer: HEPF 5'-ATA CAT GAG CAA AAT CTC AAC-3' and reverse primer: HEPR 5'-CTT ATT ATT CCA TGC TGC AG-3'. Amplification was performed in a final volume of 50 µl containing 25 µl of premix, 1 µl of each forward and reverse primer and 1 µl of DNA. PCR was run for initial denaturation, 95ºC for 12min, 34 cycles (95º for 30s, 57ºC for 30s and 72ºC for 1min 30s) followed by a final extension for 5 min at 72ºC (Otranto et al., 2011).

### 3.13 Sequencing and data analysis

Purified DNA fragments were sent to a commercial company for DNA sequencing using an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Inc.). All sequences were analyzed and reconciled using ChromasPro Version 2.1.6 (Figure 3.7) (Technelysium Pty Ltd., Qld, Australia) and BioEdit Version 7.0.9.0 (Figure 3.8) (Hall, 1999).

The haplotype network of Malaysian *R. sanguineus* was analyzed using TCS 1.13® (Clement et al., 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with > 95% confidence. To assess the genetic divergence among the haplotypes, uncorrected (p) pairwise genetic distances were calculated using PAUP 4.0B10 (Swofford, 2002).


**Figure 3.7:** Forward and reverse sequences were complemented using ChromasPro

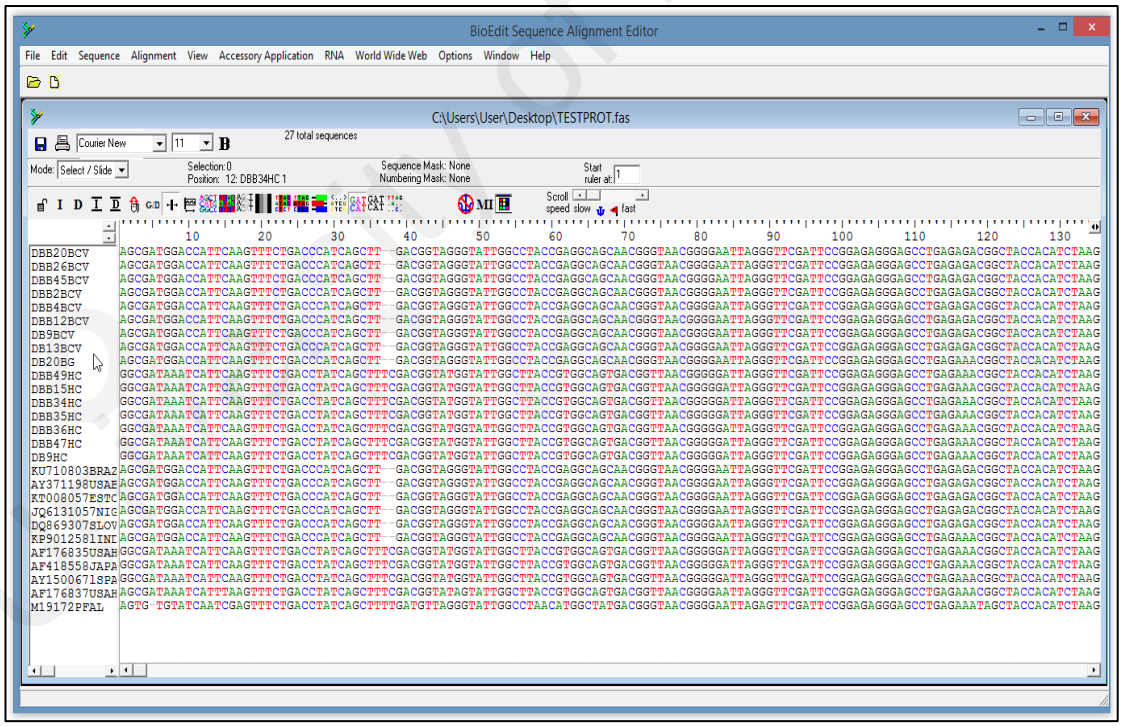


**Figure 3.8:** Nucleotide sequences were analyzed using Bioedit

For phylogenetic analysis, the unique 16S and COI haplotypes identified in the Malaysian *R. sanguineus* were aligned with reference sequences of the closely related *Rhipicephalus* taxa available in NCBI GenBank. *Rhipicephalus bursa* was used as an outgroup for the construction of phylogenetic tree A neighbor-joining (NJ) phylogenetic tree was plotted using MEGA7 (Kumar et al., 2016). The NJ bootstrap values were estimated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance) (Figure 4.1; Figure 4.2). TCS analysis was used to derive a haplotype network (Figure 4.3).

Phylogenetic trees were constructed for the tick-borne pathogens detected in this study. For the construction of phylogenetic tree of *A. platys* and *E. canis*, the sequences were aligned with reference sequences of the closely related *Anaplasma* spp. and *Ehrlichia* spp. taxa available in NCBI GenBank. *Rickettsia rickettsi* was used as an outgroup for the construction of phylogenetic tree (Figure 4.4). For phylogenetic tree of *Rickettsia* spp., the sequences were aligned with reference sequences of the closely related *Rickettsia* spp. taxa. *Rickettsia belli* was used as an outgroup (Figure 4.5). As for the phylogenetic tree of *H. canis*, the sequences were aligned with reference sequences of the closely related *Hepatozoon* spp. available in NCBI GenBank. *Coccidia* spp. was used as an outgroup (Figure 4.6). For phylogenetic tree of *Babesia* spp., the sequences were aligned with reference sequences of the closely related *Babesia* spp. available in NCBI GenBank. *H. canis* was used as an outgroup (Figure 4.7). Prevalence and the infection pattern of tick-borne pathogens in *R. sanguineus* s.l ticks and dogs were recorded.

# CHAPTER 4: RESULTS

## 4.1 Genetic characterization of *R. sanguineus* using 16S rRNA and COI genes

The 16S and COI phylogenetic trees revealed three *R. sanguineus* s.l. genetic lineages, corresponding to the known tropical, temperate and southeast European lineages. Both genes unambiguously assigned Malaysian material into the tropical lineage.

The tropical lineage is genetically closer to *Rhicephalus* species such as *R. camicasi*, *R. leporis* and *R. guilhoni*; and showed a sister relationship with the southeast European lineage. On the other hand, the temperate lineage formed a distinct clade consisted of two previously defined sub-lineages.

The 16S sequences were highly conserved as there is no variation site was observed. In contrast, the COI sequences revealed slightly higher variation by recovering four haplotypes, one of which is restricted to the state of Perlis, the northernmost of Peninsular Malaysia. The TCS haplotype network of the COI gene revealed four haplotypes (Figure 4.3). Haplotype H1 differed from haplotypes H2 and H3 with one mutation change: thymine to cytosine at position 73, and guanine to adenine at position 366, respectively. Haplotype H1 differed from haplotype H4 with two mutation changes where guanine is substituted by adenine at positions 90 and 366 (Table 4.1).

The uncorrected ‘p’ distance based on COI sequences ranged from 0.21 to 0.63%. Haplotype H1 differed from haplotypes H2, H3 and H4 by 0.21%, 0.21% and 0.42%, respectively. Haplotype H2 differed from haplotype H4 by 0.63% (Table 4.2).

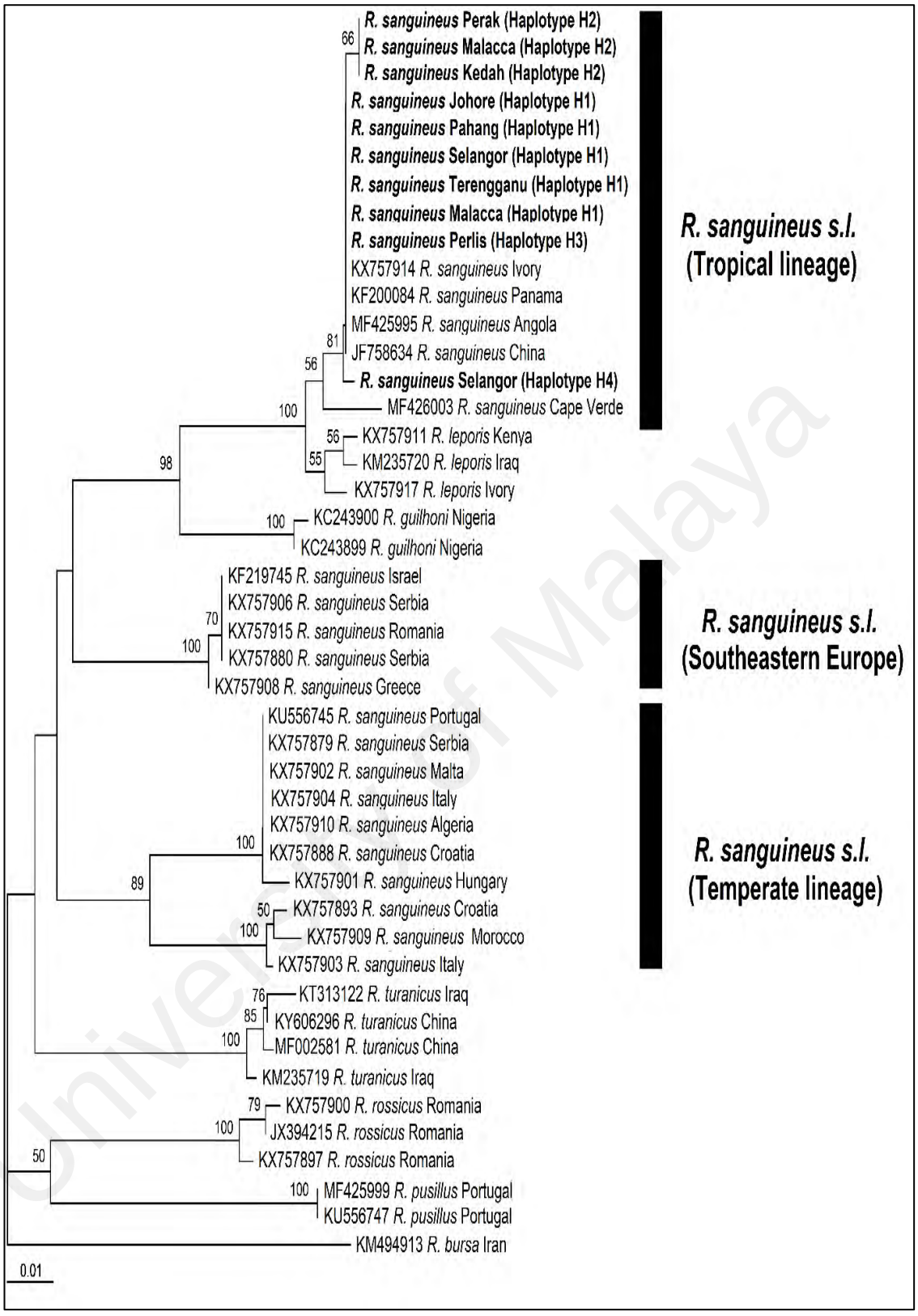


**Figure 4.1:** Neighbour-joining phylogenetic tree of *Rhipicephalus* species based on the COI sequences. New sequences generated from this study are highlighted in bold.

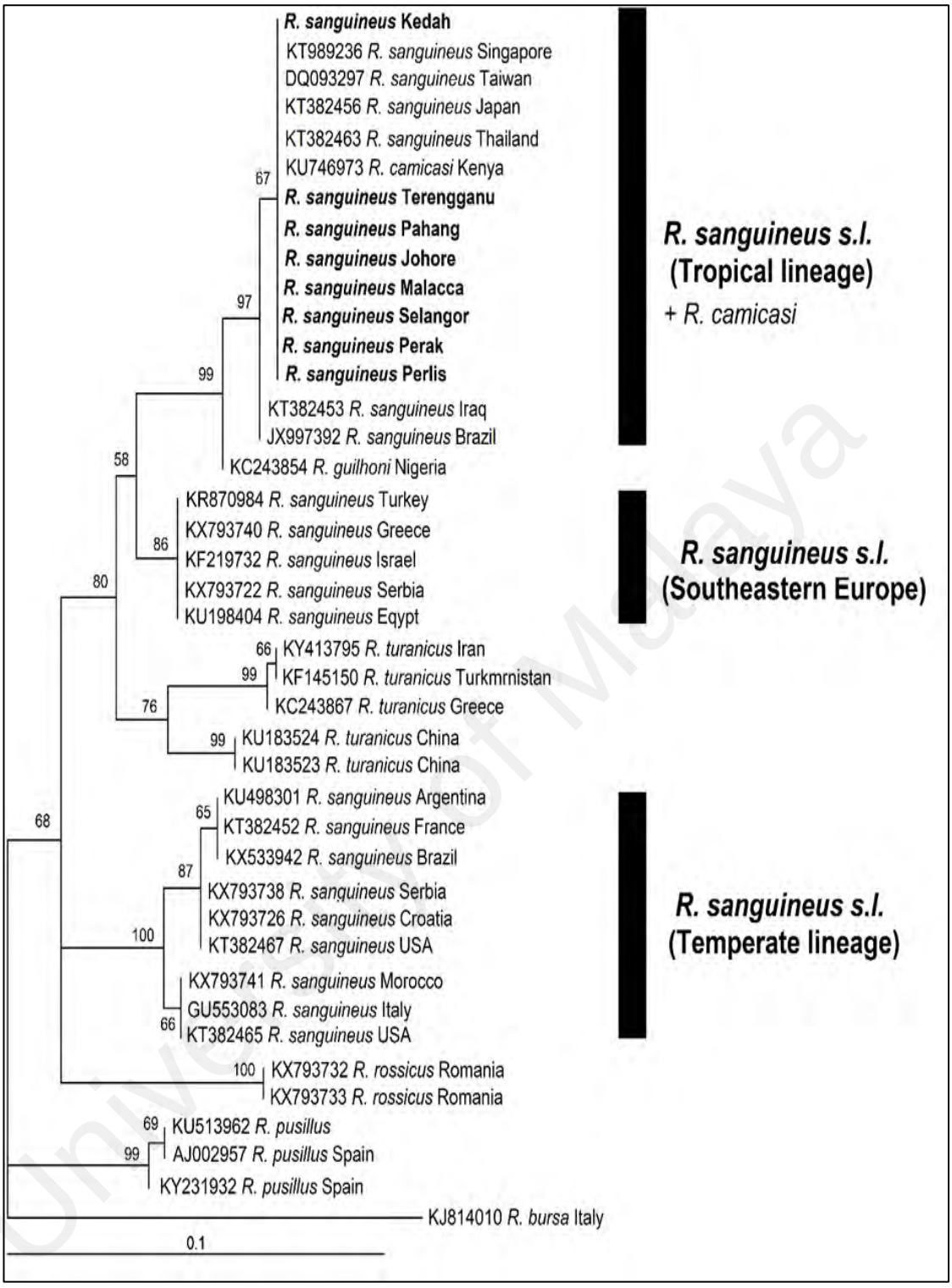


**Figure 4.2:** Neighbour-joining phylogenetic tree of *Rhipicephalus* species, based on the 16S rRNA sequences. New sequences generated from this study are highlighted in bold.

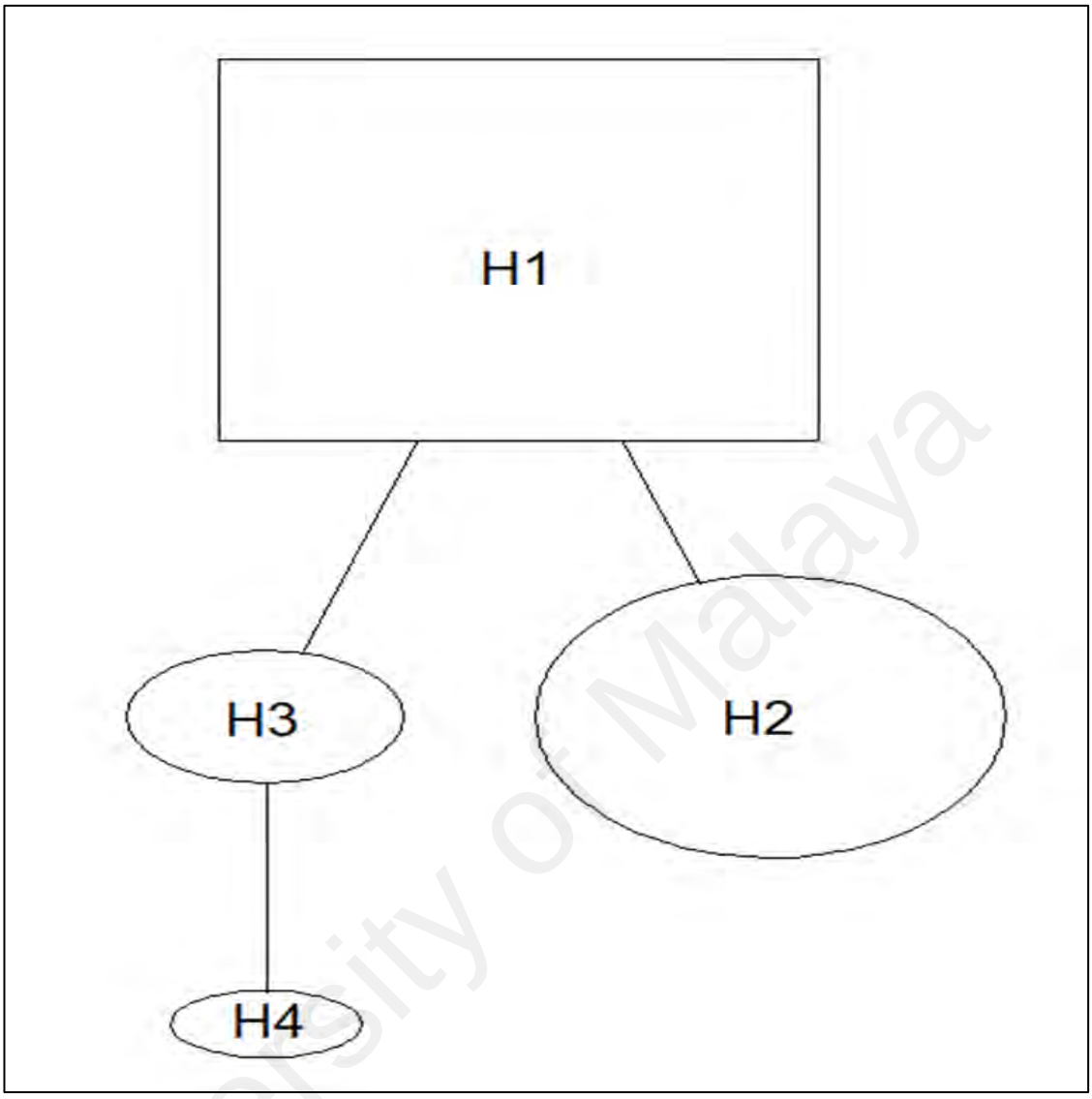


**Figure 4.3** Statistical parsimony network for COI haplotypes of *Rhipicephalus sanguineus*. Line lengths represent the number of mutational steps between haplotypes. The size of the square and ovals corresponds to the haplotype frequency.

**Table 4.1:** Variation sites of *R. sanguineus* haplotypes (H1-H4) based on COI sequences.

| Haplotype | State | Variable sites | | |
|---|---|---|---|---|
| | | 73 | 90 | 366 |
| H1 | Selangor | T | G | G |
| | Pahang | | | |
| | Terengganu | | | |
| | Johor | | | |
| | Malacca | | | |
| H2 | Kedah | C | . | . |
| | Perak | | | |
| | Malacca | | | |
| H3 | Perlis | . | . | A |
| H4 | Selangor | . | A | A |

**Table 4.2:** Uncorrected "p" distance matrix among haplotypes based on COI sequences.

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| **1. Haplotype H1** | - | | | |
| **2. Haplotype H2** | 0.21 | - | | |
| **3. Haplotype H3** | 0.21 | 0.42 | - | |
| **4. Haplotype H4** | 0.42 | 0.63 | 0.21 | - |

## 4.2 Prevalence of tick-borne pathogens in dogs and *R. sanguineus* ticks

A total of 240 dog blood samples and 140 *R. sanguineus* ticks from Peninsular Malaysia were subjected to PCR assays for detection of tick-borne pathogens. Of 240 blood sample, 36 (15.00%) were positive with at least one tick-borne pathogen. (Table 4.3). On the other hand, at least one pathogen was detected in 11 individual ticks (7.86%) (Table 4.4).

The highest prevalence of tick-borne pathogen in dogs was *E. canis* with a total of 31 positive individual ticks (12.92%), followed by *H. canis* with 8 positive individuals ticks (3.33%), *A. platys* with 8 positive individual ticks (3.33%) and *B. canis* with 5 positive individual ticks (2.08%). *Rickettsia* DNA was not detected in any of the dog blood samples (Table 4.5).

The highest prevalence of tick-borne pathogen in *R. sanguineus* ticks was *A. platys* with four positive samples (2.86%), followed by *R. asembonensis* 2 samples (1.43%), *B. gibsoni* 2 samples (1.43%), *B. vogeli* 2 samples (1.43%), *E. canis* 1 sample (0.71%) and *H. canis* 1 sample (0.71%) (Table 4.6).

**Table 4.3:** Overall prevalence of tick-borne pathogens in dogs.

| | Dog blood (N = 240) | |
|---|---|---|
| | n | % |
| Positive | 36 | 15.00 |
| Negative | 204 | 85.00 |

**Table 4.4:** Overall prevalence of tick-borne pathogens in *R. sanguineus* ticks.

| | *R. sanguineus* (N = 140) | |
|---|---|---|
| | n | % |
| Positive | 11 | 7.86 |
| Negative | 129 | 92.14 |

**Table 4.5:** Prevalence of tick-borne bacteria and protozoa in dogs from pet shelter and veterinary hospital.

| | Dog Blood (N = 240) | | | |
|---|---|---|---|---|
| | n | % | Pet shelter (A = 51) | Veterinary hospital (B=189) |
| *Ehrlichia canis* | 31 | 12.92 | 31 | 0 |
| *Anaplasma platys* | 8 | 3.33 | 8 | 0 |
| *Hepatozoon canis* | 8 | 3.33 | 8 | 0 |
| *Babesia vogeli* | 5 | 2.08 | 5 | 0 |

**Table 4.6:** Prevalence of tick-borne bacteria and protozoa in *R. sanguineus* ticks.

| | *R. sanguineus* (N=140) | |
|---|---|---|
| | n | % |
| *Ehrlichia canis* | 1 | 0.71 |
| *Anaplasma platys* | 4 | 2.86 |
| *Babesia gibsoni* | 2 | 1.43 |
| *Babesia vogeli* | 2 | 1.43 |
| *Hepatozoon canis* | 1 | 0.71 |
| *Rickettsia asembonensis* | 2 | 1.43 |

Phylogenetic analysis using the 16S rRNA gene suggested the presence of *E. canis* and *A. platys* in both dog and *R. sanguineus* tick samples, each shared 99 – 100% similarity with the published references sequences from the NCBI GenBank (Figure 4.4).

Phylogenetic analysis using the gltA gene suggested the presence of *R. asembonensis in R. sanguineus* tick, each shared 99 – 100% similarity with the published reference sequences from the NCBI GenBank (Figure 4.5).

Phylogenetic analysis of the 18S rRNA gene from eight samples demonstrated 100% similarity to *H. canis* sequences from the NCBI GenBank (Figure 4.6).

Phylogenetic analysis using the 18S rRNA gene suggested the presence of two *Babesia* species*, B. gibsoni* and *B. vogeli,* each shared 99 – 100% similarity with published reference sequences from the NCBI GenBank (Figure 4.7).

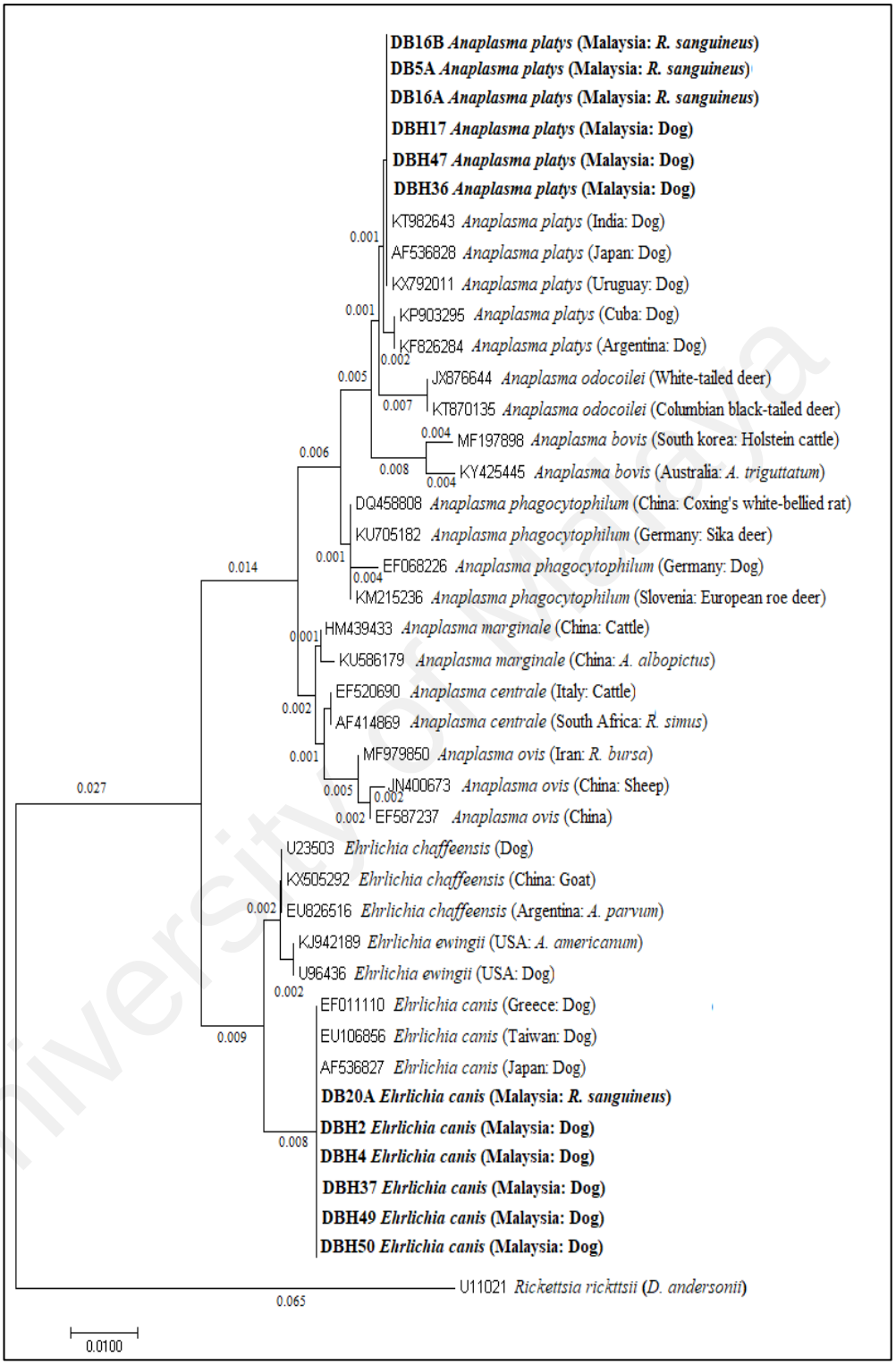


**Figure 4.4:** Neighbour-joining phylogenetic tree of *Ehrlichia* and *Anaplasma* species, based on the 16S rRNA sequences. New sequences generated from this study are highlighted in bold.

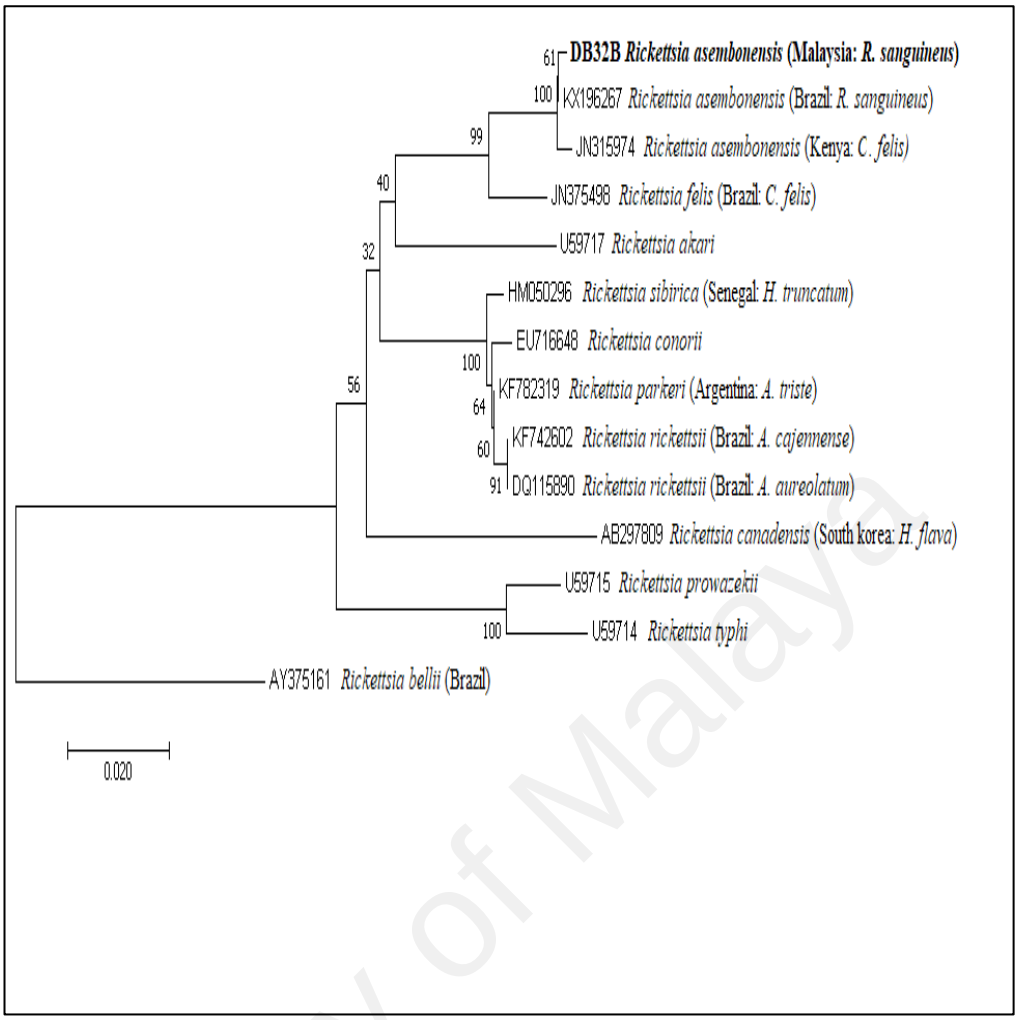


**Figure 4.5:** Neighbour-joining phylogenetic tree of *Rickettsia* species, based on the *gltA* gene sequences. New sequence generated from this study is highlighted in bold.

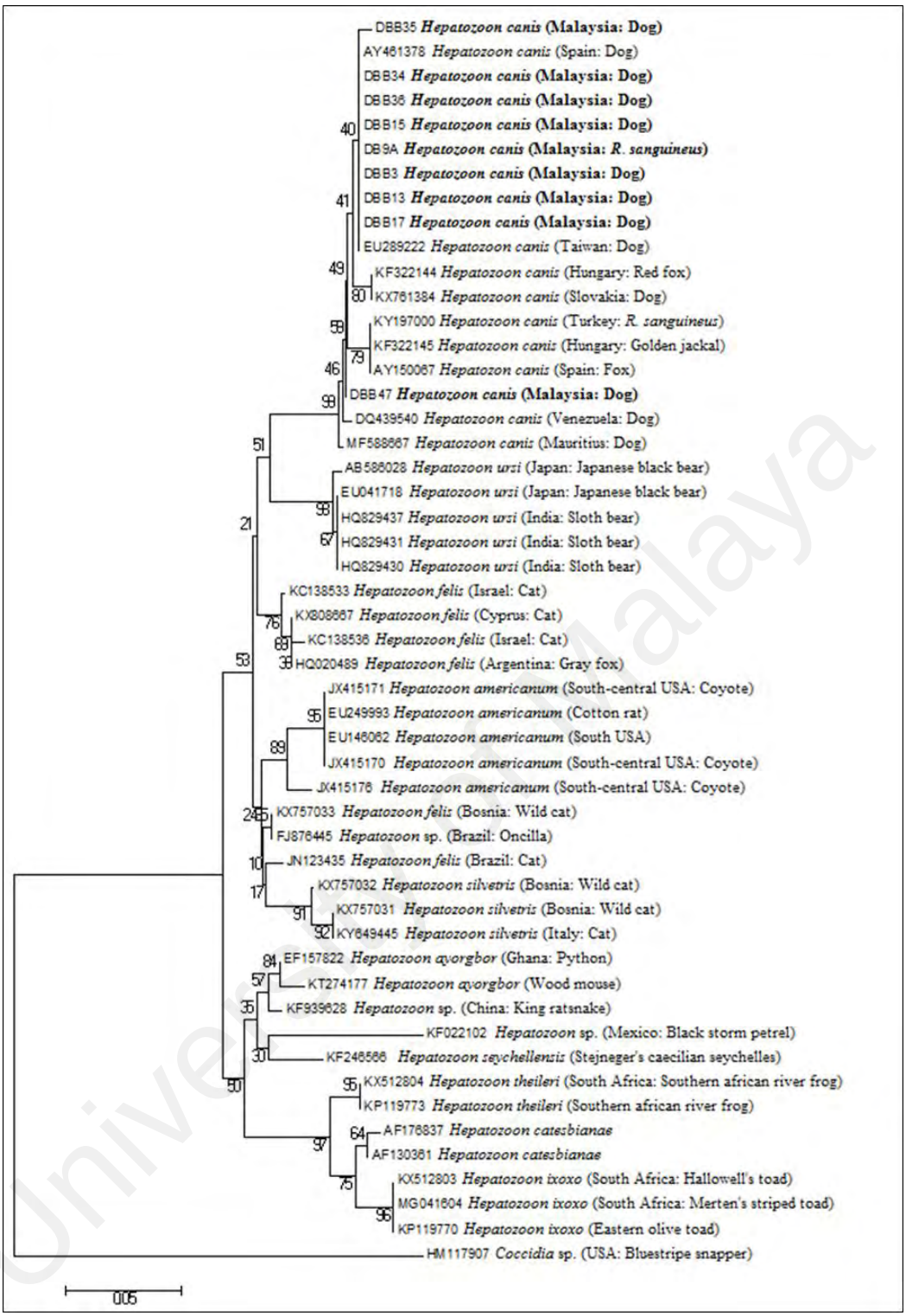


**Figure 4.6:** Neighbour-joining phylogenetic tree of *Hepatozoon s*pecies based on the 18S rRNA sequences. New sequences generated from this study are highlighted in bold.

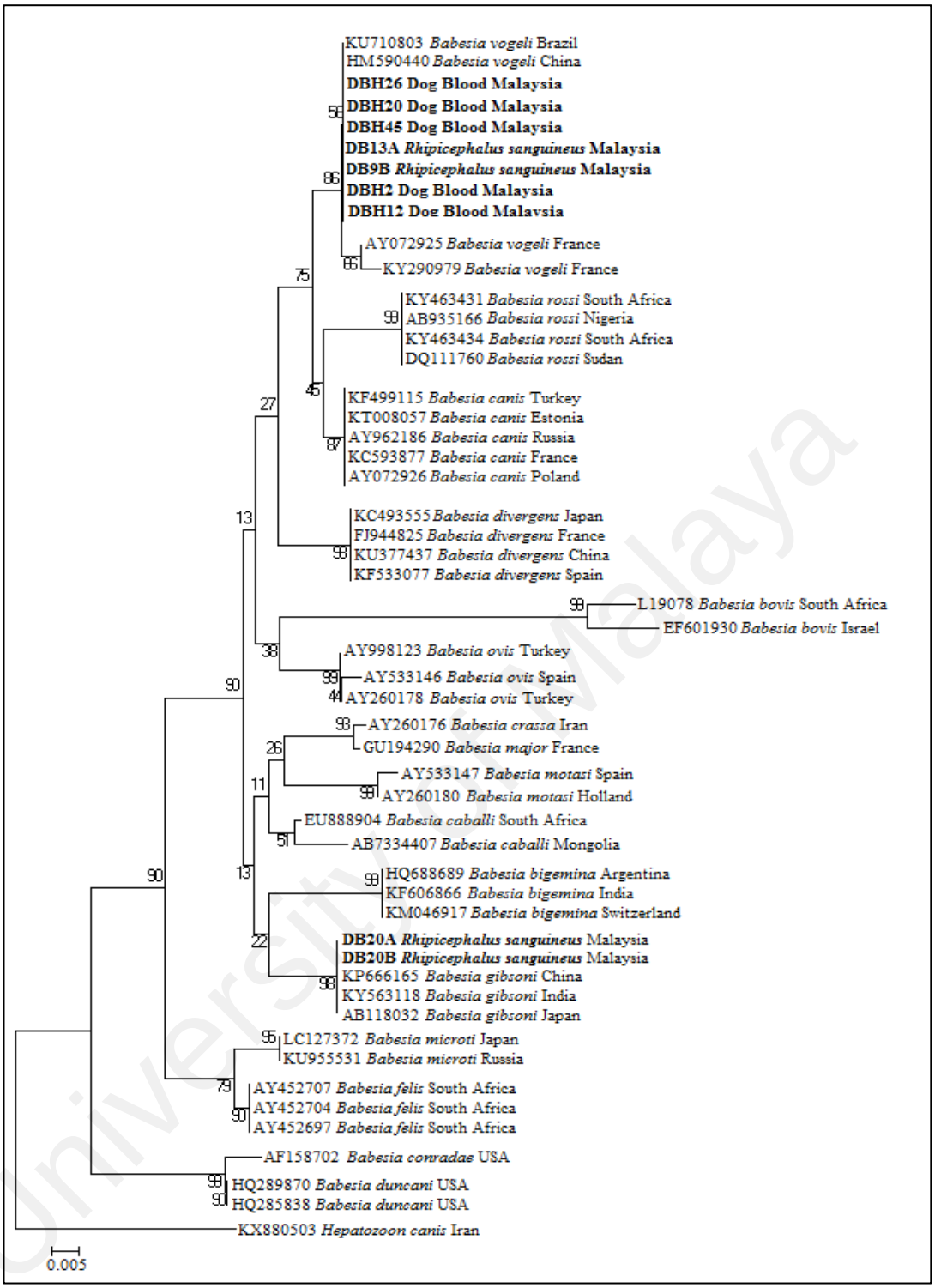


**Figure 4.7:** Neighbour-joining phylogenetic tree of *Babesia* species, based on the 18S rRNA sequences. New sequences generated from this study are highlighted in bold.

Out of 240 dog blood samples examined, 22 indicated the occurrence of monoinfection and 12 samples indicated presence of coinfection. Monoinfection was divided into two categories, which are either a single bacterial infection or a single protozoa infection in the sample. Bacterial monoinfection in dog blood sample showed the highest infection with 21 (8.75%), followed by one protozoa monoinfection (0.42%). Coinfection in dog was divided into two categories, Bacteria + Bacteria and Bacteria + Protozoa. The coinfection between Bacteria + Protozoa showed the highest infection rate (4.17%), followed by Bacteria + Bacteria (0.83%). Protozoa + Protozoa coinfection was not found in the dog blood samples examined (Table 4.7)

*Ehrlichia canis* showed the highest monoinfection rate (7.92%) in dogs followed by A. platys (0.83%) and H. canis (0.42%). Coinfection in dogs consisted of double infection and triple infection. The double infection is comprised of four different pairs of tick-borne pathogens whereas the triple infection is comprised of two different combinations of tick-borne pathogen (Table 4.8).

Out of 140 *R. sanguineus* tick examined, 10 samples (7.14%) showed monoinfection and 1 (0.71%) sample showed coinfection. Bacterial monoinfection in *R. sanguineus* showed the highest infection with six samples (4.29%), followed by protozoa monoinfection, four samples (2.86%). Coinfection between Bacteria + Protozoa was found in one tick sample (0.71%), which tested positive with both *E. canis* and *B. gibsoni.* Bacteria + Bacteria or Protozoa + Protozoa coinfection was not found in any ticks (Table 4.9).

**Table 4.7:** Prevalence of monoinfection and coinfection in dogs.

| Dog blood (N = 240) | | |
|---|---|---|
| Type of infection | N | % |
| **Monoinfection** | 22 | 9.17 |
| Bacteria | 21 | 8.75 |
| Protozoa | 1 | 0.42 |
| **Co-infection** | 12 | 5.00 |
| Bacteria + Bacteria | 2 | 0.83 |
| Protozoa + Bacteria | 10 | 4.17 |
| Total positive infection | 34 | 14.17 |

**Table 4.8:** Tick-borne pathogens with monoinfection and coinfection in dogs.

| Dog blood (N=240) | | |
|---|---|---|
| Type of infection | n | % |
| **Monoinfection** | 22 | 9.17 |
| **Bacteria** | | |
| *Ehrlichia canis* | 19 | 7.92 |
| *Anaplasma platys* | 2 | 0.83 |
| **Protozoa** | | |
| *Hepatozoon canis* | 1 | 0.42 |
| | | |
| **Coinfection** | | |
| **Double infection** | 12 | 5.00 |
| **Bacteria + Bacteria** | | |
| *Ehrlichia canis + Anaplasma platys* | 2 | 0.83 |
| **Bacteria + Protozoa** | | |
| *Ehrlichia canis + Hepatozoon canis* | 4 | 1.67 |
| *Ehrlichia canis + Babesia vogeli* | 4 | 1.67 |
| *Anaplasma platys + Hepatozoon canis* | 2 | 0.83 |
| | | |
| **Triple infection** | 2 | 0.83 |
| *Ehrlichia canis + Anaplasma platys + Babesia vogeli* | 1 | 0.42 |
| *Ehrlichia canis + Anaplasma platys + Hepatozoon canis* | 1 | 0.42 |

**Table 4.9:** Tick-borne pathogens with monoinfection and coinfection in *R. sanguineus* ticks.

| *R. sanguineus* (N = 140) | | |
|---|---|---|
| Type of infection | n | % |
| **Monoinfection** | 10 | 7.14 |
| Bacteria | 6 | 4.29 |
| Protozoa | 4 | 2.86 |
| **Co-infection** | 1 | 0.71 |
| Protozoa + Bacteria | 1 | 0.71 |
| Total positive infection | 11 | 7.86 |

# CHAPTER 5: DISCUSSION

## 5.1 Genetic characterization of *R. sanguineus* using 16S rRNA and COI genes

Molecular characterization is the alternative way to understand the diversity of the brown dog tick species. In the past few years, genetic diversity of *R. sanguineus* from different geographic regions has been mainly investigated with the mitochondrial encoded 12S rRNA (12S) and 16S rRNA (16S) genes (Burlini et al., 2010; Levin et al., 2012; Nava et al., 2012; Chao & Shih, 2016; Hekimoğlu et al., 2016; Chitimia-Dobler et al., 2017; Dantas-Torres et al., 2017; Jones et al., 2017). The DNA barcoding cytochrome c oxidase subunit I (COI) gene, on the other hand, has been relatively little studied in *R. sanguineus* (Dantas-Torres et al., 2013; Almeida et al., 2017; Hornok et al., 2017), despite its value in discovering novel lineages in other species of *Rhipicephalus* (Burger et al., 2014; Low et al., 2015). Nevertheless, based on the data from these three mitochondrial genes, *R. sanguineus* represents three main divergent lineages: the southern lineage (temperate species), the northern lineage (tropical species), and a recently discovered the southeast European lineage. The lineage of Malaysian population, however, remains unknown.

Low amplification success rates (<10%) were found from four pairs of primers designed by Folmer et al. (1994), Shao et al. (2001), Chitimia et al. (2010) and Lv et al. (2014b), all of which have been used for amplification of the COI gene of the Ixodidae, including *R. sanguineus*. In contrast, the COI sequences of all 40 individuals of *R. sanguineus* were successfully amplified and sequenced at~550 bp region using the newly developed primers.

Failure in the COI gene amplification has been a common phenomenon in the family Ixodidae. Previous studies published elsewhere opted a nested PCR and successfully amplified the COI gene sequences of *R. microplus* and several *Haemaphysalis* species

(Low et al., 2015; Ernieenor et al., 2017). Accordingly, a nested PCR was attempted in the present study using the primers from Folmer et al. (1994), Shao et al. (2001), Chitimia et al. (2010) and Lv et al. (2014b), but the amplification success rates were not satisfactory (<10%). To solve this issue, the present study developed a new pair of primers, and successfully amplified and sequenced the COI gene fragment of *R. sanguineus* in Malaysia. Nevertheless, the efficiency of these new primers has not been tested on the *R. sanguineus* from other regions of the world. Further specificity and sensitivity studies of the primers on other populations of *R. sanguineus*, including the temperate and southeast European lineages, are warranted.

The genetic lineages of *R. sanguineus* in Southeast Asia have not been fully discovered. Based on available information, the tropical lineage of *R. sanguineus* from Thailand and Vietnam has only been identified based on limited sample size (Dantas-Torres et al., 2013). One 16S sequence of *R. sanguineus* from Singapore (unpublished data retrieved from the NCBI GenBank) is included in the present analysis and results showed that it is indeed a tropical lineage of *R. sanguineus*. In Malaysian context, the present study provided new mitochondria-encoded 16S and COI sequences of the *R. sanguineus* from eight states, representing four distinct geographic regions. Based on phylogenetic analysis, all Malaysian samples are clustered together with the tropical lineage of *R. sanguineus*, verifying the presence of this lineage, for the first time, in Malaysia.

Identification of genetically distinct taxa is of paramount importance in disease control strategies because different populations or lineages of vectors may present different susceptibility levels toward pathogen infections (Burlini et al., 2010; Low et al., 2017). Previous study demonstrated that *R. sanguineus* from certain populations of Brazil has little or no importance in the transmission of *H. canis* (Demoner et al., 2013), though

this species has been incriminated as the vector of *H. canis* worldwide. It is known that both tropical and temperate lineages of *R. sanguineus* are present in Brazil (Figure 4.1), suggesting one of the lineages is responsible for *H. canis* transmission. In Malaysian context, the *H. canis* DNA was reported in *R. sanguineus* in the present study, hence, its role in the transmission of *H. canis* in dogs cannot be ruled out.

The COI marker provides better resolution than the 16S marker in revealing genetic variability of *R. sanguineus* in Malaysia. The 16S gene is highly conserved with no genetic variation detected across all populations. The COI gene, on the other hand, revealed four unique haplotypes, namely H1, H2, H3 and H4. This result is not surprising because previous studies demonstrated the value of the COI gene in identifying novel cryptic lineage of *R. microplus* while the 16S gene showing poor resolution (Burger et al., 2014; Low et al., 2015). The genetic distances among the COI haplotypes, however, are relatively low (0.21–0.63%), confirming the occurrence of only one taxon of *R. sanguineus* in Malaysia. On the contrary, the genetic distances for COI haplotypes of *R. microplus* in Malaysia were exceptionally high (>9%), verifying the presence of multiple taxa in this species complex (Low et al., 2015).

Based on TCS analysis, haplotype H1 is inferred as the basal haplotype for *R. sanguineus* because of its prevalence in east coast, west coast and southern regions in Peninsular Malaysia. It is suggested that haplotype H1 is the common ancestor of *R. sanguineus* and evolved over time into H2, H3 and H4. The haplotype H3 is primarily confined to the state of Perlis, the northernmost of Peninsular Malaysia bordering Thailand. Thus, additional sampling efforts with increased sample size from wider geographic regions, including those from the southern Thailand will be beneficial in revealing the origin of *R. sanguineus*.

## 5.2 Prevalence of tick-borne pathogens in dogs and *R. sanguineus* ticks

*Anaplasma platys* and *E. canis* showed infection rates of 3.3 and 12.9%, in dogs, respectively. However, none of the dogs from the veterinary hospital, were tested positive with any tick-borne pathogens. The infection rates were much higher (15.7% for *A. platys* and 60.8% for *E. canis*) at the surveyed dog shelter. Previous studies using molecular approaches reported varying frequencies for *A. platys* (13–38%) and *E. canis* (0–47%) in dogs in Malaysia (Mokhtar et al., 2013; Nazari et al., 2013; Koh et al., 2016; Konto et al., 2017; Mohammed et al., 2017). Taken together, the prevalence of *E. canis* was consistently higher than *A. platys* in dogs in Malaysia, though contrasting observations have been reported in other countries such as Brazil and Cape Verde (Lasta et al., 2013; Lauzi et al., 2016).

*Anaplasma platys* (2.9%) and *E. canis* (0.7%) were found at relatively low frequencies in *R. sanguineus*. Nevertheless, a previous study showed that *E. canis* has been detected in *R. sanguineus* in Malaysia at a higher frequency (42.4%), though only 33 individuals were examined (Koh et al., 2016). Moraes-Filho et al. (2015) showed that only one of the four tested populations of *R. sanguineus* was shown to be a competent vector of *E. canis*. Hence, vector competency test on *R. sanguineus* is required to suggest its possible role in the transmission of *E. canis* in dogs in Malaysia.

In the present study, *A. platys* was detected in *R. sanguineus* for the first time, in Malaysia. Of the four positive ticks, three females were collected from two infected dogs, whereas one male was removed from a non-infected dog which suggests the source of the *A. platys* DNA may be originated from the tick itself. Nevertheless, it is also possible that the tick could also have obtained *A. platys* DNA from a previous blood meal. Earlier study was not successful in demonstrating that *R. sanguineus* could serve as the vector for *A. platys* (Simpson et al., 1991).

*R. asembonensis* with contrasting infection rates in the cat flea *C. orientis* was also reported in Low et al. (2017) and *R. sanguineus* that were collected from the same dog shelter. *R. asembonensis* was detected at very high frequency (92.3%) in *C. orientis*, while the infection rate was relatively low (1.43%) in *R. sanguineus*. The representative gltA sequence from *C. orientis* was identical to the recently reported *R. asembonensis* strain Tapes (GeneBank accession no. KX196267) from Brazil (Dall'Agnol et al., 2017), confirming the circulation of this newly reported strain in Southeast Asia. Nevertheless, the representative gltA sequence (GeneBank accession no. MF281711) from *R. sanguineus* differs from that of strain Tapes with one nucleotide change. *R. asembonensis* has been commonly reported in a wide range of insect vectors including *C. felis* and *C. orientis* (Mokhtar & Tay, 2011; Hii et al., 2015). Strikingly, this species has recently been found for the first time in *R. sanguineus* from Brazil (Dall'Agnol et al., 2017). Both *R. asembonensis* sequences generated from the present study were also highly similar (99%) to that of strain RF2125 (GenBank accession no. AF516333), a genotype that has recently been isolated from monkeys (Tay et al., 2015) and humans (Kho et al., 2016). The risk of zoonotic transmission of this genotype is yet to be determined.

In Malaysia, studies on babesiosis have been mainly on the detection of *Babesia* spp. in dogs (Rajamanickam et al., 1985; Zulkifli et al., 2011; Mokhtar et al., 2013; Mohammed et al., 2017). These studies have recorded low to moderate prevalence of *B. gibsoni* (3.3–17.7%) in Peninsular Malaysia. In contrast, a relatively high prevalence of *B. vogeli* (92.9%) has been reported from East Malaysia (i.e., Sabah). Nevertheless, occurrence of *Babesia* spp. in their potential vector *R. sanguineus*, has not been thoroughly assessed.

Using PCR assay targeting the 18S rRNA gene, only DNA of *B. vogeli* was amplified from five blood samples from dog shelter (2.08%) while DNAs of *B. vogeli* and *B. gibsoni*

were detected in two *R. sanguineus* ticks each (1.43%) from different hosts. The ticks that contained babesial DNA were removed from dogs whose blood was negative for *Babesia* infection. Hence, the source of the babesial DNA was unlikely from the parasitized blood. Neither *B. vogeli* nor *B. gibsoni* was detected in the dog blood samples obtained from veterinary hospital.

The present study provides the evidence of *B. gibsoni* and *B. vogeli* infections in both adult and nymphal stages of *R. sanguineus*, for the first time in Malaysia. Nevertheless, the mechanism of transstadial transmission of *Babesia* spp. from nymphal stage to the adult stage merits further investigation, which has also been pointed out by Chao et al. (2016, 2017).

The prevalence of *B. vogeli* in dogs from the shelter was 9.8%, whereas none of the samples from the veterinary hospital tested positive for *Babesia*. Similar findings have also been observed in a previous study in Malaysia (Zulkifli et al., 2011), but the prevalence rate in stray dogs was lower than the present study (5.8%). Stray dogs are more likely to be infected with canine babesiosis, probably because of the exposure to the environment and the tick vectors. This suggestion concurs to that proposed by da Silva et al. (2016) in which dogs with access to outside environment could have higher risk of *Babesia* infection. Further, preventive practices such as acaricidal treatment given by the dog owners may reduce the tick infestation on owned dogs. On the other hand, control of tick at a satisfying level may not feasible in a dog shelter although routine treatment has been given to our surveyed dogs from the shelter.

As far as the vectorial capacity is concerned, *R. sanguineus* is primarily known as a vector of *B. canis* (Dantas-Torres, 2008), though *Dermacentor reticulatus* has also been incriminated as a reservoir in addition to its vector role, which can maintain *B. canis* locally for several tick generations even without a vertebrate reservoir host (Földvári et

al., 2016). *B. gibsoni* has a worldwide distribution, and *R. sanguineus* has been incriminated as the main vector (Dantas-Torres, 2008), although DNA of *B. gibsoni* has also been detected in *Haemaphysalis* sp. and *Ixodes ovatus* in Japan (Inokuma et al., 2003). *B. vogeli* has also been reported in *R. sanguineus* from Taiwan, Tunisia and France (René et al., 2012; M'ghirbi & Bouttour, 2008; Chao et al., 2016). Nevertheless, *B. vogeli* has also been found in *Haemaphysalis flava* and *Haemaphysalis longicornis* in Japan (Inokuma et al., 2003), further complicating the assessment of the main vector for canine babesiosis in this region.

The present study highlights the detection of *B. gibsoni* and *B. vogeli* in both adult and nymph stages of *R. sanguineus* ticks from Malaysia, suggesting their role in the circulation of *Babesia* spp. in this region. However, different strains of ticks may have different vectorial capacity, zoonotic potential, and mechanism of transstadial transmission; hence, further study is required to clarify these issues.

While several studies have incriminated *R. sanguineus* as the main vector of *H. canis* (Baneth et al., 2007; Aktas & Özübek, 2017), a study from Brazil suggested *that R. sanguineus* may not be the vector or has little importance in the transmission of *H. canis* (Demoner et al., 2013). In addition, several tick species can also be infected with *H. canis* including *A. ovale*, *H. flavas*, *H. longicornis*, *R. microplus*, and *R. turanicus* (Murata et al., 1995; Forlano et al., 2005; de Miranda et al., 2011; Demoner et al., 2013; Giannelli et al., 2016).

*H. canis* is known to infect dogs worldwide at varying frequencies (Jittapalapong et al., 2006; de Miranda et al., 2014; Qamar et al., 2017). Although *H. canis* infection in dogs has been found in Peninsular and East Malaysia, the prevalence of this protozoan parasite in ticks has not been completely understood. One of the earlier studies on *H. canis* in Peninsular Malaysia was conventionally diagnosed using microscopic

examination of stained blood films (Rajamanickam et al., 1985). This conventional approach, however, has limited use when the occurrence of *Hepatozoon* gamonts is low because it is less sensitive in comparison to the molecular approaches (Karagenc et al., 2006). Furthermore, to diagnose the presence of *Hepatozoon* in ticks, molecular detection is the only feasible way. Using a molecular approach, *H. canis* DNA has been described in a study from an island which lies >1,400 km east of Peninsular Malaysia (Mohammed et al., 2017).

While the prevalence of *H. canis* has been documented from dogs in Malaysia (Rajamanickam et al., 1985; Mohammed et al., 2017), occurrence of *H. canis* in tick species has never been reported. The present study is a first report on the detection of *H. canis* in *R. sanguineus* in Malaysia. Of three tick samples removed from a noninfected dog from shelter, only one female tick was positive for *H. canis*, hence, the source of the *Hepatozoon* DNA may be originated from the tick itself. Generally, the present results suggested that the prevalence of *H. canis* was relatively low in both ticks and dogs. Further studies are warranted to determine the vector density, geographic distribution and host immunity against *H. canis* infection.

Tick natural life cycle which may include multiple hosts could predispose it to be coinfected with several pathogens, with high likelihood of co-transmission to humans or animals (Moutailler et al., 2016). In the context of canine tick-borne diseases, awareness of co-infection is of paramount importance in clinical practice because simultaneous or sequential infection with multiple pathogens can alter various pathophysiological parameters in dogs (Gaunt et al., 2010). Furthermore, infection by two or more pathogens may lead to overlapping or atypical clinical signs (Otranto et al., 2010). While the co-infection of *Anaplasma* and *Ehrlichia* has been commonly reported in dogs (Diniz et al., 2010; Silveira et al., 2015), co-infection of these bacteria with other tick-borne protozoa

such as *Hepatozoon* and *Babesia* species has been scarcely reported in their primary ectoparasite—*R. sanguineus*. Given that co-infection in dogs and ticks might be more complicated than we thought, co-occurrence of Anaplasmataceae with other tick-borne protozoa in dogs and ticks is reported in this study.

The co-occurrences of these Anaplasmataceae agents with other tick-borne protozoa such as *E. canis*+*B. vogeli* (Rotondano et al., 2015; Alho et al., 2017), *A. platys* + *B. vogeli* and *A. platys*+*H. canis* (Cardoso et al., 2016; Licari et al., 2017) have also been documented. In the present study, co-occurrence of tick-borne bacteria and protozoa up to triple infection (*E. canis*+ *A. platys*+*B. vogeli* and *E. canis*+*A. platys* +*H. canis*) was observed in the examined dogs.

Co-infection of pathogens in ticks has not been extensively studied. There have been only few studies reported the co-infections of *E. canis*+ *A. phagocytophilum*, *E. canis*+*E. chafeensis* and *Leishmania infantum*+*E. canis*+*H. canis* in *R. sanguineus* (Gonçalves et al., 2014; Koh et al., 2016). In the present study, co-infection of *E. canis*+ *B. gibsoni* was detected in one tick sample removed from the *E. canis* infected dog. Nevertheless, the babesial DNA positive tick was removed from a dog whose blood was negative for *Babesia* infection.

Our results also highlight the importance of co-infections of tick-borne pathogens in dogs because some of the clinical signs of infection might be overlapped and the treatments could be different, especially in the case of infection with both bacteria and protozoa. Further studies on their clinical signs and treatment efficacy are warranted. Moreover, co-infection of pathogens in ticks is of great concern, but the possibility of co-transmission to the host is also worthy of further investigation.

# CHAPTER 6: CONCLUSION

In conclusion, the present study has successfully amplified and sequenced the COI gene fragment of *R. sanguineus* using a novel pair of primers, providing an alternative solution for COI gene amplification in the family Ixodidae. The COI marker provides better resolution than the 16S marker in revealing genetic variability of *R. sanguineus* in Malaysia. The 16S gene is highly conserved with no genetic variation detected across all populations. The genetic distances among the COI haplotypes are relatively low (0.21–0.63%), confirming the occurrence of only one taxon of *R. sanguineus* in Malaysia. The COI gene, on the other hand, revealed four unique haplotypes. The data presented in the study also validated the presence of a single taxon belonging to the tropical lineage also known as the northern lineage, for the first time, within this species complex in Peninsular Malaysia.

In this study, four different tick-borne pathogens were observed in dog blood samples and six tick-borne pathogens were observed in *R. sanguineus* sample. The highest prevalence of tick-borne pathogen in dogs was *E. canis* with a total of 31 positive (12.92%), followed by *H. canis*, *A. platys*, and *B. canis*. Rickettsia DNA was not detected in any dog blood samples. The highest prevalence of tick-borne pathogen in *R. sanguineus* ticks was *A. platys* with four positive samples (2.86%), followed by *R. asembonensis, B. gibsoni*, *B. vogeli*, *E. canis* and *H. canis.* In this study, *A. platys* and *R. asembonensis* was detected in *R. sanguineus* for the first time, in Malaysia. This study also represents the first evidence of both *B. gibsoni* and *B. vogeli* infections in *R. sanguineus* in Malaysia. None of the dogs from the veterinary hospital were tested positive with any tick-borne pathogens. In this study, 22 dog blood samples indicated the occurrence of monoinfection and 12 sample indicated the presence of coinfection. Of *R. sanguineus* tick examined, 10 samples (7.14%) showed monoinfection and 1 (0.71%) sample showed coinfection. The research objectives of this study were achieved.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Publications

1. Low, L. V., **Prakash, B. K**., Lim, Y. A. L., Tan, T. K., Vinnie-Siow, W. Y., Sofian-Azirun, M., & AbuBakar, S. (2018). Detection of Anaplasmataceae agents and co-infection with other tick-borne protozoa in dogs and *Rhipicephalus sanguineus* sensu lato ticks. *Experimental and Applied Acarology*, *75*, 429-435.

2. Low, V. L., & **Prakash, B. K.** (2018). First genetic characterization of the brown dog tick *Rhipicephalus sanguineus* sensu lato in Peninsular Malaysia. *Experimental and Applied Acarology*, *75*, 299-307.

3. **Prakash, B. K.**, Low, V. L., Tan. T. K., Vinnie-Siow, W. Y., Lim, Y. A. L., Morvarid, A. R., … & Sofian-Azirun. M. (2018). Detection of *Hepatozoon canis* in the brown dog tick and domestic dogs in Peninsular Malaysia. *Journal of Medical Entomology, 55*(5), 1346-1348.

4. **Prakash, B. K.**, Low, V. L., Vinnie-Siow, W. Y., Tan. K., Lim, Y. A. L., Morvarid, A. R., … & Sofian-Azirun, M. (2018). Detection of *Babesia* spp. in dogs and their ticks from Peninsular Malaysia: Emphasis on *Babesia gibsoni* and *Babesia vogeli* infections in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae). *Journal of Medical Entomology*, *55*(5), 1337-1340.

5. Low, V. L., **Prakash, B. K.**, Tan. T. K., Sofian-Azirun, M., Anwar, F. H. K., Vinnie-Siow, W. Y., & AbuBakar, S. (2017). Pathogens in ectoparasites from free-ranging animals: Infections with *Rickettsia asembonensis* in ticks, and a potentially new species of *Diplylidium* in fleas and lice. *Veterinary Parasitology*, *245*, 102-105.

**Off-shoot publications during the candidature**

**1.** Low, V. L., Norma-Rashid, Y., Ubaidillah, R., Yusoff, A., **Prakash, B. K.**, Tan, T. K., … & Sofian-Azirun, M. (2018) Does colour matter? Genetic differentiation of colour morphs of the Asian golden web spider *Nephila antipodiana* (Araneae: Nephilidae). *Animal Biology*, *68*, 417-427.

**2.** Low, V. L., Norma-Rashid, Y., Yusoff, A., Vinnie-Siow, W. Y., **Prakash, B. K.**, Tan, T. K., … & Sofian-Azirun, M. (2017). Pleistocene demographic expansion and high gene flow in the Globe Skimmer dragonfly *Pantala flavescens* Fabricius (Odonata: Libellulidae) in Peninsular Malaysia. *Zoologischer Anzeiger*, *266*, 23-27.

**3.** Low, V. L., Tan, T. K., **Prakash, B. K.**, Vinnie-Siow, W. Y., Tay, S. T., Masmeatathip, R., … & Sofian-Azirun, M. (2017). Contrasting evolutionary patterns between two haplogroups of *Haematobia exigua* (Diptera: Muscidae) from the mainland and islands of Southeast Asia. *Scientific Reports*, *7*, 5871.

**Poster Presentation**

**1.** Low, V. L., **Prakash, B. K.**, Tan, T. K., Sofian-Azirun, M., Anwar, F. H. K., & Abu Bakar, S. (2017). Pathogens associated with ectoparasites from stray animal: highlighting the infections of *Rickettsia asembonensis* in ticks, and a potentially new *Dipylidium* tapeworm species in fleas and lice, 9th Tick and Tick-borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. 2017-08-27 to 2017-09-01, TTP9 Convenors. (International).