ความหลากหลายและการติดเชื้อในเห็บ ภายในเขตห้ามล่าสัตว์ป่าเขาชีโอนและ
สถานีเพาะเลี้ยงสัตว์ป่าบางละมุง จังหวัดชลบุรี ประเทศไทย

นางจันทรา วัฒนะเมธานนท์

จุฬาลงกรณ์มหาวิทยาลัย
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TICK DIVERSITY AND PATHOGEN INFECTION IN KHAO CHI-ON NON HUNTING AREA AND BANGLAMUNG WILDLIFE BREEDING CENTER, CHONBURI PROVINCE, THAILAND

Mrs. Juntra Wattanamethanont

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
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Thesis Title	TICK DIVERSITY AND PATHOGEN INFECTION IN KHAO CHI-ON NON HUNTING AREA AND BANGLAMUNG WILDLIFE BREEDING CENTER, CHONBURI PROVINCE, THAILAND
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Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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จันทรา วัฒนะเมธานนท์ : ความหลากหลายและการติดเชื้อในเห็บ ภายในเขตห้ามล่าสัตว์ป่าเขาชีโอนและสถานีเพาะเลี้ยงสัตว์ป่าบางละมุง จังหวัดชลบุรี ประเทศไทย (TICK DIVERSITY AND PATHOGEN INFECTION IN KHAO CHI-ON NON HUNTING AREA AND BANGLAMUNG WILDLIFE BREEDING CENTER, CHONBURI PROVINCE, THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สนธยา เตียวศิริทรัพย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. มรกต แก้วธรรมสอน, 88 หน้า.

เห็บแข็งเป็นพาหะนำโรคที่สำคัญของมนุษย์และสัตว์ โดยมีสัตว์ป่าเป็นแหล่งรังโรค การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อสำรวจความหลากหลายของชนิดเห็บ ตลอดจนเชื้อโปรโตซัวและแบคทีเรียที่ตรวจพบในเห็บภายในป่าที่เป็นแหล่งอาศัยของสัตว์ป่าด้วยวิธีทางอณูชีววิทยา (PCR และ DNA sequencing) ในปี พ.ศ. 2558 ดำเนินการเก็บตัวอย่างเห็บในระยะ questing ในบริเวณพื้นที่เขตห้ามล่าสัตว์ป่าเขาชีโอนและสถานีเพาะเลี้ยงสัตว์ป่าบางละมุง จ.ชลบุรี ได้ตัวอย่างเห็บทั้งสิ้น 12,184 ตัว จำแนกได้ 3 ชนิด คือ *Haemaphysalis lagrangei* (92.82%), *Haemaphysalis wellingtoni* (0.13%) และ *Rhipicephalus* (*Boophilus*) *microplus* (7.05%) ด้วยวิธีการจำแนกจากรูปร่างลักษณะภายนอก ร่วมกับวิธี PCR ที่จำเพาะต่อยีน mitochondrial 16S rRNA ของเห็บ จากตัวอย่างเห็บจำนวน 419 ตัวอย่าง (ระยะเต็มวัยเพศผู้และเพศเมีย ระยะกลางวัย และกลุ่มของระยะตัวอ่อน) ได้ทำการตรวจหาเชื้อโปรโตซัวด้วยวิธี PCR ที่จำเพาะต่อยีน 18S rRNA ของ *Babesia* และ *Theileria* และตรวจหาเชื้อแบคทีเรียด้วยวิธี PCR ที่จำเพาะต่อยีน 16S rRNA ของแบคทีเรียในแฟมิลี่ Anaplasmataceae โดยตรวจพบเชื้อทั้งหมดจำนวน 5 สกุล คือ *Anaplasma* โดยพบอัตราการติดเชื้อในเห็บสูงที่สุดคือ 55.61% (233/419) และพบว่ามี 3 genotype group ได้แก่ *Anaplasma* ที่มีความใกล้เคียงกับ *A. bovis*, *A. platys* และ unidentified *Anaplasma* รองลงมา คือ *Theileria* โดยพบอัตราการติดเชื้อในเห็บ 4.30% (18/419) ซึ่งมี 3 genotype group ได้แก่ *Theileria* ที่มีความใกล้เคียงกับ *T. cervi*, *T. capreoli* และ unidentified *Theileria* และอีก 3 สกุลนั้นพบในเห็บเพียงจำนวน 1 ตัวอย่าง (1/419; 0.24%) ได้แก่ *Babesia*, *Ehrlichia* และ *Wolbachia* จากการศึกษานี้ทำให้ทราบความหลากหลายของชนิดเห็บ และความสัมพันธ์กับการตรวจพบเชื้อโปรโตซัวและแบคทีเรียที่นำโดยเห็บ เพื่อบ่งชี้ถึงความเป็นไปได้ในการเป็นพาหะนำโรคของเห็บภายในป่าที่เป็นแหล่งอาศัยของสัตว์ป่าในประเทศไทย ข้อมูลเหล่านี้สามารถนำมาใช้ประโยชน์ในการนำไปสู่กระบวนการรักษา ป้องกัน และควบคุมโรคติดต่อที่นำโดยเห็บต่อไป

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5775303631 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: TICKS, PROTOZOA, BACTERIA, WILDLIFE

JUNTRA WATTANAMETHANONT: TICK DIVERSITY AND PATHOGEN INFECTION IN KHAO CHI-ON NON HUNTING AREA AND BANGLAMUNG WILDLIFE BREEDING CENTER, CHONBURI PROVINCE, THAILAND. ADVISOR: ASSOC. PROF. SONTHAYA TIAWSIRISUP, Ph.D., CO-ADVISOR: ASST. PROF. MORAKOT KAEWTHAMASORN, Ph.D., 88 pp.

Ixodid ticks are important vectors in the transmission cycle of various tick-borne diseases in both humans and animals. Wildlife are considered important reservoirs of these diseases. This study aims to examine tick diversity, and tick-borne protozoal and bacterial infection in questing ticks in wildlife habitats by using PCR and DNA sequencing techniques. In 2015, questing ixodid ticks were collected from Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center, Chonburi province. All 12,184 ticks were identified using morphological characteristics and representative ticks were confirmed species by PCR that targeted tick mitochondrial 16S rRNA gene. Three tick species were identified including *Haemaphysalis lagrangei* (92.82%), *H. wellingtoni* (0.13%), and *Rhipicephalus* (*Boophilus*) *microplus* (7.05%). Totally, 419 tick samples (Individual adult and nymph, and pooled larvae) were examined by using PCR which amplified a fragment of 18S rRNA gene of *Babesia* and *Theileria*, and 16S rRNA gene of bacteria in family Anaplasmataceae. Out of 419 analyzed tick samples, four pathogens and one bacterial symbiont were detected, with the highest infection rate of *Anaplasma* spp. at 55.61% (233/419) with three genotype groups including *Anaplasma* closely related to *A. bovis*, *A. platys*, and unidentified *Anaplasma*. While *Theileria* spp. had lower infection rate at 4.30% (18/419) with three genotypes consisted of *Theileria* closely related to *T. cervi*, *T. capreoli*, and unidentified *Theileria*, and only one sequence (1/419; 0.24%) of *Babesia* spp., *Ehrlichia* spp. and *Wolbachia* spp. were detected. These findings provided the information of tick diversity in wildlife habitats, and the association with detected tick-borne protozoa and bacteria. It suggested the evidences that these tick species are possible vectors to transmit tick-borne diseases in forest of Thailand. Furthermore, these data would be useful for designing treatment, prevention, and control strategies of tick-borne diseases.

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Department:	Veterinary Pathology	Student's Signature	.....
Field of Study:	Veterinary Pathobiology	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CONTENTS

Page

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Page

Page

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF FIGURES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
bp	=	Base pair
CI	=	Confidence interval
°C	=	Degree Celsius
mm	=	Millimeter
PCR	=	Polymerase chain reaction
RH	=	Relative humidity
Temp	=	Temperature

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CHAPTER I
INTRODUCTION

1. Importance and rationale

Ticks in family Ixodidae (hard ticks) are blood-sucking arthropods, second only to mosquitoes in their medical and veterinary importance (Sonenshine et al., 2002). The wide variety of ixodid tick species comprises more than 700 species and distribution worldwide (Russell et al., 2013). Ixodid ticks are often the causes of health problems in various vertebrate host and occasionally in humans. Moreover, Ixodid ticks are important biological vectors in the transmission cycle of various diseases, including viral, bacterial, protozoal, and rickettsial diseases, in both humans and animals and most of which are zoonotic diseases. The incidence of tick-borne diseases is highly due to the global climate changes leading to increased survival and reproductive rates of ticks that influence rapidly and broadly transmission dynamics of tick-borne disease worldwide (Ogden and Lindsay, 2016).

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Ixodid ticks are ectoparasites that have four stages in their life cycle including egg, larva, nymph, and adult. Both male and female ticks require a blood meal for their development in each stage and for oviposition in female adults. Long time life cycle of ticks may spend from several months to even years and can survive a long time without having a blood meal and quite resistant to eradicate. Most ticks spend the majority of their life cycle off the host in environment (Sonenshine et al., 2002).

Ticks have the ability to widely spread diseases via blood sucking on various hosts (Slodki et al., 2011) and they can feed on different host species in their life (Nava and Guglielmone, 2013). By the time that ticks attach to their host and continue long time blood feeding, ticks modulate host hemostatic, inflammatory, and immune systems via the secreted components in tick saliva to facilitate blood feeding and influence transmission of tick-borne diseases (Anderson and Valenzuela, 2008). Wild animals are considered to be reservoirs of tick-borne diseases and may remain asymptomatic, but only if an immune suppression occurs, animals become ill and causing death (Fyumagwa et al., 2011). These reservoirs could act as a source of infection for humans and domestic animals (Jongejan and Uilenberg, 2004; Duscher et al., 2015). In another way, livestock could also transmit the diseases to wildlife (Daszak et al., 2001). Tick-borne diseases are recognized in both veterinary and medical importance that could affect animal and human health problems, including economic loss to treatment and control the diseases as well as wildlife conservatives because some species likely to be extinct.

Tick-borne diseases are distributed worldwide, including Thailand (Ahantarig et al., 2008). Tick-borne protozoa and bacteria can cause the diseases in various vertebrate animals, including livestock, domestic, wild animals, and humans. Ixodid ticks are the main biological vectors in disease transmission which allow the pathogen to multiply in their body and could be both transstadial and transovarial transmission, which all life stages of tick could involve in disease transmission cycle (Klompen, 2005).

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There were the evidences indicated that Ixodid ticks have been associated with the transmission of tick-borne protozoal and bacterial diseases (Dantas-Torres et al., 2012). Tick-borne protozoal and bacterial diseases of veterinary importance, including protozoa of the genera *Babesia* and *Theileria*, and bacteria of the genera *Ehrlichia* and *Anaplasma*, can cause severe anemia and death in infected animals (McQuiston et al., 2003; Atif, 2016). Some tick-borne protozoa and bacteria considerable medical importance, including *Babesia microti* and *Babesia divergens* that cause human babesiosis, *Ehrlichia chaffiensis* that cause human monocytic ehrlichiosis (HME), and *Anaplasma phagocytophilum* that cause human granulocytic anaplasmosis (HGA). Wild animals are considered important reservoirs of these diseases (McQuiston et al., 2003; Penzhorn, 2006; Yabsley and Shock, 2013; Mans et al., 2015).

As ticks have the ability to widely spread diseases to other hosts; therefore, diagnostic techniques are the important tools for demonstrate disease situations. Recently, polymerase chain reaction (PCR) was a comprehensive technique that amplify of target DNA. It was applied to use for species identification and pathogen detection in ticks with rapid, high sensitivity, and specificity, even though the small amounts of pathogen in ticks, destroyed specimens, or mixed pathogen populations (Sparagano et al., 1999).

Khao Chi-On Non Hunting Area is the protected forest area for nature and wildlife conservation that various wild animals live inside with the high abundance of deer (Aemsang, 2008) that may serve as reservoir hosts. Moreover, there are several

human outdoor activities in this area, including camping and meditation that may have a possibility to contact among wild animals, human, and ticks.

Although much work had been done in foreign countries, but a few data about pathogens detected in questing ticks in Thailand have been reported. This study aims to examine tick diversity and tick-borne pathogen infection in questing ticks in Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center, Chonburi province, Thailand by using PCR and DNA sequencing techniques to test the hypotheses that tick-borne protozoa and bacteria can be detected in questing ticks. The findings will provide the information of ixodid tick species in wildlife habitats and tick-borne protozoa and bacteria detected, including seasonal relationships which yields a better understanding of tick ecology and will be valuable for predicting association of questing ticks and important tick-borne protozoal and bacterial diseases and their possibility to play an important role in disease transmission cycle. Furthermore, this data will be useful for designing treatment, prevention, and control strategies.

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2. Objectives of study

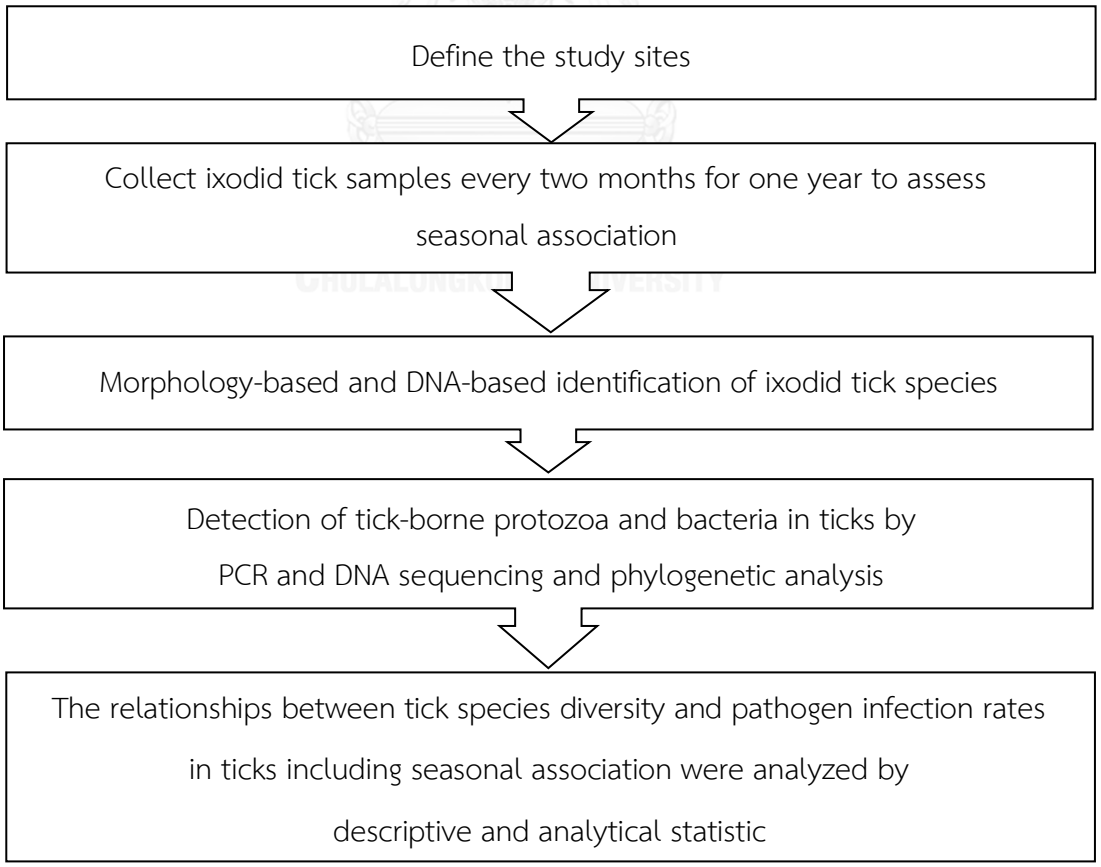
To examine tick diversity and tick-borne pathogen infection in questing ticks in Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center, Chonburi province, Thailand by using PCR and DNA sequencing techniques.

3. Hypothesis

Tick-borne protozoa and bacteria can be detected in questing ticks by using PCR and DNA sequencing techniques.

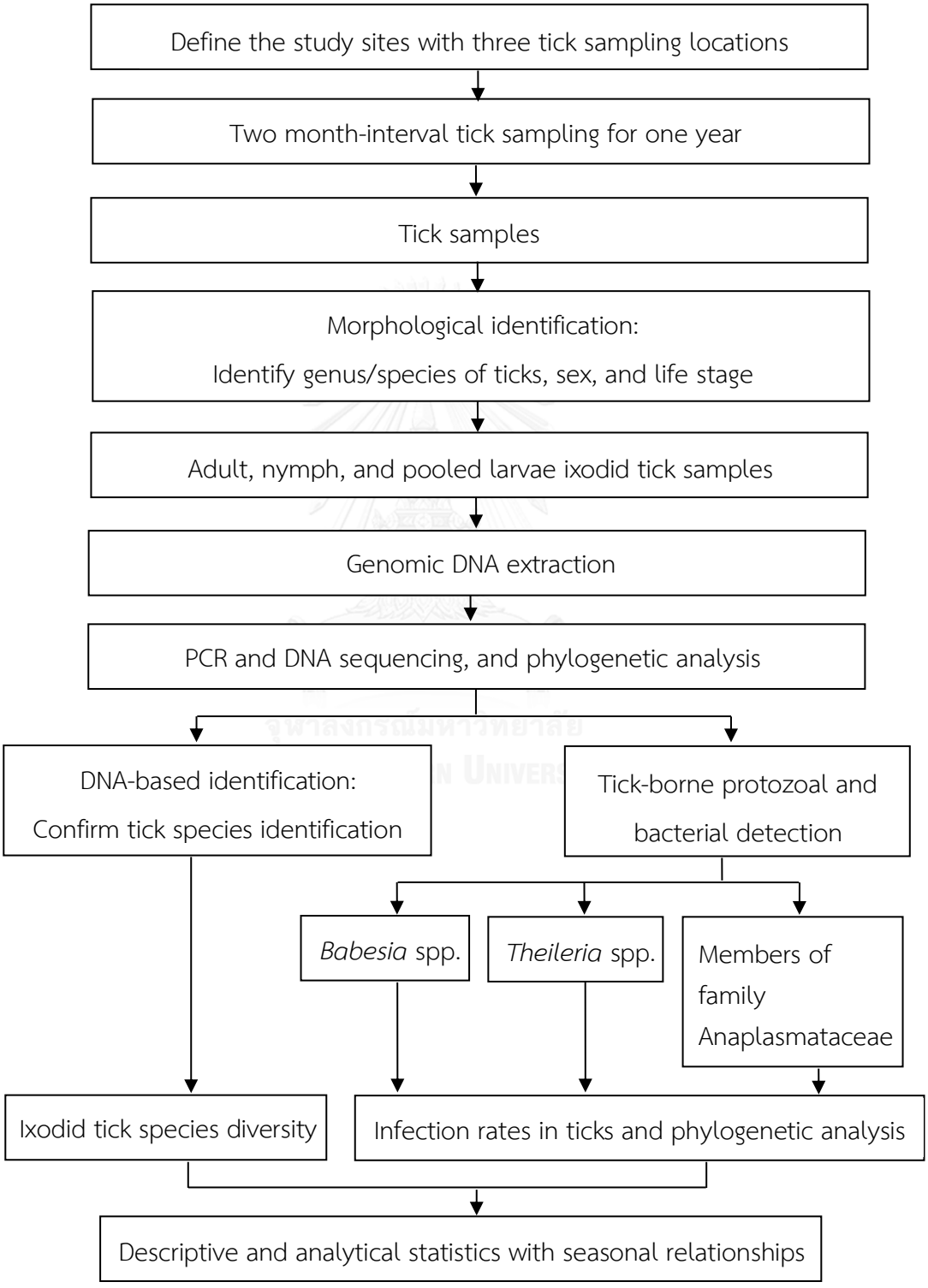
4. Conceptual framework

"Tick diversity and pathogen infection in Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center, Chonburi province, Thailand"



5. Research plan

"Tick diversity and pathogen infection in Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center, Chonburi province, Thailand"



CHAPTER II
LITERATURE REVIEW

1. Ixodid ticks

1.1 Tick biology and life cycle

Ticks are ectoparasites belonging to phylum Arthropoda same as insects but classified in class Arachnida and subclass Acari. Ticks, especially in family Ixodidae (hard ticks) commonly cause the problems to livestock, domestic, and wild animals, including humans. Ticks can harm their hosts directly by blood sucking causing discomfort and irritation, injury, allergic reaction, and tick paralysis. In heavy tick infestation, animals will be anemia due to severe blood loss, causing poor health and loss of production. Moreover, the significant medical and veterinary importance of ticks is indirectly serve as a vector to transmit tick-borne diseases, including viral, bacterial, protozoal, and rickettsial diseases (Sonenshine and Roe, 2013).

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There are four stages in a tick's life cycle; egg, larva, nymph, and adult. Both male and female ticks require blood meal from host for development in each stage and for oviposition in female adults. The long life cycle of ticks may take up to several months or even years, depending on species of ticks and environmental factors such as temperature and humidity. They can survive for a long time without having blood meals and quite resistant to eradicate (Sonenshine et al., 2002). Ixodid ticks have three basic life cycles (Figure 1). i) Three-host life cycle which larva, nymph, and adults feed on different hosts to complete their life cycle. After egg laying, the larvae disperse into

the vegetation to seek hosts. The larvae attached to the host, blood-feed until they are engorged, drop off the host, and molt to nymph in ground. Questing nymph in vegetation will seek the second host. After blood feeding, the engorged nymph will drop off the host and molt to adult in environment. Questing adult will seek and attach the third host for blood-feeding and mating, and female will drop off for oviposition in environment. This pattern was found in most of ixodid ticks, including most species of *Amblyomma, Haemaphysalis, Ixodes, Rhipicephalus*, and *Hyalomma*. ii) Two-host life cycle is the pattern that requires two different hosts in their life cycle, which both larva and nymph feed on the same host, while questing adult will seek and feed on the another host. This pattern was found in some species of *Rhipicephalus*. iii) One-host life cycle, which all stages of ticks, including larva, nymph, and adult are blood-feeding, developing and mating on a single host. This pattern was found in some species of *Rhipicephalus* (*Boophilus*) (Arthur, 1962; Sonenshine et al., 2002).

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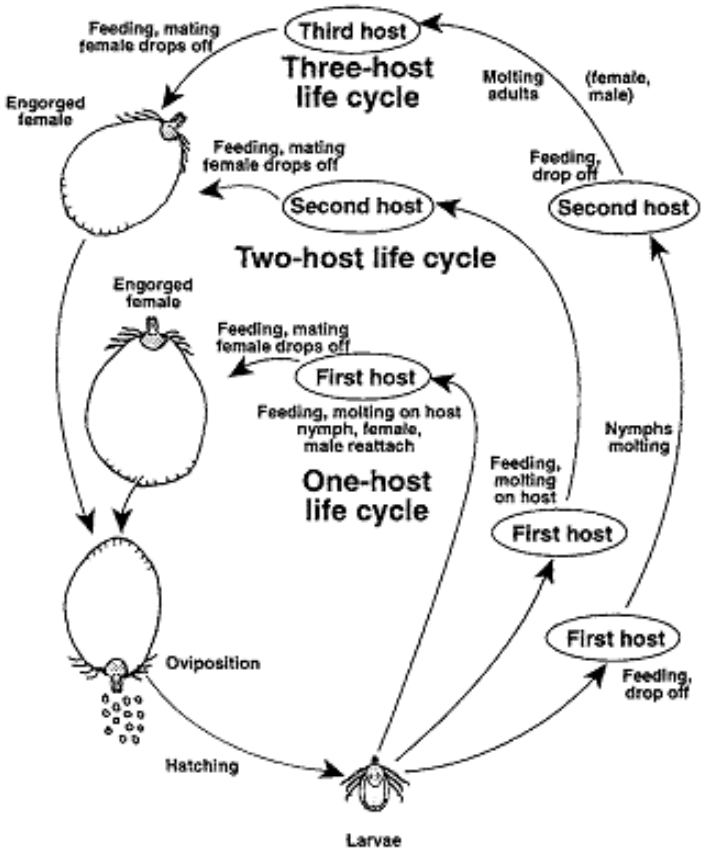

Figure 1. Three basic life cycle of ixodid ticks (Sonenshine et al., 2002)

1.2 Tick diversity in Thailand

Over 100 species of ticks were recognized in Southeast Asia, including Thailand (Petney, 1993). The listing of all ixodid tick species found in Thailand, comprise 10 genera and define into 53 species were gathered up and published by Tanskul et al. (1983). Some previous studies have reported the ixodid tick species diversity in forest in Thailand. Hirunkanokpun et al. (2003) studied the tick species in Khao Yai National Park, Nakhon Ratchasima province and Khao Rue Nai Wildlife Sanctuary, Chachoengsao province. Species of ticks found in this study, including *Amblyomma testudinarium, A. javanense, A. geoemydae, Haemaphysalis ornithophila H. shimoca, H. Cornigera,*

H. obesa, H. lagrangei, Dermacentor auratus, and *D. astrosignatus*. Grassman et al. (2004) reported the six species of ixodid ticks found in wild carnivores of the Phu Khieo Wildlife Sanctuary, Chaiyaphum province, including *Amblyomma testudinarium*, *Haemaphysalis asiatica, H. hystricis, H. semermis, Rhipicephalus haemaphysaloides*, and *Ixodes granulatus*. According to Cornet et al. (2009), the spatial distribution maps of various tick species in the genera *Amblyomma*, *Rhipicephalus*, *Rhipicephalus* (*Boophilus*), *Dermacentor*, and *Aponomma* were demonstrated in this study. Gogkhuntod et al. (2013) explored a variety of hard tick species in the dry evergreen forest in Nakhon Ratchasima province, five genera of ixodid ticks were found, including *Haemaphysalis, Dermacentor, Rhipicephalus, Ixodes,* and *Amblyomma*.

1.3 Tick species identification

The techniques used for tick species identification consist of morphology-based and DNA-based techniques. Morphology-based technique can be used to identify the ticks in genus and species level but it has its limitations because of many variations in morphology of ticks, morphologically similar species, and detailed morphological descriptions of the immature stages remain unknown for most tick species. Recently, DNA-based technique was defined more accurately than morphology-based technique in case of physical damaged ticks, engorged with blood, the variation within and among tick species, and immature stage of ticks (egg, larvae or nymphs) which were difficult to identify by using only morphological characteristics

(Jizhou et al., 2014). Many molecular markers such as mitochondrial 12S/16S rDNA, cytochrome oxidase subunit I (COI), nuclear ribosomal internal transcribed spacer 1 (ITS1), and nuclear ribosomal internal transcribed spacer 2 (ITS2) were used. However, mitochondrial 16S ribosomal RNA gene have been widely used in species identification of ticks to confirm morphological data, even though morphology similar species of ticks (Black and Piesman, 1994; Caporale et al., 1995)

2. Tick-borne protozoa and bacteria

Tick-borne protozoal and bacterial haemoparasites of veterinary importance, including *Babesia*, *Theileria*, *Ehrlichia*, and *Anaplasma*. Wild animals are considered important reservoirs of these diseases (McQuiston et al., 2003; Yabsley and Shock, 2013; Mans et al., 2015). Ixodid ticks are the main biological vectors for disease transmission which the pathogen can multiply in their body (Klompen, 2005). *Babesia* and *Theileria* are usually transmitted biologically, while mechanical transmission is another possible mode by fomites and mechanical vectors such as biting flies but the multiplication of pathogen do not occurred in these vector (Hofmann-Lehmann et al., 2004).

2.1 Tick-borne protozoa

Babesia and *Theileria* are tick-borne protozoan haemoparasites that infect a wide variety of vertebrate host. Both *Babesia* and *Theileria* are belong to the phylum Apicomplexa, class Piroplasmea and order Piroplasmida. The differences between

Babesia and *Theileria* are that *Babesia* sporozoite penetrate erythrocytes directly and all the parasitic stages develop in erythrocytes, whereas *Theileria* sporozoite enter lymphocytes (or macrophages) in which reproduce and infect another erythrocytes (Uilenberg, 2006). Early studies were differentiated between *Babesia* and *Theileria* mainly based on basic life cycle and size of trophozoite stage, however concordance with phylogenetic classification based on 16S rRNA gene, Piroplasmida were divided into 6 distinct clades (Schnittger et al., 2012) (Figure 2).

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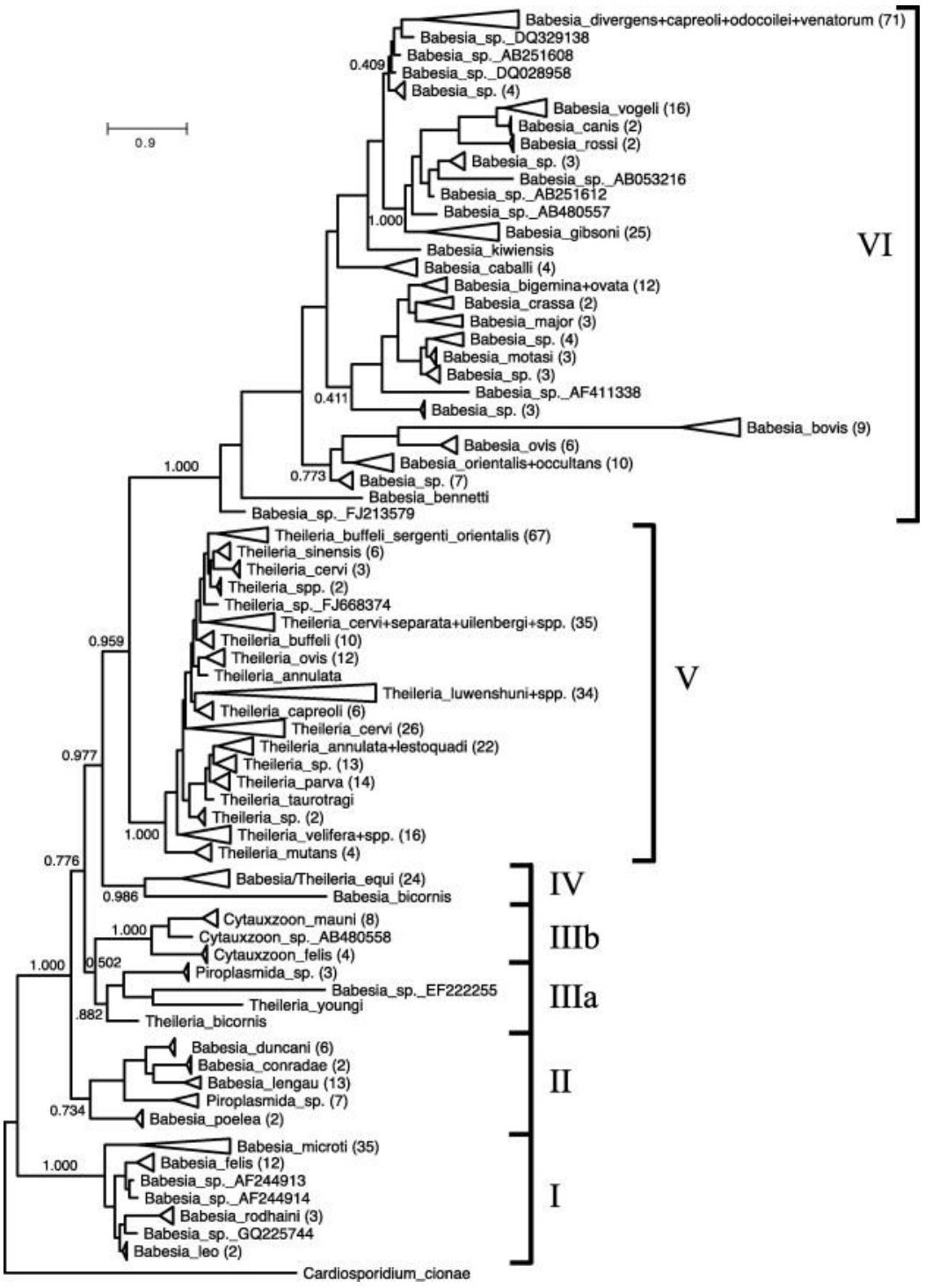

Figure 2. Molecular phylogenetic tree of 18S rRNA sequences of the piroplasmida (Schnittger et al., 2012)

2.1.1 *Babesia* spp.

Babesiosis is caused by piroplasmids in the genus *Babesia* belonged to the phylum Apicomplexa, which has pear-shaped appearance within infected erythrocytes. The multiplication of *Babesia* merozoites in erythrocytes, result in changing erythrocyte membrane fragility which causes hemolytic anemia and some clinical signs such as hemoglobinemia and hemoglobinuria (Kocan and Waldrup, 2001). Babesiosis can affect a wide ranges of livestock, domestic, and wild animals, and occasionally humans. Babesial infection in wildlife varies from subclinical to have clinical symptoms of fever, hemolytic anemia, and be fatal in low immunity or stress animals (Schnittger et al., 2012). *Babesia bovis* and *B. bigemina* are the two most important species infecting domestic and wild ruminants. Members of the genus *Babesia* are biologically transmitted by ixodid ticks in the genus *Rhipicephalus* (*Boophilus*), *Dermacentor, Haemaphysalis, Hyalomma, Ixodes,* and *Rhipicephalus* (Telford III et al., 1993)

Life cycle of *Babesia* consists of merogony, gametogony, and sporogony (Figure 3). *Babesia* sporozoites enter host blood streams to invade erythrocytes and develop to trophozoites. Trophozoites divide by binary fission (merogony) into two merozoites, which leave the host cells to infect another erythrocyte. Some merozoites develop to gametocytes, which transfer to tick vector during blood feeding. In the tick gut, gametocyte develop into ray bodies or "Strahlenkörper" (gametogony). Fusion of two different types of ray bodies to form a zygote and develop to motile kinetes that migrate to hemolymph and invade various organ of tick. The members of *Babesia*

microti-like groups are only transmit from one stage to the next stage (transstadially). However members of *Babesia* sensu stricto groups, which compose mainly *Babesia* spp., including *Babesia* spp. in ungulate and cattle, can be both transstadially and transovarially transmitted through their kinetes. They can infect ovary and eggs to transmit to the next generation of ticks. A kinete invades salivary gland and multiplies to form multinucleated sporoblast (sporogony). At the time tick attaches the new host, mature sporozoites will be transmit to another host via tick saliva during blood feeding (Uilenberg, 2006; Hunfeld et al., 2008).

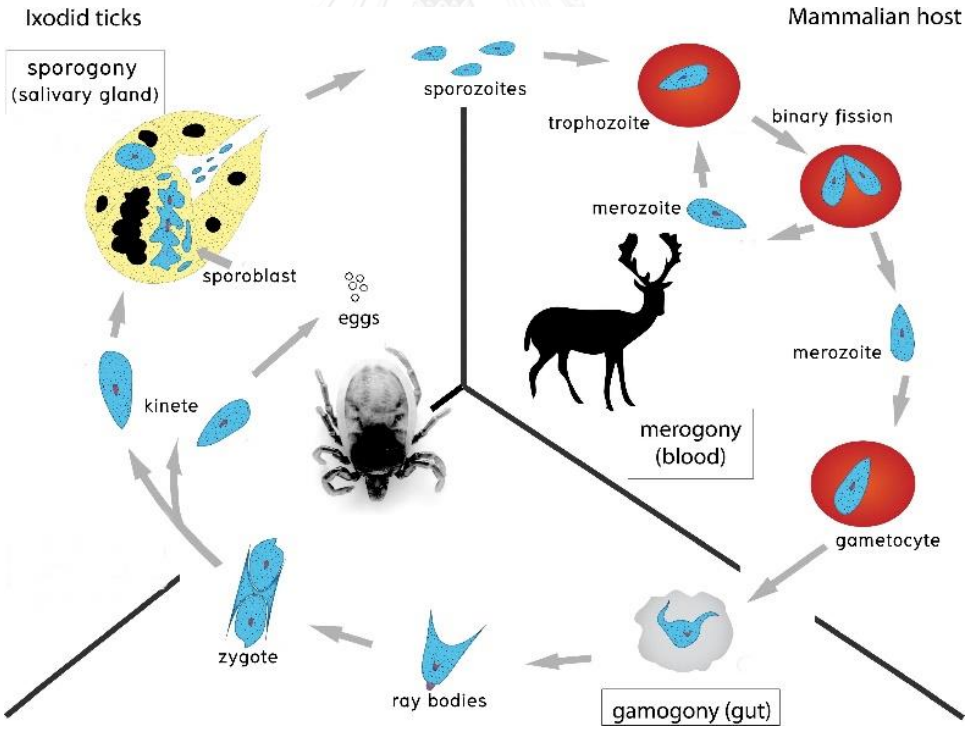

Figure 3. Life cycle of *Babesia* spp. (Hunfeld et al., 2008)

2.1.2 *Theileria* spp.

Theileria are piroplasms which polymorphic and vary in size and shape within infected host erythrocytes and the primary hosts were wild and domestic ruminants (Bishop et al., 2004). Clinical signs depend on species of *Theileria*. In Asia including Thailand, they are non-transforming *Theileria* species, which clinical sign are benign than transforming group (Sugimoto and Fujisaki, 2002). *Theileria* has been economically important in domestic ruminants due to anemia and loss of production, whereas wild ruminants are usually asymptomatic and serve as a source of infection (Bishop et al., 2004). *Theileria cervi* and *Theileria capreoli* were *Theileria* species originally found in a wide variety of deer and might be a reservoir for infection in domestic animals with varies in clinical signs from benign to moderately pathogenic (Li et al., 2014; Liu et al., 2016). Although *T. cervi* was considered a benign infection in cervids and commonly found in healthy deer, but in case of severe parasitemia clinical sign could be occur including anemia and icterus (Yabsley et al., 2005). Ticks in the genus *Rhipicephalus, Amblyomma, Hyalomma*, and *Haemaphysalis* are known to be the vector of this pathogen (Mans et al., 2015) and only transstadial transmission of *Theileria* known to be occur in biological vector ticks.

Life cycle of *Theileria* consists of merogony, gametogony, and sporogony (Figure 4). *Theileria* sporozoites enter lymphoblast to form a schizont. In transforming *Theileria* species, during dividing of lymphocytes in the transformation process, the schizont is also divided. Some schizont differentiate into numerous merozoites

(merogony) and merozoites are then released to infect erythrocytes. When infected erythrocytes are ingested by tick vector, the parasites will differentiate into male and female gamonts (gametogony) in tick gut and they will fertilize to form a zygote. The zygotes invade gut epithelial cell and differentiate to a kinete. Kinetes escape from the gut cells to hemolymph and invade salivary gland to form multinucleated sporoblast (sporogony). During blood feeding, sporoblast will multiply to produce numerous sporozoites for infecting the new host (Mans et al., 2015).

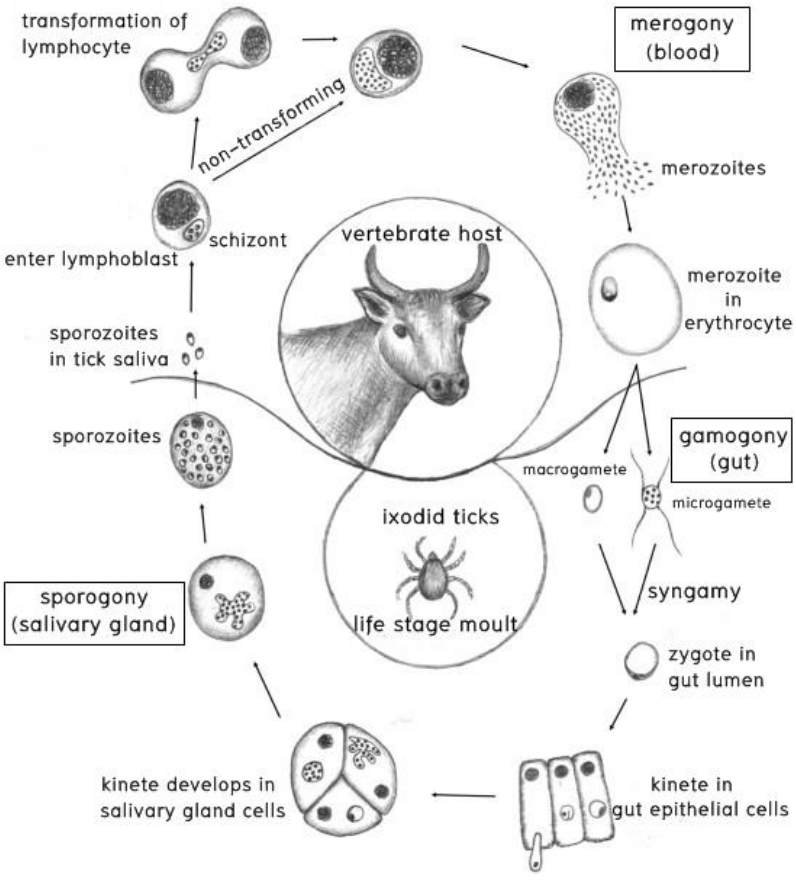

Figure 4. Life cycle of *Theileria* spp. (Mans et al., 2015)

2.2 Tick-borne bacteria

Obligate intracellular α-proteobacteria belonging to family Anaplasmataceae of the order Rickettsiales are tick-borne bacterial parasites that includes members in the genera *Ehrlichia*, *Anaplasma*, *Wolbachia*, and *Neorickettsia* (Kocan et al., 2010). The Anaplasmataceae family comprises pathogenic and non-pathogenic obligate intracellular bacteria that infect host by localized in vacuoles of host cell cytoplasm. They have different host cell types which depend on the species of bacteria. *Ehrlichia* and *Anaplasma* are important tick-borne bacterial parasites which a natural enzootic cycle involves ixodid ticks and vertebrate host (Pruneau et al., 2014). The life cycles of *Neorickettsia* species involve trematodes rather than ticks. All genera in this family, infect vertebrate host, except *Wolbachia* infect only arthropod and nematode (Werren et al., 2008). For the genetic classification based on 16S rRNA, the Anaplasmataceae were divided into four distinct clades of each four genera (Figure 5).

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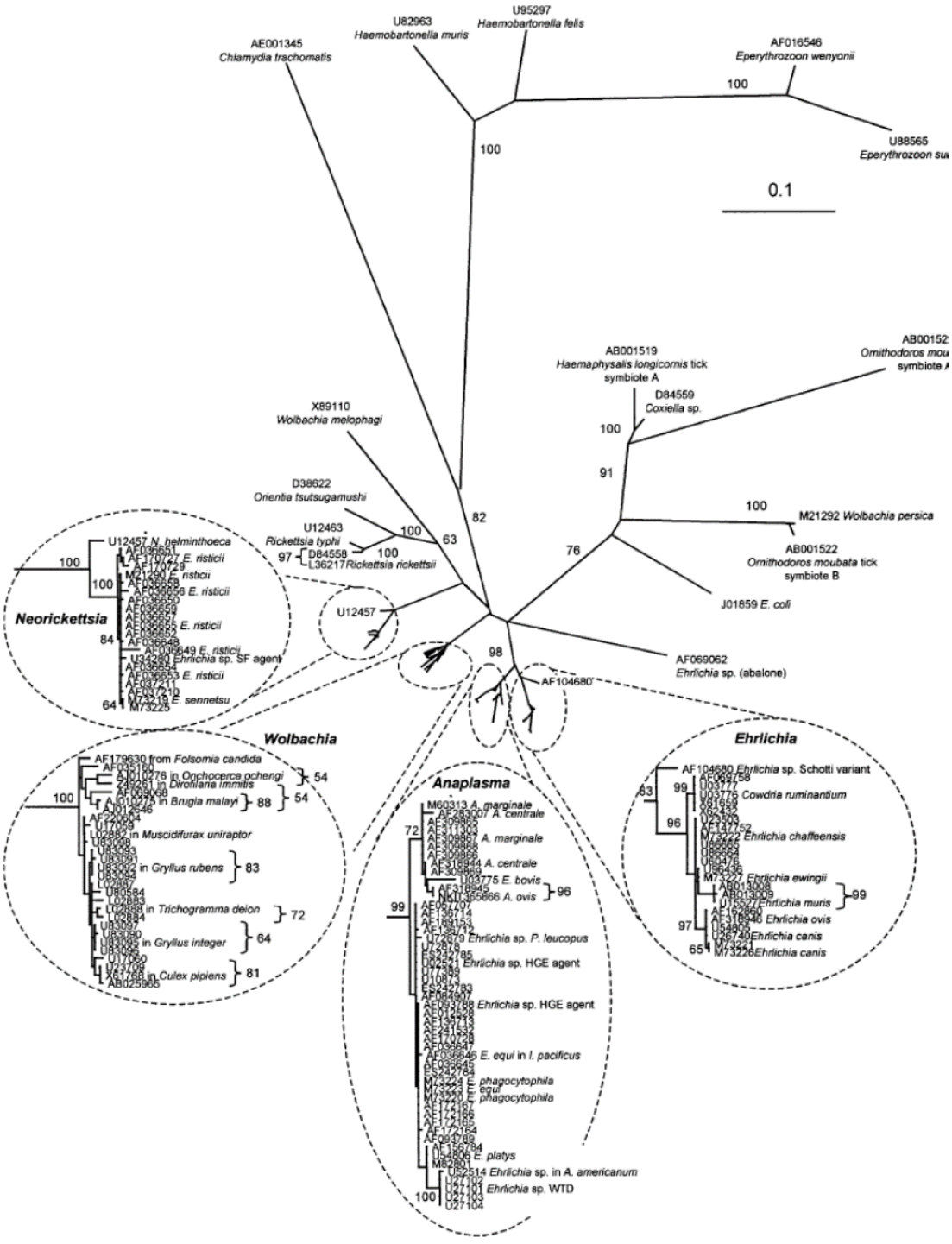

Figure 5. Molecular phylogenetic tree of the family Anaplasmataceae, based on 16S ribosomal RNA gene sequences (Dumler et al., 2001)

2.2.1 *Anaplasma* spp.

Anaplasma is an obligate intra-erythrocytic bacterial parasite commonly occurs in tropical areas that can infect a wide variety of animals and humans. *Anaplasma* and *Ehrlichia* have different primary target cells. *Anaplasma* can infect neutrophil, monocytes, and erythrocytes, however depends on its species (Rar and Golovljova, 2011). Bovine anaplasmosis caused by bacteria in the genus *Anaplasma*. *Anaplasma marginale*, *A. centrale*, *A. ovis,* and *A. bovis* are species that mostly infect both domestic and wild ruminants. Deers are usually reservoir hosts for this parasite (Rymaszewska and Grenda, 2008). Clinical signs of bovine anaplasmosis include fever, anemia, icterus, and death (Aubry and Geale, 2011). *Anaplasma bovis* can cause the disease in ruminants and small mammals and infected cattle is usually subclinical, however some clinical symptoms may appear including fever, anemia, lymphadenopathy, drowsiness, and convulsion (Dumler et al., 2005). *Amblyomma*, *Haemaphysalis*, *Rhipicephalus (Boophilus), Dermacentor, Ixodes,* and *Rhipicephalus* ticks are the vectors of this disease (Dumler et al., 2005; Rymaszewska and Grenda, 2008; Aubry and Geale, 2011).

A. marginale life cycle shows in Figure 6. Ticks ingest infected erythrocytes during blood feeding. The bacteria develop membrane-bound vacuoles or colonies within the tick gut cells and various organs, including salivary gland. Two forms of bacteria are found within colonies. The reticulated (vegetative) forms are found firstly which they are divided by binary fission. Later, the reticulated forms change to dense

forms which are infective forms for infecting the new host via blood feeding of ticks. *Anaplasma* are transmitted either biologically or mechanically (Kocan et al., 2003; Baldridge et al., 2009).

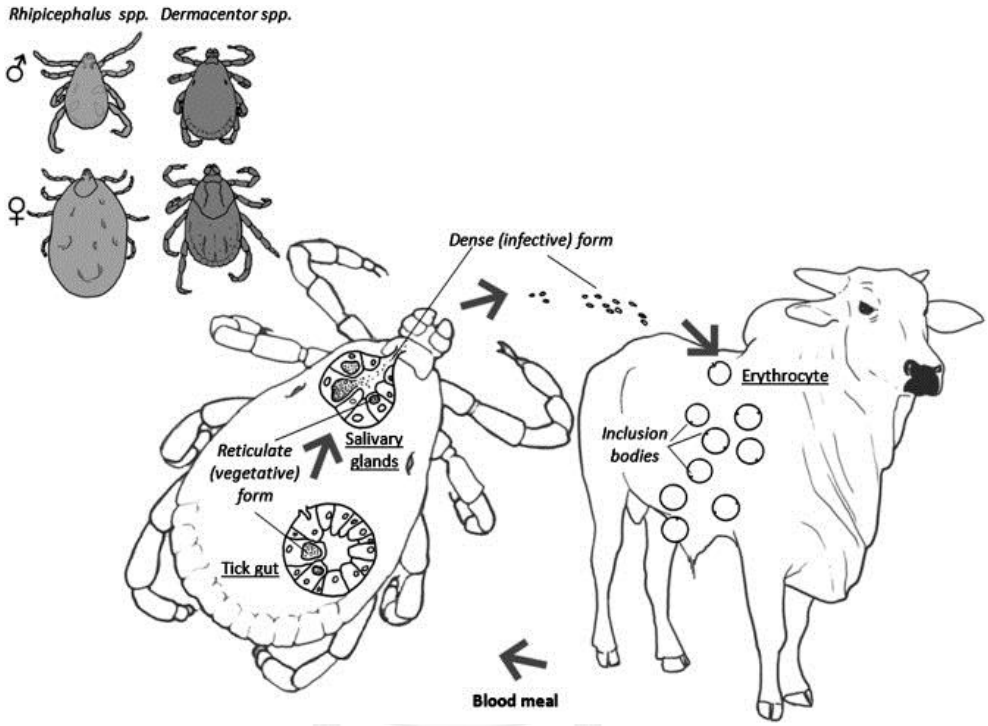

Figure 6. Life cycle of *Anaplasma marginale* (Marcelino et al., 2012)

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2.2.2 *Ehrlichia* spp.

Ehrlichia spp. are intracellular bacteria that predominantly infect mononuclear phagocytes (monocyte, macrophage), neutrophil, platelet, and endothelial cells depending on the particular species of animals and human (Rar and Golovljova, 2011). Morphology of *Ehrlichia* are different from *Anaplasma*, which *Ehrlichia* form a large morulae containing many cells, whereas *Anaplasma* form smaller morulae in which low numbers of bacteria. A wide variety of wildlife are

reported to infect with *Ehrlichia* with no clinical symptoms, however, some species of *Ehrlichia* in wild animals are pathogenic or zoonotic. *Amblyomma, Haemaphysalis, Ixodes,* and *Rhipicephalus* are reported to be a vector of *Ehrlichia* (Rikihisa, 1991; Kim et al., 2003; Kawahara et al., 2006). *Ehrlichia ruminantium*, the causative agent of heartwater in domestic and wild ruminants, is transmitted by *Amblyomma* ticks. *E. ruminantium* life cycle initially develops in gut epithelial cells of ticks and invades to tick salivary glands. During blood feeding, elementary bodies of *E. ruminantium* enter cell macrophage, neutrophil, and endothelial cells of blood capillaries via phagocytosis to form membrane bound vacuole and divide into large colonies of reticulate form. Intracytoplasmic vacuole of infected cells release the elementary bodies to tick gut and continue the transmission cycle during tick feeding (Marcelino et al., 2012) (Figure 7).

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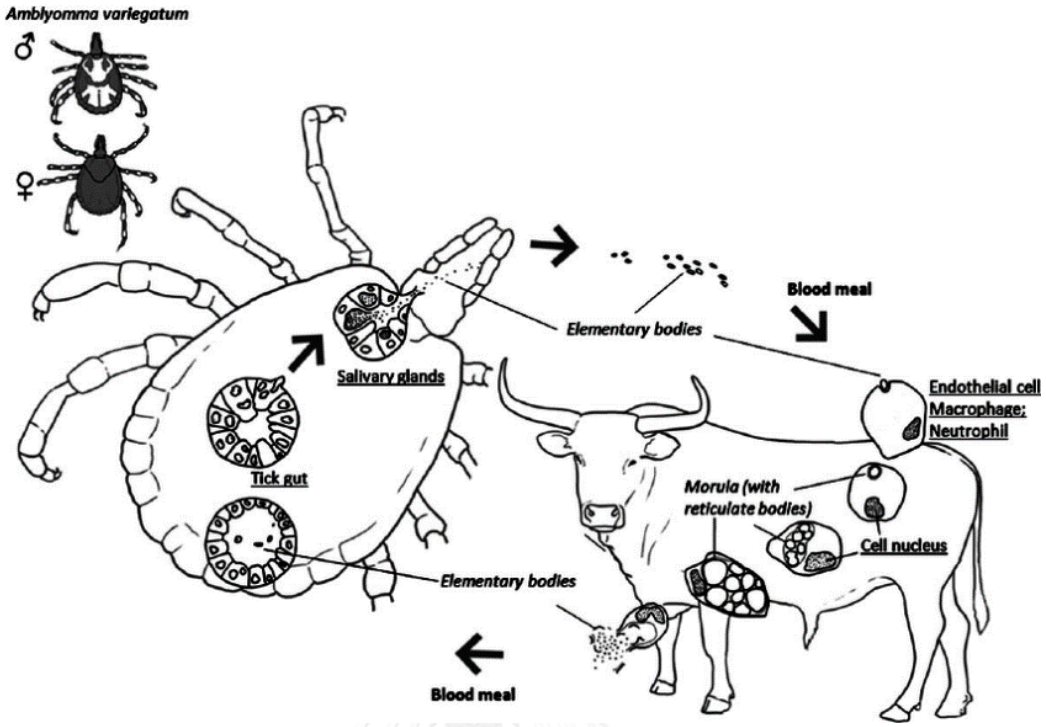

Figure 7. Life cycle of *Ehrlichia ruminantium* (Marcelino et al., 2012)

2.2.3 *Wolbachia* spp.

Wolbachia is one of the most abundant intracellular bacterial endosymbionts that commonly found in arthropods and nematodes (Brownlie and O'Neill, 2006). *Wolbachia* live within the host cells by symbiotic relationships with their host. Although *Wolbachia* are non-pathogenic species for vertebrate host, but they live in various arthropods and ticks with parasitism association as well as reproductive parasites. *Wolbachia* usually infect malpighian tubule and ovary of invertebrate host and the infection will alter reproductive capabilities of their host by four mechanisms, comprise feminization of genetic male, parthenogenesis induction to develop unfertilized eggs, the killing of male progeny by infected female, and sperm-egg

incompatibility (Werren et al., 2008). *Wolbachia* are transmitted by both transovarially and transstadially. *Wolbachia* are capable of altering reproductive system of host and highly adapt for living in various host, resulting in wide distribution in invertebrate. Nowadays, many researchers attempt to use *Wolbachia* as a biological control of pest and especially vector-borne disease transmission control in vector (Werren et al., 2008).

3. Diagnostic methods for tick-borne protozoa and bacteria in ticks

The identification of a pathogen in ticks is difficult because of a low level of infection in ticks, therefore, the diagnostic technique is the crucial step in order to demonstrate disease situation. Diagnostic methods used to detect the pathogens in ticks include conventional and molecular techniques. Conventional methods such as staining test, histology, indirect immunofluorescent test, and pathogen isolation would be difficult, complicated procedures, required skilled personnel, and specialized laboratory (Brouqui et al., 2004). Currently, molecular techniques are increasingly used to diagnose the pathogens in ticks in consequence of rapid, high sensitivity, and specificity. They can be used with death pathogens, uncultured microorganisms, destroyed specimens, or mixed pathogen populations (Sparagano et al., 1999). There are some previous studies about pathogens detection in wild tick in Thailand. Parola et al. (2003) reported the detection of *Ehrlichia*, *Anaplasma*, and *Bacteria* in wild ticks in Thai-Myanmar border in Sangkhlaburi district, Kanchanaburi province by using PCR. Malaisri et al. (2015) explored the vegetation in ten locations of tourist nature trails.

Rickettsia spp. and *Anaplasma* spp. were detected in wild questing ticks in Thailand. Kawahara et al. (2006) also reported the detection of new *Ehrlichia*, *A. bovis,* and *A. centrale* in *Hemaphysalis longicornis* ticks from wild deer and environment by PCR and sequencing. According to Fyumagwa et al. (2011), *Anaplasma*, *Babesia*, and *Theileria* were found in ticks collected from the wild animals and questing ticks in forest in Tanzania by using PCR technique. Berggoetz et al. (2014) surveyed thirteen species of ticks in wildlife and livestock in South Africa, PCR could detect the pathogen in the genus *Babesia, Theileria, Anaplasma,* and *Ehrlichia* in the salivary glands of ticks and co-infection could also be found in infected ticks. Shock et al. (2014) reported the detection of *Theileria*, *Babesia*, and *Cytauxzoon felis* in ticks collected from wild animals and questing ticks in the United States.

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CHAPTER III
MATERIALS AND METHODS

1. Study sites

Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center are under the Department of National Parks, Wildlife and Plant Conservation. Khao Chi-On Non Hunting Area covers an area of Huay Yai sub-district in Banglamung district and Nong Chub Tao sub-district in Sattahip district, Chonburi province, with an area approximately 909 acre. It locates in the geographical coordinates of latitude 12°45'32" to 12°46'31" N and longitude 100°58'22" to 100°59'28" E. The terrain's characteristics is hills alternating with plains with a heights of 90-318 meters above sea level and most areas are dry evergreen forest. Wild animals live inside this area include wild mammals, reptiles, amphibians, and birds with the high abundance of deer especially sambar deer, hog deer, and Eld's deer (Aemsang, 2008). Khao Chi-On Non Hunting Area connected with Banglamung Wildlife Breeding Center which covers an area approximately 190 acre. This center is responsible for captive wildlife conservations and wild animal rescue from illegal wildlife trade. Three sampling locations with different terrain characteristics (Table 1) (Figure 9, 10 and 11) were chosen to provide coverage both areas of Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center (Figure 8), except location C in the first time visit in February that do not collected ticks, then excluded from the study. This study was permitted by the

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Department of National Parks, Wildlife and Plant Conservation to conduct the research in these areas.

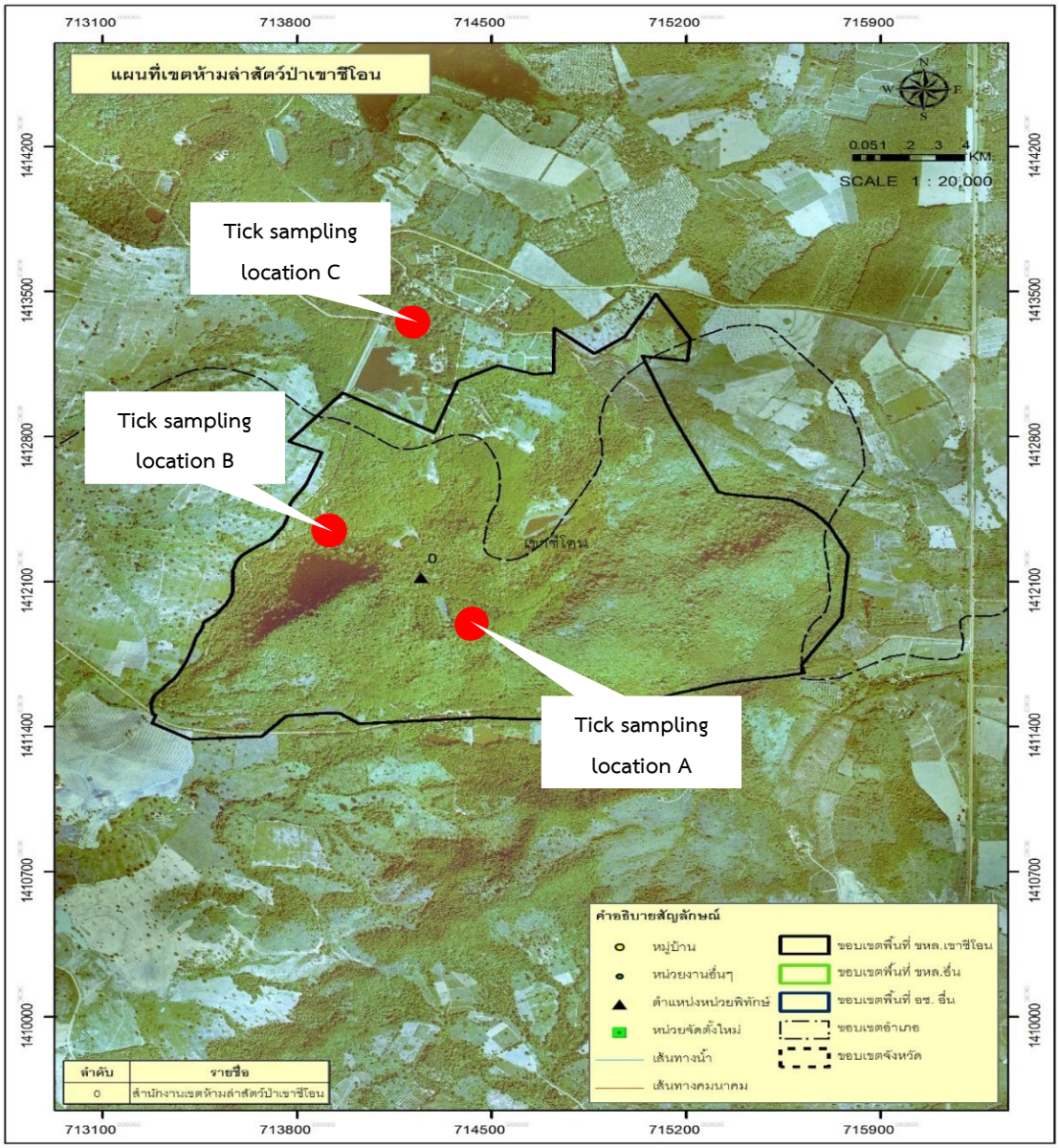

Figure 8. Map of Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center includes three sampling locations (Department of National Parks, Wildlife and Plant Conservation)

Table 1. Latitude, longitude, and level above the sea of three different locations within the study sites

Location	Latitude	Longitude	Level above the sea (meters)	Terrain characteristics
A	12°45'53.2" N	100°58'28.9" E	116.248	Plain
B	12°46'03.9" N	100°58'13.8" E	87.230	Foothill slop
C	12°46'37.8" N	100°58'23.1" E	88.448	Plain near water reservoir

Figure 9. Terrain characteristics of sampling location A

Figure 10. Terrain characteristics of sampling location B

Figure 11. Terrain characteristics of sampling location C

2. Sample collections

The ixodid ticks were sampled in six visits at two month-interval for one year, in February, April, June, August, October, and December, 2015. In each of three sampling locations, ixodid ticks were collected from vegetation by dragging with a white cloth combined with eye searching for questing ticks on the tips and beneath of the leaves by randomly walk in area of animal trails that have evidences of deer activities including droppings and footprints. It was dragged for a minimum of one hour per location. All tick samples were then kept in 70% ethanol.

This study was conducted in compliance with Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol No. 1631030) and Chulalongkorn University, Faculty of Veterinary Science Biosafety Committee (Biosafety Use Protocol No. 1631011)

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3. Tick species morphology-based identification

All ixodid tick samples were individually examined under light microscope to count and identify genus and/or species, sex, and stage of development by using morphological identification keys (Nuttall and Warburton, 1915; Arthur, 1960; Yamaguti et al., 1971; Hoogstraal et al., 1973; Tanskul et al., 1983; Tanskul and Inlao, 1989). Morphologically similar ticks in each life stage was grouped and chose individual representative of each species, each life stage of ticks was examined to confirm tick species with DNA-based identification.

4. Tick sample size for pathogen detection

Because of high numbers of collected larvae, larvae were pooled 1-50 larvae per pool, according to each species, locality, collection times, and collectors. For gathering representative data from each stage of ticks, location, and sampling months, quota sampling was used to select the ticks for detection of pathogen. For each location and sampling time, ten of each male adults, female adults, nymphs, and larval pools were the quota to selected representative tick samples from all collected ticks. If numbers of collected ticks are not sufficient to reach this quota, it would take as much as possible.

5. Genomic DNA extraction

Individual representative of each tick was examined to confirm tick species. Individual adult and nymph samples, and pooled larvae samples were chosen for pathogen detection. Prior DNA extraction step, ticks were placed individually in sterile microcentrifuge tube. Ticks were washed in 70% ethanol, rinsed three times in sterile distilled water to remove any external contaminations, air dried, and then stored at −20°C until DNA extraction. The ticks were manually homogenized by using sterile disposable needle and/or sterile tissue grinder prior to DNA extraction procedure. Genomic DNA was extracted from each tick sample by using DNeasy® blood and tissue kit (Qiagen, Germany) according to manufacturer's procedure.

6. Polymerase Chain Reactions

6.1 Tick species DNA-based identification

Genomic DNA from individual representatives of each tick and each stage of development were performed polymerase chain reaction using primer target tick mitochondrial 16S rRNA gene according to Black and Piesman (1994) and Takano et al. (2014) with slight modifications. Primer list and thermal cycling conditions used are shown in Table 2 and 4, respectively. Positive control of *Rhipicephalus sanguineus* (obtained from raring in our facility) and deionized distilled water as negative control were included in this study.

Table 2. Primers for tick species identification by PCR

Target gene	Primer names	Oligonucleotide primer (5'→3')	Product sizes (bp)	References
Mitochondrial 16S rRNA gene of ticks	16S+1	5'- CTGCTCAATGATTTTTTAAATTGCTGTGG -3'	401-416	(Black and Piesman, 1994; Takano et al., 2014)
	16S-1	5'- CCGGTCTGAACTCAGATCAAGT -3'		

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6.2 Protozoa and bacteria detection in ticks

To detect protozoa and bacteria, PCR were performed to detect *Babesia*, *Theileria* (Guerrero et al., 2007), and members of family Anaplasmataceae (Parola et al., 2000) with slight modifications. Primer list and thermal cycling conditions used are shown in Table 3 and 4, respectively. The variation of PCR product sizes of *Babesia* and *Theileria* depended on species. Positive controls of *Babesia bovis*, *B. bigemina*, *Theileria* spp. and *Anaplasma marginale* (provided by Parasitology section, National

Institute of Animal Health) and deionized distilled water as negative control were included in PCR reactions to detected *Babesia* spp., *Theileria* spp. and bacteria in family Anaplasmataceae.

Table 3. Primers for protozoa and bacteria detections by PCR

Target gene	Primer names	Oligonucleotide primer (5'→3')	Product sizes (bp)	References
18S rRNA gene of *Babesia* spp. and *Theileria* spp.	KB-16 KB-17	5'-CATCAGCTTGACGGTAGGG-3' 5'-GTCCTTGGCAAATGCTTTC-3'	*Babesia:* ~531-596 *Theileria*: ~603-622	(Guerrero et al., 2007)
16S rRNA gene of members of family Anaplasmataceae	EHR16SD EHR16SR	5'-GGTACCYACAGAAGAAGTCC-3' 5'-TAGCACTCATCGTTTACAGC-3'	345	(Parola et al., 2000)

6.3 PCR reactions and gel electrophoresis

All PCR reactions were performed in a final volume of 25 µl containing 1X High Fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1U of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), and 2 µl of template DNA. The optimized thermal cycling conditions adapted from manufacturer's procedure are shown in Table 4.

Table 4. Thermal cycling conditions used in this study

Steps	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	30 sec	1
Denature	94	15 sec	45
Annealing	55	30 sec	
Extension	68	30 sec	
Final extension	68	7 min	1
Hold	4	∞	1

Following amplification, the PCR products were examined by agarose gel electrophoresis in 1.5% agarose in 1X Tris-acetate-EDTA buffer, containing 0.05% ethidium bromide. The electrophoresis was run for 30-60 minutes at 110 volts. After electrophoresis, the DNA bands were visualized in ultraviolet transilluminator.

7. DNA sequencing

After agarose gel electrophoresing, DNA bands at the expected product size were cut out of the gel and purified using GenepHlow™ Gel/PCR Cleanup Kit (Geneaid, Taiwan) according to manufacturer's procedure. The DNA purify product was submitted to a commercial service for DNA sequencing (First Base Laboratories, Kuala Lumpur, Malaysia).

8. Molecular analysis

The obtained nucleotide sequencing results of mitochondrial 16S rRNA gene of ticks, 18S rRNA gene of *Babesia* spp. and *Theileria* spp., and 16S rRNA gene of family Anaplasmataceae members were aligned and trimmed using ClustalW multiple alignment of BioEdit (Thompson et al., 1994). The aligned sequences were compared with available sequences in the GenBank database to defined genus and/or species of ticks and pathogens using the Basic Local Alignment Search Tool (Altschul et al., 1990)

For phylogenetic analysis, nucleotide sequences of 18S rRNA gene of *Babesia* spp. and *Theileria* spp., and 16S rRNA gene of family Anaplasmataceae members were

constructed Maximum Likelihood tree using Kimura's two-parameter model with 1,000 bootstrap replications by Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar et al., 2016). Prior to constructed phylogenetic tree, the sequences were estimated the optimal model of nucleotide substitution by using MEGA.

9. Data analysis and statistical analysis

The tick diversity was analyzed by descriptive statistic and analytical statistic using one-way analysis of variance (one-way ANOVA) with p-value $<$ 0.05 (JMP7 statistical software, SAS, Cary, NC, USA) to determine the difference between collection times and locations on average numbers of collected tick species in each life stage. The associations between average numbers of collected ticks in each tick species and life stage per location distributed by collection time with trends of climatological data of means temperature, means relative humidity, and total rainfall data from the Thai Meteorological Department (Patthaya station, Chonburi province) were demonstrated by using line-stacked bar chart. The associations between average numbers of collected ticks in each tick species and life stage per collection time distributed by locations were exhibited by using stacked bar chart. Infection rates of tick-borne protozoa and bacteria in each tick species distributed on locations of each collection time were demonstrated by using stacked bar charts.

CHAPTER IV
RESULTS

1. Tick species morphology-based identification

All 12,184 collected ticks belong to family Ixodidae using criteria of extending of capitulum from anterior end of body and presented dorsal scutum. Female ticks have scutum which partly covers the body while male ticks have scutum which completely covers the body and smaller in size than female. The distinguishing among tick's stages of development is mainly based on some characteristics. For instance, both adults and nymphs have eight legs, while larvae have six legs. Adult ticks have well-developed genitalia, whereas undeveloped genitalia were found in nymphs and larvae. Nymph looks very similar to adult ticks, but smaller in size and larvae are the smallest. There are some species that adult stage was not able to identified to species level because some morphologically similar species that might contribute to the misidentification (Table 5). Due to the limitation of taxonomic data of immature stage, the individual representative of each similar morphology and life stage of ticks were examined by using DNA-based identification.

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The morphological differences among tick species were mainly in term of size, body length, appearance of palpi, spur of palpi, and coxal spur. *Haemaphysalis* ticks were different from other genera by the laterally produced of 2nd segment of palpi and the present of posterior anal groove. They were identified into two species as

followed. All *Rhipicephalus* samples collected in this study were larval stage so they can only be identified to genus level.

The adult of *Haemaphysalis lagrangei* characteristics were campanulate palpi, elongately triangular shaped postero-ventral spur of 3rd segment of palpi which overlap anterior 2/3 of 2rd segment of palpi, and broadly triangular of postero-dorsal spur of 3rd segment of palpi which median overlap at least 1/3 of anterior part of 2nd segment of palpi. All coxae had prominent spur, as well as lanceonate shaped of 1st coxa and broadly triangular spur of 2nd, 3rd and 4th coxa. The adult of *Haemaphysalis wellingtoni* characteristics were strongly laterally projecting of 2nd segment of palpi, distinctly postero-dorsal spur of 3rd segment of palpi at inner angel, and posterointernally directed ventral spur. The larva of *Rhipicephalus* (*Boophilus*) characteristics were broadly rounded ventrally capitulum, short palpi, and broaded internal spur. The morphology of ixodid ticks found in this study, including *Haemaphysalis lagrangei*, *Haemaphysalis wellingtoni*, and *Rhipicephalus microplus* were demonstrated in Figure 12-16.

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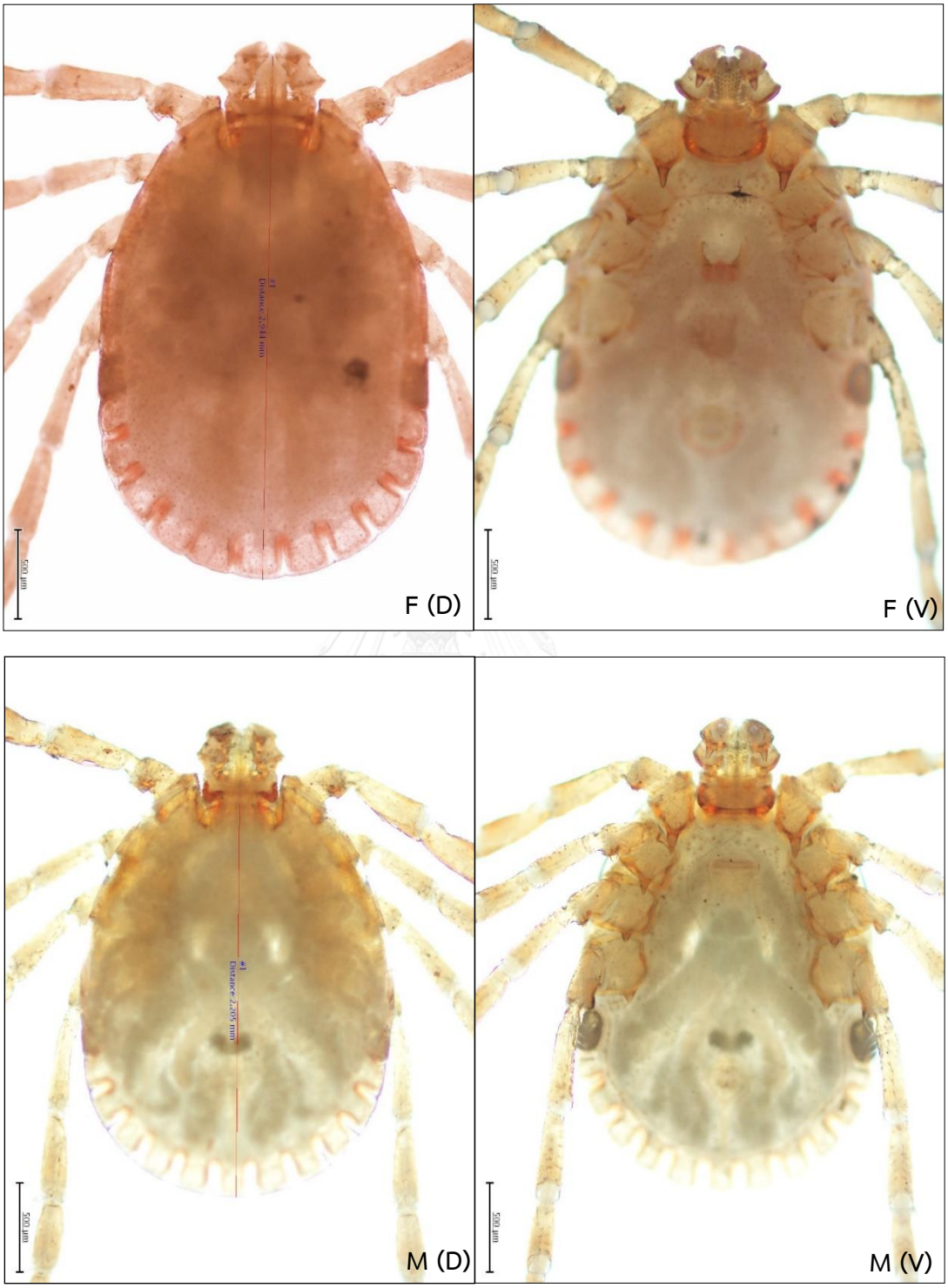

Figure 12. Morphology of *Haemaphysalis lagrangei*, F: female adult; M: male adult. (D): dorsal aspect; (V): ventral aspect

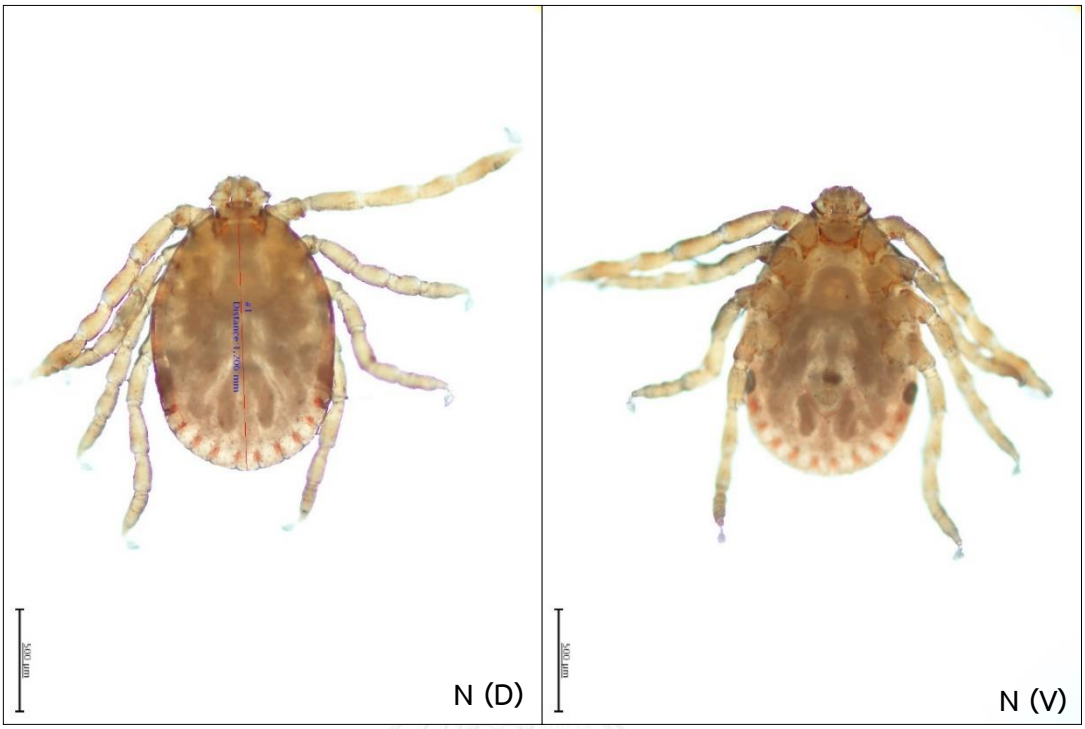

Figure 13. Morphology of *H. lagrangei*, N: nymph; (D): dorsal aspect; (V): ventral aspect

Figure 14. Morphology of *H. lagrangei*, L: larva. (D): dorsal aspect; (V): ventral aspect

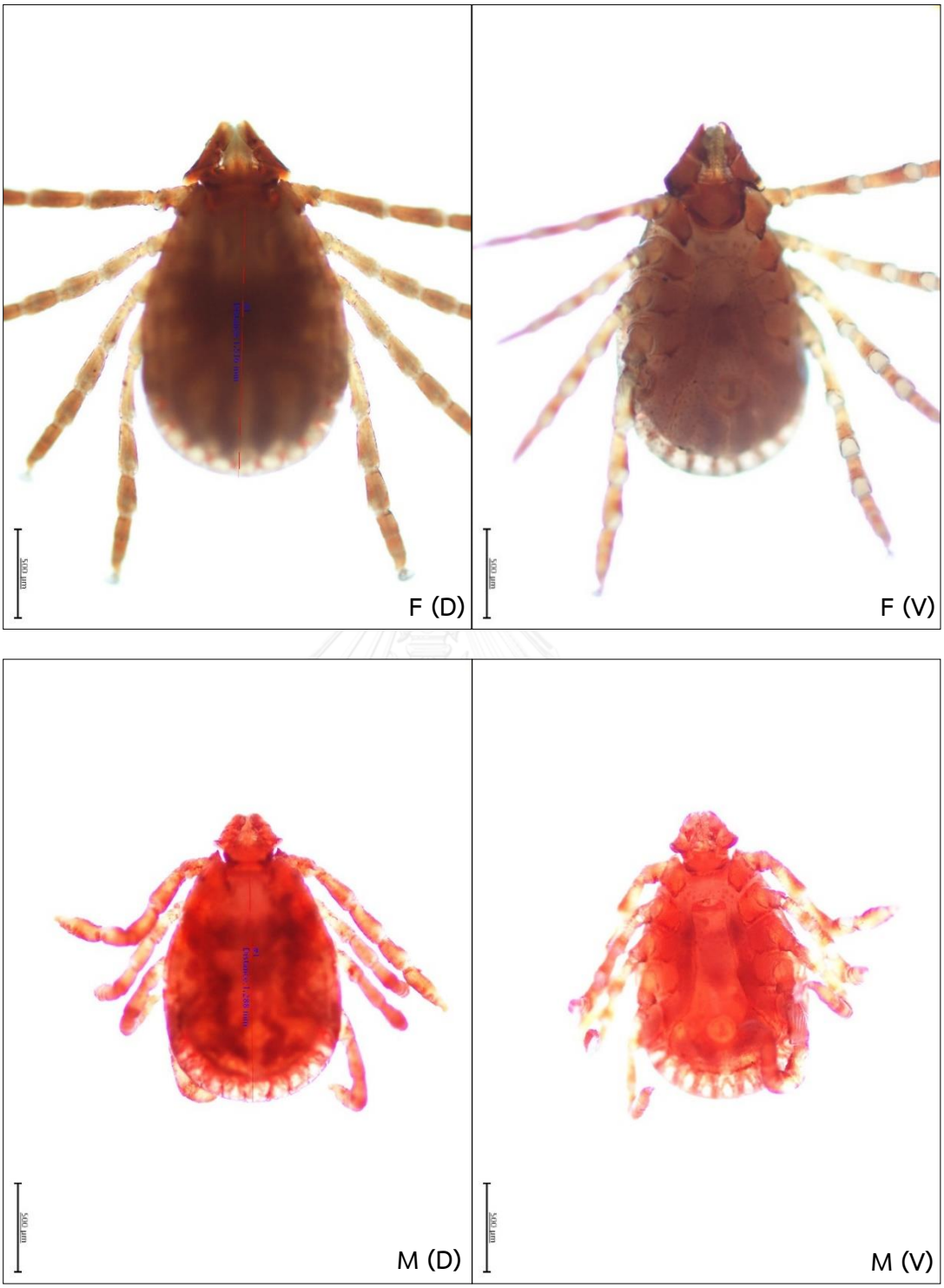

Figure 15. Morphology of *Haemaphysalis wellingtoni*, F: female adult; M: male adult. (D): dorsal aspect; (V): ventral aspect

Figure 16. Morphology of *Rhipicephalus* (*Boophilus*) *microplus*, L: larva. (D): dorsal aspect; (V): ventral aspect

2. Tick species DNA-based identification

A 401 to 416 base pairs fragment of the mitochondrial 16S rRNA gene of ticks was amplified in eight individual representatives of each morphological similar and each stage of ticks (Figure 17). The purified DNA was sequenced, analyzed, and blasted with the data in Genbank. The BLAST search results showed 99-100% identical to mitochondrial 16S rRNA gene sequences of *Haemaphysalis lagrangei*, *H. wellingtoni*, and *Rhipicephalus microplus*. The results demonstrated that morphology-based findings can be differentiated the ticks at the genus level, while DNA-based technique can be identified into species level of all representative ticks. Morphology-based findings and

molecular identification using mitochondrial 16S rRNA gene sequencing of eight representative ticks are presented in Table 5.

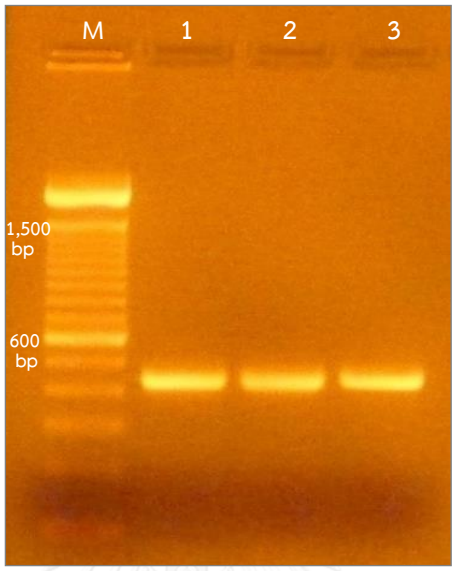

Figure 17. Amplified PCR products of mitochondrial 16S rRNA gene of ticks at approximately 401-416 base pairs length size, lane M: 100 bp DNA Marker, 1-3: tick sample

Table 5. Morphology-based and DNA-based species identifications of eight individual ticks.

No.	Morphology-based results	Stages	DNA-based results (Highest BLAST result)		
			Description	Accession	%Ident
1	*Haemaphysalis lagrangei*	Male	*Haemaphysalis lagrangei*	KC170731	99
2	*Haemaphysalis lagrangei*	Female	*Haemaphysalis lagrangei*	KC170731	99
3	*Haemaphysalis* spp.	Nymph	*Haemaphysalis lagrangei*	KC170731	99
4	*Haemaphysalis* spp.	Larva	*Haemaphysalis lagrangei*	KC170731	99
5	*Haemaphysalis* spp.	Male	*Haemaphysalis wellingtoni*	AB819221	99
6	*Haemaphysalis* spp.	Female	*Haemaphysalis wellingtoni*	AB819221	100
7	*Rhipicephalus* spp.	Larva	*Rhipicephalus microplus*	KP143546	100
8	*Rhipicephalus* spp.	Larva	*Rhipicephalus microplus*	KP143546	99

3. Tick species diversity

A total of 12,184 questing Ixodid ticks, including 200 male adults (1.64%), 187 female adults (1.53%), 107 nymphs (0.88%), and 11,690 larvae (95.95%) were collected from vegetation along six time visited in the year 2015 and all of them were un-engorged ticks. According to morphological and genetic identification, collected ticks were identified into three species belonged to two genera which consisted of *Haemaphysalis lagrangei*, *H. wellingtoni*, and *Rhipicephalus* (*Boophilus*) *microplus*. Overall, the predominant of *H. lagrangei* (192 male adults, 179 female adults, 107 nymphs and 10,831 larvae) accounted for 92.82%, followed by *R. microplus* 7.05% (859 larvae) and *H. wellingtoni* 0.13% (8 male adults, 8 female adults) (Table 6). From all 12,184 tick samples examined in this study, larvae (95.95%; 11,690/12,184) were the most abundant stage collected more than male adults (1.64%; 200/12,184), female adults (1.53%; 187/12,184), and nymphs (0.88%; 107/12,184), and all of them were un-engorged ticks.

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Table 6. Numbers of collected questing ticks for six collection times during two-month interval and three geographic locations

	Numbers of ticks (%)									Total
	Haemaphysalis lagrangei					*Haemaphysalis wellingtoni*			*Ripicephalus microplus*	
	M	F	N	L	Total	M	F	Total	L	
Months										
Feb	4 (0.21)	4 (0.21)	9 (0.46)	1870 (96.49)	**1887 (97.37)**	0 (0)	0 (0)	**0 (0)**	**51 (2.63)**	**1938**
Apr	41 (6.20)	38 (5.75)	7 (1.06)	273 (41.30)	**359 (54.31)**	3 (0.45)	0 (0)	**3 (0.45)**	**299 (45.23)**	**661**
Jun	59 (1.18)	61 (1.22)	27 (0.54)	4753 (94.89)	**4900 (97.82)**	4 (0.08)	7 (0.14)	**11 (0.22)**	**98 (1.96)**	**5009**
Aug	25 (0.72)	13 (0.37)	23 (0.66)	3023 (86.99)	**3084 (88.75)**	1 (0.03)	1 (0.03)	**2 (0.06)**	**389 (11.19)**	**3475**
Oct	24 (11.59)	32 (15.46)	15 (7.25)	118 (57.00)	**189 (91.30)**	0 (0)	0 (0)	**0 (0)**	**18 (8.70)**	**207**
Dec	39 (4.36)	31 (3.47)	26 (2.91)	794 (88.81)	**890 (99.55)**	0 (0)	0 (0)	**0 (0)**	**4 (0.45)**	**894**
Total	**192 (1.58)**	**179 (1.47)**	**107 (0.88)**	**10831 (88.90)**	11309 (92.82)	**8 (0.07)**	**8 (0.07)**	16 (0.13)	859 (7.05)	**12184**
Locations										
A	65 (0.95)	6 (0.09)	63 (0.92)	6199 (90.25)	**6333 (92.20)**	2 (0.03)	0 (0)	**2 (0.03)**	**534 (7.77)**	**6869**
B	97 (2.07)	102 (2.17)	36 (0.77)	4248 (90.50)	**4483 (95.50)**	4 (0.09)	6 (0.13)	**10 (0.21)**	**201 (4.28)**	**4694**
C	30 (5.30)	16 (2.83)	8 (1.41)	384 (67.84)	**438 (77.39)**	2 (0.35)	2 (0.35)	**4 (0.71)**	**124 (21.91)**	**566**
Total	**192 (1.58)**	**179 (1.47)**	**107 (0.88)**	**10831 (88.90)**	11309 (92.82)	**8 (0.07)**	**8 (0.07)**	16 (0.13)	859 (7.05)	**12184**

Note. M, male adults; F, female adults; N, nymphs; L, larvae

The line-stacked bar charts of average numbers of collected *H. lagrangei*, *H. wellingtoni*, and *R. microplus* in each life stage per location distributed by six collection times with trends of mean temperature, mean relative humidity and total rainfall showed in Figure 18, 19 and 20, respectively. A numbers of all life stages of *H. lagrangei*,

and *H. wellingtoni* were highest in June which were consistent with the highest temperature and rainfalls in this month, while highest numbers of *R. microplus* were found in August. Owning to high rainfall in October, the flood occurred in all three sampling areas which caused inconvenience in ticks sampling procedure. Average numbers of collected *H. lagrangei*, *H. wellingtoni*, and *R. microplus* in each life stage per collection times distributed by three locations were demonstrated in stacked bar chart in Figure 21, 22 and 23, respectively. Although average numbers of collected *H. lagrangei* and *H. wellingtoni* adults were highest in location B, one-way ANOVA to test effect of variables of collection times and locations on the tick species of each life stage indicated the significantly difference between locations effect on nymph of *H. lagrangei* (p-value = 0.024). The statistical difference (95% CI) was found in average numbers of collected *H. lagrangei* nymphs in location A (10.50; 95% CI 6.058-14.942) was higher than those collected in location C (1.33; 95% CI -3.108-5.775).

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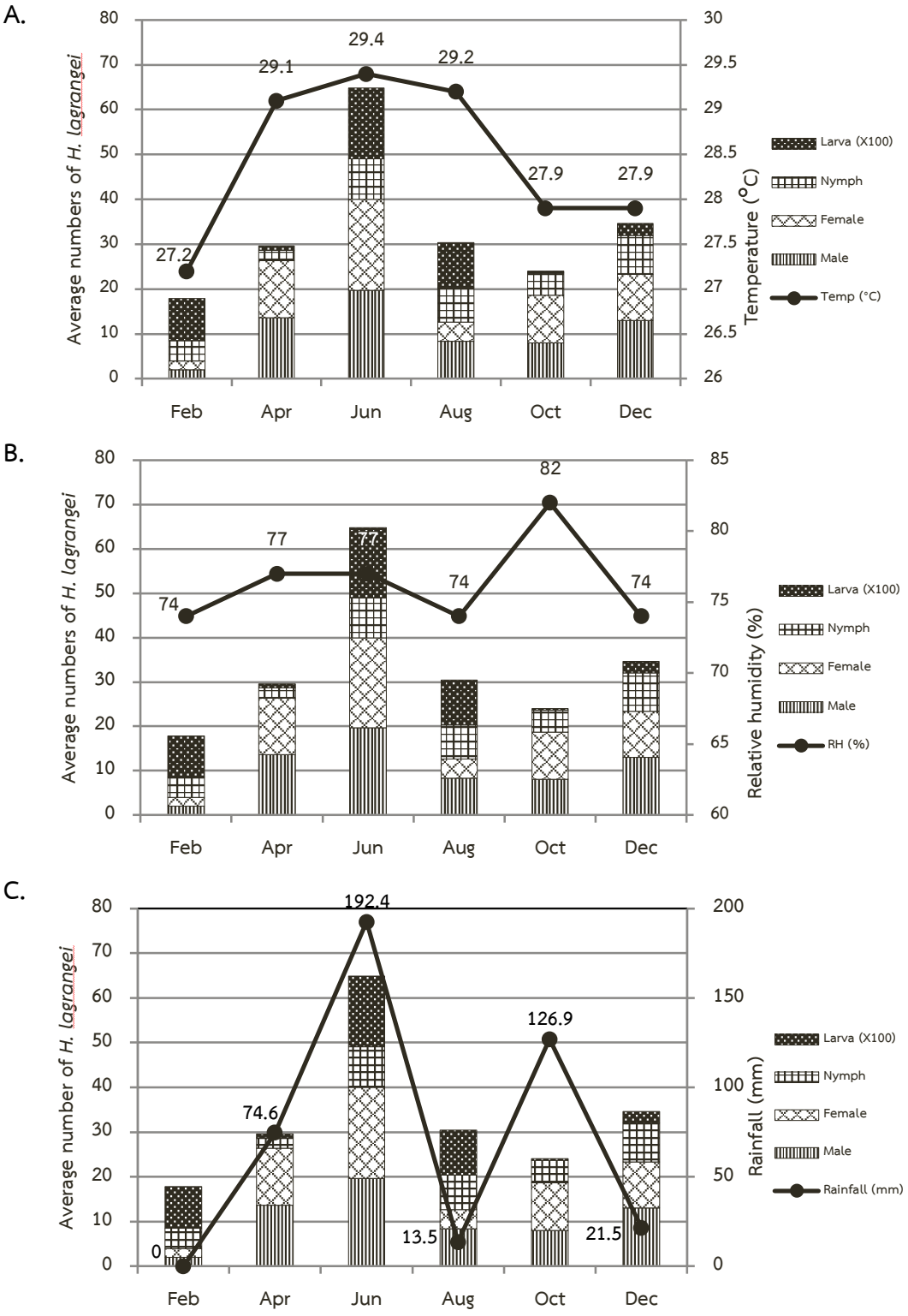

Figure 18. Average numbers of *H. lagrangei* per location distributed by collection times with trends of temperature (A), relative humidity (B), and rainfall (C)

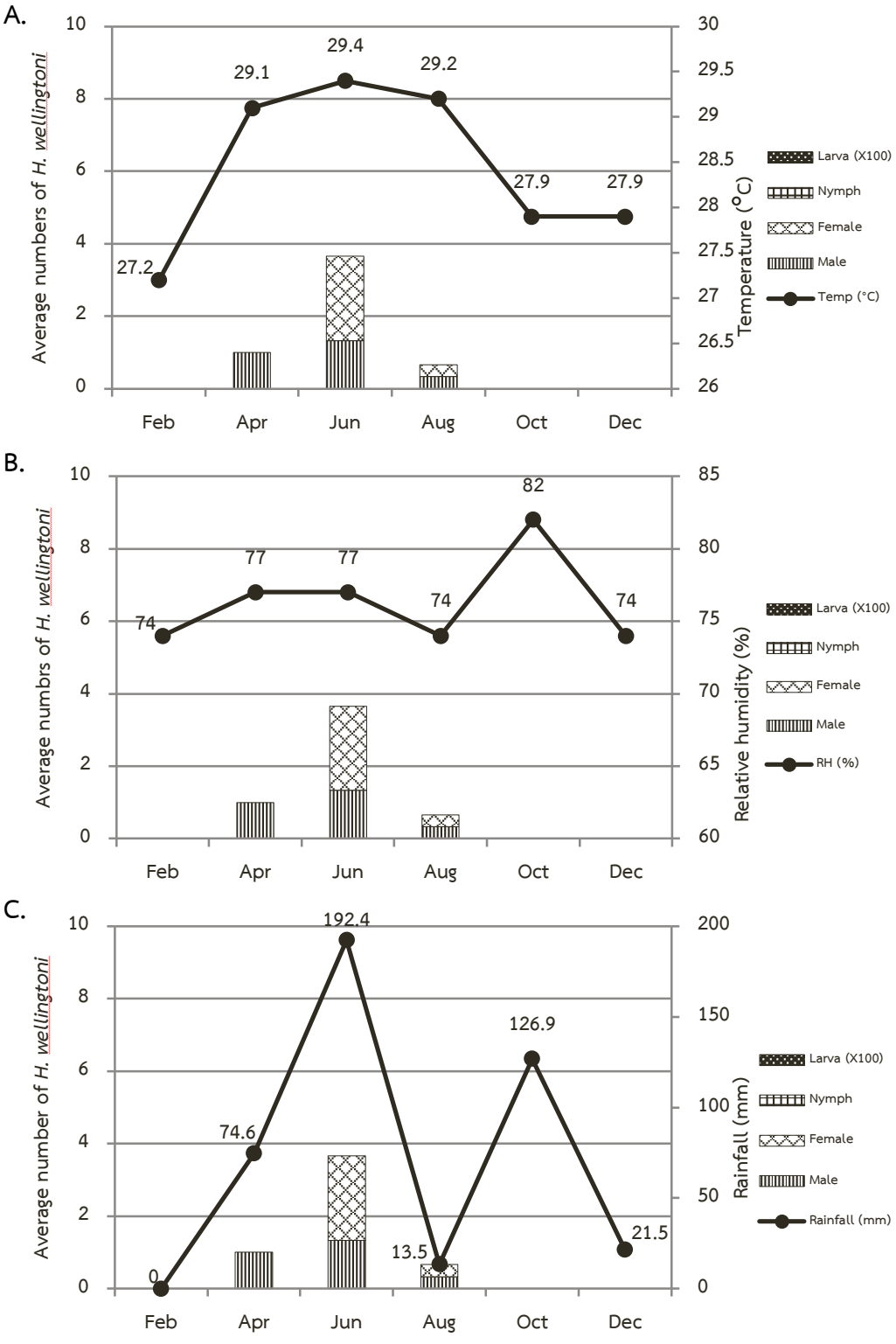

Figure 19. Average numbers of *H. wellingtoni* per location distributed by collection times with trends of temperature (A), relative humidity (B), and rainfall (C)

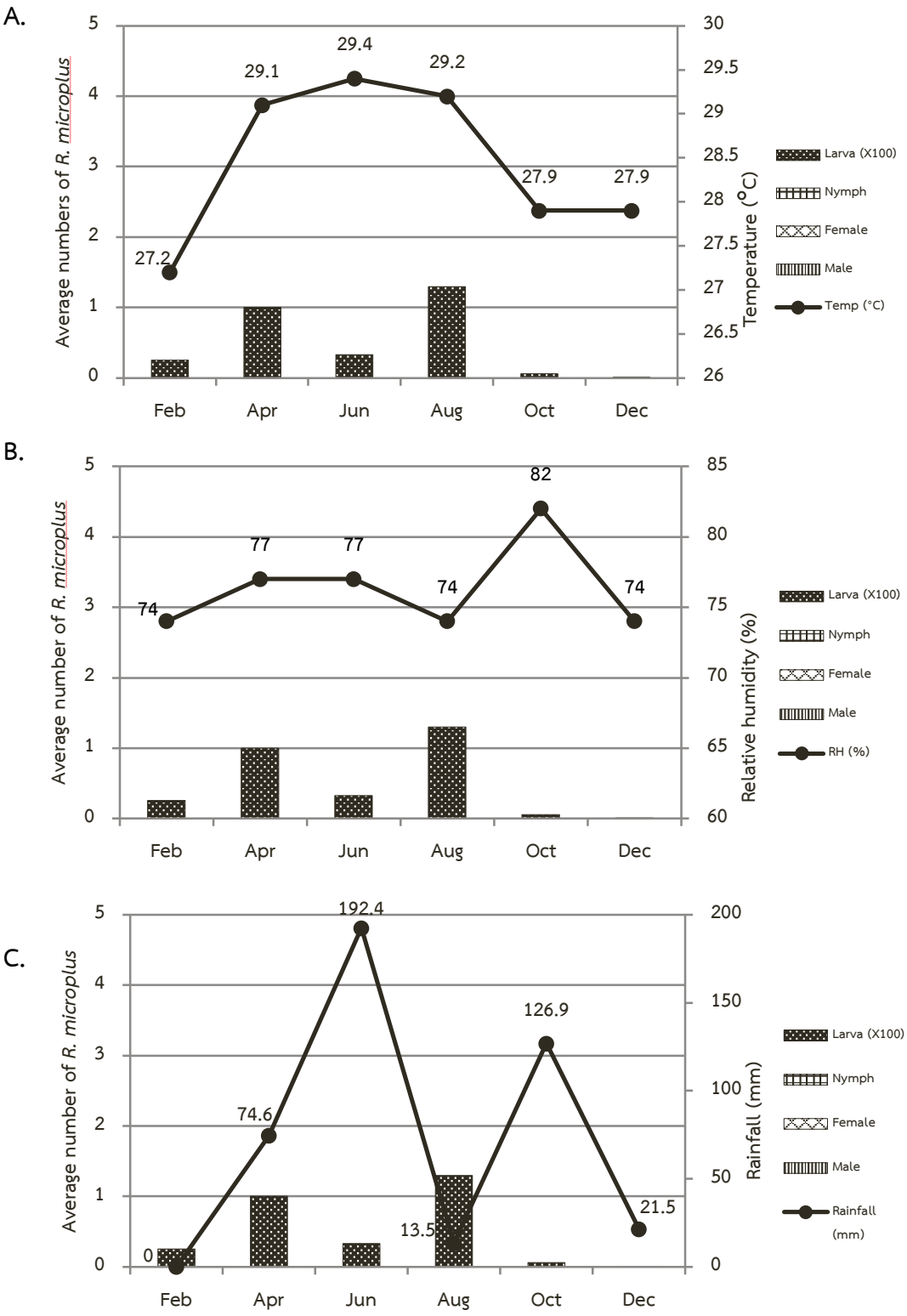

Figure 20. Average number of *R. microplus* per location distributed by collection times with trends of temperature (A), relative humidity (B), and rainfall (C)

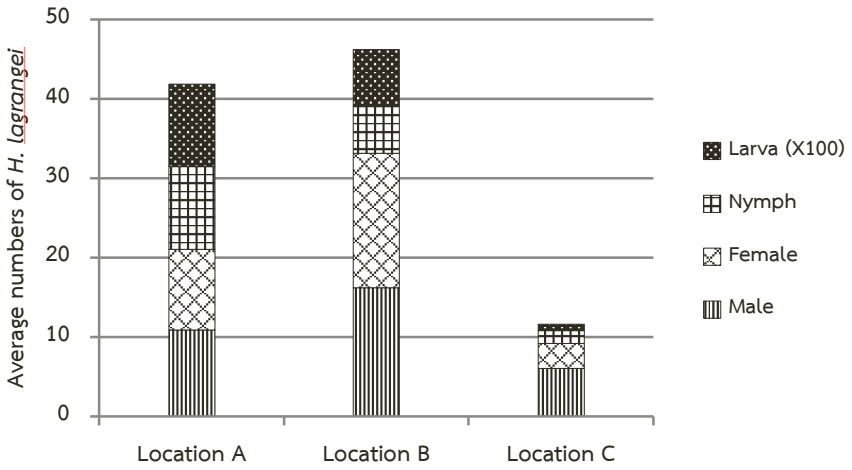

Figure 21. Average numbers of *H. lagrangei* per collection times distributed by locations

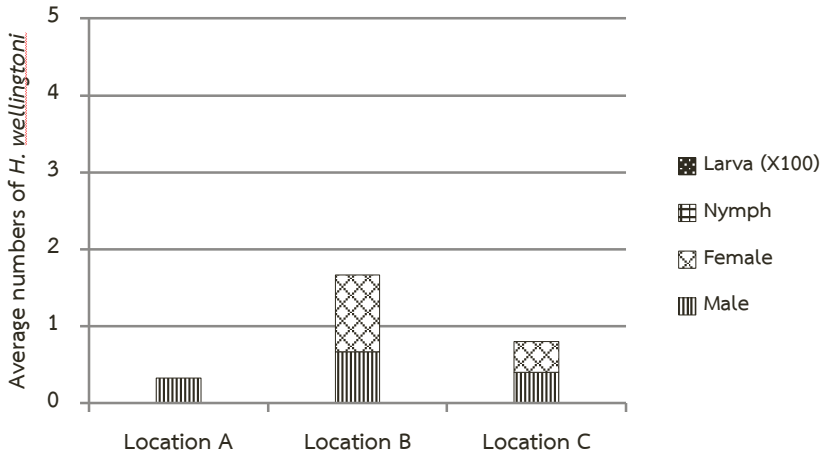

Figure 22. Average numbers of *H. wellingtoni* per collection times distributed by locations

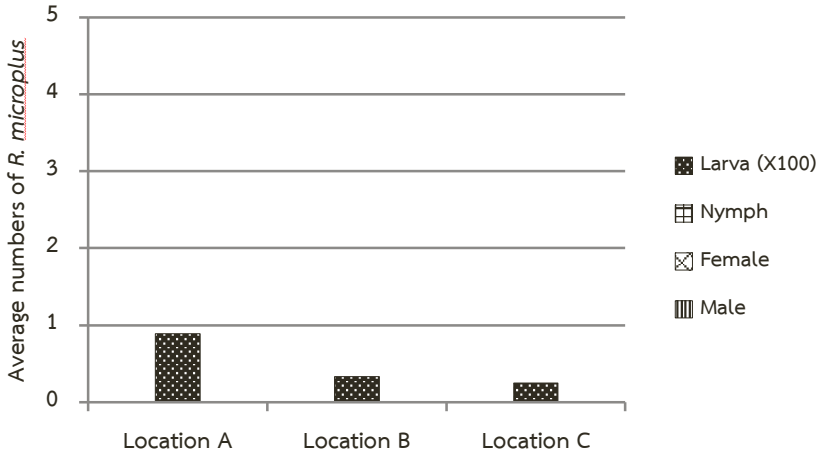

Figure 23. Average numbers of *R. microplus* per collection times distributed by locations

4. Detection protozoa and bacteria in ticks by PCR and DNA sequencing techniques

There were 419 tick samples tested for protozoa and bacteria in this study which were 364 *H. lagrangei* samples (80 male adults, 78 female adults, 79 nymphs and 127 larval pools), 13 *H. wellingtoni* samples (6 male adults and 7 female adults) and 42 *R. microplus* samples (42 larval pools). They were quota sampling for detecting of protozoa in genera *Babesia* and *Theileria* with KB-16 and KB-17 primers and detecting of bacteria in family Anaplasmataceae with EHR16SD and EHR16SR primers by PCR and DNA sequencing techniques. Purified DNA of all positive PCR products at expected product size were submitted to DNA sequencing, aligned, trimmed and analyzed the sequences.

Overall 419 tick samples tested, 44 (10.50%) were positive with KB-16 and KB-17 primers. The BLAST results showed the partial 18S rRNA gene of protozoa, including one (0.24%) *Babesia* spp. sequence and 18 (4.30%) *Theileria* spp. sequences were identified. Unexpectedly, 25 (5.97%) sequences of uncultured eukaryote were detected. Among 419 tick samples analyzed, 244 (58.23%) were positive with EHR16SD and EHR16SR primers. The BLAST results showed 233 (55.61%) *Anaplasma* spp. sequences, One (0.24%) of each *Ehrlichia* spp. and *Wolbachia* spp. sequences and 9 (2.15%) of uncultured bacterium sequences were detected. The NCBI BLAST results of 254 nucleotide sequences obtained from this study are shown in Table 7. Amplified PCR product of partial 18S rRNA gene of *Babesia* spp. and *Theileria* spp. and 16S rRNA

gene of bacteria in family Anaplasmataceae were demonstrated in Figure 24. Percentages of tick infection rate and co-infection rate categorized by collection times, tick species and locations are shown in Table 8. The results showed that *Anaplasma* could infect all three tick species, but the four remaining microorganism were only found in *H. lagrangei* ticks. Out of 239 positive ticks, 15 (3.58%) of them were found co-infection with two organisms, as *Anaplasma* co-infected with *Babesia* (1 sample), *Theileria* (12 samples), *Wolbachia* (1 sample), and *Ehrlichia* co-infected with *Theileria* (1 sample). Tick infection rates of pathogen/microorganism in each tick species distributed by collection times and locations were demonstrated in Table 9. The stacked bar chart of pathogen infection rates of *H. lagrangei, H. wellingtoni* and *R. microplus* distributed by times and locations are shown in Figure 25, 26, and 27, respectively. Pathogen infection rates of *H. largrangei* and *R. microplus* were the highest in June and August, respectively that are accordance with the highest collected ticks in these months. While *H. wellingtoni* were only detected the pathogen in August.

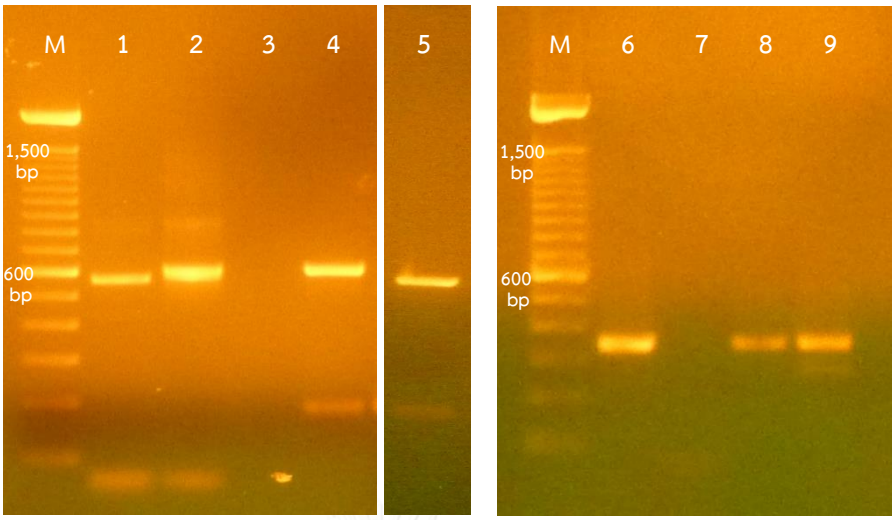

Figure 24. The amplified PCR product of *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. lane M: 100 bp DNA Marker; 1: Positive control of Protozoa (*B. bovis*); 2: Positive control of Protozoa (*B. bigemina*); 3: Negative control; 4: *Theileria* spp. positive sample (~600 bp product size); 5: *Babesia* spp. positive sample (~500 bp product size); 6: Positive control of Bacteria (*Anaplasma marginale*); 7: Negative control; 8-9: *Anaplasma* spp. positive sample (345 bp product size)

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Table 7. NCBI BLAST results of 254 nucleotide sequences retrieved from this study

No.	Sequence name	Source			Length	Highest BLAST result		
		Species	Stage	Numbers		Closely related species	Accession	% ident
1	*Babesia* spp. HL-1	*H. lagrangei*	Female	1	478	*Babesia* sp.	DQ159074	97
2	*Theileria* spp. HL-2 to 4	*H. lagrangei*	Male	3	575	*Theileria cervi*	KT959227	99
	Theileria spp. HL-5 to 8	*H. lagrangei*	Female	4	575	*Theileria cervi*	KT959227	99
9	*Theileria* spp. HL-9	*H. lagrangei*	Nymph	1	573	*Theileria cervi*	KT959227	99
10	*Theileria* spp. HL-10 to 11	*H. lagrangei*	Male	2	576	*Theileria capreoli*	KJ451470	99
	Theileria spp. HL-12 to 14	*H. lagrangei*	Female	3	576	*Theileria capreoli*	KJ451470	99
15	*Theileria* spp. HL-15	*H. lagrangei*	Nymph	1	576	*Theileria capreoli*	KJ451470	99
16	*Theileria* spp. HL-16	*H. lagrangei*	Male	1	579	*Theileria* sp.	AB602881	98
	Theileria spp. HL-17	*H. lagrangei*	Female	1	581	*Theileria* sp.	AB602881	97
	Theileria spp. HL-18	*H. lagrangei*	Male	1	590	*Theileria* sp.	AB602881	97
19	*Theileria* spp. HL-19	*H. lagrangei*	Female	1	546	*Theileria* sp.	AB602881	97
20	*Ehrlichia* spp. HL-20	*H. lagrangei*	Female	1	315	*Ehrlichia* sp.	KJ410253	100
21	*Anaplasma* spp. HL-21 to 61	*H. lagrangei*	Male	41	305	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-62 to 109	*H. lagrangei*	Female	48	305	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-110	*H. lagrangei*	Female	1	291	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-111	*H. lagrangei*	Female	1	271	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-112 to 155	*H. lagrangei*	Nymph	44	305	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-156 to 216	*H. lagrangei*	Larva	61	305	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HL-217	*H. lagrangei*	Nymph	1	272	*Anaplasma bovis*	KP314253	99
	Anaplasma spp. HW-218	*H. wellingtoni*	Male	1	305	*Anaplasma bovis*	KP314253	100
	Anaplasma spp. HW-219 to 221	*H. wellingtoni*	Female	3	305	*Anaplasma bovis*	KP314253	100
229	*Anaplasma* spp. RM-222 to 229	*R. microplus*	Larva	8	305	*Anaplasma bovis*	KP314253	100
230	*Anaplasma* spp. HL-230	*H. lagrangei*	Larva	1	305	*Anaplasma platys*	KU500914	99
231	*Anaplasma* spp. HL-231 to 232	*H. lagrangei*	Male	2	305	*Anaplasma* sp.	KX417200	100
	Anaplasma spp. HL-233 to 234	*H. lagrangei*	Female	2	305	*Anaplasma* sp.	KX417200	100
	Anaplasma spp. HL-235 to 236	*H. lagrangei*	Nymph	2	305	*Anaplasma* sp.	KX417200	100
	Anaplasma spp. HL-237 to 245	*H. lagrangei*	Larva	9	305	*Anaplasma* sp.	KX417200	100
	Anaplasma spp. RM-246 to 248	*R. microplus*	Larva	3	305	*Anaplasma* sp.	KX417200	100
	Anaplasma spp. HL-249	*H. lagrangei*	Female	1	305	*Anaplasma* sp.	KX417200	99
	Anaplasma spp. HL-250	*H. lagrangei*	Female	1	278	*Anaplasma* sp.	KX417200	99
253	*Anaplasma* spp.HL-251 to 253	*H. lagrangei*	Larva	3	305	*Anaplasma* sp.	KX417200	99
254	*Wolbachia* spp. HL-254	*H. lagrangei*	Larva	1	305	*Wolbachia* endosymbiont	KM404238	100

Table 8. Tick infection rates and co-infection rate categorized by collection times, tick species and locations

	No. of ticks analyzed	No. of pathogen detection (% tick infection rate)							
		Babesia	*Theileria*	*Ehrlichia*	*Anaplasma*	*Wolbachia*	Single infection	Co-infection	Total
Months									
Feb	52	0 (0)	0 (0)	0 (0)	6 (11.54)	0 (0)	6 (11.54)	0 (0)	6 (11.54)
April	41	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
June	103	1 (0.27)	5 (4.85)	1 (0.97)	65 (63.11)	0 (0)	62 (60.19)	5 (4.85)	67 (65.05)
August	81	0 (0)	1 (1.23)	0 (0)	62 (76.54)	1 (1.23)	60 (74.07)	2 (2.47)	62 (76.54)
October	60	0 (0)	6 (10.00)	0 (0)	44 (73.33)	0 (0)	42 (70.00)	4 (6.67)	46 (76.67)
December	82	0 (0)	6 (7.32)	0 (0)	56 (68.29)	0 (0)	54 (65.85)	4 (4.88)	58 (70.73)
Total	419	1 (0.24)	18 (4.30)	1 (0.24)	233 (55.61)	1 (0.24)	224 (53.46)	15 (3.58)	239 (57.04)
Tick species									
H. lagrangei	364	1 (0.27)	18 (4.95)	1 (0.27)	218 (59.89)	1 (0.27)	209 (57.41)	15 (4.12)	224 (61.54)
H. wellingtoni	13	0 (0)	0 (0)	0 (0)	4 (30.77)	0 (0)	4 (30.77)	0 (0)	4 (30.77)
R. microplus	42	0 (0)	0 (0)	0 (0)	11 (26.19)	0 (0)	11 (26.19)	0 (0)	11 (26.19)
Total	419	1 (0.24)	18 (4.30)	1 (0.24)	233 (55.61)	1 (0.24)	224 (53.46)	15 (3.58)	239 (57.04)
Locations									
A	194	0 (0)	5 (2.58)	1 (0.52)	120 (61.86)	0 (0)	116 (59.79)	5 (2.58)	121 (62.37)
B	160	1 (0.63)	10 (6.25)	0 (0)	83 (51.88)	1 (0.63)	77 (48.13)	9 (5.63)	86 (53.75)
C	65	0 (0)	3 (4.62)	0 (0)	30 (46.15)	0 (0)	31 (47.69)	1 (1.54)	32 (49.23)
Total	419	1 (0.24)	18 (4.3)	1 (0.27)	233 (55.61)	1 (0.24)	224 (53.46)	15(3.58)	239 (57.04)

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Table 9. Infection rates of pathogen/microorganism in each tick species distributed by times and locations

Month	Location	No. of ticks analysed	Numbers of Pathogen/microorganism (% tick infection rate of each ticks)							*Total
			Babesia	*Theileria*	*Ehrlichia*	*Anaplasma*	*Wolbachia*	Uncultured eukaryote	Uncultured bacterium	
H. lagrangei										
Feb	A	26	0	0	0	3 (0.82)	0	0	0	3 (0.82)
	B	16	0	0	0	3 (0.82)	0	0	0	3 (0.82)
	C	ND	ND	ND	ND	ND	ND	ND	ND	ND
Apr	A	9	0	0	0	0	0	2 (0.55)	0	0
	B	22	0	0	0	0	0	0	0	0
	C	1	0	0	0	0	0	0	0	0
Jun	A	48	0	2 (0.55)	1 (0.27)	41 (11.26)	0	1 (0.27)	0	44 (12.09)
	B	34	1 (0.27)	3 (0.82)	0	24 (6.59)	0	1 (0.27)	0	28 (7.69)
	C	6	0	0	0	0	0	0	0	0
Aug	A	27	0	0	0	26 (7.14)	0	0	0	26 (7.14)
	B	12	0	1 (0.27)	0	9 (2.47)	1 (0.27)	3 (0.82)	0	11 (3.02)
	C	24	0	0	0	17 (4.67)	0	0	2 (0.55)	17 (4.67)
Oct	A	29	0	1 (0.27)	0	22 (6.04)	0	5 (1.37)	1 (0.27)	23 (6.32)
	B	21	0	4 (1.10)	0	13 (3.57)	0	1 (0.27)	1 (0.27)	17 (4.67)
	C	8	0	1 (0.27)	0	7 (1.92)	0	1 (0.27)	1 (0.27)	8 (2.20)
Dec	A	37	0	2 (0.55)	0	24 (6.59)	0	5 (1.37)	1 (0.27)	26 (7.14)
	B	34	0	2 (0.55)	0	29 (7.97)	0	4 (1.10)	1 (0.27)	31 (8.52)
	C	10	0	2 (0.55)	0	3 (0.82)	0	0	0	5 (1.37)
Total		364	1 (0.27)	18 (4.95)	1 (0.27)	221 (60.71)	1 (0.27)	23 (66.32)	7 (1.92)	242 (66.48)
H. wellingtoni										
Feb	A	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0
	C	ND	ND	ND	ND	ND	ND	ND	ND	ND
Apr	A	1	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0
	C	2	0	0	0	0	0	0	0	0
Jun	A	1	0	0	0	0	0	0	0	0
	B	5	0	0	0	0	0	0	0	0
	C	2	0	0	0	0	0	0	0	0
Aug	A	0	0	0	0	0	0	0	0	0
	B	2	0	0	0	1 (7.69)	0	0	1 (7.69)	1 (7.69)
	C	0	0	0	0	0	0	0	1 (7.69)	0
Oct	A	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0
Dec	A	0	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0
Total		13	0	0	0	1 (7.69)	0	0	2 (15.38)	1 (7.69)
R. microplus										
Feb	A	4	0	0	0	0	0	0	0	0
	B	6	0	0	0	0	0	0	0	0
	C	ND	ND	ND	ND	ND	ND	ND	ND	ND
Apr	A	3	0	0	0	0	0	0	0	0
	B	1	0	0	0	0	0	0	0	0
	C	2	0	0	0	0	0	0	0	0
Jun	A	1	0	0	0	0	0	0	0	0
	B	1	0	0	0	0	0	0	0	0
	C	5	0	0	0	0	0	0	0	0
Aug	A	6	0	0	0	3 (7.14)	0	0	0	3 (7.14)
	B	5	0	0	0	3 (7.14)	0	1 (2.38)	0	3 (7.14)
	C	5	0	0	0	3 (7.14)	0	1 (2.38)	0	3 (7.14)
Oct	A	1	0	0	0	1 (2.38)	0	0	0	1 (2.38)
	B	1	0	0	0	1 (2.38)	0	0	0	1 (2.38)
	C	0	0	0	0	0	0	0	0	0
Dec	A	1	0	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0
Total		42	0	0	0	11 (26.19)	0	2 (4.76)	0	11 (26.19)
The sum		419	1 (0.24)	18 (4.30)	1 (0.24)	233 (55.61)	1 (0.24)	25 (5.97)	9 (2.15)	254 (60.62)

Note. ND, not determined (not collected in this location); *Total, total of *Babesia*, *Theileria*, *Ehrlichia*, *Anaplasma*, and *Wolbachia*

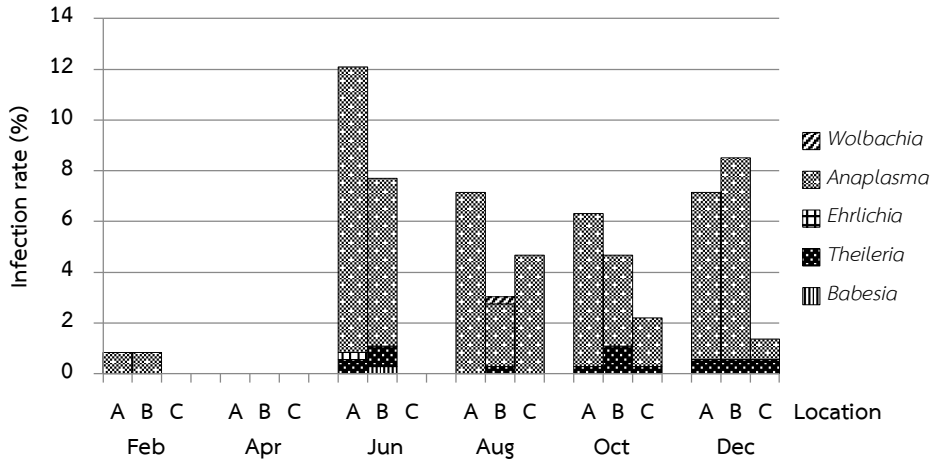

Figure 25. Pathogen infection rates of *H. lagrangei* distributed by collection times and locations

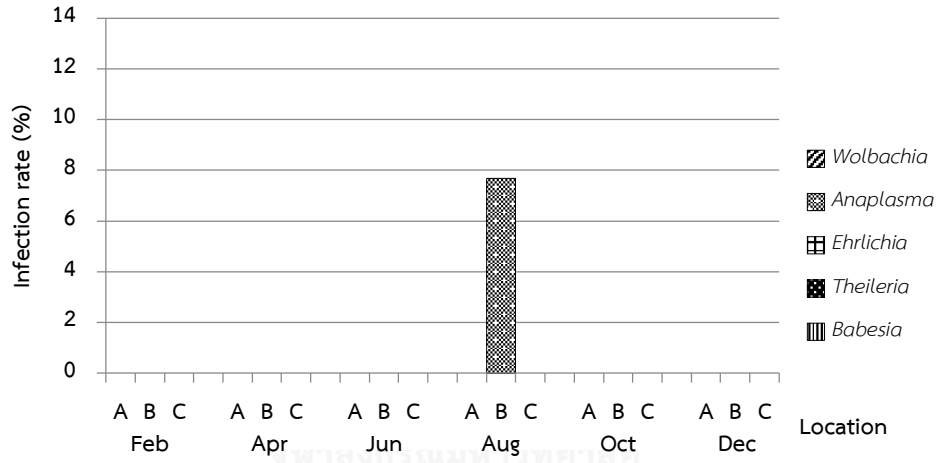

Figure 26. Pathogen infection rates of *H. wellingtoni* distributed by collection times and locations

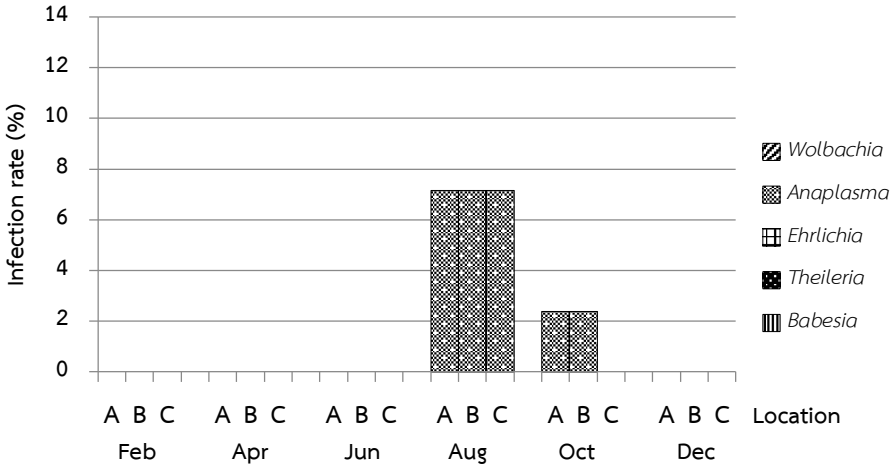

Figure 27. Pathogen infection rates of *R. microplus* distributed by collection times and locations

5. Phylogenetic analysis of protozoa and bacteria in ticks

Out of 419 analyzed tick samples, 254 positive DNA fragments were obtained from this study. All sequences were performed the BLAST search and further analyzed by constructed phylogenetic tree. The NCBI BLAST results of all sequences were shown in Table 7.

After the BLAST search, amplified DNA sequences of KB-16 and KB-17 primers were defined to two genera of *Babasia* and *Theileria*. In this study, one partial 18S rRNA gene of *Babesia* spp. from one female adult *H. lagrangei* (*Babesia* spp. HL-1) was retrieved in this study, which had 97% identity with *Babesia* spp. sequences available in Genbank. For BLAST results of *Theileria*, three groups of 18S rRNA gene of *Theileria* spp. obtained from 18 ticks were categorized. Firstly, *Theileria* spp. HL-2 to -9 identified from eight *H. lagrangei* ticks (3 male and 4 female adults, and 1 nymph) were identical each other and were closely related (99% similarity) to *T. cervi* from deer sequences, secondly *Theileria* spp. HL-10 to -15 were obtained from six ticks (2 male and 3 female adults, and 1 nymph), which identical each other and were closely related (99% similarity) to *T. capreoli* from deer sequences, and lastly, *Theileria* spp. HL-16 to -19 isolated from four ticks (2 male and 2 female adults), which showed 97-98% identity with *Theileria* sp. sequences available in Genbank database.

All 19 sequences of 18S rRNA gene of *Babesia* spp. and *Theileria* spp. detected in this study were further analyzed by constructed maximum likelihood phylogenetic tree using Kimura's two-parameter model with 1,000 bootstrap

replications to compare with representative of 19 *Babesia* and 15 *Theileria* strains available in Genbank database as shown in Figure 28. Phylogenetic analysis of *Babesia* spp. HL-1 were situated in the same cluster, at 80% bootstrap value, with several *Babesia* spp. sequences, including *Babesia* sp. Hebei-2005 from sheep in China (DQ159074), *Babesia* sp. Irk-Hc130 from *H. concinna* (KJ486569), *Babesia motasi* in sheep (AY260180), and with the phylogenetically nearest with *Babesia* sp. KO1 from human case in Korea (DQ346955).

All 18 *Theileria* spp. sequences retrieved from this study were placed in three phylogenetically clusters. Firstly, *Theileria* spp. HL-2 to 9 (8 sequences) were form a cluster with *T. cervi* from sika deer in China (KT959227), *Theileria* sp. CS-2012 from *H. lagrangei* collected from sambar deer in Thailand (JQ751277), *Theileria* sp. MT593 from *H. flava* in Japan (LC169091), and *Theileria* sp. Iwate from Japanese serow in Japan (AB602881). Secondly, *Theileria* spp. HL-10 to 15 (6 sequences) were formed a cluster with several *T. capreoli* strains including *T. capreoli* in white-lipped deer in China (KJ451470), and *T. capreoli* in roe deer from Spain (AY726011). Finally, *Theileria* spp. HL-16 to 19 (4 sequences) were formed individual cluster which not cluster with any *Theileria* species sequences. Due to *Theileria* spp. HL-16 to 19 were formed a separate cluster which appear to be a sister taxa with *T. capreoli*, the polymorphic base position were further analyzed to compare among this group of *Theileria* and *Theileria* spp. HL-10 to 15 and two *T. capreoli* sequences in Genbank database as demonstrated in Figure 29.

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For BLAST analysis of 235 amplified sequences of EHR16SD and EHR16SR primers, three genera, including *Ehrlichia*, *Anaplasma*, and *Wolbachia* were identified in this study. One of 16S rRNA sequence of *Ehrlichia* spp. was identified from one female adult of *H. lagrangei* which showed 100% identity with *Ehrlichia* spp. sequence available in Genbank. The BLAST results of 233 tick samples were identical with *Anaplasma* spp. sequences in Genbank database and were classified into three groups. Firstly, *Anaplasma* spp. HL-21 to 216, HW-218 to 221 and RM-222 to 229 from 208 tick samples were identical each other and matched (100%) identity with *A. bovis*, and one sequence of *Anaplasma* spp. HL-217 from one nymph of *H. lagrangei* was 99% identity with *A. bovis*. Secondly, *Anaplasma* spp. HL-230 was identified from one *H. lagrangei* larval pool that identical 99% with *A. platys* sequences. Finally, a total of 23 sequences of *Anaplasma* spp. HL-231 to 245, RM-246 to 248, and HL-249 to 253 were identical each other and 99-100% similarity with more than one *Anaplasma* spp., including *A. marginale, A. centrale* and *A. ovis*. Furthermore, this study was also found one sequence of *Wolbachia* spp. HL-254 from larval pool of *H. lagrangei*, which 100% identical with *Wolbachia* endosymbiont strains available in database.

All of sequences of 16S rRNA gene of bacteria in genus *Ehrlichia*, *Anaplasma* and *Wolbachia* were generated maximum likelihood phylogenetic tree using Kimura's two-parameter model with 1,000 bootstrap replications to compare with representative of 10 *Ehrlichia*, 11 *Anaplasma*, and 4 *Wolbachia* strains available in Genbank database which showed in Figure 30. For the phylogenetic analysis,

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Ehrlichia spp. HL-20 was pended among the branch of *E. ewingii* from dog (U96436), *E. ruminantium* from ruminant (DQ647615), and several *Ehrlichia* spp., including *Ehrlichia* sp. Yonaguni 206 from *H. longicornis* (HQ697589), *Ehrlichia* sp. TC251-2 from *Dermacentor nuttalli* (KJ410253) and *Ehrlichia* sp. SS15-E-L from wild deer (AB211162). *Anaplasma* sequence groups were phylogenetically characterized into three clusters. Firstly, *Anaplasma* spp. HL-21 to 216, HW-218 to 221 and RM-222 to 229, including diverge sequence of *Anaplasma* spp. HL-217, were placed in the same cluster with several *A. bovis* from *H. longicornis* (AB983376), *A. bovis* from sika deer (KJ659040), *A. bovis* from goat (KP062958) and *Anaplasma* sp. BL126-13 from *Hyalomma asiaticum* (KJ410243). Secondly, *Anaplasma* spp. HL-230 which situated in the same cluster with *Anaplasma platys* from dog (KU500914 and AY821826). Lastly, *Anaplasma* spp. HL-231 to 245, RM-246 to 248 and HL-249 to 253 were situated in the same cluster with *A. marginale* (FJ226454), *A. centrale* (KC189842), and *A. ovis* (KJ639880) which more divergence were found in *Anaplasma* spp. HL-249 to HL-253. For *Wolbachia* spp. HL-254, phylogenetic analysis was found the genetic relationships at 99% bootstrap value among many *Wolbachia* strains in arthropods such as *Wolbachia pipientis* in *Aedes fluviatilis* (GQ981315), *Wolbachia* endosymbiont of *Aedes albopictus* (KX611380), *Culex pipiens* (KJ512994) and brown planthopper (KT362741).

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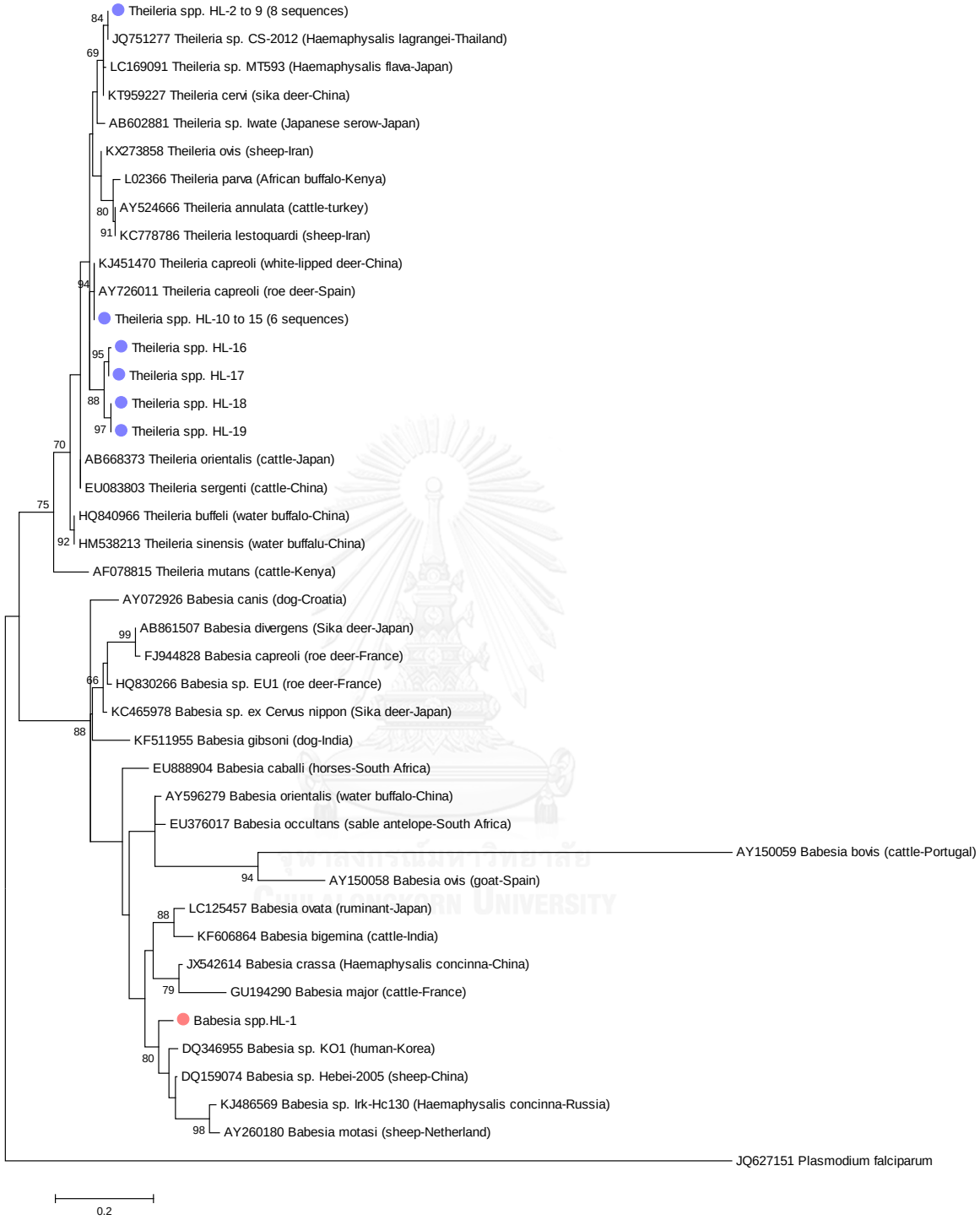
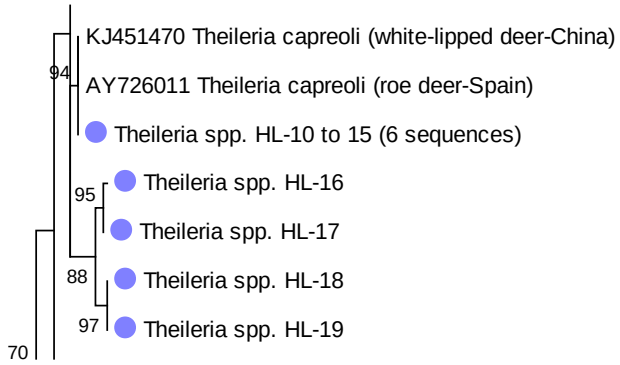


Figure 28. Maximum likelihood phylogenetic tree of 603 bp partial 18S rRNA gene of one *Babesia* spp. (Pink dot) and 18 *Theileria* spp. (Blue dot) sequences from tick samples using Kimura's two-parameter model with 1,000 bootstrap replications using *Plasmodium falciparum* as outgroup



Base position	1 2 7	2 7 4	3 1 4	3 1 9	3 2 9	3 3 1	3 3 3	3 3 4	3 4 8	3 5 9	3 6 1	3 6 3	3 6 4	3 6 5	3 6 6
Theileria capreoli (KJ451470)	T	C	G	G	C	A	A	C	C	A	T	C	G	G	T
Theileria capreoli (AY726011)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Theileria spp. HL-10 to 15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Theileria spp. HL-16	A	.	A	A	T	T	T	A	T	.	A	T	T	C	G
Theileria spp. HL-17	A	.	A	A	T	T	T	A	T	.	A	T	T	C	G
Theileria spp. HL-18	.	T	A	A	T	.	T	A	T	T	A	T	T	C	G
Theileria spp. HL-19	.	T	A	A	T	.	T	A	T	T	A	T	T	C	G

Base position	3 9 3	3 6 7	3 6 8	3 7 1	3 7 6	3 7 7	3 8 0	3 8 3	3 8 5	3 8 6	3 8 7	3 8 8	3 8 9	3 9 0	3 9 1
Theileria capreoli (KJ451470)	G	T	A	A	T	A	T	A	T	T	C	C	G	G	A
Theileria capreoli (AY726011)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Theileria spp. HL-10 to 15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Theileria spp. HL-16	T	G	T	T	C	T	G	T	.	C	.	G	A	A	T
Theileria spp. HL-17	T	G	T	T	.	T	G	T	.	C	.	G	A	A	T
Theileria spp. HL-18	T	.	T	T	.	.	A	T	C	G	A	A	A	T	T
Theileria spp. HL-19	T	.	T	T	.	.	A	T	C	G	A	A	A	T	T

Figure 29. Polymorphic base positions of unidentified *Theileria* spp. HL-16 to 19 (green letters) compared with two strains of *T. capreoli* and *Theileria* spp. HL-10 to 15 which closely related to *T. capreoli*

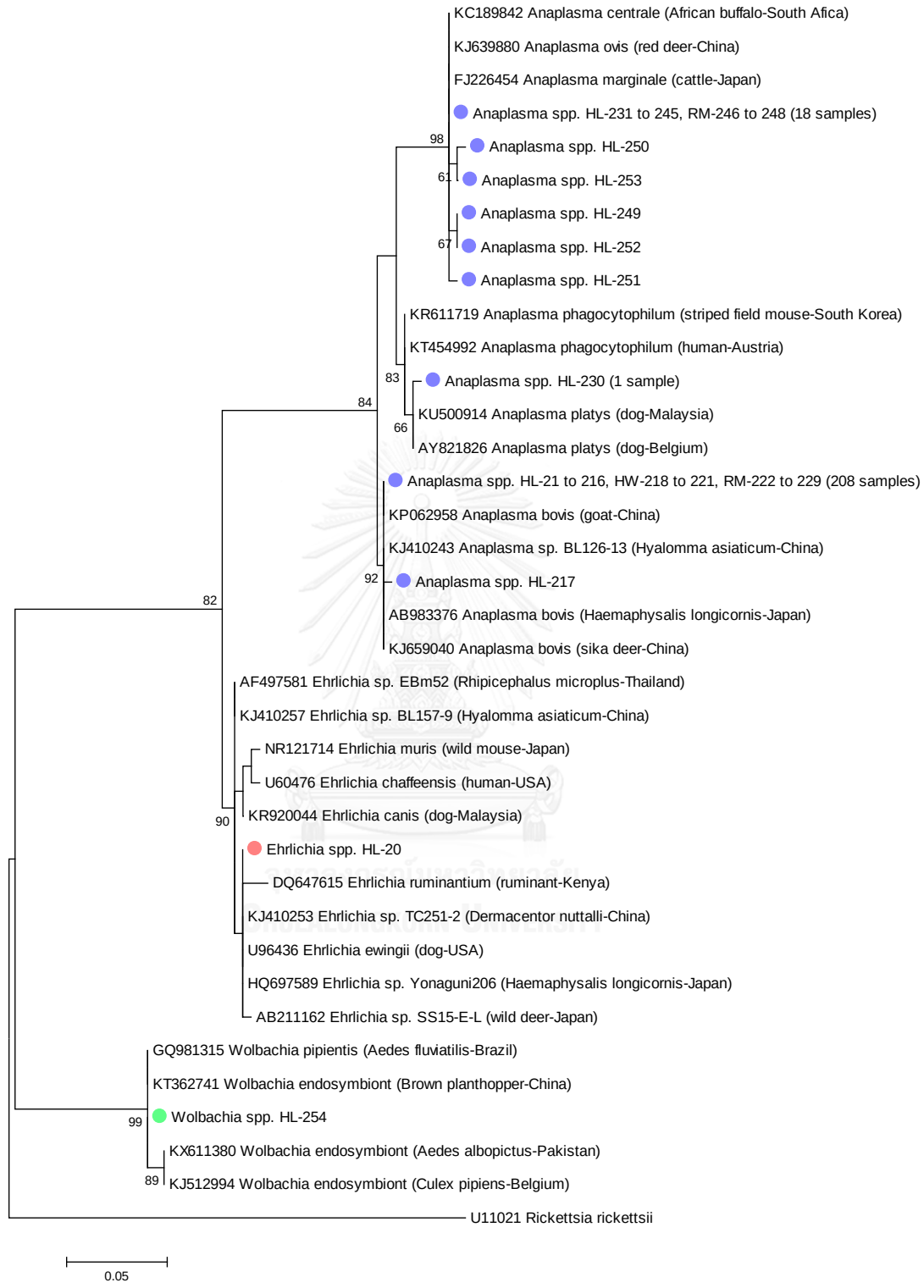


Figure 30. Maximum likelihood phylogenetic tree of 252 bp partial 16S rRNA gene of one *Ehrlichia* spp. (pink dot), 233 *Anaplasma* spp. (blue dot) and one *Wolbachia* spp. (green dot) sequences from tick samples using Kimura's two-parameter model with 1,000 bootstrap replications using *Ricketsia rickettsii* as outgroup

CHAPTER V
DISCUSSIONS

Nowadays, vector-borne diseases are important because of their emerging and re-emerging globally. Ticks are important biological vectors in the transmission cycle of various diseases and wild animals are considered important reservoirs of these pathogens. This study provided the information of questing ixodid tick species in wildlife habitats and the detection of tick-borne protozoal and bacterial pathogens. It yielded a better understanding of the tick ecology, including seasonal relationships and important tick-borne pathogen, and the association between tick species and these pathogens. These findings would support the evidences that tick-borne protozoa and bacteria can be detected in questing ticks by using PCR and confirmed through DNA sequencing technique.

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In the present study, a total of 12,184 ticks belongs to two genera and identified into three species which comprise *Haemaphysalis lagrangei*, *H. wellingtoni*, and *Rhipicephalus* (*Boophilus*) *microplus*. For the morphological identification of ticks, *H. wellingtoni* adults would be difficult to identify to the species level due to several species in this genus have similar morphology. In addition, *Rhipicephalus* samples collected in this study were only the larval stage and they could not be identified to the species level because of the limitations of taxonomic keys of immature stage ticks. Accordingly, molecular techniques should be performed to confirm the morphological

findings for more accurate results. The primers which target tick mitochondrial 16S rRNA gene used in this study have been reliable and extensively used in the species identification. Owning to strictly maternal inherited of mitochondrial gene with high numbers of copy gene and genetic highly conserved, therefore, the mitochondrial 16S rRNA gene was suitable to discriminate tick species even though closely related species with high sequencing quality (Caporale et al., 1995; Cruickshank, 2002). The finding from this study suggested that morphology-based identification can be differentiated some ticks at the genus level, while DNA-based technique can be identified into the species level of all representative ticks.

From all 12,184 tick samples examined in this study, larvae (95.95%) were predominantly collected more than male adults (1.64%), female adults (1.53%), and nymphs (0.88%), and all of them were un-engorged ticks. For tick sampling procedures, dragging combined with eye searching used in this study were the appropriate techniques for tick collection in fields. The study by Ramos Vdo et al. (2014) reported dragging and eye searching under the leave was the highest efficiency to collect the variety and various stages of ticks because they distributed in an environment which eye searching was increase the opportunity to collect adult ticks. However, a great number of collected larvae in this study might occur from a numerous larvae which were hatched from an egg batch under the leaves.

H. lagrangei was the predominant species found in this study area (92.82%) which all life stages were collected. Only the larval stage of *R. microplus* (7.05%) and

a minority of the adult stage of *H. wellingtoni* (0.13%) were collected. This might be due to its one host life cycle of *R. microplus* which only questing larval stage was found, whereas all life stages of *Haemaphysalis* ticks which are the three host ticks were found in this study. All three tick species addressed in this study have been previously reported in many parts of Thailand (Tanskul et al., 1983; Parola et al., 2003; Cornet et al., 2009). A variety of *Haemaphysalis* species was widely distributed in Southeast Asia, which are able to infest animals and human (Petney, 1993). *H. lagrangei* was originally found in forest and frequently parasitizes deer (Hoogstraal et al., 1973; Tanskul et al., 1983). In accordance with this study, *H. lagrangei* was the most abundant tick species found in the high deer density area as Khao Chi-On Non Hunting Area. According to the tick survey in the northeastern part of Thailand, *H. lagrangei* was the most plentiful tick species in Khao Yai National Park (Ariyakulwong, 2006). *H. wellingtoni* was the tick species generally reported in forest and commonly infested many species of avian. Furthermore, *H. wellingtoni* has been reported to be a vector of human Kyasanur Forest disease virus in India (Mehla et al., 2009). *R. microplus* is the common species which parasitizes domestic and wild ungulates, and it is the most significant vector of cattle tick fever in Southeast Asia.

Climate change is more likely to impact the tick life cycle, including transmission cycle of tick-borne disease (Ogden and Lindsay, 2016). There was no statistically significant difference between each six sampling months in average numbers of the collected ticks in this study. However, the highest number of

Haemaphysalis ticks was found in June and it was related with the highest total rainfall and mean temperature in this month. A small number of ticks were collected in October although the highest mean relative humidity was found in this month. The flood throughout the sampling area in October caused the difficulty of tick sample collection and a small number of collected ticks. The mean temperature in June was 29.4°C appears to nearly optimal temperature for ticks. The study by Diehl et al. (1982) revealed the optimal temperature for maximum conversion efficiency of ovipositing *R. microplus* was 29°C. Accordance with Ogden and Lindsay (2016), the increasing temperature, rainfalls, and relative humidity would increase the tick activity and reduce the tick mortality rate. On the other hand, intense rainfall and persistent flood would inhibit activity and increase mortality rate of ticks.

In different sampling locations, average numbers of *H. lagrangei* nymphs collected in location A was higher than those collected in location C and it was statistically significant difference. According to the bar charts, collected adult ticks in location B were higher than location A and C, respectively, while collected nymphal and larval ticks were higher in location B. However these differences might occur from the different terrain characteristics that location A and C were plains while location B was foothill slope.

All collected ticks in this study were un-engorged, therefore, there was no interference from undigested host blood to the pathogen detection. Four pathogens

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and one bacterial symbiont were detected in wild questing ticks with different levels of infection rates. 55.61% (233/419) and 4.30% (18/419) of tested tick samples were infected with *Anaplasma* spp. and *Theileria* spp., respectively. Only 0.24% (1/419) of tested tick samples were infected with *Babesia* spp., *Ehrlichia* spp. and *Wolbachia* spp. Uncultured eukaryote and uncultured bacterium were also detected at 5.97% (25/419) and 2.15% (9/419) of ticks, respectively. In this study, *H. lagrangei* was the most tick species which were able to infect with all five microorganisms, whereas two remaining tick species were only infected with *Anaplasma* spp.

Single and co-infection of the pathogens in ticks can be found in this study. 3.58% (15/419) of ticks had co-infection of the pathogens which were the co-infection between *Anaplasma* and *Babesia*, *Anaplasma* and *Theileria*, *Anaplasma* and *Ehrlichia*, *Anaplasma* and *Wolbachia*, and *Ehrlichia* and *Theileria*. Co-infection in ticks might due to their ability to harbor both pathogenic and symbiotic microorganisms because they fed on more than one host species during their life stages. According to the report by Moutailler et al. (2016), co-infection was frequently found between pathogen and symbiont which do not affect each other but it may be difficult to diagnosis.

Two genera of tick-borne protozoa were detected in this study, including *Babesia* spp. and *Theileria* spp. Only one sequence of *Babesia* spp. (*Babesia* spp. HL-1) from female adult *H. lagrangei* was identified in this study. According to phylogenetic analysis, *Babesia* spp. HL-1 sequence was situated in the same clade with several ovine

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Babesia spp., including *B. motasi* and it was phylogenetically nearest with *Babesia* sp. KO1 from human case in Korea. Previous study has been revealed phylogenetically similarity between ovine babesiosis and *Babesia* sp. KO1 from human (Kim et al., 2007). A great variety of wildlife species, including deer have been reported to be a common reservoir of *Babesia* spp. as well as zoonotic *Babesia* (Penzhorn, 2006; Yabsley and Shock, 2013). According to previous studies, many species of *Haemaphysalis* ticks, including *H. punctata*, *H. qinghaiensis* and *H. longicornis* were proposed to be a vector of ovine *Babesia* (Ahmed et al., 2006; Liu et al., 2007; Guan et al., 2010). These findings suggested that *H. lagrangei* might be a possible vector of *Babesia* spp. and could be concern with zoonotic *Babesia*.

In our study, three genotype groups of *Theileria* spp. were detected in 18 adults and nymphs *H. lagrangei* tick samples. Firstly, *Theileria* spp. were closely related to *T. cervi* (*Theileria* spp. HL-2 to -9) obtained from eight tick samples. *T. cervi* was piroplasms found in many cervid species that were reported in many countries (Inokuma et al., 2004; Liu et al., 2016). *Theileria* spp. HL-2 to -9 sequences were genetically correspond with *Theileria* spp. closely related to *T. cervi* which recently reported in Thailand in *H. lagrangei* and *R. microplus* collected from sambar deer (Sumrandee et al., 2015). Secondly, *Thileria* spp. closely related to *T. capreoli* (*Thileria* sp. HL-10 to -15) that attained from six tick samples. *T. capreoli* was piroplsms mostly found in wild cervids, accordingly to the finding of *T. capreoli* in deer in many countries (Garcia-Sanmartin et al., 2007; Li et al., 2014). Lastly, unidentified *Theileria* spp.

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(*Theileria* spp. HL-16 to -19) achieved from four tick samples had 97-98% similarity with *Theileria* Iwate from Japanese sarrow in Japan and these sequences were not cluster with any *Theileria* species sequences. It is speculated that there was a new variant of *Theileria* spp., which had nucleotide substitution in the thirty base positions when compared with *T. capreoli*. Recently report in Thailand by Poolkhetkit et al. (2015) described that *Theileria* spp. were closely related to *T. sinensis* and *T. orientalis/sergenti/buffei* detected in *R. microplus* ticks which were collected from domestic cattle in the buffer zone of Sai Yok National park. Both of them were *Theileria* species found in domestic ruminants which were distinct from *Theileria* spp. sequences attained from this study. It has been assumed that three groups of *Theileria* identified from *H. lagrangei* ticks might be concerned with the deer live around the study sites and should be monitored for health status of the wildlife in this area. These results supported the possibility that *H. lagrangei* might be a vector of *T. cervi* and *T. capreoli* in Thailand.

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This study has been demonstrated three different genera of bacteria in the family Anaplasmataceae including *Ehrlichia* spp., *Anaplasma* spp., and *Wolbachia* spp. Only one *Ehrlichia* spp. sequence (*Ehrlichia* spp. HL-20) which had 100% identical with *Ehrlichia* spp. detected in *Dermacentor nuttali* and *Haemaphysalis longicornis* and 99% similarity with *Ehrlichia* spp. in wild deer. In accordance with other studies that detected in different tick species, *Ehrlichia* spp. closely related to *E. ruminantium* was detected in questing *Amblyomma americanum* ticks in USA (Loftis et al., 2008).

Kawahara et al. (2006) also reported *Ehrlichia* spp. from *H. longicornis* ticks collected from deer. However, the previous report showed the detection of *Ehrlichia* spp. in *R. microplus* from cattle in Thailand (Parola et al., 2003) but it had phylogenetically far from our *Ehrlichia* spp. sequences. It suggested that species of *Ehrlichia* in wild *H. lagrangei* ticks were different from *Ehrlichia* spp. in *R. microplus* ticks from domestic cattle.

From phylogenetic tree, *Ehrlichia* spp. HL-20 was placed among the branch of *E. ewingii* and *E. ruminantium* due to the short length about 305 base pairs of this partial 16S rRNA gene which cannot distinguish between these two species of *Ehrlichia*. However, using full length gene or other gene might receive the better result in microorganism species differentiation. Because some species of *Ehrlichia* in wildlife are pathogenic or zoonotic pathogen, the monitoring of wildlife health status in this area would be beware. Furthermore, this study proposed the different tick vectors of *Ehrlichia* which *H. lagrangei* might be a potential vector in Thailand.

Anaplasma infection rate in ticks was highest in this study. It can be found in all three tick species and all life stages. The high prevalence of *Anaplasma* infection in ticks might be due to ticks are the vectors that have a greater variety of microorganisms more than other blood feeding insect (Jongejan and Uilenberg, 2004; Telford III and Goethert, 2004). Both biological and mechanical transmission of this pathogen in ticks seem to support a widely transmission in host population (Kocan et al., 2003; Baldridge et al., 2009). Three genotype groups of *Anaplasma* were achieved

in this study. Firstly, *Anaplasma* spp. closely related to *A. bovis* (*Anaplasma* spp. HL-21 to 217, HW-218 to 221 and RM-222 to 229 sequences) attained from totally 209 tick samples. Some species of *Haemaphysalis* ticks, including *H. lagrangei*, *H. megaspinosa*, and *H. longicornis* are the possible vectors of *A. bovis* reported in Asia (Kim et al., 2003; Parola et al., 2003; Yoshimoto et al., 2010). Previous studies reported the infection of *A. bovis* in cattle may be associated with the infection of deer, suggested that wild deer may serve as the natural reservoir for anaplasmosis in cattle (Jilintai et al., 2009). *Anaplasma* spp. which closely related to *A. bovis* was previously reported in Thailand in *H. lagrangei* collected from bear and sambar deer (Parola et al., 2003; Sumrandee et al., 2016). This study reported possible potential vectors of this agent in Thailand which were *H. lagrangei*, *H. wellingtoni*, and *R. microplus*.

Secondly, *Anaplasma* spp. closely related to *A. platys* (*Anaplasma* spp. HL-230) which was identified from one larval pool of *H. lagrangei*. *A. platys* is the causative agent of infectious canine cyclic thrombocytopenia which usually found in dog. Some studies reported other animal species such as cattle and foxes could be infected with this pathogen and served as a reservoir host. However, pathogenesis in these hosts are unclear (Rar and Golovljova, 2011; Cardoso et al., 2015; Dahmani et al., 2015). *R. sanguineus* ticks are reported to be a common vector of *A. platys* in dog. *A. platys* infections in other tick species, including *Ixodes persulcatus, Dermacentor nuttali,* and *Haemaphysalis longicornis* in forest of Mongolia and Korea has been reported (Kim et al., 2006; Javkhlan et al., 2014). Concordance with Sumrandee et al.

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(2016), *Anaplasma* spp. closely related to *A. platys* was detected in *H. lagrangei* collected from sambar deer which supported the evidence that *H. largrangei* might be a potential vector of *A. platys* in Thailand. The finding of *A. platys* in this study was possible due to the infection in domestic and wild canine surrounding in this area.

Lastly, unidentified *Anaplasma* spp. sequences (*Anaplasma* spp. HL-231 to 245, RM-246 to 248 and HL-249 to 253) determined from 20 *H. lagrangei* and three *R. microplus* tick samples were pended among the branch of *A. marginale*, *A. centrale*, and *A. ovis* and five sequences (HL-249 to 253) were diverged within the clade. *A. marginale, A. centrale,* and *A. ovis* are *Anaplasma* species that infected domestic and wild ruminant host, and tick vectors. Genetic variations of these sequences might be occurred from evolution of this pathogen. The previous reports suggested that genetic variation in MSP gene of *Anaplasma* may be changed in vector-pathogen interaction which affect the capability of non-vector tick species to become a vector (Estrada-Pena et al., 2009; de la Fuente et al., 2010). Due to the close homologies of 16S rRNA gene among these three species of *A. marginale, A. centrale*, and *A. ovis*, it might be required further analyzed by using full length of 16S rRNA gene or other specific gene such as MSP gene and groEL gene to studies genetic characterization and genetic variation of *Anaplasma*. The purpose of using primer EHR16SD and EHR16SR was for screening bacteria in family Anaplasmataceae to examine the infection in tick, however, the limitation to distinguish between some species of *Ehrlichia* and *Anaplasma* in this study might be required the full length of 16S rRNA genes or other

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specific genes to support the discrimination among them and for successfully genetic characterization species of these bacteria.

Interestingly, the present study showed the larvae of both *H. lagrangei* and *R. microplus* were infected with *Anaplasma* spp. that seem to be caused by transovarial transmission from infected female adults to their offsprings. Although transstadial transmission of *A. marginale* has been confirmed (Stich et al., 1989), however, transovarial transmission only reported to occur in *A. phagocytophilum* (Baldridge et al., 2009).

Wolbachia spp. sequence (*Wolbachia* spp. HL-254) achieved from one larval pool of *H. lagrangei* in this study was closely related at 100% similarity and 99% bootstrap value of genetic relationship with *Wolbachia* in a wide variety of arthropods such as ticks, mosquitoes, and other insects including *Wolbachia pipientis* sequences available in Genbank database which was suggested the widely spread of this symbiont in ticks and other arthropods. Furthermore, *Wolbachia* spp. was detected only in the larval pool of *H. lagrangei* which possible related with transovarial transmission of this microorganism. In this study, the finding of *Wolbachia* sequence supported the evidences that *Wolbachia* was symbiosis living within tick populations in Thailand and might be required further studies for using *Wolbachia* as a biological control of ticks and tick-borne pathogen in the future. For the only one sequence of *Babsia*, *Ehrlichia*, and *Wolbachia* detected, PCR followed by direct DNA sequencing used for pathogen detection in this study might be incapable to differentiate mixed PCR products of

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multiple microorganisms within ticks. However, additional molecular technique such as DNA cloning approach might be required to identify individual species in mixed microorganisms infected ticks.

This study provided the information of tick diversity in wildlife habitats with the seasonal association which yielded a better understanding of tick ecology. In addition, our results suggested that several pathogen, including bacterial endosymbiont were detected in questing ticks in forest area with prominently with deer as Khao Chi-On Non-Hunting area by using PCR and DNA sequencing techniques. The finding could support the relationships between ticks, pathogen and host as deer in the study sites because most detected pathogen were associated with deer. There was no blood sample collected from wild animals in this study to indicate the infection in these animals. However, a variety of pathogen could be detected in wild questing ticks and it can be used for monitoring the health status of wildlife and the surveillance of disease transmission between domestic and wild animals in this area. In addition, several human outdoor activities in these areas will increase the possibilities to contact among wild animals, human, and ticks, and the awareness about zoonotic diseases should be considered. Furthermore, these might be the useful data for designing the treatment, prevention, and control strategies for tick borne diseases. However, further experimental studies are needed to assess the tick vector competence for fully understand the interaction of these tick borne pathogens and tick vectors.

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In conclusions, our data revealed that the tick diversity in Khao Chi-On Non Hunting Area and Banglamung Wildlife Breeding Center including *H. lagrangei*, *H. wellingtoni*, and *R.* (*Boophilus*) *microplus*. In this study, four genera of pathogens included *Anaplasma* spp., *Theileria* spp., *Babesia* spp. and *Ehrlichia* spp. and one *Wolbachia* spp. endosymbiont were detected in wild questing ticks by using PCR and DNA sequencing techniques. These findings suggested the evidences of these tick species are possible potential vectors to transmitted tick borne diseases in forest of Thailand and they may play an important role in disease transmission cycle.

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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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VITA

Mrs. Juntra Wattanamethanont was born on December, 25, 1979 in Bangkok. She got the degree of Doctor of Veterinary Medicine, Faculty of Veterinary Medicine, Kasetsart University, Thailand in the year 2005. She has worked as veterinary officer in Northern Veterinary Research and Development Center, Upper zone, Lampang province in the year 2006-2008. Since 2008 until now, she was veterinary officer in Parasitology Section, National Institute of Animal Health, Thailand. She enrolled the degree of Master of Science Program in Veterinary Pathobiology in the Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University since academic year 2014.

ACADEMIC PRESENTATION

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